Genetic and Biochemical Factors Involved in the Progression of Mouse Skin Tumours

Jennifer Liddell

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Abbreviations

bp	base pairs
kb	kilobases
kd	kilodaltons
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
dNTP	deoxyribonucleoside triphosphate
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetra-acetic acid
aFGF	acidic fibroblast growth factor
FITC	fluorescein isothiocyanate
HEPES	hydroxy ethyl piperazine ethane sulphonic acid
HRP	horseradish peroxidase
IgG	immunoglobulin G
PBS	phosphate buffered saline
PE	PBS + EDTA
PMSF	phenyl methyl sulphonyl fluoride
RITC	rhodamine isothiocyanate
SDS	sodium dodecyl sulphate
SLM	special liquid medium
TBS	tris-buffered saline
TEMED	tetramethyl ethylene diamine
TGFβ	transforming growth factor beta
ECL	enhanced chemiluminescence
LOH	loss of heterozygosity
PCR	polymerase chain reaction

Abstract

A dramatic change in cell phenotype is sometimes seen in an advanced stage of mouse skin carcinogenesis when a relatively well-differentiated squamous cell carcinoma progresses to a less differentiated spindle cell carcinoma. This thesis describes a study of some genetic and epigenetic events that may be responsible for the squamous to spindle cell transition using a panel of fixed morphology and plastic morphology cell lines.

The experimental approaches used include genetic and cell biology-based studies. An allelotype of clonally related but morphologically distinct cell lines, A5, B9 and D3 was compiled using 118 informative markers and genetic alterations distinguishing the differing cell types have been described. Alterations distinguishing the squamous and spindle cells were found on chromosomes 1, 4, 5, 6, 8, 12, 13, 16, 18 and 19. The most interesting of these with respect to tumorigenesis are on chromosomes 4 and 6; these have been shown to be involved in carcinogenesis in other studies. Although no specific allelic losses were shown to correlate with the squamous/spindle transition, this study has provided information on the lineage of clonally related cell lines from the same tumour and has provided some clues to the gross alterations that may be central to this process.

A wide range of biochemical studies have also been used to characterise the fixed morphology cell lines plus additional cell lines which have more plastic morphologies i.e. the SN161 single cell clones. It has been possible to induce the transition from a squamous morphology to a more fibroblast-like phenotype *in vitro* and *in vivo* and some of the candidate effectors of this change have been analysed.

When the SN161 cell lines are injected into nude mice, they all produce spindle tumours regardless of their morphology *in vitro*. Explants from tumours induced by squamous cell lines can regain their squamous morphology when grown in normal culture conditions. It seems that some factor present *in vivo* is encouraging the spindle

phenotype; alternatively some factor present *in vitro* could be encouraging the squamous phenotype.

Specific extracellular factors were shown to influence the morphology of SN161 derived cell lines. Growth on fibronectin coated plates made the spindle cells more fibroblast-like but had no effect on the squamous cells. The same was true for Matrigel coated plates.

The most interesting result was found with TGFβ treatment of the cells. This induced the squamous cell lines, E1N and E4, to become fibroblast-like and caused their E-cadherin to become delocalised and its expression reduced. This observation was consistent with the appearance of the spindle morphology. This system can now be used to study the immediate consequences of changes of morphology rather than secondary changes which can complicate results based on the study of spindle tumours.

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Thanks are due to the Cancer Research Campaign and Medical Research Council for funding my work. I am also grateful to members of R7 and numerous other Beatsons for their technical support and general assistance and to various Mediterranean types for providing entertainment and extending my vocabulary. I must also credit my supervisor, Allan Balmain, for his magic pot of ideas that kept refilling every time my back was turned. I think I should be grateful for this.

My family must be congratulated for being supportive but the biggest thank you goes to Greg for encouraging my insanity.

:-)

Chapter 1

Introduction

1.1 Introduction

Age is widely recognised as an important risk factor in the development of cancer since a long latency period is characteristic of many types of neoplasia. The typical increase in susceptibility with age may be partly explained by a requirement to accumulate sufficient genetic alterations that allow the uncontrolled growth that causes neoplasia. The long-term aim of cancer research is to identify such genetic events and the biochemical changes that allow normal cells to proliferate abnormally and progress through various stages to malignancy. Some genetic alterations may be mediated through gross chromosomal changes and therefore may be cytogenetically detectable. The corollary is that characterisation of such chromosomal rearrangements should lead to the identification of genes involved in cancer.

However, mutational (genetic) events in cancer cells are not the only factors affecting cancer development. Environmental (epigenetic) factors also affect the evolution of malignancy e.g. changes in tissue biology with increasing age may provide an environment that contributes to tumour progression. Indeed, the pattern of metastasis in solid tumours is strongly influenced by the surrounding normal tissue environment, presumably reflecting the nature of interactions with the extracellular matrix and presence of local growth factors (Liotta, 1986). These are important non-genetic considerations. The involvement of both of these aspects of tumorigenesis i.e. genetic and epigenetic factors will be discussed.

1.2 Genetic alterations in carcinogenesis

The idea that carcinogenesis is a multistep process which starts with a single initiated cell that proceeds to form a neoplastic growth has been generally accepted for many years based on information from the epidemiology of human cancers and animal model systems. Much cancer research work is focused on the nature and consequences

of the genetic alterations that lead to deregulation of cellular proliferation and differentiation control mechanisms.

During the progression of tumours, neoplastic cells are thought to accumulate increasing numbers of genetic alterations that are generated by random, somatic mutational events (Nowell, 1976) and it has been proposed that the more genetic changes tumours undergo, the more malignant they become (Vogelstein et al., 1989). Natural selection presumably favours the development of a progressively more malignant phenotype, aided by an increased rate of mutational events as the genetic instability of the tumour cell population increases.

Evidence to support this hypothesis comes from analyses of mouse skin malignancies which consist of aneuploid cells with genetic changes indicative of increasing genetic instability (Aldaz et al., 1987). Gross chromosomal abnormalities such as translocations and large deletions are frequently observed in various human cancers also, especially in tumours of progressed stages (Holliday, 1989). Some of these changes will simply be a consequence of genomic instability and will have no causal role in tumorigenesis. However, some presumably play a central role in tumour development. Some genetic losses may cause a change in cell morphology related to the loss of differentiation markers while others may confer malignant properties related to metastasis such as invasiveness and motility. The purpose of the work described here was to identify such changes and test their relevance to the altered phenotype of mouse epithelial cells in late stages of experimentally induced skin carcinogenesis.

Such phenotypic changes are often a result of mutations affecting two major classes of gene; the proto-oncogenes and tumour suppressor genes. Mutations that potentiate the functions of proto-oncogenes create oncogenes that promote the growth of tumour cells. Conversely, genetic lesions that inactivate tumour suppressor genes liberate cells from the normal growth constraints imposed by these genes, yielding unconstrained growth of cancer cells. The complexity and diversity of cellular pathways dictate that progression of most tumours to full malignancy requires both

types of change in the tumour cell genome. The mouse model used for this study is ideally suited to the examination of genetic alterations.

1.2.1 Oncogenes

The study of oncogenes began some time ago and now much is known about their causal role in carcinogenesis. Proto-oncogenes and their viral counterparts, first discovered as the transforming genes of retroviruses, have been established as frequent causes of the neoplastic transformation of mammalian cells (Bishop, 1981; Bishop and Varmus, 1982).

One of the most significant advances in cancer research was the discovery that the retroviral oncogenes had normal cellular counterparts - proto-oncogenes (Stehelin et al., 1976; Bishop, 1981). In transformed cells, oncogenes encode proteins that function abnormally, inappropriately or at increased concentrations, resulting in escape from the normal growth controls that regulate cell division and differentiation. Therefore, mutations in proto-oncogenes that alter their function can cause transformation e.g. activation of the H-ras oncogene by an A-T transversion at codon 61 results in initiation of skin carcinogenesis in mice. Interestingly, there is a good correlation between the type of carcinogen used to initiate tumour formation and the particular activating mutation of H-ras (Quintanilla et al., 1986). Increased expression of the oncoprotein due to amplification or translocation can also be the cause of tumorigenesis e.g. as seen with c-myc (Garte, 1993).

Oncogenes can generally be divided into 4 groups according to the point where they interfere with cell growth control: growth factors (e.g. sis), growth factor receptors (e.g. erbB, fms, kit), cytoplasmic transducers of growth factor responses (e.g. src, ras, raf) and transcription factors that mediate growth factor-induced gene expression (e.g. jun, fos) i.e. they can be involved in the disruption of normal growth factor related signalling at any point on that pathway. These examples all lie at different positions on the same pathway but equivalents can be found on other, as yet independent, pathways.

Due to these multiple levels of control, it is not surprising that a single oncogene is unable to cause transformation alone; complete transformation usually requires the cooperation of oncogenes from different categories (Hunter, 1991). This lends support to the multistage model of carcinogenesis (see section 1.4).

1.2.2 Tumour suppressor genes

The second category of genes central to studies of carcinogenesis, tumour suppressor (TS) genes was discovered more recently but has now been studied in great detail too. As would be expected, molecular analysis of TS genes is revealing that their products are involved in diverse cellular functions e.g. cell adhesion, signal transduction and cell cycle control. However, it is surprising that most of the TS gene products now known had not been previously identified in studies of normal cells; consequently their analysis is contributing not only to understanding of tumorigenesis but also basic cell biology. One of the aims of the work described here was to identify putative TS genes involved in a specific stage of mouse skin carcinogenesis with a view to learning more about genetic changes involved in the acquisition of a metastatic phenotype. Previous work had suggested that TS genes may be affected (Stoler et al., 1993).

The earliest evidence suggesting the existence of tumour suppressor genes came from cell fusion studies (Harris et al., 1969a). Hybrids between tumour cells and normal cells were no longer tumorigenic upon injection into animals and there seemed to be some kind of tumour suppressing signal present in the normal parent cell line since loss of specific chromosomes from the normal parent correlated with re-expression of tumorigenicity in hybrid cells (Harris and Klein 1969b).

Although these observations were first made 25 years ago, progress has since been slow because TS genes are, by definition, lost during carcinogenesis. This characteristic property underlies the experimental difficulties hindering their analysis and explains the 10 year lag of this research behind that of oncogenes; it is more difficult to study a loss of function rather than a gain of function. A major breakthrough came from the method

by which the RB gene was detected. It has provided an important basis for the techniques that are now used to search for TS gene loci, including those presented in this thesis.

1.2.3 Retinoblastoma and mechanisms of loss of heterozygosity

Human genetics provided the first clues to the existence of TS genes. Studies of retinoblastoma, a childhood cancer of the retina, showed that it occurred both in sporadic and familial forms. A basic model of how TS gene loss led to tumour formation was first proposed by Knudson from a statistical analysis of retinoblastoma (Knudson, 1971). The incidences of unaffected carriers, unilateral and bilateral retinoblastoma led Knudson to propose a " two hit " model for development of the cancer. His hypothesis was that in the dominantly inherited form, the first mutation is passed through the germline while the second mutation occurs in the somatic cells. In the sporadic form, both mutations are somatic.

Several chromosomal mechanisms which could lead to the phenotypic expression of a recessive allele have been discussed (Cavenee et al., 1983). They are depicted in figure 1. The first copy of the TS gene is inactivated by somatic or germline mutation. The region carrying the surviving wild type allele may then be replaced by a duplicated copy of the homologous chromosomal region that has been mutated. This process causes a reduction to homozygosity at the RB locus i.e. a loss of heterozygosity (LOH). The mechanisms involved can be chromosomal non-disjunction, mitotic recombination or gene conversion. These steps that lead to homozygosity of a mutant suppressor allele usually involve flanking chromosomal regions as well and unrelated DNA markers mapping to nearby sites also become homozygous. Therefore, repeated observation of LOH of a specific chromosomal marker in cells from a particular tumour type suggests the presence of a closely mapping TS gene, the loss of which is involved in tumorigenesis.

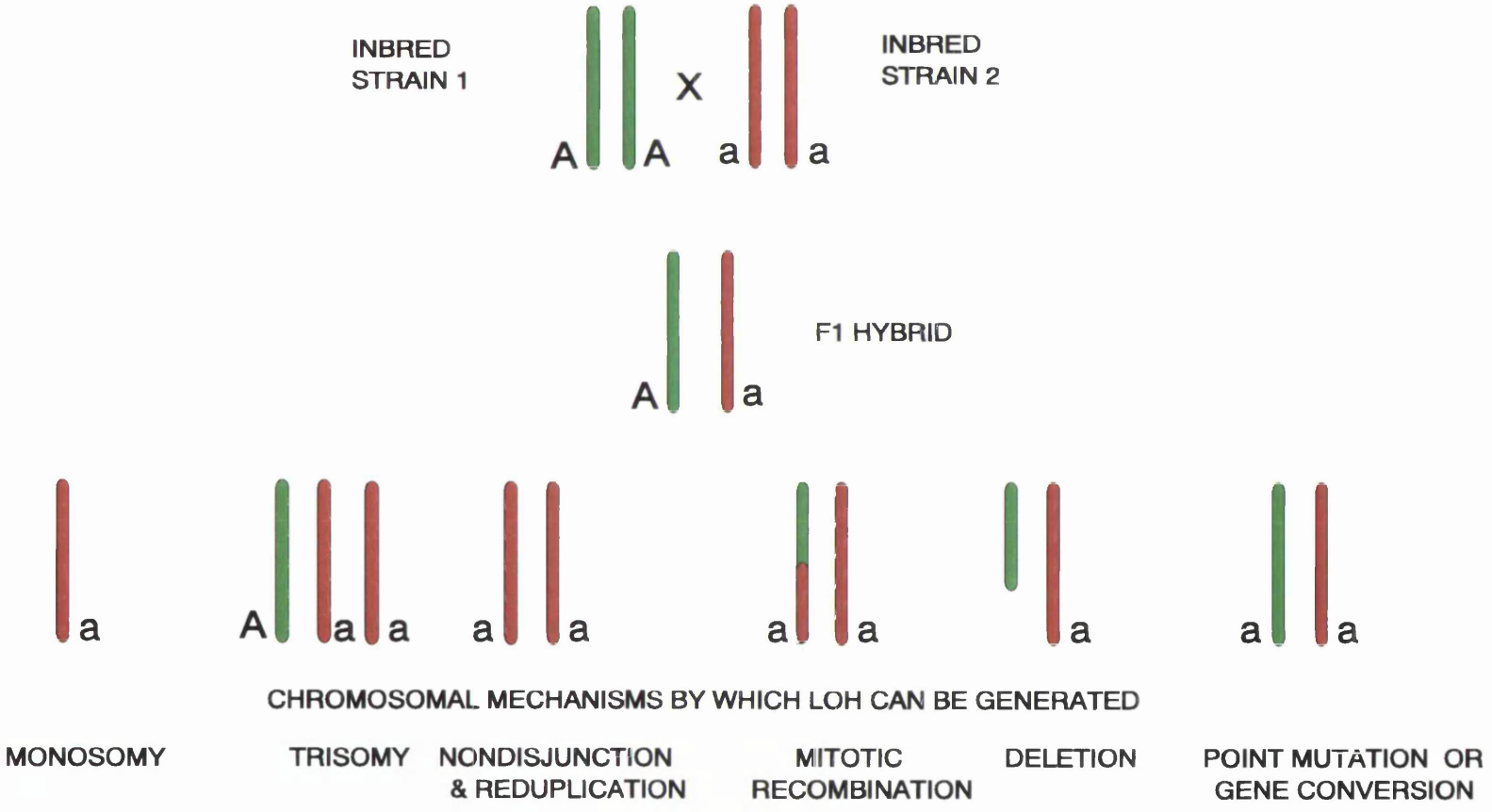


Figure 1: Use of F1 hybrids to characterise chromosomal alterations. F1 hybrid mice produced by crossing distantly related inbred strains have a high degree of heterozygosity. Loss of heterozygosity at a particular locus can be detected using a PCR-based technique which amplifies microsatellites.

Using RFLP analysis, Cavenee et al (Cavenee et al., 1983), found evidence for non-disjunction and chromosome loss, non-disjunction and reduplication and mitotic recombination on chromosome 13 in retinoblastoma DNA when compared to normal DNA from the same patient. It was later shown that in hereditary cases of retinoblastoma, the copy of chromosome 13 which remained in the tumours was inherited from the afflicted parent and the chromosomal changes detected had uncovered a recessive mutation (Cavenee et al., 1985).

These reports resulted in a great leap forward in the understanding of cancer predisposition and soon more inherited cancer syndromes were being analysed in this way.

1.2.4 p53 mutation is a common event in tumours

The most intensively studied tumour suppressor , p53, was originally found as a complex with large T antigen and has subsequently been the subject of many LOH studies. Mutations of the p53 gene are the most common mutations found to date in tumours of widely ranging origins (Hollstein et al., 1991). In most cases, missense mutations occur accompanied by loss of the remaining wild-type allele, leading to complete loss of wild-type p53 expression.

This loss of expression through recessive genetic alterations is consistent with the categorisation of p53 as a tumour suppressor gene but the situation can become more complicated. Unlike many other genetic changes in tumours, mutations of the p53 gene do not always result in complete loss of protein function. Some mutants may act as dominant negatives which inactivate any remaining wild-type protein while others may have acquired their own DNA-binding properties which were not typical of the wild-type form (Vogelstein and Kinzler, 1992).

The normal functions of p53 have been the focus of a great deal of attention. The precise mechanism by which p53 inactivation contributes to tumorigenesis is still not clear but p53 is now known to be a regulator of apoptosis (reviewed in Oren, 1994). It

is a nuclear phosphoprotein which appears to play a role in cell cycle checkpoint control, inducing G1 arrest in reponse to DNA damage (Kuerbitz et al., 1992).

It has been proposed that p53 may serve as a "guardian of the genome" by preventing proliferation of cells that have sustained some form of genetic damage (Lane, 1992). Cells lacking wild type p53 fail to arrest in G1 and the resulting genetic instability permits the accumulation of many genetic alterations which would normally not be allowed to persist. Although the consequences of these links have not been explained as yet, it is clear that p53 has a crucial role to play in normal cell processes and its link with tumorigenesis is undisputed.

Evidence for the importance of p53 mutations in tumorigenesis comes from the study of human tumours, animal tumour models and cell culture. The first evidence for germ-line mutations in p53 came from studies of 5 families with Li Fraumeni syndrome (Malkin et al., 1990) which showed that germline p53 mutations could be detected in all of the families tested. This work established a solid link between p53 mutations and susceptibility to Li-Fraumeni syndrome.

Further evidence for the importance of p53 at specific stages of tumour development has come from the mouse skin model. Loss of heterozygosity for markers on mouse chromosome 11 where the p53 gene is located and mutation of the p53 gene occurred in some carcinomas but not in papillomas in mouse skin carcinogenesis studies (Burns et al., 1991; Ruggeri et al., 1991).

A study of tumour development in p53 knockout mice also placed p53 mutations at a late stage. A comparison of the occurrence of p53 mutations in chemically-induced skin tumours in normal mice that have two wild-type alleles with that in genetically altered mice carrying either one or no p53 alleles showed that the papilloma yield was similar in wild-type and hemizygous mice but reduced in null mice (Kemp et al., 1993). Null mice developed a larger number of carcinomas instead. Most significantly, the rate of appearance of carcinomas in the hemizygous mice was associated with the loss of the remaining p53 allele. Loss of wild type p53 does not seem to increase the incidence or shorten the latency of appearance of papillomas. It does, however, enhance the rate of

malignant progression as reflected by the dramatic increase in the rate of appearance of carcinomas. Furthermore, the majority of carcinomas from the hemizygous and null mice were undifferentiated squamous carcinomas in contrast to the carcinomas in the normal mice which were mainly well-differentiated tumours.

p53 has also been shown to contribute to many types of neoplasia including colorectal cancer (reviewed in Fearon and Vogelstein, 1990). p53 mutations typically occur in late stages of tumour progression; they are rarely observed in benign adenomas but are common in malignant carcinomas. Perhaps p53 mutation provides a selective advantage during tumour progression once other genetic alterations have taken place.

1.2.5 Other human tumour suppressor genes

The field of tumour suppressor genes has undergone rapid expansion in the past 20 years and there are now tumour suppressor genes known to affect all major cellular processes.

After retinoblastoma, Wilms tumour was the second cancer which was shown to have hereditary and non-hereditary forms. In a similar way to the identification of the Rb gene, LOH and deletions at 11p13 were the first clue to the site of the WT1 gene which has since been cloned (Call et al., 1990; Gessler et al., 1990). However, the genetics of this disease are more complicated than retinoblastoma and different loci seem to be associated with sporadic and inherited forms of this cancer (van Heyningen and Hastie, 1992).

The neurofibromatosis gene, NF1, and the familial adenomatous polyposis gene, APC, have similar patterns of inheritance and allele loss associated with tumours. The NF1 gene codes for a GTPase activating protein that regulates the activity of ras. It has been mapped to chromosome 17q11 and cloned (Xu et al., 1990). It will be discussed in more detail in section 1.5.2.

Mutations of the APC (Adenomatous Polyposis Coli) gene appear to cause sporadic and familial forms of colorectal cancer. In fact, such mutations seem to be the initiating event for the development of colon cancer.

The APC protein associates with α and β catenin which are thought to link the E-cadherin/catenin complex to the cytoskeleton (Su et al., 1993; Rubinfeld et al., 1993). Therefore, APC may be involved in cell adhesion through its association with catenins. If this is true, mutations in the APC gene may result in production of mutated protein unable to interact with catenins and hence in a loss of APC-catenin-cadherin complex formation. Consequently, a loss of cell-cell interaction could lead to a lack of contact growth inhibition, resulting in hyperplasia. The subject of altered cell-cell and cell-matrix adhesion will be discussed further in section 1.6 with respect to tumour invasion and metastasis.

Germline mutations of the APC gene which has been mapped to chromosome 5q21 and cloned, predispose individuals to familial adenomatous polyposis (FAP), a major cancer of adults (Groden et al., 1991; Nihisho et al., 1991). FAP begins with the formation of numerous benign polyps in the colonic mucosa, all of which have APC mutations. In addition to these alterations, progression to malignancy requires further mutations in the K-ras oncogene, p53 and the DCC TS gene.

The DCC (Deleted in Colorectal Carcinoma) gene was isolated as a result of its location on chromosome 18 which showed loss of heterozygosity in human colorectal cancer (Fearon et al., 1990). It encodes a protein with a high degree of homology to N-CAM, a cell adhesion molecule of neural cells, and other cell surface glycoproteins. DCC expression is most commonly lost in late adenomas as they progress to an invasive phenotype. Loss of cell adhesion molecules is a common feature of invasive and metastatic tumours and will be discussed in more detail in section 1.6.5.

Other candiate TS genes have been identified by a variety of means. These include MCC (Mutated in Colorectal Cancer) (Kinzler et al., 1991), PTPγ, a protein tyrosine phosphatase (LaForgia et al., 1991), nm23, a nucleoside diphosphate kinase (Steeg et al., 1991) and K-rev1, a GTP-binding protein (Kitayama et al., 1989). In most

cases, the evidence for their being TS genes centres on their loss or inactivation during tumour development, although more formal proof comes from a specific tumour suppressing effect when the wild type form of the gene is reintroduced into tumour cells. K-rev1 was identified as a result of its ability to revert K-ras transformed cells to a less transformed phenotype (see section 1.5.4).

New putative TS genes are rapidly being described as technical hurdles are being overcome. The most productive approach to date has come from a combination of recent advances in genetics and molecular biology i.e. the analysis of microsatellite markers by the polymerase chain rection (PCR).

1.3 Microsatellites as genetic markers

The use of PCR-analysed microsatellite markers as a method for chromosomal mapping in the mouse has rapidly become well established. This technique has been adapted and used in the search for TS gene loci, revolutionising this research field. It is the method which was used to analyse the genetic alterations in mouse skin tumours described in this thesis.

Microsatellites are simple dinucleotide repeats, usually 15-40 copies of (CA) or (GA), which are within the size range of Taq polymerase and can therefore be amplified by PCR using oligonucleotide primers complementary to unique DNA sequences flanking the microsatellite (figure 2). They are ideally suited to the search for TS gene loci because they are highly polymorphic and occur frequently in mammalian genomes so they can be used to screen tumour cell genomes and identify regions of LOH. They are inherited in Mendelian fashion in the same way as RFLPs but this approach has several advantages over the more traditional method of RFLP analysis which detected DNA polymorphisms at restriction enzyme sites. These, by their nature, are rare and their detection can require the use of 20-30 different enzymes followed by blotting and hybridisation protocols which are time-consuming. Microsatellites, in contrast, are

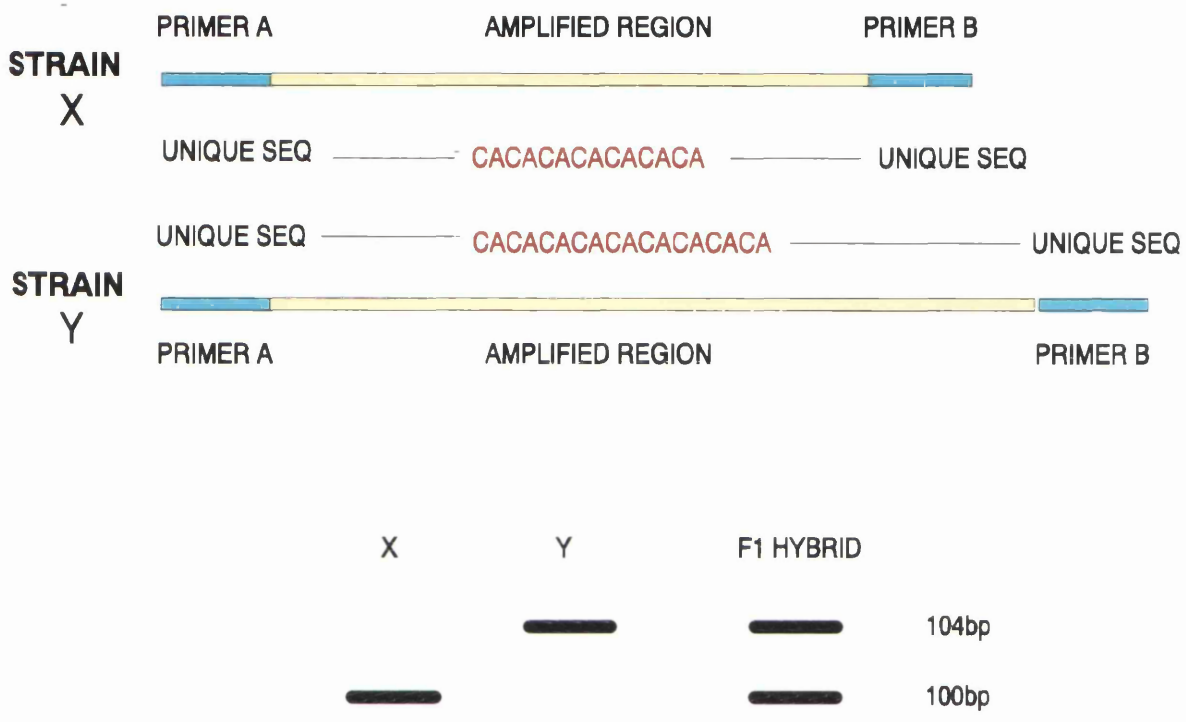


Figure 2: PCR analysis of polymorphic microsatellites. The products generated by PCR amplification of microsatellites in different strains of mouse often differ in size. This size difference can be detected by polyacrylamide gel electrophoresis.

highly polymorphic and can be easily analysed by agarose and polyacrylamide gel electrophoresis. Most importantly, they are very common - it has been estimated that there are at least $5x10^4$ sequences that contain $(CA)_n$ repeats in the mouse and human genomes (Hamada et al., 1982; Weber and May, 1989) so there are potentially enough microsatellites within the mouse genome to have a marker every 1cM.

1.3.1 LOH as means of locating TS genes

The hallmark of TS genes is that they are deleted, or suffer LOH, at high frequency in certain tumour types. These deletions often involve gross genetic alterations which can be detected by screening with polymorphic markers. LOH analysis has been widely used both alone and in conjunction with other techniques such as cell fusion and micro-cell mediated chromosome transfer to look for candidate TS genes. These approaches have succeeded in identifying common regions of LOH in most types of human cancer (Lasko et al., 1991).

Reproducible LOH at a particular locus is a good indicator of the likely presence of a TS gene in this region, as discussed with respect to RB (section 1.2.3). A summary of the main regions of LOH in a wide range of human tumours is given in table 1. Most studies have been analyses of human tumours, but there is also good data emerging from animal model systems. Syntenic regions between mouse and human chromosomes have been defined (Copeland et al., 1993) and it is interesting that the same regions are frequently included in the lists of LOH.

For example, the human 9p21 region contains chromosomal alterations in glioma cell lines, non-small cell lung cancer lines, leukaemia lines and melanoma lines. Certain 9p21 markers are deleted in more than half of all melanomas (Isshiki et al., 1994) and LOH affecting chromosome 9 has been seen in 86% of DNAs from melanoma patients (Fountain et al., 1992). The MTS1 (multiple tumour suppressor) gene maps to 9p21 and there is some evidence that it is a familial melanoma gene (Hussussian, 1994). It encodes p16, a cell cycle-related gene, which may have a role in controlling cell

CHROMOSOME	TUMOUR TYPE
1	lung, liver, stomach
2	lung
3	lung, kidney, bladder, breast, head & neck, cervical
4	liver, ovarian
5	lung, liver, stomach, colorectal, kidney, ovarian, cervical
6	breast, ovarian
7	breast
8	lung, liver, colorectal, prostate, breast
9	lung, bladder, breast
10	kidney
11	parathyroid, lung, liver, colorectal, kidney, bladder, breast, prostate, ovarian, cervical
13	lung. liver, stomach, kidney, ovarian
14	liver
16	liver, breast, prostate
17	lung. liver, stomach, colorectal, bladder, breast, ovarian
18	lung, stomach, colorectal, kidney, breast, prostate, ovarian
19	lung
22	liver, colorectal, breast, ovarian
X	ovarian

TABLE 1: LOH in human epithelial tumours (summary of published data)

proliferation. Human chromosome 9p21, where p16 is localised, is syntenic with part of mouse chromosome 4. This region is altered in mouse tumours (Kemp et al., 1993a).

Other areas of research also point to the importance of a gene on chromosome 9. LOH at 9q31 is frequently seen in Gorlin syndrome which predisposes individuals to basal cell carcinoma of the skin plus other types of tumour and developmental defects (Gailani et al., 1992). This implies that the gene involved is inactivated in tumorigenesis and may therefore be classed as a tumour suppressor gene. The gene for Ferguson-Smith syndrome, which causes multiple epidermal tumours, (Goudie et al., 1993) also maps to the same location, suggesting that perhaps both syndromes are caused by mutation of the same gene.

Contamination of primary tumours with normal tissue tends to reduce the sensitivity of LOH analysis, resulting in underscoring of LOH frequency but this is not a problem when working with cell lines isolated from tumour biopsies. These originate solely from tumour cells which are much more readily immortalised in culture compared to their normal counterparts.

One important advantage of LOH analysis over traditional techniques such as cytogenetics is that certain chromosomal mechanisms for allele loss can be distinguished e.g. mitotic recombination. The first demonstration of mitotic recombination and non-disjunction events in mouse tumours focused on chromosome 7 (Bremner and Balmain, 1990). This study showed that allelic imbalance on chromosome 7 was a very common event in mouse skin tumours which had been initiated by mutational activation of H-ras. H-ras itself may be the main factor involved in tumorigenesis in this case but other alterations are also required at each stage of tumour development.

1.4 Multistep carcinogenesis

The multistep model of carcinogenesis was first proposed by Foulds in 1954 (Foulds, 1954) and since then it has been possible to identify specific genetic alterations that correlate with defined stages in several human tumour types including malignant melanoma (Balaban et al., 1986; Williams et al., 1993), glioma (James et al., 1988) small-cell lung carcinoma (Naylor et al., 1987) and colon cancer (Vogelstein et al., 1988). Hereditary conditions such as retinoblastoma provided the first evidence for TS gene involvement in tumorigenesis and are useful for the study of mechanisms involved in tumour development where a single locus plays a central role in the process. However, it is more common for the tumour to be caused by a number of contributory factors.

Colon cancer offers the best clinical illustration of multiple somatic mutations underlying multistage carcinogenesis and genetic changes from the earliest to the most advanced stages have been studied in detail (reviewed in Fearon and Vogelstein, 1990 and Stanbridge, 1990). It is now apparent that an accumulation of mutations in several genes correlates with progression via epithelial dysplasia, adenoma and carcinoma *in situ* to complete malignancy. Furthermore, an association has been found between the number of allelic losses at multiple chromosomal sites and a poor prognosis.

The same is true for tumours induced in experimental animals and rodent models have been particularly informative.

1.4.1 Mouse skin structure

One of the most commonly used models is the mouse skin system. In a stratified epithelium, such as epidermis, keratinocytes in the basal layer adhere to the basement membrane and adjacent cells on either side and above. This layer is where proliferation takes place and epidermal cells are renewed. About 60% of the cells remain here and will divide again; the other 40% are programmed to differentiate and are pushed out into

the granular layer (Fuchs and Byrne, 1994). These suprabasal cells have lost adhesion to the matrix and instead adhere to similar cells on all sides. They have lost proliferative ability also and begin to express markers of differentiation such as keratins 1 and 10. In the granular layer proteins are cross-linked to form the insoluble cornified envelope and the cells enlarge and flatten out. Finally, at the cornified layer, the nucleus begins to disintegrate, the cells die and become squames and are eventually brushed off. Therefore, the various stages of *in vivo* keratinocyte differentiation and proliferation take place in well-defined compartments. In carcinogenesis this regulation is disrupted.

1.4.2 Mouse skin model system

Work with animal models, particularly the mouse skin, has demonstrated that initiation, which is irreversible and involves chemical carcinogen interaction with DNA, is a somatic event that may occur in a single target cell. In contrast, promotion, the clonal expansion of initiated cells to a benign lesion such as a papilloma, has been associated mainly with epigenetic factors activated by phorbol esters used in experimental tumour induction (Furstenberger et al., 1981; Hecker et al., 1982).

The next stage, progression, is the transition from a benign premalignant lesion to a malignant tumour. It has most commonly been studied using human tumours. Clinically, this stage poses most problems. Phenotypic alterations typical of progression include drug resistance, angiogenesis, invasiveness and metastasis which are usually the reasons for failure of treatment of human cancer and cause of death. Many of these new properties may be due to the acquisition of new mutations but epigenetic factors are important also.

The development of skin tumours in mice can easily be followed. Individual tumours can be measured and signs of malignant change in benign tumours can be seen with the naked eye. Gross pathological changes such as ulceration and hyperkeratosis can also be detected. After a single application of an initiator and multiple applications of a promoter, numerous benign papillomas arise in the back of the treated mouse; about

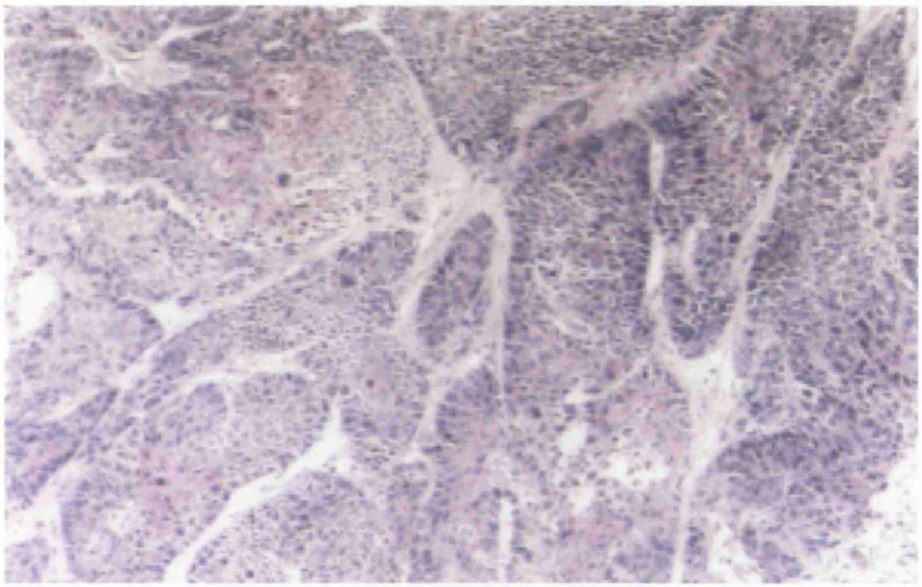
5-10% of these then progress towards malignancy. These carcinomas may be squamous cell carcinomas which still retain many of the differentiation features of the original tissue e.g. E-cadherin and keratin expression or they may be a more advanced stage spindle cell carcinoma which has lost these epithelial markers and consists of fusiform cells instead (figure 3).

