Characterisation of Clonal Variants in a Human Lung Carcinoma Cell Line: Investigations into Control of Growth and Differentiation

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Shirley McBride Date: 24th July 1995.

This thesis is dedicated to my parents, Brian and Claire McBride
and to my Grandparents.

ABSTRACT

Characterisation of Clonal Variants in a Human Lung Carcinoma Cell Line: Investigations into Control of Growth and Differentiation

Analysis of many human tumours reveals the presence of two or more heterogenous cell subpopulations. Lung cancer in particular exhibits a large degree of intrinsic heterogeneity. DLKP, a human lung cell line established from a tumour which was histologically diagnosed as a 'poorly differentiated squamous carcinoma', appears to consist of three morphologically distinct populations. In this study, three clones apparently corresponding to these populations were established from the parental DLKP cells. The growth patterns of these isolated populations in monolayer culture, soft agar, spinner flasks and serum-free medium were investigated and it was found that in all but the latter assay, the parental DLKP cells grew faster than each of the clones and that the growth of the clones themselves varied under the different assay conditions. One clone appears to behave similarly to a multipotent, stem cell-like population, capable of giving rise to the two other clonal morphologies. Variation between the clones was also seen in their respective chromosome numbers and in their ability to adhere to extracellular matrix proteins.

An immunohistological characterisation of the DLKP cells and clones was carried out. While each cell line was found to be negative for the expression of keratin and all other epithelial markers examined, the presence of neuron-specific enolase, protein gene product 9.5 and neurofilament reactivity indicated a degree of neuroendocrine (NE) differentiation. These results suggest that the DLKP cell line should be classified as either variant small cell lung carcinoma (SCLC-V) or non-small cell lung carcinoma with NE differentiation (NSCLC-NE).

Studies employing the differentiation-inducing agents, 5-bromodeoxyuridine (BrdU) and retinoic acid (RA), resulted in induction of keratin expression in DLKP cells and in each of the clonal subpopulations. A similar effect was found in another keratin-

negative lung carcinoma cell line in response to BrdU and this agent also appeared to increase keratin expression in cell lines which inherently expressed keratin intermediate filaments.

Multidrug resistance (MDR) is a phenomenon which plays a significant role in the fatality of lung cancer. The proliferative capabilities of DLKP were compared with those of an MDR variant cell line, DLKP-A, and no significant differences were found. In addition, a clonal subpopulation of DLKP-A were transfected with a ribozyme capable of targetting and reducing the expression of p-glycoprotein, a membrane efflux pump involved in drug resistance. Drug resistance was successfully reversed in transfected cells to levels approaching those of parental DLKP sensitive cells.

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ABBREVIATIONS

^{3}H-Td	Tritiated Thymidine
Ab	Antibody
Adr.	Adriamycin
ATCC	American Type Culture Collection
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CAM	Cellular Adhesion Molecule
CFE	Colony Forming Efficiency
CM	Conditioned Medium
CsCl	Cesium Chloride
CVDE	Crystal Violet Dye Elution
DMEM	Dulbeccos Minimum Essential Medium
dUMP	Deoxyuridylate
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immuno Sorbent Assay
GRP	Gastrin-Releasing Peptide
HAT	Hypoxanthine-Aminopterin-Thyroxine
HCl	Hydrochloric Acid
hr.	hour
ICAM	Intercellular Adhesion Molecule
IC_{50}	Inhibitory Concentration 50%
IL-1	Interleukin-1
IL-6	Interleukin-6
kD	Kilodalton
Ker.	Keratin
M.W.	molecular weight
MAb	Monoclonal Antibody
MDR	Multiple Drug Resistance
MEM	Minimum Essential Medium

$MgCl_2$	Magnesium Chloride
min.	minute
MMLV-RT	Moloney Murine Leukemia Virus-Reverse Transcriptase
NCAM	Neural Cell Adhesion Molecule
NCTCC	National Cell & Tissue Culture Centre
NE	Neuroendocrine
NEB	Neuroepithelial Bodies
NSCLC	Non-Small Cell Lung Carcinoma
NSE	Neuron-Specific Enolase
P	Passage
PBS A	Phosphate Buffered Saline A
PCR	Polymerase Chain Reaction
PD	Poorly Differentiated
PDGF A	Platlet Derived Growth Factor A
PDGF B	Platlet Derived Growth Factor B
RA	Retinoic Acid
r.p.m.	Revolutions per minute
RNase	Ribonuclease
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Rz	Ribozyme
SCLC	Small Cell Lung Carcinoma
SCLC-C	Classic Small Cell Lung Carcinoma
SCLC-V	Variant Small Cell Lung Carcinoma
SDS	Sodium Doedecyl Sulphate
sec.	second
SFM	Serum-Free Medium
TBS	Tris Buffered Saline
Td	Thymidine
TEMED	N,N,N',N'-Tetramethyl-Ethylenediamine
TGFα	Transforming Growth Factor α
TGFß	Transforming Growth Factor ß
TK	Thymidylate Kinase
TMP	Thymidine Monophosphate
Tris	Tris(hydroxymethyl)aminomethane
TS	Thymidylate Synthatase

TV	Trypsin Versene
VCAM	Vascular Cellular Adhesion Molecule
Vcr.	Vincristine
v/v	volume/volume
w/v	weight/volume
w.r.t	with respect to
WHO	World Health Organisation

1.0 INTRODUCTION

1.0 INTRODUCTION

The lung functions as the principal organ of gas exchange in the body. It also carries out a range of nonventilatory functions including humidification, thermal regulation, mucociliary clearance and elimination of volatile substances.

The adult mammal lung contains over 40 different cell types, at least 8 of which are found in the epithelial lining of the tracheobronchial airways. These epithelial cells include ciliated cells, basal cells, brush cells, mucous goblet cells, serous cells, Clara cells, type I and type II cells and neuroendocrine cells, (the latter 5 of which function as secretory cells), and a variety of partially differentiated cell types. The distribution of these cells varies throughout the airways and marked species variation is also seen.

1.1 LUNG DEVELOPMENT

The primordial lung arises as a bulb-shaped extension of the wall of the primitive endodermal digestive tract. This lung bud divides into 2 bronchial buds which proceed to differentiate into the bronchi. During the 5th week of human development, each bronchial bud enlarges to form the primordium of a primary bronchus. The primary bronchi subsequently subdivide into secondary bronchi and progressive branching then occurs such that by 24 weeks, approximately 17 orders of branches have formed and respiratory bronchioles have developed. A further 7 orders of airways develop after birth.

In humans, the development of the lung may be characterised in general by 4 stages, the last of which is completed at around 8 years of age.

The *pseudoglandular period* (5 to 17 weeks) is so-called because the lung resembles an exocrine gland during this time. The epithelium, which is derived from the pharyngeal endoderm, is separated from surrounding mesodermal elements by a basement membrane. The epithelial cells are tall columnar in appearance at the early stages of this period and become low columnar (cuboidal) later on. These epithelial cells are undifferentiated and contain large amounts of glycogen which is indicative of immature lung epithelial cells.

The first cell type to differentiate in human fetal lung is the neuroendocrine cell which appears in the larger bronchi of pseudoglandular lungs around 8 weeks of gestation. At first, these cells appear short and glycogen-depleted with a few dense core granules visible in the cytoplasm. These immature endocrine cells usually occur in clusters and occupy only the basal part of the epithelium at this stage.

Ciliated cells begin to appear after 10 weeks and differentiating mucus-secreting cells are present from week 13, along with submucosal glands. Basal cells also begin to differentiate at this stage, but mature basal cells are the last mature cell type to appear in the epithelium.

The *canalicular period* (16 to 25 weeks) sees the emergence of the respiratory bronchioles. Type I alveolar epithelial cells appear at this stage as determined by thinning and stretching of the periphery of the cytoplasm of these cells. Differentiation of type II epithelial cells also takes place. This occurs when the undifferentiated cuboidal cells begin to develop osmiophilic inclusion bodies, organelles characteristic of type II cells, within the cytoplasm. Clara cells begin to mature in the 19th week of development.

By 24 weeks, each terminal bronchiole has given rise to 2 or more respiratory bronchioles, some of which have developed terminal sacs which are well vascularised.

The *terminal sac period* (24 weeks to birth) sees the development of large numbers of terminal sacs. The epithelium of these primitive alveoli is very thin and capillaries begin to bulge into them. Terminal sacs are lined mainly by squamous type I cells, scattered among which are rounded, secretory type II cells. These are mature type II cells which contain large numbers of inclusion bodies and no glycogen. These cells secrete surfactant which forms a film over the epithelium and serves to counteract surface tension and facilitates expansion of the terminal sacs.

By the beginning of the *alveolar period* (late fetal period to about 8 years), the lungs are capable of respiration as the alveolar epithelial membrane is thin enough to allow gas exchange. Before birth, the immature alveoli appear as small bulges in the walls of the terminal sacs and the respiratory bronchioles. After birth, the primitive alveoli enlarge and for up to a further 8 years, the number of alveoli continues to increase.

The sequence of appearance of the various epithelial cell types during development has been shown to be similar in other species including mouse (Have-Opbroek, 1991), hamster (McDowell *et al.*, 1985) and monkey (Plopper *et al.*, 1986).

1.2 LUNG EPITHELIAL CELL TYPES

As mentioned in Section 1.0, up to 8 epithelial cell types have been identified in the adult lung and these are believed to arise from a common precursor stem cell (Figure 1.2.1). Features of these epithelial cells will be discussed here.

Ciliated cells

The ciliated cells of the lung epithelium are terminally differentiated. Their primary function is to sweep layers of mucus, together with trapped particles of dust and dead cells, up towards the mouth where they are swallowed and eliminated. The cilia are approximately 0.25μm in diameter and are constructed from microtubule cytoskeletal filaments.

Basal cells

Basal cells are morphologically characterised as a small pyramidal element attached to the basal lamina (Lentz, 1971, McDowell and Beals, 1986) and were long considered to be the stem cell population of the lung epithelium. 2 different morphological types of basal cells have been described (Baldwin *et al.*, 1991) (Figure 1.2.2). Additional analysis of these cells (Baldwin, 1994) revealed that the 'typical' basal cell is the most abundant and represents the established morphological description for basal cells. The 'atypical' basal cell has an elongated shape and a spindle-shaped nucleus. Both cell types are attached firmly to the basement membrane by junctions. Any progenitoral role of the atypical basal cell has yet to be elucidated, as does its relationship to the typical basal cell.

A possible function for both basal cell types has been proposed by Evans and Plopper (1988), who outlined the role the basal cell may play in the adhesion of columnar epithelia to the basement membrane. While airway columnar cells (ciliated and nonciliated) form desmosomal attachments with neighbouring columnar and basal cells, they do not form hemidesmosomal attachments with the basement membrane (Tandler

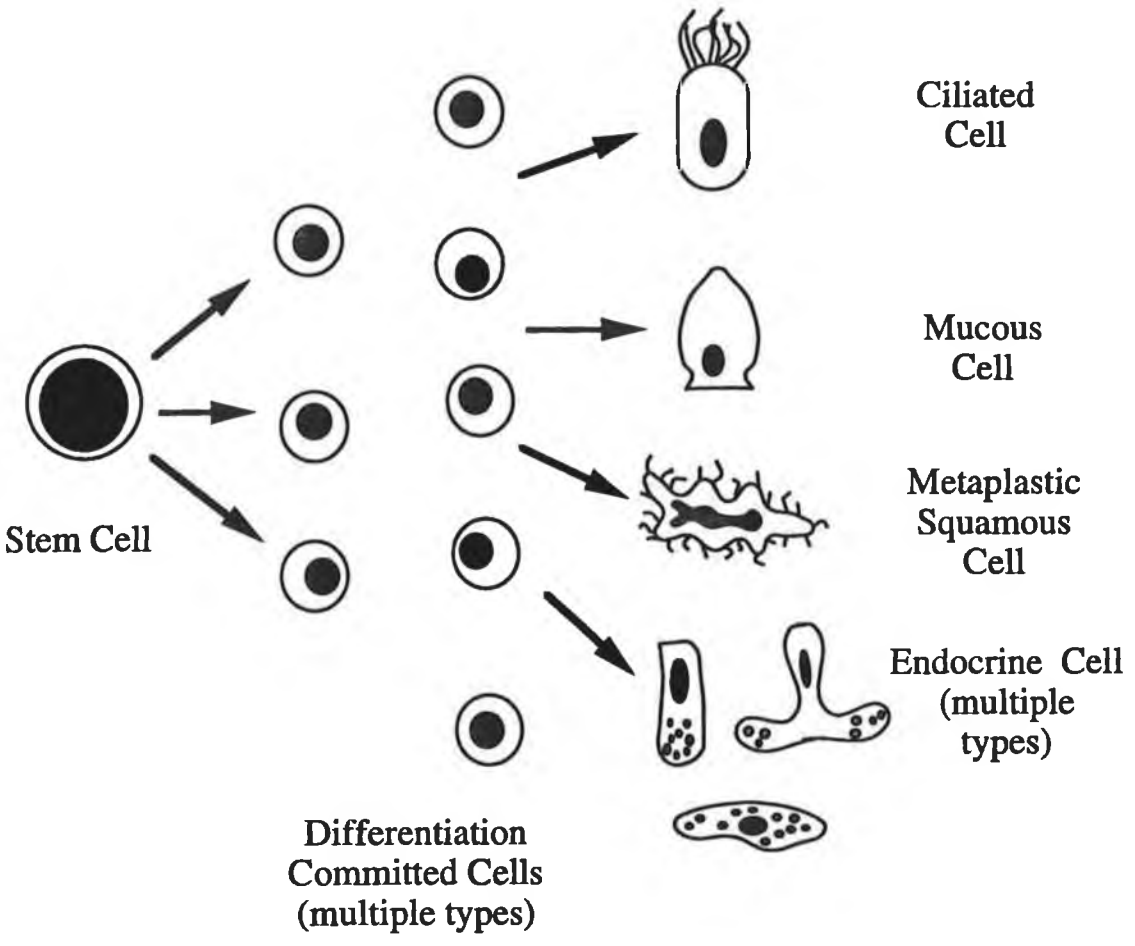


Figure 1.2.1 Unitarian theory of the origin of lung epithelial cells.

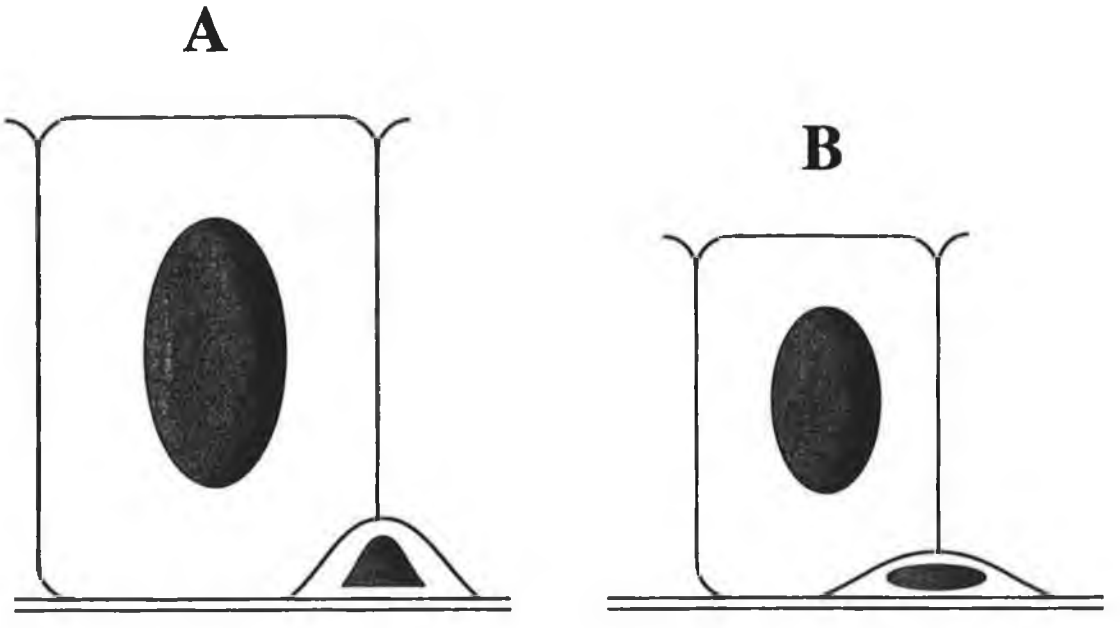


Figure 1.2.2 Diagrammatic representation of **A.** typical and **B.** atypical basal cells.

et al., 1981). Basal cells may thus serve as a means of attachment of columnar cells to the underlying basement membrane.

As mentioned earlier, basal cells were once believed to be the stem cell population of the lung epithelium. However, during development, basal cells appear last and so are at least not the ontogenic precursors of secretory and ciliated cells (Plopper *et al.*, 1986, McDowell *et al.*, 1985). In the adult lung, the stem cell compartment has yet to be identified. Donnelly and co-workers (1982) demonstrated that basal cells were capable of DNA synthesis and cell division, thus confirming their proliferative potential, but the pluripotentiality of these cells remains contentious.

Inayama *et al.*, (1988 and 1989), examined the ability of clones of tracheal basal cells to proliferate *in vitro* and to reepithelialise denuded tracheal grafts with a mucociliary epithelium. When cultured *in vitro*, on feeder layers or on plastic, the basal cells were found to proliferate and underwent 15 to 20 population doublings. After 5 days in culture, the cells changed from colonies of tightly packed, small, round cells to colonies of mixed morphology containing larger cells with abundant cytoplasm. After 20 days in culture, all colonies contained a mixture of small and large cells with the larger cells usually predominating. Squamous differentiation was demonstrated by the presence of cross-linked envelopes in some of the cells. When inoculated into denuded tracheal grafts, the basal cells gave rise to a mucociliary epithelium.

Reports from Randell's group (Liu *et al.*, 1994) also provide evidence for the role of basal cells as progenitor cells in the lung epithelium. In their studies, the binding site for *Griffonia simplicifolia* 1 (GS1)-B_4 lectin and keratin 14 were used as markers for basal cells which were sorted by flow cytometry. The resulting population, which was >95% pure basal cells, was used to inoculate denuded tracheal grafts. A subfraction highly enriched with secretory and ciliated cells and depleted of basal cells was also analysed. Both fractions gave rise to a 'poorly differentiated' (PD) epithelial lining. The PD cells were structurally unlike basal, secretory or ciliated cells and contained lipid droplets and glycogen accumulations. These cells were positive for keratin 14. The PD epithelia established by both subfractions subsequently gave rise to a mucociliary

epithelium. These findings indicate that both basal and secretory cells 'dedifferentiate' into a PD cell that is an intermediate in the regeneration process of this model. However, it was noted that pure populations were not used and the model system employed is one of extreme mechanical injury which is often not the case *in vivo*.

In contrast to these observations, evidence against the role of lung basal cells as progenitor cells comes from Johnson and Hubbs (1990). These workers found that in an isolation of tracheal epitheal cells analysed by flow cytometry, secretory cells accounted for 86% of cycling cells while the remainder consisted of basal cells. The basal cell fraction also had a lower colony forming efficiency than secretory cells. A later report from this group (Johnson *et al.*, 1990) stated that the secretory cell-rich fraction could repopulate tracheal grafts with all major cell types, while the basal cell fraction merely generated basal and ciliated cells. Additional reports from Plopper *et al.* (1986) and McDowell *et al.* (1985) also refute the progenitor role of basal cells in epithelial regeneration.

Brush Cells

Brush cells are non-ciliated epithelial cells, distinguished by the presence of unusually long, straight microvilli and epitheliodendritic synapses with nerve processes that reach in from the connective tissue (Weiss, 1983). These cells are located in the trachea and are counterparts to similar cells located in the nasal lining (which may be involved in sensory input for the sneeze reflexes).

Brush cells are not easily seen in all mamalian species, but are conspicuous in the tracheas of rats where they have been shown to possess glycogen granules as well as a preponderance of agranular recticulum in the cytoplasm.

Mucous (Goblet) Cells and Serous Cells

The mucous cell (or goblet cell) is one of the 2 types of lung surface secretory cells known to secrete mucous, the 2nd type being the less abundant serous cell. Most mucous cells contain high molecular weight mucous acidic glycoprotein and electronlucent confluent granules. Serous cells possess much rough endoplasmic recticulum and in contrast to mucous cells, contain few electron-dense granules. They are believed to contain neutral mucin and also possibly a non-mucoid substance, probably lipid. In general, the mucous-secreting cells are columnar in shape but some goblet-shaped cells occur as cells become distended by their intracellular secretion which compresses the nucleus to the base of the cell.

The progenitor potential of secretory cells was briefly alluded to previously during the discussion on basal cells. Johnson *et al.*, (1990) found that isolated secretory cells had a higher colony forming efficiency than basal cells, accounted for the majority of cycling cells in the rat tracheal epithelium and were capable of repopulating tracheal grafts with all the major cell types. Studies on the effects of mechanical injury and vitamin A deficiency on adult tracheal epithelium (McDowell *et al.*, 1984 and Keenan *et al.*, 1982) revealed that when the epithelium is injured, secretory cells divide to form a keratinising, metaplastic epithelium. When the injurant is removed, secretory cells divide and give rise to presecretory and preciliated cells, shortly after which, a complete mucociliary epithelium is reestablished. Liu and co-workers (1994) also found that isolated secretory cells regenerated a mucociliary epithelium on tracheal grafts via a poorly differentiated intermediary cell type.

Submucosal glands are responsible for producing most of the mucous present in adult human trachea and bronchi. Each gland is tubular with a duct opening into the airway lumen. A gland usually consists of 4 regions (Meyrick *et al.*, 1969): (i) a narrow ciliated duct, the lining cells of which are continuous with the surface epithelium, (ii) an expanded collecting duct lined with cells of intermediate morphology, (iii) mucous cells and (iv) serous cells.

Clara Cells (non-ciliated bronchiolar cells)

In adult human lungs, Clara cells are found only in the epithelium of the terminal bronchioles, in contrast to the mucous or serous cells which are found mainly in the larger conducting airways of the trachea and bronchi (Plopper *et al.*, 1980). Clara cells contain electron-dense secretory granules and very little glycogen is present in adult cells. The apical membrane (facing into the lumen) of the Clara cell protrudes into the airway lumen and this periphery of the cell contains many secretory granules in position for release (Massaro *et al.*, 1979).

Clara cells serve as stem cells in the bronchiolar epithelium. They divide to give rise to other Clara cells and also terminally differentiate into ciliated cells (Ayers and Jeffery, 1988). Hook and co-workers (1987) demonstrated that isolated Clara cells were capable of establishing a complete bronchiolar epithelial lining.

Clara cells contain the cytochrome P-450 monooxygenase pathway involved in the metabolism of foreign compounds and the concentration of cytochrome P-450 isozymes in these cells appears to exceed greatly that of other pulmonary cell types (Domin *et al.*, 1986). The resulting activation of xenobiotic compounds implicates the Clara cell as a possible progenitor cell for the development of some pulmonary tumours.

The nature of the secretory product(s) produced and released by Clara cells is as yet unclear. The evidence for these cells having a significant secretory role in the lung is largely circumstantial and based mainly on ultrastructural and morphological characteristics. Clara cells contain ample rough endoplasmic recticulum, a well-developed Golgi apparatus and membrane-bound inclusions (storage granules), all of which are indicative of secretory cells. In addition, the cells appear to empty the contents of their secretory granules into the lumen of the airways (Kuhn and Callaway, 1975) and elements of these secretions appear to be similar to those of other lung secretory cells (type II cells) (Ward *et al.*, 1985).

Alveolar Type I and Type II cells

Alveolar type I and type II cells are the main cells which comprise the bronchiolar epithelium (Penney, 1988). Type I cells are squamous in morphology, extremely flattened and thin, facilitating the exchange of gases in the alveoli. Type II cells, in contrast, have a cuboidal shape and contain characteristic lamellar inclusion bodies. Functions of type II cells include the synthesis, storage and secretion of surface active material (surfactant) (Chevalier and Collet, 1972), transepithelial solute transport to regulate the volume and composition of alveolar fluid (Goodman *et al.*, 1983) and re-epithelialisation of the alveoli after lung injury by differentiation into type I cells which are terminally differentiated (Adamson and Bowden, 1975).

Type II cells contain the cytochrome P-450 monooxygenase pathway involved in the metabolism of xenobiotics (Devereux *et al.*, 1989), although the P-450 isozymes are present at lower concentrations than those of Clara cells. Nonetheless, type II cell damage has been demonstrated following metabolism of exogenous compounds (Wadell and Marlow, 1980). Under normal conditions, type II cells proliferate relatively slowly in the adult lung. The turnover is believed to occur approximately every 4-5 weeks (Kauffman, 1980). In response to alveolar lining injury however, type II cell proliferation increases dramatically (Smith and Brody, 1981) to replace damaged type I and type II cells. Because of their large surface area and relative inability to repair damage due to their low levels of energy-producing organelles such as mitochondria, endoplasmic recticulum and Golgi apparatus, the type I cells are often the major site of damage in the lung following a pulmonary insult. Subsequent to destruction of type I cells, type II cells proliferate to restore the alveolar epithelium (Penney, 1988).

Considerable diversity exists amongst reports concerning the role of Clara cells and type II cells in the development of pulmonary tumours in laboratory animals exposed to carcinogens. Several reports state that either type II cells (Rehm *et al.*, 1988 and 1991a and Khalil *et al.*, 1994) or Clara cells (Ward *et al.*, 1985, Rehm *et al.*, 1991b and Belinsky *et al.*, 1991) or both (Thaete and Malkinson, 1991), depending on the nature of the insult, are the origins of spontaneous and induced mouse and/or rat lung tumours.

Both must be candidates for the originating cell(s) of pulmonary carcinomas in human due to their suseptibility to toxic and carcinogenic injury and their innate functions as progenitor cells of the lung epithelium.

Neuroendocrine Cells

As mentioned earlier, neuroendocrine (NE) cells are the first differentiated cell type to appear in the developing lung. These cells belong to the diffuse endocrine system of the body that includes the polypeptide hormone-secreting enterochromaffin cells of the gastrointestinal tract and cells of the adrenal and thyroid glands.

Mature NE cells in human lungs are usually tall and pyramidal in appearance and contain numerous characteristic membrane-bound dense core granules. These granules contain a range of neural-related amines and peptides. Serotonin, neuron-specific enolase (NSE), bombesin-like peptides (gastrin-releasing peptide - GRP), calcitonin and calcitonin gene-related peptide have all been demonstrated in the NE cells of human fetal lungs. Both serotonin (Cutz, *et al.*, 1985) and NSE (Sheppard *et al.*, 1984) are present in immature NE cells at 8 weeks. GRP is the first peptide to appear and mRNA levels peak between 16-25 weeks (Spindel *et al.*, 1987). Calcitonin reactivity occurs later at 20-22 weeks (Sheppard *et al.*, 1984) but levels never reach those of GRP.

NE cells can occur singly in the lung epithelium or in clusters called neuroepithelial bodies (NEBs). The paucity of NE cells in the lungs of normal human adults led to the belief that the most crucial role of these cells is in fetal pulmonary development (Gosney, 1992). Hoyt *et al.*, (1991 and 1993), have demonstrated the proliferative effect of fetal pulmonary NEBs on local endoderm. Attempts to demonstrate proliferation of NE cells in normal adult lung have been largely unsuccessful. 2 reports of ^{3}H-thymidine incorporation into normal NE cells (Linnoila, 1982 and Hoyt *et al.*, 1988) indicated that these cells differentiate slowly from precursor cells or that differentiated NE cells can divide, although infrequently. The role of pulmonary NE cells in adult lung thus remains unclear. The demonstration of innervation and synaptic contact in some NE cells and NEBs (Lauweryns and Cokelaere, 1970) suggests a possible sensory role for these cells but no such function has been proven.

The overlap in markers expressed in SCLC and NE cells (Hattori *et al.*, 1968, Tateishi *et al.*, 1978, Baylin *et al.*, 1978 and Gazdar *et al.*, 1980) led to the belief that NE cells were the specific precursor cells of SCLC (Pearse, 1969). However, the increasing demonstrations of markers common to several lung cancer subtypes has led to the common stem cell thoery for lung cancer. This theory is discussed in more detail in the next section.

1.3 CLASSIFICATION OF LUNG CANCERS

Lung cancer is the leading cause of cancer death in the western world (Silverberg and Lubera, 1988). In 1982, the World Health Organisation revised the histological classification of lung tumours (World Health Organisation, 1982) which had originally been published in 1967 (Kreyberg, 1967). Malignant epithelial tumours are the most common lethal lung tumours. These have been broadly classed into non-small cell lung cancer (NSCLC) comprising squamous, adeno- and large cell carcinomas and small cell lung cancer (SCLC). The 2 classes account for approximately 75% and 25% of lung cancers respectively. NSCLC is usually treated by surgical resection, due to a poor response to anticancer drugs, with a 15% cure rate while chemotherapy, with or without radiotherapy, is the treatment commonly used for SCLC, with a 5% cure rate (Minna *et al.*, 1989). The poor success rate in lung cancer treatment is due to several factors including early systemic spread, inability to detect early stages of the disease, ineffective treatment agents and the development of drug resistance following exposure to chemotherapeutic agents.

The WHO classification of lung tumours is based entirely on conventional light microscopic determination and subsequent treatment is frequently based solely on the resulting diagnosis. However, there is accumulating evidence that the current working criteria for tumour classification may be misleading. Heterogeneity within most individual tumours has been recognised for some time now (Weber, 1980, Fidler and Hart, 1982 and Owens *et al.*, 1982). Evidence that expression of SCLC-related biochemical markers on NSCLC cells may result in improved response to chemotherapy (Ruckdeschel *et al.*, 1991) suggests that knowledge of the biochemical composition of lung cancer cells may provide information about the clinical behaviour of these cells that is not apparent in routine histological analysis.

The classification of malignant epithelial lung tumours will now be discussed in more detail.

SQUAMOUS CELL CARCINOMA

The 1982 WHO classification of lung tumours describes squamous cell carcinoma as a 'malignant epithelial tumour with keratinisation and/or intercellular bridges' and the presence of trace amounts of intracellular mucin should not exclude tumours from this category. 3 histological degrees of differentiation of squamous cell carcinoma are described: (i) 'well differentiated' which exhibits orderly stratification, obvious intercellular bridges (desmosomes) and keratinisation with pearl formation, (ii) 'moderately differentiated' with features intermediate between well differentiated and poorly differentiated and (iii) 'poorly differentiated' when keratin and/or bridges are present in a largely undifferentiated tumour or when these features are detected with difficulty.

Squamous cell carcinomas are the commonest form of lung cancer. They usually occur in the larger bronchi and are believed to arise from foci of atypical squamous metaplasia. As with all cancer types, cytogenetic analysis has been carried out on squamous cell carcinomas (Buchmann, *et al.*, 1991, Testa and Siegfried, 1992 and Rodenhuis and Slebos, 1992) in an attempt to indentify any specific chromosomal abnormalities or alterations that may have clinical significance in therapy and prognosis. It is hoped that this work may also elucidate some of the events involved in malignant transformation. However, because of the relatively low mitotic index of many primary NSCLC tumours and the often complex karyotypes, identification of consistent chromosomal abnormalities has proven difficult. Despite these complications, genetic aberrations have been reported including chromosome deletions and allelic losses (Weston *et al.*, 1989, Shiseki *et al.*, 1994 and Sato *et al.*, 1994), near-triploid and near-tetraploid karyotypes (Testa and Seigfried, 1992), enhanced expression of *ras* oncogenes (Miyaki *et al.*, 1985) and differential expression of genes in normal lung and NSCLC tissue (Schraml *et al.*, 1994).

The presence of additional copies of specific chromosomes and genes has been linked in theory to the overexpression of stimulatory growth factors and their receptors in NSCLC. Testa and Siegfried (1992) demonstrated partial gains in chromosome 7 of

NSCLCs in the region where the epidermal growth factor (EGF) receptor gene is located (Spurr *et al.*, 1984). Reports of increased expression of the EGF receptor protein and the EGF ligand itself in squamous cell lung cancers (Hendler and Ozanne, 1984 and Ozanne *et al.*, 1986) and overexpression in cell lines (Sakiyama *et al.*, 1986 and Haeder *et al.*, 1988) have led to speculative links between increased chromosome numbers and chromosome gains and overexpression of growth regulatory proteins, but no real link has yet been proven.

Several protein markers have been used in immunocytochemical studies to determine squamous differentiation. One such marker is transglutaminase. Transglutaminases are a group of enzymes that covalently link peptide-bound glutamine to primary amines such as lysine (Folk, 1985) and as such, these enzymes catalyse the formation of cross-linked envelopes during squamous differentiation (Green, 1977). The expression of this enzyme during squamous differentiation of tracheal epithelium has been demonstrated (Jetten and Shirley, 1986).

Specific keratin intermediate filament proteins are expressed in squamous differentiated cells and can be used as markers for this phenotype. Broers and colleagues (1988) demonstrated that highly differentiated human lung squamous carcinomas expressed high levels of keratins 4, 10 and 13. In addition, reduction of squamous differentiation correlated with a loss of these specific keratins and an increase in keratins 7, 8, 18 and 19. A serum marker for patients with NSCLC has recently been evaluated (van der Gaast *et al.*, 1994). Cyfra 21.1 is a fragment of keratin 19 (Pujol, *et al.*, 1993) and was shown to be a sensitive marker for squamous cell carcinoma.

Cholesterol sulfate (Rearick *et al.*, 1987a) and sulfotransferase (Rearick *et al.*, 1987b) have also been described as markers for squamous cell differentiation in lung epithelial cells.

ADENOCARCINOMA

A second category of NSCLC is adenocarcinoma. This tumour type is described in the WHO classification as malignant epithelial with tubular, acinar (glandular) or papillary growth patterns and/or mucous production by the tumour cells. In moderately differentiated adenocarcinomas, cells have rounded nuclei, coarse chromatin and large, prominent nucleoli. The cytoplasm is moderate in amount and may contain fine or coarse mucin-like vacuoles.

Adenocarcinomas are subdivided according to the WHO classification as follows: (i) adenocarcinoma which has a predominance of glandular structures with or without papillary or solid areas and occurs predominantly in the larger bronchi, (ii) papillary adenocarcinoma with a predominance of papillary structures, (iii) bronchioalveolar carcinoma in which cylindrical tumour cells grow upon the walls of preexisting alveoli and (iv) solid carcinoma with mucous formation which is a poorly differentiated adenocarcinoma lacking acini, tubules and papillae but with mucin-containing vacuoles with many tumour cells.

Chromosomal aberations similar to those of squamous cell carcinomas have been detected in adenocarcinomas including increased numbers of chromosomes and partial gains of chromosomes (Testa and Siegfried, 1992) and amplification of *myc* and *ras* protooncogenes (Slebos *et al.*, 1989, Rodenhuis *et al.*, 1987, Saksela *et al.*, 1986 and Yokota *et al.*, 1986). A putative tumour suppressor gene has been identified on chromosome 4 and Herzog *et al.* (1994) have demonstrated allelic loss of this gene in 48% of lung adenocarcinomas.

As with squamous carcinomas, adenocarcinomas are often positive for EGF receptor expression (Haeder *et al.*, 1988).

Morphologically and biochemically, adenocarcinomas often demonstrate differentiation toward Clara cells and type II cells. Ultrastructural studies on adenocarcinoma lung cell lines (Gazdar *et al.*, 1990 and Linnoila *et al.*, 1992) have revealed cytoplasmic

inclusions characteristic of Clara cells and type II cells and in addition, these cells also expressed the surfactant-associated proteins SP-A, SP-B and to a lesser extent SP-C. Adenocarcinoma cell lines also often produce and secrete Clara cell 10-kDa protein (Gazdar *et al.*, 1990).

LARGE CELL CARCINOMAS

The WHO classification of lung cancer (1982) describes large cell carcinomas as malignant epithelial tumours with large nuclei, prominent nucleoli, abundant cytoplasm and usually well-defined cell borders, without the characteristic features of squamous cells, small cell or adenocarcinomas. 2 variants of large cell carcinomas are described; (i) giant cell carcinoma which consists of a significant number of pleomorphic, multinucleated cells and (ii) clear cell carcinoma which comprises elements with clear or foamy cytoplasm without mucin and they may or may not contain glycogen.

Large cell carcinomas have no distinguishing features visible by light microscopic analysis. Giant cell carcinomas are very aggressive and highly malignant and are usually found at the later stages of malignancy (Yesner and Carter, 1982). Clear cell tumours are rare and most behave in a benign fashion.

Most large cell carcinoma cell lines grow as attached epitheloid cells without evidence of squamous, glandular or papillary differentiation. However, immunocytochemical demonstration of gastrin-releasing peptide reactivity in some large cells carcinomas (Hamid *et al.*, 1990) suggests a degree of NE differentiation in such tumours.

In vivo, large cell carcinomas may be categorised as 2 subtypes, a compact growth type and a loose structure type. Ishida *et al.* (1990) have proposed that the compact growth type represents a slightly more differentiated cell type due to the higher incidence of markers such as keratin, epithelial membrane antigen, secretory component, lactoferrin and carcinoembryonic antigen on these cells compared with the loose structure type.

SMALL CELL CARCINOMA

Small cell carcinomas are a subtype of neuroendocrine tumour. Other subtypes include classic carcinoid and atypical carcinoid (or well-differentiated NE carcinoma) which are discussed later here.

3 categories of small cell carcinoma were described in the WHO classification (1982). (i) Oat cell carcinoma which is a tumour composed of uniform small cells, having dense round or oval nuclei, diffuse chromatin, inconspicuous nucleoli and very sparse cytoplasm. This tumour type is also reported to be associated with the secretion of a variety of substances including adrenocorticotropic hormone (ACTH), serotonin, antidiuretic hormone, calcitonin, growth hormone and oestrogens. (ii) Small cell carcinoma, an intermediate type which is also composed of small cells but with more abundant cytoplasm. Morphologically, these cells are less regular in appearance than those of oat cell carcinoma and may be polygonal or fusiform (spindle-shaped). Secretions similar to those of oat cell carcinoma are also prevalent in this category. (iii) Combined oat cell carcinoma, in which there is a definite component of oat cell carcinoma with adeno- and/or squamous cell carcinoma.

Of the 3 NE tumour subtypes mentioned above (small cell carcinoma, classic carcinoid and atypical carcinoid), small cell carcinoma is the most aggressive. This carcinoma metastasises early in its course (Churg, 1988) and is not usually amenable to surgical resection. Chemotherapy and radiotherapy are the usual methods of treatment but multiple drug resistance (MDR) is a problem which often results from this course of action (discussed in Section 1.7).

Small cell carcinomas exhibit many features found in neuroendocrine cells of the lung epithelium. Original opinion was that these NE cells were derived from the neural crest rather then being endodermal in origin (Pearse, 1969) and that NE tumours, including SCLC, subsequently arose from these cells. However, the current working hypothesis is that NE cells and their tumours originate from undifferentiated, totipotent bronchial epithelial cells *i.e.* there is a common stem cell origin for all lung epithelial cells

(Yesner, 1978 and Gazdar *et al.*, 1981). This theory is discussed further at the end of this section.

Several aberations have been detected at the gene level in SCLCs. High expression of *ras*, *myb*, *raf*-1 and other *myc* family protooncogenes has been demonstrated in SCLC tumour tissue and cell lines (Nakano *et al.*, 1984, Griffin and Baylin, 1985, Kiefer *et al.*, 1987, Nau *et al.*, 1985 and Rapp *et al.*, 1988). Amplification and overexpression of the *myc* gene family appears to be a unique feature of SCLCs (Carbone and Minna, 1992). Plummer *et al.* (1993), have shown a correlation between expression of c-*myc* and suppression of c-*kit*, a gene which encodes a tyrosine kinase growth factor receptor for hemopoietic stem cell factor, in SCLC cells. Chromosome 3p deletions have been found to be common in SCLC cells also (Graziano *et al.*, 1991) and Levin *et al.* (1994) have identified several genetic alterations in SCLC.

As mentioned above, small cell lung cancers express a number of NE features in common with pulmonary NE cells. Both normal and tumour NE cells have been demonstrated to contain dense core vesicles or neurosecretory granules (Ghadially, 1988 and Dardick, 1993). These granules can either store amines or take up precusor substances and decarboxylate them to amines (Pearse, 1969 and 1974) and hence cells with these properties have been termed APUD (amine precursor uptake and decarboxylation) cells. The enzyme L-dopa decarboxylase (DDC) is involved in this system and is used as a marker for APUD cells, both normal and malignant (Gazdar *et al.*, 1988). Chromogranin A (CgA) is a high molecular weight protein present in NE granules and believed to be a secretory protein, although its function is not known (O'Connor *et al.*, 1983). CgA production has been demonstrated in cell lines established from NE cell lines (Bergh *et al.*, 1989) and this is considered to be a marker for NE differentiation.

Serotonin production within the dense core vesicles of NE cells and tumour cells has also been demonstrated (Lauweryns *et al.*, 1973 and Newman *et al.*, 1993).

Peptides such as gastrin-releasing peptide (GRP), leucine-enkephalin and calcitonin have been immunolocalised to pulmonary NE cells and their tumours (Wharton *et al.*, 1978,

Tsutsumi *et al.*, 1983, Cutz *et al.*, 1981, Becker *et al.*, 1980, Gazdar, 1984, Shimosegawa and Said, 1991, Ghillani *et al.*, 1989 and Woll, 1991).

Enolase enzymes are dimers composed of α, β or γ subunits which convert 2-phosphoglycerate to phosophoenolpyruvate. The γ, γ- and α, γ-enolases are expressed mainly in neurons and NE cells (Kato *et al.*, 1982) and the γ-subunit of enolase is therefore known as neuron specific enolase (NSE). High levels of NSE have been detected in NE-related tumours, including SCLC (Odelstad *et al.*, 1981, Ishiguro *et al.*, 1983 and Tapia *et al.*, 1981) and this enzyme is used as a marker for NE-like cells.

Neural cell adhesion molecule (NCAM) is a major cell-cell-contact protein in adult neuronal tissue (Edelman, 1986) and immunoreactivity for this protein has been demonstrated in almost 100% of all SCLC (Berendsen *et al.*, 1988 and Schol *et al.*, 1988). Serum NCAM and NSE appear to have potential as *in vivo* tumour markers for SCLC (Jaques *et al.*, 1993).

Another marker used in the evaluation of NE differentiation in cells is Leu 7 which is an antigen present on a subpopulation of large granular lymphocytes which have natural killer cell and antibody-dependent cell-mediated cytolytic functions (Abo and Balch, 1981). Leu 7 immunoreactivity has been demonstrated in normal nervous tissue and also in tumours of neuroectodermal origin (Caillaud *et al.*, 1984 and Mechtersheimer *et al.*, 1991).

Protein gene product 9.5 (PGP 9.5) is a member of the family of ubiquitin carboxyl-terminal hydrolases (Wilkinson *et al.*, 1989). First isolated from human brain (Jackson and Thompson, 1981), expression of the PGP 9.5 antigen has been demonstrated on neuronal and neuroendocrine tissue (Thompson *et al.*, 1983) and on tumours of neuronal and neuroendocrine origin (Brook *et al.*, 1988 and Reubi *et al.*, 1990).

Additional markers reported to be present on NE-tumour cells are creatine kinase (Watanabe *et al.*, 1988), somatostatin receptors (Reubi *et al.*, 1990), protein 1A-1 (unidentified protein) (Lan *et al.*, 1993), neuroendocrine-specific protein (NSP) gene proteins (van de Velde *et al.*, 1994) and pro-gastrin-releasing peptide (31-98) (Miyake

et al., 1994). Recently, Bcl-2 expression was detected on 65% of small cell carcinomas examined by Ben-Ezra et al. (1994) and the authors hypothesise that this oncoprotein may play a role in the pathogensis of SCLC.

VARIANT SMALL CELL LUNG CARCINOMA

In 1985, Carney and colleagues described 2 major classes of SCLC cell lines: (i) classic SCLC lines which expressed elevated levels of 4 NE biomarkers examined, namely L-dopa decarboxylase, bombesin-like peptides, NSE and creatine kinase and (ii) variant SCLC (SCLC-V) lines which did not express one or more of the 4 NE markers (L-dopa decarboxylase and/or bombesin-like peptides) but did express elevated levels of NSE and creatine kinase. Also described were 4 morphological categories of SCLC cell lines. Type 1 grew as tightly packed floating aggregates with central necrosis, type 2 grew as relatively densely packed floating aggregates of amorphous cells lacking central necrosis, type 3 grew as very loosely adherent floating aggregates and type 4 grew attached to the substrate and consisted of polygonal overlapping cells, lacking an epithelioid appearance. There appeared to be a correlation between loss of NE marker expression and increased substrate adherence. The authors suggested that these findings may be clinically relevant and related to their previous findings that the loss of NE features observed in large cell variants derived from SCLC cell lines may be linked with the increased radiation and chemotherapy sensitivity observed in the large cell variants (Carney *et al.*, 1983).

A higher cloning efficiency and a faster doubling time have been reported for SCLC-V lines compared with classic SCLC (Gazdar *et al.*, 1985) and Broers *et al.* (1988) reported that while classic SCLC cell lines contain keratin and lack neurofilament proteins, most SCLC-V lines do not contain detectable amounts of keratins but partly express neurofilaments and vimentin. Most SCLC-V lines are also positive for NCAM and Leu 7 expression (Carbone *et al.*, 1991). Higher levels of c-*myc* expression have also been found in SCLC-V lines (Little *et al.*, 1983). Several SCLC-V cell lines have now been reported (Bepler *et al.*, 1987c, Watanabe *et al.*, 1988, Doyle *et al.*, 1990 and Yokose *et al.*, 1991).

Transition of classic SCLC cell lines to the variant phenotype after prolonged cultivation (months to years) has been reported (Gazdar *et al.*, 1985 and Bepler *et al.*, 1987a) and it has been postulated that this *in vitro* phenomenon parallels the situation *in vivo* as a tumour progresses from a classic SCLC to a mixed morphology (Bepler *et al.*, 1987b).

While all of the SCLC markers described previously have been demonstrated in NE tumour cells, it is becoming increasingly clear that few markers are actually specific for SCLC. The first suggestion of this came in 1981 when McDowell *et al.* described non-small cell neuroendocrine lung carcinomas which had been diagnosed by light microscopy as squamous, large cell or adenocarcinomas. Electron microscopic examination revealed dense core neurosecretory granules within the tumour cells. Since this report, much evidence of overlap in markers expressed by different lung tumour types has become apparent. Neal and co-workers (1986) suggested that up to 9% of non-small cell carcinomas of the lung contain neurosecretory granules. NCAM has been reported in 20-30% of NSCLC (Schol *et al.*, 1988). NSE, chromogranin A, bombesin, L-dopa decarboxylase, Leu 7 and synaptophysin were all once believed to be exclusive to SCLCs but have been demonstrated in NSCLCs (Graziano *et al.*, 1994, Kayser *et al.*, 1988, Pahlman *et al.*, 1986, Yokose *et al.*, 1991, Gazdar *et al.*, 1988, Hamid *et al.*, 1990, Bergh *et al.*, 1989 and Cole *et al.*, 1985). Where 2 or more NE markers are present in cancer cells with otherwise NSCLC features, these cells have been termed NSCLC with neuroendocrine differentiation or NSCLC-NE (Linnoila *et al.*, 1988a and 1988b).

Studies by Gazdar *et al.* (1991) indicated that the drug sensitivity of NSCLC-NE cell lines was increased with respect to NSCLC and similar to that of SCLC lines. This suggests that NSCLC-NE could possibly be successfully treated with chemotherapy. However, no clinical correlation has yet been demonstrated. Indeed, a trial involving individualised chemotherapy for NSCLC patients based on the identification of NE markers proved unsuccessful (Shaw *et al.*, 1993). A retrospective study by Graziano and colleagues (1994) also found that the presence of markers for NSE, Leu 7 and chromogranin were not of prognostic significance in a group of patients studied. However, there is evidence that the presence of the NE marker NCAM is indicative of

a poor prognosis in NSCLC (Schol *et al.*, 1988). In addition, Sundaresan *et al.* (1991) reported that patients with NSCLC-NE had increased levels of metastases compared with NSCLC patients and therefore detection of NE markers on NSCLC may be clinically relevant.

OTHER LUNG EPITHELIAL CARCINOMAS

PULMONARY CARCINOID TUMOURS

Carcinoid tumours are rare and make up only 1-6% of all primary lung tumours (Yousem, 1991). These tumours were originally believed to be derived from embryoinc neural crest precursor cells but are currently thought to arise from a pleuripotent bronchial epithelial cell, as are all epithelial lung cancers (see end of section for 'stem cell theory'). Histologically, carcinoids are composed of sheets of small round cells with uniform nuclei and cytoplasm. Neurosecretory granules are often present (Hage, 1973) and a distinction between SCLC which also contains neurosecretory granules may be made on the basis of the morphology of the intracytoplasmic secretory granules (Hage 1980). In addition, Komminoth *et al.* (1991) were able to distinguish NCAM present on SCLCs from that of carcinoids by immunological examination of the polysialic chains of NCAM.

Immunocytochemical analysis has demonstrated numerous peptides contained within carcinoids including NSE, serotonin, insulin, growth hormone, neurotensin and bombesin (Linnoila *et al.*, 1988a and Barbareschi *et al.*, 1992).

Carcinoids are a less aggressive tumour type than SCLC and Barbareschi and co-workers (1992) found a decrease in NE features from typical carcinoid to SCLC which was paralleled by an increase in proliferative activity and by an altered expression of tumour suppressor gene products. This indicates that loss of differentiation features may be related to an increase in aggressive growth in tumours.

PULMONARY BLASTOMA

Pulmonary blastomas occur infrequently (< 1% of all lung tumours) and usually consist of a mixture of immature mesenchymal and epithelial elements. These tumours are believed to recapitulate the pseudoglandular stage of the developing lung (Francis and Jacobsen, 1983 and Spencer, 1961).

Yousem *et al.* (1990) carried out an immunohistochemical study to compare the lung antigens expressed by pulmonary blastomas and immature human lungs at 10-16 weeks gestation *i.*e. pseudoglandular lungs. The authors found extensive similarities in protein marker expression between the cells in their expression of keratin, epithelial membrane antigen, carcinoembryonic antigen, chromogranin-positive neuroendocrine cells and surfactant-producing and Clara cell antigen-expressing cells. Mesenchymal features present in both pulmonary blastomas and immature lung included smooth muscle and myofibroblastic differentiation. Chejfec *et al.* (1990) have also demonstrated neuroepithelial bodies and keratin, chromogranin, NSE, gastrin, calcitonin, bombesin, somatostatin and serotonin immunoreactivities in pulmonary blastoma.

BASAL CELL (BASALOID) CARCINOMA

Brambilla and colleagues (1992) classified a unique form of lung tumour as basal cell (basaloid) carcinoma. This tumour type presents an immunophenotype similar to that of basal bronchial epithelial cells with a low level expression of keratins 5, 6, 7, 8, 13, 16, 18 and 19, infrequent and inconsistent staining for NE markers, and absence of neuroendocrine granules and the presence of some squamous and/or glandular differentiation. The authors believe that this tumour type resembles multipotent basal cells with the potential for multidirectional differentiation along squamous, glandular or neuroendocrine pathways.

'STEM CELL THEORY'

The demonstration of overlapping features between lung cancer types supports the concept of a common stem cell origin for lung epithelial carcinomas, a theory which was proposed almost 20 years ago (Yesner, 1978 and Fialkow, 1979). Gazdar *et al.* (1981) discussed the theory further and outlined how all the cells of the bronchial epithelium and the tumours which arise from them could have a common stem cell origin. Gazdar postulated that the transforming events which led to carcinogenesis affect undifferentiated or partially differentiated cells. Depending on the level of differentiation at which the transformation occurs, the resulting tumour will be differentiated or undifferentiated and will present as a squamous, large cell, adenocarcinoma or SCLC accordingly (see Figure 1.3.1).

Numerous reports of evidence for the presence of stem cell populations in tumour cell lines have been forwarded. Olsson *et al.* (1984), Spang-Thomsen *et al.* (1986) and Khan *et al.* (1991) described differences in nude mouse tumourigenicity, colony forming efficiency, reactivity with monoclonal antibodies and radiosensitivity between populations in cell lines, suggesting that stem cell-like populations were present. Others (Gazdar *et al.*, 1988 and Bergh *et al.*, 1989) have suggested that the overlapping expression of markers between different tumour types is indicative of a common stem cell origin for lung epithelial tumours.

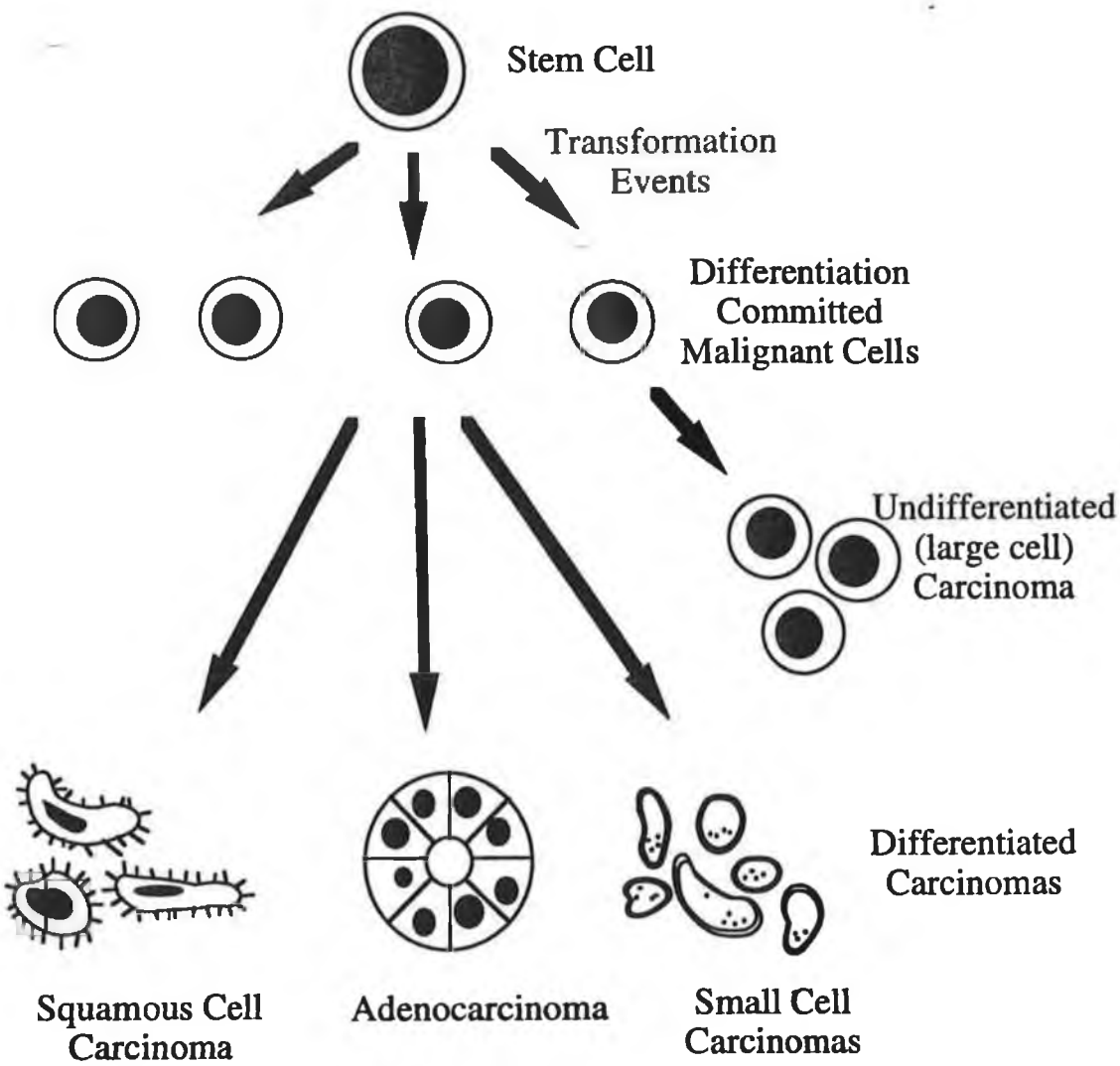


Figure 1.3.1 Common stem cell theory for origins of lung cancer cell types.

1.4 HETEROGENEITY IN LUNG TUMOURS

The concept of tumour heterogeneity was reviewed by Heppner in 1984. The author defined the term tumour heterogeneity as 'those cases in which tumour cell differences are believed to be due to differences in cell lineage, *i*.e., due to the presence of distinctly different subpopulations capable of breeding true', and stated that this did not contradict the single-cell origin of tumours as most organisms begin as single cells but soon become heterogenous. Heppner also stated that tumour heterogeneity may arise as a consequence of those mechanisms that result in heterogeneity in normal tissue as well as tumour-specific mechanisms such as genetic instability. The characteristics in which diversity between subpopulations of tumour cells may be exhibited include cellular morphology, metastatic potential, karyotype, growth rate, antigen expression and response to chemotherapeutic drugs. Lung cancer is particularly noted for its heterogeneity. In 1959, Azzopardi described a SCLC as containing tubular structures, squamous nests, rosette formation, giant cells and mucin secretion.

Diversity among lung tumour cell populations is evident at the genetic level. Studies carried out on tumour heterogeneity based on differences in DNA content among cell subpopulations have demonstrated that NSCLC appear to exhibit a large degree of heterogeneity with most tumours analysed containing at least 2 subpopulations (Leith and Michelson, 1989). During tumour progression, cancer cells are believed to accumulate increasing random alterations (Nowell, 1986). These cytogenetic abnormalities are heterogenous, not only between lung tumours, but also within individual tumour cell populations (Morstyn *et al.*, 1987 and Burholt *et al.*, 1989a). Oncogenes such as c-*raf*-1 and c-*myc* have been implicated in the acquisition of a multidifferentiated phenotype by lung tumours. When Pfiefer and co-workers (1991) inoculated mice with normal human bronchial epithelial cells which had been transformed by the c-*raf*-1 and c-*myc* protooncogenes, the resulting tumours contained desmosomes, microvilli and dense core granules, indicative of squamous, glandular and neuroendocrine differentiation respectively.

While spontaneous gene mutations occur at high rates in tumour cells (Cifone and

Fidler, 1981), the rates of variant formation in biochemical, immunological, enzymatic and biological properties found in cancer cells occur at much higher rates (reviewed in Nicolson, 1987). Histologic heterogeneity, *i.e.* the appearance of different histological types in different areas of a tumour, was demonstrated by Mooi *et al.* (1990) to be a common occurrence in lung tumours as judged by electron microscopic examination. Lung cell lines may also exhibit morphological heterogeneity with many lines containing cells which grow attached to the substrate and in suspension (Khan *et al.*, 1991 and Terasaki *et al.*, 1984).

Heterogeneity in antigen expression by lung tumour cells has been described. Moss and colleagues (1991) reported that antibodies directed against NCAM and cytokeratins reacted differentially with cells in both lung tumour sections and in serous effusions. Gorgoulis *et al.* (1992) observed considerable intratumour heterogeneity in the expression of epidermal growth factor, transforming growth factor and epidermal growth factor receptor proteins in squamous cell lung carcinomas and Walker and Wright-Perkins (1992) demonstrated that a subline derived from a classic small cell cancer cell line expressed an antigen profile different to that of the parental cells.

Diversity in the metastatic potential of lung carcinoma cells has also been observed. Brodt (1986) described 2 metastatic variants of a Lewis lung carcinoma which had different organ specificities and Young *et al.* (1987) reported on different *in vivo* metastatic potentials and *in vitro* migratory potentials of Lewis lung carcinoma clones. Overgrowth of primary tumours by metastatic subpopulations was demonstrated by Waghorne *et al.* (1988) indicating diverse behavioural patterns amongst tumour cells.

Heterogeneity in tumour cell sensitivity to anticancer drugs is a major clinical problem. In a study using an artificially heterogenous small cell lung cancer xenograft, Aabo *et al.* (1994) found that the fractional size of a resistant tumour subpopulation may determine the extent of any clinical response to a chemotherapeutic treatment. Differential sensitivities of clones from an individual tumour to anticancer drugs have been demonstrated by Barranco *et al.* (1988). Further studies from this group (1994) found a large histological variability from site to site within individual tumours and recommended the abandonment of all single site testing of tumours, as is the norm at

the moment, and the implimentation of sample testing from 3-7 different sites within each tumour.

1.5 GROWTH CONTROL IN LUNG CANCER CELLS

The processes involved in normal cellular proliferation and differentiation are highly co-ordinated and are influenced by several variables including extracellular matrix components, cell adhesion molecules and membrane junctional complexes between adjacent cells. A pivotal role in these processes is played by a diverse group of soluble, secreted regulatory molecules known as the polypeptide growth factors. Observations of growth factor mRNA and protein expression in embryogenesis (Peters *et al.*, 1992), tissue injury (Adamis *et al.*, 1991) and disease (Thornton *et al.*, 1992) and regulation of proliferation and differentiation *in vitro* have all contributed to the understanding of the complexity and extent of growth factor networks.

Growth factors exert their effects by interacting with specific receptors on the surface of target cells. In general, these target cells are within a short extracellular distance from the growth factor-producing effector cells in contrast to the long distances over which endocrine hormones travel in the body. However, low concentrations of some growth factors can be found in serum (Antoniades, 1981). The binding of a growth factor to its receptor results in a cascade of intracellular events including protein phosphorylation, inositol-lipid breakdown, ion fluxes (reviewed in Rozengurt, 1986) and changes in gene expression (Antoniades, 1992). Upon binding to its receptor, the growth factor is internalised by receptor-mediated endocytosis. Transport of the receptor-ligand complex to the lysosmal compartment results in degradation of the complex and subsequent down-regulation of the receptors and thus limits the response of the target cell to the growth factor-induced stimulation.

As their name infers, growth factors cause cells to enter and proceed through the cell cycle (reviewed in Aaronson, 1991). Firstly, 'competence' factors such as EGF, CSF-1, PDGF and FGF stimulate quiescent cells to enter the G_1 phase. The length of the G_1 phase varies greatly between cells and a continuous signal must be present if the cells are to advance into the S phase of the cycle and become committed to DNA synthesis. This second phase of the cycle is brought about by 'progression' factors. The absence of a progression factor such as IGF or insulin means that upon depletion or removal of

the competence factor, the cell will return to G_0 and will not enter S phase.

Growth factors may be grouped into families based on structural similarities (Heldin and Westermark, 1989). These families of factors interact with one or more specific transmembrane receptor types. Most growth factor receptors are protein tyrosine kinases which become activated after ligand binding. The ligand binds to an extracellular domain of the receptor and induces formation of receptor dimers, which in turn leads to autophosphorylation and activation of an intracellular tyrosine kinase domain. This domain is highly conserved and is necessary for receptor signalling, while the transmembrane domain does not directly affect signal transduction but appears to anchor the receptor in the membrane.

The autophosphorylation sites in growth factor receptors are recognition sites for specific target proteins containing Src homology 2 (SH2) domains (Lowenstien *et al.*, 1992). These SH2 domains are highly conserved sequences of up to 100 amino acids found in many signalling molecules and oncogenic proteins. The interaction of SH2 domains with autophosphorylated regions of growth factor receptors is believed to provide the link whereby diverse regulatory and enzymatic proteins can interact specifically with transmembrane receptors and thus transmit an external signal to the multiple intracellular signalling pathways.

The PDGF post-membrane signal transduction system serves as the prototype tyrosine kinase network (reviewed in Cantley *et al.*, 1991). A range of substrates become phosphorylated by the PDGF receptor kinase following physical association with the activated receptor. These proteins include phospholipase C (PLC-γ), phosphatidylinositol 3' kinase (PI-3K), Ras guanosine triphosphatase (GTPase) activating protein (GAP) and Src and Src-like tyrosine kinases. PLC-γ hydrolyses phosphatidylinositol 4,5-bisphosphate. This results in the formation of 2 second messengers: diacylglycerol which activates protein kinase C which in turn is thought to mediate the actions of a number of tumour promoters, and inositol trisphosphate which causes the release of stored intracellular calcium. PI-3K phosphorylates the inositol ring of phosphatidylinositol, while GAP regulates the function of the Ras protein. Ras is a small guanine nucleotide-binding protein and is a requisite component

of intracellular mitogenic signalling pathways. PI-3K is a negative regulator of Ras function and acts by stimulating its GTPase activity. Mutations of the *ras* gene can lead to an accumulation of active Ras due to the inability of GAP to inactivate it. Raf, a serine/threonine kinase, also becomes phosphorylated following physical association with the PDGF receptor, although it lacks SH2 domains. The substrates of Raf are as yet unknown.

Observations of the decreased serum and growth factor requirements of transformed neoplastic cells compared with their normal counterparts (Temin, 1966 and Dulbecco, 1970) along with the discovery that many oncogenes are identical or similar to growth factors, their receptors or elements of the signalling transduction pathways (Doolittle *et al.*, 1983, Downward *et al.*, 1984 and Mulcahy *et al.*, 1985) has led to widespread interest in the role of grwoth factors in tumourigenicity. It has also fuelled the belief that abnormal expression of growth factors, their receptors or their signalling pathways may result in the inappropriate growth of cells, thus contributing to tumourigenesis (reviewed in Cross and Dexter, 1991 and Goustin *et al.*, 1986). An additional aspect of abnormal growth control is the phenomenon of autocrine growth whereby cells are stimulated by growth factors they have produced themselves. It has been proposed that the autocrine production of growth factors is an essential element of the tumourigenic process (Todaro *et al.*, 1976 and De Larco and Todaro, 1978). In theory, the inappropriate expression of a growth regulatory protein could confer growth factor autonomy on cancer cells.

The first indication of an interaction between cell growth and transformation came with the discovery that the normal counterpart of the oncogene v-*sis* of simian sarcoma virus is the cellular gene encoding the B-chain of platelet-derived growth factor (PDGF) (Doolittle *et al.*, 1983 and Waterfield *et al.*, 1983). It was subsequently demonstrated that transformation by the *sis* gene occurs by an autocrine mechanism via PDGF (Johnsson *et al.*, 1985 and reviewed in Westermark and Heldin, 1991). Expression of PDGF has been demonstrated in most NSCLC cells examined (Safi *et al.*, 1992, Bravo *et al.*, 1991, Bergh, 1988 and Sariban *et al.*, 1988). Soderdahl *et al.* (1988) reported the absence of PDGF proteins in SCLC cell lines but Bravo *et al.* (1991) reported the production of PDGF by SCLC cell lines and tissues. Antoniades *et al.* (1992)

demonstrated the coexpression of PDGF and the PDGF receptor in primary human lung carcinomas suggesting a possible autocrine growth mechanism and Fitzer-Attas *et al.* (1993) also localised PDGF receptors in lung carcinoma cells.

Epidermal growth factor (EGF) is found in normal human plasma and most human body fluids and the EGF receptor is present in a wide range of normal epithelial tissues (Kasselberg *et al.*, 1985 and Gusterson *et al.*, 1984). The EGF receptor is encoded by the c-*erb* B protooncogene (Downward *et al.*, 1984). The EGF receptor is also the receptor through which transforming growth factor α (TGFα) exerts its effects (Marquardt *et al.*, 1984). When normal cells are exposed to EGF, various responses result which are associated with neoplastic transformation (reviewed in Stoscheck and King, 1986) including a partial loss of density dependent inhibition of growth, increased levels of phosphotyrosines in proteins, expression of c-*fos* and c-*myc* cellular protooncogenes and potentiation of growth in soft agar.

As mentioned earlier (Section 1.3), overexpression of the EGF receptor has been associated with NSCLC. Cherny *et al.* (1986) reported that expression of the EGF receptor was exclusive to NSCLCs as all SCLCs examined were negative while over 80% of NSCLCs were positive for expression of the receptor. Veale *et al.* (1987) also demonstrated that expression of the EGF receptor was significantly upregulated in NSCLC tissue compared with normal tissue and that squamous carcinomas reacted significantly stronger with the antibody against the receptor than other types of NSCLC. There also appeared to be a correlation between high intensity of receptor expression and advanced tumour stage, suggesting that the EGF receptor may be a useful marker for prognosis and determination of the degree of differentiation of NSCLC tumours. Studies carried out by Gorgoulis *et al.* (1992) substantiated these findings with evidence that squamous cell lung carcinomas expressing the EGF receptor along with either or both of its ligands (EGF and TGFα) displayed pathologic features of more aggressive disease. Garcia de Palazzo and co-workers (1993) reported that a mutated EGF receptor which had been identified in glioblastomas (Humphrey *et al.*, 1990) may be a specific marker for squamous cell carcinomas as only malignant squamous epithelial cells expressed the mutant protein while adenocarcinomas did not.

Many lung tumour cell lines have also been shown to express EGF, TGFα and EGF receptor proteins (Imanishi *et al.*, 1989, Damstrup *et al.*, 1992 and Soderdahl *et al.*, 1988). Soderdahl *et al.* (1988) also reported the absence of these 3 proteins from SCLC cell lines while Damstrup *et al.* (1992) found that a relatively high proportion of SCLC cell lines express EGF receptors.

Transforming growth factor β (TGFβ) consists of a family of growth regulatory peptides which are considered to be prototypical growth inhibitors (reviewed in Barnard *et al.*, 1990). In the normal human lung, bronchial epithelial cells appear to be the main location of TGFβ (Mangan *et al.*, 1994) and TGFβ has been shown to growth inhibitory for most normal epithelial cells (Roberts and Sporn, 1988 and Moses *et al.*, 1990). In lung epithelial cells, growth inhibition by TGFβ correlates with terminal squamous differentiation (Masui *et al.*, 1986). In contrast, lung carcinoma cells are often insensitive to induction of differentiation or negative regulation by TGFβ (Coffey *et al.*, 1988). In lung tumour cells, the belief that TGFβ production was restricted to NSCLC cell lines (reviewed in Pelton and Moses, 1990) was disputed by Damstrup *et al.* (1993) who detected the expression of TGFβ receptors and ligands in a high proportion of SCLC cell lines.

Norgaard *et al.* (1994) demonstrated that TGFβ was capable of inhibiting the growth of SCLC cell lines. The growth inhibitory effect of TGFβ on normal lung epithelial cells and some lung tumour cell lines led to the consideration of the antitumour effects of this growth factor. Twardzik *et al.* (1989) reported antitumour effects of TGFβ on A549 (lung adenocarcinoma cell line) tumours *in vivo*. However, a contrasting report from Robinson and Rose (1992) describes a lack of *in vivo* antitumour activity of TGFβ on A549 tumours.

Another group of cellular regulatory molecules which have effects on cell proliferation are cytokines. This group includes interleukins, interferons and colony stimulating factors. These peptides and proteins were originally discovered as modulators of immune cell functions and proliferation but have since been shown to regulate non-immune cell growth (Berdel *et al.*, 1992). As is the case with growth factors, cytokines influence cell behaviour by binding to surface receptors on target cells (Pelligrini and

Schindler, 1993 and Taniguchi and Minami, 1993). The receptors are linked to a complex array of intracellular signalling pathways which modulate cell function.

In recent years, the growth inhibitory effects of several cytokines on cancer cells has led to a focusing on the antitumour effects of these molecules. Interleukin 1 (IL-1) has been shown to inhibit proliferation in cultured lung carcinoma cells (Nakane *et al.*, 1990) and primary human lung tumours *in vitro* (Hanauske *et al.*, 1992) and to have antitumour activity *in vivo* (Iigo *et al.*, 1990). Interleukin 2 (IL-2) has been examined clinically as an antitumour agent (reviewed in Foa *et al.*, 1992) and responses are promising but have been limited by toxicity.

Interleukin 4 (IL-4) receptors have been found to be coexpressed with EGF receptors on approximately 33% of NSCLCs and appear to be absent from SCLCs (Tungekar *et al.*, 1991).

Interleukin 6 (IL-6) inhibits growth of lung cancer cell lines *in vitro* (Takizawa *et al.*, 1993) and systemic administration of this cytokine was found to mediate the regession of pulmonary nodules in mice (Mule *et al.*, 1992). Transfection of the IL-6 gene into Lewis lung carcinoma cells led to the reversal of a malignant towards a benign phenotype *in vivo* (Porgador *et al.*, 1992).

Interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) have also been demonstrated to have anti-proliferative effects on lung carcinoma cell lines (Schuger *et al.*, 1990).

The proliferative potentials of the neuropeptides and hormones found in lung cancer cell lines with NE differentiation has been explored, though not extensively. Bombesin, and its human analogue gastrin releasing peptide (GRP), have been shown to regulate epithelial cell proliferation in the lung during development and after injury (Mabry *et al.*, 1988 and 1991) and also to act as autocrine growth factors for some SCLC cell lines (Cuttitta *et al.*, 1985 and Mahmoud *et al.*, 1991). NSCLC cells have been shown to secrete and respond to GRP also (Siegfried *et al.*, 1994).

Other neuropeptides have also been demonstrated to modulate cell proliferation (Zachary *et al.*, 1987). Bradykinin, cholecystokinin, galanin, neurotensin and vasopressin may also have roles as autocrine growth factors in SCLC (Bunn *et al.*, 1990 and 1992, Sethi and Rozengurt, 1991 and reviewed in Sethi *et al.*, 1992).

Increased understanding of the roles of cell signalling molecules in the development and progression of carcinomas has led to interest in the targetting of these pathways in an attempt to halt and reverse tumourigenicity. Considered modes of therapy include antibodies directed against growth factors and growth factor receptors, growth factor agonists and antagonists, negative growth factors, growth factor-linked toxins, disruption of signal transduction and gene therapy (Shaw *et al.*, 1991 and reviewed in Kelly *et al.*, 1991). Clinical trials are underway already with suramin (Van Oosterom *et al.*, 1990), an agent which inhibits the receptor binding of various growth factors (Huang and Huang, 1988) but extensive research still remains to be carried out on these potential anticancer strategies.

1.6 CELL ADHESION AND LUNG CANCER

Cells express an array of cell surface receptors which enable them to adhere to each other and to the extracellular matrix (ECM) in a highly specific manner, thus permitting the development and maintenance of tissue structure and function. Mammalian organisms are divided into a series of tissue compartments separated by the ECM which consists of the basement membrane (BM) and its underlying interstitial stroma (Yurchenco and Schittny, 1990). Epithelial basal cells are attached to the BM and on the opposite side, the interstitial stroma contains stromal cells, fibroblasts, myofibroblasts, smooth muscle cells and macrophages. The nervous system, muscle cells and blood vessels are also surrounded by a continuous basement membrane. Under normal conditions, and even during benign tissue remodelling, proliferative disorders and carcinoma *in situ*, the integrity of the BM is largely maintained and the cell populations on either side of the connective tissue do not mix. However, during the progression from *in situ* to invasive cancer, the tumour cells invade the epithelial BM, through to the interstitial stroma (reviewed in Liotta *et al*, 1991). It is usually this invasion of organ tissue and metastasis to other body tissues that causes cancers to be fatal.

1.6.1 Extracellular Matrix

As mentioned above, the ECMs penetrated by invasive cells can be classified into 2 major categories: basement membranes and interstitial connective tissue.

1.6.1.1 Basement membrane

Basement membranes consist of collagens, glycoproteins and proteoglycans and their basic composition is similar in different tissues. Type IV collagen is the major protein present, accounting for up to 60% of BM proteins (Tryggvason *et al.*, 1987). Laminin is the major noncollagenous glycoprotein present along with entactin (Timpl *et al.*,

1983) and fibronectin (Steinman and Vaheri, 1978) and chondroitin sulfate and heparin sulfate are the predominant proteoglycans of the BM (Hassel *et al.*, 1980). These molecules interact to form an acellular, amorphous, sheet-like structure that separates epithelium or endothelium from underlying mesenchymal tissue. This structure is highly resistant to cellular penetration and contains a large amount of binding proteins which allow cells to anchor and also provides traction for migration.

1.6.1.2 Interstitial connective tissues

While interstitial connective tissue also consists of collagens, glycoproteins and proteoglycans, in contrast to BMs, the distribution of these components and their particular types can vary greatly among tissues. Again, collagens are the major proteins present with types I, II and III varying in abundance depending on location (Piez, 1984). The expression of other collagen proteins (types V-X) also occurs (Miller and Gay, 1987). Fibronectins (Hynes, 1986), vitronectin (Suzuki *et al.*, 1984), osteonectin (Termine *et al.*, 1981) and chondronectin (Hewitt *et al.*, 1981) are the glycoproteins present in various connective tissues, while the proteoglycans present include chondroitin, heparin, dermatin and keratin sulfate (Poole, 1986).

1.6.2 Cell Attachment

Cell-cell and cell-matrix interactions are mediated to a large extent by cell surface receptors called integrins and families of adhesion molecules called cadherins and cell adhesion molecules.

1.6.2.1 Integrins

Integrins are membrane glycoproteins consisting of 2 subunits, α and β. Most integrins are expressed on a wide variety of cells and most cells express several integrins (reviewed in Hynes, 1992 and Ruoslahti, 1991). Many integrins bind to ECM proteins and the specificity of binding is determined, though not solely, by the identity of the α and β chains. For example, $\alpha_1\beta_1$ and $\alpha_7\beta_1$ bind to laminin (among others) while $\alpha_v\beta_5$ binds vitronectin. However, the specificity of binding also depends on a given cell as

$\alpha_2\beta_1$ on platelets will not bind laminin (Staatz *et al.*, 1989) while this integrin on other cell types can bind laminin (Elices and Hemler, 1989).

Some integrins bind to cell membrane proteins on the surfaces of other cells and thus mediate cell-cell adhesion, *eg.* $\alpha_l\beta_2$ (also known as CDIIA/CD18) binds to intercellular adhesion molecules (ICAMs) (Springer, 1990) and $\alpha_4\beta_1$ binds to both an ECM protein and a cell membrane protein, fibronectin and vascular cell adhesion molecule (VCAM-1) respectively (Mould *et al.*, 1990).

Other integrins found on platelets mediate the binding of platelets to each other via soluble molecules such as fibrinogen, thus playing a major role in blood clotting (Ginsberg *et al.*, 1988).

The recognition site for integrin-binding on many ligands is an RGD (arginine, glycine, aspartic acid) tripeptide (Ruoslahti and Piersbacher, 1987). In addition to their roles in attachment, there is accumulating evidence that integrins may be capable of transmitting external signals into cells. Tyrosine phosphorylation (Shattil and Brugge, 1991) and cytoplasmic alkilinisation (Schwartz *et al.*, 1991) have been observed following integrin binding of ligands and integrins have been shown to regulate processes such as cell proliferation and apoptosis (Agrez and Bates, 1994).

