Characterisation of KIM 2 murine mammary epithelial cells and reversion of the transformed phenotype via Tiam1: a model for suppression of transformation in breast cancer?

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I confirm that this thesis has been composed by myself.

I acknowledge that the work reported was done by myself, with the exception of analysis of endogenous levels of Tiam1, performed by Dr. R. Van der Kammen of the Netherlands Cancer Institute, Amsterdam. I am however indebted to several people for advice, instruction and assistance in the preparation and processing of materials, as presented in the acknowledgements.

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Abstract

Cell line models are of considerable value in anticancer research in terms of defining the biological features of normal and transformed cells, and allowing genetic manipulation in vitro. Recent evidence has shown that phenotypic reversion of transformed lines to a non-transformed phenotype can be effected in vitro, for example via overexpression of the guanine nucleotide exchange factor, Tiam1. A scientific understanding of these reversion pathways may eventually provide targets amenable to therapeutic manipulation.

The KIM2 mammary cell line, containing a temperature-sensitive SV40 large T antigen, was used as a model for normal and transformed mammary epithelial cells. At 37°C the cell line appears epithelial and showed mammary differentiation (NKIM2), whereas at 33°C the cell line appears spindle-shaped and transformed (TKIM2). The aims of this study were i) to determine the biological features of the NKIM2 and TKIM2 lines, ii) revert the TKIM2 to a non-transformed phenotype by retrovirally transducing Tiam1 into the cell lines and iii) assess the reverted cells in terms of the originally defined biological features.

The NKIM2 cell line showed features of epithelial differentiation including cobblestone morphology, E-cadherin and ZO-1 expression, junctions and polarisation. In addition, a specialised myoepithelial cell type was identified among cultured cells. The NKIM2 cells also showed mammary differentiation in terms of milk protein expression in response to lactogenic hormones. In contrast, the TKIM2 line demonstrated accepted features of transformation including spindle-shaped morphology, lack of contact inhibition and serum dependence, anchorage-independence in soft agar and tumour formation in scid mice. Although unexpectedly, the NKIM2 lines also formed tumours in scid mice. There was no difference in the chromosomal locus for p53 and SEN6, which have been implicated in transformation and immortalisation respectively, in both cell lines.

Retroviral transduction of control and active Tiam1 constructs into both NKIM2 and TKIM2 lines was successful, with Tiam1 protein expression for three months following transduction. This was associated with a reversion of TKIM2 cells to an epithelioid phenotype with E-cadherin expression and junction formation, although cells were not polarised and some showed membrane ruffling. The reverted cells also demonstrated milk protein expression, contact inhibition and serum dependence, but retained anchorage-independent growth in soft agar.

In conclusion, both NKIM2 and TKIM2 mammary epithelial cells show features of normal and transformed cell lines respectively. Introduction of Tiam1 into cell lines results in a partial reversion of transformed TKIM2 cells to an epithelial phenotype.

Introduction

Breast cancer is the commonest cancer affecting females living in the developed world [1]. The age-standardized incidence rates in the U.K., North America, Australasia, and Scandinavia indicate that 60 to 70 per 100,000 women per annum will present with the disease [2]. Although the incidence rates of the disease are rising, in part due to screening [3] and longer life expectancies [4], mortality rates appear unchanged [5]. The cause of most breast cancer remains unknown, but hormonal, environmental and genetic factors are involved in its aetiology.

A significant feature of breast cancer is that although tumours appear slow-growing, by the time they present clinically metastasis to distant sites may have already occurred [6, 7]. Thus, the overall 5-year survival rate for invasive breast carcinoma is 60% for clinically localised disease, whereas for metastatic disease is 34% [1, 8]. The current strategies employed to tackle the disease include early mammographic detection by screening and a combination of surgery, systemic hormonal and chemotherapy for clinically established disease [9].

This study undertakes to understand at a cellular and molecular biological level certain processes involved in breast carcinogenesis, by studying a cell culture model for breast cancer and then manipulating it to 'cure' the disease in laboratory conditions [10].

In order to set a background to the experimental approach, breast cancer aetiology and the relevance of in vitro breast cancer models will be discussed. In particular SV40 virus, which is the immortalising agent of the KIM2 murine mammary epithelial cell line, will be considered in detail. The introduction then assesses the relevance of the biological markers which are used to characterise the normal and transformed KIM2 lines in this study. This is followed by an overview of methods of gene transfer, with particular reference to the retroviral approach undertaken. Finally, the phenomenon of phenotypic reversion is considered and in particular current understanding of Tiam1, which is transduced into KIM2 cells in this study.

Aetiology

It is clear that although several risk factors for breast cancer have been identified, the reason for a particular individual developing the disease remains unknown.

Hormonal factors

The female hormonal milieu appears to be associated with an increased risk of female breast cancer [11]. This view developed from original observations about the difference in frequency between male and female breast cancer. Male breast cancer is very rare, accounting for less than 0.1 per cent of cancer deaths in men and occurring 100 times less often than in women [12]. From meta-analysis, it is clear that gonadal ablation significantly reduces the risk of female breast cancer, while actually increases the risk of male breast cancer [13, 14].

Further epidemiological studies tend to support the view that female sex hormones play a role in breast cancer, although the mechanism for this increased risk remains controversial [15]. Certainly an early menarche, which is reported to be associated with a raised oestradiol level into early life, confers an increase risk of breast cancer [16]. Early menarche is likely to be triggered by a threshold level of adipose tissue, which is reached sooner in Western societies [17]. The raised and prolonged exposure to oestrogens such as oestradiol, is described as the 'oestrogenic period' [16]. How pregnancy effects this period remains contraversial. It is reported that pregnancy at an early age, decreases breast cancer risk [18]. One possible explanation is that the breast contains contains stem cells which are at neoplastic risk before they have been stimulated to differentiate during pregnancy [19]. Previously it has been suggested that if women actually become pregnant after a diagnosis of breast cancer, they have poorer survival rates than their non-pregnant counterpart. However the most recent evidence shows that there is no difference in mortality between the two groups [20]. If the 'oestrogenic period' is prolonged either by delayed menopause or exogenous oestrogen such as Hormone Replacement Therapy (H.R.T.) or the oral contraceptive pill, there is reported to be increased cancer risk [16]. Certainly, studies linking exogenous oestrogen to breast cancer have raised public concern. The evidence concerning the pill remains unclear [21]. Certain studies have suggested an increased risk in women below the age of 35, the risk increasing the longer the pill is used and with preparations containing high levels of oestrogen [22].

However any possible risk with the use of the newer combined pill, which contains lower dose oestrogen and progesterone, is thought to be small [23]. The most recent meta-analysis studies on H. R. T. suggest that there is a small but significant increase in cancer risk with long-term use [24]. At present, patients who have had previous breast cancer and those at increased risk, are advised to be offered H.R.T. only with caution and full information of risks, while the need for results from a large, longitudinal, randomized control trial remain [25, 26].

Perhaps the most significant recent evidence in relation to the influence of hormones on breast cancer have been treatment based [27]. In the 1950's the use of anti-oestrogens was explored in order to control advanced breast cancer. This was particularly of interest because the existing alternatives: oophorectomy and adrenalectomy had undesirable side-effects [28]. The current most widely used anti-oestrogen tamoxifen, has been shown to induce regression of tumour in a significant proportion of advanced breast cancers [29].Further to this, it has been shown by meta-analysis that post-surgical adjuvant tamoxifen therapy actually improves survival [30]. An alternative strategy includes use of long-acting LHRH agonists such as Zoladex which eventually suppress gonadotrophin release and block gonadal function.However the most recent anti-oestrogen therapies under trial are the aromatase inhibitors, which reduce the conversion of androgens to oestrogen [33].

Environmental factors

There is a striking variability in the geographic distribution of female breast cancer [2]. In contrast with the age-standardised incidence rate quoted earlier of 60-70 cases per hundred thousand per annum for some Western countries, the rate in Asian countries may be only 15 cases per hundred thousand [34]. This difference in risk appears to have a strong environmental component because those who migrate from a low risk area to high risk area, increase their risk [35, 36]. This increased risk is also continued in their descendants. To identify the principles involved in environmental factors is notoriously difficult. There is a reported increased risk of breast cancer in urban rather than rural areas, with certain groups in Western countries, such as Mormons, having a decreased risk [37]. Another often quoted example, is the large excess of dietary fat consumed in Western countries compared to some Asian countries [38].

The mechanistic theories put forward are controversial [39]. It is possible that increased dietary fat triggers early menarche, as previously mentioned, and therefore increases breast cancer risk indirectly, by prolonging exposure to oestrogen. It may also be that increased fat affects oetrogen metabolism and therefore increases circulating levels of oestradiol [40]. It has also been proposed that the fatty acid profile of the Western diet as well genetic influences, predisposes to earlier hyperinsulinaemia [41]. Hyperinsulinaemia, which may result in insulin resistance at puberty, is associated with abnormal aromatase activity in the ovaries, as well as increased levels of insulin-like growth factor (IGF) [17]. It is thought that abnormal aromatase activity resulting in increased oestrogen levels, synergises with IGF to stimulate proliferative activity in mammary epithelium [17]. These theories have translated into attempts to modify diet and exercise in families at increased risk of breast cancer. Indeed certain studies suggest that a very low fat intake reduces breast cancer risk [42].
The studies on obesity, which intuitively would follow diet, differ slightly. Most studies show obesity in Western women is associated with increased cancer risk in postmenopausal women [17]. However, obesity in teenage girls, actually appears to reduce the cancer risk in premenopausal women [41]. The explanation for this particular decreased risk is unclear. However after the menopause, adipose tissue becomes the major site of aromatase activity and source of oestrogen, so that the protective effect of early obesity is replaced by an increased risk of postmenopausal cancer if obesity persists beyond teenage years [41].

Genetic factors

While most cases of breast cancer are sporadic and not familial, genetics appears to play a role in certain scenarios. For example, if one or more first or second degree relatives have breast cancer, the increased risk to an individual can be two to five times the average risk [43]. Further to this, the identification of two inherited breast cancer susceptibility genes namely BRCA1 [44] and BRCA2 [45], by world wide analysis of approximately 200 breast cancer prone families, underlined the importance genetic disposition in breast cancer.

Although the mechanism by which mutations in these genes prediposes to cancer is not yet full understood, these discoveries may help to identify biology peculiar to breast cancer. BRCA1 has been shown in studies of the prevalence and penetrance of germline mutations, to be present in populations unselected for a family history of breast cancer [46]. For example, 13% of women diagnosed with breast cancer under 30 and 7% diagnosed under the age of 35 have germline BRCA1 mutations, although the significance of these mutations is unclear [47, 48]. Somatic mutations of BRCA1 are seen very infrequently in sporadic breast cancer [46]. The function of BRCA1 is difficult to elucidate from its primary structure with the only clue being a conserved RING finger domain, which is a type of zinc finger domain whose function is not well understood [46].

Studies show that mouse BRCA1 shares only 58% amino acid homology with human BRCA1, therefore weakening the power of experimental studies in mice [49]. Mouse homozygous BRCA1 knockouts die in utero with multiple developmental abnormalities, however the heterozygotes have shown no evidence of tumour development at 6 months [50]. The most recent study using the Cre-Lox system to conditionally express a mutation of BRCA1 in the mammary gland, resulted in blunted ductal morphogenesis and tumour formation [51].

The development of antibodies to the BRCA1 protein has yielded opposing observations regarding the cellular location of BRCA1. One proposal has been that BRCA1 is not present and therefore excluded from the nucleus of breast cancer cells, and so is unable to function [52]. However others have detected BRCA1 in the nucleus of both normal and cancer cells associated with membranes and propose that BRCA1 is a granin protein, whose secretion by definition is regulated by signals from the extracellular environment [53]. This theory remains contraversial.

There have been attempts to demonstrate the functional biology of BRCA1 in sporadic breast cancer cell lines. The introduction of a normal BRCA1 cDNA expression construct, results in a reduction in proliferation and tumour forming ability of the cell lines [54]. Unfortunately these cell lines already contain a normal BRCA1 copy, so it is unclear whether the construct raises BRCA1 levels (mRNA or protein) to 'normal' levels present in the original cancer progenitor cell, or highly overexpresses BRCA1.

Interestingly when mutated BRCA1 constructs are introduced, they lose the ability to suppress the tumour forming ability of these breast cancer cell lines, although they retain the ability to suppress ovarian cancer cell lines. It is tempting to draw parallels with particular human mutations of the BRCA1 protein which are high risk for breast cancer, but low for ovarian cancer [55].

BRCA2 germline mutations were identified as a common factor in a proportion of the remaining cancer prone families, with their risk of breast cancer estimated at 70% by age 70 [45]. Interestingly the male breast cancer risk conferred by BRCA2 is 200 fold that of the general population [56]. Evidence regarding BRCA2 is still unfolding. Structurally, BRCA2 also contains a granin motif as well as homology with a predicted protein in C. Elegans, but the functional significance of both observations is still unclear [57].

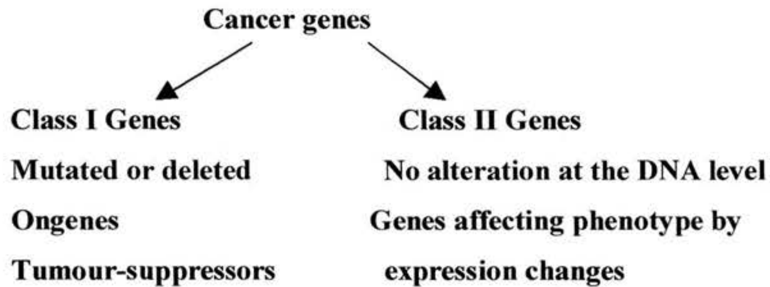
The most recent evidence has implicated both BRCA1 and BRCA2 in DNA repair. It is clear that both BRCA1 and BRCA2 can bind the transactivation domain of Rad51 [57a]. Rad51 is known to be involved in DNA recombination and repair. This is further supported by the induction of phosphorylation of BRCA1 in response to DNA damage. It is likely that BRCA1 and 2 function as transcription factors to DNA repair genes. As well as Rad51, BRCA1 can bind several specific trancriptional regulators like p53, myc, CtIP and E2F.Recent experiments with knockout mice support the role of BRCA1 and BRCA2 protens in DNA recombination and repair of double-strand DNA breaks [57a].

The tumour suppressor gene p53 may also play a role in inherited genetics predisposing to breast cancer. In studies of patients with Li Fraumeni's syndrome, who have germline mutations in p53, the majority of these patients develop cancers by early middle age [58]. Of these, breast cancer appears to be one of the most frequently developed cancers. Other mutations which confer an inherited risk for breast cancer include rare mutant alleles of the androgen receptor [59] and mutations present in ataxia-telangiectasia [60]. Current studies are underway to study the disease implications of the Cowden disease gene PTEN/MMAC1 [61].

Gene effects in breast cancer

In discussion of genes involved in cancer in general a dichotomy has been recognised. Some genes are altered in expression by mutation, others by changes in their regulation (see below).

In contrast to human breast carcinogenesis spontaneous tumours in mice have a viral aetiology such as the mouse mammary tumour virus (MMTV) [98], while in humans, tumour aetiology remains largely unknown. Human MMTV-like elements known as human endogenous retroviral elements (HERVs) have been observed in breast cancer [99], but their biological significance remains unknown. This discussion will compare the gene effects in spontaneous human and mouse breast cancer. A detailed discussion of the relevance of mouse models to human breast carcinogenesis is included in the next section.



Class I

In studies of human breast cancers the common oncogenes affected appear to be c myc, c-erbB-2 (neu/HER2) and PRAD-1/cyclinD1 [65]. Of particular interest is c-erbB-2, which encodes a transmembrane protein with tyrosine kinase activity [66]. Amplification of c-erbB-2 is seen in almost all cases of an in situ cancer known as comedo intraductal carcinoma, as well as 10% to 40% of invasive ductal carcinomas [67, 68]. Certain studies have shown that amplification and overexpression of c-erb B-2 correlates with decreased survival from breast cancer [69]. However in multivariate analysis, this feature is overshadowed by morphological parameters, specifically tumour grade [70]. It is also suggested that patients that overexpress c-erbB-2 show a reduced response to adjuvant chemotherapy and hormonal treatment [70]. In contrast, in studies of murine cancers MMTV activates putative proto-oncogenes which appear to be important in mouse carcinogenesis. These include two members of the Wnt gene family

(Wnt-1 and Wnt-3), three members of the fibroblast growth factor (Fgf) gene family, and int-3, a mammalian Notch gene, for which in vivo human counterparts have not yet been identified [100].

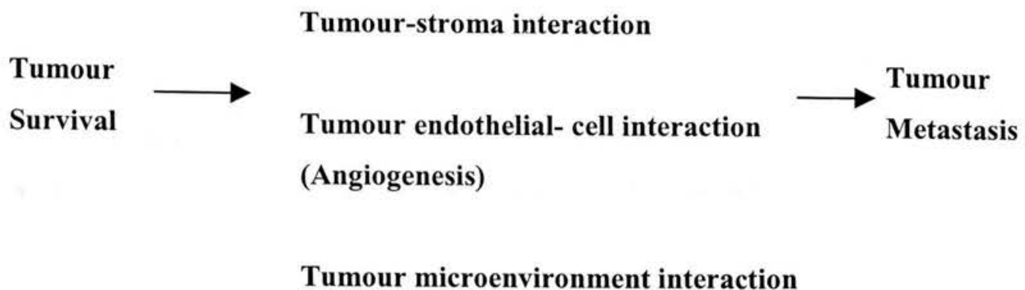
In mouse mammary epithelial cells, MMTV integration activates Wnt-1 by increasing transcription of Wnt-1 mRNA leading to increased (tumourigenic) levels of the normal Wnt-1 protein.While the normal function of Wnt- family is not completely elucidated, Wnt expression is maximal during periods of morphological change and is lost during the terminal differentiation that accompanies lactation. The best current model to explain Wnt induced neoplasia is the theory that Wnt-1 and Wnt-3 functionally substitute for one or more endogenous Wnt genes and drive inappropriate cellular proliferation [337]. It has been shown that members of the FGF family co-operate with MMTV-Wnt-1 in the generation of mouse mammary tumours [338]. Data on the role of int-3 in mammary carcinogenesis is limited.

Human breast cancers are prone to mutations in tumour suppressor genes [65], whereas this is not a prominent feature in mouse mammary tumour formation. p53, the tumour suppressor mutated in the majority of human cancers, is mutated or deleted in approximately 20-40% of sporadic breast cancer [71]. It has been shown that p53 mutations are present in carcinoma-in situ lesions which are at increased risk of progressing to cancer, but not in simple hyperproliferative lesions. In mouse transgenic models, the loss of p53 is not sufficient to cause mammary tumours alone, but it appears to cooperate with a second oncogene, such as Wnt 1 to greatly increase the rate of mammary carcinogenesis [72, 73]. While loss of p53 is not a key feature in spontaneous MMTV tumourigenesis it has been described in relation to hyperplastic alveolar nodules (HAN). These lesions are a loosely equivalent group of preneoplastic lesions to human DCIS identified by De Ome et al [101]. If HAN lines are perpetuated by serial transplantation into cleared mammary fat pads of syngeneic virgin mice, a proportion form tumours [95, 102, 102]. HAN have been further subdivided according to their degree of immortalisation, degree of hyperplasia and tumour-forming potential. There appears to be progression between these stages of HAN in the mouse, which is associated with the loss of functional p53 and alteration in cyclin function [105].

The retinoblastoma (Rb) tumour suppressor gene appears to undergo germline mutations in some human breast cancers to a lesser extent than p53, while somatic mutations have been reported in certain cases [74]. Loss of heterozygosity in human breast cancers, which may point to loss of other tumour suppressor genes has also been detected on chromosomes 1p, 1q, 3p, 11p, 13q, 17p, and 18q [65].

Class II

The Class II genes include those involved in invasion and metastasis and have been extensively evaluated in human carcinogenesis. Data on these genes in mice is derived from mouse models
rather than spontaneous tumours. These 'metastogenes' are key players in physiological processes such as inflammation, embryogenesis, placentation, organogenesis, lymphocyte activation and stem cell differentiation [328].
Tumour metastasis is the result of a plethora of cell- cell interactions (see below).



In both human and mouse, tumour-stroma reactions result in localized proteolysis mediating tumour invasion and metastasis. A number of well-described genes which are clinically relevant include those controlling extracellular matrix, plasminogenolytic and angiogenesis pathways. Components of the plasminogenolytic systems such as uPA, uPAR, PAI-1 and Matrix

Metalloproteinases (MMP's) are expressed in a complementary fashion [329]. Angiogenesis is brought about by a paracrine interplay between tumour cells and endothelial cells. In particular Vascular endothelial growth factor (VEGF) is synthesised by tumour cells, whereas its receptors VEGFR-1,2 and 3 are expressed by proliferation endothelial cells[330].

Genes with evidence for involvement in breast cancer invasion and metastasis , which may become more clinically relevant include S100A4, mta-1 (metastasis associated gene-1), and MUC 1 [331].

S100A4 is upregulated in rodent metastatic mammary carcinoma models. Moreover S100A4 promotes invasion in human breast cancer lines and has been identified in association with distant metastasis in breast cancer surgical specimens [333]. It is hypothesised that S100A4 regulates cytoskeletal dynamics of metastatic cells through modulation of myosin phosphorylation. Similarly, Mta-1 was isolated in a rat mammary adenocarcinoma model system, with an increase in metastatic phenotype [334],and expression of mta-1 is correlated with metastatic capacity in two human model systems. Mta-1 seems to be a component of intracellular signalling pathways since it contains domains which are involved in protein-protein interactions and associated with cytoskeletal components. Inhibition of mta-1 by an antisense nucleotide results in inhibition of human and mouse breast cancer cell line motility and proliferation. An additional Class II 'metastogene' is MUC-1, a heavily glycosylated type 1 transmembrane protein [335]. An underglycosylated form of MUC-1 is expressed in invasive human breast tumours. Data from mouse models is awaited.The effects of MUC1 include inhibition of cell-cell and cell-matrix interactions, immunosuppressive effects and induction of apoptosis in activated T cells.

Models of mammary gland

Before discussing mammary models, it is relevant to assess the similarities between normal structure and physiology of human and mouse mammary gland, in order to determine the relevance of models to human disease. In both the mammary gland is a secretory organ, composed of glandular tissue and ducts contained in a stroma of supporting fat and fibrous tissue [75]. The primary function is to manufacture and deliver milk during lactation, under the control of steroid hormones, peptide hormones and growth factors.There are similarities between human and mouse in terms of corresponding stages of mammary development. The development of human and mouse mammary glands is promoted by puberty or the onset of ovarian cycling [75]. However, functional maturity with differentiation of luminal epithelial cells for milk production, only occurs during pregnancy. After weaning in the mouse, the gland is remodelled and has the appearance of that of a mature virgin within a few weeks [76]. In humans, mammary involution takes approximately three months and there is a return to a more ductular system, as seen before pregnancy.

Structurally the glandular units in both human and mouse are lobules with sac-like ends known as alveoli. A coalescing system of ducts drains the lobules, eventually emerging on to the surface at the nipple [77]. The lobules are lined by an inner layer of luminal epithelial cells and a second outer layer of myoepithelial cells. The characteristic cuboidal epithelial cells are able to secrete milk proteins, and it is from this layer that the majority of breast cancers arise [78]. In both human and mouse the outer layer of myoepithelial cells have specialised contractile actin elements, which are thought to contract to help express milk during suckling [79, 80]. These structural and physiological similarities have lead to a number of mouse models being used experimentally [81]. However perhaps the main advantage of the mouse is that its genome is extensively mapped.

In order to model the normal mammary gland in vitro a number of approaches have evolved. Whole organ or explant cultures can be employed [82]; however their main disadvantage is the inability to distinguish the effects of epithelial cells from stromal components. In order to examine epithelial-stromal interactions in a more controlled environment, a coculture system, in which a monolayer of stromal adipocytes or fibroblasts are seeded with mammary epithelial cells has been developed [83].

This system is complicated experimentally by the differing serum requirements of stromal cells and epithelial cells. However, with improved separation techniques it is possible to develop mammary epithelial cell primary cultures which are fibroblast- free [84]. The particular difficulty of primary cell culture ie growing cells directly from tissue, is that the cells have a limited number of doublings before they senesce or enter crisis [85]. It has been observed however that very rare cell lines could overcome crisis and be cultured indefinitely. These spontaneously immortalized mammary epithelial lines originally derived from mid-pregnant Balb-c mice were termed the COMMA-1D line [86]. This parental line gave rise to the CID9 [87] and HC11 lines [88]. The model line for 'normal breast' quoted extensively in the literature is the HC11 cell line [88]. These spontaneously immortalized cells, differentiate in the presence of lactogenic hormones to produce early diffentiation markers such as the milk protein beta casein (β-casein). However, they have a limited ability to express late differentiation markers such as Whey Acidic Protein (WAP) and alpha lactalbumin. The increased lifespan of HC11 cells is attributed to the lack of wild type p53, but whether this leads to alteration of the differentiation pathways being examined in the cells, remains unclear [89].
The random derivation of spontaneously immortalized cell lines remains a disadvantage. Genetic manipulation can therefore 'immortalize' cells in a controlled manner. The most effective strategies appear to be introduction of the nuclear oncogene myc, the GTP binding protein Ras or DNA tumour virus immortalizing genes [90]. A notable example of the effect of DNA virus is work by Jat et al, who developed the transgenic immortomouse, which carries a temperature-sensitive Simian Virus 40 (SV40) large T antigen under the control of a housekeeping gene promoter H-2Kb [91]. This promoter directs expression to a wide range of tissues and can be induced in vitro using gamma interferon. At low levels, SV40 large T antigen allows the immortalization of various cell lines including thymocytes, colon crypt and bone precursors [91, 92]. Unfortunately mammary epithelial cell lines have not been derived from the original immortomouse because there was insufficient expression of the transgene large T antigen in the mammary secretory epithelium.

Mammary epithelium may be targetted specifically by using milk protein gene promoters such as beta casein, whey acidic protein and lactoglobulin or the mammary mouse tumour virus promoter (MMTV) [93]. This approach has been used successfully to introduce a temperature-sensitive SV40 large T antigen in the KIM 2 mammary epithelial cell line, which is targeted via the beta lactoglobulin promoter [94]
An alternative in vivo model to transgenic mice and cell lines, is to use cleared fat pads [95]. This involves the genetic manipulation of mammary epithelial cells in culture before transplantation into the cleared fat pad of syngeneic mice. The epithelial cells then regenerate to form a mammary tree which varies in phenotype according to the gene introduced. A possible disadvantage is the immune response of the host to the cells transplanted, but this can be overcome using immunodeficient animals [96] .The cleared fat pad approach has been used successfully, particularly with reference to tumourigenesis experiments [97].

Models of mammary carcinogenesis

Spontaneous tumours in mice tend to have a viral aetiology such as the mouse retrovirus-(MMTV) [98], while in humans, tumour aetiology remains largely unknown. Human MMTV-like elements known as human endogenous retroviral elements (HERVs) have been observed in human breast cancers [99], but their biological significance is still unclear. In the mouse the MMTV is passed from mother to offspring in milk and is dependent on glucocorticoid expression for transmission, whereas no analagous agent is identified in humans. MMTV activates putative proto-oncogenes which appear to be important in mouse carcinogenesis. These include two members of the Wnt gene family (Wnt-1 and Wnt-3), three members of the fibroblast growth factor (Fgf) gene family, and int-3, a mammalian Notch gene, for which in vivo human counterparts have not yet been identified [100].
While accepting that the aetiology of spontaneous tumours may differ, it is relevant to assess the stages of development in mouse and human tumourigenesis. In the human, preneoplastic lesions (atypical hyperplasias) have been identified which may be associated with increased cancer risk. In the mouse a loosely equivalent group of preneoplastic lesions (hyperplastic alveolar nodules (HAN)) have been identified by DeOme et al [101].

When transplanted into the cleared mammary fat pads of syngeneic virgin mice, HAN grow and fill the fat pad with alveolar cells similar to the normal pregnant gland. However, if these lines are perpetuated by serial transplantation into cleared fat pads, a proportion form tumours [95, 102, 103]. HAN have been further subdivided according to their immortalization, degree of hyperplasia and tumour- forming potential. In human lesions a similar subdivision has been described into ductal hyperplasias, atypical hyperplasia and carcinoma in situ [104]. There appears to be progression between stages of HAN in the mouse, which is associated with loss of functional p53 and alteration in cyclin function [105].

Apart from spontaneous models of tumourigenesis in the mouse, there are a number of transgenic models to study breast cancer. The most common promoters used to target the mammary epithelium include mouse mammary tumour virus (MMTV), whey acidic protein (WAP) and beta lactoglobulin (BLG). In broad terms the transgenic models currently studied are grouped into those with altered growth regulators, cell cycle proteins, matrix proteinases, apoptosis proteins and viral oncoproteins [106]. Although some models are applicable to gene defects observed in human breast cancer, others are more useful for studying mechanisms relevant to carcinogenesis in general. Moreover, although such female transgenic animals may develop mammary tumours, many require additional manipulations before cancers appear [107]. The group of transgenic models most relevant to this study are those involving viral oncoproteins, in particular SV40 large T antigen.

SV40 virus-clinical perspective

In 1960, SV40 virus was originally identified in rhesus monkey kidney cells, which were also being used to prepare polio and adenovaccines [110]. The virus caused no cytopathic effects in rhesus monkey kidney cells, but when the infected cells were injected into newborn hamsters, sarcomas appeared at the subcutaneous site of innoculation [111]. Hamsters also developed ependymomas when SV40 virus was injected into the brain [111]. These experiments first pointed to SV40's oncogenic potential and since then the debate over the effect of SV40 on humans has continued. It is clear that SV40 virus had contaminated both the Salk and Sabin poliovaccines [112]. These contaminated vaccines were used in a number of experiments which are ethically questionable today. For instance, it was observed that when human adults were injected with the contaminated Salk polivaccine, they developed neutralizing antibodies against SV40 [112]. Although there was no evidence of symptoms in these patients, it became clear that the virus was replicating in humans. For example, children given the contaminated SV40 oral Sabin vaccines excreted virus in the stools for approximately 5 weeks following administration [113].

Despite this, large scale epidemiological studies have not demonstrated a general increased risk of tumour formation (including ependymomas and sarcomas) in the populations receiving contaminated vaccines [115]. The only striking observation is an increased incidence in mesotheliomas, but this would correspond to the relatively small numbers of adults vaccinated with poliovaccines [115]. Other studies have identified SV40 virus in tumours such as meningiomas, but contaminated vaccine-exposure was not confirmed in these patients [117].

However, although no marked association with tumour incidence has been demonstrated, the effects of SV40 in cell culture are striking and have underpinned numerous studies of carcinogenesis. It was found that SV40 virus transformed a proportion of human cells in culture [118]. They were termed semi-permissive because SV40 virus could both replicate in human cells and transform them. Rodent cells are non-permissive because they do not support SV40 replication, although they do transform. In contrast, monkey cells are permissive because they support SV40 virus replication. Since then numerous studies have shown that SV40 virus can alter normal cell growth in rodent cultures and in its role has been examined in over 20 distinct cell types in transgenic mice [119].

Mechanism of immortalization and transformation

SV40 is a small circular DNA virus (5432bp), whose immortalization and transforming ability is attributed to two early gene products - small and large tumour T antigen [120]. Both small T and large Tantigen are transcribed from the same transcriptional unit but are differentially spliced. The large T antigen binds to tumour suppressor genes p53 and pRb which are normally involved in cell cycle regulation [121]. It may be that abrogation of these checks allows a cell in culture to divide in an uncontrolled manner, and that accumulation of chromosomal damage predisposes to either immortalization or transformation. Both p53 and pRb may be mutated in human cancers [122]. Indeed mutations of the tumour suppressor gene p53 are the most common genetic alterations observed in human malignancies. p53 has been termed the 'guardian of the genome' due to its ability to induce cell cycle arrest or apoptosis in reponse to DNA damage caused by ionising radiation or cytotoxics [123]. pRb was originally described in childhood retinoblastoma and is mutated in far fewer human malignancies than p53 [128]. It binds to certain cell cycle regulatory proteins depending on its phosporylation status [129]. In G0 when Rb is dephosphorylated, it inhibits the transcription of genes such as fos and myc that are necessary for proliferation. Growth factors relieve the inhibition exerted by Rb, by causing the protein to become phosphorylated on multiple serines and threonines.

In the virus lifecycle, large T antigen allows the virus to bypass these normal controls and thereby replicate faster than the host cell [120]. Large T also binds other proteins such as the pRb-related proteins p107 and p130, and inactivates them at its J domain [130]. The J domain has homology to the DNA J-family of molecular chaperone proteins, but the functional significance of the interaction remains unclear [130]. The role of small T antigen is still contraversial, although it is known to regulate protein phosphatase 2a and is thought to aid the transformation of specific cell types, eg murine ductal epithelial cells in liver and kidney [131].

In order to examine the function of large T antigen in mammary gland a number of transgenic mice have been developed resulting in different phenotypes. Mice created using a WAP-SV40 large T hybrid gene produced lines which developed mammary carcinomata coinciding with lactation [93], or those who showed a high level of p53-independent apoptosis during pregnancy followed by impaired milk protein production [132].

Whether large T antigen acting through p53 and pRb alone, causes cell immortalization or transformation remains uncertain. Transgenic mice with a T antigen-MMTV promoter in a tetracycline-responsive system, developed cellular transformation and salivary gland hyperplasia by 4 months of age. If T antigen was silenced at this stage, the hyperplasia and cell changes regressed. However, if T antigen was silenced at 7 months of age or later, the hyperplasia and cell changes remained [133]. It has also been shown that cells originally transformed with T antigen remain transformed even when negligible levels of T antigen are present, and there is no evidence of binding to p53 or pRb. It has therefore been proposed that chromosome rearrangements as a result of inadequate policing of the cell cycle play a role in maintaining the transformed or immortalized state [115]. The most recent evidence is from normal human fibroblasts in culture which, unlike rodents cultures, rarely if ever spontaneously immortalize. Comparison between fibroblasts which immortalize with SV40 virus and those which do not, has identified mutation of a gene on chromosome 6 (6q26-27), designated SEN6, which is thought to be key for immortalization [134-136]. Inactivation of SEN6 may be responsible for immortalization of tumours including non-Hodgkins lymphoma, mammary carcinoma and ovarian carcinoma which show loss of heterozygosity in 6q26-27 [134].

Why certain cells retain normal characteristics and simply divide indefinitely, whereas others fulfill the criteria for transformation with T antigen remains unknown. It has been proposed that low levels of T antigen are more likely to simply immortalize cells, whereas high levels result in full transformation [120]. T antigen remains the basis for immortalization of a number of 'normal' cell lines [120]. In this respect a frequently used technique is to use a temperature- sensitive mutant of T antigen. At a permissive temperature thermolabile mutants have the same properties as wild-type T antigen, whereas at the restrictive temperature T antigen is inactive. One of these mutants is tsA58 which has a cytosine changed to thymidine [91], resulting in the substitution of an alanine residue for valine within an ATP ase binding domain. There is still controversy as to whether at the restrictive temperature there is a conformational change in tsA58 which renders it inactive [137] or whether mRNA is rapidly degraded [138].

KIM2 cells are mammary epithelial cells developed using the the tsA58 targeted to the mouse mammary gland using the ovine beta lactoglobulin promoter (BLG) [94].
At a semi-permissive temperature of 37 °C, when T antigen is active at low levels, KIM2 cells appear epithelial and are stable in phenotype for over 60 passages. However cells derived at the permissive temperature of 33 °C -when T antigen is active at high levels, appear spindle shaped and transformed. Very early passage cells appear to be able to switch phenotypes according to temperature, whereas this ability is lost once cell lines are established in culture [94].
In summary there are a number of hormonal, environmental, and genetic influences in human breast carcinogenesis. Mouse mammary cell lines have certain benefits as well as disadvantages as experimental models of this process. One example of a mechanism for immortalizing and transforming cells is a temperature-sensitive SV40 virus large T antigen, which provides a model for examining both normal and transformed mammary epithelial cells in vitro.

Aims of the study

The KIM2 mammary epithelial cell line immortalized by SV40 virus may be a useful cell culture model of normal and transformed mammary epithelia. Genetic manipulation of this model may result in the reversion of the transformed phenotype to a non-transformed phenotype.

The aims of this study were therefore to:

i. examine the biological features of epithelial and mammary differentiation in 'normal' mammary KIM2 epithelial cells
ii. examine the features of transformation in 'transformed' KIM2 cells
iii. revert the 'transformed' cells to a normal phenotype by introducing Tiam1 constructs using retroviral methods
iv. confirm the extent of the reversion in terms of the biological reference points established in i,ii

Potential markers of differentiation and transformation

Much research in cell biology is devoted to determining the difference between normal and cancer cells [139]. The difficulty with this approach is that many of the differences in vitro, may not be relevant to malignancy. The so-called 'normal' cellular phenotype that will be considered, is subject to the reservation that cell lines may have been immortalized. The phenomenon of immortalization results in an indefinite lifespan in culture, which will almost certainly effect numerous normal cell signalling pathways. The 'transformed' cellular phenotype refers to tumour cells in culture in vitro, and is thought to be analagous to 'tumourigenicity' in vivo.

Certain criteria have been put forward based on experimental observations, to define normal and transformed cells [140]. These criteria will be considered and their relevance to normal and cancer cells discussed.

Morphology and contact inhibition

Cells in culture may change their morphology depending on their physical, biological and biochemical environment [141]. For instance, scanning electron microscopic studies have shown that cells radically change morphology during the cell cycle [141]. Despite this, it appears possible to distinguish between normal and transformed cells in culture using morphological features, even though these differences may be as a result of artefact. Moreover, morphological changes in culture are among the first alterations described, when normal cells transform due to the action of specific viral genes [142, 143]. Classically, normal epithelial cells form a cobblestone, cuboidal shape in culture and grow as a flattened monolayer. Interdigitation of the cytoplasm of certain epithelial cell types has been observed under electron microscopy [144]. Normal fibroblasts have an elongated spindle-shaped appearance and they similarly grow as a flat monolayer with interdigitation, although overlapping of cells is sometimes observed [144]. Transformed cells in culture are elongated and spindle-shaped similar to fibroblasts, but are pleomorphic and more rounded in cross section on electron microscopy. The rounded appearance is thought to be reflective of reduced adhesion to the substratum [145]. Transformed cells also show a lack of contact inhibition, which results in multilayering of cells in an anarchic fashion. Contact inhibition of movement was first described in normal cells, which, when they collide, turn aside and move away in different directions [146].

This lack of positional control in transformed cells, as opposed to lack of growth control, may parallel the invasive properties of cancerous cells in vivo. For example, it is clear that epithelial stem cells can proliferate in vivo, but the progeny tend not to cross the boundary to penetrate tissue of a different type. However, cancerous epithelial cells move outside their territory and in some cases, also cause breakdown of surrounding tissues [147]. From these observations it has been proposed that a number of the molecular targets for transformation, are normally responsible for organisation of the cytoskeleton and cell-cell contacts [148]. At an ultrastructural level, normal and transformed cells may differ in terms of cell surface glycoproteins and cytoskeleton including actin filaments and microtubules [149, 150]. However exceptions include certain cell lines that are transformed in terms of loss of growth control, and retain a normal cytoskeleton. Furthermore, cells lose cytoskeletal structure during mitosis and when removed from a solid substrate, but do not transform [151].

Epithelial junctions and polarity.

Given the lack of contact inhibition of transformed cells, it follows that they do not form complete cell junctions. In simple terms, cell junctions have been classified into tight junctions, communicating junctions and anchoring (adherens) junctions [152]. Studies have shown it is the anchoring junctions, mediated by the transmembrane protein E-cadherin, which are characteristic of the sheets formed by epithelia [153]. Cadherins are responsible for calcium-dependent homophilic binding in vertebrates, which links actin cytoskeletons between cells [154]. A highly conserved cytoplasmic domain of cadherins interacts with the actin cortex by means of atleast three intracellular attachment protein known as catenins [155]. At a molecular level, transformed epithelial cells in vivo and in vitro lose these functional cadherin-catenin complexes which underpin the anchoring junctions [148]. The presence of anchoring junctions termed adherens junctions, desmosomes and hemidesmosones respectively, can be identified by electron microscopy [152].

The tight junction is an alternative junction which may be detected in epithelia [156], although these junctions undergo changes in junctional integrity in the pregnant mammary gland in vivo [156]. The specific immunohistochemical marker of these junctions is the protein Zonula Occludens 1 (ZO-1), which lies on the cytoplasmic aspect of the junction [156].

Although E-cadherin and ZO-1 are useful markers of epithelial junctions, the intermediate filament keratin, is an alternative marker that allows the identification and classification of epithelial tumours which have lost junctional specialisiations [157, 158]. It is also used for tracing back the origin of metastatic carcinomas [159].

Cell polarity also remains the domain of the normal rather than transformed cell [160]. It is vital to mammary epithelial cells, which secrete milk proteins across the apical cell membrane into the lumen of alveoli in vivo. In cell culture polarity can be assessed on electron microscopy by the identification of apical microvilli which form a continuous surface between cells [161]. An alternative method involves identifying Na K ATP ase channels by immunohistochemistry which are present on the basolateral aspect of the cell only [161].

Mammary differentiation

Mammary differentiation of cell lines is assessed in terms of milk protein secretion in response to lactogenic hormones. However, the feature of milk protein secretion has been described in both normal epithelial and certain transformed mammary cell lines in culture [108]. The proteins can be secreted in response to lactogenic hormones such as prolactin, insulin and dexamethasone [162]. The two groups of proteins which may be produced include caseins, which precipitate under acidic conditions, and whey proteins which remain in milk serum at low pH [163]. Caseins are a family of phosphoproteins which constitute 80% total milk protein. Alpha and beta casein are serine phosphorylated to permit interactions with calcium phosphate resulting in formation of micelle structures in milk [164]. These micelles transport calcium, inorganic phosphate, trace amounts citrate and magnesium from mother to neonate. Beta casein is commonly identified in studies on mammary epithelial differentiation in culture [165]. However studies on knock-out mice have shown that beta casein is not essential for mammary development or survival of neonate [163]. Of the whey proteins, alpha lactoglobulin is present in the milk of many species. It is involved in synthesis of lactose from glucose and galactose and is thought to be a marker of late mammary differentiation [166]. In the rodent mammary epithelial model, an alternative late differentiation marker is the cysteine rich whey acidic protein (WAP) [166].

