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THE DERIVATION AND CHARACTERISATION OF A CONDITIONALLY IMMORTAL MOUSE MAMMARY EPITHELIAL CELL LINE USING A TRANSGENIC APPROACH

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DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts, and has not been submitted in any previous application for another degree. The work on which it is based is my own except where stated in the text or in the acknowledgements.

Katrina Ellen Gordon

ABSTRACT

The induction of proliferation and differentiation in mammary epithelium during pregnancy is quite remarkable and results in the synthesis of copious amounts of milk at parturition to feed the offspring. Understanding the key factors involved in the tissue-specific activation of the milk protein genes and their relationship to tissue organisation have been important areas of mammary gland research. Epithelial cell lines can provide *in vitro* model systems in which both the growth and differentiation of the epithelium can be investigated under defined conditions. This thesis describes the isolation and characterisation of a conditionally immortalised mouse mammary epithelial cell line.

The approach adopted utilised transgenic mice harbouring an enhancerless thermolabile mutant of SV40 T-antigen (tsA58) construct driven by the ovine β-lactoglobulin (BLG) milk protein gene promoter as a source of mammary cells. It was envisaged that the expression of T-antigen would be limited to the secretory epithelium of the mammary gland and its immortalising properties active only at the permissive temperature of 33°C. However several of the founder mice developed tumours at ectopic sites due to leaky expression of T-antigen from the BLG promoter.

Mammary tissue from the five surviving transgenic lines of mice were used to generate mammary cultures. A novel isolation procedure, exploiting the ability of explant cultures to generate epithelial outgrowths, was used. One cell line, designated KIM-2, isolated from the lowest copy transgenic line at a semi-permissive temperature of 37°C was characterised further. These cultures, established from midpregnant glands, are highly enriched with luminal cells as assessed by their strong positive staining with secretory epithelial markers (keratins 18 and 19) and have retained a stable phenotype for over 60 passages. Investigation into the functional differentiation of KIM-2 cells has shown that the induction of β-casein is similar to existing mammary cell models. However the KIM-2 cell line has retained the ability to express a late differentiation marker, whey acidic protein (WAP) on plastic unlike other cell lines which require quite complex culture conditions to induce further differentiation. Initial transfection studies in this cell line with foreign DNA constructs has also proven to be successful. Therefore, the KIM-2 cell line has the potential to provide a good *in vitro* model to study factors involved in mammary epithelium development.

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ABBREVIATIONS

AAT	human alpha-1-antitrypsin
Amp	ampicillin
BLG	Beta-lactoglobulin
bp	base pair(s)
°C	degrees Celsius
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
cm	centimetres
cpm	counts per minute
CsCl	caesium chloride
DAPI	4, 6-diamino-2phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH_20	double distilled water
DEAE	diethylaminoethyl
dGTP	2'-deoxyguanosine 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
dNTP(s)	deoxynucleotide triphosphate(s)
DTT	dithiothreitol
dTTP	2'-deoxythymidine triphosphate
EDTA	ethylenediamine-tetra-acetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)-N, N'-tetra-acetic acid
EtBr	ethidium bromide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	gram

x g	gravitational force
HCl	Hydrochloric acid
HEPES	N-2-hydroethylpiperazine-N'-2-ethane sulfonic acid
hrs	hours
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
KCl	potassium chloride
kD	kilodaltons
l	litre(s)
LB	Luria broth
LMP	low melting point
M	molar (moles/litre)
mAb	monoclonal antibody
μCi	microcurie
μg	microgram
mg	milligram
$MgCl_2$	magnesium chloride
MGF	mammary gland factor
$MgSO_4$	magnesium sulphate
min	min(s)
μl	microlitre(s)
ml	milliltres
mM	millimolar
MMTV LTR	mouse mammary tumour virus long terminal repeat
MOPS	3-N-(morpholino) propane sulfonic acid
MPBF	mammary protein binding factor
mRNA	messenger ribonucleic acid
mRNA	messenger RNA
NP-40	Nonidet P-40

OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PVA	polyvinyl alcohol
Rb	retinoblastoma
RNA	ribonucleic acid
rpm	revolutions per minute
s	second(s)
S	Svedberg unit of sedimentation coefficient
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC	standard saline citrate (0.15M NaCl, 15mM tri-sodium citrate, pH7.0)
STAT	signal transducer and activator of transcription
SV40	Simian virus 40
TAE	Tris/Acetate/EDTA
TBE	Tris/Boric/EDTA
TE	Tris/EDTA
TEMED	N, N, N, N, tetramethylethylenediamine

The following abbreviations are used throughout this work:

Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol ($C_4H_{11}NO_3$)
UV	ultraviolet
V	volt(s)
WAP	Whey acidic protein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

The female mammary gland provides a unique experimental system which has proved a challenge to scientists for over a century. It has attracted the attention of physiologists studying milk protein composition and its secretion, developmental biologists investigating organ development and cell commitment, endocrinologists investigating hormones and their actions and molecular biologists trying to gain a better understanding of tissue-specific gene expression and its regulation. In addition, biotechnologists are exploiting the mammary gland as an *in vivo* bioreactor to produce therapeutically valuable proteins. Aberrant development of the gland is of interest to the cancer research field since it may aid in the understanding of the initial stages and progression of tumourogenesis.

It is the unique developmental pattern and function of the mammary gland which has drawn such interest. Most developmental changes occur in the gland postnatally with full maturation occurring after the onset of pregnancy, resulting in drastic remodelling of the tissue. The structural and functional changes which occur result in the synthesis and secretion of copious amounts of milk which are essential for the nourishment of the neonate. After weaning milk protein production is down-regulated and the entire alveolar epithelium is lost by a process of programmed cell death termed apoptosis. The regressed gland remains in a resting state similar to that observed in cycling virgins until the next pregnancy.

The study of the cyclical changes in growth, differentiation and regression both during normal and aberrant development of the gland have been active areas of research for a number of years. This work has lead to the identification of important regulatory signals which are required for normal development and has provided clues to key cellular targets which are altered during aberrant development and carcinogenesis. The challenge ahead is to build on this basic signalling framework by establishing which signalling pathways are crucial, the interconnections between them and how these signals become integrated to alter gene expression in the nucleus and remodel the gland. An *in vitro* system which could be manipulated, under

defined conditions, to mimic the mammary gland would provide a useful tool to try to address some of these fundamental questions.

This chapter describes the morphological and functional changes which occur during various stages of rodent mammary gland development and the key roles played by circulating hormones, locally acting growth factors, cell-cell and cell-ECM (extracelullar matrix) interactions. The contributions made by *in vitro* approaches, using different mammary culture systems, and *in vivo* approaches, exploiting transgenic technology to identify key regulators involved in mammary-specific gene expression and differentiation is assessed. Finally, the establishment of conditionally immortal mammary epithelial cell lines and their potential value as a more accurate *in vitro* model of mammary development is discussed.

1.1 MAMMARY GLAND DEVELOPMENT

Mammary gland development can be divided into four main stages: foetal, postnatal, postpubertal and adult. The gland grows slowly during embryonic and neonatal life and only matures fully during pregnancy and lactation. The morphological changes which take place during these developmental periods, from the early embryo to the fully functional gland at lactation and the subsequent regression of the gland after weaning, have been well defined histologically.

1.1.1 Embryonic development

The appearance of the mammary streak at day 10-11 of embryonic life is the first signs of mammary development in the mouse (Turner and Gomez, 1933) and has been described also in rats and humans (Myers, 1917; Raynaud, 1961). The migration of epidermal cells results in 5 discreet pairs of lens shaped glands located between posterior and anterior limbs on both sides of the embryo (Propper, 1978).

Histologically, the two distinct mesenchymal components are apparent at day 14 of embryogenesis. One is the dense mammary mesenchyme consisting of 2-3 layers of fibroblasts which surrounds the epithelium. These cells contain testosterone and oestrogen receptors (Wasner, 1983) which are induced by interactions with the

epithelium (Heuberger, 1982). The other is the fat pad precursor, consisting of preadipocytes located under the mammary rudiments. This condensed tissue is visible at day 14 of gestation and gives rise to the future fat pad. At this stage the mammary anlage becomes bulb-shaped and the sex is determined.

The work of Kratochwil and his colleagues elegantly demonstrated that the mammary mesenchyme was the target tissue for the action of testosterone. At day 12 of embryogenesis in males, unknown signals from the epithelium trigger synthesis of androgen receptors in fibroblasts (Kratochwil, 1986). The testosterone-sensitive fibroblastic mesenchyme condenses around the mammary stalk, severing its connection with the nipple and preventing further development of the gland. Therefore, hormone responsiveness is in part modulated by epithelial-mesenchymal interactions during embryogenesis.

In females, the gland enters a resting phase (day 11) where very little or no growth occurs for about 5 days. By day 16 the bud begins to rapidly proliferate forming a mammary sprout which infiltrates the mammary fat pad precursor tissue. The mammary sprout branches and goes on to form the mammary ductal tree system. The number of primary branches extending from the nipple is species-dependant, with rodents and ruminants having only one per nipple, dogs ten and humans up to twenty (reviewed by Medina, 1996). At birth the virgin gland consists of a mammary tree with approximately 15-20 branchings composed of a lumina surrounded by a single or multiple layers of epithelial cells with a basal layer of myoepithelial cells. The epithelial and mesenchymal components are separated by a basement membrane which contains components synthesised by both the fibroblastic dense mesenchyme (fibronectin and tenascin) and the fat pad precursor cells which make the basement membrane components (laminin and proteoheparin sulphate).

1.1.2 Postnatal development of the mammary ductal system

The extent of mammary tree growth between birth and puberty is again species dependant, with ductal elongation and branching occuring at a slow rate until the ovarian hormones induce growth during puberty. During this phase, proliferation and

elongation occur in the terminal end buds giving rise to lateral buds which are evenly spaced along the ducts. Growth is halted when the confinements of the fat pad are reached. At this stage the terminal end buds regress to blunt-ended club-shaped structures which have a slow proliferation rate (Imagawa *et al.*, 1994; Williams and Daniel, 1983) (Diagram 1.1). During oestrus the lateral buds divide and differentiate resulting in small alveolar structures which fill the mammary fat pad (Daniel and Silberstein, 1987; Russo *et al.*, 1989). However in some strains of mice (e.g BALB/c) which lack a luteal phase there is no alveolar development and the gland consists entirely of ducts at this stage.

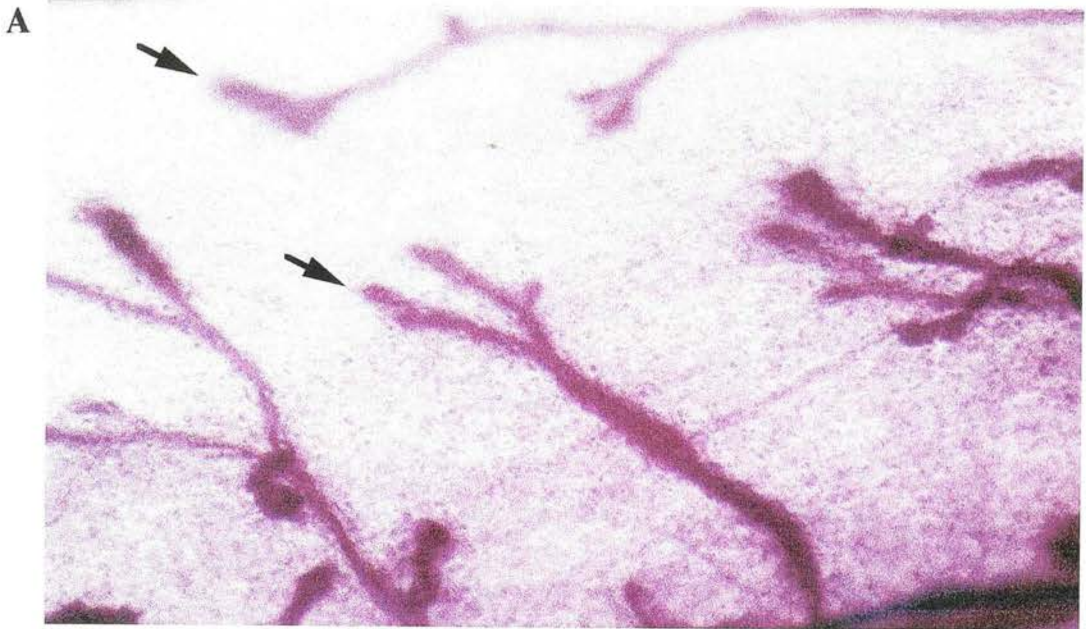
1.1.3 Structural and functional changes during pregnancy and lactation

The most dramatic changes take place in the gland after the onset of pregnancy. Proliferation index studies have shown that there is a peak of proliferation at day 4 and again at day 12 of pregnancy which co-incides with an expansion and increase in the number of alveoli to form lobuloalveolar structures (Traurig, 1967).

Several steroid and peptide hormones are associated with the ductal and lobuloalveolar growth observed during pregnancy. The exact role of the individual hormones is still unclear however the ovarian hormones (oestrogen and progesterone) are required for ductal growth (Korach, 1994) and alveolar proliferation (Lydon *et al.*, 1995) respectively. Alveolar proliferation and the induction of terminal differentiation requires the presence of the pituitary hormone, prolactin (Topper and Freeman, 1980, Vonderhaar, 1987., Pittius *et al.*, 1988; Imagawa *et al.*, 1990; Burdon *et al.*, 1991).

The lobuloalveolar structures consist of a single layer of epithelial cells surrounded by myoepithelial cells and become the sites of milk protein synthesis. The main lactogenic hormones are prolactin and the adrenal steroids (glucocorticoids). The basement membrane, rich in laminin and type IV collagen, is deposited between the two cell layers and is thought to play a modulatory role in the morphological and functional changes which lead to a fully differentiated secretory phenotype capable

A



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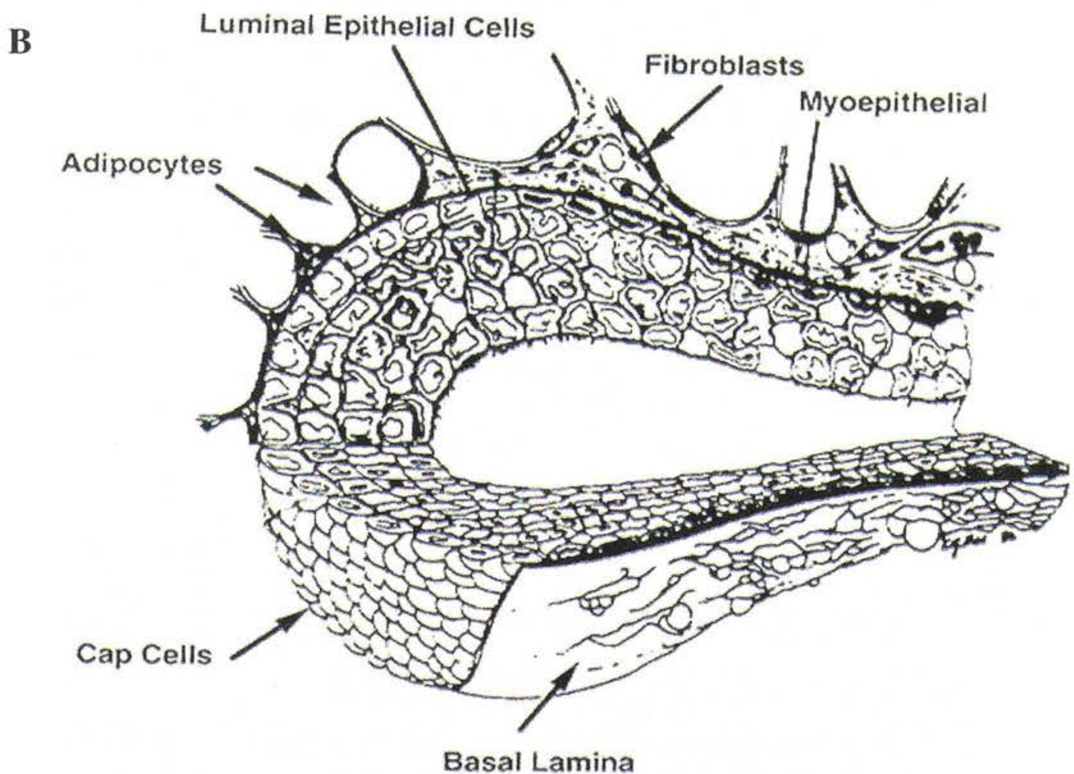


Diagram 1.1 A whole mount showing the end buds from a 4 week virgin mammary gland and an illustration of the end bud structure

(**A**) The large end buds (arrows), are located at the tips of the growing ducts. Note also the lateral branching (kindly provided by Dr. C. Watson).

(**B**) An illustration of the end bud showing the position of the cap cells in relation to the luminal and myoepithelial cells. A basal lamina surrounds the end bud with the cellular stroma comprised of fibroblasts and adipocytes.
[Adapted from Williams and Daniel (1983)].

of synthesising milk proteins in a specific temporal expression programme (reviewed by Rosen, 1987; Robinson *et al.*, 1995).

During lactation, in response to oxytocin release, the basket-like network of myoepithelial cells surrounding the alveoli contract resulting in the expulsion of milk into the small and large ducts. The contraction of the surrounding myoepithelial sheath is again the driving force to finally release the milk at the teat in response to suckling (Diagram 1.2).

1.1.4 Milk protein gene expression during pregnacy and lactation

The underlying function of the mammary gland, in all species, is to supply sufficient amounts of milk containing all the essential nutrients to support the growth of their young during the preweaning period. The major constituents of milk include:- water, lipids, carbohydrates, minerals and proteins with the relative amounts varying between species depending on the needs of the neonate.

The protein content of milk is approximately 30mg/ml (Schmitt-Ney *et al.*, 1992) and consists mainly of two groups of proteins; those which are precipitated under acidic conditions (pH 4.5-pH 4.6 for bovine milk) and the whey proteins which remain in the milk serum at low pH (see Table 1.1).

1.1.4.1 Caseins

The caseins are the most abundant milk proteins which together constitute 80% of total milk protein. This family of phosphoproteins, αs1, αs2-, β-, and κ-casein (bovine casein nomenclature in Bonsing and Mackinlay, 1987) has been shown in some quite closely related species to display considerable divergence at the nucleic acid level, for example rat (Blackburn *et al.*, 1982; Hobbs and Rosen, 1982), mouse (Hennighausen and Sippel, 1982) and guinea pig (Hall *et al.,* 1984). They do however retain 3 conserved domains, namely the 5'noncoding region, the signal peptide and the casein kinase phophorylation sequence. The α- and β-caseins are serine phosphorylated on a cluster of residues permitting interactions with calcium phosphate resulting in the formation of micelles. κ-casein is insensitive to calcium

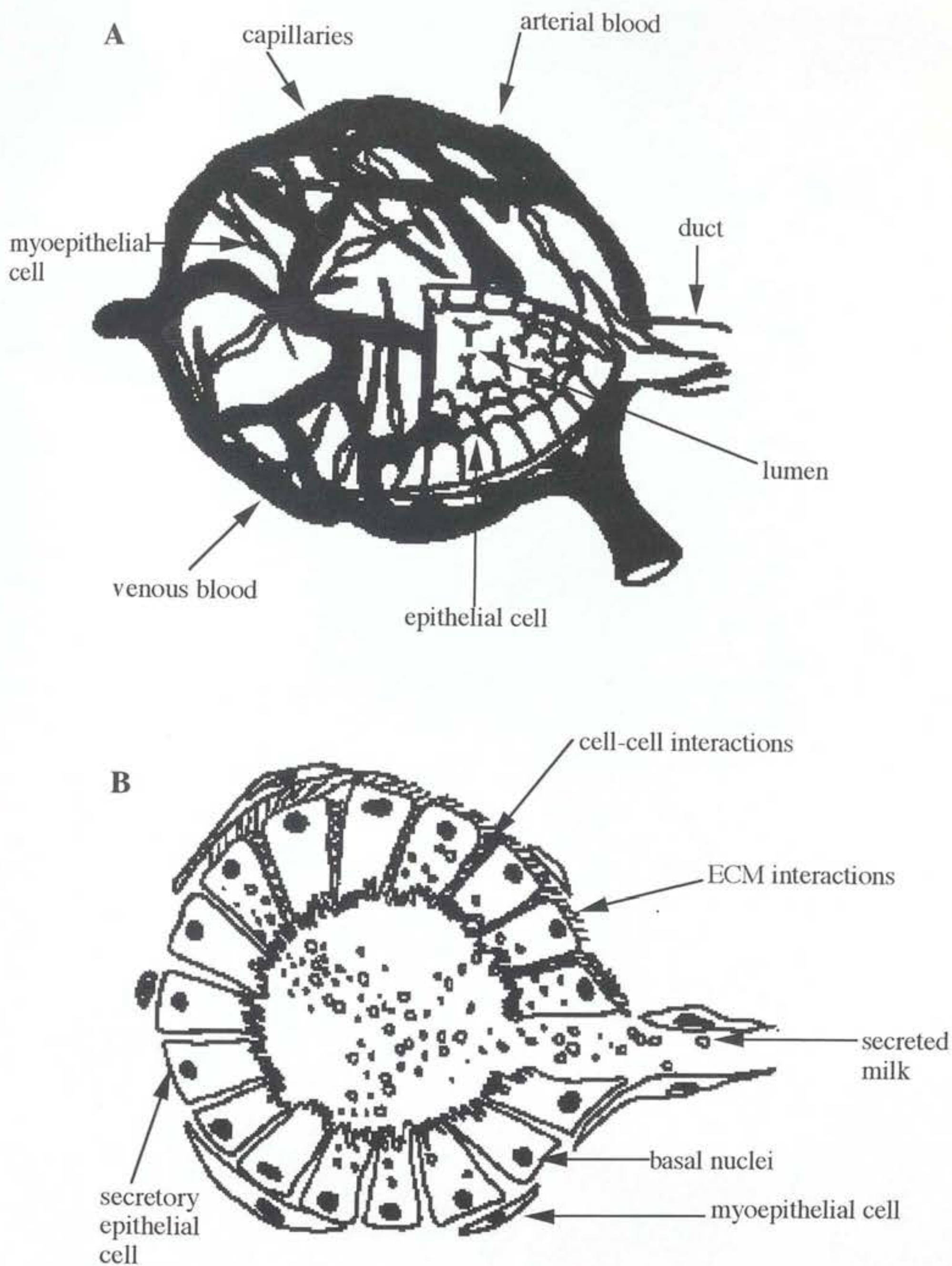


Diagram 1.2 Stucture of mammary alvelolus
(**A**) Illustration of a mammary alveolus
(**B**) Alveolus cross-section
Notice the single layer of mammary epithelial cells surrounding an enclosed lumen and the apical secretion of the milk proteins into the lumen and from there into the ducts. The ECM is deposited between the epithelium and the myoepithelial cells. There are also extensive interactions between adjacent cells.
[Reproduced from Rillema, J.A.(1994) and Streuli,C.H., (1993)]

and has a role in stabilizing the growing micelle by limiting its size (Schmidt, 1982). These micelle structures transport calcium, inorganic phosphate and trace amounts of citrate and magnesium from the mother to the neonate. Interestingly "knockout mice" which lack β-casein in their milk form smaller micelles but retain the ability to feed their offspring, although the preweaned pups are slightly growth retarded. This suggests that the expression of β-casein in rodent milk is not essential for mammary development or for the survival of the neonate (Kumar *et al.*, 1994).

1.1.4.2 Whey proteins

α-Lactalbumin (α-Lac) is the major whey protein found in the milk of many species (Brew and Hill, 1975) and is involved in the synthesis of lactose from glucose and galactose. α-Lac associates with galactosyl transferase increasing its affinity for glucose and the resulting complex, lactose synthetase, catalyses the production of lactose.

In ruminants, β-lactoglobulin (BLG) is the most abundant whey protein but its function is still unclear. The crystal structure of bovine BLG is known and shows a resemblance to plasma retinol-binding protein suggesting a possible role for BLG in vitamin A transport (Papiz *et al.*, 1986). BLG is not expressed in rodent milk but when expressed in transgenic mice it has been shown to be expressed in a tissue-specific, hormone-dependant manner (Simons *et al.*, 1982).

In rodents the most abundant whey protein is the cysteine-rich whey acidic protein (WAP). WAP has also been identified in rabbits, however the sequence homology between rat and rabbit at the nucleic acid level is only 28%, and 15% at the protein level suggesting very little conservation between species. Overexpression of murine WAP in transgenic mice results in altered mammary development and a *milchlos* phenotype (Burdon *et al.*, 1991). These lines of mice were unable to nurse their litters and showed impaired alveolar development and premature expression of both WAP and α-Lac (day 10 compared to day 16 and 21 respectively in nontransgenic mice) (Robinson *et al.*, 1995). The premature overexpression of WAP in these mice therefore resulted in an alteration in both the differentiation programme and structural development of the gland.

Table 1.1 Milk protein composition of milks from various species

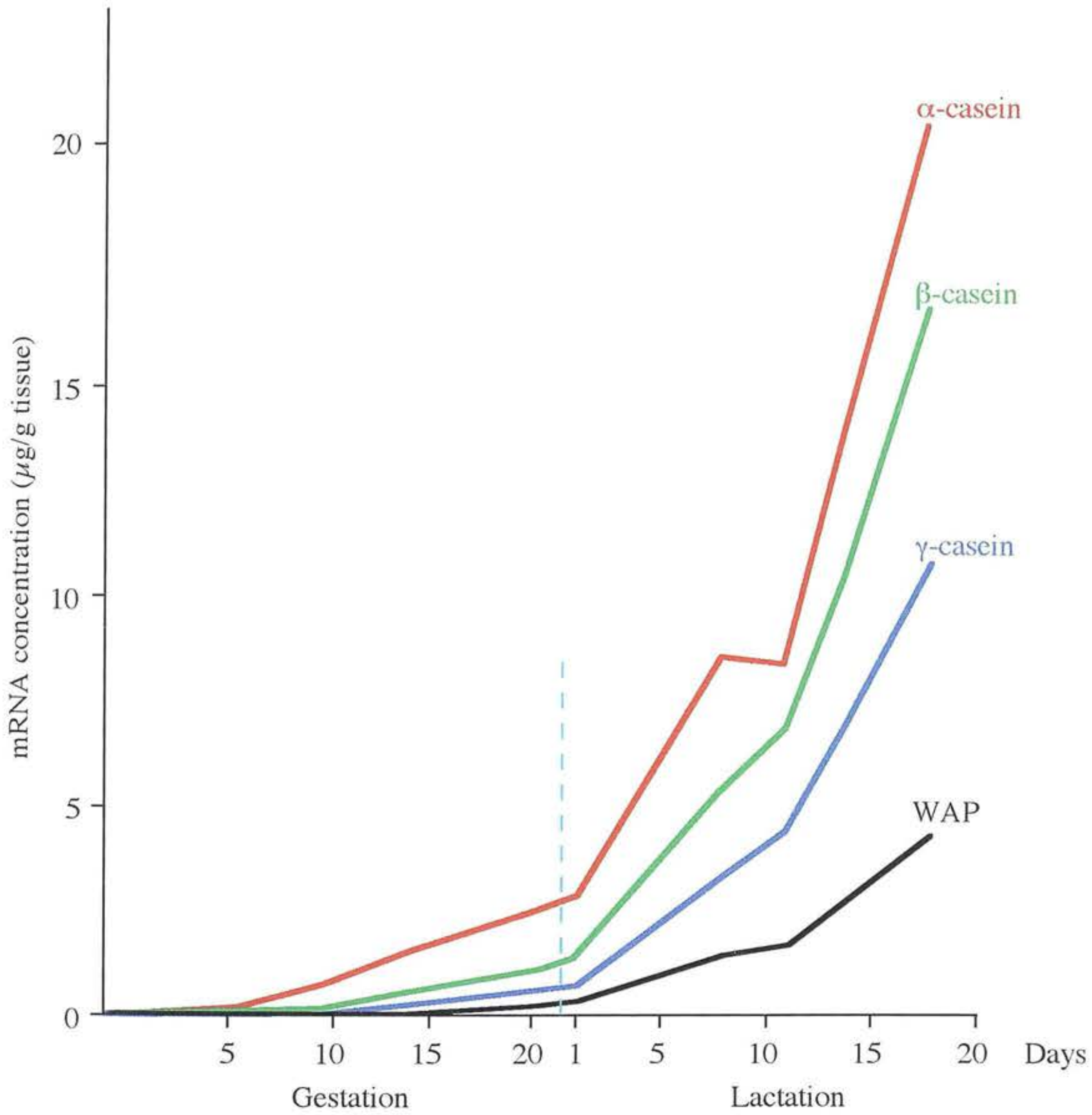
(modified from Lathe *et al.*, 1986 and Kumar, 1993).

MILK PROTEIN	CONCENTRATION IN MILK (g/l)		
	Mouse	Sheep	Human
caseins			
α_s1-casein	28.0	12.0	0.4
α_s2-casein	NDA	3.8	NDA
β-casein	21.0	16.0	3.0
κ-casein	2.4	4.6	1.0
whey proteins			
α-lactalbumin	trace	0.8	1.6
β-lactoglobulin	none	2.8	none
Whey acidic protein	2.0	none	none
Serum albumin	NDA	NDA	0.4
Lysozyme	NDA	NDA	0.4
Lactoferrin	NDA	NDA	1.4
Immunoglobulins	NDA	NDA	1.4

NDA: No data available

1.1.4.3 Expression profile of milk protein genes during pregnancy and lactation

The expression of milk protein genes in the developing gland follows a specifically timed activation programme (Graph 1). Using *in situ* hybridisation, Robinson and co-workers (1995) demonstrated low expression levels of milk protein genes in cycling virgins during oestrus within a few alveolar cells. With the onset of pregnancy the levels increase dramatically in a mosaic asynchronous manner (Rosen, 1987). In early pregnancy (day10), β-casein and the recently identified WDMN1 (Morrison and Leder, 1994) are expressed sporadically throughout the gland within pockets of alveolar cells. As the lobuloalveolar structures develop and expand, the expression of these early differentiation markers becomes more uniform and the expression of late differentiation markers, WAP and α-Lac are activated in a similar mosiac fashion.



Graph 1 Differential accumulation of casein and WAP mRNA during rat mammary development
Taken from Rosen , J.M. , 1987.

1.1.5 Remodelling and regression of the gland after weaning

After the pups are weaned the gland undergoes a massive round of epithelial cell death and tissue remodelling (Ossowki *et al.*, 1979; Pitelka, 1988). The first histological and ultrastructural signs of involution are the breakdown of tight junctions, the disruption of lobuloalveolar structures by the shedding of epithelial cells into their collapsing lumen and the breakdown of basement membrane (Wellings and DeOme, 1963; Martinez-Hernandez *et al.*, 1976; Warburton *et al.*, 1982). Ultrastructural studies indicate that the regression and remodelling of the gland shows features consistent with apoptotic cell death e.g nuclear compaction, cytoplasmic condensation and autolysis of epithelial cells (Strange *et al.*, 1992).

1.1.6 Mammary stem cells and lineage specific markers

Based on morphology and immunohistochemical staining patterns there appears to be considerable heterogeneity in the luminal cell population in the virgin glands of mice and rats. At least 10 different cell types (including the myoepithelial cells) have been identified, using a diverse range of antibody markers along the ductal structures and end buds of the gland. The keratin intermediate filaments are commonly used to determine the different cell types present *in vivo* in the developing mammary gland (see Table1.2) and to cell-type mammary culture systems.

In addition, various other markers have been described. Rudland's group for example, has described three lectins, pokeweed mitogen and *Griffonia simplicifolia*-1 (GS-1) which are markers for myoepithelial cells (Rudland and Hughes, 1991) and peanut lectin (Rudland, 1992) which is a marker for epithelial cells in the rat. Gusterson's laboratory has described two antibodies raised against the cell surface antigens, epithelial membrane antigen (EMA) (Gusterson *et al.*, 1985) and common acute lymphoblastic leukemia antigen (CALLA) (Gusterson *et al.*, 1986) which have been used to distinguish and separate populations of human and rat epithelial and myoepithelial cells respectively (O'Hare *et al.*, 1991; Gomm *et al.*, 1995; Dundas *et al.*, 1991). As well as antibody markers, antisense oligonucleotides (oligos) to WAP, WDNM1 and β-casein, have been generated and used as probes for *in situ* hybridisation (Robinson *et al.*, 1995).

Table 1.2 Cytokeratin antibody markers of the different mammary cell types in the developing adult rodent gland

(modified from Talyor-Papadimitriou and Lane, 1987)

ANTIBODY	ANTIGEN	SPECIFICITY	REFERENCE
Guinea pig polyclonal	Human skin keratins	Preferentially stains myoepithelial cells in mouse	Asch *et al.*, 1981
mAb LP34	Human psoriatic skin	Stains only myoepithelial cells in mouse and rat	Talyor-Papadimitriou *et al.*, 1983
mAb1A10	Bovine muzzle keratin	Stains only myoepithelial cells in rat	Allen *et al.*, 1984
mAb 24B42	Bovine muzzle keratin	Stains luminal cells throughout rat development	Allen *et al.*, 1984
mAb LE61	PtK1 cell detergent insoluble extract	Stains luminal cells in mouse and rat	Lane, 1982

There is at present considerable debate as to the location, and indeed the existence, of a stem cell population which could give rise to all the different cell types found in the mammary gland. This controversy is in part due to the lack of intermediate lineage-specific markers and stem cell markers. A candidate stem cell population has been proposed by Williams and Daniels (1983) as being the cap cells which are undifferentiated cells which lie on the outer layers of the growing end buds (see Diagram 1.1). A similar population of cells has been reported in rats (and may have been cloned by Rudland *et al.*, 1986). However these cells at the terminal end buds cannot be the only source of stem cells since it is possible to transplant any part of the mammary tree into the cleared fat pad of syngeneic mice and regenerate a fully functional mammary gland. The regenerated gland can differentiate normally during pregnancy but since the nipple is removed, the mice cannot feed their offspring.

A second candidate was proposed by Smith and his colleagues (1988 & 1990) who described a pale staining, keratin 6-positive cell found in the cap cells and throughout the mammary tree. They showed that this cell was found as early as day 16 of

embryonic life and throughout development of the mammary gland. It may be that keratin 6 is a mammary stem cell marker, however more conclusive proof could be achieved by looking at proliferation rate and keratin 6 expression simultaneously in the developing mammary gland. The identification of a stem cell marker would be useful in determining the fate of these cells during pregnancy and involution. The ultimate proof of stem cells in the gland would require successful cloning of a cell population and reinjection of these cells into a cleared fat pad to regenerate a functional mammary gland.

1.2 *IN VITRO* MAMMARY MODEL SYSTEMS

Although the structural development of the mammary gland is well defined in the rodent system, the actual molecular details of the signals and signalling pathways involved in altering both form and function are still largely unknown. Systemic hormones and locally-acting growth factors have been known to play a primary role in mammary development for many years. However there is now mounting evidence which has highlighted the importance of both cell-cell and cell-substrata interactions as important modulators of proliferation and differentiation in the mammary gland and in other tissues (reviewed by Roskelley *et al.*, 1995). The combination of both *in vivo* and *in vitro* approaches has been invaluable in improving our basic understanding of some of the events which lead to tissue-specific gene expression and the mechanisms involved in its regulation.

Early evidence for the involvement of the endocrine system in mammary development was obtained by assessing the effects of removing endocrine glands, administering hormones to intact animals or by hormone replacement therapy in endocrinectomised animals (summarised by Topper and Freeman, 1980). Although these test systems were rather crude they did indicate central roles played by hormones released by the ovaries, pituitary and adrenal cortex in mammary growth and morphogenesis in rodents.

Clearly, further progress in identifying and understanding the regulatory mechanisms involved in mammary proliferation and differentiation required the

development of a simpler *in vitro* system. Researchers began to focus on developing *in vitro* systems in which mammary function could be maintained under defined culture conditions and the effect of individual hormones assessed.

1.2.1 Whole organ and explant cultures

The first *in vitro* systems developed were whole organ or explant cultures using tissue initially from midpregnant mice (Elias, 1957 & 1959) and postpubertal rats (Trowell, 1959). These culture systems verified some of the *in vivo* data and extended our knowledge of the local growth factor requirements for proliferation and differentiation under conditions where the normal architecture of the gland is maintained. It became clear that the mammary gland responds to a multitude of cues which often act in a synergistic manner. In addition, different parts of the mammary tree have different hormonal and growth factor requirements for proliferation and differentiation depending on strain (Singh *et al.*, 1970) and age (Prop, 1966). Ductal elongation and branching, for example, is primarily regulated by oestrogen and growth hormone (GH) whereas the minimum growth requirement for lobuloalveolar development in mice (3-5 weeks old) requires initial priming *in vivo* with oestrogen and progesterone before the growth of lobule structures can be induced with a combination of insulin, prolactin and glucocorticoid (Banerjee, 1976; Imagawa *et al.*, 1990 & 1994). A second round of development was achieved in whole organ cultures by Tonelli and Sorof (1980) by removing prolactin and glucocorticoid and allowing the mammary tree to regress in the presence of insulin before inducing a second round of development with the same cocktail of hormones supplemented with epidermal growth factor (EGF).