1.6.2.2 Cadherins

Cadherins are calcium-dependent transmembrane proteins and the activity of these molecules involves interactions on both sides of the cell membrane. On the intracellular side, cadherins interact with catenins, cytoplasmic proteins that link cadherin proteins to the actin-based cytoskeleton (Kemler, 1993 and Gumbiner, 1993). Extracellularly, cadherins act as cell-cell adhesion molecules.

The 3 main cadherins are E-cadherin, found on many types of epithelial cells (Takeichi, 1991), N-cadherin, found on nerve, heart and lens cells (Takeichi, 1991) and P-cadherin, found in the placenta and epidermis (Kemler *et al.*, 1989). The muscle-specific M-cadherin (Donalies *et al.*, 1991) and the desmosomal proteins desmoglein

and desmocollin (Buxton and Magee, 1992) are also members of the cadherin family. The expression pattern of these molecules during embryogenesis suggests that they play an important role in this process (Takeichi, 1991). In adult tissues, E-cadherin is detected in epithelial cell types derived from all 3 germ cell layers but not in other cell types, and in polarised epithelial cells, it is expressed on the basolateral surface (Boller *et al.*, 1985).

1.6.2.3 Cellular adhesion molecules

Cellular adhesion molecules (CAMs) are a family of cell surface proteins that mediate adhesion between cells of the same type (homotypic intercellular adhesion). In contrast to cadherins, CAMs function via a calcium-independent mechanism. CAM molecules belong to the immunoglobin superfamily and the extracellular segments of these proteins are folded into several domains which are homologous to the immunoglobin domains characteristic of antibody molecules. Neural cell adhesion molecule (NCAM), intercellular adhesion molecule (ICAM) (Montefort *et al.*, 1993) and vascular cell adhesion molecule (VCAM) are all members of this family.

1.6.3 Tumour Invasion of the ECM

Benign tumours are characterised by a continuous BM surrounding the epithelial cells and separating them from the stroma. In contrast, malignant tumours actively invade the BM, enter the stroma and from there, gain access to the lymphatic and vascular systems.

3 main steps are involved in tumour cell interaction with the BM: attachment, matrix dissolution and migration. During the first step, the tumour cell attaches to elements in the BM such as type IV collagen, laminin and fibronectin via integrin and non-integrin receptors (Hynes, 1987, Humphries *et al.*, 1986 and Aznavoorian *et al.*, 1990). The second stage of the process begins within hours of attachment when the tumour cells begin to secrete degradative enzymes or induce the host to release proteinases to degrade the matrix (Gottesman, 1990). Lysis of the matrix proceeds close to the tumour cell surface where the amount of active enzyme outbalances the natural *in situ*

proteinase inhibitors (Brown *et al.*, 1990). Translocation is the third step of invasion when the cell migrates across the BM in a pseudopodial manner (Luna *et al.*, 1989).

While these events may occur under non-malignant conditions such as angiogenesis and wound healing, cancer cells can manipulate 'normal' processes in order to invade tissue and metastasise. Loss or reduction of epithelial differentiation in carcinomas and the subsequent higher motility and invasiveness of cells is often as a result of reduced intercellular adhesion (Nigam *et al.*, 1993). A substantial reduction in epithelial differentiation has been observed, particularly at invasion fronts where the cells break into the mesenchymal tissue (Gabbert *et al.*, 1985). Often, the invading cells lose their epithelial appearance and become spindle-shaped and fibroblastoid. In fact, it has been suggested that tumour cell progression to an invasive state involves a process similar to the epithelial-mesenchymal conversion which takes place during development (Valles *et al.*, 1991). Epithelial cells and mesenchymal cells are quite distinct, both morphologically and functionally. While epithelial cells express intercellular junctions and are polar and largely nonmobile, mesenchymal cells are generally loosely associated, nonpolar and surrounded by ECM. The latter phenotype resembles that of invading, metastatic cells (Liotta *et al.*, 1991).

Alterations in the adhesion properties of invasive tumour cells, including lung cells, have been observed. Integrin expression on chemically transformed cells has been shown to be different to that of the parental untransformed cells. Dedhar and Saulnier (1990) reported increased expression of integrins which bind collagens and laminin and decreased expression of those which bind vitronectin on such cells. Increased expression of a laminin receptor has also been demonstrated on human carcinoma cells (Wewer *et al.*, 1986). In addition, the distribution of integrins on transformed cells may alter, with a loss of preferential location to the basal membrane (Horan *et al.*, 1985) and a high expression of laminin and fibronectin receptors in pseudopodial extensions which develop during cell migration (Guirguis *et al.*, 1987).

In the normal lung, adhesion molecules including ICAM and several β integrin subunits have been detected on isolated type II cells (Guzman *et al.*, 1994) and the ability of bronchial epithelial cells to migrate on ECM components has also been demonstrated

(Rickard *et al.*, 1992). Hirasawa *et al.*, (1994) found that integrins present on lung cancer cells bind the same ECM proteins as their normal counterparts and determined the arrays of integrins expressed by a panel of lung cancer cell lines. Falcioni *et al.* (1994) studied the expression of β_1, β_3, β_4 and β_5 integrins in lung cancer and reported that while NSCLC expressed all 4 integrin types examined, SCLC expressed only the β_1 integrin which appeared to function as a laminin receptor on these cells. High levels of integrin expression were found on SCLC cells by Feldman and colleagues (1991) and the receptors appeared to mediate laminin binding by the cells. Laminin receptors have also been demonstrated on SCLC cells by Tagliabue *et al.* (1991) and Pellegrini *et al.* (1994) and laminin has been shown to induce anchorage-dependent growth and slight neuronal differentiation in SCLC cell lines (Giaccone *et al.*, 1992). Bohm *et al.* (1994) found that E-cadherin expression was reduced in most moderately differentiated, and reduced or absent in most poorly differentiated lung squamous cell carcinomas. In addition, all lymph node metastases and SCLCs had reduced E-cadherin expression (reviewed in Birchmeier and Behrens, 1994). BM components have also been shown to stimulate the growth of lung tumour cells *in vivo* (Topley *et al.*, 1993).

It is apparent therefore, that the adhesion properties of normal and tumour cells mediate both the immobilisation and migration of these cells and that alterations in adhesion properties play a role in the acquisition of a metastatic phenotype by tumour cells.

1.7 DIFFERENTIATION-INDUCING AGENTS

By definition, cellular differentiation is the process by which 'a cell acquires or displays a new stable phenotype without changing its genotype' (Ham and Veomett, 1980). This process usually involves the induction of genes that characterise and are responsible for the differentiated state, and the repression of genes for cell proliferation (reviewed in Davila *et al.*, 1990 and Sell and Pierce, 1994). The principle cells which differentiate are stem cells and progenitor cells (Figure 1.7.1). Stem cells are cells which are capable of rapid cell growth and division and are mulitpotent *i.e.* have the potential to differentiate into two or more different cell phenotypes. These cells typically possess virtually unlimited proliferative potential but may remain quiescent under certain conditions. Progenitor cells, in contrast, are the progeny of stem cells and possess more limited proliferation and differentiation potentials. These cells usually only give rise to one specific differentiated cell type. Genetic programmes regulate the destiny of cells during development and restrict the number of lineages that stem cells have the potential to form and thus, 'determination' is the process by which a cell becomes committed to differentiate to a specific lineage (Ham and Veomett, 1980).

A cell may differentiate to give rise to a phenotype which either retains or irreversibly loses its proliferative potential. Such cells are 'nonterminally' or 'terminally' differentiated respectively. The term 'dedifferentiation' refers to the process whereby a cell loses its differentiated phenotype, while 'transdifferentiation' occurs when a cell dedifferentiates and then redifferentiates into a new distinct cell type.

1.7.1 Models for Differentiation

Davila and colleagues (1990) have proposed a model for the control of proliferation and differentiation in mesenchymal stem cells (Figure 1.7.2). In this system, rapidly proliferating stem cells may undergo growth arrest as a result of growth factor deficiency or when they enter a predifferentiation state. The former state is reversible and does not lead to differentiation. The latter stage however, while also being reversible, can lead to a non-terminal differentiation state if the cells are not first

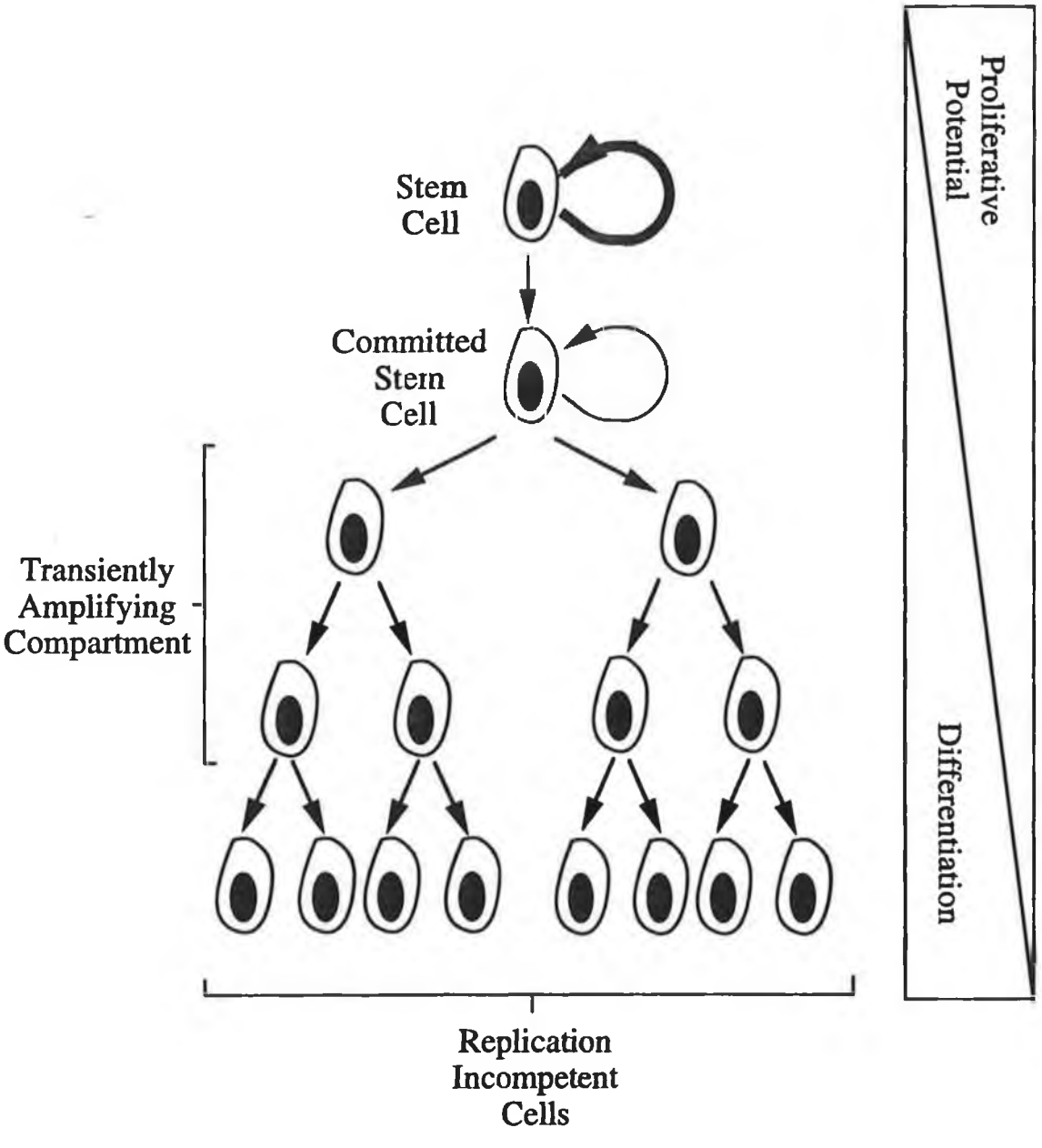


Figure 1.7.1 A theoretical model of cell lineages. Stem cells are capable of unlimited self renewal (thick curved arrow) and give rise to transiently amplifying cells that retain some self renewal ability (thin curved arrow). Transiently amplifying cells form most of the new cells in the tissue compartment (straight arrows). The most differentiated cells become incapable of further rounds of cell division.

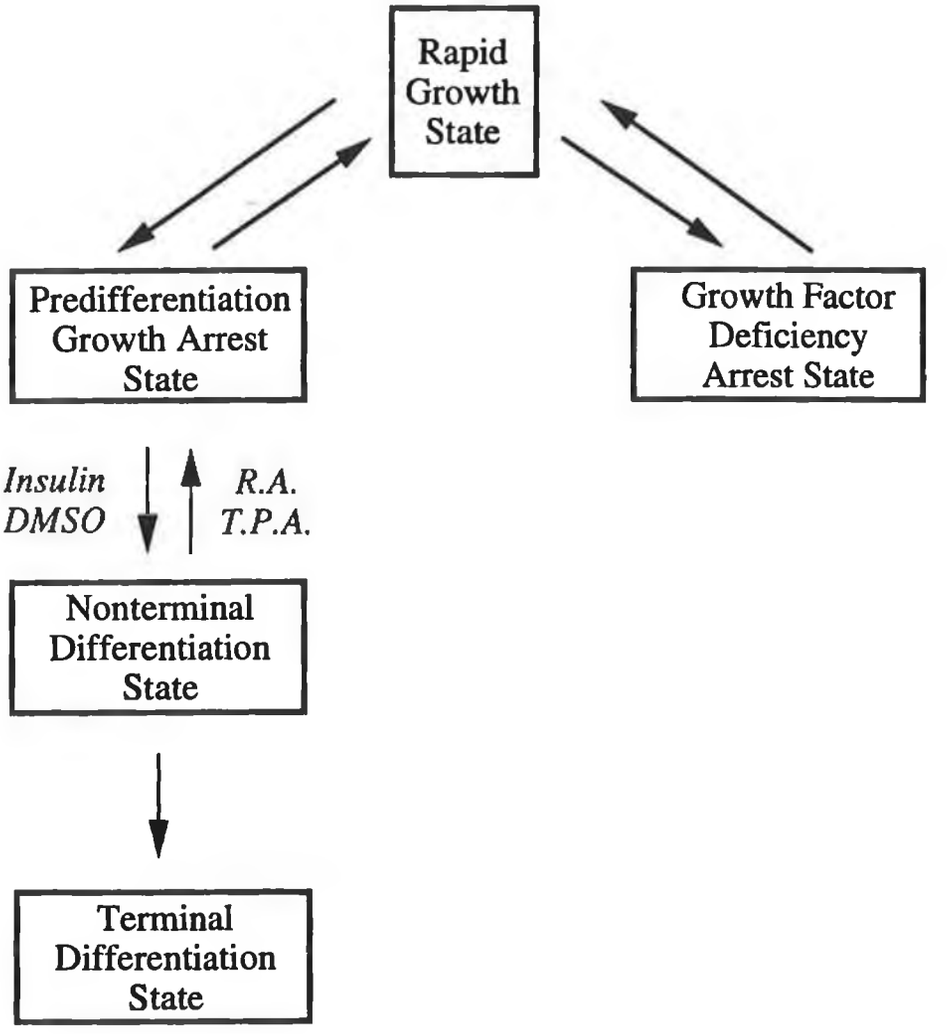


Figure 1.7.2 Model for the integrated control of proliferation and differentiation in mesenchymal stem cells showing that prior to terminal differentiation, cells must undergo predifferentiation growth arrest at a distinct biological state and then, non-terminal differentiation which is reversible.

induced to reinitiate proliferation (Scott *et al.*, 1982). The non-terminal differentiation state can be induced by factors including insulin, DMSO and serum factors while TGFβ and TPA inhibit this state (Sparks and Scott, 1986 and Yun and Scott, 1983). In addition, retinoic acid and TPA can induce dedifferentiation in these cells (Wier and Scott, 1986). Non-terminally differentiated cells acquire a completely differentiated phenotype and become less responsive to growth factors and in the final step in this model, the mesenchymal cells terminally differentiate and irreversibly lose their proliferative potential.

The fact that the proliferative potential of a cell diminishes with increasing differentiation means that two conceivable scenarios exist for the possible origins of cancer: from dedifferentiation of mature cells which retain the capacity to divide or from maturation arrest of immature stem cells. Sell and Pierce (1994) propose that the latter situation is that which gives rise to all cancers. The authors discuss a model in which normal cell renewal requires a determined stem cell to divide and the resulting daughter cells to comprise of one stem cell and one cell which differentiates, thus achieving steady state. During carcinogenesis however, a block occurs, the nature of which is as yet unknown, in the maturation process which allows neoplastic stem cells to accumulate, resulting in an increased number of cycling cells of a restricted phenotype. In this model, an individual tumour will retain the basic phenotypic potential of the tissue-determined stem cell from which it arose, but will express this potential to different and varying extents compared with those seen in normal tissue.

The pathways involved in the haematopoietic differentiation system, and their disruption leading to leukemia, have been studied in depth (reviewed in Sawyers *et al.*, 1991). Leukemia is defined as the uncontrolled proliferation or expansion of haematopoietic cells that do not retain the capacity to differentiate normally to mature blood cells. The pluripotent haematopoietic stem cell is the progenitor cell of all the cellular elements of the blood and lymph including platelets, red blood cells, neutrophils, macrophages, B lymphocytes and T lymphocytes (Figure 1.7.3). In the adult, pluripotent stem cells are located in bone marrow where they are present in low numbers and most of which are not actively cycling. Soluble factors known to induce proliferation and differentiation of committed progenitor cells include GM-CSF and IL-7 (reviewed in

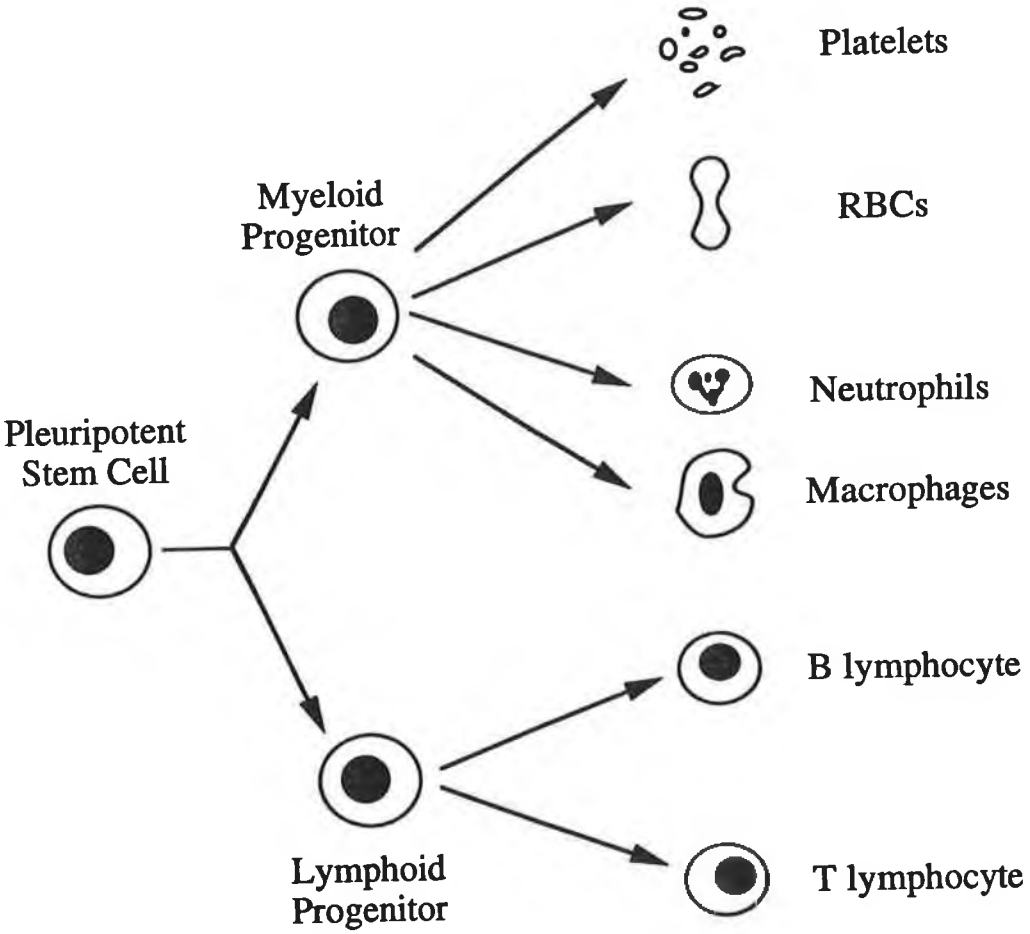


Figure 1.7.3 The cell lineages of the haematopoietic system

Metcalf, 1989). One of the genes found to have a role in differentiation in this cell lineage is that coding for the retinoic acid receptor (RAR). This gene is disrupted in many cases of acute myeloid leukemia (de The *et al.*, 1990a) and these cases can achieve complete remission of leukemia when treated with all-*trans* retinoic acid, probably because of induced differentiation of the leukemic cells (Meng-er *et al.*, 1988). Bromodeoxyuridine is another agent known to induce differentiation of myeloid cells, causing promyelocytic cells to terminally differentiate into granulocytes or macrophages-like cells (Keoffler *et al.*, 1983). Retinoic acid (RA) and bromodeoxyuridine (BrdU) as differentiation-inducing agents will now be discussed further.

1.7.2 Retinoic Acid

Members of the retinoid family of molecules have long been known to be potent agents for control of both cellular proliferation and differentiation. Wolbach and Howe (1925) first reported on the effects of vitamin A deficiency on the proliferation and differentiation of epithelial cells. They found that normal differentiation of stem cells into mature epithelial cells failed to occur and abnormal cellular differentiation was evident with excessive accumulation of keratin and increased cellular proliferation. Since these observations were made, the role and mechanism of action of retinoids in the control of cellular proliferation and differentiation has been intensively examined to the extent that over 200 different gene products have been shown to be influenced by RA (Chytil, 1992).

Cytosolic retinoic acid- and retinol-binding proteins (CRABP and CRBP respectively) have been identified (Chytil and Ong, 1983). The roles of these proteins are not clear but they may be involved in storage of retinoids or in mediating retinoid metabolism (Yost *et al.*, 1988). Two major families of nuclear RA receptors, RA receptors (RARs) and retinoid X receptors (RXRs), have been demonstrated (Petkovich *et al.*, 1987, Brand *et al.*, 1988, Krust *et al.*, 1989, Zelent *et al.*, 1989 and Yu *et al.*, 1991). These receptors display a structural organisation similar to that of the steroid hormone receptor family. Binding of RA to its receptor results in the formation of a homo- or heterodimeric complex which then binds to specific RA response elements in the

genome, leading to alterations in gene transcription. RAR genes themselves have been found to be among the targets of these transcription factors (Hoffmann *et al.*, 1990 and de The *et al.*, 1989 and 1990b). A RA response element has also been identified in the laminin B1 gene (Vasios *et al.*, 1989).

As mentioned above, retinoids play an important role in the proliferation and differentiation of epithelial cells. Normal adult lung has been shown to express RARs and RXRs (Mangelsdorf *et al.*, 1990) and normal bronchial epithelial cells show growth inhibition after RA treatment (Lechner *et al.*, 1982). The accumulation of cholesterol sulfate (Rearick and Jetten, 1986) and cholesterol sulfotransferase (Rearick *et al.*, 1987b) during confluency-induced squamous differentiation of normal rabbit tracheal epithelial cells is inhibited by retinoids. Phorbol ester-induced squamous differentiation in normal human tracheobronchial epithelial cells is also inhibited by retinoids, as determined by cholesterol sulfate and transglutaminase levels (Jetten *et al.*, 1989) and regulation of type II cell surfactant protein gene expression in human fetal lung has also been demonstrated (Metzler and Snyder, 1993). Ochiai (1992) found that undifferentiated human fetal bronchial cells could be induced by retinoids to undergo differentiation into a Clara cell type.

The ability of retinoids to inhibit squamous differentiation indicated a possible connection between squamous cell carcinoma and deregulated retinoid responsiveness. The link appears to have been made with the demonstration that most human lung cancer cell lines, both NSCLC and SCLC, exhibit resistance to RA treatment showing little growth inhibition, and also with the discovery of abnormalities in lung cancer cell RA receptor genes (Geradts *et al.*, 1993 and Zhang *et al.*, 1994).

While the proliferative response of lung carcinoma cells to RA appears altered to that of normal cells, retinoids maintain their ability to affect differentiation of cancer cells. Squamous differentiation in a SCLC cell line was induced by vitamin A deficiency (Terasaki *et al.*, 1987). Doyle *et al.* (1989) reported that variant SCLC cell lines differentiated to a classic morphology following RA exposure and Feyles and co-workers (1991) observed upregulation of neurondocrine markers on SCLC cells cultured in RA.

Expression of keratin intermediate filament proteins in epithelial cells is also regulated by retinoids (Kim *et al.*, 1984). Exposure of epidermal cells to retinoids results in the down-regulation of the terminal differentiation-associated keratins K1 and K10 (Schweizer *et al.*, 1987) and epidermal keratins K5, K6, K14 and K16 (Stellmach *et al.*, 1991).

1.7.3 Bromodeoxyuridine

The literature on BrdU-induced cellular differentiation is much less extensive than that of RA. BrdU is a thymidine (Td) analogue which competes with Td for incorporation into DNA and which is capable of modulating differentiation in a number of tissue types. Low concentrations of BrdU can reversibly block or inhibit differentiation in cells without significant alteration in cell proliferation (Rutter *et al.*, 1973, Keoffler *et al.*, 1983 and Tapscott *et al.*, 1989).

It appears that BrdU must be incorporated into DNA to have its effect, possibly implicating the involvement of inhibition of expression of differentiation-specific genes (Harding *et al.*, 1978), but relatively little is known concerning the mechanism of action of this compound. Kidson and De Haan (1990) provided evidence supporting the inhibition of differentiation-specific proteins by BrdU by comparing the effects of the Td analogues 5-iododeoxyuridine and 5-flurodeoxyuridine (IdU and FdU) on melanoma cell differentiation and proliferation with those of BrdU. IdU and FdU had no apparent effect on differentiation while BrdU significantly decreased the expression of a differentiation-specific protein in the melanoma cells. In a model of myogenic differentiation in a mouse myoblast line, BrdU blocked myogenic differentiation by specifically down-regulating the expression of the myogenic determination gene MyoD1 (Tapscott *et al.*, 1989). While BrdU was incorporated into the muscle structural genes, these genes were apparently transcribed normally. BrdU was thus blocking myogenesis at the specific level of a myogenic regulatory gene.

The mechanism behind the selective inhibition of such genes by BrdU remains to be explained. Lin and Riggs (1972) demonstrated enhanced binding of *lac* suppressor by the *lac* operon in BrdU-substituted DNA compared with unsubstituted DNA, suggesting

that BrdU may exert its effect by altering the binding of regulatory proteins. Hill *et al.* (1974) proposed that BrdU causes an altered reading of the DNA template resulting in abnormal mRNA that is unable to code accurately for differentiation-associated proteins. The ability of BrdU to block cellular differentiation is related to the degree of DNA substitution and the effect is usually reversible following removal of BrdU and replacement with Td (Ashman and Davidson, 1980). This indicates that BrdU is not acting as a mutagen but is specifically blocking differentiation pathways.

BrdU has been shown to induce schwannian differentiation in neuroblastoma (Sugimoto *et al.*, 1988 and Esumi *et al.*, 1989) and terminal differentiation in leukemic (Yen and Forbes, 1990) cell systems. In the latter system, BrdU has been shown to induce the early events of the metabolic cascade leading to onset of terminal differentiation along either myeloid or monocytic pathways. Decreased c-*myc* expression was demonstrated in these BrdU-treated cells and this oncoprotein is also down-regulated during terminal differentiation induced by agents such as RA and DMSO.

BrdU suppresses differentiation in a differentiated hamster melanoma cell line and promotes invasiveness by increasing the levels and subtypes of integrins expressed and elevating the levels of metolloproteinases present in the cells (Thomas *et al.*, 1993). Malignant melanoma cells treated with BrdU differentiate into cells which can no longer proliferate anchorage-independently in soft agar (Valyi-Nagy *et al.*, 1993). Feyles *et al.* (1991) examined the effects of BrdU on a SCLC cell line and discovered that substrate-adherent growth was induced, along with an epitheloid morphology and increased expression of neuroendocrine markers.

It thus seems likely that induction or inhibition of differentiation by BrdU depends on the cell type involved and the target genes.

1.8 MULTIPLE DRUG RESISTANCE

Clinical treatment regimes for cancer currently comprise principally of surgical resection, chemotherapy or radiation treatment, or a combination of these. Surgery is the usual course of action for NSCLC because such tumours are often amenable to resection and also because of the lack of effective drugs available against this cancer type. SCLCs in contrast, have a reasonable response rate to chemotherapy, and in particular to combination chemotherapy where several drug types are administered. Chemotherapy combined with radiation therapy proves to be even more beneficial in some cases to the extent that it is recommended that surgery should not be performed routinely on most patients with SCLC (Cook *et al.*, 1993). VP-16, adriamycin and vincristine have among the most effective single-agent responses of the anticancer drugs used (Cook *et al.*, 1993) and studies involving these drugs found that 2-drug and 3-drug combination chemotherapeutic regimes were increasingly superior to those of single agents (Bunn *et al.*, 1977 and 1992). However, the phenomenon of multiple drug resistance (MDR) is often a major problem in the treatment of cancers and one of the main reasons for the high fatalities associated with the disease.

While nearly 90% of SCLC tumours initially respond to chemotherapy, the majority of responders will relapse with drug-resistant disease. The innate drug resistance of NSCLC tumours is thus mirrored by the acquired drug resistance of SCLCs, but it is not clear if the mechanisms involved in each are similar. Following the acquisition of MDR, cells may be resistant to a wide range of drugs that are structurally and mechanistically unrelated to each other. VP16 is an etopiside which inhibits topoisomerase II activity leading to increased DNA damage, adriamycin is an intercalating agent which also inhibits topoisomerase II activity and blocks DNA synthesis and RNA transcription and vincristine is a tubulin inhibitor which disrupts spindle formation during cell division.

Despite the diversity in the modes on action of these drugs, MDR cells may be resistant to all three. Mechanisms of drug resistance include reduced levels or activity of topoisomerase II leading to reduced DNA damage (Binaschi *et al.*, 1992), increased

production of target reductase enzymes (Curt *et al.*, 1983) and increased expression of P-glycoprotein, a membrane efflux pump which removes various hydrophobic compounds from the cytosol, thus preventing their intracellular accummulation (Gottesmann, 1993).

1.8.1 Phenotypic Alterations in MDR Cells

In vitro studies have been important in contributing to the understanding of MDR mechanisms and in the development of circumvention strategies. Many MDR variant cell lines have been established by culturing cells in increasing concentrations of drug, with the resulting cells exhibiting MDR features such as the overexpression of P-glycoprotein (Jensen *et al.*, 1993) and the reduction in activity of topoisomerase II (Long *et al.*, 1991). Other phenotypic alterations have been observed in several MDR variant cell lines including overexpression of a putative membrane transporter, MRP, and alterations in enzymes of glutathione metabolism (Clynes *et al.*, 1993). Biedler *et al.* (1975 and 1981) noted alterations in cell morphology, *in vitro* growth behaviour and reduced *in vivo* oncogenic potential during selection of drug-resistant sublines with actinomycin D, vincristine and adriamycin. An increase in the number of EGF receptors of up to 10-fold has been observed in some MDR lines (Meyers *et al.*, 1986) possibly suggesting an alteration in growth control in these cells compared with their sensitive counterparts. These *in vitro* results appear to correlate with *in vivo* observations of morphological changes following chemotherapy where SCLCs were found to have altered pathologically towards NSCLC phenotypes (Abeloff and Eggleston, 1981).

Multidrug resistant neuroblastoma cells show altered intermediate filament protein expression compared with sensitive control cells and the alterations vary with different selection agents (Biedler *et al.*, 1991). The changes in intermediate filament expression appear to correlate with differentiation of the neuroblastoma cells along 2 distinct pathways. Retinoic acid was found to promote further differentiation along the same pathways. High levels of P-glycoprotein are expressed in MDR neuroblastoma cells (Biedler *et al.*, 1991) and also in the more differentiated cells of normal human tissues such as kidney or colon and in the more differentiated human tumours (Cordon-Cardo *et al.*, 1990 and van Kalken *et al.*, 1991). There therefore may be a relationship

between P-glycoprotein expression and cellular differentiation. Treatments with differentiation-inducing agents such as RA have also been shown to result in increased expression of P-glycoprotein (Mickley *et al.*, 1989 and Bates *et al.*, 1989). Biedler (1994) suggests that while the development of MDR has obvious negative effects, positive effects may also occur from the induction of tumour cell differentiation and a subsequent reduction of tumourigenic potential.

1.8.2 Ribozyme Approach to Reversal of MDR

One of the more novel approaches to the reversal of MDR utilises *MDR*1-specific ribozymes. Ribozymes are RNA molecules which have enzymatic, catalytic activity. Naturally occurring ribozymes are capable of self-catalysed cleavage (Cech, 1987) and the cleavage reactions are believed to result from RNA conformations bringing reactive groups into close proximity (reviewed in Symons, 1992). These catalytic RNAs have been found in several systems from bacteria to plants to humans (Cech, 1988 and Cech and Bass, 1986) and do not appear to require an exogenous energy source. The first ribozymes discovered were self-catalysing (Kruger *et al.*, 1982). These molecules are therefore limited to a single turnover and are modified during the reaction. Other ribozymes exist however, which act in *trans* with a potential for unlimited turnover.

Models for the design of ribozymes have been proposed (Haseloff and Gerlach, 1988) for use in the *in vitro* manipulation of RNAs. Ribozymes can be designed to target specific RNA molecules thus reducing the expression of a particular protein. Ribozymes which target *MDR*1 gene transcripts which encode P-glycoprotein have been created and have been successful in increasing drug sensitivity in MDR cells (Kobayashi *et al.*, 1994 and Kiehntopf *et al.*, 1994), but to date, no reported reversal of drug resistance in a lung cell line has been accomplished by ribozyme transfection.

1.9 AIMS OF THESIS

DLKP is a human lung cancer cell line which was established by Dr.Geraldine Grant during the course of routine primary culture of human lung tumours. The DLKP cell line was derived from a lymph node metastasis of a primary lung tumour from a 52-year old patient who smoked approximately 40 cigarettes for most of his adult life (Law *et al.*, 1992). The tumour was histologically diagnosed at the time as a 'poorly differentiated squamous cell carcinoma' and in a subsequent review of the original histology, the tumour was described as a 'poorly differentiated and necrotic carcinoma without obvious keratinization, but of larger size and with more cytoplasm than typical "oat" small cell carcinoma', and poorly differentiated NSCLC was deemed the most appropriate diagnosis.

The karyotype of DLKP has been determined (Law *et al.*, 1992) and cytological analyses have been carried out (Gilvarry *et al.*, 1990). In addition, multiple drug resistant variants of DLKP have been established (Clynes *et al.*, 1992) as models for MDR in NSCLC cells.

1.9.1 Isolation and Characterisation of DLKP Clones

Law *et al.* (1992) found that at passage 5, the DLKP cell line contained 2 chromosomal subpopulations with 65% of the cells being hyperdiploid (56 chromosomes) and 35% being hypertetraploid (95-115) chromosomes. It was also observed during routine culture of these cells that at least 3 morphologically distinct subpopulations were present. These heterogenous populations were evident in DLKP cultures which had been passaged up to 100 times. As most lung tumours contain heterogenous populations, it was thus possible that the DLKP cell line could serve as a model to closely examine such heterogeneity. It was decided to attempt to isolate the different morphological cell types of DLKP in a cloning procedure in an effort to study the individual growth characteristics of tumour cell subpopulations in isolation and also the growth effects of such populations on each other.

As outlined in Section 1.3, properties such as metastatic potential and response to chemotherapy are also often heterogenous within lung tumours and a model containing subpopulations with these characteristics would be useful in investigations into these properties of cancer cells which cause the disease to be fatal.

If the DLKP cells were to serve as models for *in vivo* phenomena such as tumour heterogeneity, tumour growth and progression and drug resistance *etc.*, it would be important to characterise the cells as fully as possible in order to allow extrapolations and comparisons to be made with *in vivo* situations. An extensive characterisation study was therefore carried out on DLKP with chromosomal, growth, physiological and biological features of the cells examined to establish their relationship to normal and other tumour lung cells *in vivo* and *in vitro*.

1.9.2 Search for Specific Markers of DLKP Clones

Studies on the isolated DLKP clonal subpopulations would be aided by the identification of markers specific to one or more of the clones. Distinct individual clonal morphologies can vary slighlty under different conditions, are not always objectively discernible and do not facilitate quantitative analyses. Specific markers however, would be measurable and would allow precise identification of the individual clonal populations. The DLKP clones were therefore included in the characterisation studies carried out on DLKP and in addition, monoclonal antibodies were raised to DLKP in an attempt to identify specific markers for the clones.

1.9.3 Examination of Interconversion and Differentiation of Clones

The inter-relationship between the isolated clonal subpopulations of DLKP became interesting when it was observed that, in terms of morphological appearance, the clones appeared to be interconverting. The cell(s) of origin of lung cancer is as yet unknown, as is the true stem cell(s) of the lung epithelium. It was possible that if the distinct DLKP morphologies represented distinct phenotypes, the interconverting clones could be used as a model to examine the progression of lung cancer cell types, including those with stem cell-like properties, and their adaptation to their environment. In addition,

manipulation of the interconversions by differentiation-inducing agents could give insight into the cellular origins of lung cancer and normal differentiation *in vivo* by elucidating pathways of differentiation open to the individual and/or collective DLKP clones.

Comparison of the growth and differentiation characteristics of DLKP cells with their MDR variants could also contribute to the understanding of lung cell differentiation as MDR cells are often more differentiated than their sensitive counterparts (Section 1.6), as well as revealing some of the effects of the acquisition of the MDR phenotype on aspects of lung cell biology.

2.0 MATERIALS AND METHODS

2.1 WATER

Ultrapure water was used in the preparation of all media and solutions. This water was purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12 - 18 megaohms/cm resistance.

2.2 GLASSWARE

Most solutions pertaining to cell culture and maintenance were prepared and stored in sterile glass bottles. These bottles (and lids) and all other glassware used for any cell-related work were prepared as follows:- all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least 1hr. after which they were scrubbed and rinsed several times in tap water. They were then washed by machine using Neodisher detergent which is an organic, phosphate-based acid detergent, rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving.

2.3 STERILISATION

Water, glassware and all thermostable solutions were sterilised by autoclaving at 120^{0}C for 20 min. under pressure of 1bar. Thermolabile solutions were filtered through a 0.22μm sterile filter (Millipore, millex-gv). Low protein-binding filters were used for all protein-containing solutions.

2.4 MEDIA PREPARATION

The basal media used during routine cell culture were prepared according to the formulations shown in Table 2.4.1. 10X media were added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7.45 - 7.55 using sterile 1.5M NaOH and 1.5M HCl. The media were then filtered through sterile 0.22μm bell filters (Gelman; G.1423S) and stored in 500ml sterile bottles at 4^{0}C. Sterility checks were carried out on each 500ml bottle of medium as described in Section 2.5.6.

The basal media were stored at 4^{0}C up to their expiry dates as specified on each individual 10X medium container. Prior to use, 100ml aliquots of basal media were supplemented with 2mM L-glutamine (Gibco; 043-05030) and 5% foetal calf serum (Sigma; F7524, Batch #13H 3389) and this was used as routine culture medium. This was stored for up to 2 weeks at 4^{0}C, after which time, fresh culture medium was prepared.

Table 2.4.1 Preparation of basal media

	DMEM (Gibco 042-02501M)	**Hams F12** (Gibco 042-01430M)	**MEM** (Gibco 074-01700N)
10X Medium	500ml	500ml	Powder
Ultrapure H_20	4300ml	4700ml	4300ml
1M HEPES* Sigma H9136	100ml	100ml	100ml
7.5% $NaHCO_3$ BDH 30151	45ml	45ml	45ml

* HEPES = N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

2.5 CELL LINES

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) and any work which involved toxic compounds was carried out in a cytoguard (Gelman). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the cabinet. Each cell line was assigned specific media and waste bottles and only one cell line was worked with at a time in the cabinet which was allowed to clear for 15min between different cell lines. The cabinet itself was cleaned thoroughly each week with industrial detergents (Virkon; Antec. International, TEGO; TH.Goldschmidt Ltd.), as were the incubators.

The cell lines used during the course of this study, their sources and their basal media requirements are listed in Table 2.5.1. Basal medium was supplemented with 5-10% serum and 2mM L-glutamine. Lines were maintained in 25cm^2 flasks (Costar; 3050) or 75cm^2 flasks (Costar; 3075) at 37^{0}C and fed every two to three days.

2.5.1 Subculture of Adherent Lines

During routine subculturing or harvesting of adherent lines, cells were removed from their flasks by enzymatic detachment. Waste medium was removed from the cells which were then rinsed with a pre-warmed (37^{0}C) trypsin/EDTA (TV) solution (0.25% trypsin (Gibco; 043-05090), 0.01% EDTA (Sigma; EDS) solution in PBS A (Oxoid; BR14a)). The purpose of this was to sequester the naturally occurring trypsin inhibitor which would be present in any residual serum. Fresh TV was then placed on the cells (2ml/25cm^2 flask or 4ml/75cm^2 flask) and the flasks were incubated at 37^{0}C until the cells were seen to have detached (5 - 10 min). The trypsin was deactivated by addition of a equal volume of growth medium (*i.e.* containing 5% serum) and the entire solution was transferred to a 30ml sterile universal tube (Sterilin; 128a) and centrifuged at 1,000 r.p.m. for 5 min. The resulting pellet of cells was resuspended in pre-warmed (37^{0}C) fresh growth medium, counted (Section 2.5.3) and used to re-seed a flask at the required cell density or to set up an assay.

Table 2.5.1 Cell lines used during the course of this study

Cell line	Basal medium	Cell type	Source
DLKP (and all its derivatives)	ATCC[1]	Human lung poorly differentiated squamous carcinoma	Dr.Grant NCTCC
SK-MES-1	MEM[2]	Human lung squamous carcinoma	ATCC[3]
SK-LU-1	MEM	Human lung adenocarcinoma	ATCC
A549	ATCC	Human lung adenocarcinoma	ATCC
H82*	RPMI 1640	Human lung SCLC-V	ATCC
H69*	RPMI 1640	Human lung SCLC-C	ATCC
HTB 120*	RPMI 1640	Human SCLC-C	ATCC
SK-N-SH	MEM	Human neuroblastoma	ATCC
HL60*	RPMI 1640	Human leukemic line	ATCC
RPMI 2650	MEM	Human nasal squamous line	ATCC
HEP-2	ATCC	Human laryngeal epithelial line	ATCC
Sp2*	RPMI 1640	Mouse hybridoma line	ATCC
T84	ATCC	Human colonic epithelial line	ATCC
Fibroblasts	ATCC	Normal human lung fibroblasts	Dr.Woodman NCTCC
703D4/704A1*	DMEM	Mouse hybridomas	ATCC
EP16*	RPMI 1640	Mouse hybridoma	ATCC

* These cells grow in suspension, all others are adherent.

[1]ATCC consists of a 1:1 mixture of DMEM and Hams F12.

[2]MEM medium was supplemented with 1% (v/v) MEM Non-essential amino acids (NEAA) (Gibco; 043-01140) and 1mM sodium pyruvate for all lines

[3]ATCC = American Type Culture Collection

2.5.2 Subculture of Suspension Lines

While cell lines that grew in suspension did not require enzymatic detachment, it was occassionally necessary to detach some cells which had adhered loosely to the flask surface by giving the flask a sharp tap with the hand. The cell suspension was removed from the flask to a sterile tube, centrifuged as in Section 2.5.1 and the cells were treated identically to adherent cells from this stage on.

2.5.3 Cell Counting

Cell counting and viability determinations were carried out using a trypan blue (Gibco; 525) dye exclusion technique. An aliquot of trypan blue was added to a sample from a single cell suspension in a ratio of 1:5. After 3 min. incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor being taken into account and final cell numbers were multiplied by 10^4 to determine the number of cells per ml (volume occupied by sample in chamber is 0.1cm x 0.1cm x 0.01cm *i.e.* 0.0001cm^3 therefore cell number x 10^4 is equivalent to cells per ml). Non-viable cells were those which stained blue while viable cells excluded the trypan blue dye and remained unstained.

2.5.4 Cell Freezing

To allow long term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures below -180^{0}C. Once frozen properly, such stocks should last indefinitely.

Cells to be frozen were harvested in the log phase of growth (*i.e.* actively growing and approximately 50 - 70% confluent) and counted as described in Sections 2.5.1 - 2.5.3. The pelleted cells were re-suspended in serum and an equal volume of a DMSO/serum (1:9, v/v) solution was slowly added dropwise to the cell suspension to give a final

concentration of at least $5x10^6$ cells per ml. 1.5ml aliquots of this suspension was placed in cryovials (Greiner; 122 278) which were then quickly placed in the vapour phase of liquid nitrogen containers (approximately -80°C) for 2.5 - 3.5 hr. After this time, the cryovials were transfered down into the liquid nitrogen where there were stored until required.

2.5.5 Cell Thawing

Immediately prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells to reduce their exposure time to the DMSO freezing solution which is toxic at room temperature. The suspension was then centrifuged at 1,000 r.p.m. for 5 min., after which , the DMSO-containing supernatent was removed and the pellet was re-suspended in fresh growth medium. A viability count was then carried out (Section 2.5.3) to determine the efficacy of the freezing/ thawing procedures and the thawed cells were placed into tissue culture flasks with the appropriate volume of medium (10ml/25cm^2 flask and 15ml/75cm^2 flask) and allowed to attach over night. After 24 hr, the cells were re-fed with fresh medium to remove any residual traces of DMSO.

2.5.6 Sterility Checks

Sterility checks were routinely carried out on all media, supplements and trypsin used for cell culture. Samples of basal media were inoculated into Columbia (Oxoid; CM331) blood agar plates, Sabauraud (Oxoid; CM217) dextrose and Thioglycollate (Oxoid; CM173) broths which should between them detect most contaminants including bacteria, fungus and yeast. Growth media (*i.e.* supplemented with serum and L-glutamine) were sterility checked at least 2 days prior to use by incubating samples at 37°C which were subsequently examined for turbidity and other indications of contamination.

2.6 *MYCOPLASMA* ANALYSIS

Mycoplasma examinations were carried out routinely (at least every 3 months) on all cell lines used in this study. These analyses were performed by Una Gilvarry, Cathy Halligan and William Nugent.

2.6.1 Indirect Staining Procedure

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells. As such, these cells were incubated with supernatent from test cell lines and then examined for *Mycoplasma* contamination. NRK cells were used for this procedure because cell integrity is well maintained during fixation. A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cell in addition to any *Mycoplasma* DNA present. A *Mycoplasma* infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and sometimes outside the cells.

NRK cells were seeded onto sterile coverslips in sterile Petri dishes at a cell density of $2x10^3$ cells per ml and allowed to attach over night at 37^0C in a 5% CO_2, humidified incubator. 1ml of cell-free (cleared by centrifugation at 1,000 r.p.m. for 5 min) supernatent from each test cell line was then inoculated onto a NRK Petri dish and incubated as before until the cells reached 20 - 50% confluency (4 - 5 days). After this time, the waste medium was removed from the Petri dishes, the coverslips were washed twice with sterile PBS A, once with a cold PBS/Carnoys (50/50) solution and fixed with 2ml of Carnoys solution (acetic acid:methanol-1:3) for 10 min. The fixative was then removed and after air drying, the coverslips were washed twice in deionised water and stained with 2ml of Hoechst 33258 stain (BDH) (50ng/ml) for 10 min. From this point on, work proceeded in the dark to limit quenching of the fluorescent stain. Following three rinses in PBS, the coverslips were mounted in 50% (v/v) glycerol in 0.05M citric acid and 0.1M disodium phosphate and examined using a fluorescent microscope with a UV filter.

2.6.2 Direct Staining

The direct stain for *Mycoplasma* involved a culture method where test samples were inoculated onto an enriched *Mycoplasma* culture broth (Oxoid; CM403) - supplemented with 16% serum. 0.002% DNA (BDH; 42026), 2μg/ml fungizone (Gibco; 042 05920), $2x10^3$ units penicillin (Sigma; Pen-3) and 10ml of a 25% (w/v) yeast extract solution - to optimise growth of any contaminants and incubated at 37^0C for 48 hr. Sample of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid; CM401) which had also been supplemented as above and the plates were incubated for 3 weeks at 37^0C in a CO_2 environment. The plates were viewed microscopically at least every 7 days and the appearance of small, 'fried egg'-shaped colonies would be indicative of a mycoplasma infection.

2.7 ESTABLISHMENT OF DLKP CLONES

The three DLKP clones were established from a limiting dilution assay, one from a single procedure and two from a re-cloning procedure.

2.7.1 Limiting Dilution Assay

A single cell suspension of DLKP cells (passage 3) was plated onto 3 x 96-well tissue culture plates (Falcon; 3072) at a cell density of 5 cells per ml. The plates were incubated at 37^0C in the presence of 5% CO_2 in a humidified atmosphere. After 5 days, each well was examined microscopically and those wells identified as containing a single cell or single colonies made up of two or three cells were marked. The remaining wells contained no cells, had more than one colony or contained cells lying close to each other but it was not clear if they were clones or not. All such wells were disregarded.

2.7.2 Culture of Clonal Populations

The marked wells were fed every 6 - 7 days until they became confluent. The cells were then removed from their wells by trypsinisation. This involved the aspiration of waste medium from each well and the addition of 50μl of TV (Section 2.5.1). The plates were then incubated at 37^{0}C for approximately 5 min. After this time, the cells had not completely detached from the well surfaces but agitation and resuspension with a micropipette was successful in completing detachment. Each clonal population was then directly transferred into an individual well in a 24-well plate (Greiner; 662160) without centrifugation. Each well contained 1ml of growth medium which was sufficient to inactivate the trypsin and centrifugation and subsequent resuspension may have led to the loss of some cells.

These wells rapidly became confluent (after 6 - 7 days) and the clones were then transferred into 6-well plates (Costar; 3516) in a similar manner to that of the previous transfer. When these wells became confluent, the cells were transferred to 25cm^2 flasks and then to 75cm^2 flasks. The cells were harvested as normal (Section 2.5.1) for these manipulations.

2.7.3 Recloning Procedure

The recloning procedure was identical to that of the above procedure, except that a lower initial cell density of three cells per ml was used to seed the 96-well plates to further reduce the possibilities of obtaining more than one cell per well.

2.8 CHROMOSOMAL ANALYSIS

Colcemid is a compound which disrupts spindle formation and so prevents cells from advancing through the cell cycle and halts them at metaphase. This agent is used in cytogenetic studies to allow examination of metaphase chromosomes when they are less densly aggregated than at other times of the cell cycle. For these studies, colcemid (Gibco; 890-30141D) was reconstituted to a working stock of 0.2μg/ml in sterile PBS A and stored at 4^{0}C.

Each line analysed was harvested and inoculated into 2x75cm^2 flasks at a density of 4x10^4 cells per ml. This inoculum level was such that each cell line would have entered the log phase of growth 24 hr later, thus optimising the number of metaphase cells which could then be collected.

24 hr after seeding, colcemid was added to each flask to give a final concentration of 0.02μg/ml and the flasks were incubated at 37^{0}C for 2 hr. Metaphase cells, which were rounded and loosely attached, were then harvested by shaking each flask vigorously and decanting the supernatent. Each flask was rinsed thoroughly with sterile PBS A, the washings were pooled with their respective supernatents and all were centrifuged in clean, glass centrifuge tubes at 1,000 r.p.m. for 5 min. The pellets were resuspended in 5 - 10 ml of pre-warmed 0.075M KCl, a hypertonic solution which caused the cells to swell and become fragile, and incubated at 37^{0}C for 20 min. The samples were centrifuged again as before and the resulting pellets were resuspended in Carnoys fixative (Section 2.6.1) which was added slowly, dropwise and the cells were incubated at 4^{0}C for 1 hr. After this time, the cells were centrifuged as before and resuspended in fresh fixative. This was repeated three times. After the final centrifugation, the cells were resuspended in 1ml of Carnoys fixative.

Using a pasteur pipette, drops of cell suspension were dropped onto clean glass slides and when they had dried (seconds), the slides were stained for 5 min with a 5% Giemsa (BDH; 35014) solution in pH 6.8 Gurr buffer (BDH; 33199), rinsed in Gurr buffer and examined microscopically. The number of chromosomes present per spread was determined by counting by eye.

2.9 GROWTH ASSAYS

A range of growth assays was carried out using a variety of conditions and end points.

2.9.1 Monolayer Assay

Cells which were in the log phase of growth were fed 24 hr prior to harvesting for the assay. Cells were harvested and resuspended vigorously in growth medium (*i.e.* serum

supplemented) after centrifugation to ensure that a single cell suspension was obtained. The cell suspension was diluted in growth medium to a concentration of $1x10^4$ cells per ml and this was then plated onto 96-well plates at 100μl per well *i.e.* $1x10^3$ cells per well using a multi-channel micropipette (supplied by Brownes). These cells were allowed to attach over night at 37^0C, in a CO_2, humidified incubator. Plates were wrapped in 'Parafilm', as were all unsealed tissue culture plates and dishes during incubation periods in the course of these studies, to prevent evapouration.

When the cells had attached, medium was removed from the wells by inverting and tapping the plates over an open vessel which had been swabbed with IMS. The wells were then rinsed three times with sterile PBS A, ensuring to remove as much PBS as possible after the final rinse.

For the serum dose response assays, medium containing the appropriate concentration of serum was plated as required. Plates were then incubated at 37^0C in a 5% CO_2 incubator for 6 days. After this time, the medium was removed from the plates and they were rinsed with PBS A as before. Growth was determined by crystal violet dye elution assay (Section 2.9.2).

2.9.2 Crystal Violet Dye Elution Assay

Following removal of waste medium and rinsing three times with PBS A, a 0.2% solution of crystal violet (BDH; 34024) in PBS A which had been filtered through a Whatman No. 1 filter (Whatman; FB105-32) immediately prior to use, was dispensed onto the assay plates at 100μl per well. Plates were incubated at room temperature for 10 min, the dye was removed and the plates were rinsed thoroughly in tap water and then inverted and allowed to dry on tissue paper.

When completely dry, 100μl of 33% glacial acetic acid was placed in each well to elute the dye and the plates were tapped gently to ensure that the dye had gone evenly into solution. The absorbance of each well was determined on an ELISA plate reader at a wavelength of 570nm which was specific for maximum absorbance of the crystal violet dye and at 620nm which was a reference wavelength.

2.9.3 Serum-Free Subculture Assay

The serum free medium (SFM) used in these assays was adapted for use with DLKP cells by Paula Meleady (NCTTC) from a SFM developed for CHOK1 cells (Chinese hamster ovary line) by Mendiaz *et al.* (1986) which had been further refined by Dr.Joanne Keenan (NCTTC). The medium consisted of basal Hams F12 medium which was supplemented with a number of trace elements, insulin and transferrin (see Table 2.9.1).

Routinely growing cells were harvested by trypsinisation but because they were to be cultured in SFM, serum-supplemented growth medium, which contains a naturally occurring trypsin inhibitor, could not be used to inactivate the trypsin. Instead, purified trypsin inhibitor (Sigma; T6522) was used. A volume of trypsin inhibitor at 1.388mg/ml will inactivate an equal volume of the 0.25% trypsin solution used here. When the cells had been centrifuged, they were resuspended in SFM and inoculated into $25cm^2$ vented flasks (Costar; 3056) at a density of $2.5x10^5$ cells per flask and incubated at 37^0C in a CO_2 incubator. Vented flasks and a CO_2 incubator were used as opposed to sealed flasks and a normal incubator as this was believed to aid in buffering the cells, one of the functions normally carried out by serum.

Subculturing of these cells was similar to that of serum-supplememted, adherent cells (2.5.1), except that trypsin inhibitor was used to inactivate the trypsin, a slightly higher speed of 1,500 r.p.m. was employed to pellet the cells and cells were always re-seeded into fresh flasks, which was not necessarily the case in serum-supplemented subculturing.

The extent of proliferation of cells under SFM conditions was determined by haemocytometer cell counts (Section 2.5.3).

Table 2.9.1 Supplememts in serum free medium

Supplement	Final concentration (μM)
$FeSO_4$	5.0
H_2SeO_3	$3.0x10^{-2}$
$CuSO_4.5H_2O$	$1.0x10^{-3}$
$MnSO_4.5H_2O$	$1.0x10^{-3}$
Na_2SiO_3	$5.0x10^{-2}$
NH_4VO_3	$5.0x10^{-3}$
$(NH_4)_6Mo_7O_{24}.4H_20$	$1.0x10^{-3}$
$NiCl_2.6H_2O$	$5.0x10^{-4}$
$ZnSO_4.7H_2O$	$5.0x10^{-2}$
$SnCl_2.H_2O$	$5.0x10^{4}$
$CaCl_2$	$6.0x10^{-2}$
Linoleic acid	$3.0x10^{-2}$
NEAA	$1.0x10^{-2}$
Transferrin	5.0μg/ml
Insulin	10.0μg/ml
L-glutamine	2mM

2.9.4 Soft Agar Assay

The agar for these assays was prepared as follows:-
1.548g of agar (Bacto Difco) was dissolved in 100ml of ultrapure water and autoclaved. This agar was then melted in a microwave oven immediately prior to use and incubated in a water bath at 44^{0}C.
Agar medium (AgM) was prepared as follows and equilibrated to 44^{0}C:-

2xDMEM*	50ml
HEPES (1mM)	2ml
$NaHCO_3$	1ml
Pen/Strep	1ml (Gibco; 15070-022)
Growth medium	14ml

*2xDMEM was prepared as follows:-

10xDMEM	20ml
Ultrapure water	76ml
HEPES (1mM)	4ml

(adjust to pH 7.4 with 1M NaOH)

The thermo-labile components L-glutamine (1ml) and serum (10ml) were added last to the AgM. 50ml of the melted agar was then added to AgM, mixed well and quickly dispensed onto 35mm sterile Petri dishes (Lux Scientific Corp.; 5217). The plates were allowed to set at room temperature and the remaining AgM was returned to the water bath and the temperature was reduced to 41^{0}C.

The cells to be assayed were harvested and resuspended in medium without serum, ensuring tha a single cell suspension was obtained. The cells were diluted to give a concentration of $2x10^4$ cells per ml in a total of 5ml. 5ml of agar (41^{0}C) was added to each cell suspension, mixed well and 1.5ml was quickly dispensed onto each pre-set agar plate, in triplicate, giving a final cell concentration of $1.5x10^4$ cells per plate. The plates were placed on trays which contained a small volume of water to prevent the agar from drying out and incubated at 37^{0}C, 5%CO_2 for 10 days.

After this time, colonies were counted using an inverted microscope (CK Olympus, Tokyo) at 40x. The Petri dishes used had grids marked on their bases which aided in the counting process. Colonies greater than 20μm in diameter were scored using an eye-piece graticule. 10 areas were viewed per plate and the total number of colonies present was extrapolated from this.

The percentage colony forming efficiency (CFE) was determined by expressing the number of colonies formed as a percentage of the number of cells plated.

2.9.5 Spinner Flask Assay

Clean spinner flasks (Techne) were coated with a siliconising agent, dimethyldichlorosilane solution (BDH; 33164) to prevent cell attachment and allowed to dry in a fume hood. The flasks were then rinsed at least three times with ultrapure water and autoclaved. Flasks were rinsed again with sterile PBS A prior to use to remove any residual silicone.

Cells were harvested and inoculated into the spinner flasks at a concentration of $2x10^5$ cells per 50ml per flask. The flasks were placed on magnetic stirrer boxes in a 37^0C constant temperature room and the stirrers caused a magnetic rod inside the flasks to rotate, thus keeping the cells in suspension. The rods were rotated at a speed of 35rpm.

Every 7 days, the cells were decanted from the flasks, an aliqout was counted using a haemocytometer and the remainder were used to re-seed the flasks at the original density of $2x10^5$ cells per flask. For the first 5 weeks of the assay, cells were not fed between counts as there appeared to be sufficient medium present to maintain their growth. However, over the course of the first 5 subcultures, the rate of growth of the cells was found to be increasing and it was necessary to add a further 50ml of medium to each flask 4 - 5 days after re-seeding.

2.9.6 Growth Factor Assay

Growth factor assays were carried out on 96-well plates. These assays proceeded as described in Section 2.9.1 up to the point where the attached cells were rinsed with PBS A. After this, 50μl of DMEM containing twice the final concentration of L-glutamine and also twice the final concentration of background serum or insulin, depending on which was being used in the assay, was plated onto the cells using a multi-channel micropipette. Growth factor stocks (see Table 2.9.1) were diluted in Hams F12 medium to twice the final concentration of their respective biologically active ranges, as recommended by the suppliers, and 50μl aliquots were plated onto the cells also. All growth factors were aliquoted and stored at -20^{0}C at high stock concentrations to minimise loss of activity.

Plates were incubated at 37^{0}C, 5% CO_2 for 6 days, after which growth levels were determined by CVDE assay as outlined in Section 2.9.2.

Table 2.9.1 List of growth factors used in these studies

Growth factor	Supplier	Cat.No.
TGFβ	R & D Systems	100-B
EGF	Promega	G5021
Insulin	Sigma	I1882
Serotonin	Sigma	H4511
Histamine	Sigma	H7125
PDGF-AA	Promega	G5181
PDGF-BB	R & D Systems	220-BB
IL-1α	R & D Systems	220-LA
IL-6	R & D Systems	206-IL

2.9.7 ^{3}H-Thymidine Incorporation Assay

All experiments involving radioactivity were carried out in a designated cabinet in a laboratory allocated specifically to such work. When solutions or vessels containing radiolabel were being handled, 2 pairs of gloves were worn and all waste solutions and materials were deactivated in a strong detergent solution such as RBS. Waste was disposed of according to University guidelines.

Cells were seeded into 96-well plates at a density of $1x10^3$ cells per well in growth medium and allowed to attach over night. The next day, waste medium was removed and the cells were rinsed 3 times with sterile PBS A. Medium without serum was then plated onto the cells and the plates were incubated over night again at 37^0C, 5%CO_2. The purpose of this was to serum starve the cells, inducing quiescence and halting them at the G_0 phase of the cell cycle. Subsequent addition of growth factors should then either induce the cells to proceed through the cell cycle and thus stimulate DNA synthesis or inhibit DNA synthesis in those cells still cycling or have no effect if the cells do not express the appropriate growth factor receptors.

100μl of fresh medium containing the growth factors to be tested were then plated and incubated for 4 hours at 37^0C, 5% CO_2. 5μl of ^{3}H-thymidine was added (100μCi) to each well and the plates were incubated for a further 24 hr.

Waste medium was then removed, the plates were rinsed once with PBS A and 200μl 1M KOH was added to each well to lyse the cells. After incubating at 37^0C for 1 hr, the contents of each well was mixed thoroughly, 100μl aliquots were added to 5ml scintillation fluid (Ecolite; ICN 882475) and the number of disintegrations per minute were determined using a scintillation counter.

2.10 CONDITIONED MEDIUM ASSAY

Medium in which cells have been growing can be termed 'conditioned medium' (CM), so-called to recognise that cells may release substances into their medium including waste products of metabolism, growth factors and other secretory products. This CM can be collected and analysed to examine various aspects of the biology of a particular cell. In these studies, CM was analysed for growth stimulatory properties.

2.10.1 Collection of Conditioned Medium

Cells were harvested from 75cm^2 flasks and seeded into tissue culture roller bottles (Falcon; 3027) at concentrations of 2x10^6 cells per ml in 100 ml growth medium. The roller bottles were incubated at 37^{0}C on a roller bottle apparatus which rotated the vessels at a speed of 0.25 r.p.m. for the first 24 hr to allow cell attachment and at 0.50 r.p.m. after this time. The medium was replaced every 2 - 3 days until the cells were approximately 50% confluent. The medium was then removed, the cells were rinsed twice with sterile PBS A and then incubated for 1 hr with 100ml PBS A to remove as much serum-containing medium as possible. After removal of the PBS A, 100ml of Hams F12 supplemented with 2mM L-glutamine but without serum was placed into the roller bottles which were incubated over night as usual.

This 100ml Hams F12, called CM1, was then discarded and replaced with fresh Hams F12 (with L-glutamine), CM2, and the cells were incubated and allowed to condition this medium for 48 hr. CM2 was then decanted, centrifuged at 1,000 r.p.m. for 10 min to remove any cell debris and was stored at 4^{0}C. A fresh 100ml of Hams F12 was placed in the roller bottles, incubated for a further 48 hr and harvested in the same manner as CM2. CM2 and CM3 were then combined and stored at 4^{0}C until assayed.

CM was collected from cells cultured in 75cm^2 flasks in the same way.

2.10.2 Ultrafiltration of Conditioned Medium

The membranes used in the ultrafiltration procedures were a YM3 (An 06177A) which had a molecular weight cut-off of 1,000kD. The membrane was pre-treated by rinsing for 1 hr in ultrapure water, with 3 changes of water in that time, to remove residual sodium azide which was used as a preservative. The membrane was then placed in an ultrafiltration cell and rinsed again by running 100ml of PBS A or ultrapure water through the system 3 times. Pressure of 50psi was provided by N_2 gas.

The sample to be filtered was then placed in the ultrafiltration cell and pressure of 50psi was applied. The sample was stirred continuously by a magnetic bar to prevent protein accumulating on the membrane. The volume of the sample was reduced the required amount, 10-fold for these studies, and that which was retained by the membrane was called a 10x sample. The 10x sample was stored at 4^0C until required.

At the end of the procedure, the membrane was rinsed with ultrapure water or PBS A, removed from the cell, soaked in water for a further 30 min and stored in a 1.5M NaCl solution at 4^0C until required again. Each membrane could be used up to 5 times.

2.10.3 Conditioned Medium Assay

Cells were plated on 96-well plates, allowed to attach over night and rinsed as outlined in Section 2.9.1. 100μl of CM was then applied to the appropriate wells. Controls for these assays consisted of Hams F12 (as this was the medium used to collect CM) which had been incubated for 24 hr at 37^0C to mimic any alterations that may have occurred to Hams F12 components during the incubation periods with the cells.

The plates were incubated at 37^0C, 5% CO_2 for 6 days and then the cells were examined using the CVDE assay (Section 2.9.2).

2.11 EXTRACELLULAR MATRIX ADHERENCE ASSAYS

Collagen type IV (Sigma; C5533), fibronectin (Sigma; F2006) and laminin (Sigma; L-2020) were reconstituted in PBS A and collagen and fibronectin stocks were stored at -20^{0}C while laminin stocks were stored at -80^{0}C.

2.11.1 Coating of Plates

Each ECM protein was diluted to 50μg/ml with PBS A and 250μl aliquots were placed into the wells of a 24-well plate and the plates were tapped gently to ensure that the base of each well was completely covered with solution. The plates were then incubated at 4^{0}C over night. The ECM solutions were then removed, the wells were rinsed twice with sterile PBS A and 0.5ml of a 0.1% BSA/PBS A solution was dispensed into each well. The plates were incubated at 37^{0}C for 15min and then rinsed twice again with PBS A.

2.11.2 Adherence Assay

The cells were harvested and resuspended in Hams F12 medium without serum. The cells were then plated at 1x10^4 cells per well in triplicate and incubated at 37^{0}C for either 15 or 30 min. Controls wells were those which had been coated but contained no cells. At each time point, the medium was removed from the wells and the attached cells were rinsed gently with PBS A. The cells were then stained with crystal violet dye for 10 min, after which, the plates were rinsed and allowed to dry.

The dye was eluted with 200μl 33% glacial acetic acid and 100μl aliquots were transferred to 96-well plates and the absorbance was determined on an ELISA plate reader (Section 2.9.2). Exent of attachment to the different substrates was determined by comparing the absorbancies in wells containing cells with those which had been coated but contained no cells.

2.12 TOXICITY ASSAY

Due to the nature of the compounds tested in the assays, precautions were taken to limit the risks involved in their handling and disposal. All work involving toxic compounds was carried out in a Gelman 'Cytoguard' laminar air flow cabinet (CG Series). Adriamycin, vincristine and VP16 waste was disposed of by hyperchlorite inactivation, autoclaving and inceration, respectively.

Cells were harvested and plated into 96-well plates at densities of $1x10^3$ cells per 100μl per well in growth medium and allowed to attach over night at 37^0C, 5% CO_2. After this time, drug dilutions were prepared at twice their final concentration and 100μl aliquots were dispensed directly onto the 96-well plates, giving final concentrations of 1xdrug.

The plates were incubated for 6 days and cell survival was determined by CVDE assay (Section 2.9.2).

2.13 PREPARATION OF CELLS FOR ELECTRON MICROSCOPY

Cells were harvested by trypsinisation and washed by centrifuging and resuspending 3 times in PBS A. The pellets were then resuspended gently in a 10% glutaraldehyde/PBS A solution (v/v) and stored at 4^0C overnight. The cells were centrifuged again (1,000 r.p.m., 5 minutes) and the pelleted cells were sent to Dr.Daved Dinsdale, Leicester, England for electron microscopic analysis.

2.14 ELECTRICAL RESISTANCE STUDIES

Cell monolayers were cultured on tissue culture plate inserts which could be removed, assayed and replaced at will to enable continuous assessment of the electrical resistance exhibited by the monolayers. The base of the insert was a transparent, porous membrane which allowed the monolayer to be viewed microscopically and also allowed the free movement of solutes between the insert and the outer well.

2.14.1 Preparation of Cell Monolayers

Cells were harvested and seeded into inserts (Falcon; 3180) which were suspended in the wells of a 12-well plate (Falcon; 3043). Cells were seeded at densities of $1x10^6$ cells per insert to enable rapid formation of a confluent monolayer. The well contained 1ml of growth medium while the insert contained 0.5ml. The plates were incubated at 37^0C, 5% CO_2 for the duration of the assay.

2.14.2 Determination of Electrical Resistance

The electrical resistance measurements were carried out by Stephen Keely, Pharmacology Department, University College Dublin using the facilities of Dr.Alan Baird. An Endohm apparatus was employed to determine resistance across the monolayers.

The measurements were carried out in a laminar flow cabinet and the apparatus was swabbed thoroughly with IMS before use. An insert was removed from its 12-well plate using a sterilised tweezer and placed in the chamber of the Endohm which contained 4 - 5 ml medium. An electrode was placed into the insert and a current of electricity was passed through the monolayer. The Endohm then registered the resistance presented by the monolayer to the electric current. The insert was then returned to its well. This procedure was repeated every 3 - 4 days until the resistance of the positive control cell line was seen to plateau.

2.15 IMMUNOCYTOCHEMISTRY

The immunocytochemical techniques carried out here followed standard procedures with only slight adaptations in the cell preparations which were analysed.

2.15.1 Preparation and Fixation of Cells

The cell preparations varied and depended on the cellular location of the antigen of interest, whether the cells grew in suspension or were adherent and whether the cells being studied were normal, untreated cells or had been exposed to differentiating agents.

The fixation procedure, however, remained constant, regardless of the cell preparation. For fixation, cells were rinsed 3 times with PBS A and then incubated at -20^{0}C for 7 min. in pre-chilled methanol. The methanol was then removed from the cells and the cells were air-dried over night and stored at -20^{0}C until required. This method was successful for every antibody used in this study.

2.15.1.1 Cytospin cell preparations

This procedure was employed if the cell being examined grew in suspension.

Cells were harvested, centrifuged at 1,000 r.p.m. for 5 min and resuspended in PBS A. The cells were rinsed a further 2 times in PBS A and were finally resuspended in cytospin buffer (Table 2.15.1). The cells were then cytospun onto clean glass slide using a cytocentrifuge. The cells were spun at 500 r.p.m. for 6 min, after which, they were allowed to air dry and were fixed as outlined in Section 2.15.1.

Table 2.15.1 Components of cytospin buffer

Component	g/L
NaCl	8.00
KCl	0.40
CaCl	0.14
MgCl	0.10
$MgSO_4$	0.10
Na_2HPO_4	0.06
KH_2PO_4	0.06
$NaHCO_3$	0.35
Glucose	1.00
Ultrapure water	1,000ml

2.15.1.2 Multiwell slide cell preparations

Multiwell glass slides (Dynatech) were utilised in most cases when the cells to be examined were adherent. These slides were used in preference to cytospins because they enabled cells to be cultured and analysed *in situ*, allowing cell morphology and antigen localisation to be examined after the immunocytochemical procedure. Each slide contained 8 or 10 wells, each of which could be analysed with a separate antibody, thus reducing the number of slides to be manipulated and the volumes of washing buffers required. In addition, cytospin preparations do not allow cell recovery after trypsinisation which may damage some cell surface antigens.

The multiwell slides were prepared as follows:-
Each slide was soaked in RBS detergent for 1 hr, then scrubbed, rinsed thoroughly with ultrapure water and wrapped in aluminium foil. The slides were then sterilised by baking for at least 2 hr at 180°C.

Cells were harvested and seeded onto the wells of the sterilised slides at densities of $1x10^4$ cells per 50μl per well. Each slide was placed into a sterile Petri dish which was sealed with 'Parafilm' and incubated at 37^{0}C, 5% CO_2 until the confluency required was achieved. The cells were then fixed in methanol (Section 2.15.1).

2.15.1.3 Tissue culture plate cell preparations

This method of cell preparation was employed for the analysis of BrdU- and RA-treated cells. The differentiation assays were carried out in 6-well tissue culture plates and because of the low cell numbers involved and the possibility of damaging antigens by transferring the cells to glass slides or making cytospin preparations, the immunocytochemical analyses on these cells was carried out *in situ*.

Following removal of waste medium, each well was rinsed with PBS A and fixed with methanol (Section 2.15.1).

2.15.1.4 EP16 and 703D4/704A1 Hybridomas

Most of the antibodies used in the immunocytochemical studies were commercial products, sold in purified form. Three of the antibodies, however, were aquired from hybridomas which were bought from the ATCC and cultured in the laboratory. These were the Ep16, 703D4 and 704A1 monoclonal antibodies. In order to obtain these antibodies, the hybridomas were cultured in their respective growth media (Table 2.5) as outlined for suspension cells in Section 2.5.2. Hybridomas secrete their antibodies into the medium, therefore, the cells were fed with fresh medium wich was then collected 3 - 4 days later. The supernatents were cleared by centrifugation and could then be used neat in immunocytochemical studies as primary antibodies. The supernatents were aliquoted and stored at -20^{0}C until required.

2.15.2 Immunocytochemical Procedure

The avidin-biotin-peroxidase complex (ABC) immunoperoxidase technique (Hsu *et al.*, 1981) combined with the diaminobenzidine (DAB) visualisation procedure was employed here to indicate primary antibody binding. The ABC method involves application of a biotin-labelled secondary antibody, followed by the addition of avidin-biotin-peroxidase complex which results in a high staining intensity due to the formation of an avidin-biotin lattice which contains several peroxidase molecules. The peroxidase enzyme reacts with a DAB solution to give an insoluble, brown-coloured precipitate. Therefore, observation of a brown precipitate following this procedure is indicative of primary antibody reactivity.

The immunocytochemical procedure used was as follows:-
(note:- the word 'slide' will be used to describe all cell preparations (Sections 2.15.1.1 -2.15.1.3)).
Frozen, fixed cell preparations were allowed to equilibrate to room temperature. A grease pen (DAKO; S2002) was used to encircle cells on cytospin slides and in tissue culture dishes to contain the various solutions involved. This was not necessary on the multiwell slides as the wells were indented and capable of holding a drop of solution. The cells were incubated for 5 min with a 3% H_2O_2 solution in ultrapure water to quench any endogenous peroxidase activity that may have been present in the cells and would give rise to false positive results. The slides were then rinsed with distilled water and placed in Tris-buffered saline (TBS) (0.05M Tris/HCl, 0.15M NaCl, pH 7.6) for 5 min. Following this, the slides were incubated for 20 min at room temperature with normal rabbit serum (DAKO; X902) diluted 1:5 in TBS, to block non-specific background. This was then tapped off, 50μl of optimally-diluted primary antibody was placed on the cells and the slides were placed on a tray containing moistened tissue paper and incubated at 37^{0}C for 2 hr. The primary antibodies, their sources and dilutions are listed in Table 2.15.1. The slides were then rinsed in TBS, x3 in 10 min, and then incubated for 30 min with biotinylated rabbit anti-mouse immunoglobins (DAKO; E354). The slides were rinsed as before and incubated with streptABComplex/ Horse Radish Peroxidase (HRP) (DAKO; K377) for 30 min at R.T., after which they

were rinsed and incubated with a DAB solution (DAKO; S3000) for 10 - 15 min.

The slides were then counterstained with a 2% methyl green solution, dehydrated in alcohol and mounted in DPX (BDH; 36029) mounting solution.

Table 2.15.1 Primary antibodies used in immunocytochemical studies

Antibody	Dilution/ Concentration	Supplier	Catalogue No.
Cam 5.2	25μg/ml	Becton Dickinson	
Pan keratin	1/100	Sigma	C2562
Keratin 8			
Keratin 10	Undiluted	DAKO	47048
Keratin 14	1/200	Sigma	C8791
Keratin 17	1/400	Sigma	C9179
Keratin 18	1/200	Sigma	C8541
EMA	*		
EP16	Neat CM	NCTCC	
Desmoplakin	Neat CM	Gift[1]	
Transglutaminase	1/20	Biogenesis	5560-6006
703D4/704A1	Neat CM	NCTCC	
NSE	*	Immunon	401400
PGP 9.5	*	Ultraclone	
Leu 7	*	Becton Dickinson	
Neurofilaments	1/20	Immunotech	0168
NCAM	1/10	DAKO	M779
5-HT	1/5	DAKO	M0758
GFAP	Undiluted	DAKO	U7038

P.T.O.

Table 2.15.1 Primary antibodies used in immunocytochemical studies (contd.)

Antibody	Dilution/ Concentration	Supplier	Catalogue No.
Chromogranin A	Undiluted	DAKO	U7030
Vimentin	*	DAKO	
LCA	*	DAKO	M701
P-glycoprotein	0.25μg/ml	Centicor Diagnostics	
α_2 integrin	1.8mg/ml	Gift[2]	

* Work carried out by Dr.Colma Barnes, Royal Victoria Eye and Ear Hospital, Dublin.

[1] Gift from Dr.David Garrod, University of Manchester, England.

[2] Gift from Dr.Fiona Watt, Imperial Cancer Research Fund, London, England.

2.16 MONOCLONAL ANTIBODY PRODUCTION

Hybridomas which secreted monoclonal antibodies were produced by immunising mice with DLKP cells, harvesting the mouse spleen cells and fusing them to mouse myeloma Sp2 cells to immortalise the antibody-producing cells.