It has been demonstrated that premature overexpression of WAP results in disruption of the differentiation programme and structural development of rodent mammary gland [167]. Rodents also produce lesser amounts of the whey protein beta lactoglobulin (BLG) in their milk, however its function remains unclear [168]. Both the BLG and WAP promoters have been used to target the mammary gland in transgenic animals [93, 169]. An alternative more contraversial marker of mammary differentiation is mammosphere formation, which occurs when mammary epithelial cells are cultured on extracellular matrix with lactogenic hormones. These mammospheres are thought to resemble alveoli in vivo, and one group has proposed that they are a prerequisite for WAP secretion in this system [170].

Serum-independence, anchorage-independence and tumourigenicity

As discussed earlier normal cells in tissue culture proliferate until they form a monolayer and show contact inhibition. The particular density at which they stop proliferating seems partly dependent on the serum concentration of the medium, which provides necessary growth factors [171]. If normal cells are plated at a lower density in the same serum concentration, they again proliferate to a certain saturation density. Although intuitively one would expect immortalization which extends the lifespan of normal cells to increase the serum requirement of cells, this appears not to be the case [172]. In contrast, transformed cells show an increased saturation density and a reduced serum requirement [173]. Growth in a serum-free or low serum environment is a recognised feature of transformation cells in culture. It is clear from the literature that there is a relationship between factors controlling growth ie serum-dependence and those controlling adhesion to matrix ie integrin-mediated anchorage-dependence [174, 175]. Early experimental evidence showed that oncogenes could activate integrin-mediated pathways in suspended cells- which are anchorage-independent [176]. One proposed model is that oncogenes can constitutively activate pathways before or after convergence of serum-dependent and anchorage-independent pathways [177]. Thus activation before convergence results in either anchorage or serum-independence in transformed cells. However, activation after convergence would result in transformed cells that were both anchorage and serum-independent. To an extent this model is supported by experimental evidence. For example overexpression or activation of oncogenic rho, FAK, CDC42, ILK and c-abl results in cells that are anchorage-independent but not serum-independent [177].

Activation of pathways by v-fos,v-Ras , v-src and SV40 large T antigen results in transformed cells which are both anchorage and serum-independent [177]. To date, there is no evidence for oncogenic activation causing serum-independence only without anchorage-independence. Anchorage-independence is assessed formally in vitro by the ability of cells to form colonies in a semi-solid medium-usually soft agar [178]. Most normal cells will not proliferate in suspension, with the exceptions of blood cell precursors and chondrocytes, both of which are in suspension in vivo [179]. Several studies have correlated the ability to grow in suspension eg growth in soft agar, with tumourigenicity in immunodeficient mice [180, 181]. Since many cell lines are derived from non-inbred animals which may elicit an immune response in a mouse from a different background it has become necessary to assay cellular tumourigenicity , in immune suppressed animals. Further more, even if a cell line is descended from an inbred strain, it may express a new cellular antigen and then elicit immunologic rejection when injected into the animal of origin.

Immunodeficient mice fall into two broad categories the nude or scid (severe combined immunodeficiency) mouse. The nude mouse lacks a thymus congenitally [182] and is deficient in thymus-dependent immunologic functions [183]. Nude mice can support the growth of certain human tumour transplants as well as a wide range of xenogeneic skin grafts [184]. Injection of certain malignant tumours can give rise to metastasizing tumours in nude mice. Mice homozygous for the scid mutation are severely deficient in functional B and T lymphocytes [109]. The mutation appears to impair the recombination of antigen receptor genes and thereby cause an arrest in the early development of B and T lineage-committed cells: other haemopoetic cell types appear to develop and function normally. Young adult mice are leaky and generate a few clones of functional B and T cells [185]. By 10-14 months all mice are leaky. Scid mice support the growth of allogeneic and xenogeneic tumours [109]. However transplantation of rat and human mammary carcinomas into the mammary fat pad has been unsuccessful, with an acceptance rate less than 15% [186]. The reason for this unclear, however it has been shown that the take rate can increase to 50% with the coinjection of Matrigel, a mixture of extracellular matrix molecules which enhances both tumour growth and metastasis [96, 187].

Although the ability to form tumours in immunodeficient nude athymic or scid mice seems an attractive gold standard for assessing transformation, it only assays for one aspect of malignancy, namely uncontrolled proliferation. A serious weakness in this assay is that it fails to demonstrate the complex in vivo properties of malignancy, namely invasion and metastasis. Indeed the 'normal' HC11 mammary cell line forms tumours when injected into immunodeficient animals, but it is unlikely to metastasise [88]. One assay of metastasis, is to score cell lines by their propensity to seed in various organs of an immunodeficient mouse. For example a melanoma line metastasizing to lung is easy to detect because of the characteristic pigment of the metastatic deposits [188]. This is a time and animal intensive approach and alteratives are still being sought. One cell culture approach used in lymphoma cells is to monitor their ability to infiltrate monolayers of hepatocytes or fibroblasts [189]. This feature apparently correlates well with the ability of lymphoma cells to form experimental metastases.

Chromosome content

Apart from assessing the biological characteristics mentioned previously, it is possible to assess cell lines in terms of chromosome and DNA content [190]. For example, DNA flow cytometry has been used to show that continual passage in cell culture results in alterations in DNA content. Furthermore, immortalisation procedures, in particular the addition of a viral antigen to cell lines may affect their chromosomal content. However in broad terms transformed cells or tumour populations are more likely to show aneuploidy than normal cell populations [190, 191]. Karyotype analysis is one method to identify the gross chromosomal aberrations in normal versus transformed lines [192]. An alternative method for identifying individual chromosomes or loci is fluorescence in situ hybridisation (FISH) [193].

Gene transfer

The choice of method for transferring DNA into target cells depends to a large extent on the research setting, the nature of the target cell and the degree to which the DNA construct is required to integrate with host DNA. Broadly speaking the choice lies between non-viral and viral methods, and the processes are termed transfection and transduction respectively.

The non- viral approaches in general terms rely on using the ability of the cell to phagocytose foreign molecules, increasing the permeability of the cell membrane or direct physical transfer of DNA into the cell. A routinely used non-viral method, is transfer of DNA to the target cell using calcium phosphate precipitation [188]. It is thought that co-precipitation of the DNA construct with calcium phosphate results in phagocytic uptake by target cells, although the precise chemical mechanism of this is unclear. Using modifications to the originally described procedure, high transfection efficiencies have been described. An alternative method using DEAE dextran with chloroquine, is useful in transient transfections requiring the analysis of large numbers of DNA constructs [188]. The DEAE dextran is mixed with the DNA construct and overlaid onto target cells, resulting in expression of the DNA within 24-48 hours. At chemical level, it is thought that the chloroquine neutralises the pH of lysosomes preventing degradation of DNA en route to the nucleus. However a number of studies report higher rates of DNA mutagenesis in DEAE dextran transfections compared with calcium phosphate [194]. Lipid- based techniques, in particular cationic liposomes, utilise the phagocytic capabilities of the target cells, similar to calcium phosphate and dextran [195]. Fatty droplets containing the DNA construct are engulfed into the cell. This approach has had success in gene therapy research, particularly with respect to cystic fibrosis [196]. The electroporation approach, in contrast to calcium phosphate and DEAE dextran, involves exposing a cell suspension with added DNA construct, to a high voltage electric field [197]. The field is thought to create large pores within the cell membrane, to allow the DNA to enter the cytoplasm. A proportion of this DNA enters the nucleus to stably integrate with the host cell genome, however the dynamics of this process are unknown.

The target cells are incubated at low temperatures in order to reseal their membranes. Similar to electroporation, chemical detergents can also partially disrupt target cell membranes to make it more them more permeable to DNA constructs, however they have been shown to also damage intercellular membranes [197].
The physical approach involving direct transfer of DNA into individual target cells via a micropipette, is termed microinjection. This approach has been used in a number of studies, however it is limited in that only a few hundred cells can be examined at one time. A significant problem encountered by all non-viral systems is that certain cell types such as epithelial cells, primary cell culture sytems and cells in suspension have intrinsically low transfection efficiencies. The reasons for this are still unclear

Viral techniques of gene transfer involve inserting DNA constructs into viral vectors, to utilise the viral method of entry into the cell and possible integration with the host genome. These techniques provide a useful, but perhaps more technically demanding alternative to the non-viral methods discussed. However, an overriding concern when using this technology is to prevent uncontrolled infection of target cells. This is achieved by engineering replication-defective viral vectors [198], which are most frequently adenoviral or retroviral. Alternative vectors include adeno-associated virus (AAV), herpes virus, vaccinia virus and several RNA viruses [199].
Adenoviral-mediated transfer is a routinely used technique, which has achieved high transduction efficiencies particularly in transient assays [200]. Although integration of adenoviral DNA with target cell DNA can occur, it is relatively inefficient because stable integration is not part of the adenoviral life cycle. The adenoviral vector has an advantage over retroviral methods, with the exception of lentiviral vectors, in that it can be used to transduce non-dividing cells [200]. The most critical advance in the development of adenoviral vectors has been the observation that replication-defective adenoviruses lacking portions of the E1 region of the viral genome could be propagated by growth in cells engineered to express the E1 genes [201]. Since then it has been shown that replication-defective adenoviral genomes carrying foreign DNA sequences can be propagated in the same way.

The main alternative category of viral vector is the retrovirus [202]. The main advantage over adenovirus, is that retroviral DNA stably integrates into the target cell genome in a defined manner, so that the gene introduced is passed on and expressed in all daughter cells [199]. Retroviruses contain elements which enhance transcription, therefore promoting high levels of gene expression in all target cells [203]. Certain vectors have been reported to achieve stable transduction of close to 100% of target cells [198]. Experimentally, retroviruses have small genomes compared to adenoviruses and therefore can be easily manipulated to introduce foreign genes, however this does limit the size of DNA which can be transduced. Recently it has been shown that high titres of retrovirus can grown relatively easily in cell culture [204].

The principle of vector technology depends on harnessing and manipulating the normal retroviral lifecycle. The defining step of the lifecycle of the retrovirus family, is the conversion of their RNA genome into DNA by a virus- encoded reverse transcriptase [199]. The retrovirus consists of two copies of a single- stranded RNA genome surrounded by a glycoprotein 'envelope'. These surface glycoproteins interact with receptors on the surface of the target cell, in order to gain entry [205]. When inside the cell, the single-stranded viral genome is converted into a linear double-stranded DNA by a virus- encoded reverse transcriptase. As the target cell undergoes mitosis, the viral DNA integrates randomly with the target cell DNA- at which point it is known as a provirus [206]. It is this proviral DNA which is manipulated to form retroviral vectors for gene transfer. The provirus then undergoes transcription and translation with the rest of the genome, resulting in the assembly of new viral particles which bud off the surface of the target cell in order to infect others.

The single most important advance in the development of retroviral vectors has been the development of the packaging cell which allows the assembly of virus particles with the gene of interest [198]. This packaging- cell method reduces the liklihood of producing a virus that is replication competent and therefore able to infect cells in an uncontrolled fashion. Nevertheless rare 'outbreaks' of wild-type virus from recombinant virus-producing packaging cell lines have been reported. In a primate bone marrow transplantation experiment which targetted haemopoetic cells, stocks were contaminated with replication-competent virus resulting in several of the transplanted animals developing lymphomas [207].

The principles behind choosing a particular packaging cell line are based on the probability of producing contaminating replication competent virus, the desired host range of the virus vector and the required titre of the virus vector. Several Murine Leukaemia Virus-based (MLV) packaging cell lines which are typically fibroblast derivatives, are available which have been modified to improve safety an prevent recombination events [198]. They contain sequences of independently coding DNA sequences known as DNA plasmids, that express viral gene products. The viral gene products include: gag- group antigen polyprotein, Pol-reverse transcriptase and Env-envelope protein [199]. In these systems viral genes are deleted for cis-acting sequences at the 3' end to reduce homology between helper and vector sequences and gag-pol and env are present on separate plasmids, having been transfected sequentially.
The system which is used in this study is based on the Nolan Stanford University Phoenix system (www.leland.stanford.edu/group/nolan) and is represented in Figure 1. It differs from other packaging cell lines in that it is not a stable cell line. In terms of procedure, the first step involved in gene transfer is the modification of the retroviral vector so that it contains the gene of interest. This vector is then transfected into the packaging cell line, using traditional non-viral DNA transfer techniques.
When the vector is introduced into these packaging cells, it is packaged into virus particles by means of the gene products just described. These virions bud off into the culture medium and are placed on the dividing target cells. The crucial event in infection appears to be the attachment of the viral particle to a specific cell- surface receptor [205]. This is followed by a complex set of comformational rearrangements which triggers fusion between viral and cytoplasmic lipid bilayers. The viral vector stably integrates into the target genome, and then expression of the gene of interest is passed to daughter cells. In order to target retroviral vectors to a specific cell type, two strategies can be adopted : i) allow infection of all cell types and regulate gene expression at a transcriptional level through tissue specific or inducible promoters[208] or ii) manipulate cell surface receptor so that regulation is introduced at the level of infection.

Safety considerations are very important when using this technology, as the main hazard is the product of a replication- competent virus which can infect humans. One way of combatting this is to use an ecotrophic virus, which means that virions can only infect mouse and rat cells, due to the specificity of their cell surface receptors [205]. In contrast, amphotrophic viruses have a range of hosts including bird, rodent, mammal and human [16], and their use is more hazardous.

Another potential problem with the system is recombination of the plasmids producing viral gene products in the packaging cell. In order to assess this, an added feature in the packaging cell plasmids is an Internal Ribosomal Entry Site or IRES sequence [209]. The IRES sequence ensures that the Gag and Pol are transcribed together with CD8. This means that packaging cells can be sorted by CD8 on a FACS machine, thus checking the integrity of the plasmids expressing Gag and Pol.

The Nolan system has been further modified for a high efficiency, so that from transfection of packaging cells to infection of target cells takes approximately 48 hours [209]. The modification uses the Epstein Barr virus which has been used to maintain retroviral vectors episomally ie outside the genome, within human-based packaging cell lines [210]. This system results in a much higher output with titres of virions as high as 10^7 Colony Forming Units per ml, produced for atleast 30 days. Alternative, less efficient systems include a tetracycline- regulated system for allowing inducible gene expression using retroviral vectors [208].

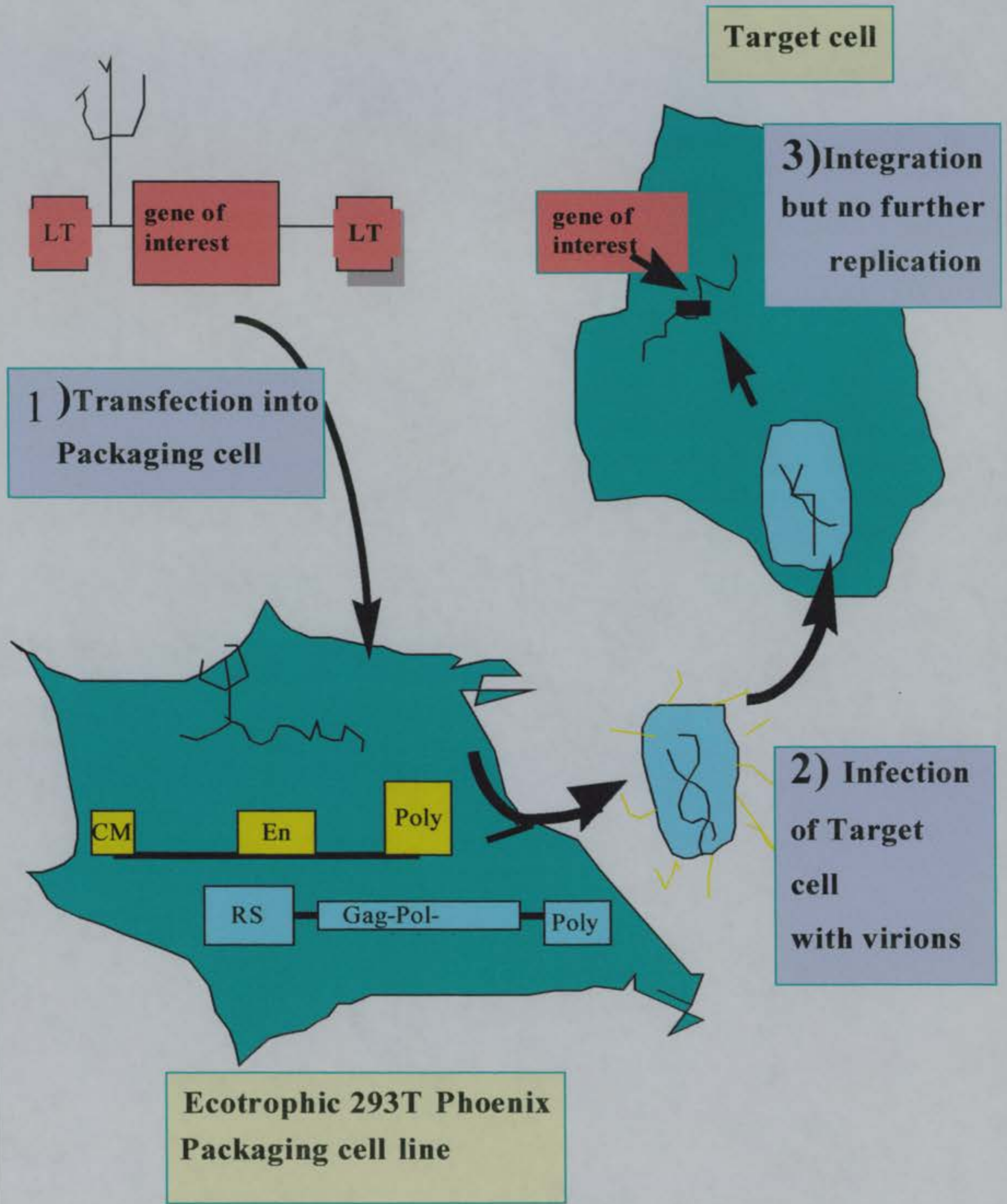


Figure 1 production of retrovirus and infection of target cell
Target cell
LT
gene of interest
LT
gene of interest
3)Integration but no further replication
1)Transfection into Packaging cell
CM
En
Poly
RS
Gag-Pol-
Poly
2) Infection of Target cell with virions
Ecotrophic 293T Phoenix Packaging cell line

Phenotypic reversion

Many studies have effected a reversion of the transformed state to a normal cellular phenotype in cell culture. In general they involve manipulation of E-cadherin, alternative components of the junctional complex, integrins or cytoskeletal assembly [212]. More upstream regulators include nm23, the E1A viral antigen, and Tiam1 [214, 216, 238]. It has also been demonstrated that the introduction of chromosomes, in particular human chromosome 6, can have a similar effect [220, 223-225].

Direct evidence about the importance of E-cadherin has been demonstrated in a number of discrete epithelial cell systems, including mouse mammary gland, and under different oncogenic stimuli including Ras and MMTV [211]. Results have shown that highly invasive epithelial tumour cells can be rendered noninvasive by the introduction of a single E-cadherin cDNA, and that invasion can be reinstated by treatment of such transfected cells with monoclonal anti-E-cadherin antibodies [212]. Moreover, invasiveness could be induced in noninvasive cells by downregulating the endogenous E-cadherin expression with antisense RNA [212, 213]. As E-cadherin has been implicated in the Tiam1 pathway of reversion it will be discussed later in more detail.

Candidates for upstream regulators of the reversion pathway remain more speculative. The nm (nonmetastatic) 23 gene is one such putative, suppressor gene which was originally identified with reduced expression in highly metastatic cell lines [214].

In vivo evidence has shown that expression of nm 23 is reduced in breast cancer patients with lymph node metastasis, and furthermore that loss of nm 23 mRNA is associated with poor survival in breast cancer patients [215]. Experimentally, introduction of nm 23 cDNA into metastatic murine melanoma cells reduces their metastatic potential [214]. It appears that the nm 23 gene is a nucleoside diphosphate kinase which is involved in microtubule organisation and signal transduction through G proteins [214]. It has been proposed that genetic instability in metastatic cells may result from reduced nm 23, allowing aberrant mitosis to occur on an aberrant mitotic spindle. This model does not explain the phenomenon of supression of invasive cell-lines, which have already sustained numerous genetic abnormalities.

More recent studies aimed at discovering events upstream, have shown that certain adenovirus E1A proteins can revert the transformed phenotype of a wide variety of cell lines, including those transformed by Ras and erbB/neu oncogene [216]. This is remarkable because the E1A protein was first identified as a viral product that could immortalize primary rodent cells, and transform them in cooperation which a second oncogene-including Ras [218].

However there is no evidence to suggest that E1A plays a role in any human cancers [217]. The primary function of the E1A viral protein in the normal lifecycle is to activate viral gene transcription as well as re-program cellular gene expression to support viral replication. This ability to re-program, allows E1A to both activate and repress gene transcription, as well as induce entry and passage through the cell cycle, and block dedifferentiation. Detailed analysis of the protein has identified regions which are responsible for suppression of transformation, tumourigenicity and metastasis [216]. It has been proposed in the case of phenotypic reversion of lines transformed by c-erb2, that E1A mediates repression of c-erb2 transcription occurs through pRb binding [218]. In the case of suppression of Ras transformation, a protein known as CtBP which has homology to enzymes of D-2-hydroxy acid dehydrogenase family, may modulate the activities of other proteins bound to the transforming regions of the same E1A molecule [216].

Another interesting observation is that expression of E1A in human A204 rhabdomyosarcoma cells induces transcription of E-cadherin, although the region responsible for this is unclear [219]. E1A also represses the expression of matrix degrading proteases through the AP-2 transcription factor, and represses expression of CD44 adhesion molecule, which could help inhibit metastasis. Moreover, E1A can induce expression of nm 23 [216].

Diverse phenotypes may be obtained upon the introduction of different chromosomes into a cell line. These include in vitro growth supression, in vivo tumour suppression and the induction of terminal differentiation [220, 221] The induction of in vitro growth suppression may represent replicative senescence ie the cells remain metabolically active but cease to proliferate [222]. In murine breast cancer cells chromosome 6 causes growth suppression [223].

There have also been reports localising the growth suppressor activity in human MCF7 breast cancer cells to chromosome 17q24-25 [224]. Interestingly the introduction of human chromosome 6 into human melanoma cell lines results in microcell hybrids which have lost their ability to form tumours in nude mice [225], and the subsequent loss of chromosome 6 in the hybrids results in reversion to a tumourigenic phenotype. Further analysis of model by DNA chip analysis has identified significant differences in gene expression between wild-type and reverted melanoma cells; however the biological significance of these differences requires exploration [226, 227].

Tiam1

Tiam1 which stands for T lymphoma invasion and metastasis gene, is a guanine nucleotide exchange factor for Rac, a member of the Rho GTP ase family [228]. Rho GTP ases have emerged as key regulators of the cytoskeleton, and through interaction with target proteins can mediate gene transcription and cell adhesion [229].
As its name suggests Tiam1 was originally described as an invasion-inducing gene in lymphoma cells. It was identified by insertion of provirus into lymphoma cell lines and then selecting for those lines which became invasive [230]. In 40% (12 out of 30) of generated invasive variants, proviral insertions were found within the coding exons of the Tiam1 gene. These insertions resulted in the majority of cases with both truncated 5'- end and 3'- end transcripts that gave rise to N- and C- terminal Tiam1 protein fragments. In one invasive variant, there was an amplication of the Tiam 1 locus with an increase in normal protein levels. It is clear that truncation of other members of the guanine nucleotide exchange factor family such as vav, Dbl and Ect-2 are also required for oncogenic potential [230]. At a functional level, the truncations result in C-terminal Tiam-1 proteins which lack N-terminal PEST domains and a potential myristorylation site (Figure 2). This could result in increased protein stability and or affect their subcellular distribution. It is clear that the N-terminal Pleckstrin homology domain is required for targetting Tiam1 to the plasma membrane [231, 232]. This domain also appears necessary for Rac- dependent membrane ruffling and c-jun NH2-terminal kinase activation [233]. Experiments to date with Tiam1 use the stabilised, truncated protein. The full length Tiam1 protein has not been successfully stably expressed in cell lines [234].

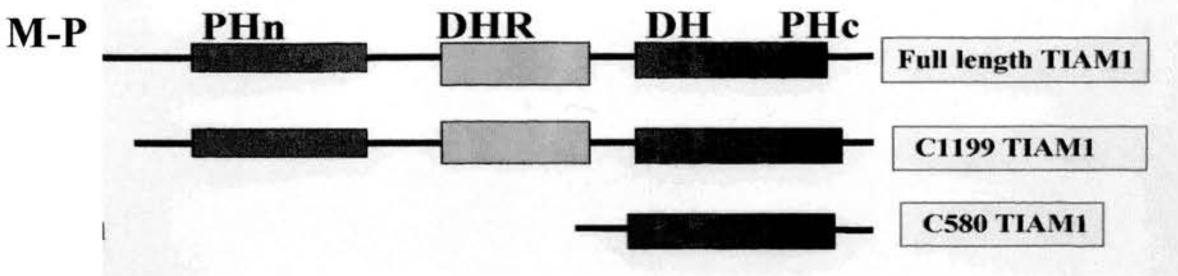


Figure 2 Tiam 1 constructs showing control C580 Tiaml active C1199 Tiaml

Tiaml and the truncated constructs are shown schematically in Figure 2. PHn and PHc represent NH2 and COOH Pleckstrin homologous domains which are present primarily insignaling molecules are thought to determine the cellular localization and/or activity of the protein by interacting with proteins or phospholipids [235].
DHR represents Discs-Large homology regions which have been implicated as protein-protein interaction motifs [236]. DH represents a Dbl homology domain, which is present in guanine nucleotide exchange protein and is considered to be the catalyic domain [237]. M--P represents a potential myristorylation signal and PEST sequences.

Transfection of truncated Tiaml constructs increases the oncogenic potential of fibroblasts, similar to lymphoma cells [234]. The precise mechanism of this remains unclear. However it has been suggested that Tiaml via Rac, effects changes in the cytoskeleton and promotes adhesive contacts between cell and its environment, thus promoting invasion. Certainly ectopic Tiaml expression results in striking morphological and cytoskeletal changes, including flattening of cells and membrane ruffling [238]. In neuroblastoma cells, Tiam 1 promotes adhesion and spreading, possibly mediated by recruitment of the alpha 6 beta 1 integrin, which is a laminin receptor [239]. The potential importance of Tiaml and interactions with the environment in neuronal cells, has been highlighted during murine central nervous system development where the gene is differentially expressed [240, 241]. However, the notion that Tiaml is an oncogene, may be questionable because in epithelial cells, truncated Tiam 1 contructs suppress invasion.

In transformed Madin Derby Canine Kidney (MDCK) epithelial cells, ectopic expression of Tiam1 results in localisation of Tiam1 with E-cadherin at adherens junctions, a remarkable phenotypic reversion to a normal epithelial phenotype, and loss of invasion [242]. These changes occur via Rac, which is involved in adherens junction assembly in keratinocytes [229]. One possible explanation for cell- type specific phenotypes with Tiam1 is the differing abilities of cells to form junctions with each other and with matrix. During invasion, epithelial cells downregulate E-cadherin expression which mediates the strong cell- cell junctions [243]. This is not necessary in fibroblasts and lymphoma cells, which do not have these junctions. After down regulation of E-cadherin, all cell types modulate attachments via integrin receptors, in order to attach to the environment and invade. It has been proposed that Tiam1 inhibits invasion in epithelial cells by upregulating E-cadherin, but promotes invasion in fibroblasts and lymphoma cells by stimulating cell-matrix contacts; the outcome may be influenced by the type of matrix present [244]. The idea that altering structure and cell adhesiveness, can overcome or promote the main features of malignancy, namely uncontrolled growth and invasiveness are not confined to cell culture models (discussed in section E-Cadherin).

The relevance of Tiam1 in humans and mice in vivo remains unclear. Mouse Tiam1 maps to the distal end of murine chromosome 16, while the human homologue of Tiam1 maps to 21q22, centromeric of the Aml (acute myeloid leukaemia) gene. Both are 95% identical at the amino acid level [245]. In normal human and murine tissues Tiam1 is expressed at very low levels, except in brain and testis [245]. The reason for this difference is still unclear, but presumably the low levels in most tissues are due to PEST instability domains in Tiam1. In contrast, Tiam1 is expressed in most human tumour cell lines including those derived from breast carcinoma, lung, ovary, bladder, pancreas, lymphomas, melanomas and neuroblastoma [245]. Whether Tiam1 levels show any association with invasive or metastatic capacity in human tumours remains to be demonstrated. The suggestion that Tiam1 is involved normally in cell signalling processes, comes from its strong evolutionary conservation and broad patterns of expression in normal tissues and tumourigenic cells [245]. Current knowledge of the upstream and down stream components of the Tiam1 signalling pathway remains limited.

Upstream modulation of the family of guanine nucleotide exchange factors has been reported to be either via phosphorylation or cellular relocalisation [246].
Rapid threonine phosphorylation of Tiam1 in Swiss 3T3 fibroblasts is stimulated by lysophosphatidic acid, a mitogen which is an important component of serum [246]. This occurs via protein kinase C. Other growth factors including platelet-derived growth factor, endothelin-1, bombesin and bradykinin (but not epidermal growth factor) have a similar but reduced effect, without evidence of relocalisation of Tiam 1 in any case [246]. However when serum is added to NIH3T3 fibroblasts, Tiam 1 specifically relocates to become associated with the membrane fraction [231]. It is still not clear whether phosphorylation of Tiam 1 or relocation to the membrane affects its nucleotide exchange activity of Rac1.
The downstream activity of Tiam1 which involves exchanging bound GDP for GTP on Rac, has been observed in fibroblasts and MDCK epithelial cells, among other cell types [230]. Rac1 has been implicated in stress fibre formation in fibroblasts, as well as interaction with other pathways controlled by the Rho family of GTPases [247]. Members of this family have provided a link between growth factor signalling and reorganization of the actin cytoskeleton, focal complex formation, endocytosis, cytokinesis, Ras transformation and cell proliferation [229]. Most of these studies used fibroblasts [229]. In terms of epithelial cells, recent evidence has demonstrated that Rac and Rho in concert are necessary for the establishment of cadherin dependent contacts in keratinocytes [229]. Most recent evidence has implicated IQGAP1 [248], a target of Cdc42 and Rac1, as a possible mediator of the effect on E-cadherin, using colocalisation and inhibition assays [249]. In these studies, IQGAP needs also to associate with beta-catenin to function.

E-cadherin

While Tiam1 may play a role in general signalling, there is clear evidence that E-cadherin plays a key biological role in tumourigenesis [243]. The only other cadherin implicated is H-cadherin which is lost in certain breast cancers [254]. As discussed earlier E- cadherin physiologically forms part of adherens junctions, but may also be involved in transduction of transmembrane signals which regulate gene expression.
Mutation or deletion of the CDH1gene coding for human E-cadherin or loss of protein expression are seen amongst a host of human malignancies including breast, colon, lung, stomach and liver [211].

In familial gastric cancer, germ-line mutations in CDH1 alone are sufficient to promote carcinogenesis [255]. In cases where E-cadherin expression appears unchanged, other components of the junctional complex may be affected, in particular mutations in the beta catenin gene are found in colon cancers [256]. Beta catenin is known to act as a transcription factor for pathways promoting growth [257]. Whether these growth pathways are inappropriately activated when junctional complexes are disrupted during carcinogenesis, remains contraversial [258]. Less common mechanisms by which E-cadherin activity is disrupted include chromatin rearrangement, hypermethylation and loss of transcription factor binding [259-261].

Aside from tumourigenesis, E-cadherin is essential for development. Mice deficient for E-cadherin die in utero because of defective development of the trophoectoderm [262]. The developmental process can be affected by the Rho family of GTP ases [263], integrin linked kinase [264], growth factor- receptor signalling [265] and matrix metalloproteases [266]. It has been suggested that misregulation of these processes is also involved in tumourigenesis.

Consistent with the observation that loss of E-cadherin is biologically significant, it has been demonstrated that restoration of E-cadherin function can suppress transformation in a number of cell culture systems [211]. The question remained in the majority of cancers in vivo whether loss of E-cadherin was causal or resulted as a consequence of tumour progression. However an elegant approach using a transgenic mouse model for pancreatic Beta cell carcinogenesis showed that crossing these mice with those that maintain E-cadherin expression, halted the process of tumourigenesis at the adenoma stage [267]. Therefore unravelling the regulation of E- cadherin expression remains a key question. In addition to mutation and post-translational modification, changes in gene expression may affect the phenotypic changes seen in culture and in vivo. For example overexpression of c-erbB2 in mammary epithelial cells inhibits E-cadherin gene expression, which may contribute to erbB2 oncogenic potential [268].

It has also been shown that oestradiol regulates E-cadherin mRNA levels in the ovary [269]. In more general terms the epithelium-specific human papilloma viruses (HPV) 16 and 18 contain several binding sequences for ubiquitous cellular transcription factors, and certain transcription factors such as NF1 show specificity of function with regard to epithelial versus mesenchymal cell types [261]. It has therefore been proposed that a variety of transcription factors could bind the E-cadherin promoter, although studies in this area still limited [261].

In summary, therefore there are several biological features used to define epithelial and mammary differentiation in normal cell lines, as well as assays to define transformation. There are also a number of methods used to transfer genes into epithelial cells, of which retroviral transduction is a useful option. Furthermore, one strategy to revert transformed epithelial cells to a non-transformed phenotype involves expressing the guanine nucleotide exchange factor Tiam1, which appears to act through E-cadherin.

Methods-contents

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Study design

Characterisation of cell lines

To examine the two KIM2 cell lines derived in the Watson lab. termed NKIM2 and TKIM2 respectively. These cells had been previously derived from transgenic mice targeted with a temperature-sensitive ts a58 SV40 large T antigen, at 37°C and 33°C respectively. In order to characterise the cell lines, they were assessed in terms of a number of accepted biological markers at 37°C and 33°C respectively. The extent of epithelial characteristics and mammary differentiation of the cell lines were studied: by the cell morphology under phase and electron microscopy; the presence of other cell types using myoepithelial and fibroblast markers; epithelial marker expression and junctional presence; the presence of polarisation; and milk protein expression. Furthermore, the extent to which cells were transformed was examined: by morphology under phase and electron microscopy; serum starvation; growth in soft agar; injection into scid mice; p53 and T antigen expression, chromosome spread, and FISH analysis of chromosome 11 and chromosome 17.

Retroviral transduction

To use retrovirus to transduce the NKIM2 and TKIM2 cell lines with control and active Tiam1, and confirm the expression in target cells.

Study of transduced cell lines

To the determine the effect of Tiam1 on the four transduced cell lines in terms of the biological markers studied in the characterisation of the cell lines, mentioned above.

Materials

Cells

KIM2 cells were derived by Katrina Gordon from transgenic mice with a fusion gene consisting of 4.2 kb of 5' flanking promoter sequences, including the transcriptional start site, of the ovine beta-lactoglobulin (BLG) gene and the temperature-sensitive variant of SV40 large T antigen (tsA58) [94]. The transgenic mice were derived from microinjection of the fusion gene into pronuclear eggs from C57BL/6 x CBA F1 mice after mating with male studs. The KIM2 cell lines used in this study were derived from mammary gland explants from mid-pregnant transgenic mice, enriched for epithelial cells and derived at the semi-permissive temperature of 37° C and 33 °C respectively. The cell lines were maintained at 37°C and 33°C respectively, with a stable epithelial and spindle morphology (over 60 passages), termed NKIM2 and TKIM2 respectively. Cultures were grown on plastic in maintenance medium: Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL), supplemented with 10% fetal calf serum, 5 μg/ml insulin, 10 ng/ml EGF and 5 μg/ml linoleic acid. Cells were passaged as clumps to help maintain their morphology, and divided 1:3 or 1:4 every 3-4 days.

In order to induce differentiation in KIM2 cells, cells were grown on plastic until confluent. After 2 days at confluency, lactogenic hormone induction media was then added consisting of maintenance medium without EGF, supplemented with 2% dexamethasone and 1% ovine prolactin. Cultures were maintained for up to 12 days with media changes every 2 days.

Retrovirus

Retroviral vectors were kindly supplied by John Collard and were based on the Nolan, Stanford University Phoenix system. The development of these vectors is detailed elsewhere [209]. The episomal retroviral backbone is outlined in Figure 1.
The packaging cell system used in conjunction with the LZRS vector are NIH-3T3 cells [282]as part of the Nolan, Stanford University Phoenix System [209] which produces transient titres of infectious particles in excess of 10^6 infectious units/ml.

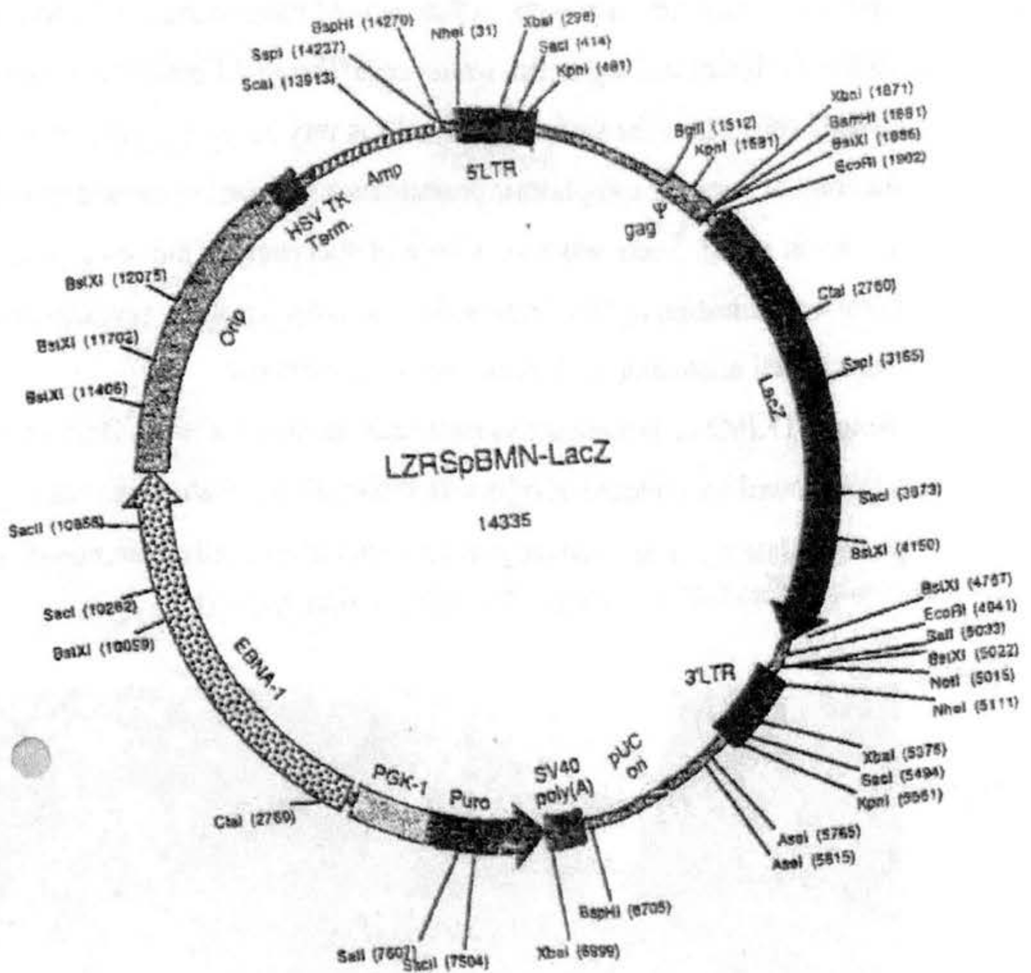

Figure 1 Retroviral vector backbone

The oligo-polylinker for the LZRS-IRES-LacZ/Zeo/Neo constructs, which allows the Tiam1 constructs to be inserted into the retroviral backbone is shown in Figure 2 (for reviews see [280, 281]).

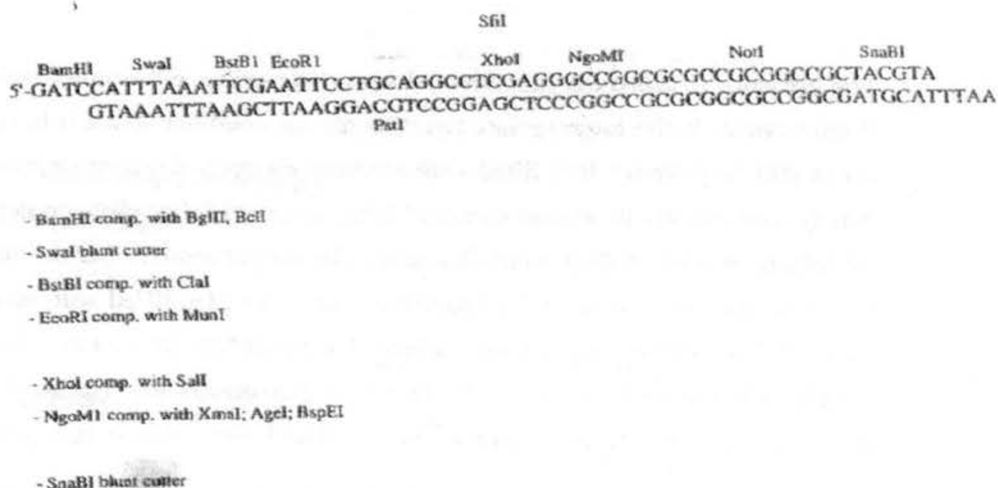

Figure 2 Retroviral Oligo-polylinker for LZRS-IRES-LacZ/Zeo/Neo constructs

Tiam1 constructs

Tiam1 constructs active C1199 Tiam1 and control C580 Tiam1 were kindly provided by John Collard. A schematic representation of the constructs is shown in Figure 3.
A full description of Tiam1 constructs is available [234].

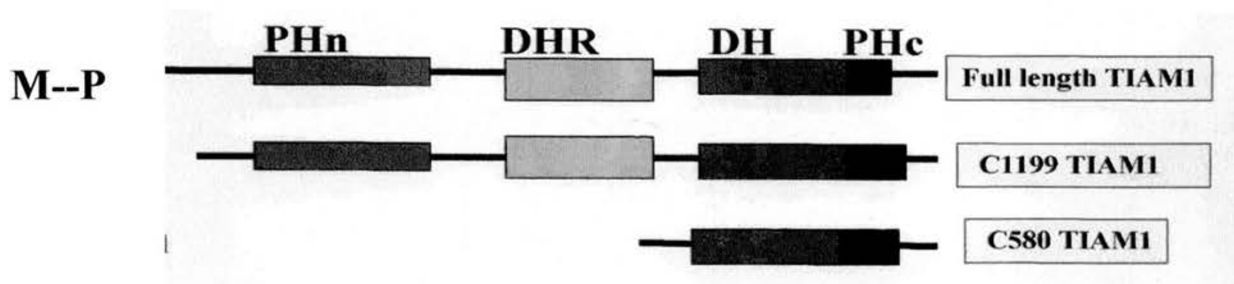

Figure 3 Schematic diagram of active C1199 Tiam1 construct (expected size 160 kD) and C580 control construct (expected size 81 kD)

Antibodies

The antibodies used are listed below, with the relevant dilution, incubation period and supplier. All were monoclonal with the exception of the polyclonal β-casein antibody.