Animal models have been used for the detection and characterisation of mutations in positive effector genes. Data from the mouse skin model have strongly suggested that genetic events have a causative role in tumour progression e.g. non-genotoxic tumour promoters such as TPA do not enhance the conversion of mouse skin papillomas to carcinomas whereas mutagenic or genotoxic carcinogens such as MNNG do (Margison and O'Connor, 1978). Chromosomal aberrations involving gross alterations of DNA sequences such as deletion, amplification and translocation are frequently observed in mouse skin carcinogenesis (Conti et al., 1986; Aldaz et al., 1989) and several stage-specific genetic alterations have already been identified in mouse skin tumours (summarised in figure 4).

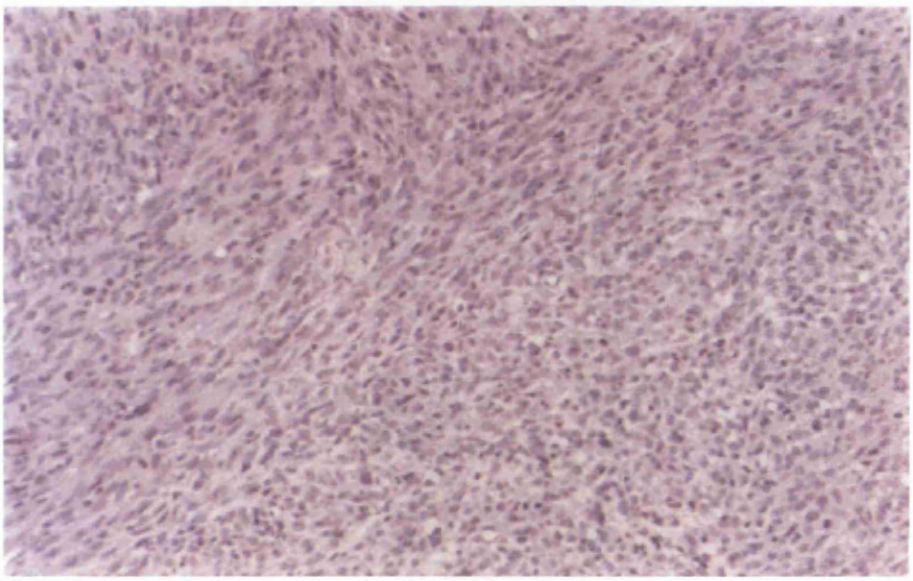
The main advantage of the mouse skin system is that the tumour aetiology can be controlled and cell lines can be obtained at sequential stages of tumour development; these explants retain many of the characteristics of the original tumour. However, from the point of view of TS gene analysis, the most useful aspect is the availability of inbred mouse strains which can be cross-bred to give highly polymorphic F_1 hybrids with a high degree of heterozygosity. Such mice have proved to be invaluable in the analysis of genetic alterations in skin tumours.

1.5 The ras superfamily and tumorigenesis

Ras mutations and perturbations of the signalling pathways it is involved in are common alterations in tumours. Ras proteins are components of receptor-mediated signalling pathways controlling cell proliferation and differentiation. This means that



SQUAMOUS CARCINOMA



SPINDLE CARCINOMA

Figure 3: Typical histology of a squamous and a spindle carcinoma. (micrographs courtesy of A. Balmain)

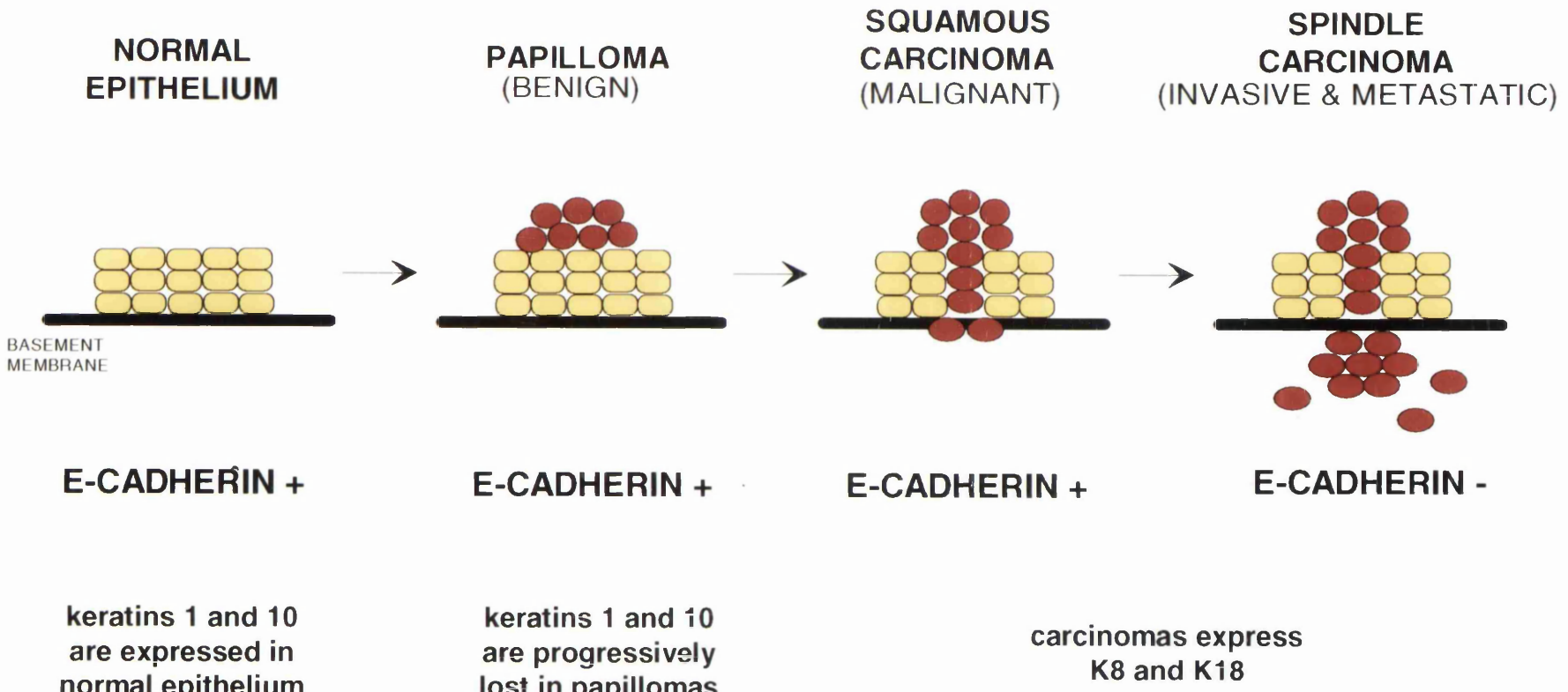


Figure 4: Mouse skin tumorigenesis. The expression of several markers can be correlated with particular stages of tumorigenesis.

activated forms of various components of the ras pathway can contribute to cancer by bypassing the regulation of cell growth by growth factors. Ras itself is a common target for mutation; more than 30% of all human carcinomas have mutant ras (reviewed in Bos, 1989).

1.5.1 Ras mutations and mouse skin tumorigenesis

Genetic changes affecting H-ras have been reported in the mouse skin model system (Quintanilla et al., 1986; Bremner and Balmain, 1990). Activation of H-ras is an early event. Indeed, a correlation between the type of carcinogen used to initiate tumour formation and the particular activating mutation of H-ras has been demonstrated (Bremner and Balmain, 1990). Although ras gene mutations are probably an important initiating event, they are insufficient for the formation of benign papillomas. These only appear after treatment with a tumour promoter such as TPA which allows the clonal selection of rare initated cells that are surrounded by normal cells. Subsequent genetic alterations accumulate over time and may encourage the malignant conversion of benign papillomas.

Amplified mutant ras has been shown to be associated with spindle cell carcinomas (R. Crombie, PhD thesis, 1994). The mutant ras content of squamous tumour cells increases as they become less differentiated and this increase may encourage the switch to a spindle carcinoma but the complete transition requires the alteration of another locus. Experiments in which the ratio of mutant to normal ras in squamous and spindle cells was altered showed that when the mutant ras allele is lost from spindle cells, only their tumorigenicity and not their morphology is affected (R. Crombie, PhD thesis, 1994).

1.5.2 NF1 and p120GAP are ras effector molecules

The products of the mammalian ras proto-oncogenes, Harvey-ras (H-ras), Kirsten-ras (K-ras) and N-ras are members of a family that includes at least 40 ras-related small GTP-binding proteins (reviewed in Barbacid, 1987) which share the ability to interconvert between an inactive (GDP-bound) and an active (GTP-bound) state (Bourne et al., 1990). The ratio of GTP to GDP on ras can be regulated by either altering the rate of GTP hydrolysis or by altering the rate of nucleotide exchange. Although ras proteins possess intrinsic GTPase and nucleotide exchange functions, several proteins exist that can stimulate GTPase activity (GTPase activating proteins, GAPs) and stimulate exchange (guanine nucleotide dissociation stimulators, GDSs), acting synergistically to control ras activity. The NF1 and GAP proteins both stimulate the GTPase activity of ras.

Deletion or abnormal function of NF1 can contribute to tumours of the central nervous system, indicating that normal NF1 can act as a tumour suppressor (Li et al., 1992; Basu et al., 1992). NF1 defects have also been implicated in other types of sporadic cancer e.g. neuroblastomas (Seizinger, 1993) and melanomas (Andersen et al., 1993) but the action of abnormal NF1 in these tumours is not understood.

The NF1 protein has a high degree of homology with the C-terminal portion of p120GAP and both proteins regulate ras by altering the ratio of rasGDP to rasGTP. In addition to their roles as negative regulators of ras, both GAPs seem to possess a second role as downstream effectors of ras activity (reviewed in McCormick, 1990). NF1 binds ras with a much higher affinity than GAP, indicating that it could be a more significant regulator of ras than GAP, considering the concentration of ras in the cell (Bollag and McCormick, 1991). Indeed, the reduced levels of functional NF1 in Schwannomas result in an elevation of GTP-bound ras although the expression of GAP is normal (Basu et al., 1992; DeClue et al., 1992). However, NF1 is not universally expressed so its effector function may be more important in some cells than others. Whether NF1 acts

as a negative regulator or downstream effector of ras may depend on the cell type or stage in development.

The effector function of p120GAP has been localised to the N-terminal domain which binds to receptor and non-receptor tyrosine kinases e.g. the EGF receptor, PDGF receptor and src (Ellis et al., 1990). Binding of rasGTP to the catalytic domain of p120GAP may cause a conformational change that exposes the N-terminal SH2/SH3 effector domains, thereby permitting their interaction with downstream targets and relaying the ras signal to transcription factors in the nucleus.

Such downstream targets may include two tyrosine phosphorylated proteins, p62 and p190, which have been shown to bind the N-terminal domain of GAP through its SH2 domains (Wong et al., 1992; Settleman et al., 1992). In cells stimulated with EGF or transformed by v-src, GAP forms complexes with p62 and p190 (Ellis et al., 1990; Moran et al., 1991).

p190 contains at least two distinct functional domains; an N-terminal GTPase domain and a C-terminal RhoGAP domain. It is a specific GAP for the rho family, therefore interaction between rasGAP and p190 may coordinate the ras and rho signalling pathways (Hall, 1992).

The second p120GAP associated protein, p62, has significant homology to a hnRNP protein and binds RNA (Wong et al., 1992) therefore it may have a role in RNA processing. However, where this fits in with p120GAP and ras signalling is still not clear since it now seems that binding of GAP to p62 and p190 is independent of ras signalling (Pronk et al., 1993).

p62 may be closely related to p68, another RNA-binding protein. p68 also binds to src and becomes tyrosine phosphorylated during mitosis in normal and src-transformed cells (Taylor and Shalloway, 1994; Fumagalli et al., 1994). It has been suggested that p62 and p68 may both coordinate RNA stability; p62 by regulating cytoskeletal components and p68 by regulating nuclear components. A link between expression levels of these proteins and cell morphology was investigated in this study.

1.5.3 Ras-related proteins as tumour suppressors

Activated rasGTP activates a kinase cascade including raf, MAPKK (mitogen-activated protein kinase kinase) and MAPK (MAP kinase) leading to phosphorylation of transcription factors such as the jun proto-oncogene (reviewed in Roberts, 1992). The entire signalling pathway is conserved in the nematode and Drosophila and its most important elements are found in yeast so it seems to have a central role to play in signalling in eucaryotes.

To date, two mammalian GAPs for ras have been identified; p120GAP which stimulates the GTPase activity of ras proteins by at least 10,000 times (Trahey and McCormick, 1987) and NF1, the gene product of the NF1 tumour suppressor gene, which also increases the rate of GTP hydrolysis. Oncogenic ras mutants have a reduced GTPase activity which is no longer stimulated by these GTPase activating proteins. Consequently, mutants are trapped in the active GTP-bound state and no longer require ligand-induced activation. Downstream signalling leading to the activation of transcription factors is therefore continuously stimulated. While mutations in ras proteins can deregulate their GDP/GTP cycling and lead to constitutive activation of ras function, the inactivation of ras GTPase activators or permanent activation of rasGDP dissociation stimulators may also indirectly lead to constitutive activation of ras. Therefore, GAPs may be putative TS genes while GDSs may be putative oncogenes. Evidence for these possibilities comes from the observation that loss of NF1 function in some human tumours results in elevated rasGTP levels (Basu et al., 1992; DeClue et al., 1992) and overexpression of p120GAP in NIH3T3 cells can suppress transformation by normal H-ras, (Zhang et al., 1990a). It can also reduce the level of rasGTP in unstimulated and PDGF-stimulated cells (Gibbs et al., 1990). However no losses of p120GAP have been identified in tumours.

Molecules which bind rasGTP tightly but do not permit signalling are also candidate TS genes. One such protein is K-rev.

1.5.4 K-rev1 is a ras antagonist

The K-rev1 gene was isolated by its ability to produce morphologically untransformed revertants when transfected into Kirsten sarcoma virus-transformed NIH 3T3 cells. In order to identify new genes affecting the ras signalling pathway, human cDNA libraries were transfected into ras-transformed fibroblasts and cDNAs able to alter the morphology of these cells were isolated (Kitayama et al., 1989). Further analysis of one clone, K-rev1, revealed that its product is identical to rap1, a member of the ras superfamily of GTP-binding proteins (Pizon et al., 1988). K-rev1 induced morphological and biological changes in transfected cells i.e. partial reversion of the malignant phenotype. Its overexpression can revert ras-transformed cells (Zhang et al., 1990b) and can inhibit ras-stimulated gene expression in transient transfections (Sakoda et al., 1992) but the method by which K-rev1 interferes with ras function is not yet clear.

1.6 Epithelial-mesenchymal transitions and invasion are linked

As described earlier, the specific stage of mouse skin carcinogenesis being studied was the advanced stage of tumorigenesis when squamous cell carcinomas progress to become spindle cell carcinomas. This change in phenotype has similarities to alterations seen during tissue development and can be influenced by extracellular factors as well as intracellular signalling via ras-related pathways.

1.6.1 Squamous v spindle carcinoma

In human carcinogenesis, spindle cell carcinomas tend to be recurrent or metastatic tumours and invasion and metastasis of these tumours are major causes of therapeutic failure. The transformed epithelial cells grow in an uncontrolled fashion and break through the basement membrane then invade the underlying mesenchyme. They

can then settle at distant sites and form secondary tumours. Spindle cell carcinomas are relatively rare but analysis of the mechanisms by which they lose almost all of the differentiation markers of the original cell may provide information regarding the control of epithelial differentiation processes, in addition to providing insights into the process of metastasis.

Morphological and functional characteristics of metastasis were recognised years ago and the state of differentiation and degree of invasiveness of a tumour are known to determine cancer prognosis. However, although the epithelial origin of spindle cell carcinomas is well-documented, many questions regarding their development remain to be answered and the underlying molecular basis is only now being investigated.

Epithelial cell tumours i.e. carcinomas, account for 90% of human malignancies. Within this category of tumour there are two distinct types with important phenotypic differences; squamous and spindle carcinomas. Well-differentiated squamous carcinomas retain epithelial tissue structures, have well-developed intercellular junctions and are usually weakly invasive but they can become more invasive and metastatic and produce secondary tumours which are also squamous. This process may be facilitated by the involvement of spindle intermediates which appear transiently and act as a migratory form of the squamous carcinoma cells. A more stable epithelial organisation may be recovered once these cells have reached another site where conditions suit this type of growth. True spindle carcinomas, in contrast, are always poorly-differentiated and have an amorphous tissue structure, few cell-cell junctions and are more invasive. They have lost so many of the typical epithelial markers that their histology can sometimes be misleading and they may be mistaken for fibrosarcomas, but *in situ* they are still identifiable as carcinomas and some evidence of their epithelial origin can still be detected. They have been described in numerous organs in humans e.g. lung (Matsui and Kitagawa, 1991) breast (Kaufman et al., 1983) cervix (Steeper et al., 1983; Evans and Smith, 1980) and are also seen in experimentally induced skin neoplasia (Klein-Szanto et al., 1989).

One interesting marker that helps to differentiate squamous and spindle carcinomas is the type of intermediate filament protein they express. Studies of tumours induced in mice have demonstrated changes in the cytokeratin differentiation markers associated with neoplasia in general (Klein-Szanto et al., 1983; Roop et al., 1988). Cytokeratins 8 and 18 are the pair of keratins associated with simple epithelia and early stages of embryogenesis. They are not normally found in adult epidermis but have been shown to be expressed in human skin carcinomas, mouse tumours and in mouse keratinocytes transfected with oncogenic H-ras (Diaz-Guerra et al., 1992). Papillomas express keratins characteristic of basal cells (K5 and K14), suprabasal cells (K1 and K10) and hyperproliferative skin (K6 and K16) and squamous carcinomas show a dramatic reduction in expression of K1 and K10 but not the other keratins (Knapp et al., 1987) .

Studies by this group have shown a similar loss of intermediate filament differentiation markers in a number of mouse spindle cell lines (Stoler et al., 1993). In fact, spindle carcinomas usually express vimentin which is more typical of fibroblasts than epithelia. Vimentin expression provides a marker for the spindle cell phenotype but this altered intermediate filament switch alone does not give rise to the spindle phenotype (Sommers et al., 1992). These results are consistent with the altered differentiation state of malignant tumours. Many of these differences in cytoskeletal structure echo changes that occur during the initial formation of tissues and organs in the embryo.

1.6.2 Epithelial-mesenchymal transitions

The first tissues to emerge in the early vertebrate embryo are epithelial in nature and the mesenchymal cells of the body all derive from these embryonic epithelia in an epithelial-mesenchymal transition (EMT). Both tissue types are found in nearly every organ where they cooperate and support each others growth.

The two cell types are very different phenotypically. Epithelial cells are closely associated with their neighbours by forming tight intercellular junctions and are thus immobile. Mesenchymal cells are, in contrast, only loosely associated and are therefore more mobile. During organ development, transitions from epithelia to mesenchyme occur and vice versa e.g. in neural crest development, neural crest cells change from an epithelial to mesenchymal phenotype (Thiery et al., 1982) and during kidney development, mesenchymal cells shift to an epithelial phenotype, (Vestweber et al., 1985; Ekblom, 1989). This plasticity of cell phenotype is also evident in tumour development. As described in section 1.6.1, carcinomas can exist in squamous or spindle forms. These are reminiscent of epithelial and mesenchymal tissues respectively.

With a view to elucidating the factors controlling this switch in normal tissues and in disease states, numerous experimental approaches have been used to induce or revert the epithelial-mesenchymal transition. The conversion of epithelial cells into cells expressing mesenchymal properties can be studied *in vitro* e.g. in lens epithelium (Greenburg and Hay, 1986), in Madin-Darby canine kidney cells (Zuk et al., 1989) and in certain carcinoma cell lines (Boyer et al., 1989). Thus, many modulators of the epithelial phenotype have been identified. These include motility and growth factors that activate receptor tyrosine kinases; scatter factor (SF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) can all convert epithelial cells to a fibroblastoid phenotype (Barrandon and Green, 1987; Stoker et al., 1987). Even simple treatments such as growing certain cell lines in low calcium conditions can change their morphology (Hennings et al., 1980).

Extracellular matrix molecules e.g. syndecan and laminin also affect cell-cell adhesion and differentiation, as do some epithelial junction complex proteins. Oncogenes may also be involved; transfection of cells with oncogenes such as ras (Garcia et al., 1986), expression of an inducible c-fos construct (Reichmann et al., 1992) or a temperature sensitive v-src protein (Behrens et al., 1993; Hamaguchi et al., 1993)

can convert epithelial into mesenchymal cells which now have an altered differentiation program and express fibroblast proteins.

1.6.3 Control of epithelial morphology and motility

Epithelial morphogenesis is almost certainly controlled by a gene which is lost or inactivated in spindle cells rather than one which is activated or amplified. Evidence supporting this hypothesis has come from cell fusion studies which showed that the morphology of hybrids between spindle cells and epithelial cells was very similar to that of the epithelial parent (Stoler et al., 1993). Identification of the gene controlling epithelial differentiation characteristics is of great importance to carcinogenesis and development studies.

Changes in differentiation programs are typical of tumour cells but they are also necessary for various normal cellular processes. An essential requirement for tissue morphogenesis and remodelling is changes in cell adhesion molecules. Cells of any particular type possess a set of adhesive properties that may be both spatially and temporally regulated. A considerable amount is now known about the molecular mechanisms that mediate these properties both in normal tissue and in carcinogenesis.

1.6.4 Invasion and metastasis

Clinically, histological criteria of malignancy are cellular polymorphism i.e. variation in size, shape and staining pattern of cells and nuclei, a high proportion of mitotic cells, invasive growth and metastasis. Invasion is a hallmark of malignancy and a prerequisite for metastasis. In 1986, Liotta put forward a 3 step theory of invasion: locomotion, adhesion to basement membrane and dissolution of basement membrane (Liotta, 1986).

Defective cell adhesion is a common feature of tumours and is thought to play a part in the invasive and metastatic behaviour of neoplastic cells. Invasive cells break

through the basement membrane and invade the underlying mesenchyme, spread into the tissues surrounding the primary tumour, enter blood vessels, lymphatics or body cavities and become dispersed to distant sites. Some may become trapped at new sites, extravasate and form new secondary tumours. This is a complex series of events that may involve various altered cellular properties such as secretion of proteolytic enzymes, alterations in cell motility, growth properties and adhesiveness; even before invading other tissues, the metastasising cells must loosen their contact with neighbours and pass through the basement membrane barrier.

Various oncogenes have been implicated in invasion and metastasis processes (Ki-ras and HER2/neu) and various TS genes have been shown to be deleted or mutated at different stages of carcinogenesis including the benign/malignant transition e.g. RB, WT, p53 and DCC. The existence of specific metastasis suppressor genes has even been suggested by analyses with cell fusion and microcell-mediated chromosome transfer experiments (Ramshaw et al., 1983; Ichikawa et al., 1991; Ichikawa et al., 1992b; Ichikawa et al., 1992a). Metastatic potential is suppressed when metastatic cells are fused with various non-metastatic cells or receive exogenously introduced chromosomes, suggesting that the loss of genetic information is a recessive trait.

1.6.5 Cell adhesion molecules as tumour suppressors

Likely candidates for the role of metastasis suppressors are cell adhesion proteins. In contrast to normal, highly organised epithelial tissues, carcinomas show reduced differentiation and a loss of normal tissue structure, partly due to impaired adhesion to their environment. In extreme cases, i.e. malignant tumours, there is invasion into surrounding normal tissues. *In vitro* properties of some transformed cells such as loss of contact inhibition and the ability to grow in soft agar suggest that tumour cells have lost the ability to respond normally to growth-regulatory signals induced by cell-cell and cell-extracellular matrix interactions. Cellular processes that control these characteristics can be influenced by soluble extracellular factors e.g. hormones and

growth factors or structural interactions between neighbouring cells or between cells and the extracellular matrix.

Cell-cell and cell-matrix interactions involve cell surface adhesion molecules i.e. integrins, cadherins, members of the immunoglobulin superfamily and selectins. These molecules, in addition to providing physical contacts, may act as receptors that transduce extracellular signals and influence tumorigenesis via growth, differentiation and invasion pathways. Therefore, they could be classed as potential tumour suppressor genes. Indeed, the DCC protein that is deleted in colorectal cancer is thought to be a N-CAM-like cell adhesion molecule (Fearon et al., 1990) and the *fat* gene implicated in imaginal disc hyperplasia in Drosophila is a cadherin-like molecule (Mahoney et al., 1991).

1.6.6 E-cadherin as an invasion suppressor

In most tissues a major contribution to cell-cell adhesion is made by calcium-dependent cell adhesion molecules, the cadherins. E-cadherin is found exclusively in epithelial cells and is lost during the transition to the mesenchymal phenotype e.g. during early development when mesoderm separates from ectoderm (Takeichi, 1988).

A number of reports have discussed the correlation of loss of E-cadherin expression and acquisition of a fibroblastoid morphology and invasive properties in epithelial tumour cells (Behrens et al., 1989; Chen and Obrink, 1991; Frixen et al., 1991). In a series of human tumour cell lines, invasion into collagen gels was inversely related to expression of E-cadherin and invasion was inhibited by transfection with a vector expressing E-cadherin (Frixen et al., 1991). The same was true for dog kidney or mouse mammary cells (Vleminckx et al., 1991). This suggests a possible "invasion suppressor" function for this cell adhesion molecule which may be a master molecule in the organisation of epithelial junction complexes.

Consistent with this hypothesis are the numerous observations of a correlation between loss of epithelial differentiation and gain of invasiveness with absent or non-

functional E-cadherin protein. However, in some dedifferentiated tumours, E-cadherin expression was apparently normal, despite reduced intercellular adhesiveness (Shimoyama and Hirohashi, 1991). The permanent loss of E-cadherin expression is not essential for malignancy and metastasis since tumour cells present in various carcinomas and their metastases, or in metastasis-derived cell cultures are found to re-express E-cadherin. Instead, the transient down-regulation, rather than irreversible loss of E-cadherin function, is thought to be a major factor contributing to the malignancy of carcinomas.

E-cadherin function may be abolished by biochemical modifications. The most likely explanation is that E-cadherin itself or one of the junctional complex proteins, perhaps one of the catenins, is phosphorylated and is therefore rendered non-functional. The reversibility of phosphorylation means that the proper structures could reform in metastases under different growth conditions. Some immunohistochemical studies of carcinomas have shown that although they express E-cadherin, the protein is not found in the correct location i.e. at regions of plasma membrane touching adjacent cells (Umbas et al., 1992). Transformation of chick embryo fibroblasts has provided evidence to support this hypothesis (Hamaguchi et al., 1993). It was shown that Rous sarcoma virus suppressed N-cadherin-mediated cell-cell adhesion without inhibiting its expression. In fact, the effect on adhesion was due to tyrosine phosphorylation of catenins and this was transformation specific.

Further evidence for the involvement of E-cadherin in tumorigenesis has also come from genetic analysis of tumours. Loss of heterozygosity (LOH) analysis has demonstrated that a particular region of human chromosome 16q is often deleted in several tumour types, including prostate and hepatocellular carcinomas (Tsuda et al., 1990; Carter et al., 1990). LOH of 16q has been correlated with tumour progression and the deleted region has been narrowed down to 16q22.1, close to the E-cadherin gene locus. However, the best evidence has come from two recent studies which screened 135 endometrial and ovarian carcinomas or tumour cell lines and found a small number of E-cadherin mutations (Risinger et al., 1994; Oda et al., 1994). Altogether, the

functional analysis of E-cadherin *in vitro*, its altered expression in human tumours and the loss of 16q suggest that E-cadherin may be a tumour suppressor involved in the development of several types of carcinoma.

A similar correlation was described for another cell surface marker involved in cell-cell and cell-matrix interaction - syndecan (Inki et al., 1992). It is lost in poorly differentiated tumours but expressed in well-differentiated tumours. There are likely to be several surface molecules which maintain the complex three-dimensional structure of the stratified epithelial tissues and are absent in mesenchymal cells and epithelial cells which have lost differentiation markers. Whether losses of these molecules are the initiating events causing loss of differentiation or are merely consequences remains to be seen.

1.7 Tyrosine kinases and phosphorylation events affect cell morphology and motility

An important function of cell adhesion molecules in addition to adhesive binding is transmission of signals across cell membranes. This will have relevance to the altered growth state of neoplasia. Study of this process has until recently been limited but it is now a subject that is receiving a great deal of attention and advances have been made regarding the involvement of adhesion molecules in signal transduction.

Members of all three of the large families of cell adhesion molecules, the cadherins, integrins and immunoglobulin-like superfamily, have been shown to participate in signalling. Signals can be transmitted from the outside to the inside of the cell in response to ligand binding, thus affecting second messengers and gene transcription. They can also pass from the inside to the outside of cells, modulating the binding affinity of cell adhesion molecules.

Three major enzymatic activities are important in growth factor signalling cascades:

- tyrosine-specific phosphorylation
- phospholipase C activation
- cAMP production by adenylate cyclase

It is the first of these that is most relevant to signalling by adhesion molecules and modulation of the epithelial phenotype. Some effectors of epithelial junction signalling are receptor tyrosine kinases e.g. the met receptor, FGF receptors and pp60src kinase. In addition, some motility and growth factors that activate receptor tyrosine kinases (RTKs) disrupt epithelial cell-cell adhesiveness e.g. scatter factor which binds to met and aFGF which binds to the FGF receptor. Junctional complexes are major sites of tyrosine phosphorylation (reviewed in Lo and Chen, 1994), so altered growth conditions can have an effect on these sites.

While cadherins are major participants in cell-cell adhesion, the most important complex with respect to cell-matrix contact is the focal adhesion. This complex comprises a large number of linked proteins whose activities are modulated by tyrosine phosphorylation, a recurring means of control where transmission of signals is concerned.

1.7.1 Focal adhesions link the ECM and cytoskeleton

Focal adhesions are specialised regions of plasma membrane where cells adhere to the substratum and where actin filament bundles terminate. They are the site where integrins and many other proteins are clustered, forming a link between the ECM and actin cytoskeleton (figure 5). The involvement of focal adhesions in cell adhesion, shape and motility suggests that effectors of focal adhesion may be affected during transformation.

Focal adhesions are relatively stable structures but frequently have to be assembled and disassembled e.g. during mitosis and migration when cells change shape rapidly and become more round. Major reorganisations of focal contacts are thought to be responsible for the loss of cell adhesion and accompanying morphological changes

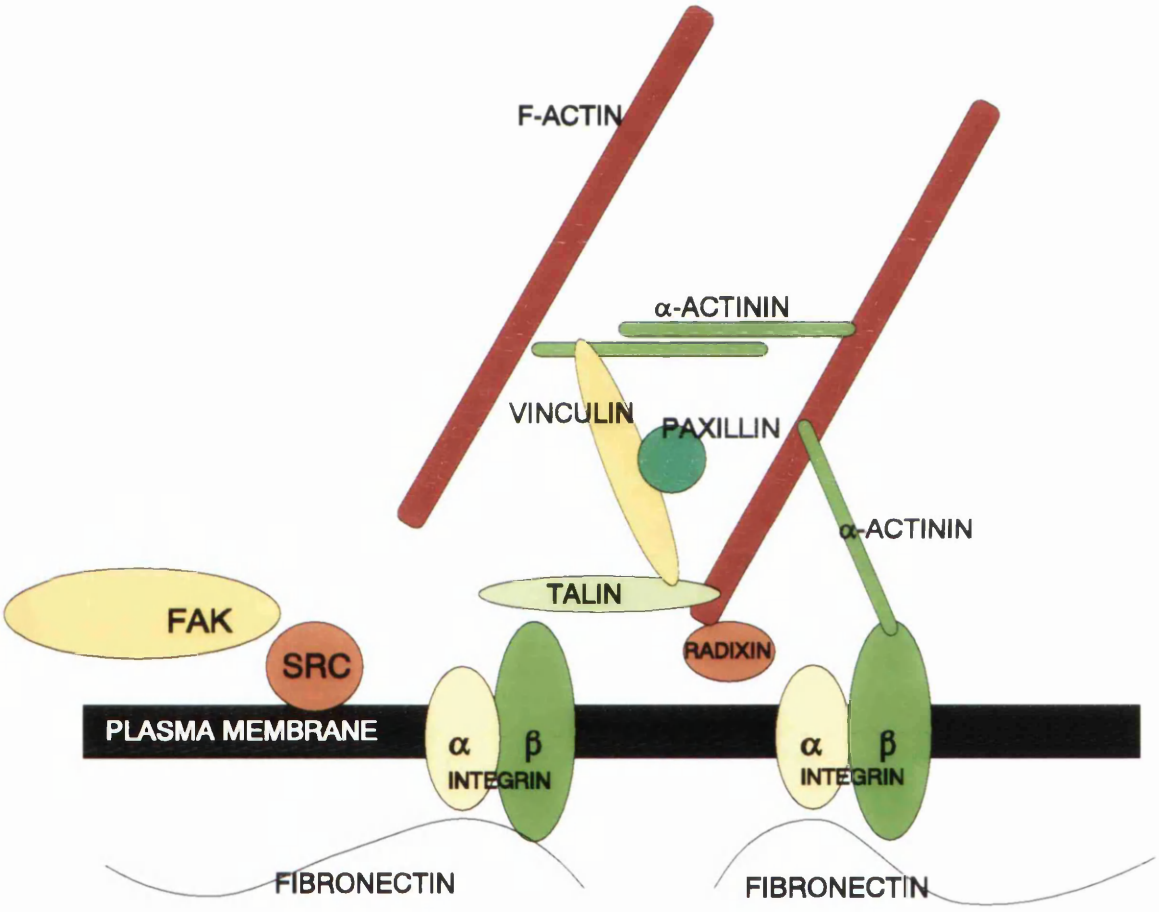


Figure 5: Relationships between focal adhesion proteins and cytoskeletal proteins.

during tumorigenesis since, in general, transformed cells are more rounded and less adherent than normal cells. These changes are accompanied by a reduced amount of focal adhesion and alterations to actin fibre assembly (reviewed in Kellie, 1988).

Focal adhesions might be sites where external signals such as mitogenic stimulation are transduced into the interior of cells. Various stimuli such as growth factors and viral transformation have been used to study the signals involved in regulating cytoskeletal interactions at focal adhesions. A common feature of these factors affecting the cytoskeleton is their activation of kinase activities, suggesting that phosphorylation of focal adhesion components could be involved in focal adhesion assembly and disassembly. The localisation of v-src and increased phosphotyrosine levels in focal adhesion after RSV-induced transformation, (section 1.7.4), imply that protein modification may have direct effects on the cytoskeleton but the consequences of phosphorylation of focal adhesion proteins remain to be established. One possibility is that they provide a binding site for the src homology 2 (SH2) domains of other proteins, thus transferring the signal downstream.

1.7.2 Integrins mediate adhesion and signalling

Integrins are the main signalling components of focal adhesions. Many of the adhesive interactions of tumour cells are mediated by the integrin family of cell surface receptors (reviewed in Hart and Saini, 1992 and Honn and Tang, 1992). Recently they have also been implicated in mediating signalling from the ECM to intracellular pathways that affect cell proliferation and morphology (reviewed in Damsky and Werb, 1992 and Schwartz and Ingber, 1994).

Integrins are the predominant family of cell surface receptors for ECM and basement membrane components including fibronectin, collagen, laminin and vitronectin. They are composed of an α and β subunit and grouped into families according to their β subunit (reviewed in Hynes, 1992). The $\beta 1$ and $\beta 3$ subfamilies bind ECM molecules and some integrins show further specificity for individual ECM

components which are present in the basement membrane of the skin. The basement membrane forms the junction between epidermis and underlying connective tissue (mesenchyme) and provides the attachment site for the basal layer of the epidermis.

By providing interactions between cells and their ECM, the integrins are believed to play a crucial role in tissue formation and maturation in the epidermis (Adams and Watt, 1990; Hertle et al., 1991). *In vitro* studies showed that leaving the basal layer is accompanied by a change in integrin function, followed by a loss of expression of $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 5\beta 1$ (Adams and Watt, 1990).

There have been reports of altered integrin expression in various cell types e.g. the decreased expression of integrin $\alpha 5\beta 1$ in some transformed cells (Hynes, 1992), the link between $\alpha 6\beta 4$ and malignant squamous carcinomas (Tennenbaum et al., 1993) and up-regulation of $\alpha 5\beta 1$ integrin in spindle cell carcinomas (Gomez et al., in press). Overexpression of $\alpha 5\beta 1$ integrins in transformed cells can inhibit aspects of their transforming behaviour such as slightly increased adhesion to fibronectin, decreased growth in soft agar and reduced ability to form tumours in nude mice (Giancotti and Ruoslahti, 1990).

These results suggest that changes in the levels of integrin expression are important in the interaction of cells with ECM components and impairment of interactions between the ECM and its receptors may mediate cell transformation *in vitro* and *in vivo*. There does not seem to be a simple loss of integrins as tumours progress and down-regulation of integrins is not a general characteristic of malignancy. It may instead be linked to the acquisition of a more fibroblast-like phenotype and invasive capability.