2.16.1 Immunisation Procedure

Two Balb/c mice were injected 3 times in 7 weeks with $1x10^7$ DLKP cells in 1ml of PBS A per injection. A final booster injection of cells was administered in week 8, 3 days prior to the fusion.

A sinus bleed was carried out at the end of week 7 to determine if the mice were producing anti-DLKP antibodies. Approximately 0.25ml of blood was collected and allowed to clot in an Eppendorf at 4^0C for 2 - 3 hr. The blood was then centrifuged at 10,000 r.p.m. to pellet the red blood cells and the serum was decanted and stored at -20^0C until its reactivity with DLKP cells was analysed by ELISA as described in Section 2.16.4.

2.16.2 Fusion

Of the 2 mice immunised, the animal whose sinus bleed had demonstrated greater reactivity with DLKP cells was chosen and sacrificed by cervical dislocation. The animal was swabbed with 70% IMS and brought into a laminar flow cabinet where it was disected using sterilised instruments and its spleen was removed. The spleen was homogenised by forcing it through a sterile tissue dissociation sieve (Sigma) using the plunger from a 20ml syringe. The resulting cell suspension was collected into a 50ml sterile centrifuge tube and any large clumps present were allowed to settle. The supernatent was then transferred to a fresh tube and the cells were centrifuged at 1,000 r.p.m. The cells were resuspended in DMEM Hybrimax (Sigma; D5660) without serum and centrifuged again (note:- the DMEM referred to throughout these antibody procedures is DMEM Hybrimax. This medium contains no HEPES buffer which is toxic to hybridomas). Red blood cells were lysed by resuspending the cells in 10ml pre-warmed (37^0C) red cell lysis buffer (Sigma; R1129) and after 5 min, an equal volume of DMEM was mixed well into the suspension to wash the cells. The cells were centrifuged at 1,000 r.p.m. for 5 min, a viability count was carried out (see Section 2.5.3) and the cells were washed a further 2 times in DMEM (without serum).

The Sp2 myeloma cells were prepared for cell fusion by harvesting from a $75cm^2$ flask rinsing twice in serum-free DMEM and counting.

A universal of 50% (w/v) PEG-1500 (Boehringer; 783641) and a universal containing 20ml of serum-free DMEM were incubated at 40^0C. The isolated splenocytes and the Sp2 cells were mixed in a 10:1 ratio in a 50ml centrifuge, using $4.5x10^7$ splenocytes (a minimum of $1x10^7$ were required). The pooled cells were washed twice with serum-free DMEM and the cells were pelleted at 2,000 r.p.m. After ensuring that all supernatent had been removed, 1ml of pre-warmed PEG solution was gently added to the cells with a plastic pipette and the cells and PEG were mixed by aspirating up and down. After 30 sec., the pipette was removed and the cells were gently swirled for a further 75 sec., after which, 0.5ml of plating medium *i.e.* DMEM containing HAT (Sigma; H0262) and 10% serum, was added slowly down the side of the universal while

swirling gently. A further 8ml plating medium was added slowly over the next 5 min. while continuously swirling the cells and the cells were then centrifuged at 500 r.p.m. for 5 min. The supernatent was then removed, the cells were resuspended in 10ml plating medium and incubated at room temperature for 15 min. 90ml plating medium was then added to the 10ml cell suspension and the cells were plated into 96-well plates at 100μl per well. A further 100μl plating medium was then added to each well and the plates were incubated undisturbed for 12 days at 37^{0}C, 5% CO_2.

2.16.3 Screening of Hybridomas

After the 12 day incubation period, the plates were viewed microscopically for colony formation and positive wells were marked. Over the next 10 - 14 days, those supernatents which changed colour from pink/purple to orange/yellow, indicating cell growth, were removed and stored at -20^{0}C and the cells were re-feed. The supernatents were screened for reactivity with DLKP cells as follows:-
DLKP cells were plated onto 96-well plates at 2x10^4 cells per well and allowed to attach over night. The plates were then washed 3 times with PBS A and fixed with 70% acetone at room temperature for 5 min. The acetone was then removed, the plates were air dried and the non-specific activity was blocked by incubating the cells with 1% BSA/TBS (see Section 2.15.2) for 1 hr. 100μl supernatent (or diluted serum from sinus bleed) was then incubated with the cells at 37^{0}C for 1.5 hr, the plates were washed 3 times with TBS/0.1% Tween 20 (Sigma; P1379) and incubated at 37^{0}C for 1.5 hr. with 1/10,000 dilution of anti-mouse IgG/IgM secondary antibody (Pierce). The plates were washed as before and then incubated with a substrate solution (1mg/ml p-nitrophenyl phosphate (PNPP) (Sigma; 104-0) in 0.1M-glycine, 0.001M-$MgCl_2.6H_2O$, 0.001M-$ZnCl_2$, pH 10.4) at 37^{0}C for 0.5 - 1 hr. Absorbances were read on an ELISA plate reader at 405nm and positive reactivity was determined by comparing supernatent-containing wells with those which had been incubated with PBS A instead of supernatent.

Positive clones were cultured further into 25cm^2 and 75cm^2 vented flasks and were gradually weaned off the HAT-containing medium (which selectively killed unfused Sp2 myeloma cells) into DMEM without HAT. Unfused splenocytes were unable to

replicate and so their numbers gradually diminished.

2.16.4 Isotype analysis

Isotype classifications were determined using a Serotec kit (MMMT RC1). The principle of the kit is based on red cell agglutination. A positive, agglutinated result is produced when a highly specific antibody recognises and binds to the particular isotype to which it is directed. This binding forms a lattice of agglutination when the reagents fall to the bottom of a microtitre plate. A negative result is produced when the reagent cells are put into a class of antibody which they do not recognise.

Dilutions of hybridoma supernatent were made and 30μl aliquots were incubated with 30μl aliquots of each specific isotyping reagent at room temperature for 1 hr. After this time, the plates were examined to determine which well contained agglutinate and the isotypes of the antibodies were revealed accordingly.

2.17 WESTERN BLOT ANALYSIS

Proteins for western blot analysis were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Resolving and stacking gels were prepared as outlined in Table 2.17.1 and poured into clean 10cm x 8cm gel cassettes which consisted of 1 glass and 1 aluminium plate, separated by 0.75cm plastic spacers. The resolving gel was poured first and allowed to set. The stacking gel was then poured and a comb was placed into the stacking gel in order to create wells for sample loading. Once set, the gels could be used immediately or wrapped in aluminium foil and stored at 4^{0}C for 24 hr.

Equal cell numbers were lysed in loading buffer (2.5ml 1.25M-Tris/HCl, 1.0g SDS, 2.5ml mercaptoethanol (Sigma; M6250), 5.8ml glycerol and 0.1% bromophenol blue (Sigma; B8026) made up to 25ml with distilled water) and boiled for 5 min. 20μl samples were loaded onto the stacking gels, as were molecular weight protein markers, and the gels were run at 250 volts, 45 mAmps. for approximately 1.5 hr.

Table 2.17.1 Preparation of electrophoresis gels

	Resolving gel (12%)	Stacking gel
Acrylamide stock*	5.25ml	0.8ml
Distilled H_2O	6.45ml	3.6ml
1.875M-Tris/HCl, pH 8.8	3.0ml	-
1.25M-Tris/HCL, pH 6.8	-	0.5ml
10% SDS (Sigma; L4509)	150μl	50μl
10% Ammonium persulphate (Sigma; A1433)	50μl	17μl
TEMED (Sigma; T8133)	9μl	6μl

* Acrylamide stock = 29.1g acrylamide (Sigma; A8887) and 0.9g NN'-methylene bis-acrylamide (Sigma; N7256) made up to 100ml with distilled water.

When the bromophenol blue dye front was seen to have reached the end of the gels, electrophoresis was stopped, the electrophesis apparatus was disassembled and the gels were removed and equilibrated for 10 min. in transfer buffer (24mM-Tris and 192mM-glycine in 250ml water, pH 8.3 without adjusting). 3 - 4 sheets of Whatman 3mm filter paper (Whatman; 1001824) were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus to act as a buffer reservoir. A sheet of nitrocellulose filter paper (Amersham; Hybond-C), also pre-soaked in transfer buffer, was placed on the filter paper and the gel was placed on top of this. Additional pre-soaked sheets of filter paper were placed on the gel and after trapped air bubbles had been removed by rolling a glass pipette over the stack of filter paper, the protein was transferred from the gel to the nitrocellulose filter at 15volts and a current of 0.34 mAmps. for 20 min. The nitrocellulose paper was then removed and incubated in blocking buffer (3% non-fat dried milk (Cadbury; Marvel skimmed milk) in TBS pH

7.5/0.1% Tween 20) for 1 hr at room temperature, to block non-specific binding. All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus to ensure even exposure of the nitrocellulose blot to all reagents.

After blocking, the blot was rinsed with PBS A and incubated in 10ml primary antibody (conditioned medium from hybridomas) at 4^0C, overnight. The blots were then washed 3 times in TBS/0.1% Tween 20 for 20 min and incubated for 1.5 hr. in secondary antibody (1/9,500 dilution of rabbit anti-mouse IgG (Sigma; A1902). The blots were then washed as before and developed by incubating in 10 - 20ml substrate buffer (70μl BCIP (Boehringer; 1383 221) and 67.5μl NBT (Boehringer; 1383 213) in 20ml of 100mM-Tris, 100mM-NaCl and 50mM-$MgCl_2$ in distilled water at pH 9.5).

2.18 DIFFERENTIATION STUDIES

Differentiation studies were carried out using 5-bromodeoxyuridine (BrdU) (Sigma; B5002) and all-*trans* retinoic acid (RA) (Sigma; R2625). All manipulations involving these agents were carried out in subdued light, as both were light sensitive.

2.18.1 Reconstitution of BrdU and RA

BrdU powder was dissolved in ultrapure water to a stock concentration of 10mM, was sterilised by filtering through a sterile 22μm filter (Millipore) and was aliquoted into sterile Eppendorfs and stored at -20^0C for up to 1 year.

RA was reconstituted in 95% ethanol to a stock concentration of 1mM and was aliquoted into cryovials and stored at -80^0C.

2.18.2 Differentiation Assays - Adherent Cells

Cells were plated into 6-well plates at densities of $5x10^3$ cells per 2ml per well and allowed to attach, spread and begin to form colonies by incubating at 37^0C, 5% CO_2, for 48 hr. The medium was then removed from the cells and replaced with BrdU or RA which had been diluted to the required concentrations with normal growth medium

appropriate to the cell type involved. Plates were wrapped in aluminium foil and incubated as before. Medium was replaced every 2 - 3 days over the course of the assay and all waste medium was retained for disposal by incineration due to the hazardous nature of BrdU and RA.

At the conclusion of each assay, the plates were rinsed 3 times with PBS A, fixed with methanol and analysed by immunocytochemistry as outlined in Section 2.15.

2.18.3 Differentiation Assays - Suspension Cells

Cells were plated into 6-well plates and incubated as described in Section 2.18.2. In order to change the medium in these plates, it was necessary to remove the cells and their supernatents to sterile universals and centrifuge at 1,000 r.p.m. for 5 min to pellet the cells. The cells were then resuspended in 2ml of the appropriate medium and returned to their wells.

When the assays were completed, the suspension cells were removed and cytocentrifuged and any cells which had adhered during the course of the assay were fixed as described in Sections 2.15.1 and 2.15.3.

2.19 RIBOZYME TRANSFECTIONS

A plasmid which contained the ribozyme *MDR* 1 gene and a gene coding for geneticin resistance (received as a gift from Dr.Kevin Scanlon, City of Hope Medical Centre, Los Angeles) was used to transfect clonal subpopulations of DLKP and DLKP-A, DLKP-I and DLKP-A-2B respectively. A calcium phosphate transfection technique was employed.

2.19.1 Transfection of Cells

Cells were harvested and seeded into 10cm diameter sterile Petri dishes at a density of $5x10^5$ cells per dish. The dishes were wrapped in 'Parafilm' and incubated overnight at 37^0C, 5% CO_2. 10μg DNA was diluted in 410μl sterile water and stored overnight

at 4^0C.

The next day, the DNA solution was incubated at 37^0C for 1 hr. and 480μl of 2xHBS (280mM-NaCl, 1.5mM-Na_2HPO_4, 50mM-HEPES, pH 7.12, filtered through a 22μm filter and stored at 4^0C) was incubated in a sterile tube at room temperature. The rest of the procedure was carried out in a laminar flow cabinet. 60μl 2M-$CaCl_2$ was added dropwise into the tube containing the DNA, being continually mixed using a vortex mixer. This was immediately added to the HBS, dropwise with continuous mixing, and then allowed to stand for exactly 30 min. The DNA-$CaPO^4$ mixture was then added dropwise to the dishes of cells which were swirled gently to ensure even mixing. The cells were then incubated at 37^0C for 4 hr. After this time, a fine, opalescent precipitate was seen to cover the cells. The medium and precipitate were aspirated off the cells and 5ml 10% glycerol in 1xHBS was placed on the cells. After exactly 3 min., the glycerol was removed, the cells were rinsed twice with serum-free medium and then fed with fresh growth medium. The dishes were then incubated at 37^0C, 5% CO_2, for 2 days.

2.19.2 Selection of Plasmid-containing Cells

2 days after being transfected, the cells were fed with geneticin-containing medium *i.e.* growth medium with 400μg/ml geneticin (Sigma; G9516). Untransfected, control cells were also fed with this medium each time the transfectants were fed in order to indicate when any untransfected cells would be dead. Therefore, it could be assumed that when all control cells were dead, any cells remaining in transfected dishes should countain the plasmid and its geneticin-resistance gene. Geneticin levels were increased to 600μg/ml after 11 days, and 17 days after the transfection, all control cells were dead.

2.19.3 Culture of Transfected Clones

When the control cells were found to have died after exposure to geneticin, the remaining transfectants were cloned in a limiting dilution assay in 96-well plates (Section 2.7.1). As a result of this, 5 DLKP-I and 2 DLKP-A-2B transfected clones were obtained. These were cultured further from 96-well plates into 6-well plates and into 25cm^2 and 75cm^2 flasks and frozen stocks were prepared (Section 2.5.4).

2.20 DNA EXTRACTION

A crude method of extraction was used to obtain DNA from transfected cells:-
A 70% confluent, 25cm^2 flask of cells was harvested by trypsinisation and pelleted by centrifugation as normal (Section 2.5.1). The cells were resuspended in 10ml sterile PBS A and centrifuged again and this was repeated twice. The cells were then resuspended in 1 - 2ml PCR buffer (50mM-KCl, 10mM-Tris/HCl (pH 8.3) 2.5mM-$MgCl_2$, 0.1mg/ml gelatin, 0.45% Nonidet P-40 (Sigma; N0896), 0.45% Tween 20 - autoclave and store at -20^{0}C and before use, thaw and add 0.6μl of 10mg/ml Proteinase K (Sigma; P2308) per 100μl of solution) in sterile eppendorfs and incubated in a water bath at 55^{0}C for 1 hr. The eppendorfs were then incubated at 95^{0}C for 10 min. to inactivate the proteinase K and the samples were stored at -20^{0}C until required.

DNA concentration was calculated by determining its optical density (OD) at 260nm and using the following formula:-

$$OD_{260nm} \times \text{Dilution factor} \times 50 = \text{mg/ml DNA}$$

2.21 RNA EXTRACTION

The labile nature of RNA required that strict procedures were followed in the course of work involving RNA. This procedures included the wearing of gloves at all times that RNA-containing vessels were being used, the baking of all glassware and foil used to make up solutions and weigh compounds and most solutions and plastics were treated with 0.1% diethyl pyrocarbonate (DEPC) which is a strong inhibitor of RNases.

RNA was then extracted from cells as follows:-
Cells were grown in 135mm diameter Petri dishes to approximately 80% confluency. Five dishes of each cell line were grown at 37^{0}C, 5% CO_2. Waste medium was then removed, the cells were washed twice with DEPC-PBS A and were lysed in 5ml of a 4M-guanidinium thiocyanate (GnSCn) solution (50g guanidinium thiocyanate (Sigma; G6639), 0.5g N-lauroyl sarcosine (Sigma; L5125) and 5ml of 1M-sodium citrate (RDH; 32320), pH 7.0 - brought up to 100ml with DEPC-water and filtered through a 0.45μm

filter and supplemented with 700μl/ml β-mercaptoethanol and 330μl/100ml antifoam A (Sigma; A5758) prior to use).

Lysates from the 5 dishes were pooled and centrifuged at 1,000 r.p.m. for 5 min. 5.5ml of a 5.7M-cesium chloride solution (95.8g CsCl (Sigma; C3032) and 2.5ml of 1M-sodium citrate, pH 7.0 in 100ml water, filtered through a 0.22μm filter, treated with 0.1% DEPC and autoclaved) was placed into polyallomer ultracentrifuge tubes and the cell lysates were then layered onto these CsCl cusions. These were then centrifuged at 27,000 r.p.m. for 21 - 24 hr. at 15^{0}C in a swinging bucket centrifuge. The tubes were then brought into a laminar flow cabinet and the supernatent was removed from the tubes, leaving approximately 1ml of CsCl in the bottom of the tube, below which lay the RNA pellet. The tube was inverted and the bottom of the tube was cut away using a heated scalpel blade. The pellet was rinsed with 95% ethanol and resuspended in 200μl DEPC-water. The solution was transferred to an eppendorf and the bottom of the tube was rinsed with a further 200μl water which was added to the eppendorf. 40μl of 3M-sodium acetate was added to the RNA solution to give a final concentration of 0.3M-sodium acetate, and 2 volumes of ice-cold absolute ethanol were also added. The eppendorfs were stored at -80^{0}C overnight and the RNA was then pelleted at 4^{0}C at maximum speed in a microfuge. The resulting pellet was washed with 70% ethanol, the supernatent was removed and the pellet was resuspended in 50μl DEPC-water.

RNA concentration was calculated by determining its OD at 260nm and using the following formula:-

$$OD_{260nm} \times \text{Dilution factor} \times 40 = \mu g/ml \text{ RNA}$$

The purity of the RNA extraction was calculated by determining its OD at 260nm and 280nm. An $A_{260} : A_{280}$ ratio of 2 is indicative of pure RNA and only those samples with ratios between 1.8 and 2.1 were used.

2.22 POLYMERASE CHAIN REACTION

A standardised polymerase chain reaction (PCR) procedure was followed in this study. The eppendorf tubes used (Eppendorf; 0030 121 023) and the sterile water were DEPC-treated. All reagents had been aliquoted and were stored at -20^{0}C and all reactions were carried out in a laminar flow cabinet.

Each PCR tube contained the following:-
24.5μl water
5μl 10x buffer* (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100)
3μl 25mM-$MgCl_2$*
8μl dNTPs (1.25mM each of dATP, dCTP, dGTP and gTTP) (Promega; U1240)
1μl each of first and second strand target primers (250ng/μl)
1μl each of first and second strand endogenous control primer (250ng/μl)
0.5μl of 5U/μl *Taq* DNA polymerase enzyme*
5μl DNA or cDNA
*(Promega; N1862)

A drop of autoclaved mineral oil was placed in each reaction tube to prevent evaporation and the DNA was amplified by PCR (Techne; PHC-3) as follows:-
95^{0}C for 1.5 min - to denature double-stranded DNA
30 cycles: 95^{0}C for 1.5 min. - denature
55^{0}C for 1 min - anneal
72^{0}C for 3 min. - extend
72^{0}C for 7 min. - extend
The reaction tubes were then stored at 4^{0}C until analysed by gel electrophoresis as described in Section 2.24.

2.23 REVERSE TRANSCRIPTASE REACTION

Reverse transcriptase (RT) reactions were carried out in laminar flow cabinets using micropipettes which were specifically allocated to this work.

cDNA was formed using the following procedure:-
1μl oligo $(dT)^{12-18}$ primers (1μg/μl) (Promega; C1101)
1μl total RNA (1μg/μl)
3μl water
were mixed in an eppendorf, heated to 70^{0}C for 10 min and then chilled on ice. To this, the following were added:-
4μl of a 5x buffer (250mM-Tris/HCl pH 8.3, 375mM-KCl and 15mM-$MgCl_2$)
2μl DTT (100mM) (Gibco; 510-8025 SA)
1μl RNasin (40U/μl) (Promega; N2511)
1μl dNTPs (10mM of each dNTP)
6μl water
1μl Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) (40,000U/μl) (Gibco; 510-8025 SA).
The solutions were mixed and the RT reaction was carried out by incubating the eppendorfs at 37^{0}C for 1 hr. The MMLV-RT enzyme was then inactivated by heating to 95^{0}C for 2 min. The cDNA was stored at -20^{0}C until required for use in PCR reactions as outlined in Section 2.22.

2.24 ELECTROPHORESIS OF PCR PRODUCTS

A 3% agarose gel (NuSieve;GTG) was prepared in TBE buffer (5.4g Tris, 2.75g boric acid, 2ml 0.5M-EDTA pH 8.0 in 500ml water) and melted in a microwave oven. After allowing to cool, 0.003% of a 10mg/ml ethedium bromide solution was added to the gel which was then poured into an electrophoresis apparatus (BioRad). Combs were placed in the gel to form wells and the gel was allowed to set.

10μl loading buffer (50% glycerol, 1mg/ml xylene cyanol, 1mg/ml bromophenol blue,

1mM EDTA) was added to each 50μl PCR sample and 10 - 20μl was run on the gel at 80mVolts for approximately 1 hr. When the dye front was seen to have migrated the required distance, the gel was removed from the apparatus and examined on a transilluminator.

3.0 RESULTS

3.1 CLONAL VARIATION IN DLKP

During routine culture of the DLKP cells , it appeared that at least 3 morphologically different populations were present within the cell line. The largest population ('A'; ~65%) were squamous in appearance with distinct cell boundaries and were relatively large in size with a high cytoplasmic to nuclear ratio. The second population ('B'; ~30%) were smaller (approx. 1/4 area of 'A' cells) and grew in colonies with indistinct cell boundaries. The third population ('C'; ~5%) were of intermediate size with an irregular, fibroblastoid-like morpholgy and did not appear to form colonies. It was not clear at this stage whether the 3 speculative populations were (i) merely different stages of the cell cycle of one parental cell type - although this seemed unlikely as specific colonies of the different populations A, B and C, when present in a culture, appeared to be constant rather than transient, (ii) a mixture of different cell types - this also seemed unlikely as the different morphologies were still present in cultures at high passage numbers (>passage 80) where one would expect the faster growing cell type in an initially mixed population to have overtaken and out-grown its slower counterparts or (iii) various differentiation states of one (or more) parental cell(s) - this would seem the most likely situation. Clonal subpopulations were isolated from the DLKP cell line in an attempt to isolate, if possible the morphologically diverse populations seen in culture.

3.1.1 Isolation of DLKP Clones

Clones of the DLKP cell line were obtained by limiting dilution assay (Section 2.7.1). Initial attempts to clone using cloning rings proved to be practically difficult and prone to fungal contamination, although this technique has often been successful for other workers. The endpoint for both techniques is the same as in each case, the presence of a true single colony is determined by eye. However, the limiting dilution assay procedure is only suitable for cells such as DLKP which are not density dependent and can proliferate at low seeding densities. To clone cells without this capability, methods such as cloning rings or cloning in soft agar may be more suitable.

In a limiting dilution assay, very low seeding densities are used to facilitate isolation of clonal populations and for the DLKP cells, 5 cells/ml (or 1 cell/2 wells of a 96-well plate) was the density used. Seeding densities higher than this were not successful in obtaining single colonies of cells. The number of clones obtained using this method is therefore not usually high.

The lowest obtainable passage number of DLKP cells, passage 3, were used in the cloning assay in an attempt to obtain clones with characteristics as close as possible to cells in the original tumour. The first cloning assay resulted in 5 clones, each of which appeared to be a homogenous population. The clones were named DLKP 1-5. DLKP-1, -3 and -5 had morphologies similar to that described as 'A' in Section 3.1 while DLKP-2 and -4 had morphologies similar to 'B' and 'C' respectively. However, due to the frequent manipulations (feeding, trypsinising *etc.*) and the suseptibility of unsealed plates to contamination, unfortunately clones DLKP-1 and -5 became contaminated with fungus and had to be discarded. As is the case in all routine cell culture carried out here, no anti-bacterial or anti-fungal agents were used at any stage in the cloning process to avoid deleterious effects on the cells and/or their behaviour.

In order to ensure the clones were true clonal populations, DLKP-2 (passage 12), -3 (passage 16) and -4 (passage 20) were recloned as in the original cloning assay except that a lower seeding density, 3 cells/ml (or 1 cell/3 wells) was used. DLKP-2 and -4 were recloned successfully and named DLKP-2A and DLKP-4A respectively. DLKP-3 however, did not reclone successfully at this density. Following seeding into the 96-well plates, wells containing 1 cell were marked as usual and fed with the same medium as the other reclones, but the DLKP-3 cells failed to proliferate. Parental DLKP cells (passage 15) were cloned at the same time as the recloning and this resulted in a clone, DLKP 7, with an apparently homogenous population, similar to that of morphology 'A' described in Section 3.1. At a later stage, the nomenclature of the 3 clones was changed to avoid any possible confusion with other DLKP variants such as DLKP-A. Names were chosen which reflected the morphology and/or behaviour of each clone; DLKP 2A was renamed DLKP-I (intermediate), DLKP 4A was changed to DLKP-M (mesenchymal-like) and DLKP 7 became DLKP-SQ (squamous) (see Figure 3.1.1). From now on, the clones will be refered to as DLKP-I, -M or -SQ.

While initially each clone appeared to be a homogenous population, after about 20-25 passages in culture, interconversion between the clones was observed. After this time, colonies with DLKP-I morphology began to appear in cultures of DLKP-M and DLKP-SQ (approximately 5-10% of total population) and similarly, colonies of DLKP-SQ morphology, and to a lesser extent DLKP-M morphology, could be seen in cultures of DLKP-I (approximately 5-10% and <5% of total population respectively. No interconversion was observed between DLKP-M and DLKP-SQ). It was therefore necessary to establish large stocks of each clone and to strictly confine experiments with the cells to specific passage numbers. Unless otherwise stated, all work here was carried with the clones at passage numbers from 20 to 35.

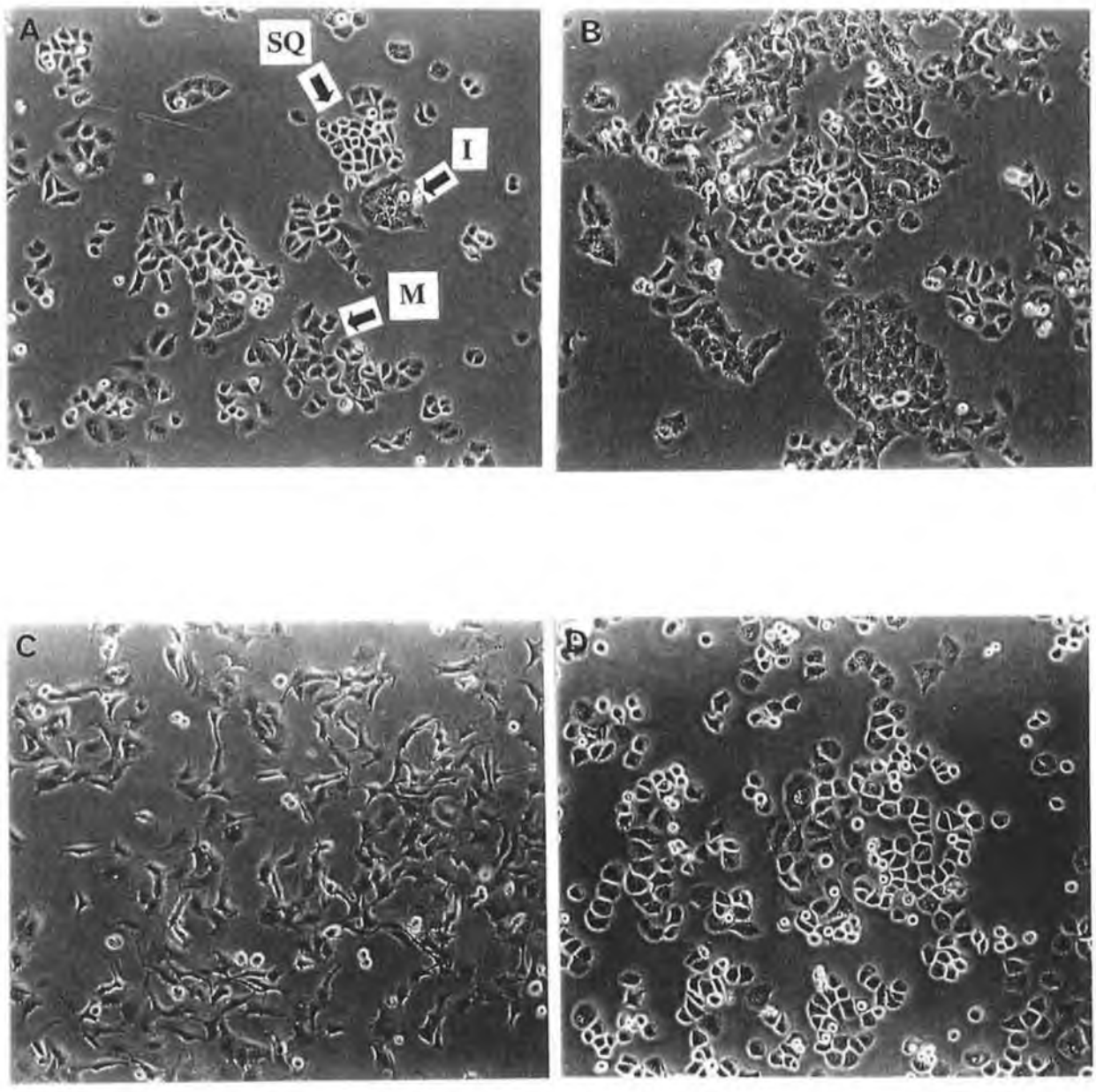


Figure 3.1.1 Morphological appearance of **A.** DLKP (I=DLKP-I-like, M=DLKP-M-like and SQ=DLKP-SQ-like morphologies), **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cultured in DMEM:Hams F12 (1:1), supplemented with 5% serum. x100.

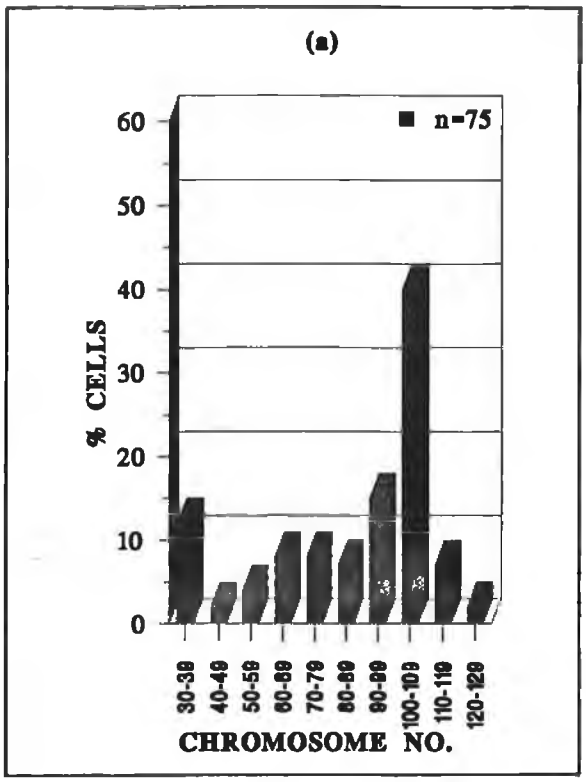
3.1.2 Analysis of Chromosome Distribution in DLKP and Clones

The clones, DLKP-SQ, -I and -M appeared morphologically different to each other but as yet, no physiological or behavioural differences had been explored. The chromosome distribution in DLKP (passage 5) had previously been determined by Law et al., (1992) in a cytogenetic analysis of the cell line. Law concluded that 2 chromosomal populations were present in DLKP; one (65% of cells) consisting of hyperdiploid cells with a modal number of 56 chromosomes and the other (35% of cells) being a hypertetraploid population with a chromosome distribution between 95 and 115.

In order to determine if chromosomal as well as morphological differences existed between the DLKP clones, or if any of the clones represented either of the 2 chromosome populations observed by Law, the chromosomal distributions of the cells were compared (see Section 2.8). DLKP cells at passage 22 were used here (note, passage 5 used by Law) to compare with DLKP-SQ, -I and -M which were used at passages 29, 26 and 32 respectively (Figure 3.1.2).

A range of chromosome populations was seen in all lines with prominent populations evident in each case (Table 3.1.1). The largest DLKP chromosomal population was hypertetraploid with 40% of the cells containing 100-110 chromosomes. A single peak was also evident at 30-39 chromosomes. 36% of DLKP-I cells were roughly tetraploid (90-100 chromosomes) and DLKP-M exhibited the closest to normal complement with 60% of cells being hyperdiploid (50-60 chromosomes). DLKP-SQ cells had the most varied chromosome numbers. 36% of the cells had chromosome numbers between 90 and 100 while 27% had numbers between 100 and 110.

A



B

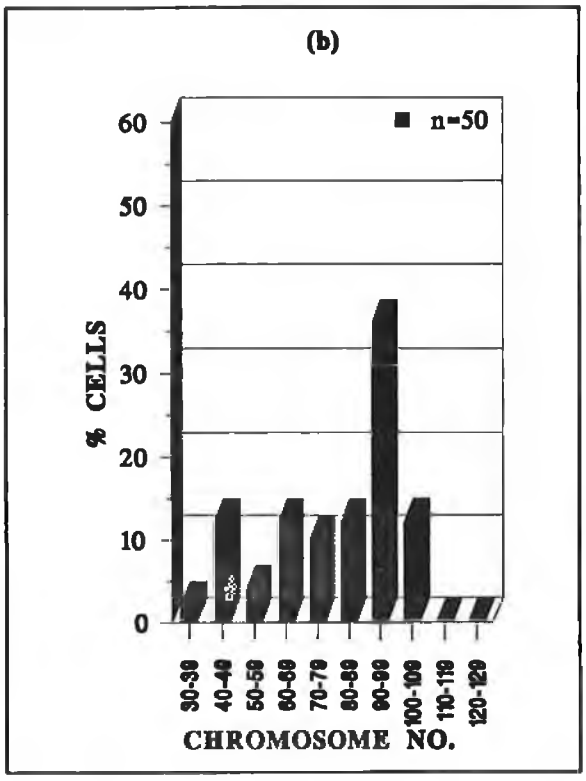
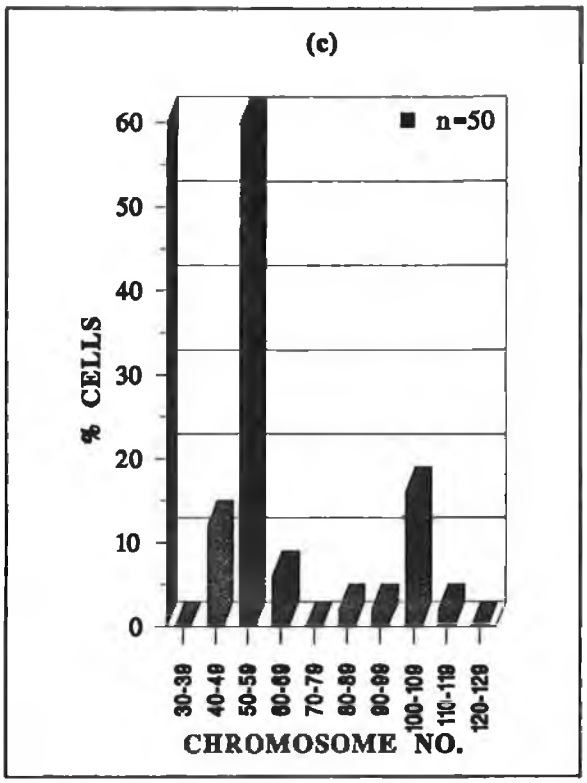


Figure 3.1.2 (continued on next page)

C



D

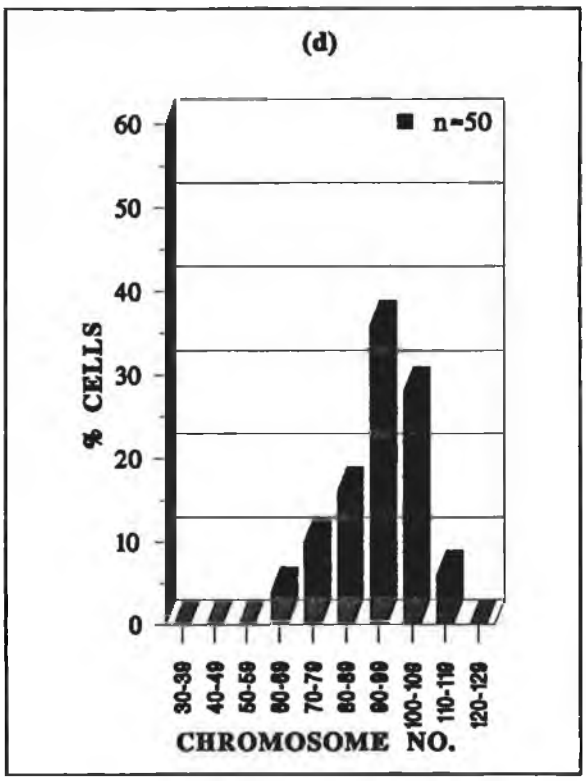


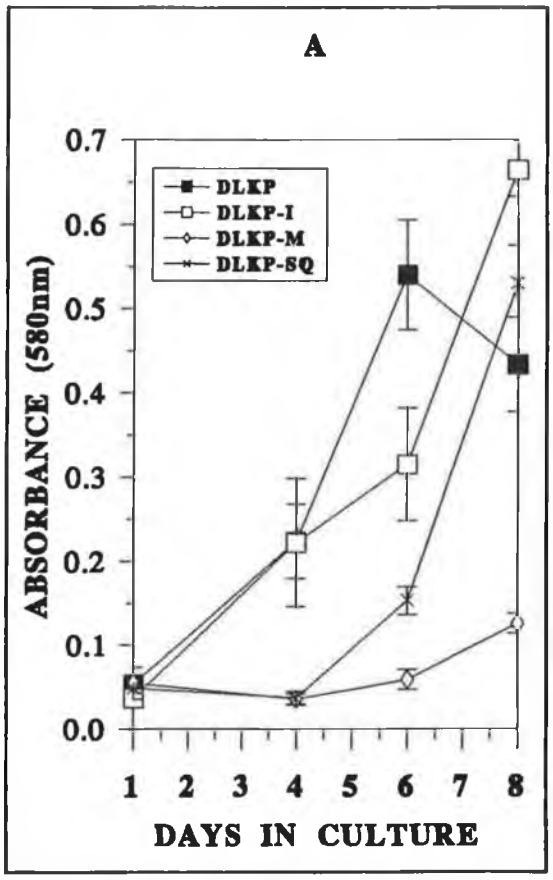
Figure 3.1.2 Range of chromosomal populations determined in **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ.

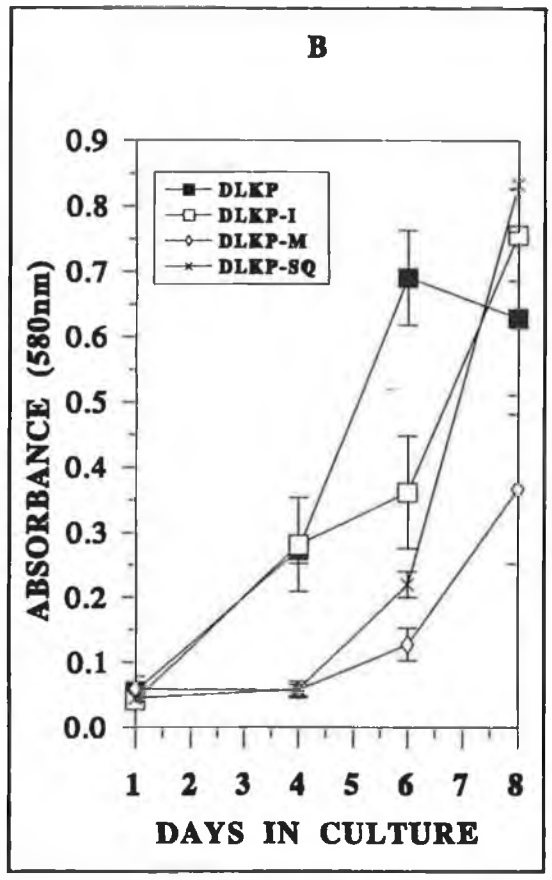
3.1.3 Analysis of Growth of DLKP and Clones

As mentioned in Section 3.1, the 3 morphological cell types present in DLKP and apparently represented by DLKP-I, -M and -SQ can be seen in cultures of DLKP up to passage 80 and above. The ratios of the cell types also appear to remain constant. It therefore seems that a balance exists between the growth potentials of each of the clones and it presumably is of benefit to the mixed parental population that this balance is maintained. Because of this, it was of interest to study the growth capabilities of these isolated clonal populations in comparison to each other and the parental DLKP cells in an attempt to understand the growth properties of individual tumour subpopulations and what role they may play in tumour formation, growth and progression.

3.1.3.1 Growth in serum

The growth curves of DLKP, DLKP-SQ, -I and -M in monolayer culture were determined in the presence of 0%, 1% and 5% serum (see Section 2.9.1). At each serum concentration, the parental DLKP cells proliferated faster than any of the clones (Figure 3.1.3). Of the clones themselves, DLKP-I were the fastest growers in this assay having growth curves almost equal to those of DLKP. DLKP-SQ and DLKP-M grew relatively poorly in the first few days of culture, appearing to be density dependent, but after this this slow start, their growth rates increased also.





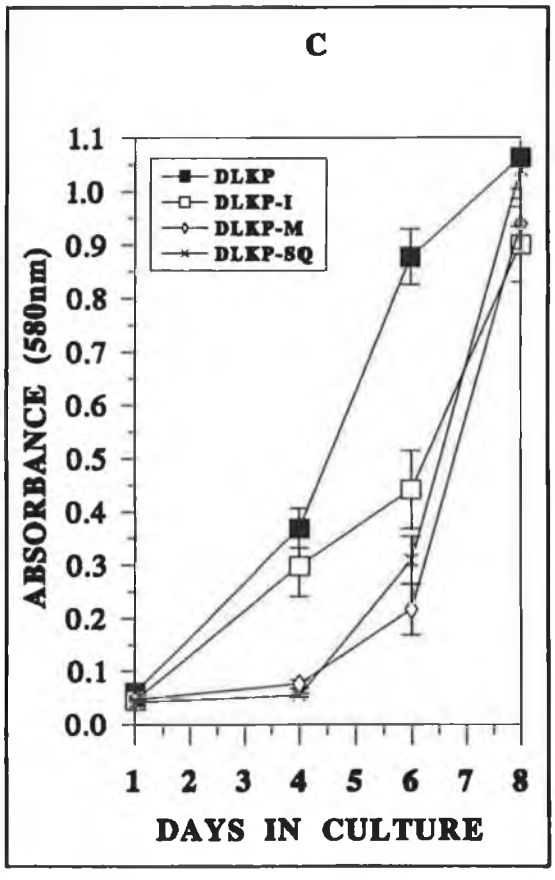


Figure 3.1.3 Growth of DLKP and clones in a monolayer assay in **A.** 0% serum, **B.** 1% serum and **C.** 5% serum.

3.1.3.2 Growth in serum-free medium

Analysis of the growth of cells in defined serum-free medium (SFM) is often of greater interest and more informative than their growth in serum-containing media as a more precise knowledge of the growth requirements of the cells is gained. The SFM used in these experiments was an adaptation of that used by Mendiaz et al., (1986). The only mitogen present in this medium is insulin which is supplemented with transferrin, non-essential amino acids, trace elements and L-glutamine (see Section 2.9.3).

When DLKP and the clones were placed in SFM, all cells were seen to attach rapidly (within 1 hr.) to the surface of the flask. After 5-6 days, the appearance of each of the 4 lines differed from each other (Figure 3.1.5). In the case of DLKP, most of the cells, although attached to the flask surface, had not spread but remained rounded and so the squamous morphology they exhibited in serum-supplememted medium was not evident. However, about 25% of the cells were growing as colonies which resembled the morphology of DLKP-I colonies under normal culture conditions (*i.e.* medium + 5% serum). The DLKP-I cells began to form colonies within 3-4 days of transferral to SFM, and these colonies were also very similar to those of DLKP-I in serum-containing medium. DLKP-M cells in SFM also resembled their serum-supplemented counterparts. These cells were elongated with neurite-like processes present on some cells, and no colony formation occurred. The entire DLKP-SQ population, when placed in SFM, remained rounded in appearance, failed to form colonies and resembled the round phenotype present in DLKP-SFM cultures.

The proliferation of the cell lines was determined every 6 - 7 days. The numbers of DLKP-SQ and -M cells failed to increase above seeding densities at any stage, regardless of how often they were fed or how long the cultures were maintained for. In contrast, DLKP and DLKP-I grew well under the SFM conditions and were cultured for up to 10 passages at a time. The extent of growth of the 2 lines varied, but in general, the numbers of DLKP-I cells were higher than DLKP (Figure 3.1.4).

As described above, the morphologies of DLKP and DLKP-I when cultured in serum-

free medium were different to their normal, serum-supplemented counterparts. Increasing numbers of cells growing in suspension appeared with time in both cell lines. In order to compare the morphologies of the cells grown in serum-containing and serum-free media and to determine if culture in serum-free medium affected the morphologies of the DLKP and DLKP-I cells, samples of each cell line were placed into flasks containing serum-supplemented medium after 5 and 10 passages in serum-free medium. The cells were allowed to attach and after 6-7 days, when reasonably large colonies had formed, the morphologies of the cells were examined under the microscope. No change in morphology was apparent in cells which had been cultured for 5 passages in SFM compared with their morphologies prior to serum-free culture (Figure 3.1.6). However, changes were apparent in those cells which had been cultured for 10 passages in SFM (Figure 3.1.6). The DLKP cells had become more homogenous with the majority of cells (>90%) being squamous in appearance, similar to the DLKP-SQ morphology. Relatively few colonies of DLKP-I-like morphology, and no cells of DLKP-M-like morphology were evident. The morphology of the DLKP-I cells had also altered after 10 passages in SFM. The population had become more heterogenous, with large numbers of DLKP-SQ-like cells evident.

Culture in serum-free medium therefore seemed to influence the morphologies of the 2 cell lines and drive them towards a squamous-like morphology similar to DLKP-SQ. However, since DLKP-SQ cells failed to proliferate in serum-free medium, the squamous-like cells appearing in serum-free culture are not identical to DLKP-SQ.

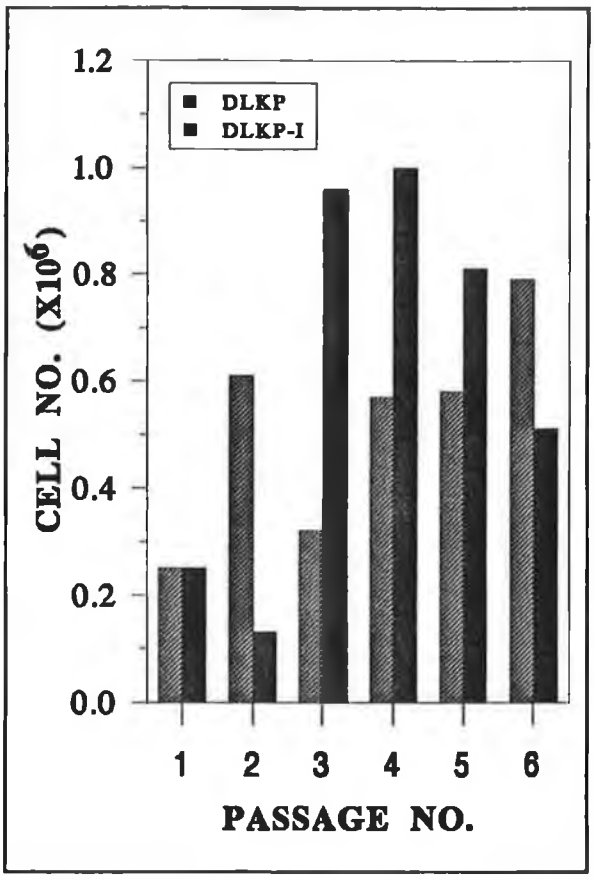


Figure 3.1.4 Growth of DLKP and DLKP-I in SFM. Flasks were inoculated with $2.5x10^5$ cells and growth was determined every 7 days by counting. Flasks were then re-seeded at original inoculating densities.

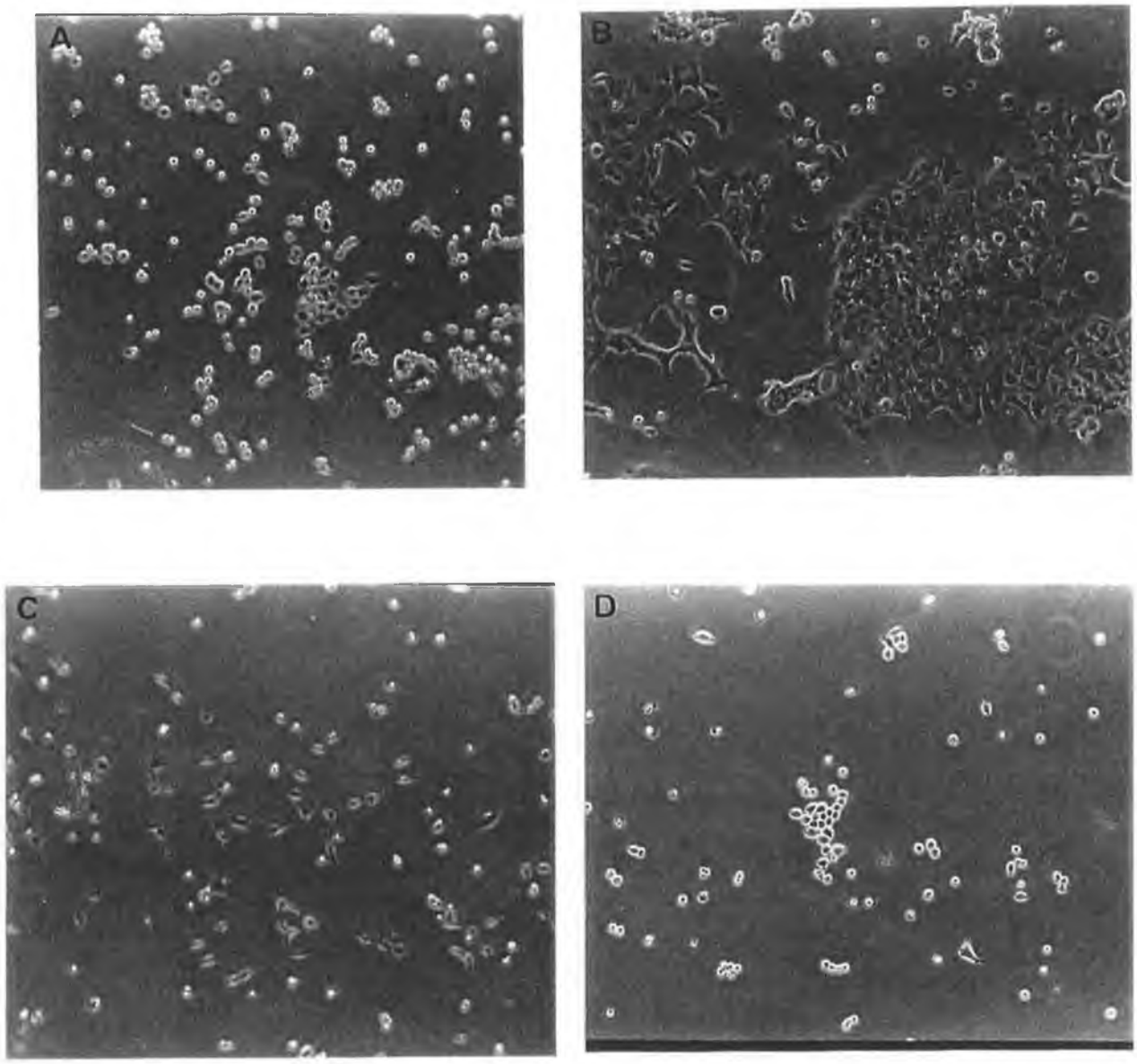


Figure 3.1.5 Appearance of cells when cultured in SFM: **A.** DLKP, passage 5 in SFM, **B.** DLKP-I, passage 6 in SFM, **C.** DLKP-M, passage 1 in SFM and **D.** DLKP-SQ, passage 1 in SFM. x100.

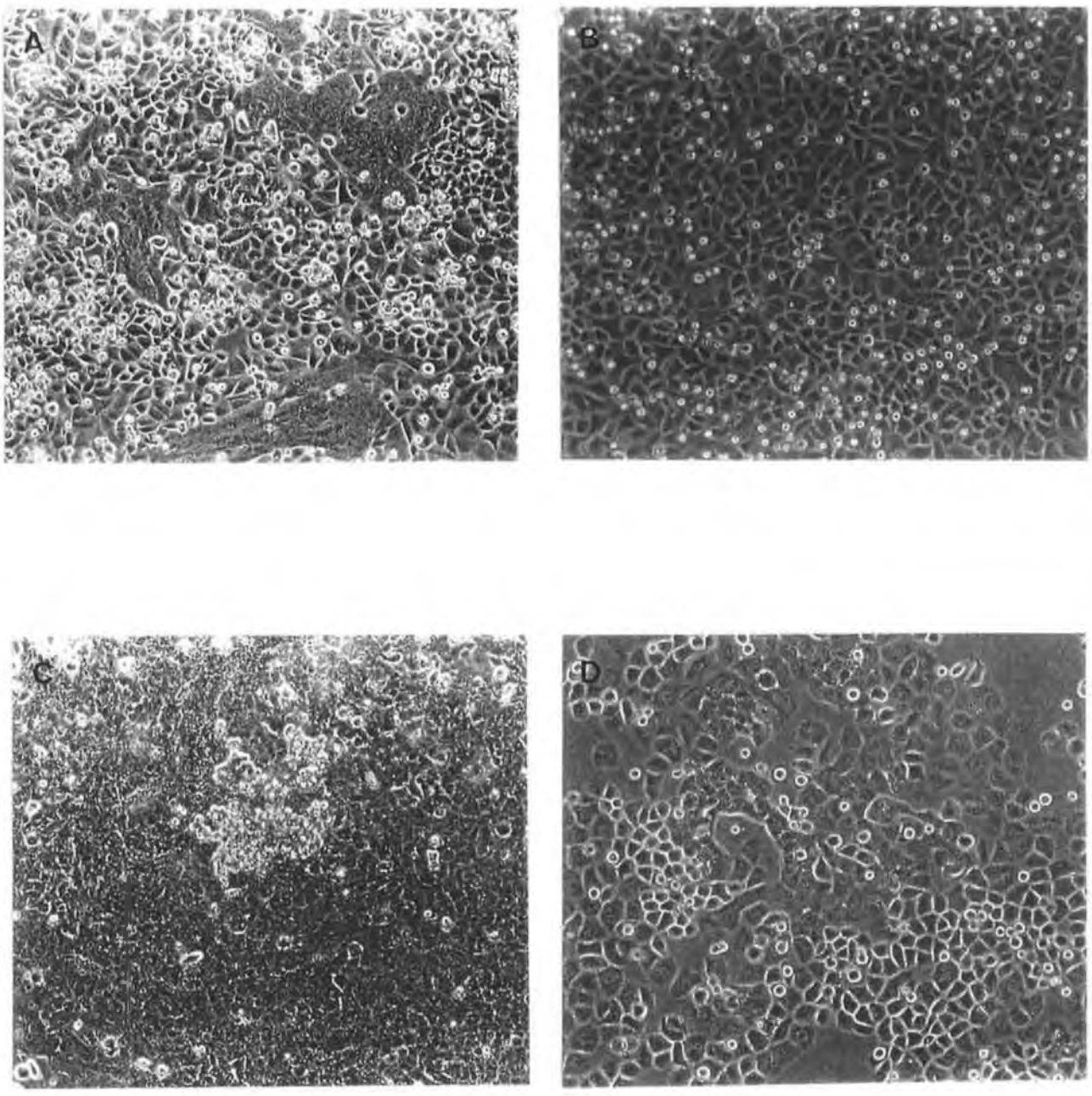


Figure 3.1.6 Appearance of DLKP cells in serum-containing medium after **A.** 5 passages (x100 magnification) and **B.** 10 passages (x200) in SFM and appearance of DLKP-I cells after **C.** 5 passages (x100) and **D.** 10 passages (x200) in SFM. x100.

3.1.3.3 Growth in soft agar

Many tumour cells have the ability to form 3-dimensional colonies in soft agar and this characteristic reflects their malignant clonogenic potential. The ability of DLKP and each of the clones to form colonies in 0.3% soft agar assays was determined (see Section 2.9.4). Colonies greater than 20μM in diameter were counted and a large difference in the CFEs of the lines was observed (Table 3.1.2).

The parental DLKP cells proved to have the highest CFE with a value of 37.58% $\pm$ 7.72%. Of the 3 clones, DLKP-SQ had the best CFE (26.78% $\pm$ 0.44%), with DLKP-M having an intermediate CFE value of 13.08% $\pm$ 0.27% and DLKP-I having effectively no ability to form colonies in soft agar (2.45% $\pm$ 2.04%). The poor growth of DLKP-I cells in agar is surprising, given their rapid growth in monolayer and in serum-free medium.

During the course of this assay, the cells (normally maintained at 37^{0}C) were suspended in agar at 41^{0}C. Therefore, a relatively heat-sensitive cell type may perform deceptively poorly in this assay and fail to form colonies as a result their exposure to increased temperatures. To examine this possibility with regard to the low CFE of DLKP-I compared with the other cell lines, the following experiment was carried out:- suspensions of DLKP, DLKP-I, -M and -SQ cells were incubated in a water bath at 42^{0}C for 15 min. (note soft agar assay conditions less severe; agar was pre-warmed to 41^{0}C, transfered to a laminar flow and would have been cooling towards room temperature when cells were suspended in it). The cells were then placed in normal tissue culture flasks and incubated at 37^{0}C, as in routine cell culture. After 5 days, the flasks were examined microscopically and all 4 lines appeared to be growing normally (Figure 3.1.7). All lines were subsequently passaged successfully and no deleterious effects of the temperature treatment were apparent in any of the lines, including DLKP-I. Therefore, these cells did not appear to be more adversely affected than the other 3 lines by increased temperatures up to 42^{0}C.

Cell line	Largest chromosomal population	% Total cells counted
DLKP*	100-110	40
DLKP-I	90-100	36
DLKP-M	50-60	60
DLKP-SQ	90-100 100-110	36 27

Table 3.1.1 Chromosomal distribution in DLKP and clones (n = 50, *n = 75).

Cell line	CFE (%)
DLKP	37.58 ± 7.72
DLKP-I	2.45 ± 2.04
DLKP-M	13.08 ± 0.27
DLKP-SQ	26.78 ± 0.44

Table 3.1.2 Colony forming efficiency (CFE) of DLKP and clones in soft agar. Colonies greater than 20μM in diameter were counted microscopically and expressed as a percentage of the total number of cells plated. The results shown are mean results from 2 separate experiments.

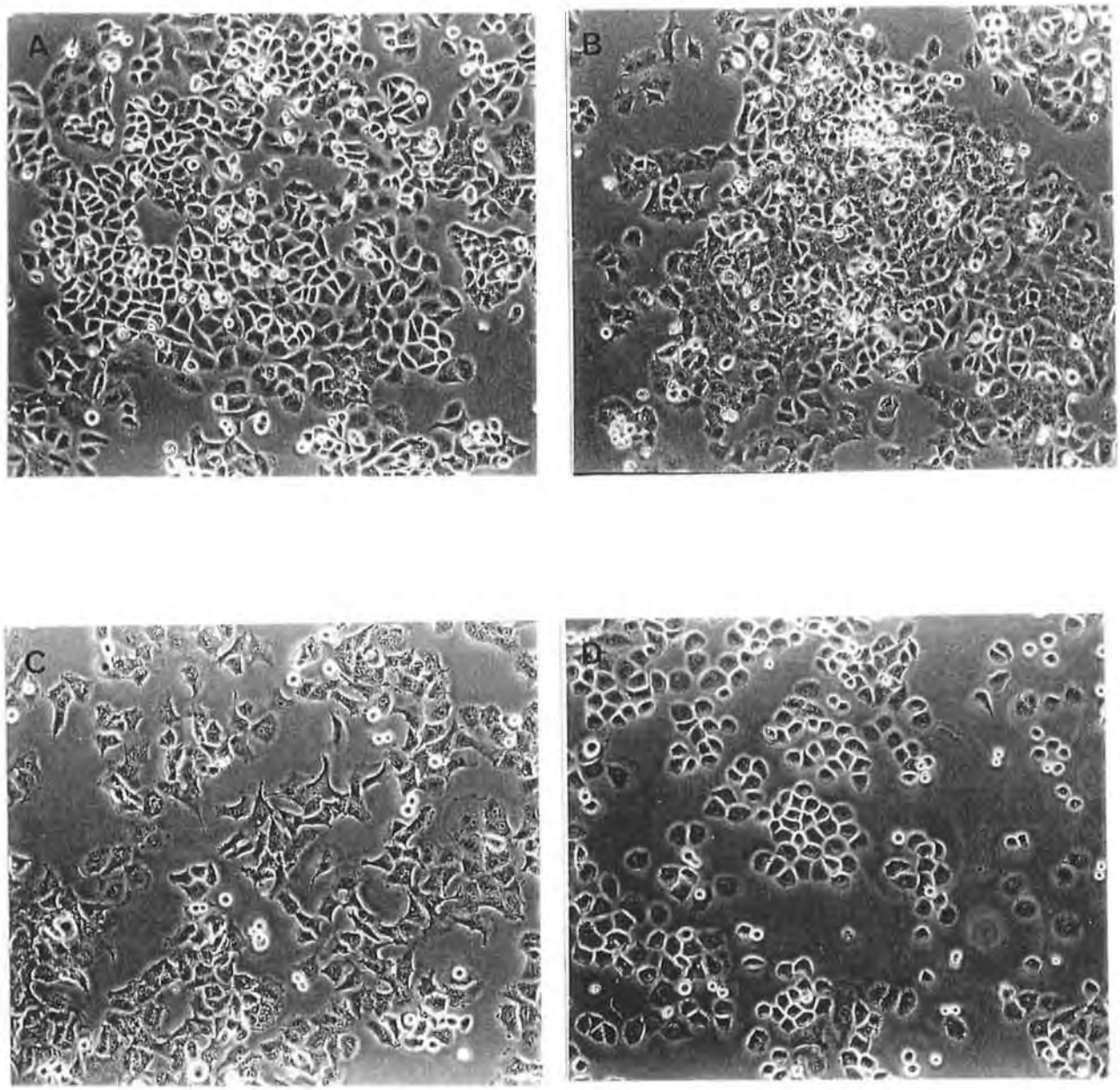


Figure 3.1.7 Morphologies of **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells when cultured following incubation at 42^{0}C for 15 min. x100.

3.1.3.4 Growth in suspension

It had been observed during routine culture that a small percentage of DLKP cells (~1%) grew in suspension in normal culture flasks at any given time. Whenever these cells were decanted and placed in new culture flasks, all cells were seen to attach to the flask. It therefore seemed that growth in suspension was possible for some, if not all, DLKP cells and that these suspension cells could also attach to a substrate.

The ability of DLKP and clones to grow in suspension in spinner flasks was examined as outlined in Section 2.9.5. Flasks were coated with a siliconising agent to prevent cell attachment to the flask surface and magnetic stirrers rotated a rod inside each flask which kept the cells in suspension.

The DLKP cells were found to be the most efficient of the 4 lines at growing in suspension in spinner flasks (Figure 3.1.8). DLKP-SQ were found to grow better than the other 2 clones, paralleling the result in soft agar. All lines grew in clumps up to ~1mm in diameter.

To determine if the lack of substrate adherence and enforced growth in suspension affected the morphology of the cells in any way, aliqouts of each cell line were placed in tissue culture flasks after passages 5 and 10 in spinner flasks. After a few days, morphologies of the resulting attached cells were examined under the microscope. The morphologies of cells examined after 5 passages in spinner flasks seemed unchanged. However, after 10 passages it appeared that the DLKP population had become homogenous with all the cells having the squamous phenotype of DLKP-SQ. DLKP-SQ itself appeared unchanged while DLKP-I appeared mixed, having colonies of both its own and the squamous phenotype (Figure 3.1.9). DLKP-M also appeared unchanged. It would thus seem that growth of these cells in suspension causes an interconversion towards the squamous phenotype represented by DLKP-SQ.

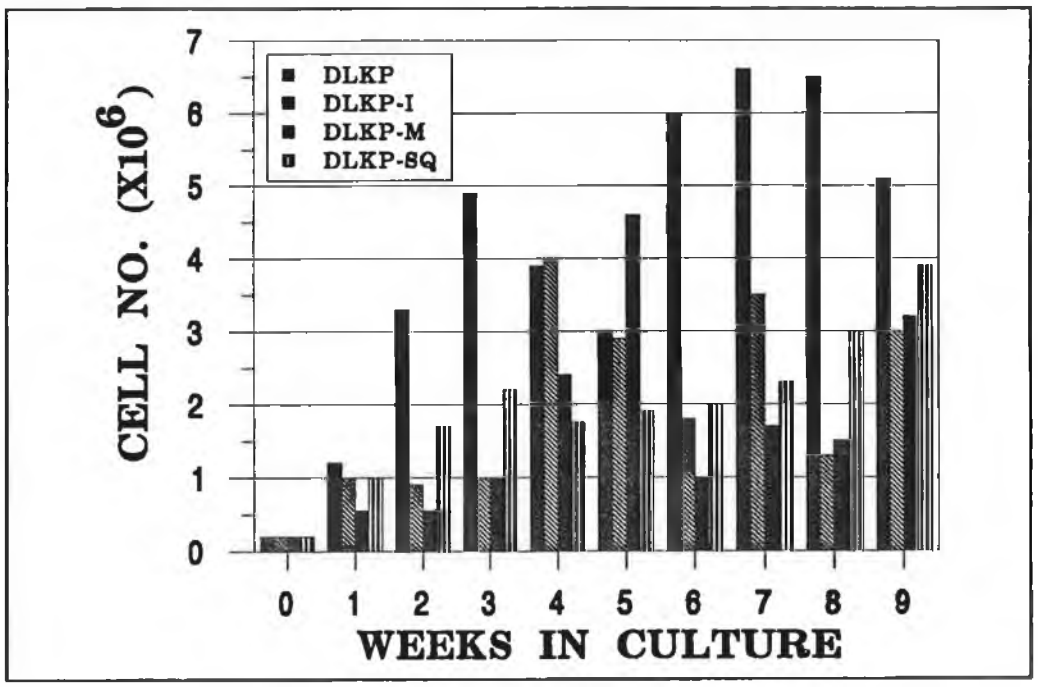


Figure 3.1.8 Growth of DLKP and clones in spinner flasks. Flasks were initially inoculated with $4x10^4$ cells/ml and growth was determined every 7 days by counting. Flasks were then re-seeded at original inoculating density.

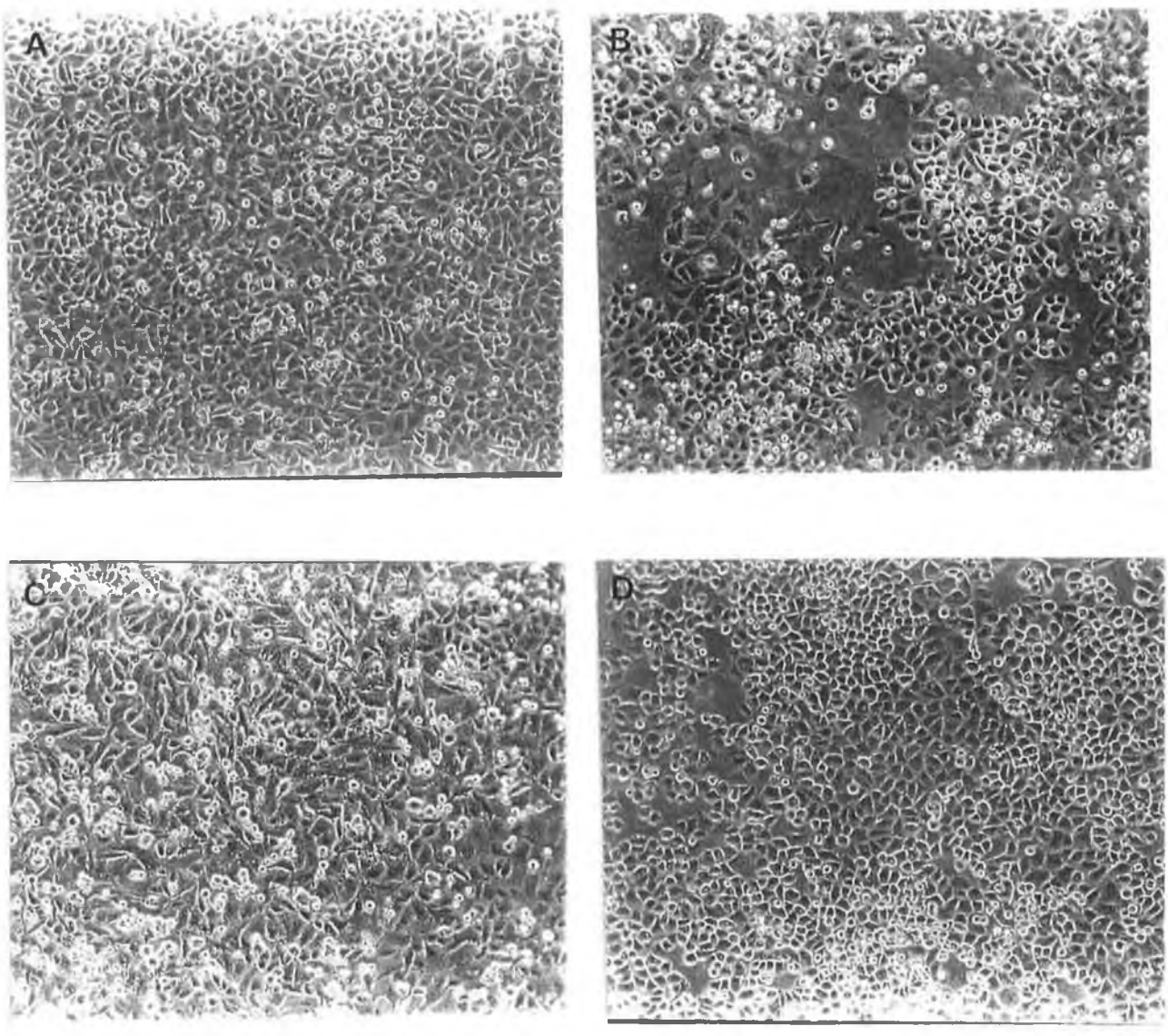


Figure 3.1.9 Appearance of **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ in monolayer culture after 10 passages growing in suspension in spinner flasks. x100.

3.1.3.5 Growth factor assays

Enhanced sensitivity to stimulatory growth factors and/or reduced sensitivity to inhibitory growth factors, as mentioned in Section 3.1.3.4, is an aspect of altered growth control often found in tumour cells. To further explore the growth characteristics of DLKP and the clones, the proliferative responses of the cells to a selection of exogenous growth factors were examined.

TGFβ, EGF, IL-4, insulin, serotonin and histamine were all assayed for their effects on the growth of DLKP and its clones, as described in Section 2.9.6. However, no clear patterns of response were clear for any of the growth factors. Despite strict adherence to standard procedures before, during and after assays such as confluency of cells harvested to set up assays, feeding of cells on the day prior to harvesting, use of specific multichannel pipette to avoid inter-pipette differences, same stocks of serum and growth factors used during course of assays, same length of time assays incubated for and consistent staining procedure, extensive variation was found both in the nature of response *i.e.* stimulatory, inhibitory or no effect, and in the extent of such responses (results not shown). Various assays were carried out using 0.5% serum background, 0% serum background and 0% serum + 50ng/ml insulin background in an attempt to achieve more consistent results, but none were successful.

3.1.4 Examination of Autocrine Growth Factor Production

As mentioned in Section 3.1.3.4, the inappropriate expression of growth factors and/or their receptors by tumour cells is one way in which such cells achieve enhanced growth and proliferate at faster rates than normal cells. In an attempt to detect any such autocrine growth stimulatory activity in the DLKP cell line or its clones, a range of conditioned medium (CM) assays were carried out. CM was collected as outlined in Section 2.10.

3.1.4.1 Initial detection of DLKP autocrine activity

Preliminary experiments with CM collected from DLKP cells grown in Hams F12 with 1% L-glutamine and without serum (as outlined in Section 2.10), were carried out on DLKP cells plated at 2 different seeding densities ($4x10^2$ and $5x10^2$ cells/ml) and with 2 different background serum concentrations (1% and 2% serum). The results (Figure 3.1.10) suggested that a growth stimulatory activity was present in the CM. The stimulatory effect ranged from 51.7% to 14.6% increase in growth over control levels, depending on the assay parameters.

3.1.4.2 Further examination of DLKP autocrine activity

In the light of the results found in Section 3.1.4.1, further work was carried out examining the apparent autocrine activity present in DLKP CM. Assays carried out with DLKP CM on another cell line, Hep 2 (a human cell line derived from a carcinoma of the larynx) consistently resulted in stimulation of the growth of the Hep 2 cells (Figure 3.1.11). In addition, neat CM collected from another cell line, RPMI 2650 (a human nasal carcinoma cell line) was capable of stimulating the growth of DLKP (Figure 3.1.12). This assay was carried out with DLKP cells plated at 2 different densities:- $3x10^2$ and $7x10^2$ cells/ml.

However, when DLKP CM was subsequently assayed for autocrine activity on DLKP cells, no stimulation of growth was seen, contrasting with initial experiments (Section 3.1.4.1). In an attempt to detect any stimulatory activity, the DLKP CM was concentrated by ultrafiltration through an R1,000 membrane (see Section 2.10.1). Figure 3.1.13 shows the results of a typical experiment. Contrary to stimulation, inhibition of growth was seen with CM that was 10-fold more concentrated than untreated neat CM (10x CM). Dilutions of the 10x CM were made with Hams F12 and the inhibitory activity was found to decrease with increasing dilution from 7x to 1/10x.

CM collected from both flasks and roller bottles was assayed (Figure 3.1.14) to allow for possible differences in CM collection methods that may affect the CM, such as movement or greater cell numbers in roller bottles *etc.* No stimulation of growth was detected in CM collected by either method.

Up to this point, untreated Hams F12 had been used as a negative control (CM collected in Hams F12). An ultrafiltered Hams F12 control had not been assayed because the largest molecule present in this basal medium is phenol red (<700kD) and as all molecules less than 1,000 kD should pass through the filtration membrane, the Hams F12 should merely reduce in volume (*eg.* 100ml → 10ml) without becoming concentrated at the same time (*i.e.* 1x → 1x). However, when a sample volume of Hams F12 was ultrafiltered to a 10-fold reduction in volume and assayed in the DLKP CM assay, it was found to be inhibitory to the growth of the cells when compared with

Hams F12 that was not ultrafiltered (Figure 3.1.15). 10x-Hams F12 was also inhibitory to Hep 2 cells (Figure 3.1.15), compared with non-ultrafiltered Hams F12. It therefore appeared that the inhibitory effect of 10x-CM, concentrated through a 1,000 MW cut-off filter, on DLKP cells was probably due, at least in part, to concentration of the Hams F12 basal medium.

An alternative explanation for the inhibitory activity of the ultrafiltered samples was that despite the pre-treatment of the membranes themselves (Section 2.10.2), contaminants were still present on them which entered the filtered samples and were either inhibitory or toxic to the cells. To examine this possibility, 2 separate batches of Hams F12 ('A' and 'B') and a volume of a different basal medium, MEM, were ultrafiltered through a single membrane as follows:- after normal membrane pre-treatment, Hams F12 'A' was filtered, 100ml → 10ml; 100ml ddH_2O was then run through the membrane to clear it; MEM was filtered, 100ml → 10ml; membrane was cleared with 100ml ddH_2O; Hams F12 'B' was filtered, 100ml → 10ml. The resulting 10x-media and dilutions thereof were assayed on DLKP cells (Figure 3.1.16). Both batches of Hams F12, 'A' and 'B', were found to be inhibitory to the growth of DLKP cells with the inhibition being diluted out at 5x and 1x, while the 10x-MEM had no inhibitory effect on the cells compared with untreated MEM. It would therefore appear that the growth inhibitory effects were a characteristic of ultrafiltered Hams F12 samples and not of all basal media.

It was subsequently decided to focus attention on possibly improving the DLKP CM assay in an attempt to detect stimulatory activity in CM that was not concentrated *i.e.* neat or diluted.

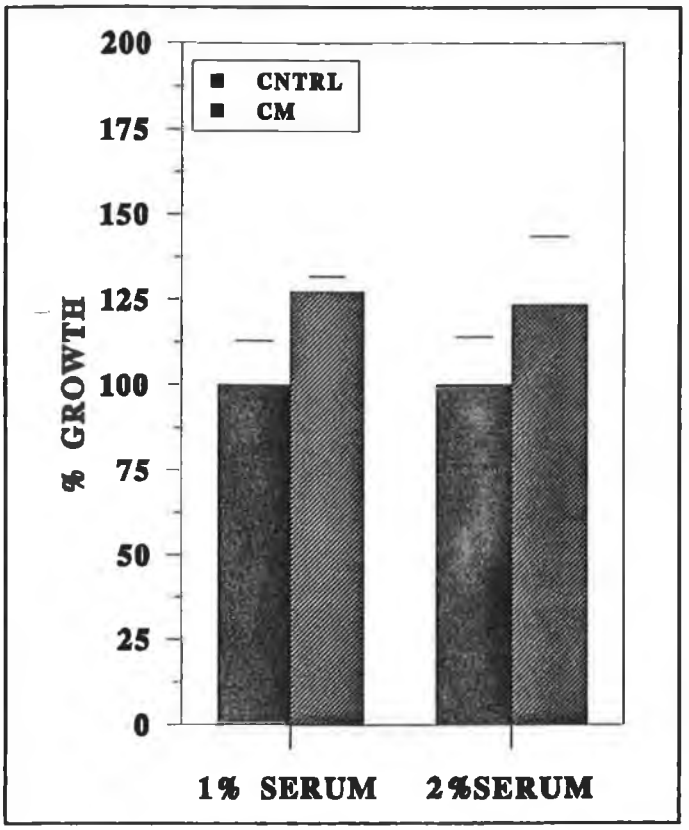
Firstly, the DLKP seeding density was lowered. Normally, cells were seeded at $7x10^2$ cells/ml/well. When seeded at 5, 3 and $1x10^2$ cells/ml, increasing inhibition of growth was found with decreasing seeding densities in the presence of neat DLKP CM compared with neat Hams F12 controls (Figure 3.1.17).