	Dilution	Incubation	Supplier (clone)
primary			
E-cadherin	1:1,600	1hr room temp.	Sigma (DECMA-1)
α- actin(smooth muscle)	1:400	1hr room temp.	Sigma (1A4)
Vimentin	1:800	1hr room temp.	Sigma (VIM 13.2)
β- casein	1:1000	1hr room temp.	Sigma (CAS-1)
SV40 large T antigen	1:400	1hr room temp.	Sigma (Pab-101)
Tiam-1	1:200	1hr room temp.	Santa Cruz (C-16)
ZO-1	1:400	1hr room temp.	Santa Cruz (C-16)
p53	1:400	1hr room temp.	Sigma (BP53-12)
secondary			
fluorescein-conjugated goat anti mouse	1:100	1hr room temp.	Sigma
fluorescein-conjugated goat anti rabbit	1:100	1hr room temp.	Sigma

The fluorescein conjugate for the secondary antibodies is fluorescein isothiocyanate (FITC).

Reagents and commercial suppliers

A list of general reagents and the addresses of the commercial suppliers is given below. Any additional specific reagents are mentioned in context with the relevant supplier.

Reagents	Supplier
Alcohol	Fisons
Agar (Sea Plaque)	Sigma
DMSO (dimethylsulfoxide)	NBS biologicals
Eosin	Sigma
Foetal calf serum	Gibco
Goat serum	Sigma
Haematoxylin	Sigma
HEPES (see below)	Sigma
NaCl	Sigma
NaOH	Sigma
Oligonucleotide	Oswell DNA
PBS (Phosphate buffered Saline)	Sigma
Protease inhibitors	Sigma
TBS (0.05M Tris-buffered Saline pH 7.5)	Sigma
Trypsin (1 in 250)	Difco
Xylene	Fisons

HEPES: N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

Addresses of commercial suppliers:

Becton Dickinson UK Ltd. Between Towns Road. Cowley.Oxford.U.K.

Boehringer Mannheim GmbH. Mannheim. Germany

CAMBIO Drawer 1630, Silverthorne.USA 80498

DAKO Limited. 16 Manor Courtyard, Hughenden Avenue. High Wycombe. Bucks. UK

DIFCO Laboratories, Detroit. Michigan.USA

FISONS Scientific Equipment Ltd.. Bishop Meadow Road. Loughborough. UK

GIBCO Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley,

Pierce 600 Main Street, Bar Harbor, ME, USA.

OSWELL DNA service, University of Southampton, Boldrewood, Southhampton,UK

NBS Biologicals, New Brunswick Scientific (UK) Limited, Edison House, 163 Dixons Hill Road, North Mymms, Hatfield, UK.

SIGMA UK Technical services, Fancy Road, Poole, DORSET, UK

SANTACRUZ, Autogen Bioclear UK ltd, Holly Ditch Farm, Mile Elm, Calne, Wiltshire,UK

Methods

Immunofluorescence

Semi-quantitive detection of antigen expression was carried out by immunohistochemistry. Its main advantage over more quantitative methods such as Western blotting is that the site of expression can be visualised. The methodology used is based on the original description which has been refined to use with an immunofluorescent secondary antibody [284]. Immunofluorescence is more convenient to use on fixed cells in terms of clarity of morphology, as opposed to horse radish peroxidase techniques which are traditionally used on tissue sections. The problem of fading of the immunofluorescent signal is overcome by addition of antifade solutions (Sigma) prior to viewing. Where available all runs included a slide known to be positive, (usually mammary gland). Furthermore all runs included a negative control by omission of the primary antibody. Any run in which the positive control did not stain or the negative control did stain was rejected. Exact controls are described in the appropriate results section and all incubations were carried out at room temperature.

Cells were plated (1 x 10^3 cells) on slide flasks (Nunc) and washed in TBS (0.05M Tris-buffered Saline) for 5 minutes before fixing to remove any floating cells in the medium.They were fixed with methanol: acetone at a ratio of 1:1 for 10 minutes and washed with TBS Ph 7.6 to remove any cell debris created during fixation. Non-specific antibody binding was reduced by 1 hour incubation in TBS + 20% Goat serum. Then cells were incubated with primary antibodies (see Antibodies in Materials section) diluted as appropriate with TBS + 20% Goat serum. Negative controls were incubated with TBS + 20% Goat serum only. After three 5 minute washes in TBS + 20% Goat serum, cells were incubated with fluorescein-labelled secondary antibodies, diluted as appropriate with TBS + 20% Goat serum (see Antibodies in Materials section). After a further three 5 minute washes in TBS + 20% Goat serum only, antifade solution was added to cells for 2 minutes, before the flask portion was detached from the slide flask. After the addition of vector mount and coverslips to the slides, images were analysed by fluorescence microscopy using a Zeiss microscope. Phase and fluorescence images were recorded separately and subsequently merged. Slides were stored at -70°C.

Electron Microscopy

Detailed introductions to the principles and practice of electron microscopy have been reviewed [151, 285]. The technique is useful for examining cell-cell contacts and ultrastructure. The cells to be examined were grown in culture, trypsinised, spun and fixed in 3% glutaraldehyde in 0.1M Sodium Cacodylate/HCL buffer pH 7.2-7.4 at 4 °C for 48 hours. The subsequent processing of electron microscopy sections was carried out by Frank Donnelly (F.D.). After washing with deionised water for 20 minutes, the samples underwent secondary fixation in 1% Osmium Tetroxide in deionised water for 45 minutes at room temperature. Samples were then dehydrated with methylated spirits and absolute ethanol, before linking to propylene oxide for 10 minutes and impregnation in Emix resin (supplied by Fison's, Leicester) overnight at room temperature. After polymerisation for 18-24 hours at 70 °C, 90 nanometer sections were mounted on 300 mesh copper grids and stained using the uranyl acetate/lead citrate method. Finally sections were examined and photographs taken using a Jeol 100CXXII Transmission Electron Microscope. The results were examined in 100 electron microscopic fields.

Cell counting for estimation of growth kinetics

For cell counting a standard hemocytometer was used, consisting of two chambers in which each chamber is ruled into 9 major squares. When coverslipped the volume of each square is 0.1 mm^3 or 1.0×10^{-4} m. Cells were trypsinised and resuspended in maintenance medium. Using pasteur pipettes two aliquots of cell suspension were delivered into each side of the coverslipped haemocytometer by capillary action. The total number of cells in 5 of the 9 large squares in each of the two sides of the haemocytometer were counted. Cells that overlapped two sides of the square were included in the count and not counted on the other two sides. The number of cells in a total of 10 chambers were added to give the number of cells in 1×10^{-3} m. The total number of cells was multiplied by 1000 to give the number of cells per ml in the sample counted. Each growth experiment was repeated on three independent occasions.

Western blot analysis

Western blot analysis to determine the relative levels of protein expression in cell samples was carried out was performed according to standard protocols, based on a description by Laemmli (1970) [325].

Sample preparation

Cells were washed and scraped in 5ml ice cold PBS (phosphate-buffered saline)/ sodium vanadate (ratio of 1:1) in order to prevent protein degradation. The cells were then spun at 1000 rpm for 5 minutes to disrupt cell membranes, the supernatant was aspirated and discarded, and the pellet snap frozen in liquid nitrogen and stored at -70°C until assayed. In order to prepare protein samples, the pellets were defrosted and 200ul RIPA buffer (see below) added, then vortexed until the pellet was dispersed. The DNA in the sample was sheared by passing cells 3 times through a green needle (21G) or smaller and transferring cells to an eppendorf. The cells were spun at 10,000 rpm at 4°C for 15 minutes, the supernatant removed and aliquoted. The protein concentrations of the samples were determined using the BCA Protein Assay Reagent kit (Pierce) so that samples with equal concentrations could be compared on gel electrophoresis.

SDS-PAGE

One dimensional SDS-PAGE (sodium dodecyl sulfate -polyacrylamide gel electrophoresis) in 10-15% polyacryamide gels was performed with a 3% stacking gel [325]. Proteins were transferred from gels to nitrocellulose filters at a current of 0.8mA/cm^2 for 1-3 hours using a transfer kit or a semi-dry electroblotter.

After blocking non-specific binding (endogenous peroxidase) with 1% BSA (bovine serum albumen) in TBS/ Tween (0.1%) overnight, the nitrocellulose filter was exposed to primary antibodies eg primary β- casein, Tiam 1 or E-cadherin at a dilution 1: 5000 in blocking solution, for 45 minutes. Filters were then washed twice for 7 minutes with TBS-T (Tween 0.05%) and incubated with horse radish peroxidase-conjugated secondary diluted 1: 800 with blocking solution for 30 minutes. The filters were further washed with TBS-T for 3 x 5 minutes and incubated with ECL reagents for 1 minute. Filters were then processed in the dark room and bands visualised.

RIPA buffer: 50mM Tris HCl pH 7.5, 150mM NaCl, 1% Nonidet p40, 0.5% sodium deoxycholate 0.1% SDS in water, and stored at 4°C. Immediately before use the protease inhibitors aprotinin 10ug/ml, pepstatin 1uM, leupeptin 1 uM, sodium vanadate 1mM and phenylmethylsulfonylfluoride (PMSF) 1mM were added to the RIPA buffer.

Chromosomal analyses

The isolation of metaphase chromosomes allows both biochemical and morphological analysis.The method used attempts to maintain in vivo chromosome structure without perturbation by using semi-physiological conditions, similar to that described by Lewis and Laemmli 1982 [326].

Preparation of metaphase spreads for chromosome counting and FISH analyses

Cell cultures were fed 24 hours before harvesting to achieve partial synchronisation and then harvested when approximately 50% confluent and thus in log phase growth.
Two hours before harvesting colcemid (Boehringer Mannheim) was added to the culture medium to a final concentration of 0.1 micrograms/ml, in order to arrest cells in mitosis. Cells were then harvested and spun down at 1000 rpm for 5 minutes. The pellets were then gently resuspended in hypotonic 0.075M KCl to promote cell swelling and incubated for four minutes at room temperature. Cells were re-centrifuged and gently resuspended in a single drop of supernatant before fixing by the addition of 10ml of ice-cold fix (60% methanol, 40% glacial acetic acid). The cell suspension was left on ice for 30 minutes, centrifuged and resuspended in 5ml of fresh fix to assist the dissociation of mitotic complexes. After five minutes on ice, the cell suspension was spun again and resuspended in a smaller volume of fix (depending on the cell density).
Glass slides were prepared by washing in 70% ethanol, rinsing thoroughly in tap water and storing in double distilled water (DDW). Three drops of the cell suspension was dropped onto each slide from a height of approximately 40cm, and the slides air dried. Slides were stored in a dessicator and the metaphase spreads viewed with a light microscope within one month.

Chromosome counts

For chromosome counts, slides were stained in 5% Giemsa prepared in PBS. Twenty metaphase spreads were selected at random and the number of chromosomes counted in each spread, and counts were repeated three times on each slide.

FISH analysis

FISH (fluorescence in situ hybridisation) analyses were performed using a biotinylated murine chromosome 11 and 17 paint (Cambio) [134]. Chromosome paints often contain repeat elements that can be used to detect clustered repetitive DNA in heterochromatin blocks and centromeric regions of individual chromosomes, and therefore are particularly useful in determining numerical chromosome aberrations.

Specimen preparation

Glass slides with metaphase spreads were warmed at 60°C for 30 minutes and denatured in 70% formamide at 65 °C for two minutes, before being dehydrated through ice cold 70 %, 90% and 100% ethanol for 2 minutes each. Then the slides were air dried, prewarmed to 42°C and briefly vortexed.

Paint preparation

The chromosome paint was denatured at 70°C for 8 minutes and pre-annealed at 37°C for 15 minutes. Whilst the paint was pre-annealing, coverslips were prewarmed to 37°C. 15 μl of the paint was pipetted onto each coverslip.

Hybridisation

The coverslips were lowered onto the slides with metaphase spreads and sealed with rubber solution. Slides were then incubated overight at 37°C. The rubber seal was removed from the coverslip and the coverslip allowed to fall off in the first wash. Slides were washed 4 times, for 3 minutes each in 2x standard sodium citrate solution (SSC), 50% formamide at 45°C. Then a similar wash was performed in 2x SSC only, before slides were transferred to 4x SSC, 0.1% Tween 20.

Detection

Freshly prepared, 40 μl aliquots of avidin FITC, biotinylated anti-avidin and blocking buffer were spun for 15 minutes at 4 °C to remove antibody conjugates. Each specimen slide was incubated for 5 minutes, at room temperature, with 40 μl of blocking buffer. The blocking buffer was replaced with 40 μl of avidin FITC, and slides incubated for 30 minutes at 37°C. Then slides were washed 3 times, for 2 minutes each in 4x SSC, 0.1% Tween 20 at 45 °C, before incubation with 40 μl of biotinylated anti-avidin for 30 minutes, at 37°C. The wash process was repeated, and the slide incubated for a further 30 minutes with 40 μl of avidin FITC. Finally, the slides were washed and mounted with 40μl of vectormount, for viewing by fluorescence microscopy.

Retroviral transduction

The retroviral system used was the Phoenix system [209] which had been modified and produced by R.K. at the Netherlands Cancer Institute. Transduction of the Tiam1 cells was carried out with substantial help from and under the supervision of R.K .
Packaging cells were passaged in hygromycin and diptheria toxin one week before transduction and sorted by FACS for expression of CD8 and surface expression of envelope protein with 83A25 antibody, to ensure that plasmids had not undergone recombination (see Introduction). The whole transduction procedure took 4 days.

Day 1 Packaging cells were plated in producer growth media containing DMEM, 10% FCS, 1% Penecillin-Streptomycin, 1% Glutamine. Cells were maintained at subconfluence because confluency reduces their transfection efficiency.
Day 2 Packaging cells were transfected with the Tiam1 DNA constructs using calcium phosphate precipitation:-

Preparation of DNA

Firstly, DNA was prepared in HBSS (Hank's balanced salt solution) immediately before transfection: 5-10 μg DNA was added in a dropwise fashion to a 15 ml tube at room temperature. 438 μl of double distilled water was added to wash the DNA to the bottom of the tube and 61 μl 2M $CaCl_2$ was added and mixed thoroughly by finger tapping. Then 0.5ml of 2x HBSS (see below) was added quickly and bubbled vigourously with an automatic pipettor for 3-15 secs.

Transfection of DNA into packaging cell line

Chloroquine was added to the Phoenix packaging cell line 5 minutes before transfection to inhibit lysosomal DNAases, by neutralizing vesicle pH.
The HBSS/DNA solution was then added dropwise onto the media gently and quickly. After adding the HBSS/DNA to the media very small black particles were visible under the light microscope. The plate was put in a 37°C incubator and rocked a few times to ensure even distribution of the DNA/CaPO4 particles.

2x HBSS: 8.0g NaCl, 6.5g HEPES (see Reagents) and 10ml Na_2HPO4 stock solution (5.25g in 500ml water). The pH of the 2 x HBSS was adjusted to 7.0 using NaOH or HCl and the volume adjusted to 500mls.

Day 3 Target cells were infected with the retroviral virons containing Tiam1 constructs. The target cells had been prepared so that they were subconfluent, in a dividing state. The supernatant from transfected packaging cells containing virions was pipetted into a 15ml tube and centrifuged at 1500 rpm for 5 minutes to pellet cell debris.

1ml of media was removed from each target cell plate and 3 μl of polybrene (100x at 5 mg/ml) added to each plate to assist uptake of the virions. 1ml of viral supernatant was then added to each plate at 37°C with gentle shaking.

Day 4 24 hours post infection the media was removed and fresh DMEM and 10% fetal calf serum was placed on the target cells at 37°C.

Day 5 The cells were assessed for morphological change, 24-48 hours post infection because the actual process of reverse transcription and integration takes place within 24-36 hours, depending on cell growth kinetics.

Anchorage-independent growth

The principle of the assay was to suspend the cells in soft agar and monitor their growth in suspension [180]. This involved suspending a top layer of agar containing cells on a bottom layer of agar only, to prevent cells obtaining sites for anchorage. A cell suspension was prepared at 10^4-10^5 cells per ml in media, and kept at room temperature. The bottom layer of agar was prepared by adding 1.5% agar to double distilled water (100ml total voume) at 44°C in a water bath for 30 minutes. Then 33ml of the heated agar was added to a 100 ml medium mix (50ml 2x maintenance medium, 10ml 10 % fetal calf serum and 7 ml double distilled water). The solution was mixed well and 15mls poured into 6cm glass petri dishes, and allowed to set at room temperature for 30 minutes.

The top layer of agar was prepared by adding agar to the cell suspension at 2:1. 1.5ml of this was poured onto the set bottom layer in the petri dishes. The dishes were fed with maintenance medium (2ml) every two days. Colonies were counted using after 7 to 14 days when they were approximately 5 mm in diameter.

Injection of scid mice

These studies were carried out with the help and guidance of Alison Ritchie.

Matrigel

Matrigel had been previously thawed overnight at 4° C, aliquotted (250µl) on ice into precooled microcentrifuge tubes and then frozen at -20°C until ready for use [187]. All procedures involving Matrigel were carried out on ice to prevent polymerisation.

Cells

KIM2 cells had been grown in maintenance medium in $75cm^2$ flasks until near confluence. The medium was removed and cells were washed in PBS and cells were harvested using trypsin (5ml). The cell suspension was transferred to a universal container and 20ml DMEM /10% fetal calf serum was added to deactivate the trypsin. The cells were then centrifuged at 2,500 r.p.m. for 5 minutes to obtain a cell pellet and the supernatant was discarded. The cell pellet was then resuspended in DMEM minus fetal calf serum and the cell number counted on a haemocytometer. The cell number was adjusted to 5×10^7/ ml. Cells were then resuspended in 250µl DMEM (minus fetal calf serum) chilled on ice, and made up to 500µl with Matrigel before being injected.

The cell suspension (100 µl) was injected subcutaneously into both flanks of a female scid mouse (Dept. Oncology) at 4-6 weeks of age. All mice were regularly examined for tumour development for atleast 4-5 months following the injection.

Results

1 Epithelial characteristics and mammary differentiation

1.1 Morphology
1.2 Myoepithelial and fibroblast marker expression
1.3 Serum starvation of NKIM2
1.4 Electron microscopy of myopithelial filaments
1.5 Epithelial marker expression
1.6 Electron microscopy of tight and adherens junctions
1.7 Polarisation
1.8 Milk protein marker expression
1.9 Western analysis of milk protein expression
1.10 Electron microscopy of differentiated NKIM2
1.11 Discussion

2 Features of transformation

2.1 Morphology
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1 Epithelial Differentiation

This section reports the data obtained by cell culture of original KIM2 cell lines before genetic manipulation, in order to determine the epithelial and mammary characteristics of the lines. Most of the data refers to normal KIM2 cells (NKIM2) derived and grown at 37°C, and NKIM2 cells stimulated to show mammary differentiation with lactogenic hormones (NKIM2-diff). The data referring to transformed KIM2 cells (TKIM2), derived and grown at 33°C are shown in more detail in section 2 (Transformation). All the data presented are consistent from passage 10 through to 30. For ease of description the cell lines will be referred to using the abbreviations outlined in Table 1.

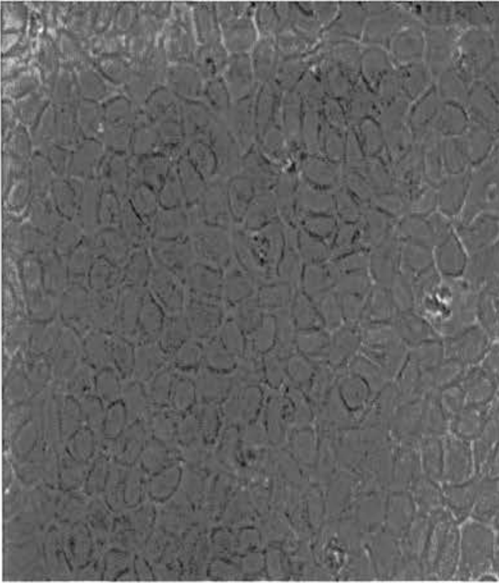
Abbreviation	Cell line
NKIM2	Normal KIM2 cell line Grown at 37°C in maintenance medium only
NKIM2-diff	Normal KIM2 cell line stimulated to show mammary differentiation in terms of milk protein production. Derived by growing NKIM2 cells 2 days at confluency in maintenance medium, and then exposing cells to differentiation medium containing lactogenic hormones (at 37°C)
TKIM2	Transformed KIM2 cell line Grown at 33°C in maintenance medium only

Table 1 Cell line Abbreviations and Terminology

1.1 Morphology on phase microscopy

Confluent NKIM2 showed a cobblestone epithelial morphology whereas TKIM2 were spindle-shaped and showed overlapping of cells. Figure 1.1 illustrates the morphological differences between the two cell lines. The TKIM2 cells are pictured semi-confluent and at a higher magnification than NKIM2, because the TKIM2 cells were multilayered when fully confluent and therefore poorly resolved by phase microscopy. A formal comparison of the growth kinetics of NKIM2 and TKIM2 is shown in section 2 (Transformation).

NKIM2



TKIM2

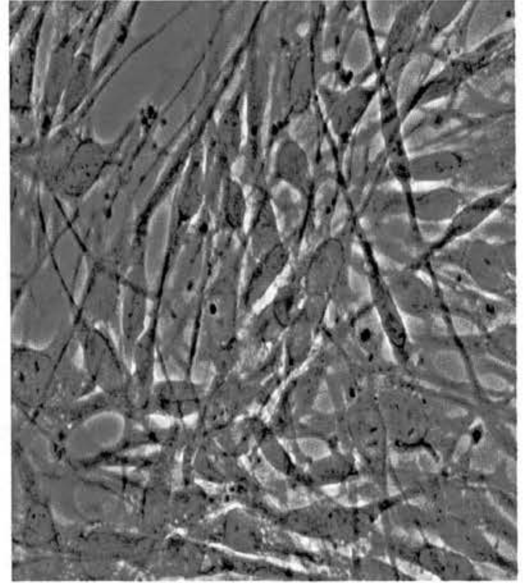


Figure 1.1 Morphological comparison of a, normal (NKIM2) (x10) and b, transformed KIM2 (TKIM2) (x25) by phase microscopy

The morphology of NKIM2-diff is illustrated in Figure 1.1.1, showing the presence of a dome-like structure surrounded by a confluent monolayer. Each confluent T25 flask of NKIM-2 diff cells contained approximately 10-20 domes of differing size and random distribution. Analysis of 20 flasks showed that the mean time to develop domes after the addition of differentiation medium was 12 days with a range of (10-14 days). Control NKIM2 cells left in maintenance medium only for the same period did not form domes.

Examination of the dome-like structures in NKIM2-diff showed that many were associated with cells with spindle-shaped, elongated cell processes, which were different in appearance from the remaining epithelial cells. Two of these spindle cells are seen in Figure 1.1.1 This raised the possibility that another cell type was present among the NKIM2 cultures.

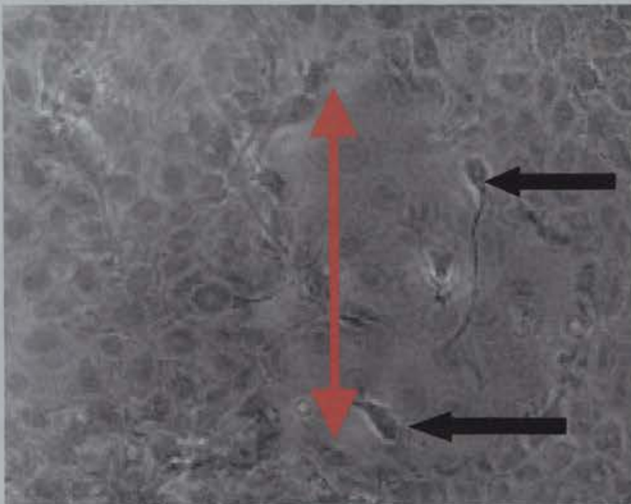


Figure 1.1.1 NKIM2-diff morphology on phase microscopy (x 40)
The red arrow spans a dome-like structure and the black arrows identify two cells with spindle-shaped cell processes

1.2 α-actin and vimentin expression by immunofluorescence

In order to investigate the possibility of an additional cell type among KIM2 cells, immunofluorescence experiments were performed using α-actin and vimentin, which are recognised markers for myoepithelial and fibroblast cells respectively.
α-actin-positive cells were identified in NKIM2 at a low frequency (< 1%), after four repeat immunofluorescence experiments. Figure 1.2 illustrates α-actin-positive cells amongst the cobblestone NKIM2 monolayer. In order to generate the image, the green immunofluorescent signal is superimposed on the phase microscopy background. In addition, a computer-generated red background has been added to enhance the appearance of the green immunofluorescent signal. The disappearance and reappearance of the signal along the length of the cell processes suggested that the cell processes were woven above and below the confluent monolayer.

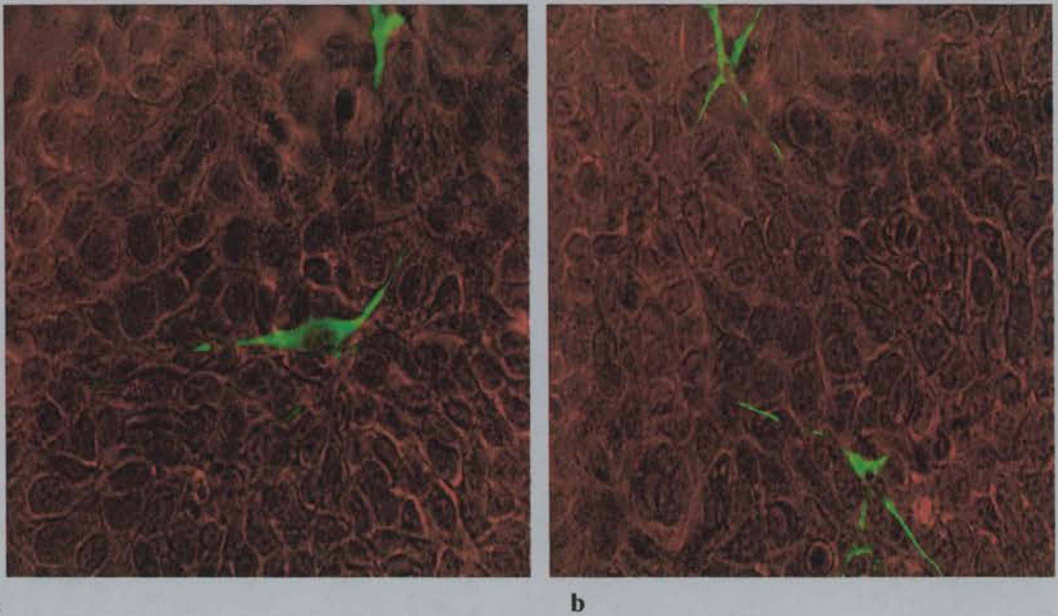


Figure 1.2 α-actin-positive myoepithelial cells in NKIM2 (x 40)

In contrast, no vimentin-positive cells were identified among NKIM2 cells, in the presence of a fibroblast positive control for the vimentin antibody. A negative result on immunofluorescence consisted of a black field only, similar to the background seen in Figure 1.2.2, and therefore will not be illustrated.

Similar to NKIM2 cells, NKIM2-diff contained α-actin-positive cells at a frequency of <1%, after 4 repeat experiments. Figure 1.2.1 a illustrates an α-actin-positive cell in NKIM2-diff associated with a dome-like structure. Examination of this dome at different focal planes revealed that all the other spindle- shaped cell processes seen in the background are also α-actin-positive. Figure 1.2.1 b demonstrates that a neighbouring cell process shows a clear green immunofluorescent signal in a different focal plane.

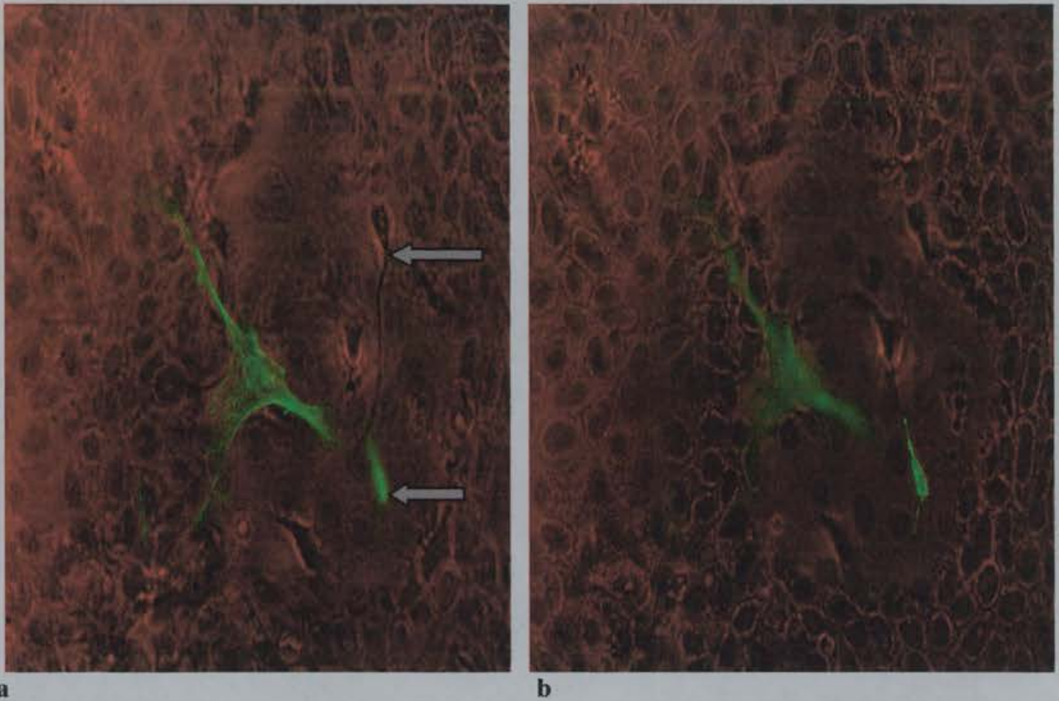
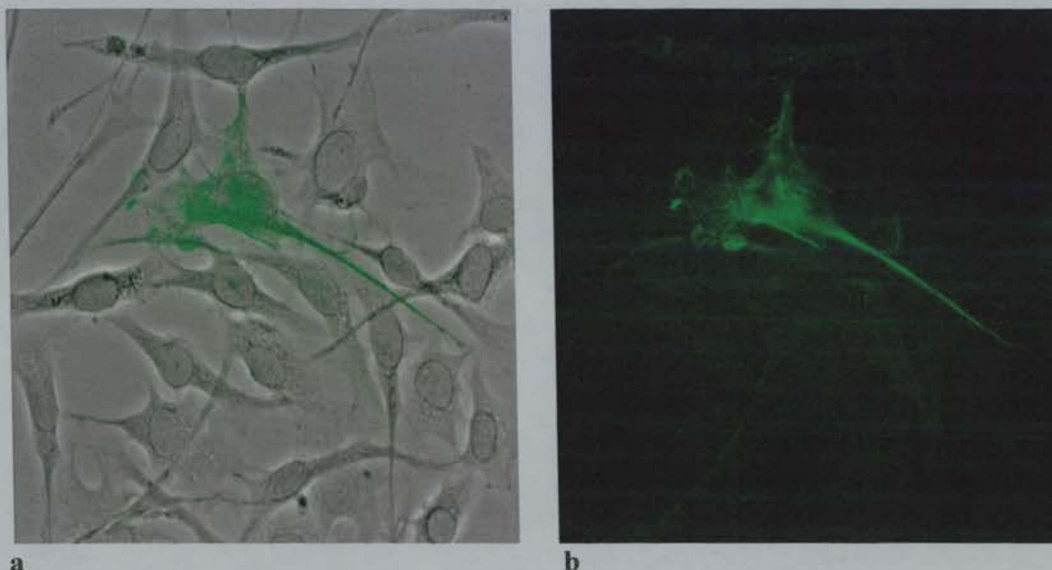


Figure 1.2.1 a, α- actin-positive myoepithelial cell in NKIM2-diff (x 40) b, different focal plane on the dome. The grey arrows indicate background spindle-shaped cells which immunofluoresce in different focal planes.

There was no evidence of vimentin-positive cells among NKIM2-diff on repeat immunofluorescence, in the presence of a positive control for the vimentin antibody.

α-actin-positive cells were present at a very low frequency in TKIM2 cells (< 0.1%) compared with NKIM2 and NKIM2-diff cells. Figure 1.2.2 a illustrates the only α actin positive cell identified among TKIM2 cells after four repeat experiments, superimposed on the phase microscopy background, and b illustrates the corresponding raw data signal on a black negative background.



a b

Figure 1.2.2 α-actin expression in TKIM2 cells (x40) a, superimposed on phase microscopy background, b, raw data

The TKIM2 cells were vimentin-negative after experiments with positive controls, similar to the NKIM2 and NKIM2-diff cells. A summary of the α-actin and vimentin expression data is presented in Table 1.2.3. There was no significant difference in the α-actin staining between the NKIM2, NKIM2-diff and TKIM2 lines.

Cell line	α-actin (myoepithelial marker)	vimentin (fibroblast marker)
NKIM2	9 cells per 1000 positive (< 1%)	vimentin-negative
NKIM2-diff	9 cells per 1000 positive (<1%)	vimentin-negative
TKIM2	1 cell per 1000 positive (<0.1%)	vimentin-negative

Table 1.2.3 α-actin and vimentin expression in cell lines

1.3 Serum starvation of NKIM2

In order to assess the growth kinetics of NKIM2 cells in response to serum deprivation, a recognised protocol was followed [69].NKIM2 cells were seeded at the same density (1x 10^5 cells) in maintenance medium for 24 hours, then washed with serum-free medium before being seeded in either control maintenance medium (containing 10% fetal calf serum) or maintenance medium with 0% serum. At 24 hour intervals culture plates within each series were harvested and the cells counted, and medium replaced in remaining plates. Figure 1.3 illustrates the growth curves (mean and standard deviation) of NKIM2 cells from three independent experiments. It can be seen that after 5 days of serum starvation the majority of NKIM2 cells appear to die with only ~1x10^2 cells surviving, whereas control NKIM2 cells continued to proliferate. However this assay did not assess rates of apoptosis or necrosis, which may contribute to differences in cell number.

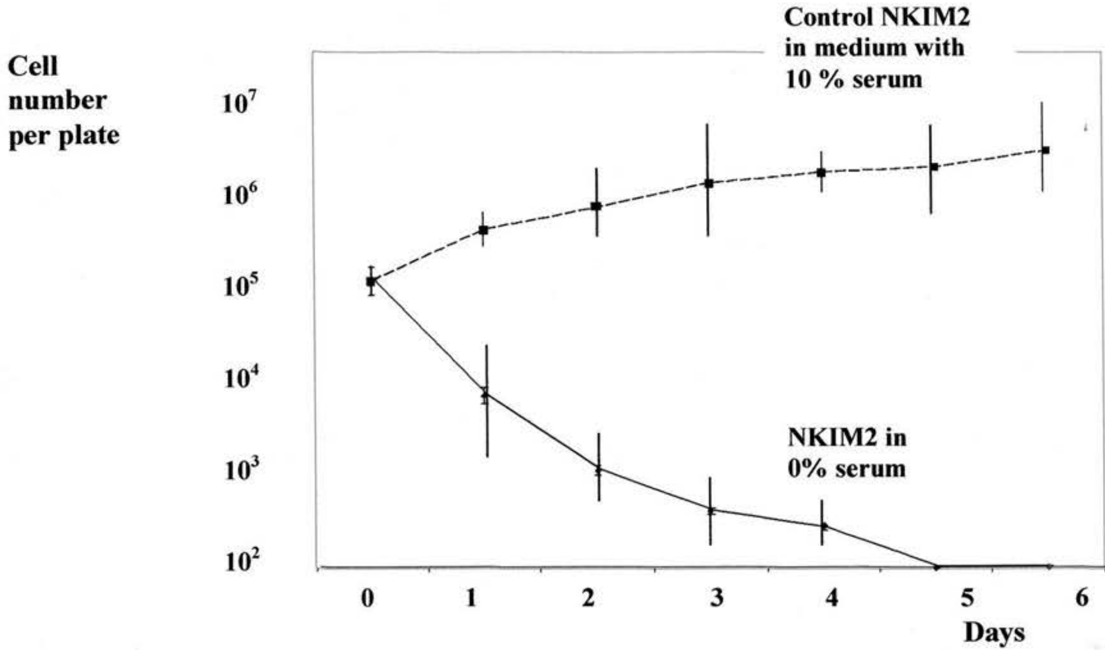


Figure 1.3 showing the growth curve (mean and standard deviation from 3 experiments) of NKIM2 cells after serum starvation. 1x 10^5 cells were plated at Day 0 and after 5 days, ~ 1x10^2 cells survive

Myoepithelial cells are thought to be resistant to stress than epithelial cells [95], therefore immunofluorescence for α-actin was performed on the NKIM2 cells remaining after 5 days serum starvation. Figure 1.3.1 illustrates two α-actin-positive myoepithelial cells seen among surviving NKIM2 cells. Above the myoepithelial cells a rounded cell is seen which is either in the process of dividing or dying.

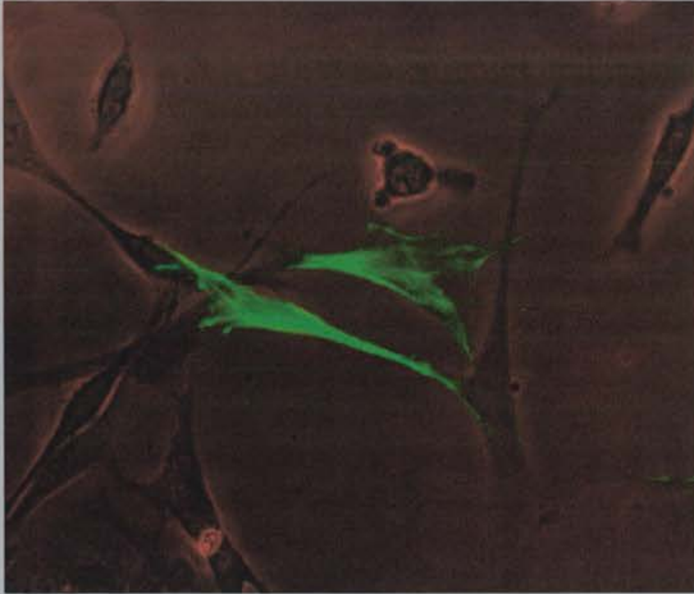


Figure 1.3.1 Serum starvation of NKIM2 for 5 days and α-actin immunofluorescence of surviving cells. (x40)

1.4 Electron microscopy of myoepithelial cells

Electron microscopy of the NKIM2 cells allowed identification of the α-actin-positive cells as myoepithelial, due to the presence of characteristic myofilaments. Examination of 100 fields of NKIM2 cells confirmed < 1% of cells were myoepithelial, corresponding to the proportion detected on the immunofluorescence data. Figure 1.4 illustrates a myoepithelial cell at lower power with the characteristic myofilaments highlighted with a red arrow.

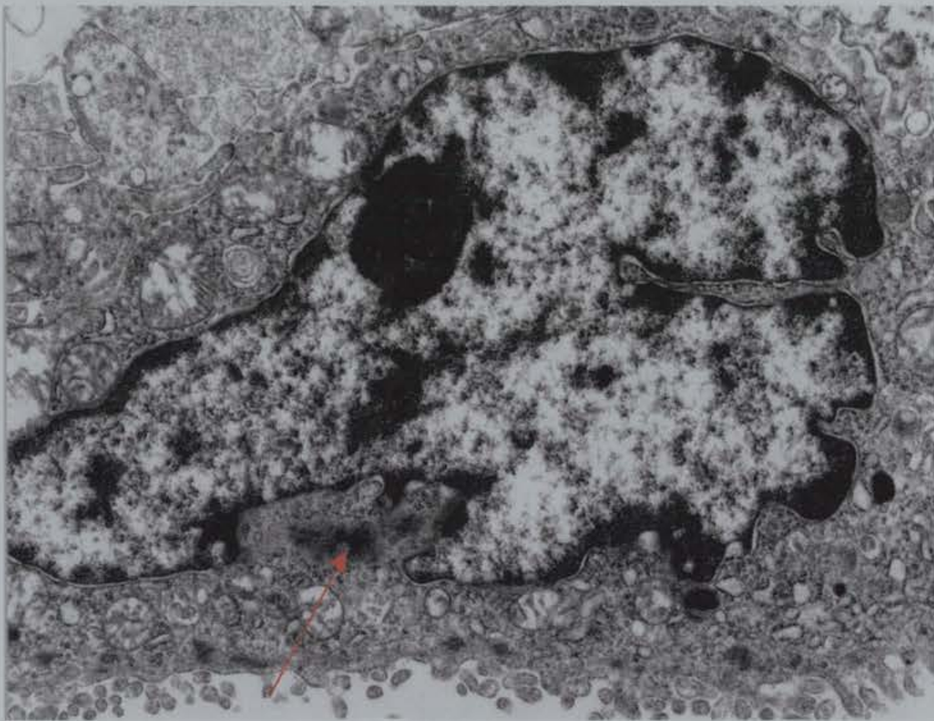


Figure 1.4 Morphology of a myoepithelial cell identified among NKIM2 cells on electron microscopy (neg.mag x6750, print mag.x13500). The red arrow highlights an area of cytoplasmic filaments which is depicted at higher power in 1.4.1

Figure 1.4.1 illustrates the myofilaments at higher power, showing the characteristic electron densities along their length which are indicative of actin-myosin assemblies at a molecular level.