It has become clear that integrins, as components of focal adhesion complexes, can participate in signal transduction processes as well as in adhesion. Integrin cytoplasmic domains are known to mediate inside-out signal transduction (O'Toole et al., 1994) but current knowledge of the signalling through focal adhesions is mostly from outside to inside. Integrin-mediated cell adhesion or clustering of integrins on the cell surface using antibodies results in the enhanced tyrosine phosphorylation of certain

intracellular proteins, suggesting that integrins may play a role in a signal tranduction cascade (Guan and Shalloway, 1992; Burridge et al., 1992). In cultured fibroblasts, carcinoma cells and platelets a cytoplasmic tyrosine kinase (p125 FAK) has been implicated in integrin-mediated tyrosine phosphorylation (reviewed in Schaller and Parsons, 1994 and Lo and Chen, 1994).

1.7.3 FAK is a focal adhesion signalling protein

Focal adhesion kinase (FAK) is a major target for tyrosine kinases during various cellular events associated with cell adhesion and growth control. It is a cytoplasmic tyrosine kinase itself but, unlike most other tyrosine kinases, does not have any SH2 or SH3 domains. FAK has been localised to focal adhesions by immunofluorescence experiments (Schaller et al., 1992) and shown to be phosphorylated following clustering of integrins with anti-integrin antibodies or by plating cells on fibronectin (Hanks et al., 1992; Kornberg et al., 1992). This increase in tyrosine phosphorylation is accompanied by an increase in FAK activity measured *in vitro* (Guan and Shalloway, 1992), suggesting that FAK is a downstream transducer of an integrin-mediated signal. Attention is now focused on identifying the targets of FAK.

Paxillin and tensin are good candidates. They colocalise with FAK at focal adhesions (Davis et al., 1995; Turner et al., 1990) and are phosphorylated in a FAK-dependent manner (Schaller et al., 1993). Tyrosine phosphorylation may regulate their interactions with other proteins which could be other components of focal adhesions.

FAK has also been implicated as a substrate for v-src and therefore may be an important protein in transformation processes mediated by src. The tyrosine phosphorylation of FAK is increased in cells transformed by pp60src (Guan and Shalloway, 1992) and most of it exists as a complex with pp60src (Cobb et al., 1994). However, nothing is known about the way in which the association of these two non-receptor kinases contributes to transformation. It may cause increased tyrosine phosphorylation of focal adhesion proteins which then leads to reorganisation of

cytoskeletal structures as observed in src-transformed cells (Burridge et al., 1992). Indeed, increased tyrosine phosphorylation of tensin, paxillin, vinculin, talin and β1 integrin have been reported in src-transformed cells (reviewed in Lo and Chen, 1994). The complex of activated FAK and src in focal adhesions of transformed cells may also promote intracellular signals through pathways normally under the control of cellular tyrosine kinases.

1.7.4 V-src causes epithelial-mesenchymal transitions and increased invasiveness

V-src is localised in cell-cell and cell-ECM junctions in fibroblasts transformed by Rous sarcoma virus (Rohrschneider, 1980). This location is relevant to some of the transformation-related effects of v-src.

Activation of temperature-sensitive v-src causes changes in cell morphology and increased invasiveness as shown by collagen invasion assays (Behrens et al., 1993) although over-expression of c-src alone does not alter cell-cell adhesion (Iba et al., 1984). V-src causes reversible cell dissociation and its effect is seen in 15min, therefore rapid enzymatic reactions rather than changes in transcription or translational control are likely to be involved in initial steps. Indeed, the morphological effects of src are linked to increased tyrosine phosphorylation of E-cadherin and β-catenin in MDCK cells (Behrens et al., 1993). The same is true in chick embryo fibroblasts (Hamaguchi et al., 1993). Therefore, tyrosine kinases may regulate intercellular adhesion via modification of catenins. This supports the hypothesis discussed previously (section 1.6.6) that loss of E-cadherin function, not necessarily expression, is required for transformation.

Src also interferes with cell-substratum adhesion by phosphorylation of integrin receptors (Tapley et al., 1989). It is therefore involved in the regulation of formation and activity of focal contact complexes. Both forms of src (cellular and viral) are physically associated with junctional complex proteins such as focal adhesion kinase,

FAK; in pp60src-transformed cells, more than 80% of FAK is found as a stable complex with pp60src (Cobb et al., 1994).

The link between src transformation and changes in cytoskeletal and membrane-bound signalling proteins is an area of intense investigation at the moment but the physiological targets of activated c-src have not yet been identified.

Transformation and mitosis both involve major reorganisation of the cytoskeleton, possibly by similar pathways. Therefore, studies of the effects of src transformation on cell morphology and cytoskeletal components may provide clues to the events that take place during mitosis also.

1.8 Growth factors can induce the epithelial-mesenchymal transition

1.8.1 Acidic fibroblast growth factor and epidermal growth factor modulate epithelial cell morphology

Other types of transmembrane signalling can cause changes in cell morphology and motility. These are mediated by growth factor receptors which bind acidic FGF or EGF. aFGF and EGF both induce the squamous to spindle transition in certain cell lines and both act through receptors at the plasma membrane.

As with focal adhesion signalling, tyrosine kinase activity is central to the two biological responses to aFGF. Treatment of subconfluent cultures of NBT-II cells makes them dissociate and produces individual elongated, motile cells (Valles et al., 1990a), while confluent cultures enter mitosis (Valles et al., 1990b). Inhibitors of tyrosine phosphatases can reproduce the two effects of aFGF, thus providing evidence for the importance of tyrosine phosphorylations in this pathway.

EGF has also been reported to induce confluent cultures of rat epithelial cells to proliferate and subconfluent cultures to migrate (Blay and Brown, 1985). This observation has been extended to human bladder carcinoma cells which also disperse in response to treatment with physiological concentrations of EGF (Tucker, pers. comm.).

A prerequisite for carcinoma spreading and metastasis is the dissociation of the tumour mass, allowing some cells to escape and eventually migrate away from the primary site of tumour growth. The acquisition of cell motility is accompanied by dramatic changes in the program of cell differentiation. Although keratinocytes can be motile, the motile cells more often resemble mesenchymal cells which no longer express cytokeratin filaments but express vimentin instead. The cell-cell adhesion typical of epithelial cells mediated by molecules such as E-cadherin is also reduced (Boyer et al., 1992), encouraging motility. Cells which have broken through the basement membrane are in close contact with connective tissue rich in extracellular components where local growth factors, such as EGF and aFGF, produced by endothelial cells may then play a crucial part in tumour cell motility by enhancing cell scattering. However, these are not the only growth factors that induce scattering.

1.8.2 Scatter factor induces the epithelial-mesenchymal transition

Tyrosine kinase activity is also involved in changes in cell morphology caused by scatter factor (SF). SF was initially described as an activity secreted by human embryo fibroblasts that caused epithelial cell colonies to dissociate or scatter (Stoker and Perryman, 1985). It is identical to HGF which was originally identified as a potent mitogen for hepatocytes in culture (Nakamura et al., 1987) and is also mitogenic for melanocytes, endothelial cells and epithelial cells (Rubin et al., 1991).

The effects of scatter factor are varied. They include increasing cell motility and scattering (Gherardi et al., 1989), induction of the epithelial-mesenchymal transition, growth stimulation and growth inhibition (Stoker et al., 1987). SF also promotes the invasiveness of human carcinoma cell lines into collagen matrices, suggesting that this factor may be involved in tumour metastasis (Weidner et al., 1990).

The c-met proto-oncogene product, a constitutively phosphorylated intracellular tyrosine kinase, has been identified as the oncogenic form of the SF receptor (Bottaro et al., 1991). It transduces motility, proliferation and morphology signals of SF in

epithelial cells (Weidner et al., 1993). *In situ* hybridisation has shown that met is expressed in epithelial cells (Chan et al., 1988; Iyer et al., 1995), whereas SF is expressed in mesenchyme (Stoker and Perryman, 1985; Weidner et al., 1990). This is an example of a paracrine agent mediating the interaction between adjacent epithelial and mesenchymal tissues, thus supporting each other's growth requirements. Cell dissociation and motility triggered by SF are mediated by the met receptor tyrosine kinase (Weidner et al., 1990), in particular its cytoplasmic domain (Komada and Kitamura, 1993).

Although expression of SF cDNA in MDCK cells alters cell morphology, motility and anchorage-independent growth properties (Uehara and Kitamura, 1992), NIH3T3 fibroblasts cotransfected with the c-met receptor tyrosine kinase and with its ligand, SF, can form tumours with epithelial characteristics (Tsarfaty et al., 1994). Therefore, a role for SF in the differentiation and invasion of tumour cells is still uncertain.

1.9 ECM components influence tumour cell morphology

Extracellular signals affecting tumour cell morphology do not always act through growth factors; the extracellular matrix itself can be an important influence. Extracellular interactions, including the formation of cell-cell junctions and contacts with extracellular matrix (ECM) and tissue stroma, are important in regulating development and stabilising normal tissues. They are also important in tumours. The extracellular environment of tumours is determined by the matrix synthesised by normal and tumour cells, as well as host stromal components secreted by surrounding or infiltrating host fibroblasts and other normal cells. Components of this ECM and stroma can produce signals that are transduced into cells and lead to alterations in gene expression and cell phenotype. Therefore, unravelling the molecular pathways by which the ECM controls differentiation in epithelial cells is of importance in

understanding the changes in differentiation states that are a feature of tumorigenesis and metastasis.

During tumorigenesis, drastic alterations in intercellular and cell-substratum interactions take place; such alterations are particularly evident in tumours of epithelial origin. Neoplastic cells are often altered in their response to ECM and stroma (reviewed in Ruoslahti, 1992) and when cells are transformed, contact-mediated cell communication systems are altered (Mesnil and Yamasaki, 1993). The growth of cancer cells is often independent of adhesion to the ECM and a characteristic of transformed cells is a reduction in adhesion to solid substrates. This is thought to reflect the invasive and metastatic potential of these cells *in vivo*.

In summary, the environment of tumour cells still influences their characteristics and behaviour. It is accessible to soluble growth factors and hormones that modulate processes such as differentiation and proliferation. Furthermore, several genes e.g. DCC and *fat* which were defined as tumour suppressors by virtue of their loss during tumour progression code for proteins that are constituents of the ECM (Hedrick et al., 1993). Therefore, it seems that the actions of oncogenes and growth factors are insufficient to explain carcinogenesis and the effects of ECM components must also be considered.

1.9.1 TGFβ modulates interactions of cells with their extracellular matrix

The influences of growth factors with their associated tyrosine phosphorylation events and the effects of ECM components on epithelial cells have already been described. These different aspects of cell-ECM signalling can be connected by one growth factor, TGFβ, which can modulate interactions of cells with their environment by inducing synthesis of ECM components.

TGFβ was first described as a soluble factor capable of transforming normal rat kidney cells in the presence of TGFα or EGF (Roberts et al., 1981). It is now known to have different effects on cell growth depending on the particular cell type and growth conditions and has numerous properties including:-

- inhibition or stimulation of mitogenesis
- cellular transformation
- induction of ECM component synthesis
- stimulation or reduction of expression of metalloproteinases
- induction of synthesis of proteases inhibitors
- stimulation of integrin expression
- induction of scatter factor expression

The 3 isoforms of TGFβ - 1, 2 and 3 - are expressed in distinct but overlapping patterns in most tissues where their biological activities are mediated by the type I and type II TGFβ receptors. Thus TGFβ1 exerts a wide variety of biological activities on many cells both in culture and *in vivo*.

The growth of most normal cell lines, whether primary or immortalised, is completely inhibited by TGFβ. In contrast, most malignant cells are refractory to its antiproliferative effects *in vitro*; malignant carcinomas require much higher levels of TGFβ to achieve a similar degree of growth suppression and some are only partially inhibited, whatever the concentration (Shipley et al., 1986; Wakefield et al., 1987). Resistance seems to be a late event in the process of transformation *in vitro* and experimental data suggest that tumour cells *in vivo* also become refractory at a late stage (Haddow et al., 1991; Manning et al., 1991).

Loss of sensitivity to TGFβ may be linked to the development of invasive properties since overexpression of TGFβ1 in carcinoma cells is associated with tumorigenesis and metastatic spread (Walker and Dearing, 1992; Walker et al., 1994; Gorsch et al., 1992).

The crucial properties of TGFβ with respect to invasion are connected with its involvement in cell-ECM interactions. TGFβ can modulate interactions of cells with their ECM partly by inducing the synthesis of various ECM proteins. It induces the deposition of individual ECM components such as fibronectin and collagens (Ignotz et al., 1987) and modifies cell-matrix interactions via integrins (Gailit et al., 1994; Ignotz et al., 1989), (Heino et al., 1989). It can also block matrix degradation by reducing the

synthesis of proteases, while increasing the synthesis of protease inhibitors (Edwards et al., 1987). These functions are important in normal cell processes such as wound repair and tissue remodelling. TGFβ plays an important role in embryogenesis (Heine et al., 1987) and there is an abundant supply of TGFβ1 in wounded tissue where it is spatially and temporally regulated (Kane et al., 1991).

One major role of TGFβ in these normal situations seems to be the control of integrin expression. If this relationship between TGFβ activity and integrin expression becomes deregulated e.g. in a carcinoma, affected cells may become more motile at inappropriate times and locations. As a result, the tumour may have acquired the capacity to invade surrounding tissue. Increased degradation of existing matrix components may aid this process. Studies of rat mammary adenocarcinoma cells have shown that exogenous TGFβ may modulate the metastatic potential of mammary tumour cells by controlling their ability to break down and penetrate the barrier normally formed by the basement membrane (Welch et al., 1990).

1.9.2 Extracellular proteases

As discussed previously, the extracellular matrix influences the development and morphogenesis of vertebrate embryos and influences basic cellular processes such as proliferation, differentiation, migration and adhesion. A dynamic equilibrium exists between synthesis and degradation of the matrix. Degradation is mediated by extracellular proteases which digest the ECM on the cell surface (Chen, 1992) and metalloproteinases which may have substrate-specific activity e.g. collagenase or more general activity e.g. stromelysin. The activity of these enzymes is tightly regulated partly by specific tissue inhibitors of metalloproteinases (TIMPs).

Degradation of ECM components by matrix-degrading metalloproteinases (MMPs) is normally strictly controlled at multiple levels. The action of MMPs and TIMP can be regulated by growth factors and hormones e.g. TIMP expression is induced by EGF (Edwards et al., 1987). In neoplasia, regulation can be lost and

abnormally high levels of expression of some MMPs can be caused by growth factors, tumour promoters, oncogenes and hormones (Matrisian and Hogan, 1990).

Other factors can have the opposite effect e.g. TGFβ is a potent inducer of ECM components and has been shown to affect MMP and TIMP levels in some systems (Edwards et al., 1987). TGFβ selectively reduces expression of collagenase and stromelysin but cooperates with other growth factors to elevate TIMP expression beyond its normal level. Therefore, the effects of TGFβ are partly due to changes in the control of MMP and TIMP expression. This combines with the effects of TGFβ on matrix synthesis induction to maintain matrix structure.

However, exposure of normal cells to TGFβ has the opposite effect to that found in transformed cells where TIMP expression is repressed and MMP expression is constitutively elevated. Remodelling of the matrix may be required for changes in the proliferation, differentiation or migratory state of tumour cells. The changes are needed for complex physiological processes such as wound healing or morphogenesis and are also evident in cancer. Tumour cell invasion has been proposed to involve a 3 step process; tumour cell adhesion, release of ECM-degrading enzymes and tumour cell motility. Clearly, extracellular proteases and TIMPs may be involved in the control of these processes.

2.1 Materials

2.1.1 Chemicals

All chemicals were of AnalaR grade and were obtained from BDH Chemicals Ltd., Poole, Dorset or Sigma Chemical Co. Ltd., Poole, Dorset except those obtained from the suppliers listed below.

Amersham International PLC, Amersham, Bucks

all radioisotopes

rainbow protein markers

ECL Western blotting detection kit

Beatson Institute Central Services

L-broth

sterile PBS

PE

Becton Dickinson Labware, Bedford, MA

rat tail collagen, type I

BDH

Repelcote

Biogenesis Ltd., Bournemouth

RNazol B

Boehringer Mannheim UK Ltd., Lewes, East Sussex

proteinase K

Taq polymerase

DOTAP transfection reagent

TGFβ-1

BRL (UK), Gibco Ltd., Paisley

all restriction enzymes

Taq polymerase

J. Burrough (FAD) Ltd., Witham, Essex

ethanol

Difco Labs., Detroit, Michigan

Bacto-tryptone

FMC

Nusieve agarose

Gateway plc, Glasgow

Marvel dried non-fat milk powder

Gibco Europe, Life Technologies Ltd., Paisley

SLM

10xDMEM

foetal calf serum

200mM glutamine

2.5% trypsin

penicillin

streptomycin

geneticin (G418)

Rathburn Chemicals Ltd., Walkerburn

phenol (water saturated)

Severn Biotech, Kidderminster

acrylamide (30% and 40%)

Sigma Chemical Co Ltd., Poole, Dorset

Bicinchoninic acid kit for protein concentration determination

epidermal growth factor, EGF (tissue culture grade)

acidic fibroblast growth factor, aFGF (tissue culture grade)

fibronectin

agarose

NP40

Tween20

ethidium bromide

TEMED

β-mercaptoethanol

PMSF

aprotinin

Na deoxycholate

EGTA

orange G

bromophenol blue

xylene cyanole

protein A sepharose

Stratech Scientific, Luton, Beds

Matrigel

Vector Laboratories Inc., Burlington USA

Vectashield mounting medium

horse serum

2.1.2 Equipment and plasticware

Amersham International PLC, Amersham, Bucks

Hybond-N membrane

Becton Dickinson Labware, Plymouth, Devon

tissue culture dishes (60,100mm)

Bibby-Sterilin Ltd., Stoney, Staffs

bacteriological dishes (60mm)

Biorad

Zetaprobe membrane

Cambridge Electrophoresis

EV400 gel tank

Eastman Kodak Co., Rochester, New York

x-ray film (XAR-5)

duplicating film

pan F b/w film

Ektachrome colour slide film

Fuji Photo Co. Ltd., Japan

x-ray film (RX)

Gibco Europe, Life Technologies Ltd., Paisley

Nunc 1ml cryotubes

Nunc 8-well chamber slides (permanox)

Griener Labortechnik Ltd., Dursley

Eppendorf tubes

Labsystems, Basingstoke

pipette tips

Molecular Bioproducts, San Diego CA

aerosol-resistant tips

Pharmacia Ltd., Milton Keynes

Nick columns

Schleicher & Schuell, Inc. Dassel, Germany

SA85 nitrocellulose membrane

Whatman International Ltd., Maidstone

3MM paper

2.1.3 Antibodies

Affiniti Research Products Ltd., Nottingham

FAK (mouse monoclonal for immunoblotting only)

HRP-linked phosphotyrosine

Amersham International plc, Bucks

HRP-linked mouse Ig

HRP-linked rat Ig

HRP-linked rabbit Ig

New Brunswick Scientific

p62 (rabbit polyclonal)

Sigma Chemical Co. Ltd., Poole, Dorset

pan keratin (mouse monoclonal)

vimentin (mouse monoclonal)

vinculin (mouse monoclonal)

talin (mouse monoclonal)

RITC-linked phalloidin

FITC-linked rat IgG

rabbit anti-mouse IgG

Tissue Culture Services

FAK (mouse monoclonal for immunoprecipitation only)

Upstate Biotechnology Inc.,

phosphotyrosine (mouse monoclonal)

Vector Laboratories

FITC-linked mouse IgG

a gift from Dr Hans Bos, Utrecht University, Netherlands

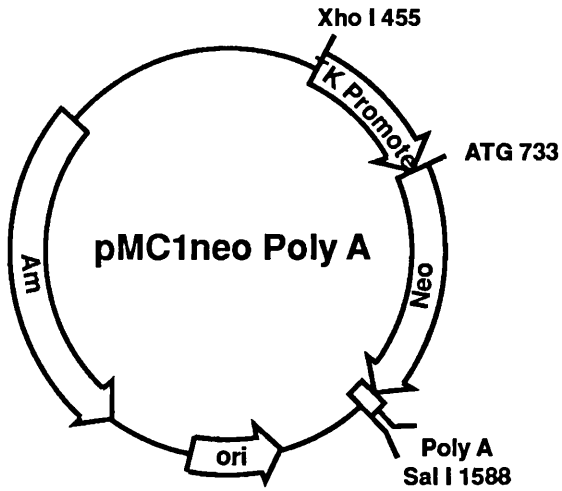
Rap1 (rabbit polyclonal)

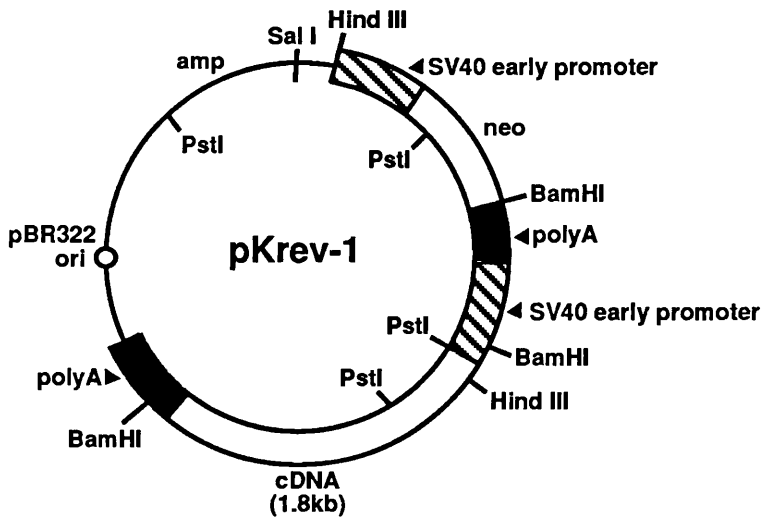
a gift from Prof M. Takeichi, Kyoto University, Japan

E-cadherin, ECCD-2 (rat monoclonal)

2.1.4 Plasmids (figure 6)

$p^{K\text{-}rev1}$-1	a gift from M. Noda, Japan
pMC1neo	Stratagene, San Diego, USA
pECE-GAP-N	a gift from T. Pawson, USA





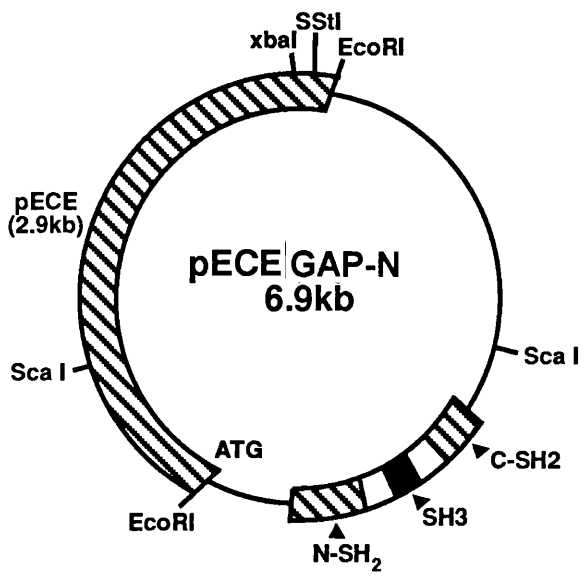


FIGURE 6: Plasmids

2.2 Cell culture

All cell lines were maintained in plastic petri dishes or sealed plastic flasks containing Special Liquid Medium (SLM) supplemented with 2mM glutamine and 10% foetal calf serum (FCS). Cells were passaged when subconfluent by aspirating the growth media then adding a minimum volume of trypsin solution (10% v/v trypsin, 0.01% EDTA in PBS) and incubating at 37°C until the cells detached. Trypsin was inactivated by resuspending the cells in serum-containing medium and the suspended cells were then reseeded at the appropriate dilution.

2.2.1 Cell lines

A5	spindle explant from the MSC11 carcinoma produced by multiple DMBA treatment of a *mus spretus/mus musculus* F_1 hybrid mouse (single cell clone)
D3	" "
B9	squamous explant from the MSC11 carcinoma produced by multiple DMBA treatment of a *mus spretus/mus musculus* F_1 hybrid mouse (single cell clone)
C5N	squamous, non-tumorigenic immortalised cell line isolated by single cell cloning of MCA3D cells derived by Kulesz-Martin et al., 1983 from a Balb/c mouse
SN161	mixed population cell line (at least 75% squamous) derived from a lymph node metastasis in a 129/NIH F_1 hybrid mouse
carB	highly aggressive spindle cell line isolated from a tumour produced in an NIH mouse after DMBA/TPA treatment

A5, D3, B9, SN161 and carB were isolated in this lab.

Cells were photographed at 100x magnification using a Nikon Diaphot inverted microscope with phase contrast optics and a Nikon 35mm camera back mounted on the microscope. The film used was Kodak pan F.

2.2.2 Assessment of tumorigenicity in nude mice

Adherent cells were trypsinised, washed in PBS and resuspended to give 10^7 cells/ml then injected subcutaneously into nude mice using 10^6 cells per injection site. Tumours were harvested when they reached 1cm in diameter in accordance with Home Office guidelines.

Tumours were fixed in 10% formalin, embedded in paraffin wax and 7μm sections were stained with haematoxylin and eosin according to standard procedures. This was carried out by the Glasgow University Veterinary School histology service.

2.2.3 Generation of cell lines from tumours

Tumours were harvested when they reached 1cm in diameter in accordance with Home Office guidelines. They were immediately placed in tumour explant medium (SLM, 0.1mg/ml streptomycin, 50units/ml penicillin, 2.5ug/ml amphotericin). Under sterile conditions the tumour was finely chopped and pieces placed in a dry 100mm dish at 37°C to allow the fragments to adhere to the dish. Explant medium was then slowly added taking care not to wash the tumour pieces off the dish and the explants were incubated at 37°C until sufficient cells had grown out from the original tumour mass. Cell lines were obtained by trypsinising and expanding the growing tumour cells.

2.2.4 Cloning

Single cell cloning

Single cell clones were selected by limiting dilution from the uncloned SN161 cell line. A suspension of trypsinised cells was diluted to give less than 5 cells/ml. A 96 well microplate was seeded with this suspension using 200µl per well. Wells containing only 1 cell were marked the next day and the rest discarded. Selected clones were chosen for expansion.

Ring cloning

Subclones were isolated with cloning rings after microscopic selection for an epithelial or fibroblastic phenotype. Cloning rings were placed over the chosen colony and held in place using a vaseline seal. Trypsin could then be added to remove only this isolated colony.

2.2.5 Long-term storage of cells

All cell lines were stored at -70°C. Subconfluent F175 flasks (175cm^2 surface area) were trypsinised and the cells pelleted then resuspended in 2.5ml growth medium. An equal volume of freezing medium (20% DMSO, 25% FCS, 55% growth medium) was slowly added and 1ml aliquots of the cell suspension frozen at -70°C in freezing vials. The next day cells were transferred to liquid nitrogen for long-term storage.

2.2.6 Soft agar assay

Soft agar assays were used to test the ability of various cell lines to grow under anchorage independent conditions. Bacto-agar (40ml of 2.5%) was melted in a microwave and cooled to 45°C then 20ml of foetal calf serum and 100ml of

1xDMEM were added. This 0.5% agar solution was kept liquid at 45°C until required. Base plates were prepared by pouring approximately 5ml of this solution into each 60mm dish. Once the base plates had set, the required number of cells was resuspended in 0.5ml DMEM and mixed with 1ml cooled base agar. This agar/cell mixture was poured onto a base plate and allowed to set at room temperature before transfer to an incubator.

Triplicate plates were used for each cell line and colonies were counted after 2-3 weeks incubation.

2xDMEM was made with :-

20ml	10xDMEM
2ml	200mM glutamine
2ml	100mM Na pyruvate
10ml	7.5% $NaHCO_3$
4ml	1M HEPES
58ml	sterile H_2O
	antibiotics

2.2.7 GAP-N transfection of B9 and C5N

$5x10^5$ cells were seeded in 100mm dishes containing SLM + 10%FCS 24-48hr prior to transfection. Cell lines expressing the GAP-N protein were produced by cotransfection of B9 or C5N cells with pECE.GAP-N and pMC1neo. 5μg or 10μg of DNA per 100mm dish was introduced by lipofection using DOTAP by the recommended procedure. After incubation at 37°C for 24-48hr, the DNA-containing medium was removed and the cells were trypsinised and split into 10 dishes then grown in selection medium (standard medium plus 400μg/ml G418 (B9) or 300μg/ml (C5N)).

Selected neomycin resistant colonies were expanded and expression of the GAP-N protein was analysed by Western blotting of whole cell lysates using anti-GAP antibodies.

2.2.8 K-rev1 transfection of carB, A5 and D3

$5x10^5$ cells were seeded in 100mm dishes containing SLM + 10%FCS 24-48hr prior to transfection. Cell lines expressing the K-rev1 protein were produced by transfection of carB cells with pK-rev1. 5μg or 10μg of DNA per 100mm dish was introduced by lipofection using DOTAP by the recommended procedure. After incubation at 37°C for 24-48hr, the DNA-containing medium was removed and the cells were trypsinised and split into 15 dishes then grown in selection medium (standard medium plus 300μg/ml G418).

Selected neomycin resistant colonies were expanded and expression of the K-rev1 protein was analysed by Western blotting of whole cell lysates using anti-K-rev1 antibodies.

Clones which obtained a flatter morphology upon transfection were selected for further analysis.

2.2.9 ECM component assays

Growth on fibronectin

Fibronectin coated plates were prepared by diluting stock fibronectin (1mg/ml) to 30ng/μl in sterile PBS then adding 2ml of this solution to each plate and allowing it to coat for 2-3hr at room temperature. Excess fibronectin was removed and the plates were then rinsed with PBS and stored at 4°C until required. Trypsinised cells resuspended in growth medium were plated at the required density and the effect of growth on fibronectin monitored for one week.

Growth on Matrigel

An aliquot of Matrigel was thawed slowly at 4°C overnight and kept on ice before use. Since the matrix gels irreversibly at temperatures above 22°C, all pipette tips, plates and tubes were cooled to 4°C prior to dispensing Matrigel. Equal volumes of Matrigel and PBS were mixed and minimum volumes of the diluted Matrigel dispensed into 24 well plates. The matrix was allowed to set for several hours at room temperature then rinsed with PBS before adding cells which had been trypsinised and resuspended in growth medium. The multi-well plates were incubated at 37°C and the effect of Matrigel on cell morphology monitored for one week.

Growth on collagen I

Rat tail collagen type I coated plates were prepared by diluting stock collagen I (2.87mg/ml) to 50μg/ml in sterile PBS then adding a minimum volume to each plate and allowing it to coat for several hours at room temperature. The plates were washed with PBS and stored at 4°C until used. Trypsinised cells resuspended in growth medium were plated at the required density and the effect of growth on collagen I monitored for a week.

2.2.10 Growth factor treatment

Cells were cultured in SLM containing 2mM glutamine and 5% FCS. Growth factors were added to this medium at the appropriate concentrations:

EGF 10ng/ml

aFGF25ng/ml plus 10ng/ml heparin

TGFβ 5-10ng/ml

The growth factor containing medium was changed every 2-4 days and any morphological effect of treatment was analysed further at various time points by immunoblotting, immunofluorescence and photography.

2.2.11. Chromosome spreads

Colchicine was added at 1μg/ml final concentration to an F25 flask of cells at 75% confluence for 1hr at 37°C. The medium was then removed, the cells trypsinised and resuspended in PBS then pooled with the medium to inactivate the trypsin and maintain the metaphase block. The cells were pelleted and carefully resuspended in 0.5ml hypotonic solution (75mM KCl) which had been prewarmed to 37°C. After incubation for 5-10min at 37°C, the cells were again pelleted and the KCl aspirated. Ice-cold 3:1 methanol : acetic acid fixative was added to resuspend the cells and this suspension was left for 20min at room temperature. The cells were pelleted and resuspended in 3 changes of fixative and finally resuspended in 0.5ml of fixative. Spreads were prepared by dropping the cell suspension from a great height onto ice-cold microscope slides. Once dry, the spreads were stained with 10% Giemsa.

2.3 Immunoblotting

2.3.1 Preparation of total protein extracts

Whole cell extracts were prepared without trypsin treatment by either of 2 methods.

(a) Extracts containing known numbers of cells per ml were obtained by plating four 100mm dishes with equal numbers of cells. When subconfluent, the total number of cells in one of these dishes was counted. The remaining 3 dishes were washed with ice-cold PBS then the cells were lysed in 1ml of lysis buffer (10%glycerol, 5% SDS,

0.08M tris pH6.8) plus 40µl/ml β-mercaptoethanol added just before use. The viscous lysate was removed from the dish using a cell scraper. After boiling, sonication (2x10sec) and centrifugation at 14000rpm for 15min, the lysates were adjusted using lysis buffer to give $3x10^6$ cells/ml. Lysates were stored at -20°C.

(b) Extracts containing equal protein concentrations were prepared by washing 100mm dishes with ice-cold PBS then lysing in MQ buffer (1% Triton X100, 0.5% Na deoxycholate, 0.1% SDS, 0.1M NaCl, 0.05M trisHCl pH7.4, 0.005M $MgCl_2$, 3mM PMSF). The lysate was scraped off the dish and, after centrifugation at 4°C for 15min, the supernatant protein concentration was measured using the BCA method (section 2.3.2) and adjusted to give 600µg/ml.

2.3.2 Protein concentration determination

The protein concentration of whole cell lysates was determined by the BCA method which depends on a colour change produced by the reaction between $CuSO_4$, bicinchoninic acid and protein. Protein samples and standards were plated on a 96 well microtitre plate and the absorbances read at 562nm automatically. The protein content of the samples could then be calculated.

2.3.3 Immunoblotting

Whole cell lysates were prepared as described.

Proteins were separated according to the method of Laemmli (1970) by electrophoresis on 8% SDS polyacrylamide gels size 14x14cm.

Gels were loaded with 30µg protein or $0.15x10^6$ lysed cells per lane plus 5µl of rainbow coloured molecular weight markers in 30µl western lysis buffer. All samples were denatured by boiling for 3min followed by rapid cooling on ice prior to loading. Separated proteins were transferred onto nitrocellulose membranes as described by Towbin et al., (1979) for 1h20 using a semidry electroblotter.

PROTEIN	SOURCE	CLONE/CATALOGUE NO.
E-cadherin	M. Takeichi	ECCD-2
keratin	Boehringer	Lu5
vimentin	Sigma	VIM13.2
talin	Sigma	8d4
vinculin	Sigma	VIN-11-5
phosphotyrosine	UBI	05-321
phosphotyrosine	Affiniti	RC20H
p62	Santa Cruz Bio.	sc-108
GAP	M. Frame	
K-rev1	H. Bos	
FAK	Affiniti	F15020

After completion of transfer, the blot was blocked with blotto (5% powdered milk in TBS, 0.05% Tween-20) for at least 2hr. It was then incubated with a minimum volume of primary antibody diluted in fresh blotto for the required time. Filters were washed 3x10min in blocking solution and incubated with the appropriate horseradish peroxidase conjugated secondary antibody for up to 1h at room temperature. Blots were then washed thoroughly 2x10min in blotto followed by 2x10min washing with TBS/tween then immunodetection of antigens on the nitrocellulose was performed using enhanced chemiluminescence (ECL).
3% BSA in PBS/tween was used in place of blotto for anti-phosphotyrosine blotting.

The following primary antibodies were used:

E-cadherin	mouse monoclonal	1/50000 dilution
keratin	mouse monoclonal	1/300000
vimentin	mouse monoclonal	1/20000
talin	mouse monoclonal	1/20000
vinculin	mouse monoclonal	1/20000
phosphotyrosine	mouse monoclonal	1/1000 (UBI)
"	"	1/1000 (Affiniti)
p62	rabbit polyclonal	1/1000
GAP	mouse monoclonal	1/250
K-rev1	rabbit polyclonal	1/2000
FAK	mouse monoclonal	1/1000

These were detected using the following HRP-linked secondary antibodies:

mouse	1/2000-4000 dilution
rat	1/300-1000
rabbit	1/2000-4000

2.3.4 Immunoprecipitation

GAP immunoprecipitation

Subconfluent monolayers were washed with PBS then lysed in radioimmunoprecipitation assay (RIPA) buffer (1%NP40, 0.5% Na deoxycholate, 0.1% SDS in PBS) containing 1x aprotinin, 1μM Na fluoride, 1mM dithiothreitol, 1μM β-glycerophosphate, 1.25mM PMSF, 1μM Na vanadate, 1mM Na pyrophosphate for 15min at 4°C, scraped off the dish and centrifuged at 14000rpm for 15min at 4°C.