The effect of increased seeding densities on CM activity was then examined. No difference was found between seeding densities up to $1.9x10^3$ cells/ml (Figure 3.1.18).

Assays were carried out on DLKP CM which was diluted up to 1/1,000 to determine if it was necessary to dilute the autocrine activity before it became detectable. No difference in activity was seen over the range of dilutions tested (Figure 3.1.19).

The normal background serum levels for these asays was 1%. To determine if additional factors present in higher concentrations of serum were necessary to detect autocrine activity, the assays were carried out in 2.5, 5 and 7% serum but again, no stimulation was seen (Figure 3.1.20).

A



B

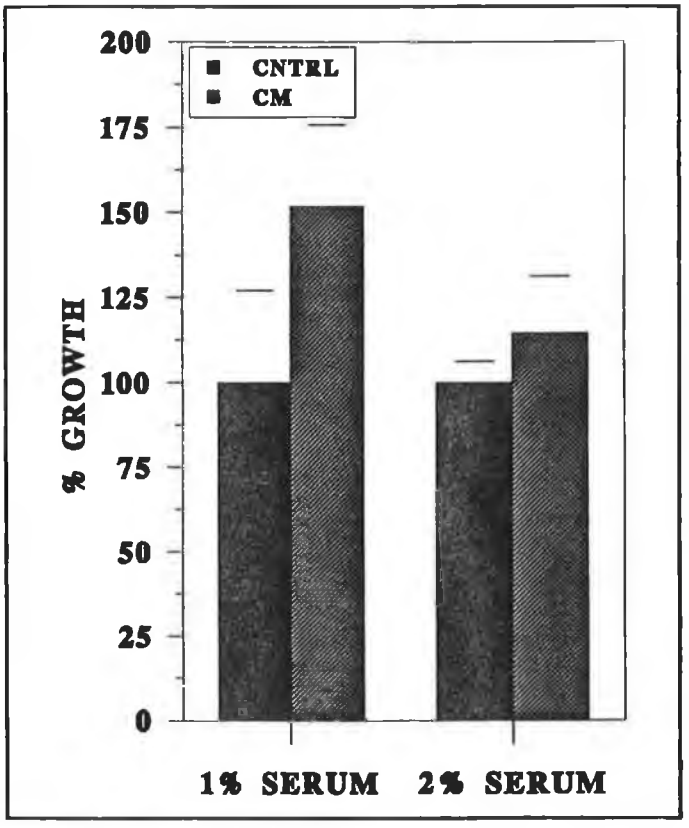


Figure 3.1.10 Effect of DLKP CM on the growth of DLKP cells seeded at **A.** $4x10^2$ and **B.** $5x10^2$ cells per well in 1% and 2% background serum.

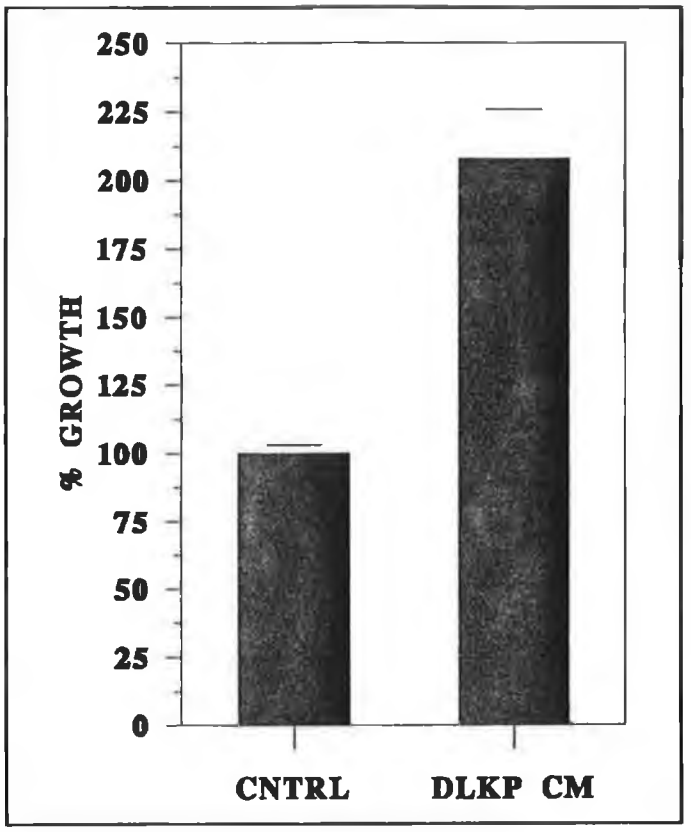


Figure 3.1.11 Effect of DLKP CM on the growth of Hep 2 cells.

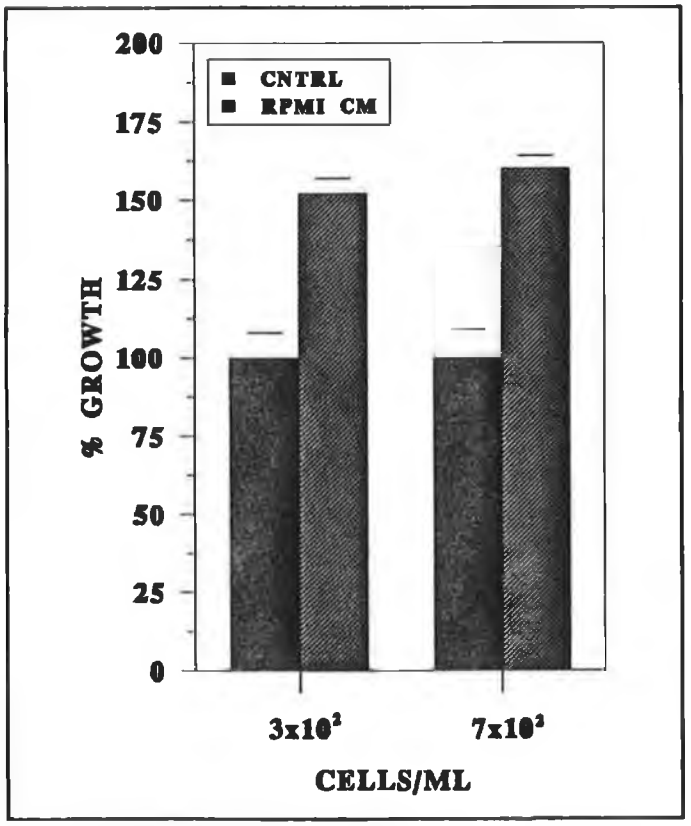


Figure 3.1.12 Effect of RPMI 2650 CM on the growth of DLKP cells.

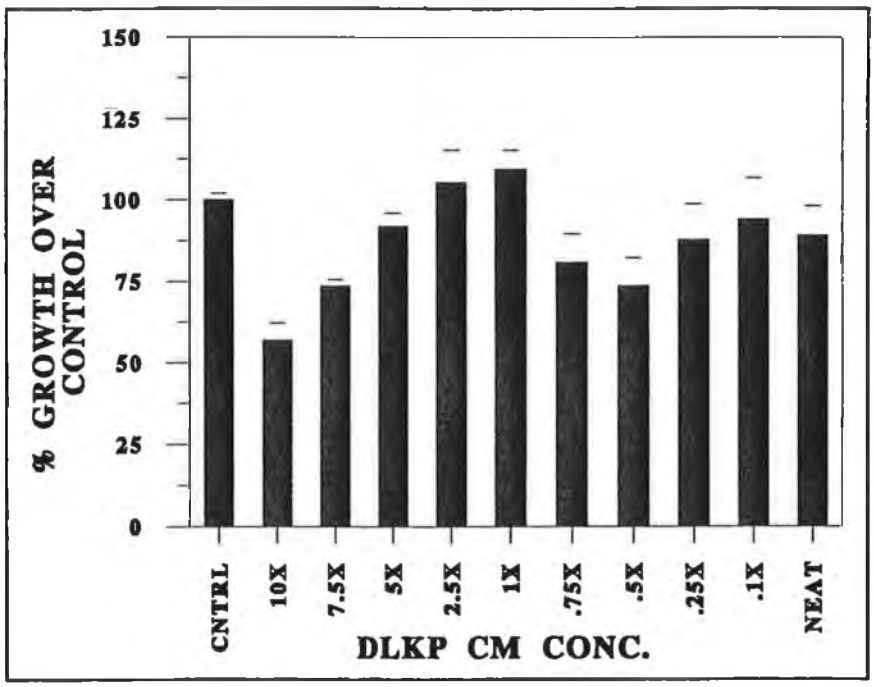


Figure 3.1.13 Effect of varying DLKP CM concentration on growth of DLKP cells.

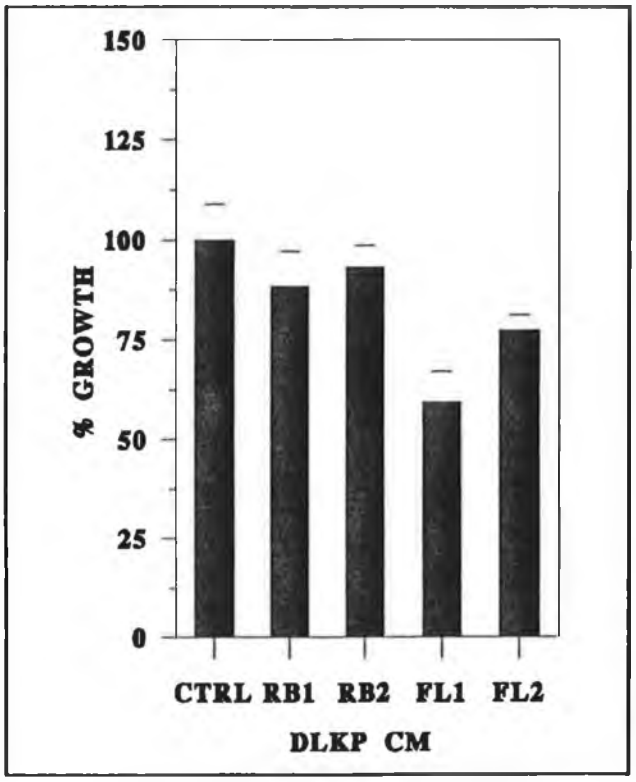


Figure 3.1.14 Effect of mode of collection, roller bottle (RB) or flask (FL), on DLKP CM activity

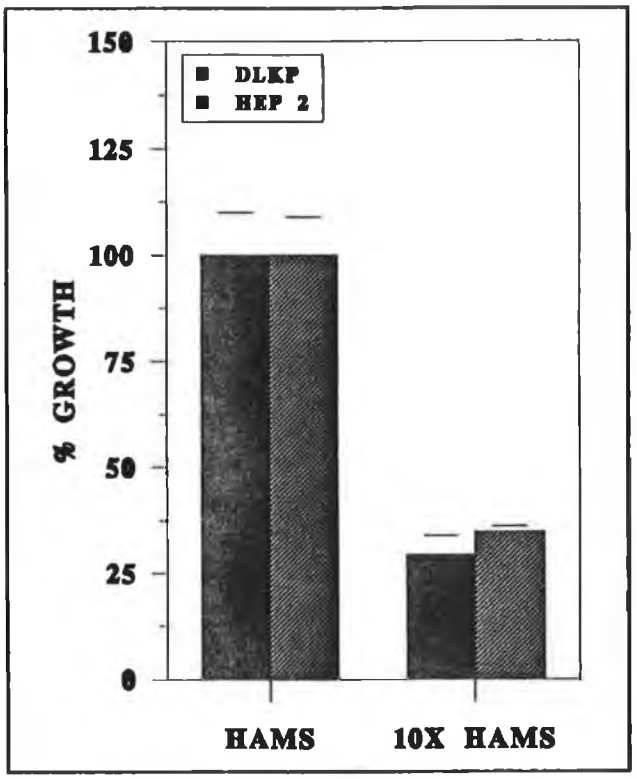


Figure 3.1.15 Effect of 10x Hams F12 on the growth of DLKP cells and Hep 2 cells.

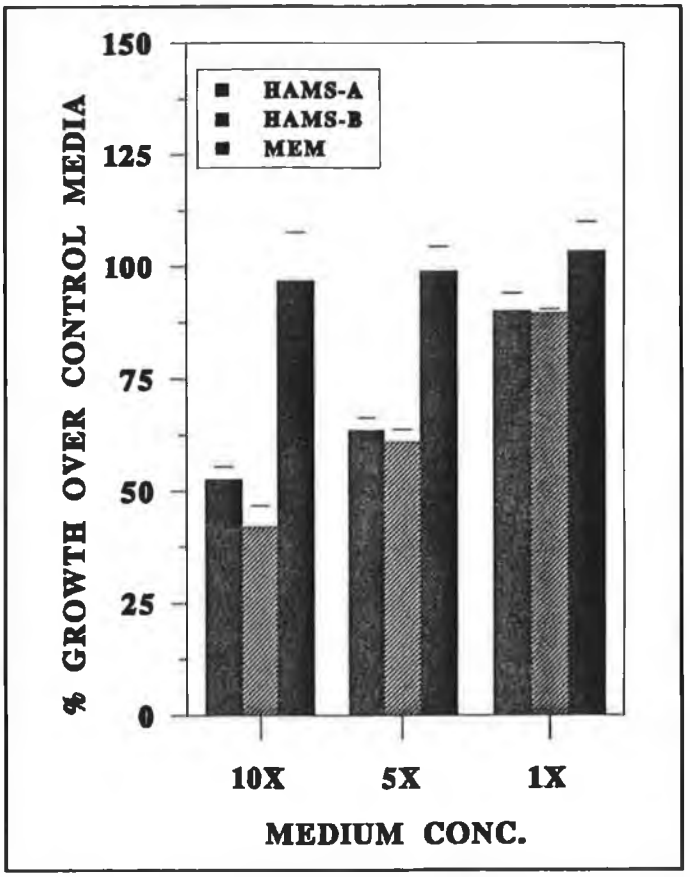


Figure 3.1.16 Effect of ultrafiltered basal media on the growth of DLKP cells.

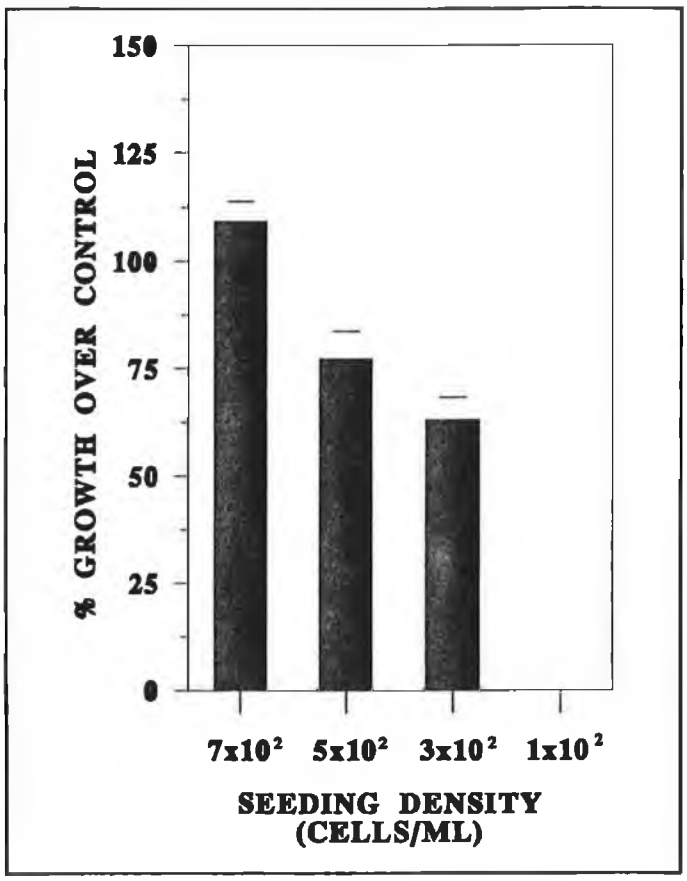


Figure 3.1.17 Effect of lower seeding densities on DLKP CM autocrine activity.

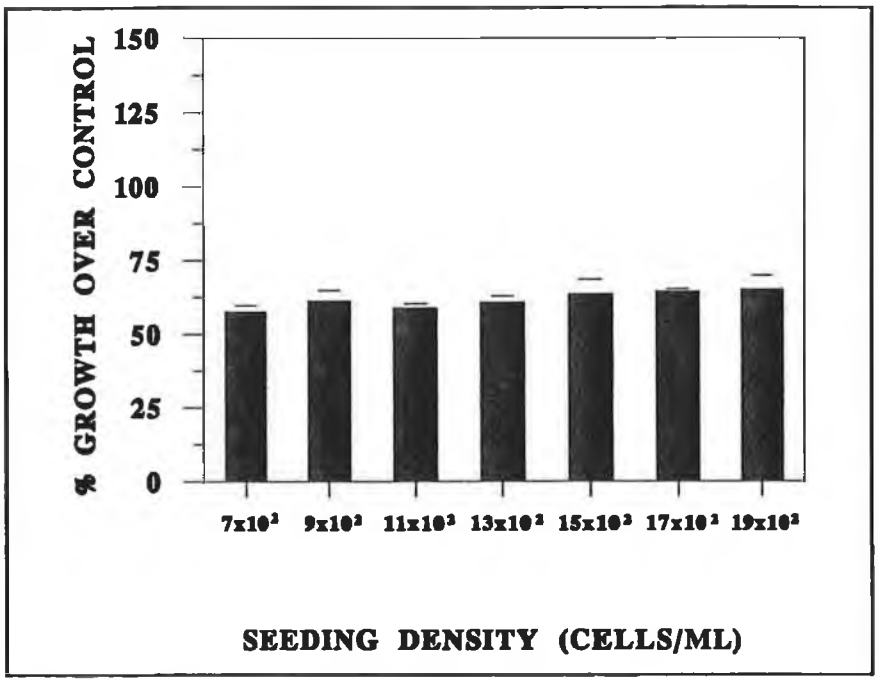


Figure 3.1.18 Effect of higher seeding densities on DLKP CM autocrine activity.

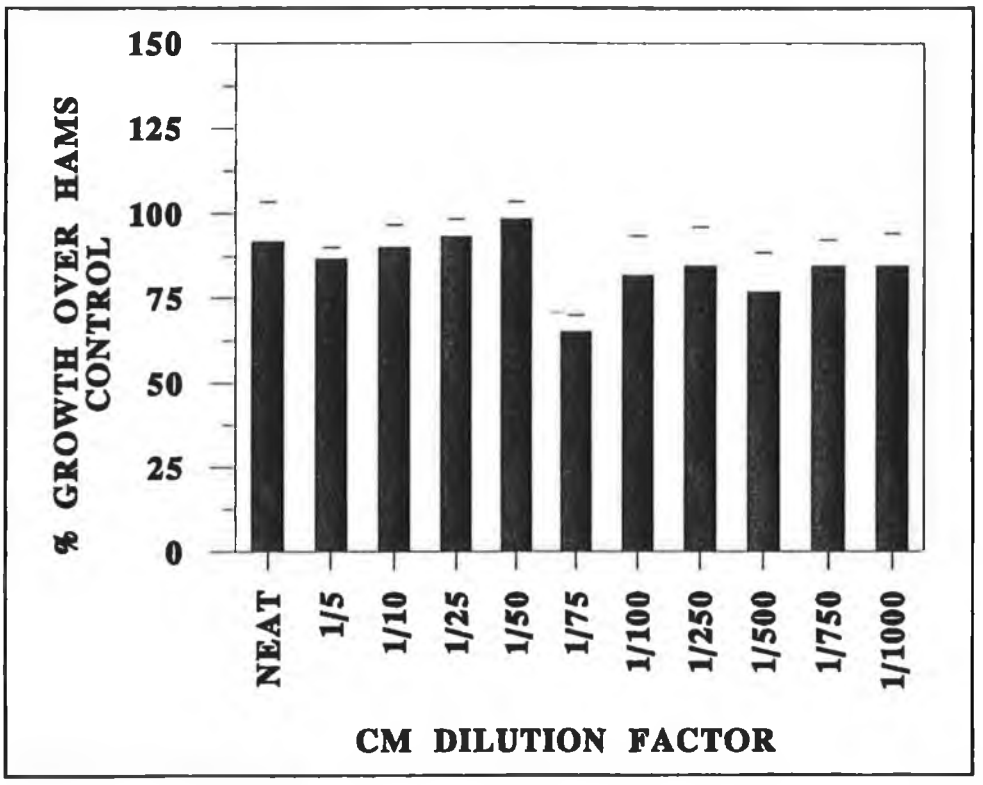


Figure 3.1.19 Effect of dilution of CM on DLKP autocrine activity.

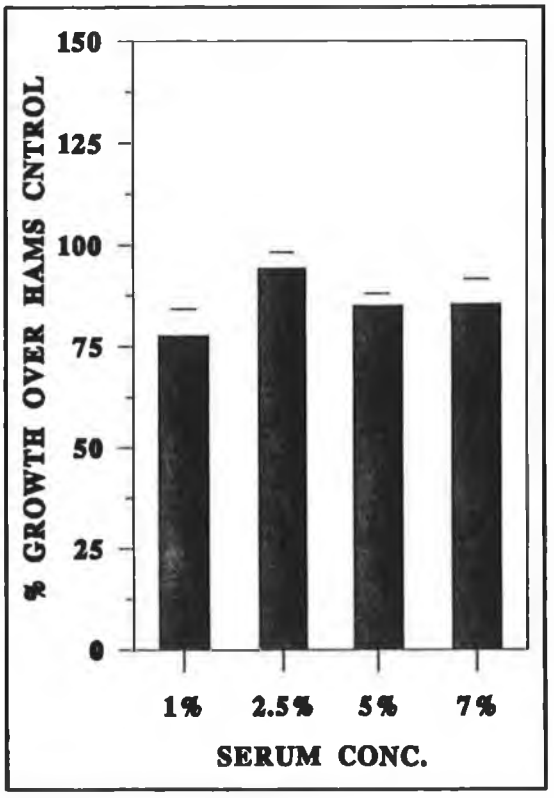


Figure 3.1.20 Effect of serum concentration on DLKP CM autocrine activity

3.1.4.3 Analysis of DLKP clones CM for autocrine activity

Serum-free CM was collected from each of the DLKP clones, as was collected from DLKP, and examined in monolayer assays for growth stimulatory activity (results not shown). The various CM were assayed on the cells from which they were collected and also on the other clones and on DLKP.

Again, as was found with the DLKP CM assays, the results varied with different batches of CM resulting in stimulation, inhibition or no effect in each assay carried out. No correlation was seen between modes of collection (roller bottle *versus* 75cm^2 flask) or length of storage time of CM (always at 4^{0}C) before assaying.

The relatively rapid and aggressive growth of DLKP and its clones in growth assays (Figure 3.1.3) and soft agar assays (Table 3.1.2), their ability to proliferate in the absence of serum (Figure 3.1.3), (particularly in the cases of DLKP and DLKP-I in defined serum-free medium (Figure 3.1.4)) led to the belief that some, if not all, of these cells were producing stimulatory autocrine growth factors. However, no such activity was detectable under the assay conditions described above in Section 3.1.4.

3.1.5 Extracellular Matrix Adherence Assays

In vivo, normal tissue cells interact intimately with the ECM proteins that surround them through cell surface adhesion molecules. The ECM proteins not only anchor the cells and allow them to organise themselves spatially, but also act as important signalling molecules involved in the induction and maintenance of differentiated phenotypes. Tumour cells *in vivo* also interact with ECM proteins but often these interactions are deregulated and alterations in tumour cell adhesion molecules can result in a metastatic cell phenotype which is motile and capable of migration and invasion.

The attachment properties of DLKP and the clones to ECM substrates were compared to determine if differences in their adherence profiles existed. Wells in 24-well plates were coated with the various substrate proteins, after which uncoated sites were blocked with 0.1% BSA (described in Section 2.11). Cells were plated in medium without serum and attachment was determined after 15min. and 30min.

3.1.5.1 Adherence to plastic

The attachment of the cells to uncoated plastic blocked with 0.1% BSA was determined. Here, as with the ECM-coated wells, wells without cells were used as controls. After each time point, all wells were stained with crystal violet dye which, after drying, was eluted with 33% acetic acid (as in CVDE assay, Section 2.9.2) and the absorbances of the resulting solutions were determined spectrophotometrically on an ELISA plate reader. Attachment was expressed as a percentage of control wells which did not contain cells (Figure 3.1.21).

After 15 min. incubation, it was found that the DLKP-M cells had attached to the plastic to a greater extent than the other 3 lines which themselves showed effectively no attachment at this time (Figure 3.1.21.A).

After 30 min., DLKP and DLKP-I showed increased attachment , while DLKP-SQ still showed little adherence (Figure 3.1.21.B).

3.1.5.2 Adherence to collagen type IV

After 15 min. incubation in collagen type IV-coated wells, very little adherence was seen in any of the cell lines (Figure 3.1.21.A).

However, after 30 min., DLKP-M showed increased attachment (160% of control) while DLKP, DLKP-I and DLKP-SQ still remained unattached to the substrate (Figure 3.1.21.B).

3.1.5.3 Adherence to fibronectin

Incubation of the cells in wells coated with fibronectin had the most dramatic effect on cell attachment. After 15 min., DLKP, DLKP-I and DLKP-SQ had each attached 200-250%, while DLKP-M had attached 450% compared with controls (Figure 3.1.21.A).

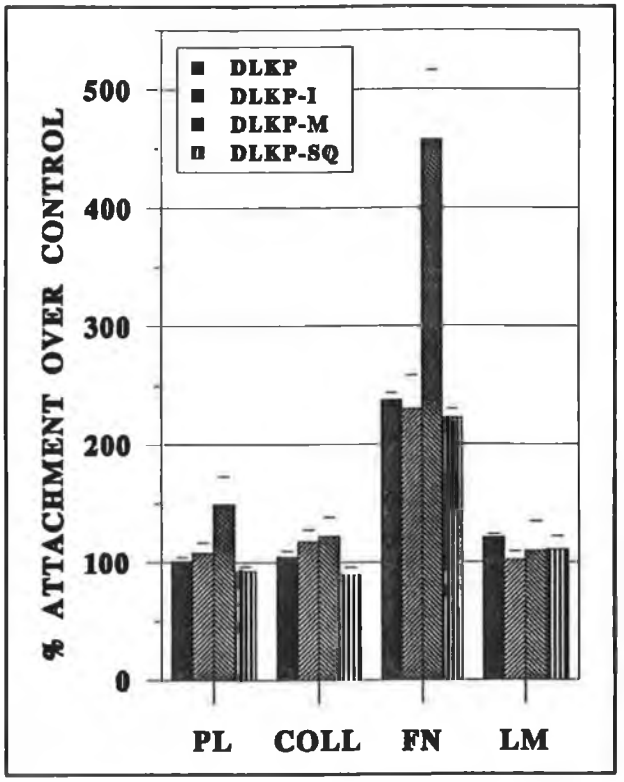
After 30 min., DLKP, DLKP-I and DLKP-SQ had continued to attach up to 300% compared with controls while DLKP-M showed decreased attachment (compared to 15min.) at 340% (Figure 3.1.21.B).

3.1.5.4 Adherence to laminin

Little attachment was seen with any cell line after 15 min. incubation in laminin-coated wells (Figure 3.1.21.A).

However, after 30 min., DLKP, DLKP-M and DLKP-SQ had adhered up to 200% of control values, while DLKP-I still showed no great increase in attachment after this time (Figure 3.1.21.B).

A



B

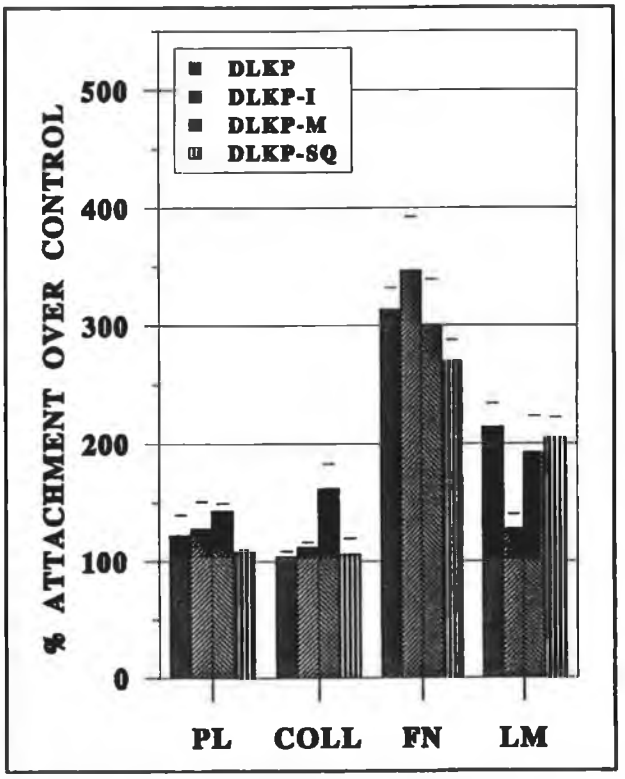


Figure 3.1.21 Attachment of DLKP and its clones to ECM proteins after **A.** 15 minutes and **B.** 30 minutes. (Pl=plastic, Coll=collagen type IV, FN=fibfonectin, LM=laminin).

3.1.6 Sensitivity to Chemotherapeutic Drugs

Several multiple drug resistant (MDR) variants of the DLKP cell line have previously been selected by culturing the cells in increasing concentrations of chemotherapeutic drugs until highly resistant lines were established. These variants not only exhibit a marked increase in drug resistance compared with their sensitive parents but are also cross-resistant to a range of drugs that are structurally unrelated to the original selective agent. For example, the DLKP-A variant line, established by Dr.Alice Redmond, was selectively grown in increasing concentrations of adriamycin but is also several fold more resistant to vincristine and VP16 than DLKP (see Table 3.6.2). These variants have been established as models for the MDR phenotype which can occur *in vivo* following combination chemotherapy and are used to study mechanisms of MDR and possible modes of circumvention.

While it is considered likely that the MDR variants of DLKP arose via adaptation to the progressively increasing concentrations of selecting agent, it is possible that a subpopulation of the sensitive parental DLKP cells were inherently highly resistant and it was these which were selected out. To examine this possibility and to determine if variation existed between the DLKP clones with regard to their MDR profiles, the sensitivity of the clones to the 3 structurally different chemotherapeutic drugs, adriamycin, vincristine and VP16, was determined.

3.1.6.1 Sensitivity to adriamycin

DLKP, DLKP-I, -M and -SQ were exposed to concentrations of adriamycin in the range from 0.6ng/ml to 25ng/ml (see Section 2.12). This was the range over which the toxicity profile of DLKP had previously been determined. The IC_{50}s for DLKP and the clones are shown in Table 3.1.3. The results are the mean results from 4 separate experiments and each experiment was carried out in replicates of 8. Table 3.1.4.1(ii) shows the mean fold difference of each clone w.r.t. the DLKP cell line.

Taking standard deviations into account, no real difference was seen between DLKP, DLKP-M and -SQ. DLKP-I was the most sensitive of the clones to adriamycin w.r.t. DLKP, but the difference is less than 0.5-fold.

3.1.6.2 Sensitivity to vincristine

The vincristine toxicity assays were carried out over a range of concentrations from 0.023ng/ml to 2.5ng/ml. The results shown in Tables 3.1.3 and 3.1.4 are the mean results from 3 experiments. In this case, DLKP-M had an IC_{50} value similar to that of DLKP, as did DLKP-I, although the standard deviation was rather high. DLKP-SQ was approximately 0.5-fold more sensitive than DLKP.

3.1.6.3 Sensitivity to VP16

The toxicity assays with VP16 were carried out over a range of 2.4ng/ml to 250ng/ml VP16. The results shown in Tables 3.1.3 and 3.1.4 are the mean results from 6 experiments. Once more, taking standard deviations into account, very little difference was seen between the 4 lines. DLKP-I had the lowest IC_{50} but this was again less than 0.5-fold sensitive w.r.t. DLKP.

The toxicity profiles of the DLKP clones were thus found to be very similar for the 3 drugs examined and in addition, were found to vary less than 0.5-fold from those of DLKP. Therefore, putative highly resistant populations in DLKP were not represented by any of these clones.

Cell line	Adraimycin (IC_{50}, ng/ml)	Vincristine (IC_{50}, ng/ml)	VP16 (IC_{50}, ng/ml)
DLKP	11.78 ± 5.19	0.52 ± 0.17	30.7 ± 9.8
DLKP-I	5.71 ± 2.72	0.39 ± 0.31	19.2 ± 6.7
DLKP-M	8.67 ± 2.76	0.51 ± 0.26	22.5 ± 12.8
DLKP-SQ	9.55 ± 5.58	0.25 ± 0.09	33.5 ± 8.9

Table 3.1.3 Sensitivity of DLKP and clones to MDR drugs.

Cell line	Adriamycin (fold w.r.t.DLKP)	Vincristine (fold w.r.t.DLKP)	VP16 (fold w.r.t.DLKP)
DLKP	1	1	1
DLKP-I	0.54 ± 0.14	0.65 ± 0.33	0.61 ± 0.12
DLKP-M	0.83 ± 0.26	0.95 ± 0.16	0.74 ± 0.34
DLKP-SQ	0.96 ± 0.45	0.48 ± 0.11	1.10 ± 0.29

Table 3.1.4 The fold differences in IC_{50} values for each clone with respect to (w.r.t.) DLKP shown for each drug examined.

	Adriamycin				
Cell line	Assay 1 (fold)	Assay 2 (fold)	Assay 3 (fold)	Blinded* (fold)	Mean (fold)
DLKP	1	1	1	1	1
DLKP-I	0.50	0.48	0.78	0.42	0.54 ± 0.14
DLKP-M	0.69	0.72	1.28	0.65	0.83 ± 0.26
DLKP-SQ	1.05	0.64	1.67	0.50	0.96 ± 0.45

Table 3.1.5 Results from individual assays carried out with adriamycin showing fold IC_{50} values obtained for each clone w.r.t. DLKP.

	Vincristine			
Cell line	Assay 1 (fold)	Assay 2 (fold)	Blinded* (fold)	Mean (fold)
DLKP	1	1	1	1
DLKP-I	0.49	0.51	1.07	0.65 ± 0.33
DLKP-M	0.84	0.87	1.14	0.95 ± 0.16
DLKP-SQ	0.59	0.38	0.46	0.48 ± 0.11

Table 3.1.6 Results from individual assays carried out with vincristine showing fold IC_{50} values for each clone w.r.t. DLKP.

	VP16						
Cell line	Assay 1 (fold)	Assay 2 (fold)	Assay 3 (fold)	Assay 4 (fold)	Assay 5 (fold)	Blinded (fold)	Mean (fold)
DLKP	1	1	1	1	1	1	1
DLKP-I	0.78	0.68	0.57	0.39	0.65	0.62	0.61 ± 0.12
DLKP-M	0.50	0.44	0.61	0.58	1.31	1.00	0.74 ± 0.34
DLKP-SQ	1.31	0.84	1.50	0.77	1.31	0.84	1.10 ± 0.29

Table 3.1.7 Results from individual assays carried out with VP16 showing fold IC_{50} values for each clone w.r.t. DLKP.

3.2 CHARACTERISATION OF DLKP AND CLONES

If the DLKP cells were to be used as a model for lung cells, malignant or otherwise, it would be necessary to characterise the DLKP cells and each of the clones as thoroughly as possible. To fully understand the relevance and significance of any experiments carried out with these cells, it would be crucial to know as accurately as possible, what class of tumour the cells belonged to. In addition, knowledge of the stage of differentiation the DLKP cells were at before, during and after experiments would allow for a clearer perception of the behaviour of this particular tumour cell type, *in vivo* and *in vitro*.

Therefore, to understand fully the cell system being examined, a broad characterisation study of the DLKP cells was carried out. This study encompassed functional, ultrastructural and biological aspects of lung epithelial cell biology.

3.2.1 Electron Microscopy

Electron microscopy (EM) is the most definitive method presently available to study and characterise a cell type. The organelles contained by a cell can be determined and localised and staining procedures can be carried out to identify material contained within a cell. The organelle complement of a cell is specific to the function of that cell and therefore, EM allows accurate diagnosis of cell type.

Suspensions of each cell line - DLKP (P7), DLKP-I (P21), DLKP-M (P34) and DLKP-SQ (P34) - were fixed in glutaraldehyde (Section 2.13). Subsequent processing and EM analysis was carried out by Dr.David Dinsdale, University of Leicester.

The cells were found to contain no characterising organelles such as dense core granules or lamellar bodies which are indicative of NE cells and type II cells respectively (see Figure 3.2.1). In fact, the cells contained very little ultrastructural detail apart from the nucleus, mitochondria *etc*. No differences were found between any of the 4 DLKP lines examined. Significantly, no difference was seen between the relatively early passage DLKP cells (P7) and the later passage numbers of DLKP-M and DLKP-SQ (P34). The results indicated that the cells were at a very early stage of differentiation, similar to basal or stem cells which would also lack differentiation-specific organelles.

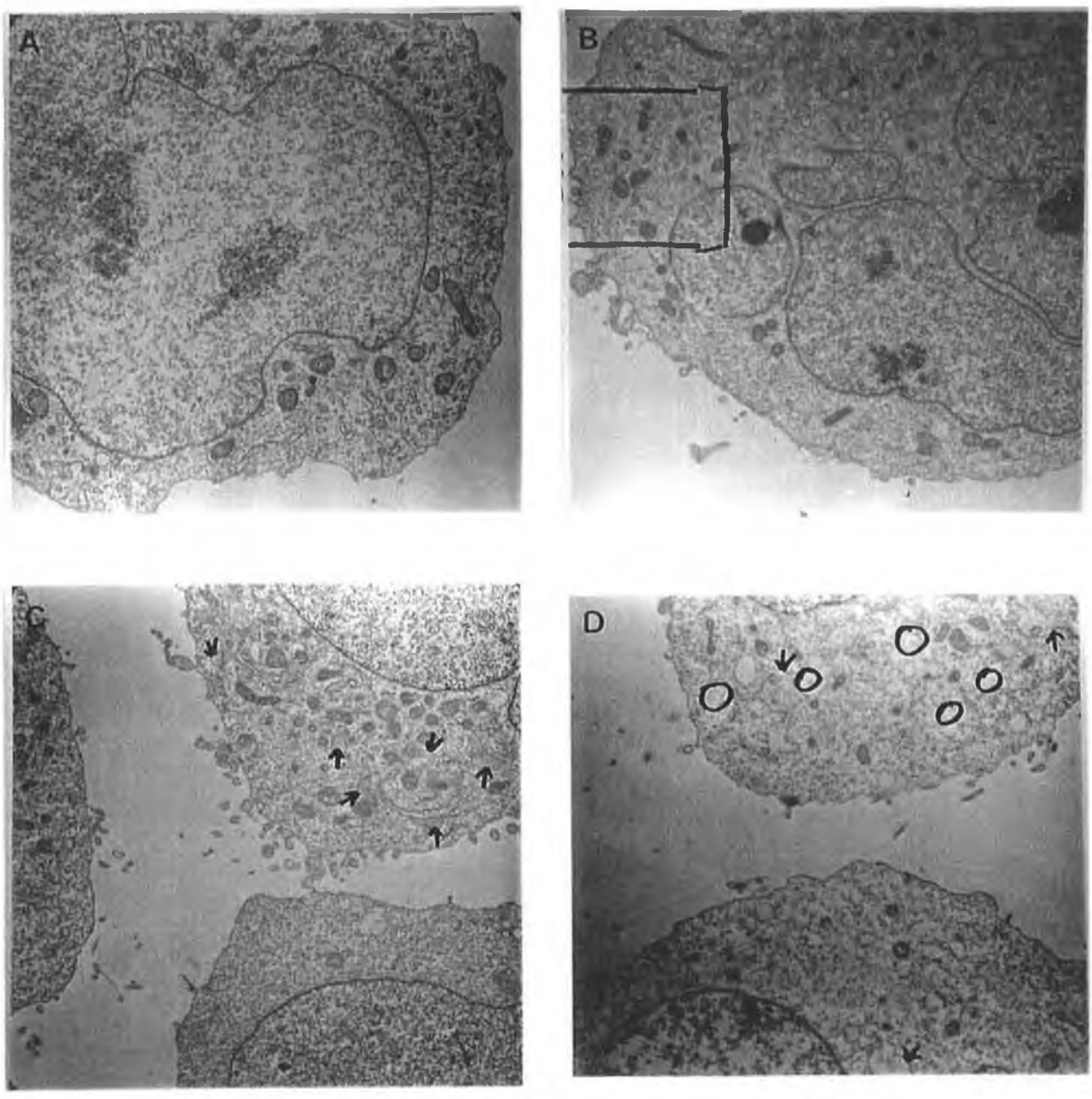


Figure 3.2.1 Ultrastructural features of **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells demonstrated by electron microscopic analysis. Arrows indicate possible dense core vesicles but these occurred extremely infrequently among all of the cells examined. (Magnification unknown).

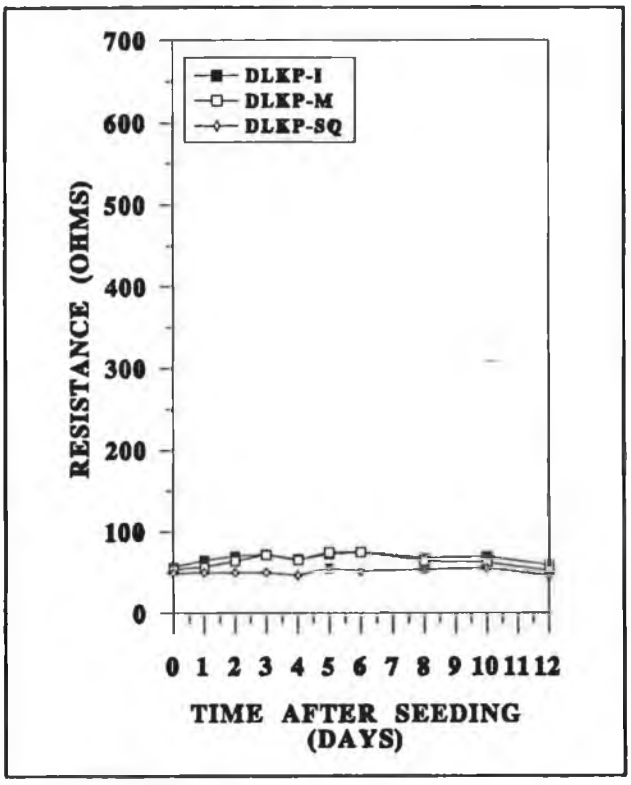
3.2.2 Electrical Resistance Studies

The cells in epithelial tissues are tightly bound together enabling the epithelium to function as a lining or barrier between the body and the outside world. The lining also facilitates compartmentalisation of cell and tissue types. Epithelial cells contain tight junctions which serve to maintain the selective barrier function of epithelial sheets. They also prevent diffusion of epithelial cell membrane proteins which results in the polarisation of epithelial cells.

To determine if DLKP cells or any of the clones were capable of forming an epithelial sheet with selective-barrier functions, the electrical resistances of confluent monolayers of the cells were determined as described in Section 2.14.2.

Cells were seeded onto millipore filters at confluent densities and grown in 12-well plates, as described in Section 2.14. Every 3-4 days, the electrical resistances of the cell monolayers were measured using an Endohm apparatus (electrical resistance measurements were determined by Stephen Keely and facilities provided by Dr.Alan Baird, Pharmacology Dept., University College Dublin). Increased electrical resistance was indicate of tight junction formation as the cells grew tighter together and the epithelial barrier formed. T84 cells, a human colonic cell line classically used in these experiments, were used as a positive control. The resistance of these cells was seen to increase over 8 days from 80 ohms to over 600 ohms (Figure 3.2.2). No increase in resistance was seen in any of the DLKP cells, indicating that these cells do not contain tight junctions and so are not polarised epithelial cells.

A



B

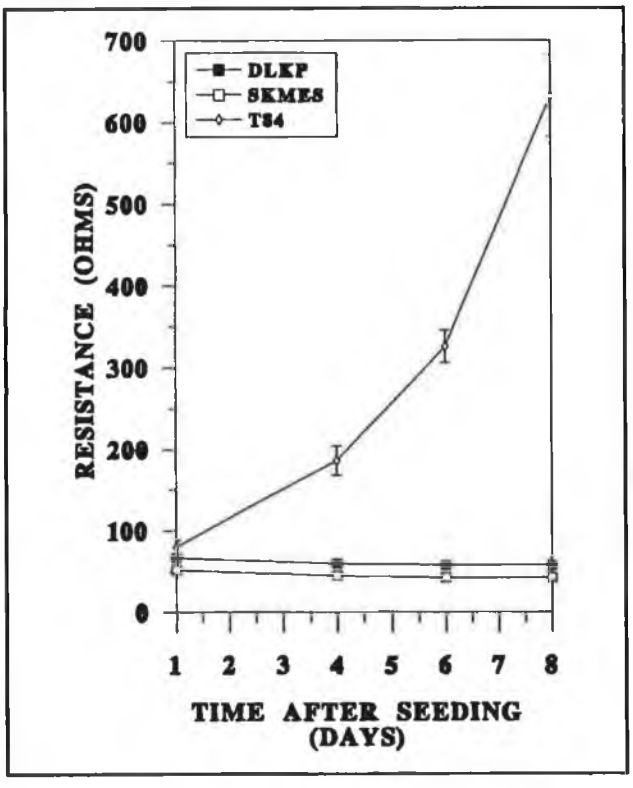


Figure 3.2.2 Electrical resistance exhibited by confluent monolayers of **A.** DLKP-I, DLKP-M and DLKP-SQ cells and **B.** DLKP, SKMES-1 and T84 cells.

3.2.3 Immunocytochemical Analysis

An extensive immunocytochemical study was undertaken to determine the profile of protein markers expressed by DLKP cells. This profile would enable comparisons to be made between DLKP and their normal and malignant counterparts *in vivo* and *in vitro*. It would also serve as a means of qualitative analysis during experiments *i.e.* did a particular treatment of the cells result in an up- or down-regulation of a marker, or switch on or off marker expression and allow for surmise on possible implications of such events. An additional aim was to detect differences in marker expression between the DLKP clones. Differences in growth, morphology and chromosome distribution had been established (Section 3.1) and detection of contrasting marker expression could identify possible differences in differentiation states of the 3 clones. It would also allow identification of specific clones in a mixed population and would enable the interconversion process to be observed more closely.

While some markers are not specific for particular cell types, especially tumour types, if a broad range of markers is examined, it is possible to substantially characterise tumour cells.

An immunocytochemical survey was therefore carried out on DLKP and its clones using specific Abs to determine the expression of protein markers. In some cases, the original paraffin-embedded tumour block was available for study.

3.2.3.1 Epithelial markers

A number of markers exist which are specific for normal epithelial cells. It has been shown that of a possible 29 or more keratin proteins, each type of epithelial cell contains a characteristic combination of 2 to 10 cytokeratin intermediate filaments. Specific Abs are available which react with individual or subsets of cytokeratins, making it possible to determine cellular origins and degrees of differentiation of tumour cells. Several additional Abs which react specifically with epithelial cells have been raised but as yet their antigens remain unidentified.

Dr.Geraldine Grant had previously established the absence of cytokeratin expression in DLKP cells using an anti-pan keratin Ab.

3.2.3.1.1 Cam 5.2

Clone Cam 5.2 is a murine monoclonal antibody which reacts with keratin peptides 8 and 18. These peptides are found in most epithelial cells with the exception of stratified squamous epithelium.

Sectioned agar blocks of cultured DLKP and clone cells and the original tumour block were examined for reactivity with Cam 5.2. All samples were found to be negative (Figure 3.2.3).

3.2.3.1.2 Pan cytokeratin

The reactivity of the cells with a pan cytokeratin antibody which reacts with cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19 was also examined. The tumour block was not available for study here.

Again, DLKP and the 3 clones were all found to be negative for keratin expression (Figure 3.4.4 - Section 3.4).

3.2.3.1.3 Epithelial membrane antigen

The epithelial membrane antigen (EMA) antibody is specific for epithelial cells but the antigen it recognises is as yet unknown.

Only the tumour block was examined with this antibody and was found to be negative (Figure 3.2.3).

3.2.3.1.4 EP16

The antigen which the EP16 antibody recognises is also unidentified but it is specific for epithelial cells.

The tumour block was not studied here. DLKP and each of the clones failed to react with this antibody (Figure 3.2.4).

3.2.3.1.5 Desmoplakin

Desmoplakin is a protein present in the desmosomes of epithelial cells. Desmosomes serve as anchoring sites for intermediate filaments and join epithelial cells together.

No desmoplakin was detected in DLKP cells or in any of the clones (Figure 3.2.5).

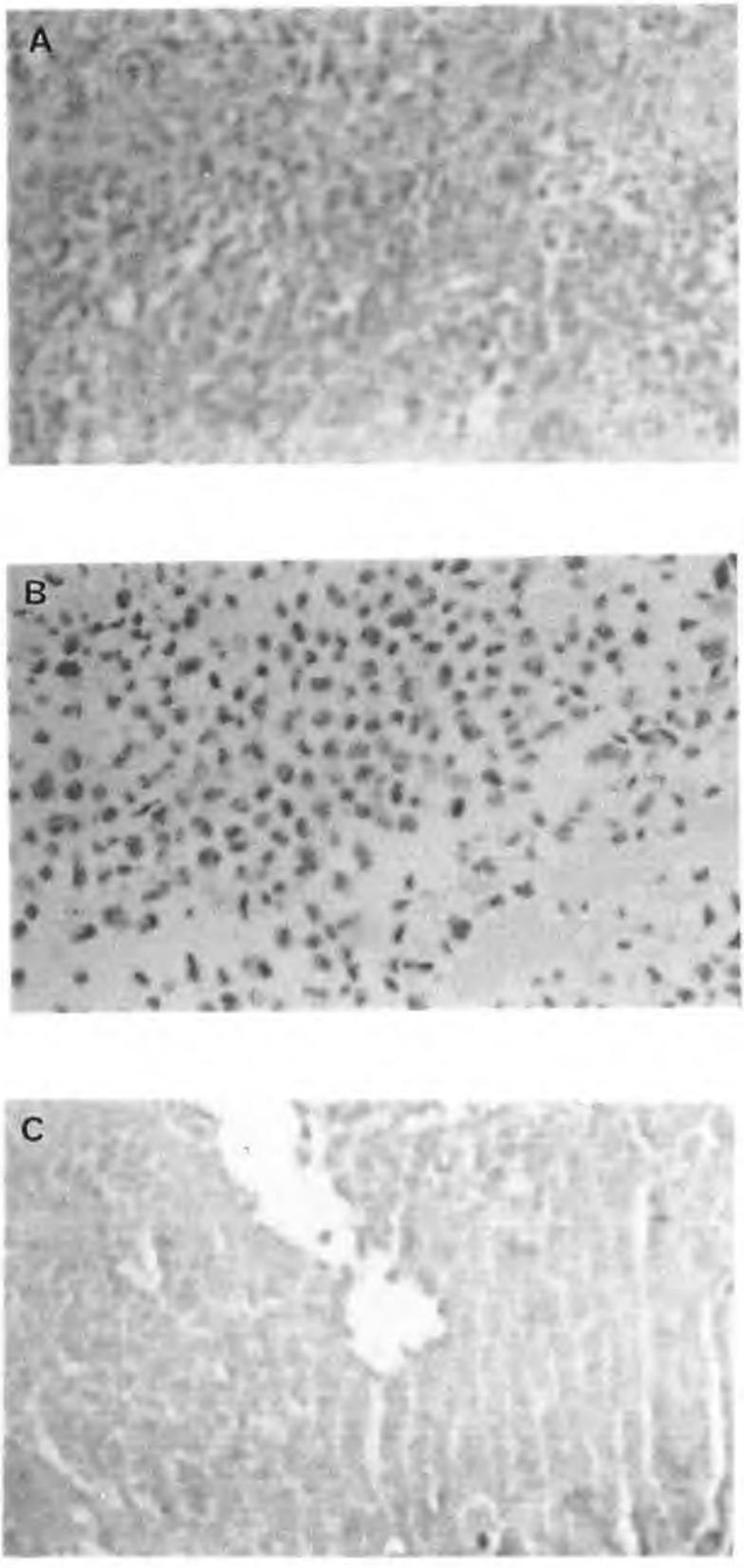


Figure 3.2.3 Reactivity of Cam 5.2 anti-keratin Ab. with **A.** DLKP original tumour block and **B.** DLKP, passage 6 cells. In both cases, cells were negative. Also shown is reactivity of anti-EMA Ab. with **C.** DLKP tumour block. Again, cells were negative. x100.

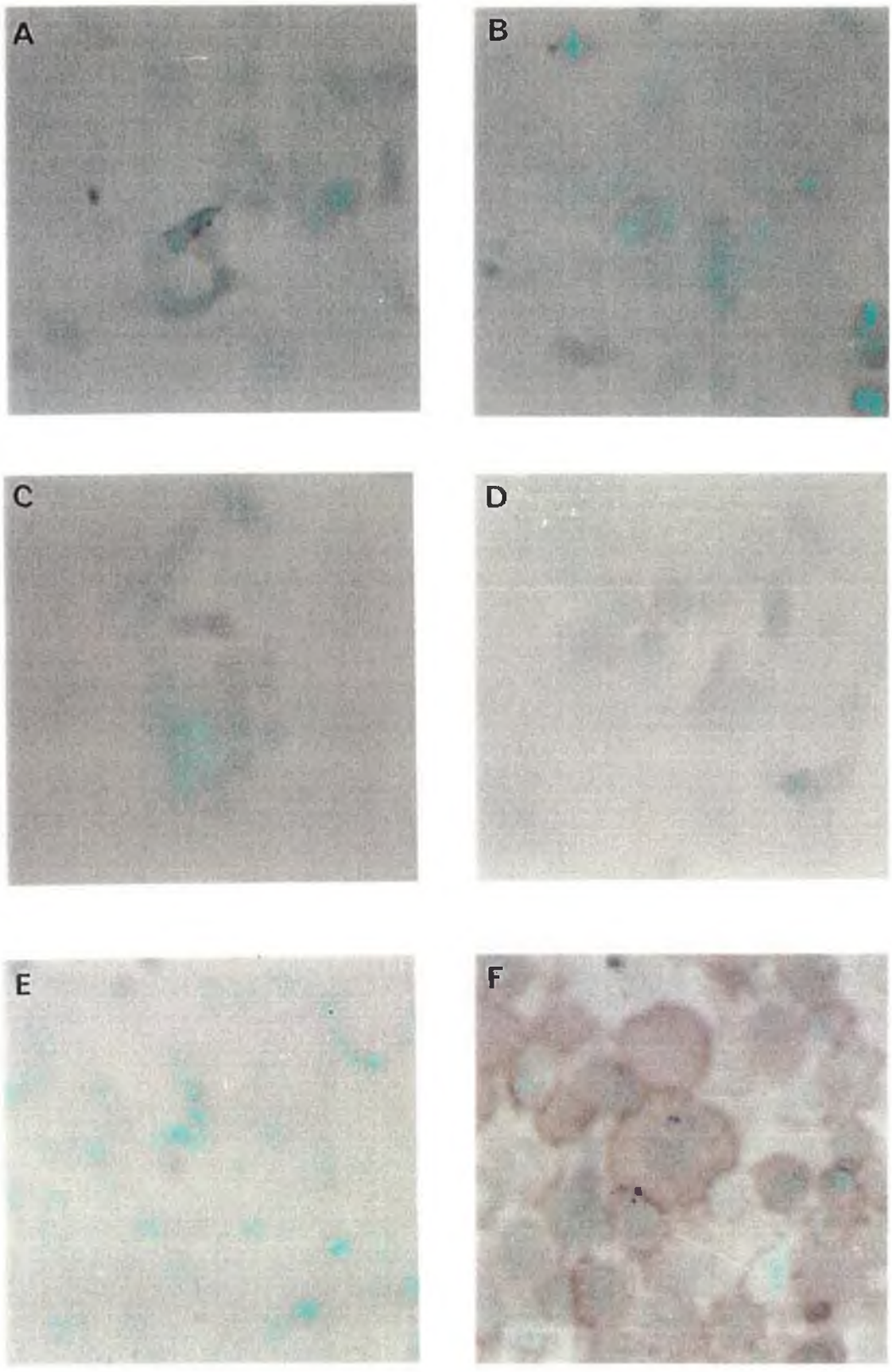


Figure 3.2.4 Reactivity of EP16 Ab. with **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M, **D.** DLKP-SQ and **E.** Hep 2 cells, all of which were negative. **F.** SCC-9 cells were used as a positive control. x100.

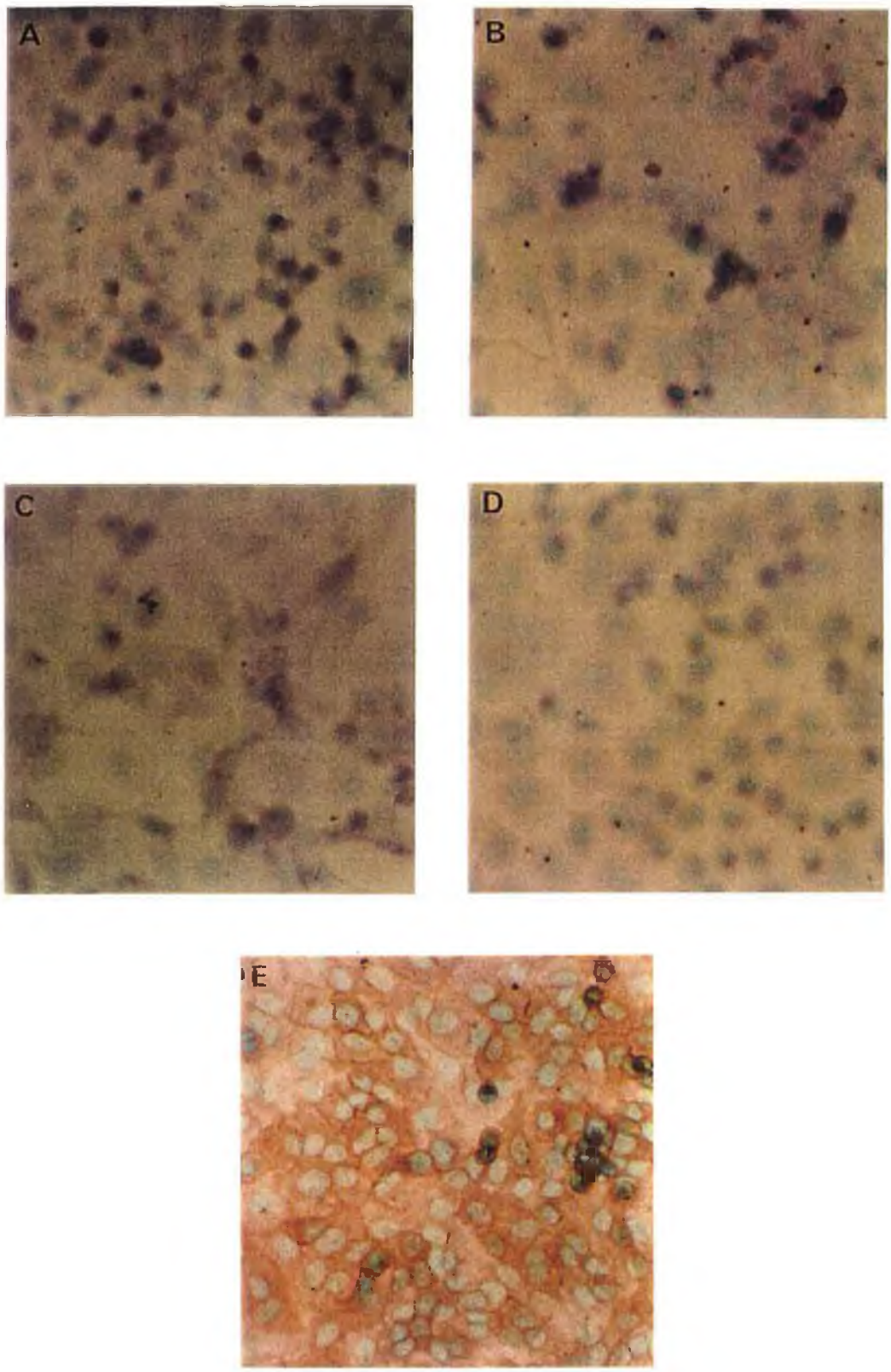


Figure 3.2.5 Reactivity of anti-desmoplakin Ab. with **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells, all of which were negative. **E.** A549 cells were used as a positive control. x100.

3.2.3.2 NSCLC markers

NSCLC is broadly sub-divided into squamous, adenocarcinoma and large cell carcinoma based on morphological and biochemical criteria. A marker specific for squamous differentiation and 2 antibodies which react with NSCLC and not with SCLC were examined.

3.2.3.2.1 Transglutaminase

Transglutaminases are a group of enzymes involved in the formation of cross-linked envelopes which are present in squamous differentiated cells.

DLKP and the 3 clones were negative for expression of this enzyme (Figure 3.2.6).

3.2.3.2.2 703D4/704A1

703D4 and 703D1 are antibodies which are believed to react only with NSCLC and not with SCLC, breast, colon, neuroblastoma or lymphoid tumours. Mulshine, *et al.*, who raised these antibodies believe that the antigen detected by the 703D4 antibody is expressed in cells early in the tumourigenic process, before any morphologic chage can be detected (personal communication, Dr.James Mulshine, National Cancer Institute, Rockville, Maryland, USA.).

DLKP and the 3 clones reacted positively with both of these antibodies (Figure 3.2.7).

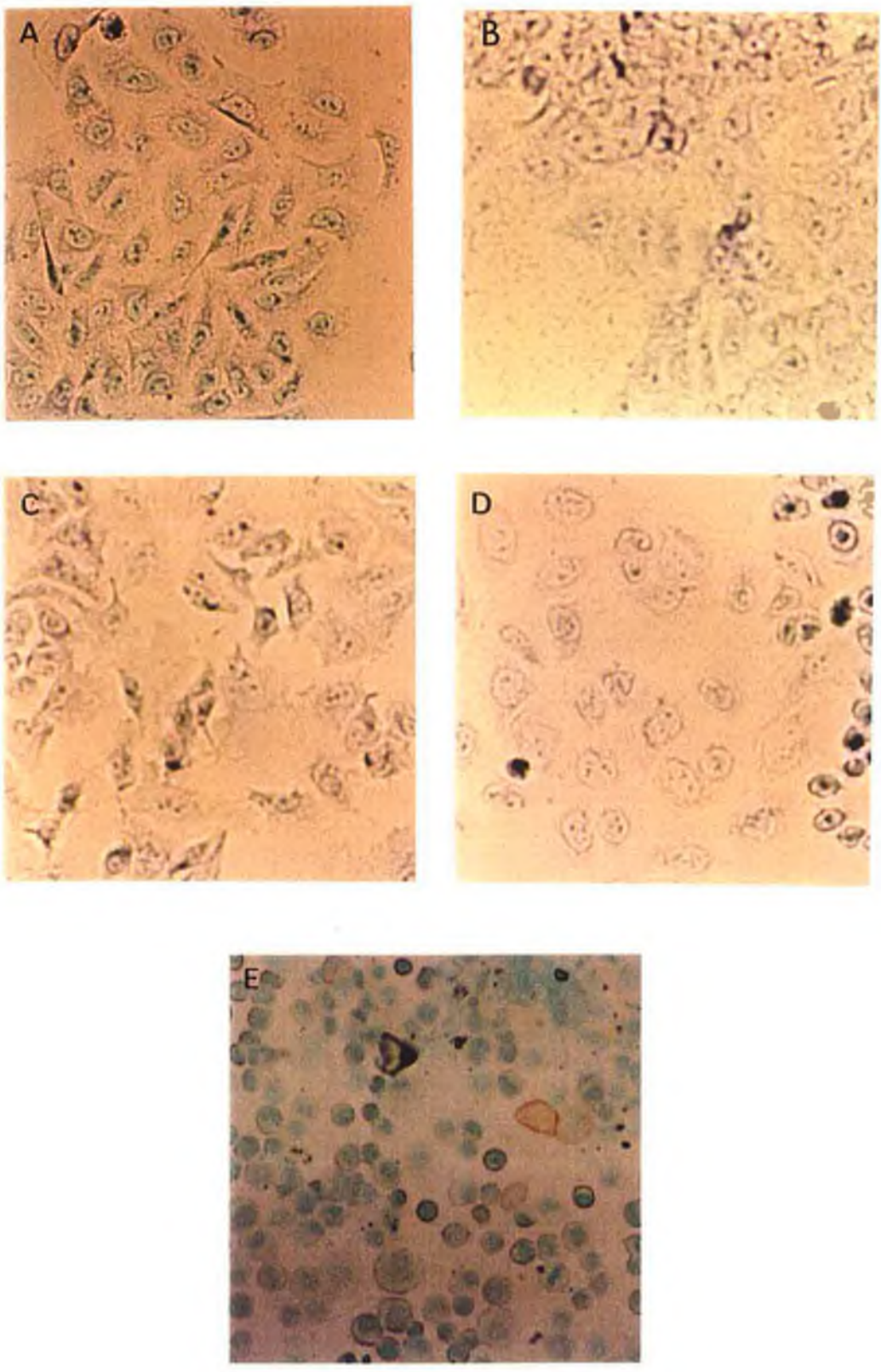


Figure 3.2.6 Reactivity of anti-transglutaminase Ab. with **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells, all of which were negative. **E.** SCC-9 cells were used as a positive control. x100.

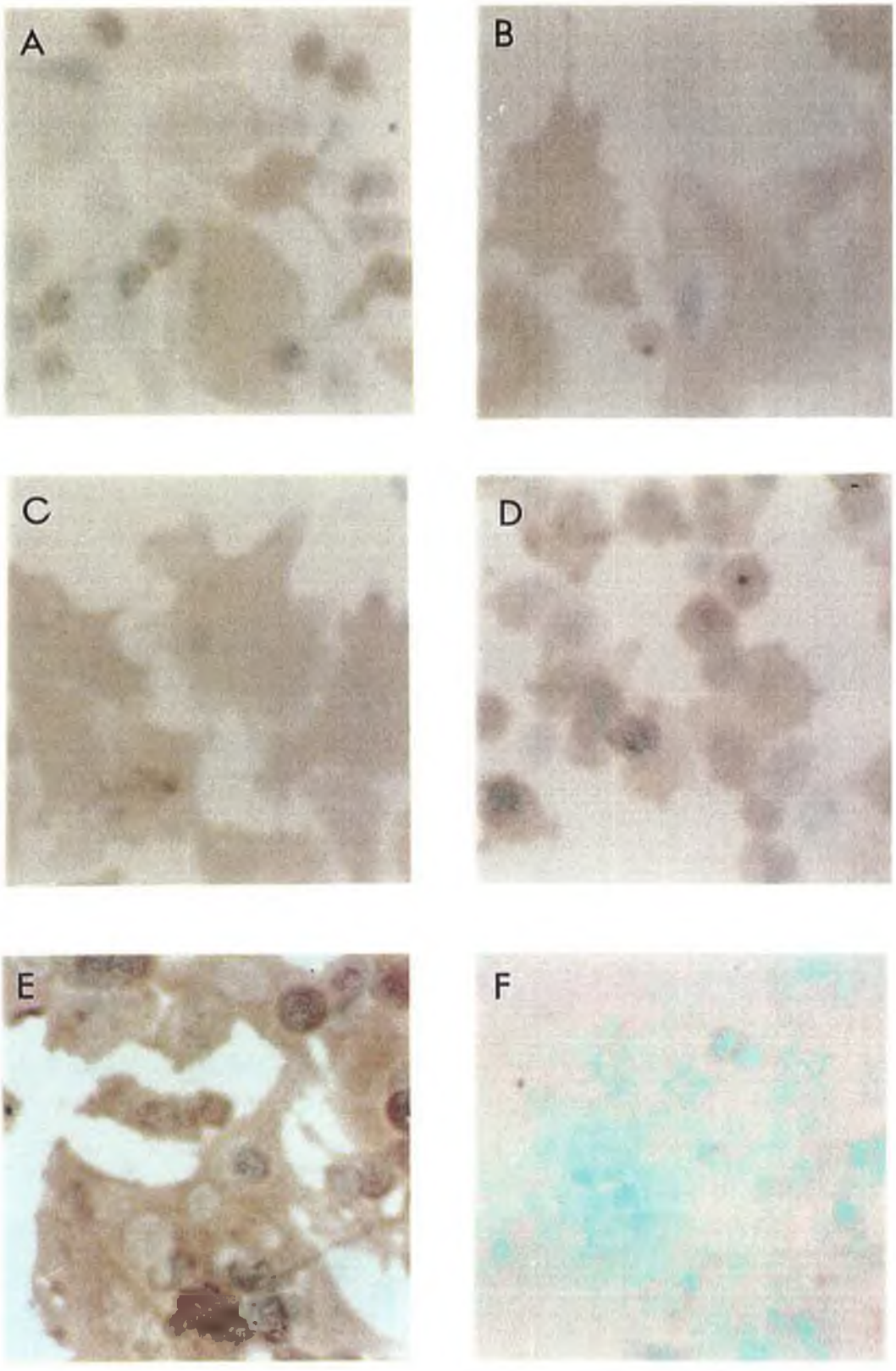


Figure 3.2.7 Reactivity of 703D4 Ab. with **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M, **D.** DLKP-SQ and **E.** SKMES-1 cells, all of which were positive. **F.** HTB 120 cells were used as a negative control. x200.

3.2.3.3 SCLC markers

SCLCs can be characterised by the presence of neuroendocrine markers. A combination of morphological examination and marker expression can allow determination of the degree of SCLC differentiation of a tumour. However, the identity and number of these markers can vary and in addition, the markers are not confined to SCLC and are found in other tumour types including NSCLC.

3.2.3.3.1 Neuron specific enolase (NSE)

NSE is a homophilic adhesion molecule. It exists as a homo or heterodimer of alpha, beta or gamma subunits. The gamma subunit is expressed primarily in neurons and in neuroendocrine cells. Originally believed to be specific for SCLC, NSE reactivity has been found in other tumour types.

Only the tumour block was studied for NSE gamma subunit expression and was found to be strongly positive (Figure 3.2.8).

3.2.3.3.2 Protein gene product 9.5 (PGP 9.5)

The PGP 9.5 antigen is as yet unknown, but the antibody has been shown to react with SCLC.

The tumour block, DLKP and each of the clones reacted strongly with this antibody (Figure 3.2.8).

3.2.3.3.3 Leu 7

Leu 7 is one of 3 antibodies which comprise the CD57 cluster of leukocyte differentiation antigens corresponding to the NK3 cell-associated molecule, HKN-1. In normal tissue, CD57 is restricted to thick nerve fibres and can be expressed by some neural-related tumours.

Only the tumour block was examined for reactivity to Leu 7 and some cells were seen to react positively (Figure 3.2.8).

3.2.3.3.4 Neurofilaments

Neurofilaments are intermediate filaments found only in neural cells of normal tissue. Their expression in tumours indicates neural origins or neural differentiation.

The tumour block was not examined here but DLKP and each of the cells were strongly positive for neurofilament expression (Figure 3.2.9).

3.2.3.3.5 Neural cell adhesion molecule (NCAM)

NCAMs are a family of cell surface sialglycoproteins which mediate homotypic and heterotypic cell-cell interactions. They are expressed on nerve cells and glial cells and the antibody used reacts with the 125kD isoform.

No NCAM expression was detected in DLKP cells or in any of the clones (Figure 3.2.10).

3.2.3.3.6 Serotonin (5-hydroxy-tryptamine, 5-HT)

5-HT is a monoamine neurotransmitter which has marked excitatory or inhibitory actions on a variety of organs and physiologic functions through an action on smooth muscle and on glandular and neuronal tissue. Normal tissues which contain 5-HT-positive cells include the central nervous system, adrenal medulla and neuroendocrine cells of the bronchial epithelium.

DLKP and the clones contained no detectable levels of 5-HT. SK-N-SK, a neuroblastoma cell line and H69, a SCLC cell line, were used as positive controls for this antibody. However, reactivity, while positive, was weak on the cells and was not visible when photographed. Therefore, no results are shown here for this antibody.

3.2.3.3.7 Glial fibrillary acidic protein (GFAP)

Glial fibrillary acidic protein is a type II intermediate filament protein present in astrocytes and some Schwann cells.

DLKP and the 3 clones were negative for expression of this protein (Figure 3.2.11).

3.2.3.3.8 Chromogranin A

Chromogranin A is a member of the secretogranin/chromogranin class of proteins that occur in secretory granules of a wide variety of endocrine cells and neurons. In normal tissues, chromogranin A is widely expressed in neuronal tissues and in the secretory granules of endocrine cells. Expression is also found in a variety of neuroendocrine tumours.

No chromogranin A reactivity was detected in DLKP or any of the clones. As was found with the anti-serotonin antibody, reactivity was weak on positive control cell lines used with this antibody (SK-N-SH and H69) and it was not possible to photograph the results satifactorily. Therefore, the results are not shown.

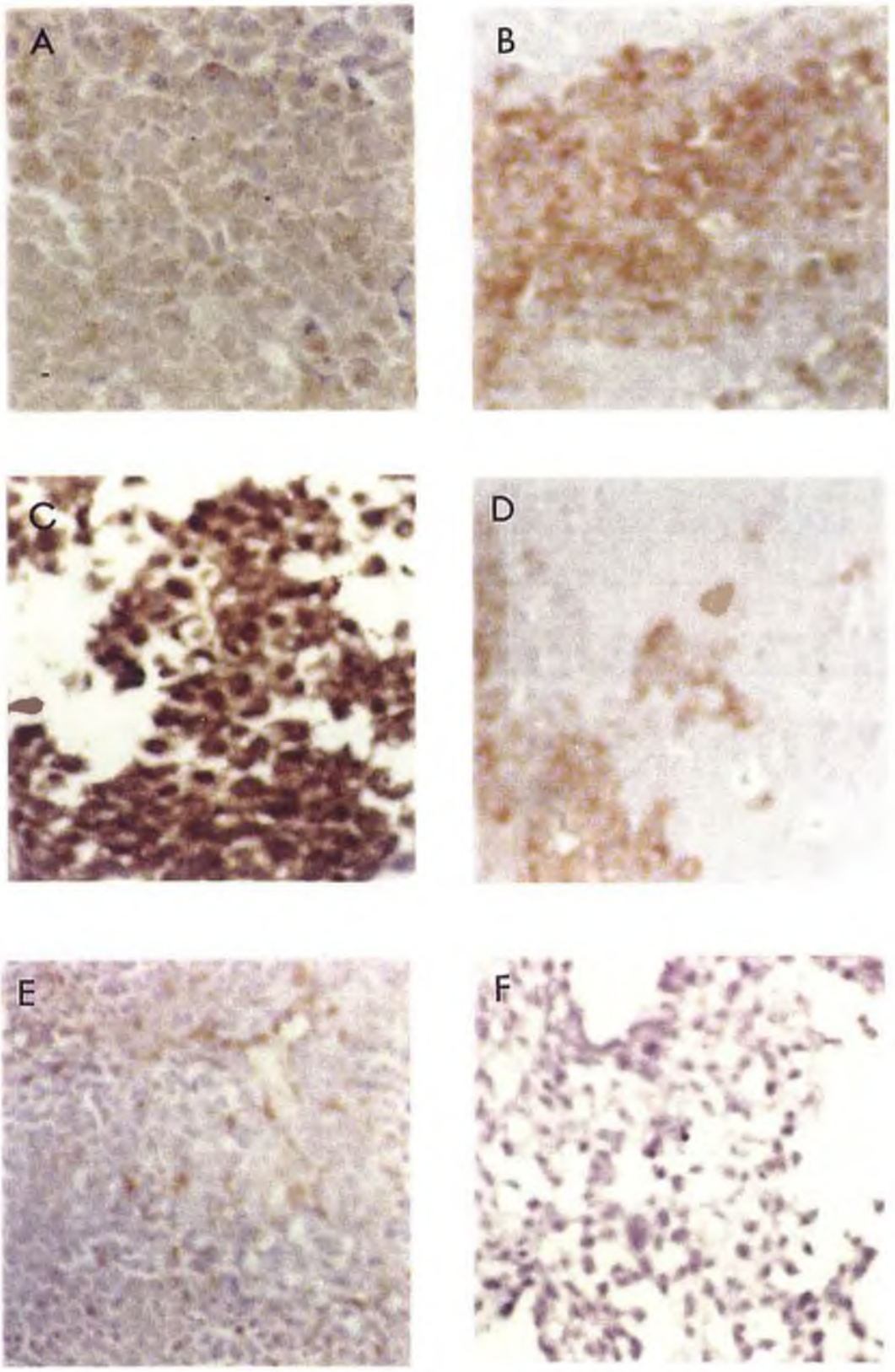


Figure 3.2.8 Shown here are:- **A.** the detection of NSE immunoreactivity in DLKP tumour block, x200; the detection of PGP 9.5 immunoreactivity in **B.** DLKP tumour block and **C.** DLKP-SQ cells, x100; the detection of **D.** Leu 7 and **E.** vimentin immunoreactivity in a small number of cells in the DLKP tumour block, x100;
and **F.** the absence of LCA immunoreactivity from DLKP cells, x100.