The extensive work carried out in the laboratories of Nandi and Banerjee resulted in organ culture systems, isolated from different developmental stages, which could undergo functional and developmental changes similar to those observed *in vivo* such as morphogenesis, lactogenesis and involution under defined culture conditions. These early *in vitro* systems were crucial in verifying directly the involvement of hormones and identifying locally acting growth factors in mammary gland

development. However the limitation of these systems became clear when the painstaking research was begun to decipher the molecular and cellular targets involved in proliferation and functional differentiation of the mammary epithelia. In these systems only the overall growth of the mammary tree could be determined and not the actions or targets of particular hormones or growth factors. It was impossible to determine if these factors acted directly on the epithelial cells or indirectly via an interaction with stromal components.

1.2.2 Co-cultures of mammary epithelial cells with stromal cells

During embryogenesis and thoughout postnatal mammary development mesenchymal-epithelial interactions are crucial. In the developing female embryo, the fibroblastic mesenchyme induces growth of the epithelial bud and the fatty stroma plays a role in branching morphogenesis during puberty (Kratochwil *et al.*, 1987; Sakakura *et al.*, 1991). A clue to the inductive capacity of the mesenchymal component in determining morphological and functional changes originated from early developmental studies. Transplantation of embryonic adipose precursor mesenchyme into adult virgin glands resulted in typical ductal branching whereas transplantion of fibroblastic mesenchyme resulted in nodular hyperplasia. Conversely transplantation of embryonic epithelium into adult mesenchyme produced normal alveolar development and normal differentiation during pregnancy. These transplantation experiments elegantly demonstrated that both adult mesenchyme and epithelium retain their inductive capacity. In addition to influencing normal development, alterations in stromal-epithelial interactions could potentially lead to the progression and/or development of cancer. Further credence for a role in carcinogenesis includes (i) suppression of a tumour phenotype achieved in rat primary Dimethylbenz[a]anthracene (DMBA) tumours when transplanted into a normal gland or under the fat pad (Rivera *et al.*, 1982; Ethier *et al.*, 1987; Welsch *et al.*, 1987) (ii) Cocultures of mammary tumour cells with embryonic mammary mesenchyme decreased proliferation and increased differentiation (DeCosse *et al.*,

1975) (iii) there is increased evidence that stromal cells are altered in breast cancer (Cullen *et al.*, 1991).

Coculture systems were developed to complement *in vivo* transplantation studies to try to assess directly the role of stromal and epithelial cell interactions in directing normal mammary differentiation and aberrant development. These coculture models usually consisted of stromal fibroblast or adipocycte monolayers with mammary cells seeded on top. These systems could be easily manipulated depending on the question being addressed. The feeder layers could be left untreated or irradiated to prevent proliferation so that the requirement for reciprocal interactions between the stroma and the epithelium could be examined. Alternatively, the epithelial cell cultures could be incubated with conditioned media from growing fibroblasts or adipocytes to test for the presence of soluble factors released by the stroma which affect the epithelial phenotype. Essentially these systems established that both substrate and soluble growth factors, supplied by the fibroblasts and fat cells, stimulated mammary epithelial cell proliferation, morphogenesis and expression of milk proteins. For example growth of mouse mammary epithelium was promoted when cultured on irradiated preadipocytes (3T3-L1) or on differentiated adipocytes (Wiens *et al.*, 1987). Modifications to these coculture systems include the use of permeable membrane (polyethyleneterephthalate-PET) inserts which permitted the investigation of possible paracrine interactions between the two cell types without cell-cell contact. Typically, the stromal cells are cultured on the bottom of the dish and the mammary cells seeded on top of the insert directly, or on inserts which have first been coated with matrigel or another substratum (Ip and Darcy, 1996). This system permits the investigation of the influences of stromal cells on epithelial proliferation, morphogenesis and differentiation.

The major advantage of coculture systems over others is the possibility of establishing and characterising the role of stromal cells (from different developmental stages) and epithelial cells (either normal, preneoplastic or transformed) in inducing reciprocal functional and morphological changes. Already, several possible mechanisms have been hypothesised for the interaction of stromal

and epithelial cells. These include secretion of diffusable growth modulators, modification of ECM and direct communication through gap junctions. Both growth stimulatory and growth inhibitory factors are expressed and/or secreted by stromal cells including insulin like growth factor-1 and 2 (IGF-1, IGF-2), platelet derived growth factor-A (PDGF-A), basic fibroblastic factor, (bFGF), fibroblastic growth factor-5 (FGF-5), transforming growth factor-β1 (TGFβ-1), transforming growth factor-α (TGF-α) and hepatocyte growth factor (HGF) which can modulate mammary epithelial cell proliferation (Cullen and Lippman, 1991; Imagawa *et al.*, 1994). Additionally, stromal cells can modulate ECM either directly or via epithelial cells by synthesising and secreting matrix proteins. Alternatively they can regulate basement membrane turnover by modulating the secretion of metalloproteinases and their inhibitors, tissue inhibitors of metalloproteinases (TIMPS).

The major disadvantage to the currently used coculture systems is the serum requirement. Stromal cells grow much better in serum and consequently undefined factors present in the serum may be problematic when investigating effects of stromal-epithelial interactions. The serum could interact with diffusible factors released by the stromal cells directly or in conditioned media. This problem could be overcome by using defined media which supports the growth of stromal cells.

1.2.3 Mammary epithelial cell enriched primary cultures

Without the development of *in vitro* systems whereby the epithelial and stromal components could be separated, further progress at the molecular and cellular level would be impossible. To this end investigators concentrated on trying to separate and culture the epithelial component.

The ground work in determining the minimal requirements for growth and maintainance of mammary cells *in vitro* had already been established from the experiments with organ systems. The ability to separate the mammary epithelium from the fat pad by collagenase digestion of minced mammary gland had been described by Lasfargues (1957a/b). However, it was the development of purification techniques such as differential centrifugation, separation through percoll/ficoll

gradients and/or fluorescent-activated cell sorting (Barcellos-Hoff and Bissell, 1989; Kidwell *et al.*, 1984; Darcy *et al.*, 1991) which allowed the isolation of enriched populations of epithelial cells. The ability to maintain these cultures relatively free of fibroblasts provided the first tools which could be used to identify factors involved in proliferation and functional differentiation of the mammary epithelium.

1.2.3.1 The role of epithelial-substrata interactions in determining the extent of epithelial differentiation

Primary cultures have been the system of choice for a number of years since these systems are thought to most closely mimic epithelial cells *in vivo*. The first primary culture models developed used routine culturing on tissue culture plastic which meant that the cells often lost their differentiation potential and showed abnormal chromosome numbers. This setback was overcome by Emerman and Pitelka in the late 1970s when they discovered that primary epithelial cell cultures had the potential to undergo morphological and functional changes when cultured on typeI collagen gels and that hormone-dependant casein expression could be induced by floating the gels (Emerman and Pitelka, 1977; Emerman *et al.*, 1977 & 1979). These findings were reinforced by Shannon and Pitelka (1981) and Lee *et al.*, (1984) and extended by Nandi's group in 1990. Although fixed collagen gels supported proliferation there was limited differentiation. The other extreme was achieved by floating the gels i.e differentiation at the expense of proliferation. Both proliferation and formation of 3-dimensional ductal structures in mammary epithelial cultures (MEC) was accomplished from virgin and midpregnant mice by culturing the cells within typeI collagen gels. A modification of this procedure permitted proliferation and differentiation to be studied independantly within the same system under serum free conditions (Flynn *et al.*, 1982). This two-step approach involved the induction of proliferation by the addition of EGF and insulin to MEC plated on attached collagen gels. Functional differentiation could then be induced in the second step by switching to differentiation media containing prolactin, glucocorticoid and insulin and releasing the gels to float. These systems were used to optimise serum-free conditions for growth of MEC derived from different stages of development of rodents and other

species (reviewed by Imagawa *et al.*, 1990 & 1994). However, even under optimal conditions these cultures could only be induced to undergo ductal development and limited differentiation, with only β-casein being expressed at significant levels.

The realisation that the basement membrane played an active role in determining the differentiation phenotype of cultured mammary cells was an important discovery. Circumstantial evidence for the involvement of basement membrane in mammary differentiation originated from studies which showed that the composition of the extracellular matrix (ECM) changes during different developmental stages and inhibition of ECM synthesis caused mammary differentiation to be halted (reviewed by Barcellos-Hoff and Bissell, 1989). The first direct evidence was obtained by Wicha's group who cultured MEC on a reconstituted basement membrane derived from the mammary glands of midpregnant rats. They demonstrated that a greater degree of functional differentiation could be achieved in these MEC when cultured on basement membrane. These cultures expressed higher levels of β-casein when induced with lactogenic hormones compared to cultures induced on floating collagen gels (Wicha *et al.*, 1982). This finding resulted in researchers trying to determine the importance of basement membrane in directing the degree of functional differentiation of the mammary epithelium *in vitro* and its relevance to mammary development in the whole animal. To this end, the work of Bissell's group and others has produced unequivocal evidence of the active role of ECM in phenotypic differentiation in culture systems. These *in vitro* studies revealed that MEC derived from midpregnant mice cultured on basement membrane derived from Engelbreth-Holm-Swarm (EHS) tumour (Kleinman *et al.*, 1986) or the commercially available Matrigel could undergo a limited degree of proliferation but crucially this substratum could support alveolar development (Blum *et al.*, 1987; Barcellos-Hoff *et al.*, 1989; Neville *et al.*, 1991). Culturing MEC on EHS caused morphological changes which resulted in the formation of cell clusters surrounded by EHS and within 4-6 days these "mammosphere" structures showed remarkable resemblance to alveolar structures seen in the pregnant and lactating gland *in vivo* (Aggeler *at al.*, 1991). This *in vitro* system demonstrated the potential of primary cultures, given the correct

hormonal and ECM cues, to mimic mammary differentiation *in vivo* and express both early differentiation markers such as β-casein and late differentiation markers such as WAP which had previously not been observed in culture systems (Chen *et al.*, 1989).

An *in vivo* role for ECM was investigated by generating transgenic mice which inappropriately expressed an ECM-degrading enzyme stromelysin-1 in the mammary gland. Stromelysin-1 appears to be involved in remodelling of the gland during involution (Talhouk *et al.*, 1991; Strange *et al.*, 1992) and slow releasing implants containing tissue inhibitors of metalloproteinases (TIMP-1) delays involution (Talhouk *et al.*, 1992). Expression was directed to the secretory epithelium of the mammary gland using both the rat WAP promoter (Sympson *et al.*, 1994) and mouse mammary tumour virus promoter (MMTV) (Witty *et al.*, 1995). The WAP promoter is active midway through pregnancy and increases during lactation (Andres *et al.*, 1987; Schonenberger *et al.*, 1988) whereas the steroid sensitive MMTV is active throughout mammary gland development (Pattengale *et al.*, 1989). It was anticipated that the mammary glands in these mice would be either nonfunctional since ECM signalling would be disrupted or that the gland may be partially functional but there would be premature involution due to overexpression of stromelysin-1. However the transgenic lines generated displayed an unexpected mammary phenotype. The WAP-stromelysin mice exhibited premature maturation, with virgin glands resembling midpregnant gland. The virgin glands showed alveolar development and expressed similar levels of β-casein mRNA as midpregnant normal glands. However during pregnancy and lactation the basement membrane disintegrated and β-casein and WAP mRNA levels decreased significantly.

The MMTV transgenic mice showed a similar premature development of virgin glands however these animals could still lactate and feed their offspring. Although the phenotypes of these mice was unexpected, it does demonstrate that alteration in ECM does have a profound effect on mammary development both structurally and functionally *in vivo*. Clearly the full development of the mammary gland requires a multitude of cues to act at specified developmental stages.

The endeavours by many laboratories has lead to the development of complex primary culture systems which have proven to be effective tools in building up a general picture of the diverse signals required to achieve full mammary differentiation. The time consuming work involved in modifying this system has been rewarded by providing an *in vitro* tool which more closely mimics morphologically and functionally the developmental changes observed *in vivo* during pregnancy. Bissell's group have hypothesised a hierarchy of ECM-mediated control of differentiation based on results from their *in vitro* model systems (Diagram 1.3). The first level of control is structural, resulting in changes in cell shape which permit lactoferrin expression. The second level of control is mediated by the ECM component laminin, and results in the activation of an ECM-responsive element (BCE1) and the induction of β-casein expression. The third level of control involves basement membrane mediated "mammosphere" formation which resembles the alveolus *in vivo* (see Diagram 1.3) and permits the synthesis of WAP. The fourth level of control results in the degradation of basement membrane by metalloproteinases and results in the loss of differentiated function.

Primary culture systems are, for many, the systems of choice to look at normal mammary gland development *in vitro*. However these systems have several limitations. A major disadvantage is the inability to maintain and passage the cells in an undifferentiated state. Normal mammalian cells will only undergo a limited number of doublings before entering a state of senescence (Hayflick and Moorhead, 1961; Martin, 1977; Hayflick, 1980) thus imposing a time limitation on experiments. Another disadvantage of primary culture systems is the inability to efficiently transfect the cells. Transient transfections into primary cultures has been attempted, however the recovery of the cells is poor and the levels of DNA uptake are highly variable (Yoshimura and Oka, 1990). This problem limits the usefulness of primary cultures in the identification and manipulation of signal transduction pathways activated by developmental and enviromental cues. For example, this system cannot be used directly to establish which transcription factor bindings sites within milk protein promoters are important in the activation of gene expression at

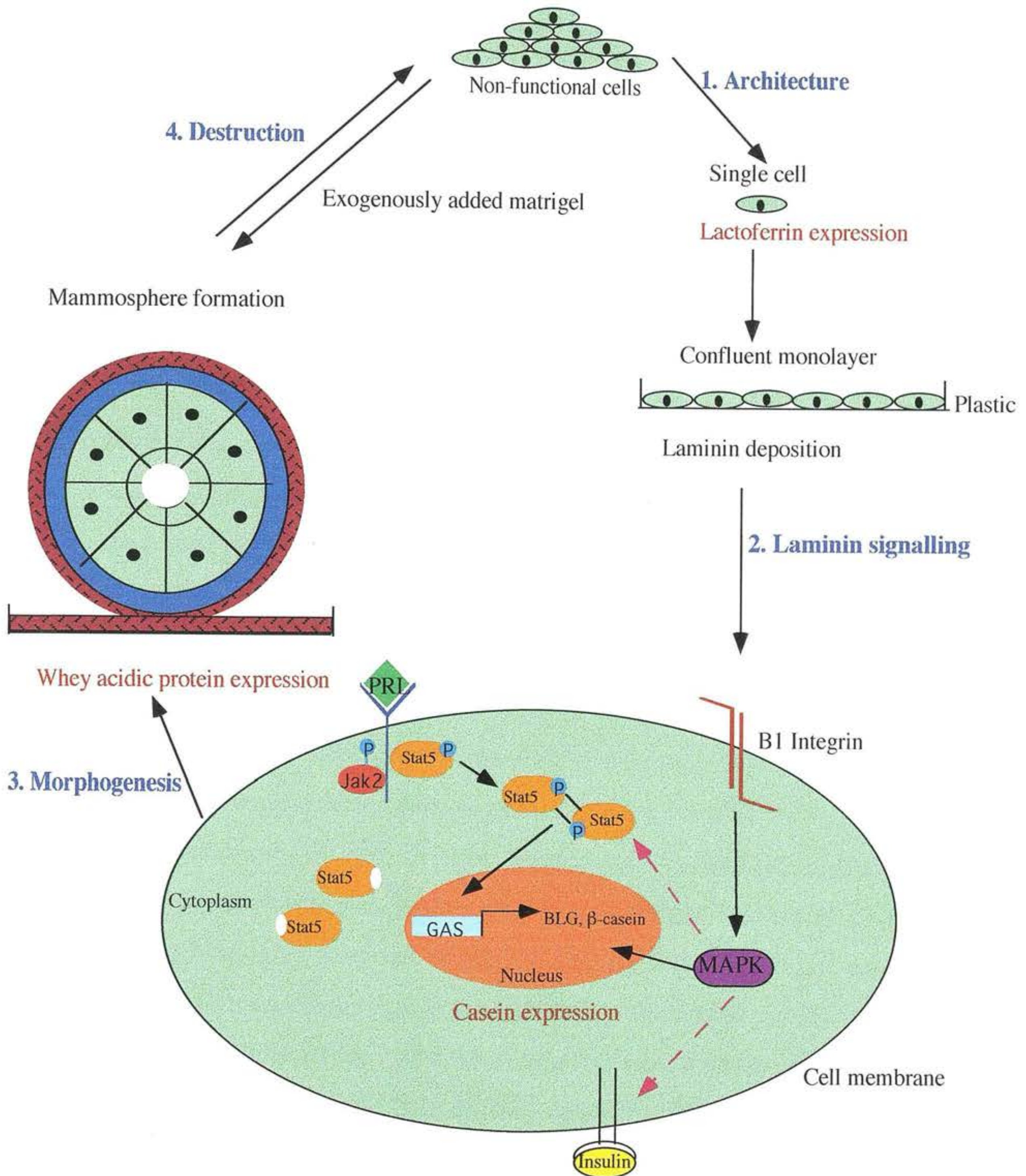


Diagram 1.3 Hierarchy of control in primary mouse mammary cultures undergoing functional differentiation.

Level 1: Architectural changes which lead to lactoferrin expression. Level 2: Laminin deposition leading to β-casein expression. Level 3: Basement membrane mediated morphogenesis resulting in mammosphere formation leading to WAP expression. Level 4: Matrix metalloproteinase-mediated involution.

(Modified from Roskelley *et al.,* 1995).

particular stages in development. In addition, it is not feasible to manipulate upstream signalling events in this system, such as altering the expression of components of the prolactin signalling pathway. Consequently, at present most of these experiments are done in transgenic animals which is both expensive and time consuming. Ideally an *in vitro* assay system in which the performance of the transgene constructs could be assessed in culture to optimise expression levels, would prove less costly than directly generating transgenic colonies of mice.

Matrigel itself can pose a problem when using defined culture conditions since it can act as a 'growth factor sink' for transforming growth factor-β (TGF-β) and basic fibroblastic growth factor (bFGF) which cannot be removed by conventional methods of dialysis or ammonium sulphate treatment. There are also problems with the possible presence of metalloproteinase activity and components of the ECM interfering with biological assays.

1.2.4 Spontaneously immortalised mouse mammary epithelial cell lines

Improvements in establishing and maintaining enriched cultures of epithelial cells resulted in primary cultures which could be maintained *in vitro* for defined periods of time. On rare occasions investigators found that after an initial crisis some cultures became spontaneously immortalised and could be cultured indefinitely. One of the first cell lines to be generated in this way was the COMMA-1D line which was established from midpregnant BALB/c mice under conditions of prolonged culture in low serum (Danielson *et al.,* 1984). This parental cell line has been a useful source of clonal cell lines such as HC11 (Ball *et al.*, 1988) and CID9 (Schmidhauser *et al.*, 1990).

The HC11 cell line has proven to be a valuable *in vitro* system to study the regulation of β-casein expression. Treatment of confluent HC11 cells, grown on plastic, with lactogenic hormones, results in the rapid induction of endogenous β-casein expression (Ball *et al.,* 1988). Synergistic interaction between prolactin and glucocorticoids was found to be necessary for high level β-casein expression since individually the hormones had only a weak effect on expression. This inductive

property, along with the ability to easily transfect foreign DNA constructs into HC11 cells has been exploited to define cis-acting DNA sequences within the promoters of both the rat β-casein gene (Doppler *et al.*, 1989) and the ovine BLG gene (Burdon *et al.*, 1994a) which are required for expression. The introduction of promoter-chloramphenicol acetyltransferase (CAT) reporter constructs demonstrated that 338bp 5' of the β-casein transcriptional start site or 408bp of the BLG promoter was sufficient to confer hormonal responsiveness. Using nuclear extracts prepared from hormonally induced and uninduced cells, a number of common transcription factor binding motifs have been identified which have both positive and negative effects on transcription (Altiok and Groner, 1993; Li and Rosen, 1994; Meier and Groner, 1994).

The putative mammary-specific transcription factor mammary gland factor (MGF) or milk protein binding factor (MPBF) was identified independantly through its interaction with the β-casein and BLG promoters respectively (Schmitt-Ney *et al.*, 1991; Watson *et al.*, 1991). *In vitro* mutational analysis of MGF sites in the β-casein promoter revealed that the MGF site at -90bp was essential for hormone dependant induction of transcription (Schmitt-Ney *et al.*, 1991). In the BLG promoter, mutation of the two proximal MPBF sites had a similar effect, abolishing hormonally induced transcription (Burdon *et al.*, 1994b). *In vivo* analysis of the same MPBF sites in transgenic mice showed that mutations did not abolish transcription but did result in considerable reduction of expression of the transgene when all 3 MPBF sites were mutated (Burdon *et al.*, 1994a). This transgenic data indicated that MPBF binding was not essential for mammary expression but was necessary for maximal transcription of the BLG gene *in vivo*. It also illustrated the importance of validating *in vitro* data in the context of the whole animal.

The cloning and sequencing of sheep MGF by Groner's group (Wakao *et al.*, 1994) identified MGF/MPBF as the fifth member of the signal transducers and activators of transcription (STAT) famiiy (Darnell *et al.*, 1994). Despite the fact that HC11 cells express only low levels of the prolactin receptor and attempts to isolate clones which express higher levels of the recepter have failed (Gouilleux *et al.*, 1994) they have

still proved informative in examining prolactin signalling (Welte *et al.*, 1994). HC11 cells have provided a model system best adapted for the study of regulation of β-casein as well as some β-casein driven hybrid genes.

However, the major limitation of this cell line is its inability to undergo full differentiation and express milk proteins which are induced late in pregnancy, for example WAP and α-Lac. The identification of cis-acting regulatory sequences in the promoters of these genes has mainly been achieved using trangenic animals. In addition it has recently been shown that HC11 cells lack functional p53 (Merlo *et al.*, 1994). The main consequence of this is that these cells are prone to mutations. Since p53 acts as 'the guardian of the genome' (Lane, 1992), in its absence any mutations which occur in the DNA remain uncorrected and can be replicated and passed on to daughter cells after mitosis thereby increasing the genetic instability of these cells. It may well be that this absence of p53 is responsible for the increased oncogenic potential observed in Comma-1D at later passage (Medina *et al.*, 1986).

The CID9 cell line was derived from the Comma-1D cell line to provide an *in vitro* model system which could be easily transfected but still required exogenously added basement membrane to induce differentiation. This cell line has proved useful in defining the molecular mechnisms involved in ECM-dependant differentiation. Using this model system a matrix dependant transcriptional enhancer, BCE1, was identified 1.8kb upstream of the bovine β-casein transcriptional start site (Schmidhauser *et al.*, 1990). CID9 cells and its derivatives (SpC2) have provided a useful tool to start to decipher the molecular details of ECM signalling and how these signals result in changes in gene expresion in the nucleus.

Another cell line IM-2 isolated by Reichmann *et al.*, (1989), is responsive to lactogenic hormones on plastic and expresses β-casein but when clonal epithelial (Cl 31 E) or fibroblatic (Cl 31 F) cell lines were isolated this differentiation capacity was lost in monocultures. The induction of differentiation could only be achieved in the epithelial clonal cell line if it was cocultured with either a fibroblastic cell line (Cl 31 F or NIH 3T3) or with typeI collagen. Using this system Reichmann *et al.*, (1989) demonstrated that epithelial-fibroblastic cell contact led to the deposition of laminin

regardless of hormonal induction and that substitution of the fibroblasts with typeI collagen had the same effect. This system provides an *in vitro* tool to investigate the role played by epithelial-fibroblastic interactions in mammary differentiation.

Spontaneously immortalised cell lines have overcome some of the problems encountered with primary cultures. These culture systems can be maintained in an undifferentiated state for prolonged periods of time, stored, resuscitated and still retain some differentiation characteristics of primary cultures. In addition, the ability to transfect these cell lines allows questions to be addressed about important regulatory elements required for expression of a certain gene construct.

Taken together the properties within the cell lines presently available do mimic mammary development but taken individually none show both full functional differentiation and amenability to genetic manipulation. Additionally, an important caveat with spontaneously immortalised cell lines is that the normal proliferation and differentiation pathways may themselves be altered due to the immortalisation event itself. Since the nature of this immortalisation event is often unknown it may be difficult to predict the pathways involved.

1.3 *IN VIVO* MAMMARY MODEL SYSTEMS

1.3.1 Transgenic mice

The limited usefulness of the available *in vitro* systems to answer questions pertaining to normal and aberrant growth of the mammary gland has led to the development and utilisation of transgenic approaches. The ability to specifically target expression to the mammary gland using tissue-specific promoters and the development of transgenic technology has allowed significant progress to be made. Resection analysis of the promoter regions of the milk protein genes, β-casein, WAP and BLG has identified minimal promoter regions which are required for high-level tissue-specific expression in the mammary glands of transgenic mice. Using nuclear extracts prepared from both mammary tissue at different stages in development and HC11 cells induced with lactogenic hormones, a number of common transcription

Table 1.3 Advantages and Disadvantages of mammary culture models (modified from Ip and Darcy, 1996).

Model	Advantages	Disadvantages
Organ Culture	• Architecture of the gland is retained • Model for proliferation, morphogenesis, lactogenesis and involution • Toxicity testing of chemotherapy and carcinogenic drugs	• Epithelial cell specific effects cannot be investigated • Cannot be used for *in vitro* reconstitution experiments • Limited life span in culture (2-3 weeks)
Explants	• Can study functional differentiation and its regulation • Architecture of the gland is retained • Serum-free conditions can be used	• Carry over of hormones • Epithelial cell specific effects cannot be investigated • Cannot be used for *in vitro* reconstitution experiments • Limited life span (4-5 days) and no proliferation
Primary Cultures Plastic	• Limited proliferation can be achieved • Enriched epithelial populations so can study epithelial specific effects • Can study synthesis and secretion of ECM proteins • Serum-free conditions can be used	• In general, there is no functional differentiation • Response to hormones and growth factors inconsistent • No morphogenesis • Genotypic and phenotypic instability
collagen gels	• Proliferation • Epithelial specific effects can be investigated • Limited functional differentiation and secretion • Culturing cells within gels can result in ductal morphogenesis	• No WAP synthesis • No alveolar development
Reconstituted basement membrane (EHS)	• Maintenance and induction of alveolar development • Functional differentiation and expression of milk proteins and lipids • Vectorial secretion of proteins	• Possible presence of growth modulators in EHS • EHS interferes with many biological assays
Cocultures	• Different types of stromal-epithelial interactions can be examined • *In vitro* reconstitution studies can be done • Synthesis, assembly and degradation of ECM proteins can be studied	• Most coculture systems require serum

factor binding motifs have been identified within the different milk protein promoters.

Nuclear factor I (NF-I) and glucocorticoid receptor have been shown to be bind to milk protein gene promoters (Watson et al., 1991; Li and Rosen, 1995) but their fucntion is still unclear. On the other hand, STAT5 recognition sites have been identified in the promoter region of the caseins, WAP, BLG and α-lac genes, suggesting STAT5 is an important regulator of milk protein gene expression. The mutational analysis of STAT5 binding sites in β-casein, BLG and WAP promoters demonstrated the importance of STAT5 in prolactin signalling and its requirement for maximal transcription activation (see section 1.2.4). This work has been extended using *in vitro* approaches to determine the signal transduction pathway activated by prolactin (reviewed by Groner and Gouilleux, 1995; Watson and Burdon, 1996). Essentially, binding of prolactin to its receptor induces receptor homodimerization and the activation of the kinase JAK2 which tyrosine phosphorylates the receptor. The latent form of STAT5 is transiently associated with the active receptor complex through its SH2 domain (Src-homology-2) and becomes activated through tyrosine phosphorylation by JAK2. The active STAT5 can then dissociate from the receptor complex, dimerize and translocate into the nucleus. Once in the nucleus it can bind to its recognition site in gene promoters and activate transcription (Diagram 1.3). Since the milk protein genes are expressed at different stages of development it is envisaged that other transcription factors e.g. NFI and glucocortiod receptor also play a part in transcriptional regulation at the appropriate point in development.

Two murine STAT5 homologues have recently been identified STAT5a and STAT5b (Lui *et al.*, 1995). These closely related genes show 96% identity at the amino acid level but have different C-termini. Heterogeneity is increased further by alternative splicing to create at least two polypeptides from each gene (Azam *et al.*, 1995 & Lui *et al.*, 1995).

Genetic disruption of the prolactin signalling pathway has recently been achieved in mice by inactivation of the genes encoding the prolactin receptor [PRLR] (Ormandy *et al.*, 1997), STAT5a (Liu *et al.*, 1997) and STAT5b (Davey *et al.*, 1997).

Since the PRLR is expressed in many tissues during embryogenesis and STAT5 is a component of the cytokine signal transduction pathway in many cell types it was predicted these knockouts would result in an embryonic lethal phenotype. However mice deficient in PRLR or STAT5a or STAT5b survived to adulthood with defects in specific tissues.

Females with one intact PRLR allele failed to lactate during the first lactation due to reduced mammary development. However mammary development after the second pregnancy was sufficient for normal lactation, suggesting that functional development does eventually occur with continued hormonal stimulation. Homozygous females were infertile with multiple reproductive abnormalities. Similarly 50% of the homozyous males displayed fertility problems suggesting a key role for PRLR in mammalian reproduction.

STAT5a-deficient mice develop normally but fail to lactate after parturition due to lack of terminal differentiation. Although STAT5b has a 96% similarity and shows a similar expression pattern to STAT5a during mammary development it cannot substitute for the absence of STAT5a. The different C-termini of the two STAT5 homologous obviously have unique non-overlapping activities. This is supported by the different phenotype observed in the STAT5b knockouts which exhibit reduced growth of males and severe fertility problems in the females. The reproductive problems in the STAT5b deficient mice and the PRLR deficient mice are similar suggesting that prolactin signalling through STAT5b is a key regulator of mammalian reproduction.

Transgenic technology has also proven to be useful in the cancer research field and has lead to an increased understanding of both the development and progression of neoplasia. In particular, the ability to direct overexpression of candidate oncogenes in a particular tissue and to inactive gene function through homologous recombination has proven to be a very powerful tool.

Over the past decade, the transgenic mouse model system has been extensively used to investigate the actions of nuclear oncoproteins (e.g Fos, Jun, Myb and Myc families), nuclear tumour suppressor proteins (e.g p53, pRb and Wilms tumour [WT-

1]) and growth factors (e.g EGF, TGF-β and TGF-α). A number of these transgenic lines develop tumours with remarkable similarities to human cancers. In many cases the expression of oncogenes in transgenic mice has lead to hyperplastic lesions but not a fully transformed phenotype (neoplasia). It is now clear that many oncogenes require the cooperation of additional genes to make the transition to a full neoplastic phenotype (Kudson, 1985). One approach which has been used to identify cooperative effects between genes is to cross transgenic mouse lines which overexpress two different genes of interest in the same target cell, resulting in increased incidence of tumours and/or a more aggressive tumour phenotype. In the mammary gland this approach has been effective in determining key genes involved in mammary tumourogenesis. For example co-expression of H-*ras* and c-*myc*, (Sinn *et al.*, 1987) or *int*-1 plus *int*-2 (Kwan *et al.*, 1992) in mammary epithelial cells by generating double transgenic lines of mice results in an earlier appearance and a higher frequency of solid tumours as compared to expression of either of the transgenes alone.

The identification of key genes which are aberrantly expressed during the different stages of cancer has given researchers a starting point to try to establish the signalling mechanisms involved during its progression. Again the transgenic mouse model provides a system to determine and dissect the cellular pathways which are controlled, for example by tumour suppressor genes or those which are altered by oncogenes. However careful analysis of multiple founder mice and families are required to obtain reliable data which again is time consuming and expensive. A cell culture model system which could be manipulated *in vitro* to mimic some of the *in vivo* events would be beneficial in both defining the molecular mechanisms by which these pathways function and a useful complementary approach which could be utilised to guide whole animal experiments. Therefore using a combined transgenic and *in vitro* approach as opposed to a transgenic approach alone would provide a more efficient strategy to answer questions pertaining to the mechanisms involved in cancer progression. A more in depth review describing the contribution made by

transgenic mice to cancer research can be found in Transgenic Mice in Cancer Research ; Seminars in Cancer Biology; Ed by G. Merlino (1994).

The pharmaceutical industry has also exploited these advances in transgenic technology and gene targeting. In particular the production of human recombinant proteins in transgenic livestock has been used as an alternative to large scale mammalian cell culture production. By using the mammary gland as a bioreactor several human proteins with therapeutic potential have been produced in the milk of transgenic mice (Gordon *et al.*, 1987; Yu *et al.*, 1989; Archibald *et al.*, 1990; Meade *et al.*, 1990), rabbits (Buhler *et al.*, 1990) and sheep (Clark *et al.*, 1989). However the success of this work has been tempered by the reports of low frequency and/or low levels of transgene expression. Poor performance of transgenes is one of the major drawbacks faced by pharmaceutical companies utilising transgenesis. Since large scale production of proteins requires large transgenic livestock to be used e.g. sheep, goats or cattle, the problem of poor performance must be overcome to make this approach more cost effective.

Research into the regulatory elements required for high level expression of heterologous proteins in the mammary gland is presently being pursued using transgenic mice. In general, genomic constructs work better than cDNAs however the performance of cDNA constructs can be improved using intronic sequences (Brinster *et al.*, 1988; Palmiter *et al.*, 1991). A cell line which could be used as a reliable test system to screen the expression performance of constructs would be a valuable assest to optimise expression levels of transgenes before going to the expense of generating transgenic animals.

1.3.2 Cleared fat pad

A less expensive route towards introducing transgenes *in vivo* is to use the cleared fat pad technique (reviewed by Edwards *et al.*, 1996). This approach requires the manipulation of mammary epithelial cells in culture before transplantation into the cleared fat pad of syngeneic mice. It relies on the unique postnatal development of the mammary gland and the ability of any piece of mammary tissue to repopulate the gland. The mammary fat pad is cleared by removing the nipple of 3 week old female

mice which prevents any epithelial development. Regeneration of the mammary tree can then be achieved by injecting primary epithelial cells into the cleared fat pad. The mammary tree which is produced from these reconstitution techniques responds in the same manner as normal mammary tissue up to the time of parturition. However, these females cannot feed their offspring because the nipple connection is severed during the procedure. Genetic manipulation of the epithelium is done *in vitro* by infecting donor cells with nonreplicating viruses carrying the gene of choice and then these cells can be injected into a cleared fat pad of a recipient mouse. After approximately 10 weeks the fully grown reconsistuted gland is removed along with its non-manipulated partner which has its nipple intact and is an inbuild control for the procedure. The entire epithelium is normally analysed using whole mounts.

The transplantation approach provides a model system to study the early stages of tumour development. Unlike germline transgenesis where every cell contains the transgene, the reconstitution model offers a system to investigate clonal expansion of a population of cells and the interactions and/or competition between abnormal cells and normal cells during the early stages of tumourigenesis. Several oncogenes have now been expressed in reconstituted mammary epithelium. In general, expression of oncogenes in the mammary gland results in aberrant growth of the epithelium. This alteration in growth can be quite subtle with only minor changes to the pattern of growth or can result in gross morphological changes and complete hyperplasia. Edwards and his colleagues have successfully used this technique to try to model the development of neoplasia and in particular the preneoplastic changes which occur in human breast tissue.

ErbB2, a growth factor receptor which belongs to the ErbB/type I family of receptor tyrosine kinases (Ullrich and Schlessinger, 1990) is known to be activated in many breast tumours (King *et al.*, 1985; Slamon *et al.*, 1987; van der Vijver *et al.*, 1987; Slamon *et al.*, 1989; Gullick, 1990). Around 20-30% of breast carcinomas express elevated levels of ErbB2 which is usually associated with amplification of the gene (Berger *et al.*, 1988). Gusterson *et al.*, (1988) found that some ductal

carcinomas *in situ* overexpress ErbB2 suggesting that they could be precursors of tumours which overexpress the protein.

Transgenic mice have been generated using the MMTV promoter to drive expression of c-*erb*-B2 (*neu)* the rat equivalent *neu* protooncogene and *neu* oncogene. Activated *neu* was originally identified as a transforming gene in chemically induced rat neuroectodermal tumours (Shih *et al.*, 1981). Oncogenic activation can occur through a point mutation in the transmembrane domain (Bargmann *et al.*, 1986), deletion of the extracellular domain (Bargmann and Weinberg, 1988) or overexpression of the protein (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987; DiMarco *et al.*, 1990) which is thought to result in constitutive dimerization and activation of the receptor (Gullick *et al.*, 1992). Amplification and overexpression of the human homologue of *neu* (c-*erb*-B2) has been observed in a large percentage of primary breast cancers. However no equivalent point mutation (at position 664-valine to glutamic acid substitution) in the the human homologue or anywhere else in the transmembrane domain of the human gene has been discovered yet in human cancers (Slamon *et al.*, 1989; Lemione *et al.*, 1990). It has been shown however that genetic introduction of this point mutation at the homologous position in the human gene *in vitro* causes oncogenic activation of human c-*erb*-B2 (Hynes *et al.*, 1990).