The clarified extract was incubated with 5μl of normal rabbit serum and 60μl of protein A Sepharose with gentle agitation for one hour at 4°C. After a brief spin to remove the protein A sepharose, the total protein concentration of the precleared extracts was determined using BCA reagents. Immunoprecipitation was carried out with 2μl of anti-GAP antibody per mg protein overnight at 4°C. 60μl protein A sepharose was added then pelleted by centrifugation after 1hr. The pellet was washed 3 times with RIPA buffer then resuspended in 2-fold concentrated Laemmli sample buffer and boiled for 5min. The eluted proteins were separated on an 8% SDS polyacrylamide gel with molecular weight standards and transferred to Hybond-C filters.

The filters were blocked by incubation in phosphate-buffered saline plus 0.05% Tween-20 (PBST) containing 3% BSA then the primary antibody, rabbit polyclonal antiserum raised against p120GAP, was added at a 1/4000 dilution for 12 hours. Following several washes with 3% BSA in PBST, the filter was incubated with anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody at a 1/4000 dilution for 1hr.

After further washes with PBST, ECL detection (Amersham) was performed and the filter exposed to x-ray film (Fuji).

FAK and phosphotyrosine immunoprecipitation

A similar method was used for FAK and phosphotyrosine immunoprecipitation but the lysis buffer was 1% Triton, 150mM NaCl, 10mM tris pH 7.4, 1mM EGTA, 1mM EDTA, 0.2mM $NaVO_4$, 0.2mM PMSF, 0.5% NP40. The lysate was scraped off the dish, clarified by centrifugation at 14000rpm for 15min and the pellet discarded. The protein concentration of the supernatant was measured by the BCA method (section 2.3.2) and 200-500μg of protein was incubated with 1-5μg of antibody and immunoprecipitation buffer for up to 24h. 60μl of protein A sepharose was added and incubation at 4°C continued with agitation for 1h. In the case of the FAK mouse monoclonal antibody, the protein A sepharose had to be preincubated with 1/10 volume of rabbit anti-mouse IgG. The protein A sepharose-protein-antibody complex was precipitated by brief centrifugation and the pellet was washed 3 times with immunoprecipitation buffer. It was finally resuspended in western lysis buffer, boiled and the released proteins separated by SDS-PAGE.

2.3.5 Immunofluorescence

For immunodetection of E-cadherin, $5x10^4$ cells were seeded in 8-well permanox chamber slides. Cells were washed in ice-cold PBS then fixed and permeabilised with methanol at -20°C for 10min. They were then washed thoroughly in PBS and, after blocking with horse serum for 20min, cells were incubated with a rat monoclonal anti-E-cadherin antibody, ECCD-2 (Takeichi), at a 1:500 dilution for 1h at room temperature. The slides were again washed in PBS/0.15M NaCl/ 0.05% Tween-20 and an anti-rat FITC-linked secondary antibody was applied (40 fold dilution) for 20min. After further washing in PBS/NaCl/Tween and rinsing in water, the slides were mounted with Vectashield antifade using nail varnish to seal the coverslips and viewed at 40x magnification using a Leitz fluorescence microscope.
The same procedure was followed for staining with pan keratin and vimentin antibodies. These were used at 1:300 and 1:200 dilutions respectively and the

secondary antibody was an anti-mouse IgG-fluorescein isothiocyanate conjugate used at a 200 fold dilution.

2.3.6 Focal contact staining

Cells were grown in the same way as for E-cadherin immunodetection described above and the procedure was the same except that methanol was replaced with acetone for cell permeabilisation and fixing. Antibodies against the focal contact proteins vinculin and talin (both diluted 1/100) were applied for 1hr and, after washing, the secondary antibody, a mouse IgG-FITC conjugate in both cases, was added for 30min. After incubation with the secondary antibody and washing with PBS/salt/Tween, TRITC-linked phalloidin (diluted 1/10) was applied for 30min. The slides were washed and mounted in the usual way and viewed using a confocal microscope. The images were then manipulated to superimpose the FITC and RITC fluorescence staining patterns.

2.4 DNA and RNA manipulations

2.4.1 Agarose gel electrophoresis

Flat bed apparatus was used in all cases. 0.5-1% gels (w/v) were cast in 1xTAE buffer (40mM Tris base, 16mM acetic acid, 1mM EDTA, pH8) containing 0.5μg/ml ethidium bromide. Low melting point agarose was used for gels from which plasmid inserts were to be isolated for use as labelled probes.

Gels were submerged in 1xTAE buffer and samples were loaded with 0.25% bromophenol blue, 0.25% xylene cyanole, 50% glycerol, 1xTAE. Gels for Southern blots were run at 30-50V for 15-25hr and low melting point agarose gels were run at 100V for 2hr. The appropriate molecular weight markers were included on each gel.

After electrophoresis, the size-fractionated DNA was visualised by ultraviolet light illumination and photographed

2.4.2 Restriction enzyme digestion of DNA

Plasmid and eucaryotic DNA were digested in the same way. 5 units of enzyme per μg DNA were incubated with the appropriate reaction buffer, 1mM spermidine and 100μg/ml BSA as recommended by the enzyme supplier at 37°C for at least 5hr.

2.4.3 Isolation of plasmid insert DNA for use as radiolabelled probes

40μg of plasmid DNA was digested and the insert DNA separated from the plasmid by electrophoresis in 1% low melting point agarose. The insert band could be cut from the gel when visualised using shortwave UV illumination. The agarose slice was mixed with 2x vol water and stored at -20°C until needed for radiolabelling. The concentration of insert DNA was estimated using the amount of uncut plasmid loaded as a guide.

2.4.4 Extraction of genomic DNA from adherent cells

Subconfluent cells were trypsinised and pelleted at 1000rpm. The trypsin was then removed and the cells resuspended in 1ml of lysis buffer (100mM tris pH8.5, 5mM EDTA, 200mM NaCl, 0.2% SDS, 100μg/ml proteinase K). This suspension was rotated for several hours at 37°C until clear. DNA was precipitated by adding an equal volume of isopropanol then spooled onto a sealed pasteur and washed in ethanol. Once dry, the DNA could be resuspended in TE and its concentration measured.

2.4.5 Extraction of RNA from adherent cells

Total RNA was isolated using RNazol, a commercial version of the guanidinium-phenol single step RNA isolation method. 3ml of RNAzol was added directly to a subconfluent 75cm^2 flask of adherent cells, and the lysate scraped off using a sterile scraper. This was placed in a 15ml Falcon tube and one tenth volume of chloroform was added. After shaking, the tube was placed on ice for 5min then centrifuged at 4000rpm at 4°C for 15 minutes. The colourless upper phase was transferred to a fresh tube, and the RNA precipitated using an equal volume of isopropanol. The RNA was then pelleted and washed with 75% ethanol and allowed to air dry. The pellet was then resuspended in 50% ethanol in diethylpyrocarbonate (DEPC) treated water. Care was taken to ensure that all solutions used were ribonuclease free. RNA was stored at -20°C.

2.4.6 Bulk preparation of plasmid DNA

A 10ml starter culture was prepared overnight. This was then added to 500ml of L-broth containing 100µg/ml ampicillin and incubated for at least 16 hrs. The culture was centrifuged in 500ml Sorvall bottles at 5000rpm for 5min at 4°C. The pellet was resuspended in 25ml of ice cold lysis solution (50mM glucose, 2mg/ml lysozyme, 25mM trisCl, 10mM EDTA). After 15 minutes on ice, 50ml of alkaline solution (0.2M NaOH, 1%SDS) was added and the solution mixed vigorously by vortexing. Finally 37.5ml of potassium acetate solution (3M KAc, 5M acetic acid) was added and the bottles incubated on ice for approximately 15min. The bottles were centrifuged at 6000rpm, 4°C for 15min. The supernatant was filtered through gauze and precipitated with 0.6 volumes of isopropanol. The pellet was resuspended in TE.

The plasmid DNA was then further purified by equilibrium centrifugation in a continuous CsCl gradient. 1.55g/ml CsCl was dissolved in the DNA solution.

Ethidium bromide was added to a final concentration of 1mg/ml. The refractive index of the solution was checked to ensure the density of the solution was correct (1.55g/ml; refractive index 1.3860). The DNA sample was then loaded into polycarbonate tubes and centrifuged at 80000 for 16h at 20°C. After centrifugation, the lower red band containing the plasmid was removed from the gradient with a syringe. The ethidium bromide was removed from the DNA by extraction with butanol. This was repeated until the red colour of the ethidium bromide had disappeared.

2.4.7 Southern blot transfer of DNA

10μg of restriction enzyme digested DNA was separated by agarose gel electrophoresis and transferred to Zetaprobe nylon membrane by the method of Rigaud et al., (1987). Following ethidium bromide staining and photography, the size-fractionated DNA was denatured by soaking the gel in 1.5M NaCl, 0.5M NaOH, pH13 (2x20min) then neutralised in 1M ammonium acetate, 0.02M NaOH (2x20min). The transfer of DNA from the gel to membrane was carried out on a raised platform covered with a wick of 3MM paper soaked in transfer buffer. The ends of this wick rested in a reservoir of transfer buffer. The gel was placed on the wick with Saranwrap surrounding it to minimise evaporation and ensure that all capillary action was through the gel and membrane. Zetaprobe membrane was placed on top of the gel then covered in 3 sheets of 3MM paper soaked in transfer buffer, a stack of paper towels and a weight. Transfer took place overnight then the membrane was washed in 2xSSC and crosslinked using a Stratagene UV Stratalinker 1800.

2.4.8 Northern blot transfer of RNA

Total RNA from adherent cells was isolated by the RNazol method (section 2.4.5).

Denatured RNA (10μg) was fractionated on 1% agarose gels using formaldehyde/ phosphate buffer and transferred to nylon membranes (Zetaprobe) using 10xSSC as described (Sambrook et al., 1989).

2.4.9 Generation of random-primed radiolabelled probes

Labelled probes from double-stranded DNA were made by the random priming method. Approximately 50ng of purified insert DNA was boiled for 10 minutes to ensure denaturation then cooled and added to a reaction mixture containing oligonucleotide labelling buffer, 1.85MBq of α-^{32}P dCTP and 5 units of Klenow enzyme and the reaction incubated at room temperature for at least 1h.
The unincorporated nucleotides were removed by running the probe through a NICK-column (Pharmacia), and its specific activity estimated using a scintillation counter.

2.4.10 Hybridisation of labelled probes to Northern and Southern blots

Blots were prehybridised in 0.25M Na_2HPO_4 pH7.2, 7% SDS by brief incubation at 65°C. Prehybridisation fluid was replaced with hybridisation solution (also Na_2HPO_4 pH7.2, 7% SDS) containing the labelled probe and hybridisation was carried out at 65°C for approximately 16h. After the required time, hybridisation solution was removed and washes with 20mM Na_2HPO_4 pH7.2, 5% SDS (2x30-60min) then 20mM Na_2HPO_4 pH7.2, 1%SDS (2x30-60min) were carried out at 65°C. Washes were repeated until background counts were minimised.

2.5 PCR

2.5.1 PCR amplification using microsatellites

PCR was performed as described by Love et al (1990). Each 25µl reaction was performed with 100ng of genomic DNA in a reaction mixture with 250µM each of dCTP, dGTP, dATP, dTTP, 9.25kBq α-^{32}P dCTP, 2 oligonucleotide primers at 0.1µM each and 0.5 units of Taq DNA polymerase (from a variety of suppliers) with the appropriate buffer containing $MgCl_2$ at 2µM.

All reactions were conducted using a Perkin Elmer GeneAmp PCR thermal cycler under the following standard conditions in thin-walled reaction tubes (Perkin Elmer Cetus): an initial denaturation step at 95°C for 1min followed by 30 cycles of denaturation (95°C, 30s), annealing (50°C or 55°C, 30s) and extension (72°C, 30s) then a final elongation step (72°C, 4min). The annealing temperature was varied to give the best amplification at highest stringency.

Amplimers were obtained from 3 main sources. Microsatellites most likely to be informative were selected using data from the work of Todd et al (Cornall et al., 1991; Love et al., 1990; Hearne et al., 1991) and the primers synthesised at this institute. Others were obtained from the Human Genome Mapping Project Research Centre, Harrow or from Research Genetics, Huntsville, Alabama.

2.5.2 Separation of PCR-amplified products

PCR products were resolved on either 4% agarose or 6% acrylamide gels.

A quick test of the PCR reaction success was carried out using 4% agarose gels made with 3% NuSieve (FMC), 1% agarose (Sigma), 0.5µg/ml ethidium bromide and TBE buffer. Amplified DNA was loaded with 0.25% orange G in 50% glycerol, 50% 1xTBE and the gels were run for 30min at 100V then photographed.

6% non-denaturing acrylamide gels formed using 20x38cm glass plates (EV400 gel tank, Cambridge Electrophoresis) coated with Repelcote were used for quantitative analysis. PCR reaction products were loaded with 50% glycerol, 1xTBE, 0.25% bromophenol blue, 0.25% xylene cyanole and electrophoresis was conducted at room temperature for 2-4 hours at 15W depending on the sizes of PCR fragments. The gel was blotted onto 3MM paper, wrapped in Saranwrap and exposed to x-ray film (Fuji, RX) overnight at -70°C with intensifying screens.

Chapter 3

Results

3.1 Derivation of cell lines with a range of morphologies

A dramatic change in cell phenotype is sometimes seen in an advanced stage of mouse skin carcinogenesis when a relatively well-differentiated squamous cell carcinoma progresses to a less differentiated spindle cell carcinoma. As described in section 1.4.2, the development of papillomas and carcinomas in stratified epithelium can be followed using the mouse skin model system. Most of the carcinomas are squamous and retain many of the differentiation features of the tissue of origin e.g. E-cadherin and keratin expression. These well-differentiated squamous carcinomas maintain typical epithelial tissue structures such as well-developed intercellular junctions and therefore are weakly invasive. Spindle cell carcinomas, in contrast, have altered their differentiation program and now express many proteins more often linked with fibroblasts than epithelia e.g. they have lost E-cadherin and keratin expression and express vimentin, an alternative intermediate filament protein, instead.

It is possible that a factor which controls the normal differentiation program of epithelial cells is lost in the carcinogenesis process. This is unlikely to be a simple switch; it will probably involve a number of stages through intermediates which are neither squamous nor spindle. A panel of cell lines has been built up with the aim of studying these stages. There are cell lines which have stable squamous or spindle morphologies and a number of related cell lines whose morphologies can vary depending on their growth conditions i.e. they have a degree of plasticity not seen with the fixed squamous or spindle cell lines.

This thesis describes a study of some genetic and epigenetic events that may be responsible for the squamous to spindle cell transition using this panel of fixed morphology and plastic morphology cell lines.

These studies were designed to provide specific information relating to the control of the morphological differences existing between the cell lines tested. It was anticipated that such information would provide some clues to the forces controlling the squamous to spindle cell carcinoma transition seen in mouse skin tumorigenesis. These

findings may also have relevance to the control of differentiation in mouse skin epithelia. This chapter describes the experimental approaches employed.

3.1.1 Isolation of clonally-related squamous or spindle cell lines with fixed morphologies

Two cell lines, A5 and B9, which represent late stages of carcinoma development have been isolated (originally identified and isolated by M. Clarke and S. Haddow). They were both derived from the same skin tumour, MSC11 (figure 7), which was induced in an MSC mouse by DMBA/TPA treatment (carcinogen treatment carried out by R. Bremner). This mouse was an F_1 hybrid produced by crossing a *mus musculus* female (CBA strain) and a *mus spretus* male. The use of two distantly related strains of mice ensures that their offspring will have a high degree of heterozygosity which is ideally suited to genetic analysis and allows the inheritance of maternal and paternal alleles to be followed.

A cell line derived from the MSC11 tumour, MSC11a, was seen to be a mixed population of squamous and spindle cells (figure 8). These distinct components were separated by single cell cloning to give 3 clonally-related cell lines: A5 (spindle), B9 (squamous) and D3 (spindle). The morphologies of these lines have been stable *in vitro* over a long period of time. The relationship between them was demonstrated when it was shown that they all possessed the same 3 p53 mutations and one ras mutation (Burns et al., 1991). It could therefore be assumed that they originated from the same progenitor cell and are probably representative of 2 sequential stages in the carcinogenesis pathway. A genetic analysis of the 2 morphologically distinct cell types will be discussed in detail in section 3.3.

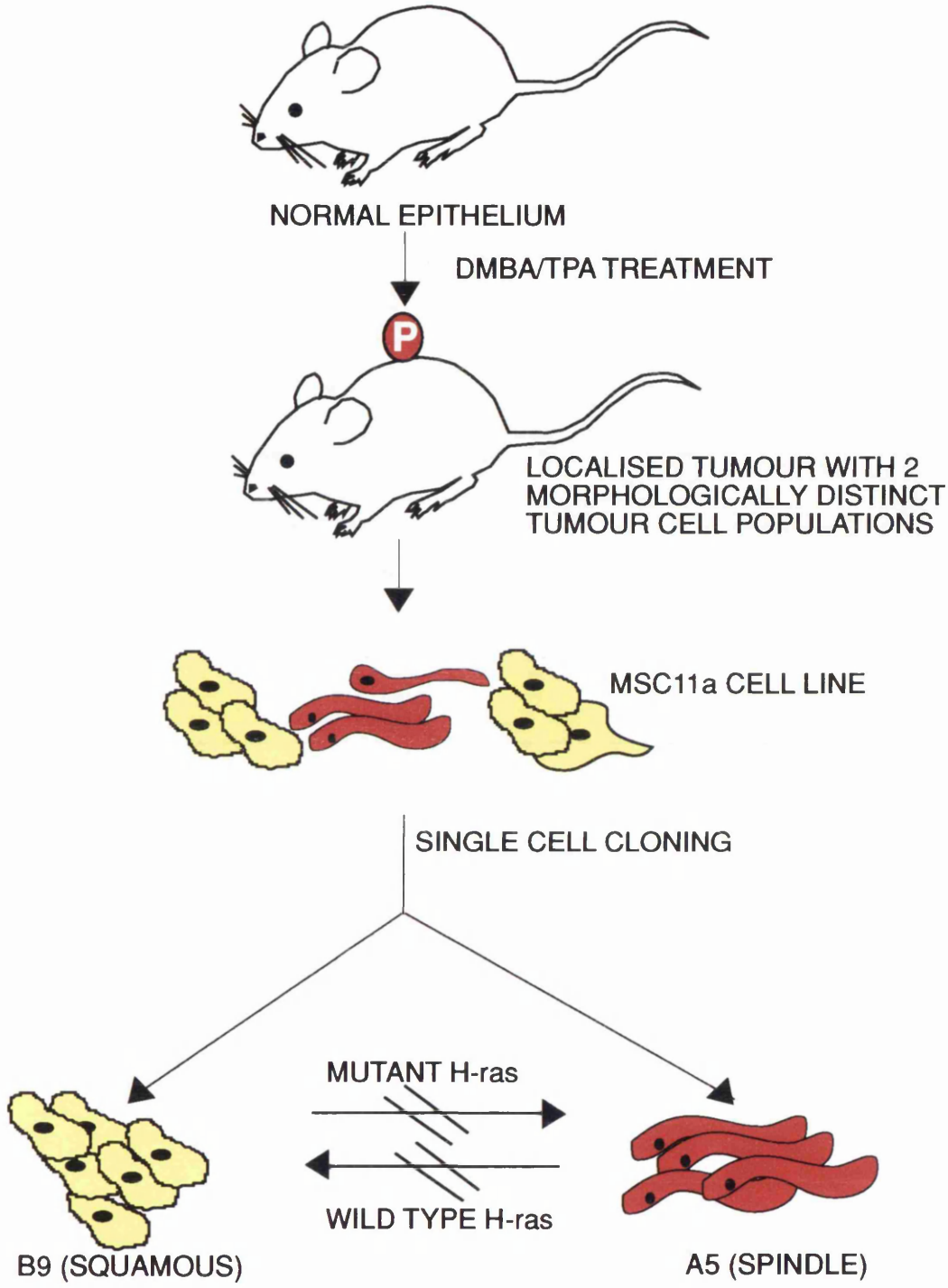
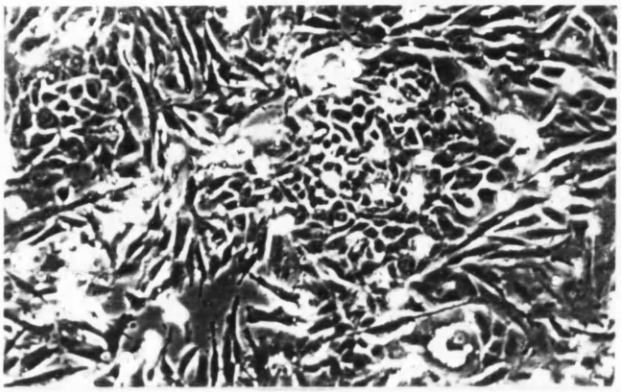
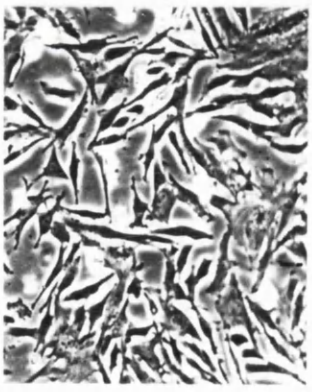


Figure 7 : Clonal relationship between squamous and spindle cells derived from the same primary tumour

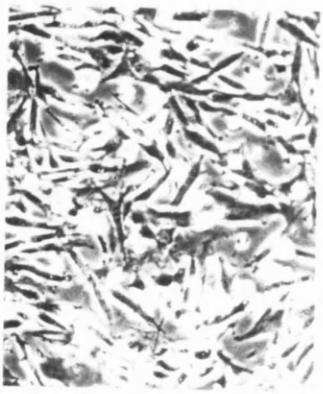


MSC11cl

B9 (squamous)



A5 (spindle)



D3 (spindle)

Figure 8: Morphology of the parent MSC11 cell line which consists of squamous and spindle components and 3 single cell clones derived from it.

3.1.2 Isolation of eleven single cell clones with variable morphologies

Another source was used for the isolation of cell lines whose morphologies were not fixed. A lymph node metastasis from a primary skin tumour in a 129/NIH hybrid mouse which had been treated with DMBA and TPA was the source of the SN161 cell line (carcinogen treatment and cell line isolation carried out by R. Bremner). This cell line comprised a mixed population of cells with morphologies ranging from squamous to spindle.

The different morphologies were initially separated by single cell cloning, resulting in the production of six new cell lines (figure 9). Three cell lines, G5, SN4 and SN8 were uniformly spindle but E1 and C11 still seemed to contain a number of different morphologies (figure 10). Further single cell cloning of E1, which had a small proportion of spindle cells, yielded two more lines, E1N and E4, both of which are entirely squamous (figure 11). E4 has the most squamous morphology of all the single cell clones and most closely resembles a normal keratinocyte.

The C11 cell line was subjected to ring cloning in an attempt to separate the squamous and spindle components; this produced the C11c and C11d cell lines both of which still had a variety of cell types (figure 10). Further single cell cloning of C11d produced the B8, H9 and H11 cell lines (figure 11). B8 is similar to C11c and C11d i.e. it exists as squamous colonies surrounded by dissociated spindle cells; H9 is flat but not truly epithelial; H11 is spindle but, after long periods in culture, small squamous colonies begin to appear.

It was therefore concluded that some of these cells are truly plastic i.e. it is not always possible to isolate a line with a uniform morphology. Indeed, the morphology of these cell lines can be altered experimentally by methods which will be discussed in this chapter.

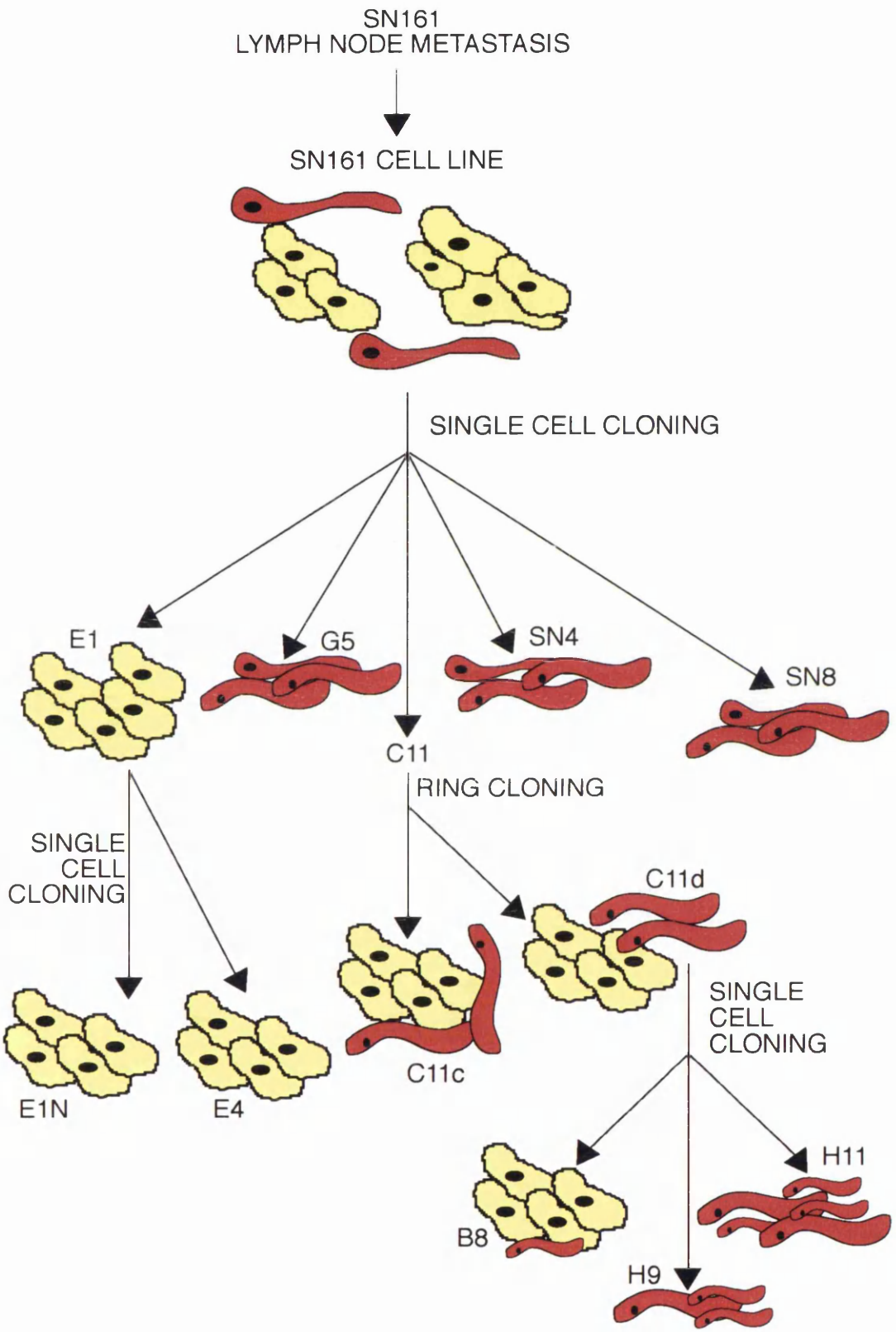
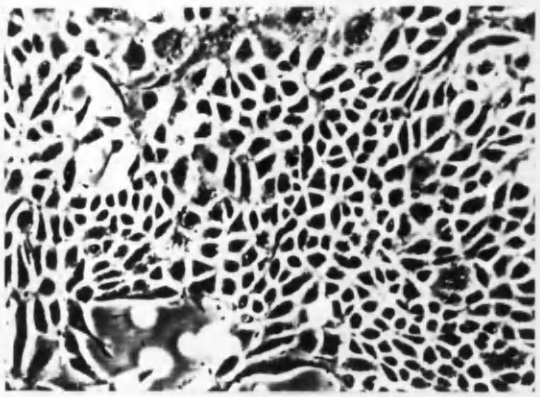
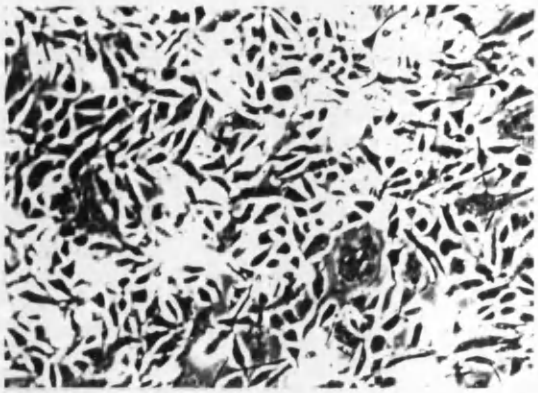


Figure 9 : Derivation of SN161 cell line and isolation of 11 single cell clones

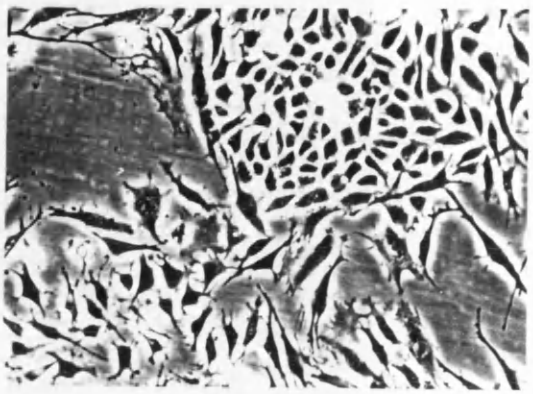


E1

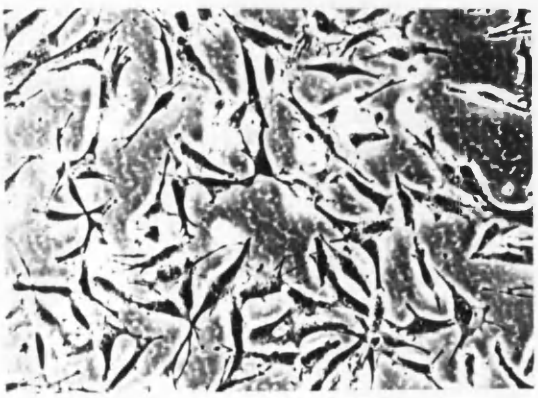


G5

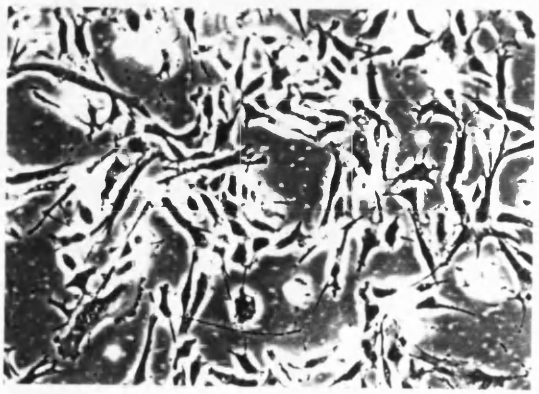
C11c



C11d

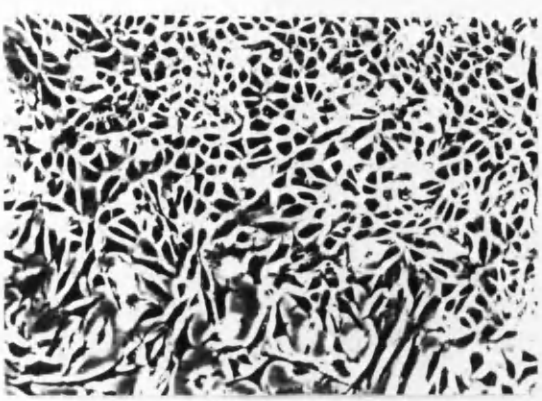


SN4

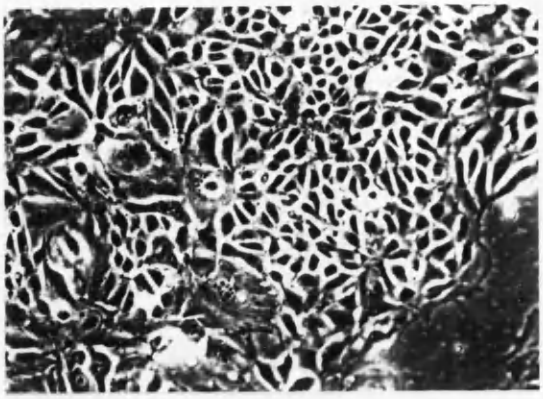


SN8

Figure 10: Morphologies of single cell clones derived from the SN161 cell line.

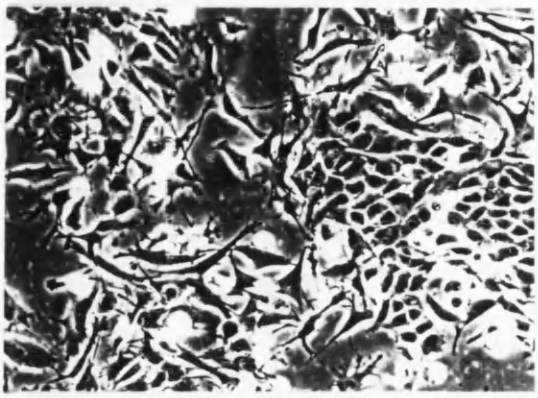


SN161

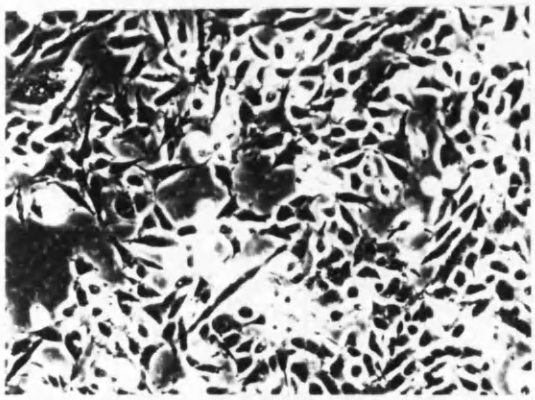


E1N

E4



B8



H9

H11

Figure 11: Morphologies of single cell clones and the parent SN161 cell line from which they were derived.

3.2 Biological characterisation of cell lines

3.2.1 Detection of E-cadherin, vimentin and keratin in A5 and B9

The expression of markers that distinguish epithelial and fibroblast-like cells was determined in A5 and B9 by immunocytochemistry and Western blotting. As would be expected, the squamous cell line, B9, expresses typical epithelial markers such as E-cadherin and cytokeratins (figures 12, 16 and 17).

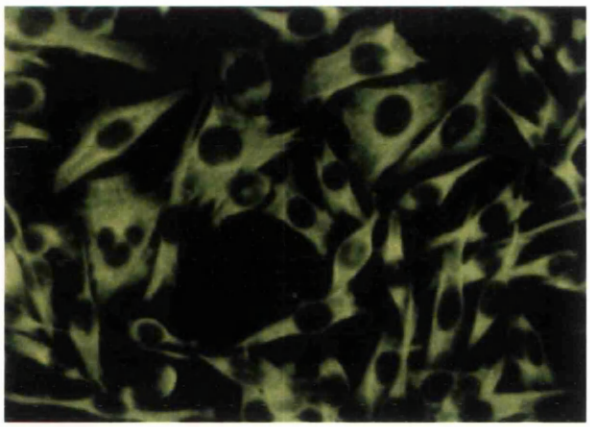
These cytokeratins are probably K5 and K8; A5 was previously shown to express K8 by using a K8 specific antibody (Stoler et al., 1993). K5 is a typical marker of epithelial cells but K8 is not normally expressed in adult epidermis; it is usually associated with the early stages of embryogenesis. It can, however, be re-expressed in skin carcinomas induced by expression of mutant ras as a result of chemical carcinogenesis protocols.

A5 is a spindle cell line that has lost epithelial markers such as E-cadherin and instead shows a more fibroblast-like program of differentiation e.g. by expressing vimentin as shown by immunocytochemistry and Western blotting. It still retains some expression of cytokeratins but produces only the lower molecular weight cytokeratin (figures 12 and 17).

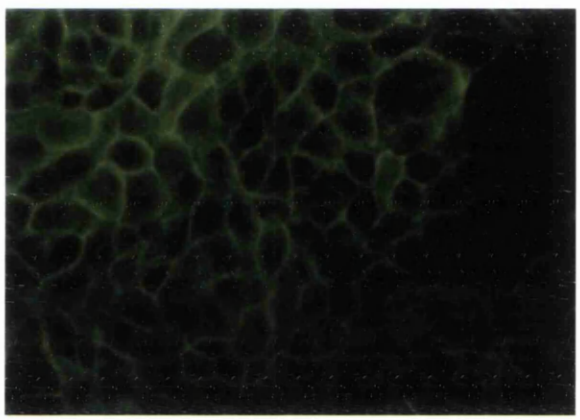
The C5N cell line has been included in some cases as a control for epithelial-specific markers. It more closely resembles a normal keratinocyte than B9 as shown by its expression of only the higher molecular weight cytokeratin (figure 17). The C5N cell line is not related to the MSC cell lines or the panel of SN161-derived cell lines.