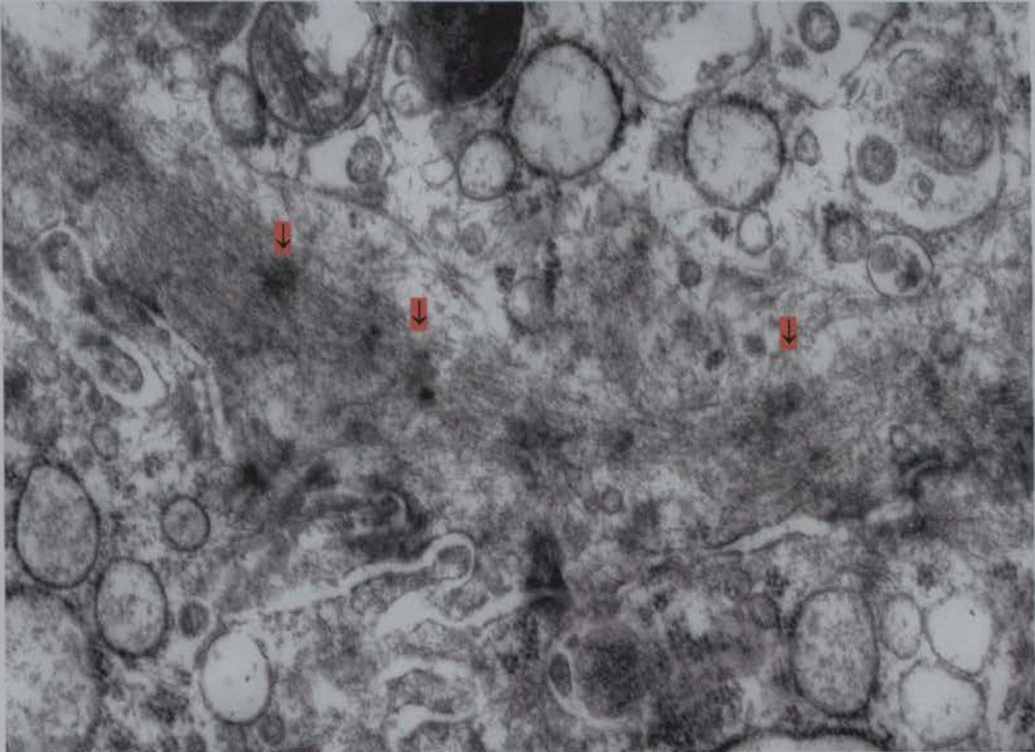


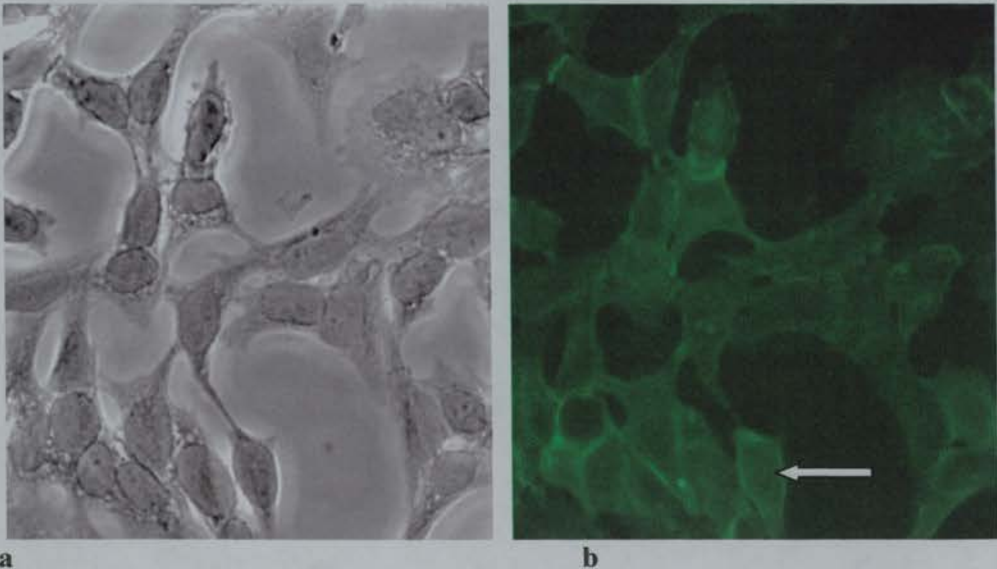
Figure 1.4.1 Higher power view of myofilaments (neg. mag. x19799, print mag. x39598). The red arrows indicate the characteristic electron densities along their length

1.5 Epithelial marker expression : E-cadherin and ZO-1

E-cadherin and ZO-1 are recognised markers for adherens and tight junctions respectively, which are unique to epithelia [153].

E-cadherin was identified at areas of cell-cell contact in NKIM2 in both pre-confluent and confluent cells, on four repeat experiments. Figure 1.5 a,b illustrates E-cadherin expression occurring before NKIM2 cells reach confluency, at individual cell borders. Growing cells were assessed for E-cadherin localisation every 12 hours in order to determine when best to assess the lines for this marker, and the earliest expression of E-cadherin was identified at 48 hours after splitting NKIM2 cells. Although in general expression was identified at areas of cell contact, occasional cells lacking cell contact expressed E-cadherin at their cell borders, as illustrated in Figure 1.5 b.

One possible explanation identified on time-lapse video microscopy of growing KIM2 cells, is that cells form and lose temporary attachments during growth before they reach confluence. Therefore the lone cell that expresses E-cadherin at its borders may have recently lost contact with neighbouring cells.



a b

Figure 1.5 a, Phase contrast image and b, E-cadherin expression in growing NKIM2 cells (x40) The white arrow indicates E-cadherin expression at the boundaries of a cells without contact with neighbouring cells

Figure 1.5.1 illustrates the immunofluorescent E-cadherin signal between all NKIM2 cell boundaries in a confluent culture, highlighting the cobblestone arrangement of cells.

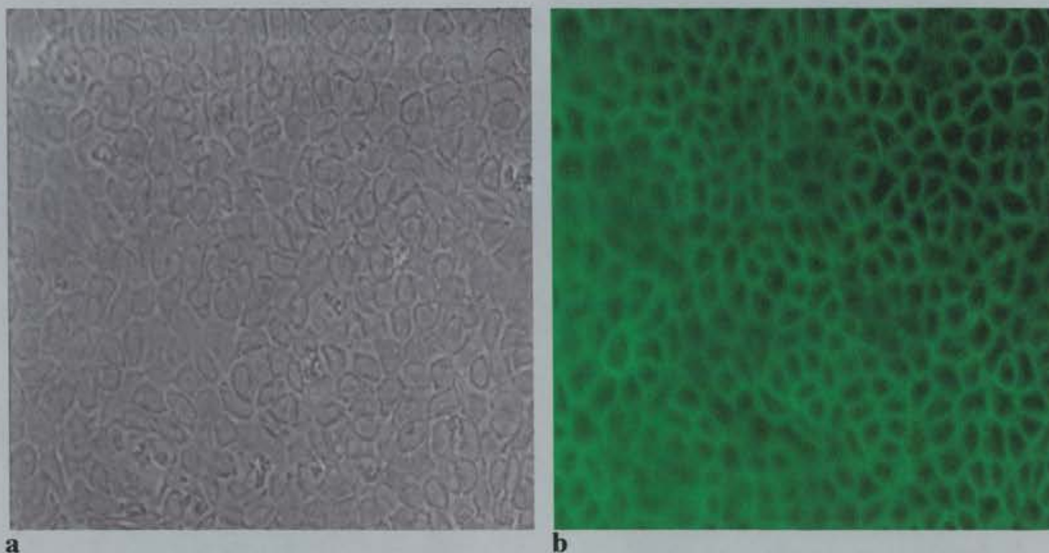


Figure 1.5.1 a, Phase contrast image and b, E-cadherin expression in NKIM2 cells (x25)

Examination of confluent NKIM2-diff cells similarly confirmed E-cadherin expression at cell boundaries. Figure 1.5.2 illustrates E-cadherin expression in NKIM2-diff cells with a dome-like structure surrounded by a flat monolayer. Study of the dome in different focal planes confirmed that the immunofluorescence signal was continuous between cells on the monolayer and the dome.

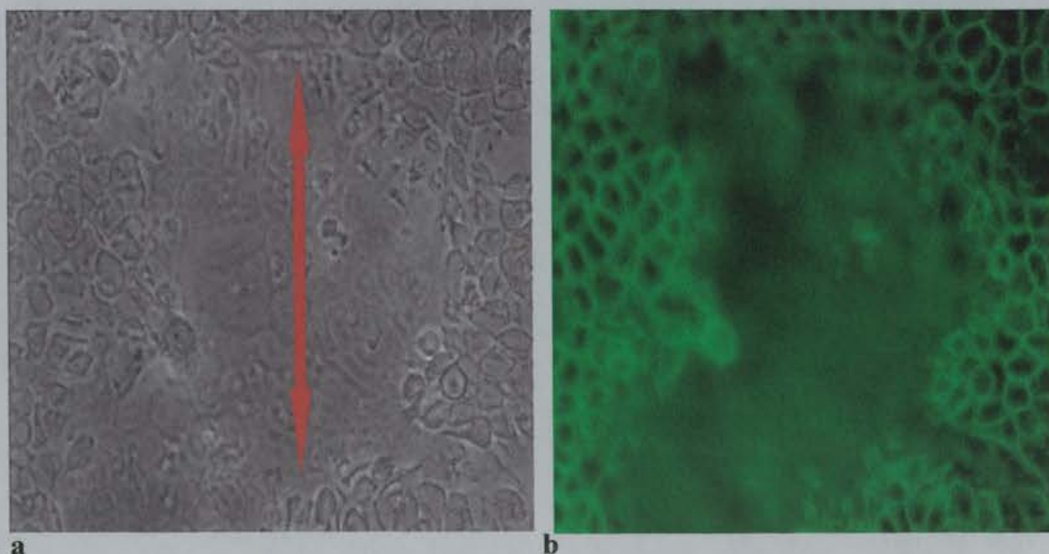
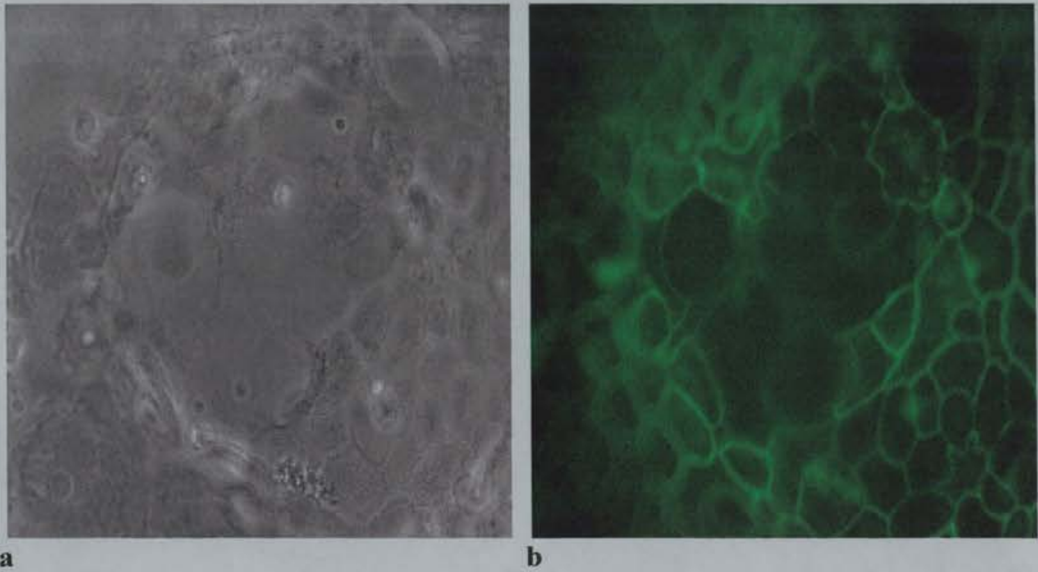


Figure 1.5.2 a, Phase microscopy image and b, E-cadherin expression in NKIM2-diff cells (x25). The red arrow spans the dome-like structure.

The ZO-1 expression pattern on repeat immunofluorescence, was identical in appearance to the E-cadherin expression in both growing and confluent NKIM2 cells and is summarised in Table 1.5.3 below. Figure 1.5.3 demonstrates ZO-1 expression in NKIM2-diff cells at the boundaries between cells in a flat monolayer surrounding a dome structure. When viewed in different focal planes, ZO-1 expression is continuous between all cells forming the dome, similar to E-cadherin.



a **b**

Figure 1.5.3 a Phase contrast image and, b ZO-1 expression in NKIM2-diff cells (x 40)

Epithelial marker expression in the NKIM2 cells is summarised in Table 1.5.3.

Cell line	E-cadherin	ZO-1
Growing NKIM2	expression occurs after cells meet approximately 48 hours after splitting cells	expression occurs after cells meet approximately 48 hours after splitting cells
Confluent NKIM2	present between all cell boundaries	present between all cell boundaries
NKIM2-diff	present between all cells including dome structures	present between all cells including dome structures

Table 1.5.3 Epithelial marker expression in NKIM2

1.6 Electron microscopy of tight and adherens junctions in NKIM2 cells

Both tight and adherens junctions were confirmed in NKIM2 cells by electron microscopy. The presence of tight junctions correlated with the ZO-1 expression seen by immunofluorescence. Figure 1.6 illustrates a tight junction between two NKIM2 cells, which is denoted by the red arrow. The junction is the electron dense area situated apically, where the outer leaflets of the apposing cell membranes are fused becoming a single leaflet. The cell coat or glycocalyx is characteristically absent in this area. Also present are apically arranged microvilli forming a continuous surface from one cell to the next, which is the defining feature of polarised cells on electron microscopy.

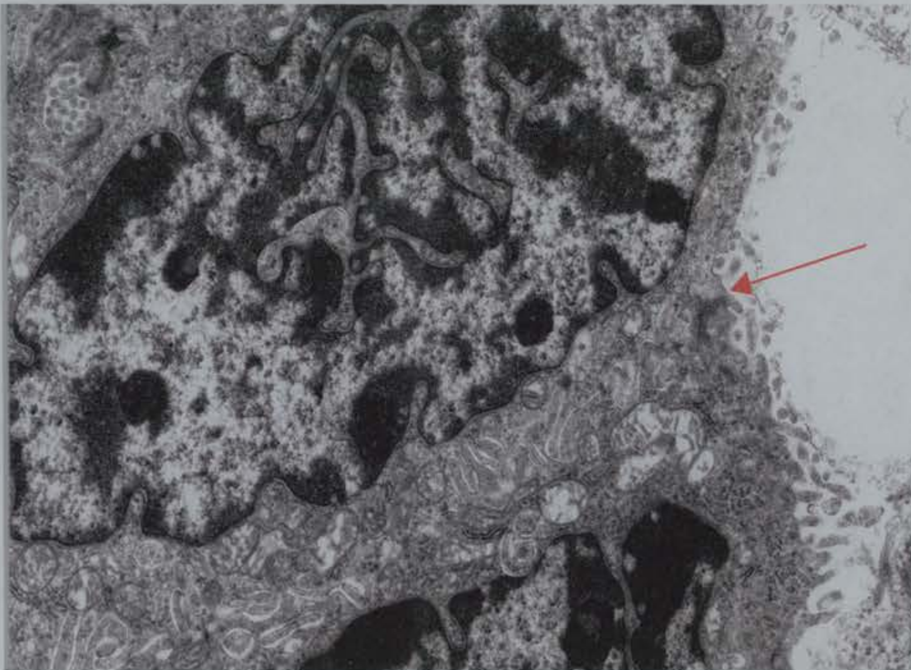


Figure 1.6 Electron microscopy demonstrating a tight junction (red arrow) between two neighbouring NKIM2 epithelial cells (neg. mag. x 6750, print mag. x 13500). On the apical surface, microvilli are seen forming a continuous layer between cells.

The identification of adherens junctions between NKIM2 cells correlated with E-cadherin expression seen by immunofluorescence.The adherens junctions identified were either intermediate junctions or desmosomes. Intermediate junctions were identified as areas between cells where the intercellular space was larger than normal (about 200 Angstroms (A)) filled with medium electron-dense amorphous material, and the adjacent cytoplasm was filled with minute filaments.
The desmosome or hemidesmosome were identified as button-like structures with an intercellular space of about 250 A, also filled with electron-dense material. However in contrast to the intermediate junction,

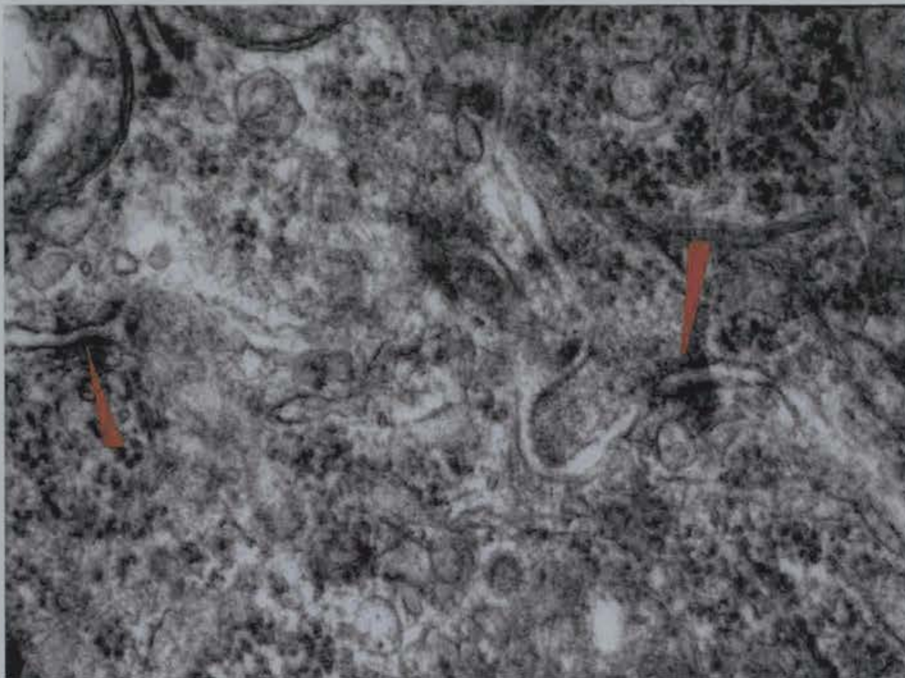


Figure 1.6.1 Two desmosomes between NKIM2 cells on electron microscopy (neg. mag. x 41580, print mag. x 83160)

the paradesmosomal cytoplasm contained plate-like structures to offer anchorage for cytoplasmic filaments. Figure 1.6.1 illustrates two desmosomes between two NKIM2 cells, denoted by the red pointers. The border between the two cells is not continuous because it has been disrupted during preparation of the block for electron microscopy.

Interdigitation of NKIM2 cells

As well as identifying tight and adherens junctions, an additional feature was identified on electron microscopy between NKIM2 cells which is known as interdigitation, ie the cytoplasm of both cells was very closely apposed in finger-like projections to increase the surface area for communication between the cells. Figure 1.6.2 illustrates interdigitation between the cytoplasm of neighbouring NKIM2 cells.

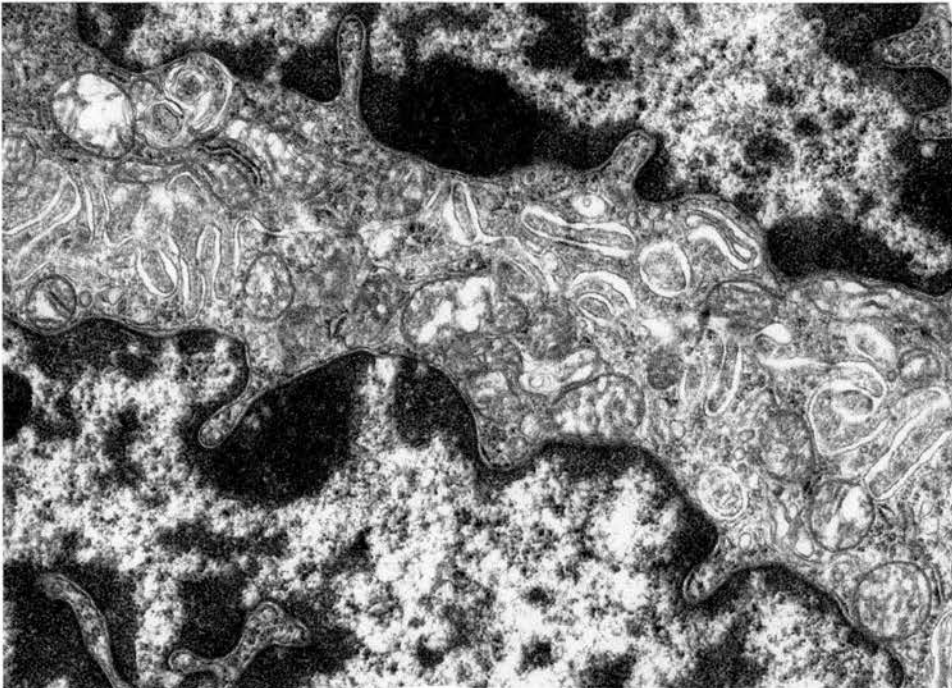


Figure 1.6.2 Neighbouring NKIM2 cells show interdigitation of their cytoplasm on electron microscopy (neg.mag. x10368, print mag. x20736)

1.7 Polarisation

Examination of NKIM2 cells by electron microscopy demonstrated apically arranged microvilli which formed a continuous surface between neighbouring cells, thus confirming cells were polarised. This is demonstrated at low power in Figure 1.7 where the microvilli are continuous between the central cells. The remaining peripheral edges of cells in the figure do not show this feature because their relation to one another has been disrupted during the cell-block preparation process. The continuity of microvilli between cells at higher power was illustrated previously in Figure 1.6 which also illustrated tight junctions between NKIM2 cells.

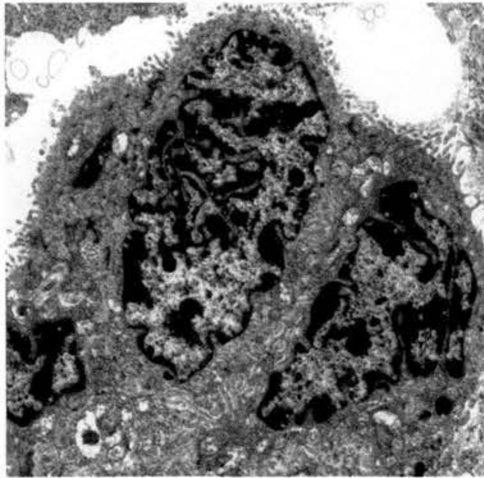


Figure 1.7 Polarisation of NKIM2 cells demonstrated by the presence of apical microvilli running continuously between the centrally placed cells (neg.mag. x2777, print mag. x5554)

1.8 Milk protein expression

The potential for mammary differentiation in NKIM2 cells was assessed in terms of the milk protein β-casein production, in response to the lactogenic hormones present in differentiation medium. Figure 1.8 illustrates the immunofluorescent staining pattern of the polyclonal β-casein antibody on a control histological section of late lactation /early involution mammary gland. Although the β-casein antibody stains alveoli and milk-filled ducts, there appears to be some non-specific staining on fat cells and vessels, which may be due to cross reactivity of the antibody or milk protein present on these cells.

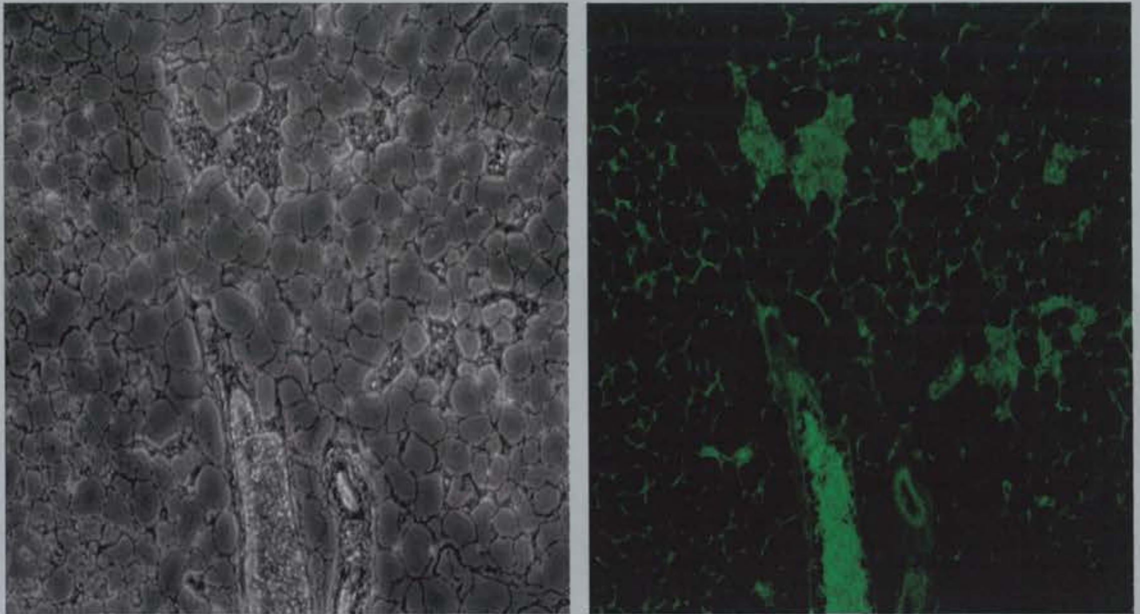
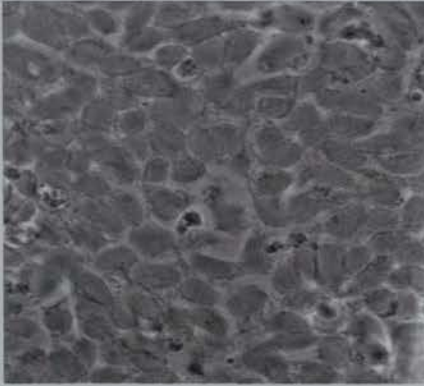


Figure 1.8 β- casein immunofluorescence in a histological section of control mammary gland (x 10) Apart from staining alveoli, some staining of fat cells is seen

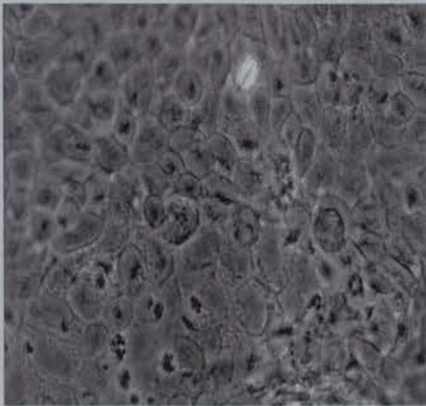
Despite this reservation regarding the β-casein antibody, immunofluorescence experiments were carried out because the cell lines did not contain fat cells. NKIM2 cells had very low levels of β- casein expression on four repeat experiments, whereas NKIM2-diff had high levels of expression.

Figure 1.8.1 illustrates the difference in β-casein expression by immunofluorescence in NKIM2 cells compared to NKIM2-diff cells. The undifferentiated NKIM2 cells in Figure 1.8.1 a,b show low level cytoplasmic speckling, whereas differentiated NKIM2-diff cells in c,d have strong cytoplasmic staining. Moreover, it is clear that β-casein expression does not occur simultaneously in every NKIM2-diff cell.

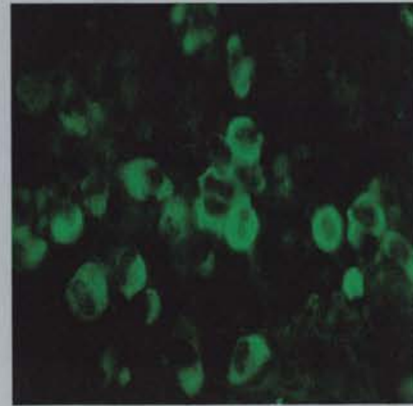


a

b



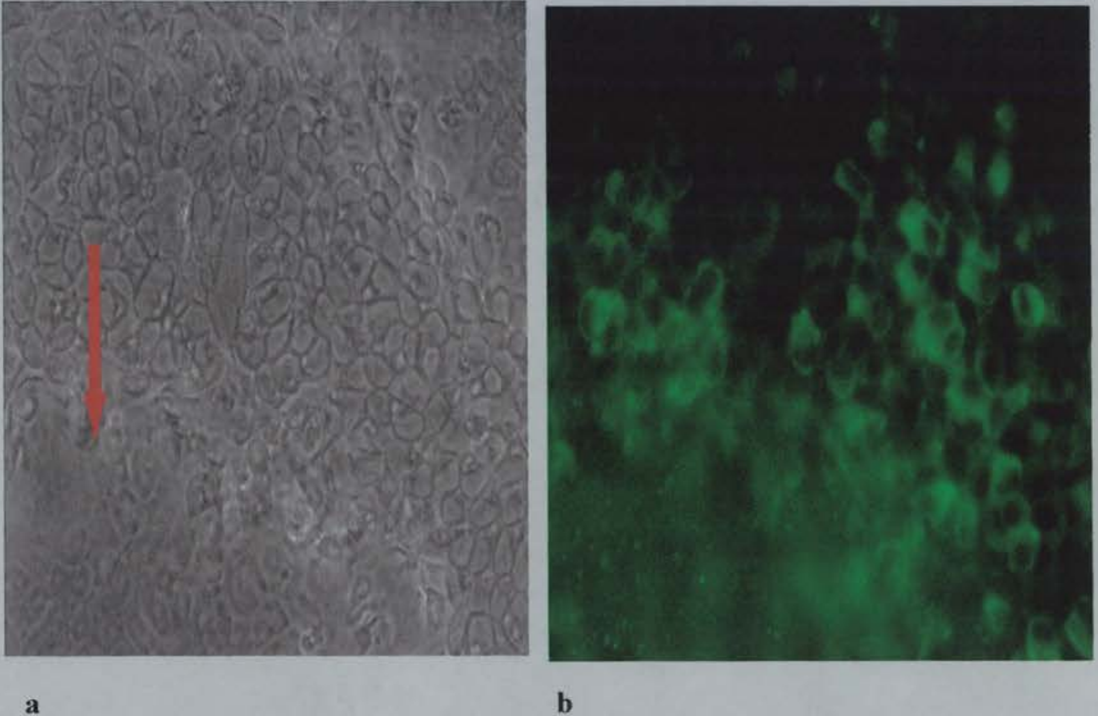
c



d

Figure 1.8.1 Comparison of β-casein expression in a,b NKIM2 cells and c,d NKIM2-diff cells (x 40)

Further study of NKIM2-diff cells at different focal planes,
identified that β-casein expression was randomly distributed between the dome-like structures and the monolayer. Figure 1.8.2 a,b. illustrates cytoplasmic β-casein expression adjacent to a dome-like structure.



a b

Figure 1.8.2 β-casein expression in NKIM2-diff cells (x25). a, phase microscopy image, b, immunofluorescence The red arrow indicates the corner of a dome-like structure

1.9 Western analysis of β-casein expression

In order to confirm the observations obtained by immunofluorescence, β-casein protein levels determined by repeat Western analysis. Samples of equal protein concentration were loaded into each lane. Figure 1.9 shows that protein levels are very low in NKIM2 cells and high in NKIM2-diff cells. Unexpectedly,TKIM2 cells in maintenance medium alone also show β-casein expression, but at a lower level than NKIM2-diff cells. Lactating mammary gland was used as a positive control and the molecular weights in kilodaltons of protein markers are indicated parallel to the positive control lane.

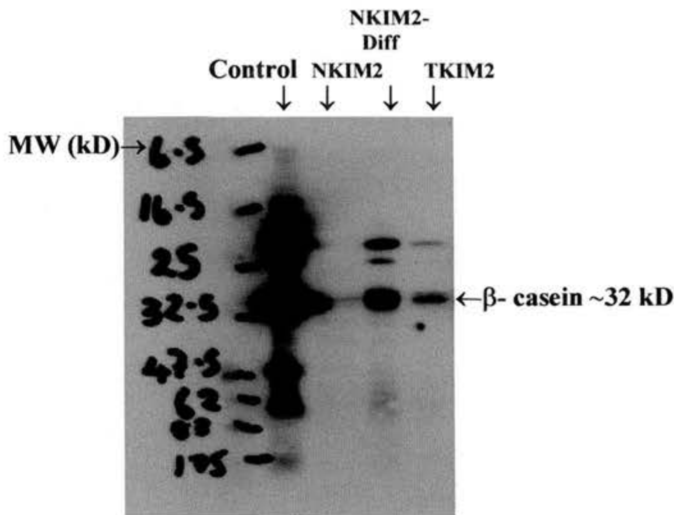


Figure 1.9 Western analysis comparing β-casein protein production in NKIM2 cells, NKIM2-diff cells and TKIM2 cells

1.10 Electron microscopy of milk production in NKIM2-diff

Electron microscopy of NKIM2-diff cells to identify milk components lipid and protein (caseins), correlating with the β-casein expression seen on immunofluorescence data. The remaining components of milk ie sugar (lactose) and mineral salts are not readily identifiable on electron microscopy. Figure 1.10 illustrates NKIM2-diff cells which contain large numbers of lipid droplets varying in size. The smaller black electron dense elements represent protein.

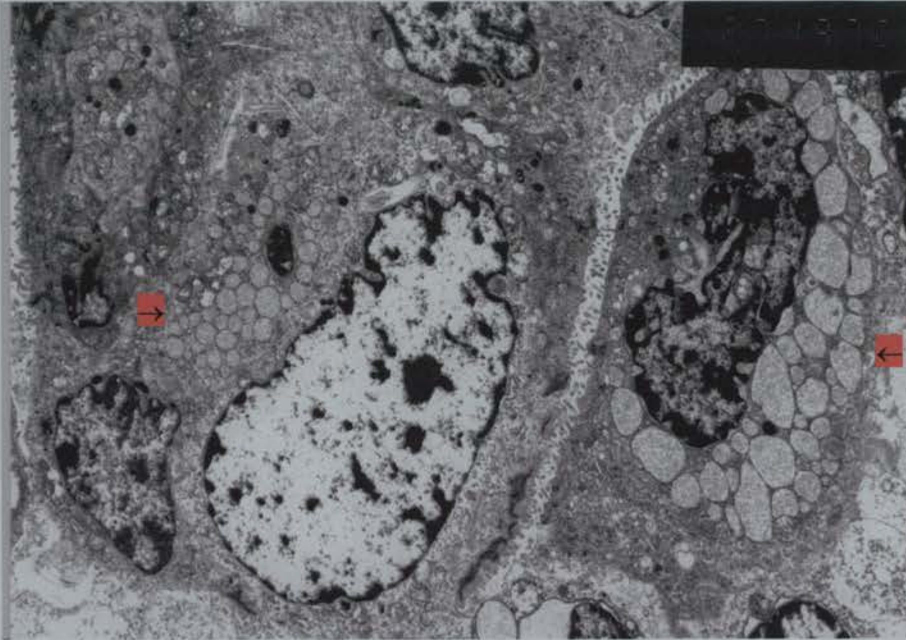


Figure 1.10 Milk production in cytoplasm of NKIM2-diff cells on electron microscopy (neg.mag. x 2777, print mag. x5554). The arrows indicate cytoplasmic lipid vacuoles.

Further examination of NKIM2-diff cells revealed that only small numbers of protein elements were identified in comparison to lipid vacuoles. Figure 1.10.1 illustrates the lipid vacuoles in NKIM2 cells at higher magnification, with some fragmentation of their contents caused by the preparation process. One dense black protein particle is visible.

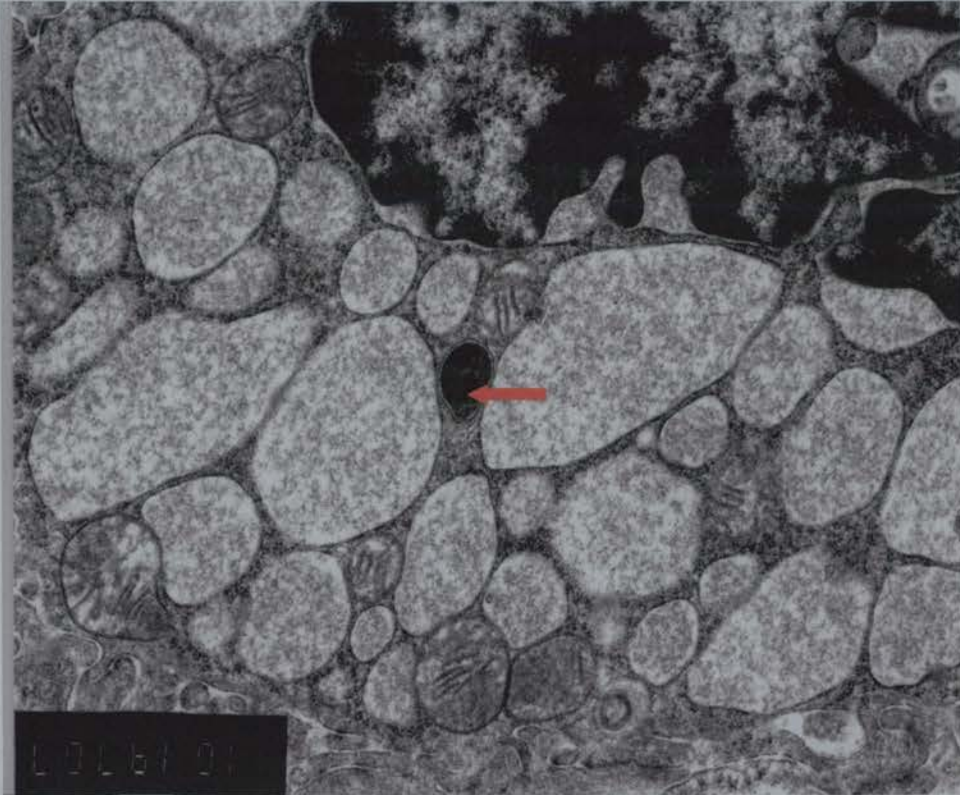


Figure 1.10.1 Cytoplasmic lipid in NKIM2-diff cells on electron microscopy (neg.mag. x 10368, print mag. x 20736). The red arrow denotes a protein particle

Figure 1.10.2 illustrates a protein particle at higher magnification which is composed of minute globules, each 25 A in diameter arranged in a crystalline array. This structure is known as a protein micelle, which help transport ions in milk in vivo. A neighbouring lipid vacuole is also present.

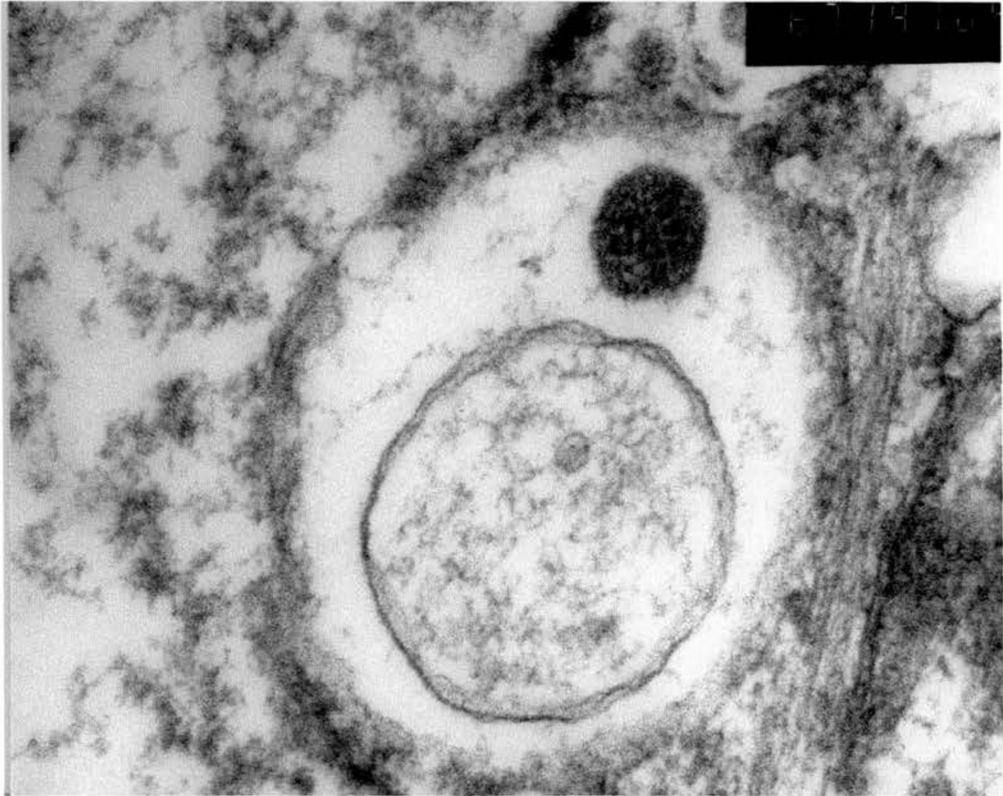


Figure 1.10.2 Protein micelle and lipid vacuole on electron microscopy of NKIM-diff cells (neg. mag. x 68040, print mag. x 136080)

1.11 Discussion

A considerable body of data already exists as to the relevance of epithelial characteristics and mammary differentiation in model cell lines, and the data presented on NKIM2 cells conform with some of these findings.
In particular the morphological data (Figure 1.1) at a phase microscopy and electron microscopic level are characteristic of epithelia [87, 88]. The overall epithelial morphology of NKIM2 is supported by the presence of epithelial markers E-cadherin and ZO-1. E-cadherin is a traditional marker of epithelia, and has specific relevance to suppression of transformation through adherens junctions [154] and therefore was chosen over alternative markers such as keratins [157]. The data show that E-cadherin is expressed when NKIM2 cells meet during growth, and this expression persists in confluent NKIM2 cells and NKIM2-diff cells (Figure 1.5.1 and 1.5.2). This E-cadherin expression in NKIM2 cells is consistent with previous studies in classic epithelial cell lines such as the MDCK line [154]. Although dome-like structures are a unique feature to the NKIM2-diff cells, MDCK cells form cyst-like structures in collagen which similarly show E-cadherin expression [302].
The presence of the ZO-1 marker is not always assessed in classic epithelial cell lines, although the tight junction is thought to be present in most epithelia together with adherens junctions, [152]. However it is relevant in mammary cells where the tight junction plays a specialised function in pregnancy [300]. In vivo, the tight junction impedes the passage of small molecular weight substances between epithelial cells [301], but during pregnancy before full lactation, tight junctions become leaky and allow components of the interstitial space, such as inflammatory cells to enter the milk [300].
The expression of ZO-1 among NKIM2 cells (Table 1.) showed a similar distribution to E-cadherin on immunofluorescence in NKIM2 cells. However there was no loss of ZO-1 immunofluorescence in NKIM2-diff cells (Figure 1.5.3) to parallel the in vivo leakiness of tight junctions during pregnancy [156].
In order to further support the evidence regarding the epithelial nature of NKIM2 cells, both adherens (Figure 1.6.1) and tight junctions (Figure 1.6) were identified between NKIM2 cells on electron microscopy. Although conventional transmission electron microscopy provides a high level of structural detail, the monolayer is disturbed- because preparation of the block involves spinning cells to form a pellet and consequently not all cells remain attached or orientated to each other [299].