Transgenic lines of mice carrying activated *neu* driven by the MMTV promoter initially appear normal but at approximately 3 months of age every transgenic mouse examined developed multi-focal mammary adenocarcinoma. These tumours arose synchronously and affected the entire epithelia suggesting that expression of activated *neu* requires few if any additional genetic events to lead to a transformed phenotype (Muller *et al.*, 1988). Overexpression of the proto-oncogene using the same promoter resulted in focal mammary tumours with longer latency than the activated *neu* transgenics (5-10 months compared to 3 months) with 72% showing metastatic tumours in the lungs at 8 months (Guy *et al.*, 1992). No point mutations were found in the transmembrane domain of tumours expressing *neu*, however small in-frame deletions were detected in the region coding for the extracellular

juxtamembrane domain of the receptor (Siegel *et al.*, 1994). Screening for comparable mutations in the extracellular domain in human tumours is now underway.

Expression of *neu* in the mammary gland using the cleared fat pad technique resulted in a similar phenotype. However, under closer examination, focal clusters of alveoli similar to those normally found during pregnancy were seen in the virgin gland. Such subtle changes in mammary growth were not observed in the germline derived transgenic equivalents. Edwards *et al.*, suggests that *c-erb*-B2/*neu*, which is normally expressed in pregnancy and lactation, (Dati *et al*, 1990), has a role to play in the development or maintenance of alveoli for these transplant experiments.

One of the major drawbacks of the reconstitution approach is the possiblity of an immunological response to the introduced gene product. For example two viral proteins MC29 *gag-myc* fusion protein (Edwards *et al.*, 1988) and SV40 large T-antigen when introduced into the cleared fat pad resulted in lymphocyte infiltration around the epithelium. This problem could be overcome by using nude mice or by inducing tolerance to the introduced protein in mice before trying to repopulate the gland. Also the identification of cells which express the introduced gene is problematic. Usually only a small number of cells in the transplant express the introduced gene since retroviruses will only integrate into dividing cells and primary cultures only divide slowly. The levels of expression within these cells is often rather low and the available antibodies are not of sufficient quality to be used on sections for detection. *In situ* hybridisation may be the answer but the mammary gland tends to give high background when looking at rare transcripts, and the retrovirus transcripts may be below the level of detection using current protocols (Matsui *et al.*, 1990; Weber-Hall *et al.*, 1994). An alternative solution to this problem would be to use β-galactosidase to "tag" the transfected cells. Edwards *et al.*, (1996) has already constructed an expression vector for this purpose. The construct has been designed so that two genes can be transcribed from the same transcript using the internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV). The coexpression of genes with β-galactosidase should aid in the interpretation of results.

In particular, in experiments where transplants do not result in an obvious phenotype, it will be possible to rule out whether cells which were manipulated in culture contributed to the transplant or not. To date only overexpression of genes has been achieved using this technique. Clearly it would be advantageous to be able to inhibit gene expression or knockout a gene. It would be particularly useful in cases where a germline knockout mouse dies in late gestation or perinatally, since embryonic mammary epithelium could be transplanted into a normal host and the development of the mammary gland assessed.

The development of several *in vitro* and *in vivo* systems has been an integral part in improving our understanding of how the mammary gland functions and some of the key genes which are involved in both normal and aberrant development of the gland. The emerging picture from these studies is one where mammary differentiation occurs through the activation of specific genes and is controlled on several levels by peptide and steroid hormones in conjunction with cell-cell and cell-matrix interactions. This hierarchy of control leads to a fully functional gland at lactation and orchestrates the changes which takes place as the gland regresses via an apoptotic route during involution.

1.4 THE DEVELOPMENT OF NEW CONDITIONALLY IMMORTAL MOUSE MAMMARY CELL LINES

Cellular proliferation is normally under tight regulatory control with cellular division dependant on progression through a series of steps termed the "cell cycle". These steps involve a period of DNA synthesis (S-phase), a period of mitosis (M-phase), with gaps in between these two phases termed G1 and G2 (Diagram 1.4). Although some cells remain in the cell cycle the majority of cells remain for prolonged periods of time in a resting or nonproliferative/senescent state termed G0. A number of checkpoints which reside in G1 have been identified which regulate the entry, from G0, and allow the progression of cells through the cell cycle (reviewed by Sherr, 1994). However, it is possible to interfere with this process to produce cultures with an infinite lifespan. Spontaneous immortalisation can occasionally occur in rodent cells, for example the loss of p53, a checkpoint control in G1, in

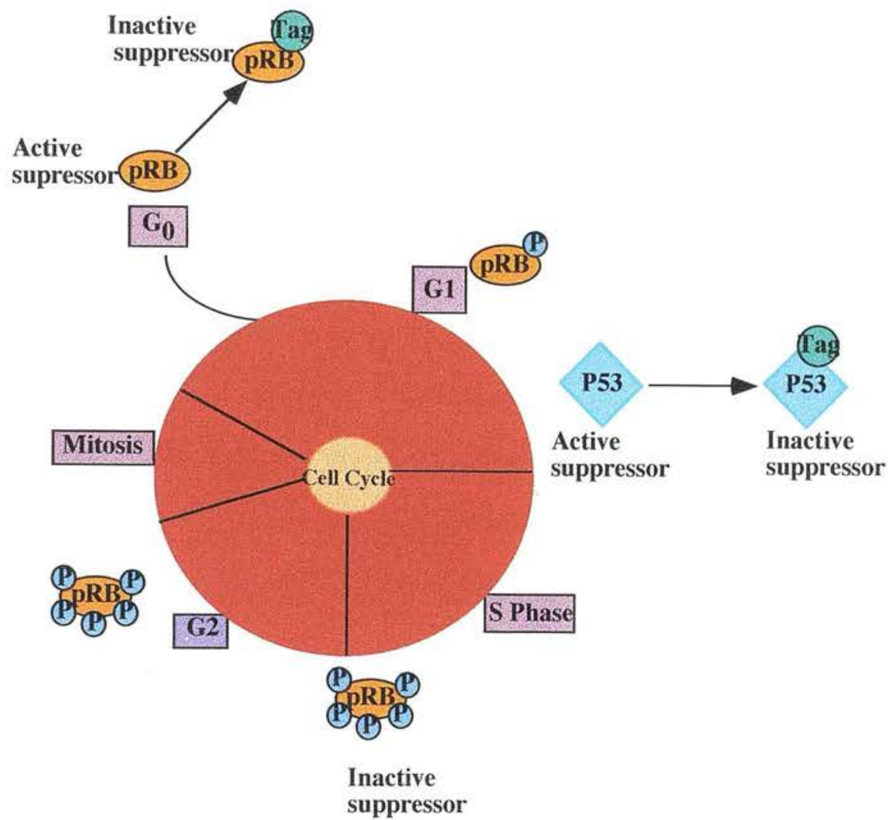


Diagram 1.4 Interactions of T-antigen with the cell cycle regulators pRb and p53

Actively cycling cells contain predominately phosphorylated pRb, whereas non-cycling (G0) cells contain the active supressor. T-antigen complexes with this unphosphorylated form of pRb and with p53, effectively removing these "brakes" normally imposed on the cell cycle resulting in cells which constantly proliferate. [Adapted from Sherr, C. J. (1994)]

HC11 cells. However, immortalisation is more efficiently achieved by introducing a known immortalisation agent such as the nuclear oncogene myc, the GTP binding protein ras, or DNA tumour virus immortalising genes. The immortalisation ability of the viral genes are discussed in more depth with particular reference to T-antigen.

Viruses are very effective at exploiting their host cells' DNA replication machinery and have evolved mechanisms to stimulate the growth of quiescent cells. Depending on the requirements for viral production, the cells can be maintained in a constantly proliferative phase or can lead to a transformed state. The DNA tumour virus genes, human papillomavirus type16E6/E7, adenovirus-5 E1A/E1B and Simian virus 40 (SV40) T-antigen have proven to be particularly effective at disrupting normal cell growth. Recent studies on host-protein interactions has revealed that these viral proteins interact with the same subset of cellular proteins. The identification of two tumour suppressor proteins, p53 and retinoblastoma gene product (pRB), involved in cell cycle contol (Levine, 1990; Nevins, 1992; Moran, 1993) are targets for these viral proteins. This has lead to the hypothesis that these proteins interfere with the regulatory pathways involved in normal cell growth by targeting genes which control the cell cycle by inhibiting cell growth. Unfortunately these viral proteins also have transforming abilities both in culture and in transgenic animals. For example expression of wild type SV40 T-antigen results in tumour formation and a dedifferentiated phenotype in transgenic animals (Choi *et al.*, 1988). However the prospect of exploiting the immortalising property of these genes has been reassessed since the isolation of temperature senstive mutants of T-antigen. Clearly it would be advantageous to have an *in vitro* system in which cells could be switched from a constantly proliferating state (immortalised) to one in which they could, given the correct stimuli, fully differentiate. Conditionally immortal mammary cell lines may provide a model system which can express the full repertoire of milk proteins.

1.4.1 Structure and function of wild type SV40 T-antigen and a thermolabile mutant tsA58.

SV40 is a small circular DNA virus (5243bp) whose natural host is primates but it can also infect a variety of mammalian cell types without multiplying. The

transforming ability of SV40 is attributed to the early gene products which are transcribed soon after infection. The two early gene products, small and large tumour antigen (T-antigen) are transcribed from the same transcriptional unit but are differentially spliced. Small t-antigen has only a small intronic sequence removed but retains a translation stop codon which results in a small 20kD protein. Large T-antigen, on the other hand, has more intronic sequences removed including the translation stop codon resulting in a larger protein of 94kD. Consequently both proteins share 80 amino acids at the amino-terminus but have completely different carboxyl termini (Diagram 1.5).

The actual role of small t-antigen is still unclear but it appears to regulate protein phosphatase 2a (Pallas *et al.*, 1990; Walter *et al.*, 1990; Yang *et al.*, 1990; Scheidtmann *et al.*, 1991). In culture it is not necessary for viral replication but it does appear to aid in the transformation of specific nonpermissive cell types e.g. murine ductal epithelial cells in liver and kidney (Choi *et al.*, 1988).

Large T-antigen is responsible for the control of viral infection and the necessary alteration in cellular processes for viral replication. Its ability to disrupt normal cell growth has been well documented in rodent cultures (Tegtmeyer, 1975; Tooze, 1980) and in over 20 distinct cell types in transgenic mice (Hanahan 1988 &1989; Adams and Cory 1991). Its expression *in vivo* is generally associated with tumourigenesis however this response appears to be cell type specific. For example the MMTV promoter was used by Choi *et al.*, (1988) to direct expression of T-antigen (and small t-antigen) to a variety of epithelial cells in transgenic mice but did not result in tumour formation in all the tissues which expressed the protein. In particular the mammary gland appeared to be relatively resistant to T-antigen transformation since even during lactation when the MMTV promoter is most active no neoplasia was observed in these mice.

The molecular mechanisms employed by T-antigen to alter normal cellular growth have recently become clearer. As mentioned before, the stimulation of host cell replication depends on the ability of T-antigen to interact with a subset of cellular proteins which are involved in negatively regulating entry into S-phase of the cell

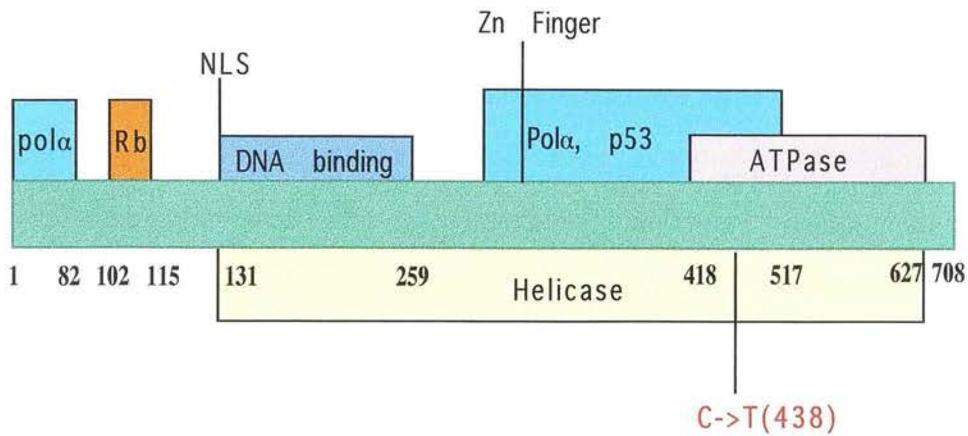


Diagram1.5 Functional domains of SV40 T-antigen and the site of mutation in the tsA58 mutant
The minimal regions of the protein which retain binding to DNA polymeraseα-primase (polα), ATPase and Helicase. The retinoblastoma (pRb) and p53 tumour suppressor proteins binding sites are also indicated and the single point mutation found in tsA58.

cycle. The binding of T-antigen to these proteins removes these cellular "brakes" on the cell cycle resulting in the stimulation of DNA synthesis and cell growth. Its transforming ability is thought to be mediated by its interaction with the tumour suppressor proteins p53 and pRb (Schreier *et al.*, 1990). A third protein p107 which is a member of the pRb family also interacts with T-antigen in the same domain as pRb. The binding of these proteins to T-antigen is illustrated in Diagram 1.5. The function of p107 is still unclear but p53 and pRb have been extensively studied. Both these proteins are involved in cell cycle control (Bookstein and Lee 1991; Lane 1992; Levine *et al.*, 1991; Marshall 1991; Weinberg 1991; Hamel *et al.*, 1992; Hollingsworth *et al.*, 1993) and mutations in these genes are associated with cancer. Both genes have been knocked out in mice and display a phenotype. The p53 null mice develop normally, however 90% develop tumours between 3-6 months of age (Donehower *et al.*, 1992; Harvey *et al.*, 1993; Jacks *et al.*, 1994). Mice homozygous for a pRb mutation die *in utero* between 13.5-15.5 days and display neurogenic and haematopoietic defects (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). It has been postulated that T-antigen in effect acts as a double "knockout" complexing with p53 and pRb preventing the brakes on the cell cycle which is normally imposed by these proteins resulting in cells which are allowed to progress unchecked through the cell cycle (Diagram 1.4). This implies that any errors in DNA replication or segregation during mitosis will not be corrected and will result in increased genetic instability and possible tumour formation.

Temperature-sensitive mutants of T-antigen have been isolated which have the same properties as wild type T-antigen at a permissive temperature (33 °C) but at the restrictive temperature (39°C) T-antigen is inactive. One of these mutants is tsA58 which has a cytosine changed to thymidine resulting in the substitution of an alanine residue for valine at position 438 within the ATPase binding domain (Diagram 1.5). At the restrictive temperature of 39°C there is some controversy as to whether there is a conformational change in this thermolabile T-antigen mutant (Deppert *et al.*, 1991) which renders it inactive or whether the mRNA is rapidly degraded (Tegtmeyer, 1975). Clearly this thermolabile mutant could be used to generate

constant proliferating cell lines at 33°C but by moving the cells to 39°C the cells lose this immortalisation. In many cell types constant proliferation is incompatible with differentiation. This is observed in a number of immortalised cell lines which dedifferentiate and can no longer undergo functional differentiation. Consequently establishing conditionally immortalised cell lines potentially provides a culture system in which the cells can be switched from a proliferative state to a fully differentiated state given the correct differentiation stimuli.

1.4.2 Introduction of immortalising genes into mammary cells

To exploit the properties of SV40 T-antigen and its thermolabile mutants to generate mammary cell lines requires stable integration of the DNA into the genome. This can be done *in vitro* using DNA mediated gene transfer or retroviral gene insertion. The efficiencies obtained using each method is highly dependant on the cell type used. Alternatively the generation of transgenic animals which express T-antigen in the mammary gland using tissue-specific promoters could be used to stably integrate T-antigen into the genome.

1.4.2.1 Non-viral mediated transfection methods

There are now a variety of different gene transfer methods which can be used to introduce DNA into mammalian cells. The nonviral methods include; calcium phosphate precipitation, DEAE-dextran, electroporation and several lipid based transfection systems which are commercially available e.g. Lipofectamine, Dotap. The most commonly used method is still calcium phosphate co-precipitation of the DNA construct and addition of the precipitate to the cells. Using this technique DNA entering the cell is taken up into phagocytic vesicles (Graham and van der Eb, 1973) but sufficient DNA enters the nucleus and is integrated into the genome to allow expression of the gene construct. Using a variety of cell types, transfection efficiencies of up to 10^{-3} have been obtained and calcium phosphate transfection methods are still routinely used to generate stably expressing mammary cell lines. A number of modifications to the original procedure have been made over the years which have been reviewed by Gorman (1985).

DEAE dextran is more often used when transient transfections of mammalian cells in culture are required. It is useful when large numbers of transfected constructs are required to be analysed since the DEAE dextran mix is prepared and the DNA construct added, overlaid onto the cells and assayed for expression 24-48 hrs later.

The chemical mechanism of cellular uptake is unclear (as is the case for calcium phosphate) but transfection efficiencies of this method can be improved by adding chloroquine. It is thought that the chloroquine neutralises the pH of the cells' lysosomes thus inhibiting the degradation of the DNA on its way to the nucleus. However DNA introduced into cells using this method are reported to undergo a high rate of mutagenesis when compared to calcium phoshate transfections (Calos *et al.*, 1983; Razzaque *et al.*, 1983; Ashman and Davison, 1985).

Recently lipid based transfection methods have been successfully used to transfect mammalian cell lines. However there has been no reports of this method being used to transfect mammary cell lines.

Electroporation is another method which has been devised to introduce foreign DNA into mammalian cells. This procedure involves mixing a cell suspension with the DNA construct and exposing the cell/DNA mixture to a high voltage electric field. This creates pores within the cell membrane which allows the DNA into the cytoplasm where some of the DNA reaches the nucleus and becomes stably integrated into the host cells' genome. The resealing of the membrane occurs in a time and temperature(low)-dependant manner therefore incubating the cells at 0°C for a period will increase cell survival. Again the efficiency of transfection is dependant on the correct parameters being set and is different for different cell types.

1.4.2.2 Viral-mediated gene transfer

Viral mediated gene transfer exploits several characteristics of retroviruses which are suitable for gene transfer including:- efficient transmission to recipient cells, integration into host chromosomal DNA, plasticity of the genome which allows the insertion of foreign DNA. The gene construct is introduced into a nonreplicating retrovirus. The virus infects cells by attaching to cell surface receptors before becoming internalised and uncoated. The RNA can then be reverse transcribed by the

viral reverse transcriptase and the DNA is transported into the nucleus where it integrates into the host genome through two viral long terminal repeats (LTR). However this method requires cells to be proliferating and growing to achieve transfection efficiencies of 1%.

All the transfection methods described above require a large number of cells to be transfected and grown under selective pressure to obtain sufficient cells for experimentation. Optimising one of these transfection methods could be used to generate conditionally immortal primary mammary epithelial cultures by stable integration of the thermolabile T-antigen mutant (tsA58) under the control of a suitable promoter. In addition to the usual difficulties of working with primary cultures (see section 1.2.2) there are additional problems with efficiencies of transfections. Primary cultures do not readily integrate foreign DNA, probably due to their low growth potential in culture, therefore large numbers of primary cultures would have to be generated to establish some clones which have stably integrated and express T-antigen. Additionally within the same pool of cells there can be a number of different sites of integration which could affect expression levels of T-antigen. Despite these problems, tsA58 has been successful introduced into bovine primary cultures by transfection and a cell line designated MAC-T established which can differentiate and express casein proteins (Huynh *et al.*, 1991). Human luminal epithelial cells have also successfully been cultured from milk using wild type T-antigen (Bartek *et al.*, 1991) and retroviral mediated gene transfer.

1.4.3 Transgenesis

Another option is to exploit transgenic technology and direct expression of the T-antigen mutant to the mammary glands of transgenic mice using a mammary specific promoter (see section 1.3.1). Generating transgenic mice using either a milk protein promoter or the MMTV promoter should direct expression of the SV40 T-antigen to the secretory epithelium. This would allow a single integration site to be looked at in a given line of mice. The temperature-sensitive mutant should not be active in the animals thus overcoming the transforming properties which were previously

encountered when wild type T-antigen was targeted to the mammary gland using the WAP promoter (Tzeng *et al.*, 1993).

Jat *et al*, (1991) used this approach to generate the "immortomouse" which carries the temperature-sensitive T-antigen mutant tsA58 under the control of a housekeeping gene promoter H-2K^b. This promoter directs expression to a wide range of tissues and can be induced *in vitro* using γ-interferon. Although the immortomouse shows thymic hyperplasia these mice do undergo normal development and have successfully been used to establish a number of conditionally immortal cell lines. Cell lines, to date which have been established from this transgenic line of mice include thymocytes (Jat *et el.*, 1991), crypts from the colon and small intestinal epithelial cells (Whitehead *et al.,* 1993), osteoclast precursor cells and bone marrow stromal cells (Chambers *et al.,* 1993), astrocyte lines which express *in vitro* many of the cell properties of glial scar tissue clonal cultures (Groves *et al.*, 1993) and myogenic cell lines which can be genetically modified *in vitro* and transplated into a suitable donor to study mutant phenotypes of clinical importance (Morgan *et al.*, 1994). However no mammary cell lines have been generated from "immortomouse". The lack of success could be attributed to insufficient expression of the transgene in the secretory epithelium *in vitro*. Using a mammary specific promoter which expresses highly in the secretory epithelium at specific stages of development should allow the derivation of conditionally immortal cell lines.

1.5 AIMS OF PROJECT

The aim of this project was to use a transgenic approach to derive a conditionally immortal mouse mammary epithelial cell line by directing expression of the temperature-sensitive T-antigen, tsA58 to the secretory epithelial cells using a milk protein promoter BLG. It was envisaged that conditional immortalised cell lines would provide an elegant *in vitro* system whereby the immortalisation gene could be switched off by raising the temperature and the cultures, given the correct stimuli, induced to differentiate. It was anticipated that such a system would overcome some

of the problems presently encountered with the available cell lines and provide a more accurate model of mammary gland development during pregnancy and lactation. Characterisation of the cell types present in the generated cultures and assessing functional differentiation in response to lactogenic hormones should determine their usefulness as a differentiation model system. An *in vitro* tool which could express the full repertoire of milk proteins in culture would provide a valuable research tool to define the molecular pathways activated during the differentiation process. It would also be a useful assay system to test out expression constructs prior to generating transgenic animals.

MATERIALS AND METHODS

2.1 DNA MANIPULATION

2.1.1 Restriction digestion of plasmid DNA

Plasmid DNA was digested in the appropriate enzyme buffers suggested by the manufacturers (Boehringer Mannheim, New England Biolabs) at 37°C for approximately 2 hours using 3-5 fold excess of the recommended amounts of enzyme. Double digests were carried out together unless the salt concentrations were incompatible, in which case the lower salt concentration digest was carried out first and then the salt concentration increased for the second digest.

2.1.2 Isolation of DNA fragments from agarose gels

Plasmid DNA fragments were isolated on low melting point agarose gels and recovered using Agarase (New England Biolabs) as indicated by the manufacturers. Typically, 2-5μg plasmid DNA was digested with the appropriate restriction enzyme and electrophoresed in low melting point agarose gels (see section 2.3). The DNA was visualised using long-wave UV light and the appropriate fragment excised from the gel into an eppendorf tube and incubated for 1hr at 42°C with 1/10 vol of enzyme buffer and 5 units of Agarase. Once the agarose was completely digested the DNA was either used directly in a ligation or alternatively the DNA was concentrated by precipitation with 2.5 volumes of isopropanol and 0.1 volumes of 3M sodium acetate at -20°C for 1 hr. The DNA was pelleted by centrifugation (14 000g for 15 minutes) then washed with 70% ethanol to remove the sodium acetate and resuspended in TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) or ddH_20. Typically 50-60% of the original DNA fragment was recovered using this method.

2.1.3 Filling in reaction

The filling in of a 5' DNA overhang to create a blunt-ended DNA fragment was carried out as follows:-

0.5μg DNA fragment (in 10μl TE buffer)
2μl 10x T4 DNA polymerase buffer
1μl 2mmol dNTPs
1μl T4 DNA polymerase
7μl ddH_2O
Total 20μl

The reaction was incubated at 37°C for 5 minutes and then stopped by adding 1μl of 0.5M EDTA. A phenol/chloroform extraction was performed to remove the protein and the DNA precipitated with 2 volumes of 100% ethanol and 0.1 volumes of 3M sodium acetate at -20°C for 1h. The DNA was pelleted by centrifugation at 14 000g for 20 minutes, washed with 70% ethanol, air dried and resuspended in 20μl TE buffer.

2.1.4 Ligation reactions

"Ready-To Go" T4 DNA Ligase (Pharmacia) was used as follows:- In a cohesive end ligation 50-100ng of vector and insert DNA (molar ratio 1:3) in 20μl of TE buffer were added to a tube of "Ready-To-Go" T4 DNA Ligase which contained a minimum of 6 units of FPLC pure T4 DNA Ligase, 66mM Tris-HCl pH 7.6, 6.6mM $MgCl_2$, 0.1mM ATP, 0.1mM spermidine, 10mM DTT, and stabilisers stored lyophilized. The ligation was incubated at room temperature for 5 minutes and mixed by pipetting several times and the contents collected by a brief centrifugation. The ligation reaction was carried out at 16°C for 30 minutes and then 2μl used to transform _E.coli_ DH5α competent cells (see section 2.1.5). In blunt-end ligations ~300ng of DNA was used and incubation time extended to 45 minutes. The

incubation time could be extented to overnight if the ligation had not gone to completion.

2.1.5 Transformation of competent cells with plasmid DNA

E.coli DH5α (competent cells (Genotype:F⁻, endA1, hsdR17[rk,⁻ mk⁺], supE44, thi-1, λ,⁻ recA1, gyrA96, relA1, Δ[argF-lacZYA]U169, φ80dlacZΔ[M15Δ] purchased from Life Technologies, Inc.) were stored at -70°C. The φ80dlacZΔ[M15Δ] marker provides α-complementation of the β-glacatosidase gene permitting blue/white colour selection of recombinant/nonrecombinant plasmids (see section 2.1.6.2). Transformations were performed as described by the manufacturers. Typically, 50μl of competent cells were transformed with 1ng of purified plamid DNA or 2μl of ligation reaction mixture.

2.1.6 Preparation of agar plates

2.1.6.1 Antibiotic plates

LB bottom (1% w/v, 0.5% w/v yeast extract, 1.5% w/v agar, 0.1M NaCl) was melted and cooled to 37°C before adding (100μg/ml) ampicillin. Agar plates were poured using aseptic technique and allowed to set. Unused plates could be stored for approximately 2 weeks at 4°C.

2.1.6.2 Blue/white colour selection

Blue/white colour selection exploits a phenomenon called α-complementation (Jacobs and Monod, 1967) and is used to select recombinant plasmids. For example the pUC and Bluescript series of vectors encodes the α-fragment of the lacZ gene with a polylinker inserted. The nonrecombinant plasmids produce a functional α-fragment (N-terminus) and when the host cell encodes (lacZ[ΔM15], an ω fragment (C-terminus) then a functional β-galactosidase is produced. When colonies are grown on medium containing IPTG, which inactivates the lac repressor, and Xgal, which

acts as a substrate for β-galactosidase, the colonies are blue. However, when, DNA is inserted into the polylinker of the plasmid, the α-fragment is disrupted and α-complementation does not occur and recombinant colonies are white.

2.1.7 Small-scale preparation of plasmid DNA

The alkali lysis method as described by Sambrook *et al*., (1989) was used. 2mls LB medium (Sambrook *et al*., 1989), containing 50μg/ml ampicillin (Sigma) in 15ml sterilin tubes were inoculated with single bacterial colonies and incubated overnight at 37°C in a shaking incubator (Gallenkamp, 200rpm). 1.5mls of each culture were decanted into eppendorf tubes and centrifuged at 14 000g for 10 seconds to pellet the cells. After removal of the supernatant the pellets were resuspended in 100μl of ice-cold TGE solution (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0). 200μl of 0.2N NaOH/1% (w/v) SDS was added to lyse the cells and denature the DNA. The tubes were inverted several times and stored on ice. 150μl of ice-cold potassium acetate/glacial acetic acid solution (3M and 2M, respectively) were added, mixed, incubated on ice for 5-10 minutes and then centrifuged at 14 000g for 5 minutes. The supernatants were decanted into fresh tubes and the DNA precipitated with 2 volumes of ethanol at room temperature. The DNA was pelleted by centrifugation for 10 minutes and then washed twice with 70% (v/v) ethanol. The pellets were air dried and dissolved in TE buffer supplemented with DNase-free pancreatic RNase (20μg/ml).

2.1.8 Large-scale preparation of plasmid DNA

Plasmid DNA was prepared from large scale bacterial cultures by the method of Birnboim and Doly, (1979) and followed by either caesium chloride gradient centrifugation or purification through ion-exchange plasmid purification columns supplied by Promega.

0.5mls of a bacterial culture (grown overnight) was inoculated into a 2 litre flask containing 500mls of LB medium supplemented with 50μg/ml ampicillin and shaken overnight at 37°C. The bacteria were pelleted by centrifugation at 6 000rpm at 4°C for 10 minutes in a Sorvall GS3 rotor. The cells were resuspended in 50mls of ice-cold TGE buffer and lysed by adding 120mls of 0.2N NaOH/1% (w/v) SDS. The viscous solution was mixed thoroughly and left on ice for 10 minutes to complete the lysis and allow the DNA to denature. Chromosomal DNA and proteins were precipitated by adding 60mls of ice-cold potassium acetate/glacial acetic acid solution (3M and 2M respectively), incubated on ice for 10 minutes and centrifugated for 10 minutes as above. The plasmid DNA containing supernatant was filtered through 4 layers of nylon gauze and precipitated with 0.6 volumes of isopropanol for 10 minutes at room temperature. The plasmid DNA was pelleted by centifugation at 8 000rpm for 15 minutes and the pellet washed twice with 70% (v/v) ethanol, air dried and resuspended in ~2mls of TE buffer.

Purification of plasmid DNA through Caesium Chloride/Ethidium Bromide Density Gradients

9.5 g of caesium chloride (CsCl) was added to DNA/TE (from 500ml starting culture) and volume made up to 10mls with TE buffer. The caesium chloride was dissolved and 500μl of 10mg/ml ethidium bromide added and mixed carefully. The solution was transferred to Beckman quickseal tubes and centrifuged at 50 000rpm for 16-18 hours at 20°C. Three distinct bands could be seen after centrifugation. The lower band which contained closed circular and supercoiled DNA was removed into tubes using a needle and syringe making sure that the upper chromosomal and open circular DNA was left. The ethidium bromide was removed from the CsCl/DNA mix by increasing the volume to 5 mls with ddH_20 and extracting with an equal volume of butan-1-ol. The upper aqueous layer containing the DNA was removed into a fresh sterilin tube and the pink organic phase discarded. The extraction was repeated

until the upper layer was clear (usually four times). The DNA was precipitated by adding 2.5 volumes of 70% (v/v) ethanol, 0.1 volumes of 3M sodium acetate and incubating at -70°C for 30 minutes. The DNA was pelleted by centrifugation at 8 000rpm for 10 minutes at 4°C in a HB4 rotor. The pellet was air dried and resuspended in 500µl of TE buffer. In general caesium chloride plasmid preparations were used when good quality highly pure DNA was required, for example, transfections into cultured cells or isolation of fragments for microinjection.

"Magic" Maxipreps DNA purification (Promega)

10mls of purification resin was added to the DNA solution and mixed by swirling. The mixture was added to a magic maxicolumn and a vacuum applied to draw the DNA/resin mixture into the column. The DNA bound to the ion-exchange column was washed with 25mls of column wash solution (200mM NaCl, 20mM Tris-HCl, pH 7.5, 5mM EDTA, diluted 1:1 with 95% (v/v) ethanol). 5mls of 80% (v/v) ethanol was applied to the column to wash the DNA. The resin/DNA was dried by drawing a vacuum for an additional 10 minutes. 1.5mls of preheated (70°C) TE buffer was applied to the column, left for 1 minute and the DNA eluted by centrifugation at 2 500rpm for 5 minutes in a swing-out bucket rotor.

The DNA concentration and purity was estimated by measuring absorbance ratio at 260nm/280nm. In general highly pure DNA has a 260nm/280nm ratio of >1.8, while protein contamination lowers this ratio. The quality of the DNA was checked by running a mini-ethidium bromide agarose gel (see section 2.3). Typically a 5µl aliquot of the plasmid and 5µl loading dye (40mM EDTA, 0.1%SDS (w/v), 30% (w/v) ficoll and 1.2mg/ml bromophenol blue) was loaded and run on the gel.

2.1.9 Purification of transgene fragment for microinjection

The transgene construct (pBS-4.2BLG-tsA58) was purified free from plasmid sequences as described in section 2.1.2. Approximately 100µg of DNA was used in

the digestion. The transgene was resuspended in a Low salt buffer (0.2M NaCl, 20mM Tris-HCl pH 7.4, 1mM EDTA) and purified using an Elutip-d column (Schleicher & Schuell) as described by the manufacturers. Briefly the column was prewashed with 5mls of a High salt solution (1M NaCl, 20mM Tris-HCl pH 7.4, 1M EDTA). The column was then equilibrated with 5mls of Low salt buffer before the DNA fragment was filtered through a 0.45μm cellulose acetate filter (Schleicher & Schuell) attatched to the column. This removes any particulates from the sample e.g residual gel fragments. The filter was disconnected from the column and 5mls of Low salt loaded onto the column. The DNA was eluted by loading 5mls of High salt onto the column and collecting the eluant in a 1.5ml eppendorf tube. The DNA was precipitated using 2 volumes of isopropanol at 4°C for 15 minutes and pelleted by centrifugation (14 000g for 15 minutes). The DNA fragment was resuspend in TE buffer at a final concentration of 50ng/μl.

2.2 Nucleic Acid Isolation

2.2.1 Isolation of mouse tail genomic DNA

Five week old mice were anaesthetised (Flecknell, 1983) and 1-2cm tail biopsies taken for preparation of genomic DNA. The biopsies were incubated in 0.75 mls digestion buffer (10mM Tris-HCl pH 7.9, 1mM EDTA pH 8.0, 0.3M sodium acetate, 1% SDS and 200μg/ml proteinase K) overnight in a shaking incubator at 37°C. Tail residues were centrifuged for 5 minutes at 14 000g in a microfuge at 4°C and the supernatant decanted into 1 ml of ethanol, inverted several times until threads of DNA were visible. The DNA was pelleted by centrifugation at the same speed and temperature as above for 15 minutes. The ethanol was removed and the pellets washed with 70% ethanol and air dried. The DNA was resuspended in 500μl of TE buffer, incubated at 65°C for 10 minutes and tubes flicked to disperse the DNA. The DNA was then stored at -20°C and used for PCR and/or Southern blotting.

2.2.2 Isolation of liver genomic DNA

Approximately 0.5g of tissue was homogenised in 1ml of solution D (4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% [v/v] sarcosyl, 0.1M [v/v] 2-mercaptoethanol) Chomcyzynski and Saachi (1987). The samples were incubated for 1 hour at 50°C with 100μl of proteinase K (200μg/ml) and 20μl of RNase (50μg/ml) added to digest protein and RNA respectively. 1ml of 100% ethanol was added and mixed carefully by inverting a few times. The DNA was "spooled " out on glass pasteur pipettes and washed with 70% (v/v) ethanol, air dried and dissolved in 500μl of TE buffer. 20μl of proteinase K was added and incubated overnight at 50°C to ensure that the DNA was completely dissolved and proteins completely digested. The samples were phenol/chloroform extracted to remove any protein and then stored at -20°C.

2.2.3 Isolation of total RNA from mouse tissue.

All plastics used for RNA analysis were double autoclaved.

Female mice were sacrificed by cervical dislocation. Approximately 0.5g of tissue was homogenised in 2mls of RNazol (Biogenesis). 0.1 volumes of chloroform was added to the homogenate, mixed well and incubated on ice for 15 minutes. Protein was removed from the samples by centrifugation (14 000g for 15 minutes at 4°C). The upper aqueous layer containing the RNA was carefully removed avoiding the protein contained at the white interphase and inorganic bottom layer. The RNA was precipitated with 1 volume of isopropanol at 4°C for 15 minutes. The RNA was pelleted by centrifugation (14 000g for 15 minutes at 4°C), washed with 70% (v/v) ethanol, dissolved in deionised formamide (Chomczynski 1992) and stored at -20°C.

2.2.4 Isolation of total RNA from cultured cells.

Confluent flasks of cells (normally T80cm^2 flasks with approximately 1 x 10^7 cells) were washed once in 1x PBS and cells harvested by trypsinization (see section

2.8.2). The cells were pelleted by centrifugation at 1000rpm in a bench top centrifuge for 5 minutes and the supernatant removed. The cells were lysed by resuspending in 2mls of RNA-zol. The RNA was extracted as above and dissolved in deionised formamide and stored at -20°C.

The concentation and purity of the RNA was estimated by measuring the O.D. ratio at wavelength 260nm/280nm. Approximately 100µg of RNA was normally obtained from this method and the ratio of isolated RNA >1.8.