3.2.2 Detection of E-cadherin, vimentin and keratin in SN161 clones

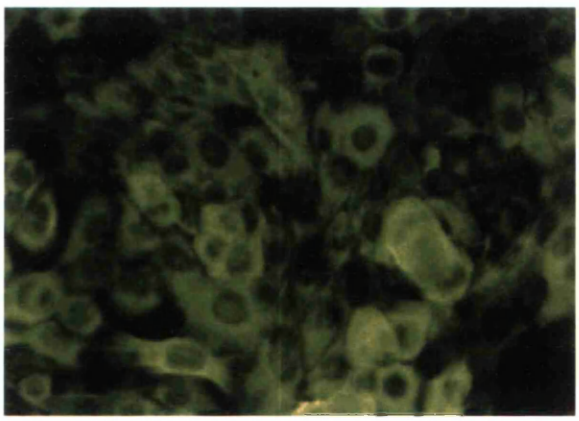
The same analysis was carried out for the eleven SN161 clones. Immunofluorescence studies showed the E-cadherin, vimentin and keratin expression patterns that would be expected on the basis of their morphologies. Cell lines which



A5 VIMENTIN



B9 E-CADHERIN



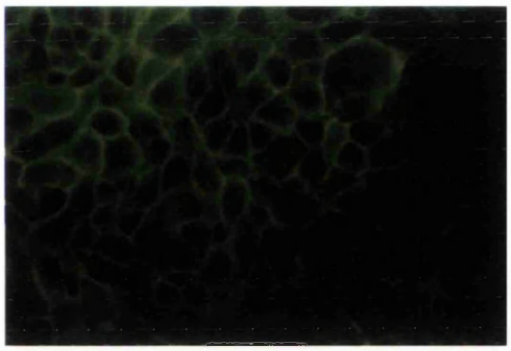
B9 KERATIN

Figure 12: Characterisation of A5 (spindle) and B9 (squamous) by immunocytochemistry. A5 expresses vimentin while B9 expresses keratin and E-cadherin. These expression patterns have been confirmed by Western blotting (figures 16 and 17).

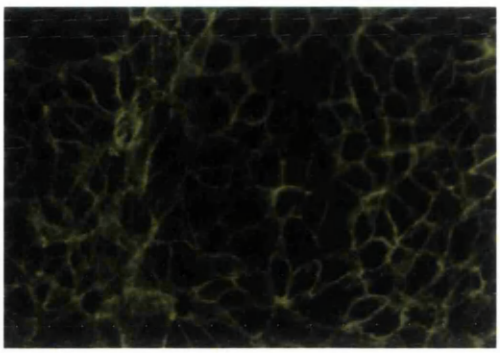
consisted of varying proportions of squamous colonies surrounded by individual spindle cells showed areas of positive staining for E-cadherin surrounded by negative areas (figure 13). Positive immunofluorescent staining was dependent on the existence of close cell-cell contacts and was therefore observed only in confluent cultures of epithelial cells or within small squamous colonies in low density cultures. It was absent where there were no neighbouring cells or where adjacent cells were fibroblast-like.

Staining patterns for keratin and vimentin were reverse images of each other. Keratin intermediate filaments were present in squamous cells whether in close contact with their neighbours or sparse (figure 14), while a vimentin network could be seen in fibroblast-like cells (figure 15). Some cross-reactivity between the keratin antibody and vimentin expressing cells was occasionally observed. High background staining due to an excess of antibody resulted in low level staining of cells which would normally be negative e.g. C11d as shown in figure 14. Cell lines comprising of a range of morphologies had distinct areas of positive staining for each type of intermediate filament; squamous colonies were positive for keratin while surrounding spindle cells within the same culture were positive for vimentin. This indicated that the two different cell types had adopted alternative differentiation programmes and keratin and vimentin intermediate filament networks are, in this case, mutually exclusive.

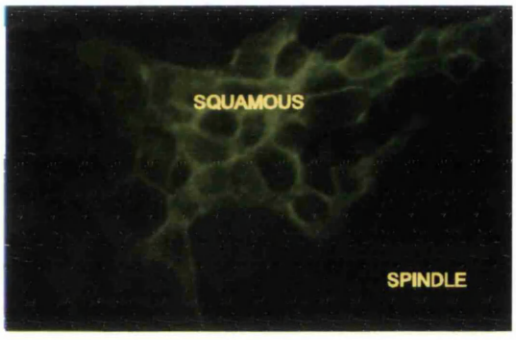
Immunoblotting studies supported these findings. Squamous SN161 cell lines expressed E-cadherin and the lower molecular weight cytokeratin while spindle lines expressed vimentin and had lost E-cadherin expression (figures 16 and 17). The lower molecular weight bands in B9, C11c and E1 lanes on E-cadherin probed blots (figure 16) are due to partial degradation of the protein in some cell lysates. Vimentin expression in SN161 and B8 is not seen in figure 17 due to the low percentage of spindle cells in the lysate but it can be detected with a longer exposure (not shown). It is interesting that all of the SN161 clones that express keratin produce only the lower and not the higher molecular weight keratin which is the major keratin seen in the epithelial lines B9 and C5N. This may indicate that the SN161 clones are representative of a more advanced stage of tumorigenesis.



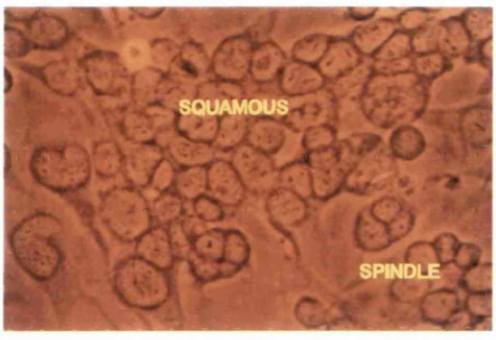
E4



EIN

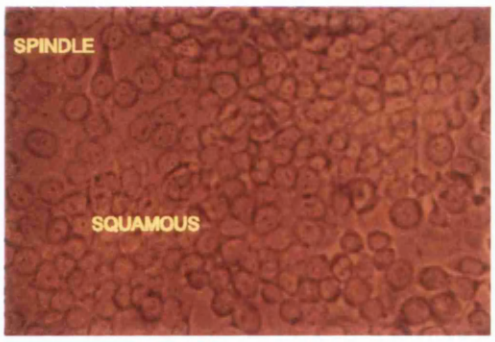


H9



H9

B8



B8

Figure 13: Detection of E-cadherin by immunocytochemistry. E4 and E1N are positive throughout the population where cells are confluent. After long periods in culture, B8 and H9 begin to show regions of positively staining squamous cells surrounded by negative spindle cells.

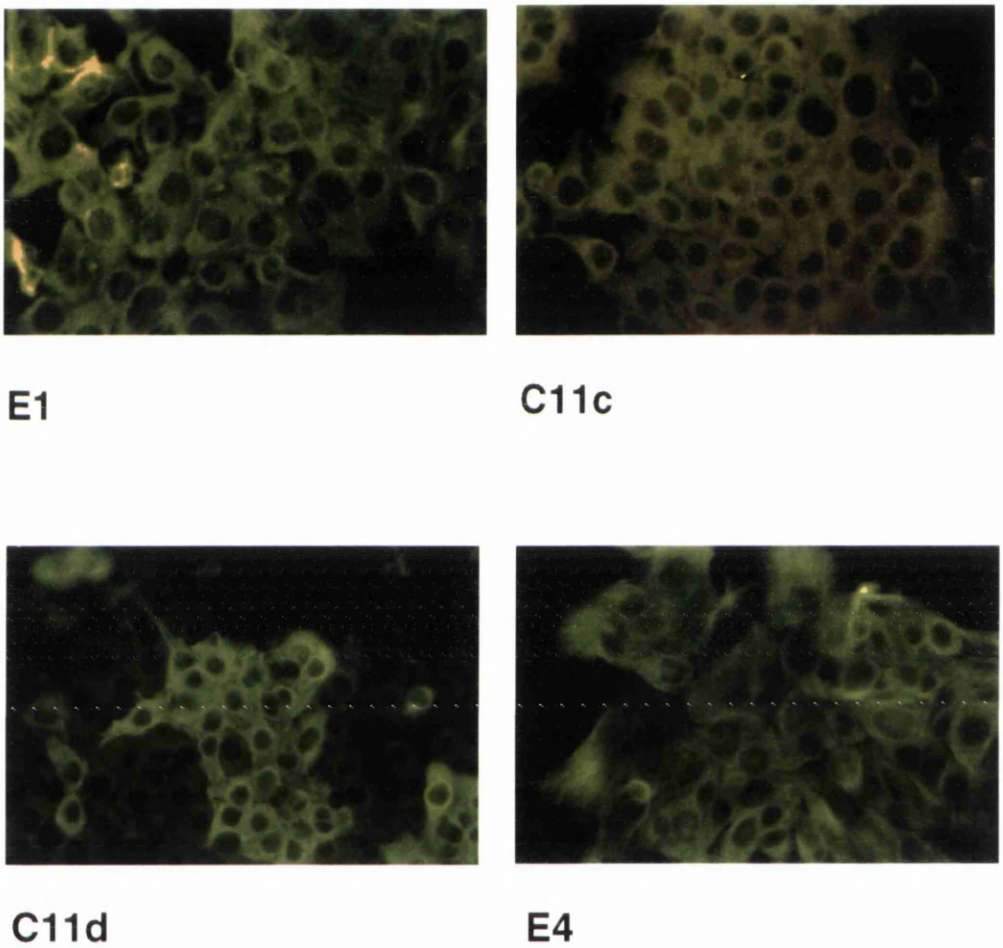
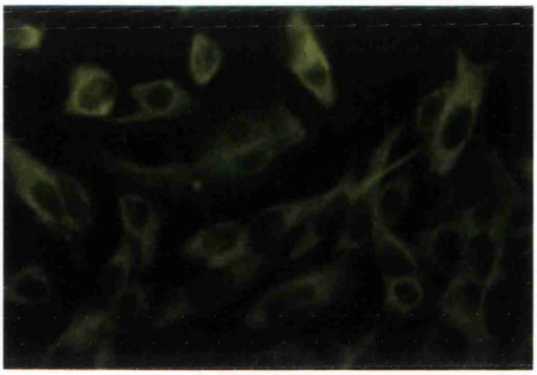
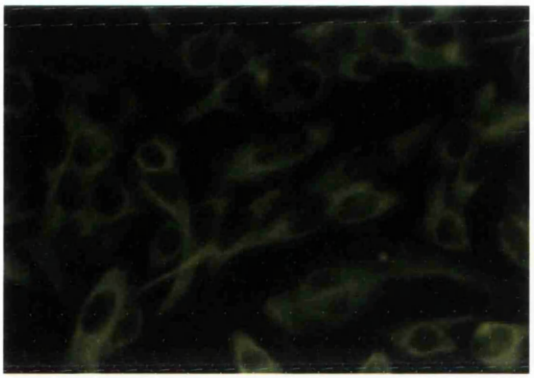


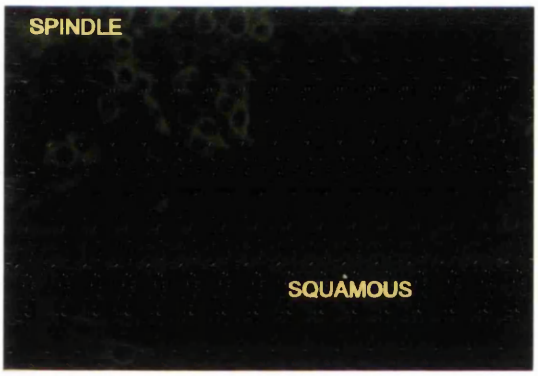
Figure 14: Detection of keratin intermediate filament network by immunocytochemistry. E1 and E4 are positive. C11c and C11d have positively staining squamous colonies surrounded by negative spindle cells.



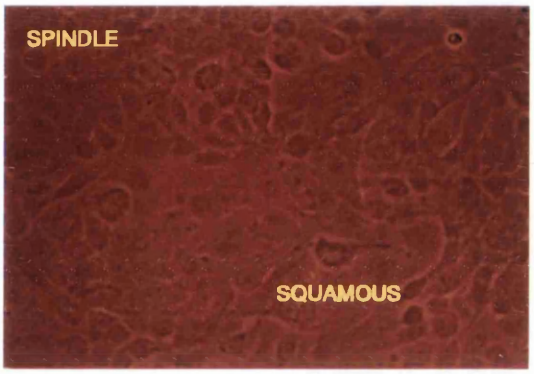
SN4



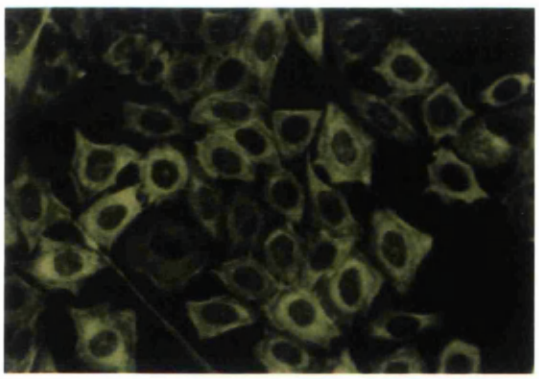
SN8



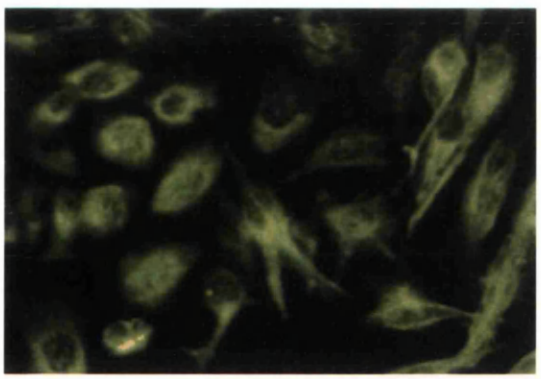
SN161



SN161



G5



H11

Figure 15: Detection of vimentin intermediate filament network by immunocytochemistry. SN4, SN8, G5 and H11 express vimentin. SN161 has positive and negative regions depending on the cell morphology; squamous colonies are negative while surrounding spindle cells are positive.

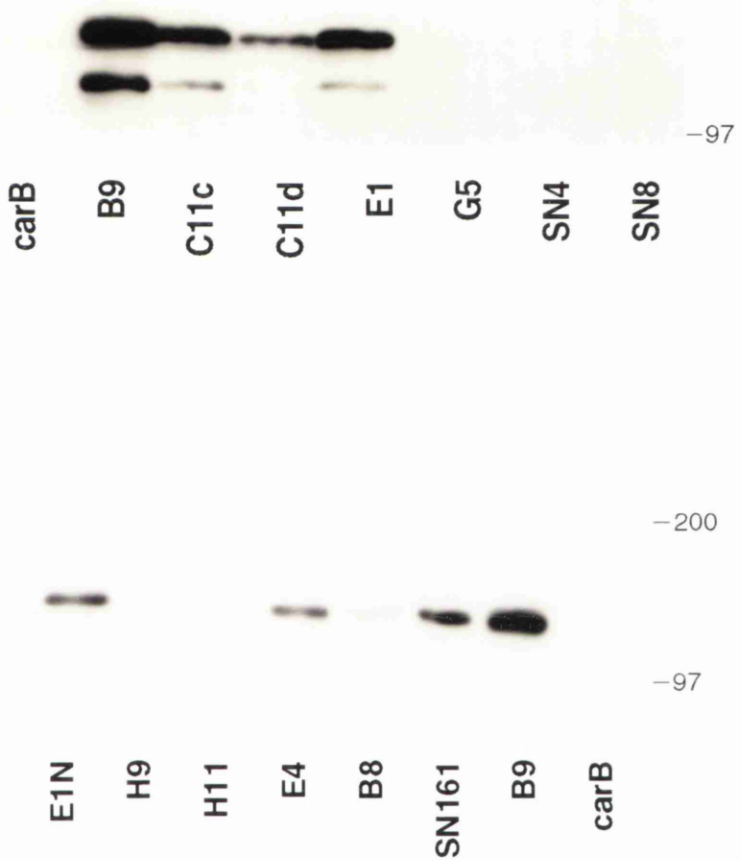


Figure 16: Expression of E-cadherin protein in SN161 clones and other squamous and spindle cell lines derived from mouse skin tumours. The expression pattern is consistent with that predicted by the morphology of each cell line i.e. squamous cells are positive and spindle cells are negative.
The lower molecular weight bands in the upper gel are degradation products of the E-cadherin protein.

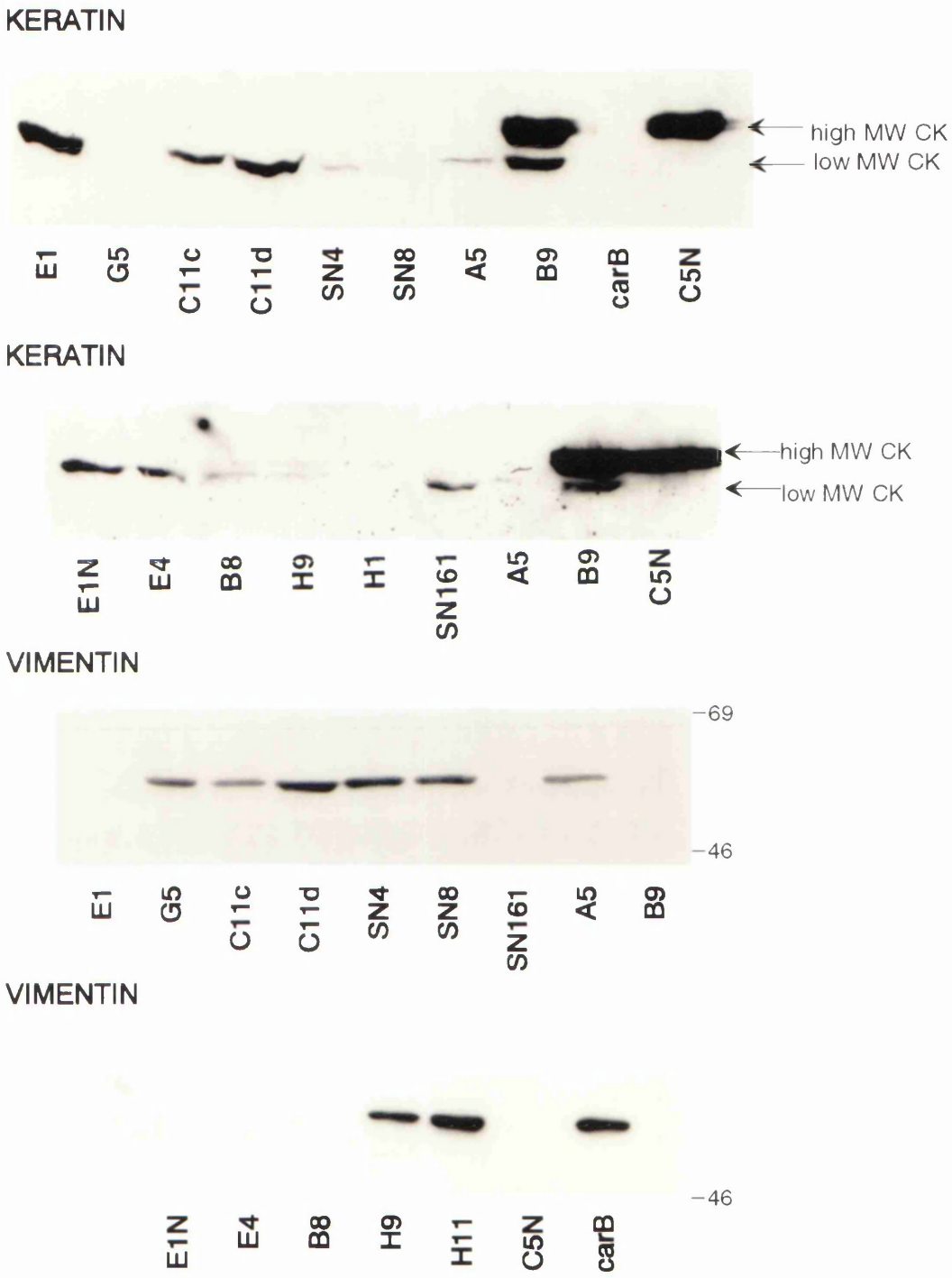


Figure 17: Expression of cytokeratin (CK) and vimentin intermediate filament proteins in SN161 clones and other squamous and spindle cell lines derived from mouse skin tumours. Squamous cell lines express keratin while spindle cells express vimentin. Cell lines with mixed morphologies express both.

3.2.3 Assessment of tumorigenicity and anchorage-independent growth

It has been frequently observed that spindle cells are more tumorigenic than their squamous equivalents and the more advanced a cell type is with respect to its position on the carcinogenesis pathway, the more tumorigenic it tends to be. Each of the SN161 cell lines was injected into nude mice in order to test this relationship between tumorigenicity and cell shape. The results obtained show a general trend linking morphology and latency (table 2). The most spindle clones (H11, SN4 and SN8) produced tumours of 10mm diameter in 35-43 days, while the most squamous of the clones, E4, grew much more slowly and took 76 days to produce a tumour of similar size. These figures are, however, only a rough guide since there is a degree of error involved in deciding when a particular tumour is of the appropriate size; some tumours protrude from the skin surface more than others. With the exception of E4, all of the cell lines induce tumour formation in a relatively short time when compared with the B9 cell line which was derived from a squamous cell carcinoma (6 weeks).

A common property of transformed cells is their ability to grow in semi-solid media. This property often correlates with a spindle morphology so 5 of the SN161 clones were tested for their ability to grow in soft agar (table 3). As would be predicted, the squamous clones (E1N and E4) did not grow. In contrast, lines that contain a proportion of spindle cells (H9, H11 and B8) had varying abilities to grow and the most spindle of these, H11, grew as well as the spindle cell line, A5. Two explants derived from nude mouse tumours induced by SN161 clones (E1-3 and G5-23) were also tested. E1-3 (a squamous cell line) grew poorly despite being derived from a spindle tumour, although it may have an enhanced growth compared to the original E1 cell line which was not tested. G5-23, a mainly spindle explant from a tumour induced by injection of the spindle cell line, G5, grew slightly better but still did not produce the same number and size of colonies as other spindle cell lines.

It seems that a growth-promoting factor which is present *in vivo*, is absent in the conditions used to test anchorage-independent growth in culture.

CELL LINE	MORPHOLOGY	LATENCY (DAYS)	TUMOUR HISTOLOGY	EXPLANT MORPHOLOGY
SN161	mixed	26	spindle	some squamous
E1	squamous	43	spindle	mostly squamous
G5	spindle	26	spindle	spindle
C11c	mixed	26	spindle	mixed
C11d	mixed	35	spindle	mixed
SN4	spindle	43	spindle	spindle
SN8	spindle	35	spindle	spindle
E1N	squamous	33	spindle	mostly squamous
E4	squamous	76	spindle	squamous
B8	mixed	35	spindle	mixed
H9	mixed	17	spindle	mixed
H11	spindle	35	spindle	spindle

TABLE 2: Assessment of tumorigenicity in nude mice. There is a general trend linking morphology and latency ie. spindle cell lines have shorter latencies than the squamous cell lines.

CELL LINE	ORIGIN & MORPHOLOGY	COLONY FORMATION IN SOFT AGAR
A5	spindle clone from skin tumour	+++
EIN	squamous clone from metastasis	-
E1-3	squamous explant from tumour induced by injection of E1 (squamous)	+
G5-23	spindle explant from tumour induced by injection of G5 (spindle)	++
H9	mixed morphology clone from metastasis	++
H11	spindle clone from metastasis	+++
E4	squamous clone from metastasis	-
B8	mixed morphology clone from metastasis	+
C5N	squamous cell line	-

TABLE 3:
Assessment of anchorage independent growth of various cell lines with a range of morphologies. The ability to grow in soft agar was determined by measuring 2 factors : the number of colonies and their size. Cell lines which were unable to grow under these conditions are indicated -. Cell lines which produced approximately 50 colonies of 100 cells are indicated + (weakly positive), those which produced more than 100 colonies of about 100 cells are indicated ++ and those which grew as well as the positive control, A5, (more than 100 colonies of more than 100 cells each) are marked +++.

3.2.4 Demonstration of morphological plasticity of metastasis-derived cell lines

As described in section 3.2.3, SN161 parental cells produce spindle tumours when injected into nude mice despite being comprised of a majority of squamous cells. It was not known if this was due to dominance of the minority of spindle cells *in vivo* or if the whole population had switched to a more spindle phenotype induced by environmental factors in the host tissue. This question was addressed by injection of the SN161 clones which have a variety of morphologies.

The histology of all tumours induced by SN161 single cell clones was spindle even when the cell line injected was uniformly squamous e.g. E1N. However, when explants were grown from these tumours, squamous cells could be recovered if the cell line injected had a squamous component; explants from tumours induced by spindle cell lines were mostly spindle (figure 18).

The origin of the tumour explants was confirmed by PCR. The cell lines injected were initially isolated from a 129/NIH hybrid mouse tumour, a genetic background which can be distinguished from the nude mice used to test tumorigenicity. Four microsatellite markers (D2MIT22, D2MIT26, D9MIT9 and D9MIT11) showed amplification of 129 and NIH alleles using explant DNA (figure 18) so it can be assumed that the tumours are derived from injected cells and their spindle morphology is not due to host fibroblast recruitment.

This supports the theory that the cell lines have a plastic morphology that can be modulated by factors in the subcutaneous environment where they were injected. These factors presumably are not present *in vitro* because a significant proportion of the cells which were altered *in vivo* regain their original squamous morphology in culture. It is also possible that *in vitro* growth conditions encourage the squamous morphology.

A valuable resource has now been created which will be extremely useful in the study of both carcinogenesis and epithelial differentiation. A great deal is known about the phenotypes of squamous and spindle cell lines such as A5 and B9 and various events

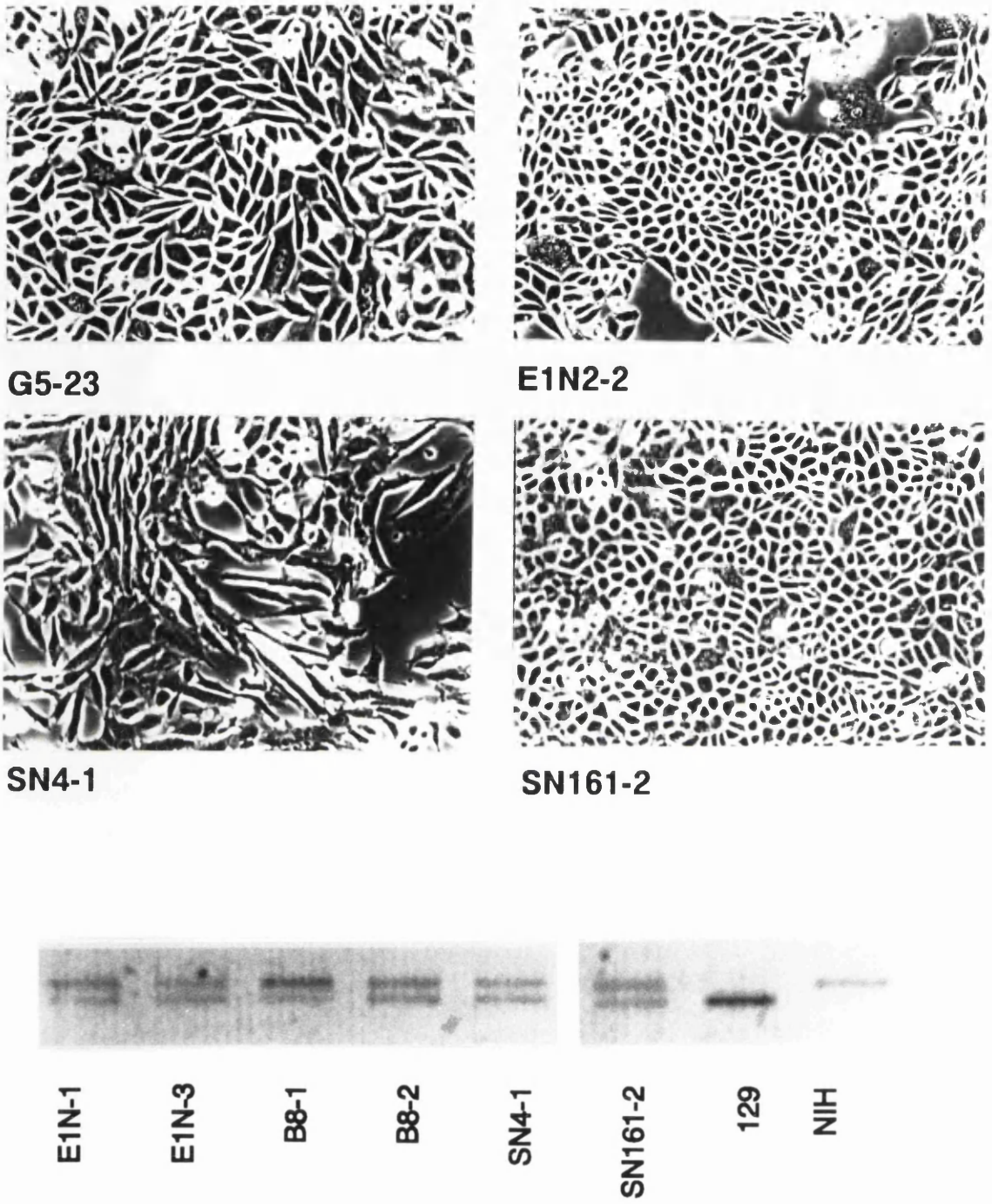


Figure 18 : Explants from nude mouse tumours. Squamous cells can be recovered from spindle tumours if the inoculated cell line had a squamous component. The relationship of the explants to the injected cells is proven by PCR of 129/NIH markers.

that occur during carcinognesis. Now another aspect has been introduced; cell lines which are capable of altering their morphologies in response to signals from their environment. These cell lines may represent an intermediate stage which lies between the squamous and spindle extremes and possesses the ability to alter its morphology transiently. Using all of the cell lines now characterised, it should be possible to identify both genetic and biochemical factors central to the squamous/spindle transition.

3.3 Genetic analysis of fixed morphology cell lines

Numerous studies have indicated the importance and causal nature of genetic alterations in carcinogenesis. Neoplastic cells accumulate increasing numbers of genetic alterations generated by random somatic mutational events (Nowell, 1976) and the more genetic changes tumours undergo, the more malignant they become (Vogelstein et al., 1989). Specific alterations have been associated with particular stages e.g. ras and p53 mutations and it is now evident that many of these alterations can be detected as gross chromosomal changes.

The recently developed technique of mapping using microsatellites as markers has been adapted to provide a means of allelotyping mouse skin tumours. It is much more sensitive than karyotyping and can detect relatively small genetic alterations. It has been applied to the mouse skin model system with the aim of identifying alterations specific to the squamous/spindle transition.

3.3.1 Summary of allelotype

On the basis of previous work carried out on A5 and B9, it was thought that some of their phenotypic differences were due to the loss of a gene with tumour suppressing properties. Indirect evidence from cell fusion studies showed that the spindle phenotype was recessive and the tumorigenicity of spindle cells could be suppressed to some extent

depending on the morphology of the cells they were fused with (Stoler et al., 1993). This suggested that a loss of information had occurred during the progression from squamous to spindle carcinoma.

A5 and B9 were derived from a tumour induced in a CBA/*mus spretus* F1 hybrid mouse. The hybrid has a high degree of heterozygosity which can be exploited to search for loss of heterozygosity which is often a good indication of the presence of a tumour suppressor gene at that locus. The purpose of this study initially was to identify gross chromosomal alterations, not specific genetic changes relating to particular genes. Genetic changes affecting specific genes e.g. the H-ras oncogene have already been reported in this system (Quintanilla et al., 1986; Bremner and Balmain, 1990) and will not be discussed here.

Many markers were screened under different reaction conditions to find those which were most informative i.e. were polymorphic for MSC mice. Typical results of microsatellite analysis using a variety of DNA samples and polymorphic markers are shown in figure 19. Using D7MIT17, *spretus* and *musculus* DNA give products of different sizes so this marker can be used for *spretus/musculus* hybrids e.g. MSC. The same is true for 129/NIH and *spretus*/NIH crosses since each parental product can be seen. The marker is not informative for sencar/Balb crosses since both of these strains give products of the same size.

Examples of balanced and imbalanced alleles are also shown in figure 19. The DNA samples tested were isolated from cell lines and spleen tissue. The A5, B9 and D3 cell lines were single cell cloned from the MSC11 cell line (MSC11cl), a cell line derived from the MSC11a tumour (figure 7). MSC11cl exists as a population of mixed morphologies, approximately 75% squamous and 25% spindle. It was therefore anticipated that the results for B9 and MSC11cl should be very similar and the allelic ratios found for MSC11cl should equal the combined ratios for A5, B9 and D3.

Primer D9MIT9 shows that A5, B9 and D3 have the same result as control spleen DNA i.e. the lower *spretus* allele amplifies better than the CBA allele. Primer D13MIT14 shows that genetic alterations have taken place in A5 and B9. The spleen,

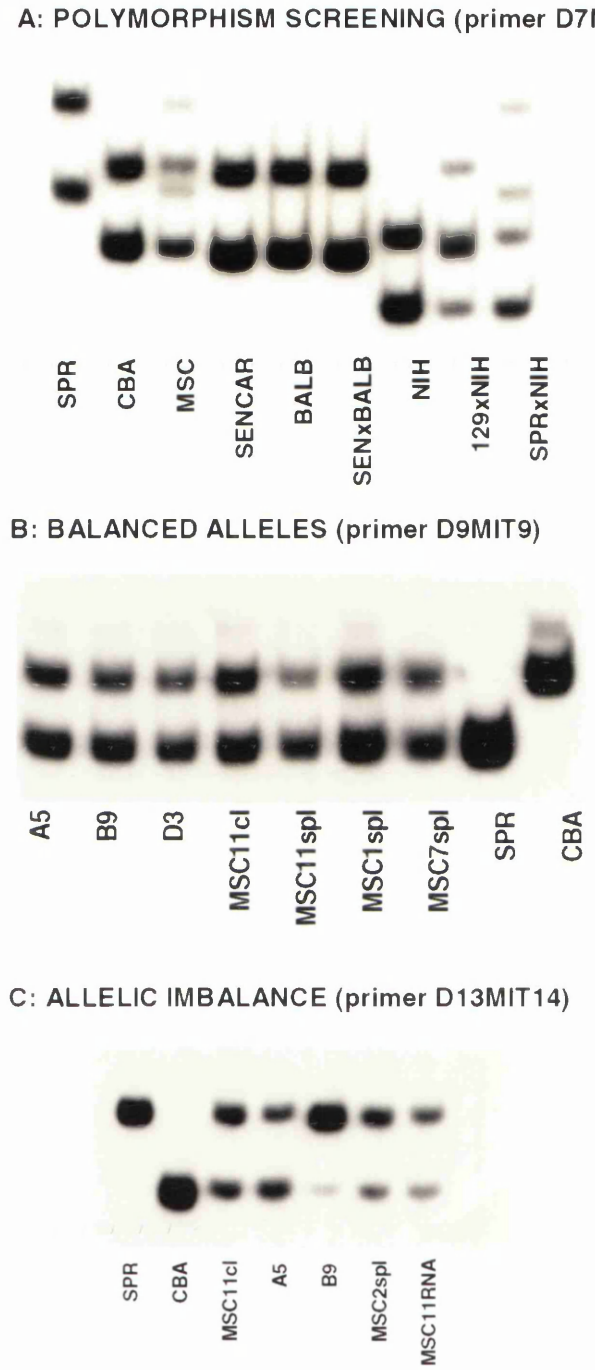


Figure 19: Typical results from PCR amplification of polymorphic microsatellites.
A: Screening for polymorphisms using primer D7MIT17 showed that it was informative for MSC, 129/NIH and SPR/NIH crosses.
B: Primer D9MIT9 shows that all DNA samples give the same ratio of amplified products
C: Primer D13MIT14 shows that the B9 cell line has an imbalance in favour of the spretus allele

MSC11 tumour DNA (MSC11T) and MSC11cl DNA samples show preferential amplification of the larger *spretus* allele but A5 has the opposite ratio and B9 has lost nearly all of the CBA product. It is important that results are always compared to spleen samples because amplification of one marker more than the other may be due to the reaction conditions and primer design rather than the genetic composition of the DNA sample.