An alternative method to preserve the monolayer when using transmision electron microscopy, involves growing cells on specially designed coverslips in a glass bottomed microwell. After dehydration and infiltration with resin, the coverslips are removed leaving cells embedded in the resin and ready for further processing, with the monolayer intact [151]. Unexpectedly, electron microscopy also identified interdigitation between the cytoplasm of certain neighbouring NKIM2 cells (Figure 1.7.1). Although this has previously been described in cell types which require large surface areas for cell/cell communication, such as renal tubular epithelial cells [299], it has not been observed previously in equivalent mammary cell lines such as the HC11 or COMMA D lines. It is possible that the combination of interdigitation, adherens and tight junctions contributed to NKIM2 remaining attached during the preparation process and allowed the assessment of polarisation by electron microscopy.The polarisation of NKIM2 lines was defined by the presence of a continous apical microvilli layer between cells (Figure1.7) [302]. Apart from being an epithelial feature, it is key for mammary function, in terms of milk secretion [303]. In vivo, polarised mammary epithelial cells transport proteins to apical or basolateral surfaces [302]. An alternative method to determine polarisation of cells is to identify Na/K/ATPase channels by immunofluorescence [267], which are only present on the baso-lateral surfaces of polarised cells.

While the data discussed so far supports the epithelial nature of NKIM2, the identification of small numbers of myoepithelial cells among the NKIM2 cells via α-actin (Figure 1.2), does introduce a small variable, particularly with reference to the proposed gene transduction and analysis of the cell line. Classic epithelial cell lines such as the MDCK line are pure epithelial, however the close association between epithelial and myoepithelial cells, as well as the extracellular matrix in the mammary setting is well recognised [95]. Although myoepithelial cells have not been described among the frequently- used HC11 or COMMA D mammary cell lines, there are rat mammary cell lines in use which are morphologically intermediate between mammary epithelial and myoepithelial cell lines [294]. The inclusion of the myoepithelial cell in NKIM2 may have occurred during isolation of the NKIM2 cell line, however, two alternative possibilities may explain the appearance of the specialised cell types within mammary cultures. One is the presence of a mammary stem cell population within the cells which retains the ability to differentiate into different cell types [295, 296]. However this remains contraversial partly due to the lack of intermediate lineage-specific markers to follow cell differentiation [293].

The second less likely explanation is that in rare situations mammary cells have a degree of plasticity and can alter cell lineage in response to certain stimuli. [297]. This phenomenon- termed transdifferentiation, which is common during development, is still poorly understood. It has been described very rarely in mammary epithelial cells, one example being the transdifferentiation of cells in response to TGF-beta stimulation [297]. The α-actin immunofluorescence data also demonstrated that myoepithelial cells were randomly arranged on the flat monolayer (Figure 1.2) in NKIM2, as well as being associated with dome-like structures (Figure 1.2.2) among NKIM2-diff cells. In vivo myoepithelial cells form a basket-like mesh around alveoli, and therefore it is tempting to speculate that the myoepithelial cells in NKIM2-diff cultures are attempting to recapitulate these structures in vitro. However three-dimensional cultures of NKIM2-diff in Matrigel would be necessary to examine this question in detail.
Although α-actin and other markers including cytokeratin and S-100 [293], are reliable indicators of myepithelial cells, the recognition of the cytoplasmic myofilament on electron microscopy provides definitive evidence of their presence (Figure1.4.1). Studies have shown that myofilaments are composed of actin, tropomyosin and myosin arranged in a similar pattern to smooth muscle, resulting in characteristic electron densities [299].
Apart from the myoepithelial cell, fibroblast contamination of epithelial cultures is a recognised problem during the development of epithelial cell lines, although this can reduced by various separation techniques [84, 291, 292]. However the lack of vimentin expression on immunofluorescence (Table 1.2) and of fibroblast cells on electron microscopy made it unlikely that these were present among NKIM2 cells.

While serum-dependence is not specific to epithelial cells and is more a feature of non-transformed cells, the data provides a baseline for experiments in later sections. The growth curve data demonstrate that the majority of NKIM2 epithelial cells are highly serum dependent (Figure 1.3.), and is in keeping with other epithelial cell lines [89]. In the mammary setting in vivo, it has been proposed that the death induced on withdrawal of serum -which contains growth factors, may be analagous to the apoptotic removal of the lobuloalveolar compartment in the mammary gland following weaning, when there are great fluctuations in growth factor and hormone levels [94]. However although there was a reduction in cell number in response to the serum deprivation experiments, there was no formal measurement of death rates in the cells.

The α-actin immunofluorescence data from the serum starvation experiments on NKIM2 cells (Figure1.3.1), show that certain myoepithelial cells survive starvation. This may support a theory that the myoepithelial cell has a protective function in the mammary gland in vivo and is more resistant to environmental stress [81]. However a formal comparison of equal numbers of myoepithelial and epithelial cells would be necessary to test this hypothesis.

A discussion of mammary differentiation in NKIM2-diff cells revolves around the response of cells to lactogenic hormones, particularly in terms of milk protein expression [305]. The NKIM2-diff cells formed dome-like structures in response to lactogenic hormones (Figure 1.1.1), and the significance of these structures is unclear. The formation of similar 'mammospheres' has been previously recognised in other mammary cultures grown on basement membrane or Matrigel [287, 288]. It has been proposed that the mammospheres show a resemblance to alveolar structures seen in pregnant and lactating mammary gland in vivo [289], and that their formation occurs as part of a hierarchy of changes mediated by the extracellular matrix [290]. In the NKIM-diff cells, however, domes form in the absence of extracellular matrix. One possibility is that the NKIM2 cells secrete laminin a component of the basement membrane, allowing cells to form a dome structure which is a forerunner of the mammosphere [94].Experiments growing NKIM2-diff cells in three-dimensional cultures in Matrigel are necessary to explore this question further.

However the ability of NKIM2-diff to secrete β-casein in response to lactogenic hormones (Figure 1.8.1) confirms that they exhibit mammary differentiation. This is analogous to the HC11 mammary epithelial cell line which is similarly grown on plastic, and also shows a rapid induction of endogenous β-casein with the addition of lactogenic hormones [88]. Although the polyclonal β-casein antibody used for both immunofluorescence and Western analysis may have cross-reactivity with other cells such as adipocytes (Figure 1.8), the likelihood of cross-reactivity was small because NKIM2 cells are comprised mainly of epithelial cells.

As expected, low levels of β-casein were present in NKIM2 cells on immunofluorescence, compared with high levels in NKIM2-diff cells (Figure 1.8.1). Previous studies in cycling virgin mice using in situ hybridisation, have shown that expression of milk protein genes are identified within a few alveolar cells only [304]. It may be that the low levels of β-casein seen in NKIM2 cells, parallels this observation. In contrast, similar in situ hybridisation studies in pregnant mice, show that milk protein gene expression increases dramatically in a mosaic asynchronous manner, so that by early pregnancy (day 10), β-casein is identified sporadically within pockets of alveolar cells throughout the gland [166]. In NKIM2- diff cells, high level β-casein expression was present only in a proportion of cells, after 12 days in differentiation medium (Figure 1.8.1), perhaps mirroring the mosaic asynchronous expression seen in vivo. However, β-casein-expressing cells were distributed randomly, and not concentrated in dome-like structures in NKIM2-diff cells (Figure 1.8.2). If the dome- like structures had paralleled the in vivo alveoli, as previously suggested, one would expect the expression to be mainly in the domes.

Several studies have identified β-casein expression as an early marker of mammary differentiation [305]. As pregnancy progresses, the expression of late differentiation markers Whey Acidic Protein (WAP) and α-Lactoglobulin (α-Lac) are activated in a mosaic fashion, in vivo [166]. However mammary epithelial cell lines are often unable to express these late differentiation markers in vitro, and therefore they were not assessed in NKIM2-diff cells.

Previous studies on β-casein have identified a difference in size between intracellular β-casein (~29kDa) and the secreted protein (~32kDa), and have proposed that the discrepancy may be due to differences in phosphorylation [305]. The β-casein identified in our study was approximately 29 kDa (Figure 1.9) and is presumed to be the intracellular protein. The other bands on identified on Western analysis have been recognised previously with the same antibody and are thought to represent other caseins [94] As expected, there was increased β-casein protein expression in NKIM2-diff cells compared with NKIM2 cells (Figure 1.9), however the presence of β-casein among TKIM2 cells was surprising and will be discussed.

Both immunofluorescence and Western analysis data are supported by the detection of the milk components lipid and protein, in NKIM2 diff cells on electron microscopy (Figure 1.10). However, although abundant cytoplasmic lipid was identified, only scant milk protein was present among NKIM2-diff cells which had been exposed to lactogenic hormones for 12 days (Figure 1.10.). This contrasts with previous studies of lactating mouse mammary gland which identified large amounts of milk protein using electron microscopy [299]. Moreover β-casein protein was readily identified on Western analysis of equivalent NKIM2-diff cells (Figure 1.9). It is possible that the NKIM2-diff cells studied by electron microscopy represented a small sample of cells with low milk protein expression, similar to the non-staining NKIM2-diff cells present on immunofluorescence for β-casein (Figure 1.8.1)

While most of the data presented aims to determine whether NKIM2 cells show epithelial or mammary differentiation, a number of observations regarding TKIM2 were made. Firstly, in transformed TKIM2, only one myoepithelial cell was identified by immunofluorescence (Figure 1.2.1). The reason for the difference between the NKIM2 and TKIM2 cell line in terms of frequency of myoepithelial cells remains unclear, although it may be that transformation in TKIM2 occurred in a clone of epithelial cells which outgrew other cells. The data contrasts with a previous study which describes differentiation of SV-40 transformed mammary epithelial stem-cell lines to myoepithelial-like cells, in association with increased expression of SV40 virus large T-antigen [298].

Secondly, the presence of β-casein protein in TKIM2 cells was unexpected, because it was assumed that transformed cells would be less likely to express markers of differentiation. It may be that in TKIM2 cells, innapropriate stimulation of pathways results in endogenous β-casein expression. For example, studies have shown that one of the factors (STAT5) which activates β-casein in normal cells, is upregulated in certain transformed mammary cell lines [327]. However expression of STATs was not explored in the TKIM2 cells.Thirdly, the general lack of vimentin and α-actin staining on immunofluorescence, combined with β-casein expression on Western analysis (Figure 1.9) tend to indicate that the transformed cells were originally epithelial rather than another cell type. Although in general, it is difficult to identify the origin of cell lines once they are transformed, particularly on morphological grounds alone.

So in conclusion, to what extent do NKIM2 cells show both epithelial and mammary differentiation? It is clear that both epithelial and mammary differentiation are intertwined –previous studies have shown that cell-cell interactions promote mammary epithelial cell differentiation [307]. The NKIM2 cells satisfy a number of criteria for epithelia in that they form appropriate junctions, express appropriate markers and are polarised. One potential disadvantage is the presence of the specialised myoepithelial cell, which may have different properties to the epithelial cell and would be difficult to investigate separately, due to the small numbers present in the culture. However the presence of both cell types is more representative of the in vivo situation.

In terms of mammary differentiation, the NKIM2 cells express the milk protein β-casein at high levels in response to lactogenic hormones. The relevance of the formation of dome-like structures in response to lactogenic hormones is still unclear.

2 Transformation

This section reports the data relating to the original cell lines before genetic manipulation, in order to assess recognised features of transformation.

The experiments regarding in vitro growth kinetics plus and minus serum, growth in soft agar, and p53 and T-antigen expression are carried out both at 37°C and 33°C (the respective culture temperatures of NKIM2 and TKIM2) to control for any effect of temperature on growth.

2.1 Morphology

TKIM2 consisted of spindle-shaped cells which showed multilayering ie no contact inhibition, which have been shown previously (Figure 1.1). Further assessment of TKIM2 by electron microscopy, revealed cells with irregular cell surfaces and gross pleomorphism in terms of cell size and cellular organelles. Figure 2.1 illustrates a representative field of TKIM2 cells where there are large numbers of irregular cytoplasmic projections and flaps, but no evidence of apical microvilli formation as seen previously in NKIM2 (Figure 1.6 and 1.7). Formal examination of 100 fields under electron microscopy revealed no evidence of polarisation in terms of cell orientation and apical microvilli formation [311].

Furthermore, no myoepithelial cells with cytoplasmic myofilaments were identified in TKIM2 under electron microscopy, supporting previous data which found <0.001% α-actin positive cells by immunofluorescence (Table 1.2). This was similarly assessed by the examination of 100 fields under electron microscopy.

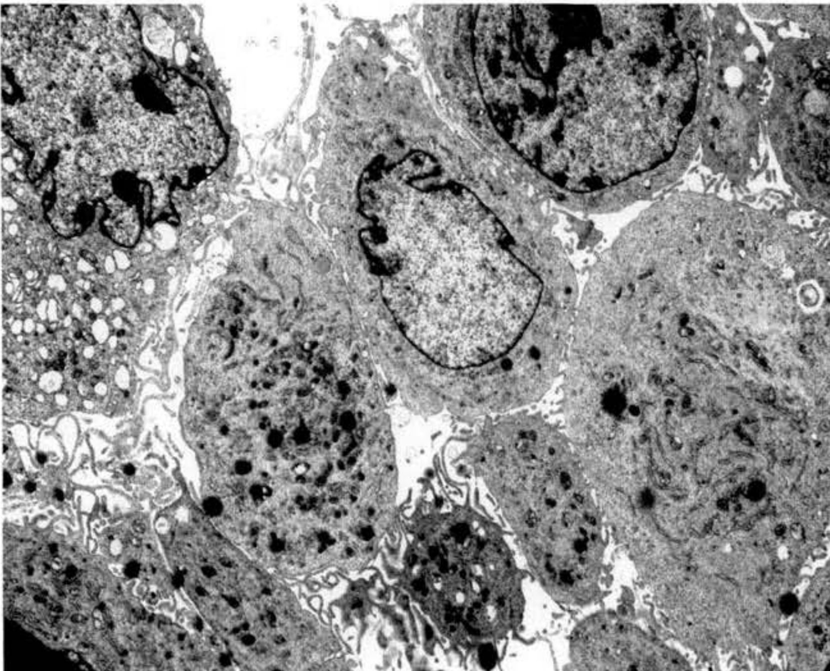


Figure 2.1 A representative field of TKIM2 cells on electron microscopy showing pleomorphism of cells, with no evidence of polarisation (neg.mag. x1963, print mag.x3297)

2.2 Electron microscopy to assess junctional formation

Further examination of TKIM2 cells, showed no evidence of junctional specialisations, in 100 fields examined. Figure 2.2 illustrates a representative field of TKIM2 with two neighbouring cells lacking junction formation.

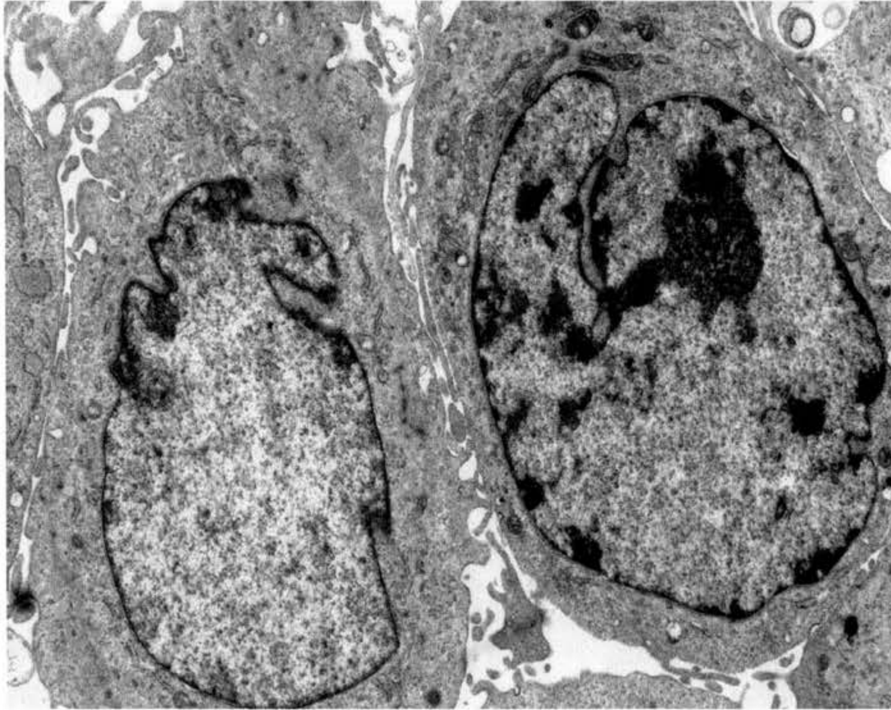
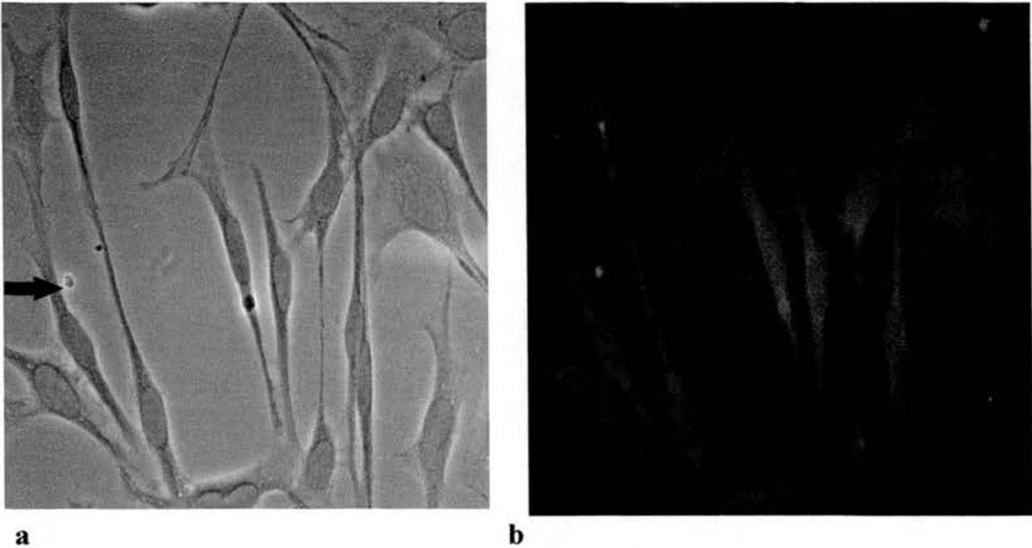


Figure 2.2 Representative field of TKIM2 on electron microscopy (neg.mag. x1963, print mag. x3297)

2.3 Epithelial Marker Expression

The lack of junctions on electron microscopy corresponded with a lack of expression of E-cadherin in TKIM2 cells at cell boundaries on immunofluorescence. Figure 2.3 illustrates the lack of organised E-cadherin expression seen in TKIM2 at a preconfluent stage. When TKIM2 cells are confluent and cells overlap they similarly retain a diffuse distribution, with no evidence of membrane localisation.

Occasional spots of bright autofluorescence seen adjacent to cells are caused by cell debris. As expected, there was no evidence of ZO-1 expression in TKIM2 cells on immunofluorescence, in the presence of a positive control. This appeared as a black field with no signal and therefore is not illustrated.



a b

Figure 2.3 a Phase microscopy image, and b E-cadherin expression in TKIM2(x25) The black arrow indicates cell debris which autofluoresces

2.4 In vitro growth kinetics

In order to compare growth between cell lines, cells were counted according to a recognised protocol [69]. The TKIM2 and NKIM2 cells were seeded at the same density (1 x 10^5 cells) in maintenance medium at 37° and 33°C. At 24 hour intervals culture plates within each series were harvested and the cells counted. Figure 2.4 shows the growth curves drawn from three independent experiments. It can be seen that TKIM2 cells appear to grow faster than NKIM2 regardless of temperature, but there is a greater increase in cell number at 37°C compared with 33°C.

The greatest increase in cell number occurs between day 0 and 3 in all cell lines, followed by a slower rate of increase between day 3 and 5. However the TKIM2 lines appear to increase in cell number faster than NKIM2 between day 0 and 3, and it may be that NKIM2 lines show a degree of growth latency. This experiment does not assay for apoptotic rates in individual cell lines, which may also contribute to differences in cell number.

Cell number per plate

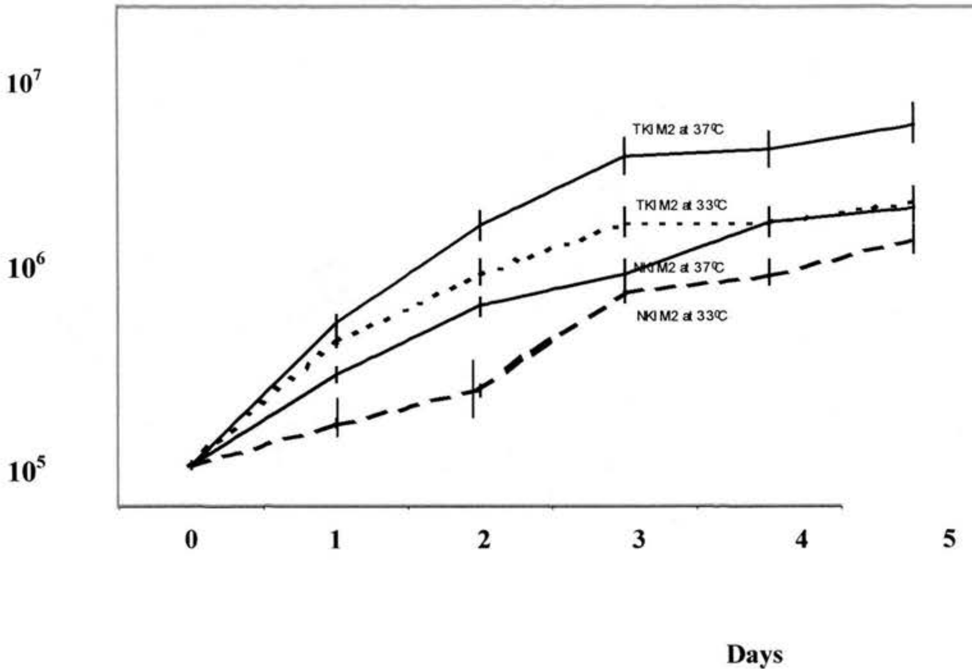


Figure 2.4 Comparison of growth curves (mean and standard deviation from 3 experiments) of NKIM2 and TKIM2 at 37°C and 33°C.

2.5 Serum-dependence

Previous studies have shown that transformed cells can survive in the absence of serum [291]. From the previous chapter, it was observed that in conditions of serum starvation, < 1% of NKIM2 cells survive after 5 days (Figure 1.3). In order to investigate the growth kinetics of both lines without serum , they were seeded at the same density (1×10^5 cells) at 33°C and 37°C in normal maintenance medium for 24 hours, then washed and seeded in serum-free maintenance medium. They were harvested and cells counted, similar to the previous experiment. Similarly no measure of apoptosis was undertaken. Figure 2.5 illustrates the ability of TKIM2 cells to grow in serum-free conditions compared to NKIM2 cells, ~ 97% of which died after 5 days. The TKIM2 cells at 37°C increase in cell number at a faster rate than TKIM2 cells at 33°C, as expected.

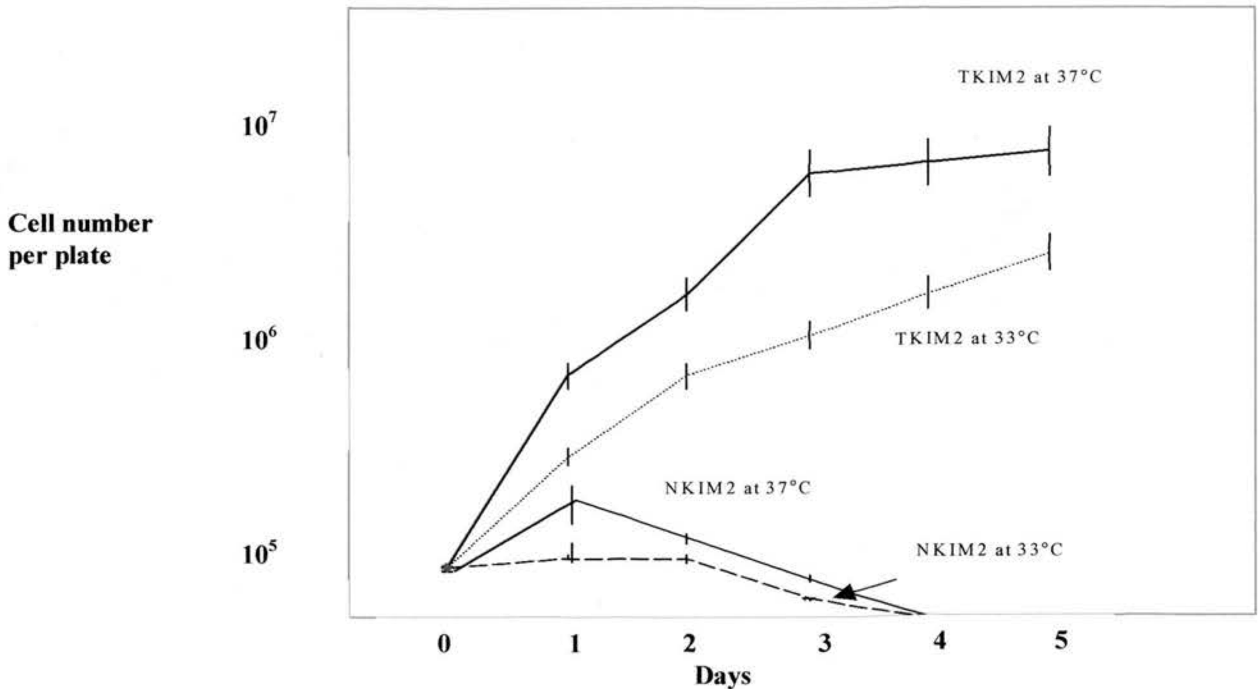
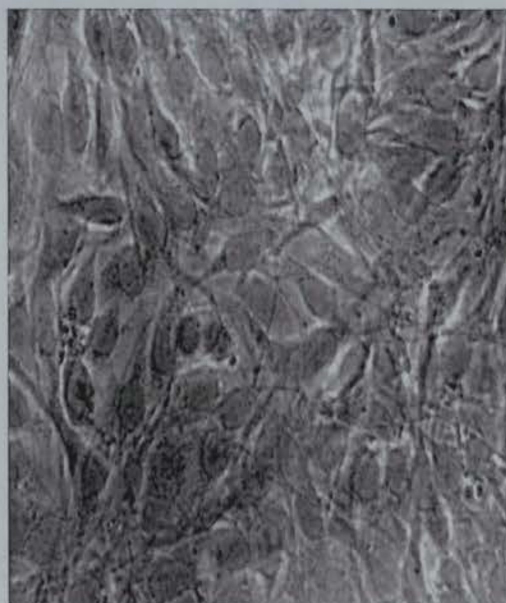


Figure 2.5 Comparison of growth curves (mean and standard deviation from 3 experiments) of TKIM2 and NKIM2 in serum-free conditions at 37°C and 33° C.

To confirm that TKIM2 cells grow in serum-free conditions, Figure 2.5.1 illustrates the growth of TKIM2 cells after 1 day and 5 days in 0% serum. The cells at 5 days after seeding show considerable multilayering, and it is difficult to identify individual spindle-shaped cell bodies.



a **b**

Figure 2.5.1 Representative fields of TKIM2 cells in 0% serum at a, 1 day and b, 5 days after seeding at 37°C(x10)

2.6 Anchorage-dependence in Soft Agar

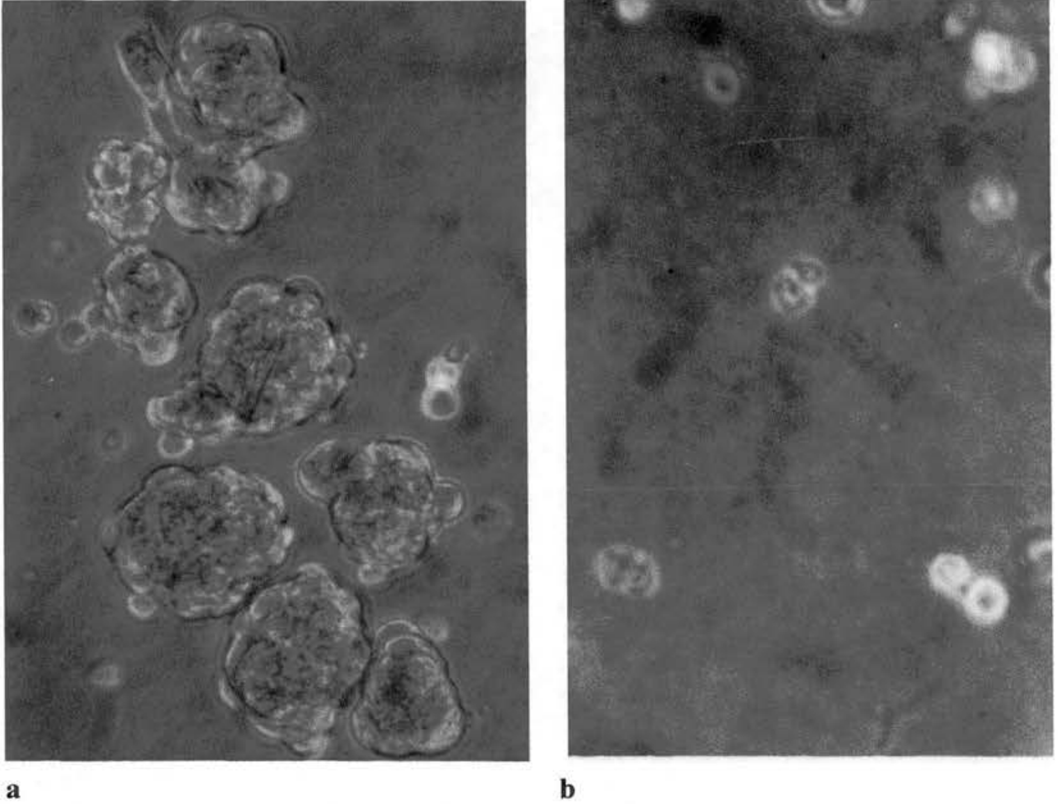
To assess anchorage-independent colony formation in soft agar, TKIM2 and NKIM2 were seeded into soft agar at varying cell densities at 37°C and 33°C according to a recognised protocol [220], as shown in Table 2.6. A colony was defined as a spherical growth of cells over 5 mm in diameter. Positive control Ras-transformed and negative control non-transformed cell lines were supplied by D.B.

As expected, TKIM2 cells formed colonies at both 37°C and 33°C, whereas NKIM2 did not form colonies at either temperature. TKIM2 cells formed more colonies at 37°C compared with at 33°C, corresponding to previous growth data.

Cells seeded	**TKIM2 at 37°C**	**NKIM2 at 37°C**
$2x10^4$	12.6 +/- 1.5	0
$1x10^4$	11.0 +/- 2.0	0
$4x10^3$	9.2 +/- 1.2	0
$2x10^3$	8.4 +/-2.0	0
$1x10^3$	6.4 +/-1.7	0
	TKIM2 at 33°C	**NKIM2 at 33°C**
$2x10^4$	9.0 +/-1.6	0
$1x10^4$	7.4 +/- 3.0	0
$4x10^3$	7.0 +/- 1.4	0
$2x10^3$	6.3 +/- 1.2	0
$1x10^3$	5.2 +/- 1.4	0

Table 2.6 Comparison of Colony formation in soft agar in TKIM2 and NKIM2. Values are means and standard deviations from triplicated experiments

The difference between TKIM2 and NKIM2 in terms of colony formation in soft agar is illustrated in Figure 2.6.1. The TKIM2 cells form large anchorage-independent colonies, while among NKIM2 cells only small fragments of cell debris are visible.



a b

Figure 2.6.1 Growth in soft agar of a, TKIM2 and b, NKIM2 (x25) at 37°C

2.7 Injection into scid mouse

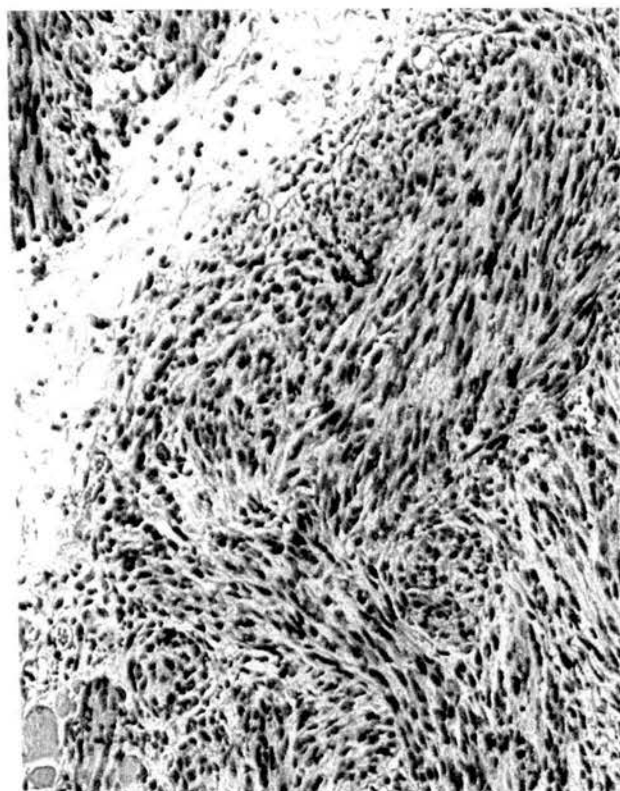
Unexpectedly injection of the NKIM2 and TKIM2 cell lines in to scid mice resulted in subcutaneous tumour formation in both lines at an overall identical take rate of 25%, as illustrated in Table 2.7.

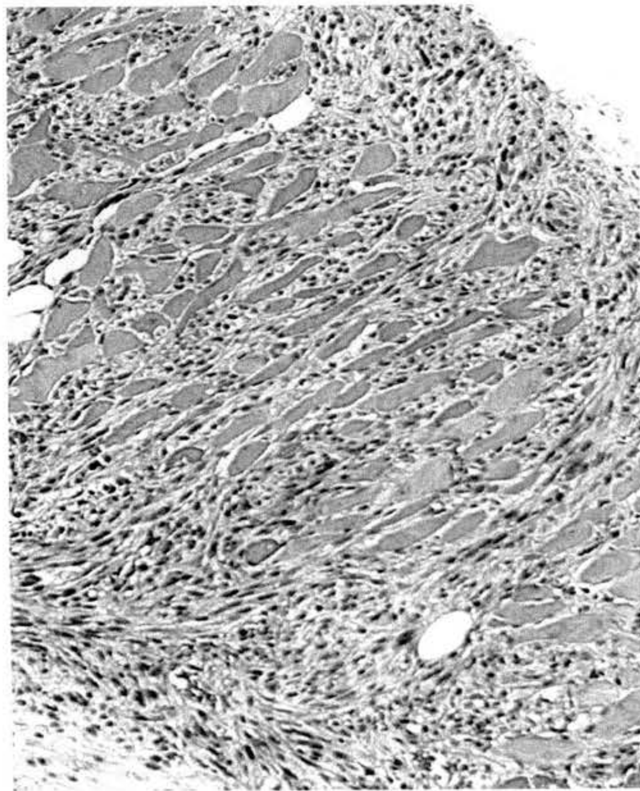
The size of the tumour harvested at 4 months did not significantly differ between cell lines, although the mean tumour volume had not been compared during the 4 month period.

Cell line	Incidence of tumours (Take rate)	Size of tumours after 4 months (Diameter in mm)
NKIM2	2/8 (25%)	32mm and 29mm
TKIM2	2/8 (25%)	30mm and 35mm

Table 2.7 Incidence of tumours in NKIM2 and TKIM2 cell lines

The histology of all tumours formed by both TKIM2 and NKIM2 lines was similar, consisting of pleomorphic spindle-shaped cells, with few mitotic figures were visible, as illustrated in Figure 2.7.1. By light microscopy alone, there was no evidence of special tumour differentiation eg muscle striations, however immunohistochemistry was not carried out to explore this further.





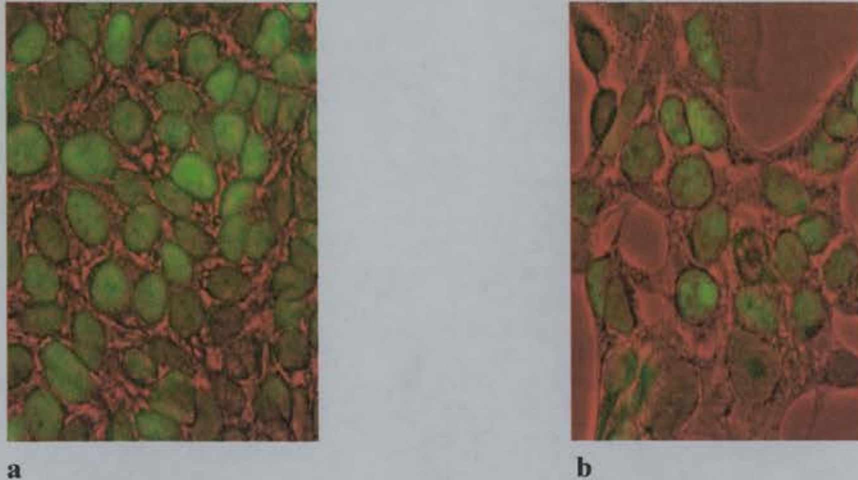
a b

Figure 2.7.1 Histology of tumours formed in scid mice of a, TKIM2 cells and b, NKIM2 cells. In b, tumour cells are penetrating subcutaneous muscle.

2.8 p53 and T antigen expression

Immunofluorescence for p53 and T antigen resulted in strong nuclear staining in NKIM2 cells (Figure 2.8) at 37°C and 33°C in passage 20 cells. T antigen is normally found in the nucleus [93], however the presence p53 in the nucleus rather than the cytoplasm suggested that T antigen was binding p53.

The positive control for p53 was the mammary HC11 cell line, which is immortalised by mutant p53 and negative control was omission of the primary antibody. The positive control for T antigen was SV40 T antigen-immortalised canine kidney epithelial cells donated by C.S and the negative control was omission of the primary antibody.



a b

Figure 2.8 Comparison of a, p53 and b, T antigen distribution in representative fields of NKIM2 cells at 37°C (p20) (x40)

Similarly, immunofluorescence for p53 and T antigen resulted in nuclear staining in TKIM2 cells (Figure 2.8.1) at 37°C and 33°C in passage 20 cells.

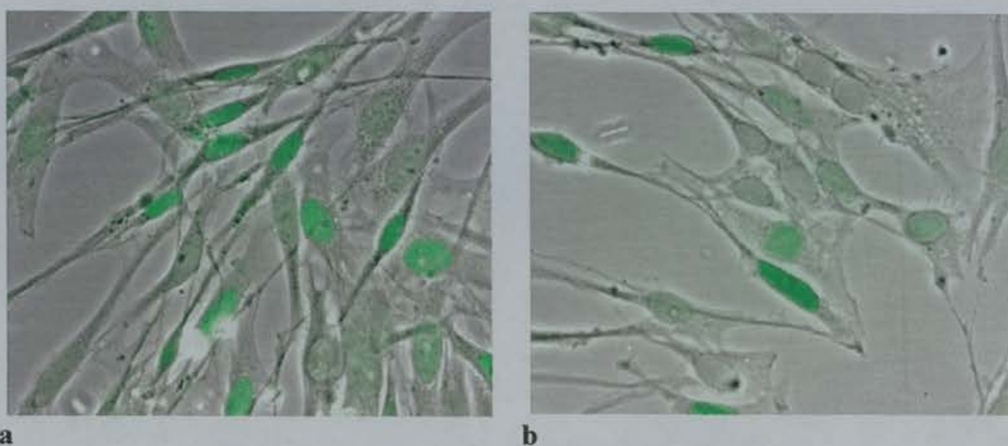


Figure 2.8.1 Comparison of a, p 53 and b, T antigen distribution in representative fields of TKIM2 cells at 33°C (p20) (x40)

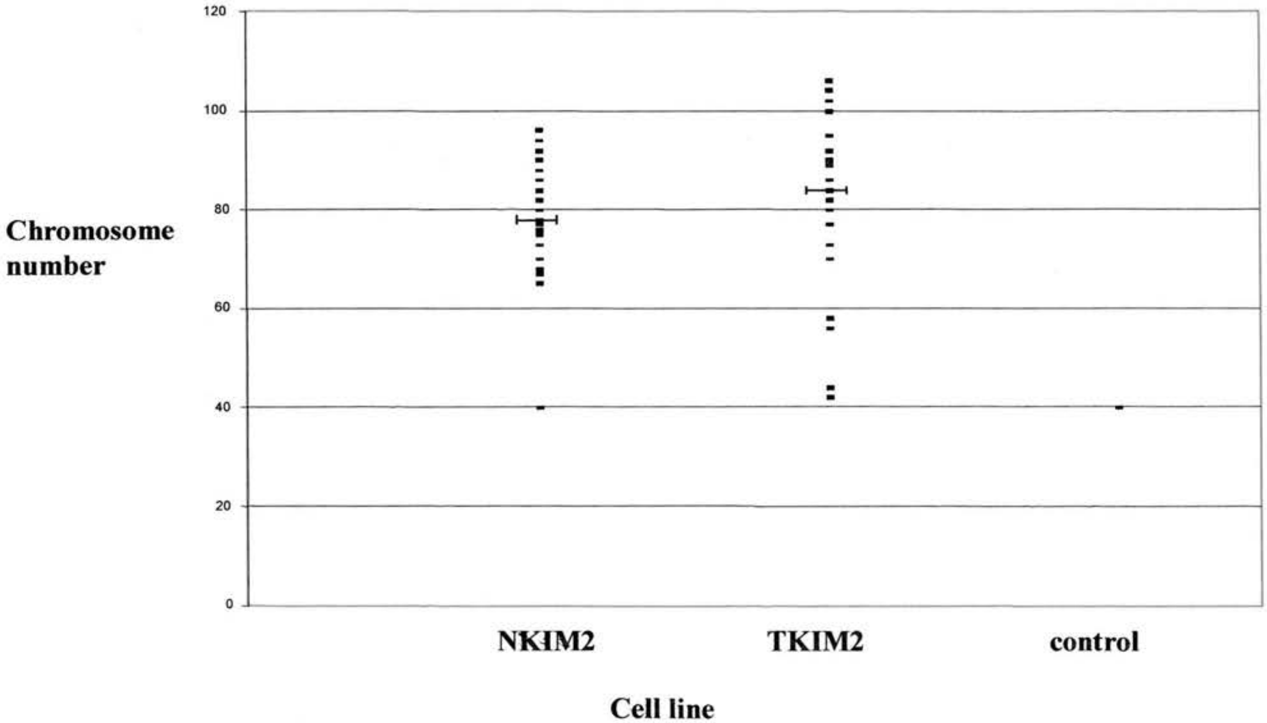
Although the nuclear localisation of p53 and T antigen suggests that T antigen is binding p53, the ideal method to prove that they are bound is to carry out an immunoprecipitation analysis. This involves using beads to isolate molecules bound to p53 antibody, and then probing this isolated sample with the T antigen antibody (or vice verse) to confirm that the two are associated. However this methodology did not work with the p53 and T antigen antibodies used in this study.