2.3 ELECTROPHORETIC TECHNIQUES

2.3.1 Agarose gel electrophoresis of genomic DNA

0.8g of agarose was dissolved in 1x TAE buffer (40mM Tris-acetate, 2.5mM EDTA pH 7.7), allowed to cool to 45-50°C and ethidium bromide added to a final concentration of 1µg/ml. The gel was poured and allowed to set before submerging in 1x TAE running buffer. 0.1 volumes of loading dye was added to 10µg equivalents of genomic DNA and samples either electrophoresed overnight at 1-2 volts/cm or at 4 volts/cm for 3-5 hours. The DNA was visualised under long-wave UV light and photoghraphed.

2.3.2 Formaldehyde gel electrophoresis of total RNA

Total RNA samples were electrophoresed on a 1% (w/v) agarose gel prepared with MOPS buffer (0.02M 3-N-[morpholinol] propanesulfonic acid, 5mM sodium acetate containing 6.8% (v/v) formaldehyde essentially as described by Sambrook *et al.*, (1989). The solution was swirled to mix and the gel poured in a fume hood and allowed to set. 19µl of Northern loading buffer (10µl ddH_2O, 3.5µl formaldehyde, 2µl 10xMOPS, 4µl of loading dye and 0.25µl of 10mg/ml ethidium bromide) was added to 10µg equivalents of RNA, denatured at 65°C for 5 minutes, to remove secondary structures and immediately loaded on to the gel. The samples were

electrophoresed in 1xMOPS, overnight at 1-2volts/cm, visualised by long-wave UV light and photographed.

2.3.3 Native polyacrylamide gel electrophoresis

Native 6% polyacrylamide gels and nuclear extracts were prepared as described by Watson *et al.*, 1991 with some modifications (see section 2.9.2). Loading dye (25mM HEPES pH 7.5) was added to the DNA/protein complex and the gel electrophoresed at 4volts/cm for 3hrs in 1xTAE buffer (6mM Tris, 1mM EDTA, 7.5mM sodium acetate pH 7.5). The buffer was recirculated using a peristaltic pump and water cooled to prevent dissociation of protein/DNA complexes. The gels were fixed in 10% (v/v) acetic acid and dried by vacuum pump before being exposed to phosphorimager screens or X-ray film (AGFA:CUPRIX RP1).

2.3.4 Denaturing (SDS/PAGE) polyacrylamide gel electrophoresis

One dimensional SDS/PAGE gel electrophoresis was performed as described by Laemmli, (1970). Using a premixed polyacrylamide solution (30%) supplied by Scotlabs, a 15% running gel with a 3% stacking gel was cast. Protein samples in electrophoresis sample buffer (see section 2.9.1) were boiled for 3 minutes to ensure complete denaturation of the proteins before loading on the gel. Gels were electrophoresed at a constant current of 30mA for about 1hr and then at 48mA for 5hrs.

2.3.5 Sequencing gels

6% (w/v) polyacrylamide gels with 7M urea was used for DNA sequencing. The samples were denatured at 65°C for 5 minutes before loading on to the gel. The samples were electrophoresed at constant power (60 watts) in 1x TBE (0.13M Tris, 4mM boric acid, 0.25mM EDTA) and fixed in 10% acetic acid then dried by vacuum pump before being exposed to X-ray film (AGFA:CUPRIX RP1).

2.4 NUCLEIC ACID TRANSFER

2.4.1 Transfer of DNA from agarose gels to nylon membrane.

The DNA was transferred from the agarose gel onto Hybond-N membrane (Amersham) using the blotting method described by Southern (1975). The DNA was firstly depurinated by gently shaking in two changes of 0.2N HCl for 15 minutes (required when fragments >8kb are to be transferred). Then the DNA was denatured in two changes of denaturing solution (1.5M NaCl, 0.5M NaOH) for 15 minutes each, to hydrolyse the phosphodiester backbone at the site of depurination and rinsed in distilled water before being neutralised (ceases hydrolysis), in two changes of neutralising solution (1.5M NaCl, 0.5 M Tris-Cl pH 7.5, 1mM EDTA).

The gel was placed on a wick consisting of two sheets of Whatman 3mm paper on a glass plate and soaked in 20x SSC (3M NaCl, 0.3M sodium citrate). A piece of Hybond-N membrane cut to the size of the gel was prewetted in 2x SSC and placed on to of the gel. This was followed by 2 pieces of Whatman 3mm cut to the same size and prewetted in 2x SSC. Air bubbles were removed using a sterile glass pipette. Paper towels were placed on top of the 3mm paper followed by a glass plate and finally a weight (approx. 0.5kg) and the DNA allowed to transfer to the membrane by capillary action as the SSC is drawn through the paper towels. After 3hrs the wet paper towels nearest the gel were replaced by dry towels and the transfer allowed to proceed for approximately 20 hrs in total. The membrane was removed, rinsed in 3x SSC and the DNA cross-linked onto the membrane using an automatic UV-linker (Stratagene).

2.4.2 Transfer of RNA from formaldehyde gels to nylon membranes

A similar procedure as above was used but the depurination, denaturation and neutralisation steps were omitted. The transfer of the RNA was done with 10x SSC, membrane and Whatman paper prewetted with sterile water as this gave the most efficient transfer of RNA from the gel.

2.5 RADIOLABELLING TECHNIQUES

2.5.1 Random priming

Essentially this procedure exploits the exonuclease activity of DNA polymerase I. The C-terminal, Klenow fragment retains 3'->5' exonuclease activity but lacks 5'->3' exonuclease activity and can therefore incorporate nucleotides which are complementary to 5' overhangs. Radiolabelled DNA probes were prepared by the methods described by Feinberg and Vogelstein (1983 & 1984).

25-50ng linearised double stranded DNA in 23µl of ddH_2O was added to an eppendorf tube and 10µl of random primers from a Prime-it II kit (Stratagene) added and boiled for 2 minutes. The following components from the kit were added:

10µl 5x dCTP primer buffer

1µl Klenow fragment of DNA polymerase I (5 units/µl)

5µl ^{32}P labelled α- dCTP (Amersham: Specific Activity 3000Ci/mM).

The tube was incubated at room temperature for 30 minutes and incorporation of radioactivity measured by TCA precipitation as described by Sambrook *et al* ., (1982).

The unincorporated label was removed by applying the probe (made up to 100µl with ddH_2O) to a G50 Sephadex column (5 Prime->3 Prime Inc) as described by the manufacturers. Briefly the column was inverted several times and the column buffer removed by centrifuging the column at 1000rpm in a swing out rotor bench top centrifuge for 2 minutes. The probe was applied to the column and centrifuged again for 2 minutes at 1000rpm. The unincorporated nucleotides remained trapped in the column and the labelled fragment eluted. The probe was denatured by adding 1/4 of a volume of 2M NaOH and leaving for 5 minutes before adding to the hybridisation solution.

2.5.2 End labelling

Double stranded oligonucleotides, synthesised by Oswel DNA systems, King's Buildings, Edinburgh, were radiolabelled by phosphorylating the 5' end using Polynucleotide kinase (Boehringer Mannheim) and ^{32}P labelled γATP (Amersham:Specific Activity >5000 Ci/mM). The following components were added, in order, to a microfuge tube:-

2μl (50ng) double stranded oligonucleotide

1μl 10x kinase buffer

4μl ddH_20

2μl ^{32}P γ-ATP (10 units/μl)

1μl Polynucleotide kinase

The labelling reaction was incubated at 37°C for 30 minutes and unincorporated nucleotides removed by applying the probe (made up to 100μl) to a Sephadex G50 or G25 column depending on the size of the oligonucleotide (5 Prime->3 Prime Inc) as described above.

2.6 HYBRIDISATION OF DNA AND RNA PROBES TO NYLON MEMBRANES

The hybridisation conditions described by Church and Gilbert (1984) were used with some minor modifications.

Membranes were prehybridised for 30 minutes with 10mls of hybridisation solution (0.5M phosphate buffer pH 7.2, 7% SDS, 1mM EDTA) in a roller oven (Techne) at 65°C before adding the probe (see section 2.5). Hybridisation was carried out overnight at 65°C and the excess probe removed by washing with different stringency washes. A high stringency wash was performed using two washes with 0.2x SSC/1%SDS for 15 minutes. This was followed by a 15 minute wash in 0.1x SSC/0.1% SDS if background was high. A low stringency wash

consisted of 2 washes at 65°C with 2x SSC/1% SDS for 15 minutes each, followed by 1 wash with 1x SSC/0.1% SDS. Membranes were wrapped in Saran Wrap and either exposed to phosphorimager screen or to X-ray film (AGFA:CURIX RP1) at -70°C with intensifying screens.

To strip the membrane for reprobing, it was placed in a boiling solution of 0.1% (w/v) SDS and the solution allowed to cool to room temperature. The membrane was re-exposed to X-ray film or phosphoimager screens to check the blot was stripped of signal.

2.7 DNA SEQUENCING

Double stranded DNA sequencing was performed as described by manufacturers using a T7 Sequencing kit (Promega). Briefly 1.5µg of template DNA in 8µl of TE buffer was denatured with 2µl of 2M NaOH for 10 minutes. 3µl of 3M sodium acetate pH 4.8 and 7µl of ddH_20 was added and the DNA precipitated with 3 volumes of 100% ethanol at -20°C for 15 minutes. The DNA was pelleted by centrifugation at 14 000g for 10 minutes and washed once with ice-cold 70% ethanol. The pellet was dried for 10 minutes under vacuum and redissolved in 10µl ddH_20. The primer and DNA template were annealed by adding 2µl (0.8µM) of primer and 2µl of annealing buffer to the DNA template at 37°C for 20 minutes. The annealed primer and DNA were left for a further 10 minutes at room temperature before proceeding to the sequencing reactions. 2.5µl of A, T, C and G mix-short were dispensed into labelled tubes and an enzyme premix made up in another tube.

The enzyme premix contained:-

ddH_20	n µl	
Labelling Mix-dATP	3n µl	
Diluted T7 DNA polymerase	2n µl (1.5 units/µl)	
[α-^{35}S]dCTP	n µl (10µCi)	
Total volume	7n µl	n=number of DNA templates

The components were mixed gently by pipetting and the contents collected by a brief centrifugation. 6μl of the premix was added to each tube containing the annealed template and primer, mixed and left at room temperature for 5 minutes to complete the labelling reaction.

The nucleotide mixes were prewarmed at 37°C and 4.5μl of the labelling reaction transferred into another tube before heating to 75-80°C for 2 minutes. 3μl of each reaction was loaded on to a 6% (v/v) polyacrylamide gel and run at 60 watts for 1.5 hrs before loading a second set and leaving for another 1.5-2hrs. Typically between 200-300bp could be read from two loadings on the same sequencing gel.

2.8 GENOTYPING OF TRANSGENIC MICE BY TAIL DNA PCR

Using filtered tips throughout to avoid contamination a master mix was made up containing:-

x1 MIX

5μl 10X PCR buffer (Boerhinger)

0.5μl 10mM dNTP mix(dCTP, dATP, dGTP and dTTP, Pharmacia)

1μl 20μM *BLG primers (transgene specific)

1μl 20μM §WAP primers (endogenous control)

0.5μl Taq polymerase (Boerhinger)

42μl ddH_20

*BLG primer set:- 5'-TCGTGCTTCTGAGCTCTGCAG-3'

5'-GCTTCTGGGGTCTACCAGGAA-3'

WAP primer set:- 5'-CCTCCTCAGCATAGACA-3'

5'-GGTGATCAGTCACTTGCCTGA-3'

50μl of the master mix were dispensed into each tube and overlaid with 50μl of mineral oil to avoid evaporation. 1μl of crude tail DNA was added to each tube and a known positive and negative tail sample set up alongside to act as controls for the

reaction. The samples were placed in a PCR machine and the following program executed:-

Denature 5 mins at 95°C (1 cycle)

Denature 1min at 95°C
Anneal 1min at 42°C
Extention 2 minutes at 72°C
] 30 cycles

Extention for 5 minutes at 72°C (1cycle)

20μl of the amplified DNA samples were electrophoresed in a 2.5% (w/v) 1x TBE agarose gel until the BLG specific band (246bp) and the WAP (207bp) specific band could be distinguished under U.V light.

2.9 CULTURING OF MOUSE MAMMARY CELLS

2.9.1 Culturing mammary explants and establishing outgrowths

Two pairs of dissection scissors and forceps (per mouse) were sterilised in a 180°C glass beaker for 6hrs, the day prior to removing the mammary glands. Media and collagen coated flasks were also prepared as follows:-

Dissection Medium:

M199 (Gibco/BRL: Cat.No. 22340-012)

2x antibiotic/antimycotic (Gibco/BRL:Cat.No. 15240-039)

50μg/ml gentamycin (Sigma:Cat.No. G-1397)

Explant growth medium

(1:1) DMEM/F12 (Gibco/BRL:Cat.Nos. 4196-039/21765-029)

5μg/ml bovine insulin (Gibco/BRL:Cat.No. 13007-018)

5μg/ml ovine prolactin (Sigma:Cat.No. L-6520)

5μg/ml hydrocortisol (Sigma:Cat.No. H-0888)

5ng/ml estradiol (Sigma:Cat.No. E-2758)

10ng/ml EGF (Sigma:Cat.No. E4127)

50μg/ml gentamycin

T25cm^2 flasks were coated with 0.1% (v/v) typeI collagen (Sigma:Cat.No. C8919) diluted in 0.1N acetic acid and left overnight at 4°C.

Mammary glands were asceptically removed from midpregnant mice (13.5-14 days of pregnancy) either transgenic mice (4.2BLG-tsA58 and H2K^b/SV40 tsA58 [kindly provide by D. Kiouiss]) or nontransgenic control mice. One set of forceps and dissection scissors were used to cut and pin back the skin to expose the mammary tissue. The glands were removed using the other set of forceps and scissors, avoiding the muscle which lies across the second thoracic glands. The glands were washed several times in Dissection medium and transferred to a glass petri dish. The tissue was cut using scalpels until pieces were approximately 1mm in size. Dissection media was changed several times during the course of cutting the explants to prevent the explants from sticking together. The collagen coated flasks were washed several times with 1X PBS (Ca^{2+} and Mg^{2+} free tablets supplied by Oxiod, dissolved in AnalaR water [BDH] and autoclaved) to neutralise the collagen prior to the addition of the explants. Typically 300 explants could be obtained from one midpregnant mouse. Approximately 30 explants were seeded into each T25cm^2 flask and cultured in a 5% CO_2 incubator for two weeks at either the fully permissive temperature (33°C) or at a semi-permissive temperature (37°C) with daily medium changes using Explant growth medium. In general the Explant growth medium did not contain serum but where indicated 10% (v/v) serum was used. Explants were removed after two weeks and the outgrowths cultured in Complete medium.

Complete medium:

(1:1) DMEM/F12

10% (v/v) FCS

5μg/ml bovine insulin

10ng/ml EGF

5μg/ml Linoleic acid

50μg/ml gentamycin.

These primary cultures were maintained for several weeks and passaged when the size of the islands stopped increasing or the flask became almost confluent.

2.9.2 Passaging and maintenance of KIM-2 cultures

To passage the cells, the medium was aspirated and the cells washed with 1X PBS. Cells were passaged as clumps of 5-10 cells by a gentle trypsinization using TEG:-

TEG (Trypsin EGTA):

100mls 2.5% (v/v)Trypsin (Flow Laboratories)

0.5g EGTA (Sigma)

150mg PVA (Sigma)

7.5g NaCl

0.12g Na_2HPO_4

0.37g KCl

1.0g D-Glucose

3.0g Tris

1.0mls 1% (v/v) Phenol Red

Cells were incubated with TEG for 1 minute before Complete medium was added to the flask to deactivate the trypsin. The cells were harvested using a cell scraper and broken into clumps of 5-10 cells by pipetting up and down in a 10 mls glass pipette 3 times. The clumps of cells appeared to survive better than a single cell suspension. The cells were pelleted by centrifugation at 1 000rpm for 5 minutes, the supernatant aspirated and resuspended in an appropriate amount of Complete media. Cells were split 1:2 for 3 passages on to collagen coated flasks until the cells became used to the culture conditions. Cells are now routinely passaged as clumps of 5-10 cells and split 1:4 every 3-4 days on to plastic coated flasks and incubated at 37°C with medium changed every 2 days.

2.9.3 Freezing and resuscitation of KIM-2 cells

Cells were harvested from a T25cm^2 flask as described above and resuspended in 0.75mls of Complete medium and placed on ice. An equal volume of ice-cold Freezing mix was added dropwise with continual mixing of the contents of the tube.

Freezing mix:-

60% (v/v) Complete medium

20% (v/v) FCS

20% (v/v) dimethyl sulfoxide (Sigma:Cat.No. D-2650)

The mixture was dispensed into 3x 0.5ml aliquots into prechilled screw cap tubes (Sarstedt), placed in a polystyrene box and incubated at -70°C overnight before transferring to liquid nitrogen for long term storage.

To resuscitate the cells, the vial was transferred from the liquid nitrogen to a beaker of water preheated to 37°C and agitated until the mixture had thawed. The contents of the tube were added to 10mls of Complete medium and the tube rinsed with 1mls of medium. The cells were pelleted by centrifugation at 1 000 rpm for 5 minutes, the supernatant removed. The pellet was resuspended in 5mls of Complete medium, transferred to a T25 cm^2 flask and placed in a CO_2 incubator at 37°C. The cells adhere to the plastic or collagen after approximately 3-4 hrs.

2.9.4 Induction of differentiation in KIM-2 cells

Cells were grown on collagen or plastic in Complete medium until confluent and rinsed with PBS. The medium was changed to Complete medium without EGF for 2 hrs and then changed to Induction medium for the times indicated. Differentiation of KIM-2 cells on Matrigel was achieved by seeding the cells at $4x10^4$ cells/cm^2 onto precoated 6-well dishes (Becton and Dickinson) in Complete medium until "mammospheres" formed then differentiating with serum-free Induction medium.

Induction medium:-

(1:1) DMEM/F12

10% (v/v) FCS

5μg/ml bovine insulin

5μg/ml Linoleic acid

50μg/ml gentamycin

5μg/ml ovine prolactin (Sigma:Cat.No. L-6520)

1μM dexamethasone (Gibco/BRL:Cat.No. D-4902)

2.9.5 Transfection of KIM-2 cells

KIM-2 cells were transfected using a variety of transfection methods as described by manufacturers or standard protocols. The conditions for each transfection method was optimised by transient transfection with either pCH110 (Pharmacia) or βgeo (kindly provided by Dr. W. Skarnes) reporter LacZ constructs. The optimal amount of transient transfectants was assayed by counting the number of positive LacZ staining cells (see section 2.10)

2.9.5.1 Lipofectamine

KIM-cells were transfected using the liposome based deliver of plasmid DNA into the cells as outlined in the manufacturers guidelines (Gibco/BRL). The optimal conditions for transient transfection of KIM-2 cells were as follows:-
Cells were plated at a density of 2x 10^5 cells per well of a 6-well plate the day before the transfection. For each well to be transfected 15μl of Lipofectamine was added to 100μl of Optimem (basal media recommended by Gibco/BRL) in an Eppendorf tube and 2μg of circularized plasmid DNA added to 100μl of Optimem in another tube. The tubes were mixed and incubated at room temperature for 45 minutes with occassional mixing by flicking the tube. The cells were washed with Complete medium without serum. 0.8 mls of serum free medium was added to the tube

before assaying for β-gal expression. Typical transfection efficiencies using calcium phosphate ranged from 0.09%-0.29%.

For stable transfections a similar procedure was followed but on a larger scale. A confluent T80cm^2 flask of KIM-2 (1.7x 10^7 cells) was split 1:3 the day prior to transfection and incubated overnight in Complete medium.. Approximately 5.6x10^6 cells per T80cm^2 flask were cotransfected with 0.6μg pSV2neo and 15μg of the test construct as described by Gorman (1985). The calcium phosphate precipitate was washed off the cells 5-6hr after the start of the transfection and the media changed to complete medium but without any antibiotics and allowed to recover for 24hrs-48hrs before selection with growth medium supplemented with 200μg/ml G418 (Sigma:Cat.No G5013). After 14 days of selection discreet G418 resistant colonies could be observed. Pools of cells were harvested by trypsinization and expanded before being used in induction experiments and frozen stocks made.

2.9.5.4 Strontifect

Strontifect phosphate (Biovation) mediated transient transfection procedure was similar to the calcium phosphate method except strontium phopshate was used instead of calcium phopshate. The precipitate was left on the cells either for 5hrs or overnight since the strontium phosphate is less toxic to the cells than calcium phosphate. The transfections which were carried out overnight were done in the Complete medium without antibiotics since this method is reported to function in the presence of serum. Typical transfection efficiencies ranged from 0.001-0.02%.

2.10 β -GALACTOSIDASE STAINING OF TRANSFECTED KIM-2 CELLS

Transiently transfected cells were washed with 1x PBS and incubated with fixative (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 10 minutes at 4°C. The cells were washed 3 times with 1x PBS to remove residual fixative which may inhibit β-galactosidase activity. The fixed cells were incubated overnight in staining solution (2mM $MgCl_2$, 5mM $K_4Fe(CN)_6$ $3H_20$, 5mM $K_3Fe(CN)_6$, 1mg/ml X-Gal). The

staining solution was removed and the cells overlayed with 1xPBS . The cells were viewed using a phase-contrast microsope and blue cells counted.

2.11 PROTEIN EXTRACTION AND ANALYSIS

2.11.1 Extraction of protein from tissue culture cells

Cells were harvested as described above and total protein cell extracts made by directly lysing the cells in 0.5mls of protein lysis sample buffer (0.125M Tris-HCl pH 6.8, 2% (w/v) SDS, 2% (v/v) 2-mecaptoethanol, 10% (v/v) glycerol). The samples were boiled for 10 minutes and stored at -20°C.

2.11.2 Preparation of nuclear protein extracts from tissue and cultured cells

Nuclear extracts were prepared from tissues and cultured cells as described by Dignam *et al.*, (1983) with minor modifications described by Watson *et al* ., (1991). Briefly mouse mammary tissue was flash frozen in liquid nitrogen and approximately 7g used to prepare the extracts. The following buffers were prepared prior to making the nuclear extracts.

Buffer A:

0.1mM Sodium orthovanadate	2mM EGTA
0.5mM PMSF	2mM DTT
0.6M Sucrose	0.2% (v/v) TritonX-100
120mM KCl	10mM HEPES pH 7.5
15mM NaCl	2mM Spermidine
0.3mM Spermine	28mM (v/v) 2-mercaptoethanol
4mM EDTA	

Buffer NT	Buffer L:
15mM NaCl	10mM NaCl
10mM HEPES pH 7.5	0.1% (v/v) NP 40
	10mM HEPES pH 7.5

Buffer NR:

400mM NaCl

10mM HEPES pH 7.5

1.5mM $MgCl_2$

0.1mM EDTA

0.1mM EGTA

0.5mM DTT

10% (v/v) Glycerol

0.5mM PMSF

0.1mM Sodium orthovanadate

Dialysis buffer:-

20mM HEPES pH 7.5

100mM KCl

0.1mM EDTA

0.1mM EGTA

0.5mM DTT

20% (v/v) Gycerol

0.5mM PMSF

0.1mM Sodium orthovanadate

The frozen tissue was ground to a fine powder and resuspended in 10mls of A/NT/L buffers in a ratio of 2:3:5 respectively and kept on ice to prevent degradation of the proteins by cellular proteases. The dispersed cells were homogenised using a Dounce homogeniser (10 strokes at speed 7) and filter through two layers of micracloth (Cambridge Bioscience). The nuclei were pelleted by centrifugation at 2 500rpm for 10 minutes (4°C) in a swing out Sorvall rotor. The supernatant was discarded and the pellet resuspended in 10ml of A/NT buffers and pelleted again at 2 500rpm for 10 minutes (4°C). The supernatant was removed and the pellet resuspended in 10mls of NR buffer and left in ice on a shaking platform ice, shaking gently for 30 minutes to lyse the nuclei. The nuclear debris was pelleted by centrifugation at
35 000rpm for 30 minutes in a swing out SW50.1 rotor at 4°C. The supernatant was removed and dialysed against 2 litres of dialysis buffer for 4 hrs at 4°C. The nuclear extracts were dispensed into 1ml aliquots and flash frozen in liquid nitrogen before storing at -80°C. Extracts were stable for at least 1 year using this method.

Cell nuclear extracts were prepared by harvesting the cells from a confluent T80cm^2 flask as described in section 2.8.3 and washing the pellet with 1xPBS. The

pellets were placed on ice and resuspended in 3mls of A/NT/L as above but cells were dispersed using Eppendorf homogeniser sticks, supplied by Anachem. The nuclei were pelleted by centrifugation for 5 minutes at 12 000g in a microfuge at 4°C. The pellets were resuspended in 3mls of A/NT and nuclei collected again and resuspended in 0.5mls NR buffer. The extracts were shaken on ice at 4°C for 30 minutes and spun for 15 minutes in a microfuge at 12 000g. The supernantant was removed and dialysed for 3-4 hrs in dialysis buffer, flash frozen and stored at -80°C.

2.11.3 Estimation of protein concentration

Protein concentration was measured using the PIERCE detection system and the Standard Protocol. Protein standards were prepared in the range 200μg/ml-1200μg/ml by diluting the bovine serum albumin stock (2mg/ml) provided by the manufactures and a blank prepared with no protein. 10μl of the nuclear extracts were diluted 10 fold with ddH_20. 0.1ml of each of the standards and the nuclear extracts were pippetted into fresh tubes and 2.0 mls of the Working Reagent added to each tube, mixed and incubated at 37°C for 30 minutes. During this incubation time there is a colour change from pale green to purple due to the reaction of protein with Cu^{2+} in an alkaline medium to yield Cu^{1+} which when complexed with bicinchoninic acid (BCA) gives a purple reaction product which exhibits strong absorbance at wavelength 562nm.

The absorbances at wavelength 562nm of the standard (blank corrected) were plotted against protein concentration. Using this standard curve the protein concentrations of the nuclear extracts was determined.

2.11.4 Western blotting

To detect β-casein, one dimensional SDS-PAGE was performed (see section 2.3.5). Proteins were transferred to nitrocellulose (Schleicher & Schuell) at $0.8mA/cm^2$ for 1 hr using a semi-dry electroblotter (Khyse-Andersen, 1984). After blocking non-specific binding with 1% (w/v) BSA in PBS/Tween (0.01% v/v) for 1hr the nitrocellulose was exposed to a polyclonal anti-rabbit β-casein antibody diluted 1:10 000 in blocking solution for 1hr (kindly provided by Dr. B. Binas). The membrane was washed twice with PBS/Tween at 15 minute intervals. The primary antibody was visualized using peroxidase-conjugated anti-IgG secondary antibody and ECL detection reagents (Amersham).

To detect T-antigen protein the same procedure was performed but 5% Marvel was used to block non-specific binding instead of BSA. The large T-antigen specific antibody Pab 416 (Calbiochem: Cat.No. DP-02) and Pab 108 (Santa Cruz: Cat.No. sc-148) which reacts with both large T and small t antigen were used at 1:500 dilution.

2.11.5 Electrophoretic mobility shift assays (EMSA)

0.5ng of end labelled double stranded DNA probe (see section 2.5) containing 10000-50000cpm was incubated with 2μg of nuclear extracts for 20 minutes in a buffer containing 20mM Hepes pH 7.5, 1mM EDTA, 1mM DTT, 10% (v/v) glycerol, 100mM NaCl, 0.05% (v/v) NP40 and 1μg poly dI-dC carrier (Boehringer Mannheim) in a 20μl reaction. The DNA-protein complexes were resolved on native 6% (v/v) polyacrylamide gels as described in section 2.3.3

Supershifts were performed by adding the antibody to the nuclear extract and buffer mix and incubating for 1hr on ice before addition of the probe as described above.

2.12 IMMUNOHISTOCHEMISTRY

2.12.1 Preparation of paraffin tissue sections for staining

The right and left inguinal (number 4) mammary glands were removed from normal and transgenic C57BL6 x CBA mice at various stages of development. The glands were fixed in formalin (10% (v/v) formaldeyde in PBS pH 7.6) for at least 24 hours before embedding in paraffin using standard procedures. 5μm sections were cut from blocks using a Leica microtome. The sections were floated in a 50°C water bath and collected on glass slides.

The sections were dewaxed and rehydrated in Coplin jars using standard procedures. Briefly, sections were dewaxed in two changes of xylene and rehydrated through graded alcohol -100%/70%/50% Ethanol/ddH$_2$0 (v/v). The sections were washed in running tap water before staining procedures were carried out.

2.12.2 Histologically staining of mammary sections

Mayer's haematoxylin-eosin staining of the first and last tissue sections cut were routinely performed to examine the morphology of the glands. The sections were placed in Mayer's haematoxylin solution (BDH) for 5 minutes and washed in running water for 3 minutes or until blue stain is visible. The sections were then counterstained for 30-60 seconds with eosin (BDH), rinsed in running tap water for 30 seconds and dehydrated through graded alcohols, cleared in xylene and mounted in DPX mounting medium.

2.12.3 Immunohistochemical staining of mammary sections

For immunohistology, optimal concentrations of the monoclonal antibodies were determined. Generally tissue culture supernatants were used neat and ascites diluted 1:50-1:500. Rehydrated mammary sections were placed in a humidifier and blocked with 5% (v/v) normal goat serum in TBS (Tris-HCl buffered saline) for 10 minutes. The excess serum was wiped from each section and the section covered with the

appropriate concentration of monoclonal antibody (diluted in the block) and incubated overnight in the humidifier box at 4°C. The excess solution was tipped off the slides and the sections briefly washed in running tap water and 2 washes in TBS. The secondary antibody, either biotinylated rabbit anti mouse (DAKO), diluted 1:400 in block or FITC rabbit anti mouse (DAKO) diluted 1:50 in block were added as above and incubated for 30 minutes. The sections were washed again in running tap water. The FITC stains were done in the dark and were mounted directly in DABCO or Mowiol mounting medium which helps prevent fading of the fluorescence.

The ABC system (DAKO)-avidin/biotinylated enzyme complex was used to visualise staining of section which had been incubated with the biotinylated secondary antibody as per manufactures instructions. One drop of avidin and biotinylated enzyme was added to 5 ml of buffer (50mM Tris-HCl) 30 minutes before use. ABComplex was added to the sections and incubated for 30 minutes before washing in tap water. Fresh diaminobenzidine (DAB) was prepared and used as substrate. Sections were incubated with DAB (Sigma) for 20 minutes in the dark, then washed in running tap water. Sections were counterstained in haematoxylin and dehydrated, cleared and mounted as described in section 2.12.2

2.12.4 Immunofluorescent staining of KIM-2 cells

KIM-2 cultures were fixed with acetone:methanol (1:1) at 4°C for 10 minutes. The fix was removed and the cells washed with TBS. The staining procedure was basically the same as that described for FITC staining of tissue sections. Table 2.1 shows the monoclonal antibodies used to stain KIM-2 cells. The cells were mounted in DABCO or Mowiol and left to dry in the dark.

2.12.5 Normal/Ultraviolet Microscopy

Sections or cells were examined using a Leitz Ortholux II microscope equipped with both normal and UV light sources, FITC and TRITC filters, and a Wild

photoautomat automatic exposure photographic unit. Sections were photographed using 64T ASA film (Kodak) for normal light and 1600 ASA film for fluorescence photography.

Table 2.1. Monoclonal antibodies used for immunohistochemical staining of tissue culture cells

Monoclonal (Isotype)	Specificity	Cell type	Concentration	Reference
LE 61 (IgG1)	keratin 18	luminal epithelial	Neat	Lane, 1982
LP2K (IgG2b)	keratin19	luminal epithelial	Neat	Stasiak, 1989
Vim 13.2	Vimentin	myoepithelial and stromal	Neat	Sigma Cat.No. V5255
IMMH-2	∝-Smooth muscle actin	myoepithelial and fibroblasts	1:1000	Sigma Cat.No. A-2547
laminin-1	laminin	basement membrane component	1:1000	Streuli, 1995
Pab 419	T-antigen		1:500	Harlow, 1982

TRANSGENIC MICE HARBOURING A THERMOLABILE T-ANTIGEN GENE DRIVEN BY THE β-LACTOGLOBULIN PROMOTER: GENERATION AND ANALYSIS.

3.1 INTRODUCTION

Remarkable progress has been made in genetic manipulation in the last 10 years. Using transgenic technology it is posssible to alter the mouse genome by stably introducing foreign DNA into the mouse germ line. This is achieved by microinjection of multiple copies of the transgene into the pronucleus of a fertilised one-cell embryo. The transgene randomly integrates into the genome (usually at a single site) and the cleaved embryos (two cell stage) are transferred to pseudopregnant females (females which have been mated with vasectomised males). The livebirths can then be screened at around 7 weeks of age for the presence of the transgene by PCR and Southern blot analysis of DNA from tail biopsies. Transgenic lines carrying exactly the same transgene insertion can be generated by mating each of the founders with F1 nontransgenic mice (C57BL/6xCBA) hybrid strain were used in this study).

The mechanisms involved in the integration event are largely unknown but result in the insertion of different copy numbers of the transgene, usually in direct orientation (Bishop and Smith, 1989), at different integration sites in each different line of mice. In the majority of cases the transgene is susceptible to "position effects," (Al-Shawi *et al.*, 1990) resulting in aberrant or different expression levels in lines carrying the same construct (Spradling and Rubin, 1983; Palmiter and Brinster, 1986). The "position effects" can result from the transgene integrating into a number of different sites in different lines which either enhance or repress expression. For example the transgene could integrate into an endogeneous gene, close to an enhancer or repressor, near a CpG island (Bird, 1986), into a region which is imprinted (Surani *et al.*, 1988), close to or in a locus control region (LCR) or heterochromatin. However, a number of transgenes have now been described which display position-independant expression i.e. they express regardless of their integration site (Grosveld *et al.*, 1987). Presumably these constructs contain strong regulatory elements which overcome any influence the site of integration

may have on expression of the transgene. These elements may act in a dominant manner to direct expression (Grosveld *et al.*, 1987). Alternatively they could target the integration of the transgene into matrix- or scaffold-attachment regions MARS or SARs (respectively). This region contains (A+T)-rich repetitive DNA elements, which are thought to establish chromatin loop structures, often found close to "open chromatin" and active genes (Blasquez *et al.*, 1989; Bonifer *et al.*, 1990). For example, coinjection of a WAP transgene with MARs from the chicken lysozyme locus resulted in all 11 lines expressing WAP compared to only 50% in its absence (McKnight *et al.*, 1992).

In this project a transgenic approach was utilised to target expression of a thermolabile mutant of SV40 T-antigen to the secretory cells of the mammary gland. This mutant should not be active *in vivo* and therefore not detrimental to the health of the animals but once the mammary glands are removed and cultured at the permissive temperature of 33°C, the T-antigen should become active and can be used to immortalise the secretory cells. Since the level of T-antigen required to immortalise mammary epithelial cells is unknown it would be an advantage to generate transgenic lines of mice with variable levels of expression of the transgene.

Several promoters have been described which have been used to direct expression of transgenes to the mammary epithelium. For example, the MMTV long terminal repeat (LTR) is regulated by steroid hormones and is expressed throughout mammary gland development with activity peaking during lactation (Varmus *et al.*, 1973, Marcus *et al.*, 1981; Hu *et al.*, 1984). However the expression of this promoter is not confined to the mammary glands of transgenic mice. Mice carrying a MMTV LTR promoter driving expression of wild type SV40 T-antigen expressed the transgene in a number of different organs which contain cells of an epithelial origin such as lungs, kidneys, prostate, salivary and mammary gland as well as cells of non-epithelial origin e.g Leydig and lymphoid cells (Choi *et al.*, 1987).

In comparison, both WAP and BLG promoters are both hormonally and developmentally regulated and have been used successfully to direct expression of heterologous proteins to the mammary glands of transgenic mice (Gordon *et al.*, 1987; Yu *et al.*, 1989; Archibald *et al.*, 1990; Meade *et al.*, 1990; Whitelaw *et al.*, 1991). The

BLG promoter was used for this project since its regulatory regions have been extensively studied in this laboratory. Additionally, BLG transgenes are less dependant on the genomic integration site for expression than WAP (Whitelaw *et al.*, 1992) and despite the fact that rodents do not have endogenous BLG it is expressed in transgenic mice throughout mammary development with a similar expression profile to β-casein. The expression of BLG is therefore compatible with dividing cells at an early stage in the differentiation pathway, which is critical for the establishment of cultures from early stages of mammary gland development.

In some transgenic lines of mice ectopic expression of BLG driven transgenes had been observed in the salivary gland (Dr. B. Whitelaw, pers. comm.) however, since this temperature-sensitive mutant of SV40 T-antigen has been reported to be active only at 33°C this drawback was considered to be negligible.

This chapter describes the construction of the BLG-tsA58 transgene, the establishment of the transgenic lines of mice and the analysis of transgene expression.