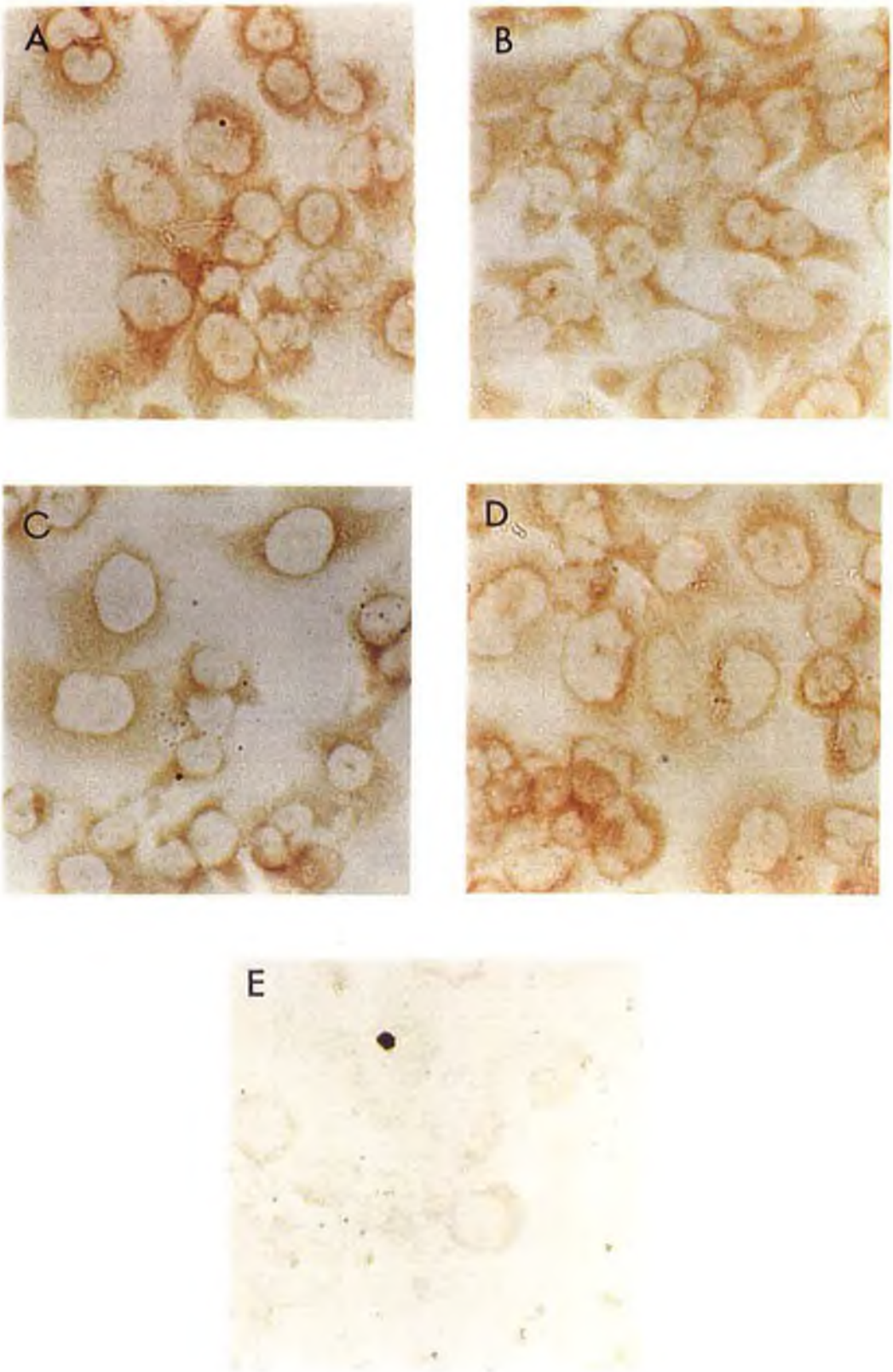


Figure 3.2.9 Positive neurofilament immunoreactivity in **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells and negative reactivity in **E.** normal lung fibroblasts. x100.

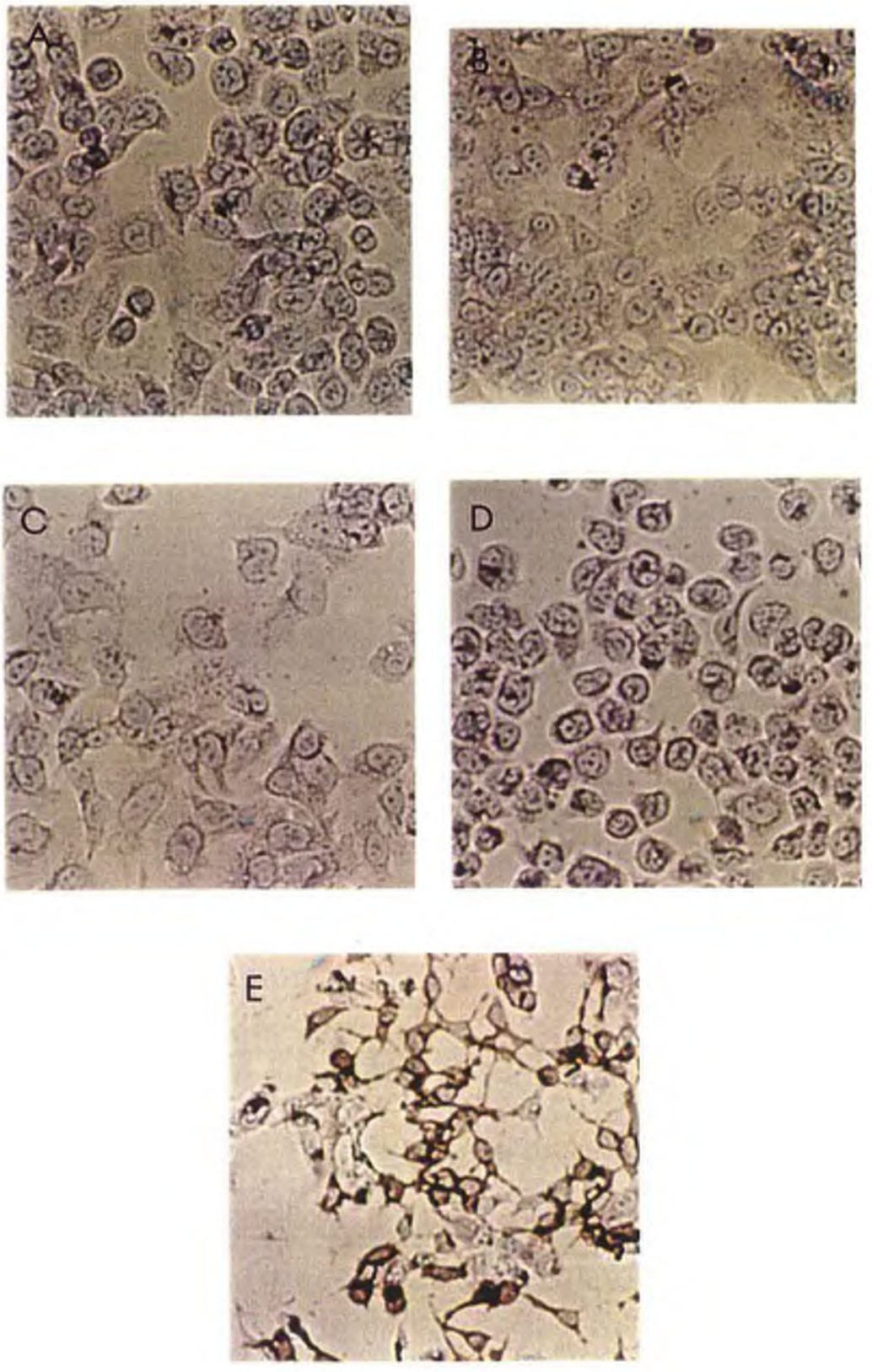


Figure 3.2.10 Negative NCAM immunoreactivity in **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells and positive reactivity in **E.** SK-N-SH cells. x100.

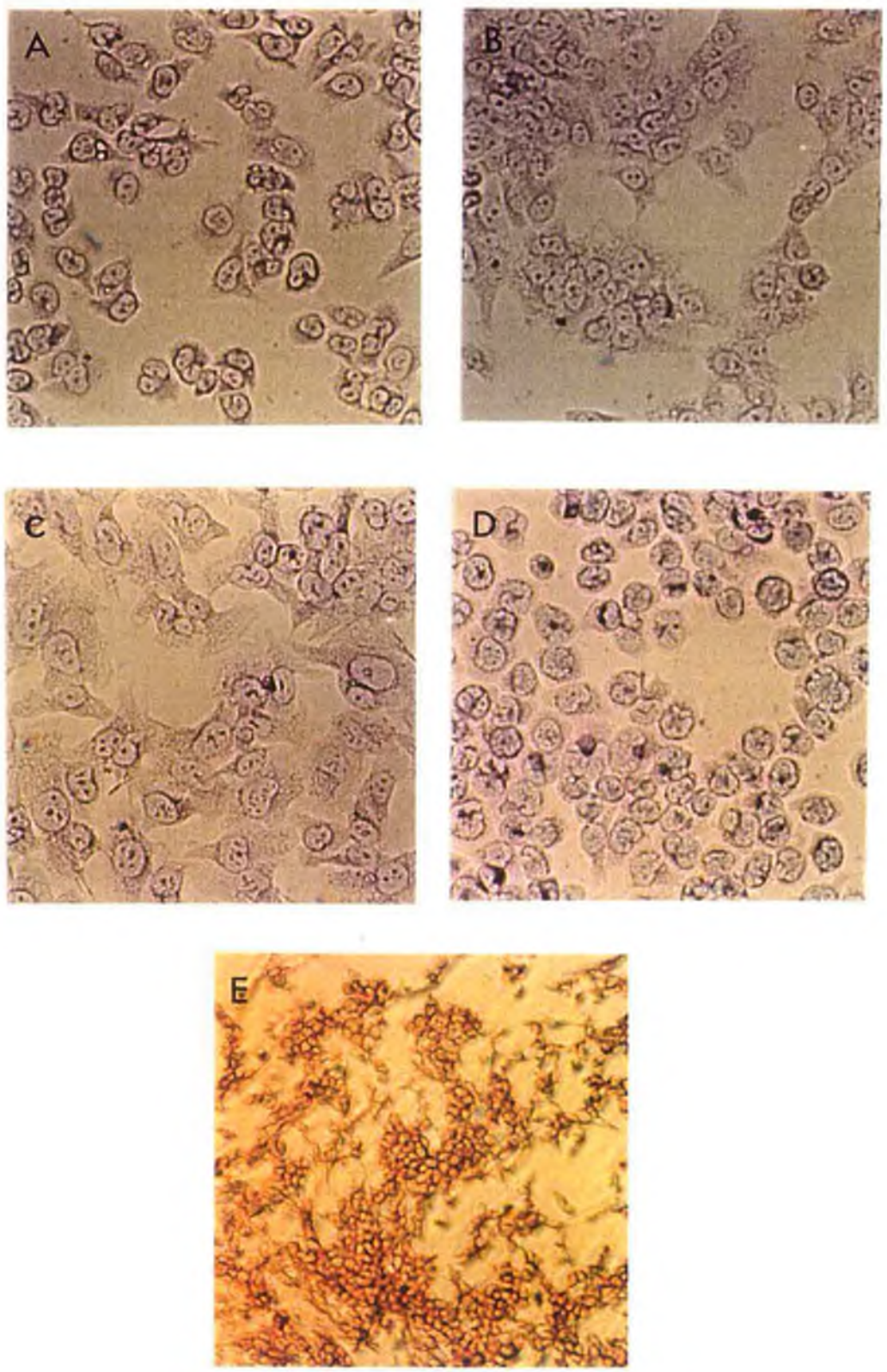


Figure 3.2.11 Negative GFAP immunoreactivity in **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells and positive reactivity in **E.** SK-N-SH cells. x100.

3.2.3.4 Other markers

DLKP cells were also examined for their expression of several other marker proteins.

3.2.3.4.1 Vimentin

Vimentin is a type II intermediate filament expressed in many cells of mesenchymal origin including fibroblasts, blood vessel endothelial cells and white blood cells. Expression of vimentin is also often induced in cultured cells.

The tumour block was examined for vimentin expression. Only scattered positives were present which were probably fibroblasts (Figure 3.2.8).

3.2.3.4.2 Leucocyte common antigen (LCA)

Leucocyte common antigen is a family of 5 or more high molecular weight glycoproteins present on the surface of the majority of human leucocytes. Normal lymphoid cells, macrophages and histiocytes express these proteins as do neoplastic B and T cells in non-Hodgkin's lymphoma. Because the DLKP cell line was established from a lymph node metastasis, it was necessary to eliminate the possibility that a lymphatic primary tumour was in fact the source of DLKP.

The tumour block was not examined but DLKP and each of the clones showed no reacivity with the LCA antibody (Figure 3.2.8).

3.2.3.4.3 P-Glycoprotein

P-glycoprotein is a membrane drug efflux pump which is highly expressed in the DLKP-A cell line, a multiple drug resistant variant of DLKP. Previous work (Dr.Alice Redmond, PhD thesis) had shown that the sensitive DLKP cells expressed very low levels of this protein. Here, the DLKP clones were examined for their expression of P-glycoprotein to determine if a population of cells in DLKP inherently expressed high

levels of P-glycoprotein which would suggest that these had been selected out to form the variant lines.

No expression of P-glycoprotein was detectable in DLKP or any of the clones (results not shown).

3.2.3.4.4 α-2 Integrin

The integrins are a large family of cell surface receptor molecules involved in cell-cell and cell-matrix interactions. α2-integrin binds to collagen I and IV, laminin and fibronectin and can influence cell adhesion and motility.

α-2 integrin expression was detected in DLKP cells and in each of the clones (Figure 3.2.12). DLKP-M cells appeared to have higher levels of expression than the 2 other clones and DLKP.

A summary of the immunocytochemical reactivities of DLKP and the clones is presented in Table 3.2.1.

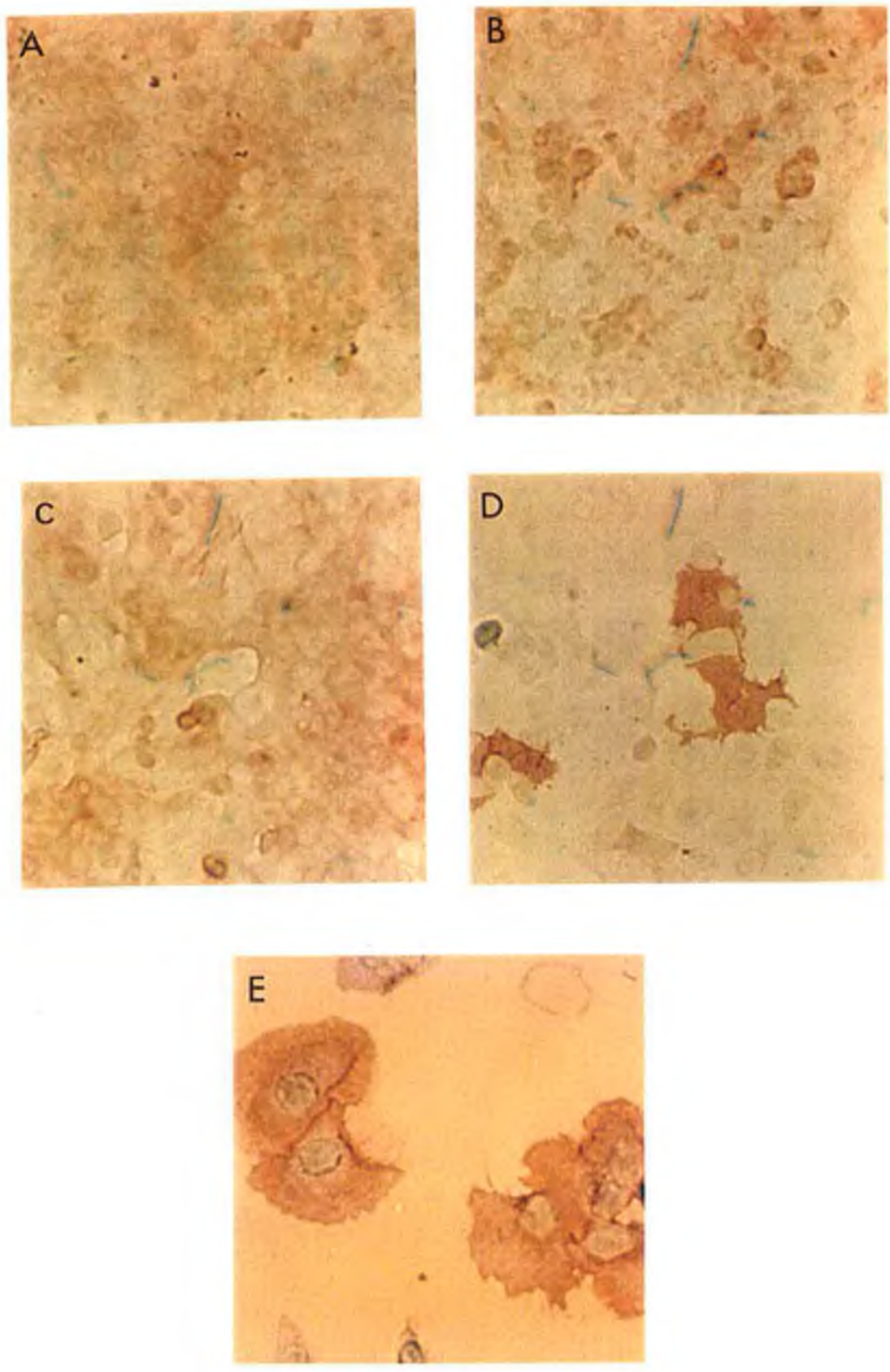


Figure 3.2.12 Positive α_2-integrin immunoreactivity in subpopulations of **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells and positive reactivity in SKMES-1 cells. x100.

Marker	Tumour block	DLKP (P6)	DLKP (P29)	DLKP-I	DLKP-M	DLKP-SQ
Epithelial						
Cam 5.2	-	-	-	-	-	-
Pan ker.		-	-	-	-	-
EMA	-					
EP16		-	-	-	-	-
Desmopl.		-	-	-	-	-
NSCLC						
Transgl.		-	-	-	-	-
703D4/ 704A1		✓	✓	✓	✓	✓
SCLC						
NSE	✓					
PGP 9.5	✓	✓	✓	✓	✓	✓
Leu 7	✓					
N.fils			✓	✓	✓	✓
NCAM			-	-	-	-
5-HT			-	-	-	-
GFAP			-	-	-	-
Crg.A			-	-	-	-
Others						
Vimentin	-					
LCA		-	-	-	-	-
P-gly.		-	-	-	-	-
α2-intgr.			✓	✓	✓	✓

Table 3.2.1 Expression of biological markers in DLKP cells and clones. (Pan ker.= pan anti-keratin, EMA = epithelial membrane antigen, Desmopl.= desmoplakin, Transgl.= transglutaminase, NSE = neuron specific enolase, PGP 9.5 = protein gene product 9.5, N.fils.= neurofilaments, NCAM = neural cell adhesion molecule, 5-HT = serotonin, GFAP = glial fibrillary acidic protein, Crg.A = chromogranin A, LCA = leucocyte common antigen, P-gly.= P-glycoprotein and α2-intgr.= α2-integrin).

3.3 PRODUCTION OF MONOCLONAL ANTIBODIES

During the characterisation studies carried out on the DLKP clones described in Section 3.2, no measureable feature had emerged, biological of immunological, that would allow for actual identification of any 1 clone in a mixed population *i.e.* no differential marker(s) had been identified. The presence of one or more markers on any of the clones would mean that the interconversion of the cells occurring in culture, which up to this point had only been observed morphologically under the microscope, could be analysed more closely and quantitated. Knowledge of differences in the expression of specific proteins between the clones may also give insight into the respective differentiation states of the cells and could be used in experiments with differentiating agents to explore the differentiating capacities of the cells. The presence of markers on 1 or more of the clones would also aid in comparisons between those particular cells and possible corresponding cells *in vivo*, normal and/or malignant. For these reasons, it was decided to raise monoclonal antibodies (MAbs) to the cells to aid in the search for markers for the clones.

3.3.1 Immunisation with DLKP Cells

Mice were immunised with DLKP whole cells. The parental population was chosen for immunisations because, in theory at least, this mixture of cells should possess the entire complement of antigens expressed by all the indivivual clones, plus any that may not be represented by the clones. For the same reasons, DLKP cells were used in the preliminary screening assays to identify antibody-producing hyrbridomas. In later screening assays, the DLKP clones were examined for differential expression of antigens.

Three immunisations were carried out over 7 weeks, followed by a final boosting injection 3 days prior to sacrifice of the animals at week 9. 2 weeks after the 3rd immunisation, sinus bleeds were carried out on the mice. The sera from these samples were analysed for immunoreactivity with DLKP cells by ELISA as outlined in Section 2.16.3. Immunoreactivity was detected in all samples indicating that the immune systems of the mice had responded to the foreign DLKP cells by producing antibodies specifically directed against DLKP antigens.

3.3.2 Fusion and Selection of DLKP-immunoreactive MAb Clones

Fusion of splenocytes from the immunised mouse with mouse myeloma Sp2 cells was carried out as described in Section 2.16.2. A successful fusion would mean that the resulting hybridomas were immortalised cells capable of producing and secreting Abs. The fused cells were plated into 96-well plates and incubated undisturbed for 7 days. After this time, the plates were examined under the microscope and any wells containing colonies of cells were marked. Approximately 50% of the wells examined contained colonies of varying sizes.

The marked wells were allowed to become confluent until the supernatent had turned from red/purple to yellow/orange, indicating cell proliferation and metabolism and thus possible Ab secretion. The supernatent was them removed and analysed for immunoreactivity with DLKP cells (see Section 2.16.3). The first batch screened consisted of 45 supernatents and these were tested 11 days after the initial fusion. Those supernatents which resulted in absorbance readings greater than approximately 20% those of control wells (*i.e.* without Ab) were designated positive and their corresponding clones were marked for further culture and were subsequently grown up into 6-well plates and then into 25cm^2 flasks. Of the 45 supernatents tested in the first batch, 15 were positive. The remaining 30 clones were thus discarded.

Six such preliminary screenings of supernatents were carried out using DLKP cells as positive indicators of reactivity. More than 200 supernatents were analysed and of these, 28 appeared to consistently react positively with DLKP cells. It was noted that the reactivity of the Abs produced by some of the hybridomas appeared to diminish with time.

3.3.3 Screening Against the DLKP Clones

Supernatents from the 28 selected hybridomas were collected and their immunoreactivities with the DLKP clones were examined. Because a marker which could differentiate between the clones was being sought, only those supernatents which differentially reacted with the clones were of interest *i.e.* those which reacted with 1 or 2 clones only or which reacted more strongly with 1 clone than another. After 7 screenings of the clones, no Ab was found to react specifically with only 1 or 2 of the 3 clones. However, 2 Abs, named 4D2 and 3E1, appeared to consistently react more strongly with DLKP, DLKP-M and DLKP-SQ than with DLKP-I. Further work was carried out to characterise these 2 Abs and to determine their efficacy as markers for subpopulations of DLKP cells.

3.3.4 Isotyping of MAbs

It was important to determine the isotypes of the 4D2 and 3E1 Abs. In humans, there are 5 classes of Abs, IgA, IgD, IgE, IgG and IgM, which are classified according to the nature of their antigen-binding chains. IgM is the first class of Ab produced by immune cells in response to a foreign antigen (Ag). The presence of 10 binding sites on these Abs gives them a high affinity for their specific Ag. However, an increase in the affinity of Abs produced with time after immunisation is often seen due to mutations in the genes which code for the Ag-binding sites. Therefore, IgG Abs, which are the major class of Ab in the blood and are produced during secondary immune responses, are more desirable for use as analytical tools.

Isotyping of the 4D2 and 3E1 Abs was carried out using a Serotec kit, described in Section 2.16.4. During the ELISA screenings with these Abs, it appeared that 4D2 had a stronger reaction with the DLKP cells than 3E1. Therefore, for the isotype analysis, 4D2 was tested at dilutions of 1/50, 1/100 and 1/200 while 3E1 was tested at 1/10, 1/50 and 1/100.

The results of the indicated that the 4D2 Ab was an IgG molecule while the 3E1 Ab was an IgM molecule. These results were consistent with the 4D2 Ab having a stronger reaction with the DLKP cells than the 3E1 Ab in the ELISAs.

3.3.5 Western Blot Analysis

In order to determine the molecular weights of the antigens which reacted with the 4D2 and 3E1 Abs, Western blots were carried out (see Section 2.17 for techniques). Western blots also allow semi-quantitative analysis as either the protein concentrations or cell numbers that are loaded onto a gel are pre-determined and equal, thus allowing the size of the resulting bands to be compared to each other. For these Westerns, whole cells were run on the gels ($1X10^4$ cells per lane).

The 3E1 Ab failed to produce a band on any Western attempted using the following cells:- DLKP, DLKP-I, -M, -SQ, DLKP-A, DLKP-A-2B, DLKP-A-5F and SKMES-ADR (Figure 3.3.1). It was therefore not possible to estimate a size for the Ag which had reacted with 3E1 in the ELISAs.

When the blots were probed with the 4D2 Ab, a single band resulted for each cell line examined. When compared with the standard molecular weight markers run simultaneously, the band produced had an apparent size of 58kD and the band was this size in all the cell lines analysed.

Consistently *i.e.* on each of 8 separate Westerns carried out, the band produced by DLKP-SQ cells was stronger than those produced by DLKP, DLKP-I and -M, indicating a higher level of expression of the 4D2 Ag in the DLKP-SQ cells (Figure 3.3.2).

Several other cell lines were examined with the 4D2 Ab - DLKP-A, DLKP-A-2B, DLKP-A-5F, SKMES-1, SKMES-1-ADR, SKLU-1 and A549 - and all produced a single band of approximately 58kD. The SKMES-1-ADR cells appeared to produce the strongest band of all the lines examined, including DLKP-SQ (Figure 3.3.3).

Figure 3.3.1 Western blot examined with 3E1 Ab. No reactivity was seen with any cell lines examined.

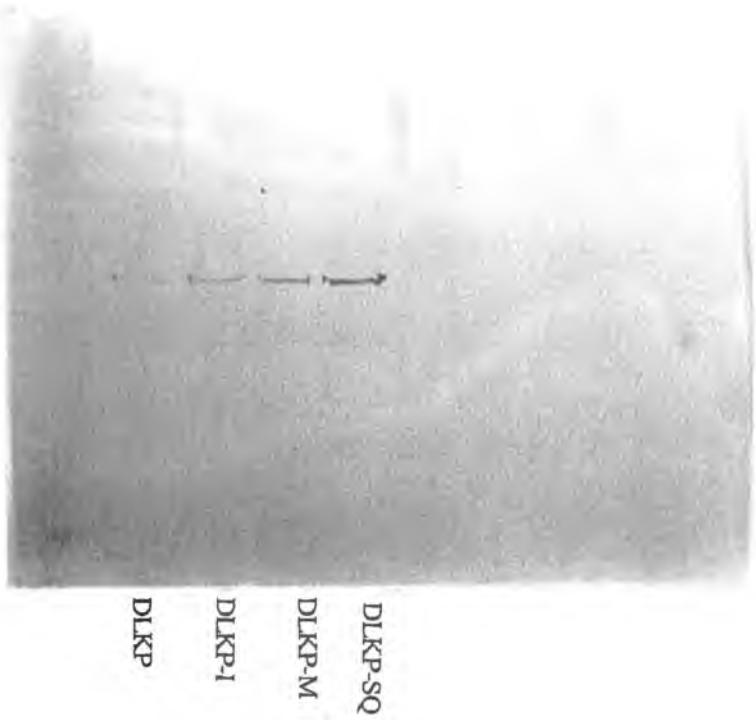


Figure 3.3.2 Western blot examined with 4D2 Ab resulting in a band of approximately 58kD in lanes containing DLKP and each of the clones. This Ab. consistently reacted most strongly with DLKP-SQ cells.

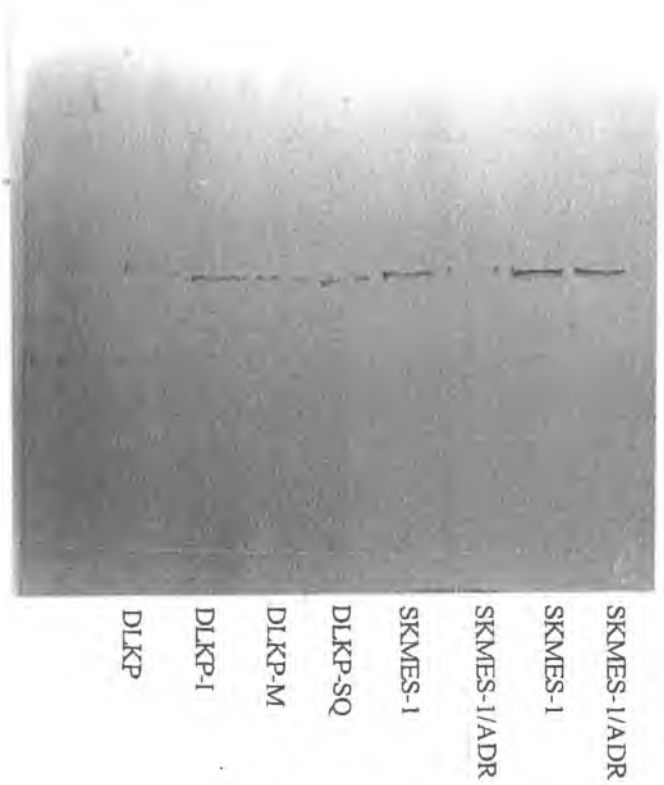


Figure 3.3.3 Western blot examined with 4D2 Ab. A single band of approximately 58kD was produced for all cell lines examined.

3.3.6 Immunocytochemical Analysis

The 4D2 and 3E1 Abs had been selected on the basis of their reactivities in ELISAs where the intensity of colour produced by a soluble substrate was used as an indicator of positivity. In order to examine more closely the nature of the interaction of the Abs with the cells, immunocytochemical analysis with the 3 stage DAB-ABC staining technique was carried out as prevoiusly described in Section 2.15.2. This results in an insoluble colour precipitate where the primary Ab reacts with its specific Ag and thus gives an indication of the location and distribution of the Ag within the cell *eg.* membranous, nuclear, cytoplasmic, filamentous, vesicular *etc.* While this is an imprecise analysis, it can be helpful in ruling in or out likely candidates for the identity of the Ag. It is also possible that a single Ag may have different distributions in different cells.

3.3.6.1 DLKP and clones

The reactions of 4D2 and 3E1 with DLKP, DLKP-I, -M and -SQ were examined by the DAB-ABC method. Neat conditioned media from the hybridomas were used in all experiments.

The 4D2 Ab had a strong reaction with DLKP and the 3 clones, appearing to be strongest in DLKP-SQ (Figure 3.3.4). Staining was localised to the cytoplasm in each of the cell lines and inasmuch as was possible to determine under the microscope, the staining appeared to be uniform throughout the cytoplasm *i.e.* not localising in structures of any kind such as vesicles or networks.

The 3E1 Ab had a similar reaction with DLKP and the clones, but the staining was much less intense than that seen with the 4D2 Ab (results not shown). Again, the staining was cytoplasmic and general, as observed with 4D2.

Cytospins were made of both subconfluent and confluent DLKP cells and these were analysed to examine the possibility that the Ag(s) was expressed only in either actively

growing cells or in contact inhibited cells. No difference was seen in Ab reactivity with such cells and all cells were positive (Figure 3.3.5).

3.3.6.2 Screening of other cell types

A wide range of cell types, normal and malignant, were screened to determine the specificity of the Abs and to assess their suitability for use as markers for any specific cell type *eg*. lung cells, tumour cells, epithelial cells *etc*. The cell types examined are listed in Table 3.3.1. The 4D2 and 3E1 Abs were found to react positively with every cell type examined. Examples of some of these results are shown in Figures 3.3.6 and 3.3.7.

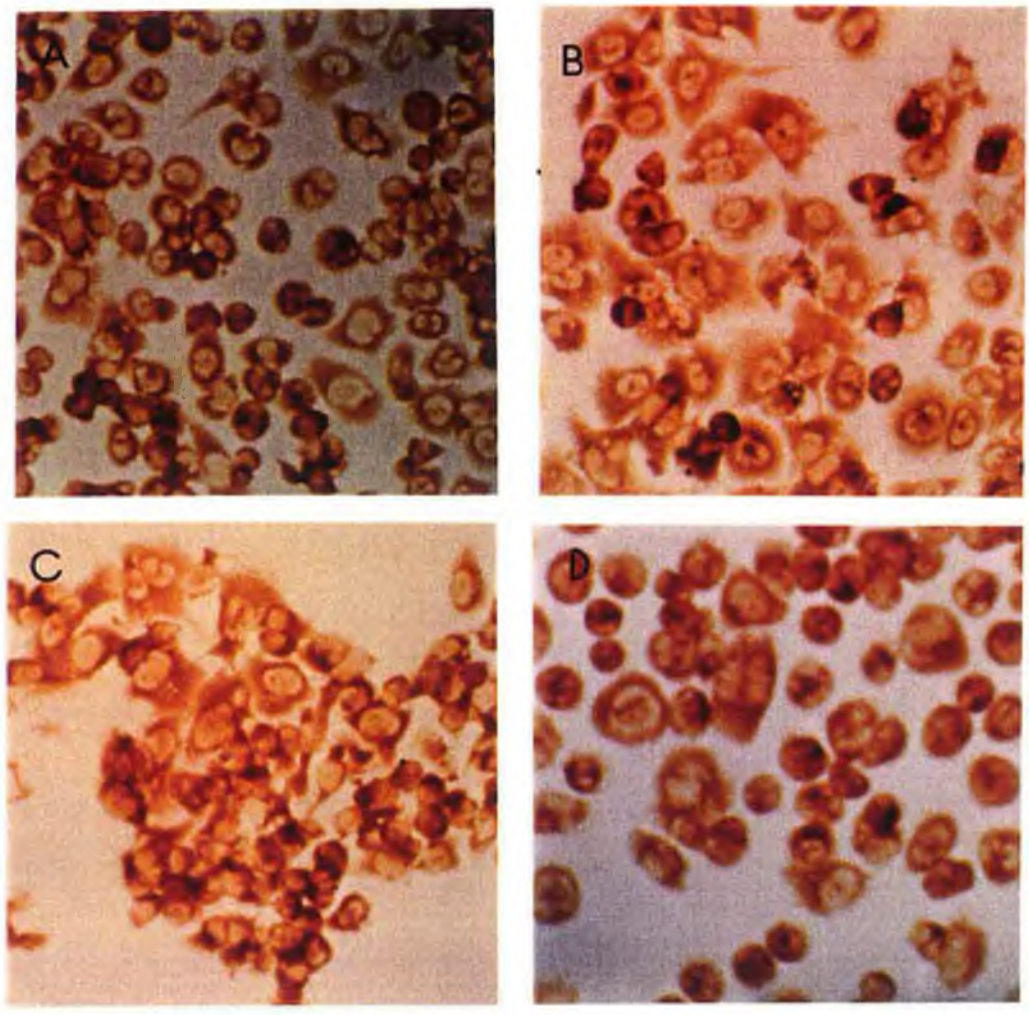


Figure 3.3.4 Positive 4D2 immunoreactivity in **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells. Staining appears to be cytoplasmic. x100.

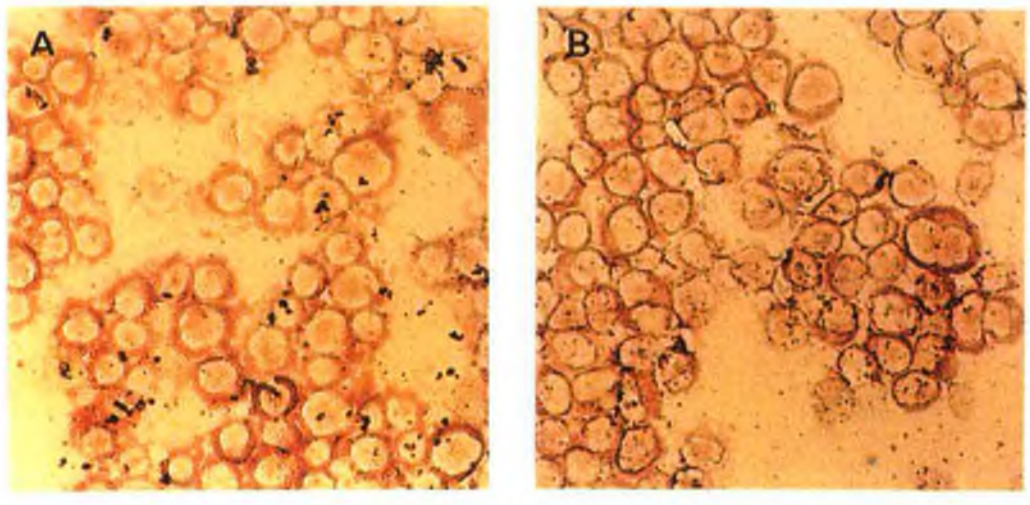


Figure 3.3.5 Positive 4D2 immunoreactivity in **A.** subconfluent and **B.** confluent DLKP cells. No difference in reactivity was apparent. x100.

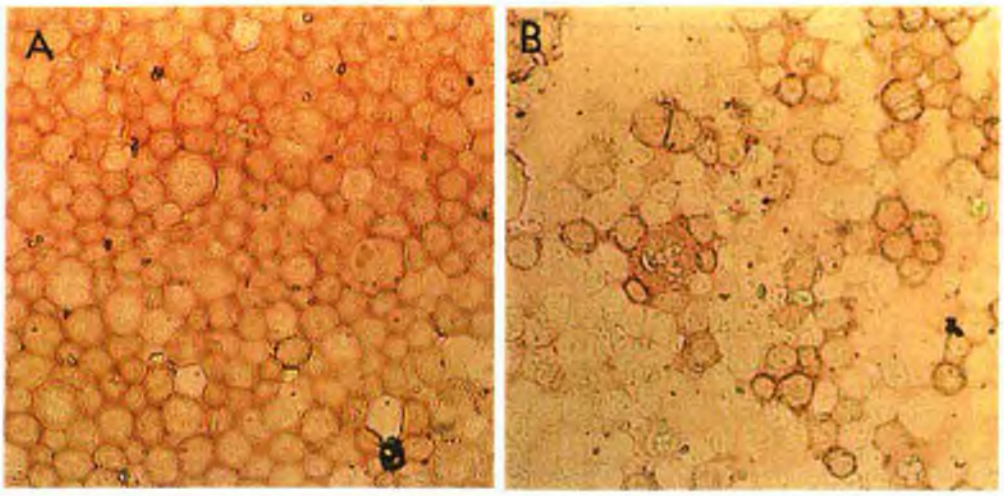


Figure 3.3.6 Positive 3E1 immunoreactivity in **A.** DLKP and **B.** Hep 2 cells. x100.

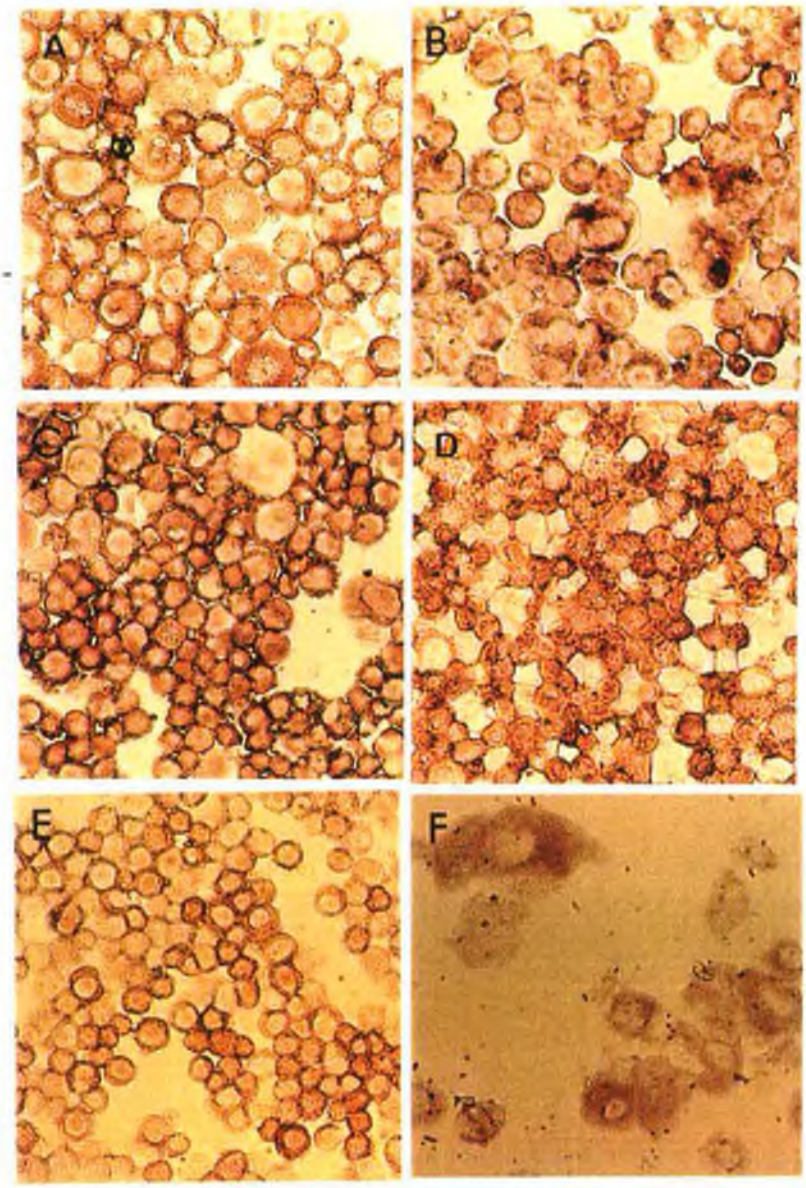


Figure 3.3.7 Positive 4D2 immunoreactivity in **A.** DLKP, **B.** SKLU-1, **C.** T84, **D.** Hep 2, **E.** RPMI 2650 and **F.** normal lung fibroblast cells. x100.

Table 3.3.1 Reactivity of a range of cell types with 4D2 and 3E1

Cells	Tumour cells	Normal cells	Origin	4D2 reactivity	3E1 reactivity
DLKP(P8)	✓		Lung	✓	✓
DLKP-A(P70)	✓		Lung	✓	✓
SKMES(P38)	✓		Lung	✓	✓
F.blast(P5)*	✓		Lung	✓	✓
A549(P7)	✓		Lung	✓	✓
SKLU-1(P31)	✓		Lung	✓	✓
T84	✓		Colon	✓	✓
Hep 2	✓		Larynx	✓	✓
RPMI 2650	✓		Nasal	✓	✓
DLKP-A-2B (P61)	✓		Lung	✓	ND
DLKP-A-2B (P61)	✓		Lung	✓	ND
SKMES-1 ADR(P73)	✓		Lung	✓	ND
Human Type II		✓	Lung	✓	ND
Rat Type II		✓	Lung	✓	ND
Pig Type II		✓	Lung	✓	ND

Note: figures in parenthesis refer to passage number of cells eg.P8 = passage 8
*F.blast = normal fibroblasts
ND = not determined.

3.4 DIFFERENTIATION STUDIES ON DLKP AND ITS CLONES

The characterisation studies carried out on DLKP and the clones and described in Section 3.2 indicated that each of the lines was poorly differentiated with no ultrastructural or biochemical features detected that would be indicative of any particular differentiated phenotype in the lung. In an attempt to gain further insight into the nature of the DLKP cells and each of the clones *i.e.* what normal and/or abnormal cells they may represent *in vivo*, differentiation studies were carried out.

2 principal aims were perceived. Firstly, the effects, if any, of differentiating agents on the interconversion of the clones was to be observed. The ability to control the interconversion may allow the process to be studied more closely and possible mechanisms to be elucidated. Secondly, the effects of these agents on marker expression in the cells may provide information on potential differentiation pathways available to each cell type and enable comparisons and contrasts to be made between these malignant cells and normal lung epithelial differentiation. Concerning the first aim here, no differential marker for the clones emerged during the characterisation work and the MAb study also failed to produce such a marker. Therefore, morphological appearance remained the criterion for identification and classification of the clones.

2 agents known to affect the differentiation of many cell types, 5-bromodeoxyuridine and retinoic acid, were examined for their effects on morphology and marker expression in DLKP and the clones. The entire experiments, including exposure to differentiating agents and immunocytochemical analysis were carried out in 6-well tissue culture plates as outlined in Section 2.18.

3.4.1 Bromodeoxyuridine

The effect of BrdU, a thymidine analogue, on the morphology and marker expression of DLKP and its clones was examined. BrdU is capable of both blocking and inducing expression of differentiated phenotypes but its mechanism of action is not understood. (For discussion see Section 4.8).

3.4.1.1 Morphological effects on DLKP and clones

Preliminary differentiation experiments were carried out with 1μM, 10μM and 50μM BrdU which was added to the cells in normal growth medium (5% serum) 24hrs after plating (as outlined in Section 2.18.2). Cells were fed with fresh BrdU-containing medium every 2-3 days. The toxic effect of BrdU on DLKP cells had been determined in a previous experiment (Section 3.5.3). The toxic effect of BrdU seen here reflected the previous findings. 10μM and 50μM BrdU were quite toxic to the cells resulting in approximately 75% and 90% kill respectively.

Table 3.4.1 lists brief descriptions of the effects of each BrdU concentration on the morphology of DLKP, DLKP-I, -M and -SQ after 14 days exposure to the compounds. 1μM BrdU had little effect on the morphology of any of the cells. Occasionally, larger than normal cells were evident, but apart from these and the slightly lower number of cells present compared with untreated controls (80-90%), no other morphological effects were apparent. At the higher BrdU concentrations (10μM and 50μM), fewer cells were present (25% and 10% of controls respectively) and these grew as single cells, not in colonies. The surviving cells were 4-6 times larger than the control cells and their cytoplasm had a stretched, fibrous appearance (Figures 3.4.1 and 3.4.2).

No interconversion of clonal morphologies was observed at any BrdU concentrations examined.

To determine if the change in morphology observed after BrdU treatment was permanent or reversible, cells treated for 2 weeks in 10μM and 50μM BrdU were fed

with normal medium without BrdU, and their morphologies were observed over 4 weeks. After this time, colonies had reformed and each clone resembled its original morphology before BrdU treatment (see Figure 3.4.3).

Cell line	Control	50μM BrdU	10μM BrdU	50μM RA	10μM RA
DLKP	- ~50% confluent - some very big colonies	- very few cells alive - flattened & stretched	- ~10% of controls - flattened & stretched	- ~30% of controls - not as distorted as BrdU	- similar % to controls -morphology normal
DLKP-I	- ~40% confluent - 'I' colonies & some 'SQ' colonies	- very few cells alive - flattened & stretched	- ~10% of controls - flattened & stretched	- ~10% of controls - normal colonies of 3-4 cells	- similar % to controls - colonies smaller but normal
DLKP-M	- ~90% confluent - cells look normal	- very few cells alive - flattened & stretched	- ~10% of controls - flattened & stretched	- ~40% of controls - most cells normal	- similar % to controls - looks like control well
DLKP-SQ	- ~40% confluent - normal 'SQ' colonies	- ~40% of controls - stretched & elongated	- ~80% of controls - stretched & elongated	- ~10% of controls - normal squamous	- ~80% of controls - squamous colonies with cells more dispersed

Table 3.4.1 Summary table of morphological effects of bromodeoxyuridine (BrdU) and retinoic acid (RA) on DLKP and the clones.

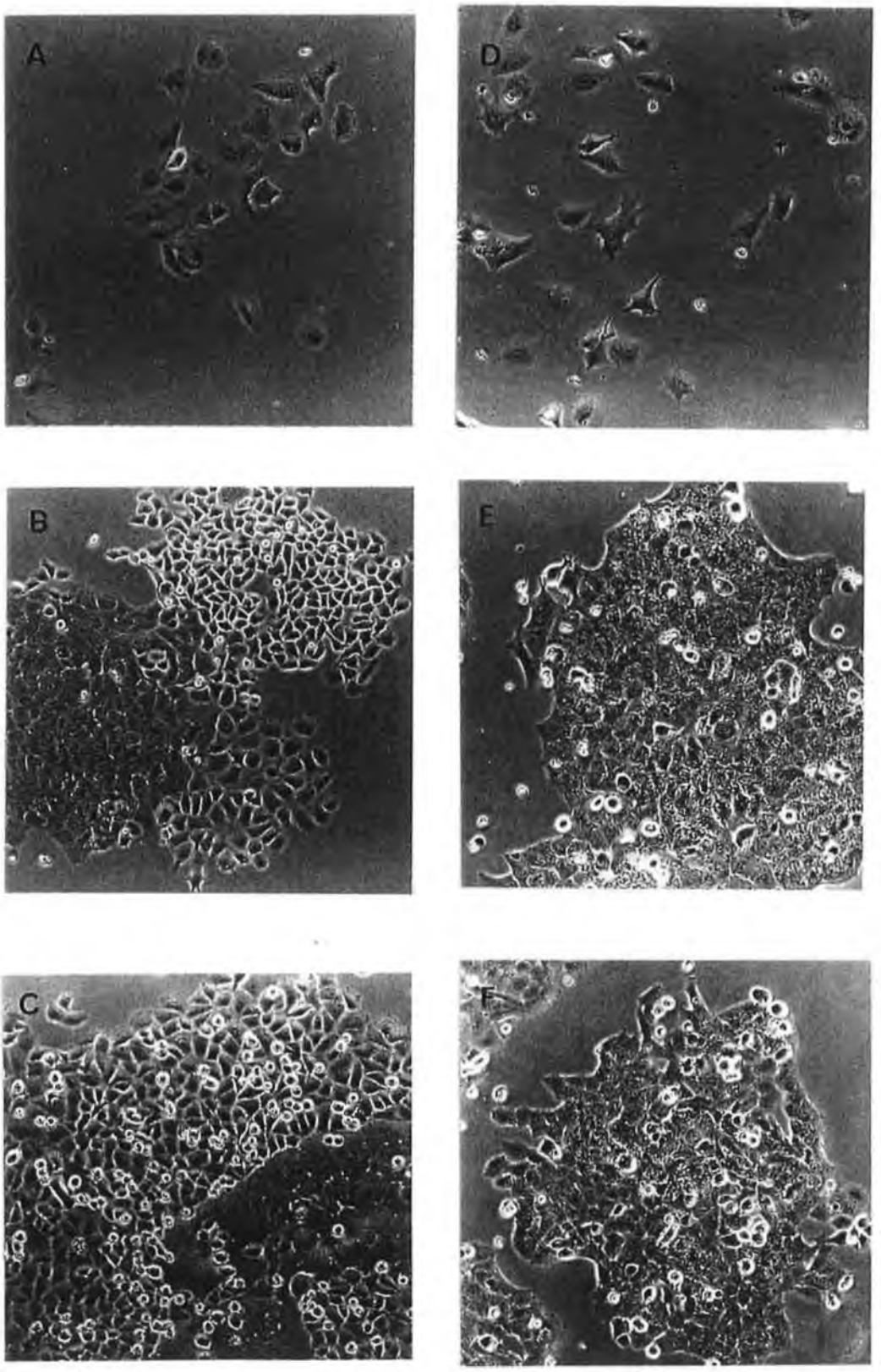


Figure 3.4.1 Effect of **A.** 10μM BrdU and **B.** 10μM RA on morphology of DLKP cells compared with **C.** untreated DLKP cells and effect of **D.** 10μM BrdU and **E.** 10μM RA on morphology of DLKP-I cells compared with **F.** untreated DLKP-I cells. x100.

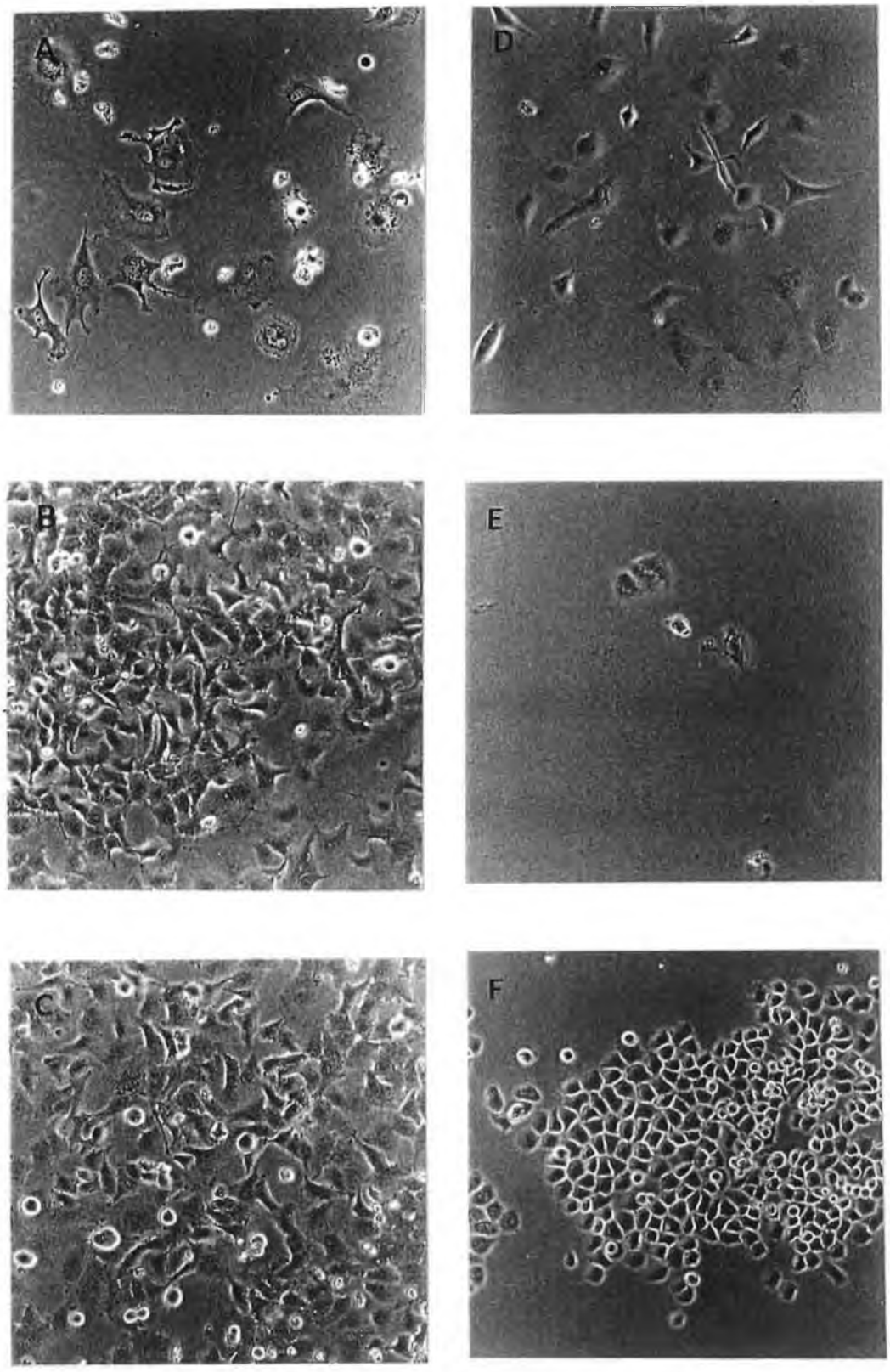


Figure 3.4.2 Effect of **A.** 10μM BrdU and **B.** 10μM RA on the morphology of DLKP-M cells compared with **C.** untreated DLKP-M cells and effect of **D.** 10μM BrdU and **E.** 50μM RA on the morphology of DLKP-SQ cells compared with **F.** untreated DLKP-SQ cells. x100.

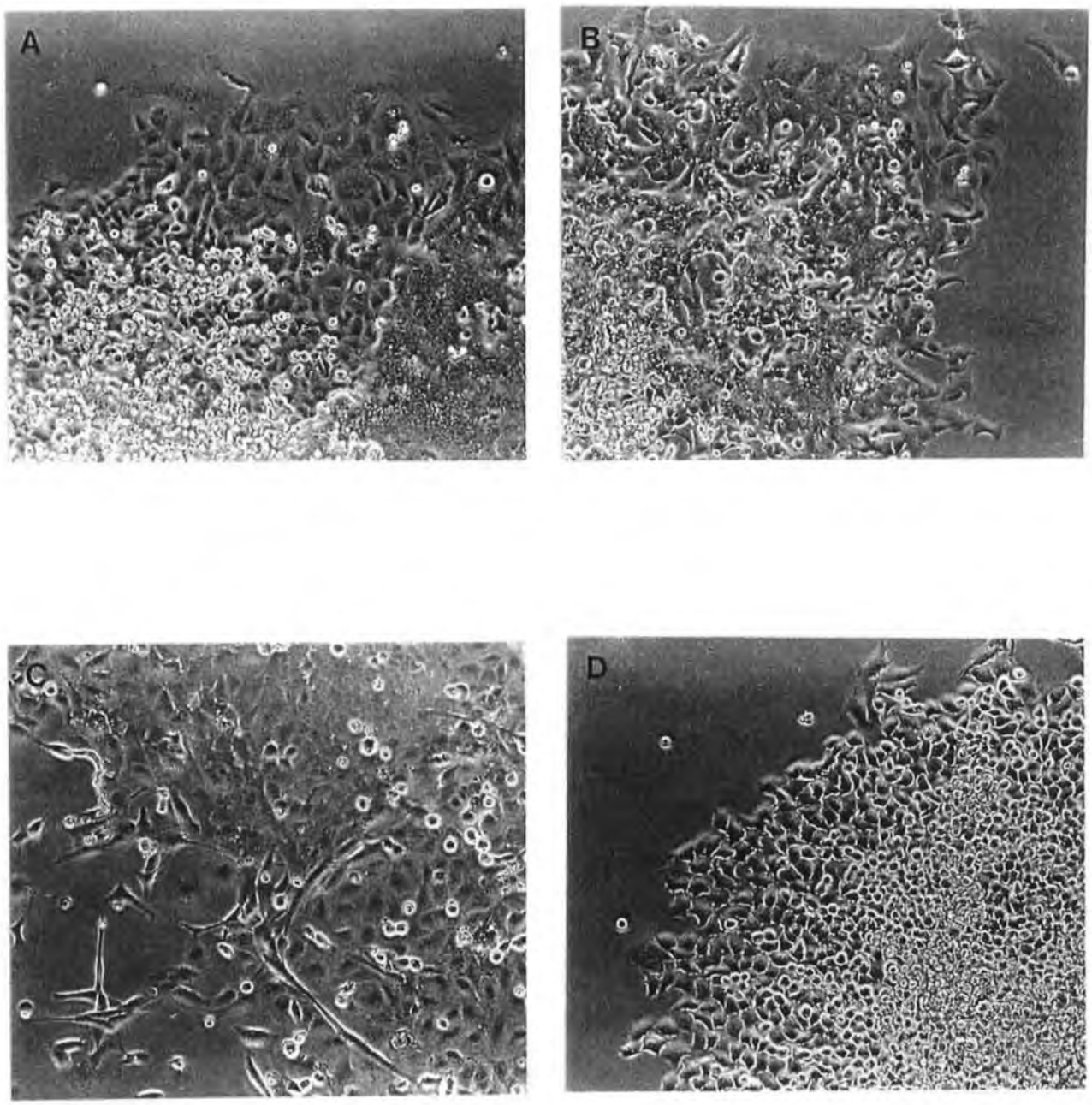


Figure 3.4.3 Recovery of **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells which had been fed in 10μM BrdU-containing medium for 2 weeks and then in BrdU-free growth medium for 4 weeks. x100.

3.4.1.2 Determination of marker expression

Cells in each of the DLKP lines had appeared enlarged, flattened and squamous-like following exposure to BrdU. These cells were now examined for their their expression of several markers whose presence/absence had previously been determined (Section 3.2.3). Emphasis was placed on epithelial markers because of the squamous appearance of the treated cells.

3.4.1.2.1 Keratins

Cells not exposed to BrdU had already been shown to express no epithelial markers including cytokeratins (Dr.Geraldine Grant, PhD thesis). Cells exposed to 1μM and 10μM BrdU for 7 days were fixed with methanol and examined for keratin (ker.) expression by immunocytochemistry (for details of methods see Section 2.15.1 - 2.15.2). An anti-pan cytokeratin Ab was used. This Ab was a broad spectrum reagent specific for kers. 1, 4, 5, 6 , 8, 10, 13, 18 and 19, and would thus react with simple, cornifying and non-cornifying squamous epithelia and pseudostratified epithelia. Approximately 20% and 50% of cells were found to react positively with this Ab following treatment with 1μM and 10μM BrdU respectively. Staining was very strong in 10μM BrdU-treated cells.

Expression of individual keratins is limited to specific epithelial cell and tissue types. It is therefore possible to identify likely origins of a cell by determining its keratin profile (for discussion see Section 4.8). Five keratin proteins were selected for analysis and BrdU-treated cells were found to be positive for ker. 8, 18 and 17 expression and negative for ker. 10 and 14 expression (Figure 3.4.4).

The time course for appearance of keratin proteins in DLKP, DLKP-I, -M and -SQ cells was determined. Multiple plates were set up and cells were grown in the presence of 10μM BrdU and fixed after 2, 6, and 8 days. Ker. 17, 18 and anti-pan cytokeratin Abs were used to probe the cells. Each Ab reacted faintly with cells exposed to BrdU for 2 days and the reactivity increased at days 6 and 8 (results not shown).

The ability of cells to retain keratin expression following removal of BrdU from the medium was examined. Cells were treated for 7 days with 50μM BrdU and then fed for another 8 days with growth medium without BrdU. Cells were then analysed for keratin expression and found to exhibit continued expression of kers. 17, 18 and also reacted positively with the anti-pan cytokeratin Ab (results not shown).

3.4.1.2.2 Neurofilaments

DLKP and the clones were exposed to 1μM BrdU for 7 days and then analysed for expression of neurofilaments which had previously been demonstrated in untreated cells (Section 3.2.3.3.4).

Neurofilament reactivity was found to be retained in all of the BrdU-treated cells (Figure 3.4.5).

3.4.1.2.3 Transglutaminase

To determine if other epithelial markers were now being expressed in the BrdU-treated cells, cells which were grown in 10μM BrdU for 7 days were examined for expression of transglutaminase, a squamous cell marker.

No transglutaminase reactivity was detected in any BrdU-treated cells (Figure 3.4.6).

3.4.1.2.4 Desmoplakin

Cells exposed to 1μM BrdU for 7 days were analysed for desmoplakin expression, a protein found in the desmosomes of epithelial cells.

All cells were negative for desmoplakin reactivity (Figure 3.4.7), as they had been shown to be in untreated cells in previous experiments in Section 3.2.3.

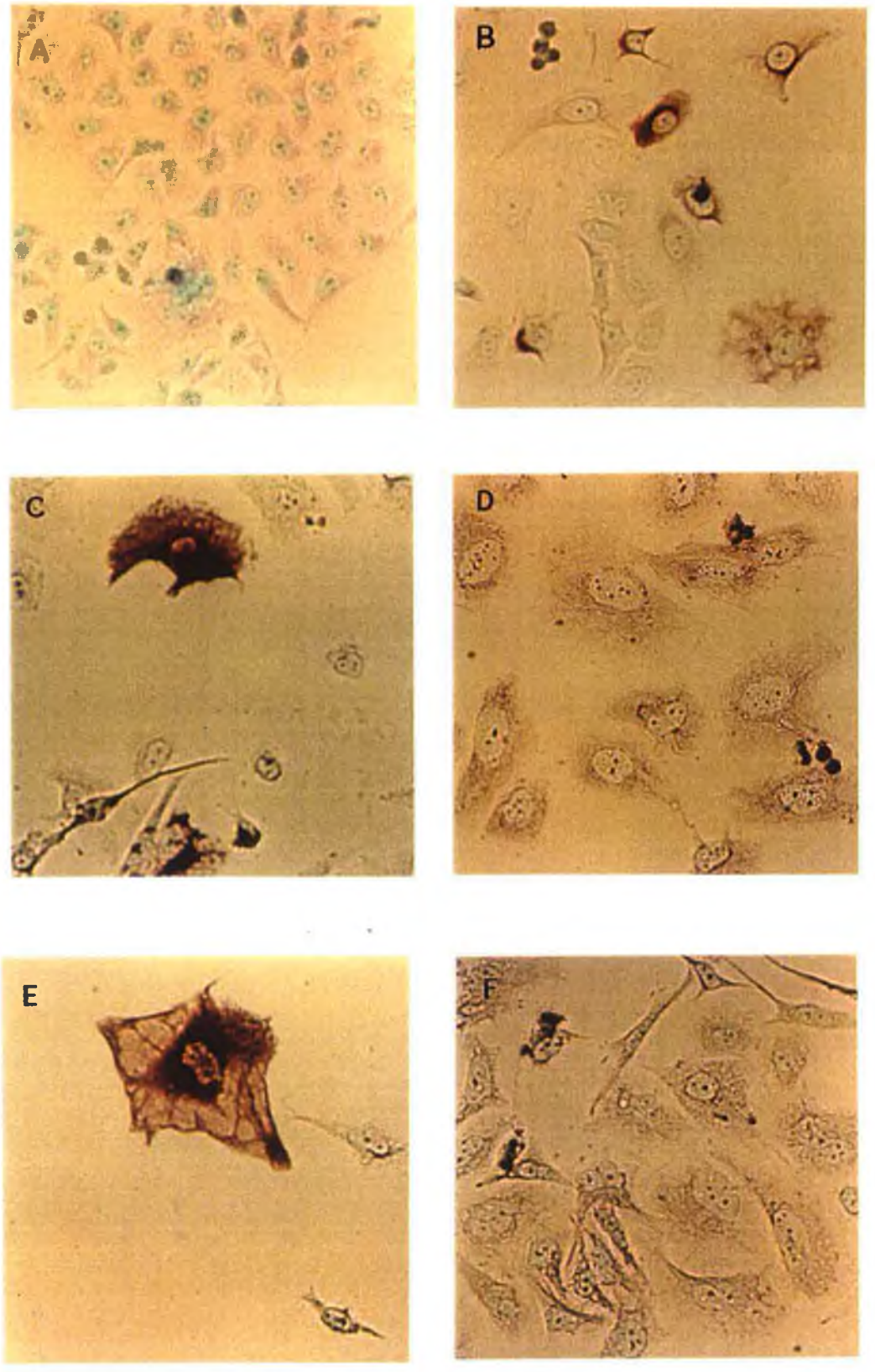


Figure 3.4.4 BrdU-induced keratin expression in DLKP cells. **A.** No immunoreactivity with anti-pan keratin Ab was detected in untreated cells .Cells treated with 5μM BrdU were found to react positively with anti- **B.** pan keratin, **C.** ker.8 **D.** ker.17 and **E.** ker.18 Abs. and negatively with **F.** anti-ker.10 Ab. x100.

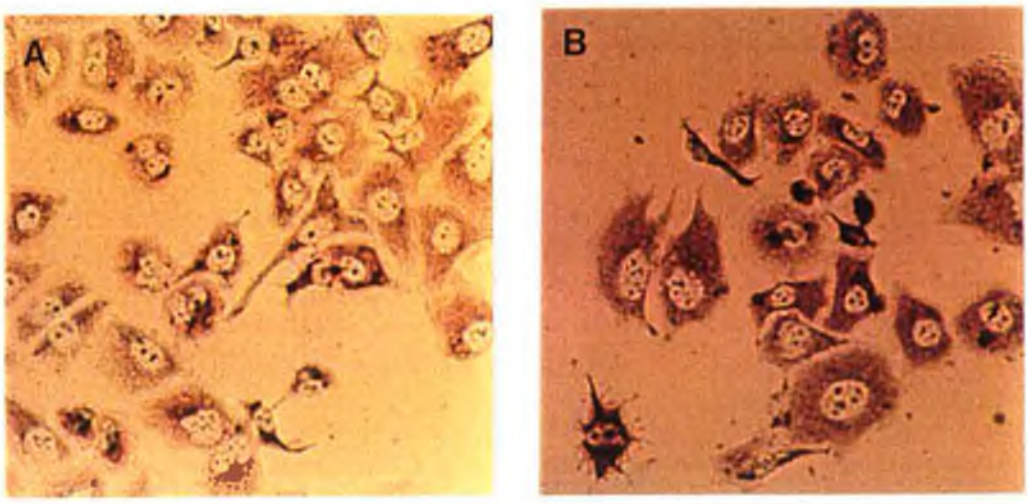


Figure 3.4.5 Neurofilament immunoreactivity is retained in DLKP cells treated with **A.** 1μM BrdU and 10μM RA. Results with DLKP clones were similar. x100.

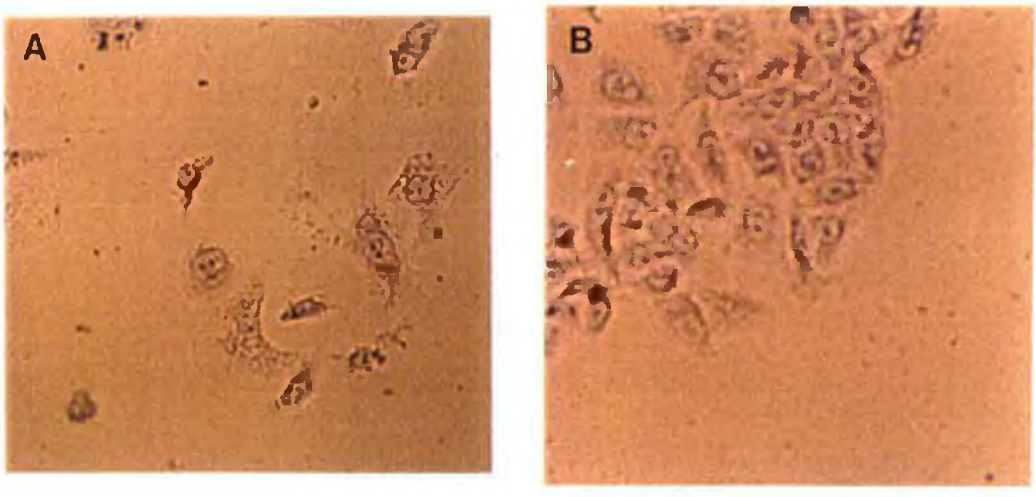


Figure 3.4.6 DLKP cells remained negative for transglutaminase immunoreactivity following treatment with **A.** 10μM BrdU and **B.** 20μm RA. Results with DLKP clones were similar. x100.

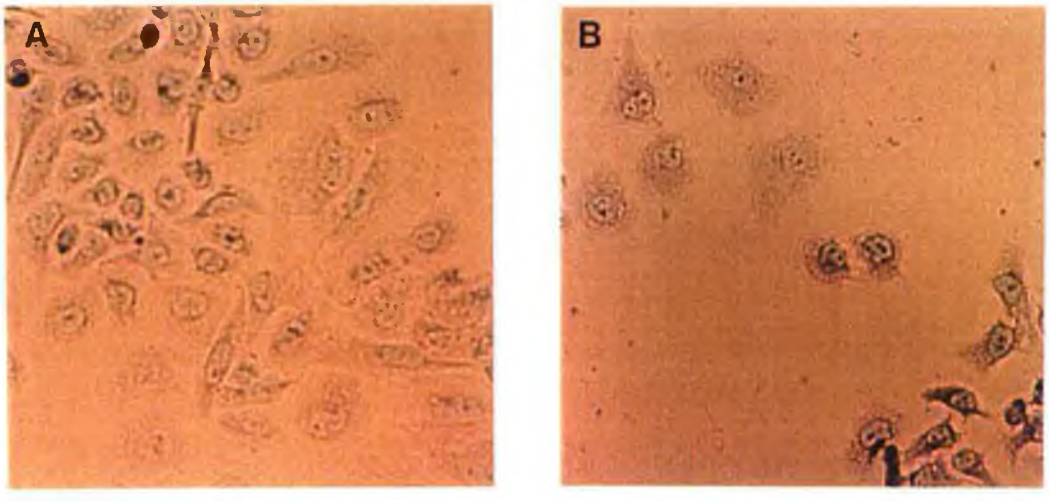


Figure 3.4.7 DLKP cells remained negative for desmoplakin immunoreactivity following treatment with **A.** 1μM BrdU and **B.** 10μM RA. Results with DLKP clones were similar. x100.

3.4.1.3 Effects of BrdU on keratin expression in other cell types

To ascertain whether the phenomenon of BrdU-induced keratin expression was unique to DLKP cells or not, a number of cell types from lineages both similar and different to that of DLKP were analysed. These cells were treated with BrdU as the DLKP cells had been and their keratin profiles before and after treatment were determined. In all assays with these lines, DLKP cells were assayed simultaneously as a positive control for the BrdU effect.

3.4.1.3.1 H82

H82 is a human cell line and is classified as variant SCLC. As such, this line expresses no cytokeratin proteins under normal growth conditions (see Table 2.5.1 for growth media), despite being presumably derived from lung epithelial cells. H82s do express a small number of NE markers such as NCAM and Leu 7, allowing them to be classified as NE cells. These cells are therefore similar to DLKP cells which also may be classified as SCLC-V, although some of the NE markers expressed by DLKP differ from those found in H82. It was therefore of interest to determine if BrdU was capable of inducing keratin expression in H82 cells.

The H82 cells were exposed to 1μM and 10μM BrdU for 7 days and the medium was replenished 3 times during that time, as was standard procedure in these assays. H82 cells grow in aggregates in suspension so it was necessary to harvest the cells each time the medium was changed. The cells were then resuspended in fresh medium. As with all cells that were grown in the presence of BrdU, decreased growth was noted with increasing BrdU concentration.

After 2 days' exposure to BrdU, it was observed that cells in the presence of BrdU had begun to attach to the surface of their wells. A greater number had attached in the 10μM wells than in 1μM BrdU (Figure 3.4.8). No cells had attached in the control wells which contained no BrdU. Attachment of H82 cells occurs in flasks under normal culture conditions *i.e.* without BrdU, but this is ususlly after more than a week in culture and less than 5% of the cells are seen to attach. After 7 days exposure to BrdU,

1μM and 10μM BrdU resulted in >50% and >90% attachment of surviving cells. Some attached cells had also begun to spread (Figure 3.4.8).

Following treatment with BrdU for 7 days, the supernatents from all H82 wells were collected, centrifuged and any cells harvested were cytospun onto glass slides and fixed (methanol as before, Section 2.15) for immunocytochemical analysis. All wells were also fixed and along with the cytospins, were tested for keratin expression.

Cytospins of control H82 cells (without BrdU) were negative for keratin expression when examined with the anti-pan cytokeratin Abs (Figure 3.4.9). None of these cells had attached to their wells during the 7 days of the assay.

Cytospins of H82s exposed to 1μM BrdU were found to be negative when analysed with anti-pan, ker.17 and ker.18 Abs. Attached cells also failed to react with these Abs (results not shown).

A very small number of H82 suspension cells were harvested from 10μM BrdU wells. These were negative when probed with anti-pan, ker.17 and ker.18 Abs. The cells which had attached to the wells in the presence of 10μM BrdU (>90% of population) were examined with anti-pan and ker.18 Abs. A small number of the cells (<5%) reacted positively with the anti-pan cytokeratin Ab and also with the anti-ker.18 Ab (Figure 3.4.9).

3.4.1.3.2 SK-N-SH

SK-N-SH is a human neuroblastoma cell line. These cells were examined because they are derived from the neural crest and therefore should not normally express keratin intermediate filaments.

The SK-N-SH cells were exposed to 5μM BrdU for 7 days. Unlike the H82 cells, these cells do not grow in suspension but attached to a substratum. Decreased growth was seen in the presence of BrdU. After 7 days, the cells were fixed and keratin expression was determined.

Unexpectedly, it was found that untreated SK-N-SH cells contained keratin proteins as they reacted positively with the anti-pan and ker.18 Abs. No reactivity was seen with the anti-ker.17 Ab (Figure 3.4.9).

Cells treated with 5μM BrdU appeared to have increased reactivity with the pan and ker.18 Abs. These cells also reacted with a ker.8 Ab but did not express keratins 10, 14 or 17 (Figure 3.4.9).

3.4.1.3.3 HL60

HL60 is a human nonlymphatic leukemic cell line and therefore is derived from a lineage distinct from epithelial and neural crest lines. These cells are a classical model for studying differentiation *in vitro* as they can be induced to undergo terminal differentiation along either the myeloid or monocytic pathway. These cells do not express keratin intermediate filaments.

HL60 cells grow in suspension and therefore, during the BrdU assays, they were handled as the H82 cells had been. During exposure to 1μM and 10μM BrdU, no attachment of the cells to their wells was seen. Following 7 days treatment, all cells were harvested, made into cytospins and fixed for keratin analysis.

When control HL60 cells (without BrdU) were analysed, no keratin reactivity was seen with the anti-pan cytokeratin Ab (Figure 3.4.10).

No reactivity was seen either with those cells which had been exposed to 1μM or 10μM BrdU (Figure 3.4.10).

3.4.1.3.4 A549

A549 cells are classified as human lung adenocarcinoma cells. They are therefore derived from the epithelium and they express keratin proteins. These cells were examined to determine the effects of BrdU on cells which already contain keratins.

The A549 cells were exposed to 5μM BrdU for 7 days. After this time, untreated control cells reacted positively with anti-pan and ker.18 Abs (Figure 3.4.10).

The cells which were exposed to BrdU showed increased reactivity with the same Abs. They also reacted strongly with anti-ker.8 and ker.17 Abs but no expression of keratins 10 or 14 was detected (Figure 3.4.10).

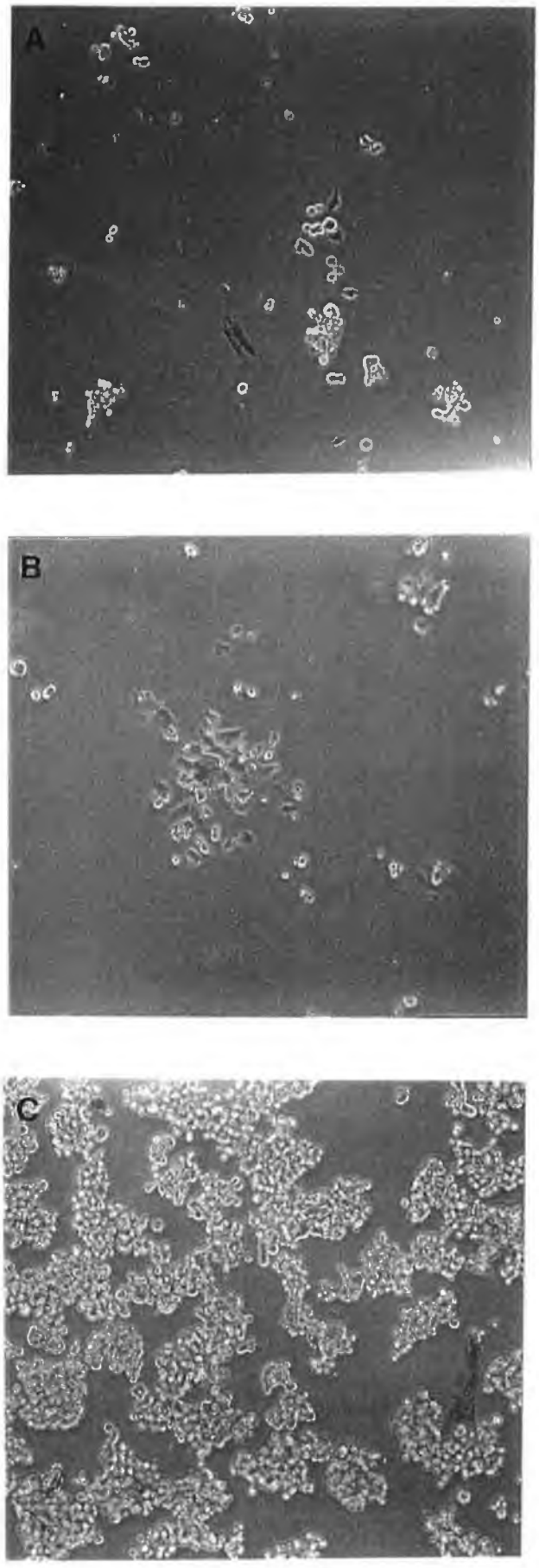


Figure 3.4.8 Effect of **A.** 1μM BrdU and **B.** 10μM BrdU on the morphology of H82 cells compared with **C.** untreated H82 cells. Increased adherence and decreased cell growth was seen with increasing BrdU concentration. x100.