2.9 Chromosome metaphase analysis

Chromosome analysis was undertaken to compare the karyotypes of NKIM2 and TKIM2. Previous DNA flow cytometry data on KIM2 cells predicted that the cells had an unstable chromosome content at an early passage (p5), however that DNA content stabilised in mid passages (p15-p30)[318]. Therefore metaphase spreads were prepared at passage 20 in both NKIM2 and TKIM2.

The control diploid rat embryo fibroblast cell line was provided by M.L., and contained the expected 40 mouse chromosomes. However both NKIM2 and TKIM2 lines contained a range of chromosome numbers with a median of 78 and 84 respectively, from a count of 20 metaphase spreads (Figure 2.9). There was no significant difference between chromosome number between NKIM2 and TKIM2 at p20, although TKIM2 did contain a greater range of chromosome counts

Figure 2.9 Chromosome number (median —, and range) from 20 metaphase spreads of NKIM2, TKIM2 and control cell lines (p20)



The numbers of chromosomes were so large in both NKIM2 and TKIM2, that it was impossible to karyotype the mouse chromosome preparation either by computer analysis packages, or using an experienced cytogeneticist (M.L). A representive metaphase spread from NKIM2 and TKIM2 is illustrated in Figure 2.91 and 2.9.2 respectively, showing the large number of chromosomes present in each.

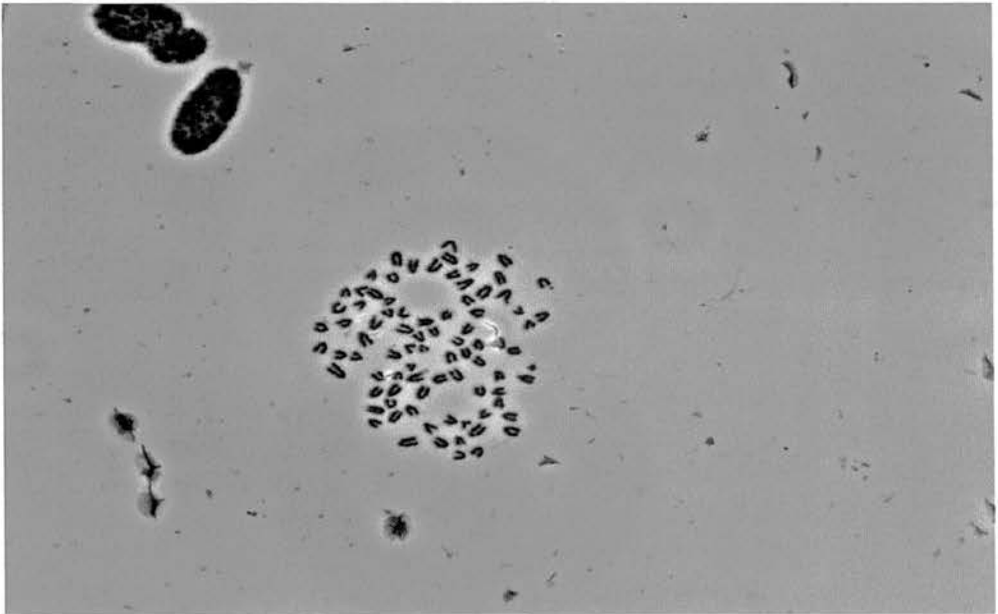


Figure 2.9.1 Representative metaphase spread from NKIM2 (p20)

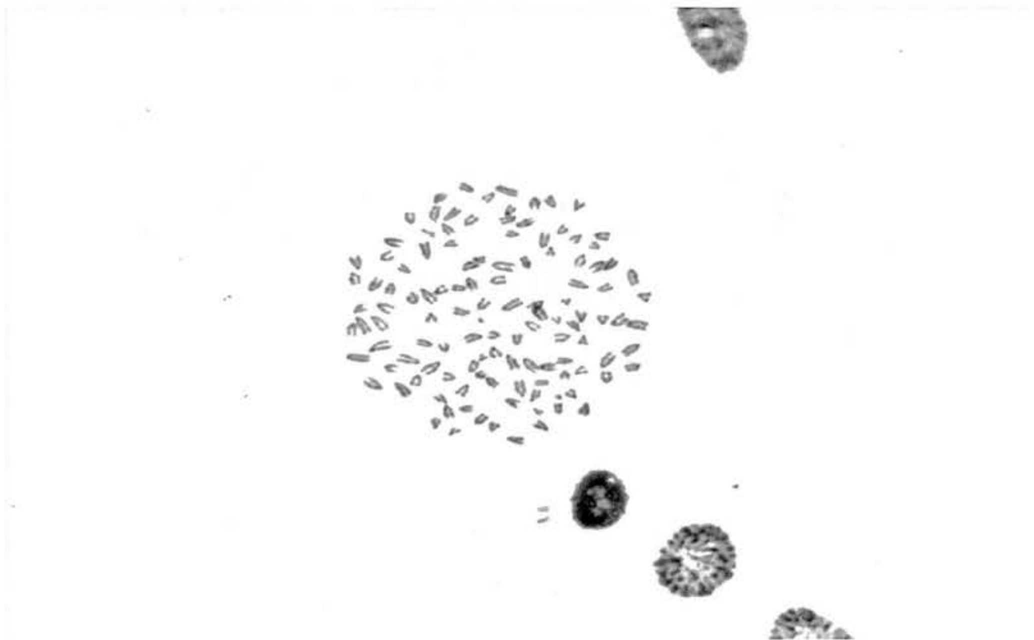


Figure 2.9.2 Representative metaphase spread from TKIM2 (p20)

2.10 FISH analysis of chromosome 11

FISH analysis with chromosome paint 11 was undertaken to compare chromosome 11 in NKIM2 and TKIM2, which is the locus of p53 in the mouse.

Figure 2.10 illustrates the range of chromosome 11 found per cell in NKIM2 and TKIM2 with median values of 5 and 6 respectively in 20 metaphase spreads. This presumably reflects the great range of total chromosome numbers found on metaphase analysis (Figure 2.9). However there was no consistent loss of chromosome 11 in NKIM2 or TKIM2. Counts were carried out similar to the previous experiment and as expected the control cell line had 2 copies of chromosome 11 per cell (not shown).

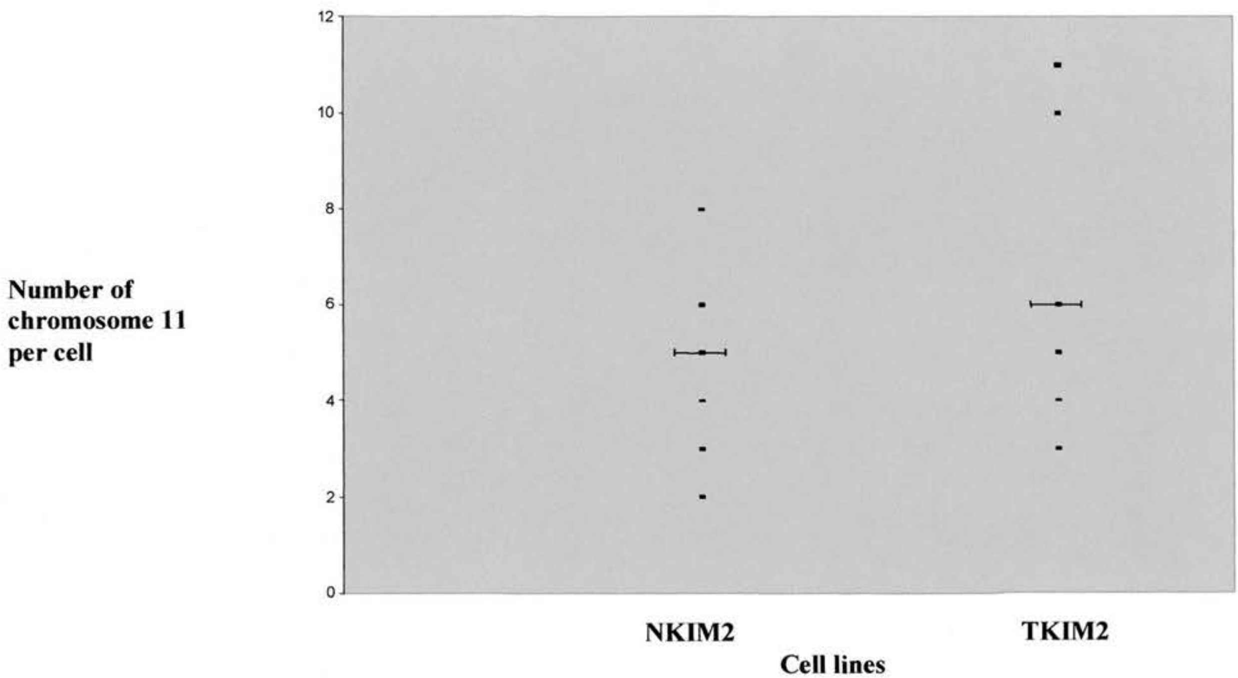


Figure 2.10 Comparison of number (median —, and range) of Chromosome 11 in NKIM2 and TKIM2 cell lines (p20)

Figure 2.10.1 illustrates FISH analyses of chromosome 11 in a, NKIM2 and b, TKIM2. Both show five chromosome 11 per cell, however the break seen in the green signal of one chromosome in 2.10.1 a is due to an overlying chromosome blocking view of the signal, determined by computer-assisted analysis.

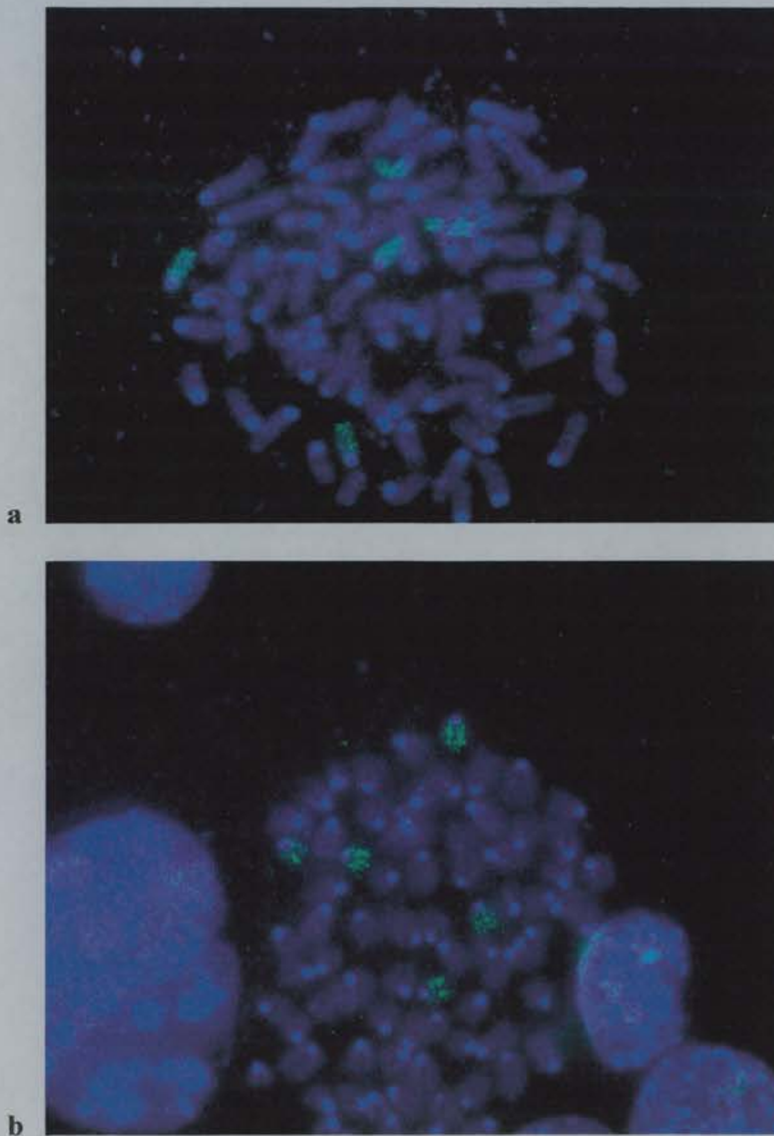


Figure 2.10.1 FISH analysis of chromosome 11 in a, NKIM2 and b, TKIM2. The break in the green signal in a, is due to an overlying chromosome.

2.11 FISH analysis of chromosome 17

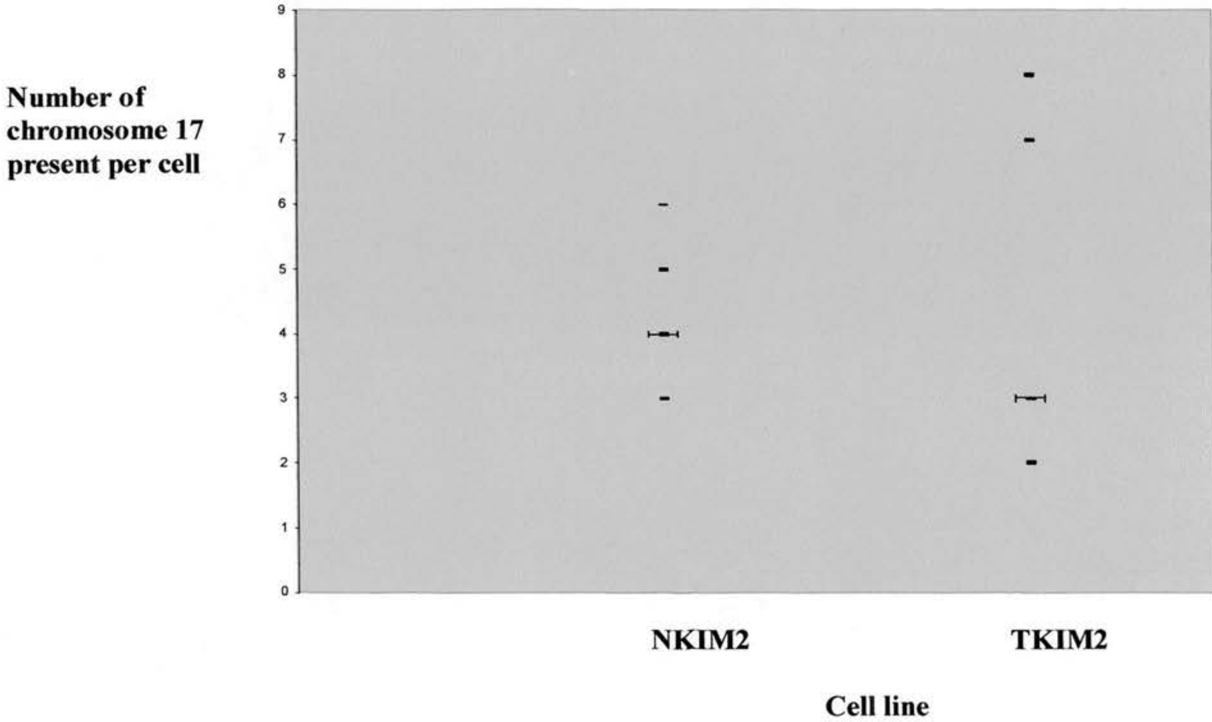
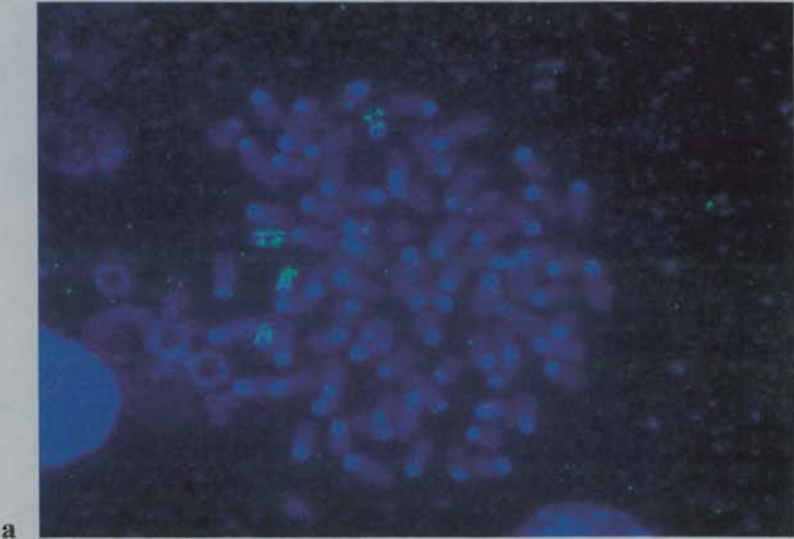


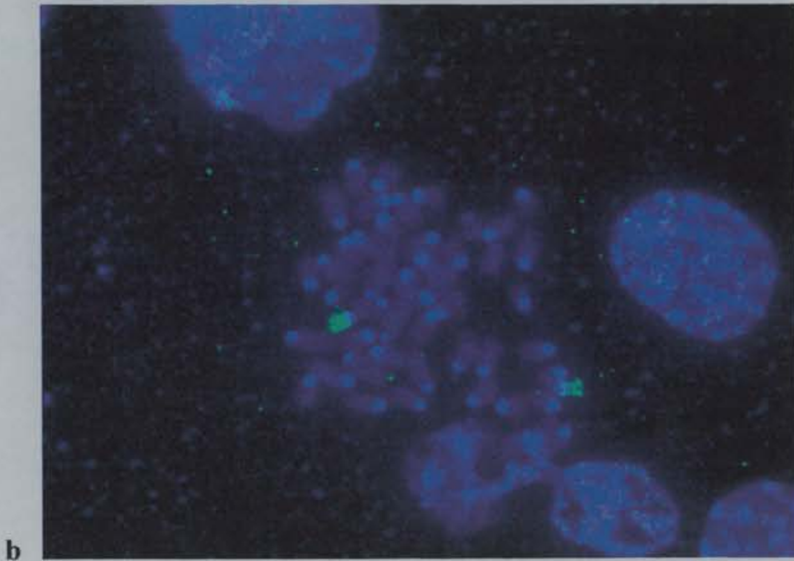
Figure 2.11 Comparison of the number (median —, and range) of Chromosome 17 in NKIM2 and TKIM2 (p20)

A similar analysis was carried out on chromome 17, the murine locus of SEN 6 which is thought to underlie immortalization in SV40 immortalised human fibroblasts [134]. This revealed similarly no consistent loss of chromosome 17 in NKIM2 and TKIM2 cells, and a range of values per cell. Figure 2.11 illustrates a comparison of chromosome number 17 in 20 cells, with a median of 4 and 3 in NKIM2 and TKIM2 cells respectively. Similar counts and controls were used as in the FISH analysis of chromosome 11.

Figure 2.11.1 illustrates FISH analysis of chromosome 17 in a, NKIM2 and b, TKIM2 cells. The NKIM2 cell contains four chromosome 17 and the TKIM2 cell contains two chromosome 17.



a



b

Figure 2.11.1 FISH analysis of chromosome 17 in a, NKIM2 and b, TKIM2

Table 2.11.2 Summarising Features of TKIM2 compared to NKIM2

	NKIM2	TKIM2
Morphology	**Epithelial**	**Spindle**
Dome formation	**Present**	**Absent**
Junction formation	**Present**	**Absent**
Epithelial marker expression	**At cell-cell junctions**	**Cytoplasmic**
In vitro growth kinetics	**Steady growth**	**Rapid proliferation**
Serum-dependence	**Present**	**Absent**
Anchorage-dependence	**Present**	**Absent**
Tumour formation in scid mice	**2/8 (25%)**	**2/8 (25%)**
p53,T antigen expression	**Nuclear expression**	**Nuclear expression**
Metaphase spreads	**Median 78 (range 40-88)**	**Median 84 (range 42-108)**
Chromosome 11	**Median 5 (range 2-8)**	**Median 6 (range 3-11)**
Chromosome 17	**Median 4 (range 3-6)**	**Median 3 (range 2-8)**

2.12 Discussion

The biological features used to define transformation are derived from an impressive amount of data on transformed lines, and in general the TKIM2 cells fulfil these criteria. In particular the spindle-shaped morphology and lack of contact inhibition of TKIM2 cells (Figure 1.1) are features shared by transformed lines in general, as well as equivalent SV40 transformed cell lines [143, 308]. Similarly, previous studies have observed that the surface of transformed cells is more irregular than normal, with a relative increase in surface microvilli and cytoplasmic flaps, and a loss of microvillous polarity on electron microscopy, as seen in TKIM2 cells (Figure 2.1) [144, 309, 310]. Although the ultrastructure of individual organelles has been extensively compared in normal versus transformed cells [139], it is rather the cumulative differences in terms of overall pleomorphism and varying organelle numbers which is thought to be significant. Moreover previous studies indicate that differences in cell surface and intercellular junctions appear more reliable markers of transformation [150].

The lack of junctional specialisations in TKIM2 on electron microscopy (Figure 2.2) is consistent with previous literature which describes either absent or a reduced number of poorly formed, junctions in transformed cell lines [155]. The lack of E-cadherin expression at cell-cell boundaries in TKIM2 cells on immunofluorescence (Figure 2.3) concurs with this data. However, a previous study examining E-cadherin expression in human carcinoma cell lines, suggests that epithelioid carcinoma lines (eg MCF7) generally express E-cadherin at cell boundaries, whereas spindle-shaped, fibroblastoid, cell lines (eg MDA-MB45s) completely lack E-cadherin expression [212]. Other studies suggest that it is a loss of E-cadherin function as opposed to expression, that is significant in the fibroblastoid cell lines [211]. It is likely that any E-cadherin protein detected in the TKIM2 cells in this study, is not playing a functional role in terms of cell-cell adhesion.

Apart from morphology and lack of junctions, certain accepted features of transformed cells stem from their lack of growth control [312, 313].
The overall lack of growth control in TKIM2 was illustrated by the growth kinetics experiments showing that TKIM2 proliferated more rapidly than control NKIM2 cells at both growth temperatures (Figure 2.4). Supporting this evidence was the ability of TKIM2 cells to proliferate when lacking serum (Figure 2.5). Transformed cells are serum-independent because they produce inappropriate amounts of the growth factors found in serum which normally regulate growth [173].
While anchorage-independence of transformed cells is thought to result from abnormal function of integrin-mediated pathways, certain studies have proposed a link between these and growth-control pathways. In particular SV40 large T antigen is thought to affect both growth and integrin-mediated pathways, and previous studies have demonstrated that SV40-transformed cells show both serum and anchorage-independent growth [177]. Therefore ability of TKIM2 cells to form anchorage-independent colonies in soft agar (Figure 2.6), and grow without serum (Figure 2.5) is consistent with previous studies.

In principle, the tumour formation in scid mice of injected TKIM2 cells, supports the theory that TKIM2 are transformed (Figure 2.7.1). However, previous studies have suggested that SV40 T antigen alone will not make cells tumorigenic, but that an additional event such as overexpression of the Ras oncogene is also required [119]. It is possible that a random additional event in the TKIM2 analysed may have occurred, as indicated by the great variation in chromosome number in the line. However the formation of tumours by NKIM2 cells at the same take rate of 25% (Table 2.7) as TKIM2 cells, was unexpected and difficult to explain. Conventionally non-transformed cells should not have the capacity to form tumours in scid mice. However 'immortalized' non-transformed lines sometimes prove an exception to this rule, for example the HC11 mammary epithelial cell line, which is immortalized by mutant p53, also appears to be tumourigenic in mice [88].
It is possible that the immortalization strategy of SV40 T antigen in both NKIM2 and TKIM2 promoted tumourigenesis, however this means that further results from scid mice experiments in manipulated lines should be interpreted with caution.

Further to this, the overall take tumour take rate in the scid mice experiments appears low. The experiments utilised Matrigel, which is a solubilised tissue basement membrane extracted from the Engelbrowth-Holm Swarm (E.H.S.) mouse tumor, which acts as a biologically active matrix material [187]. Coinjection of the cell suspension with Matrigel is known to improve the efficiency of xenografting, particularly in mammary lines [187]. Oestrogen pellet supplementation is also used to improve tumour take rates in certain mammary lines , however it has varying efficiency and in rare cases actually inhibits the growth lines, and therefore was not used in this study [187].
The histology of all tumours assessed by light microscopy in NKIM2 and TKIM2 lines appeared similar, although full investigation of tumour differentiation would require further immunohistochemistry. The spindle 'sarcoma-like' appearance of tumours caused by SV40 large T antigen has been described previously, in addition to other SV40 large T antigen induced phenotypes [142]. However analysis of each tumour for T antigen would be required to ensure that it was involved in formation of the lesions identified.

The data confirms that both p53 and T antigen both localise to the nucleus in NKIM2 and TKIM2 (Figure 2.8 and 2.8.1), suggesting that they are binding to each other. Further confirmation that p53 was in the nucleus could have been achieved by counterstaining the p53- expressing cells with a DNA stain such as DAPI (4',6-diamidino-2-phenylindole), which highlights the nucleus only. Previous studies have shown that cellular wild-type p53 is normally regulated by rapid turnover and the level present in the nuclei of normal cells is below the sensitivity of immunohistochemical detection [123]. It is also known that SV40 large T antigen complexes wild-type p53 protein and inactivates its transcriptional activity [315]. This may be one explanation for the appearance of p53 in the cell nuclei in NKIM2 and TKIM2, although another would be mutation of endogenous p53 in the cell lines. An ideal experiment would be to probe cell lines with T antigen and p53 antibodies by immunoprecipitation analysis, to see if both were complexed to each other.
An alternative way to assess whether T antigen was binding p53 and abrogating its activity, would be to assess p21WAF1/CIP1. p21 is downstream of functional p53, and is strongly upregulated after DNA damage eg ionizing radiation [316].

Previous studies have shown only very weak accumulation of p21 in human breast adenocarcinoma cell lines which have p53 inactivated by SV40 large T antigen, after ionizing radiation, [316, 317]. The presence and activity of pRb, the other main factor which SV40 T antigen binds, was not assessed in this study.
It has been previously suggested that SV40 virus immortalization or transformation may occur secondary due to chromosome instability, caused by the abrogation of p53 function by T antigen [122]. Chromosomal analysis of metaphase spreads, showed that both NKIM2 and TKIM2 had a large and variable chromosome number per cell. The median number of chromosomes per cell for NKIM2 was 78 (range 40-96) and for TKIM2 was 84 (range 46-98) (Figure 2.9) at passage 20, while the chromosome number for control diploid cells was 40. One way of assessing chromosomal content with increasing passage number would be to use DNA flow cytometry analysis. Previous analysis of NKIM2 using this method has shown initial instability at passage 5 with loss of chromosomal content with each passage, and subsequent stabilisation of content at passage 20 [318]. TKIM2 cells have not been analysed using this method.
It was not possible to identify chromosomes by karyotype analysis of cells, due to the large and variable chromosome number in KIM2 cells. Therefore FISH analysis was used to investigate whether there was chromosome loss of known senescent or tumour-suppressing loci namely: chromosome 11 - the locus of p53 and chromosome 17- the locus of SEN 6. FISH analysis demonstrated a large variation in the number of chromosome 11 present in each cell, presumably reflecting the overall chromosomal variability (Figure 2.10). There was no evidence of a consistent loss of chromosome 11 in NKIM2 cells or TKIM2 cells, suggesting that endogenous or acquired disruption of the p53 locus was not responsible for the immortalization and transformation in these cells. This evidence tends to support the immunofluoresence data, which points to an abrogation of p53 function through SV40 T antigen. However to determine definitively that the p53 locus is not altered in these cells, further detailed experiments involving restriction fragment length polymorphism (RFLP), more detailed FISH analysis and PCR would be required.

Several similar previous studies have shown that in vivo human breast cancer is associated with loss of heterozygosity of chromosome 17 p, where p53 gene is located in humans [319]. Moreover, the HC11 mammary epithelial cell line has a missense mutation in exon 5 and a point mutation at the intron 4/ exon 5 junction at the locus of p53, leading to a splicing error resulting in abnormal function of the p53 protein [89].

A potential candidate for chromosomal loss or mutation secondary to the action of SV40 T antigen is chromosome 17- the locus of the mouse equivalent of SEN6.
FISH analysis of this locus in KIM2 cells showed a large variation in the number of chromosome 17 per cell (Figure 2.11). Similar to the previous FISH analysis, there was no evidence of consistent loss of chromosome 17 in NKIM2 or TKIM2 cells, and in order to fully characterise the SEN6 locus in the KIM2 cells, a range of experiments would be required. Previous studies in human lines, have shown that loss of chromosome 6q26-27 underlie SV40 mediated immortalization of human fibroblasts [134]. In this model, expression of T antigen extends the lifespan of the fibroblasts only, but chromosome loss of SEN 6 was required to immortalize them. However a key difference between the human fibroblast model and the KIM2 model is that unlike murine cells, human fibroblasts almost never immortalize spontaneously. Therefore, it may be that human cells have a more complex hierarchy of controls that have to be breached in order for tumour formation [134].

So in conclusion, to what extent do TKIM2 cells show features of transformation?
The TKIM2 cells fulfill all the criteria for a transformed cell line including lack of junctions and contact inhibition; serum-independence; growth in soft-agar and tumour formation in immunosupressed mice. In general the NKIM2 cells do not fulfil these criteria, and could be considered non-transformed. However, the main evidence which does not fit this model, is the tumour formation of NKIM2 cells in scid mice.
Both T antigen and p53 localise to the nucleus in NKIM2 and TKIM2 cells, and it is likely that T antigen is abrogating the function of p53, resulting in general chromosomal variability, in both lines. Examination of the murine equivalent of p53 and the SEN6 locus, showed no evidence for consistent loss in either NKIM2 or TKIM2 lines.

3 Retrovirus

The data in this section refer to the retroviral transduction of NKIM2 and TKIM2 cells with Tiam 1 constructs, which was assessed by Western analysis. The Western analyses were repeated on three occasions, using cells harvested from confluent T25 flasks, with equal protein concentrations loaded in each lane. Standard protein markers were used to assess the different sizes of the truncated control and active protein Tiam1 constructs detected by the Tiam1 antibody.

3.1 Endogenous levels of Tiam1 in untransduced NKIM2 and TKIM2

Western analysis of endogenous levels of full length Tiam1 in both untransduced NKIM2 and TKIM2 identified practically zero levels of protein. Figure 3.1 illustrates Western analysis in untransduced NKIM2 (passage 20) and TKIM2 (passage 6) showing no bands at the expected size (~200kD). The positive control was neural tissue which is known to express high levels of endogenous Tiam1 and which is associated with numerous breakdown products. It may be that the very faint lower bands represent breakdown products of endogenous Tiam1 in both NKIM2 cells and TKIM2 cells (R.K. personal communication).

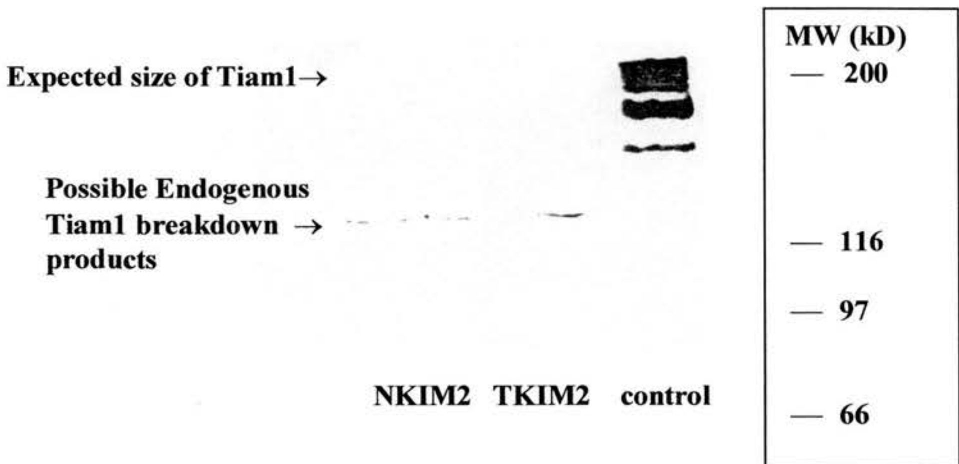


Figure 3.1 Western analysis of endogenous Tiam1 in NKIM2 and TKIM2.

3.2 Transduction of Tiam 1 into NKIM2 and TKIM2

The two retroviral constructs that were used to infect target NKIM2 and TKIM2 cells were C1199 Tiam1 - the active construct with an expected size of 160 kilodaltons (kd), and C580 Tiam1- the control construct with an expected size of 81 kd (see Materials).

The constructs contained antibiotic-selection markers, so that cells that were not transduced with the constructs did not survive when the G418 antibiotic was added to the maintenance medium following transduction.

The efficiency of transduction was judged to be high (approximately 90% of cells survived antibiotic selection). After transduction, the control and active Tiam1 constructs appeared to be strongly expressed in approximately equal amounts in all four transduced lines on Western analyses. Figure 3.2 illustrates the Western analysis showing high Tiam1 protein levels compared to the endogenous Tiam1 products seen in the untransduced lines.

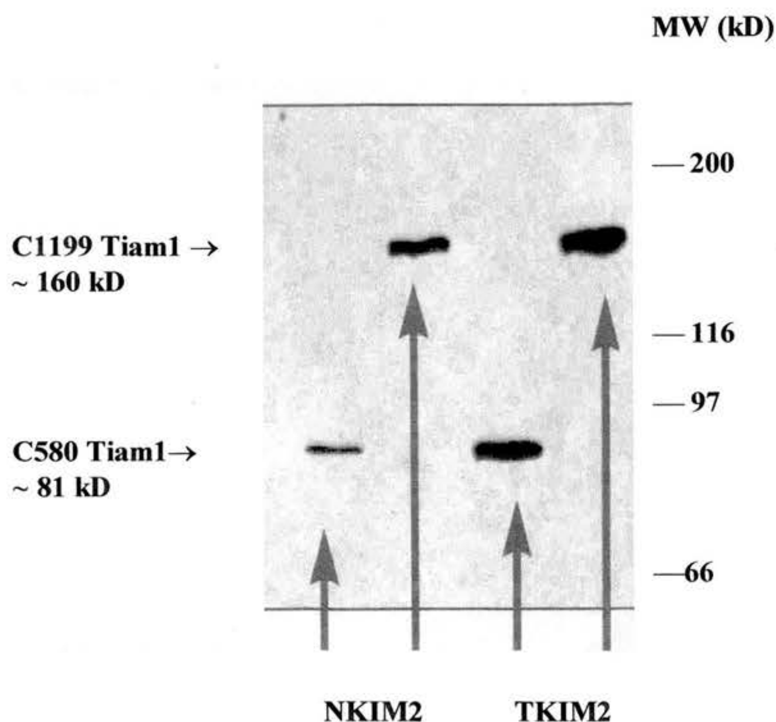


Figure 3.2 Western analysis of retroviral Tiam1 transduction

3.3 Tiam1 construct expression

Western analysis showed that transduced contructs were expressed for atleast 3 months following transduction. Figure 3.3 illustrates Western analysis repeated after both NKIM2 (passage 47) and TKIM2 cells (passage 40) had been continuous culture for 3 months. Both C1199 Tiam1 and C580 Tiam1 construct expression remained present, however the levels of expression of the active C1199 Tiam1 constructs are slightly reduced compared with control C580 Tiam1 constructs.

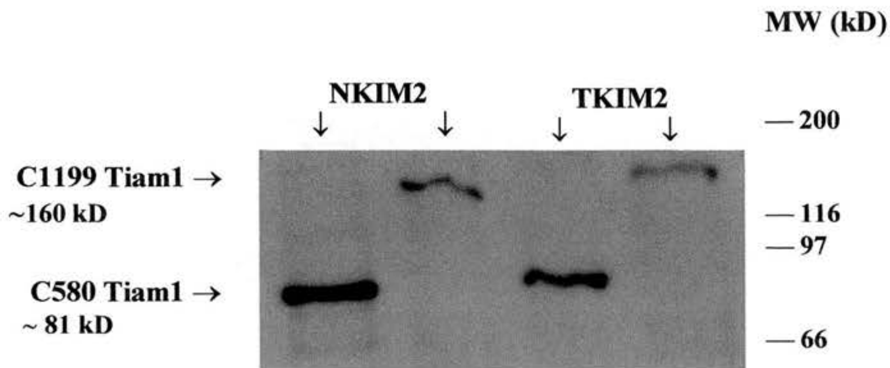


Figure 3.3 Western analysis of Tiam1 expression after 3 months passaging of NKIM2 (p47) and TKIM2 (p40).

3.4 Discussion

The data shows that endogenous levels of Tiam1 in both NKIM2 and TKIM2 are negligible (Figure 3.1), with the exception of possible faint breakdown products identified on Western analysis. The lack of endogenous full length Tiam1 in NKIM2 cells concurs with previous studies of murine Tiam1 expression, which have shown low or neglible levels in most normal tissues, except brain and testis [245]. The low expression of Tiam1 in normal tissues may be a result of the instability of the protein, which is probably mediated by the PEST domain in its structure [230]. Moreover, at a functional level guanine nucleotide exchange factor is required to continually exchange GDP to GTP on Rac, and therefore it could be predicted that protein levels would be tightly regulated. The low level of endogenous Tiam1 in TKIM2 lines is not consistent with the majority of transformed cell lines, which show in general show high Tiam1 expression [245]. In particular the human HBL-100 mammary epithelial line immortalised by SV40 large T antigen expresses Tiam1 at high levels, as does murine breast carcinoma cell line TA3 ST. However certain tumour or transformed lines have much lower levels of Tiam1 expression, for example the MCF7 cell line [245]. In all the transformed cell lines examined, the normal 7 kb Tiam1 transcript was found without evidence for major alternative splice events or truncation.

Retroviral transduction of C1199 and C580 Tiam1 constructs resulted in strong and approximately equal expression in all four cell populations (Figure 3.2). This concurs with previous studies using the same C1199 Tiam1 and control C580 constructs in phenotypic reversion of Ras-transformed epithelial MDCK cells [242].

Ideally as part of the retroviral transduction, a control empty retroviral vector should have been transduced into the KIM2 cells, together with the Tiam1 construct, but there was not sufficient virus for this. However the identical vector had been used in the Ras -MDCK study under the same conditions, showing that it was not responsible for the biological effects observed [242].

The data shows that Tiam1 construct expression remained present during three months passaging ie to passage 40 in NKIM2 cells and passage 47 in TKIM2 cells (Figure 3.3). The experiments characterising phenotypic reversion in transduced cells, were therefore carried out between passages 20-40 (Results chapter 4). One of the accepted features of retroviral transduction is that the continued retroviral gene expression might drop over a period of weeks to months depending on the cell line, site of integration, relative toxicity of the insert [203, 209].

This may explain the slightly reduced expression of the active Tiam1 construct at the end of the three month period .However in general the expression of the Tiam1 constructs remained stable over an appropriate experimental period.

So, in conclusion untransduced NKIM2 and TKIM2 has very low levels of endogenous Tiam1, and both lines were successfully transduced with active and control constructs. The expression of these constructs remained present for atleast 3 months in cell culture.

4 Phenotypic Reversion

This section contains data referring to the KIM2 cell lines after retroviral transduction with active and control Tiam1 constructs.

The Tiam1 constructs comprise the C1199 Tiam1 construct which has a N-terminal truncation and is active at areas of cell-cell contact [234], and a short C580 Tiam1 construct which is also N- terminally truncated, but has been shown in previous studies to localise to the cytoplasm [234]. These will be referred to Tiam-active and Tiam1-control respectively. Table 4 summarises the terminology used for the four transduced cell lines.

Cell line terminology	Description
NKIM2 plus Tiam1-control	Normal KIM2 cells transduced with control C580 Tiam1construct
NKIM2 plus Tiam1-active	Normal KIM2 cells transduced with active C1199 Tiam1 construct
TKIM2 plus Tiam1-control	Transformed KIM2 cells transduced with control C580 Tiam1 construct
TKIM2 plus Tiam1-active	Transformed KIM2 cells transduced with active C1199 Tiam1 construct

Table 4 Terminology referring to the four transduced cell lines

4.1 Morphology of transduced cell lines

As expected NKIM2 cells transduced with Tiam1-control constructs retained an epithelial, cobblestone morphology (Figure 4.1 a). However although the majority of NKIM2 cells transduced with Tiam1-active constructs remained epithelioid, certain cells appeared rounded and showed ruffling of their membranes (Figure 4.1 b). A cells showing membrane ruffling is identified by the red arrow.

The morphology of all transduced cells lines remained constant after intervals of 48 hours, 7 days and 14 days following transduction.