3.2 RESULTS

3.2.1 Construction of BLG-tsA58 transgene

Diagram 3.1 summarises the cloning steps used to construct the BLG driven temperature sensitive T-antigen construct. 4.2kb of the upstream sequences of the BLG promoter, which have previously been used to direct expression of α1-anti-trypsin in transgenic mice (Archibald *et al.*, 1990) was excised from plasmid pBJ39 (kindly provided by Dr. B. Whitelaw) by digesting with SalI and EcoRV (Figure 3.1A). Bluescript/KS (Stratagene) was also digested with SalI and EcoRV (Figure 3.1A). The 4.2kb and the linearised plasmid fragments were excised from the LMA gel and the agarose digested with Agarase (see section 2.3). The 4.2kb BLG fragment was directly subcloned into the cut polylinker of the Bluescript/KS vector recreating both the SalI and EcoRV sites. This intermediate, designated pBS-4.2BLG provided a more versatile polylinker which facilitated subsequent cloning steps.

pUC-tsA58 (kindly provided by Dr. P. Jat) was digested with BglI yielding 3 fragments (Figure 3.1B). The 3.9kb fragment does not contain the upstream enhancer

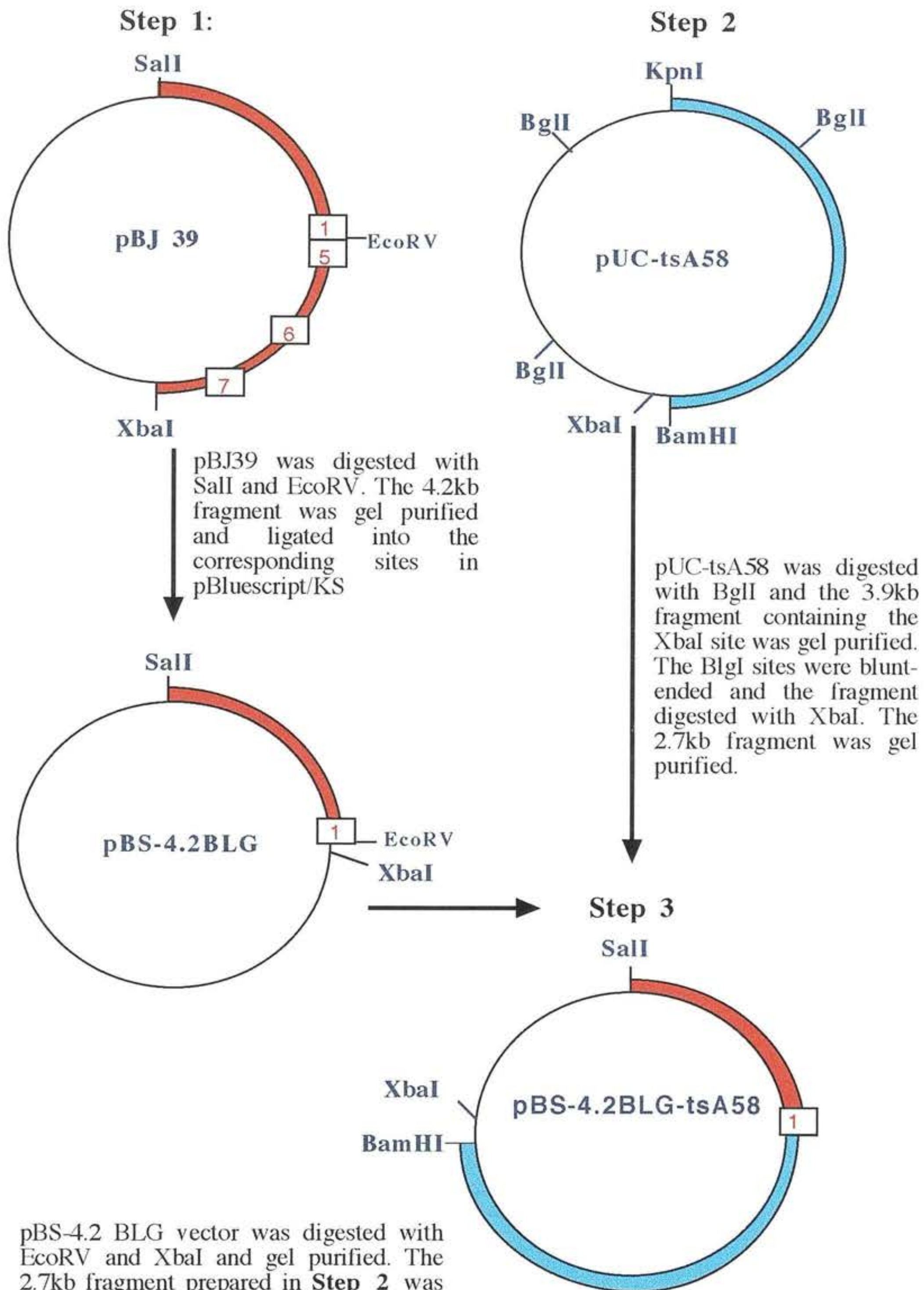


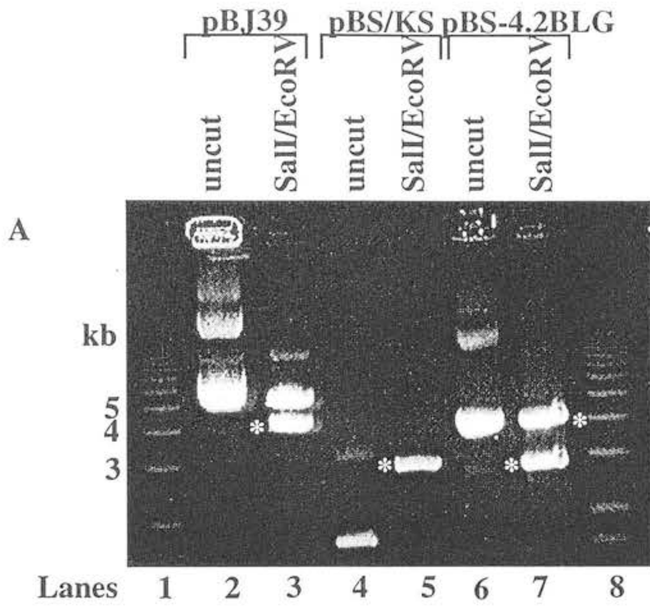
Diagram 3.1 Cloning strategy used to construct pBS-4.2BLG-tsA58 construct.
pBJ 39 plasmid was kindly provided by Dr. B. Whitelaw and J. Webster
pUC-tsA58 was kindly provide by Dr. P. Jat.
pBluescript/KS was purchased from Stratagene. (plasmids not drawn to scale)

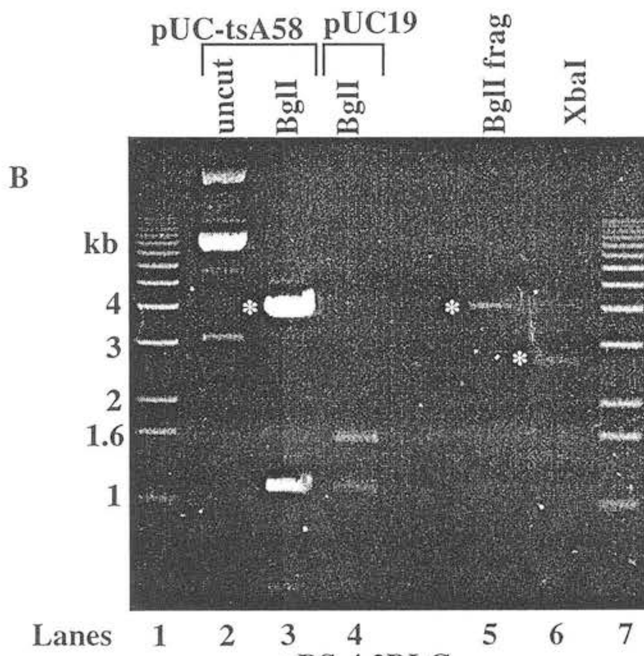
Figure 3.1 Restriction digests of plasmids used to construct pBS4.2BLG-tsA58

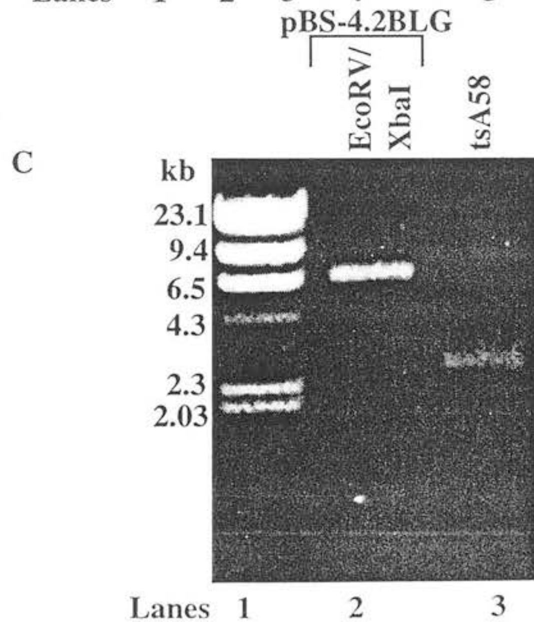
(A) Step 1: Subcloning of 4.2kb of the BLG promoter from pBJ 39 into the Bluescript KS vector (BS/KS). Lanes 1 and 8 are molecular size markers, Lanes 2, 4 and 6 are uncut pBJ 39, BS/KS and pBS-4.2BLG respectively, Lanes 3, 5 and 7 are SalI/EcoRV double digests of pBJ 39, BS/KS and pBS-4.2BLG respectively. The 4.2kb fragment indicated in Lane 3 * and the cut vector, Lane 5 * were gel purified and ligated to produce the intermediate pBS-4.2BLG plasmid. pBS-4.2BLG was digested with SalI and EcorRV to confirm that the correct size fragments (4.2kb insert+3kb plasmid band) were present in the construct (*).

(B) Step 2: Isolation of SV40 T-antigen mutant sequences without upstream enhancer elements. Lanes 1 and 7 are molecular size markers, Lane 2 is uncut, pUC-tsA58 plasmid, Lanes 3 and 4 are BglI digests of pUC-tsA58 (3.9kb+1.1kb+0.4kb) and pUC 19 (1.1kb+1.8kb). The BglI digest of pUC-tsA58 was "filled in" using T4 DNA polymerase. The 3.9kb fragment (*) was gel purified (Lane 5) and digested with XbaI (2.7kb+1.2kb, Lane 6). The 2.7kb band (*) containing T-antigen sequence free of upstream enhancer elements was gel purified.

(C) Step 3: Ligation of SV40 T-antigen downstream of the BLG promoter.
Lane 1 molecular size markers, Lanes 2 and 3 are the pBS-4.2kb BLG vector digested with EcoRV/XbaI and the 2.7kb SV40 BglI (filled-in)/XbaI insert respectively. Both vector and insert were gel purified before the ligation.
All digests were run on a 1% agarose gel.


pBJ39
pBS/KS
pBS-4.2BLG
uncut
SalI/EcoRV
uncut
SalI/EcoRV
uncut
SalI/EcoRV
A
kb
5
4
3
Lanes 1 2 3 4 5 6 7 8


pUC-tsA58
pUC19
uncut
BglI
BglI
BglI frag
XbaI
B
kb
4
3
2
1.6
1
Lanes 1 2 3 4 5 6 7


pBS-4.2BLG
EcoRV/
XbaI
tsA58
C
kb
23.1
9.4
6.5
4.3
2.3
2.03
Lanes 1 2 3

sequences of SV40, which have been shown to direct expression to the choriod plexus in the brain (Palmiter *et al.*, 1985). This fragment was gel purified, Agarase treated, "filled in" using T4 DNA polymerase (see section 2.1.3) and then cut with XbaI to remove 1.2kb of plasmid sequences (Figure 3.1B). The 2.7kb fragment was gel purified, excised from the gel and Agarase treated.

The final cloning step involved digesting pBS-4.2BLG with EcoRV and XbaI, gel purifying and Agarase treating the linearised plasmid before ligating with the 2.7kb SV40 BglI (filled-in)/XbaI fragment (Figure 3.1C and Step 4 in Diagram 3.1).

The final construct, designated pBS4.2BLG-tsA58 was cut with a panel of 10 restriction enzymes to check that no rearrangements occurred during the building of the construct. Figure 3.1D illustrates some of the diagnostic digests which were carried out to check that the components of the construct and the final construct gave the expected banding pattern (Diagram 3.2). For example the NdeI digest of pBS4.2BLG-tsA58 yields 3 fragments (lane 14) of which two bands (3.7kb+1kb) correspond to internal sites within T-antigen sequences. This was confirmed by digesting the parental plasmid pUC-tsA58 with the same enzyme which produce the same size of fragments (lane 12).

Sequencing of the 5' junction between exon1 (noncoding exon) and the start of SV40 T-antigen was carried out to verify the fidelity of the ligations. The EcoRV site and the filled in BglI site were as expected. The 3' end of the transgene was also partially sequenced (Figure 3.2B). Once the expected sequence had been confirmed, approximately 100μg of pBS4.2BLG-tsA58 was digested with SalI and XbaI and the transgene purified free of any plasmid sequences which are thought to interfere with the stable integration of transgenes. The transgene DNA was concentrated using Elutip columns (see section 2.1.8) and a 50ng/μl stock prepared in TE-buffer. The DNA was microinjected at a concentration of 1.5ng/ml into pronuclear stage eggs from superovulated (C57BL/6 x CBA F1) females by Roberta Wallace.

Figure 3.1D Restriction digests of plasmid DNA from the components and final pBS-4.2kb BLG-tsA58 construct.

Lane 1 is molecular size markers, Lanes 2, 4, 8, 10 and 13 are uncut BS/KS, pBS-4.2BLG, pUC19, pUC-tsA58 and pBS-4.2BLG-tsA58 respectively. Lanes 3, 5, 9 and 11 are BglI digests of BS/KS, BS-4.2BLG, pUC19 and pUC-tsA58 respectively. Lane 6 is a StuI digest of pBS-4.2BLG. Lanes 7 and 15 are BamH I digests of pBS4.2BLG and pBS4.2BLG-tsA58 with common bands indicated (*). Lanes 12 and 14 are NdeI digests of pUC-tsA58 and pBS4.2BLG-tsA58 with common bands indicated (*). The lower band in the NdeI pUC-tsA58 is a double (1kb+0.9kb) and could not be resolved on a 1% gel, however it was on a 2% gel (data not shown).

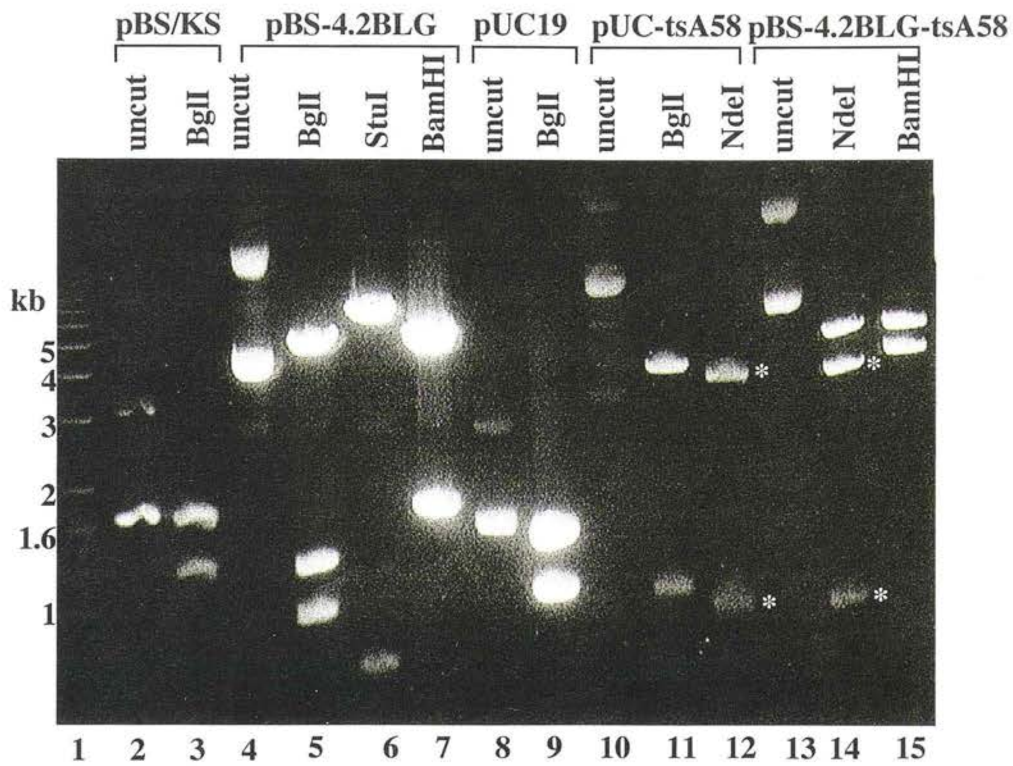
Diagram 3.2. Restriction digest map of pBS4.2BLG-tsA58 construct and its components

The structure of pBS-4.2BLG-tsA58 construct and its components with the predicted fragment sizes indicated.

(**A**) Map of pBS4.2BLG

(**B**) Map of pUC-tsA58

(**C**) Map of pBS4.2BLG-tsA58



A

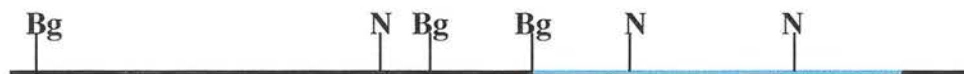
Fragments

Bg=BglI digest: 5.0kb + 1.3kb +0.9kb

St=StuI digest: 6.5kb + 0.7kb

B=BamHI digest: 5.5kb + 1.7kb

B



Fragments

Bg=BglI digest: 4.1kb +1.1kb + 0.5kb

N=NdeI digest: 3.7kb + 1kb + 0.9kb

C

Fragments

N=NdeI digest: 5.1kb + 3.7kb + 1kb

B=BamHI digest: 5.4kb + 4.4kb

key:
- plasmid sequences
- BLG sequences
- SV40 tsA58 sequences

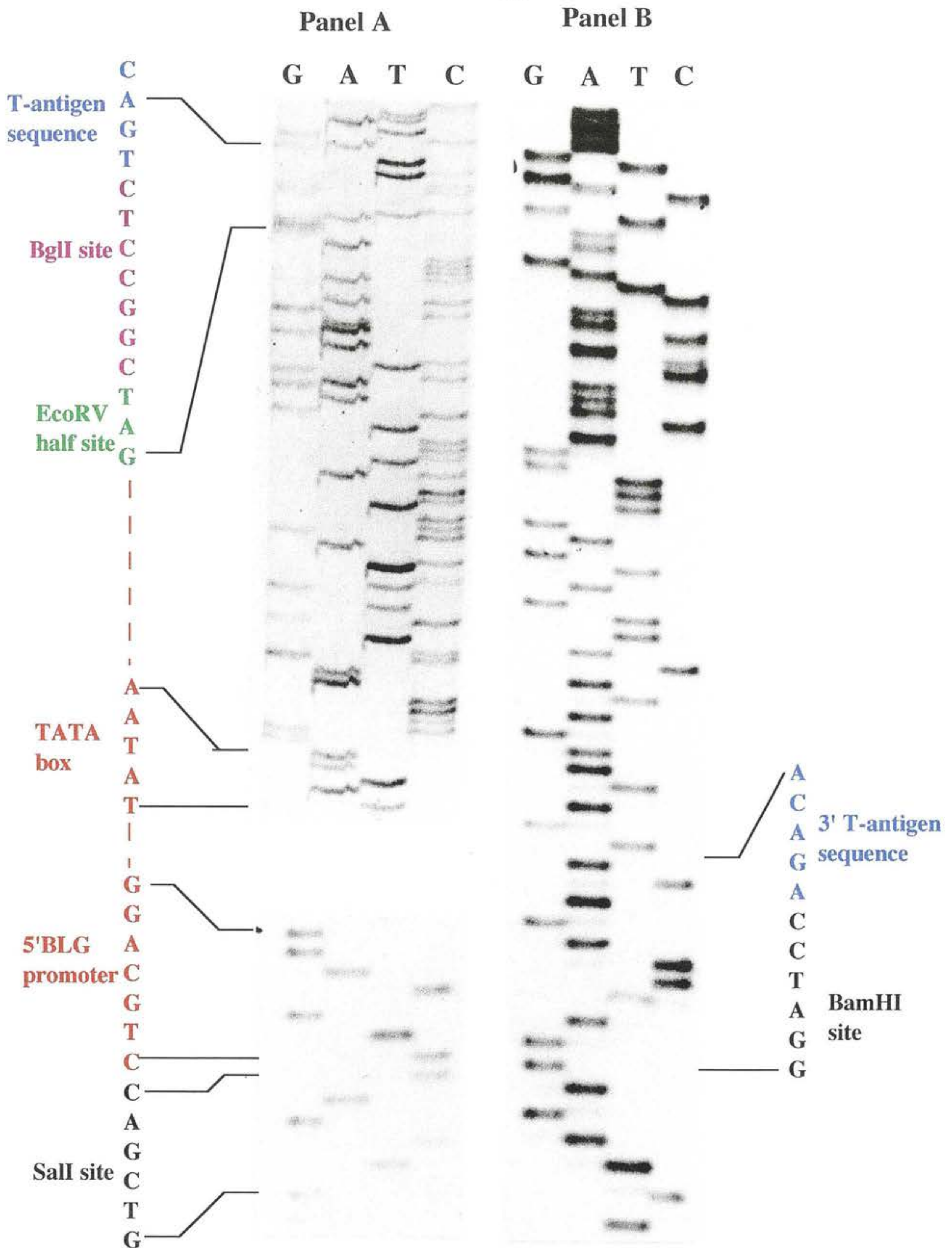


Figure 3.2 Partial sequence of pBS4.2BLG-tsA58 construct

Panel A shows the sequence of the 5' end of the construct reading through the SalI cloning site and the junction between the BLG promoter and the T-antigen tsA58 5' sequence. The junction between EcoRV and the filled in BglI site are shown.

Panel B shows the sequence of the 3' end of the construct reading through the BamHI site into the 3' end of T-antigen tsA58 sequence.

3.2.2 Analysis of founders and the establishment of transgenic lines of mice

Transgenic founders were identified by PCR and Southern analysis of mouse tail DNA. PCR analysis was done using two primer sets. The BLG primer mix was designed to amplify a 246bp internal fragment which was specific to the transgene. The other set of primers used in the reaction were WAP specific primers which amplified a 207bp fragment of the endogenous WAP gene and acted as an internal control for the PCR reaction. Figure 3.3 illustrates a typical set of PCR reactions with the positive and negative mice clearly shown. In theory the PCR reaction should result in the amplification of the endogenous WAP PCR product in the nontransgenic animals and both WAP and BLG products in the transgenic animals. However only one band was usually observed in the PCR reactions, either the endogeneous WAP band or the transgene-specific BLG band. One possible explanation for this is the BLG primers may compete more efficiently for the nucleotides in the PCR reaction than the WAP primers.

Southern blot analysis was routinely used to confirm the results of the PCR reactions (see section 2.3.1). Out of 54 live births, 12 mice were positive by both PCR and Southern blotting, an efficiency of 22%. Figure 3.4 is a Southern blot of all the founder mice obtained and Diagram 3.2 shows the genomic digest carried out and the probe used. The different intensities of the band indicates the variation in copy numbers of the transgene in the different founders. SV40-2 was under loaded and could be seen on a longer exposure (data not shown).

3.2.3 Ectopic expression of the transgene in the founder mice

It was originally envisaged that expression of the thermolabile mutant of T-antigen would not be observed in transgenic mice since it has been reported to be active *in vitro* at 33°C (Tegtmeyer, 1975; Zaret *et al.*, 1988, Jat and Sharp, 1989; Petit *et al.*, 1989; Randa *et al.*, 1989) below the body temperature of mice. However the generation of the "immortomouse" demonstrated that *in vivo* this mutant is at least partially active since these animals developed thymic hyperplasia, even in the lowest copy line (Jat *et al.*, 1991). Therefore using the BLG promoter to drive expression of the thermolabile mutant

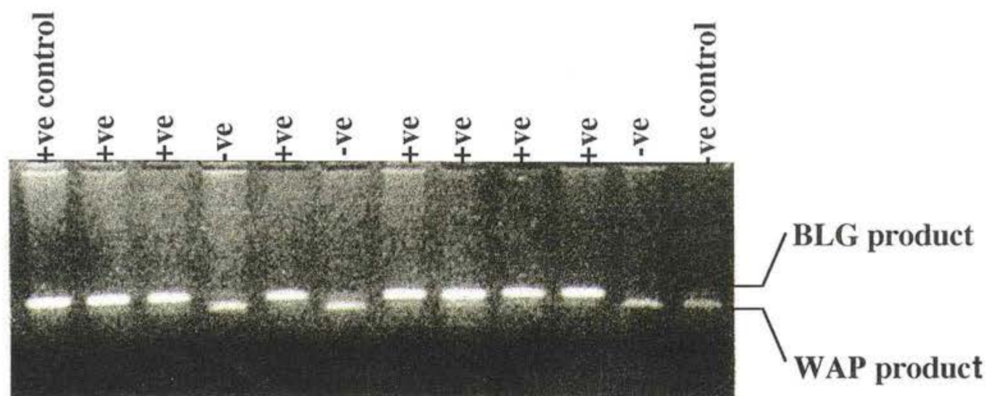


Figure 3.3 Screening of genomic mouse tail DNA for transgenic mice carrying the 4.2-BLG-tsA58 transgene
Positive and negative control DNA samples are loaded either side of genomic DNA prepared from a set of pups from one of the SV40 transgenic lines of mice

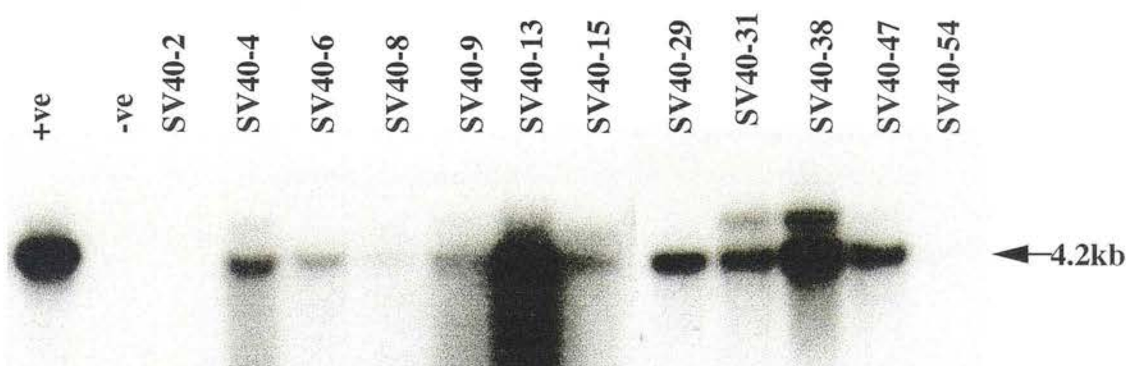


Figure 3.4 Southern blot of genomic tail DNA from transgenic founders carrying the 4.2BLG-tsA58 transgene
10μg genomic DNA was digested with BamHI and the Southern blot hybridised with probe I (see diagram 3.3).

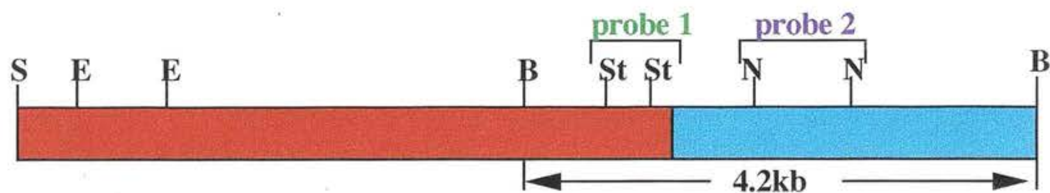


Diagram 3.3 A map of the 4.2BLG-tsA58 transgene
Probes 1 and 2 are indicated and **S**=SalI, **B**=BamHI, **St**=StuI, E=EcoRI and **N**=NdeI.

of T-antigen it was anticipated that there may well be expression of T-antigen in the mammary glands *in vivo* particularly during pregnancy and lactation when BLG is most active. Unexpectedly, however the mice did not develop mammary tumours but tumours at ectopic sites. These ectopic tumours were quite aggressive in some of the founders. Figure 3.5 shows a particularly large tumour on the leg of one of the founders which did not appear to have invaded the muscle tissue. Histological examination of the tumour confirmed it was a smooth muscle sarcoma (inset).

RNA dot blot analysis confirmed this tumour expressed high levels of T-antigen (Figure 3.6, sample lt47) and was used to rapidly screen other tumours for expression of T-antigen. Duplicate samples (2µg of total RNA) were loaded on the same blot and hybridised with a T-antigen probe (probe 2, Diagram 3.2) and then reprobed with a 28S ribosomal probe as a loading control. Figure 3.6 shows high levels of T-antigen expression in the tumours removed from some of the founders and in the spleen which was often enlarged. No T-antigen was detected in tissue which appeared normal, for example the liver sample. Unfortunately from the original 12 founders only 5 transgenic lines could be established since the other founders died before they were able to be mated.

3.2.4 Analysis of the transgene in the surviving lines of mice

3.2.4.1 Transgene copy number

The copy number of the transgene in each of the surviving lines of mice were estimated by Southern blot analysis (Figure 3.7). Genomic DNA was prepared from liver tissue from 3 transgenic mice per surviving line and from a non transgenic littermate as a negative control. Sheep genomic DNA was prepared to use as a copy control (2 copies of BLG in the genome). The DNA was digested with EcoRI which cuts twice in the construct and the Southern blot hybridised with a 1.0 kb StuI fragment probe (probe 1) which detects the 4.4 kb internal fragment (see Diagram 3.2) in a head to tail array. The other bands are presumably due to differences in the structure of the array, for example head to head or tail to tail arrays. DNA loading was corrected by reprobing the blot with the WAP probe (a single copy rodent gene; Gupta *et al.*, 1982). Table 3.1 summarises the copy numbers in the surviving lines of mice.

Figure 3.5 Ectopic expression of T-antigen in one of the founders carrying the 4.2BLG-tsA58 transgene
A smooth muscle sarcoma developed on the left forelimb of the female founder SV40-47 at 8 weeks of age. Inset histological section of tumour.

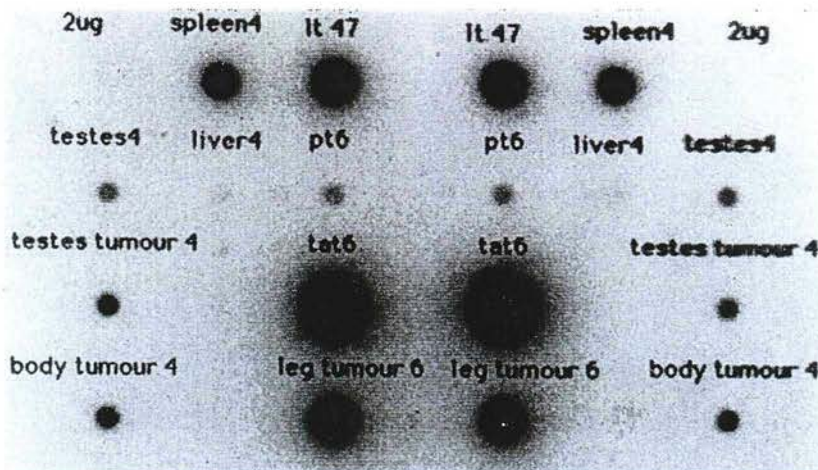


Figure 3.6 Ectopic expression of T-antigen in founder mice carrying the 4.2BLG-tsA58 transgene
Dot blot of total RNA prepared from various tissues and tumours from the transgenic founders and hybridised with a T-antigen DNA fragment (probe2:Diagram 3.2). lt47 is RNA prepared from the leg tumour seen in Figure 3.5 above.
pt6=preputial gland tumour from SV40-6, tat6=testes associated tumour from SV40-6

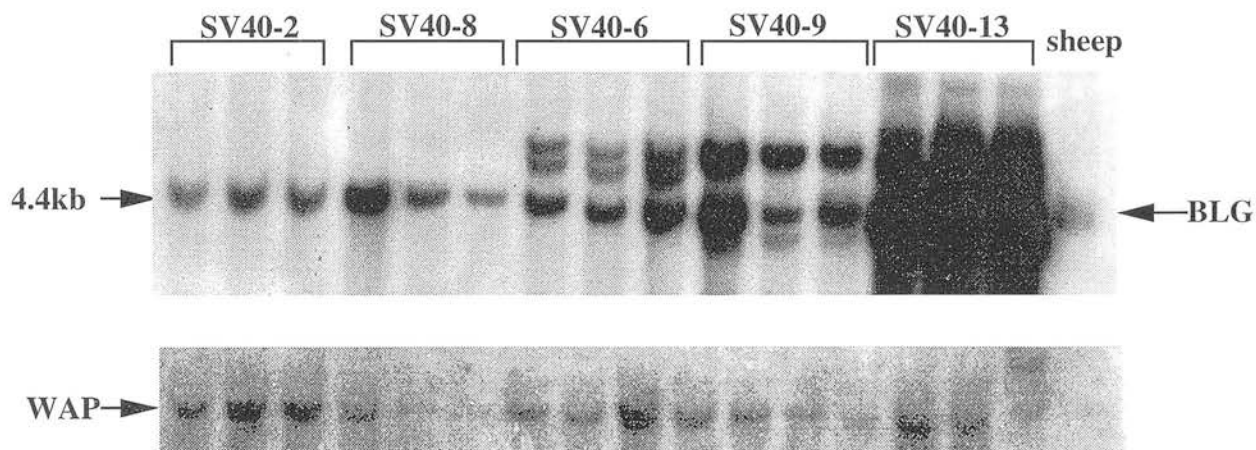


Figure 3.7 Transgene copy number in surviving SV40 lines of mice
Southern blot with 10μg of EcoRI digested genomic liver DNA from 5 independent transgenic lines of mice carrying the 4.2BLG-tsA58 transgene (3 sisters from each line) were analysed and a sheep genomic liver sample loaded as a single copy control. The blot was hybridised with probe 1 (Diagram 3.3) then stripped and rehybridised with a WAP probe as a loading control.

3.2.4.2 Ectopic expression

The ectopic expression in the five surviving lines of mice have been analysed by dot blot and the results summarised in Table 3.1. Three of the lines displayed ectopic expression (SV40-6,-9,-13) which was maintained through subsequent generations. The severity of the tumour phenotype appeared to be copy number related with the highest copy line (SV40-13) displaying a high degree of ectopic expression and shortest life span.

In SV40-6 the mice developed smooth muscle sarcomas usually attached to the leg muscle, abdominal swelling resulting from enlarged preputial glands and testes-associated tumours in males which occurred between 5-10 weeks of age. In both sexes between 15-25 weeks the animals developed breathing difficulties and rapidly lost weight. Post mortem examination revealed abnormally large hearts in these mice and possibly abnormalities in the lungs.

In the SV40-9 mice subcutaneous tumours were visible all over their bodies at 5-10 weeks of age and the skull appeared mis-shapen. Post-mortem examination of the skull showed these mice developed tumours where the two bones in the skull fuse (fontanelles). In addition these mice had enlarged or necrosed spleens and solid tumours along the intestinal tract.

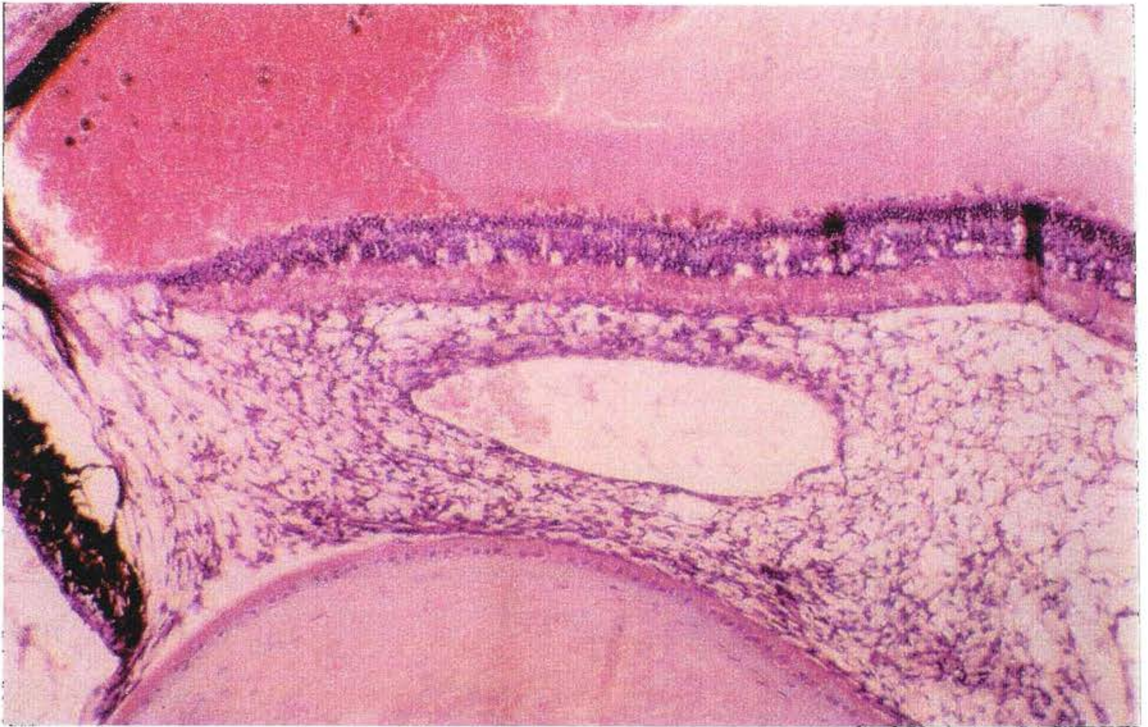
SV40-13 mice displayed a similiar ectopic expression pattern as that observed in the other two ectopic expressing lines. Athough the onset of the tumour phenotype was similar the health of these mice rapidly deteriorated with very few mice surviving beyond 18 weeks of age. This line of mice also displayed an unusual eye phenotype which correlated 100% with the presence of the transgene. The eyes were bright red at 3-4 weeks of age due to retinal bleeding and by 6-7 weeks of age a cloudy film similar to a cataract appeared on the eye ball (Figure 3.8).