Control DNAs from inbred *spretus* and CBA mice were tested with the MSC11-derived DNAs to show which allele in the hybrid samples could be attributed to which parent. However, some results were complicated by the fact that all of the primers were designed for *musculus* sequences. The most important region for Taq polymerase is the 3' end of the primer which must be a good match with the genomic sequence to which it should bind in order to allow Taq polymerase to extend the oligonucleotide. In some cases the *spretus* sequence varies significantly from *musculus*, therefore the allele does not amplify - the marker is then "not informative". In other cases the *spretus* allele does amplify but the *spretus* product size varies between control *spretus* DNA and MSC hybrid DNA. This is due to some variation between individual *spretus* mice which are not inbred. The same can be true for the *musculus* allele (CBA in this study) although it varies much less often. It is important to bear this variation in mind when analysing the PCR products. For this reason, control spleen DNA samples were included to check that individual primers were informative. These were prepared from different mice as indicated by their number i.e. MSC2spl, MSC7spl etc. These are only useful as proof that both alleles amplify since individual spleen DNAs can sometimes produce differing ratios of products with the same primers despite being "normal" controls.

Further complications can be created by shadow bands. These are caused by strand slippage during PCR and can eliminate potentially useful markers e.g. the *spretus* product shadow band may be the same size as the *musculus* band which is then masked. In many cases, however, this can be avoided by using appropriate gel running conditions.

Microsatellite allelotype analysis of A5 and B9 using polymorphic markers has identified genetic differences which distinguish the two cell lines. As shown in table 4, of the 147 markers examined (5-19 per chromosome), those on chromosomes 3, 4, 7, 13, 16 and 17 showed that all 3 cell lines differed from spleen DNA. Only the spindle cell lines, A5 and D3, differ from spleen on chromosomes 1, 5, 12 and 19 and only the squamous cell line, B9, differs from spleen on chromosomes 6, 8 and 18. There were no differences between squamous and spindle cell lines on chromosomes 2, 3, 7, 9, 10, 15 and 17. Therefore, the genetic alterations that distinguish the squamous and spindle lines involve chromosomes 1, 4, 5, 6, 8, 12, 13, 16, 18 and 19. In most cases it was either the squamous or spindle cells that differed from spleen but markers on chromosomes 13 and 16 identified different alterations in A5/D3 and B9.

This is a simplified presentation of the allelotype since certain individual markers give results inconsistent with other markers on that chromosome; these will be discussed later. Further complications are due to differences between the two spindle cell lines e.g. on chromosomes 11 and 14 where only A5 differs from spleen. For the purposes of this study, only alterations that distinguish both spindle cell lines from the squamous cell line have been regarded as significant.

Strikingly, out of all 118 informative markers analysed, there was only one complete loss of heterozygosity at D8MIT13 (figure 20). All other markers on this chromosome produced both alleles. This contrasts dramatically with the larger number of allelic imbalances found. It is even more interesting that the loss has occurred in B9, not A5. It seems to be a local deletion but the precise region of loss has not yet been investigated.

Some individual markers showed large imbalances not seen with flanking markers. For example, the D3 cell line result with D9MIT16 shows much greater amplification of the *musculus* allele than the *spretus* allele (data not shown). This is in contrast to the result for A5 and B9 at this locus and the result for all cell lines at all other markers on this chromosome. It is not possible to say with certainty whether D3 has lost *spretus* or gained *musculus* alleles since PCR amplification only shows relative

CHROMOSOME	ALL CELL LINES DIFFER FROM SPLEEN	ONLY A5 & D3 (SPINDLE) DIFFER FROM SPLEEN	ONLY B9 (SQUAMOUS) DIFFERS FROM SPLEEN
1		●	
2			
3	▲		
4	▲		■ (1)
5		●	
6			■
7	▲		
8			■ (2)
9			
10			
11		● (3)	
12		●	
13	▲ (4)		
14		● (3)	■
15			
16	▲ (4)		
17	▲		
18			■
19		●	

TABLE 4: Summary of A5, B9 and D3 allelotypes indicating gross chromosomal alterations

(1) B9 has undergone further alteration (recombination) not seen in A5 and D3

(2) B9 has complete LOH at D8MIT13

(3) only A5 differs from spleen

(4) squamous and spindle cell lines have different alterations.

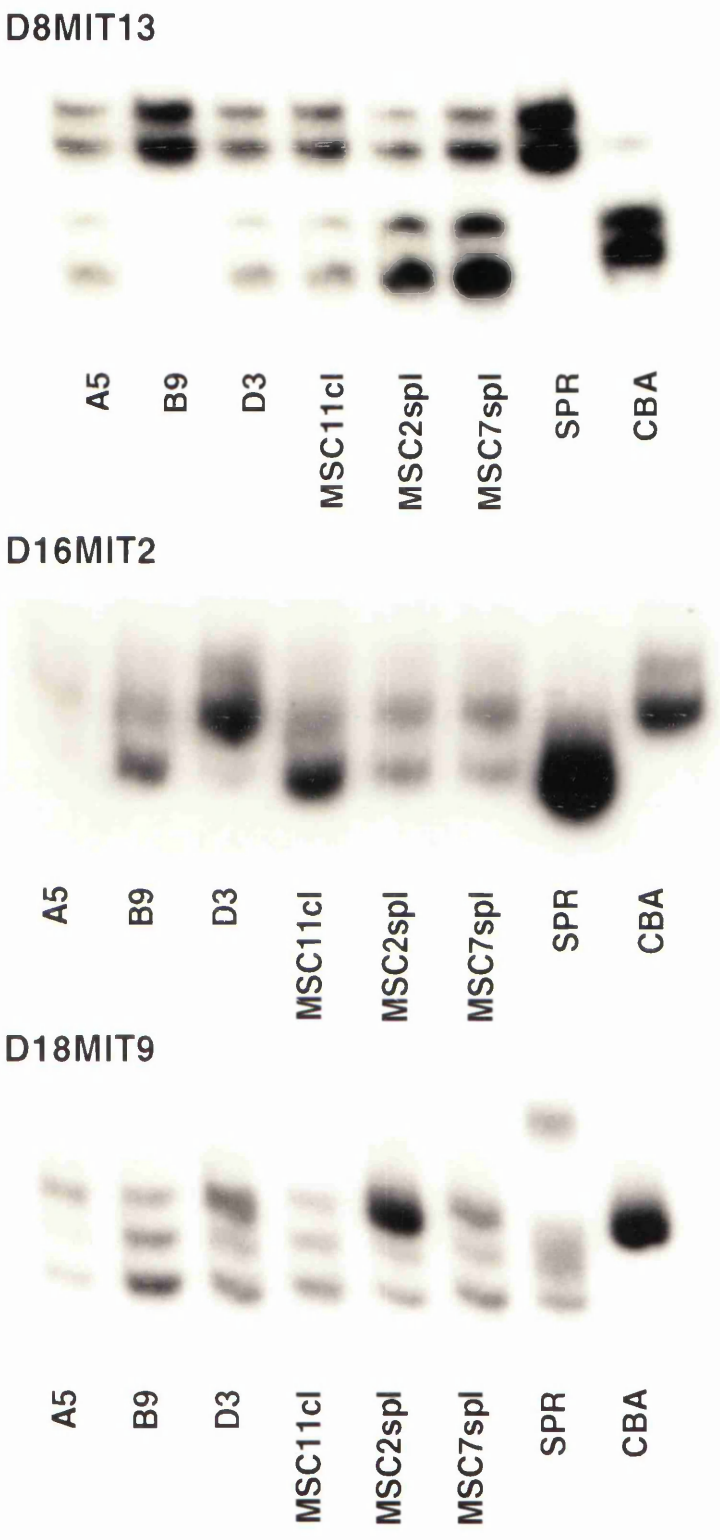


Figure 20: Analysis of chromosomes 8, 16 and 18. D8MIT13 shows the only complete loss of heterozygosity found i.e. B9 has lost the CBA allele. D16MIT2 and D18MIT9 show a difference between B9 and A5/D3; in both cases B9 has an imbalance in favour of the spretus allele while A5 and D3 have an imbalance in favour of the CBA allele.

amounts of the two alleles. The situation is further complicated by the degree of aneuploidy of the cell lines; A5 and B9 are tetraploid as determined by karyotyping (data not shown).

3.3.2 Detailed analysis of chromosomes 6, 16 and 18

Alterations found on particular chromosomes have interesting implications for the study of mouse skin carcinogenesis. Indeed some can be linked to genes already known to be directly involved in tumorigenesis. In accordance with published results from primary tumours also obtained using the mouse skin model system (Kemp et al., 1993), chromosome 6 was shown to have an imbalance at all markers tested (figure 21). In every case, the *spretus* allele was under-represented in B9. The degree of imbalance suggests that trisomy of chromosome 6 has occurred with the CBA allele being duplicated in B9: A5 and D3 were normal with respect to spleen DNA. Chromosome 6 harbours several genes which have relevance to the carcinogenesis process e.g. K-ras, raf-1 and met.

Chromosome 16 markers showed that B9 has an over-represented *spretus* allele compared to A5 and D3 (figure 20). All markers produced this result. Mouse chromosome 16 has regions of homology with human chromosomes 3, 16, 21 and 22. It is not clear what significance this has with regard to tumour development.

Chromosome 18 markers showed that B9 again has an over-represented *spretus* allele compared to A5 and D3 (figure 20). All markers produced this result. Mouse chromosome 18 has regions of homology with human chromosomes 5, 10 and 18. These are the locations of the APC, MCC and DCC genes which are known to be mutated in human tumours and may have relevance to mouse carcinogenesis also.

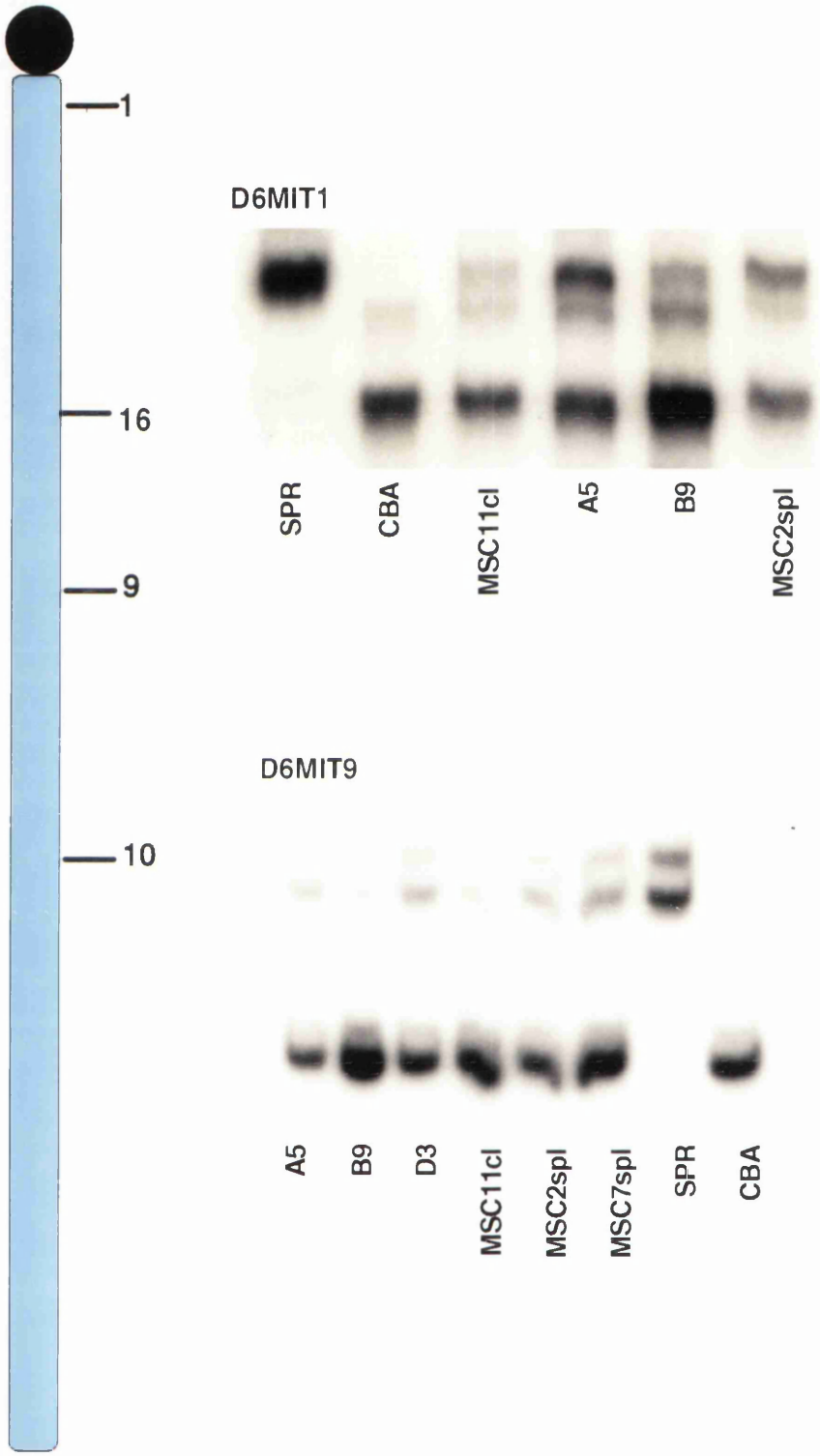


Figure 21: Markers on chromosome 6 show an imbalance in favour of the musculus allele in B9.

3.3.3 Detailed analysis of chromosome 4

The most interesting imbalance with respect to skin carcinogenesis is on chromosome 4 (figure 22). All 3 cell lines have suffered a trisomy in favour of the *musculus* allele and B9 has a further alteration (possibly a recombination) affecting a distal portion of the chromosome distinguishing it from the spindle cell lines. In each cell line, all markers between D4MIT4 and D4MIT13 show the same imbalance in favour of the *musculus* allele but, in B9, markers distal to D4MIT13 (69cM) i.e D4MIT59, T51, T81 and T35 show an increased amplification of the *spretus* allele with respect to CBA. It is reasonable to assume this indicates that a recombination has taken place between markers D4MIT13 at 69cM and D4MIT59 at 80cM in B9 and MSC11cl but not in A5 or D3.

This genetic difference is significant because other studies of mouse skin tumours have shown loss of heterozygosity on chromosome 4 in advanced carcinomas. Mouse chromosome 4 has some homology with human chromosome 9 which has been implicated in various types of cancer, but the changes found in A5 and B9 are distal to these regions. The region involved in the putative recombination is syntenic with human chromosome 1p34 and 1p36.

Marker D4MIT31 results (not shown) have been treated with caution since they show what may be an anomalous result at 48cM. All neighbouring markers show an imbalance in favour of the *musculus* allele with respect to control spleen DNA while D4MIT31 shows a relative increase in proportion of *spretus* products in A5 and D3. It is extremely unlikely that a double recombination has occurred in this small region although the imbalance may be due to a localised amplification in the spindle lines. Another possible explanation is that the marker has been incorrectly mapped.

The same analysis has been applied to the panel of SN161-derived clones using a limited number of markers. Interestingly, the genetic alterations distinguishing the squamous and spindle cell lines, A5 and B9, have not been found in these cell lines (data not shown).

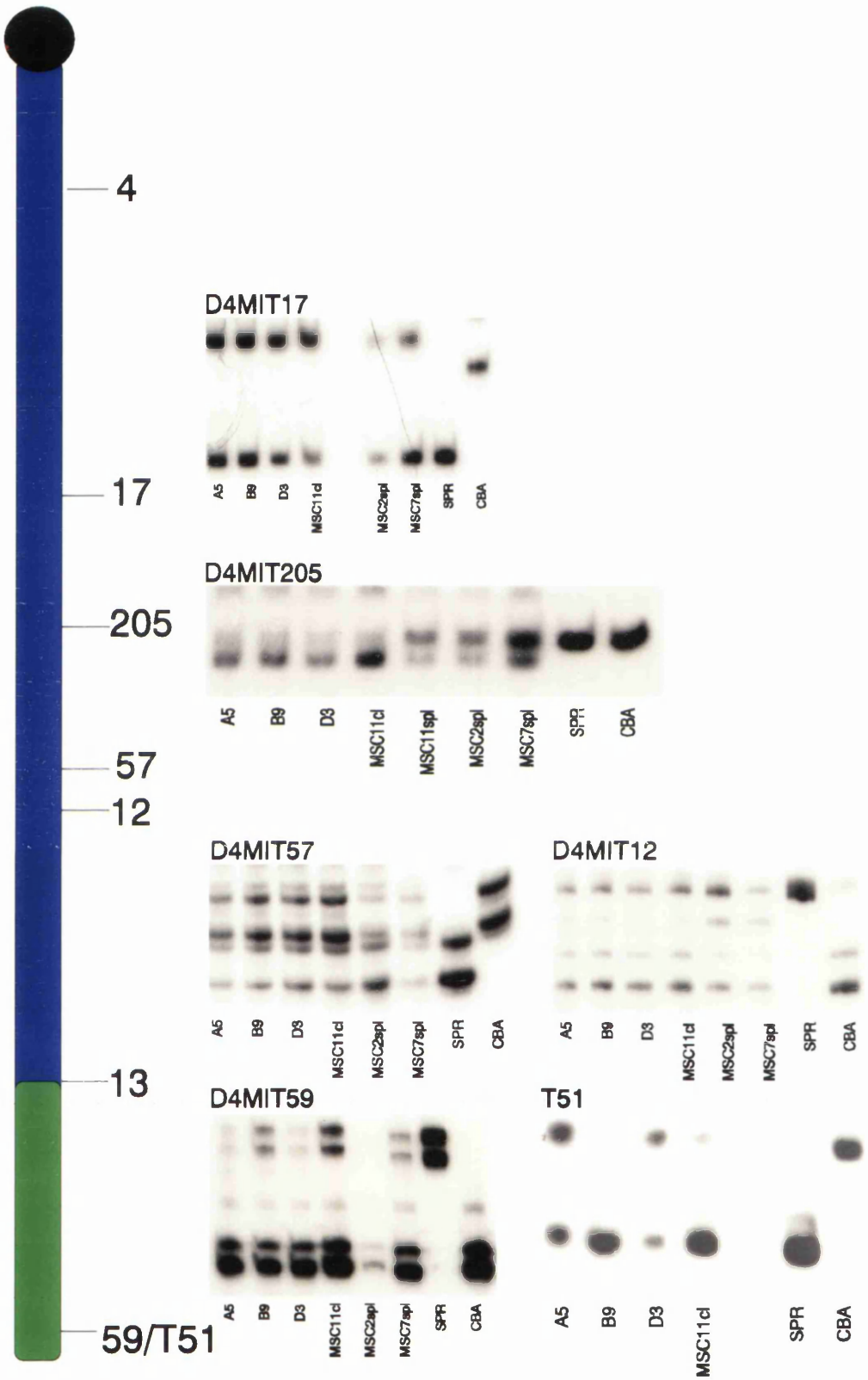


Figure 22: Detailed analysis of chromosome 4. All markers proximal to D4MIT13 show an imbalance in favour of the musculus allele in all cell lines. A recombination seems to have occurred between markers D4MIT13 and D4MIT59 in B9 but not A5 or D3.

3.3.4. Relationship between A5, B9, D3, MSC11cl and MSC11T

3.3.4A Imbalances A5 has with respect to B9

At first glance there seem to be many differences distinguishing the squamous B9 from spindle A5. On closer examination, however, these can be simplified to give an overall picture of relatively few differences. A5 differs from B9 at all markers on chromosomes 1, 5, 6, 11, 12, 13, 14, 16, 18 and 19. In some cases these differences are likely to be due to whole chromosome imbalances but this can not be assumed without further investigation. There are some interesting differences on chromosomes 4 and 8 which have already been discussed (sections 3.3.3 and 3.3.1). Isolated differences affecting only part of a chromosome are rare and can not be linked with specific candidate genes. At present there are only 4 examples of this: D3MIT16, D8MIT13, D15MIT16 and T30 (chromosome 15).

At D3MIT16, B9 has equal amplification of both alleles while all other markers on chromosome 3 show over representation of the spretus allele. A5 and D3 produce the same result at all markers i.e. preferential amplification of spretus. It is not yet clear what significance this result has.

D8MIT13 is the only primer in this study of 118 informative microsatellite markers which shows a complete loss of heterozygosity. As shown in figure 20, B9 has completely lost the CBA allele which is retained in A5 and D3. Flanking markers produce both products. It is interesting that the loss has occurred in the squamous but not the spindle cell lines. The precise region of loss has not yet been determined.

There are 2 markers on chromosome 15 which produce different results for A5 and B9. D15MIT16 and T30 show an increased amplification of the spretus allele in B9 compared with A5. This is true for two out of seven of the markers tested on this chromosome. The significance of this result, if any, has not been examined further.

3.3.4B Differences between A5 and D3

In contrast to the significant number of differences between A5 and B9, there are few differences between A5 and D3. These two cell lines differ at only 4 individual markers (D9MIT16, D12MIT11, D12MITS2 and D15MIT17) plus all 4 markers on chromosome 14 where A5 and B9 have completely lost the *spretus* allele while it is retained in D3. This small number of differences is encouraging because it suggests that the genetic differences found between A5 and B9 are likely to be related to the varying cell morphologies and are not due to genetic instability in culture over long periods of time. The same outcome is obtained with A5 and D3 at 110 out of a total 118 markers despite their being cultured separately after the initial single cell cloning. In this respect, the technique of microsatellite analysis has been successful in detecting gross chromosomal alterations while giving reliable and reproducible results.

3.3.4C Differences between B9 and MSC11cl

MSC11cl is the parent cell line from which A5, B9 and D3 were cloned. It exists as a population of mixed morphology cells, approximately 75% of which are squamous. It was therefore anticipated that the results for B9 and MSC11cl should be very similar and the allelic ratios found for MSC11cl should be equal to the combined ratios for A5, B9 and D3. In most cases this is true. However, B9 differs from MSC11cl at 5 markers (D3MIT16, D8MIT13, D15MIT16, D16MIT7 and T30 on chromosome 15) plus all markers on chromosome 14 where B9 has lost the *spretus* allele while it is retained in MSC11cl.

3.3.4D Relationship between A5, B9 and MSC11 tumour

Of the 25 markers tested, DNA from the original MSC11 tumour gives the same result as A5 in 9 cases and the same result as B9 at 3 markers. The other 13 markers

showed that the tumour DNA was different to both A5 and B9. This result is surprising because the histology of the tumour showed that it was mostly squamous. However, the sections of tumour used to prepare DNA and the cell lines would not necessarily have the same morphologies. The results can not be explained by combining the allelic ratios for A5 and B9, perhaps because there is too much interference from the presence of contaminating normal tissue DNA in the MSC11 tumour DNA sample.

3.3.5 Summary of allelotype

The technique of allelotyping using microsatellite markers has been used to compile a detailed genetic analysis of clonally-related squamous and spindle cell lines. Alterations distinguishing the two cell types have been found and gross chromosomal changes have been described. While providing information describing the genetic differences between a pair of closely-related but phenotypically divergent cell lines, this allelotype has also provided clues to their lineage and hypotheses regarding their development from normal epithelial cells can be formed. These will also be discussed in more depth in Chapter 4.

3.4 Alterations in intracellular signalling pathways

3.4.1 Status of ras-related proteins

While analysis of the genetic alterations occurring during the squamous/spindle transition was of prime importance, it was evident that biochemical alterations were also involved. Genetic changes affecting H-ras have been reported in the mouse skin system and activation of H-ras has been shown to be an early event (Quintanilla et al., 1986; Bremner and Balmain, 1990). Furthermore, amplified mutant ras has been shown to be associated with spindle cell carcinomas (R. Crombie, PhD thesis, 1994). The mutant ras

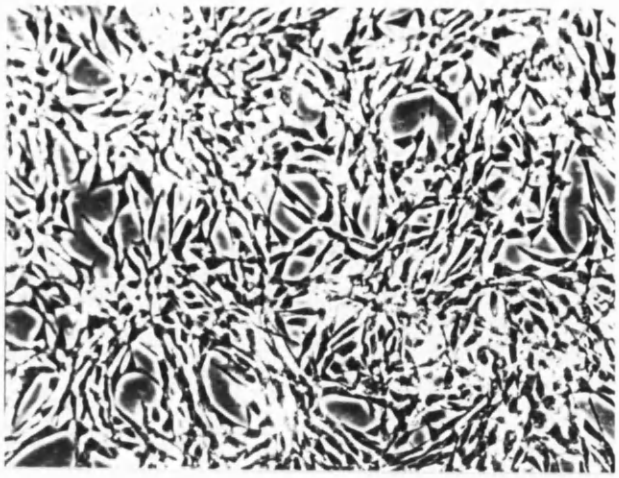
content of squamous tumour cells increases as they become less differentiated and this increase may encourage the switch to a spindle carcinoma but the complete transition requires the alteration of another locus. Experiments in which the ratio of mutant to normal ras in squamous and spindle cells was altered showed that when the mutant ras allele is lost from spindle cells, only their tumorigenicity and not their morphology is affected (R.Crombie, PhD thesis, 1994).

Ras mutations and perturbations of the signalling pathways it is involved in are common alterations in tumours. Ras proteins are components of receptor-mediated signalling pathways controlling cell proliferation and differentiation. Many of the components of this pathway have been identified and described in detail. They include raf, GAP, NF1 and K-rev. Raf, GAP and NF1 are ras effectors and as such may be targets for mutation in carcinogenesis. There is evidence from other studies showing their aberrant function in tumours (section 1.5) so their possible involvement in this system was investigated.

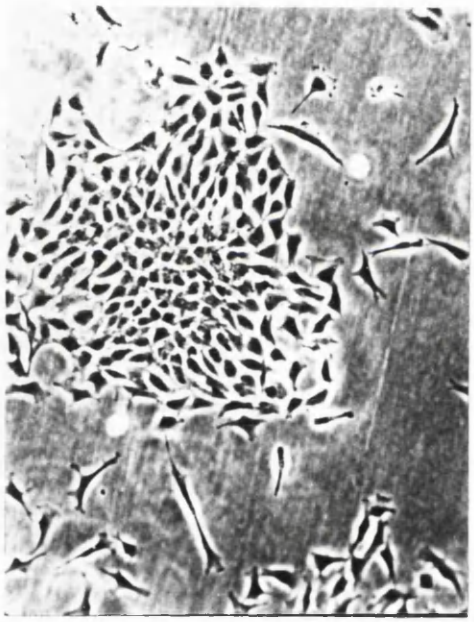
3.4.1A Transfection with K-rev

K-rev was first isolated by its ability to revert K-ras transformed cells (Noda et al., 1989). It was also shown to have a small effect on lung tumours (Caamano et al., 1992). Bearing in mind the central involvement of mutant H-ras in this system, the possibility that a downstream effector of cytoskeletal organisation and adhesion may be lost in spindle cells and the ability of K-rev to revert K-ras transformed cells, K-rev seemed an attractive candidate for an additional gene that is altered on the ras-related pathway. The available biological evidence suggested that K-rev may be an important factor in determining cell morphology.

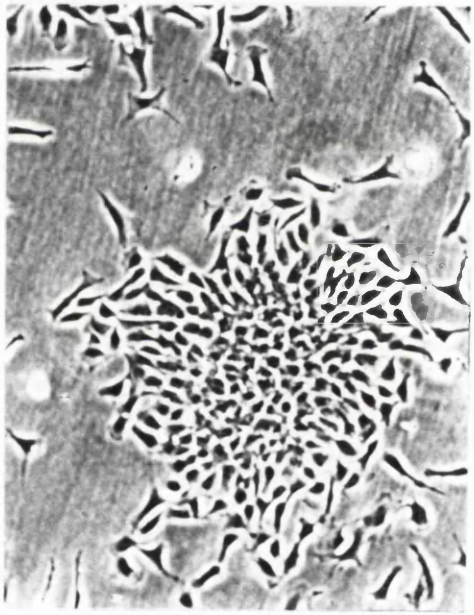
Three cell lines, A5, D3 and carB were transfected with a K-rev expression vector but only carB produced flat revertants. These were clearly different from the parental carB cell line which has a fibroblast-like morphology (figure 23). Instead they resembled epithelial cells and formed colonies of cells in close contact with each other.



carB



transfectant 1



transfectant 2

Figure 23: carB cells transfected with pKrev have a flatter, more squamous morphology when compared with the parental carB spindle cells.

However, it was not possible to maintain this more squamous morphology over a long period of time and reverted cells rapidly lost their epithelial appearance. Some immunocytochemistry and immunoblotting was carried out but the revertants showed no expression of epithelial markers (figure 24). Although the construct seemed to be present in transfected cells (data not shown), elevated levels of the exogenous protein were not detected by immunoblotting. The reverted phenotype may have been lost by the time this analysis was carried out. Further investigation would be necessary to formally prove that the transfected cDNA is being expressed.

3.4.1B Transfection with GAP-N

Another component of the ras signalling pathway has received a lot of attention. There is evidence that p120GAP, in addition to negatively regulating ras, may also play a role in the downstream signalling from ras (McCormick, 1989). p120GAP itself has 2 effectors which may be relevant to the squamous/spindle transition: p62 and p190. These are both tyrosine-phosphorylated proteins which bind to the N-terminal domain of GAP through its SH2 domains (Wong et al., 1992; Settleman et al., 1992). p190 is a specific GAP for the rho family which, together with rac, co-ordinates cytoskeletal effects (Hall, 1992) therefore it may coordinate the ras and rho signalling pathways through its interaction with GAP.

A GAP-N construct which lacks the C-terminal ras-binding domain was transfected into C5N and B9 cells. It had been shown to disrupt the cytoskeleton of transfected fibroblasts through some interaction with p190 (McGlade et al., 1993) so its effect on 2 squamous cell lines was tested. Several neo resistant lines were obtained but no morphological change could be seen and transfection of C5N with the truncated GAP-N construct had no effect on anchorage-independent growth. Expression of the truncated protein was not formally proven, however, and this experiment was not continued due to the lack of effect on morphology.

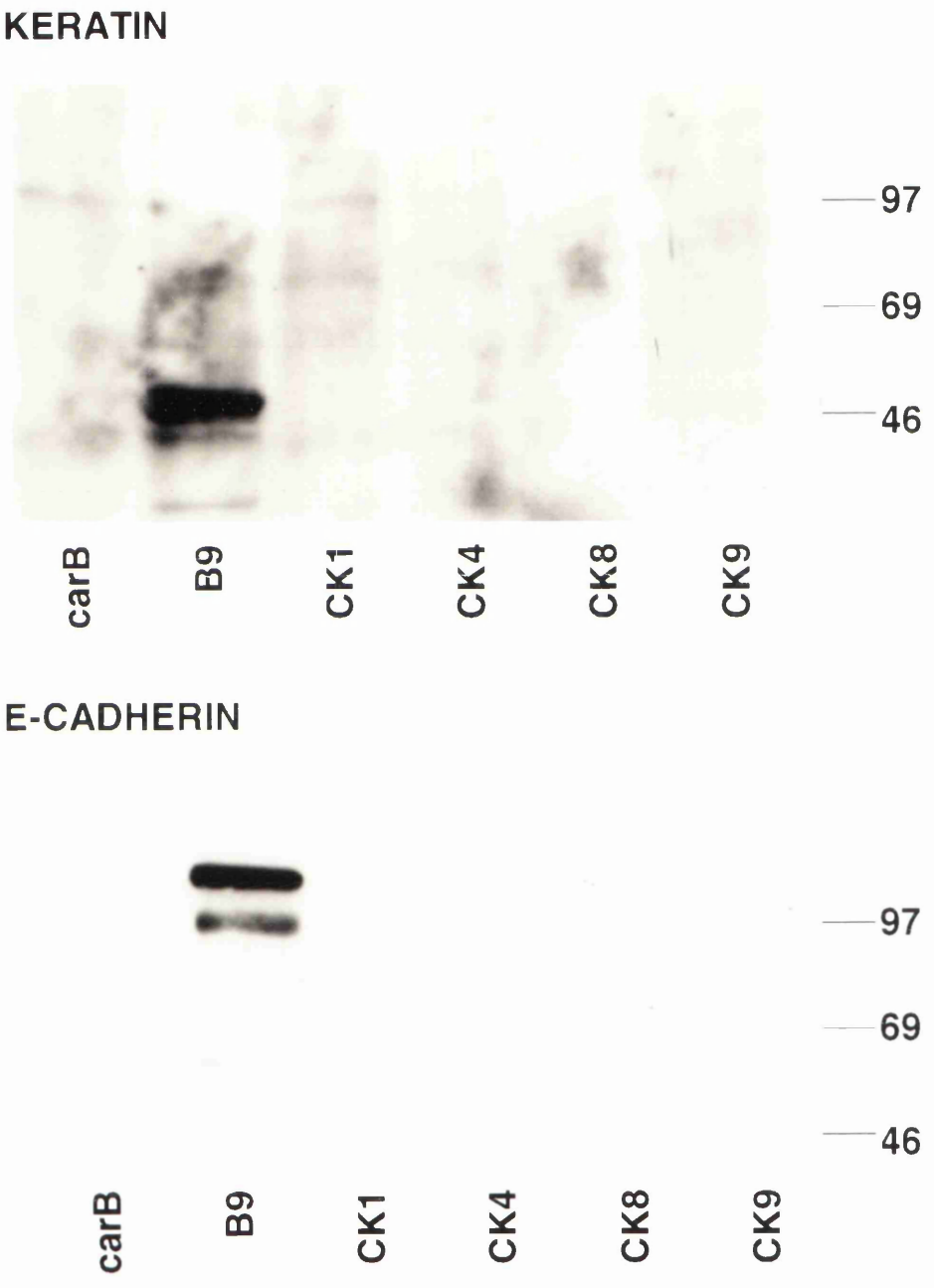


Figure 24: Characterisation of K-rev transfectants CK1, CK4, CK8 and CK9. All transfectants tested show the same lack of expression of E-cadherin and keratin seen in the parental untransfected cell line, carB.

3.4.1C Status of p120GAP and p62 proteins

On the basis of its proposed role in linking the ras signalling pathway with complexes that affect cell shape and attachment to substrates, the expression of p120GAP was investigated in squamous and spindle cell lines. Expression of the p120GAP protein was determined by immunoprecipitation (figure 25). GAP was expressed in all cell lines tested at similar levels and there was no difference between squamous and spindle lines. The same was true for p62.

p62 is a GAP-associated protein which has significant homology to an hnRNP protein and binds RNA (Wong et al., 1992). It is highly tyrosine-phosphorylated during calcium-induced keratinocyte differentiation (Filvaroff et al., 1992) and becomes tyrosine phosphorylated and exists in a complex with GAP in v-src transformed cells (Koch et al., 1992). It has therefore been proposed to be involved in cytoskeletal changes associated with transformation. However, no difference in expression associated with cell type could be seen when p62 levels were tested by Western blotting (figure 25).

The antibody used also showed some binding to p68, a closely-related protein, which also had similar expression levels in both cell types. It was concluded that variable expression levels of either of these components of the GAP pathway was not likely to be a factor determining cell morphology in the cell lines tested.