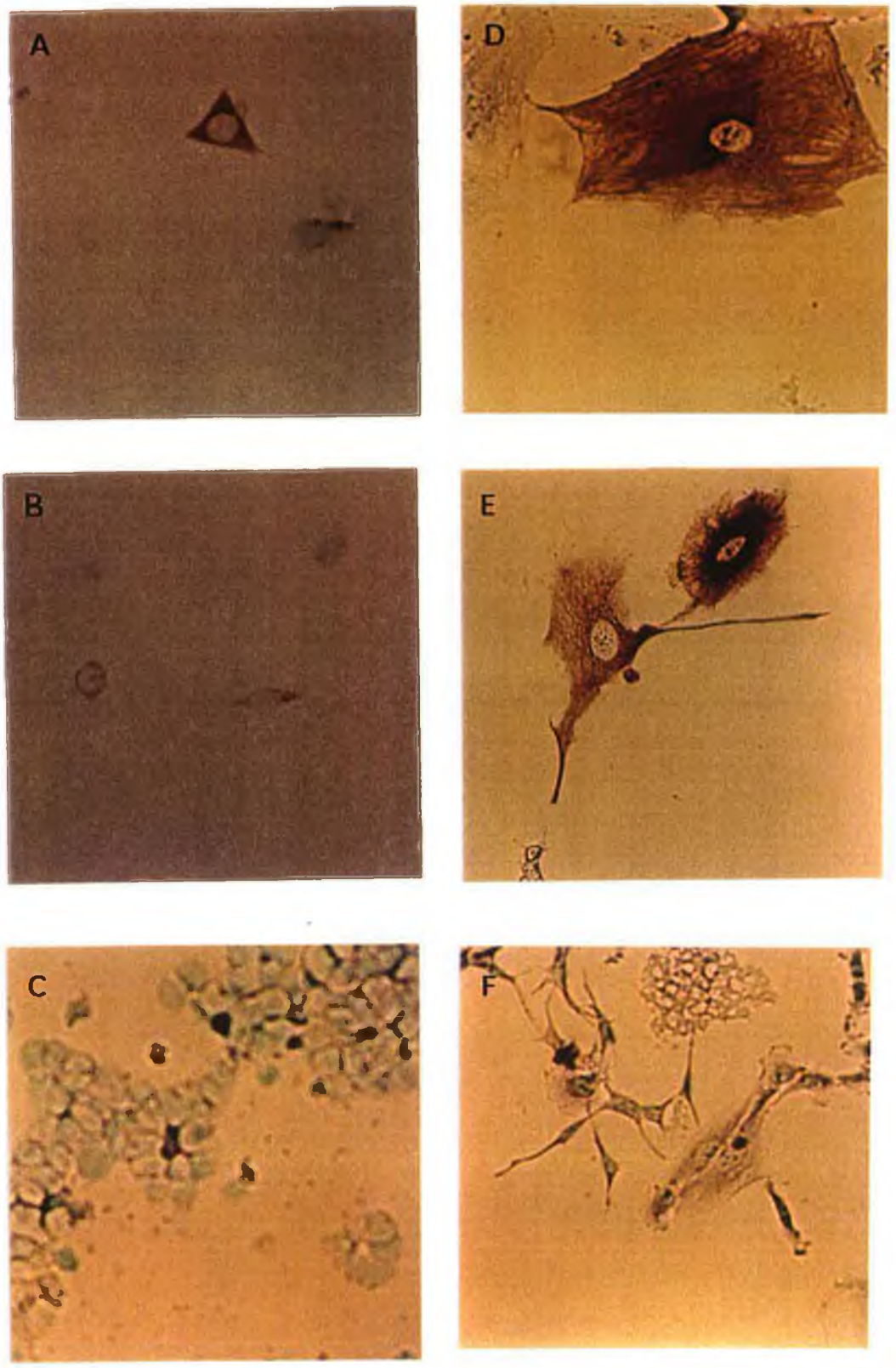


Figure 3.4.9 Induction of **A.** keratin expression (anti-pan keratin Ab.) and **B.** ker.18 expression in H82 adherent cells following exposure to 10μM BrdU. **C.** Untreated suspension H82 cells remained negative for keratin expression (anti-pan keratin Ab.). x100.
Increased expression of **D.** ker.8 and **E.** ker.18 in SK-N-SH cells following exposure to 5μM BrdU compared with **F.** ker.18 expression in untreated SK-N-SH cells. x100.

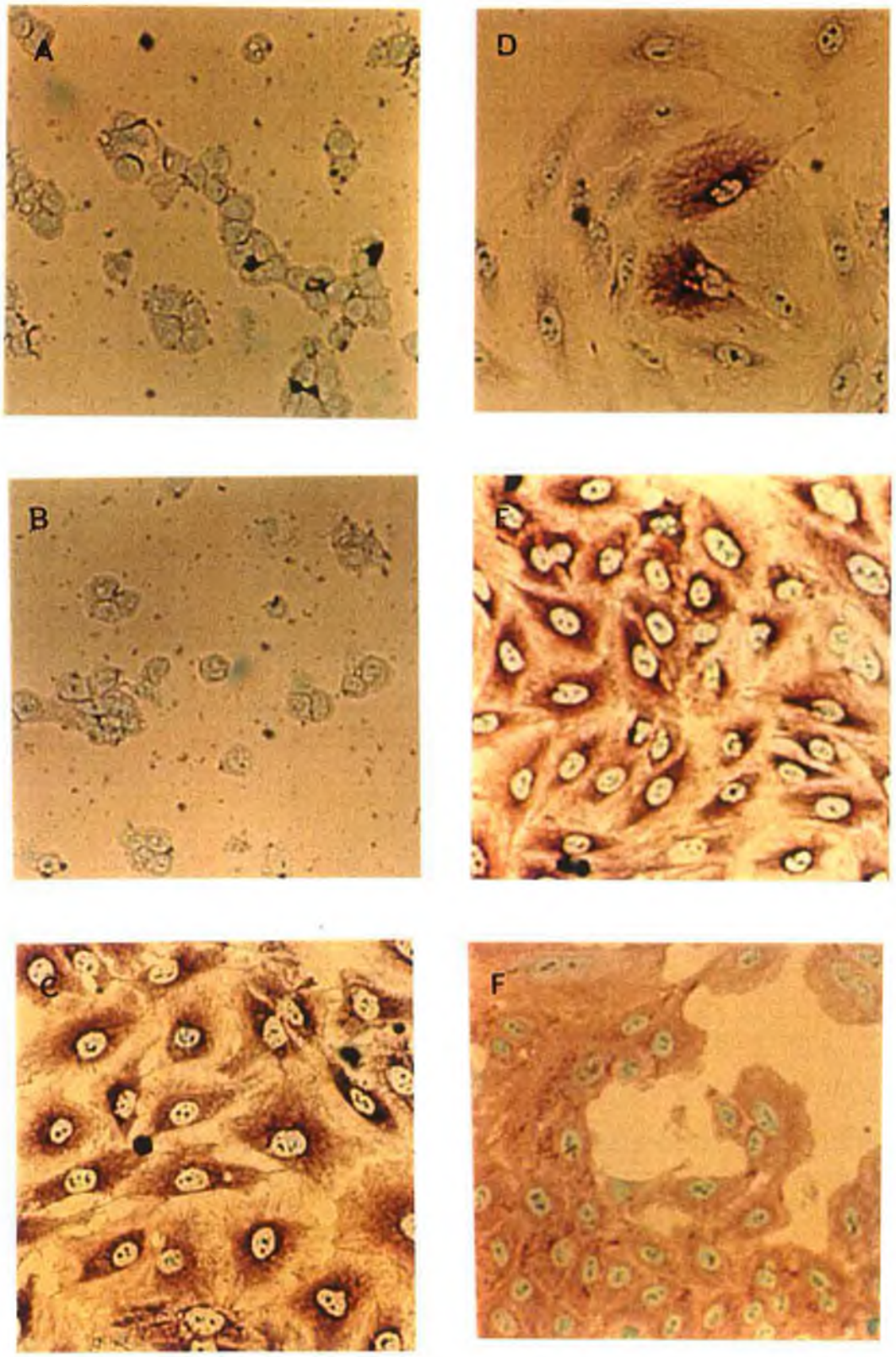


Figure 3.4.10 Reactivity of anti-pan keratin Ab. with HL60 cells **A.** before and **B.** after treatment with 10μM BrdU. No keratin was detected in either case. x100.
An apparent increase in expression of **C.** ker.8, **D.** ker.17 and **E.** ker 18 was detected in A549 cells which had been exposed to 5μM BrdU compared with **F.** untreated A549 cells. x100.

3.4.2 Retinoic Acid

Vitamin A and its analogues, the retinoid family, have been shown to modulate epithelial cell differentiation both *in vivo* and *in vitro*. These agents inhibit squamous cell differentiation and squamous metaplasia can result from vitamin A deficiency. Retinoids interact with nuclear retinoid receptors but as yet, their mechanism of action on differentiation processes is not understood. The effect of all-trans retinoic acid (RA) on the interconversion of the clones and on their marker expression was examined.

3.4.2.1 Morphological effects on DLKP and clones

DLKP and the clones were grown in 6-well plates in the presence of 10μM and 50μM RA. The medium was replaced every 2-3 days and after 14 days exposure, the cells were viewed under the microscope. The observations made are summarised in Table 3.4.1.

Only slight differences between control cells and 10μM RA-treated cells were apparent. There were approximately 10% fewer cells in the treated wells. These cells were growing in colonies which were slightly smaller than control colonies. The cells in these R.A.-treated colonies were not as tightly packed as control cells and the cells appeared separated from each other. The morphologies of each of the DLKP clones were apparently unchanged and no interconversion was observed. Very few cells remained in the 50μM RA-treated wells ($<5\%$ controls). Some colonies of cells were present and morphologically the cells resembled their normal, untreated counterparts. No giant or stretched cells similar to BrdU-treated cells were seen.

3.4.2.2 Determination of marker expression

DLKP, DLKP-I, -M and -SQ were treated with RA and then analysed for alterations in marker expression.

3.4.2.2.1 Keratin

The DLKP lines were grown in 20μM RA for 7 days and then analysed for keratin expression.

All cells reacted positively with the anti-pan cytokeratin Ab (Figure 3.4.11). More cell survival was seen in these RA-treated cells compared with BrdU-treated cells. In addition, the cells were growing in colonies and were apparently normal in size. Keratin reactivity appeared to be present in every cell.

3.4.2.2.2 Neurofilaments

Cells were exposed to 10μM RA for 7 days and then analysed for neurofilament expression.

Neurofilament reactivity was seen in all cells of each DLKP line (Figure 3.4.5).

3.4.2.2.3 Transglutaminase

Cells treated with 20μM RA were examined for expression of transglutaminase.

No transglutaminase was detected in any of the lines (Figure 3.4.6).

3.4.2.2.4 Desmoplakin

Expression of desmoplakin was analysed in cells grown in 10μM RA for 7 days.

No desmoplakin reactivity was detected (Figure 3.4.7).

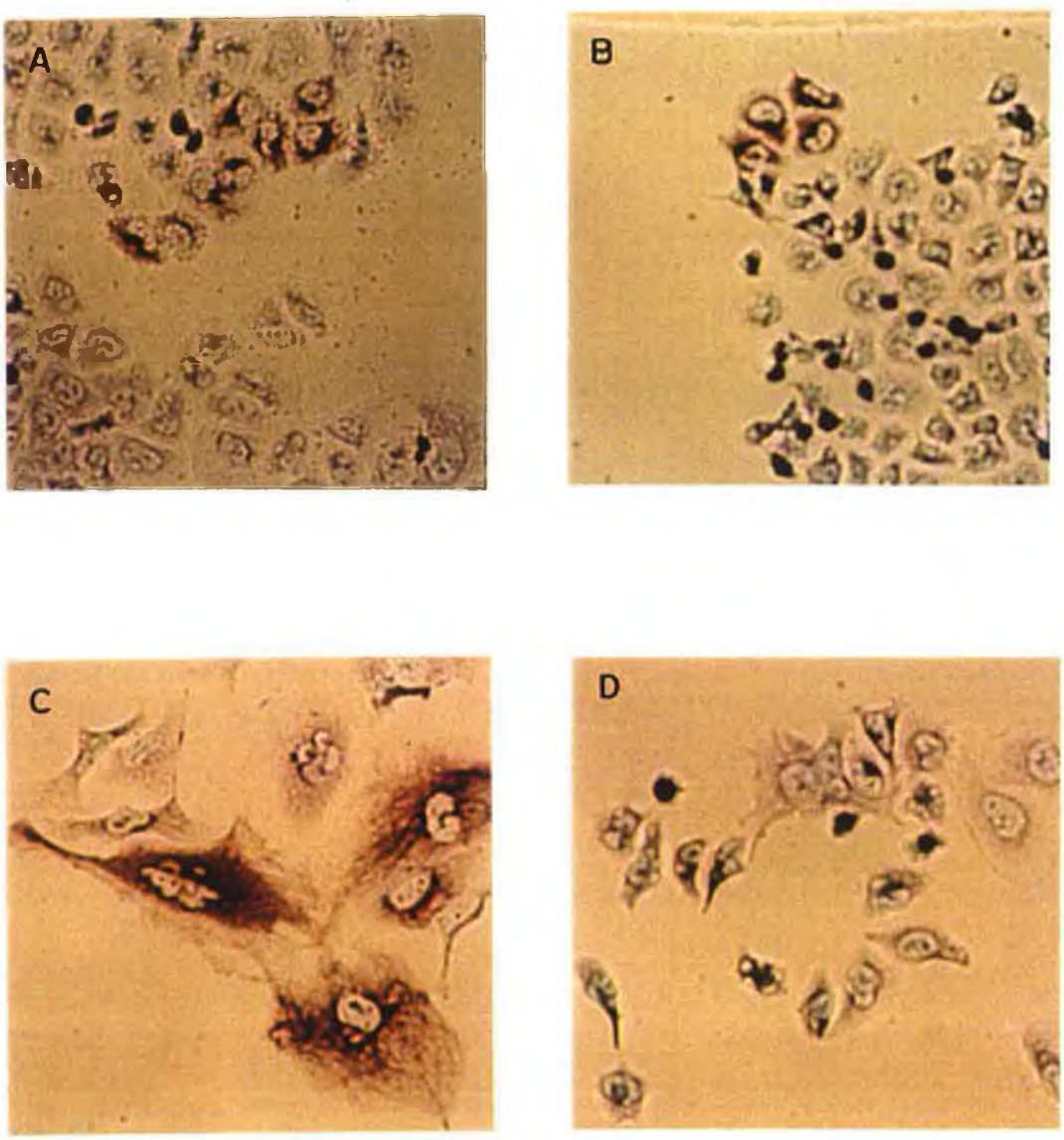


Figure 3.4.11 Detection of induction of keratin expression using anti-pan keratin Ab. in **A.** DLKP, **B.** DLKP-I, **C.** DLKP-M and **D.** DLKP-SQ cells which had been exposed to 20μM RA. x100.

3.5 COMPARISON OF GROWTH OF DLKP AND DLKP-A

DLKP-A is a MDR variant of DLKP which was selected in increasing concentrations of the chemotherapeutic drug adriamycin and is several-fold more resistant than DLKP to a range of toxic compounds (see Table 3.5.1). Biological differences between DLKP and DLKP-A have previously been detected.For example, DLKP-A expresses high levels of P-glycoprotein, a transmembrane pump, which is probably one of the mechanisms of its resistance. This protein is expressed at extremely low levels in DLKP and is often indetectable, even by RT-PCR analysis (Lorraine O'Driscoll, PhD thesis).

The growth rates and responses to growth factors of DLKP and DLKP-A were compared to determine if the aquisition of the MDR phenotype had altered the growth characteristics of DLKP-A w.r.t. DLKP *ie.* was development of the MDR phenotype accompanied by enhanced or depleted growth capabilities, or neither.

Drug	DLKP-A (fold w.r.t. DLKP)
Adriamycin	322.2
Vincristine	79.5
VP16	36.2

Table 3.5.1 Fold resistances of DLKP-A w.r.t. DLKP to several chemotherapeutic agents. From Clynes *et al., Cytotechnology* **10**:75-89, 1992.

3.5.1 Growth Responses to Serum

The rate of the growth of DLKP and DLKP-A in different serum concentrations over 9-10 days was examined.

No significant difference in growth rates between the 2 lines was apparent up to 7 days in culture. After 7 days however, the rate of growth of DLKP-A appeared to decrease relative to that of DLKP (Figure 3.5.1).

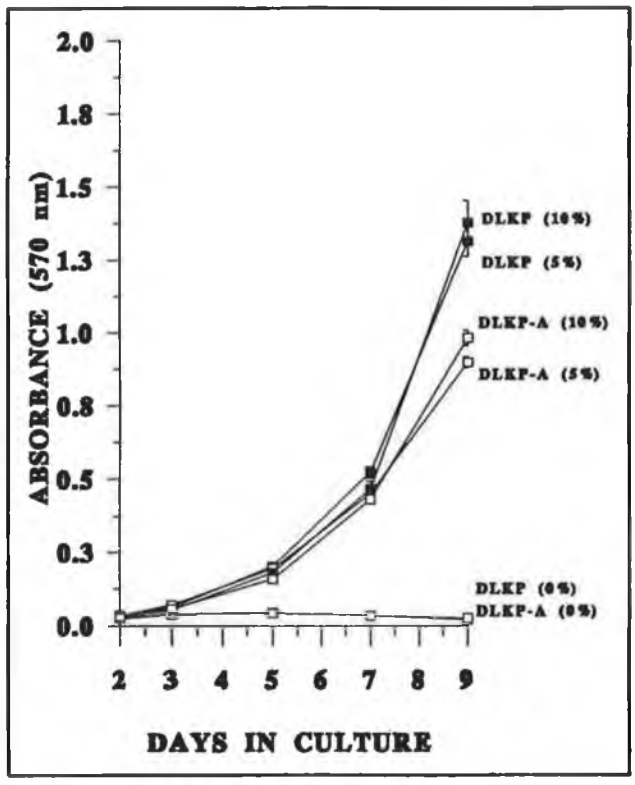


Figure 3.5.1 Proliferation of DLKP and DLKP-A cells in monolayer culture in the presence of 0%, 5% and 10% serum (as indicated in parentheses), using CVDE end-point.

3.5.2 Responses to Growth Factors

The effects of 6 growth factors (IL-1, IL-6, TGFβ, EGF, PDGF-A and PDGF-B) on the growth of DLKP and DLKP-A cells were determined. 2 end-points were employed; (i) crystal violet dye elution after 7 days in culture (see Section 2.9.2) and (ii) ^{3}H-thymidine incorporation after 24 hr in culture (see Section 2.9.7).

3.5.2.1 Crystal violet end-point

The effect of the above 6 growth factors on the growth of DLKP and DLKP-A after 7 days in culture was determined. Assay to assay variation in the extent of responses of both lines was common but the response itself *ie*. stimulatory, inhibitory or neither, was reasonably constant. Assays were carried out in 1% background serum because negligible growth occurred in the absence of serum. The concentrations of growth factors used were within the biologically active ranges for each growth factor. The results presented are the mean results from at least 3 separate experiments, therefore error bars are often large. Each individual experiment also consisted of 8 replicates. Growth was determined by CVDE assay as outlined in Section 2.9.2. The results are expressed as percentage growth over control where the control is medium without growth factor.

3.5.2.1.1 PDGF-A

PDGF-A appeared to have a slight stimulatory effect on the growth of DLKP-A cells over the range of concentrations examined (Figure 3.5.2). Maximum stimulation in the region of 130% of control was seen at concentrations of 1-25ng/ml PDGF-A. A decrease in stimulation was seen at 50ng/ml. A lesser degree of stimulation was seen for DLKP with a maximum effect at lower concentrations of PDGF-A, but large error bars are also noted here.

3.5.2.1.2 PDGF-B

PDGF-B also appeared to stimulate the growth of DLKP-A up to approximately 130% of control (Figure 3.5.3). Again, little effect was seen for DLKP at the concentrations of PDGF-B assayed.

3.5.2.1.3 TGFβ

TGFβ had a small stimulatory effect of up to 115% of control on the growth of DLKP-A (Figure 3.5.4). In contrast, this growth factor had an inhibitory effect on DLKP. Error bars are large making an accurate assessment of the extent of inhibition difficult. Nonetheless, inhibition of growth was seen at each concentration of growth factor examined, with a maximum effect of close to 65% growth of control *ie.* 35% inhibition.

3.5.2.1.4 EGF

EGF was also stimulatory to DLKP-A cells to about 140% of controls (Figure 3.5.5). Again, the extent of stimulation varied between assays resulting in large standard deviations, but the overall effect was stimulation at concentrations from 1-20ng/ml. EGF appeared to have no real effect on the growth of DLKP cells.

3.5.2.1.5 IL-1

IL-1 stimulated the growth of DLKP-A cells up to approximately 125% of controls (Figure 3.5.6). Again, no effect was seen with DLKP cells, except inhibition to approximately 80% of controls at 20ng/ml IL-1 which was the highest concentration examined.

3.5.2.1.6 IL-6

IL-6 had a slight stimulatory effect on DLKP-A growth, up to approximately 125% of controls, again with large standard deviations (Figure 3.5.7). As was the case with most of the other growth factors, no effect was observed with the DLKP cells, except at 75U/ml where stimulation up to 120% of controls was seen.

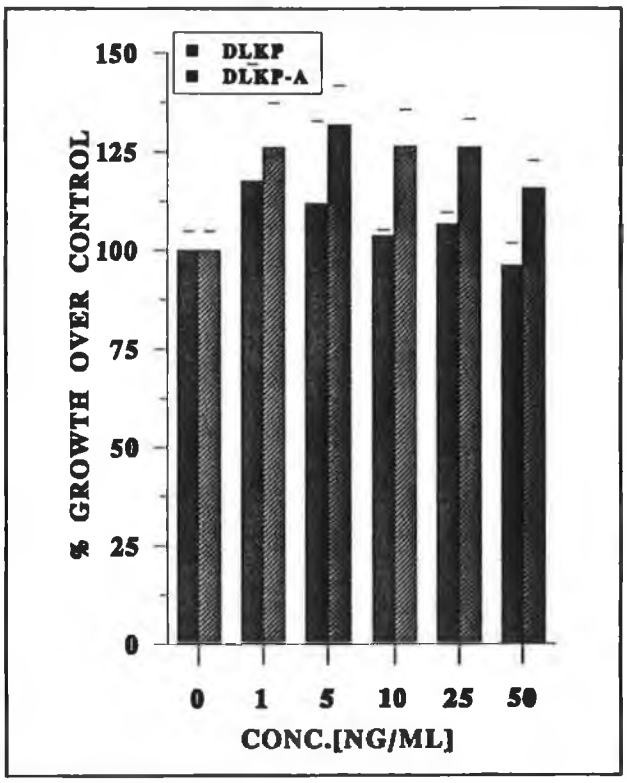


Figure 3.5.2 Effect of PDGF-A on the growth of DLKP and DLKP-A cells using CVDE end-point.

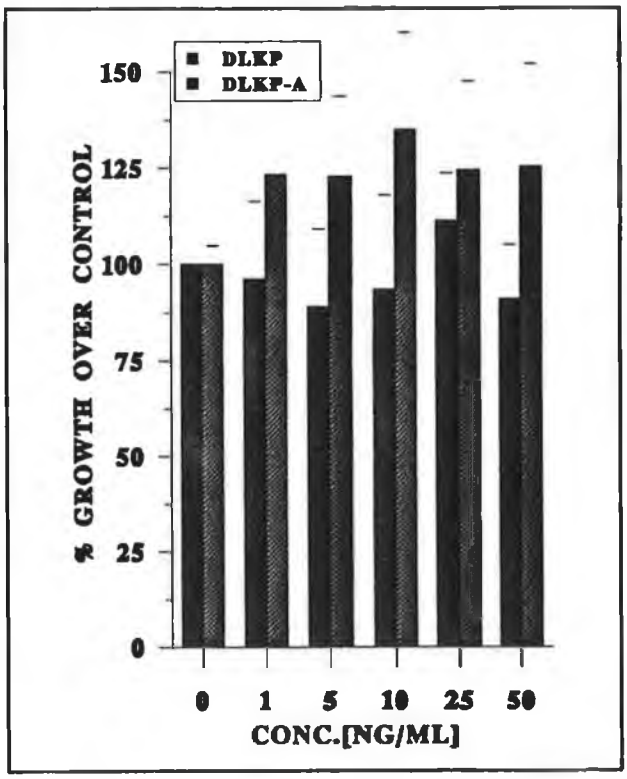


Figure 3.5.3 Effect of PDGF-B on the growth of DLKP and DLKP-A cells using CVDE end-point.

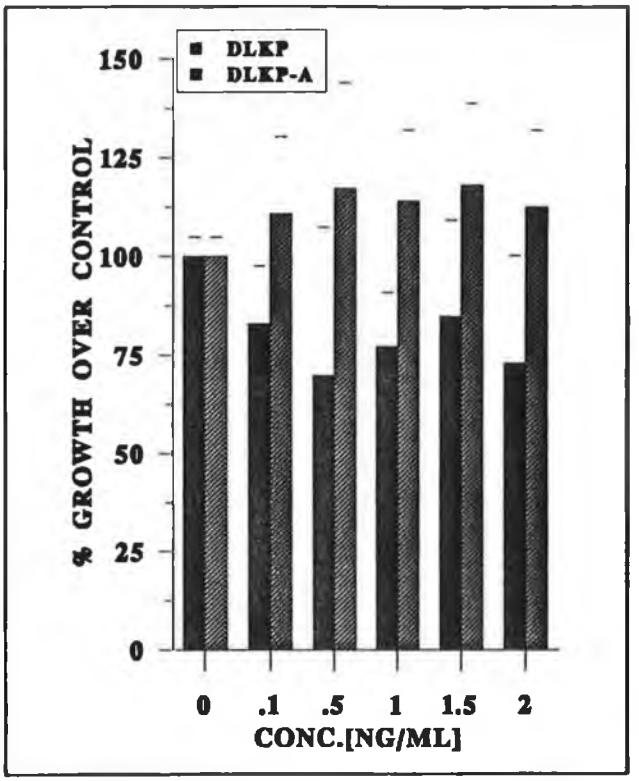


Figure 3.5.4 Effect of TGFβ on the growth of DLKP and DLKP-A cells using CVDE endpoint.

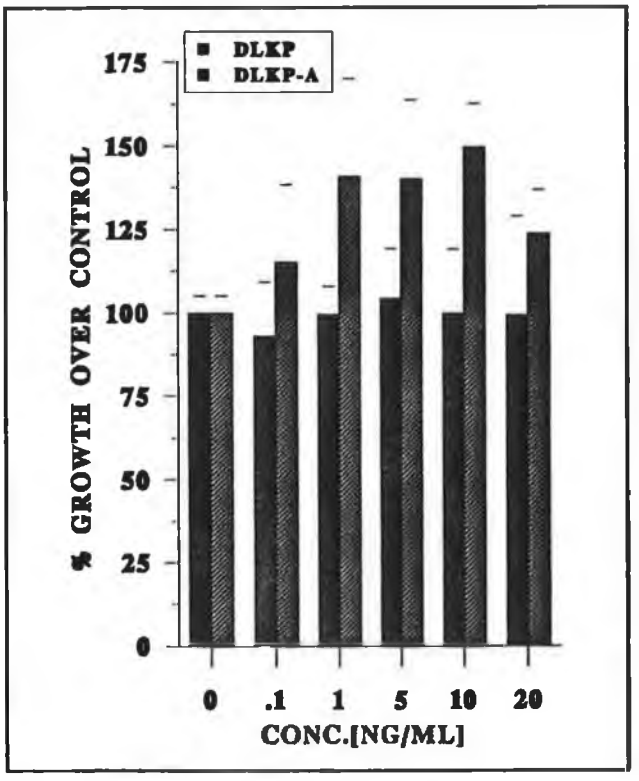


Figure 3.5.5 Effect of EGF on the growth of DLKP and DLKP-A cells using CVDE end-point.

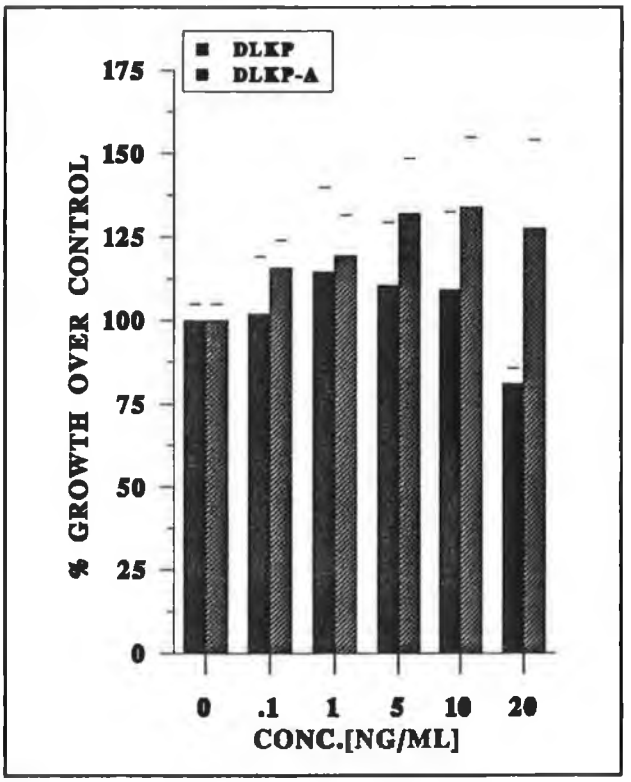


Figure 3.5.6 Effect of IL-1 on the growth of DLKP and DLKP-A cells using CVDE end-point.

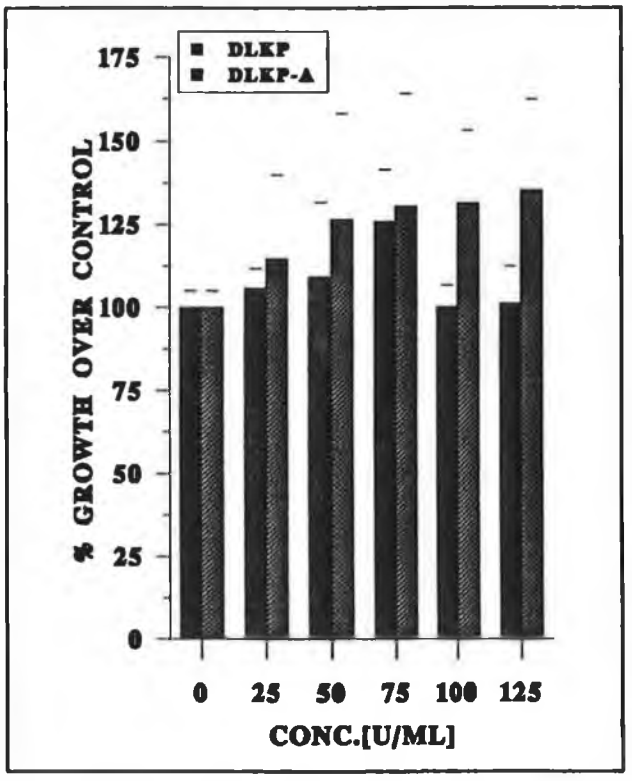


Figure 3.5.7 Effect of IL-6 on the growth of DLKP and DLKP-A cells using CVDE end-point.

3.5.2.2 ^{3}H-Thymidine incorporation

The incorporation of exogenous radio-labelled thymidine (Td) into the DNA of replicating cells, and so indicating DNA synthesis, was used as an alternative measurement of cell proliferation. As outlined in Section 2.9.7, cells were serum-starved for 24 hr prior to addition of growth factor in order to decrease cells proliferation and thus DNA synthesis. Growth factors were then added to the cells in serum-free medium and 4hrs later, ^{3}H-thymidine (^{3}H-Td) was added. Any stimulatory or inhibitory effects of the growth factors on the cells should be represented by an increase or a decrease in radio-labelled Td present in the cells. This was determined by liquid scintillation counting. As with the crystal violet end-point determinations (Section 3.3.2.1), results are presented as the mean results of at least 3 separate experiments, and each experiment contained 8 replicates.

As was the case with the crystal violet end-point assays, variation was seen between assays in the size of responses, but not in actual responses *ie.* stimulatory, inhibitory or neither. It was noted during the course of these experiments that ^{3}H-Td incorporation by positive control cells *ie.* in presence of 5 - 10% serum, was usually less than 200% and never more than 300% that incorporated by negative control cells, *ie.* in presence of 0% serum. Therefore, the 'window' of stimulation produced by individual growth factors was also likely to be relatively narrow and would not be expected to be greater than that produced by whole serum.

3.5.2.2.1 PDGF-A

PDGF-A caused an increase in DNA synthesis in DLKP cells, with increased synthesis occurring with increasing concentrations of PDGF-A from 1-50ng/ml (Figure 3.5.8). At 50ng/ml, a maximum incorporation of 3H-Td of 180% of control was seen. In contrast, PDGF-A had no effect on DNA synthesis in DLKP-A cells at the same concentrations.

3.5.2.2.2 PDGF-B

DNA synthesis in DLKP cells was also stimulated to 160% of controls by PDGF-B (Figure 3.5.9). Again, increased incorporation of 3H-Td was found with increasing concentrations of PDGF-B up to 50ng/ml where levels began to decrease. No stimulation of DNA synthesis was seen with DLKP-A, and indeed, slight inhibition seemed to be occurring.

3.5.2.2.3 TGFβ

TGFβ was found to increase DNA synthesis in DLKP cells up to 150% of control values, in the range of concentrations assayed (Figure 3.5.10). DNA synthesis in DLKP-A was reduced in response to TGFβ. Reduction to 80% of controls was seen in these cells.

3.5.2.2.4 EGF

The response of the cells to EGF varied in this assay (Figure 3.5.11). DLKP seemed to be stimulated by lower concentrations of EGF (0.1-5ng/ml) where 3H-Td incorporation up to 140% of controls was found. The response of DLKP-A cells was erratic with inhibition of DNA synthesis occurring at 1ng/ml and stimulation at 20ng/ml.

3.5.2.2.6 IL-1

3h-Td incorporation in DLKP and DLKP-A cells varied from assay to assay (Figure 3.5.12). An increase in DNA synthesis in DLKP was found across the range of IL-1 concentrations examined, but as with all of the growth factor assays, the magnitude of the response varied, reaching a maximum of 160% of controls at 5ng/ml. The response of DLKP-A cells also varied with no real increase in 3H-Td incorporation occurring at lower concentrations and up to 150% occurring at 20ng/ml.

3.5.2.2.6 IL-6

Increased DNA synthesis in DLKP cells occurred in response to IL-6, particularly at high concentrations where incorporation of up to 150% of controls was found (Figure 3.5.13). Again, slight inhibition of DNA synthesis was apparent in DLKP-A cells at 50U/ml IL-6.

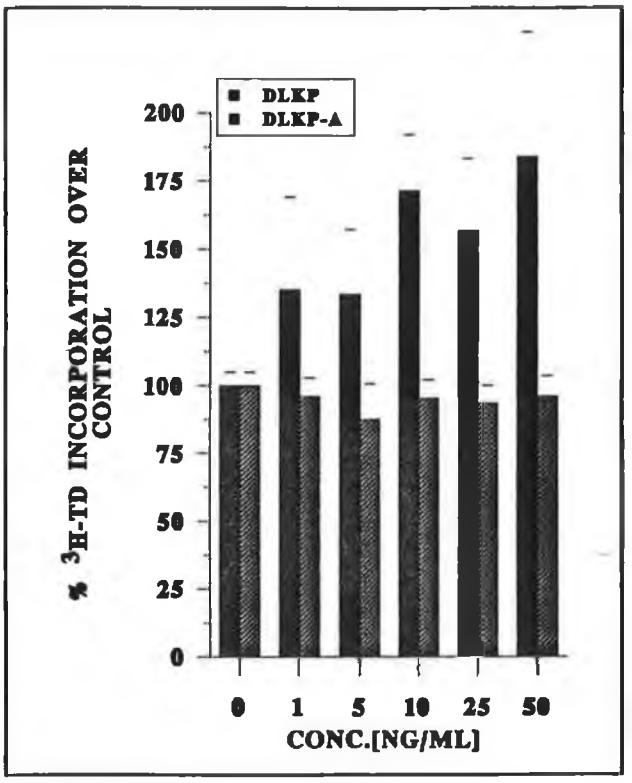


Figure 3.5.8 Effect of PDGF-A on ^{3}H-Td incorporation in DLKP and DLKP-A cells.

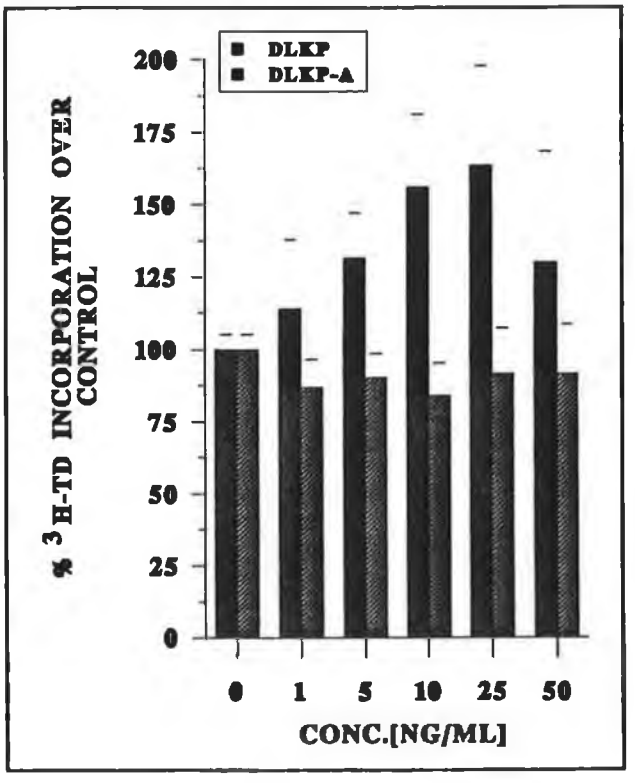


Figure 3.5.9 Effect of PDGF-B on ^{3}H-Td incorporation in DLKP and DLKP-A cells.

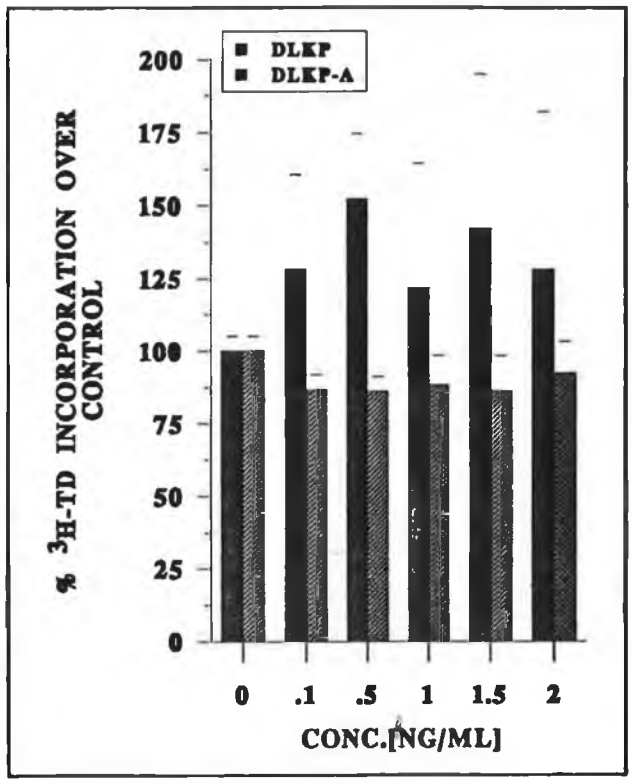


Figure 3.5.10 Effect of TGFβ on ^{3}H-Td incorporation in DLKP and DLKP-A cells.

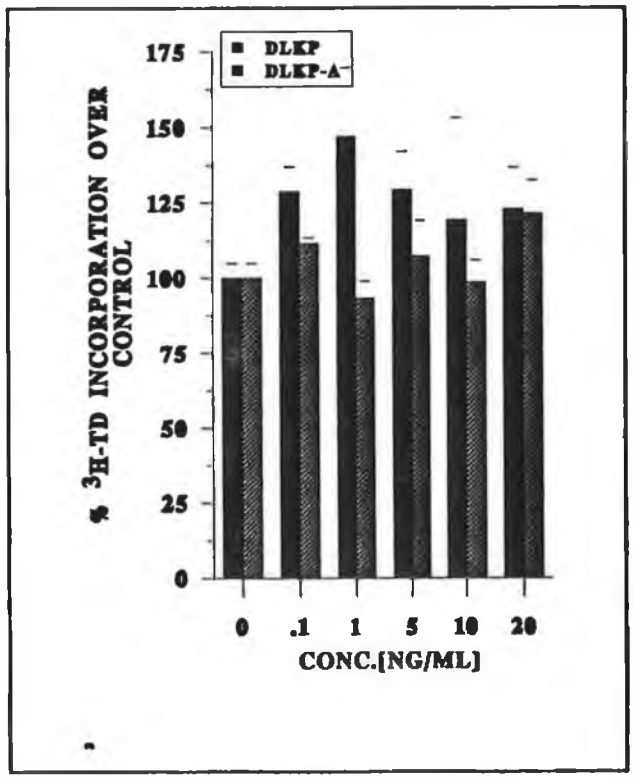


Figure 3.5.11 Effect of EGF on ^{3}H-Td incorporation in DLKP and DLKP-A cells.

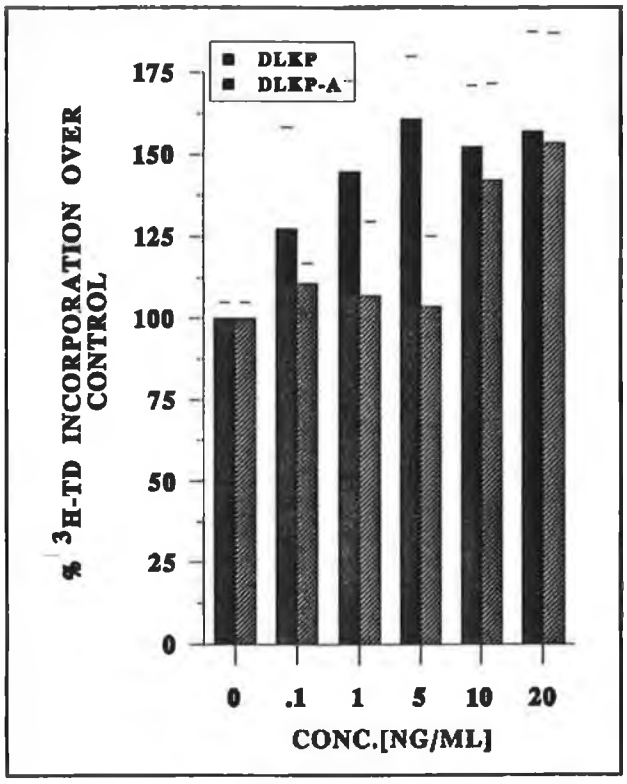


Figure 3.5.12 Effect of IL-1 on ^{3}H-Td incorporation in DLKP and DLKP-A cells.

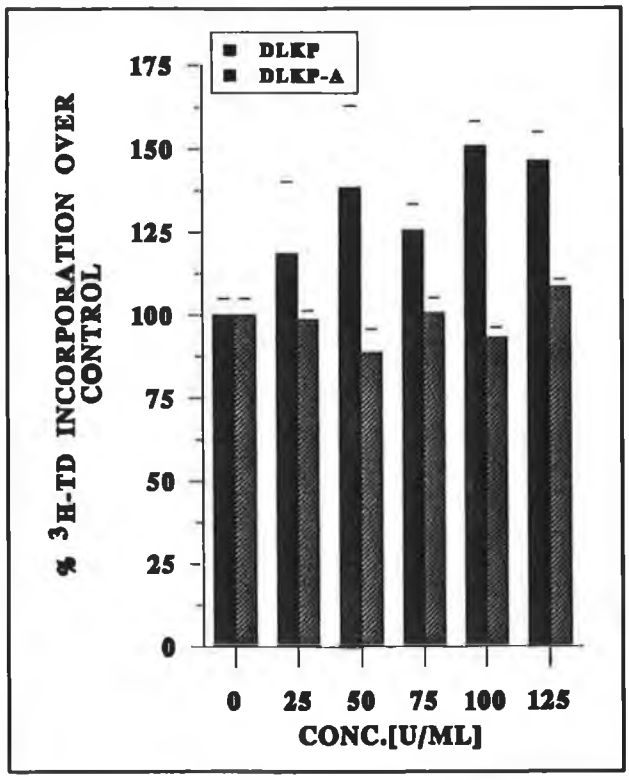


Figure 3.5.13 Effect of IL-6 on ^{3}H-Td incorporation in DLKP and DLKP-A cells.

Growth factor	DLKP	DLKP-A
PDGF-A	+/ 0	+
PDGF-B	0	+
TGFβ	0 / -	+
EGF	0	++
IL-1	+/ 0	+
IL-6	+/ 0	+

Table 3.5.2 Summary table of growth responses of DLKP and DLKP-A to growth factors as determined in the crystal violet dye elution (CVDE) assay.

+++ is stimulation >175% of control
++ is stimulation ~150-175% of control
+ is stimulation ~100-150% of control
0 is no effect
- is inhibition

Growth factor	DLKP	DLKP-A
PDGF-A	+++	0
PDGF-B	++	0 / -
TGFβ	++	-
EGF	+	+
IL-1	++	+
IL-6	+	0 / -

Table 3.5.3 Summary table of DNA synthesis responses of DLKP and DLKP-A to growth factors as determined by ^{3}H-thymidine incorporation assay. (+++, ++ *etc.* as for Table 3.5.1.).

3.5.3 Bromodeoxyuridine Toxicity Assay

The responses of DLKP-A cells to growth factors determined after 7 days in culture (CVDE end-point) indicated that the growth of these cells was capable of being stimulated by the growth factors examined but the ^{3}H-Td incorporation assays indicated otherwise. It was possible that the DLKP-A cells were responding to the growth factors and synthesising DNA but the cells were not taking up the exogenous Td to do so. During the biosynthesis of DNA precursors, cells can utilise a *de novo* route for synthesis of thymidylate (TMP) from deoxyuridylate (dUMP) via thymidylate synthetase (TS) or a salvage route from thymine and thymidine via thymidine kinase (TK) (Kornberg, 1974). Decreased levels of TK can prevent cells using the salvage pathway, thus decreasing the incorporation of exogenous Td. It was possible that DLKP-A cells lacked, or expressed reduced levels of, TK and so failed to incorporate ^{3}H-Td in the growth factor assays.

In order to examine the possibility that decreased levels of TK had caused the DLKP-A cells to incorporate very little exogenous 3H-Td compared with DLKP, a BrdU toxicity assay was carried out. If DLKP-A cells contained lower levels of of TK than DLKP, BrdU may be less toxic to DLKP-A than DLKP as less BrdU would be incorporated via the salvage pathway. Such a decrease in TK activity could explain why growth was seen in response to growth factors in the crystal violet assays (Section 3.5.2.1) while no incorporation of exogenous Td was detected in the 3H-Td assays (Section 3.5.2.2).

As can be seen in Table 3.5.3, less than a 2-fold difference in IC_{50} values between DLKP and DLKP-A was found in the BrdU toxicity assay. This would suggest that there was little difference in TS activity between the 2 cell lines and so these results do not explain the failure of DLKP-A cells to respond to growth factors in the 3H-Td assays.

Cell line	BrdU IC_{50} (μM)
DLKP	2.6
DLKP-A	1.7

Table 3.5.3 Sensitivity of DLKP and DLKP-A to BrdU toxicity.

3.6 *MDR* 1 RIBOZYME TRANSFECTIONS

The development of cross-resistance to a number of structurally and functionally unrelated drugs, a phenomenon termed multiple drug resistance (MDR), is a major problem in cancer chemotherapy, as discussed in Section 1.7. Overexpression of P-glycoprotein (pgp, p170), a drug efflux pump, is one mechanism by which MDR occurs. The P-glycoprotein drug efflux pump is encoded by a gene called *MDR* 1. Reduction of cellular levels of P-glycoprotein has become one of the routes explored in the quest for reversal of MDR. Inhibition of protein expression can be targetted at gene, mRNA or protein level. Ribozymes are catalytic RNA molecules which can be used as tool to target specific mRNA sequences. mRNAs may thus be cleaved at critical sites, rendering the resulting fragments non-functional. No functional protein molecules are then created.

DLKP-A is an MDR variant of the DLKP cell line and expresses high levels of P-glycoprotein. DLKP expresses little to no P-glycoprotein. During a study of heterogenicity in sub-populations of MDR cell lines, a number of clones were established from the DLKP-A cell line (Dr.Mary Heenan, PhD thesis). These clones display varying cross-resistance profiles to MDR drugs and also express varying levels of P-glycoprotein. A study was carried out here on the effect on toxicity profiles of transfection of an *MDR* 1 mRNA-specific ribozyme (Rz) into clonal populations of the sensitive DLKP cells and the resistant DLKP-A cells, DLKP-I and DLKP-A-2B respectively.

A plasmid containing a specific *MDR* 1 Rz gene was transfected into DLKP-I and DLKP-A-2B cells using a calcium phosphate and glycerol shock technique as described in Section 2.19. The plasmid also contained a gene encoding geneticin resistance, allowing for selection of plasmid-containing cells by growth in geneticin-containing medium.

3.6.1 Transfection and Selection of Ribozyme-containing Clones

DLKP-I and DLKP-A-2B cells were transfected with plasmids containing the *MDR* 1 Rz and geneticin resistance genes (plasmids stocks amplified by Dr.Carmel Daly). 2 days after the transfection, selection in geneticin-containing medium (400μg/ml) commenced. 11 days after this, the level of geneticin was increased to 600μg/ml. Untransfected cells of both lines were concurrently grown in geneticin-containing medium to serve as indicators for when any untransfected cells would be dead. 17 days after the transfection, these cells were dead. It was therefore assumed that any cells remaining on transfected plates contained the Rz.

The selected cells were then cloned out in a limiting dilution assay *ie*. seeded at a density of 1 cell/2 wells of a 96-well plate (see Section 2.7.1), and cultured routinely. The cloning resulted in 7 DLKP-I transfectants (1A9, 1B10, 1C9, 1G8, 2D4, 2F7 and 2G3) and 2 DLKP-A-2B transfectants (1A5 and 1C7).

3.6.2 Detection of Ribozyme DNA by PCR

The DLKP-I and DLKP-A-2B transfectants were resistant to geneticin so it could be assumed that the cells contained the plasmid. However, to prove that the plasmid was indeed present in the cells, DNA PCR was carried out. Primers specific for the plasmid were used to amplify a product of 221 base pairs. PCR was carried out on crude DNA extracted from each of the transfectants plus untransfected DLKP-I and DLKP-A-2b controls, as outlined in Sections 2.20 and 2.22.

The Rz was found to be present in all of the transfected cells and absent in the untransfected controls (Figure 3.6.1).

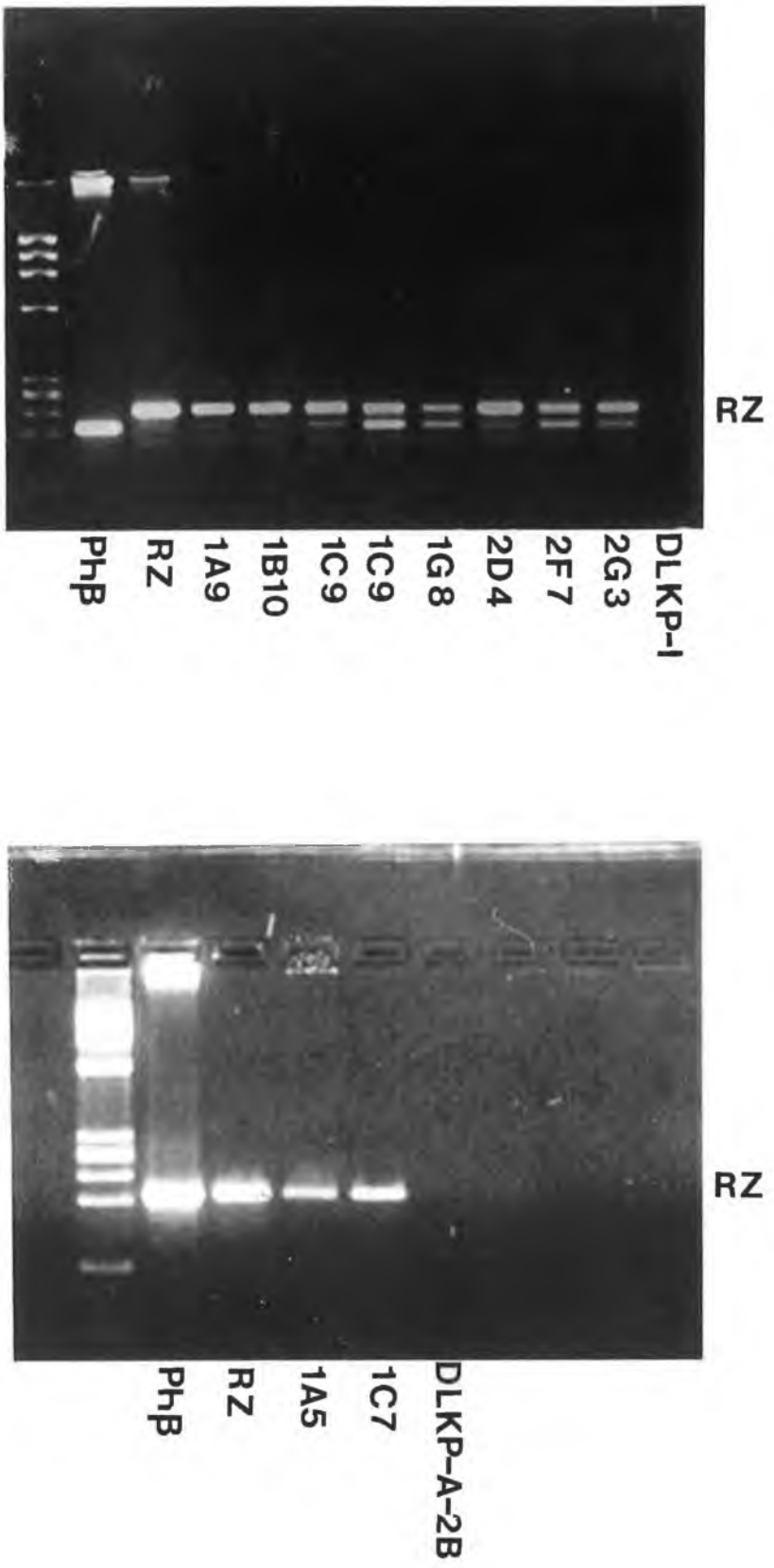


Figure 3.6.1 PCR products resulting from amplification of DNA extracted from DLKP-I and DLKP-A-2B ribozyme transfectants and their respective untransfected, parental lines. The primers selected for ribozyme DNA amplification produced a band of 221 base pairs as indicated. Phβ is plasmid which did not contain ribozyme DNA and therefore produced a smaller PCR product. Rz refers to plasmid containing ribozyme DNA which was amplified as a positive control.

3.6.3 Determination of Ribozyme Expression by RT-PCR

It was necessary to shown not only that the plasmid was present in the cells, but also that it had been incorporated into the hosts genome and was being properly transcribed.

RT-PCR was carried out on RNA extracted from the transfected cells and from control untransfected DLKP-I and DLKP-A-2B cells, as described in Sections 2.21 and 2.23. Primers specific for a sequence in the plasmid and a sequence in the Rz were used to amplify a product of 118 base pairs.

The appropriate Rz RNA band was demonstrated in all of the transfected lines indicating transcription of the Rz gene (Figure 3.6.2). No bands were seen in the DLKP-I and DLKP-A-2B untransfected controls.

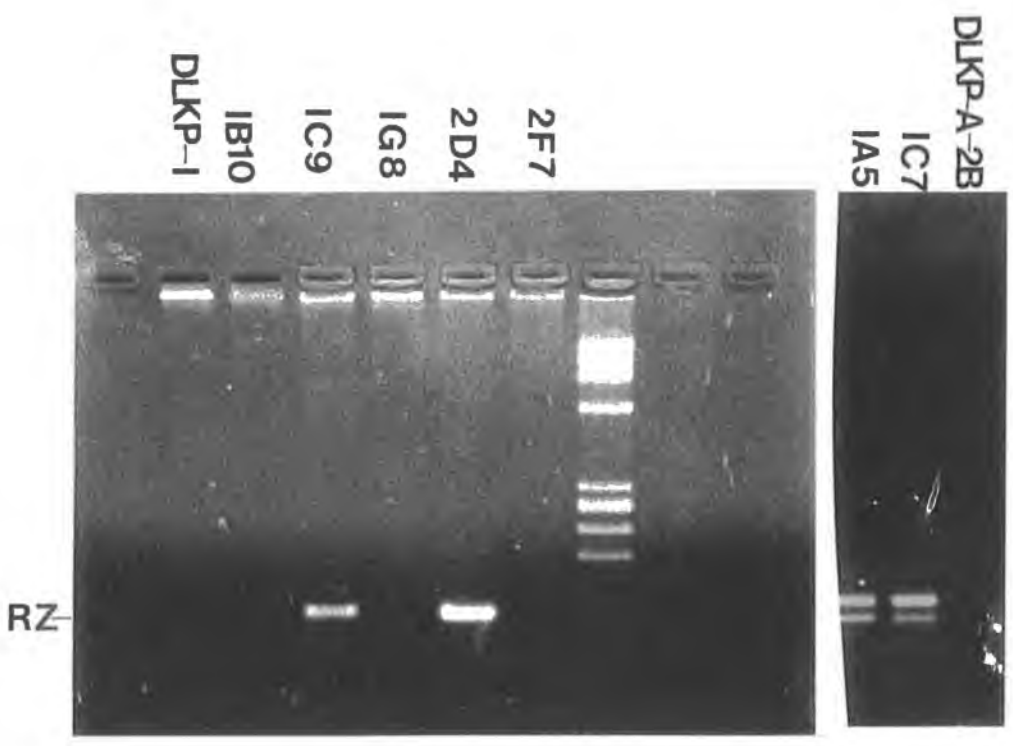


Figure 3.6.2 RT-PCR products resulting from amplification of ribozyme RNA extracted from DLKP-I and DLKP-A-2B ribozyme transfectants and their respective untransfected, parental lines. The primers selected for ribozyme RNA amplification produced a band of 118 base pairs as indicated.

3.6.4 Effect of Ribozyme on *MDR* 1 Gene Expression

The purpose of the Rz transfections was to target and reduce the levels of *MDR* 1 RNA in MDR cells, thus reducing the amount of protein expressed and reversing the drug resistance of the cells. To examine the effect of the Rz on *MDR* 1 RNA levels in transfected cells, RT-PCR was carried out on RNA extracts using primers specific for *MDR* 1. A product of 157 base pairs is produced by these primers.

DLKP-I untransfected control cells were found to contain no detectable *MDR* 1 RNA (Figure 3.6.3). The DLKP-I transfectants also produced no *MDR* 1 band.

Untransfected DLKP-A-2B cells were shown to express *MDR* 1 RNA as a band of the appropriate size was present. The DLKP-A-2B 1A5 transfectant had slightly lower levels of *MDR* 1 RNA as shown by the presence of a smaller band than that of DLKP-A-2B. The 1C7 transfectant contained no detectable levels of *MDR* 1 RNA as no band was produced by these cells.

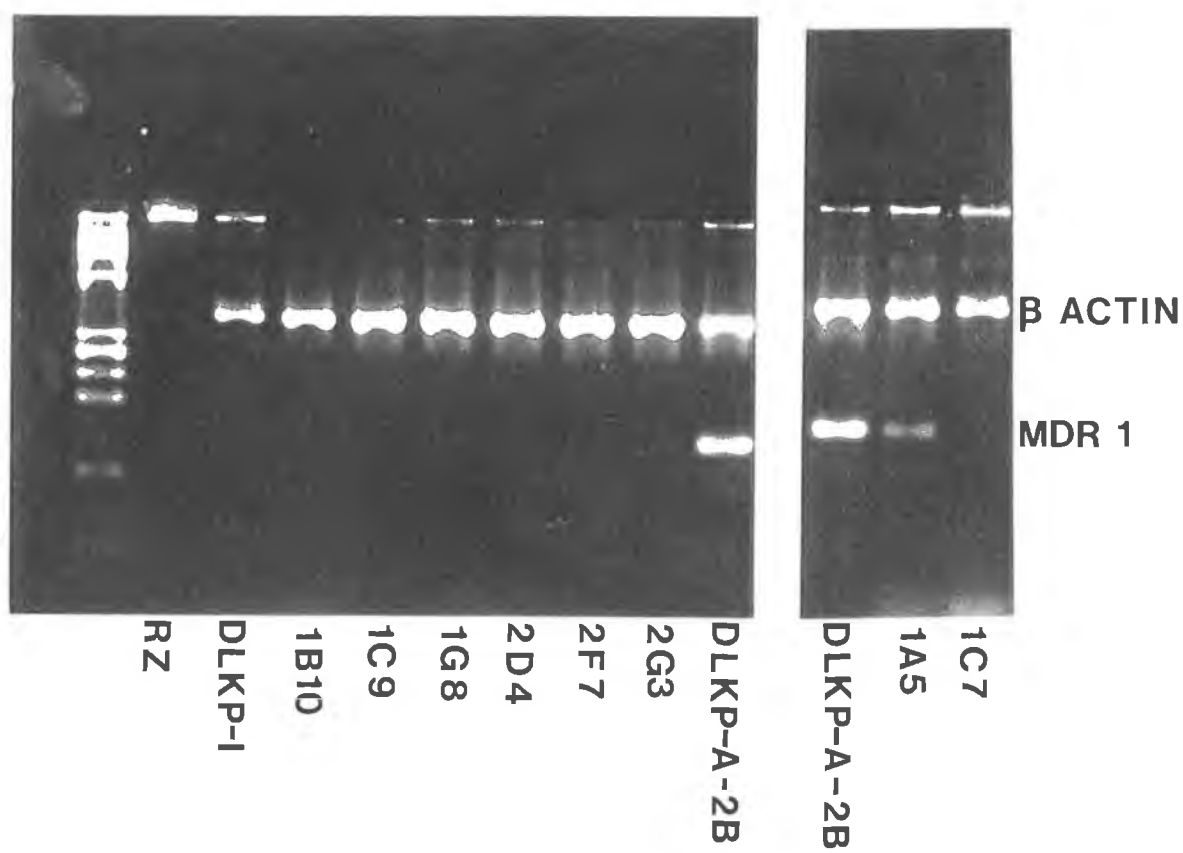


Figure 3.6.3 RT-PCR products resulting from analysis of *MDR* 1 RNA extracted from DLKP-I and DLKP-A-2B ribozyme transfectants and their respective untransfected, parental lines. The primers selected for *MDR* 1 analysis produced a band of 157 base pairs. This band was absent in DLKP-I and its transfectants and was present in DLKP-A-2B but was reduced in DLKP-A-2B 1A5 and absent from DLKP-A-2B 1C7 transfectants. β-Actin RNA was amplified as an internal control using specific primers to amplify a band of 383 base pairs.

3.6.5 DLKP-I Transfectants Toxicity Assays

Toxicity tests were carried out on the transfected cells to determine the ultimate effect of the Rz on the cells *i.e.* reversal of drug resistance.

The toxicity profiles of 3 MDR drugs - adriamycin, vincristine and VP16 - for DLKP-I and its transfectants were determined.

These cells express no detectable *MDR* 1 (Section 3.6.4) and so the IC_{50} values, shown in Table 3.6.1, were found to be relatively low, as expected. There was no more than a 2-fold difference in IC*50* values between the untransfected DLKP-I cells and any of the transfected cells. Results shown are mean values from 3 separate experiments.

3.6.6 DLKP-A-2B Transfectants Toxicity Assays

The toxic effects of adriamycin, vincristine and VP16 on DLKP-A-2B cells and their transfectants were determined to establish if the reduction in *MDR* 1 levels found in the RT-PCR analysis (Section 3.6.4) correlated with a decrease in resistance to MDR drugs.

The IC_{50} values for DLKP-A-2B, 1A5 and 1C7 are shown in Table 3.6.2 and 3.6.3 and are the mean values from at least 3 experiments. Transfectant 1A5 which showed a slight reduction in *MDR* 1 RNA expression by RT-PCR, showed no increase in sensitivity to the MDR drugs compared with DLKP-A-2B. In fact, these cells were more resistant to the 3 drugs. In contrast, transfectant 1C7 showed increased sensitivity to each drug. The greatest effect was seen for vincristine with an 26-fold increase in sensitivity compared with DLKP-A-2B. A 4.7-fold increase and a 1.3-fold increase was seen for adriamycin and VP16 respectively.

Cell line	Adriamycin (IC_{50}, ng/ml)	Vincristine (IC_{50}, ng/ml)	VP16 (IC_{50}, ng/ml)
DLKP-I	5.33 ± 0.47 (1.00)	0.275 ± 0.100 (1.00)	51.00 ± 2.94 (1.00)
1B10	4.08 ± 0.31 (1.31)	0.275 ± 0.025 (1.00)	31.67 ± 4.71 (1.61)
1C9	9.42 ± 2.20 (0.57)	0.615 ± 0.015 (0.05)	84.00 ± 17.28 (0.61)
1G8	9.42 ± 1.42 (0.57)	0.390 ± 0.065 (0.71)	78.00 ± 18.40 (0.65)
2D4	6.33 ± 1.12 (0.84)	0.462 ± 0.137 (0.59)	51.67 ± 10.27 (0.99)
2G3	3.58 ± 0.32 (1.49)	0.275 ± 0.025 (1.00)	29.00 ± 2.94 (1.76)

Table 3.6.1 Sensitivity of DLKP-I transfectants to MDR drugs. Results shown are mean results from 3 experiments. Values in parentheses refer to fold increase in sensitivity w.r.t. DLKP-I cells.

Cell line	Adriamycin (IC_{50}, μg/ml)	Vincristine (IC_{50}, μg/ml)	VP16 (IC_{50}, μg/ml)
DLKP-A-2B	0.160 ± 0.08 (1.00)	0.049 ± 0.005 (1.00)	0.43 ± 0.11 (1.00)
1A5	0.330 ± 0.05 (0.49)	0.111 ± 0.027 (0.48)	0.58 ± 0.16 (0.74)
1C7	0.034 ± 0.015 (4.71)	0.0019 ± 0.0012 (25.79)	0.32 ± 0.019 (1.34)
DLKP-A*	~2.5	~1.000	~1.000
DLKP*	~0.010	~0.00075	~0.075

Table 3.6.2 Sensitivity of DLKP-A-2B transfectants to MDR drugs. Results shown are mean values from at least 3 experiments. Values in parentheses refer to fold increased sensitivity w.r.t. DLKP-A-2B cells.

* Previously determined by Dr.Alice Redmond and shown here to allow comparison with ribozyme transfectants.

Assay No.	Adriamycin (IC_{50}, μg/ml)	Vincristine (IC_{50}, μ/ml)	VP16 (IC_{50}, μ/ml)
DLKP-A-2B			
#1	0.280	0.055	0.59
#2	0.100	0.050	0.37
#3	0.100	0.043	0.34
1A5			
#1	0.340	0.072	0.54
#2	0.380	0.130	0.41
#3	0.260	0.130	0.79
1C7			
#1	0.026	0.0021	0.60
#2	0.055	0.0006	0.26
#3	0.021	0.0012	0.18
#4	-	0.0038	0.25

Table 3.6.3 Results from individual toxicity assays (assay #1, #2 *etc.*) showing IC_{50}s determined for each cell line in the presence of MDR drugs.

4.0 DISCUSSION

population again. It is therefore possible that the ratio of clonal morphologies present in the parental DLKP population (which is relatively consistent itself) is the steady-state ratio for these particular cells and that clones isolated from DLKP will always ultimately recreate the mixed DLKP population.

No alteration in the treatment of the clones was knowingly made which would cause them to interconvert. Media, serum, culturing techniques *etc.* were all standard and this, when considered with the fact that the interconversion occurred consistently after 20-25 passages in culture, suggests that the interconversion was determined within the cells and not a result of a sudden change in external factors.

Interconversion was not observed between DLKP-SQ and DLKP-M, while both of these morphologies appeared relatively quickly in cultures of DLKP-I, the latter being the most morphologically unstable clone. Colonies of DLKP-I cells also appeared in DLKP-SQ and -M cultures. This seems to suggest that DLKP-I is behaving as a stem cell-like population which can give rise to DLKP-SQ and -M cells (see Figure 4.1.1). These cells can also revert to DLKP-I cells to give rise to the parental DLKP population again. Such phenotypic reversion and interconversion has been observed in tumour cells *in vivo* and *in vitro* (reviewed in Nicolson, 1987).

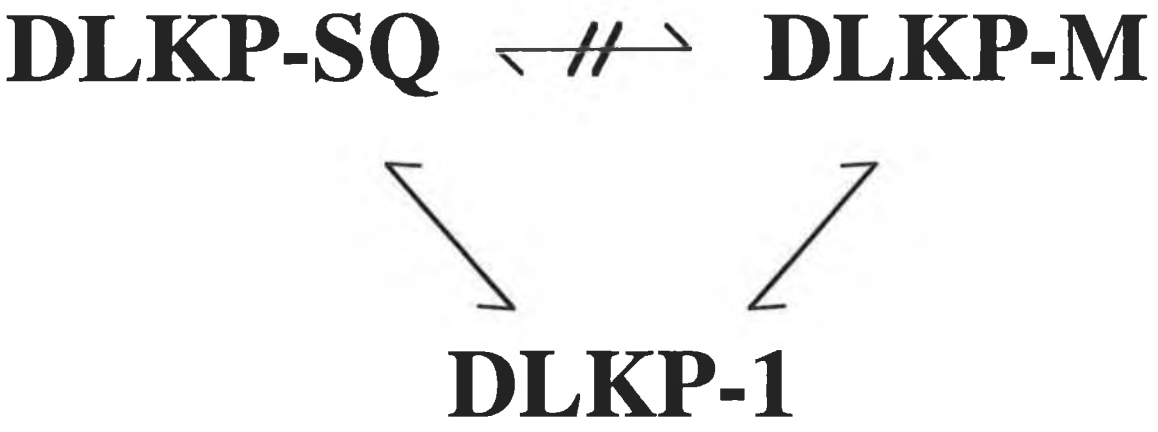


Figure 4.1.1 Proposed model for interconversion of DKLP clones. DLKP-SQ and DLKP-M cells appear to interconvert with DLKP-1 cells, but no interconversion has been observed between DLKP-SQ and DLKP-M cells.

4.2 CHROMOSOMAL ANALYSIS OF DLKP CLONES

The karyotype of DLKP, passage 5, had previously been determined (Law *et al.*, 1992) and was found to consist of a hyperdiploid subpopulation (65% of cells) and a hypertetraploid subpopulation (35% of cells). In the present study, the chromosome distributions of DLKP (passage 22), DLKP-I (passage 26), DLKP-M (passage 32) and DLKP-SQ (passage 29) were examined.

40% of DLKP cells were found to be hypertetraploid with 100-110 chromosomes and approximately 12 and 15% of cells had 30-39 and 90-99 chromosomes respectively. The remaining cells contained anywhere between 40 to 130 chromosomes. 36% of DLKP-I cells were found to contain 90-99 chromosomes (*i.e.* near tetraploid), with chromosome numbers from 40 to 110 being distributed almost equally throughout the remaining population. DLKP-M was found to be the most homogenous population with respect to chromosome number with 60% of the population having 50-59 (near diploid) chromosomes. 15% of the population had 100-109 while 12% had 40-49 chromosomes. DLKP-SQ exhibited 3 large chromosomal populations with 36%, 27% and 16% of the cells having 90-99, 100-110 and 80-89 chromosomes respectively. No DLKP-SQ cells examined contained less than 60 chromosomes.

The chromosomal distributions of the 3 clones reflected their morphological distribution in DLKP as the DLKP-M phenotype and karyotype are not substantially represented in the DLKP population, while DLKP-SQ and -I account for the majority of DLKP cells in terms of both morphology and chromosome number.

Thus, the morphological diversity of the clones was reflected in their respective chromosome numbers and indeed may be explained by them. The karyotypic evolution of several cultured undifferentiated large cell lung carcinoma lung cell lines has been found to follow an orderly and predictable pattern. Burholt *et al.* (1989a and 1989b) reported that while one such cell line exhibited extensive heterogeneity with respect to both numerical and structural chromosomal abnormalities, their results suggested that the genetic evolution of this cell line followed an orderly and predictable sequence of

events. The sequence consisted of a relatively rapid doubling of chromosome number, creating a tetraploid population, followed by a gradual loss of chromosomes and accounted for most of the observed variability in chromosome numbers per cell over time. This could explain the different chromosome distribution found in this study using DLKP passage 22 cells compared with that found by Law *et al.* (1992) using DLKP passage 5 cells. In addition, the 3 DLKP clones, with their distinct chromosomal profiles, may represent different stages of a similar process where a continuous cycle of chromosome doubling and subsequent chromosome loss is occurring. The changes in balance of gene expression accompanying the fluctuating chromosome populations could result in the diverse yet interconverting morphologies observed in the clones. Determination of chromosome distributions in the clones at various passages over the time in which interconversion is seen to take place could reveal if such changes are occurring in DLKP cells and thus give an insight into possible mechanisms by which diversity among phenotypes of tumour subpopulations can occur.

4.3 GROWTH CONTROL IN DLKP CLONES

During normal development and tissue maintenance, a balance exists between cell proliferation and differentiation. As outlined in Section 1.6, a limited number of multipotent stem cells are laid down during embryogenesis. These stem cells give rise to a much larger population of more developmentally restricted progenitor cells which become committed to differentiation. The stem cells are also capable of self-renewal to replace those which differentiate. Uncoupling of these coordinated processes of proliferation and differentiation leads to tumourigenesis.

The proliferation potential of a cell decreases with increasing differentiation (see Figure 1.7.1) and the most aggressive tumour cells are poorly differentiated and highly proliferative (Warren *et al.*, 1990). The growth properties and potentials of the isolated DLKP clones were examined to determine what roles individual tumour subpopulations may play in the progression of malignant disease *i.e.* did all DLKP subpopulations have comparable proliferative capabilities under various conditions or not? If not, did this correlate with increased differentiation and could poorer-performing clones adapt with time to assay conditions and increase proliferation and if so, how did they adapt? Also of interest was how the proliferative capabilities of the isolated clones compared with those of the parental DLKP cells.

4.3.1 Growth Assays

When assayed in monolayer culture in the presence of 0%, 1% and 5% serum, the parental DLKP cells proliferated faster than any of the clones (Section 3.1.3.1). DLKP-I were found to be the fastest growers of the clones in this assay, with DLKP-M and -SQ proliferating relatively poorly over the first 4 - 5 days. DLKP-M and -SQ appeared to be density-dependent as their growth increased rapidly after 5 days of the 8 day monolayer assay.

The growth of the cells in serum-free conditions reflected the monolayer assay results (Section 3.1.3.2). The sole mitogen present in the defined serum-free medium was

insulin and only DLKP and DLKP-I were found to be capable of proliferation in this medium. While all 4 lines attached readily to the flask surface, only DLKP and DLKP-I formed colonies (see Figure 3.1.5). This suggested either that these 2 cell lines could proliferate in the presence of insulin alone, or, what is more likely, that additional growth factors are being produced by these populations, allowing them to expand more aggressively than the other clones and relatively independently of their environment.

The colonies formed by DLKP-I in SFM were morphologically similar to those formed by this cell line in serum-supplemented medium. While approximately 75% of DLKP cells appeared to proliferate without forming colonies, remaining rounded and loosely attached, the remaining 25% also formed DLKP-I-like colonies. Presumably, these represented the DLKP-I-like cells present in the mixed parental population.

These results and observations indicate that, of the 3 clonal populations, DLKP-I was the most independent of exogenous growth and attachment/adherence signals because these cells were capable of forming characteristic colonies in the presence of either serum or insulin alone. This also would explain why the growth rate of DLKP-I in defined SFM usually exceeded that of DLKP, as in the mixed DLKP population, these self-sufficient growers account for only approximately 30% of the total population.

With regard to DLKP-M and -SQ, while all cells in each line appeared to attach successfully in this SFM, only a small percentage were seen to divide and when harvested, cell counts were always below those of initial seeding densities. In the monolayer assays (Section 3.1.3.1), the growth of these 2 lines appeared to be density dependent in the presence of 5%, 1% and 0% serum, with little proliferation occurring at the early stages of the culture, *i.e.* low cell numbers, followed by a late surge. These assays were carried out in 96-well plates with cells seeded initially in serum-supplemented medium at $1x10^3$ cells/well and growth was determined by CVDE assay. Thus, cells were not harvested and re-seeded at any stage. In contrast, in the SFM assays, cells were seeded at $2.5x10^5$ cells/25cm^2 flask, which is a similar density to the 96-well plate assay, but cells were plated in SFM and so did not spread and were fed or harvested by trypsinisation every 6-7 days. This time span was adequate to observe proliferation in DLKP and DLKP-I and may have been too short to see growth in

DLKP-M and -SQ. However, the assay succeeded in demonstrating important growth differences between the clones.