The eye phenotype was investigated further by examining its structure by routine histological sectioning. Figure 3.9 shows the phenotype of two transgenic mice from this line. Figure 3.9A is an eye section from a 11 week old female with intense tumour growth within the vitreous layer and retinal detachment. Figure 3.9B is an eye section

Figure 3.8 Characteristic eye phenotype in the highest copy surviving transgenic line

SV40-13 offspring displaying the eye phenotype which is characteristic of all the transgenic animals in this line. Notice also the raised forehead and body tumour which was observed in this line and in SV40-9 line.

A



B

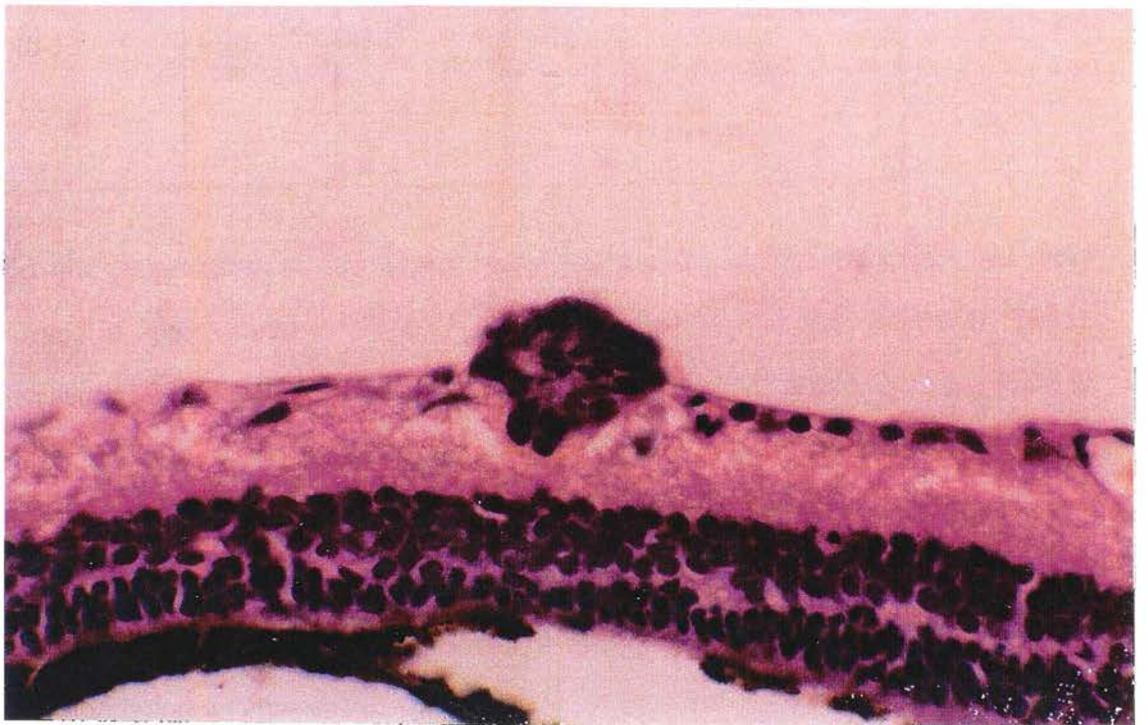


Figure 3.9 Histological sections through the eye of SV40-13 transgenic animals showing abnormal development and tumour formation

(**A**) Shows an aggressive tumour growth in the vitreous layer, retinal detachment with subretinal bleeding and infiltration of the intraocular structures by the tumour. This sample was taken from a 11 week old female.

(**B**) Shows a tumour in the ganglion cell layer, beginning to infiltrate the vitreous layer. This sample was taken from a 8 week old male.

from a 8 week old male with a tumour in the ganglion layer beginning to infiltrate the vitreous layer.

Table 3.1 Summary of the abnormalities in surviving SV40 lines of transgenic mice

Transgenic lines	Copy number	Mammary expression	Life span (weeks)	Anatomic site of abnormalities
SV40-2	1-2	Yes	normal	none
SV40-6	<5	No	15-20	testes preputial gland lungs heart muscle
SV40-8	1-2	No	normal	none
SV40-9	<5	No	15-20	subcutaneous spleen thymus gut
SV40-13	<50	Yes	10-12	subcutaneous spleen thymus gut retina

Ectopic expression of transgenes containing genomic BLG sequences has been observed in approximately 40% of the lines generated (Farini and Whitelaw, 1995). However in this study the ectopic expression in transgenic lines of mice carrying 4.2kb of 5'BLG sequences was not examined. Therefore it was not clear whether the high incidence of ectopic expression (10/12) observed in the SV40 lines was due to expression from the BLG promoter or whether sequences within the thermolabile T-antigen were directing expression. Alternatively it could be a combination of both with the ectopic expression being a consequence of the unique combination of sequences present in this hybrid construct.

To assess the extent of ectopic expression from the 4.2kb BLG promoter, RNA was prepared from several tissues from transgenic line 45. This line carries approximately 17 copies (Whitelaw *et al.*, 1992) of a 3' truncated transgene and secretes approximately 23.7mg/ml of BLG into the milk. It carries the same BLG promoter as used in the generation of the SV40 mice but drives genomic BLG expression instead of T-antigen.

Figure 3.10 is a mixed tissue Northern blot with RNA prepared from line 45 virgin mice probed with a BLG cDNA probe (kindly provide by Dr. B. Whitelaw). As expected the mammary gland expresses high levels of the BLG mRNA however ectopic expression is also observed in several tissues where tumours have been detected in the SV40 mice. In particular the highest levels of ectopic expression were detected in the lungs, spleen and lymph nodes. Lower levels of expression were observed in the heart, liver, ovaries, thymus, thyroid, salivary gland and gut. In addition to the correct size transcript (~800bp) there were higher molecular weight transcripts observed in some tissues. In the salivary gland this larger RNA transcript was the predominant species.

Although the ectopic expression pattern of line 45 correlates with the site of tumours or abnormalities in the SV40 lines, direct comparison cannot be made since they are different transgenes with their own unique integration sites and transgene arrays. However it can be concluded that integration of this promoter into an "appropriate" site for expression can lead to constitutive ectopic expression. The sites of ectopic BLG expression in transgenic mice using 4.2kb of the BLG promoter is consistent with tumour sites observed in the SV40 transgenic lines generated in this study.

3.2.4.3 Transgene expression in the mammary gland

Initially mRNA levels of T-antigen in the mammary gland were examined by Northern blot analysis. However, despite several attempts, little or no T-antigen mRNA was detected from RNA isolated from mammary glands at the peak of lactation (11 days) from any of the lines. Therefore levels of the protein were also examined in the surviving lines. Protein extracts were prepared from mammary tissue at the peak of lactation (day 11), run on a 10% SDS-PAGE gel and transferred to nitrocellulose. The Western blot was probed with a monoclonal T-antigen antibody DP-02 (Sigma) which specifically reacts with large T-antigen. Figure 3.11 clearly shows expression of T-antigen in 3 sisters from both SV40-2 and SV40-13 lines. The expression level in one of the SV40-2 sisters appears to be lower than in the other two. A duplicated gel run simultaneously and stained with Coomassie blue revealed less total protein in this track than the others and could account

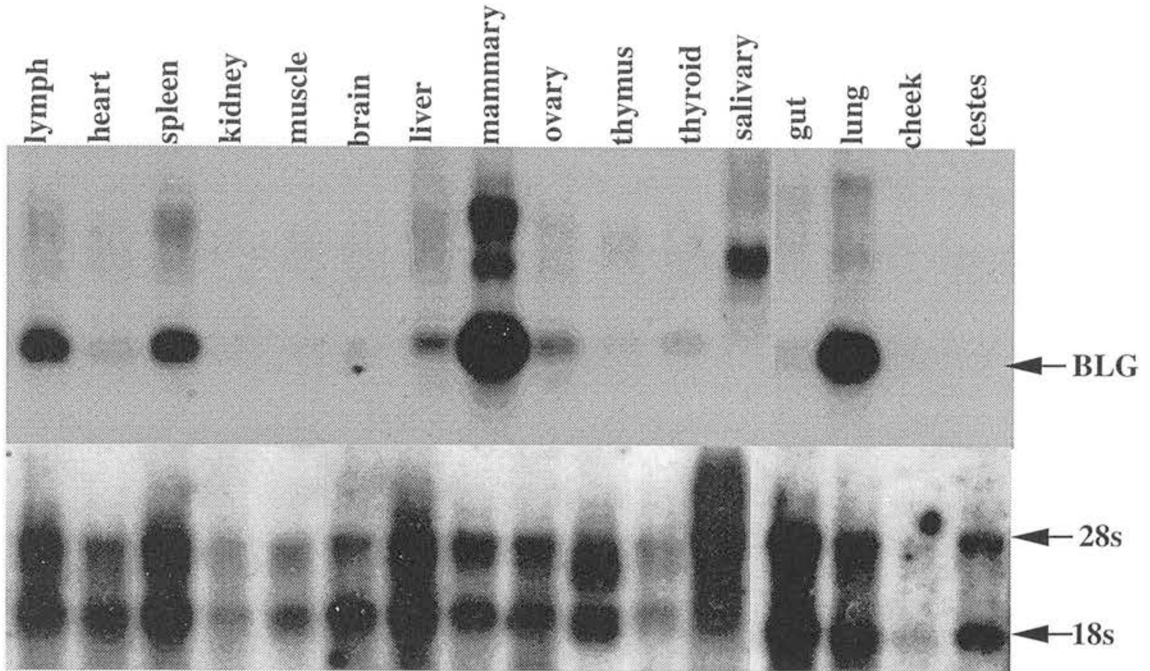


Figure 3.10 Ectopic expression of BLG mRNA in a high expressing BLG line of mice

Northern analysis of BLG expression in line 45 (the highest expressing BLG line produced to date). This line carries the same 4.2kb promoter used to generated the SV40 lines of mice. The blot was hybridised with a BLG cDNA in the upper panel and the blot stripped and hybridised with a ribosomal probe as a loading control (lower panel).

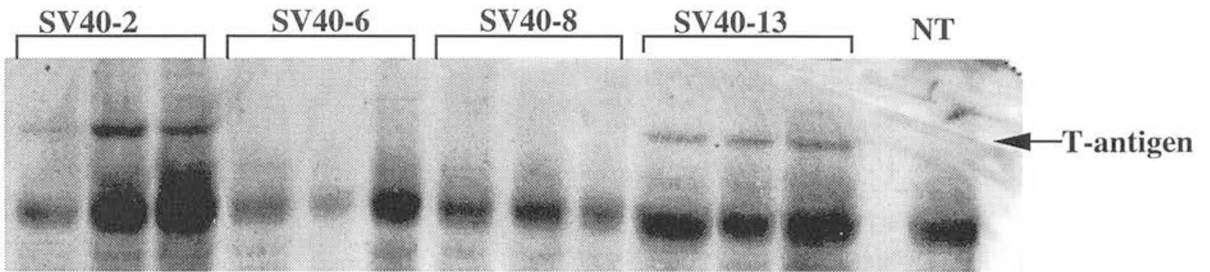


Figure 3.11 T-antigen protein expression in lactating mammary extracts from SV40 transgenic lines
Protein extracts (20µg) from day11 lactation mammary tissue were prepared from 4 of the SV40 lines of mice (3 sisters in each line). The expression levels of T-antigen protein was assessed by Western analysis. The blot was incubated with a T-antigen specific antibody, DP-O2 (Pab 416) diluted 1:500 (Calbiochem). A nontransgenic (NT) 11 day lactation sample was loaded as a negative control.

for this discrepancy. T-antigen expression could not be detected in the mammary glands of the other 3 surviving lines of mice (SV40-6, -8, -9) by Western blotting. This antibody also detected a non-specific 60-70kD protein which was present in both transgenic and control wild type mouse mammary tissue.

Immunohistochemical staining of dewaxed paraffin embedded sections from midpregnant and 11 day lactation tissue with this antibody and with the antibody Pab 108 were unsuccessful.

3.3 DISCUSSION

Micro-injection of the 4.2BLG-tsA58 transgene into single cell mouse embryos resulted in the generation of 12 transgenic founder mice from a total of 54 analysed, all with varying copy numbers of the transgene. However one of the founders was mosaic and did not pass the transgene on to her offspring and 6 of the founders died before lines could be generated due to ectopic expression of the transgene. Transgenic lines of mice were successfully generated from the remaining 5 founders. SV40-2 which has 1-2 copies of the transgene was the only line which displayed detectable expression in the mammary gland and had no visible ectopic expression. Expression of T-antigen did not alter mammary development and this line can successfully feed their litters, unlike the transgenic mice which express wild type T-antigen from the WAP promoter (Tzeng *et al.*, 1993; Li *et al.*, 1996). SV40-8 which also has 1-2 copies of the transgene did not display any ectopic expression but mammary expression was undetectable by Western or Northern blotting. Presumably this transgene has integrated into a site in the genome where the influence of the neighbouring chromatin silences the transgene (Kellum and Schedl, 1991; McKnight *et al.*, 1992). In SV40-6 and SV40-9 again no mammary expression was detected but ectopic expression in other tissues lead to tumour formation and in SV40-13 both mammary and ectopic expression was observed. The onset of the tumour phenotype in each line was retained through subsequent generations and was characteristic of the line.

Ectopic expression in a highly expressing BLG line of mice (line 45) which carries the same promoter as the SV40 lines shows, high levels of ectopic expression. The sites of

expression are consistent with some of the sites where tumours or abnormalities were observed in the SV40 lines, most notably the spleen, ovary, salivary gland, gut and lung. In addition, genomic constructs containing BLG promoter sequences have displayed ectopic expression in 40% of the lines (Farini and Whitelaw, 1995). The frequency of this ectopic expression in the SV40 lines is higher with 10/12 lines (83%) displaying ectopic expression. It may well be that the apparent high incidence of ectopic expression is due to the sensitivity of detection of the transgene i.e. tumour formation in tissues susceptible to T-antigen transformation.

The ability of T-antigen to direct ectopic expression must also be considered. In the majority of cases, using a tissue specific promoter to drive expression of wild-type T-antigen resulted in expression in the appropriate organ. For example the insulin gene enhancer resulted in T-antigen expressing Langerhans islet B-cell tumours (Hanahan, 1985; Efrat *et al.*, 1988) and pancreatic acinar cell tumours were induced by expression from the elastase promoter (Ornitz *et al.*, 1985). However there are several cases in the literature where the expected tissue-specific expression of T-antigen was not observed. For example, when the β-globin LCR was fused to SV40 T-antigen early regions, the transgenic mice generated did not express T-antigen in erythrocytes (Teitz *et al.*, 1993). Instead the phenotype of the transgenic mice was dependant on the copy number of the transgenes. Mice with 1-2 copies of the transgene developed normally but mice with 3-7 copies developed rhabdomyosarcomas in a number of anatomical sites and mice with more than 10 copies were growth retarded and died after only a few weeks. Clearly some tissue-specific genes do direct T-antigen to the appropriate cell type however there are cases where this does not occur.

Another complicating factor is that the cell type and state of differentiation may contribute to the ectopic tumour expression pattern observed in the SV40 transgenic lines of mice. Low levels of T-antigen expression are sufficient to transform particular cell types and not others. The SV40 T-antigen mutant used in this study encodes a thermolabile mutant of large T-antigen as well as encoding small t-antigen. However it does not contain any upstream enhancer sequences which have previously been shown to direct expression to the choroid plexus in the brain (Palmiter *et al.*, 1985). Although two

of the SV40 transgenic lines (SV40-9 and SV40-13) displayed a 'bulge' on their heads the tumours which are present are attached to the skull and do not appear to have invaded any brain tissue. The lack of choroid plexus tumours is consistent with the upstream enhancers directing expression to the brain. Using the MMTV LTR Choi *et al.*, (1988) targeted expression of large T-antigen alone or with small t-antigen to the ductal epithelial and lymphoid cells of transgenic mice. Mice expressing either construct developed similar malignant lymphomas. Lymphoma cells were seen in the lymph nodes, thymus, lung, liver, Peyer's patch, kidney, spleen, salivary gland and skeletal muscle. However, only the transgenic lines expressing both large and small T / t-antigen developed lung and kidney adenocarcinomas. Despite the highest level of expression of both transgenes in the mammary gland it was relatively resistant to SV40 tumourigenesis *in vivo*. The reason for this is unknown but it could be that there is a high level of p53 in the mammary gland which complexes T-antigen and prevents it leading to transformation. It appears that the lymphoid tissues are particularly susceptible to T-antigen transformation whereas the mammary gland is not. SV40 small t-antigen may have a role in tumour formation in slowly dividing cells e.g kidney and lung. Abnormalities in the spleen and the thymus were apparent in the ectopic expressing SV40 transgenic lines. In SV40-9 there were also abnormalities in lung tissue.

The molecular mechanism of the transformation induced by T-antigen is becoming clearer. Its transforming ability has been attributed to its ability to bind to and inactivate cellular genes such as p53 and pRb family members. Presumably, the expression of the "appropriate" levels of T-antigen could result in the inactivation of p53 and/or pRb function in certain tissues. Comparision of the phenotype of the p53 null mice (Donehower *et al.*, 1992) with the SV40 lines generated here shows some similarities. In particular the rapid onset of tumours occurs around the same time period (15-25 weeks). The sites and types of tumours or abnormalities which develop are similar. In particular both are prone to sarcomas and lymphomas (Dr. W. Wallace & Dr.D. Harrison pers. comm.) and the organs involved include the thymus, heart , lung, spleen, and kidney.

Mice homozygous for an Rb mutation die *in utero* between 13.5-15.5 days and display defects in neurogenesis and haematopoiesis (Clarke *et al.*, 1992; Jacks, 1992; Lee *et al.*,

retinoblastoma or secondary tumours, such as osteosarcomas or soft-tissue sarcomas which are characteristic of the human hereditary form of the disease (Weinberg *et al.*, 1992). However brain and pituitary tumours have been detected in mice at around 8-10 months of age (Jacks, 1992). Since the SV40 ectopic expressing lines are routinely sacrificed at between 6-7 months of age or earlier due to their tumour burden, it is not possible to comment on the ability of the SV40 lines to develop pituitary or brain tumours.

Interestingly, double-mutant mice generated by interbreeding the p53 homozygotes with the pRb heterozygotes gave similiar retinal defects to those seen in SV40-13. These mice displayed retinal dyplasia (abnormal architecture) as opposed to hyperplasia. Since these mice, like the double-mutants rarely survive beyond 4-5 months of age it is not possible to determine whether these lesions would develop into true retinoblastomas. However this may well be the case since targeting wild-type SV40 T-antigen to the retina does result in retinoblastomas (Windle *et al.*, 1990; al-Ubaidi *et al.*, 1992).

Although the high level ectopic expression of the BLG-tsA58 transgene was not anticipated, the sites of tumour formation which were observed in the surviving lines of mice are consistent with the ectopic expression observed in a transgenic line carrying the same promoter but driving BLG expression. In addition the onset of tumour development and the tumour types are comparable with those observed in the p53 null mice and in double mutants (pRb-/+; p53-/-).

THE ISOLATION OF MOUSE MAMMARY EPITHELIAL CELL-ENRICHED CULTURES DERIVED FROM EXPLANT OUTGROWTHS

4.1 INTRODUCTION

Mammary gland development and morphogenesis has been well defined histologically. However little is known about the specific cell lineages involved in normal development of the gland and the changes that occur during neoplasia. In part this is due to the lack of cell-specific markers to study the developmental fate of the different cell types found in the mammary gland during its development and partly due to the lack of suitable cell lines.

There are several antibodies which are specific for cellular components, in particular the keratin intermediate filaments, which can differentiate between the mesenchymal and epithelial cell lineages. However there are a lack of markers which can differentiate between the different cell types within lineages, for example, epithelial cells of ducts, terminal end buds and alveolar buds. The mammary cell lines which are presently available have either undergone spontaneous or induced immortalisation (either chemically or by transfection of known immortalising agents) leading to constantly proliferating cultures. The effects this has on the normal cellular processes is unclear and therefore it is difficult to investigate aberrant growth in these cell lines. This problem can in theory be overcome by using conditionally immortalising genes.

The "immortomouse" was the first transgenic line of mice generated as a potential source of conditionally immortal cell lines. Jat *et al.*, (1991) targeted expression of a temperature-sensitive mutant of SV40 T-antigen to a wide range of tissues using an inducible housekeeping gene promoter H-2K^b. In theory, isolating cells from any organ and culturing these cells at the permissive temperature of 33°C should result in the expression of active T-antigen and produce constantly proliferating cell lines. However, by shifting the temperature to 39°C the immortalisation properties of T-antigen are abrogated and the cells can undergo differentiation, assuming the appropriate stimuli are present. Indeed several conditionally immortal cell lines have been produced using the "immortomouse" (see section 1.4.3). However no mammary cell lines have been

the "immortomouse" (see section 1.4.3). However no mammary cell lines have been isolated either by myself or others using this transgenic line (Streuli, C.H. pers. comm.).

The transgenic mice generated during this project, which express the same temperature sensitive T-antigen under the control of the BLG promoter did prove more fruitful. This chapter describes a novel procedure used for the isolation of highly enriched mammary epithelial primary cultures from both transgenic and nontransgenic mice.

4.2 RESULTS

4.2.1 Establishment and growth of explant cultures

Similar procedures have generally been used to isolate epithelial enriched cultures. Minced mammary glands are digested with collagenase to dissociated the cells and separate the mammary tissue from the fat pad (Lasfargues, 1957). The epithelial component can then be purified using percoll/ficoll gradients (Kidwell *et al.*, 1984), differential centrifugation (Barcellos-Hoff and Bissell, 1989) or fluorescent-activated cell sorting (Darcy *et al.*, 1991). However this procedure often requires a large amount of starting tissue (12-14 midpregnant mice) to obtain the high density of epithelial cells required for the primary cultures to survive. Instead of using this standard procedure, a less orthodox approach was utilised to isolate epithelial-enriched cultures. This approach involved culturing mammary explants on typeI collagen under growth promoting conditions which resulted in epithelial outgrowths from the explant (summarised in Diagram 4.1). In general sufficient outgrowths could be obtained from a single mouse to attempt to generate cell lines.

To optimise the possibility of this procedure working it was important to isolate cells which were still proliferating and not terminally differentiated. In addition it was necessary for the BLG promoter to be transcriptionally active to ensure sufficient levels of T-antigen is expressed at the permissive temperature to immortalise the cells. Mammary tissue was initially removed from midpregnant (12.5-13 days) mice. This time-point was chosen as the most suitable starting point based on the following

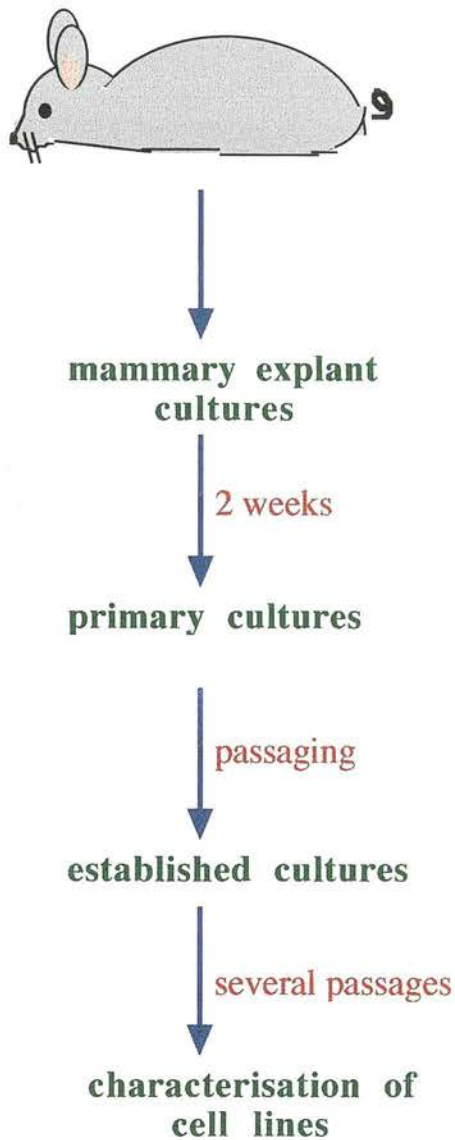


Diagram 4.1 Flow chart summarising the steps involved in the generation of the cell lines
Mammary tissue was removed from day 12.5-13 pregnant mice and explant outgrowths established on typeI collagen in Explant growth medium. The explants were removed and the primary cultures grown for approximately 5-6 weeks in Complete medium before passaging.

criteria:- Firstly, the BLG promoter is active by this stage; secondly this time-point coincides with a peak of epithelial proliferation (Traurig, 1967); and thirdly the mammary gland is in the early stages of differentiation with β-casein being the only differentiation marker expressed.

The expression level of T-antigen required to immortalise without transforming the cells was not known, so mammary explant cultures were made from the five surviving SV40 transgenic lines and "immortomouse" with nontransgenic littermates used as controls. Since the BLG-tsA58 transgene showed ectopic expression in the transgenic lines *in vivo* and resulted in hyperplasia or tumours which expressed T-antigen it was assumed that the tsA58, in this construct at least, was partially active at the body temperature of the mice. In light of this observation it was decided to culture half of the flasks at the fully permissive temperature of 33°C and the rest at 37°C, a semi-permissive temperature. Figure 4.1 (A-D) illustrated the typical outgrowths which were observed after culturing the outgrowths without serum for two weeks on collagen typeI coated flasks. All the outgrowths looked the same at this stage regardless of the presence or absence of the transgene or whether the cultures were maintained at 33°C or 37°C. In cultures which were supplemented with serum, the growth of the explants was slightly enhanced at this stage but upon removal of the explants these cultures became very heavily overgrown with fibroblasts (data not shown).

4.2.2 Morphological differences in primary cultures derived from the different transgenic lines.

The cultures were monitored daily by phase contrast light microscopy under x10 magnification. Morphological differences in the cultures became apparent only a few days after explant removal. The primary cultures which were established from the nontransgenic control tisssue did not proliferate well and after 4 weeks in culture the remaining islands of cells contained a highly heterogeneous cell population which only survived 2-3 passages (Figure 4.2).

Similarly the primary cultures which were established from the "immortomouse"and the line SV40-8 (no ectopic expression or mammary expression could be detected in this line) did not proliferate well but after 5 weeks in culture at 33°C the cultures

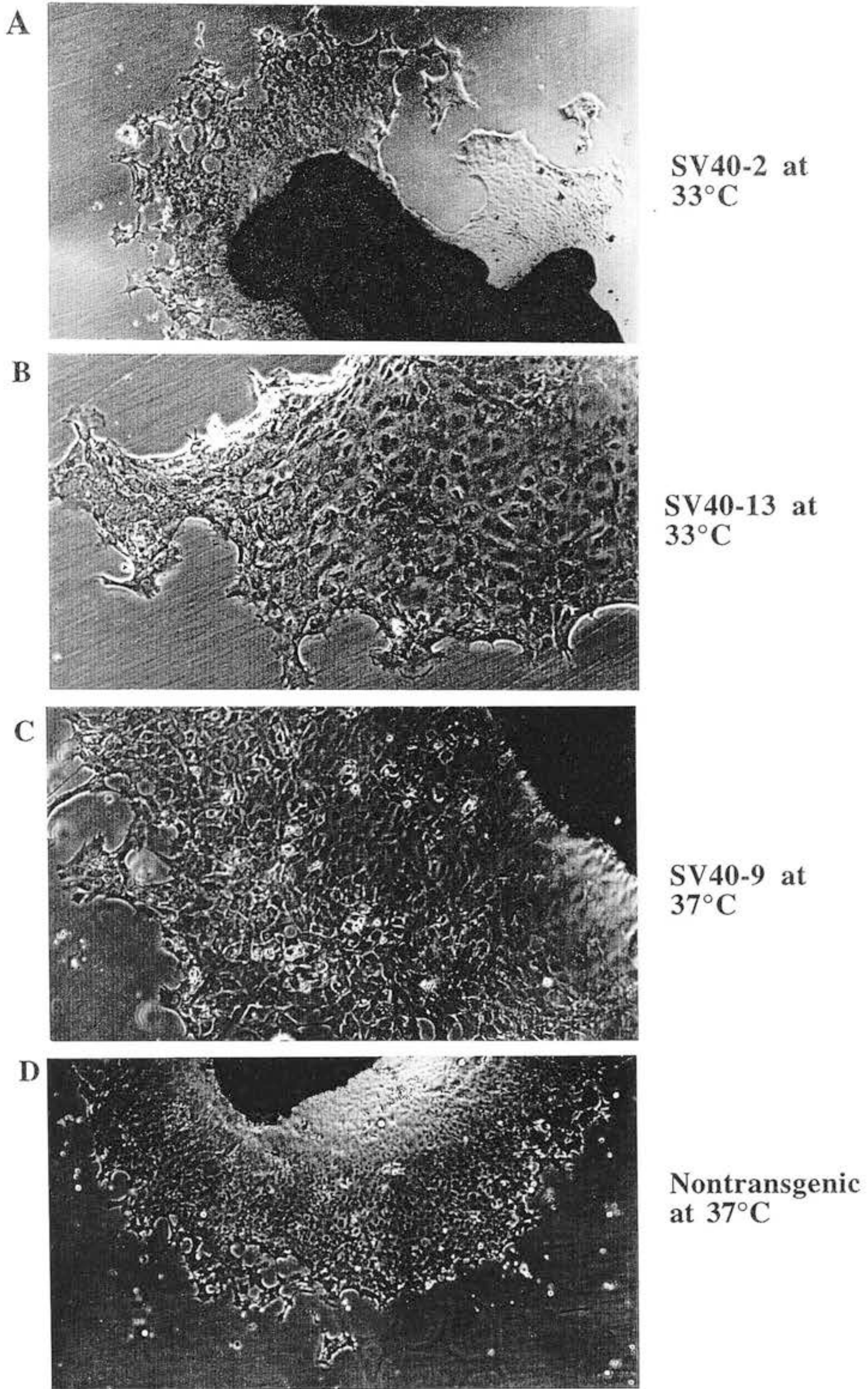


Figure 4.1 Typical outgrowths obtained from midpregnant mammary glands grown for 2 weeks on typeI collagen coated flasks

(**A-C**) are explants established from transgenic lines carrying different copy numbers of the BLG-tsA58 transgene. (**D**) is an explant established from a nontransgenic littermate.

NONTRANSGENIC CONTROL PRIMARY CULTURES
GROWN AT 37°C

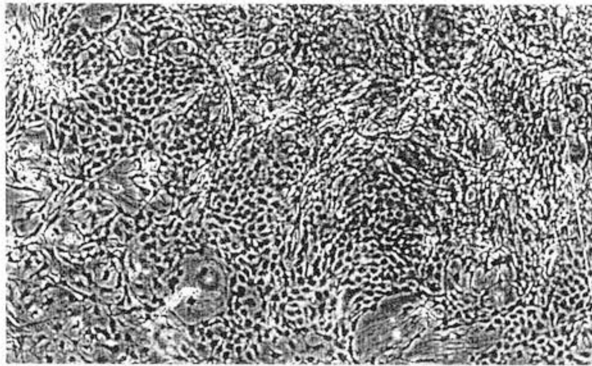


Figure 4.2 Light microscopy of primary cultures established from a nontransgenic control midpregnant mouse
Note the heterogeneity of the cultures. These cultures did not survive passaging (magnification x10).

consisted of a heterogeneous population of cells consisting mainly of large striated cells (Figure 4.3).

Mammary cultures established from the SV40 lines which displayed ectopic expression of T-antigen resulting in tumours (BLG/SV40-6, 9, 13), all showed a similar phenotype when cultured at 33°C or 37°C. The cultures initially retained the "cobblestone" morphology characteristic of epithelial cells (Figure 4.4A) but within 14 days of explant removal a second cell type was apparent. These cells were elongated, were peripheral to the epithelial island and highly refractive to light. (Figure 4.4B). This rapidly proliferating second cell type predominated at the permissive and semi-permissive temperatures resulting in the loss of the epithelial cells. At the nonpermissive temperature, when the thermolabile mutant should be inactive, a few "dome" structures formed in high density cultures (Figure 4.4C). These "dome" structures have been described as indicative of secretory epithelia (Danielson *et al.*, 1984).

The remaining SV40-2 transgenic line displayed a similar heterogeneous cell phenotype (Figure 4.5A) when cultured at 33°C with the elongated cells becoming more abundant as the cultures continued to be grown at 33°C (Figure 4.5B). However explant cultures which had been grown at 37°C without serum retained a uniform cobblestone morphology and all the primary cultures formed "dome" structures at high density after 3-4 weeks in culture (Figure 4.5C). This cobblestone morphology was retained after passaging as clumps of 5-10 cells onto flasks coated with typeI collagen. These cultures designated KIM-2 cells, were expanded and stocks cryopreserved before the cultures were characterised further.

The cultures which did become established from the other SV40 transgenic lines were frozen down and not analysed further since the epithelial cell morphology was lost even at the semi-permissive temperature of 37°C. Table 3.1 summaries the culture history of the different primary cultures which were generated from the different transgenic lines of mice.

MIDPREGNANT IMMORTOMOUSE PRIMARY CULTURE
GROWN AT 33°C

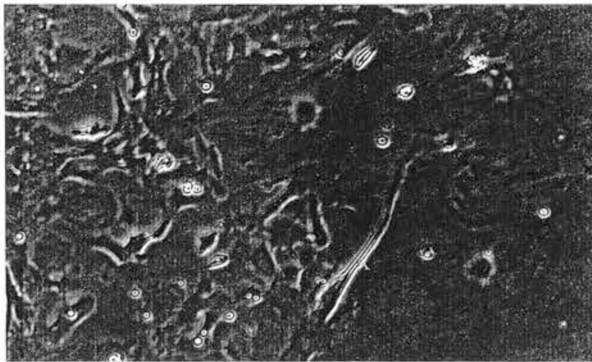


Figure 4.3 Light microscopy of primary cultures established from a midpregnant gland of immortomouse
Note the heterogeneity and sparseness of the cultures. These cultures did not survive passaging.

PRIMARY CULTURES

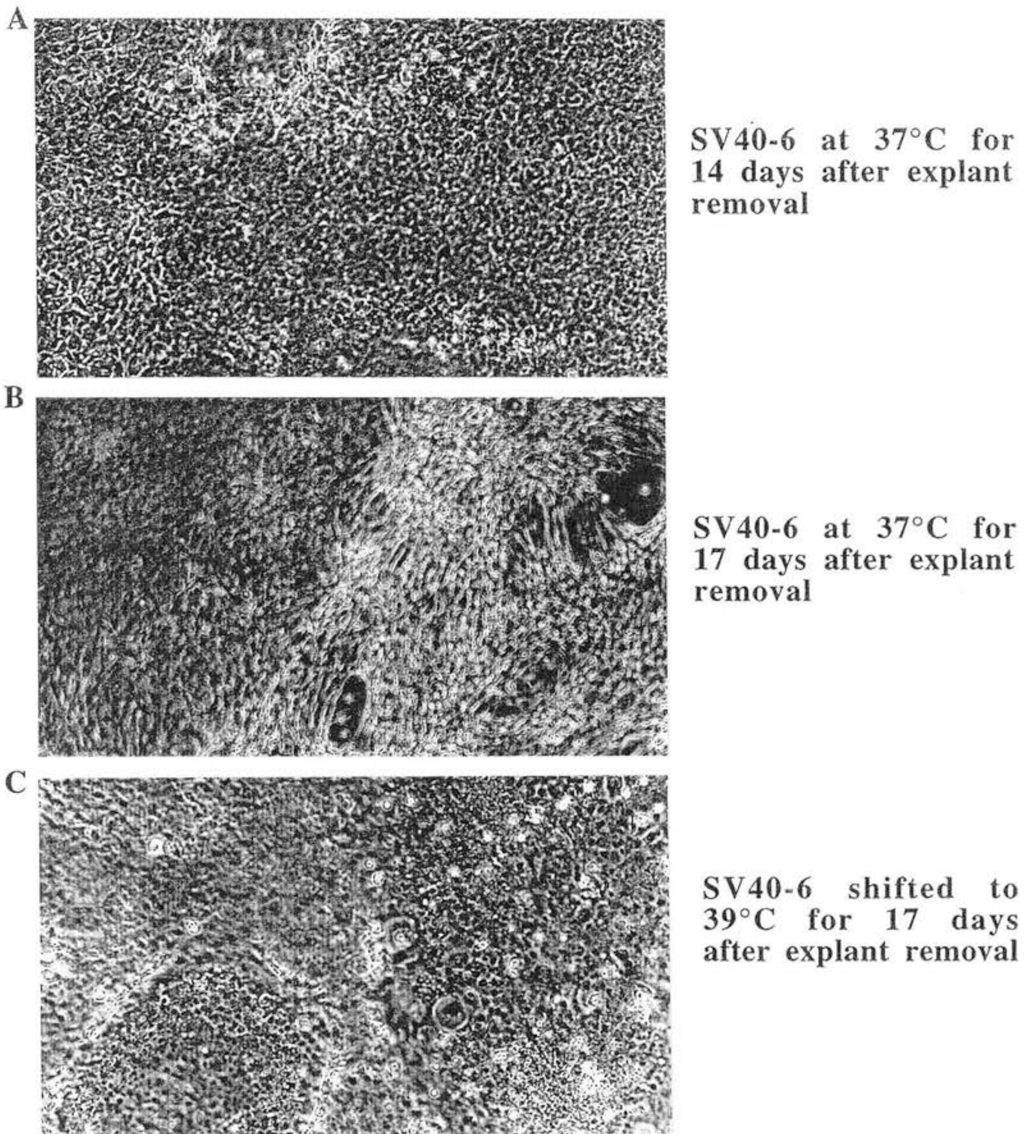


Figure 4.4 Typical morphological changes which occurred in the SV40 transgenic lines displaying ectopic expression of the transgene.