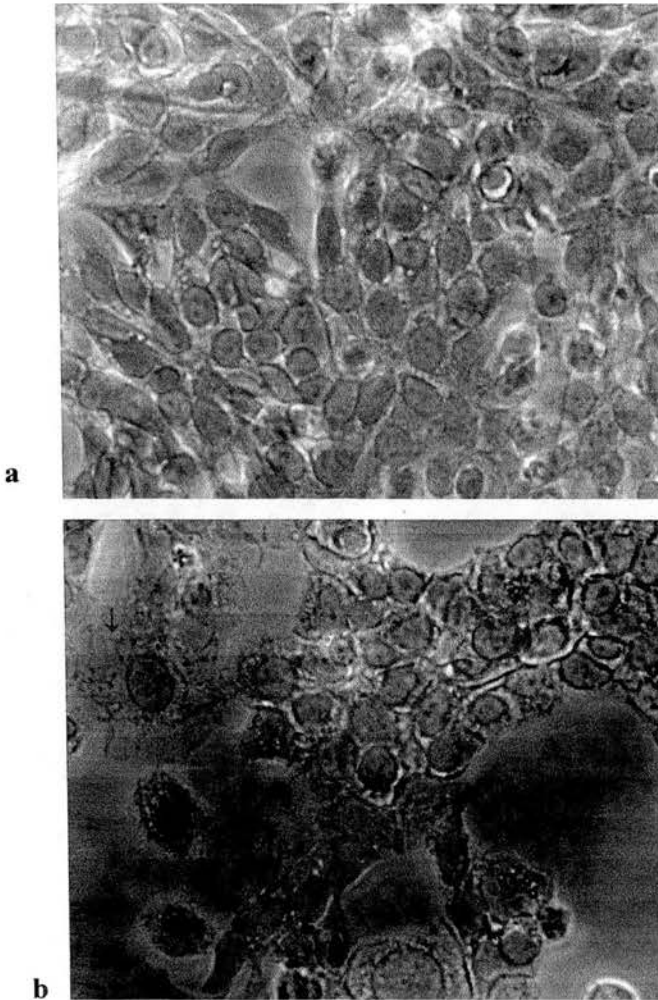


Figure 4.1 a,b Morphology of NKIM2 cells transduced with a, Tiam1-control and b, Tiam1-active (x25)

TKIM2 cells transduced with Tiam1-control remained spindle- shaped with a lack of contact inhibition, continuing to grow in multilayers (Figure 4.1.1 a), however TKIM2 cells transduced with Tiam1-active showed a remarkable change in phenotype, with cells becoming rounded and gaining an epithelioid appearance Figure 4.1.1 b). They showed contact inhibition of movement, with no evidence of multilayering. However certain cells showed ruffling of their membranes similar to NKIM2 cells in Figure 4.1 b.

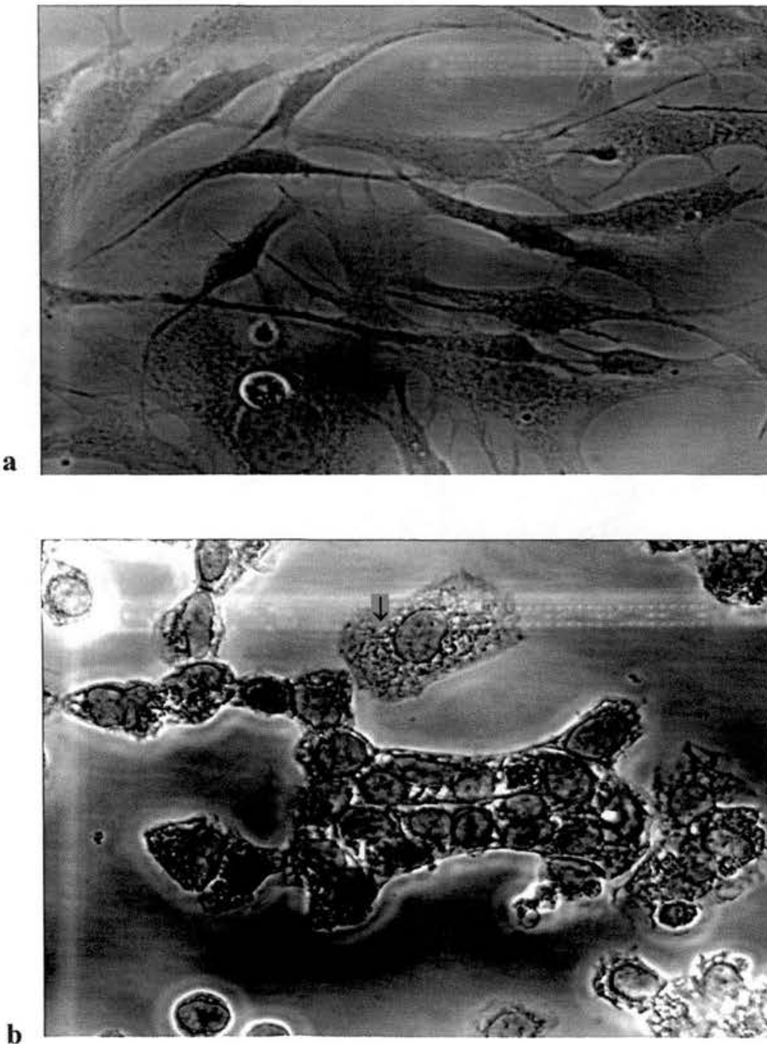


Figure 4.1.1 Morphology of TKIM2 cells transduced with a, Tiam1-control and b, Tiam1-active. b, contains cells showing membrane ruffling (↓) (x 25)

Dome formation

When all four cell lines were grown in differentiation medium for 12 days, only NKIM2 cells transduced with Tiam1-control formed domes, the remaining cell lines appeared unchanged in morphology. Figure 4.1.2 illustrates dome formation in NKIM2 cells transduced with Tiam1-control.

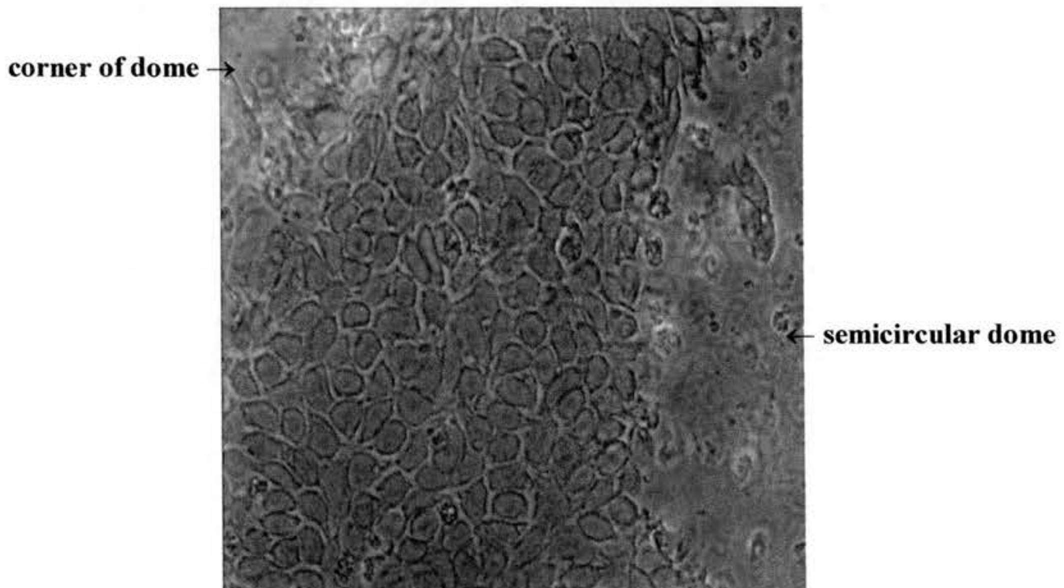


Figure 4.1.2 Dome Formation in NKIM2 cells transduced with control Tiam1 (x10)

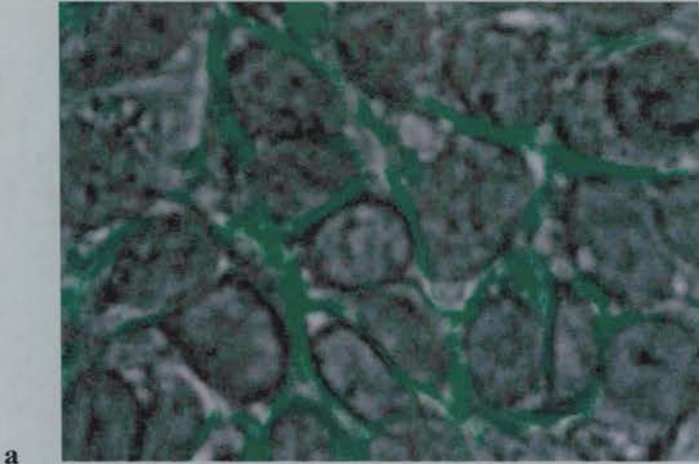
4.2 Presence of myoepithelial or vimentin marker expression

There was no evidence of α-actin or vimentin expression in any of the four cell lines on repeated immunofluorescence experiments, in the presence of positive controls. The black background seen in a negative result has been illustrated previously in Results section1 (Figure 1.2.2 b)and therefore will not be illustrated here.

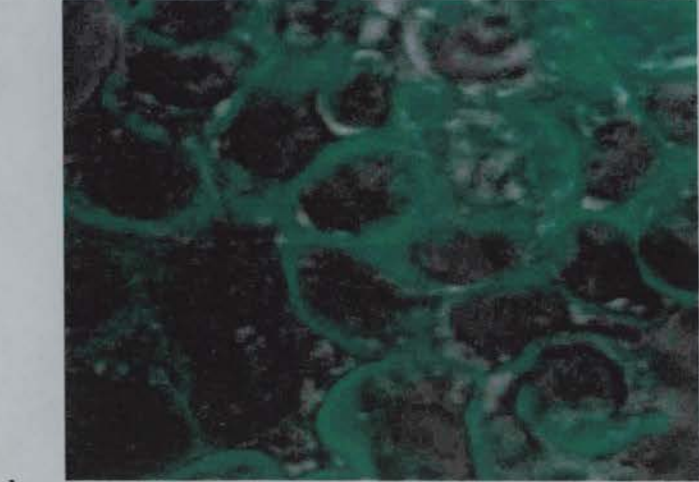
Furthermore, no myoepithelial filaments were identified on electron microscopy in all four cell lines.

4.3 Epithelial marker expression E- cadherin

E-cadherin expression occurred in both NKIM2 cell lines transduced with Tiam1-control and Tiam1-act. Figure 4.3 a,b illustrates a comparison of E-cadherin expression in both cell lines, showing that it is distributed between cell boundaries in both. There appears to be an increase in E-cadherin expression in NKIM2 transduced with Tiam1-active compared with NKIM2 transduced with Tiam1-active .

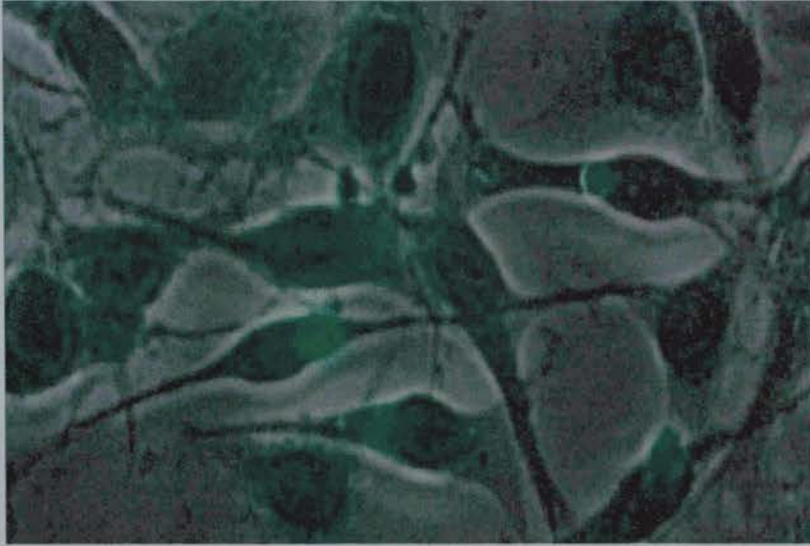


a

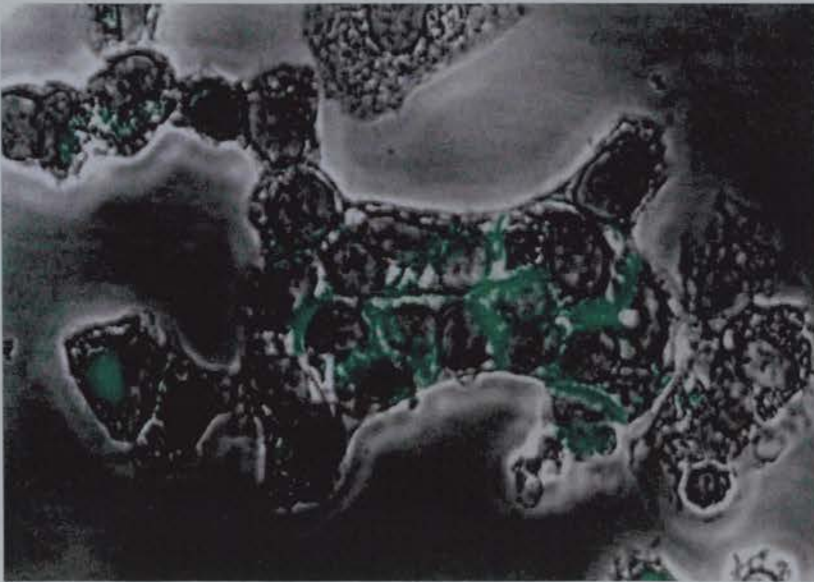


b **Figure 4.3 E-cadherin expression in NKIM2 cells transduced with a, Tiam1-control and b Tiam1-active (x40)**

E-cadherin expression in TKIM2 cells transduced with Tiam1-control showed a diffuse distribution whereas the reverted TKIM2 cells transduced with Tiam1-active, showed E-cadherin expression at certain, but not all cell-cell boundaries. Figure 4.3.1 a,b illustrates a comparison of E-cadherin expression in both the TKIM2 lines.



a



b

Figure 4.3.1 Comparison of E-cadherin expression by immunofluorescence in TKIM2 cells transduced with a Tiam1-control (x25) and b, Tiam1-active (x 40)

In order to determine whether the differences in E-cadherin expression distribution with change in morphology on immunofluorescence (Figure 4.3 a,b) was reflected by changes in protein levels,Western analysis was carried out using equal protein concentrations in each lane (Figure 4.3.2). This illustrated a large increase in levels of E-cadherin protein in NKIM2 cells transduced with Tiam1-active compared with Tiam1-control. However the increase between TKIM2 transduced with Tiam1-active compared with Tiam1-control was only slight. The positive control used was untransduced NKIM2 cells-which had high levels of E-cadherin confirmed by immunofluoresence. Therefore TKIM2 cells transduced with Tiam1-active expressed protein at lower levels to the control, whereas NKIM2 cells transduced with Tiam1-active expressed E-cadherin at much higher levels.

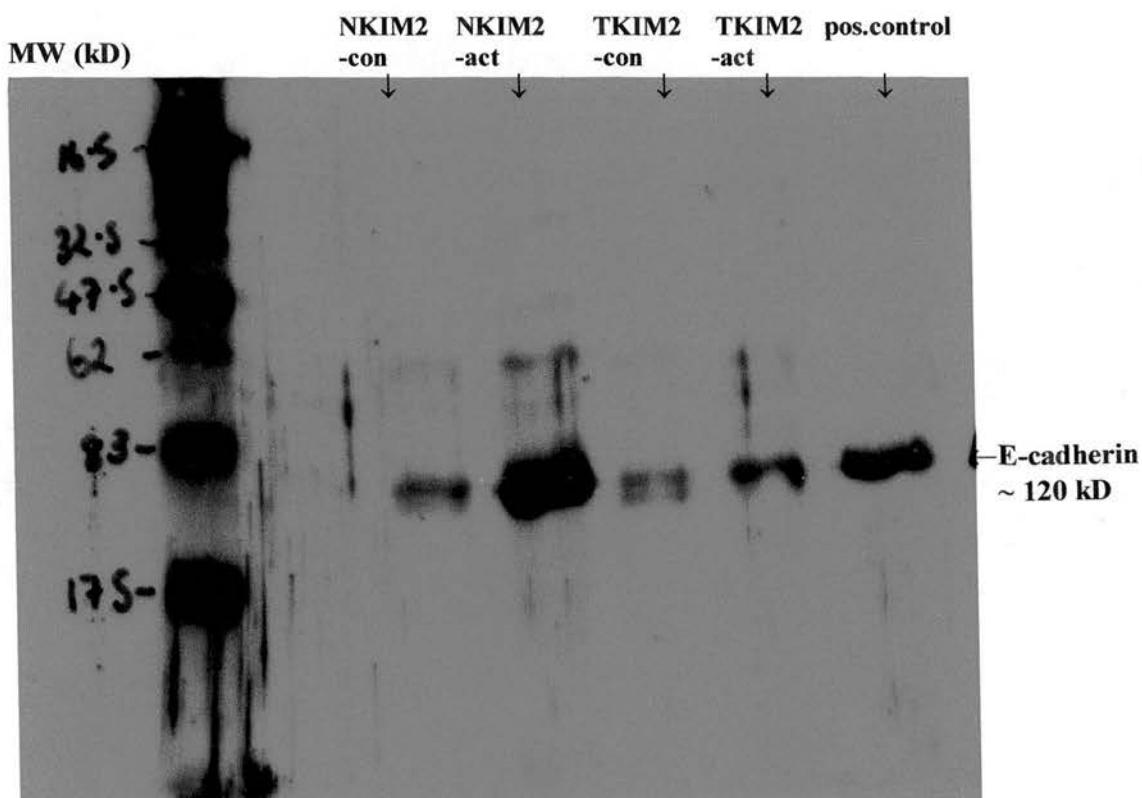
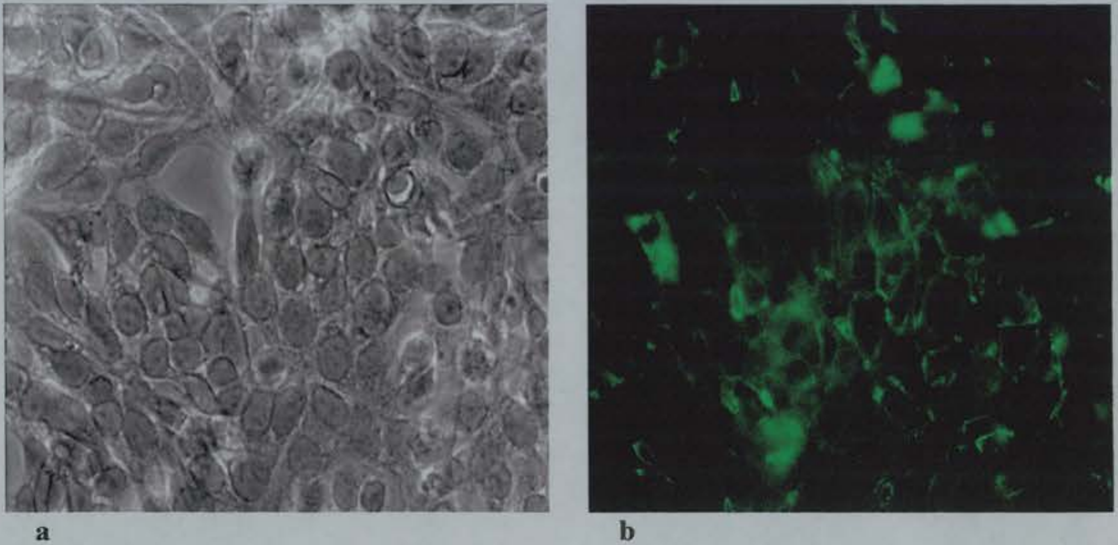


Figure 4.3.2 Western analysis of E-cadherin levels in cell lines transduced by Tiam1

ZO-1 expression was present in NKIM2 cells transduced with Tiam1-control and Tiam1-active only, but not in either TKIM2 cell line. Figure 4.3.3 illustrates ZO-1 expression in NKIM2 cells transduced with Tiam1-control. The expression differed from ZO-1 staining in untransduced cells in that it was not uniform at all cell-cell boundaries.



a b

Figure 4.3.3 Phase microscopy and ZO-1 expression in NKIM2 cells transduced with Tiam1-control

The epithelial marker expression in all four cell lines is summarised in Table 4.3.3

Cell line	E-cadherin	ZO-1
NKIM2 plus Tiam1-control	at all cell-cell boundaries	at certain cell-cell boundaries
NKIM2 plus Tiam1-active	at all cell-cell boundaries	at certain cell-cell boundaries
TKIM2 plus Tiam1-control	diffuse expression	negative
TKIM2 plus Tiam1-active	at certain cell-cell boundaries	negative

Table 4.3.3 Summary of epithelial marker expression in transduced NKIM2 and TKIM2 cell lines

4.4 Electron microscopy of junctions

Both NKIM2 cells transduced with Tiam-control and Tiam1-active had an epithelial appearance with microvilli on their apical surface. Figure 4.4 illustrates NKIM2 cells transduced with Tiam1-control. The cells show no pleomorphism or irregular features. Tight junctions are identified between certain cells.

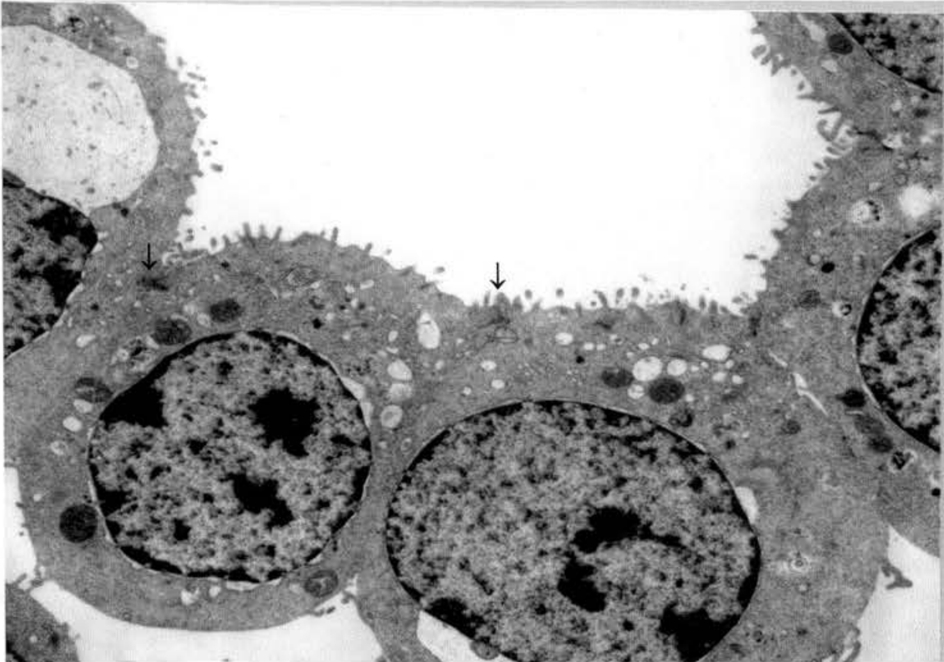


Figure 4.4 Morphology of NKIM2 cells transduced with Tiam1-control by electron microscopy (neg mag x2100, print mag x3034)
The arrows denote tight junctions which can be observed between certain cells at their apical margins

An illustration of the junctions identified in untransduced NKIM2 cells is included (Figure 4.4a) for direct comparison with junctions formed in NKIM2 cells transduced by Tiam-control and Tiam-active on the following page.

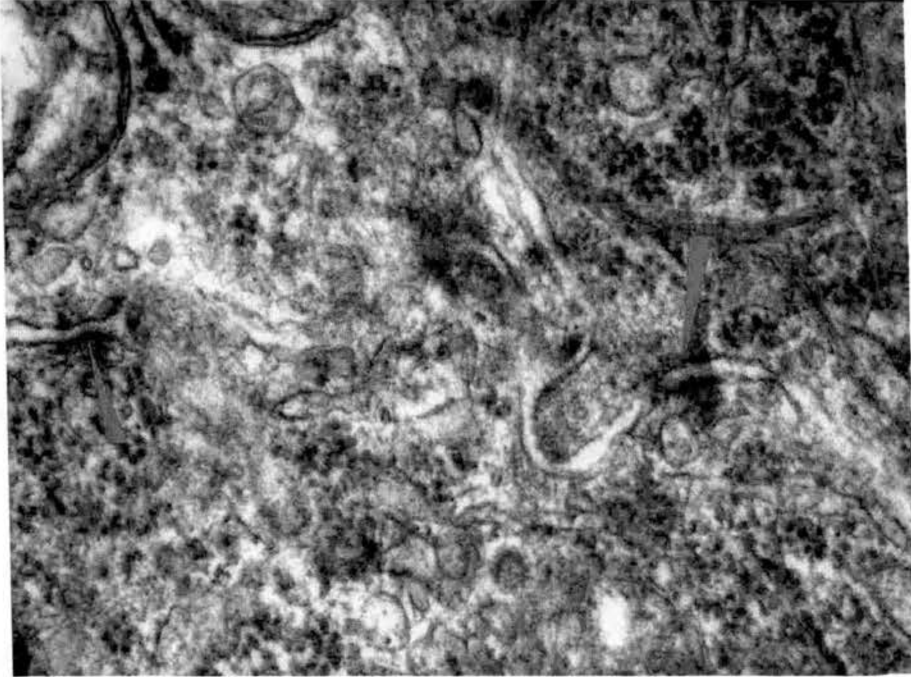
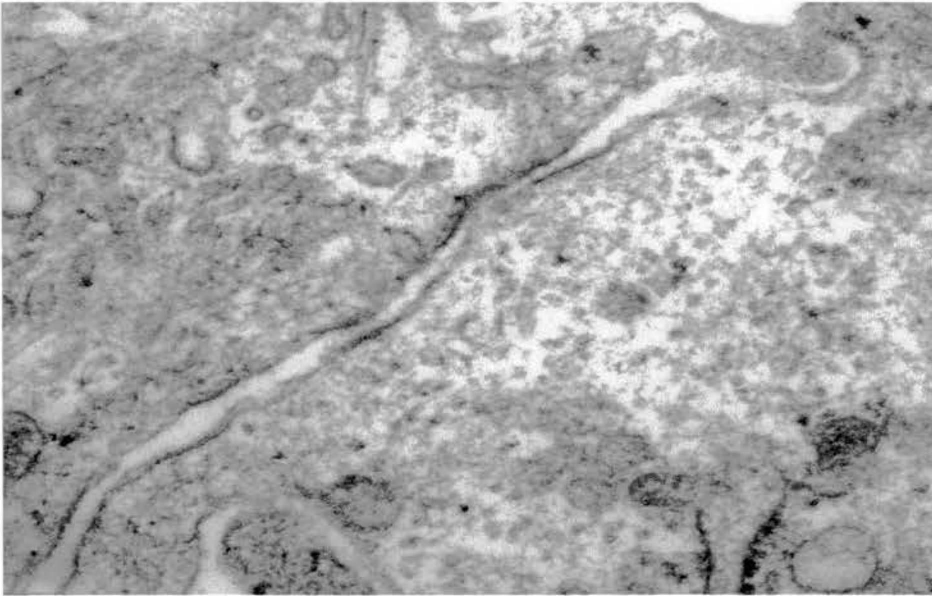
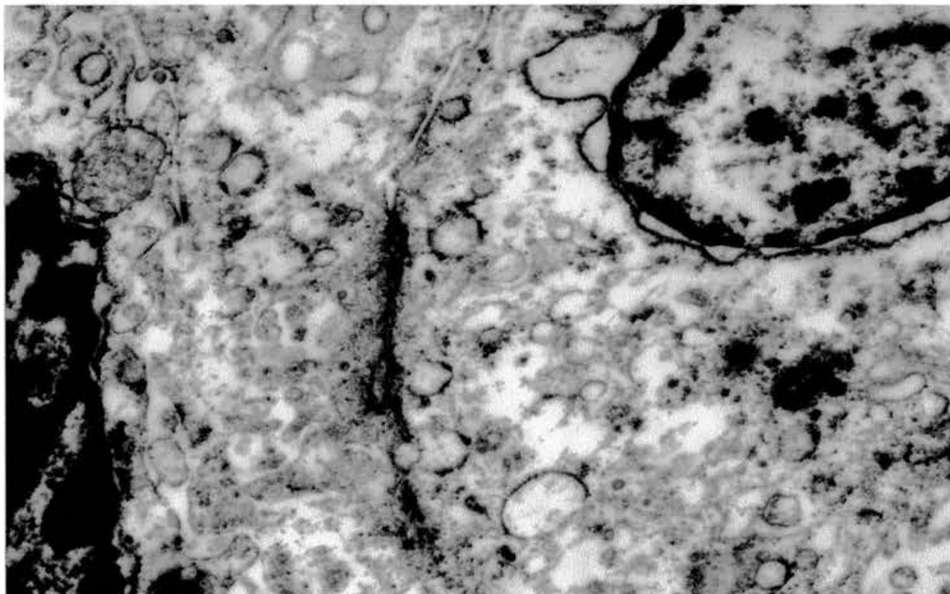


Figure 4.4a Two desmosomes between NKIM2 cells on electron microscopy (neg. mag. x 41580, print mag. x 83160) for comparison with Figure 4.4.1

Electron microscopy of junctional formations at higher power show that junctions are present in both NKIM2 transduced with Tiam1-control and Tiam1-active. The majority of junctional specialisations were adherens junctions, however these did not include desmosomes or hemidesmosomes, as due to the lack of electron dense parajunctional cytoplasmic filaments. Figure 4.4.1 a,b illustrates junctions formed in NKIM2 transduced with Tiam1-control and Tiam1-active respectively.



a



b

Figure 4.4.1 Junctional formations in NKIM2 cells transduced by a, Tiam1-control and b,Tiam1-active (neg mag x 5780, print mag x 12000)

The morphology of TKIM2 cells transduced with Tiam-control showed pleomorphic cells with irregular cell surfaces similar to untransduced TKIM2. Figure 4.42 illustrates the cells which have no junctions between cells, and no apical microvilli.

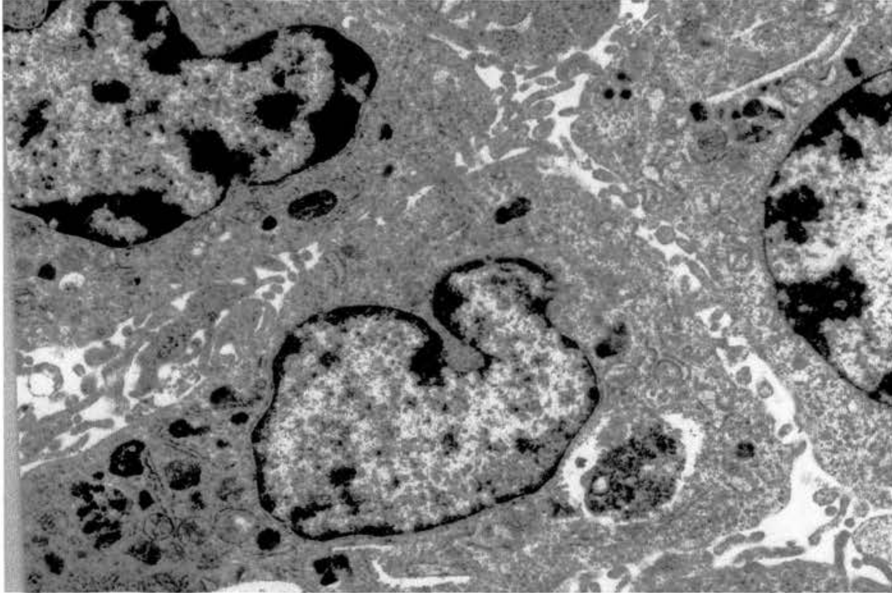


Figure 4.4.2 TKIM2 cells transduced with Tiam1- control by electron microscopy (neg mag x 6640, print mag x 12600)

TKIM2 cells transduced with Tiam1-active had an unexpected irregular appearance on electron microscopy (Figure 4.4.3). Although cells appeared more rounded than TKIM2 cells transduced with Tiam1-control (above), they were still pleomorphic and not polarised. However, the red arrow denotes a junctional formation.

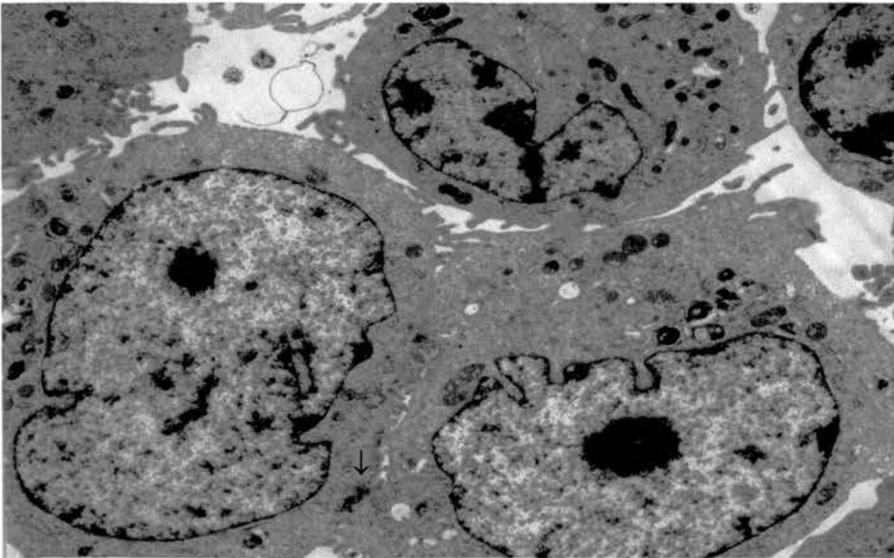


Figure 4.4. 3 TKIM2 cells transduced by Tiam1-active by electron microscopy (neg mag x 5560 , print mag x 11200). A junctional formation is denoted by (↓)

Further examination of these occasional junctional specialisations at higher power, showed that they were of adherens type, but not desmosomes or hemidesmosomes because they lacked parajunctional densities. Tight junctions were not identified in the reverted TKIM2 cells transduced with Tiam 1-active. Figure 4.4.4 illustrates a junctional specialisation seen in reverted TKIM2 transduced with Tiam1-active.

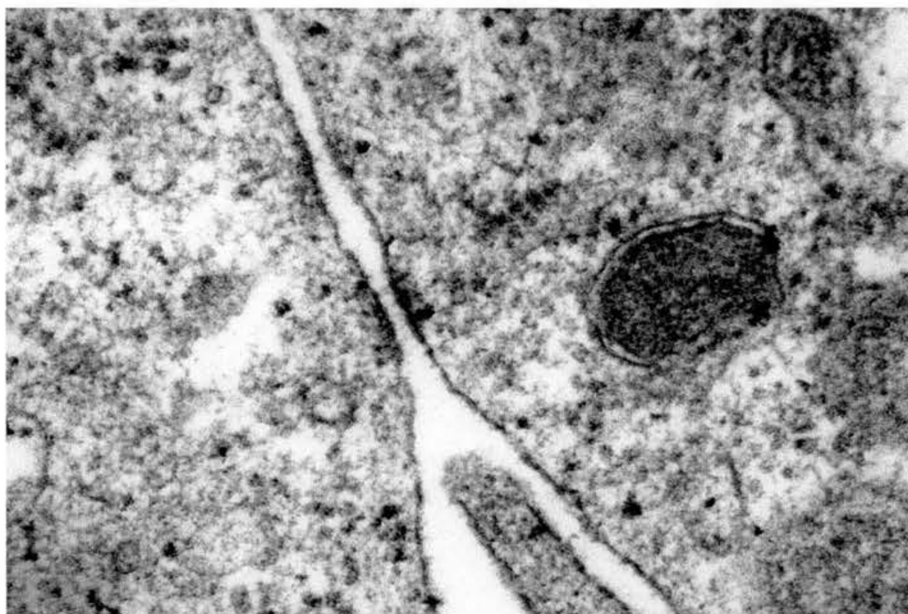


Figure 4.4.4 Adherens junctional specialisation seen in TKIM2 cells transduced with Tiam1-active (neg mag x 7075, print mag x 13500).

4.5 Polarisation

Figure 4.4 and 4.4.2 illustrated the only two populations which had apical microvilli ie showed polarisation on electron microscopy were NKIM2 cells transduced with Tiam1-control and NKIM2 cells transduced with Tiam1-active.Both TKIM2 lines transduced with Tiam1-active and Tiam1-control lacked apical microvilli (Figure 4.4.2 and Figure 4.4.3)

4.6 Milk protein expression

β-casein protein production was identified in all transduced cell lines exposed to differentiation medium, as immunoprecipitation analysis of all four transduced cell lines illustrates (Figure 4.6). Although immunoprecipitation analysis allows clean specific identification of the desired protein when using a polyclonal or less specific antibody, the relative amounts of protein produced in each population is not assessed by this assay. Lactating mammary gland was used as the positive control.

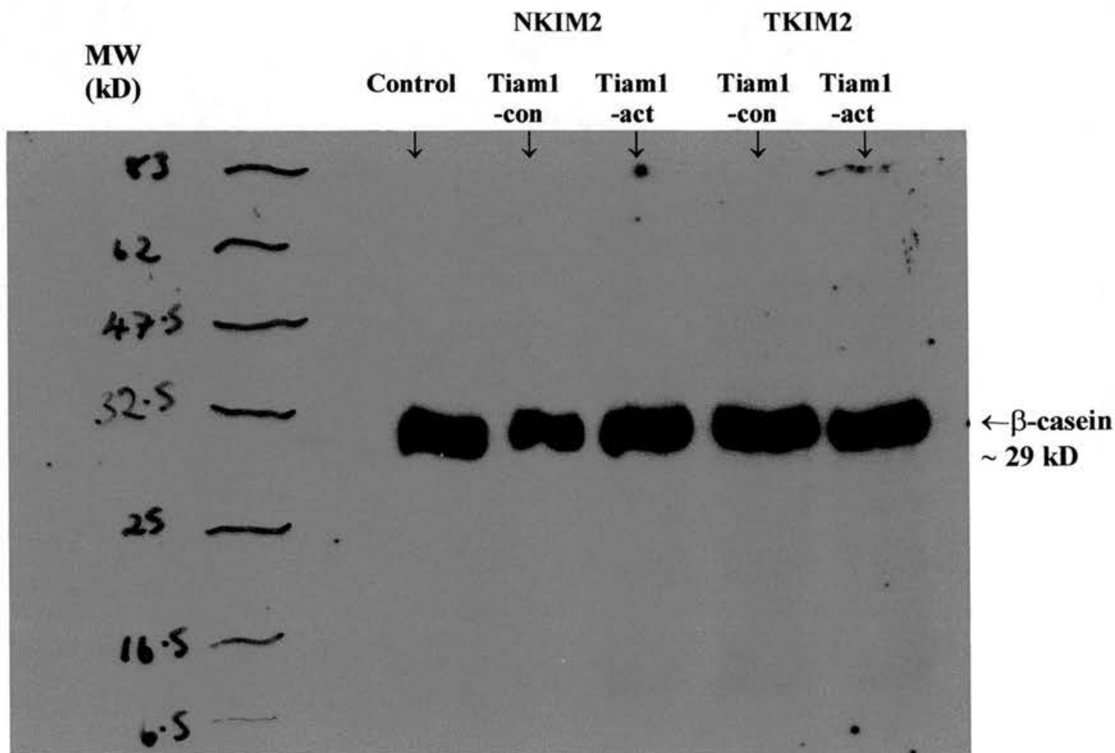


Figure 4.6 Immunoprecipitation analysis showing that all transduced cell lines express β- casein protein after incubation in differentiation medium for 12 days. Analyses were repeated three times.

Further Western analysis (Figure 4.6.1) of the relative amounts of β-casein in the cell lines demonstrated that levels were high in NKIM2 transduced with Tiam1-active and Tiam1-control, and TKIM2 transduced with Tiam1-active. However as expected β-casein protein levels were low in TKIM2 transduced with Tiam1-control. Lactating mammary gland was used as a positive control, similar to the previous experiment. The molecular weights of the protein markers used are indicated in kilodaltons parallel to the gel.

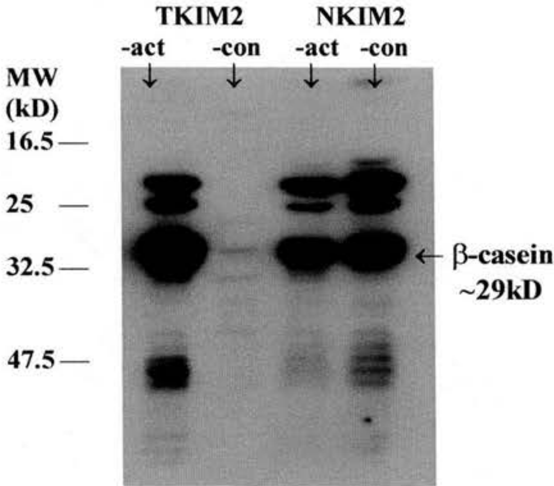


Figure 4.6.1 Western analysis of β-casein expression in transduced cell lines. Analyses were repeated three times.

4.7 In vitro growth kinetics

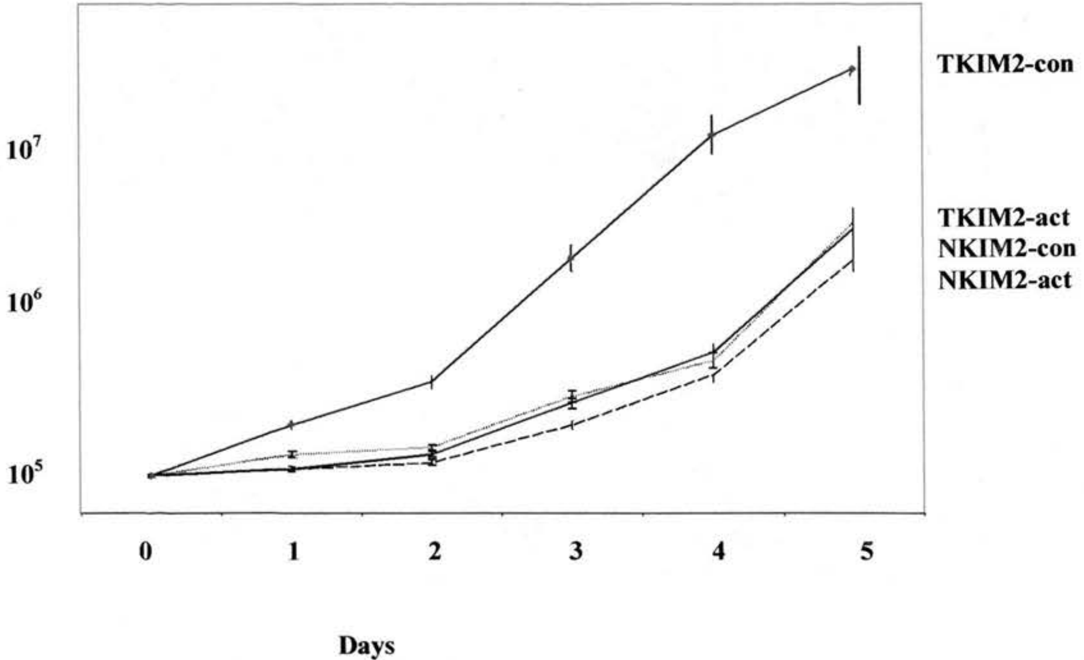


Figure 4.7 In vitro growth kinetic of transduced cell lines in standard serum and 37°C. The points represent mean values and standard deviations from triplicate expts.