DLKP and DLKP-I cells which had been cultured in SFM were placed back into serum-supplemented medium to determine if their morphologies had altered. While no change was observed in cells which had been passaged 5 times in SFM, cells at passage 10 appeared altered when plated in the presence of serum (Figure 3.1.6). DLKP cells had become more homogenous with >90% of cells being squamous-like in appearance, similar to the DLKP-SQ morphology. DLKP-I cells also appeared changed, being more heterogenous, with large numbers of squamous cells present (up to 30%).

Culture in serum-free medium thus seemed to influence the morphologies of these cell lines but the squamous-like cells which appeared are probably not identical to DLKP-SQ cells as the latter failed to proliferate in SFM. It would be interesting to compare the chromosome numbers of DLKP-SQ and the serum-free squamous cells to determine if they are similar. Increasing numbers of unattached and rounded cells had been observed in later passages of serum-free DLKP and DLKP-I cells and it was probably these which attached and became squamous-like when the serum-free cells were placed back into serum-containing medium. The emergence of a squamous cell phenotype in cell lines has been previously reported by Terasaki *et al.* (1987) who demonstrated that delipidised serum-supplemented medium (*i.e.* retinoic acid deficient) induced squamous cell characteristics in a small cell lung cancer cell line. The changes were reversible upon addition of retinoic acid to the medium. The inhibition of squamous differentiation by retinoic acid is well established (Section 1.6) and so it is possible that the absence of this agent from the defined SFM used to culture DLKP and DLKP-I caused the cells to differentiate towards a squamous phenotype which was discernable only when the cells were able to attach in serum-supplemented medium. It is also possible that the lack of attachment factors in the SFM caused the cells to differentiate into a phenotype which no longer required such factors, hence the increased numbers of cells observed in suspension in later serum-free passages. In the presence of serum, these cells then attached and spread and were squamous in appearance.

The ability to form colonies in soft agar is a characteristic of many malignant cells and is a measure of their clonogenic potential. The end-point in such assays is often based on colony number or colony diameter and varies widely from 50 cells (Hay *et al.*, 1991) to 120μm (Bepler *et al.*, 1987c) to 250μm (Tonita *et al.*, 1992). When it was found that DLKP-I cells formed effectively no colonies in this assay (Section 3.1.3.3), a relatively low diameter width of 20μm (approximately 25-30 cells) was chosen to score colonies. This resulted in high CFEs for DLKP, DLKP-M and -SQ of approximately 38%, 27% and 13% respectively.

The inability of DLKP-I cells to form colonies in soft agar was unexpected in the light of their high growth rates in serum-supplemented and serum-free medium. The possibility that these cells had been adversely affected by the high temperatures during plating of the cells was discounted (Section 3.1.3.3 and Figure 3.1.7), leaving the surprising conclusion that these cells had a very low CFE compared with the 2 other clones and the parental DLKP cells. It is possible that the DLKP-I cells require attachment to a substratum for optimum proliferation and colony formation and so did not perform well in suspension in the soft agar. Classic SCLC cell lines generally have a lower cloning efficiency in soft agar than variant types (Kiefer *et al.*, 1987 and Carney *et al.*, 1985) so it is possible that the DLKP-I cells express a phenotype closer to that of SCLC than DLKP-M and -SQ. Cell-cell attachment molecules also influence anchorage independent growth (Doyle *et al.*, 1990 and Carbone *et al.*, 1991) and the complement of attachment molecules and receptors expressed by DLKP-I may differ to those of the 2 other clones and may not facilitate colony formation in semi-solid agar.

The ability of the clones to grow in suspension in spinner flasks was inferior to that of DLKP (Section 3.1.3.5). The growth of the parental cells often exceeded that of any of the clones by over 100%. With some week-to-week variation, the DLKP-SQ clone proliferated best under these assay conditions. The rate of proliferation of each clone increased slightly most weeks, indicating adaptation to the conditions.

Observations of changes in cell morphology when re-seeded into normal tissue culture flasks again after 10 passages in spinners appeared to confirm an adaptive process. As was the case in the SFM medium assays, alteration towards a squamous morphology

was evident in DLKP and DLKP-I after 10 passages in suspension. DLKP-SQ and DLKP-M appeared unchanged, both resembling their respective original morphologies. The time-frame over which the adaptation took place was similar to the serum-free assays where no change was apparent after 5 passages but were so after 10 passages. In addition, the enforced growth in suspension in spinner flasks resembles the situation in the serum-free medium assays where attachment factors would have been absent, save those, if any, produced by the cells themselves. Therefore, when anchorage-independent growth was either enforced (spinners) or induced (SFM), the resulting phenotype was squamous-like, suggesting that this was an adaptation to allow 3-dimensional growth in suspension. These interconversions support the theory that DLKP-I is a stem cell-like population which can give rise to the other clonal phenotypes. The DLKP-I cells may be basal-like cells which *in vivo* would be attached to the basement membrane. These then could give rise to various daughter cell types including DLKP-M and -SQ, the latter of which forms the bulk of the 3-dimensional tumour (thus causing the biopsy to be diagnosed as 'poorly differentiated squamous'). Possible roles of the DLKP-M cells are discussed later in Section 4.4.

It appears that the combined growth properties of the different DLKP clones span the range of growth conditions examined here - soft agar, suspension, serum free and monolayer. Thus, *in vivo*, these cells may readily adapt to changing environmental conditions and situations, conferring plasticity on the tumour as an entity and facilitating continuous growth.

In all growth assays except the defined serum-free medium assays, the parental DLKP cells proliferated faster than each of the clones. It would thus appear that the balance of phenotypes present in the DLKP population was the best for most growth conditions. This could explain why interconversion of the clones seemed to tend towards recreation of this mixed population. Adaptation of all lines apart from DLKP-M was evident in serum-free and spinner assays and it would be interesting to determine if continued culture of adapted cells in normal, serum-supplemented conditions would bring about a return to original clonal morphologies.

The responses of DLKP and the clones to a range of exogenous, purified growth factors

were examined but no consistent effects were seen (Section 3.1.3.6). DLKP cells are stimulated by even trace amounts of serum (0.1%) so assays were carried out in the absence of serum to avoid masking any growth factor effects. However, no repeatable responses, stimulatory, inhibitory or no effect, were obtainable. When insulin was included with the growth factors to determine if the cells could be 'primed' or prepared for growth factor responses similar results were obtained. Therefore, no conclusions could be drawn regarding the exogenous growth factor requirements of the cells following these growth factor experiments.

4.3.2 Autocrine Growth Regulation

The ability of DLKP and DLKP-I to proliferate successfully in 0% serum (Section 3.1.3.1) and in defined serum-free medium (Section 3.1.3.2) suggested that these cells were producing autocrine growth factors capable of stimulating their own growth. However, despite the initial apparent detection of DLKP autocrine activity (Section 3.1.4.1), no substantial autocrine effects, stimulatory or otherwise, were demonstrable. A wide range of assay conditions were examined but none were suitable to detect autocrine growth activity in either DLKP cells or any of the clones.

It is possible that the autocrine activity was not a soluble factor secreted into the medium, thus rendering the collection of CM futile. The putative factor(s) may have been transmitted *via* mechanisms such as gap junctions or membrane-membrane contact, or may be an extracellular matrix protein which stimulated growth from the substratum (see Section 1.5).

If the factor(s) was a soluble, secreted molecule, it may have been extremely labile and as no CM was assayed on the day of its collection, activity may have been lost before it was assayed. A secreted factor may also exert its effect by accumulating locally and becoming concentrated around neighbouring cells, thus increasing the signal seen by these cells while overall levels of the factor remain low. This effect would also be lost during the collection and subsequent assaying of CM.

It may be necessary to utilise antisense or neutralising antibody 'knock-out' approaches to identify autocrine growth factors in DLKP cells. These would cover most of the aforementioned possibilities but knowing which molecules to target would be a problem. 'Transwell' assay systems are another possibility. These consist of multi-well plates with mini, well-shaped inserts suspended in each well (see Section 2.14) and the culture of 2 cell monolayers in a single well allows 1 cell line to be used as indicator cells which respond to factors released by the other. This could solve the problem of the loss of activity during storage as a factor could act on the target cells as soon as it diffuses through the medium.

4.4 EXTRACELLULAR MATRIX

The adherence properties of DLKP cells and the clones to a number of ECM proteins were found to differ from each other (Section 3.1.5). Of the 4 lines, DLKP-M attached most rapidly to plastic, fibronectin and collagen type IV. In the case of laminin, DLKP, DLKP-M and -SQ all attached at the same rate while DLKP-I attached more slowly. Interestingly, while the attachment of DLKP, DLKP-I and -SQ to fibronectin increased with time, DLKP-M cells appeared to detach from this substrate after an initial rapid rate of adherence. The DLKP-M cells have a mesenchymal-like appearance. They do not form colonies but grow in a scattered manner and have an elongated, irregular shape, with many neurite-like processes. When studied by time-lapse videomicroscopy (results not shown), DLKP-I and -SQ cells were found to be relatively immobile once attached to the substrate. DLKP-M cells however, were quite motile and though not travelling long distances, moved in a circular fashion about the substrate and often, cells were seen to move around each other and between other cells. The attachment properties of DLKP-M cells therefore appear to be quite complex and different to those of the 2 other clones.

As discussed in Section 1.5, the invading, migratory cells found at tumour invasion fronts often loose their epithelial morphology and appear more mesenchymal, becoming spindle-shaped and fibroblast-like. The down-regulation of epithelial cell-cell attachments and altering of normal cell-matrix interactions enables metastasising cells to migrate and invade the basement membrane. DLKP-M cells appear to possess many characteristics of metastasising cells including their appearance and their unusual attachment properties. The finding that these cells attached rapidly to fibronectin and then appear to detach possibly reflects their migratory nature which allows them to attach to and derive traction from a substrate but does not render them immobile.

The DLKP-M clone may therefore represent a metastatic-like subpopulation which is present at low levels in the parental DLKP population. The fact that a degree of interconversion is evident between DLKP-M and DLKP-I suggests that this metastatic phenotype is capable of evolving or devolving, depending on conditions. The DLKP-M

clone proliferated relatively slowly in the monolayer and soft agar growth assays and not at all in defined serum-free conditions suggesting that clonal expansion is not the inherent function of these cells. The relative stability of morphology of this clone compared with DLKP-I and -SQ which interconvert more readily and the failure of growth in suspension in spinner flasks to affect the morphology of DLKP-M while those of DLKP-I and -SQ were altered, indicates that this may be a more specialised, differentiated phenotype which does not readily convert back to the DLKP-I morphology.

Invasion assays and the determination of expression of matrix-degrading enzymes by DLKP-M cells could support their status as metastatic cells. Unfortunately, DLKP and each of the clones failed to form tumours when injected subcutaneously into nude mice, therefore the metastatic ability of DLKP-M cells *in vivo* is currently unknown. However, if the cells could be made to form such tumours *eg*. by injection directly into the lung, demonstration of metastasis formation by DLKP-M cells would be persuasive evidence that DLKP and its clones could serve as a good model for tumour progression, invasion and metastasis. In the interim however, quantification of the motility of DLKP-M cells by time-lapse videomicroscopy, further analysis of their ECM attachment capabilities and characterisation of their membrane adhesion molecules and receptors, in comparison with those of the non-motile DLKP-SQ and -I clones, could be useful in our understanding of tumour progression.

4.5 SENSITIVITIES OF CLONES TO CHEMOTHERAPEUTIC DRUGS

A number of multidrug resistant variants of the DLKP cell line have been established for the investigation of drug resistance in tumour cells. These include DLKP-A, a variant which was selected in adriamycin and DLKP/VP-3 and DLKP/VP-8 (established by Dr.Mary Heenan) which were selected in VP-16. These variants are not only resistant to their selective agents but also to other, unrelated, chemotherapeutic drugs.

The sensitivities of the DLKP clones to 3 such drugs - adriamycin, vincristine and VP16 - were determined and found to be similar to the sensitive parental DLKP cells (Section 3.1.6). This finding supports the theory that MDR is an acquired phenotype which occurs as a result of exposure to drugs, rather than the selection of an inherently resistant subpopulation. It is still possible however, that additional phenotypes are present in the DLKP population which are not represented by the 3 clones and which are highly multidrug resistant.

No significant differences were found in the sensitivities of the clones with each of the drugs examined. Thus, the different modes of action of the 3 drugs were equally effective on each of the clones suggesting that the topoisomerase II activities, RNA transcription, spindle formation *etc.* which the drugs effect (see Section 1.7) are broadly similar in the clones, *i.e.* if one or more of the drugs had a significantly different IC_{50} value for one or other of the clones, it could indicate differences in some of these cellular processes as well as differences in drug resistance.

It had been anticipated that the slower growing clones, DLKP-M and -SQ might have slightly lower IC_{50} values than DLKP and DLKP-I because many of the effects of the MDR drugs occur during DNA replication. Therefore, a cell line with a high turnover rate may be effected to a greater extent than one which divided at a slower rate. There was no evidence for this in the toxicity assays carried out here. It may however be possible to see such differences if the cells were exposed to the drugs for shorter lengths of time than the standard 7 day incubation period used in this study. Another alternative would be to use lower, less toxic drug concentrations with longer incubation

times. These approaches may reveal more subtle differences in sensitivities between the clones.

Also of interest would be the establishment of MDR variants of the DLKP clones. Multidrug resistant phenotypes are heterogeneously expressed in tumours (Barranco *et al.*, 1988) and the DLKP-A cell line has been shown to contain subpopulations with varying levels of drug resistance. Investigations into the acquisition of this phenotype by the individual DLKP clones could give insight into the subpopulations of tumours *in vivo* most likely to adopt this phenomenon and how and why they do so. In addition, the putative links between multidrug resistance and differentiation (Biedler, 1994) could also be further examined in the light of the differentiation studies carried out here on the DLKP clones.

4.6 CHARACTERISATION OF DLKP CLONES

One of the main aims of the work described here was to characterise the DLKP cells and each of the clones as fully as possible to determine what type of lung tumour cells DLKP resembled and whether individual phenotypic roles could be assigned to the isolated clonal subpopulations. To achieve this, ultrastructural, functional and biological features of the cells were examined.

4.6.1 Electron Microscopic Analysis

Electron microscopic analysis of cells at the ultrastructural level is one of the most useful methods currently available for examining cell structure and content, allowing cells to be characterised and classified accordingly. As mentioned in Section 1.2, it was EM studies which first revealed the overlapping features within NSCLC and SCLC cells (McDowell *et al.*, 1981), observations which have ultimately led to the now widely held 'common stem cell' theory of the origins of lung tumours.

Ultrastructural analysis frequently reveals neuroendocrine features in cells, such as dense core granules, which may otherwise be undetectable. (Hammer *et al.*, 1989). In many cases, EM analysis is the only definitive method of cell identification *eg.* determination of the presence of lamellar inclusion bodies in lung type II cells, and studies employing this technique have yielded much information on the structure and function of lung epithelial cells (Baldwin, 1994 and Have-Opbroek *et al.*, 1988).

DLKP, DLKP-I, -M and -SQ were examined by EM and were found to contain no organelles characteristic of any differentiated lung epithelial cell types (Section 3.2.1). Features such as dense core granules and lamellar bodies are indicative of NE cells and type II cells respectively, but no such features were present. The 4 lines were found to be similar to each other in their lack of cell type-specific organelles and no differences were apparent between the cells.

It therefore appears that each of the clones is poorly differentiated, containing few

ultrastructural features apart from those necessary for replication. As mentioned earlier, lack of differentiation is typical of aggressively growing tumours, and differentiation inversely correlates with proliferation. It cannot be determined however, if these DLKP tumour cells arose from already poorly differentiated cell types or dedifferentiated from a slightly more differentiated cell type. The common stem cell theory implies that the former is the case.

The EM analysis here was carried out on cells which had been harvested *i.e.* not on growing, attached cells. Examination of DLKP cells and clones *in situ* could yield significant additional information about these cells *eg.* determining the presence of cell-cell attachments such as desmosomes, the location of the nucleus in each clonal cell type (central, basal or apical), the cross-sectional shape of the cells (columnar, cuboidal or pyramidal) *etc.* Such information could be of significant benefit in further characterising the clones.

4.6.2 Electrical Resistance

Polarised epithelial cells line all body cavities in adult mammals and are also found in tissues which carry out specialised absorptive and secretory functions. These cells are characterised by a) the polarised distribution of the cytoskeleton and cytoplasmic organelles, b) the formation of 3 distinct membrane domains, apical, lateral and basal, brought about by the ordered distribution of plasma membrane proteins and lipids, c) the presence of tight junctions which separate apical and lateral membrane domains and form barriers to the intercellular diffusion of ions and macromolecules and d) cohesive cell-cell interactions formed by cell adhesion molecules and a highly developed junctional complex (reviewed in Rodriguez-Boulan and Nelson, 1989).

Bronchial epithelial cells are polarised and serve as a barrier to the movement of water and solutes between the airways and the interstitial compartments of the lung (Boucher *et al.*, 1988). Cultured Clara cells have been shown to actively transport Na^+ in an apical to basolateral (lumen to interstitial compartment) direction (Van Scott *et al.*, 1987 and 1989). Transport functions such as these are important in maintaining the depth and composition of the layer of liquid lining the airway surfaces. Abnormalities in these

pathways can result in lung diseases such as cystic fibrosis which involves defects in the transport of Na^+ and Cl^- that result in accumulation of mucoid secretions and occlusion of the small airways (Anderson and Welsh, 1991).

The colonic tumour cell lines T84 and $Caco_2$ have been employed extensively for physiological studies on epithelial ion transport (reviewed in Dharmsathaphorn and Madara, 1990). These cells can form tight junctions with high resistance which separate their apical and basolateral membranes and confer on them their electrolyte transport properties. Measurements of tight junction formation may be made by determining the electrical resistance across monolayers of cultures cells (Yu *et al.*, 1994). Electrical resistance increases over time (days) in concert with the number of tight junctions formed in a confluent monolayer. The failure of a monolayer to exhibit increased electrical resistance is indicative of the absence of tight junctions and such cells cannot be polarised and thus do not carry out vectorial secretory or absorptive functions.

Neither DLKP nor its 3 clones formed tight junctions when cultured in confluent monolayers as determined by their failure to demonstrate increased electrical resistance compared with T84 cells. Therefore, none of the 4 DLKP lines appeared to form polarised monolayers as normal lung epithelial cells would do *in vivo* and *in vitro* (Yu *et al.*, 1994) and so cannot perform electrolyte secretory functions similar to Clara cells of the bronchial epithelium. SKMES-1, a human lung squamous carcinoma cell line, also also appeared to lack tight junction formation when examined for comparative purposes and so too could be considered to be non-polar. These results indicate that none of 3 DLKP clones represent a cell type similar to differentiated Clara cells capable of electrolyte transport functions.

4.6.3 Biological Marker Expression

The importance of the correct diagnosis of lung tumour type was outlined in Sections 1.2 and 1.7. NSCLC and SCLC respond differently to chemotherapy (Gazdar and Linnoila, 1988) and can have different rates of progression with implications for prognosis (Lequaglie *et al.*, 1991 and Warren *et al.*, 1990). Clinically, the distinction between NSCLC and SCLC is still largely made on the basis of morphological, light microscopic examination. The development of antibodies which are specific for protein markers in normal and tumour cell types has resulted in a greater precision in tumour classification and has also demonstrated the frequent overlap between the standard classifications. In addition, tumour-specific markers may be useful for antibody-directed therapy. The Third International Workshop on lung tumour and differentiation antigens reported recently (Stahel *et al.*, 1994) on their progress in identifying clusters of antibodies based on similar reactivities against cell lines and tissues, in an effort to define antigens associated with lung tumours and lung differentiation. It is hoped that this internationally coordinated study will identify antibodies which can be used as tools in the elucidation of normal and tumourigenic lung cell processes.

DLKP cells were examined for the expression of a number of markers (Section 3.2.3) which it was hoped would give an insight into the tumour type from which DLKP was derived, as well as the respective differentiation states of the DLKP clones.

The finding that neither DLKP cell lines (examined at varying passage numbers from 6 to 30), nor the tumour from which they were established, expressed the epithelial markers examined was unexpected given that the tumour had been diagnosed as a poorly differentiated squamous cell carcinoma. While the antigens of EMA and EP16 are as yet unknown, both antibodies exhibit widespread reactivities with epithelial cells, but not with DLKP cells. The lack of desmoplakin reactivity indicated the absence of desmosomes and hemidesmosomes from DLKP cells, which epithelial cells would also be expected to contain. Transglutaminase expression was examined to determine if the squamous morphology exhibited by the cells could be shown to reflect underlying squamous differentiation, but no reactivity was detected.

DLKP cells were also examined for the expression of 7 of the most commonly occurring keratins with an anti-pan cytokeratin antibody. No reactivity was detected. Human fetal lungs at the pseudoglandular stage (10-16) weeks are positive for both keratin and EMA reactivity (Yousem *et al.*, 1990), so DLKP may represent cells that are either more poorly differentiated than 10 week old fetal epithelial cells, or cells which have dedifferentiated from more well-differentiated epithelial cells. The only lung epithelial tumour cells which have been shown to lack keratin expression are SCLC-V cells (Broers *et al.*, 1985) and these have been shown to express neurofilament proteins, as have DLKP cells. SCLC-V cells are thought to arise from the progression of classic SCLC cells to the more aggressive variant phenotype (Carney *et al.*, 1983 and Doyle *et al.*, 1991). SCLC-C cells usually contain keratin filaments and not neurofilaments. Keratins form complex skeletal networks throughout the cytoplasm of epithelial cells and appear to attach to the intercellular desmosomal plaques which play an important role in epithelial cell adhesion (reviewed in Steinert and Roop, 1988). Thus, the downregulation of cell-cell attachment seen in the loosely aggregated colonies of SCLC-V cells (Gazdar *et al.*, 1985, Carney *et al.*, 1985 and Bepler *et al.*, 1987b) may reflect the loss of desmosomal cell-cell attachments and the replacement of keratin proteins with neurofilament proteins, allowing a less restricted, more aggressive phenotype to emerge.

The 703D4 and 704A1 antibodies reacted positively with DLKP cells and each of the clones. These antibodies were believed to distinguish NSCLC from SCLC (Mulshine *et al.*, 1983) but are now thought to identify an antigen which is expressed in cells very early, before clinical features of neoplasia are evident, in the tumourigenic process (personal communication, Dr.J.Mulshine, NCI, Maryland, USA and Tockman *et al.*, 1988). Therefore, reactivity with these antibodies may not indicate NSCLC differentiation but may reflect the presence of an early marker for lung cancer.

The DLKP tumour block showed strong positivity for NSE expression and moderate Leu 7 immunoreactivity. The tumour block and all DLKP cell lines examined were also positive for PGP 9.5 expression. These results, along with the demonstration of neurofilament reactivity, are indicative of neuroendocrine differentiation. GFAP and

NCAM proteins were not detectable in any DLKP cells, nor were 5-HT and chromogranin A reactivities discernable. This correlates with the absence of dense core granules demonstrated in the electron microscopic analysis (Section 3.2.1). The expression of a limited number of NE markers including NSE, Leu 7 and neurofilaments but not dense core granules is characteristic of SCLC-V cells (de Leij *et al.*, 1985, Watanabe *et al.*, 1988, Deftos *et al.*, 1988 and Carbone *et al.*, 1991). As described in Section 1.1, neuroendocrine cells are the first mature cell type to appear in the developing lung and NSE immunoreactivity is one of the earliest markers observed in these cells, appearing in immature NE cells at 8 weeks before most neuropeptides can be detected (Cutz *et al.*, 1982). Therefore, the presence of NSE positivity in cells which do not contain neuropeptides or APUD elements, as is the case with DLKP, may indicate that such cells are at a very early stage of differentiation and theoretically can progress towards several distinct phenotypes. Maintaining this plasticity is of obvious benefit to tumour cells as they remain uncommitted to any single differentiation pathway and so are free to adapt to a changing environment.

The complement of marker expression determined in DLKP lines suggests that the cells should be classified as either NSCLC-NE or SCLC-V. The former subtype is defined as the expression of 2 or more NE markers in otherwise NSCLC cells while the latter is considered to be the absence of expression of some NE markers in otherwise SCLC cells which are tending towards large cell morphology. The fact that DLKP cells express NSE and neurofilaments and do not express keratins slightly favours classification as SCLC-V.

Vimentin intermediate filament proteins were not detected in the tumour block, confirming that the tumour was probably not mesenchymal in origin (Steinert and Roop, 1988) and the absence of leucocyte common antigen (LCA) reactivity suggested it was unlikely that the tumour was a lymphatic primary tumour. The fact that the biopsy was taken from a lymph node metastasis made this confirmation necessary.

None of the clones, nor parental DLKP cells (passages 6 and 29) were found to express detectable levels of P-glycoprotein, the membrane drug efflux pump. This correlated with the high sensitivities of the cells to MDR drugs (Section 3.1.6) and leant support

to the theory that the MDR phenotype, which in the case of the DLKP-A variant cells includes overexpression of P-glycoprotein, is acquired as a result of drug exposure rather than the selection of an inherently multidrug resistant subpopulation.

DLKP and each of the clones were found to express α_2-integrin. However, the reactivity appeared strongest in DLKP-M cells. These cells had exhibited unusual extracellular matrix attachment properties (Section 3.1.5) and the difference in expression of this integrin compared with the other clones could be one of the reasons for this. Metastatic cells exhibit altered integrin expression and the overexpression of α_2 integrin, which can bind collagens, laminin and fibronectin (Rouslahti, 1991), in DLKP-M cells may play a significant role in the behaviour of this mesenchymal-like cell type. Alternatively, there may be a downregulation of α_2-integrin expression in DLKP, DLKP-I and -SQ cells. Normal bronchial and alveolar epithelial cells express high levels of this integrin (Damjanovich *et al.*, 1992 and Mette *et al.*, 1993) and others which allow them to adhere strongly to their basement membrane and resist the shear forces and airway turbulence caused by normal breathing and coughing *etc*. Antibodies directed against specific integrins can be used to determine their roles in attachment and migration of different cell types (Adams and Watt, 1991). Determination of the array of integrins and other attachment molecules expressed by the DLKP cells and clones, along with the examination of the effects of neutralising antibodies, could reveal differences in the physiology and behaviour of the individual DLKP clonal subpopulations.

The characterisation studies of DLKP thus indicated that the cells were poorly differentiated with no evidence for differentiation towards any normal mature lung cell type. The absence of keratin and the presence of neurofilaments and the early fetal lung marker NSE suggested that the cells resembled immature, multipotent fetal epithelial cells with very slight neuroendocrine differentiation. The tumour type which DLKP resembles most closely appears to be variant SCLC, or perhaps NSCLC-NE, but the presence of neurofilaments and the lack of keratin, desmosomes and other epithelial markers favours the former category. No evidence was found here to support the theory that DLKP-I represented a stem cell-like population from which DLKP-M and -SQ arise because no such indicative differences in function or markers were found.

4.7 SPECIFIC MARKERS FOR DLKP CLONES

While the determination of marker expression in DLKP cells yielded much information on the tumour cell types they were similar to *in vivo*, no markers had emerged that could be used to distinguish between the individual DLKP clones. The immunological characterisations had been carried out using immunocytochemical methods which are not quantitative, as for example Western blot analysis could be. Therefore, there may have been quantitative differences in the levels of marker expression between the clones. However, using differences such as these as markers for the individual clones would not have been satisfactory as it would not allow *in situ* qualitative or quantitative examination of clonal phenotypes in mixed populations such as in interconversion or differenation studies. Only the identification of a marker which was expressed on 1 or 2 of the clones and not on all 3 would enable such studies to be carried out.

Therefore, in an attempt to identify specific markers for the clones, monoclonal antibodies were raised to the parental DLKP cells (Section 3.3.1). The rationale was that this population should contain the sum of the markers expressed by the individual clones, possibly along with some additional markers, and so the resulting monoclonal antibodies may react differentially with the clonal subpopulations. However, of the 28 hybridomas which produced antibodies directed against DLKP antigens, all were found to react to some extent with each of the clones in ELISAs, although 2 antibodies, 4D2 and 3E1, appeared to be less reactive with DLKP-I than the 2 other clones.

When the 4D2 and 3E1 monoclonal antibodies were used in Western blot analyses, 3E1 failed to produce a band for any cell lines examined. This was probably due to the antigen it recognised being altered in the reducing conditions of the SDS gel. 4D2, however, produced a tight band in Western blots which had an apparent molecular weight of approximately 58kD. The intensity of this band was consistently stronger in DLKP-SQ cells than in DLKP, DLKP-I or -M cells. The 4D2 antibody not only reacted with DLKP cells in Western blots, but also with several other lung lines examined, all of which resulted in a single band of 58kD also. Immunocytochemical analysis revealed that the 4D2 and 3E1 antigens were expressed in effectively all tissue types, both normal and tumour, and also in non-human species (Tables 3.3.6.2 - 3.3.6.4). The

staining patterns produced by both antibodies indicated that the antigen was cytoplasmic.

It would therefore appear that the 4D2 and 3E1 antibodies, far from being specific DLKP clone markers, detect ubiquitous markers for all cell types. The demonstration that the 4D2 antigen was expressed at a higher level on DLKP-SQ cells than the other clones was not sufficient to enable the antibody to be used in clone identification studies.

4.8 EFFECTS OF DIFFERENTIATION-INDUCING AGENTS ON DLKP CELLS

The effects of differentiation-inducing agents on the morphologies and marker expression of DLKP and its clones were examined in an attempt to manipulate the apparently spontaneous clonal interconversions observed in culture and also to elucidate the differentiation pathways open to the clones by determining changes in marker expression. Many differentiaion-inducing agents are described in the literature and of these, BrdU and RA were selected for study, the former partly because of the demonstration of its effect on SCLC cell morphology (Feyles *et al.*, 1991) and the latter partly because of its well-established involvement in the regulation of squamous differentiation (Terasaki *et al.*, 1987).

The 3 distinct subpopulations which have been identified in many neuroblastoma cell lines (Ciccarone *et al.*, 1989) serve as an interesting comparison to the DLKP clones and interconversion of these subpopulations has been demonstrated in response to RA and BrdU (Ross *et al.*, 1994). The neuroblastoma subpopulations consist of (i) a cell type termed 'N' which is neuroblastic in appearance with a rounded cell body and numerous neurite-like processes and possesses APUD features, (ii) a cell type termed 'S' which is larger, flattened, highly substrate-adherent and resembles epithelial cells and exhibits Schwannian rather than neuronal properties and (iii) a cell type termed 'I' with a morphology intermediate between that of N and S cells. The N and S cell types are descended from a common precursor cell type and both are capable of spontaneous interconversion to the other cell type (Ross *et al.*, 1983). The I cell type may represent a cellular intermediate in the N- and S-cell transdifferentiation process or may be a stem cell from which both N and S cells arise. Studies by Ross *et al.* (1994) found that BrdU treatment induced differentiation of I cells to S-type morphologies while RA treatment resulted in N-type colonies. However, not all neuroblastoma lines respond in this manner and some have been shown to differentiate towards an S-type morphology following RA treatment (Ross *et al.*, 1991).

The spontaneous interconversions of the DLKP clones mirrored those of neuroblastoma

subpopulations and both appeared to occur *via* an intermediate cell type. However, no interconversion was seen between the DLKP clones in response to BrdU or RA (Section 3.4). In fact, RA had very little effect on morphology except that cells in colonies seemed less tightly packed together. BrdU, in contrast, had more obvious effects on morphology and the effect increased with increasing BrdU concentration. The treated cells no longer grew in colonies but became larger and more stretched in appearance. This increase in cell surface area in response to BrdU has been previously reported for SCLC cells (Feyles *et al.*, 1991), melanoma cells (Valyi-Nagy *et al.*, 1993) and neuroblastoma cells (Ross *et al.*, 1994), all using similar concentrations of approximately 10μM BrdU.

Increasing the lengths of exposure time to RA and BrdU may lead to interconversions between the DLKP clones. The longest exposure time used here was 14 days whereas the interconversions in neuroblastoma subclones is observed after 21 days or more (Ross *et al.*, 1994). Alternatively, the DLKP clones may have greater differentiating potential than neuroblastoma cells and RA and BrdU treatment may be inducing expression of additional phenotypes rather than just inducing interconversion between 2 or 3 morphologies.

The finding that keratin expression had been strongly induced in BrdU- and RA-treated DLKP cells was surprising. While reports of alterations in keratin expression in response to differentiation-inducing agents exist in the literature (Terasaki *et al.*, 1987 and Stellmach *et al.*, 1991), no reference has yet been found to the induction of keratin expression in cells which previously contained no keratin filaments. BrdU and RA appeared to induce differentiation of DLKP cells towards a new phenotype which was not already present in the parental population, rather than causing clonal interconversion. This supports the proposal above that DLKP cells are multipotent and capable of differentiation along several pathways. This also indicates that the cells possess undifferentiated stem cell-like phenotypes which corroborates the poor differentiation demonstrated in immunocytochemical and electron microscopic analyses. It appears that DLKP cells may be cycling between restricted, undifferentiated stem cell phenotypes, incapable of advancing along normal differentiation pathways due to unknown blocks and as a result are tumourigenic. It is possible that BrdU and RA

overcame these blocks and caused the cells to differentiate along as yet unidentified pathways. Determination of the proliferative capacities of these new phenotypes may indicate if this hypothesis is accurate. Reduced growth rates and colony forming efficiencies in treated cells would strongly suggest that the malignant phenotype exhibited by untreated DLKP cells was being reverted to a less malignant state.

BrdU was found to effect other cell lines in a similar manner to that of DLKP (Section 3.4.1.3). A549, an adenocarcinoma cell line and SK-N-SH, a neuroblastoma cell line, both exhibited an increase in cell surface area. Keratin expression appeared to be increased in these cells after BrdU treatment (not examined with RA) and it was unexpected to find that SK-N-SH expressed keratins prior to treatment. It had been hoped to use these cells as a negative control to determine the effect of BrdU on other cell lineages which did not express keratin inherently. However, HL60, a leukemic cell line which grows in suspension, did not express keratin proteins before or after BrdU treatment. The cells also remained in suspension throughout the exposure time.

H82, a SCLC-V which also grows in suspension and does not express keratins, has previously been shown to differentiate towards a more tightly aggregated, classic morphology in the presence of RA (Doyle *et al.*, 1989), however, differences in marker expression were still found between the RA-treated cells and SCLC-C cells. The BrdU-treated H82 cells in this study were found to behave differently to the RA-treated cells of Doyle *et al.* (1989) and attached increasingly with increasing BrdU concentration. The attached cells spread and were epithelioid in appearance. The H82 cells which remained in suspension during BrdU treatment did not express keratins, but keratin positivity was apparent in some attached cells (Figure 3.4.9). BrdU has been shown to have similar effects in a SCLC-C cell line, H69, with floating cell aggregates becoming attached and epithelioid in appearance (Feyles *et al.*, 1991). It therefore appears that BrdU and RA each have distinct differentiation effects in multipotent cell populations such as neuroblastoma, H82 and DLKP cells.

The differentiation studies here indicate that BrdU induces keratin expression in keratin-negative SCLC-V cells such as DLKP and H82 and increases keratin content in cell lines which inherently express keratin proteins. However, keratin induction does not

occur in non-epithelial cells such as HL60 which themselves are induced to differentiate by BrdU (Yen and Forbes, 1990) suggesting that the control of keratin expression in non-epithelial cells differs significantly from that of epithelial-derived cells, which is to be expected as only epithelial cells normally express keratin. BrdU and RA thus appear to be capable of inducing cell differentiation along lineage specific pathways and will not, for example, induce keratin expression in a non-epithelial cell type.

The phenotypes induced in DLKP by BrdU and RA treatment are as yet unclear. The agents had distinct effects on cell morphology suggesting that the resulting phenotypes were not identical and that BrdU and RA had induced differentiation along different pathways. The expression of neurofilaments, transglutaminase and desmoplakin remained unchanged in treated and untreated DLKP cells, thus giving no further insight into the identity of the new phenotypes. Retention of neurofilament proteins along with keratin expression meant that BrdU-treated cells were expressing 2 sets of intermediate filament proteins. Co-expression of 2 and even 3 types of intermediate filaments can occur in both normal and tumour tissues (Virtanen *et al.*, 1989, Van Muijen *et al.*, 1987 and Lehto *et al.*, 1985) and keratin and neurofilament proteins are often co-expressed in neuroendocrine tumours (Virtanen *et al.*, 1985).

The proteins of the keratin family, which comprises at least 20 different proteins not including hair keratins, are expressed in a very cell-specific manner in epithelial tissue (Moll *et al.*, 1982). A particular epithelium or epithelial cell can be characterised by its specific pattern of keratin expression and this means that identification of the keratins being induced in DLKP cells could aid in understanding the differentiation pathways involved.

The family of keratin molecules is subdivided into type I keratins (Ker 9 - Ker 20) which are small and acidic and type II keratins (Ker 1 - Ker 8) which are large and neutral to basic (reviewed in Schaafsma and Ramaekers, 1994). All keratin intermediate filaments are heteropolymers composed of at least one type I and one type II chain both *in vivo* and *in vitro* (reviewed in Steinert and Roop, 1988). The profiles of keratin expression in normal and malignant lung epithelial tissues are shown in Table 4.8.1 (Broers *et al.*, 1988). Elias *et al.* (1988) proposed that the overlapping expression of

keratin filaments in SCLC, NSCLC and normal tissue further supports the concept of a stem cell origin of lung cancers.

Logistically, it was not feasible to determine the expression of each keratin protein induced in DLKP. 5 keratins were selected for immunocytochemical analysis of BrdU-treated DLKP cells and clones. Individual keratin expression was not examined in RA-treated cells. The keratins selected were keratins 8, 10, 14, 17 and 18. Keratins 8 and 18 are the major keratins expressed in simple epithelia, keratin 10 is expressed in cornifying epithelia, keratin 14 is found in myoepithelial cells and basal cells and keratin 17 is expressed in transitional and pseudostratified epithelia, basal and hyperproliferating cells (Schaafsma and Ramaekers, 1994).

Keratin 10 and keratin 14 induction was not detected in DLKP cells and clones exposed to BrdU. Keratin 14 is expressed in poorly differentiated squamous cell carcinomas but not in adenocarcinomas or SCLCs (Schaafsma and Ramaekers, 1994) and its absence along with that of keratin 10 suggests that differentiation was not towards squamous or myoepithelial cell types.

Strong induction was apparent, however, in the case of keratins 8 and 18 and weaker induction of keratin 17 was also evident. Keratins 8 and 18 are expressed in normal simple epithelia and their expression has been shown to increase during the progression of malignancy (Broers *et al.*, 1988). These 2 keratin types are also expressed in squamous, adenocarcinoma and SCLC cells, therefore no real conclusions as to the identity of the new DLKP phenotypes can be drawn from their appearance, except perhaps confirming that the cells were simple, undifferentiated epithelial cells and thus are likely to be highly malignant. The absence of these 2 keratins would have been indicative of a more specialised cell type which would have been unusual in poorly differentiated cells.

The detection of keratin 17 expression was more informative. This keratin is expressed in normal basal cells and in hyperproliferating cells (Schaafsma and Ramaekers, 1994). It is also present in squamous cell carcinomas but the strong expression of keratin 14 in squamous cell carcinomas also, which was absent in DLKP cells, suggests that

Keratin No.	Normal Tissue				Tumour Tissue		
	Columnar Epithelium	Basal Cells	Mixed Glands	Lung Alveoli	Adeno-carcinoma	Squamous carcinoma	SCLC
4	±	±	-	±	+	+	
5	+	+	+	+			
7	+	-	+	+	+	+	-
8	+	-	+	-	+	+	+
10	-	-	-	-	-	+	-
13	±	-	-	-	-	+	-
14						+	+
15						+	
17						+	
18	+	±	+	+	+	+	+
19	+	+	+	+	+	+	+

Figure 4.8.1 Expression of keratin proteins in normal and tumour lung tissues from Broers *et al*, (1988).

squamous differentiation is not as likely to be occurring here as the expression of a basal cell phenotype. The absence of transglutaminase reactivity in BrdU-treated cells also supported this likelihood.

Determination of the expression of other keratin and protein markers will give a clearer insight into the differentiation induced in DLKP cells. Identification of the keratin types and other markers induced in the RA-treated cells will determine if the pathways of differentiation resulting from RA exposure differ from those of BrdU exposure. Examination of other epithelial cell type markers and electron microscopic analysis should aid in the identification of the new phenotypes. Differences between the BrdU-/RA-treated DLKP clones may also be revealed in such studies.

The novel induction of keratin expression in keratin-negative cells also raises interesting questions about the mechanisms of action of BrdU and RA. The information currently available on RA-induced gene expression, and specifically keratin gene expression, is more substantial than that on BrdU (see Section 1.6). Most RA-induced alterations in keratin expression occur at the transcriptional level (Kim *et al.*, 1987 and Kopan and Fuchs, 1989) and effects can occur as early as 6hr after exposure, increasing over 24-48hr (Stellmach *et al.*, 1991). The literature on BrdU-induced differentiation is sparse and no reports of its effects on keratin expression could be found. The mechanism of action of BrdU in differentiation is unknown but the induction of keratin expression in DLKP cells may afford an excellent opportunity to investigate this mechanism. Techniques such as DNA footprinting could determine if BrdU acts *via* the specific mechanism of induction of transcription factors. This technique would involve extracting protein from treated and untreated cells and mixing them with DNA extracts from the cells. Any induced transcription factors will bind to their specific regions of DNA and upon incubation with DNase, will protect that region from digestion and will create a gap or footprint when the digested DNA fragments are run on a gel. Reproducible footprints would indicate that BrdU was inducing transcription of specific genes by activation of transcription factors.

Determination of the effects of other differentiation-inducing agents on DLKP and its clones would also be valuable in determining the differentiation potentials of the cells.

Agents such as butyric acid (Chen and Breitman, 1994), vasoactive intestinal peptide (Pence and Shorter, 1990), TGFβ (Masui *et al.*, 1986 and Jetten *et al.*, 1986), interferon-α, interleukin 6 and insulin (McCormick *et al.*, 1995), phorbol esters and diacylglycerol (Jetten *et al.*, 1989), sodium butyrate, dimethyl sulfoxide and dimethyl formamide (Mickley *et al.*, 1989 and Khan *et al.*, 1993) and forskolin (Ross *et al.*, 1991) have all been found to be differentiation-inducing agents in many cell systems including lung epithelial cells and could be used with DLKP cells to assist in the elucidation of tumour cell potentials.

4.9 COMPARISON OF GROWTH OF DLKP AND DLKP-A

As outlined in Section 1.7, alterations in phenotype and growth control such as reduced growth proliferative potential, reduced oncogenic potential and altered morphology are often evident in MDR variants of tumour cell lines (Biedler *et al.*, 1981 and 1975). These observations led to the proposal that acquisition of the MDR phenotype may be related to cell differentiation (Biedler, 1994). The growth of DLKP and one of its MDR variants, DLKP-A, was examined in the presence of serum and a range of growth factors to determine if there was evidence of altered differentiation in this MDR variant (Section 3.5).

No difference was found between the proliferation rates of DLKP and DLKP-A in the presence of 0% serum in the 9 days over which the assays were carried out. In the presence of 5% and 10% serum, the growth rates of the 2 lines were similar up to day 7, after which time, the proliferation of DLKP exceeded that of DLKP-A at both serum concentrations. The fact that the growth rates of the 2 lines were comparable for the first 7 days of the assays indicates that the cells respond similarly to the growth signals present in serum. The increase in growth of DLKP over DLKP-A after day 7 suggests that the DLKP cells may be secreting additional growth stimulatory molecules themselves that are produced in sufficient amounts to stimulate growth only at the cell densities reached by day 7. Alternatively, DLKP-A may be producing growth inhibitory factors which decrease its growth with respect to DLKP. Thus, the difference in growth responses of the 2 cell lines to serum may be indicative of a reduced growth potential of the MDR variant compared with its sensitive counterpart.

However, the responses of DLKP and DLKP-A to growth factors, PDGF-A, PDGF-B, EGF, TGFβ, IL-1 and IL-6, in the 7 day monolayer assay produced evidence contrary to this. In these assays, the growth of DLKP-A cells was stimulated by each of the 6 growth factors examined. While much assay-to-assay variation was apparent in both cell lines, a trend towards stimulation in DLKP-A cells was nonetheless evident. DLKP, in contrast, showed little responsiveness to the growth factors. It is possible that a longer incubation period would have affected the responses of DLKP , as was the case in the

serum response assays, or that combinations of growth factors, rather than single agents, would stimulate DLKP growth. However, it is not clear at the moment why individual growth factors succeeded in enhancing the growth of DLKP-A cells while serum failed, and why the reverse occurred with DLKP cells.

The ^{3}H-thymidine incorporation assays in turn seemed to contradict the monolayer assays. Here, DNA synthesis appeared to be increased in DLKP cells in response to growth factors while little effect was seen with DLKP-A cells. In these assays, responses were determined after only 24hr exposure to the growth factors which was much shorter compared with the 7 days of the growth assays. It is possible that the time scale over which the growth factors exerted their effects on the 2 lines differed and that while DLKP responded within 24 hr to growth factor signals, DLKP-A responded more slowly and so DNA synthesis had not begun before incorporation of 3H-thymidine was determined. However, no such delay in DLKP-A responses to growth signal was apparent in the 7 day growth assays which would seem to rule out this possibility. The finding that DLKP and DLKP-A had similar toxicity profiles for BrdU seemed to eliminate the possibility that DLKP-A cells expressed lower levels of thymidine kinase than DLKP cells and failed to incorporate exogenous ^{3}H-thymidine as a result.

The growth factor assays carried out here are therefore inconclusive, both as regards differences in growth regulation between MDR variants and their sensitive counterparts and the theory that acquisition of an MDR phenotype correlates with increased differentiation.

4.10 RIBOZYME TRANSFECTANTS

Ribozyme gene transfection is a relatively novel approach to the reversal of MDR in cancer cells but to date, no lung cell lines have been successfully reverted to a sensitive phenotype by this technique (see Section 1.7). In this study, clonal populations of DLKP and DLKP-A, DLKP-I and DLKP-A-2B respectively, were chosen to transfect with an *MDR*-1-specific ribozyme in an attempt to overcome multidrug resistance (Section 3.6). Clonal subpopulations were opted for because it was envisaged that a more homogenous population would take up the plasmid, express it and be affected by it in a more uniform manner than mixed parental populations and thus would be more beneficial as models during the initial establishment of the procedure. Mixed parental populations would obviously be closer to the *in vivo* situation and possibly more relevant in the long term and these were indeed successfully transfected with the *MDR*-1 ribozyme also (work carried out by Dr.Carmel Daly), but clonal populations were considered to facilitate a more precise initial assessment of the possibilities of this approach.

The ribozyme plasmid contained a geneticin-resistance gene and so, following transfection, survival of cells in geneticin-containing selection medium indicated that the cells had been successfully transfected and were expressing at least the geneticin-resistance gene in the plasmid. PCR and RT-PCR analyses confirmed that the ribozyme gene was present in the transfected cells and not in control untransfected cells, and was being transcribed.

The *MDR*-1 ribozyme was to exert its effect by enzymatically degrading *MDR*-1 RNA, thus preventing P-glycoprotein expression. DLKP-I untransfected cells contained no *MDR*-1 RNA by RT-PCR analysis and no expression was seen in DLKP-I untransfected cells either. DLKP-A-2B untransfected cells, in contrast, exhibited overexpression of *MDR*-1 RNA when examined by RT-PCR. The level of RNA expressed in DLKP-A-2B 1A5 transfectants was lower than in DLKP-A-2B parental cells, and no RNA expression was detected in the 1C7 transfectant. It thus appears that the *MDR*-1 ribozyme was successfully downregulating the expression of *MDR*-1 RNA to some extent in

transfectant 1A5 and apparently completely in transfectant 1C7.

The *MDR*-1 ribozyme was therefore capable of decreasing P-glycoprotein expression at the RNA level but it was necessary to determine what effect , if any, this had on the drug resistance of DLKP-A-2B cells. As expected, the toxicity profiles of the DLKP-I transfectants were largely unchanged from those of DLKP-I for the 3 MDR drugs, adriamycin, vincristine and VP16. Two of the transfectants, 1C9 and 1G8, appeared to have approximately a 2-fold increase in IC_{50} values over those of untransfected DLKP-I cells.

Interestingly, the DLKP-A-2B 1A5 transfectants were also found to exhibit increased resistance to each drug when compared with DLKP-A-2B cells, similar to that which had been observed in DLKP-I 1C9 and 1G8 transfectants. In the cases of adriamycin and vincristine, approximately a 2-fold increase in resistance was seen in the 1A5 cells, with a smaller increase evident in response to VP16. The transfection procedure involves cloning of the transfected cells (Section 2.19) and it is possible that inherently more resistant clones were selected at this stage from both the DLKP-I and DLKP-A-2B populations. Despite being clonal subpopulations, it is possible that variations in resistance were present. Thus, in the case of DLKP-A-2B 1A5, the ribozyme may have been having an effect on P-glycoprotein expression and indeed appeared to be doing so in the analysis P-glycoprotein RNA levels (Figure 3.6.3), but it cannot be determined if drug resistance was reduced in these cells. Alternatively, the site at which the plasmid was incorporated into the genome may have had significant ramifications for the drug resistance profiles of the cells. Incorporation at a site involved in any aspect of MDR biology could result in altered resistance and this possibility cannot be avoided.

The sensitivity of the DLKP-A-2B 1C7 transfectant was markedly increased compared with the untransfected DLKP-A-2B cells, revealing that the *MDR*-1 ribozyme had successfully affected the drug resistance of these cells. The greatest effect was in response to vincristine where a 26-fold reduction in resistance to this drug compared with DLKP-A-2B was found. When compared with sensitive DLKP parental cells, the IC_{50} value of 1C7 cells was only twice that of DLKP, indicating that the ribozyme had effectively abolished resistance to vincristine in 1C7 cells.

The resistance of 1C7 cells to adriamycin was also decreased with a 5-fold reduction evident when compared with DLKP-A-2B cells. This corresponded to approximately a 3.5-fold increase in resistance over the sensitive DLKP cells and compares with a 16-fold increase in resistance of DLKP-A-2B cells over DLKP cells. The smallest effect was seen in response to VP16 where 1C7 cells were only approximately 1.5-fold more sensitive than DLKP-A-2B cells.

The patterns of altered resistances of 1C7 cells to the 3 drugs correlated with the extent to which P-glycoprotein is involved with their respective mechanisms of action. Overexpression of P-glycoprotein is the major mechanism involved in resistance to vincristine (Clynes *et al.*, 1990). Therefore, downregulation of *MDR*-1 RNA should result in a significant decrease in resistance to this drug. This was found to be the case in the 1C7 tranfectants. Adriamycin and VP16, in contrast, operate via topoisomerase II inhibition, in addition to other mechanisms including to a lesser extent overexpression of P-glycoprotein (Osheroff *et al.*, 1994). In agreement with this, the *MDR*-1 ribozyme was less effective in reducing resistance to these drugs in the 1C7 cells. However, it is not necessary to confer on a cell sensitivity to every drug. Reversal of resistance to one drug may be sufficient to restore the benefits of chemotherapy.

5.0 CONCLUSION

5.0 CONCLUSION

The DLKP cell line appears to represent a poorly-differentiated lung epithelial population which is similar to the omnipotent cell type expressed at early stages (before 8 weeks) of fetal lung development. The absence of cell type-specific markers from the cells, as determined by electron microscopic and immunocytochemical analyses, in addition to the lack of differentiation-associated functions inferred from the inability of the cells to form polarised monolayers, indicates a low level of differentiation, typical of aggressive tumour cells.

The presence of morphologically heterogenous subpopulations within the DLKP cell line was confirmed by the establishment of the 3 clonal populations DLKP-I, -M and -SQ. The morphologies of the individual clones are distinct from each other and are homogenous for up to 20 passages after isolation. DLKP-I possesses the least morphologically stable morphology and appears to behave as a stem cell-like population by giving rise to the 2 other clonal cell types. The DLKP-I cells morphologically resemble basal cells in terms of their small size and low nuclear-to-cytoplasmic ratio and their inability to form colonies in soft agar may reflect a requirement for attachment to the basement membrane for optimum, 3-dimensional proliferation. *In vivo*, basal cells are the only epithelial cells which form attachments to the basement membrane (Tandler *et al.*, 1981).

The spontaneous conversion of DLKP-I cells towards a squamous morphology during normal culture conditions and when cultured in SFM and in suspension flasks, the observation that up to approximately 65% of parental DLKP cells are squamous in appearance and the diagnosis of the original biopsy as a poorly differentiated squamous carcinoma suggests that differentiation from DLKP-I, stem cell-like cells, towards DLKP-SQ, squamous-like cells, may be the main pathway followed by these tumour cells, both *in vitro* and *in vivo*.

In contrast, the DLKP-M phenotype appears to be more stable and this, in addition to the slower proliferation rates of these cells in the growth assays and their failure to

proliferate in SFM, suggests that the DLKP-M cells may be more differentiated or may have a more specialised function than DLKP-I and -SQ cells. Also in support of this theory are the observed similarities between DLKP-M cells and metastatic cells with regard to properties such as such as their morphology, motile behaviour, distinct ECM attachment properties and apparent altered α_2-integrin expression.

The DLKP cell subtypes may therefore serve as a useful model for examining tumour cell interactions and conversions. The ability to study isolated, characterised clonal populations with apparently distinct roles should advance our understanding of tumour cell biology.

While most tumour types contain overlapping features, it is necessary to define any tumour cell model as completely as possible to enable extrapolations to be made with other *in vitro* models, in addition to the *in vivo* situation. The biological marker profile of the DLKP cell line is consistent with that of variant small cell lung carcinoma cells. DLKP cells may now therefore be used more effectively to study aspects of the tumourigenic process with a clearer understanding of how information obtained from these cells may be applied *in vivo*.

While the DLKP clones are morphologically distinct and have different growth properties, no marker has been identified which is capable of distinguishing between the clones. The identification of such a marker is important in terms of confirming phenotypic differences between the clones and also to serve as a quantitative marker in interconversion and differentiation studies.

The failure of the differentiation-inducing agents, BrdU and RA, to induce clonal interconversion but to instead induce apparent differentiation indicates that these agents are capable of overcoming the supposed 'block' that causes the DLKP cells to remain in a poorly differentiated state and also confirms the multipotent nature of the DLKP cells. It remains to be determined if these new phenotypes are less malignant than their untreated counterparts and future characterisation work should shed light on the pathways of differentiation open to DLKP tumour cells. These cells could possibly be used in studies examining the efficacy of differentiation-inducing agents as anti-tumour

agents.

The observation that BrdU and RA are capable of inducing keratin expression in keratin-negative epithelial cells is a novel finding with important implications for both normal and abnormal differentiation processes. Further studies with DLKP cells may aid in the elucidation of control of keratin gene expression as well as facilitating investigations into the mechanisms of action of BrdU and RA as differentiation-inducing agents.

Lung cancer is the leading cause of cancer death and new treatment regimes are urgently required. *MDR* 1 ribozyme transfection experiments carried out here were successful in reversing multidrug resistance in a DLKP-A clonal population, DLKP-A-2B 1C7. No such reversal has been reported in a lung cell line to date. Therefore, while these studies are still in preliminary stages, the 1C7 cells should allow, not only further investigations of the role of P-glycoprotein in multidrug resistance, but also the assessment of ribozyme gene therapy as a strategy in the treatment of lung cancer.

6.0 BIBLIOGRAPHY

Aabo, K., Roed, H., Vindelov, L.L. and Spang-Thomsen M. (1994) A dominated and resistant subpopulation causes regrowth after response to 1,3-bis(2-chloroethyl)-1-nitrosourea treatment of a heterogenous small cell lung cancer xenograft in nude mice. Can. Res. 54, 3295-3299.

Aaronson, S.A. (1991) Growth factors and cancer. Science 254, 1146-1153.

Abeloff, M.D. and Egglestron, J.C. Morphological changes following therapy. In: Greco, F.A., Oldham, R.K. and Bunn, P.A.Jr. (eds) Small cell lung cancer. Grune and Stratton (1981) pp.235-259.

Abeloff, M.D., Eggleston, J.C., Mendelshohn, G., Ettinger, D.S. and Baylin, S.B. (1979) Changes in morphological and biochemical characteristics of a small cell carcinoma of the lung. Am. J. Med. 66, 757-764.

Abo, T. and Balch, C.M. (1981) A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J. Immunol. 127, 1024-1029.

Adamis, A.P., Meklir, B. and Joyce, N.C. (1991) *In situ* injury-induced release of basic-fibroblast growth factor from corneal epithelial cells. Am. J. Pathol. 139, 961-967.

Adams, J.C. and Watt, F.M. (1991) Expression of β_1, β_3, β_4 and β_5 integrins by human epidermal keratinocytes and non-differentiating keratinocytes. J. Cell Biol. 115, 829-841.

Adamson, I.Y.R. and Bowden, D.H. (1975) Derivation of type 1 epithelium from type 2 cells in the developing rat lung. Lab. Invest. 32, 736-745.

Agrez, M.V. and Bates, R.C. (1994) Colorectal cancer and the integrin family of cell adhesion receptors: Current status and future directions. Eur. J. Can. 30A (14), 2166-2170.

Anderson, M.P. and Welsh, M.J. (1991) Calcium and cAMP activate different chloride channels in the apical membrane of normal and cystic fibrosis epithelia. Proc. Natl. Acad. Sci. (USA) 88, 6003-6007.

Antoniades, H.N. (1981) Human platelet-derived growth factor (PDGF): Purification of PDGF-I and PDGF-II and separation of their reduced subunits. Proc. Natl. Acad. Sci. (USA) 78, 7314-7317.

Antoniades, H.N. (1992) Linking cellular injury to gene expression and human proliferative disorders: examples with the *PDGF* genes. Mol. Carcinogen. 6, 175-181.

Antoniades, H.N., Galanopoulos, T., Neville-Golden, J. and O'Hara, C.J. (1992) Malignant epithelial cells in primary human lung carcinomas coexpress *in vivo* platelet-derived growth (PDGF) and PDGF receptor mRNAs and their protein products. Proc. Natl. Acad. Sci. (USA) 89, 3942-3946.

Ashman, C.R. and Davidson, R.L. (1980) Inhibition of Friend erythroleukmic cell differentiation by bromodeoxyuridine. Correlation with the amount of bromodeoxyuridine in DNA. J. Cell. Physiol. 102, 45.

Ayers, M. and Jeffery, P.K. (1988) Proliferation and differentiation in adult mammalian airway epithelium: a review. Eur. Respir. J. 1, 58-80.

Aznavoorian, S., Stracke, M.L., Krutzsch, H., Schiffmann, E. and Liotta, L.A. (1990) Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. J. Cell Biol. 110, 1427-1438.

Azzopardi, J.G. (1959) Oat cell carcinoma of the bronchus. J. Pathol. Bacteriol. 78, 513-519.

Baldwin, F. (1994) Basal cells in human bronchial epithelium. Anat. Rec. 238, 360-367.

Baldwin, F., Hovey, A., McEwen, T., O'Connor, R., Unruh, H. and Bowden, D.H. (1991) Surface to nuclear distances in human bronchial epithelium: Relationship to penetration by Rn daughters. Health Phys. 60, 155-162.

Barbareschi, M., Girlando, S., Mauri, F.A., Arrigoni, G., Laurino, L., Palma, P.D. and Doglioni, C. (1992) Tumour suppressor gene products, proliferation and differentiation markers in lung neuroendocrine neoplasms. J. Pathol. 166, 343-350.

Barnard, J.A., Lyons, R.M. and Moses, H.L. (1990) The cell biology of transforming growth factor β. Biochem. Biophys. Acta 1032, 79-87.

Barranco, S.C., Perry, R.R., Durm, M.E., Werner, A.L., Gregorcyk, S.G., Bolton, W.E., Kolm, P. and Townsend, C.M.Jr. (1994) Intratumor variability in prognostic indicators may be the cause of conflicting estimates of patient survival and response to therapy. Can. Res. 54, 5351-5356.

Barranco, S.C., Townsend, C.M.Jr., Jenkins, V.K., Koester, S.K., Ho, B.Y. and Reumont, K.J. (1988) Treatment-induced changes in sensitivity in a multiclonal human tumor mixture model *in vitro*. Can. Res. 48, 2749-2755.

Bates, S.E., Mickley, L.A., Chen, Y-N., Richert, N., Rudick, J., Biedler, J.L. and Fojo, A.T. (1989) Expression of a drug resistance gene in human neuroblastoma cell lines: Modulation by retinoic acid-induced differentiation. Mol. Cell. Biol. 9, 4337-4344.

Baylin, S.B., Weisburger, W.R., Eggleston, J.C., Mendelsohn, G., Beaven, M.A., Abeloff, M.D. and Ettinger, D.S. (1978) Variable content of histamine, L-dopa decarboxylase and calcitonin in small cell carcinoma of the lung - Biologic and clinical implications. N. Engl. J. Med. 299, 105-110.

Becker, K.L., Monaghan, K.G. and Silva, O.L. (1980) Immunocytochemical localisation of calcitonin in Kulchitsky cells of human lung. Arch. Pathol. Lab. Med. 104, 196-198.

Belinsky, S.A., Devereux, T.R., White, C.M., Foley, J.F., Marinpot, R.R. and Anderson, M.W. (1991) Role of Clara cells and type II cells in the development of pulmonary tumours in rats and mice following exposure to a tobacco-specific nitrosamine. Exp. Lung Res. 17, 263-278.

Ben-Ezra, J.M., Kornstein, M.J., Grimes, M.M. and Krystal, G. (1994) Small cell carcinomas of the lung express the Bcl-2 protein. Am. J. Pathol. 145 (5), 1036-1040.

Bepler, G., Jaques, G., Havemann, K., Koehler, A., Johnson, B.E. and Gazdar, A.F. (1987b) Characterization of two cell lines with distinct phenotypes established from a patient with small cell lung cancer. Can. Res. 47, 1883-1891.

Bepler, G., Jaques, G., Koehler, A., Gropp, C. and Havemann, K. (1987a) Markers and characteristics of human SCLC cell lines. Neuroendocrine markers, classical tumour markers and chromosomal characteristics of permanent human small cell lung cancer cell lines. J. Can. Res. Clin. Oncol. 113, (3), 253-259.

Bepler, G., Jaques, G., Neumann, K., Aumuller, G., Gropp, C. and Havemann, K. (1987c) Establishment, growth properties and morphological characteristics of permanent human small cell lung cancer cell lines. J. Can. Res. Clin. Oncol. 113, 31-40.

Berdel, W.E., Danhauser-Riedl, S., Oberberg, D. and Zafferani, M. (1992) Effects of hematopoietic growth factors on malignant nonhematopoietic cells. Sem. Oncol. 19 (2) Suppl.4, 41-45.

Berendsen, H.H., deLeig, L., Postmus, P.E., ter Haar, J.G., Poppema, S. and The, T.H. (1988) Detection of small-cell-lung-cancer metastases in bone-marrow aspirates using monoclonal antibody directed against neuro-endocrine differentiation antigen. J. Clin. Pathol. 41, 273-276.

Bergh, J. (1988) The expression of the platelet-derived and transforming growth factor genes in human nonsmall lung cancer cell lines is related to tumor stroma formation in nude mice tumors. Am. J. Pathol. 133 (3), 434-439.

Bergh, J., Arnberg, H., Eriksson, B. and Lundqvist, G. (1989) The release of chromogranin A and B like activity from human lung cancer cell lines: A potential marker for a subset of small cell lung cancer. Acta Oncol. 28, 651-654.

Biedler, J.L. (1994) Drug resistance: genotype *versus* phenotype. Can. Res. 54, 666-678.

Biedler, J.L., Casals, D., Chang, T-d., Meyers, M.B., Spengler, B.A. and Ross, R.A. Multidrug-resistant human neuroblastoma cells are more differentiated than controls and retinoic acid further induces lineage-specific differentiation. In: Evans, A.E., D'Angio, G.J., Knudson, A.G.Jr. and Seeger, R.C. (eds) Advances in neuroblastoma research, 3. New York, Wiley-Liss (1991) pp.181-191.

Biedler, J.L. and Peterson, R.H.F. Altered plasma membrane glycoconjugates of Chinese hamster cells with acquired resistance to actinomycin D, daunorubicin and vincristine. In: Startorelli, A.C., Lazo, J.S. and Bertino, J.R. (eds) Molecular action and targets for cancer chemotherapeutic agents. Bristo-Myers Cancer Symposia, Vol.2, New York, Academic Press (1981) pp.453-482.

Biedler, J.L., Riehm, H., Peterson, R.H.F. and Spengler, B.A. (1975) Membrane mediated drug resistance and phenotypic reversion to normal growth behaviour of Chinese hamster cells. J. Natl. Can. Inst. 55, 671-680.

Binaschi, M., Giaccone, G., Gazdar, A.F., De Isabella, P., Astaldi Ricotti, G.C.B., Capranico, G. and Zunino, F. (1992) Characterization of a topoisomerase II gene rearrangement in a human small-cell lung cancer cell line. J. Natl. Can. Inst. 84, 1710-1716.

Birchmeier, W. and Behrens, J. (1994) Cadherin expression in carcinomas: Role in the formation of cell junctions and the prevention of invasiveness. Biochem. Biophys. Acta 1198, 11-26.

Bohm, M., Totzeck, B., Birchmeier, W. and Wieland, I. (1994) Differences of E-cadherin expression levels and patterns in primary and metastatic human lung cancer. Clin. Exp. Metastasis 12, 55-62.

Boller, K., Vestweber, D. and Kemler, R. (1985) Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. J. Cell Biol. 100, 327-332.

Boucher, R.C., Van Scott, M.R., Willumsen, N. and Stutts, M.J. (1988) Epithelial injury. Mechanisms and cell biology of airway epithelial injury. Am. Rev. Respir. Dis. 138, S41-S44.

Brambilla, E., Moro, D., Brichon, P.Y., Stoebner, P., Paramelle, B. and Brambilla, C. (1992) Basal cell (Basaloid) carcinoma of the lung: A new morphologic and phenotypic entity with separate prognostic significance. Hum. Pathol. 23, 993-1003.

Brand, N., Petkovich, M., Krust, A., Chambon, P., de The, H.D., Marchio, A., Tiollais, P. and Dejean, A. (1988) Identification of a second human retinoic acid receptor. Nature 332, 850-853.

Bravo, M., Vasquez, R., Rubio, H., Salazar, M., Pardo, A. and Selman, M. (1991) Production of platelet-derived growth factor by human lung cancer. Respir. Med. 85, 479-485.

Brodt, P. (1986) Characterisation of two highly metastatic variants of Lewis lung carcinoma with different organ specificities. Can. Res. 46, 2442-2448.

Broers, J.L.V., Carney, D.N., de Ley, L., Vooijs, G.P. and Ramaekers, F.C.S. (1985) Differential expression of intermediate filament proteins distinguishes classic from variant small-cell lung cancer cell lines. Proc. Natl. Acad. Sci. (USA) 82, 4409-4413.

Broers, J.L.V., Ramaekers, F.C.S., Klein Rot, M., Oostendorp, T., Huysmans, A., van Muijen, G.N.P., Wagenaar, S.S. and Vooijs, G.P. (1988) Cytokeratins in different types of human lung cancer as monitored by chain-specific monoclonal antibodies. Can. Res. 48, 3221-3229.

Brook, F.B., Raafat, F., Eldeeb, B.B. and Mann, J.R. (1988) Histologic and immunohisto-chemical investigation of neuroblastomas and correlation with prognosis. Hum. Pathol. 19, 879-888.

Brown, P.D., Levy, A.T., Margulies, I., Liotta, L. and Stetler-Stevenson, W.G. (1990) Independent expression and cellular processing of the 72-kDa type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Can. Res. 50, 6184-6191.

Buchmann, A., Ruggeri, B., Klein-Szanto, A.J.P. and Balmain, A. (1991) Progression of squamous carcinoma to spindle carcinomas of mouse skin is associated with an imbalance of H-*ras* alleles on chromosome 7. Can. Res. 51, 4097-4101.

Bunn, P.A.Jr. (1992) Clinical experiences with carboplatin (paraplatin) in lung cancer. Semin. Oncol. 19 (Suppl.2), 1-11.

Bunn, P.A.Jr., Chan, D., Dienhart, D.G., Tolley, R., Tagawa, M. and Jewett, P.B. (1992) Neuropeptide signal transduction in lung cancer: Clinical implications of bradykinin sensitivity and overall heterogeneity. Can. Res. 52, 24-31.

Bunn, P.A.Jr., Dienhart, D.G., Chan, D., Puck T.T., Tagawa M., Jewett P.B. and Braunschweiger E. (1990) Neuropeptide stimulation of calcium flux in human lung cancer cells: deliniation of alternative pathways. Proc. Natl. Acad. Sci. (USA) 87, 2162-2166.

Bunn, P.A.Jr., Cohen, M.H., Ihde, D.C., Fossieck, B.E., Matthews, M.J. and Minna, J.D. (1977) Advances in small cell bronchiogenic carcinoma: A commentary. Can. Treat. Rep. 61, 333-342.

Burholt, D.R., Shackney, S.E., Ketterer, D.M., Pollice, A.A., Smith, C.A., Brown, K.A., Giles, H.R. and Schepart, B.S. (1989a) Karyotypic evolution of a human undifferentiated large cell carcinoma of the lung in tissue culture. Can. Res. 49, 3355-3361.

Burholt, D.R., Shackney, S.E., Ketterer, D.M., Pollice, A.A., Smith, C.A., Giles, H.R. and Hartsock, R.J. (1989b) Tetraploidization, chromosome loss and the production of structural chromosomal abnormalities in a human lung adenocarcinoma cell line. Proc. Am. Assoc. Can. Res. 30, 37.

Buxton, R.S. and Magee, A.I. (1992) Structure and interactions of desmosomal and other cadherins. Semin. Cell Biol. 3, 157-167.

Caillaud, J.M., Benjelloun, S., Bosq, J., Braham, K. and Lipinski, M. (1984) HNH-1-defined antigen detected in paraffin embedded neuroectoderm tumors and those derived from cells of the amine precursor uptake and decarboxylation system. Can. Res. 44, 4432-4439.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kappeller, R. and Soltoff, S. (1991) Oncogenes and signal transduction. Cell 64, 281-302.

Carbone, D.P., Koros, A.M., Linnoila, R.I., Jewett, P. and Gazdar, A.F. (1991) Neural cell adhesion molecule expression and messenger RNA splicing patterns in lung cancer cell lines are correlated with neuroendocrine phenotype and growth morphology. Can. Res. 51, 6142-6149.

Carbone, D.P. and Minna, J.D. (1992) Molecular genetics of lung cancer. Adv. Internal Med. 37, 153-171.

Carney, D.N., Gazdar, A.F., Bepler, G., Guccion, J.G., Marangos, P.J., Moody, T.W., Zweig, M.H. and Minna, J.D. (1985) Establishment and identification of small cell lung cancer cell lines having classic and variant features. Can. Res. 45, 2913-2923.

Carney, D.N., Mitchell. J.B. and Kinsella, T.J. (1983) *In vitro* radiation and chemotherapy sensitivity of established cell lines of human small cell lung cancer and its large cell morphological variants. Can. Res. 43, 2806-2811.

Cech, T.R. (1987) The chemistry of self-splicing RNA and RNA enzymes. Science 236, 1532-1539.

Cech, T.R. (1988) Ribozymes and their medical implications. J. Am. Med. Assoc. 260, 3030-3034.

Cech, T.R. and Bass, B. (1986) Biological catalysis by RNA. Ann. Rev. Biochem. 55, 599-629.

Chejfec, G., Cosnow, I., Gould, N.S., Husain, A.N. and Gould, V.E. (1990) Pulmonary blastoma with neuroendocrine differentiation in cell morules resembling neuroepithelial bodies. Histopathology 17, 353-358.

Chen, Z. and Breitman, T.R. (1994) Tributyrin: A prodrug of butyric acid for potential clinical application in differentiation therapy. Can. Res. 54, 3494-3499.

Cherny, T., Barnes, D.M., Hasleton, P., Barber, P.V., Healy, K., Gullick, W. and Thatcher, N. (1986) Expression of epidermal growth factor receptor (EGF-R) in human lung tumours. Br. J. Cancer 54, 265-269.

Chevalier, G. and Collet, A.J. (1972) *In vivo* incorporation of choline-^{3}H, leucine-^{3}H and galactose-^{3}H in alveolar type II pneumonocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy. Anat. Rec. 174, 289-310.

Churg, A. Tumours of the lung. In: Thurlbeck, W.M. (ed) Pathology of the lung. New York, NY, Thieme Medical Publishers (1988) pp.311-423.

Chytil, F. (1992) The lungs and vitamin A. Am. J. Physiol. 262, L517-L527.

Chytil, F. and Ong, D.E. (1983) Cellular retinoid and retinoic acid-binding proteins. Adv. Nutr. Res. 5, 13-29.

Ciccarone, V., Spengler, B.A., Meyers, M.B., Biedler, J.L. and Ross, R.A. (1989) Phenotypic diversification in human neuroblastoma cells: Expression of distinct neural crest lineages. Can. Res. 49, 219-225.

Cifone, M.A. and Fidler, I.J. (1981) Increasing metastatic potential is associated with genetic instability of clones isolated from murine neoplasms. Proc. Natl. Acad. Sci. (USA) 78, 6949-6952.

Clynes, M., Heenan, M. and Hall, K. Human cell lines as models for multidrug resistance in solid tumours. In: Clynes, M. (ed) Multiple drug resistance in cancer: Cellular, molecular and clinical approaches. Kluwer Academic Publishers (1993) pp.231-256.

Clynes M., Redmond, A. and Heenan, M. (1990) Recent developments in research on multiple drug-resistance in cancer cells. Cancer J. 3 (1), 34-39.

Clynes, M., Redmond, A., Moran, E. and Gilvarry, U. (1992) Multiple drug-resistance in variant of a human non-small cell lung carcinoma cell line, DLKP-A. Cytotechnology 10, 75-89.

Coffey, R.J., Bascom, C.C., Sipes, N.J., Graves-Deal, R., Weissman, N.H. and Moses, H.L. (1988) Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. Mol. Cell. Biol. 8, 3088-3093.

Cole, S.P.C., Mirski, S., McGarry, R.C., Cheng, R., Campling, B.C. and Roder, J.C. (1985) Differential expression of the Leu-7 antigen on human lung tumor cells. Can. Res. 45, 4285-4290.

Cook, R.M., Miller, Y.E. and Bunn, P.A. Small cell lung cancer: etiology, biology, clinical features, staging and treatment. In: Ozols, R.F. (ed) Current problems in cancer. Mosby, St.Louis (1993) pp.71-141.

Cordon-Cardon, C., O'Brien, J.P., Boccia, J., Casals, D., Bertino, J.R. and Melamed, M.R. (1990) Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumour tissues. J. Histochem. Cytochem. 38, 1277-1287.

Cross, M. and Dexter, T.M. (1991) Growth factors in development, transformation and tumorigenesis. Cell 64, 271-280.

Curt, G.A., Carney, D.N., Cowan, K.H., Jolivet, J., Bailey, B.D., Drake, J.C., Chien Song, K.S., Minna, J.D. and Chabner, B.A. (1983) Unstable methotrexate resistance in human small-cell carcinoma associated with double minute chromosomes. N. Eng. J. Med. 308, 199-202.

Cuttitta, F., Carney, D.N., Mulshine, J., Moody, T.W., Fedorko, J., Fischler, A. and Minna, J.D. (1985) Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature, 316, 823-826.

Cutz, E., Gillan, J.E. and Bryan, A.C. (1985) Neuroendocrine cells in the developing human lung: Morphologic and functional considerations. Pediatr. Pulmonol. 1, (Suppl.) S21-S29.

Cutz, E., Chan, W. and Track, N.S. (1981) Bombesin, calcitonin, and leuenkephalin immunoreactivity on endocrine cells of human lung. Experientia 37, 765-767.

Damjanovich, L., Albelda, S.M., Mette, S.A. and Buck, C.A. (1992) Distribution of integrin cell adhesion receptors in normal and malignant lung tissue. Am. J. Respir. Cell Mol. Biol. 6, 197-206.

Damstrup, L., Rygaard, K., Spand-Thomsen, M. and Skovgaard Poulsen H. (1992) Expression of the epidermal growth factor receptor in human small cell lung cancer cell lines. Can. Res. 52, 3089-3093.

Damstrup, L., Rygaard, K., Spang-Thomsen, M. and Skovgaard Poulsen, H. (1993) Expression of transforming growth factor β (TGFβ) receptors and expression of TGFβ_1, TGFβ_2 and TGFβ_3 in human small cell lung cancer cell lines. Br. J. Cancer 67, 1015-1021.

Dardick, I. (1993) Reliability of criteria for ultrastructural identification of neuroendocrine granules. Ultrastruc. Pathol. 17, 37-47.

Davila, D.G., Minoo, P., Estervig, D.N., Kasperbauer, J.L., Tzen, C-Y. and Scott, R.E. Linkages in control of differentiation and proliferation in murine mesenchymal stem cells and human keratinocyte progenitor cells: The effects of carcinogenesis. In: Fischer, P.B. (ed) Mechanics of differentiation. Vol.1. CRC Press (1990) pp.1-13.

de Leij, L., Postmus, P.E., Buys, C.H.C.M., Elema, J.D., Ramaekers, F., Poppema, S., Brouwer, M., van der Veen, A.Y., Mesander, G. and The, T.H. (1985) Characterisation of three new variant type cell lines derived from small cell carcinoma of the lung. Can. Res. 45, 6024-6033.

de The, H., Chomienne, C., Lanotte, M., Degos, L. and Dejean, A. (1990a) The t(15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor α gene to a novel transcribed locus. Nature 347, 558-561.

de The, H., Marchio, A., Tiollias, P. and Dejean, A. (1989) Differential expression and ligand regulation of the retinoic acid receptor α and β genes. EMBO J. 8, 429-433.

de The, H., Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H. and Dejean, A. (1990b) Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. Nature 343, 177-180.

Dedhar, S. and Saulnier, R. (1990) Alterations in integrin receptor expression on chemically transformed human cells: Specific enhancement of laminin and collagen receptor complexes. J. Cell. Biol. 100, 481-489.

Deftos, L.J., Linnoila, R.I., Carney, D.N., Burton, D.W., Leong, S.S., O'Connor, D.T., Murray, S.S. and Gazdar, A.F. (1988) Demonstration of chromogranin A in human neuroendocrine cell lines by immunohistology and immunoassay. Cancer 62, 92-97.

DeLarco, J.E. and Todaro, G.J. (1978) Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. (USA) 75, 4001-4005.

Devereux, T.R., Domin, B.A. and Philpot, R.M. (1989) Xenobiotic metabolism by isolated pulmonary cells. Pharm. Ther. 41, 243-256.