(**A**) illustrates the initial cobblestone morphology which was observed in primary culture established at 33°C or 37°C.

(**B**) shows the same cultures losing contact growth inhibition after removal of explant for 17 days.

(**C**) shows cultures obtained from the same transgenic line of mice with explant outgrowths established at 37°C and cultures moved to 39°C once the explants were removed. Note the dome forming.

PRIMARY CULTURES

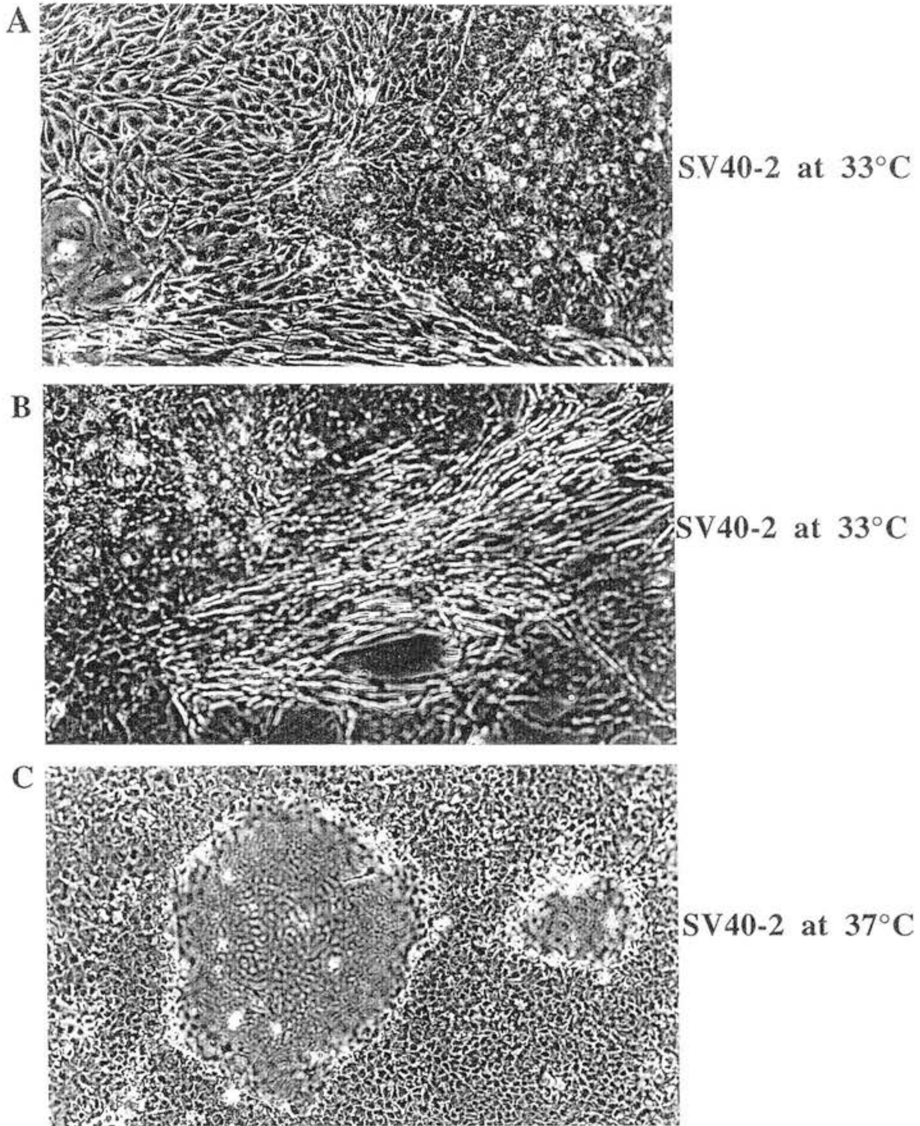


Figure 4.5 Primary cultures established from SV40-2 transgenic line cultured at 33°C or at 37°C

(**A**) illustrates the heterogeneity of cultures grown at 33°C after 21-28 days.

(**B**) illustrates a culture at an early stage of fibroblastic overgrowth after 21-28 days in culture at 33°C.

(**C**) illustrates a typical primary culture forming "domes" obtained from this transgenic line of mice after culturing for 21-28 days at 37°C.

Table 4.1 Summary of the culture history and morphology of the different primary cultures generated from transgenic and nontransgenic mice

Lines	T-antigen expression *in vivo*		Morphology of primary cultures		Successful passaging
	mammary	ectopic	33°C	37°C	
SV40-2	yes	no	heterogeneous with spindle-like and epithelial cells	mainly epithelial cells	yes
SV40-6	no	yes	heterogeneous with loss of contact inhibited growth	heterogeneous with spindle-like and epithelial cells	yes
SV40-8	no	no	heterogeneous with mainly large striated cells	heterogeneous with mainly large striated cells	no
SV40-9	no	yes	heterogeneous with loss of contact inhibited growth	heterogeneous with spindle-like and epithelial cells	yes
SV40-13	yes	yes	heterogeneous with loss of contact inhibited growth	heterogeneous with loss of contact inhibited growth	yes
$H\text{-}2K^b$	yes	yes	heterogeneous with mainly large striated cells	NDA	no
nontransgenic	no	no	heterogeneous population	heterogenous population	no

NDA: No data available

4.3 DISCUSSION

The outgrowths obtained from midpregnant mammary explant cultures has provided an efficient source of highly enriched populations of primary epithelial cells. The outgrowths established from the transgenic lines of mice and from nontransgenic control mice were morphologically identical as assessed by phase-contrast light microscopy.

It is not suprising that the nontransgenic control primary cultures did not survive through continual passages, since it has been well documented that primary cultures lose both their proliferation and differentiation potential in culture unless spontaneous inmortalisation occurs.

The primary mammary cultures which were established from the "immortomouse" did not proliferate well and the epithelial component was lost after only a few passages. It could be that there is insufficient expression of T-antigen from the $H2K^b$ promoter in the mammary gland to immortalise the cells. Alternatively, the expression of T-antigen in the mammary gland could be higher in the other mammary cell types, such as the fibroblastic cells which grow much better in culture than the epithelial component thus adding to their growth advantage.

The morphological phenotypes observed in the SV40 transgenic derived primary cultures are curious and not easily explained. When primary mammary cultures isolated from the two transgenic lines of mice, which displayed ectopic expression of T-antigen but no detectable mammary expression *in vivo* (SV40-6 and SV40-9), were incubated at 33°or 37° the cell type which predominated upon removal of the explants was a thin elongated cell type. Although expression levels of T-antigen was not examined in these *in vitro* cultures this observation is consistent with the expression of T-antigen observed by Rudland and Barraclough (1990). Using SV40-transformed human breast cell lines they demonstrated the ability of these cells to differentiate to a 'more elongated myopepithelial-like' cell type which correlates with increased expression of T-antigen. Although morphologically the elongated cell type described here and by Rudland and Barraclough shows similarities, there are differences which need to be resolved. In particular, similar cultures containing these elongated cells described here stain strongly for vimentin, a mesenchymal marker (see Chapter 5) whereas Rudland and Barraclough describe a myoepithelial staining population. Regardless of the origin of this cell type it would be interesting to examine this differentiation pathway in the SV40 cultures and the expression of T-antigen in explant and primary cultures from the nonexpressing transgenic lines of mice.

The primary cultures which were derived at 33°C from the lowest copy transgenic line (SV40-2) also displayed an elongated phenotype. However cultures which were established and maintained at 37°C maintain a uniform "cobblestone" morphology which was maintained after passaging. Presumably, this cell line synthesises sufficient levels of T-antigen at a semi-permissive temperature (37°C) to produce constantly

proliferating cultures without resulting in overgrowth of the 'elongated' cell type. This cell line, designated KIM-2, has been maintained without any obvious morphological alterations for 18 months. The cultures are split (1:3) as clumps of 5-10 cells every 3-4 days and are routinely maintained at 37°C in Growth media (section 2.8.1 and 2.8.2).

The major advantage of this isolation procedure was that cultures were relatively free of fibroblasts at the onset therefore problems with fibroblastic overgrowth was not encountered. The overgrowth of fibroblasts in relatively pure primary epithelial cultures has proven to be a problem in the past with cultures eventually losing their epithelial population. Passaging the cells onto typeI collagen for the first 5 passages during expansion prior to cryopreservation is novel to this isolation procedure. *In vivo*, following the onset of pregnancy (day 4) collagen typeI is synthesised by fibrobastic cells found immediately adjacent to the developing epithelium (Keely *et al.*, 1995). Levels then decrease and collagen typeIV and laminin increase in the latter stages of pregnancy. Culturing the epithelial cells on typeI collagen in the absence of lactogenic hormones may provide an enviroment closer to that found *in vivo* with the cells retaining epithelial characteristics which might otherwise be lost on plastic substrata.

THE CHARACTERISATION OF A MOUSE MAMMARY CELL LINE (KIM-2) ISOLATED AT 37°C

5.1 INTRODUCTION

In vitro cell culture systems have provided tools in which the growth and differentiation of mammary epithelial cells can be studied at the molecular and cellular levels. For example, the HC11 cell line has provided a useful model to study the activation of β-casein gene expression. Unfortunately it has proven to be more difficult to express late differentiation markers such as WAP and α-lactalbumin *in vitro*. In addition, the mammary cell lines which have be isolated are irreversibly immortalised and it is unclear whether this event leads to alterations in the proliferation and differentiation pathways being examined. To attempt to overcome the latter problem a mammary cell line was generated carrying a thermolabile T-antigen mutant to act as an immortalisation switch. This chapter describes the characterisation of this cell culture system, designated KIM-2. Initial studies involved cell typing of the cultures at the semi-permissive temperature (37°C) and at the fully permissive temperature (33°C) by immunohistochemical staining with cell-type specific markers. Subsequent studies were carried out to assess the ability of the cultures to undergo functional differentiation in response to lactogenic hormones. The expression of the differentiation markers β-casein (early) and WAP (late) were examined in both early and late passage cells to determine (i) the degree of functional differentiation and (ii) the ability of the cultures to undergo differentiation after prolonged periods in culture.

5.2 RESULTS

5.2.1 Expression ofcell-type specific markers in KIM-2 cells

KIM-2 cultures isolated at 37°C maintained the typical epithelial cobblestone morphology throughout passaging. Cell typing was carried out by examining the expression of lineage-specific markers using immunohistochemical staining. The cultures were approximately 95% epithelial in origin as assessed by their positive staining with keratin 18 [LE61] (Figure 5.1B) and keratin 19 monoclonal antibodies (not shown). In some cultures there were a few cells which showed positive staining for smooth muscle actin, a myoepithelial marker (Figure 5.1C) and vimentin, a fibroblastic and stromal cell marker (Figure 5.1D). However KIM-2 cultures at 37°C were predominantly epithelial in origin and the epithelial phenotype was retained after prolonged culturing (up to 60 passages).

These cultures also showed strong nuclear staining with a T-antigen specific antibody in the majority of cells at the semi-permissive temperature of 37°C (Figure 5.2).

5.2.2 Mesenchymal-epithelial cell transition triggered by a temperature switch: Immunohistochemical analysis

KIM-2 cultures isolated and grown at the permissive temperature of 33°C are morphologically different from cultures established and maintained at the semi-permissive temperature of 37°C. Cells isolated and grown at 33°C show a high percentage of elongated spindle-like cells (Figure 5.3A), whereas cultures which were isolated and grown at 37°C retained a cobblestone morphology typical of cuboidal epithelial cells (Figure 5.3G). These morphological differences were investigated further using immunohistochemical markers to identify the cell-types present in the cultures at the different temperatures. The cultures were fixed and immunostained with monoclonal antibodies specific for epithelial and mesenchymal cell lineages.

Cultures maintained at the permissive temperature (33°), where T-antigen is fully functional stained strongly with a vimentin specific antibody (Vim 13.2), a mesenchymal marker (Figure 5.3B). Only a few patches of epithelial cells remained in these cultures as can be seen morphologically (Figure 5.3A) and by the expression

Figure 5.1 Identification of cell types present in KIM-2 cultures using mammary cell type specific markers.

(**A**) phase contrast

(**B**) same field showing positive staining with keratin 18 monoclonal antibody (LE61).

(**C**) weak positive staining with a smooth muscle actin monoclonal antibody (Sigma)

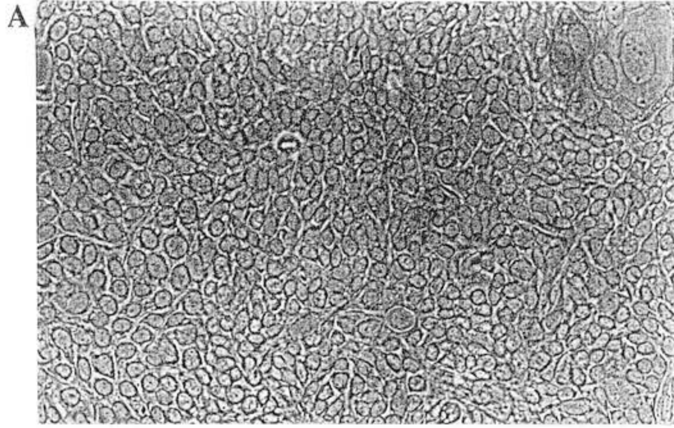
(**D**) weak positive staining with vimentin monoclonal antibody (Vim-13.2; Sigma) (magnification x10)

Vimentin staining was carried out by Dr. M. Smalley

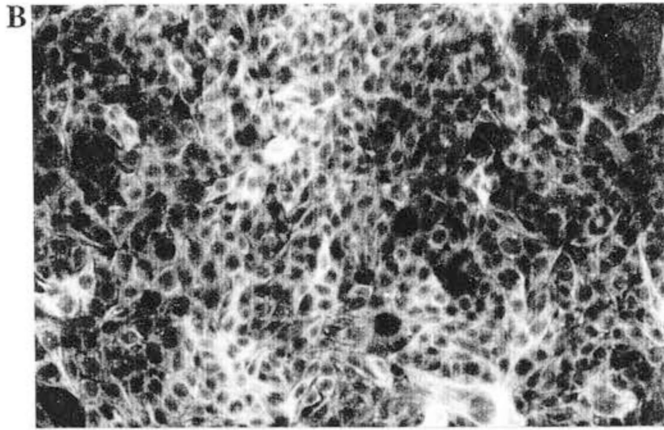
Cytokeratin antibodies were kindly provided by Prof. E.B. Lane

EXPRESSION OF SPECIFIC CELL TYPES IN KIM-2 CULTURES

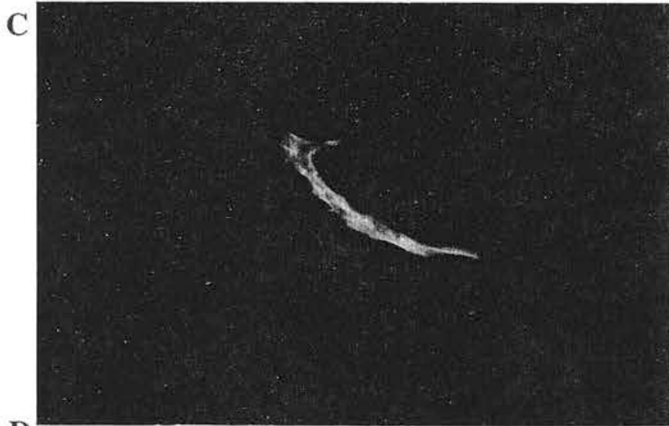
A



B



C



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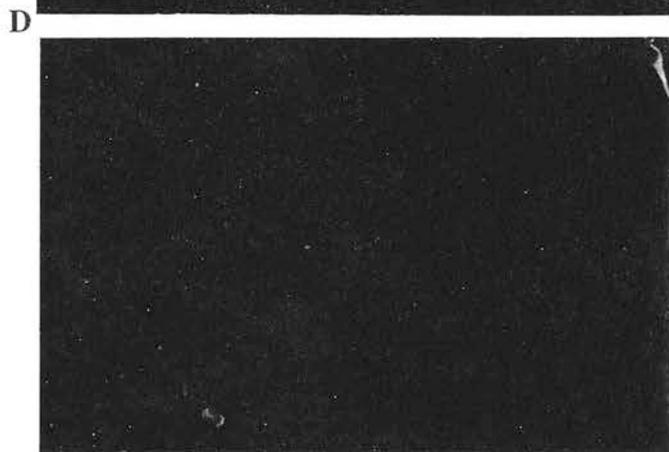


T-ANTIGEN EXPRESSION IN KIM-2 CULTURES GROWN AT 37°C

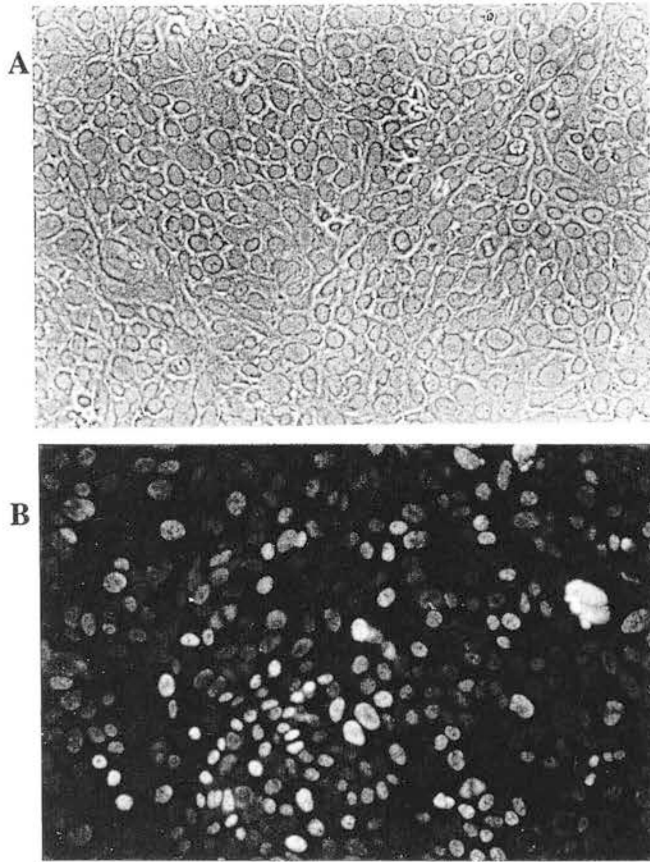


Figure 5.2 Immunohistochemical staining of KIM-2 cells grown at 37°C with a T-antigen specific antibody.

(**A**) phase contrast

(**B**) same field showing nuclear staining with a monclonal antibody specific for T-antigen, Pab419, (magnification x10).

(Staining carried out by Dr. M. Smalley)

pattern of keratin 18 (LE61), a luminal cell marker (Figure 5.3C). In contrast, cultures which had been maintained at 37°C displayed the opposite staining pattern. These cultures exhibited strong staining with the keratin 18 antibody, indicating a predominately epithelial population, and were almost devoid of vimentin expressing cells (Figure 5.3G-I). Cultures which had been isolated at the fully-permissive temperature and then switched to the semi-permissive temperature, contained a more mixed population of epithelial and mesenchymal cells with both markers being expressed (Figure 5.3D-F). In these cultures the epithelial islands of keratin 18 positive cells were more abundant and larger than those observed in cultures established and maintained at 33°C.

It is possible that at 33°C, when this thermolabile mutant of T-antigen is fully active there is preferential proliferation of a vimentin positive population of cells as opposed to the keratin 18 positive cells. However, closer inspection of the staining patterns of the two antibodies at the fully permissive temperature and in some of the switches to the lower temperature (not shown here) revealed that there were a small proportion of the cells which expressed both markers suggesting that these cells could be intermediates in a mesenchymal-epithelial conversion. At present it is unclear whether this conversion between epithelial and mesenchymal lineages is fully reversible or whether cultures devoid of keratin 18 expression remain so and cannot be rescued by raising the temperature.

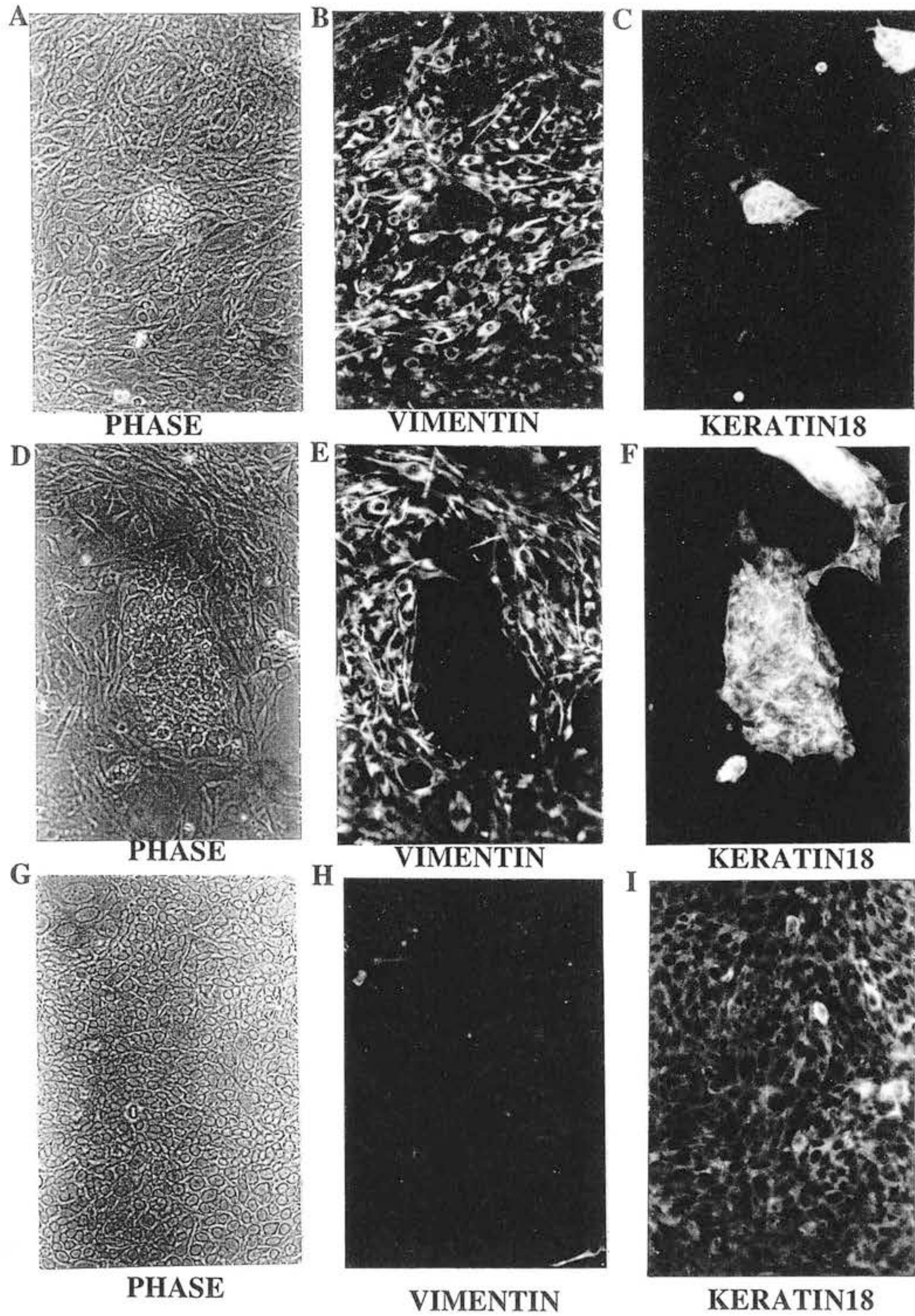
5.2.3 Expression of differentiation markers in KIM-2 cells.

Milk proteins synthesis *in vivo* takes place within lobuloalveolar structures in the developing mammary gland during pregnancy and lactation. The timing of expression of the different milk protein genes is strictly controlled by lactogenic hormones and interaction of the different cell types with each other and with components of the ECM. The primary culture systems and the mammary cell lines which are presently available as *in vitro* mammary models can undergo a degree of differentiation resulting in the transcriptional activation of some milk protein genes. The extent of this functional differentiation appears to be dependant on the substratum upon which the cells are

Figure 5.3 The expression of epithelial and mesenchymal lineage specific markers in KIM-2 cultures grown at different temperatures.

(**A-C**) KIM-2 cells were established and grown at 33°C. The same field was photographed in the order phase->vimentin->keratin18 (magnification x10). Note the patch of epithelial keratin18 positive cells in the middle of the field (**C**) but the majority of the cells are mesenchymal (fibroblasts or stromal cells) since the cultures stain strongly for vimentin (**B**) There is also some cells which express both markers (arrows).
(**D-F**) KIM-2 cells were established at 33°C and switched to 37°C for 4 days. Note the higher proportion of epithelial to mesenchymal cells in these cultures.
(**G-I**) KIM-2 cells were established and maintained at 37°C. The predominant cell type in these cultures is epithelial (**I**) with only a few mesenchymal cells present (**H**). (Staining carried out by Dr. M. Smalley)

MESENCHYMAL-EPITHELIAL CONVESION IN KIM-2 CELLS



cultured. β-casein is an early differentiation marker and can, for example, be induced by culturing cells on plastic with the addition of lactogenic hormones. Although the levels of β-casein can be increased by culturing cells on floating collagen gels and EHS the minimium requirement for its expression *in vitro* is the addition of lactogenic hormones to confluent cultures. However the expression of late differentiation markers such as WAP requires more complex cultures condition to induce expression *in vitro*. WAP appears to require cells to be grown on EHS to allow mammosphere formation before it is expressed (Chen *et al.*, 1989, Lin *et al.*, 1993). The ability of KIM-2 cells to undergo functional differentiation was therefore investigated.

The cells were routinely differentiated using the following conditions. Cultures were grown to confluency at 37°C in Growth media (see section 2.9.1) and EGF removed for 4 hours. The cells were washed washed 3 times with 1xPBS and incubated in Induction media (see section 2.9.4) containing the lactogenic hormones, prolactin and dexamethasone for the period and at the temperature indicated in each experiment.

In Figure 5.4 the cultures grown on typeI collagen were incubated at 37°C, where T-antigen is partially active, and at 39°C, were T-antigen is inactive, for 4 days and levels of β-casein protein analysed by Western blotting. β-casein was expressed in cultures grown at both temperatures suggesting that expression of wild type T-antigen is compatible with the expression of this early differentiation marker. Intracellular β-casein is detected as a doublet at approximately 29kDa. Secreted β-casein in defatted milk, on the other hand, contains a 32kDa protein. This discrepancy in size between the intracellular and secreted protein is probably due to differences in phosphorylation states and has previously been described by Durban *et al.*, 1985. In addition, the polyclonal β-casein antibody used did give quite high levels of nonspecific binding with the cell extracts and cross-reacted with α-casein in the milk. However it did show a dramatic induction of β-casein in the induced compared to the uninduced cells.

HORMONAL INDUCTION OF β-CASEIN PROTEIN IN KIM-2 CULTURES AT 37°C AND 39°C

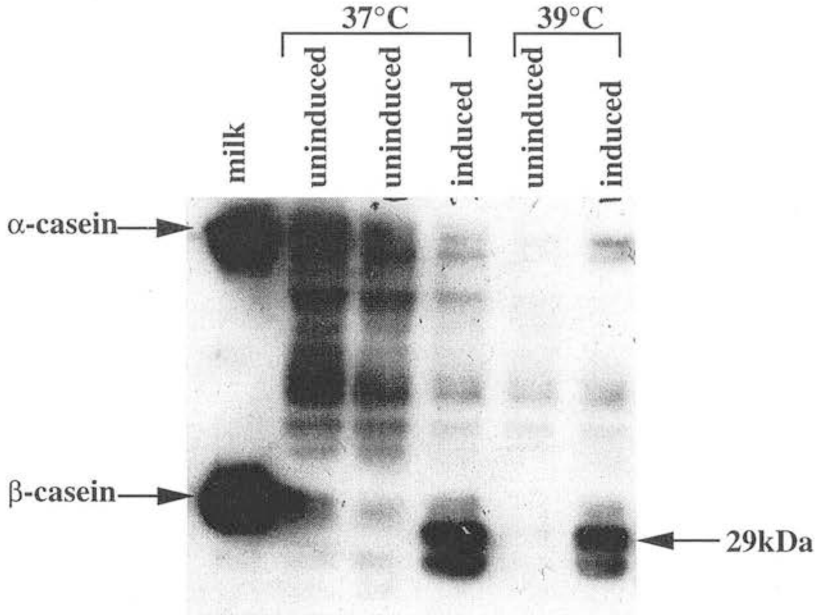


Figure 5.4 Western blot of KIM-2 cells induced with lactogenic hormones at a semi-permissive and non-permissive temperature

Total protein cell extracts (20μg) were prepared from early passage (P6) confluent KIM-2 cultures induced with the lactogenic hormones, prolactin and dexamethasone, for 4 days or uninduced (insulin) at the temperatures indicated. A defatted milk sample (20μg total protein) was used as a positive control for the antibody. The blot was probed with a murine β-casein polyclonal antibody : dilution:1:10,000 (kindly provided by Dr B. Binas).

5.2.3.1 Comparison of expression levels of β-casein protein in cultures grown on plastic and typeI collagen

KIM-2 cells which were derived and grown on typeI collagen coated flasks were passaged onto tissue culture plastic for 4-5 passages before determining whether the cells' capacity to synthesise β-casein was reduced or lost on tissue culture plastic. Figure 5.5 shows a time-course of induction of β-casein expression in KIM-2 cells grown on both substrata. Similar increasing levels of β-casein were observed over a period of 10 days induction on both substrata. This finding is consistent with the work of Emerman and co-workers who showed that increased levels of β-casein expression were observed in primary cultures only when they were cultured on floating typeI collagen gels (Emerman and Pitelka, 1977; Emerman *et al.*, 1979). However they did not observe high levels of β-casein expression on plastic.

5.2.3.2 Comparison of the levels of β-casein expression in KIM-2 cells and the HC11 clonal cell line

The level of expression of β-casein in KIM-2 cells was compared with an already established clonal mammary cell line HC11. This cell line, which was selected for its ability to express high levels of β-casein on plastic was used to assess the differentiation capacity of KIM-2 cells in response to lactogenic hormones. Higher levels of β-casein were expressed in confluent KIM-2 cultures after induction than in confluent HC11 cultures over the same time period (Figure 5.6).

5.2.3.3 Phenotypic stability of the differentiated phenotype in KIM-2 cultures

One of the drawbacks of mammary epithelial cell lines presently available is that they lose their differentiation capacity at later passages. This property was investigated in late passage (P31) KIM-2. Figure 5.7 shows the expression levels of β-casein protein obtained in KIM-2 cells after prolonged culture (3 months=P31). KIM-2 cultures therefore retain the ability to undergo functional differentiation after prolonged periods in culture as assessed by the expression of the early differentiation marker β-casein.

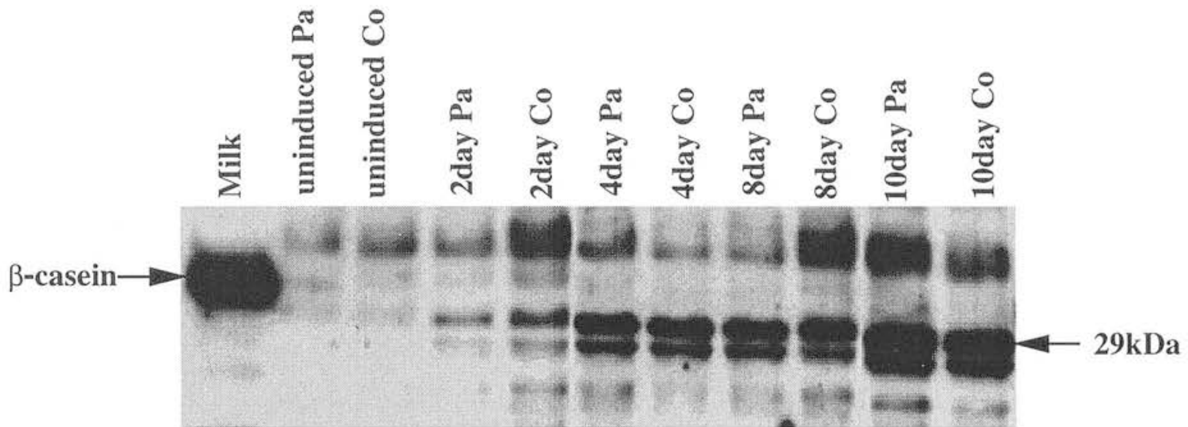


Figure 5.5 Comparison of β-casein protein induction in KIM-2 cultures grown on plastic and typeI collagen

Total protein extracts (20μg loaded) were prepared from KIM-2 cultures grown to confluency at 37°C on either tissue culture plastic or typeI collagen. The cultures were induced with lactogenic hormones, prolactin and dexamethasone for the time period indicated or uninduced (insulin). A defatted milk sample (20μg total protein) was used as a positive control for the antibody. The blot was probed with a murine β-casein polyclonal antibody:dilution 1:10,000 .

COMPARISON OF β-CASEIN EXPRESSION IN KIM-2 AND HC11 CULTURES

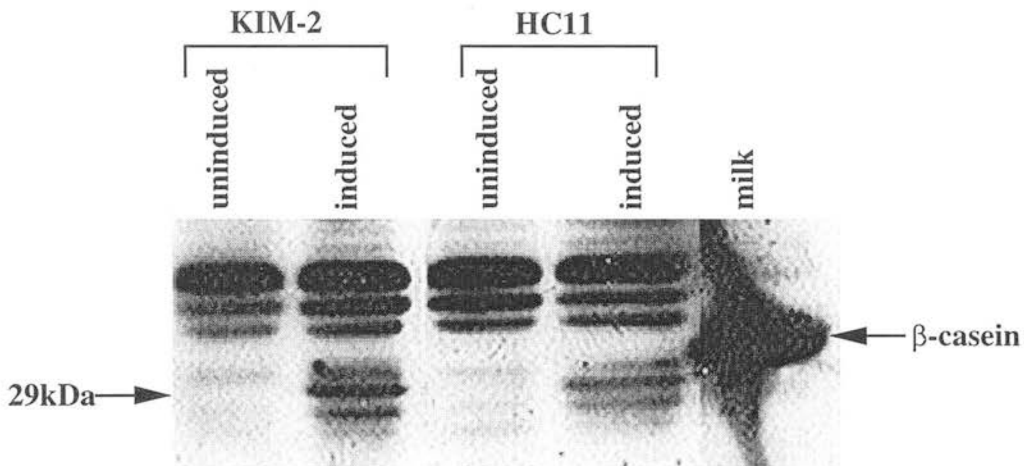


Figure 5.6 Western blot analysis of β-casein expression levels in KIM-2 cultures compared with the HC11 cell line

Total cell extracts were prepared from confluent KIM-2 cultures and HC11 cells (20μg total protein) which were induced with lactogenic hormones or uninduced. Defatted milk (20μg) was used as a positive control for the murine β-casein antibody: dilution 1:10,000.