The growth curves of the four transduced cell lines were compared, similar to previous analysis in results chapter 2. The cells were seeded at the same density (1x 10^5 cells) in regular growth medium at 33°C and 37°C to control for temperature effects. At 24 hour intervals culture plates within each series were harvested and the cells counted. The results obtained are from triplicated experiments, and a similar result was obtained at both growth temperatures. Figure 4.7 illustrates that TKIM2 cells transduced with Tiam1-control had a high growth rate. However TKIM2 cells transduced with Tiam1-active had a lower growth rate, similar to both NKIM2 cells transduced with Tiam1-control and Tiam1-active.

4.8 Serum dependence

In order to investigate the growth kinetics of the transduced cell lines without serum , they were seeded at the same density (1x 10^5cells) at 33°C and 37°C in normal medium for 24 hrs, then washed with serum free medium, before being seeded in 0% serum. They were harvested and cells counted, similar to the previous experiment. Figure 4.8 illustrates the ability of TKIM2 cells transduced with Tiam1-control to grow in serum-free conditions compared to the TKIM2 transduced with Tiam1-active, and both NKIM2 cell lines transduced with Tiam1-control and Tiam1-active. The growth of TKIM2 cells transduced with Tiam1-control is illustrated in Figure 4.8.1. Similar results were obtained at both growth temperatures.

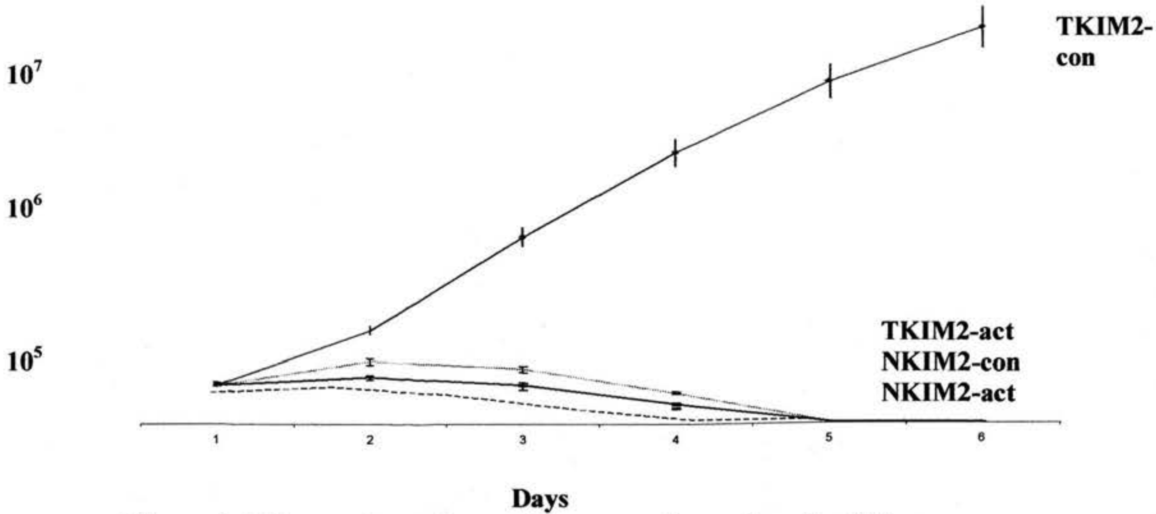


Figure 4.8 Comparison of growth curves of transduced cell lines in standard serum-free conditions (37°C).

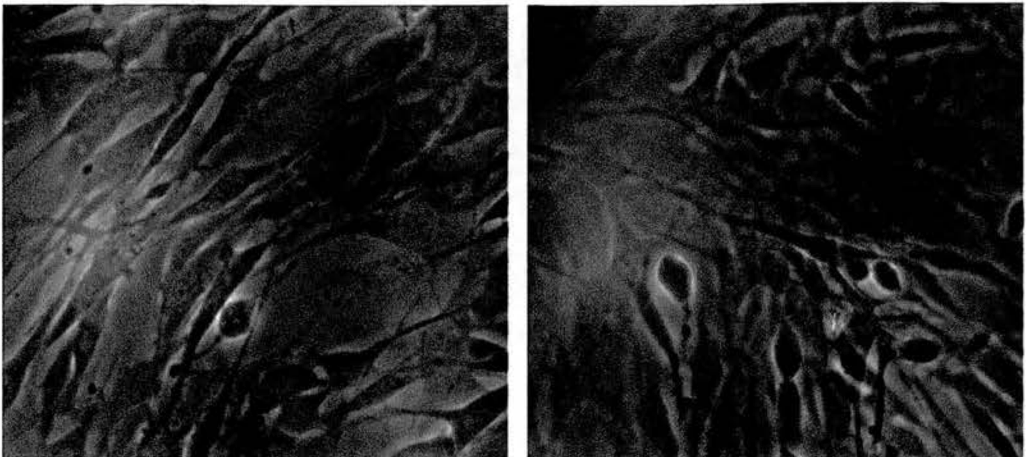


Figure 4.8.1 Representative fields showing growth of TKIM2 transduced with Tiam1-control at a, 3 days and b, 6 days after seeding in serum free medium

4.9 Soft agar formation

To assess colony formation in soft agar TKIM2 and NKIM2 were seeded into soft agar at a series of cell densities at 37°C and 33°C. Positive control Ras-transformed and negative control non-transformed fibroblast cell lines were supplied by D.B. Table 4.9.1 shows that both TKIM2 cells transduced with Tiam1-control and Tiam1-active retained the ability to form colonies in soft agar, compared with both NKIM2 transduced lines. However the TKIM2 cells transduced with Tiam1-active formed fewer colonies than the TKIM2 cells with Tiam1-control. Figure 4.9.2 illustrates the growth of both TKIM2 lines in soft agar. Similar results were obtained at both growth temperatures.

Cells seeded	TKIM2 -control	NKIM2-control
$2x10^4$	12.6 +/- 1.5	0
$1x10^4$	11.0 +/- 2.0	0
$4x10^3$	9.2 +/- 1.2	0
$2x10^3$	8.4 +/-2.0	0
$1x10^3$	6.4 +/-1.7	0
	TKIM2 -active	**NKIM2-active**
$2x10^4$	3.0 +/-1.6	0
$1x10^4$	2.4 +/- 3.0	0
$4x10^3$	1.0 +/- 1.4	0
$2x10^3$	1.3 +/- 1.2	0
$1x10^3$	1.2 +/- 1.4	0

Table 4.9.1 Comparison of Colony formation in soft agar of transduced TKIM2 and NKIM2 at 37°C. Values are means and standard deviations.

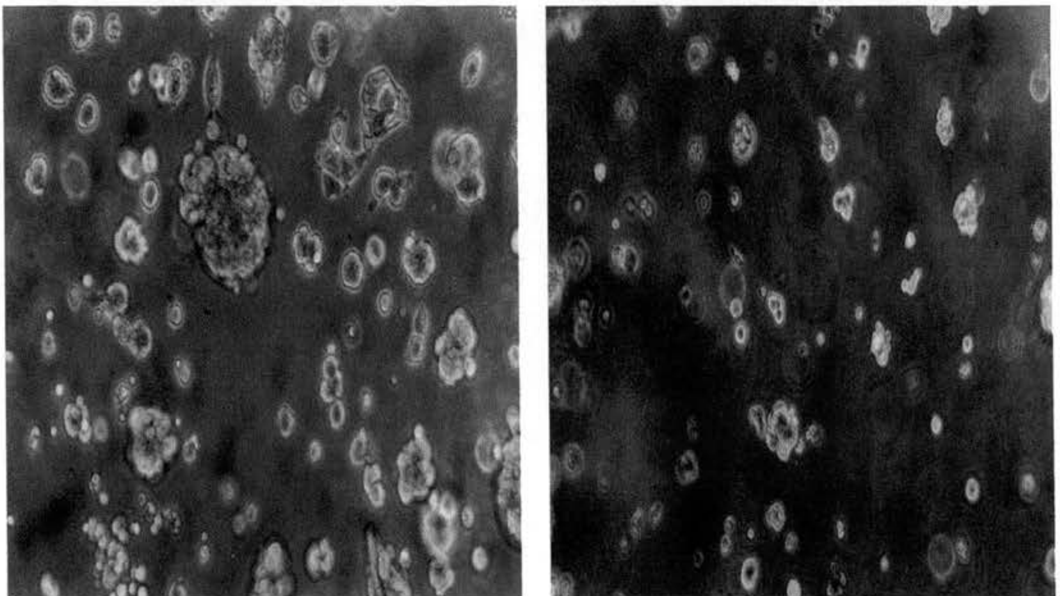


Figure 4.9.2 Growth in soft agar of a, TKIM2-control and b, TKIM2-active, 14 days after seeding (x10) Colonies of 5mm diameter and above were scored (37°)

4.10 Injection into scid mice

Injection of the transduced cell lines in to scid mice resulted unexpectedly in the formation of a single tumour in the NKIM2 transduced with control Tiam1, but not in the other 3 cell lines (Table 4.10). The size of the tumour harvested was 10mm and its histology which appeared to be composed of pleomorphic spindle cells with few mitoses is illustrated in Figure 4.10.1. The mean tumour volume had not been measured sequentially during the 4 month period.

Cell line	Incidence of tumours (Take rate0	Size of tumours after 4 months (Diameter in mm)
NKIM2-control	1/8 (12.5%)	10 mm
NKIM2-active	0/8 (0%)	—
TKIM2-control	0/8 (0%)	—
TKIM2-active	0/8 (0%)	—

Table 4.10 Incidence of tumour formation in scid mice, in transduced cell lines

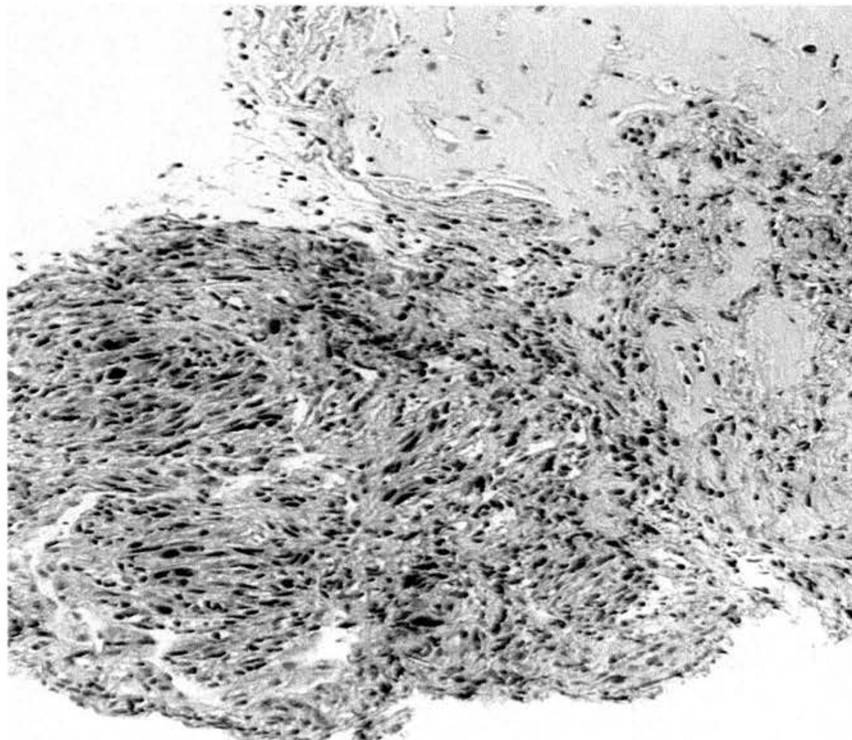


Figure 4.10.1 Single tumour formed on injection of NKIM2 transduced with Tiam1-control into scid mice (x 25)

4.11 Discussion

To date this study presents the first reversion by Tiam1 of a mammary epithelial cell line transformed by SV40 large T antigen. The only previous reversion using Tiam1, described Hordijk et al, involved reverting Madin Derby Canine Kidney epithelial (MDCK) cells, which were transformed by Ras [242]. This discussion aims to evaluate the degree of epithelial and mammary differentiation, and features of transformation in the reverted cells. For ease of discussion, the features relating to epithelial and mammary differentiation in the transduced cell lines are summarised in Table 10.

	NKIM2 plus Tiam1-control	**NKIM2 plus Tiam1-active**	**TKIM2 plus Tiam1-control**	**TKIM2 plus Tiam1-active**
Morphology	epithelial	epithelial	spindle	epithelioid
E-cadherin expression	at all cell-cell boundaries	at all cell-cell boundaries	diffuse	at certain cell-cell boundaries
ZO-1 expression	at certain cell-cell boundaries	at certain cell-cell boundaries	no expression	no expression
Junction formation	adherens and tight junctions	adherens and tight junctions	no junctions identified	occasional adherens junctions
Polarisation	apical microvilli	apical microvilli	no apical microvilli	no apical microvilli
α-actin or vimentin expression	no expression	no expression	no expression	no expression
Dome formation in differentiation medium	dome formation	no dome formation	no dome formation	no dome formation
β-casein expression	β-casein expression	β-casein expression	β-casein expression	β-casein expression

Table 10 Summary of features regarding epithelial and mammary differentiation displayed by NKIM2 and TKIM2 transduced with control and active Tiam1

In terms of epithelial differentiation, the morphological data confirm that transduction of TKIM2 cells with active Tiam1 results in a reversion of the transformed phenotype (Figure 5.11 a,b) to an epithelioid appearance, whereas TKIM2 cells transduced with control Tiam1 remain essentially unchanged. Although the morphological change is striking, it is clear that certain reverted cells show membrane ruffling –a cytoskeletal change mediated by the actin cytoskeleton [223]. Similarly, although both control and active Tiam1 do not alter the essential epithelial appearance of NKIM2 cells, the active Tiam1 causes rounding of the cells, with some evidence of membrane ruffling (Figure 5.11b). Hordijk et al. did not observe membrane ruffling after transduction of MDCK cell lines with Tiam1 [242], however previous work in fibroblasts and COS cells has shown that the Tiam1 is required for Rac-dependent membrane ruffling [233]. At a mechanistic level, membrane ruffling occurs when Rac is activated by agonists such as platelet-derived growth factor or insulin [229]. This leads to the assembly of a meshwork of actin filaments at the periphery of the cell to produce lamellipodia and membrane ruffles, which may form integrin-associated complexes [229, 247]. It may be that overexpression of Tiam1 stimulates these pathways leading to a degree of membrane ruffling via Rac, in the transduced KIM2 cells [320].

The lack of classic epithelial morphology in the reverted TKIM2 cells is underlined at an electron microscopic level where cells showed pleomorphism and irregular surfaces (Figure 4.4.3), although to a lesser degree than the fully transformed TKIM2 cells transduced with control Tiam1 (Figure 4.4.2). Hordijk et al did not examine reverted MDCK cells by electron microscopy [242]. Similarly previous reversion studies using E-cadherin have not examined reverted cells using electron microscopy [212]. It may be that the appearance of the cells illustrates the conflict in the cell between the original transforming drive and the attempts to control these by altering the morphology of the cell at a cytoskeletal level. The reverted TKIM2 cells are very different in appearance from NKIM2 transduced with control and active Tiam1, which retain an epithelial morphology (Figure 4.4). These appearances tend to suggest that the reverted cells may not function fully as normal epithelial cells.

While the morphology of the reverted cells was not classic epithelial, the epithelial marker E-cadherin was expressed at certain but not all, cell boundaries (Figure 4.3.1). Although the presence of E-cadherin confirms the epithelial nature of the reverted cells, the lack of expression at all boundaries differs from the study by Hordijk et al in which reverted cells expressed E-cadherin at every cell-cell contact [242]. It is possible that the reverted TKIM2 cells that showed membrane ruffling morphologically and thus cytoskeletal changes, were less likely to form adherens junctions containing E-cadherin. Previous studies have shown that E-cadherin is closely linked to the submembranous cytoskeleton; for example transfection of E-cadherin into fibroblasts causes the redistribution of spectrin, ankyrin and NaKATP ase to sites of cell-cell contact, similar to their arrangement in MDCK cells[302]. An alternative possibility is that formation of complex junctions containing a number of components, is regulated by a number of signalling pathways, which may be functioning abnormally in reverted cells-similar to transformed cells [150]. As expected on electron microscopy, the adherens junctions were less frequent in the reverted TKIM2 cells, and appeared to be intermediate junctions rather than full desmosomes or hemidesmosomes (Figure 4.4.4). Furthermore, ZO-1 expression was not seen in the reverted TKIM2 cells, suggesting a lack of tight junction formation, which was confirmed on electron microscopy. Previous work in MDCK cells has shown that E-cadherin is required for the formation of tight junctions as well as zonula adherens and desmosomes, because removal of calcium from the medium causes delocalisation of junctional components and subsequent treatment with anti-E-cadherin antibodies delays junction reformation [302].

However, NKIM2 cells transduced with control and active Tiam1, express E-cadherin at all cell-cell boundaries (Figure 4.3) as well as showing an epithelial morphology. This is consistent with the study carried by Hordijk et al, in which overexpression of Tiam1 in normal MDCK epithelial cells increased E-cadherin-mediated cell adhesion. This was demonstrated by increased resistance of transduced cells to scattering induced by Hepatocyte Growth Factor [242]. In order to explain the increased cell adhesion, Hordijk et al proposed that localization of Tiam 1 at adherens junctions may lead to Rac-mediated F-actin polymerisation, which may facilitate the association of E-cadherin complexes with the cortical actin cytoskeleton.

If, as Hordijk et al suggest, upregulating Tiam1 results in association of E-cadherin complexes with the actin cytoskeleton, one might predict a difference in the junction-associated proteins β- and α- catenin. However, these were not altered in their study [242]. α- and β-catenin were not individually assessed in this study, although the whole junctional formation was examined by electron microscopy.

The possibility that part of the regulation of E-cadherin by Tiam1 may be transcriptional is raised by the Western analysis showing an upregulation of Tiam1 in lines transduced with the active Tiam1 construct compared with the control, although this is more convincing in NKIM2 cells compared with TKIM2 cells (Figure 4.3.2) Supporting this evidence are previous studies examining carcinoma cell lines in vitro which have found that the relative amounts of E-cadherin protein and mRNA correspond to each other in E-cadherin expressing lines [212]. Moreover an E-cadherin promoter has been characterised, which is bound by certain transcription factors and is known to control epithelial-specific gene expression [261]. Further exploration of this transcriptional regulation is required, and will be considered in the general discussion.

It is clear from electron microscopy that polarisation was absent in reverted TKIM2 cells , although present in NKIM2 cells transduced with control and active Tiam (Figure 4.4). The lack of polarisation in reverted cells could be predicted from the junctional abnormalities and relative lack of E-cadherin already discussed. Although previous studies have shown that MDCK cells in collagen maintain their apical orientation even when their basolateral orientation is disrupted by loss of E-caderin contacts through calcium depletion [302].

In contrast with the lines before genetic manipulation, none of the transduced cell lines appeared to contain other cell types, in terms of α-actin-expressing myoepithelial cells, or vimentin-expressing fibroblasts. This was confirmed by electron microscopy, where neither cell type was identified. The original untransduced NKIM2 and TKIM2 cell lines did not contain fibroblasts (section 2), so it is reasonable to expect that the transduced cells similarly do not contain this cell type. However small numbers of myoepithelial were present in untransduced NKIM2 (Figure 1.2), and the retroviral transduction of cells occurred with high efficiency, so one would predict the presence of myoepithelial cells among transduced NKIM2 lines

.

It is possible that by chance alone the myoepithelial NKIM2 cells were not transduced, or it may be that the myoepithelial cells did not survive following retroviral transduction. In principle the retrovirus should have transduced both cell types with the same efficiency because its cell surface receptors were specific for all murine cells types [205]. Formal experiments comparing epithelial and myoepithelial cells are necessary to determine whether there is a real difference in transduction of these cell types, however the numbers of myoepithelial cells among NKIM2 cultures are too small to make meaningful comparisons. While the lack of myoepithelial cells in transduced cultures remains difficult to explain, it is convenient, because all transduced cell lines can be considered epithelial cultures.

In terms of mammary differentiation, the reverted TKIM2 cells express milk protein in response to lactogenic hormones. From the data all transduced cell lines show the ability to form β-casein on immunoprecipitation analysis (Figure 4.6.) where the bands only reveal that β-casein is present. This analysis involves specific probing and detection steps which produce a specific clean result, when using a polyclonal or less specific antibody. Further investigation of the relative amounts of β-casein in each cell line by Western analysis, revealed that the reverted TKIM2 lines produce β-casein in similar amounts to other NKIM2 lines (Figure 4.6.1). TKIM2 transduced with control Tiam1 express very small amounts of β-casein compared to other lines, and less than untransduced TKIM2 cells (Figure 1.9). To date the main players in mammary differentiation and lactogenesis appear to be prolactin and the JAK-STAT pathway [327]. Recent evidence has shown cross-talk between the JAK-STAT pathways and the Ras-mediated signalling. It is possible that Tiam 1 stimulation of Rac-which is known to effect Ras pathways, may provide a link to pathways that control milk gene expression. Alternatively, it may be the partial restoration of epithelial differentiation which is key to promoting high levels of milk protein expression.
Reverted TKIM2 cells were unable to form dome-like structures after growth in lactogenic hormones. As discussed in results (section 1) the significance of these structures to mammary differentiation requires further exploration. However it is more likely that cells were not able to form domes as a result of junctional aberrations, than any lack of mammary differentiation. This may also be responsible for the inability of NKIM2 cells transduced with active Tiam1 to form domes, compared with NKIM2 cells transduced with control Tiam1 (Figure 4.1.2).

In order to help consider whether the features of transformation are suppressed in reverted cells, Table 10.1 displays features of transformation in all the transduced cell lines

	NKIM2 plus Tiam1-control	**NKIM2 plus Tiam1-active**	**TKIM2 plus Tiam1-control**	**TKIM2 plus Tiam1-active**
Contact Inhibition	contact inhibition	contact inhibition	no contact inhibition	contact inhibition
Serum independence	serum dependent	serum dependent	serum independent	serum dependent
Growth in soft agar	no colonies	no colonies	colonies	colonies
Injection of scid mice	1 out of 8 tumour	no tumour formation	no tumour formation	no tumour formation

Table 10.1 Features of transformation in transduced cell lines

The morphological data confirms that reverted TKIM2 cells transduced with active Tiam1 regained contact inhibition and grew in a flat monolayer (Figure 4.1.1). This induction of contact inhibition in reverted cells was also observed in the previous Tiam1 reversion study by Hordijk et al [242]. As expected, both NKIM2 cell lines retained the feature of contact inhibition, similar to untransduced NKIM2 cell lines (Figure 4.1 a,b).
It is clear the rate of growth of reverted TKIM2 cells was reduced, with a similar growth rate to the two NKIM2 cell lines (Figure 4.7). The lack of growth in reverted cells would tend to suggest that Tiam1 is influencing another feature of transformation -namely uncontrolled growth. This is further confirmed by the inability of reverted cells to grow serum-free conditions (Figure 4.71), suggesting the cells have become dependent on growth factors in the serum for proliferation.

While contact inhibition and growth control appear to be reinstated in reverted TKIM2 cells, unexpectedly the reverted TKIM2 cells transduced with active Tiam1 showed anchorage-independent growth in soft agar. Fewer colonies were formed in reverted TKIM2 cells compared TKIM2 cells transduced with control Tiam1 (Figure 4.9.1).While there is considerable data on how transformed cells usurp growth control, studies on the mechanisms that govern cellular location and anchorage-dependence have lagged considerably. Evidence demonstrating that integrins transduce signals that influence intracellular pathways have provided some insight into anchorage independence [177].The disparity between induction of growth control and anchorage-dependence is interesting in the Tiam1- reverted cells, because it is known that there is conversion of integrin and growth factor pathways at certain points and that certain oncogenes can stimulate each pathway separately before conversion. Therefore it may be that Tiam1 may be reverting pathways controlled by growth factor receptors and not integrins. Although it is likely that there is some cross talk between pathways, particularly because the colony- formation is reduced in reverted TKIM2 cells compared with TKIM2 cells transduced with control Tiam1.

While reverted TKIM2 cells did not form tumours in scid mice, the most unexpected result was the formation of a single tumour in scid mice in normal NKIM2 cells transduced with control Tiam1, but in no other population (Figure 4.9). It has been shown that normal untransduced NKIM2 cells could form tumours in scid mice (Figure 2.7), so it is feasible that NKIM2 cells transduced with the control construct could do likewise. However the lack of tumour formation in TKIM2 cells transduced with the control Tiam1construct is surprising. One explanation for the low tumour take rates overall, is that both control and active Tiam1 are in general are reducing the tumour forming ability of all the cells, although the mechanism of action of the control construct would not be clear. An alternative explanation is that the process of retroviral transduction may have reduced the tumour forming ability of the cells in general. The only way to confirm whether the retroviral transduction is having this effect would be to transduce cells with an empty vector and compare their tumourigenicity in scid mice. However considering the previous low take rates in scid experiments, it is more likely that this experiment has not been optimised for these cell lines as discussed in Results chapter 2.

So, in conclusion to what extent do transformed cells which have been reverted by Tiam1, show epithelial and mammary differentiation, and to what degree have the features of transformation been reversed in these cells? The reverted cells do show a remarkable reversion to an epithelioid phenotype with restoration of the key features of epithelial differentiation-namely E-cadherin expression at certain cell boundaries and adherens junctions. However further examination shows that the cells are unable to fulfil more complex criteria such as polarisation, tight junction formation and they still show considerable morphological irregularities under electron microscopy. They are capable of expressing the milk protein β-casein and so demonstrate mammary differentiation.
In general, the reverted cells have lost their features of transformation including lack of contact inhibition, rapid growth rate and lack of serum-dependence. However they retained the ability to form anchorage-independent colonies in soft agar. Although reverted cells did not form tumours in scid mice, the results from this experiment are difficult to interpret because the only cell line which did form a tumour was NKIM2 cells transduced with the control Tiam1 construct.

General Discussion

To date this is the first description of Tiam1- reversion of mammary cells transformed by SV40 large T antigen, and a consideration of how this extends current knowledge is necessary.

Cells previously reverted by Tiam1 were transformed by an oncogenic mechanism- namely a Ras-transformed pathway [242]. In Ras- transformation, a point mutation in a Ras gene can create a Ras protein that fails to hydrolyse its bound GTP and so persists abnormally in its active state, transmitting an intracellular signal for cell proliferation. Whereas the strategy of SV40 large T antigen involves binding to the protein products of two key tumour suppressor genes of the host cell, putting them out of action and so permitting the cell to replicate its DNA and divide [120]. Although both mechanisms result in unregulated cell proliferation, it is unlikely that overexpression of Tiam1 would result in the specific reversion of these two different mechanisms of for transformation.It appears more likely that by promoting a physical morphological change- by rearranging F-actin and promoting E-cadherin contacts between cells, Tiam1 can atleast partially overcome transformation by Ras and SV40 large T antigen. This theory would be strengthened by exploring the reversion capability in cells transformed by an alternate mechanism. Initial data suggests that Tiam1 may be also able to revert src-transformed cells (J.C. personal communication). Although as a concept the suppressive effect on transformation appears surprising, it may be that cell-cell contact stimulates a number of signalling pathways which are as yet unknown. Certainly it is clear that restoration of E-cadherin and a functional cadherin/ catenin complex in various cell culture systems transformed by different mechanisms, can revert lines to a benign epithelial phenotype [243]. At a cell signalling level, it has been proposed that E-cadherin sequesters β-catenin in the E-cadherin cell-adhesion complex, and thus prevents free β-catenin from stimulating the Wnt signalling pathway which may promote uncontrolled cell proliferation in the context of tumourigenesis [257]. However while data supports a connection between E-cadherin function and β-catenin transcriptional activity in vitro, it remains uncertain whether this link is physiologically relevant.

This study raises the possibility that Tiam1 may regulate E-cadherin through a transcriptional mechanism, by demonstrating upregulation of the E-cadherin protein expression by active Tiam1. However to date knowledge of the E-cadherin promoter and possible transcriptional regulators is limited. Analysis of the E-cadherin promoter in mice has shown epithelial specificity 178 base pairs upstream of the transcription start site [270]. This promoter fragment contains a GC-rich region, a CCAAT –box and a 12 base pair palindromic element named E-Pal [261], and these components are also present in human E-cadherin. It is thought that epithelium specific regulation of E-cadherin may be controlled by two mechanisms. Firstly, the E-Pal element acts as a promoter or repressor of gene expression depending on whether or not it is in epithelial or mesenchymal cells respectively. Secondly a tissue specific enhancer (ESE) promotes transcription exclusively in epithelial cells [261]. It has been reported that both these mechanisms have been disturbed in MCF-7 breast carcinoma cell lines [271].
In terms of trancriptional regulation, E-Pal has consensus sequences for binding of helix-loop-helix (HLH) transcription factors, which are implicated tissue-specific gene expression, although functional activity has not yet been demonstrated. The CCAAT- box family of proteins CP-1 and C/EBP also have binding activity for the CCAAT box of E-cadherin, and the ESE element enhances transcription through AP2 [261]. However none of these transcription factors have been associated with Tiam1 to date.
It may be that a prime candidate for transcriptional regulation of E-cadherin via Tiam1 has not been appreciated to date in the reversion context. Recent work has shown that the Tiam1 family of guanine nucleotide exchange factors (Dbl, Ost and Vav), via the activation of the members of the Rho family of GTPases stimulate the transcription factor Nuclear Factor –kappa B (NF-κB) [251]. Although NF-κB is traditionally associated with the response to pathogens or stress, it can modulate expression of the cell adhesion molecules P, E-selectin, ELAM-1, ICAM, VCAM-1, integrins $\alpha 1\beta 2$, and vimentin [253]. Therefore NF-κB is a potential candidate for a transcriptional regulator of E-cadherin and phenotypic reversion, although a DNA binding site for NF-κB has not been previously described on the E-cadherin promoter. Moreover unlike Tiam1, abnormal NF-κB activity has been implicated in human breast carcinogenesis and therefore may be physiologically relevant.

It has been reported that highly expressed p100/p52 precursor NF-κB sequesters other NF-κB proteins in cytoplasm of human breast cancer cell lines, thus abrogating its normal function in cancer cells [277]. Other studies have reported abberant activation of NF-κB in human breast tumour lines, rat and human breast cancer specimens, although detailed analysis of sub-units is not demonstrated [278]. However, alternative data, also in tumour lines, suggests that constitutive activation of NF-κB occurs during the progression of certain human breast cancers from being antioestrogen-sensitive (ER positive) to insensitive (ER negative) [279]. The role of NF-κB in the Tiam1 reversion pathway therefore requires further exploration, and an initial approach would involve confirming by Electrophoretic Mobility Shift analysis (EMSA) whether active Tiam1 upregulated the NF-κB transcription factor in transduced KIM2 lines.

It is highly likely that Tiam1 is stimulating alternative pathways in the KIM2 system, which also require exploration. For example, in certain cell types, Tiam1 via Rac can stimulate c-jun N-terminal kinase [233] and moreover, activated Rac1 has also been shown to activate the p38/Mpk2 pathway which is induced by different stress conditions [321], as well as regulate the transcriptional activity of serum response factor (SRF) [322].

An alternative approach to considering the individual cell signalling pathways affected by reversion, is to consider the key changes triggered by overexpression of Tiam1 and the implications of these. Figure 1 depicts a scheme for considering the effects of Tiam1 on transformation and differentiation from this study.

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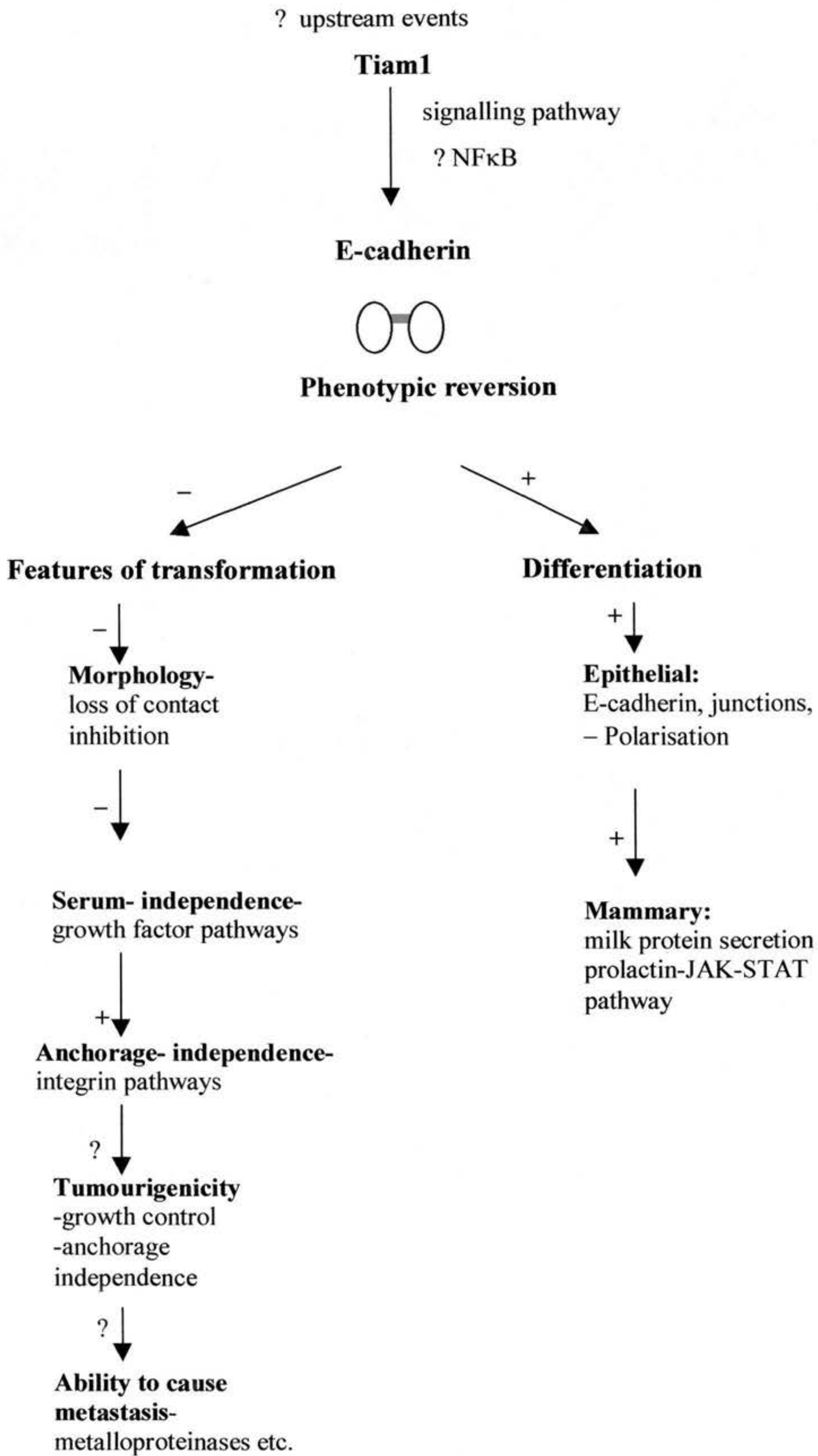
Figure 1 scheme for considering the effects of Tiam1



From Figure 1 it is clear that in terms of features of transformation that reorganisation and restoration of cell-cell contacts by Tiam1 affects morphology- and could explain a reorganisation from a spindle to a cuboidal phenotype. Similarly induction of contact inhibition may result also from generalised increased cell-cell adhesion. However it has been shown in vitro that contact inhibition is also facilitated in normal cells by increased cell-substratum adhesion at the periphery of the cell, which prevents migrating cells underlapping normal cells [175]. Cell-substratum interactions are normally mediated by certain integrins, and presumably help prevent invasion in vivo.

While Tiam1 controls serum-independent growth mediated by growth factor pathways in reverted cells, anchorage-independence mediated by integrin pathways remains present. Several studies have shown that the ability of cancer cells to proliferate in the absence of adhesion to extracellular matrix proteins ie anchorage-independence, correlates closely with tumourigenicity in animal models [181]. This property of cancer cells presumably reflects the tendency of tumour cells to survive and grow in inappropriate locations in vivo. Although tumourigenicity was explored in reverted cells-which did not form tumours, this question remains unanswered because certain control lines were tumourigenic. Furthermore perhaps the most significant question was not answeredby this study , namely can Tiam1 prevent cells from metastasizing. The key difference between tumourigenicity and metastasis, is the ability for detached cells to destroy the extracellular matrix by proteolysis and invade. Importantly the first stage of metastasis involves decreased E-cadherin adhesion, in order to allow cells to detach [32]. Therefore it may be more significant in vivo that Tiam1 prevents cell-detachment from an existing tumour rather than full reversion of the transformed phenotype. To explore this further, it would be more profitable to overexpress Tiam1 in a human breast cancer line which is known to cause metastasis, rather than in KIM2 mammary cells which may be less likely to metastasize.

Assessment of epithelial and mammary differentiation allows a prediction of how close reverted cells are to 'normal', and the effect of overexpression of Tiam1 on normal cells. From Figure 1, it is clear that Tiam1 promotes E-cadherin expression between cells, one of the hall marks of epithelia, however in this study expression was not universal, possibly due to the effect of Tiam1 on other signalling pathways such as those controlling membrane ruffling. It is probable that lack of E-cadherin or conflict between signalling pathways inhibited tight junction formation and polarisation in reverted cells.
However reverted cells exhibit mammary differentiation in terms of milk expression, which is controlled by prolactin via JAK-STAT pathways [327]. This contrasts with previous studies which show that mammary differentiation is dependent on epithelial differentiation [84]. Moreover the only similar study reversing the effects of immortalization in mammary HC11 cells, showed that reintroducing and overexpressing wild-type p53 inhibited the expression of atleast two milk proteins [89]. However the HC11 cells with wild- type p53 also exhibited generalised growth inhibition which may have contributed to the lack of milk protein expression.

Clearly it is possible to revert transformed cells in the in vitro scenario, but how does this translate to in vivo clinical objectives in the management of breast cancer ?. Perhaps the results are most relevant to the field of gene therapy. To date the most successful approach using gene therapy combined with enzyme therapy, has been the treatment of Adenosine Deaminase Deficiency- a genetic disease that leaves patients prone to infection [207]. Although many strategies are currently employed with regards to cancer gene therapy, success has been limited. They include reinjecting genetically modified lymphocytes to destroy tumours, enhancing the immune reaction to genetically modified tumour cells, corrective approaches eg restoring wild-type p53, and triggering apoptosis pathways [207]. The main problems have been related to low gene transduction of the target tissue and vector-related problems [202].
Technical problems aside, it is intriguing to speculate that stimulation of the Tiam1 pathway could be used in gene therapy treatment, to revert transformed cells.
However the data from this study clearly demonstrate that both normal and transformed Tiam1 transduced cells, show certain abnormal biological features. In vivo, Tiam1 appears tightly regulated and its precise role in vivo is still to be elucidated.

Therefore there may be significant hazards involved in overexpressing Tiam1 in normal tissue in vivo. In contrast, the targetting of cancer cells only, with Tiam1 may be a feasable longterm objective, particularly if gene therapy becomes a clinical option. It is unlikely that Tiam1 would revert tumours to normally functioning tissue, but it may be that a degree of growth control is restored, and perhaps tumourigenicity would abolished. Perhaps the most crucial question will be whether or not overexpressing Tiam1 suppresses the ability of cells to metastasize.

An alternative possibility is that other components of the reversion pathway are used in gene therapy which disrupt fewer signalling pathways in general. For example it may be that direct restoration of wild-type of E-cadherin in cancers where it is mutated or deleted proves to be as effective, as indicated by early mouse experiments [267]. These elegant experiments using a transgenic mouse model for pancreatic Beta cell carcinogenesis showed that crossing these mice with those that maintain E-cadherin expression, halted the process of tumourigenesis at the adenoma stage [267].

It may be that apart from examining cell signalling pathways in vitro, the next step in the research of Tiam1 would involve using transgenic animals to help determine the function of Tiam1 in vivo and ultimately its effect on tumourigenesis.

It could be argued that the Tiam1 reversion in murine KIM2 cells are more representative of in vivo human disease than previously reverted canine kidney epithelial cells [242]. However although the KIM2 model does inactivate p53 and pRb, which have been implicated in human breast carcinogenesis, this is only indirectly through SV40 large T antigen.

In conclusion how far were the aims of this study achieved?
The KIM2 mammary model clearly provides a model for normal and transformed cells, which is amenable to genetic manipulation with Tiam1. Although several other experimental normal breast and breast cancer cell lines exist, the advantage of this system is that both lines derived from a common origin. Therefore one could predict that the differences between the cell lines are a consequence of transformation only, rather than any intrinsic difference between the cell lines. However as discussed one potential drawback of the system is a degree of chromosomal variability shown in both normal and transformed cells, although this is present in many in vitro cell lines [73]. This variability may have been responsible for the formation of tumours in scid mice by NKIM2 cells

The retroviral transduction of NKIM2 and TKIM2 cells proved a successful method of gene transfer in these cells. Previous attempts to transfect these cells by conventional techniques have proved very inefficient [318]. Furthermore, it is clear that overexpression of the transgene occurred for atleast three months following transduction, despite presumed changes in the chromosomal content of the cells.

It is clear that Tiam1 can atleast partially revert SV40 large T antigen transformed mammary TKIM2 cells to an epithelial phenotype. This is significant because it suggests that Tiam1 is effective in reverting different mechanisms of transformation, because previous reverted cells were Ras-transformed [242]. Furthermore, the reversion also provides a model for future examination of the components of the Tiam1 signalling pathway, in particular the transcription factor NF-κB, which may be involved in the transcriptional regulation of E-cadherin.

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Dedication

This thesis is dedicated to my husband Gerard, mother, father and sister Anna, whose loving support is behind everything I do.

Publications arising from this study

Phenotypic reversion of transformed mammary epithelial cells with Tiam1
Kurian KM, Van der Kammen RA, Collard JG, Wyllie AH, Watson CJ
Journal of Pathology, 1999, Vol. 187, No.SS pA4

Induction of differentiation and apoptosis in a novel conditionally immortal
Mouse mammary epithelial cell line KIM2
Gordon KE, Binas B, Chapman RS, Kurian KM, Clark AJ, Watson, CJ.
Journal of Cell Science (in press)

DNA chip technology
Kurian KM, Watson CJ, Wyllie AH
Journal of Pathology, 1999, Vol. 187, No.3, pp267-271

Retroviral vectors
Kurian KM, Watson CJ, Wyllie AH
Journal of Pathology (in press)

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