Dharmsathaphorn, K. and Madara, J.L. Established intestinal cell lines as model systems for electrolyte transport studies. In: Methods in enzymology 192, Academic Press (1990) pp.354-364.

Domin, B.A., Devereux, T.R. and Philpot, R.M. (1986) The cytochrome P-450 monooxygenase system of rabbit lung: Enzyme components, activities and induction in the nonciliated bronchiolar epithelial (Clara) cell, alveolar type II cell and alveolar macrophage. Molec. Pharmacol. 30, 296-303.

Donalies, M., Cramer, M., Ringwald, M. and Starzinsky-Powitz, A. (1991) Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. Proc. Natl. Acad. Sci. (USA) 88, 8024-8028.

Donnelly, G.M., Haack, D.G. and Heird, C.S. (1982) Tracheal epithelium: Cell kinetics and differentiation in normal rat tissue. Kinet. 15, 119.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. (1983) Simian sarcoma virus *onc* gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221, 275-277.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984) Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. Nature 307, 521-527.

Doyle, L.A., Borges, M., Hussain, A., Elias, A. and Tomiyasu, T. (1990) An adherent subline of a unique small-cell lung cancer cell line downregulates antigens of the neural cell adhesion molecule. J. Clin. Invest. 86, 1848-1854.

Doyle, L.A., Giangiulo, D., Hussain, A., Park, H-J., Chiu Yen, R-W. and Borges, M. (1989) Differentiation of human variant small cell lung cancer cell lines to a classic morphology by retinoic acid. Can. Res. 49, 6745-6751.

Doyle, L.A., Mabry, M., Stahel, R.A., Waibel, R. and Golgstein, L.H. (1991) Modulation of neuroendocrine surface antigens in oncogene-activated small cell lung cancer lines. Br.J.Cancer 63 Suppl.XIV, 39-42.

Dulbecco, R. (1970) Topoinhibition and serum requirement of transformed and untransformed cells. Nature 227, 802.

Edelman, G.M. (1986) Cell adhesion molecules in the regulation of animal form and tissue pattern. Ann. Rev. Cell Biol. 2, 81-116.

Elias, A.D., Cohen, B.F. and Bernal, S.D. (1988) Keratin subtypes of small cell lung cancer. Can. Res. 48, 2724-2729.

Elices, M.J. and Hemler, M.E. (1989) The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. Proc. Natl. Acad. Sci. (USA) 86, 9906-9910.

Esumi, N., Imashuku, S., Tsunamoto, K., Todo, S., Misawa, S., Goto, T. and Fujisawa, Y. (1989) Procoagulant activity of human neuroblastoma cell lines in relation to cell growth, differentiation and cytogenetic abnormalities. Jpn. J. Can. Res. 80 (5), 438-443.

Evans, M.J. and Plopper, C.G. (1988) The role of basal cells in adhesion of columnar epithelium to airway basement membrane. Am. Rev. Respir. Dis. 138, 481-583.

Falcioni, R., Cimino, L., Gentileschi, M.P., D'Agnano, I., Zupi, G., Kennel, S.J. and Sacchi, A. (1994) Expression of $\beta 1$, $\beta 3$, $\beta 4$ and $\beta 5$ integrins by human lung carcinoma cells of different histotypes. Exp. Cell Res. 210, 113-122.

Feldman, L.E., Shin, K.C., Natale, R.B. and Todd III, R.F. (1991) β_1 integrin expression on human small cell lung cancer cells. Can. Res. 51, 1065-1070.

Feyles, V., Sikora, L.K.J., McGarry, R.C. and Jerry, L.M. (1991) Effects of retinoic acid and bromodeoxyuridine on human melanoma-associated antigen expression in small cell lung carcinoma cells. Oncol. 48, 58-64.

Fialkow, P.J. (1979) Clonal origin of human tumors. Annu. Rev. Med. 30, 135-176.

Fidler, I.J. and Hart, I.R. (1982) Biological diversity in metastatic neoplasms, origins and implications. Science 217, 998-1003.

Fitzer-Attas, C., Feldman, M. and Eisenbach, L. (1993) Expression of functionally intact PDGF-α receptors in highly metastatic 3LL Lewis lung carcinoma cells. Int. J. Cancer 53, 315-322.

Foa, R., Guarini, A. and Gansbacher, B. (1992) IL2 treatment for cancer: From biology to gene therapy. Br. J. Cancer 66, 992-998.

Folk, J.E. (1985) Transglutaminases. Ann. Rev. Biochem. 85, 517-531.

Francis, D. and Jacobsen, M. (1983) Pulmonary blastoma. Curr. Top. Pathol. 73, 265-294.

Gabbert, H., Wagner, R., Moll, R. and Gerharz, C.D. (1985) Tumor dedifferentiation: An important step in tumor invasion. Clin. Exp. Metastasis 3, 257-279.

Garcia de Palazzo, I.E., Adams, G.P., Sundareshan, P., Wong, A.J., Testa, J.R., Bigner, D.D. and Weiner, L.M. (1993) Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Can. Res. 53, 3217-3220.

Gazdar, A.F. The biology of endocrine tumors of the lung. In: K.L. Becker and A.F. Gazdar (eds) The endocrine lung in health and disease. Philadelphia, W.B. Saunders (1984) pp.448-459.

Gazdar, A.F., Carney, D.N., Guccion, J.G. and Baylin, S.B. Small cell carcinoma of the lung: Cellular origin and relationship to other pulmonary tumors. In: Greco, F.A., Oldham, R.K. and Bunn, P.A. (eds) Small cell lung cancer. Grune and Stratton (1981) pp.145-175.

Gazdar, A.F., Carney, D.N., Nau, M.M. and Minna, J.D. (1985) Characterisation of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological and growth properties. Can. Res. 45, 2924-2930.

Gazdar, A.F., Carney, D.N., Russell, E.K., Sims, H.L., Baylin, S.B., Bunn, P.A., Guccion, J.G. and Minna, J.D. (1980) Establishment of continuous, clonable cultures of small cell carcinoma of the lung which have amine precursor uptake and decarboxylation properties. Can. Res. 40, 3502-3507.

Gazdar, A.F., Helman, L.J., Israel, M.A., Russell, E.K., Linnoila, R.I., Mulshine, J.L., Schuller, H.M. and Park, J. (1988) Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A and dense core granules in human tumors of endocrine and nonendocrine origin. Can. Res. 48, 4078-4082.

Gazdar, A.F., Kadoyama, C., Venzon, D., Park, J.G., Tsai, C.M., Linnoila, R.I., Mulshine, J.L., Ihde, D. and Giaccone, G. (1991) Association between histological type and neuroendocrine differentiation on drug sensitivity of lung cancer cell lines. J. Natl. Cancer Inst. Mongr. 13, 191-196.

Gazdar, A.F. and Linnoila, R.I. (1988) The pathology of lung cancer - changing concepts and newer diagnostic techniques. Semin. Oncol. 15, 215-225.

Gazdar, A.F., Linnoila, R.I., Kurita, Y., Oie, H.K., Mulshine, J.L., Clark, J.C. and Whitsett, J.A. (1990) Peripheral airway cell differentiation in human lung cancer cell lines. Can. Res. 50, 5481-5487.

Geradts, J., Chen, J-Y., Russell, E.K., Yankaskas, J.R., Nieves, L. and Minna, J.D. (1993) Human lung cancer cell lines exhibit resistance to retinoic acid treatment. Cell Growth Diff. 4, 799-809.

Ghadially, F.N. Neuroendocrine (APUD) granules and uranaffin reaction in normal and neoplastic cells. In: Ultrastr.Path.Cell Matrix, London, Butterworths (1988) 1, pp.364-389.

Ghillani, P.P., Motte, P., Troalen, F., Jullienne, A., Gardet, P., LeChevalier, T., Rougier, P., Schlumberger, M., Bohoun, C. and Bellet, D. (1989) Identification and measurement of calcitonin precursors in serum of patients with malignant disease. Can. Res. 49, 6854-6851.

Giaccone, G., Broers, J., Jensen, S., Fridman, R.I., Linnoila, R. and Gazdar, A.F. (1992) Increased expression of differentiation markers can accompany laminin-induced attachment of small cell lung cancer cells. Br. J. Cancer 66, 488-495.

Gilvarry, U., Farrell, D., Lynch, V., Moriarty, M., Dooley, M., Law, E. and Clynes, M. (1990) Cytological differences between normal and malignant human cell populations in culture. Can. Res. 50, 3390-3393.

Ginsberg, M.H., Loftus, J.C. and Plow, E.F. (1988) Cytoadhesions, integrins and platelets. Thromb. Haemos. 59, 1-6.

Goodman, B.E., Fleischer, R.S. and Crandall, E.D. (1983) Evidence for active Na^+ transport by cultured monolayers of pulmonary alveolar epithelial cells. Am. J. Physiol. 245, C78-C83.

Gorgoulis, V., Aninos, D., Mikou, P., Kanavaros, P., Karameris, A., Joardanoglou, J., Rasidakis, A., Veslemes, M., Ozanne, B. and Spandidos, D.A. (1992) Expression of EGF, TGF-α and EGFR in squamous cell lung carcinomas. Anticancer Res. 12, 1183-1188.

Gosney, J.R. Pulmonary endocrine pathology: Endocrine cells and endocrine tumours of the lung. Butterworth-Heinemann, Oxford (1992).

Gottesman, M.M. (1990) The role of proteases in cancer. Cancer Biol. 1, 97-160.

Gottesman, M.M. (1993) How cancer cells evade chemotherapy. Can. Res. 53, 747-754.

Goustin, A.S., Leof, E.B., Shipley, G.D. and Moses, H.L. (1986) Growth factors and cancer. Can. Res. 46, 1015-1029.

Graziano, S.L., Pfiefer, A.M., Testa, J.R., Mark, G.E., Johnson, B.E., Hallinan, E.J., Pettengill, O.S., Sorenson, G.D., Tatum, A.H., Brauch, H., Zbar, B., Klejter, W.L., Ehrlich, G.D. and Poiesz, B.J. (1991) Involvement of the *RAF1* locus, at band 3p25, in the 3p deletion of small-cell lung cancer. Genes Chroms. Can. 3, 283-293.

Graziano, S.L., Tatum, A.H., Newman, N.B., Oler, A., Kohman, L.J., Veit, L.J., Gamble, G.P., Coleman, M.J., Barmada, S. and O'Lear, S. (1994) The prognostic significance of neuroendocrine markers and carcinoembryonic antigen in patients with resected stage I and II non-small cell lung cancer. Can. Res. 54, 2908-2913.

Green, H. (1977) Terminal differentiation of cultured human epidermal cells. Cell 11, 405-416.

Griffin, C.A. and Baylin, S.B. (1985) Expression of the c-*myb* oncogene in human small cell lung carcinoma. Can. Res. 45, 272-275.

Guirgius, R., Marguiles, I., Tarabolette, G., Schiffman, E. and Liotta, L. (1987) Cytokine-induced pseudopodial protrusion is coupled to tumor cell migration. Nature 329, 261-263.

Gumbiner, B. (1993) Proteins associated with the cytoplasmic surface of adhesion molecules. Neuron. 11, 551-564.

Gusterson, B., Cowley, G., Smith, J.A. and Ozanne, B. (1984) Cellular localisation of human epidermal growth factor receptor. Cell Biol. Int. Reports 8, 649-658.

Guzman, J., Izumi, T., Nagai, S. and Costabel, U. (1994) ICAM-1 and integrin expression on isolated human alveolar type II pneumonocytes. Eur. Respir. J. 7, 736-739.

Haeder, M., Rotsch, M., Bepler, G., Hennig, C., Havemann, K., Heimann, B. and Moelling K. (1988) Epidermal growth factor receptor expression in human lung cancer cell lines. Can. Res. 48, 1132-1136.

Hage, E. (1973) Histochemistry and fine structure of bronchial carcinoid tumors. Virchows Arch (Pathol. Anat.) 361, 121-128.

Hage, E. (1980) Ultrastructure of endocrine cells and endocrine tumours of the human lung. Abst.Int.Assoc.Study Lung Cancer, Copenhagen, Denmark, p.64.

Ham, R.G. and Veomett, M.J. Mechanisms of development. Mosby, C.V. (ed) St.Louis (1980) pp.317.

Hamid, Q.A., Corrin, B., Dewar, A., Hoefler, H. and Sheppard, M.N. (1990) Expression of gastrin-releasing peptide (human bombesin) gene in large cell undifferentiated carcinoma of the lung. J. Pathol. 161, 145-151.

Hammar, S., Bockus, D., Remington, F. and Cooper, L. (1989) The unusual spectrum of neuroendocrine lung neoplasms. Ultrastruct. Pathol. 13, 515-560.

Hanauske, A-R., Degen, D., Marshall, M.H., Hilsenbeck, S.G., Banks, P., Stuckey, J., Leahy, M. and Von Hoff, D.D. (1992) Effects of recombinant human interleukin-1α on clonogenic growth of primary human tumors *in vitro*. J. Imunother. 11, 155-158.

Harding, J.D., Przybyla, A.E., MacDonald, R.J., Pictet, R.L. and Rutter, W.J. (1978) Effects of dexamethasone and 5-bromodeoxyuridine on the synthesis of amylase mRNA during pancreatic development *in vitro*. J. Biol. Chem. 253, 7531-7537.

Harris, A.L. (1990) EGF receptor (EGFR) expression in human primary cancers. Proc. Am. Assoc. Can. Res. 31, 458-460.

Haseloff, J. and Gerlach, W.L. (1988) Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature 334, 585-591.

Hassel, J.R., Gehron-Robey, P., Barrach, H.J., Wilczek, J., Renard, S.I. and Martin, G.R. (1980) Isolation of a heparin-sulfate-containing proteoglycan from basement membrane. Proc. Natl. Acad. Sci. (USA) 77, 3705-3709.

Hattori, S., Matsuda, M., Tateishi, R., Tatsumi, N. and Terazawa, T. (1968) Oat-cell carcinoma of the lung containing serotonin granules. Gann 59, 123-129.

Have-Opbroek, A.A.W. (1991) Lung development in mouse embryo. Exp. Lung Res. 17, 111-130.

Have-Opbroek, A.A.W., Dubbeldam, J.A. and Otto-Verberne, C.J.M. (1988) Ultrastructural features of type II alveolar epithelial cells in early embryonic mouse lung. Anat. Rec. 221, 846-853.

Hay, F.G., Duncan, L.W. and Leonard, R.C.F. (1991) Establishment and characterisation of two new small cell lung cancer cell lines - one from a patient with previous familial retinoblastoma. Br. J. Cancer 63 (Suppl). XIV, 43-45.

Heldin, C-H. and Westermark, B. (1989) Growth factors as transforming proteins. Eur. J. Biochem. 184, 487-496.

Hendler, F.J. and Ozanne, B.W. (1984) Human squamous cell lung cancers express increased epidermal growth factor receptors. J. Clin. Invest. 74, 647-651.

Heppner, G.H. (1984) Tumor heterogeneity. Can. Res. 44, 2259-2265.

Herzog, C.R., Wiseman, R.W. and You, M. (1994) Deletion mapping of a putative tumor suppressor gene on chromosome 4 in mouse lung tumors. Can. Res. 54, 4007-4010.

Hewitt, A.T., Varner, H.H., Silver, M.H., Dessau, N., Wilkes, C.M. and Martin, G.R. (1981) The isolation and partial characterization of chondronectin, an attachment factor for chondrocytes. J. Biol. Chem. 257, 2330-2334.

Hill, B., Tsuboi, A. and Baserga, R. (1974) Effect of 5-bromodeoxyuridine on chromatin transcription in confluent fibroblasts. Proc. Natl. Acad. Sci. (USA) 71, 455-459.

Hirasawa, M., Shijubo, N., Uede, T. and Abe, S. (1994) Integrin expression and ability to adhere to extracellular matrix proteins and endothelial cells in human lung cancer cell lines. Br. J. Cancer 70, 466-473.

Hoffmann, B., Lehmann, J.M., Zhang, X-k., Hermann, T., Graupner, G. and Pfahl, M. (1990) A retinoic acid receptor-specific element controls the retinoic acid receptor-β promoter. Mol. Endocrinol. 4, 1734-1743.

Hook, G.E.R., Brody, A.R., Cameron, G.S., Jetten, A.M., Gilmore, L.B. and Nettesheim, P. (1987) Repopulation of denuded tracheas by Clara cells isolated from lungs of rabbits. Exp. Lung Res. 12, 311-329.

Horan, H.P., Thor, A., Scholm, J., Rao, C.N. and Liotta, L.A. (1985) Expression of laminin receptor in normal and carcinomatus human tissues as defined by a monoclonal antibody. Can. Res. 45, 2713-2719.

Hoyt Jnr., R.F., McNelly, N.A., McDowell, E.M. and Sorokin, S.P. (1991) Neuroepithelial bodies stimulate proliferation of airway epithelium in fetal hamster lung. Am. J. Physiol. 260, L234-L240.

Hoyt Jnr., R.F., McNelly, N.A. and Sorokin, S.P. (1988) [^{3}H] thymidine labeling of neuroepithelial bodies (NEB) in rat and hamster lungs. Abst. Fourth Int. Cong. Cell Biol., Montreal, Aug. 14-19.

Hoyt Jnr., R.F., Sorokin, S.P., McDowell, E.M. and McNelly, N.A. (1993) Neuroepithelial bodies and growth of the airway epithelium in developing hamster lung. Anat. Rec. 236, 15-22.

Huang, S.S. and Huang, J.S. (1988) Rapid turnover of the platlet-derived growth factor receptor in cis-transformed cells and reversal by suramin. J. Biol. Chem. 263, 12608-12618.

Humphrey, P.A., Wong, A.J., Vogelstein, B., Zalutsky, M.R., Fuller, G.N., Archer, G.E., Friedman, H.S., Kwatra, M.M., Bigner, S.H. and Bigner, D.D. (1990) Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factors in human glioblastoma. Proc. Natl. Acad. Sci. (USA) 87, 4207-4211.

Humphries, M.J., Olden, K. and Yamada, K.M. (1986) A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science 233, 467-470.

Hynes, R.O. (1986) Fibronectins. Sci. Am. 254, 42-51.

Hynes, R.O. (1987) Integrins: A family of cell surface receptors. Cell 48, 549-554.

Hynes, R.O. (1992) Integrins: Versatility, modulation and signalling in cell adhesion. Cell 69, 11-25.

Iigo, M., Nishikata, K. and Hoshi, A. (1990) Effect of recombinant interleukin-1α, recombinant interleukin-2, recombinant interferon-β and recombinant tumor necrosis factor on subcutaneously implanted adenocarcinoma 755 and Lewis lung carcinoma. J. Biol. Response Mods. 9, 426-430.

Imanishi, K., Yamaguchi, K., Suzuki, M., Honda, S., Yanaihara, N. and Abe, K. (1989) Production of transforming growth factor-α in human tumour cell lines. Br. J. Cancer 59, 761-765.

Inayama, Y., Hook, G.E.R., Brody, A.R., Cameron, G.S., Jetten, A.M., Gilmore, L.B., Gray, T. and Nettesheim, P. (1988) The differentiation potential of tracheal basal cells. Lab. Invest. 58, (6), 706.

Inayama,Y., Hook, G.E., Brody, A.R., Jetten, A.M., Gray, T., Mahler, J. and Nettesheim, P. (1989) *In vitro* and *in vivo* growth and differentiation of clones of tracheal basal cells. Am. J. Pathol. 134, (3), 539.

Ishida, T., Kaneko, S., Tateishi, M., Oka, T., Mitsudomi, T., Sugimachi, K., Hara, N. and Ohta, M. (1990) Large cell carcinoma of the lung. Prognostic implications of histopathologic and immunohistochemical subtyping. Am. J. Clin. Pathol. 93, 176-182.

Ishiguro, Y., Kato, K., Ito, T. and Nagya, M. (1983) Determination of three enolase isozymes and S-100 protein in various tumours in children. Can. Res. 43, 6080.

Jackson, P. and Thompson, R.J. (1981) J. Neurol. Sci. 49, 429-438.

Jaques, G., Auerbach, B., Pritsch, M., Wolf, M., Madry, N. and Havemann, K. (1993) Evaluation of serum neural cell adhesion molecule as a new tumor marker in small cell lung cancer. Cancer 72, 418-425.

Jensen, P.B., Christensen, I.J., Schested, M., Hansen, H.H. and Vindelov, L. (1993) Differential cytotoxicity of 19 anticancer agents in wild-type and etoposide resistant small cell lung cancer cell lines. Br. J. Cancer 67, 311-320.

Jetten, A.M., Bernacki, S.H., Floyd, E.E., Saunders, N.A., Pieniazek, J. and Lotan, R. (1992) Expression of a preprorelaxin-like gene during squamous differentiation of rabbit tracheobronchial epithelial cells and its suppression by retinoic acid. Cell Growth Diff. 3, 549-556.

Jetten, A.M., George, M.A., Pettit, G.R. and Rearick, J.I. (1989) Effects of bryostatins and retinoic acid on phorbol ester- and diacylglycerol-induced squamous differentiation in human tracheobronchial epithelial cells. Can. Res. 49, 3990-3995.

Jetten, A.M. and Shirley, J.E. (1986) Characterisation of transglutaminase activity in rabbit tracheal epithelial cells. J. Biol. Chem. 261 (32), 15097-15101.

Jetten, A.M., Shirley, J.E. and Stoner, G. (1986) Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGFβ. Exp. Cell Res. 167, 539-549.

Johnson, N.F. and Hubbs, A.F. (1990) Epithelial progenitor cells in the rat trachea. Am. J. Respir. Cell Mol. Biol., 3, 579-585.

Johnson, N.F., Wilson, J.S., Habbersett, R., Thomassen, D.G., Shopp, G.M. and Smith, D.M. (1990) Separation and characterisation of basal secretory cells from the rat trachea by flow cytometry. Cytometry 11, 395-405.

Johnsson, A., Betsholtz, C., Heldin, C-H. and Westermark, B. (1985) Antibodies against platelet-derived growth factor inhibit transformation by simian sarcoma virus. Nature 317, 438-440.

Kasselberg, A.G., Orth, D.N., Gray, M.E. and Stahlmann, M.T. (1985) Immunocytochemical localisation of human epidermal growth factor/urogastrone in several human tissues. J. Histochem. Cytochem. 33, 315-322.

Kato, K., Ishiguro, Y., Suzuki, F., Ito, A. and Semba, R. (1982) Distribution of nervous system-specific forms of enolase in peripheral tissues. Brain Res. 237, 441.

Kauffman, S.L. (1980) Cell proliferation in the mammalian lung. Int. Rev. Exp. Pathol. 22, 132-191.

Kayser, K., Schmid, W., Ebert, W., Wiedenmann, B. (1988) Expression of neuroendocrine markers (neuronspecific enolase, synaptophysin and bombesin) in carcinoma of the lung. Path. Res. Pract. 183, 412-417.

Keenan, K.P., Combs, J.W. and McDowell, E.M. (1982) Regeneration of hamster tracheal epithelium after mechanical injury. III. Large and small lesions: Comparitive stathmokinetic and single pulse and continuous thymidine labeling autoradiographic studies. Virchows Arch. [Cell Pathol.] 41, 231-252.

Keizer, H.G., Schuurhuis, G.J., Broxterman, H.J., Lankelma, J., Schoonen, W.G.E.J., van Rijn, J., Pinedo, H.W. and Joenje, H. (1989) Correlation of multidrug resistance with decreased drug accumulation, altered subcellular drug distribution and increased P-glycoprotein expression in cultured SW-1573 human lung tumor cells. Can. Res. 49, 2988-2993.

Kelly, K., Kane, M.A. and Bunn, P.A.Jr. (1991) Growth factors in lung cancer: Possible etiologic role and clinical target. Med. Ped. Oncol. 19, 449-458.

Kemler, R. (1993) From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet. 9, 317-321.

Kemler, R., Ozawa, M. and Ringwald, M. (1989) Calcium-dependent cell adhesion molecules. Curr.Opin.Cell Biol. 1, 892-897.

Keoffler, H.P., Yen, J. and Carlson, J. (1983) The study of human myeloid differentiation using bromodeoxyuridine (BrdU). J. Cell. Physiol. 116, 111-117.

Khalil, N., O,Connor, R.N., Flanders, K.C., Shing, W. and Whitman, C.I. (1994) Regulation of type II alveolar epithelial cell proliferation by TGF-β during bleomycin-induced lung injury in rats. Am. J. Physiol. 267, L498-L507.

Khan, M.Z., Freshney, R.I., McNicol, A.M. and Murray, A.M.B. (1993) Induction of phenotypic changes in SCLC cell lines *in vitro* by hexamethylene bisacetamide, sodium butyrate and cyclic AMP. Ann. Oncol. 4, 499-507.

Khan, M.Z., Freshney, R.I., Murray, A.M.B., Merry, S., Plumb, J.A. and McNicol, A.M. (1991) Identification and characterisation *in vitro* of cells with a non-SCLC cell-like phenotype derived from a continuous SCLC cell line. Anticancer Res. 11, 1687-1696.

Kidson, S.H. and DeHaan, J.B. (1990) Effect of thymidine analogs in tyrosinase activity and mRNA accumulation in mouse melanoma cells. Exp. Cell Res. 188, 36-41.

Kiefer, P.E., Bepler, G., Kubasch, M. and Havemann, K. (1987) Amplification and expression of protooncogenes in human small cell lung cancer cell lines. Can. Res. 47, 6236-6242.

Kiehntopf, M., Brach, M.A., Licht, T., Petschauer, S., Karawajew, L., Kirschning, C. and Herrmann, F. (1994) Ribozyme-mediated cleavage of the *MDR*-1 transcript restores chemosensitivity in previously resistant cancer cells. EMBO J. 13 (19), 4645-4652.

Kim, K.H., Schwartz, F. and Fuchs, E. (1984) Differences in keratin synthesis between normal epithelial cells and squamous carcinomas are mediated by vitamin A. Proc. Natl. Acad. Sci. (USA) 81, 4280-4284.

Kim, K.H., Stellmach, V., Javors, J. and Fuchs, E. (1987) Regulation of human mesothelial cell diferentiation: Opposing roles of retinoids and epidermal growth factor in the expression of intermediate filament proteins. J. Cell Biol. 105, 3039-3051.

Kobayashi, H., Dorai, T., Holland, J.F. and Ohnuma, T. (1994) Reversal of drug sensitivity in multidrug-resistant tumour cells by an *MDR*1 (*PGY*1) ribozyme. Can. Res. 54, 1271-1275.

Komminoth, P., Roth, J., Lackie, P.M., Bitter-Suermann, D. and Heitz, P.U. (1991) Polysialic acid of the neural cell adhesion molecule distinguishes small cell lung carcinoma from carcinoids. Am. J. Pathol. 139 (2), 297-304.

Kopan, R. and Fuchs, E. (1989) The use of retinoic acid to probe the relation between hyperproliferation-associated keratins and cell proliferation in normal and malignant epidermal cells. J. Cell Biol. 109, 295-307.

Kreyberg, L. (1967) Histological typing of lung tumours. International histological classification of tumours, no.1. Geneva, World Health Organisation.

Kruger, K., Grabowski, P.J., Zuag, A.G., Sands, J., Gottschling, D.E. and Cech, T.R. (1982) Self-splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. Cell 31, 147-157.

Krust, A., Kastner, P.H., Petkovich, M., Zelent, A. and Chambon, P. (1989) A third human retinoic acid receptor, hRAR-γ. Proc. Natl. Acad. Sci. (USA) 86, 5310-5314.

Kuhn, C.,III and Callaway, L.A. (1975) The formation of granules in the bronchiolar Clara cells of the rat. II. Enzyme cytochemistry. J. Ultrastruct. Res. 53, 66-76.

Lan, M.S., Russell, E.K., Lu, J., Johnson, B.E. and Notkins, A.L. (1993) 1A-1, a new marker for neuroendocrine differentiation in human lung cancer cell lines. Can. Res. 53, 4169-4171.

Lauweryns, J.M. and Cokelaere, M. (1973) Hypoxia-sensitive neuro-epithelial bodies. Intrapulmonary secretory neuroreceptors, modulated by the CNS. Z. Zellforsch. 145, 521-540.

Lauweryns, J.M., Cokelaere, M. and Theunynck, P. (1973) Serotonin producing neuroepithelial bodies in rabbit respiratory mucosa. Sci. 180, 410-413.

Law, E., Gilvarry, U., Lynch, V., Gregory, B., Grant, G. and Clynes, M. (1992) Cytogenetic comparison of two poorly differentiated human lung squamous cell carcinoma lines. Can. Genet. Cytogenet. 59, 111-118.

Lechner, J., Haugen, A., McClendon, I. and Pettis, E. (1982) Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. In Vitro 18, 633-642.

Lehto, V., Miettinen, M. and Virtanen, I. (1985) A dual expression of cytokeratin and neurofilaments in bronchial carcinoid cells. Int. J. Cancer 35, 421-425.

Lentz, T.L. (1971) Fine structure. Saunders, Philadelphia, pp.100-101.

Lequaglie, C., Patriarca, C., Cataldo, I., Muscolino, G., Preda, F. and Ravasi, G. (1991) Prognosis of resected well-differentiated neuroendocrine carcinoma of the lung. Chest 100, 1053-1056.

Levin, N.A., Brzoska, P., Gupta, N., Minna, J.D., Gray, J.W. and Christman, M.F. (1994) Identification of frequent novel genetic alterations in small cell lung carcinoma. Can. Res. 54, 5086-5091.

Lin, J.Y., Nettesheim, P. and Randell, S.H. (1994) Growth and differentiation of tracheal epithelial progenitor cells. Am. J. Physiol. 266, L296.

Lin, S. and Riggs, A.D. (1972) Lac operator analogues: Bromodeoxyuridine substitution in the lac operator affects the rate of dissociation of the lac repressor. Proc. Natl. Acad. Sci. (USA) 69, 2574-2576.

Linnoila, R.I. (1982) Effects of diethylnitrosamine on lung neuroendocrine cells. Exp. Lung Res. 3, 225-236.

Linnoila, R.I., Jensen, S., Steinberg, S., Minna, J., Gazdar, A.F. and Mulshine, J. (1988b) Neuroendocrine differentiation correlates with favourable response to chemotherapy in patients with non-small cell lung cancers. (Abstract) Lung Cancer 4, 33.

Linnoila, R.I., Jensen, S.M., Steinberger, S.M., Mulshine, J.L., Eggleston, J.C. and Gazdar, A.F. (1992) Peripheral airway cell marker expression in non-small cell lung carcinoma. Association with distinct clinicopathologic features. Am. J. Clin. Pathol. 97, 233-243.

Linnoila, R.I., Mulshine, J., Steinberg, S., Funa, K., Matthews, M.J., Cotelingam, J. and Gazdar, A.F. (1988a) Neuroendocrine differentiation in endocrine and nonendocrine lung carcinomas. Am. J. Clin. Pathol. 90, 641-652.

Liotta, L.A., Steeg, P.S. and Stetler-Stevenson, W.G. (1991) Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64, 327-336.

Little, C.D., Nau, M.M., Carney, D.N., Gazdar, A.F. and Minna, J.D. (1983) Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306, 194-196.

Liu, J.Y., Nettesheim, P. and Randell, S.H. (1994) Growth and differentiation of tracheal epithelial progenitor cells. Am. J. Physiol. 266, L296-L307.

Long, S.H., Way, L., Lorico, A., Wang, R.C.C., Brattain, M.G. and Casazza, A.M. (1991) Mechanisms of resistance to etoposide and tenoposide in acquired resistant colon and lung carcinoma cell lines. Can. Res. 51, 5275-5284.

Lotan, R., Pieniazek, J., George, M.D. and Jetten, A.M. (1992) Identification of a new squamous cell differentiation marker and its suppression by retinoids. J. Cell. Physiol. 151, 94-102.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D. and Schlessinger, J. (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to *ras* signalling. Cell 70, 431-442.

Luna, E.J., Wuestehube, L.J., Ingalls, H.M. and Chia, C.P. (1989) The *Dictyostelium discoideum* plasma membrane: A model system for the study of actin-membrane interactions. Adv. Cell Biol. 3, 1-33.

Mabry, M., Nakagawa, T., Nelkin, B.D., McDowell, E., Gelesi, M., Eggleston, R.A., Casero, R.A.Jr. and Baylin, S.B. (1988) v-Ha-*ras* oncogene insertion: A model for tumor progression of human small cell lung cancer. Proc. Natl. Acad. Sci. (USA) 85, 6523-6527.

Mabry, M., Nelkin, B.D., Falco, J.P., Barr, L.F. and Baylin, S.B. (1991) Transitions between lung cancer phenotypes - implications for tumor progression. Cancer Cells 3, 53-58.

Magnan, A., Frachon, I., Rain, B., Peuchmaur, M., Monti, G., Lenot, B., Fattal, M., Simonneau, G., Galanaud, P. and Emilie, D. (1994) Transforming growth factor β in normal human lung: Preferential location in bronchial epithelial cells. Thorax 49, 789-792.

Mahmoud, S., Staley, J., Taylor, J., Bogden, A., Moreau, J-P., Coy, D., Avis, I., Cuttitta, F., Mulshine, J.L. and Moody, T.W. (1991) [Psi^{13,14}]bombesin analogues inhibit growth of small cell lung cancer *in vitro* and *in vivo*. Can. Res. 51, 1798-1802.

Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 345, 224-229.

Marquardt, H., Hunkapillar, M.W., Hood, L.E. and Todaro, G.J. (1984) Rat transforming growth factor type I: Structure and relation to epidermal growth factor. Science 223, 1079-1082.

Massaro, G.D., Paris, M. and Thet, L.A. (1979) *In vivo* regulation of secretion of bronchiolar Clara cells in rats. J. Clin. Invest. 63, 167-172.

Masui, T., Wakefield, L.M., Lechner, J.F., LaVeck, M.A., Sporn, M.B. and Harris, C.C. (1986) Type β transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. Proc. Natl. Acad. Sci. (USA) 83, 2438-2442.

McCormick, C., Freshney, R.I. and Speirs, V. (1995) Activity of interferon α, interleukin 6 and insulin in the regulation of differentiation in A549 alveolar carcinoma cells. Br. J. Cancer 71, 232-239.

McDowell, E.M. and Beals, T.F. (1986) The bronchial airways. In: Biopsy pathology of the bronchi. Chapman and Hall, London, pp.49-76.

McDowell, E.M., Keenan, K.P. and Huang, M. (1984) Effects of vitamin A-deprivation on hamster tracheal epithelium. Virchows Arch. [Cell Pathol.] 45, 197-219.

McDowell, E.M., Newkirk, C. and Coleman, B. (1985) Development of hamster tracheal epithelium: 1. A quantitative morphologic study in the fetus. Anat. Rec. 213, 429-447.

McDowell, E.M., Wilson, T.S. and Trump, B.S. (1981) Atypical endocrine tumours of the lung. Arch. Pathol. Lab. Med. 105, 20-28.

Mechtersheimer, G., Staudter, M. and Moller, P. (1991) Expression of the natural killer cell-associated antigens CD56 and CD57 in human neural and striated muscle cells and in their tumors. Can. Res. 51, 1300-1307.

Meng-er, H., Yurchen, Y., Shu-rong, C., Jin-ren, C., Jia-Xiang, L., Long-jun, G. and Zhen-yi, W. (1988) Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. Blood 27, 567-572.

Metcalf, D. (1989) The molecular control of cell division, differentiation committment and maturation in haematopoietic cells. Nature 339, 27-30.

Mette, S.A., Pilewski, J., Buck, C.A. and Albelda, S.M. (1993) Distribution of integrin cell adhesion receptors on normal bronchial epithelial cells and lung cancer cells *in vitro* and *in vivo*. Am. J. Respir. Cell Mol. Biol. 8, 562-572.

Metzler, M.D. and Snyder, J.M. (1993) Retinoic acid differentially regulates expression of surfactant-associated proteins in human fetal lung. Endocrinology 133, 1990-1998.

Meyers, M.B., Merluzzi, V.J., Spengler, B.A. and Biedler, J.L. (1986) Epidermal growth factor is increased in multidrug resistant Chinese hamster and mouse tumor cells. Proc. Natl. Acad. Sci. (USA) 83, 5521-5525.

Meyrick, B., Sturgess, J. and Reid, L. (1969) Reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland. Thorax 24, 729-736.

Mickley, L.A., Bates, S.E., Richert, N.D., Currier, S., Tanakas, S., Foss, F., Rosen, N. and Fojo, A.T. (1989) Modulation of the expression of a multidrug resistance gene (*mdr*-1/P-glycoprotein) by differentiating agents. J. Biol. Chem. 264 (30), 18031-18040.

Miller, E.J. and Gay, S. The collagens: An overview and update. In: Cunningham, L.W. (ed) Structural and contractile proteins: Extracellular matrix. New York, Academic Press (1987) 144, pp.3-41.

Minna, J.D., Pass, H., Glastein, E.J. and Ihde, D.E. Cancer of the lung. In: De Vita, V.T., Hellman, S. and Rosenberg, S. (eds) Principles and practice of oncology. Philadelphia: J.B. Lippincott (1989) pp.591-705.

Mirski, S.E.L., Gerlach, J.H. and Cole, S.P.C. (1987) Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. Can. Res. 47, 2594-2598.

Miyake, Y., Kodama, T. and Yamaguchi, K. (1994) Pro-gastrin-releasing peptide (31-98) is a specific tumor marker in patients with small cell lung cancer. Can. Res. 54, 2136-2140.

Miyaki, M., Sato, C., Matsui, T., Koike, M., Mori, T., Kosaki, G., Takai, S., Tonomura, A. and Tsuchida, N. (1985) Amplification and enhanced expression of cellular oncogene c-Ki-*ras*-2 in a human epidermoid carcinoma of the lung. Gann 76, 260-265.

Moll, R., Franke, W.W. and Schiller, D.L. (1982) The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 31, 11-24.

Montefort, S., Holgate, S.T. and Howarth, P.H. (1993) Leucocyte-endothelial adhesion molecules and their role in bronchial asthma and allergic rhinitis. Eur. Respir. J. 6, 1044-1054.

Mooi, W.J., Dingemans, K.P., Wagenaar, S., Hart, A.A.M. and Wagenvoort, C.A. (1990) Ultrastructural heterogeneity of lung carcinomas: Representativity of samples for electron microscopy in tumor classification. Hum. Pathol. 21, 1227-1234.

Morstyn, P.C., Brown, J., Novak, U., Gardner, J., Bishop, J. and Garson, M. (1987) Heterogenous cytogenetic abnormalities in small cell lung cancer cell lines. Can. Res. 47, 3322-3327.

Moses, H.L., Yang, E.Y. and Pietenpol, J.A. (1990) TGF-beta stimulation and inhibition of cell proliferation: New mechanistic insights. Cell 63, 245-247.

Moss, F., Bobrow, L., Happerfield, L., Souhami, R. and Beverley, P. (1991) Differential expression of antigens by human small cell lung carcinoma in sections of tumour and in serous effusions. Br. J. Cancer 63 Suppl.XIV, 54-55.

Mould, A.P., Wheldon, L.A., Komoriya, A., Wayner, E.A., Yamada, K.M. and Humphries, M.J. (1990) Affinity chromatographic isolation of the melanoma adhesion receptor for the 111CS region of fibronectin and its identification as the integrin $\alpha_4\beta_1$. J. Biol. Chem. 265, 4020-4024.

Mulcahy, L.S., Smith, M.R. and Stacey, D.W. (1985) Requirement for *ras* protooncogene function during serum-stimulated growth of NIH 3T3 cells. Nature 313, 241-243.

Mule, J.J., Custer, M.C., Travis, W.D. and Rosenberg, S.A. (1992) Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice. J. Immunol. 148, 2622-2629.

Mulshine, J.L., Cuttitta, F., Bibro, M., Fedorko, J., Fargion, S., Little, C., Carney, D.N., Gazdar, A.F. and Minna, J.D. (1983) Monoclonal antibodies that distinguish non-small cell from small cell lung cancer. J. Immunol. 131, 497-502.

Nakane, T., Szentendrei, T., Stern, L., Virmani, M., Seely, J. and Kunos, G. (1990) Effects of IL-1 and cortisol on β-adrenergic receptors, cell proliferation and differentiation in cultured human A549 lung tumor cells. J. Immunol. 145, 260-266.

Nakano, H., Yamamoto, F., Neville, C., Evans, D., Mizuno, T. and Perucho, M. (1984) Isolation of transforming sequences of two human lung carcinomas: Structural and functional analysis of the activated c-K-*ras* oncogenes. Proc. Natl. Acad. Sci. (USA) 81, 71-75.

Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F. and Minna, J.D. (1985) L-*myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. Nature 318, 69-73.

Neal, M.H., Kosinski, R., Cohen P. and Ohrenstein, J.M. (1986) Atypical endocrine tumours of the lung. A clinicopathologic study of 19 cases. Hum. Pathol. 17, 1264-1277.

Newman, C., Wang, D. and Cutz, E. Serotonin (5-hydroxytryptamine) expression in pulmonary neuroendocrine cell (NE) and a NE tumor cell line. In: Data P.G. (ed) Neurobiology and cell physiology of chemoreception. Plenum Press, New York (1993) pp.73-78.

Nicolson, G.L. (1987) Tumor cell instability, diversification, and progression to the metastatic phenotype: From oncogene to oncofetal expression. Can. Res. 47, 1437-1487.

Nigam, A.K., Savage, F.J., Boulos, P.B., Stamp, G.W.H., Liu, D. and Pignatelli, M. (1993) Loss of cell-cell and cell-matrix adhesion molecules in colorectal cancer. Br. J. Cancer 68, 507-514.

Norgaard, P., Damstrup, L., Rygaard, K., Spang-Thomsen, M. and Skovgaard Poulsen, H. (1994) Growth suppression by transforming growth factor β_1 of human small-cell lung cancer cell lines is associated with expression of the type II receptor. Br. J. Cancer 69, 802-808.

Nowell, P.C. (1986) Mechanisms of tumor progression. Can. Res. 46, 2203-2207.

O'Connor, D.T., Burton, D. and Deftos, L.J. (1983) Immunoreactive human chromogranin A in diverse polypeptide hormone producing tumors and normal endocrine tissues. J. Clin. Endocrinol. Metab. 57 (4), 1084-1088.

Ochiai, A. (1992) Differentiation of Clara cell (distal type) antigen in human fetal bronchial epithelial cell line (HFBE). Exp. Toxic. Pathol. 44, 223-234.

Odelstad, L., Pahlman, S., Nilsson, K., Larsson, E., Lackgren, G., Johansson, K-E., Hjerten, S. and Grotte, G. (1981) Neuron-specific enolase in relation to differentiation in human neuroblastoma. Brain Res. 224, 69-82.

Olsson, L., Sorenson, H.R. and Behnke, O. (1984) Intratumoral phenotypic diversity of cloned human lung tumor cell lines and consequences for analyses with monoclonal antibodies. Cancer 54, 1757-1765.

Osheroff, N., Corbett, A.H. and Robinson, M.J. (1994) Mechanism of action of topoisomerase II-targeted antineoplastic drugs. Adv. Pharmacol. 29B, 105-126.

Owens, A.H., Coffey, D.S. and Baylin, S.B. (eds) Tumour cell heterogeneity: Origin and implications. New York: Academic Press (1982).

Ozanne, B., Richards, C.S., Hendler, F., Burns, D. and Gustersen, B. (1986) Overexpression of the EGF receptor is a hallmark of squamous cell carcinomas. J. Pathol. 149, 9-14.

Pahlman, S., Esscher, T. and Nilsson, K. (1986) Expression of γ-subunit of enolase, neuron-specific enolase, in human non-neuroendocrine tumours and derived cell lines. Lab. Invest. 54 (5), 554-560.

Pearse, A.G. (1969) The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. J. Histochem. Cytochem. 17, 303-313.

Pearse, A.G. (1974) The APUD concept and its implications in pathology. Pathol. Ann. 9, 27-41.

Pellegrini, R., Martignone, S., Menard, S. and Colnaghi, M.I. (1994) Laminin receptor expression and function in small-cell lung carcinoma. Int. J. Cancer (Suppl.) 8, 116-120.

Pelligrini, S. and Schindler, C. (1993) Early events in signalling by interferons. TIBS 18, 338-342.

Pelton, R.W. and Moses, H.L. (1990) The beta-type transforming growth factors. Mediators of cell regulation in the lung. Am. Rev. Respir. Dis. 142, s31-s35.

Pence, J.C. and Shorter, N.A. (1990) *In vitro* differentiation of human neuroblastoma cells caused by vasoactive intestinal peptide. Can. Res. 50, 5177-5183.

Penney, D.P. (1988) The ultrastructure of epithelial cells of the distal lung. Int. Rev. Cytol. 11, 231-269.

Peters, K.G., Werner, S., Chen, G. and Williams, L.T. (1992) Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. Development 114, 233-243.

Petkovich, M., Brand, N.J., Krust, A. and Chambon, P. (1987) A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330, 444.

Pfiefer, A.M., Jones, R.T., Bowden, P.E., Mann, D., Spillare, E., Klein-Szanto, A.J., Trump, B.F. and Harris, C.C. (1991) Human bronchial epithelial cells transformed by the c-*raf*-1 and c-*myc* protooncogenes induce multidifferentiated carcinomas in nude mice: a model for lung carcinogenesis. Can. Res. 51, 3793-3801.

Piez, K.A. Molecular and aggregate structures of the collagens. In: Piez, K.A. and Reddi, A.H. (eds) Extracellular matrix biochemistry. Amsterdam, Elsevier (1984) pp.1-30.

Plopper, C.G., Alley, J.L., Weir, A.J. (1986) Differentiation of tracheal epithelium during fetal lung maturation in the Rhesus monkey *Macaca mulatta*. Am. J. Anat. 175, 59-71.

Plopper, C.G., Mariassy, A.T. and Hill, L.H. (1980) Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung. 1. A comparison of rabbit, guinea pig, rat, hamster and mouse. Exp. Lung Res. 1, 139-154.

Plummer III, H., Catlett, J., Leftwich, J., Armstrong, B., Carlson, P., Huff, T. and Krystal, G. (1993) c-*myc* expression correlates with suppression of c-*kit* protooncogene expression in small cell lung cancer cell lines. Can. Res. 53, 4337-4342.

Poole, A.R. (1986) Proteoglycans in health and disease: Structures and functions. Biochem. J. 236, 1-14.

Porgador, A., Tzehoval, E., Katz, A., Vadai, E., Revel, M., Feldman, M. and Eisenbach, L. (1992) Interleukin 6 gene transfection into Lewis lung carcinoma tumor cells suppresses the malignant phenotype and confers immunotherapeutic competence against parental metestatic cells. Can. Res. 52, 3679-3686.

Pujol. J.L., Grenier, J., Daures, J.P., Daver, A., Pujol, H. and Michel, F.B. (1993) Serum fragment of cytokeratin subunit 19 measured by CYFRA 21-1 immunoradiometric assay as a marker of lung cancer. Can. Res. 53, 61-66.

Rapp, U.R., Huleihel, M., Pawson, T., Linoilla, I., Minna, J.D., Heidecker, G., Cleveland, J.L., Beck, T., Forchhammer, J. and Storm, S.M. (1988) Role of *raf* oncogenes in lung carcinogenesis. Lung Cancer 4, 162-167.

Rearick, J.I., Albro, P.W. and Jetten, A.M. (1987b) Increase in cholesterol sulfotransferase activity during *in vitro* squamous differentiation of rabbit tracheal epithelial cells and its inhibition by retinoic acid. J. Biol. Chem. 262 (27), 13069-13074.

Rearick, J.I., Hesterberg, T.W. and Jetten, A.M. (1987a) Human bronchial epithelial cells synthesise cholesterol sulfate during squamous differentiation *in vitro*. J. Cell. Physiol. 133, 573-578.

Rearick, J.I. and Jetten, A.M. (1986) Accumulation of cholesterol 3-sulfate during *in vitro* squamous differentiation of rabbit tracheal epithelial cells and its regulation by retinoids. J. Biol. Chem. 261 (30), 13898-13904.

Rehm, S., Devor, D.E., Henneman, J.R. and Ward, J.M. (1991a) Origin of spontaneous and transplacentally induced mouse lung tumours from alveolar type II cells. Exp. Lung Res. 17, 181-195.

Rehm, S., Lijinsky, W., Singh, G. and Katyal, S.L. (1991b) Mouse bronchial cell carcinogenesis: Histologic characterization and expression of Clara cell antigen in lesions induced by N-nitrosobis-(2-chloroethyl) ureas. Am. J. Pathol. 139, 413.

Rehm, S., Ward, J.M, Ten Have-Opbroek, A.A.W., Anderson, L.M., Singh, G., Katyal, S.L. and Rice, J.M. (1988) Mouse papillary lung tumours transplacentally induced by N-nitrosoethylurea: Evidence for alveolar type II cell origin by comparative light microscopic, ultrastructural and immunohistochemical studies. Can. Res. 48, 148-160.

Reubi, J.C., Waser, B., Sheppard, M. and Macauley, V. (1990) Somatostatin receptors are present in small-cell but not in non-small-cell primary lung carcinomas: Relationship to EGF-receptors. Int. J. Cancer 45, 269-274.

Rickard, K.A., Taylor, J., Spurzen, J.R. and Rennard, S.I. (1992) Extracellular matrix and bronchial epithelial cell migration. Chest 101, (3) (Suppl.) 18s.

Roberts, A.B. and Sporn, M.B. (1988) Transforming growth factor beta. Adv. Cancer Res. 51, 107-145.

Robinson, S.P. and Rose, W.C. (1992) Transforming growth factor β_1: Lack of *in vivo* antitumor activity on A549 and Wehi 3BD+ tumors. Anticancer Res. 12, 73-80.

Rodenhuis, S. and Slebos, R.J.C. (1992) Clinical significance of *ras* oncogene activation in human lung cancer. Can. Res. (Suppl.), 2665s-2669s.

Rodenhuis, S., van de Wetering, M.L., Mooi, W.J., Evers, S.G., van Zandwijk, N. and Bos, J.L. (1987) Mutational activation of the K-*ras* oncogene: A possible pathogenic factor in adenocarcinoma of the lung. N. Engl. J. Med. 317, 929-936.

Rodriguez-Boulan, E. and Nelson, W.J. (1989) Morphogenesis of the polarized epithelial cell phenotype. Science 245, 718-725.

Ross, R.A., Bossart, E., Spengler, B.A. and Biedler, J.L. Multipotent capacity of morphologically intermediate (I-type) human neuroblastoma cells after treatment with differentiation-inducing drugs. In: Advances in neuroblastoma research 3, Wiley-Liss, Inc. (1991) pp.193-201.

Ross, R.A., Spengler, B.A. and Biedler, J.L. (1983) Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J. Natl. Can. Inst. 71, 741-747.

Ross, R.A., Spengler, B.A., Rettig, W.J. and Biedler, J.L. Differentiation-inducing agents stably convert human neuroblastoma I-type cells to neuroblastic (N) or nonneuronal (S) neural crest cells. In: Advances in neuroblastoma research 4, Wiley-Liss, Inc. (1994) pp.253-259.

Rozengurt, E. (1986) Early signals in the mitogenic response. Science 234, 161-166.

Ruckdeschel, J.C., Linnoila, R.I., Mulshine, J.L., Kim, K., Aisner, S., Gazdar, A.F., Jensen, S.M. and Bonomi, P. (1991) The impact of neuroendocrine (NE) and epithelial (EPI) differentiation on response and survival in lung cancer: The ECOG experience. Proc. ASCO 10, 248.

Ruoslahti, E. Integrins as receptors for extracellular matrix. In: Hay, E.D. (ed) Cell biology of extracellular matrix. 2nd ed. New York, Plenum Press (1991) pp.343-363.

Ruoslahti, E. and Pierschbacher, M.D. (1987) New perspectives in cell adhesion: RGD and integrins. Science 238, 491-497.

Rutter, W.J., Pictet, R.L. and Morris, P.W. (1973) Towards molecular mechanisms of developmental processes. Annu. Rev. Biochem. 42, 601-646.

Safi, A., Sadmi, M., Martinet, N., Menard, O., Vaillant, P., Gallati, H., Hosang, M. and Martinet, Y. (1992) Presence of elevated levels of platelet-derived growth factor (PDGF) in lung adenocarcinoma pleural effusions. Chest 102, 204-207.

Sakiyama, S., Nakamura, Y. and Yasuda, S. (1986) Expression of epidermal growth factor receptor gene in cultured human lung cancer cells. Jpn. J. Can. Res. 77, 965-969.

Saksela, K., Bergh, J. and Nilsson, K. (1986) Amplification of the N-*myc* oncogene in adenocarcinoma of the lung. J. Cell. Biochem. 31, 9.

Sariban, E., Sitaras, N.M., Antoniades, H.N., Kufe, D.W. and Pantazis, P. (1988) Expression of platelet-derived growth factor (PDGF)-related transcripts and synthesis of biologically active PDGF-like proteins by human malignant epithelial cell lines. Am. Soc. Clin. Invest. 82, 1157-1164.

Sato, S., Nakamura, Y. and Tsuchiya, E. (1994) Difference of allelotype between squamous cell carcinoma and adenocarcinoma of the lung. Can. Res. 54, 5652-5655.

Sawyers, C.L., Denny, C.T. and Witte, O.N. (1991) Leukemia and the disruption of normal hematopoiesis. Cell 64, 337-350.

Schaafsma, H.E. and Ramaekers, F.C.S. (1994) Cytokeratin subtyping in normal and neoplastic epithelium: Basic principles and diagnostic applications. Pathol. Annu. (USA) 29, 21-62.

Schol, D.J., Mooi, W.J., van der Gugten, A.A., Wagenaar, S.S. and Hilgers, J. (1988) Monoclonal antibody 123C3, identifying small-cell-carcinoma phenotype in lung tumours, recognising mainly, but not exclusively, endocrine and neuron-supporting normal tissues. Int. J. Cancer, 2 (Suppl.), 34-40.

Schraml, P., Shipman, R., Colombi, M. and Ludwig, C.U. (1994) Identification of genes differentially expressed in normal lung and non-small cell lung carcinoma tissue. Can. Res. 54, 5236-5240.

Schuger, L., Dixit, V.M., Carey, T.E. and Varani, J. (1990) Modulation of squamous carcinoma cell growth, morphology, adhesiveness and extracellular matrix production by interferon-γ and tumor necrosis factor-α. Pathobiology 58, 279-286.

Schwartz, M.A., Lechene, C. and Ingber, D.E. (1991) Insoluble fibronectin activates the Na/H antiport by clustering and immobilising integrin $\alpha_5\beta_1$, independent of cell shape. Proc. Natl. Acad. Sci. (USA) 88, 7849-7853.

Schweizer, J., Furstenberger, G. and Winter, H. (1987) Selective suppression of two postnatally acquired 70kDa and 65kDa keratin proteins during continuous treatment of adult mouse tail epidermis with vitamin A. J. Invest. Dermatol. 89, 125-131.

Scott, R.E., Hoerl, B.J., Wille, J.J.Jr., Florine, D.L., Krawisz, B.R. and Yun, K. (1982) Coupling of proadipocyte growth arrest and differentiation. II. A cell cycle model for the physiological control of cell proliferation. J. Cell Biol. 94, 400-405.

Sell, S. and Pierce, G.B. (1994) Biology of disease: Maturation arrest of stem cell differentiation is a common pathway for the cellular origin of teratocarcinomas and epithelial cancer. Lab. Invest. 70 (1), 6-22.

Sethi, T., Langdon, S., Smyth, J. and Rozengurt, E. (1992) Growth of small cell lung cancer cells: Stimulation by multiple neuropeptides and inhibition by broad spectrum antagonists *in vitro* and *in vivo*. Can. Res. (Suppl.) 52, 2737s-2742s.

Sethi, T. and Rozengurt, E. (1991) Multiple neuropeptides stimulate clonal growth of small cell lung cancer: Effects of bradykinin, vasopressin, cholecystokinin, galanin and neurotensin. Can. Res. 51, 3621-3623.

Shattil, S.J. and Brugge, J.S. (1991) Protein tyrosine phosphorylation and the adhesive functions of platelets. Curr. Opin. Cell Biol. 3, 869-879.

Shaw, G.L., Gazdar, A.F., Phelps, R., Linnoila, R.I., Ihde, D.C., Johnson, B.E., Oie, H.K., Pass, H.I., Steinberg, S.M., Ghosh, B.C., Walsh, T.E., Nesbitt, J.C., Cotelingam, J.D., Minna, J.D. and Mulshine J.L. (1993) Individualized chemotherapy for patients with non-small cell lung cancer determined by prospective identification of neuroendocrine markers and *in vitro* drug sensitivity testing. Can. Res. 53, 5181-5187.

Shaw, J.P., Akiyoshi, D.E., Arrigo, D.A., Rhoad, A.E., Sullivan, B., Thomas, J., Genbauffe, F.S., Bacha, P. and Nichols, J.C. (1991) Cytotoxic properties of $DAB_{486}EGF$ and $DAB_{389}EGF$, epidermal growth factor (EGF) receptor-targeted fusion toxins. J. Biol. Chem. 266 (31), 21118-21124.

Sheppard, M.N., Marangos, P.J., Bloom, S.R. and Polak, J.M. (1984) Neuron-specific enolase: A marker for the early development of nerves and endocrine cells in the human lung. Life Sci. 34, 265-271.

Shimosegawa, T. and Said, S.I. (1991) Co-occurrence of immunoreactive calcitonin and calcitonin gene-related peptide in neuroendocrine cells of rat lungs. Cell Tiss. Res. 264, 555-561.

Shiseki, M., Kohno, T., Nishikawa, R., Sameshima, Y., Mizoguchi, H. and Yokota, J. (1994) Frequent allelic losses on chromosome 2q, 18q and 22q in advanced non-small cell lung carcinoma. Can. Res. 54, 5643-5648.

Siegfried, J.M., Han, Y-H., DeMichele, M.A., Hunt, J.D., Gaither, A.L. and Cuttitta, F. (1994) Production of gastrin-releasing peptide by a non-small cell lung carcinoma cell line adapted to serum-free and growth factor-free conditions. J. Biol. Chem. 269 (11), 8596-8603.

Silverberg, E. and Lubera, J.A. (1988) Cancer statistics. Cancer 38, 5-22.

Slebos, R.J.C., Evers, S.G., Wagenaar, S.S. and Rodenhuis, S. (1989) Cellular protooncogenes are infrequently amplified in untreated non-small cell lung carcinoma. Br J. Cancer 57, 76-80.

Smith, L.J. and Brody, J.S (1981) Influence of methylprednisolone on mouse alveolar type 2 cell response to acute lung injury. Am. Rev. Respir. Dis. 123, 459-464.

Soderdahl, G., Betsholtz, C., Johansson, A., Nilsson, A. and Bergh, J. (1988) Differential expression of platelet-derived growth factor and transforming growth factor genes in small- and non-small cell human lung carcinoma lines. Int. J. Cancer 41, 636-641.

Spang-Thomsen, M., Clerici, M., Engelholm, S.A. and Vindelov, L.L. (1986) Growth kinetics and *in vivo* radiosensitivity in nude mice of two subpopulations derived from a single human small cell carcinoma of the lung. Eur. J. Can. Clin. Oncol. 22, 549-556.

Sparks, R.L. and Scott, R.E. (1986) Transforming growth factor type β is a specific inhibitor of 3T3 T mesenchymal stem cell differentiation. Exp. Cell Res. 165, 345-352.

Spencer, H. (1961) Pulmonary blastomas. J. Pathol. Bacteriol. 82, 161-165.

Spindel, E.R., Sunday, M.E., Hofler, H., Wolfe, H.J., Habener, J.F. and Chin, W.N. (1987) Transient elevation of messenger RNA encoding gastrin-releasing peptide, a putative pulmonary growth factor in human fetal lung. J. Clin. Invest. 80, 1172-1179.

Springer, T.A. (1990) Adhesion receptors of the immune system. Nature 346, 425-433.

Spurr, N.K., Solomon, E., Jansson, M., Sheer, D., Goodfellow, P.N., Bodmer, W.F. and Vennstrom, B. (1984) Chromosome localisation of the human homologues to the oncogenes erbA and B. EMBO J. 3, 159-163.

Staatz, W.D., Rajpara, S.M., Wayner, E.A., Carter, W.G. and Santoro, S.A. (1989) The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg^{++}-dependent adhesion of platelets to collagen. J. Cell Biol. 108, 1917-1924.

Stahel, R.A., Gilks, W.R., Lehmann, H-P. and Schenker, T. (1994) Third international workshop on lung tumor and differentiation antigens: Overview of the results of the central data analysis. Int. J. Cancer (Suppl.) 8, 6-26.

Steinert, P.M. and Roop, D.R. (1988) Molecular and ceullar biology of intermediate filaments. Ann. Rev. Biochem. 57, 593-625.

Stenman, S. and Vaheri, A. (1978) Distribution of a major connective tissue protein, fibronectin, in normal human tissues. J. Exp. Med. 147, 1054-1064.

Stellmach, V., Leask, A. and Fuchs, E. (1991) Retinoid-mediated transcriptional regulation of keratin genes in human epidermal and squamous cell carcinoma cells. Proc. Natl. Acad. Sci. (USA) 88, 4582-4586.

Stoscheck, C.M. and King, L.E.Jr. (1986) Role of epidermal growth factor in carcinogenesis. Can. Res. 46, 1030-1037.

Sugimoto, T., Kato, T., Sawada, T., Horii, Y., Kemshead, J.T., Hino, T., Morioka, H. and Hosoi, H. (1988) Schwannian cell differentiation of human neuroblastoma cell lines *in vitro* induced by bromodeoxyuridine. Can. Res. 48, 2531-2537.

Sundaresan, V., Reeve, J.G., Stenning, S., Stewart, S. and Bleehan, N.M. (1991) Neuroendocrine differentiation and clinical behaviour in non-small cell lung tumours. Br. J. Cancer 64, 333-338.

Suzuki, S., Pierschbacher, M.D., Hayman, E.G., Nguyen, K., Ohgren and Ruoslahti, E. (1984) Domain structure of vitronectin: Alignment of active sites. J. Biol. Chem. 259, 15307-15314.

Symons, R.H. (1992) Small catalytic RNAs. Annu. Rev. Biochem. 61, 641-671.

Tagliabue, E., Martignone, A., Mastroianni, A., Menard, S., Pellegrini, R. and Colnaghi, M.I. (1991) Laminin receptors on SCLC cells. Br. J. Cancer 63, (Suppl.) XIV, 83-85.

Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphologenetic regulator. Science 251, 1451-1455.

Takizawa, H., Ohtoshi, T., Ohta, K., Yamashita, N., Hirohata, S., Hirai, K., Hiramatsu, K. and Ito, K. (1993) Growth inhibition of human lung cancer cell lines by interleukin 6 *in vitro*: A possible role in tumor growth via an autocrine mechanism. Can. Res. 53, 4175-4181.

Tandler, B., Sherman, J. and Boat, T.F. (1981) EDTA-mediated separation of cat tracheal lining epithelium. Am. Rev. Respir. Dis. 124, 469-475.

Taniguchi, T. and Minami, Y. (1993) The IL-2/IL-2 receptor system: A current overview. Cell 73, 5-8.

Tapia, F.J., Polak, J.M., Barbosa, A.T.A., Bloom, S.R., Marangos, P.J., Dermody, C. and Pearse, A.G.E. (1981) Neuron-specific enolase is produced by neuroendocrine tumours. Lancet i, 808.

Tapscott, S.J., Lassar, A.B., Davis, R.L. and Weintraub, H. (1989) 5-Bromo-2'-deoxyuridine blocks myogenesis by extinguishing expression of MyoD1. Science 245, 532-536.

Tateishi, R., Horai, T. and Hattori, S. (1978) Demonstration of argyrophilic granules in small cell carcinoma of the lung. Virchows Arch. Path. Anat. Histol. 377, 203-210.

Temin, H.M. (1966) Studies on carcinogenesis by avian sarcoma viruses, VI. The differential effect of serum and polyanions on multiplication of uninfected and converted cells. J. Natl. Can. Inst. 37, 167-175.

Terasaki, T., Kameya, T., Nakajima, T., Tsumuraya, M., Shimosato, Y., Kato, K., Ichinose, H., Nagatsu, T. and Hasegawa, T. (1984) Interconversion of biologic characteristics of small cell lung carcinoma depending on culture conditions. Gann 75, 1089-1099.

Terasaki, T., Shimosato, Y., Nakajima, T., Tsumuraya, M., Ichinose, H., Nagatsu, T. and Kato, K. (1987) Reversible squamous cell characteristics induced by vitamin A deficiency in a small cell lung cancer cell line. Can. Res. 47, 3533-3577.

Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L. and Martin, G.R. (1981) Osteonectin, a bone specific protein linking mineral to collagen. Cell 26, 99-105.

Testa, J.R. and Siegfried, J.M. (1992) Chromosome abnormalities in human non-small cell lung cancer. Can. Res. 52 (Suppl.), 2702s-2706s.

Thaete, L.G. and Malkinson, A.M. (1991) Cells of origin of pulmonary neoplasms in mice: Morphologic and histochemical studies. Exp. Lung Res. 17, 219-228.

Thomas, L., Chan, P.W., Chang, S. and Damsky, C. (1993) 5-Bromo-2-deoxyuridine regulates invasiveness and expression of integrins and matrix-degrading proteinases in a differentiated hamster melanoma cell. J. Cell Sci. 105, 191-201.

Thompson, R.J., Doran, J.F., Jackson, P., Dillon, A.P. and Rode, J. (1983) A new marker for vertabrate neurons and neuroendocrine cells. Brain Res. 278, 224-228.

Thornton, S.C., Robbins, J.M., Penny, R. and Breit, S.N. (1992) Fibroblast growth factors in connective tissue disease associated with interstitial lung disease. Clin. Exp. Immunol. 90, 447-452.

Timpl, R., Dziadek, M., Fujiwara, S., Nowack, H. and Wick, H. (1983) Nidogen: A new, self-aggregating basement membrane protein. Eur. J. Biochem. 137, 445-465.

Tockman, M.S., Gupta, P.K., Myers, J.D., Frost, J.K., Baylin, S.B., Gold, E.B., Chase, A.M., Wilkinson, P.H. and Mulshine, J.L. (1988) Sensitive and specific monoclonal antibody recognition of human lung cancer antigen on preserved sputum cells: A new approach to early lung cancer detection. J. Clin. Oncol. 6 (11), 1685-1693.

Todaro, G.J., DeLarco, J.E. and Cohen, S. (1976) Transformation by murine and feline sarcoma viruses specifically block binding of epidermal growth factor to cells. Nature 264, 26-31.

Tonita, N., Jiang, W., Hibshoosh, H., Warburton, D., Kahn, S.M. and Weinstein, B. (1992) Isolation and characterisation of a highly malignant variant of the SW480 human colon cancer cell line. Can. Res. 52, 6840-6847.

Topley, P., Jenkins, D.C., Jessup, E.A. and Stables, J.N. (1993) Effect of reconstituted basement membrane components on the growth of a panel of human tumour cell lines in nude mice. Br. J. Cancer 67, 953-958.

Tryggvason, K., Hoyhtya, M. and Salo, T. (1987) Proteolytic degredation of extracellular matrix in tumor invasion. Biochim. Biophys. Acta 907, 191-217.

Tsutsumi, Y., Osamura, R.Y., Watanabe, K. and Yanaihara, N. (1983) Immunohistochemical studies on gastrin-releasing peptide and adrenocorticotropic hormone-containing cells in the human lung. Lab. Invest. 48, 623-632.

Tungekar, M.F., Turley, H., Dunnill, M.S., Gatter, K.C., Ritter, M.A. and Harris, A.L. (1991) Interleukin 4 receptor expression on human lung tumors and normal lung. Can. Res. 51, 261-264.

Twardzik, D.R., Ranchalis, J.E., McPherson, J., Ogawa, Y., Gentry, L., Purchio, A., Plata, E. and Todaro, G.J. (1989) Inhibition and promotion of differentiated-like phenotype of a human lung carcinoma in athymic mice by natural and recombinant forms of transforming growth factor-β. J. Natl. Can. Inst. 81, 1182-1185.

Valles, A.M., Boyer, B. and Thiery, J.P. Adhesion systems in embryonic epithelial-to-mesenchyme transformations and in cancer invasion and metastasis. In: Goldberg, I.D. (ed) Cell motility factors. Basel, Birkhauser Verlag (1991) pp.17-34.

Valyi-Nagy, I., Shih, I.M., Gyorfi, T., Greenstein, D., Juhasz, I., Elder, D.E. and Herlyn, M. (1993) Spontaneous and induced differentiation of human melanoma cells. Int. J. Cancer 54 (1), 159-165.

van de Velde, H.J.K., Senden, N.H.M., Roskams, T.A.D., Broers, J.L.V., Ramaekers, F.C.S., Roebroek, A.J.M. and Van de Ven, W.J.M. (1994) NSP-encoded reticulons are neuroendocrine markers of a novel category in human lung cancer diagnosis. Can. Res. 54, 4769-4776.

van der Gaast, A., Schoenmakers, C.H.H., Kok, T.C., Blijenberg, B.G., Cornillie, F. and Splinter, T.A.W. (1994) Evaluation of a new tumour marker in patients with non-small-cell lung cancer: Cyfra 21.1. Br. J. Cancer 69, 525-528.

van Kalken, C.K., van der Valk, P., Hadisaputro, M.M.N., Pieters, R., Broxterman, H.J., Kuiper, C.M., Scheffer, G.L., Veerman, A.J.P., Meyer, C.J.L.M., Scheper, R.J. and Pinedo, H.M. (1991) Differentiation dependent expression of P-glycoprotein in the normal and neoplastic human kidney. Ann. Oncol. 2, 55-62.

Van Muijen, G.N.P., Ruiter, D.J. and Warnaar, S.O. (1987) Coexpression of intermediate filament polypeptides in human fetal and adult tissues. Lab. Invest. 57 (4), 359-369.

Van Oosterom, A.T., De Smedt, E.A., Denis, L.J., De Bruijin, E.A. and Mahler, C. (1990) Suramin for prostatic cancer: a phase 1/11 study in advanced extensively pretreated disease. Eur. J. Can. 26, 422-428.

Van Scott, M.R., Davis, C.W. and Boucher, R.C. (1989) Na^+ and Cl^- transport across rabbit nonciliated bronchiolar epithelial (Clara) cells. Am. J. Physiol. 256, C893-C901.

Van Scott, M.R., Hester, S. and Boucher, R.C. (1987) Ion transport by rabbit nonciliated bronchiolar epithelial cells (Clara cells) in culture. Proc. Natl. Acad. Sci. (USA) 84, 5496-5500.

Vasios, G.W., Gold, J.D., Petkovich, M., Chambon, P. and Gudas, L.J. (1989) A retinoic acid-responsive element in the 5' flanking region of the laminin B1 gene. Proc. Natl. Acad. Sci. (USA) 86, 9099-9103.

Veale, D., Ashcroft, T., Marsh, C., Gibson, G.J. and Harris, A.L. (1987) Epidermal growth factor receptors in non-small cell lung cancer. Br. J. Cancer 55, 513-516.

Veale, D., Kerr, N., Gibson, G.J. and Harris, A.L. (1989) Characterisation of epidermal growth factor receptor in primary human non-small cell lung cancer. Can. Res. 49, 1313-1317.

Virtanen, I., Heikinheimo, K., Hormia, M., Kivela, T., Laitinen, L. and Thornell, L. (1989) Expression of intermediate filaments (IF) in tissues and cultured cells. Int. J. Dev. Biol. 33, 55-61.

Virtanen, I., Miettinen, M., Lehto, V-P., Kariniemi, A. and Paasivuo, R. (1985) Diagnostic application of monoclonal antibodies to intermediate filaments. Ann. NY. Acad. Sci. 455, 635-648.

Wadell, W.J. and Marlowe, C. (1980) Tissue and cellular disposition of paraquat in mice. Toxicol.Appl.Pharmacol. 56, 127.

Waghorne, C., Thomas, M., Lagarde, A., Kerbel, R.S. and Breitman, M.L. (1988) Genetic evidence for progressive selection and overgrowth of primary tumors by metastatic cell subpopulations. Can. Res. 48, 6109-6114.

Walker, C. and Wright-Perkins, S. (1992) Expression of neuroendocrine and epithelial markers in an adherent subline derived from a classic small cell lung cancer cell line. Oncol. Res. 4 (10), 419-429.

Ward, J.M., Singh, G., Katyal, S.L., Anderson, L.M. and Kovatch, R.M. (1985) Immunocytochemical localisation of surfactant apoprotein and Clara cell antigen in chemically induced and naturally occurring pulmonary neoplasms of mice. Am. J. Pathol. 118, 493-499.

Warren, W.H., Memoli, V.A., Jordan, A.G. and Gould, V.E. (1990) Reevaluation of pulmonary neoplasms resected as small cell carcinomas: Significance of distinguishing between well-differentiated and small cell neuroencocrine carcinomas. Cancer 65, 1003-1010.

Watanabe, H., Takahashi, T., Ueda, R., Utsumi, K.R., Sato, T., Ariyoshi, Y., Ota, K., Obata, Y. and Takahashi, T. (1988) Antigenic phenotype and biological characteristics of two distinct sublines derived from a small cell lung carcinoma cell line. Can. Res. 48, 2544-2549.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C-H., Huang, J.S. and Deuel, T.F. (1983) Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304, 35-39.

Weber, G. (1980) Key enzymes and tumour cell heterogeneity. Antibiot. Chemother. 28, 53-61.

Weiss, L. Histology. Cell and tissue biology. 5th ed. Macmillan Press (XXXX).

Westermark, B. and Heldin, C-H. (1991) Platelet-derived growth factor in autocrine transformation. Can. Res. 51, 5087-5092.

Weston, A., Willey, J.C., Modali, R., Sugimura, H., McDowell, E.M., Resan, J., Light, B., Haugen, A., Mann, D.L., Trump, B.F. and Harris, C.C. (1989) Differential DNA sequence deletions from chromosomes 3, 11, 13 and 17 in squamous-cell carcinoma, large-cell carcinoma and adenocarcinoma of the human lung. Proc. Natl. Acad. Sci. (USA) 86, 5099-5103.

Wewer, U.M., Liotta, L.A., Jaye, M., Ricia, G.A., Drohan, W.N., Claysmith, A.P., Rao, C.N., Wirth, P., Coligan, J.E., Albrechtsen, R., Mudryj, M. and Sobel, M.E. (1986) Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. Proc. Natl. Acad. Sci. (USA) 83, 7137-7141.

Wharton, J., Polak, J.M., Bloom S.R., Ghatei, M.A., Solcia, E., Brown, M.R. and Pearse, A.G.E. (1978) Bombesin-like immunoreactivity in the lung. Nature 273, 769-770.

Wier, M.L. and Scott, R.E. (1986) Regulation of the terminal event in cellular differentiation: biological mechanisms of the loss of proliferative potential. J. Cell Biol. 102, 1955-1964.

Wilkinson, K.D., Lee, K., Deshpande, S., Duerksen-Hughes, P., Boss, J.M. and Pohl, J. (1989) The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 246, 670-673.

Wolbach, S.B. and Howe, P.R. (1925) Tissue changes following deprivation of fat soluble A vitamin. J. Exp. Med. 42, 753-777.

Woll, P.J. (1991) Neuropeptide growth factors and cancer. Br. J. Cancer 63, 469-475.

World Health Organisation, Geneva, Switzerland (1982) The World Health Organisation Histological typing of lung tumours: 2nd edition. Am. J. Clin. Pathol. 77, 123-136.

Yen, A. and Forbes, M.E. (1990) c-*myc* down regulation and precommitment in HL-60 cells due to bromodeoxyuridine. Can. Res. 50, 1411-1420.

Yesner, R. Spectrum of lung cancer and ectopic hormones. In: Sommers, S.C. and Rosen, P.P. (eds) Pathology Annual, part 1. New York, Appleton-Century-Crofts (1978) pp.217-240.

Yesner, R. and Carter, D. (1982) Pathology of carcinoma of the lung: Changing patterns. Clin. Chest Med. 3 (2), 257-289.

Yokose, T., Asami, H. and Ito, Y. (1991) Immunohistochemical and ultrastructural study of mixed small cell/large cell carcinoma of the lung. Acta Pathol. Jpn. 41, 540-551.

Yokota, J., Toyoshima, K. and Sigimura, T., Battifora, H. and Cline, M.J. (1986) Amplification of c-*erbB*-2 oncogene in human adenocarcinoma *in vivo*. Lancet i, 765-766.

Yost, R.W., Harrison, E.H. and Ross, A.C. (1988) Esterification by rat liver microsomes of retinol bound to cellular retinol-binding protein. J. Biol. Chem. 263, 18693-18701.

Young, M.R., Young, M.E. and Wepsic, H.T. (1987) Effect of prostaglandin E2-producing nonmetastatic Lewis lung carcinoma cells on the migration of prostaglandin E2-responsive metastatic Lewis lung carcinoma cells. Can. Res. 47, 3679-3683.

Yousem, S.A. (1991) Pulmonary carcinoid tumours and well-differentiated neuroendocrine carcinomas: Is there room for an atypical carcinoid? Am. J. Clin. Pathol. 95, 763-764.

Yousem, S.A., Wick, M.R., Randhawa, P. and Manivel, J.C. (1990) Pulmonary blastoma: An immunohistochemical analysis with comparison to fetal lung in its pseudoglandular stage. Am. J. Clin. Pathol. 93, 167-175.

Yu, X-Y., Schofield, B.H., Croxton, T., Takahashi, N., Gabrielson, E.W. and Spannhake, E.W. (1994) Physiologic modulation of bronchial epithelial cell barrier function by polycationic exposure. Am. J. Respir. Cell Mol. Biol. 11, 188-198.

Yu, V.C., Delsert, C., Anderson, B., Holloway, J.M., Devary, O.V., Naar, A.M., Kim, S.Y., Boutin, J-M., Glass, C.K. and Rosenfeld, M.G. (1991) RXRβ: A coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements. Cell 67, 1251-1266.

Yun, K. and Scott, R.E. (1983) Biological mechanisms of phorbol myristate acetate-induced inhibition of proadipocyte differentiation. Can. Res. 43, 88-96.

Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1577-1590.

Zachary, I., Woll, P. and Rozengurt, E.A. (1987) A role for neuropeptides in the control of cell proliferation. Dev. Biol. 124, 295-308.

Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P. (1989) Cloning of murine α and β retinoic acid receptors and a novel receptor predominantly expressed in skin. Nature 339, 714-717.

Zhang, X., Liu, Y., Lee, M-O. and Pfahl, M. (1994) A specific defect in the retinoic acid response associated with human lung cancer cell lines. Can. Res. 54, 5663-5669.