3.4.2 Detection of focal contact proteins

Many changes in gene expression occur during the squamous/spindle transition. Some of the most apparent changes affect the cytoskeleton. As discussed previously, squamous cells possess a cytoskeleton composed of cytokeratins, while spindle cells express vimentin. Changes in cell adhesion accompany these changes in intracellular architecture. The complexes which connect cytoplasmic systems with the membrane proteins that respond to the extracellular environment are thought to be responsible for

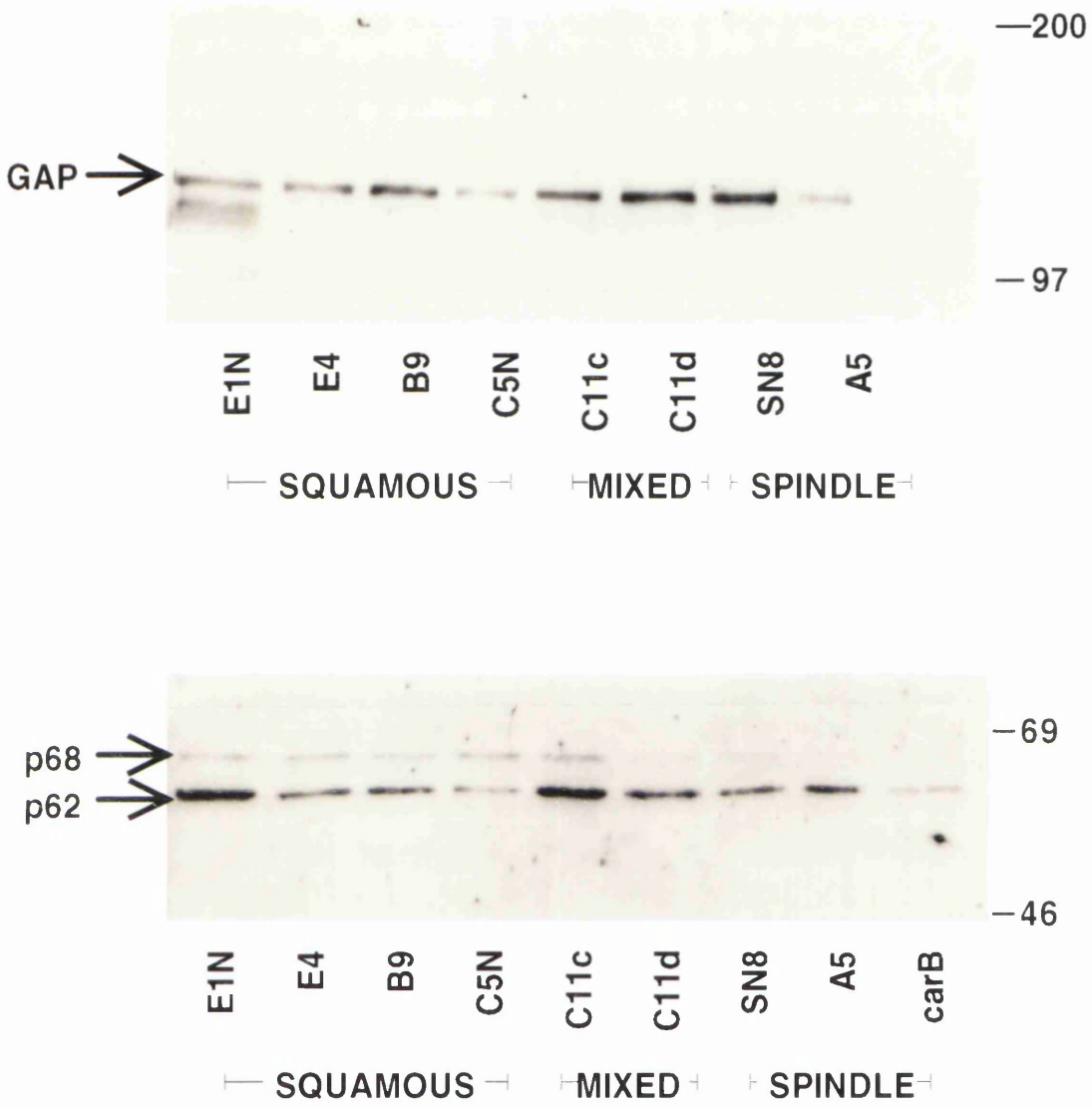


Figure 25: Status of p120GAP and p62 proteins in squamous and spindle cells. There is no correlation between morphology and expression level of either of these proteins. The p62 antibody shows some binding to the closely related protein p68. The p120GAP protein was immunoprecipitated. p62 and p68 were detected by immunoblotting whole lysates.

the loss of cell adhesion and concomitant morphological changes during tumorigenesis; in general, transformed cells are more rounded and less adherent than their normal counterparts.

Focal adhesions are complex assemblies of many proteins, some of which have signalling functions and may thus have more than just a structural role. The expression of proteins located in focal adhesion was investigated.

3.4.2A Expression of vinculin and talin

Vinculin and talin are focal adhesion components which as yet have only been demonstrated to have a structural function. Their expression in squamous and spindle cells was studied.

Western blotting of whole cell lysates showed a correlation between expression of vinculin and talin and cell morphology. As shown in figure 26, all 4 entirely spindle cell lines tested express vinculin protein at high level. The squamous cell lines also express vinculin but at a much reduced level estimated to be less than half the amount. The same trend is seen for talin. Spindle cells express talin but it can barely be seen in squamous cell lysates even after a long exposure; in fact, it is completely absent from B9 and C5N. Cell lines which are comprised of approximately equal numbers of squamous and spindle cells show significant expression of both proteins similar to that seen for the uniformly spindle lines. The conclusion is that spindle cell lines derived from mouse tumours express substantial quantities of focal adhesion proteins but squamous cell lines derived from the same tumours have much reduced expression. This has relevance to studies of the cytoskeletal architecture of the two cell types.

3.4.2B Expression of FAK (focal adhesion kinase)

FAK is a major target for tyrosine kinases during various cellular events associated with cell adhesion and growth control. It has been proposed as a downstream

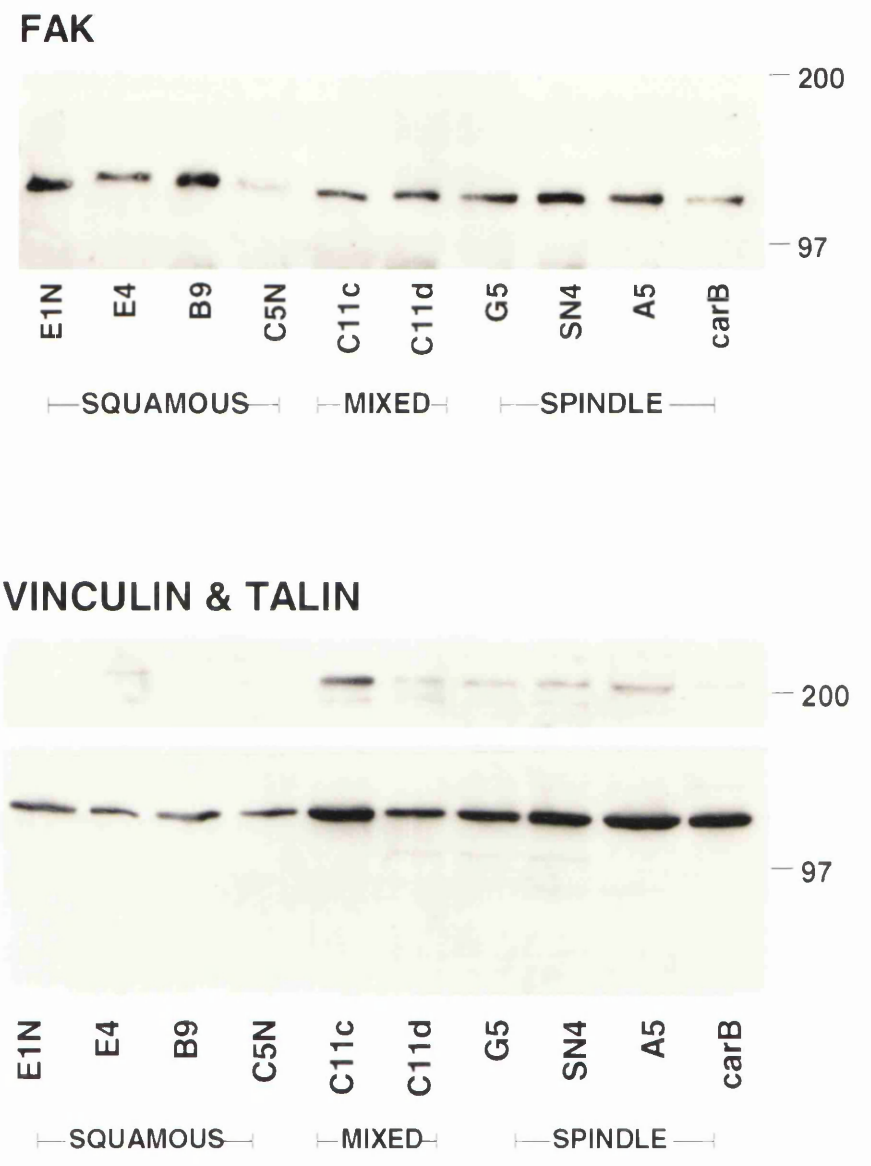


Figure 26: Status of focal adhesion related proteins in squamous and spindle cells. All cell types express FAK (125kD) at varying levels. All cell lines express vinculin (125kD) also but there is a correlation between morphology and expression level of this protein; spindle cells express more vinculin than do squamous cells. Only spindle cells or cell lines with a spindle component express significant amounts of talin (205kD).

transducer of integrin-mediated signals and has been implicated as a substrate for v-src (Guan and Shalloway, 1992) therefore any correlation between altered FAK expression and changes in cell morphology would be of great interest.

The total FAK expression pattern is not linked to cell morphology. When equal amounts of protein from whole cell lysates are loaded, all cell lines express FAK at varying levels (figure 26). This is interesting when the results from direct immunofluorescence staining of focal contacts are considered (see section 3.4.2C).

3.4.2C Detection of focal contacts by immunocytochemistry

Double immunocytochemistry using a fluorescein-linked antibody to a focal contact protein (vinculin, talin or FAK) and a rhodamine-linked phalloidin antibody reveals the localisation of focal contacts in fixed monolayers of squamous and spindle cell lines (figure 27). Spindle cells have extended processes projecting from the cell body. Focal contacts can clearly be seen where these processes presumably anchor the cell to its substrate (only vinculin staining is shown). This clear staining pattern is not so obvious in the more rounded squamous cells which lack such processes. However, localised expression of vinculin and FAK in focal contacts can still be seen in squamous cells despite expression of these proteins as shown by Western blotting (FAK immunofluorescence not shown).

3.4.2D Conclusions

Some differences in expression of focal contact proteins consistent with cell shape have therefore been established. Vinculin and talin can easily be detected in spindle cells by Western blotting and are clearly localised in focal contacts as seen by immunocytochemistry. Squamous cells express little or no talin and have a much reduced expression of vinculin compared to spindle cells.

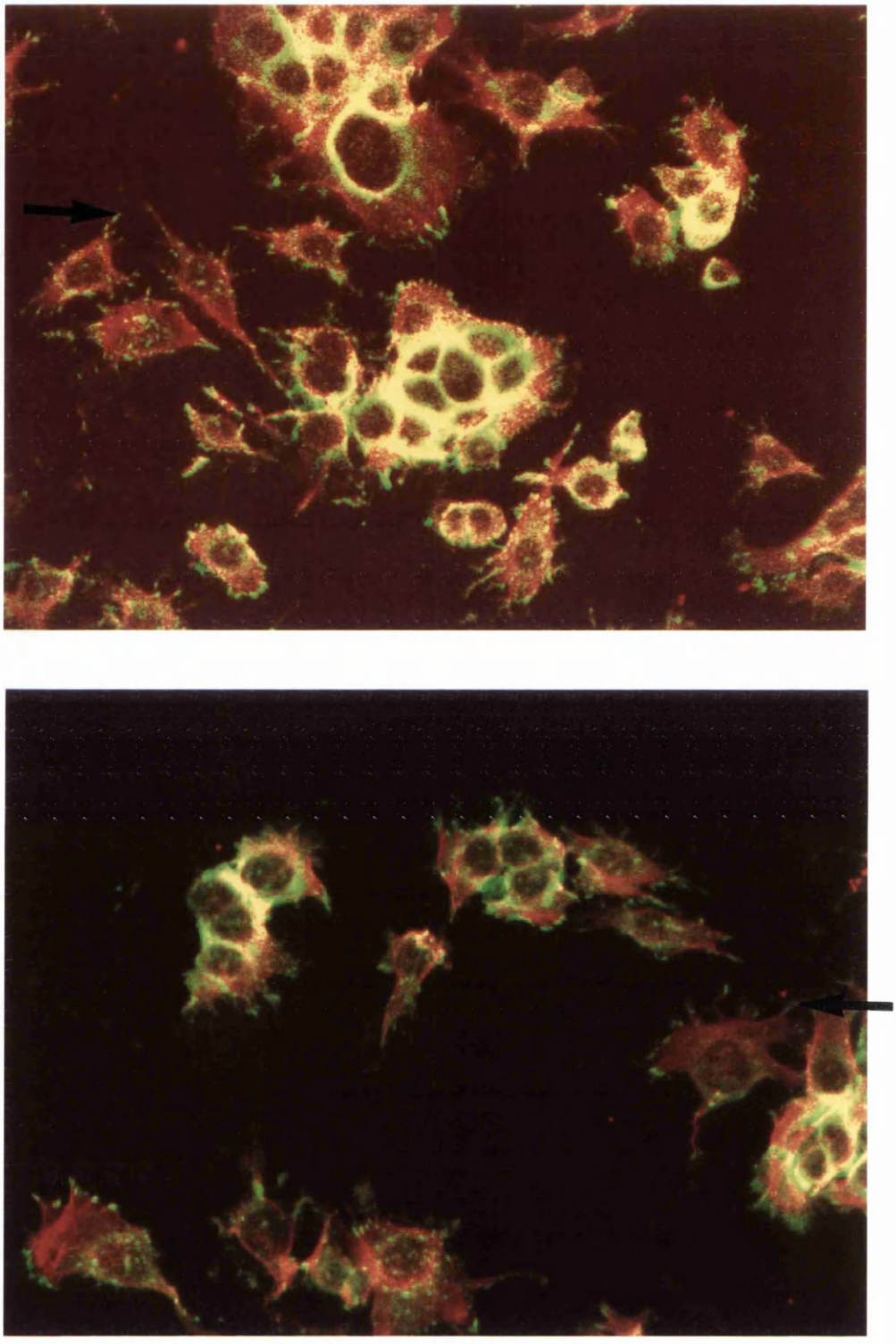


Figure 27: Visualisation of focal contacts by immunocytochemistry. The cell line is B8 (squamous and spindle components). Focal contacts are indicated by arrows. Actin is stained red and vinculin is stained green.

FAK is expressed by all cell types but can only be seen in focal contacts of spindle cells.

3.5 Influence of extracellular matrix components on cell morphology

The adhesion of cells (even tumour cells) to their extracellular environment is not merely a passive process. During tumorigenesis, drastic alterations in intercellular and cell-substratum interactions take place; such alterations are particularly evident in tumours of epithelial origin. A characteristic of transformed cells is a reduction in adhesion to solid substrates which is thought to reflect the invasive and metastatic potential of these cells *in vivo*. However, the environment of tumour cells still influences their characteristics and behaviour.

The SN161-derived cell lines have been shown to respond to growth subcutaneously in nude mice by becoming more spindle. This may be a matrix or growth factor driven process. In an attempt to modify the phenotype of these cells *in vitro*, they were cultured on plates coated with various extracellular matrix components or in medium containing additional growth factors.

During routine culture it was noticed that re-seeding trypsinised cells on plates which had previously been used for the culture of those cells induced much more rapid adherence of the suspended cells and squamous populations of such cultures became gradually more abundant. This was especially true for all mixed morphology cell lines and H9 and H11. Re-seeding squamous cells on plates previously used to grow spindle cell lines had no effect on their morphology and vice versa. However, seeding various SN161-derived cell lines on plates coated with extracellular matrix components did influence cell morphology.

3.5.1 Matrigel

Matrigel is a reconstituted basement membrane derivative composed mainly of laminin, collagen IV, heparan sulphate proteoglycan and entactin. It has been shown to greatly enhance the tumorigenicity of various malignant cells (Fridman et al., 1994; Pretlow et al., 1991). The effect of growth on this basement membrane substitute was tested for the SN161 cell lines. Squamous and spindle cell lines responded differently. Plating the cells on thin coatings of Matrigel made the spindle cell lines, SN4, H11 and B8 more fibroblast-like i.e. they were more spindle-shaped and spread more (figure 28). There was no effect on the squamous cell lines, B9, E1 and E4. Thick gels made all of the cell lines form tight ball shapes and there was no cell spreading.

3.5.2 Collagen

Collagen has been shown to promote the dispersion of aggregates of rat bladder carcinoma cells (Tucker et al., 1990) so its effect was tested on various cell lines. Plating squamous, spindle and mixed morphology cell lines on plates coated with collagen I had no effect on the morphology of any cell line tested (data not shown).

3.5.3 Fibronectin

Fibronectin coated plates were also tested for their effect on cell morphology. They had no effect on any of the squamous cell lines, B9, E1 and E4. Spindle lines, however, became much more fibroblast-like and spread much more than when grown on standard tissue culture plastic (figure 29).

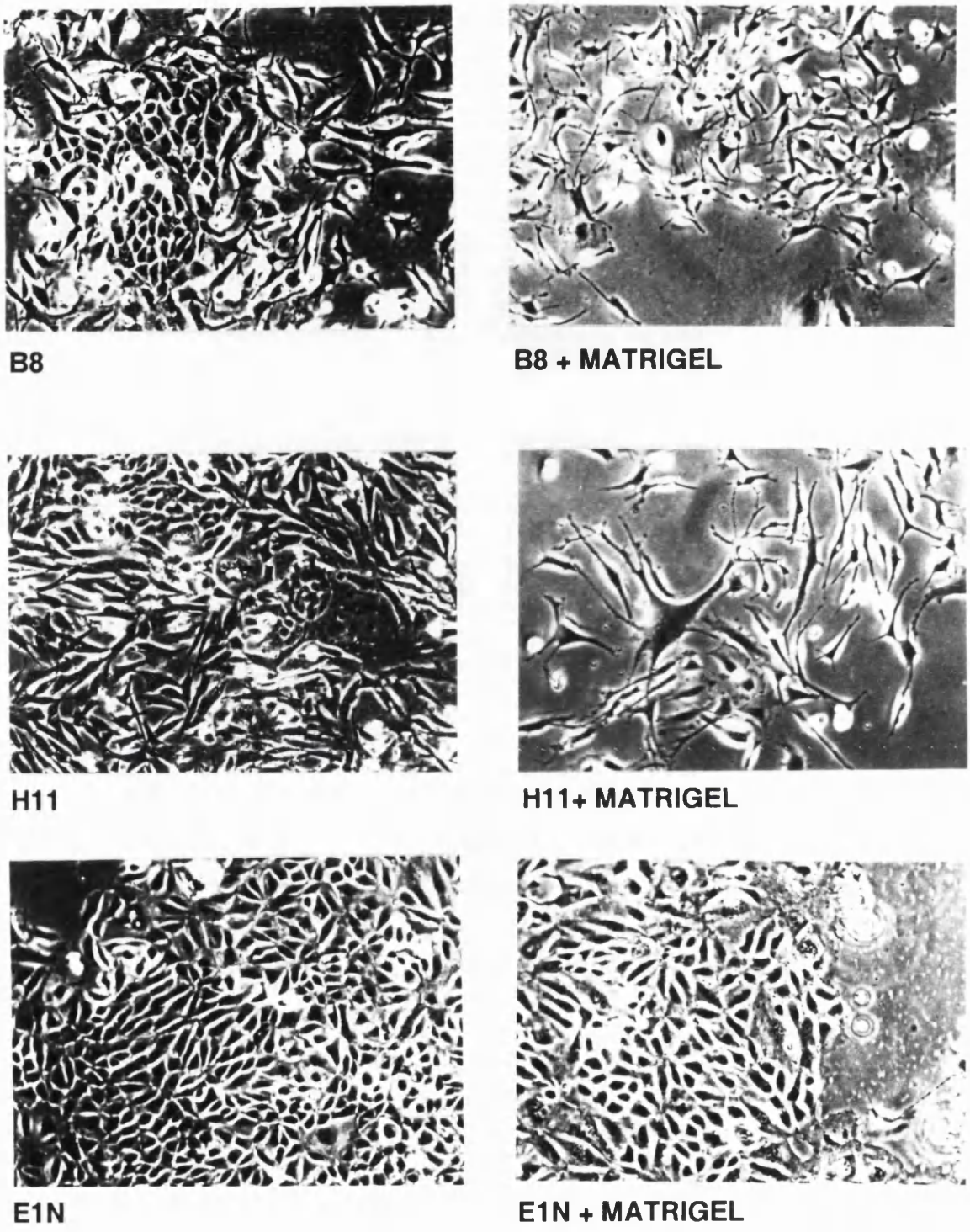


Figure 28: Effect of growth on Matrigel on cell morphology.

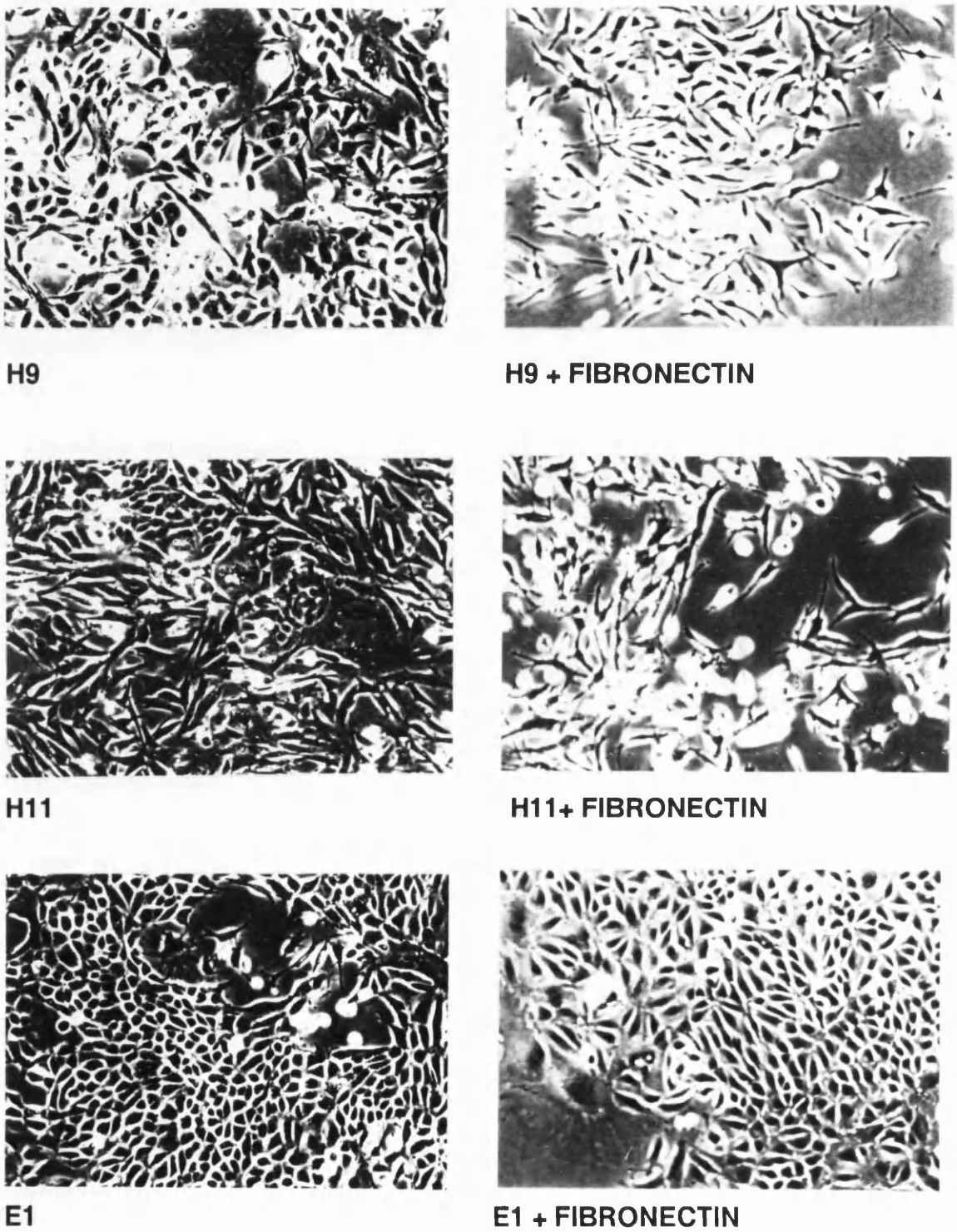


Figure 29: Effect of growth on fibronectin on cell morphology.

3.5.4 Conclusions

These experiments show that Matrigel and fibronectin, but not collagen I, can make some cell lines more fibroblast-like. Certain extracellular matrix components can therefore affect the morphology of metastasis-derived cell lines *in vitro*. This contrasts with the progressively more squamous phenotype that is seen when the same cell lines are grown on standard tissue culture plastic. The cells which become more squamous and attach more rapidly to their substrate may produce a matrix factor which aids adhesion and squamous growth but is not the same as fibronectin or any of the components of Matrigel. However, these matrices may have relevance to the squamous/spindle tendency seen when the SN161 clones are injected into the subcutaneous environment of nude mice.

3.6 Growth factor effects

3.6.1 Treatment with EGF and aFGF

Regulation of proliferation and differentiation in tissues is determined not only by interactions between cells and their extracellular matrix but also by intercellular communication via growth factors. These interactions are important in tumorigenesis especially during invasion and metastasis. Treatment of subconfluent cultures of NBT-II cells makes them dissociate and produces individual elongated, motile cells (Valles et al., 1990a). EGF has also been reported to induce subconfluent rat epithelial cultures to migrate (Blay and Brown, 1985). This observation has been extended to human bladder carcinoma cells which also disperse in response to treatment with physiological concentrations of EGF (Tucker, pers. comm.)

A prerequisite for carcinoma spreading and metastasis is the dissociation of the tumour mass, allowing some cells to escape and eventually migrate away from the

primary site of tumour growth. Cells which have broken through the basement membrane are then in close contact with connective tissue rich in extracellular components. Local growth factors, such as EGF and aFGF, produced by endothelial cells may then play a crucial part in tumour cell motility by enhancing cell scattering.

The difficulty in maintaining pure clones of entirely squamous or spindle cell lines derived from SN161 may be due to some communication between the squamous and spindle cells. Most of the single cell clones seem to prefer to exist as mixed populations. It was proposed that the production of a soluble growth factor by one cell type maintains the growth of the other. This was tested by growing each cell type in conditioned medium. No combination had any effect on cell morphology.

The effect of growth factors known to influence cell morphology in other systems was tested. Treatment with EGF and aFGF which are potent inducers of the spindle phenotype in other cells had no effect on the morphology of SN161-derived clones.

3.6.2 Effect of TGFβ treatment

One growth factor which has wide-ranging effects on most cell types is TGFβ. TGFβ was first described as a soluble factor capable of transforming normal rat kidney cells in the presence of TGFα or EGF (Roberts et al., 1981). It is now known to have different effects on cell growth depending on the particular cell type and growth conditions and has wide-ranging list of properties (see section 1.9.1).

These include stimulation of extracellular matrix formation and promotion of migration. Since ECM components were shown to promote the spindle phenotype in some cases (sections 3.5.1 and 3.5.3), the effect of growth in TGFβ containing medium was tested. The squamous cell lines, E1N, E4 and B9, all responded to incubation in medium containing 5-10ng/ml TGFβ (but not to 1ng/ml) by changing their morphology. E1N and E4 rapidly lost expression of E-cadherin at cell-cell contacts as shown by immunocytochemistry (figure 30) and by 48 hours of treatment had adopted a more spindle morphology (figure 31). Although E-cadherin was beginning to be delocalised

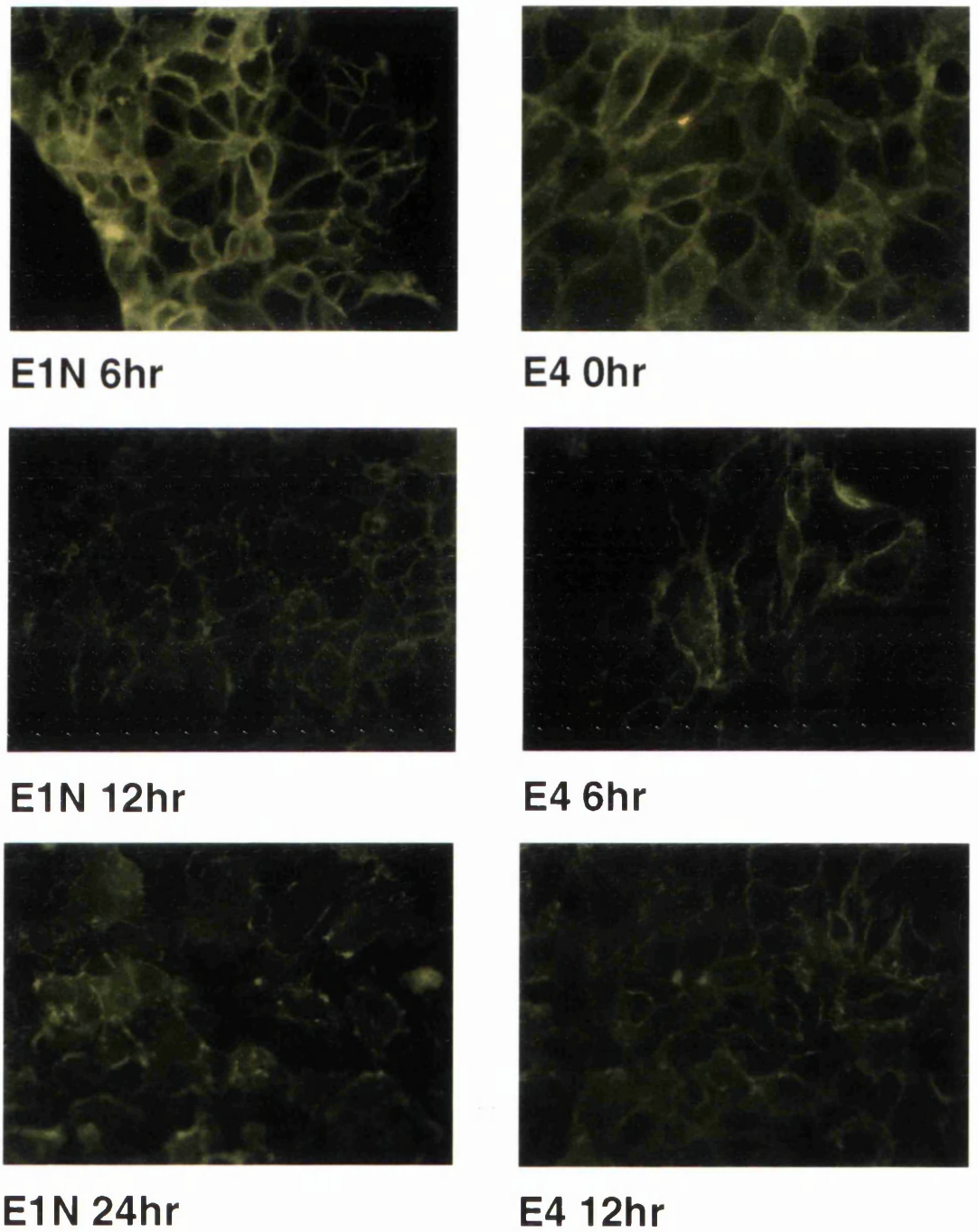


Figure 30: Effect of treatment with TGF-β on E-cadherin localisation and expression. There is a progressive loss of E-cadherin localisation at the membrane.

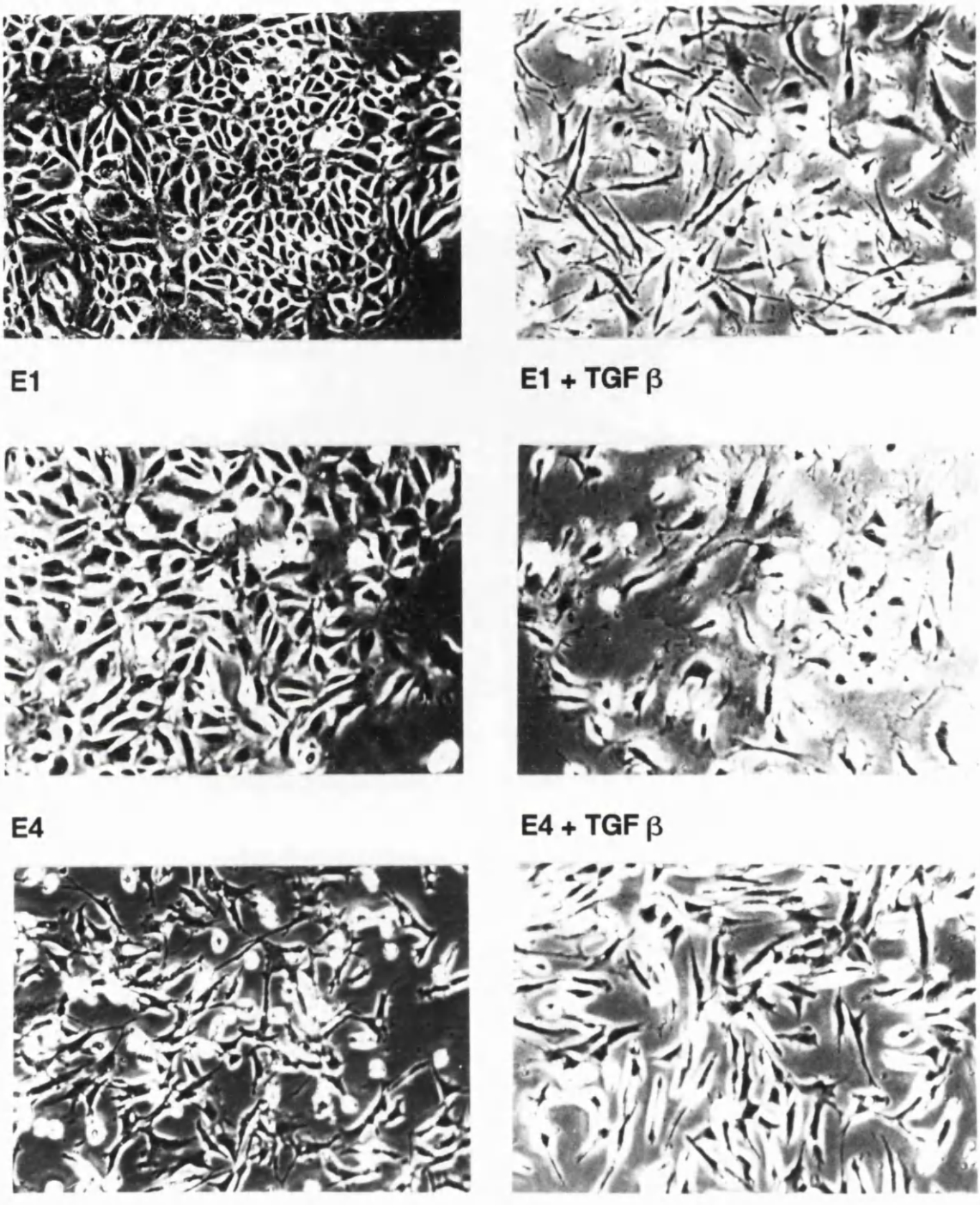


Figure 31: Effect of growth in TGFβ-containing medium for 48h on morphology of E1, E4 and H11 cells.

and lost from the cell boundaries by 6h of treatment and was mostly delocalised by 18h, immunoblotting showed that the levels of E-cadherin protein at these time points were comparable with untreated cells (figure 32). This suggests that the functional protein is simply being redistributed at early stages. At 36h from the start of treatment, E-cadherin expression became reduced to about half of its original level and remained so until 72h.

After longer periods of TGFβ treatment, E-cadherin expression was reduced further. Long-term treatment up to 16 days caused a substantial reduction in E-cadherin expression but was not completely lost even though the cells were very fibroblast-like and have no E-cadherin staining at the cell membranes (figure 32).

The effect of TGFβ was completely reversible and cells returned to their usual morphology within 48h of the growth factor being removed. It was also noticed that cells which had become spindle in response to TGFβ became more squamous as they reached confluence while still cultured in TGFβ-containing medium. This may influence results showing the expression levels of E-cadherin during treatment with TGFβ. Cultures used to prepare lysates at different time points always contained a small proportion of squamous cells because TGFβ only has an effect on cell morphology when the cells are subconfluent. It was impossible to maintain cultures at exactly the correct density throughout at all time points; there were always some confluent regions that had not responded. This means that the amount of E-cadherin being expressed during TGFβ treatment is likely to be over-estimated.

Levels of keratin expression were also determined using a pan keratin antibody. They remained constant throughout and no change in the specific types of keratin expressed was seen (data not shown). Moreover, no switch to expression of vimentin was seen; all cells were negative throughout the time course and long-term treatment also failed to induce vimentin expression (data not shown).

B9 cells did not become fibroblast-like in response to TGFβ. 5ng/ml had no effect on morphology but 10ng/ml made the cells scatter and look like fried eggs! They also had delocalised or punctate E-cadherin staining as seen by immunofluorescence (data

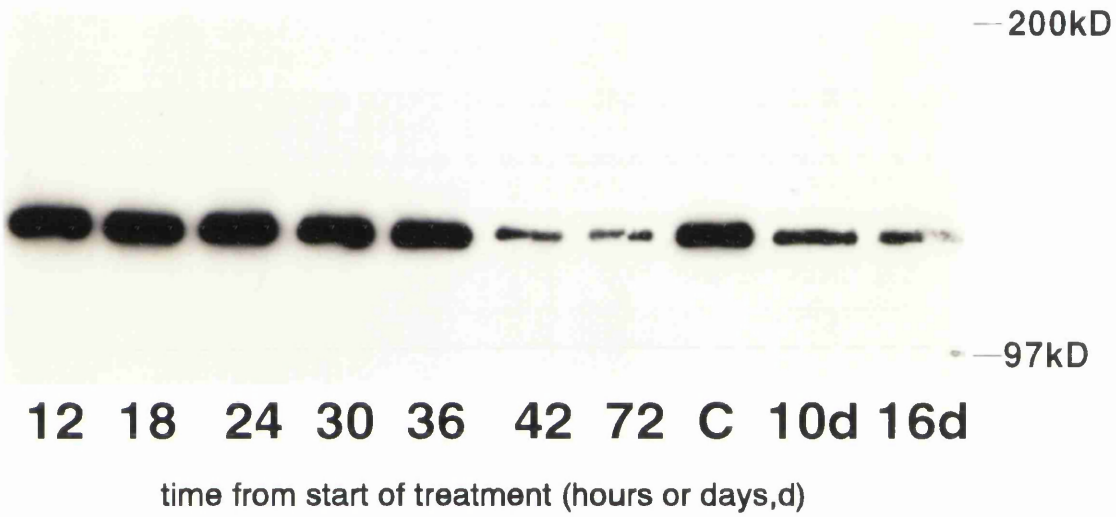


Figure 32: E-cadherin expression in E1N cells during treatment with TGFβ. E-cadherin expression is at normal levels until 36h. At 42h there is a reduction in expression. E-cadherin protein remains at this reduced level until 16 days. Lane C is a positive control.

not shown). This higher concentration of TGFβ was severely growth inhibitory and the effect on cell shape may be a consequence of this toxicity rather than a direct effect on morphology.

3.6.3 Conclusions

The crucial properties of TGFβ with respect to invasion are connected with its involvement in cell-ECM interactions. TGFβ can modulate interactions of cells with their ECM partly by inducing the synthesis of various ECM proteins. It induces the deposition of individual ECM components such as fibronectin and collagens (Ignotz et al., 1986) and modifies cell-matrix interactions via integrins (Gailit et al., 1994) (Heino et al., 1989) (Ignotz et al., 1989).

These properties may have relevance to the effects of TGFβ treatment on squamous cell lines described here. It is not clear whether these responses are due to extracellular matrix-related effects or intracellular signalling. Further experiments should be able to discriminate between these two possibilities.

The effect of TGFβ treatment on the morphology of squamous E1N and E4 cells is an exciting discovery and will be an invaluable starting point on which to base studies of early changes in gene expression associated with the change in morphology. It should now be possible to concentrate on more specific consequences of the squamous/spindle morphological change without interference from secondary changes.

Chapter 4

Discussion

4: Discussion

A large amount of evidence supports the theory that tumour progression is due to successive somatic mutations and an accumulation of epigenetic events that cooperate to progressively transform the target cells. The aim of the work described here has been to investigate the contribution of both genetic and epigenetic factors to late stages of mouse skin tumorigenesis.

One of the experimental approaches used was allelotype analysis of clonally related but morphologically distinct cell lines, A5, B9 and D3. A wide range of cell biology-based studies was also used to characterise these plus additional cell lines which have more plastic morphologies i.e. the SN161 single cell clones. These studies were designed to provide specific information relating to the control of the phenotypical differences existing between the cell lines tested. It was anticipated that such information would provide some clues to the forces influencing the squamous to spindle cell carcinoma transition seen in mouse skin tumorigenesis. The findings may also have relevance to the control of differentiation in mouse skin epithelia and to the epithelial-mesenchymal transition in development.

4.1 Allelotype of cell lines representative of squamous and spindle cell carcinomas

Large-scale chromosomal aberrations including deletions, amplifications and translocations are common features of mouse skin carcinogenesis (Conti et al., 1986; Aldaz et al., 1987) but little is known about mutations specific to the late stage of tumorigenesis when squamous cell carcinomas progress to spindle cell carcinomas. This is partly due to the lack of suitable pairs of cells representative of each phenotype. Fortunately this laboratory has an established system ideally suited to the study of this process.

Most changes may simply be consequences of the genetic instability that is characteristic of tumour cells. Others presumably play an important role in carcinogenesis since the deleted regions may contain tumour suppressor genes and the affected cells may therefore escape normal growth control mechanisms. Further genetic losses may confer malignant properties to primary tumours e.g. invasiveness. The purpose of the work described here was to analyse these changes.

Allelotype analysis has become a popular means of searching for genetic alterations in tumours. Part of the reason for this popularity is its ease of use but the main attraction is the success it has had in identifying tumour suppressor genes. p53 and DCC were both found by LOH studies and now most tumour types have had regions of LOH described (Lasko et al., 1991). This type of information is only of use if candidate genes can then be implicated in the development of that type of tumour.

In this study, cell lines characteristic of the squamous and spindle cell carcinoma stages have been subjected to genetic analysis by PCR amplification of polymorphic microsatellite markers. Previous work on these cell lines in this lab and by others had shown that the spindle phenotype is recessive (Stoler et al., 1993). It was therefore proposed that the progression from a squamous cell carcinoma to a spindle cell carcinoma may be due to a loss of genetic information, possibly causing the elimination of a tumour suppressor gene. Two cell lines, A5 and B9, were chosen for this study. As described already, (section 3.1.1) they are clonally-related cell lines derived from a single primary tumour. B9 is assumed to be characteristic of a squamous cell carcinoma since it produces squamous carcinomas when injected into nude mice and retains many differentiation markers of epithelia. A5, in contrast, produces spindle carcinomas with a short latency period and has a more fibroblast-like programme of differentiation.

4.1.1 Gross genetic alterations

It seemed likely that by screening for loss of heterozygosity (LOH) it should be possible to isolate a small number of genetic differences distinguishing the two clonally-related cell types. It was hoped that a region of complete LOH in A5 would be found to affect a gene involved in the control of epithelial differentiation.

In fact, no such losses were seen in A5; the only complete LOH was found in B9 on chromosome 8 at D8MIT13 where B9 has completely lost the CBA allele (figure 20). Since the primary tumour has both alleles at this locus and A5 and D3 have retained the *musculus* allele, this result was not thought to be relevant to the squamous/spindle transition and further investigation of this region was not pursued. Nevertheless, this result suggests an interesting possibility i.e. that a non-linear relationship exists between A5 and B9 (the *musculus* allele is lost in B9 but present in A5). B9 may have acquired this loss during progression *in vivo* or, more likely, *in vitro*. MSC11cl which is comprised of 75% squamous cells has both alleles with similar ratios to A5 and D3. There is no increase in the proportion of the CBA allele which might have been contributed by squamous cells. It is important to remember that B9 is a single cell clone from MSC11cl, therefore it may not be representative of all squamous cells in the tumour.

The relatively small number of complete losses of heterozygosity is significant in itself. Studies of human tumours show much more frequent losses. This may be due to the longer lifespan of tumours in patients which have been present for a number of years before their detection. During this time they may have accumulated numerous mutations. Experimentally-induced mouse tumours, in contrast, are usually removed before the mouse is one year old and the tumours used here are less than two months old. It is possible that mouse cells are less tolerant of LOH. LOH usually covers large regions of chromosomes and consequently affects large numbers of genes. Although monosomy is rare in mouse

and human tumours and LOH is usually accompanied by reduplication, even homozygosity of a mutant allele is not well tolerated.

This does not mean that these cell lines derived from tumours have few genetic alterations when compared to control spleen DNA. Many quantitative imbalances have been identified, affecting nearly all chromosomes as described in section 3.3.1. The most interesting alterations with respect to the squamous/spindle transition involve chromosomes 1, 4, 5, 6, 12, 13, 16, 18 and 19. Most of these chromosomal aberrations involve the whole chromosome since all markers produce the same result. The most likely explanation of the ratios found is that some chromosomes have undergone non-disjunction during mitosis, causing trisomy of the whole chromosome.

4.1.2 Chromosome 6

Strong evidence for trisomy of chromosome 6, which has also been shown to be altered in primary mouse skin tumours (Kemp et al., 1993; Aldaz et al., 1989), has been found. Duplication of a chromosome in this way could have implications for the expression of genes affected by the trisomy. Trisomy may lead to increased expression of an important growth stimulatory gene, a property that is selected for during tumorigenesis. Mouse chromosome 6 has regions of homology with human chromosome 7 (Elliot and Moore, 1992) where the met proto-oncogene is located (Bottaro et al., 1991). The gene encoding the ligand for this receptor, scatter factor, is also located on chromosome 7 (Saccone et al., 1992) so duplication of the chromosome could result in increased expression of both the ligand and its receptor. This could have a stimulatory effect on growth. This example only applies to human cells since murine scatter factor is located on chromosome 5, however the principle could apply to many other receptors and their growth factors. It is not necessary for both to be on the same chromosome so there is a great deal of potential for over-expression of ligands and their receptors.

Other genes on chromosome 6 could contribute to carcinogenesis by enhancing the growth rate of tumour cells i.e. the K-ras and raf-1 proto-oncogenes. Raf-1 is of particular interest since A5 and D3 are known to have amplified mutant H-ras while B9 has a 2:1 mutant:normal ratio (R.Crombie, PhD thesis, 1994). The crucial factor in the squamous/spindle transition may be downstream from ras since altering the ratios of mutant and normal ras has no effect on cell morphology (R.Crombie, PhD thesis, 1994). Raf-1 is an effector of ras (McCormick, 1994), so its overexpression may aid signalling from mutant ras. This may be essential to mediate the effects of amplified ras. TGFα is also on chromosome 6. It has been shown to convert epithelial cells to a more fibroblastoid phenotype (Barrandon and Green, 1987).

4.1.3 Chromosome 4

There are various lines of interest in chromosome 4. It was singled out by Harris et al (1969) as being important in suppression of tumorigenicity. It is also altered in mouse skin tumours (Kemp et al., 1993). Microsatellite analysis shows an imbalance in favour of the *musculus* allele in A5 and D3 at all markers (figure 22). The same is true for B9 at all markers between D4MIT4 and D4MIT13 but there is a further alteration distal to marker D4MIT13 causing a reversed imbalance in favour of the *spretus* allele which is almost certainly due to a recombination event between D4MIT13 and D4MIT59. MSC11cl gives the same result as B9.

This genetic difference is significant because other studies of mouse skin tumours have shown loss of heterozygosity on chromosome 4 in advanced carcinomas. Mouse chromosome 4 has some homology with human chromosome 9 (Nadeau et al., 1992) which has been implicated in various types of cancer, but the changes found in A5 and B9 are distal to these regions. The region involved in the putative recombination is syntenic with human chromosome 1p34 and 1p36. It is

not clear what relevance this has to tumour development although human chromosome 1 shows LOH in lung, liver and stomach cancers (Lasko et al., 1991).

4.1.4 Chromosome 11 and p53

One region where LOH may have been expected but was not found is chromosome 11. This is commonly altered in tumours, presumably because it harbours the p53 gene. In fact, p53 mutation is often seen in LOH studies in Li Fraumeni patients (reviewed in Lane and Benchimol, 1990). Allelotypes of mouse skin tumours have identified LOH on chromosome 11 but no difference between the squamous and spindle cell lines and control DNA was seen here. This may be due to the cells already possessing two mutant p53 alleles (Burns et al., 1991) therefore no further inactivation of the gene is required and there is no selective advantage of having LOH on chromosome 11.

The mutation of p53 is more likely to have an important effect at earlier stages of tumorigenesis before the divergence of the squamous and spindle cell carcinoma phenotypes since both A5 and B9 have the same 3 p53 point mutations. p53 does seem to have a role in tumour progression and studies using knockout mice have demonstrated a function for p53 specifically at the benign-malignant transition in mouse skin tumours (Kemp et al., 1993); p53 LOH was seen only in carcinomas, not papillomas. The stage under investigation here is even more advanced and represents the progression to a more invasive and metastatic phenotype when p53 mutations are not causal factors.

Further support for this hypothesis comes from the study of SN161 cells. They were derived from a metastasis and have an 8 base pair deletion in p53 (Burns et al., 1991). The primary tumour, SN158, has the same deletion, therefore p53 mutation in this case also preceded metastasis and invasion.

The role of mutant p53 in this model system is more likely to be related to its function as "guardian of the genome". p53 null cells become aneuploid quickly

once in culture and most cells which get gene amplifications lack p53 (Harvey et al., 1993). Therefore, a loss of p53 function may go some way to explaining the number of chromosomal abnormalities seen in both A5 and B9 compared to control normal spleen DNA.

In order to acquire the number of mutations required for carcinoma development, the mutation rate of the tumour cells must have been increased beyond that of normal cells i.e. the tumorigenic cells must have a "mutator phenotype" (Loeb, 1991; Nowell, 1976). The background mutation rate of normal cells even in the mouse (which has a lower capacity for DNA repair when compared to human cells) can account for only 2 or 3 mutations in each tumour and not the much larger number of mutations identified in tumours. However, if an early mutation causes the elimination of a DNA repair function then this mutation rate could increase dramatically.

Creating double minutes would be one way of producing the large amplifications seen. They have already been shown to harbour the amplified mutant ras gene in A5 cells (N. Keith, unpub.).

4.1.5 Other genetic changes

Putative trisomies and other genetic alterations involving chromosomes 1, 5, 12, 13, 16, 18, and 19 may also have carcinogenesis-promoting results.

As described already, the allelotype of the MSC cell lines shows a large number of genetic alterations, many of which are likely to be whole chromosome trisomies. Duplications of various chromosomes are consistent with the karyotypes of A5 and B9. Both cell lines appear to be tetraploid (data not shown) so there is the potential for more than one copy of each chromosome to be represented as indicated by the total chromosome number.

There are also some more localised changes distinguishing the squamous and spindle cell lines. It is not possible to determine whether these involve a loss or gain of function since the nature of the PCR technique allows only the relative amounts of each allele to be determined. The imbalances could therefore be support for either oncogene or TS gene involvement in the process of tumour development. Changes in specific genes which may influence the squamous-spindle transition have not yet been identified.

4.1.6 Clues to the relationship between A5, B9 and MSC11T

Since the original MSC11 tumour was mainly squamous and the MSC11 cell line derived from it was mainly comprised of squamous cells with a minority of approximately 25% spindle cells, it was thought that microsatellite results for MSC11 tumour DNA would reflect these proportions. Surprisingly, this was not the case; of the 25 markers tested, only 3 showed the same result as B9 and only 7 were the same as A5 (data not shown). The remainder differed from both A5 and B9. Combining the ratios for squamous and spindle cells did not improve the relationship. It may be that the section of tumour used for histology had different proportions of squamous and spindle cells compared to the piece used for DNA preparation or that the piece used for DNA preparation had a significant proportion of contaminating normal cells. In addition, the major clone that represented the tumour *in vivo* may not have been the one that adapted best to culture conditions.

Some interesting conclusions regarding the relationships between the various cell lines and tumour cells can be deduced from the microsatellite analysis. Initially it was thought that B9 would be more similar genetically to the primary tumour than A5 would. It was predicted that A5 would have acquired genetic alterations not seen in B9 and that there would be a linear relationship between the primary tumour, B9 and A5. This has proved not to be the case. It is more likely that A5 and B9 both derived from a common intermediate on the tumorigenesis pathway

and were the result of two separate lines diverging from this predecessor which presumably would have the three p53 mutations and one ras mutation. This would explain how A5 could retain genetic information that is lost in B9. Therefore A5 and B9 are more distantly related genetically than was first thought and the differences between them are too numerous to allow any conclusions to be drawn from this study regarding the cause of the different phenotypes.

It is interesting that only a relatively small number of changes appear to take place *in vitro*. There are few differences between A5 and D3 ; B9 and MSC11cl are also very alike genetically. This suggests that most of the alterations found are relevant to the development of the tumour and are not *in vitro* culture artifacts.

4.2 Induction of the squamous-spindle transition *in vivo* and *in vitro*

It was hoped that the morphology of A5 or B9 could be altered experimentally in order to provide a model system where a limited number of variables could be changed to switch the cell phenotype in either direction as required. The close relationship of the two cell lines made this an attractive proposition since the number of differences between them should be relatively small and, furthermore, they should be directly related to the change of phenotype. However, no attempts to alter the morphologies of A5 and B9 have been successful. These have included exposure to ionising radiation up to 5Gy (data not shown), treatment with growth factors and conditioned medium and growth on various matrix components. In contrast, the SN161-derived cell lines have responded to several types of treatment and it has been possible to alter either morphology. Other unrelated cell lines were included in some assays. One of these was CarB.

4.2.1 Reversion of spindle phenotype by transfection with K-rev

One method was successful in reverting the spindle phenotype of a cell line unrelated to A5 and B9. CarB cells which are normally very fibroblast-like were switched to a more epithelial morphology by transfection with K-rev (figure 23). Although A5 and D3 were also transfected, they did not change morphology. K-rev is known to increase the degree of differentiation of lung tumour cells (Caamano et al., 1992) and was originally identified as a result of its ability to revert 3T3 cells (Kitayama et al., 1989). It has been proposed to block the proliferation function of ras, therefore promoting its differentiating function. If this is true, the ras status of transfected cells will be important in deciding whether K-rev can cause any reversion or not. This may explain why A5 and D3 did not respond; they both have amplified mutant ras carried on double minutes while carB has homozygous mutant ras with no amplification i.e there may be a dosage effect.

Unfortunately the reverted phenotype was not stable in culture. Transfected cells which had previously been epithelial rapidly lost this morphology after only a few passages. It seems that the pressure to maintain the spindle phenotype is too great in these cells and they quickly lose any squamous features. Immunocytochemistry and immunoblotting analysis failed to show the expression of epithelial markers such as keratins and E-cadherin (figure 24) so it can be concluded that the change in morphology seen here is only a partial reversion. Expression of the K-rev protein was not formally proven although the presence of the transfected construct was confirmed by Southern blotting (data not shown).

4.2.2 Plasticity of SN161 clones

Compared with the fixed state of the MSC lines which have had no known phenotypic alterations since the time when they were single cell cloned, the SN161 cell lines show much more plasticity. They were isolated from a lymph node

metastasis derived from a skin tumour in a 129/NIH hybrid mouse that had been treated with chemical carcinogens. There are 11 single cell clones derived from the original SN161cl. Within this group there are representatives of squamous and spindle cell lines plus other intermediate morphologies. These cell lines have proved to be much less stable with respect to morphology than the MSC11 cell lines and it has been possible to alter their phenotypes by a number of methods.

4.2.3 Manipulation of cell phenotype by *in vivo* and *in vitro* factors

The first indication that the SN161 cell line could alter its morphology came from tumours induced by injection of this cell line into nude mice. It was noticed that although SN161 is 75% squamous, it produced spindle tumours *in vivo*. It was later shown that squamous cells derived from the original tumour-producing cell line could be recovered from a tumour explant *in vitro*. The identity of these cells as 129/NIH hybrids was confirmed by PCR and the possibility of recruitment of host cells was ruled out (section 3.2.4). It could therefore be concluded that the cells were capable of existing as squamous populations *in vitro* but were converted to spindle cells *in vivo* and that this process was reversible. The same was true for all of the subclones; every cell line produced spindle tumours in nude mice regardless of its morphology *in vitro*. It is not clear if the important factor causing the switch is a component of the culture conditions or is present in the subcutaneous environment of the mouse skin.

Other studies using mouse models have produced similar results indicating that the squamous phenotype of carcinomas is unstable. It was shown that the expression of several keratins is suppressed when transformed cells are injected into nude mice and they are re-expressed when the nude mice-derived tumours are re-established in tissue culture. This suggests that control of keratin expression depends not only on the degree of transformation of the cells themselves but also on host-derived factors (Banks-Schlegel and Rhim, 1986). Similar findings were

obtained in experiments where the expression of E-cadherin was being monitored (Mareel et al., 1991).

The result with the SN161 cell lines obviously merits further investigation. It is possible that extracellular factors such as matrix components or growth factors are playing an important part in this process. Some experiments were carried out to test this hypothesis.

4.2.4 Importance of tumour cell environment

It is clear that the environment of the SN161-derived cells is able to determine their phenotype. Tumour-extracellular matrix interactions are also known to be influential in growth and development and are not merely passive. Carcinoma cells, like normal cells, are surrounded by cellular connective tissue composed of fibroblasts, endothelium and other cells in a dense network of proteins which provides them all with a 3D structural framework and influences their behaviour.

It is significant that the SN161cl was derived from a lymph node metastasis, unlike the MSC cell lines which were derived from a primary squamous cell carcinoma. The plasticity of the SN161 clones may be a direct result of the type of tumour they were isolated from.

The metastatic process is regulated by a balance between adhesive forces linking cells together and cell-substratum interactions. Human tumour metastases are usually squamous but they are unlikely to have metastasised in this morphology. They may have had a degree of plasticity which has allowed them to become spindle transiently, therefore adopting more motile characteristics that allowed them to move from one position to another. This possibility has been addressed experimentally (Boyer et al., 1989) and it seems plausible although the rat bladder cells used in this work are not an ideal model. Results with the SN161 clones provide further evidence to support this hypothesis.

Metastases are rare in mice but the same principles apply as to human tumours. The SN161 tumour may have arisen as a result of cells from the original skin carcinoma (SN158) becoming motile transiently. These cells may have been spindle until they reached the lymph node where they settled and, due to local environmental factors, regained their squamous morphology. Cells from the resulting SN161 tumour may have retained this capacity to respond to certain extracellular factors. Therefore, although the MSC11 and SN158 tumours were both induced by chemical carcinogen treatment, there are clear differences in behaviour. SN161 cells may represent intermediate cell types between the extremes of A5 and B9 since they can be induced to become more spindle or squamous.

Various factors could be responsible for the induction of a fibroblast-like morphology *in vivo*. These include components of the extracellular matrix such as structural proteins and growth factors. The effects of some of these were investigated.

4.2.5 ECM proteins influence cell morphology

It is generally assumed that epithelial cells break through the basement membrane and invade the underlying mesenchymal stroma which is relatively passive in the metastasis process. However, mesenchymal cells might actively suppress carcinomas in adjacent epithelia and the loss of such active suppression might contribute to carcinogenesis. Alternatively, there may be ECM proteins present which aid the acquisition of a motile, fibroblast-like phenotype by the invading tumour cells. Matrigel (a reconstituted basement membrane derivative composed mainly of laminin and collagen) greatly enhances the tumorigenicity of various malignant cells (Fridman et al., 1994; Pretlow et al., 1991). Tumour cells premixed with Matrigel then injected into nude mice consistently produced tumours which grew faster and became much larger than tumours induced by the same cells

injected without Matrigel. Some interaction of premalignant cells with ECM components can therefore contribute to the process of tumorigenesis.

The effect of plating the various SN161 single cell clones on different matrix components was tested. Collagen I had no effect on morphology but both Matrigel and fibronectin made the spindle cell lines even more fibroblast-like (figures 28 and 29). Squamous cell lines were unaffected.

The response to Matrigel and fibronectin may be due to the presence of different matrix receptors on the surface of the spindle cells or due to their ability to activate the appropriate receptors. Signalling through such receptors e.g. the $\alpha 5\beta 1$ integrin which binds to fibronectin may trigger a growth response that involves a change in cell morphology. The expression pattern of integrins has not been determined for these cell lines but this would be recommended since they are so important in responding to the extracellular environment (section 4.2.8).

It was thought that perhaps the squamous and spindle cell lines produced different matrix components which affected their morphology; some spindle cell lines acquired isolated squamous colonies after long periods in culture and suspended cells settled much more quickly on dishes which they had previously been growing in compared with fresh dishes. Plating either squamous or spindle cells on plates which had previously been used to grow the other type of cell line had no effect on morphology, so the difference does not seem to be due to the secretion of alternative matrix proteins by the cells.

4.2.6 Growth factors influence cell morphology

The effect of treating cells with aFGF, EGF or TGFβ was tested. aFGF and EGF had no obvious effect on phenotype, but TGFβ treatment produced a dramatic response.

TGFβ regulates the proliferation and differentiation of normal cells. Both of these functions may be important in tumour formation. TGFβ has a dual role in

carcinogenesis, inhibiting papilloma development but enhancing progression once neoplastic transformation has occurred and the growth inhibitory response to TGFβ has been lost (W.Cui, unpublished). Overexpression of TGFβ1 in carcinoma cells enhances tumorigenesis and metastatic spread (Welch et al., 1990). This effect may be related to the ability of TGFβ to influence mesenchymal-epithelial interactions.

Treatment of E1N and E4 (both squamous clones) induced an epithelial-mesenchymal transition in 24-48h. At first E-cadherin became delocalised and its expression level reduced, then the cells became more spindle. High doses of TGFβ (10ng/ml) were required to produce this response but it was obvious and fairly rapid. Although the mechanism by which this occurs has not been identified, the treated and untreated cells will be invaluable for further studies of changes accompanying the squamous-spindle transition and immediate consequences of the change in morphology.

Other functions of TGFβ, besides growth inhibition, include modulation of cell-ECM interactions e.g. by inducing the synthesis of various ECM components such as collagen and fibronectin (Ignotz et al., 1987). The culture of cells on collagen I coated plates did not have any effect on morphology in this study but growth on fibronectin and Matrigel which contains laminin and collagen IV made the spindle cells spread more and become more fibroblastic. The morphology induced by growth on these substrates was similar to that induced by treatment of squamous cells with TGFβ although these squamous lines showed no response to growth on matrix components. The two effects may be unrelated but it is tempting to assume a link between them.

Perhaps a more intriguing possibility is the induction of scatter factor expression by TGFβ. TGFβ can strongly upregulate the steady-state levels of c-met mRNA, the scatter factor receptor, in human carcinoma cell lines (Moghul et al., 1994) and the expression pattern of c-met is greatly upregulated in tumour tissues and several carcinoma lines (Prat et al., 1991). Scatter factor has several well-described effects on cell morphology and motility (see section 1.8.2), all of which

are relevant to the acquisition of a more spindle, invasive phenotype during late stages of tumour progression. This may be the outcome of treatment of squamous carcinoma-derived cells with TGFβ.

Another possibility is that the high doses of TGFβ used to induce a change in morphology are not acting solely through the TGFβ receptor II; TGFβ may also be binding to the activin receptor which is closely related. Activin is a key signalling molecule in the induction of dorsal mesoderm (Hoffmann, 1991). It reduces the adhesive strength of cadherin-mediated adhesion in blastomeres, allowing cells to exchange neighbours during the complex movements of gastrulation in Xenopus. This process is reminiscent of the delocalisation of E-cadherin followed by cell scattering and gain of spindle phenotype that is seen in response to TGFβ. It will therefore be important to determine the effect of treating the same cells with activin.

4.2.7 Growth factors that affect cell morphology work via tyrosine kinases

Both E1N and E4 responded to TGFβ within 12h of the start of treatment. E-cadherin localisation was beginning to be altered at 6h and it had become fully delocalised by 24h. This was long before a change in the expression of E-cadherin protein could be seen; a change in protein levels was first detected at 36h. The implication of this timing is that existing E-cadherin complexes are being modified in response to the extracellular stimulus.

E-cadherin is a key component in the squamous-spindle transition. The E-cadherin junctional complex that includes catenins is broken down as a result of tyrosine phosphorylation. This is a common feature of the acquisition of a spindle phenotype and tyrosine phosphorylations are common responses to growth factor signalling. Indeed, tyrosine phosphorylation of E-cadherin or catenins is often seen during transformation when cells become more spindle (Behrens et al., 1993; Hamaguchi et al., 1993).

It is also possible for cells which are not expressing E-cadherin protein to regain expression of the correctly positioned and functional protein. This was observed in the spindle cell lines, H9 and H11. After some time in culture they gained a number of small squamous regions which were positive for E-cadherin expression as shown by immunocytochemistry (figure 13). Surrounding spindle cells remained negative. A similar effect was seen in cells which were being treated with TGFβ. Where the culture was subconfluent the cells were spindle and E-cadherin was delocalised; as they became confluent and made contact with their neighbours, correct E-cadherin expression reappeared.

In both cases close cell-cell contact reactivates the E-cadherin complex. It is known that the E-cadherin promoter is active in H9 and H11 even when there is no E-cadherin protein production (G. Portella, unpublished) so it seems that some post-transcriptional mechanism is capable of regulating the junctional complex either by acting on E-cadherin itself or another component such as the catenins. This phenomenon is currently being investigated.

4.2.8 ECM protein and growth factor effects can be mediated by integrins

Changes in integrin expression can have profound effects on the neoplastic phenotype since they are important mediators of cell-cell and cell-ECM interactions and changes in these interactions are characteristic of malignancy. Such changes are particularly important for invasion and metastasis (Dedhar, 1990; Plantefaber and Hynes, 1989; Schwartz and Ingber, 1994).

The expression of a particular integrin complex is tissue and cell type specific. α6β4 is the predominant integrin of squamous cell carcinomas and in skin and redistribution of α6β4 to the suprabasal compartment is a marker of high risk for malignant progression (Tennenbaum et al., 1993). Therefore, qualitative and quantitative changes in cell surface matrix receptors are linked to the malignant

conversion and may contribute to altered interactions of carcinoma cells with their surrounding normal cells and stroma. These epigenetic changes in benign tumour cells may encourage the development of characteristics generally attributed to the malignant phenotype.

4.3 Markers distinguishing squamous and spindle cells

The work presented here illustrates some phenotypic differences between squamous and spindle cells and between cells whose morphologies are fixed and those which can change morphology to some extent. The most obvious differences are the alternative intermediate filaments possessed by squamous and spindle cells and the presence or lack of E-cadherin. Other markers which are likely to be consequences rather than determinants of the phenotype are focal adhesion proteins such as vinculin and talin. These were expressed at high levels in spindle cells but were only just detectable or absent in squamous cells. Total FAK expression showed no consistent difference between the two cell types but the difference in this case could be more subtle e.g. contrasting degrees of phosphorylation. This possibility was not investigated. The presence or absence of focal contact proteins is probably a result of signalling from an upstream control of morphology which co-ordinates these and other mutually exclusive markers such as keratin and vimentin proteins.

More likely candidates for determination of cell morphology were GAP and p62 or p68 since these are closer to the control centres of the cell in terms of signalling. None of these showed any difference between squamous and spindle cells.

The alternative expression of those markers which did have some link with morphology was consistent among the fixed and plastic morphology cell lines. It may be that the switch from squamous to spindle in SN161-derived cells is influenced by epigenetic factors while it has become fixed by genetic changes in the

MSC11-derived cells. Both factors are known to be relevant to the carcinogenesis process. SN161 may have arisen from an "earlier" target cell which still has the capacity to undergo the epithelial-mesenchymal transition.

4.4 Genetic and epigenetic events in carcinogenesis

Epigenetic events and mutations are not mutually exclusive; together they account for all the heterogeneity seen between and within tumours. Tumour evolution requires a selective mechanism using genetic and epigenetic forces that combine to create a carcinogenic phenotype. Combinations of different forces are involved at each stage of tumorigenesis.

It is well documented that initiation results from genetic events. The mechanism of promotion is likely to be epigenetic since only 2 or 3 genetic changes in normal keratinocytes are sufficient to produce papillomas (Roop et al., 1986; Bailleul et al., 1990) and promoting agents aren't always mutagens. The cause of progression may also be due to epigenetic events, although genetic events are undoubtedly required for the development of true spindle cell carcinomas.

The reversible phenotypic modifications seen here may occur by similar mechanisms to those occurring in tumour progression. Cells switching between squamous and spindle morphologies, whether *in vitro* or *in vivo*, may acquire changes in expression of the same set of genes. However, the changes in tumours are probably fixed by genetic alterations. The hyperproliferative phenotype of papillomas could contribute to the spontaneous accumulation of such chromosomal changes associated with progression in skin carcinogenesis. This could then create the correct conditions for the evolution of cells which become spindle cell carcinomas through further genetic alterations or in response to epigenetic factors.

Loss of adhesion and disorganisation of cell and tissue architecture are not immediate consequences of growth deregulation due to oncogene activation or tumour suppressor loss; rapidly growing but well-differentiated tumours are

common. Genetic changes that lead to permanent activation of growth regulatory pathways occur first and allow subsequent mutations in genes that are involved in cell-ECM adhesion. Once tumour growth becomes independent of adhesion, selection for a range of different phenotypes becomes possible. Without a preceding loss of anchorage dependence, the cells without a normal adhesion system and cytoskeleton would not be viable. Once cells have become ECM independent, the cell and tissue structure can become more disorganised.

Cadherins are important in maintaining tissue organisation and have been classed as tumour suppressors since they show decreased expression or functional inactivation in tumours and their overexpression leads to reduced tumorigenicity (reviewed in Takeichi, 1993). They are important in cell-cell adhesion and connect to the cytoskeleton but there is no evidence as yet that cadherins directly regulate intracellular growth control pathways or that cell-cell adhesion contributes to cell growth in a manner similar to cell-ECM adhesion. Despite this they do have an anti-tumorigenic role, if only as a mechanical restraint on motility.

Epigenetic and genetic factors may affect the same pathways in different ways. Growth independent of both anchorage and soluble mitogens can be induced by many transforming agents e.g. src can activate the integrin-mediated pathway independent of adhesion to substrates that normally causes activation (Guan and Shalloway, 1992). Oncogenes and tumour suppressors may irreversibly activate pathways controlled by integrins as well as by growth factors. These alterations can make cells independent of particular growth factors and may constitutively activate integrin pathways. Clearly this would affect both growth and adhesion-related properties. Thus epigenetic and genetic factors can promote tumorigenesis by acting on the same pathways; the difference is that genetic alterations are fixed while epigenetic alterations are usually reversible.

4.5 Parallels between neoplasia and development

The conversion between epithelial and mesenchymal morphologies in neoplasia is similar to the epithelial-mesenchymal transition seen in embryonic development and can be mimicked *in vitro* in appropriate culture conditions (see section 1.8). Tissue remodelling, whether in morphogenesis, wound repair or pathogenic situations such as invasion and metastasis of tumour cells, requires the epithelial cells to acquire a degree of motility that is not normally a property of these cells. There are two ways in which epithelial cells can move; they can remain linked together and move as an epithelial sheet as in gastrulation and wound healing or they can dissociate and migrate as individual cells. During embryogenesis, the gain of motility is associated with a dramatic change in the programme of cell differentiation. Migrating cells acquire a mesenchymal phenotype exhibited by a change in their cytoskeletal organisation (they no longer express keratins and express vimentin instead) and decreased cell-cell adhesion. This conversion is reversible.

Analogous to the situation in development, tumour cells can also be plastic. Indeed, the squamous/spindle transition that occurs late in carcinogenesis may be a recapitulation of the epithelial-mesenchymal transition which takes place during development. Epithelial cells lose their characteristic markers e.g. keratins and adopt a more fibroblast-like phenotype which involves expressing vimentin and becoming more spindle shaped. Their reduced cell-cell and cell-substrate adhesion contributes to increased potential for motility.

Developmental processes can be influenced by a number of parameters e.g. ECM components and cell adhesion molecules but a key role is played by soluble growth factors. There are developmental abnormalities associated with alterations in growth factor or growth factor receptor function. Moreover, some growth factors and their receptors have oncogenic potential.

One such growth factor is TGFβ. It has a role in epithelial morphogenesis (including the epithelial-mesesnchymal transition) (Heine et al., 1987) and can also have various effects during carcinogenesis (section 1.9.1). The results presented here indicate that TGFβ treatment of squamous cells can induce a phenotype that resembles spindle cells. Characterisation of these cells has shown that their similarities to spindle tumour cells are striking. As the cells scatter and become more fibroblast-like, E-cadherin expression becomes delocalised. It would be interesting to know if this effect (produced by treatment with a high concentration of TGFβ) is acting through the activin receptor rather than a TGFβ receptor since the same alterations to E-cadherin are seen during gastrulation in Xenopus in response to activin signalling (Ueno et al., 1990).

4.6 Similarities between neoplasia and mitosis

Studies such as this also provoke suggestions that tumour cells may be caricatures of mitotic cells since links between tumour cell phenotypes and normal cellular processes can be found. There are many similarities between alterations that take place during transformation and mitosis. The same pathways may be involved in both cases since mitotic cells, like tumour cells, have reduced adhesion to their substrate and their cytoskeleton is disassembled. It is possible that tumour cells copy mitotic cells and some of the pathways involved are the same. Observations such as this suggest that tumour cells can be likened to cells permanently in mitosis i.e. altered cell cycle control may be involved. Therefore, evidence is now growing to suggest that tumorigenesis, development and mitosis depend on different ways of utilising the same cellular processes. The precise differences in the ways that these are regulated are the key unanswered questions.

4.7 Suitability of the mouse skin model system

Many discoveries from studies of mouse skin have been landmarks in cancer research and they have provided many insights into the processes of tumorigenesis. Evidence has been obtained for the multistep nature of skin carcinogenesis in mice and distinct stages have been described (section 1.4.2). Other findings have included evidence for the clonal origin of benign and malignant tumours, the requirement for multiple genetic changes in malignant conversion and the binding of carcinogens to DNA (rev in Slaga, 1984).

Experimental skin carcinogenesis is the favoured model for understanding the pathogenesis of squamous cell carcinomas. As mentioned previously, distinct stages have been identified and the process of tumour development can be separated into the various steps of the pathway shown in figure 4. Recent advances have linked specific genetic and biological alterations with each of these stages. The work described here has contributed to the data, especially to the final stage of progression i.e. the transition from a squamous to a spindle cell carcinoma.

Permanent conversion to a spindle cell carcinoma, which involves a complete change in cell phenotype, is not seen in all carcinomas. In fact, it is less common in human tumorigenesis than in the mouse model system. However, reversible conversion may be very common and therefore is still of great interest in understanding the processes that become disrupted in tumour development and is of central relevance to the mechanisms of invasion and metastasis. Study of these processes will not only provide information about the behaviour of tumour cells; it will also give an insight into the control of epithelial differentiation and development.

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