HORMONAL INDUCTION OF β-CASEIN EXPRESSION IN LATE PASSAGE (P31) KIM-2 CULTURES AT 37°C AND 39°C

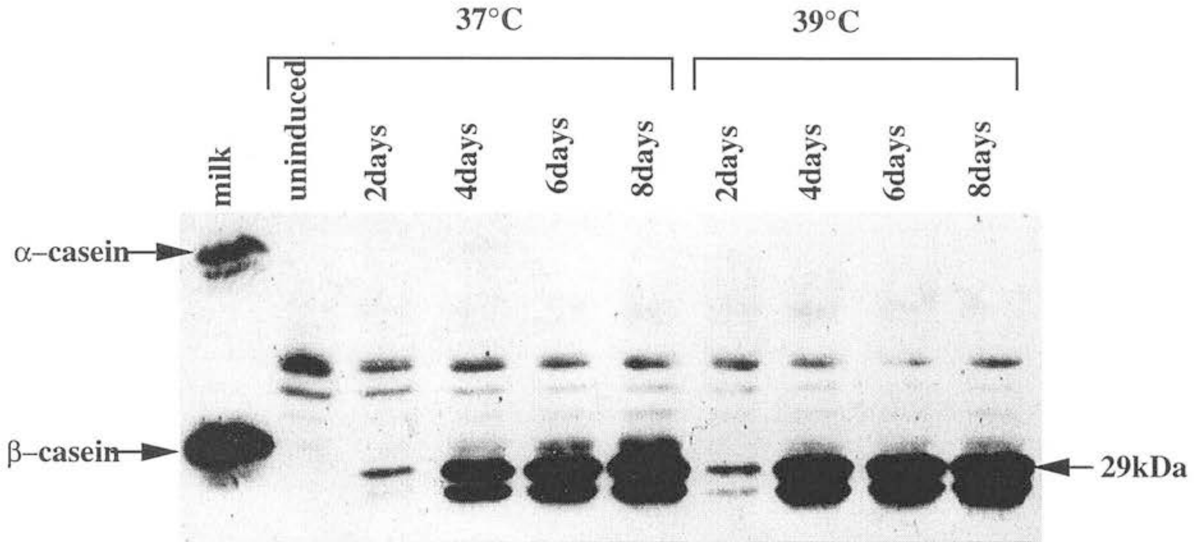


Figure 5.7 Western blot of the time course of β-casein protein induction in late passage cells

Total cell extracts (20μg loaded) were prepared from KIM-2 cultures grown to confluency at 37°C and induced with lactogenic hormones at 37°C and 39°C for the time periods indicated. A defatted mouse milk sample was used as a positive control for the murine β-casein polyclonal antibody: dilution 1:10,000.

5.2.3.4 Expression of WAP, a late differentiation marker WAP in KIM-2 cultures plated on plastic

WAP mRNA transcripts could be detected in KIM-2 cells grown on plastic and induced with lactogenic hormones. This was unexpected since primary cultures and cell lines which have previously been isolated require quite complex culture conditions before WAP expression is observed (see section 1.2.2).

Unlike β-casein RNA expression which was always detected after 2 days induction, increasing to a maximal level by 12 days, WAP expression was less predictable. In some cultures very low levels of expression could be observed 4 days after exposure to lactogenic hormones, in other cultures the onset of expression was delayed and was detectable after 8-10 days induction. Figure 5.8 shows the expression profile of β-casein and WAP mRNA in KIM-2 cells over a period of 12 days induction in one experiment.

Unfortunately it was not possible to examine the levels of WAP protein synthesised by KIM-2 cells using the WAP antibodies which were available at the time (kindly provided by Dr. L. Hennighausen and Dr. F. Schanbacher). These antibodies did detect WAP expression in dewaxed paraffin embedded tissue sections from lactating mice but did not detect WAP on a Western blot with 20μg samples of defatted milk (data not shown).

5.2.4 "Mammosphere" formation and β-casein expression in KIM-2 cells plated on reconstituted basement membrane (Matrigel)

Mammary epithelial cells appear to undergo a greater degree of functional differentiation when plated on reconstituted basement membrane (commercially available as Matrigel; Becton and Dickinson). Bissell's group and others (section 1.2.3.1) have shown that culturing MEC on reconstituted basement membrane causes morphological changes resulting in the formation of "mammospheres" which resemble alveolar structures in pregnant and lactating glands (Aggeler *et al.*, 1991). The addition of lactogenic hormones to these cultures results in the synthesis of higher levels of β-casein when compared to other substrata (Wicha *et al.*, 1982).

TIME COURSE OF mRNA EXPRESSION OF DIFFERENTIATION MARKERS IN KIM-2 CULTURES

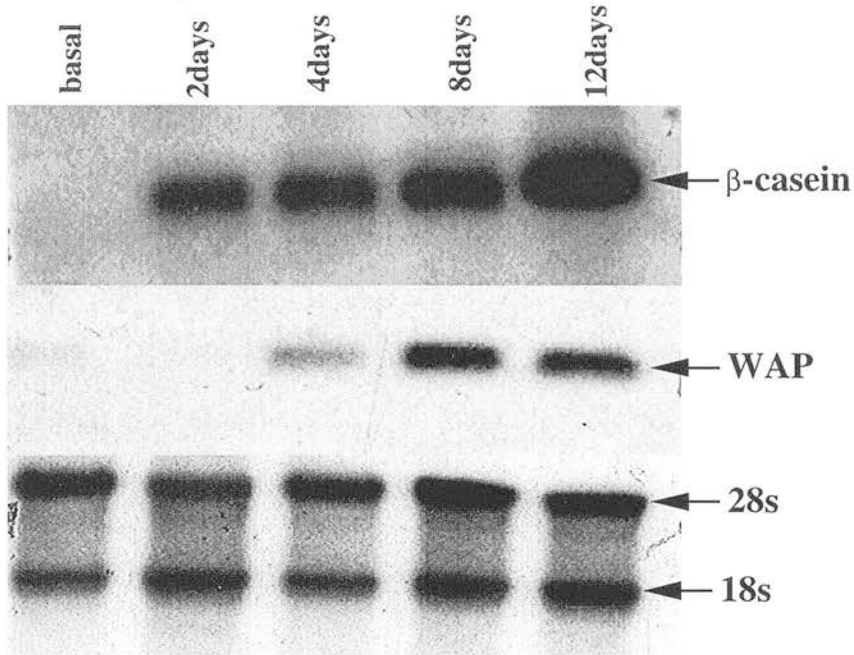


Figure 5.8 Time course of β-casein and WAP mRNA induction in KIM-2 cultures grown on tissue culture plastic.

KIM-2 cultures were grown to confluency at 37°C and induced for the period of time indicated with lactogenic hormones (prolactin and dexamethasone) or uninduced (insulin). 20μg of total RNA was loaded on a formaldeyde gel. The blot was hybridised with a WAP probe, then stripped before hybridising with a β-casein probe. The blot was stripped again before finally hybridising with a ribosomal probe as a loading control.

In addition Bissell's group claims that the formation of "mammospheres" is a prerequisite for WAP expression (Chen *et al.*, 1989).

KIM-2 cells were plated at high density ($4x10^5$ cells/cm^2) onto Matrigel in Complete medium (see section 2.9.4) to determine whether similar morphological changes could be seen. Figure 5.9 shows the morphology of KIM-2 cells when plated on Matrigel. The cells appeared to migrate towards each other forming a lattice-like network 7 hrs after plating (Figure 5.9A). After 24 hrs "mammosphere" were beginning to form, some with processes to adjacent structures (Figure 5.9B). After 48 hrs these structures appeared to be fully formed (Figure 5.9C).

Functional differentiation was induced by incubating the cultures in Induction media (omitting the serum) for 2 days. The cultures were fixed, cryosectioned and stained with an anti-β-casein antibody and counterstained with 4, 6-diamidino-2-phenyl-indole [DAPI] (carried out by Dr. N. Bailey; see Streuli *et al.*, 1991). Figure 5.10 shows immunofluorescent staining of a "mammosphere" with the β-casein antibody and counterstaining of the nuclei in the same field with DAPI. Unfortunately it was not possible to examine the levels of WAP protein synthesised by KIM-2 cells using the WAP antibodies which were available (see above). The expression of WAP mRNA has not been examined in the "mammosphere" cultures yet but it will be interesting to compare the levels observed in these cultures with the levels on plastic.

5.3 DISCUSSION

The KIM-2 cultures grown at the semi-permissive temperature of 37°C were highly enriched with luminal epithelial cells as assessed by their positive staining with keratin 18 and keratin 19 monoclonal antibodies. Under these conditions the cells were also relatively free of mesenchymal cells (vimentin) and myoepithelial cells (smooth muscle actin). This phenotype was retained for 60 passages without any obvious morphological changes.

Cultures which have been isolated and maintained at 33°C stain with the mesenchymal specific antibody, vimentin and retain only residual epithelial-specific

"MAMMOSPHERE" FORMATION IN KIM-2 CELLS PLATED ON MATRIGEL

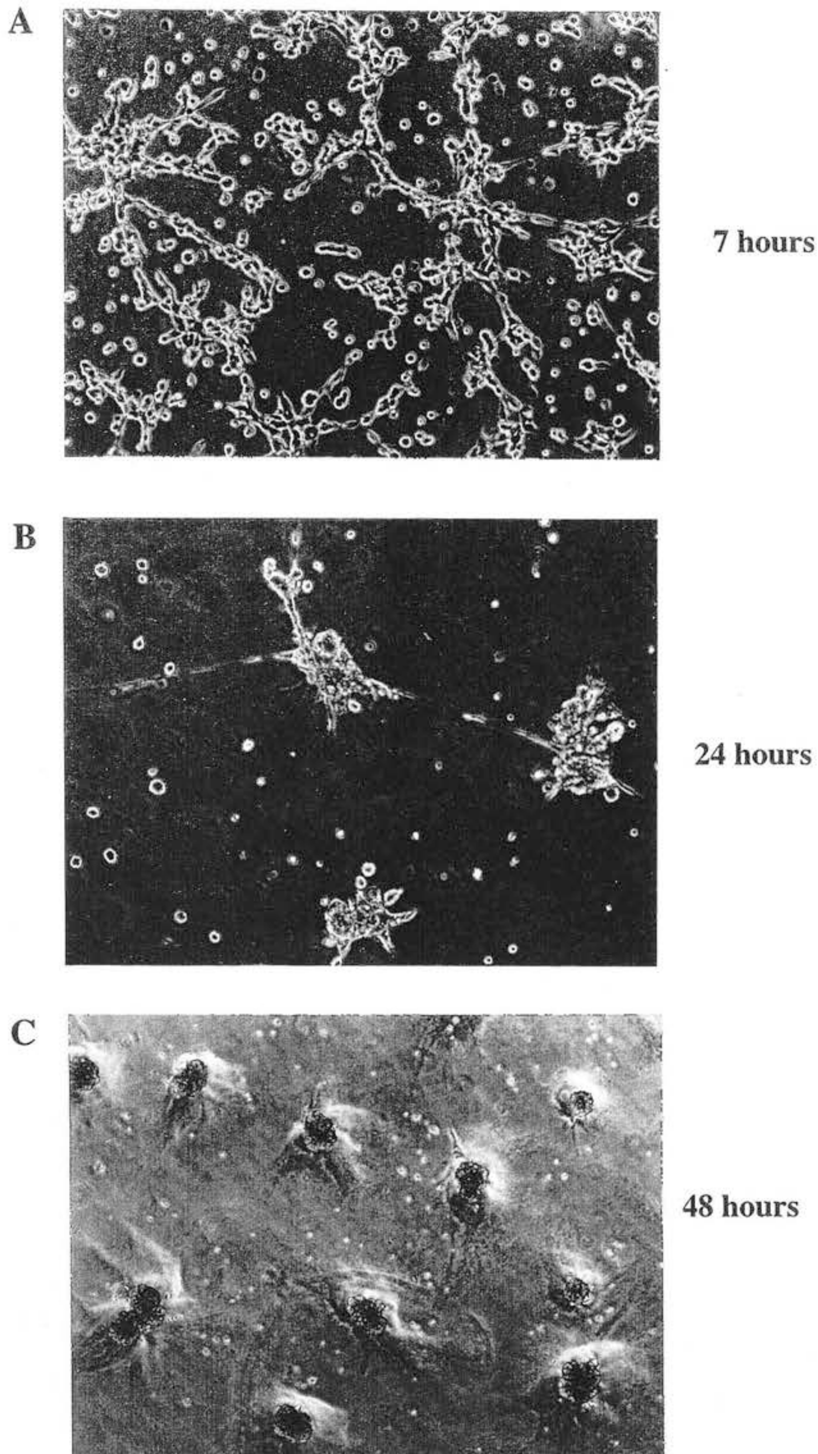


Figure 5.9 Formation of "mammospheres" in KIM-2 cells plated on Matrigel
KIM-2 cells were seeded onto dishes precoated with Matrigel and incubated in Complete medium at 37°C for 48hrs. (**A**) The cells migrated to form a lattice-like network within 7hrs of plating. (**B**) "Mammospheres" started to form after 24hrs. (**C**) "Mammospheres" were fully formed after 48hrs of plating and the Matrigel started to be pulled around these structures.

β-CASEIN SECRETION IN A "MAMMOSPHERE"

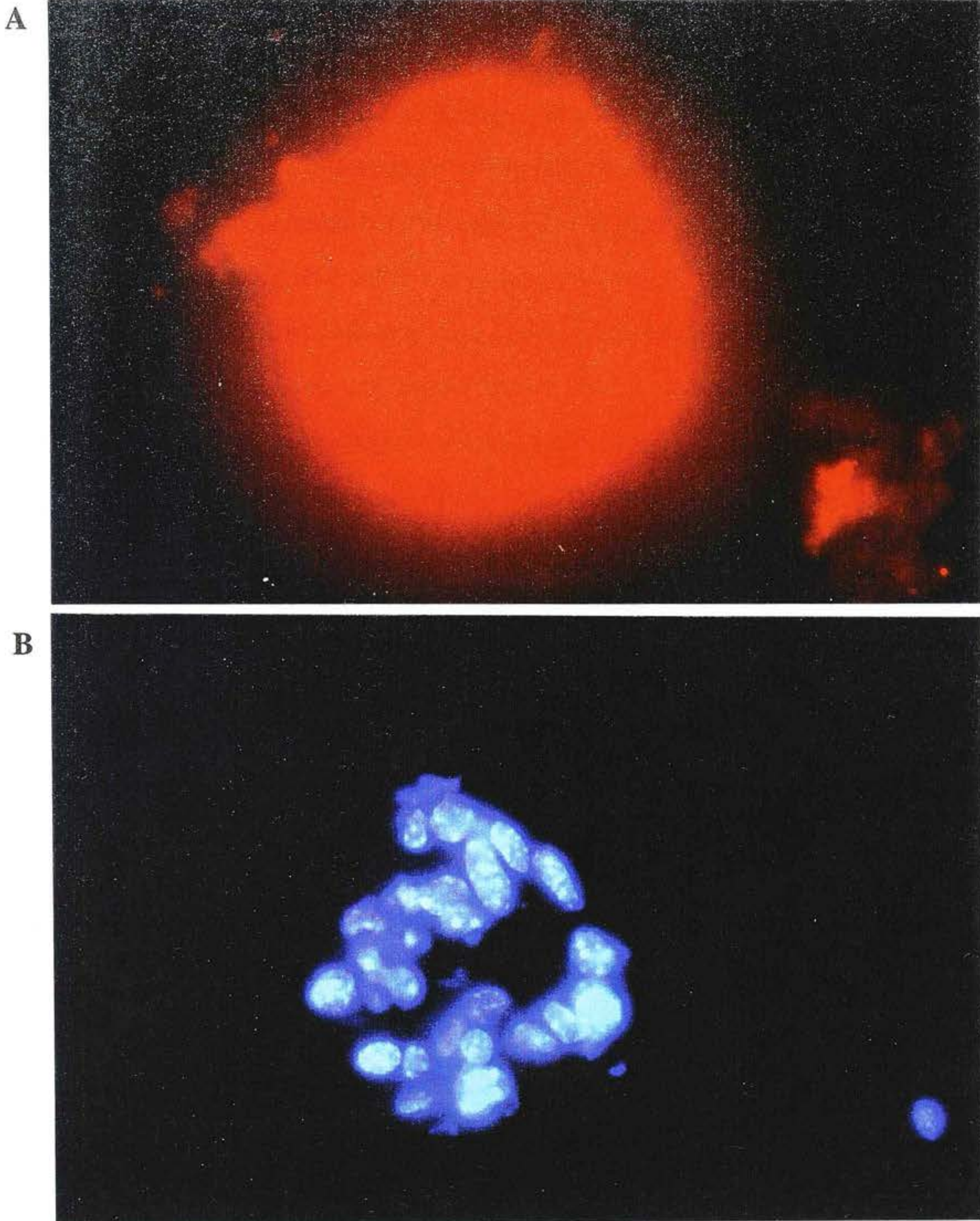


Figure 5.10 Immunofluorescent staining of a "mammosphere" cross-section with a β-casein antibody and nuclei counterstained with DAPI
(**A**) β-casein expression in a frozen section from a KIM-2 "mammosphere"
(**B**) Nuclear staining of the same cross-section with DAPI
(Kindly carried out by Dr. N. Bailey and Dr. C . Watson)

keratin 18 expression. However shifting the cultures to 37°C resulted in an increase in the number of keratin 18 positive cells. The reason for this change in the cell population at the different temperatures is unclear at present.

A similar mesenchymal to epithelial cell conversion has been reported to occur in NIH3T3 fibroblasts which have been engineered to overexpress either the murine or human Met proto-oncogene (Tsarfaty *et al.*, 1994). Transplantion of these cells into nude mice resulted in formation of tumours. Examination of these tumours by laser scanning confocal microscopy revealed that a high proportion of the tumours contained luminal structures. The authors proposed that the 'activation of Met in mesenchymal cells at the site of wounds may play a role in converting these cells to epithelial cells'. Furthermore inappropriate expression of Met in mesenchymal cells could lead to certain carcinomas which express both mesenchymal and epithelial markers. This type of carcinoma has been observed in lung (Chejfec *et al.*, 1991), kidney (Ward *et al.*, 1992) as well as ductal and muscinous adenocarcinomas (Gould *et al.*, 1990).

Rudland and Barraclough (1990) have reported the conversion of SV40-transformed human breast cell lines to a 'myoepithelial-like cell type' as expression of T-antigen is increased. Since the proportion of cells which express myoepithelial cell markers, such as smooth muscle actin was not examined in the KIM-2 cultures grown at 33°C or in the switch 33°C->37°C it is not it is not possible to exclude the presence of this cell type.

It would be worthwhile examining this mesenchymal-epithelial conversion more throughly. Firstly, it could be established whether the alterations in cell types correlates with increased expression of active T-antigen. Secondly, a time-course of switches from 33°C->37°C and from 37°C->33°C would determine: (i) which cell types are present at intermediates stages (using epithelial, myoepithelial and mesenchymal markers) (ii) whether the conversion can occur in either direction i.e from mesenchymal to epithelial and vice versa (iii) when or if the cells become irreversably commited to a specific lineage (iv) whether this conversion correlates with the activation of specific signalling pathways which have been shown to be activated during tumourigenesis. This system may provide an easily manipulated model to study cell commitment.

KIM-2 cells can undergo functional differentiation and express higher (approximately 5 fold) levels of β-casein than the clonal epithelial cell line, HC11. The requirements for expression of β-casein are similar to other mammary epithelial cell lines requiring the addition of lactogenic hormones to confluent cultures. The secretory pathway in these cultures has not be analysed yet. However, if the correct post-translational modifications can occur in this system it may be useful in studying milk secretion. In addition KIM-2 cells can be induced to differentiate after several months in culture suggesting that this differentiation property is relatively stable.

Endogeneous WAP expression has not been observed in any previously isolated mammary epithelial cell line. Therefore the expression of WAP mRNA in KIM-2 cells grown on tissue culture plastic is a novel characteristic of this cell line. This observation requires further investigation, initially to determine the full extent of differentiation in these cultures (i.e can α–lactalbumin be expressed) and secondly to examine whether, during a time course of lactogenic hormone induction, the pattern and timing of milk protein expression mimics the pregnant mammary gland.

The expression of WAP mRNA on plastic conflicts with the model proposed by Roskelley *et al.*, (1996). In their model it is proposed that a prerequisite to WAP expression is the requirement for basement membrane components and the formation of 'mammosphere' structures. Clearly in the KIM-2 cultures the formation of 'mammopsheres' is not necessary for the expression of WAP mRNA. KIM-2 cells will however form "mammopsheres" on Matrigel (commercially available basement membrane) and synthesise β-casein. There is not an obvious explanation for these differences. The cell lines and primary cultures used by Bissell and others have been isolated from the same developmental stage i.e. midpregnant glands, as the KIM-2 cultures. However the KIM-2 culture were derived differently and initially grown on typeI collagen whereas the primary cultures and cell lines examine by others were isolated and maintained on tissue culture plastic. It may well be that this isolation step is the key to this novel characteristic in KIM-2 cells. Isolating and growing cells on tissue culture plastic may result in the selection of a particular population of cells which is different from those obtained using typeI collagen.

TypeI collagen has been shown to be expressed in the early stages of pregnancy (day 4) before decreasing as typeIV collagen and laminin increases in the latter stages (Keely *et al.*, 1995) as the epithelium undergoes differentiation. It is plausible that by initially maintaining KIM-2 cells on typeI collagen there is a population of these cells which are similar to cells found during the early stages of pregnancy.

In conclusion KIM-2 cultures do mimic the differentiating mammary epithelium during pregnancy. In particular, they respond to lactogenic hormones by inducing endogenous milk protein genes. The degree of functional differentiation observed in KIM-2 exceeds that observed in other isolated mouse mammary epithelial cell lines. Expression of both β-casein and WAP on plastic substrata is unique to this culture system. Further differentiation of these cultures to express α-lactalbumin has not been assessed.

At present it is only possible to speculate on the value of a culture system which can maintain its full differentiation potential. However it is envisaged that such a tool could be utilised by both cell and molecular biologists to complement *in vivo* data from animal experiments. Additionally it is a potentially useful tool for the cancer research field to study the early stages of mammary tumour development since the cells are conditionally immortalised.

KIM-2 CELL LINE: AN *IN VITRO* MODEL OF THE MAMMARY GLAND?

6.1 INTRODUCTION

Cell proliferation and differentiation of the mammary epithelium is precisely controlled during the development and maturation of the gland *in vivo*. The molecular analysis of the events which govern the transcriptional activation of milk protein genes and the synthesis of milk during lactation has been actively pursued for many years. Using both *in vivo* and *in vitro* models it has been established that a complex interplay of steroid and peptide hormones, complemented by cell-cell and cell matrix interactions are required. The action of these regulators at a molecular level is becoming more clear with the aid of cell lines such as HC11 and CID9. However both these cell lines have advantages and disadvantages which have been discussed previously (see Chapter 1).

The KIM-2 culture system isolated and characterised here could potentially provide a more accurate model of mammary development. Functionally the cells respond to lactogenic hormones and induce milk protein gene expression. The level of differentiation observed in KIM-2 cultures exceeds that observed in other isolated cell lines with both an early (β-casein) and a late differentiation marker (WAP) being expressed on tissue culture plastic. This property could be used to decipher the intracellular signalling mechanisms involved in the specific activation of both early and late milk protein genes. Mechanistic questions concerning how epithelial cells respond to the multitude of diverse cell surface signalling molecules, e.g growth factors and hormones, and how these signals are relayed to the nucleus and ultimately lead to the co-ordinated activation of milk protein genes could be addressed.

The lactogenic hormone prolactin is known to be one of the principle players (along with insulin and glucocrticoid) in the differentiation of mammary epithelial cells. It promotes milk protein gene expression by regulating the transcription and stability of mRNA (Vonderhaar and Ziska, 1989). It exerts its function by binding to the prolactin receptor (PRLR), which is a member of the cytokine/haematopoietic growth factor

receptor family. However it is only in the past 4 years that the intracellular signalling pathway, activated by the binding of prolactin to its receptor, has been elucidated. The key intracellular components of this pathway in the mammary gland are the protein tyrosine kinase JAK2 and the transcription factor STAT5 (section 1.3.1 and Diagram 1.3). The activation of this pathway by prolactin stimulation was investigated in KIM-2 cells to asssess their value as an *in vitro* tool to study this signalling pathway.

The ability to genetically manipulate KIM-2 cells (via transfection) would also provide an opportunity to:- (i) interfere with signalling pathways activated during differentiation (ii) identify other important regulatory elements within the milk protein promoters which are necessary for expression (iii) provide an *in vitro* assay system to evaluate gene constructs at the level, and fidelity, of RNA and protein expressed and thereby screen potential transgene constructs prior to generating transgenic mice. The transient transfection of a reporter β-galactosidase construct was used to optimise transfection conditions for KIM-2 cells before examining the expression of a series of milk protein promoter based constructs.

6.2 RESULTS

6.2.1 Rapid activation of STAT5 after prolactin stimulation

The time course of STAT5 activation in KIM-2 cells in response to prolactin stimulation was assayed by EMSA (electophoretic mobility shift assay) analysis. Nuclear extracts were prepared from confluent KIM-2 cultures which had been grown to confluency in Complete medium and EGF removed for 24 hrs before the cultures were induced with prolactin for the time periods indicated (Figure 6.1A). EGF was not added to the culture media once the cells were confluent since there is evidence that activation of the EGF receptor prevents lactogenic hormone induction in HC11 cells (Hynes *et al.*, 1990). The nuclear extracts were incubated with a radioactive DNA oligonucleotide probe which corresponded to the highest affinity STAT5 binding site within the BLG promoter (Watson *et al.* 1991). The activation of endogenous STAT5 induced in KIM-2 cells was rapid. Activated STAT5 was observed within 5 minutes of prolactin stimulation (Figure 6.1, Lane 4) and peaked between 30-60 minutes (Figure 6.1, Lanes 6 and 7)

before declining. The DNA-complex observed in stimulated cells was a similar size compared to lactating mouse mammary gland and the level of binding was estimated to be approximately 50 fold lower in KIM-2 cells. (Figure 6.1, Lane 7 compared with Lane 13).

To confirm this complex was STAT5 a competition EMSA was performed using nuclear extract prepared from KIM-2 cells which were either unstimulated (no EGF) or stimulated with prolactin for 5-45 minutes (Figure 6.2). 50ng of non-radioactive competitor oligonucleotide was added to the time-points when peak activation of STAT5 had been detected (Lanes 6 and 8). The STAT5 binding activity could be competed by the unlabelled STM binding site but not by an unrelated oligonucleotide (not shown) indicating that the binding detected was specific. The level of binding in KIM-2 cells was estimated to be 150 fold lower in KIM-2 cells compared to sheep lacating mammary gland (Figure 6.2, Lane 7 compared with Lane 9). To determine whether the STAT5a or STAT5b form is activated in prolactin-stimulated KIM-2 cells a supershift experiment was performed using STAT5a and STAT5b specific antibodies (Santa Cruz). This was carried out by incubating the nuclear extracts with the antibody for 1hr on ice before carrying out a conventional EMSA. Figure 6.3 shows that the STAT5a or the STAT5b specific antibody supershifts approximately 50% of the activated complex (Lanes 2 and 3 respectively). However, when both antibodies are used together the activated complex is almost completely supershifted (Lane 4) demonstrating that both forms are activated in KIM-2 cells.

6.2.2 Genetic manipulation of KIM-2 cells

It was important to demonstrate that KIM-2 cells could be transfected with foreign DNA constructs since this would facilitate genetic manipulation of the culture system.

6.2.2.1 Transient transfections

Transient transfections have been carried out in KIM-2 cells using four different transfection methods: calcium phopshate, Strontifect (Biovation), Lipofectamine (Gibco/BRL) and Tfx-50 (Promega). A plasmid containing β-galactosidase driven by a β-actin promoter (βgeo-kindly provided by Dr. W. Skarnes) was used as a reporter.

STAT5 ACTIVATION IN PROLACTIN STIMULATED KIM-2 CELLS

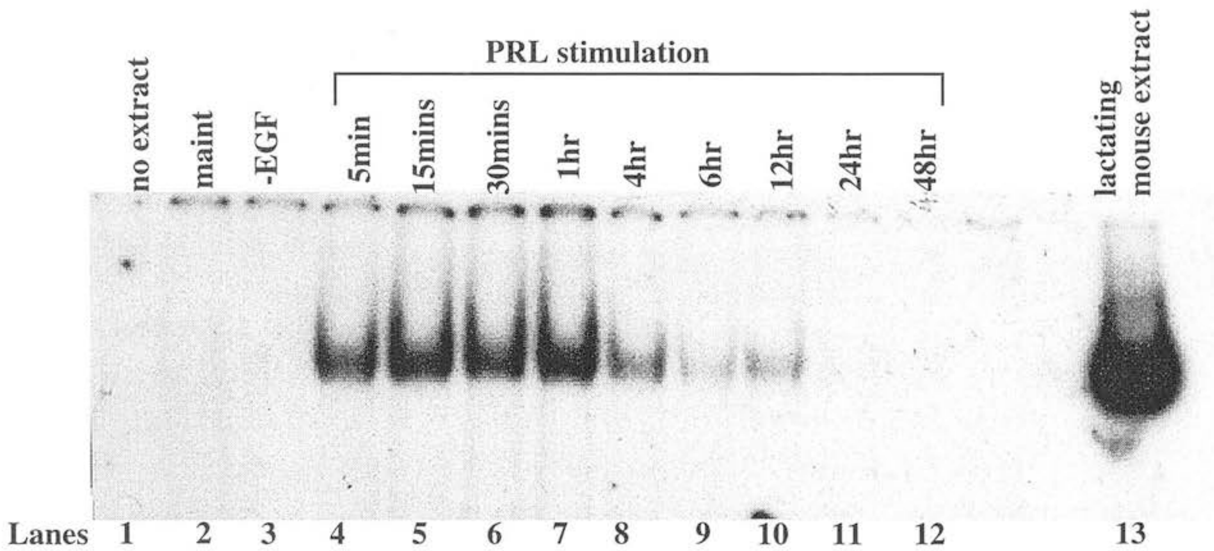
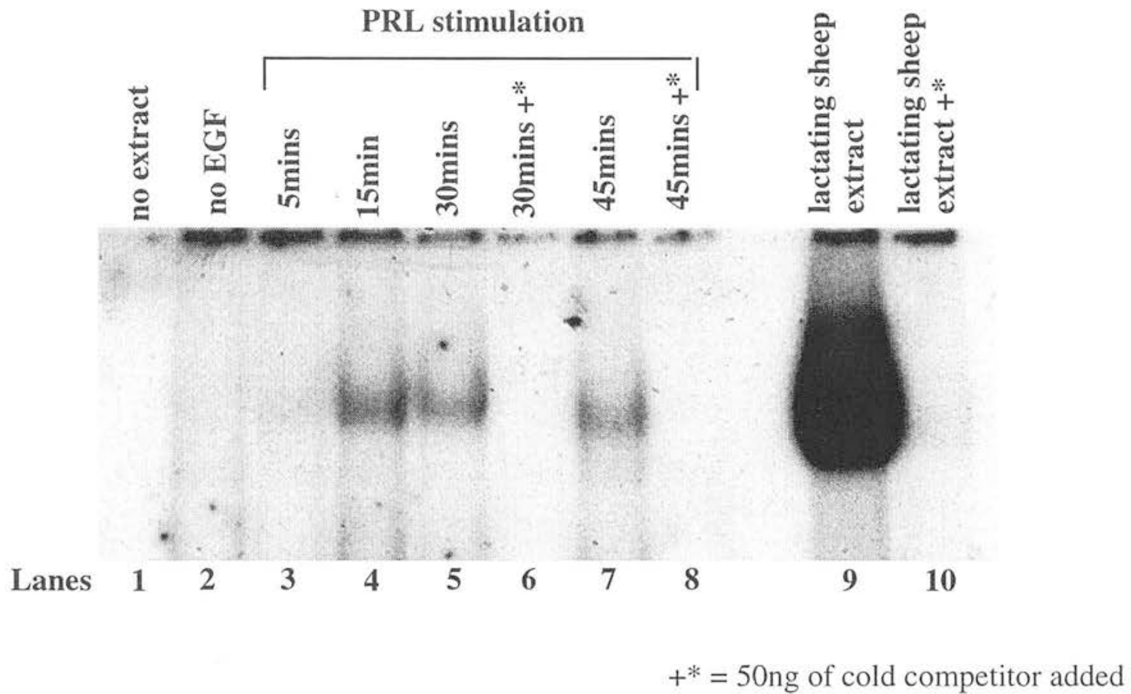


Figure 6.1 Time course of STAT5 activation in KIM-2 cells stimulated with prolactin

KIM-2 cultures were grown to confluency and stimulated with prolactin for the time period indicated in the absence of EGF. Unstimulated control cultures were grown in the presence of insulin and EGF (maint) or in the presence of insulin and the absence of EGF (-EGF). Nuclear extracts were prepared and STAT5 activity assayed by EMSA analysis. The protein-DNA complexes were resolved on a native 6% polyacrylamide gel.

COMPETITION OF STAT5 BINDING IN NUCLEAR EXTRACTS FROM KIM-2 CELLS



+* = 50ng of cold competitor added

Figure 6.2 Competition of STAT5 binding in prolactin stimulated KIM-2 cultures

KIM-2 cultures were grown to confluency and stimulated with prolactin for the time period indicated in the absence of EGF. Unstimulated control cultures were grown in the presence of insulin and the absence of EGF (-EGF). Nuclear extracts were prepared and STAT5 activity assayed by bandshift analysis. 50ng of nonradioactive 'cold' STM oligonucleotide competitor (GGGATTTGGCCAACCGC) was added to extracts in lanes 6, 8 and 10. The protein-DNA complexes were resolved on a native 6% polyacrylamide gel.

STAT5a AND STAT5b FORMS ARE ACTIVATED IN PROLACTIN STIMULATED KIM-2 CELLS

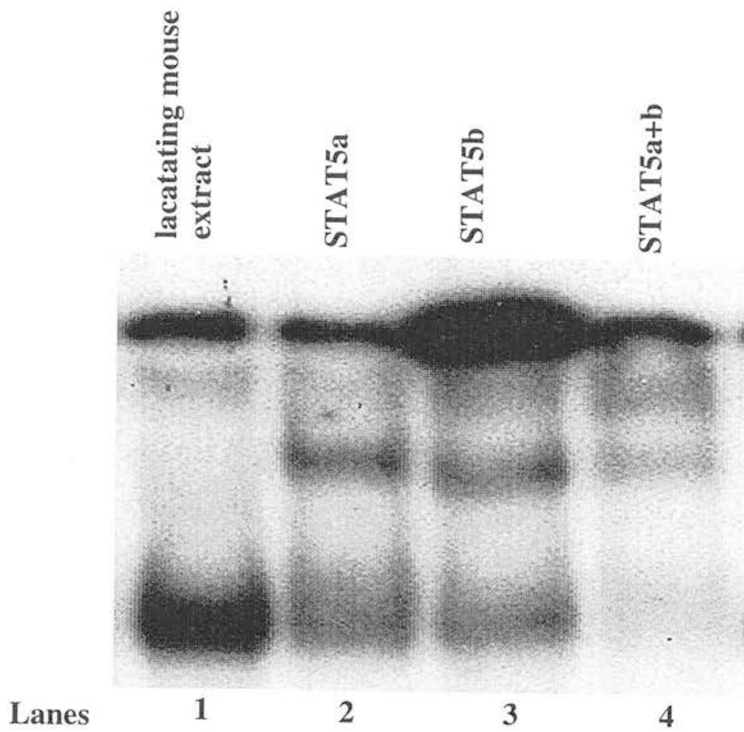


Figure 6.3 STAT5a and STAT5b forms are activated in prolactin induced KIM-2 cultures
KIM-2 cells were grown to confluency and stimulated with prolactin for 1 hr and nuclear extracts prepared. 2μg of the nuclear extract was incubated with 1μl of STAT5a (Lane 2), STAT5b (Lane 3) or both antibodies (Lane 3) for 1hr on ice before adding the radiolabelled STM oligonucleotide. The complexes were resolved on a 6% polyacrylamide gel.

The transfections were carried out as described in materials and methods (section 2.9.5) and stained for LacZ (section 2.10). Figure 6.4 shows transfected cells stained blue in the presence of X-gal.

The calcium phosphate transfection method gave the highest transfection efficiency however it was also the most variable ranging from 0.09%-0.29%. The lipid-based transfection methods were less variable ranging from 0.07%-0.1% for Lipofectamine and 0.02-0.04% for Tfx-50 but the transfection efficiencies were considerably less than that obtained with calcium phopshate. Strontifect, which is apparently less toxic to cells than calcium phosphate gave the poorest transfection efficiencies ranging from 0.001%-0.02%. Figure 6.5 summarises the transfection efficiencies obtained using the different transfection methods.

6.2.2.2 Stable transfections

To determine whether KIM-2 cells may provide an *in vitro* assay system to evaluate gene constructs for expression levels and RNA fidelity a series of β-lactoglobulin Protein C constructs (pCorp1, 6, 7 and 8, kindly provided by PPL Therapeutics) were stably transfected into KIM-2 cells. These constructs show varying levels of expression in different transgenic lines of mice. Figure 6.6 shows Protein C RNA expression in pools of uninduced or induced cells (induced=lactogenic hormones for 4 days). It is anticipated that a comparison of transgene expression in the KIM-2 culture system with data generated from the lactating mammary gland of transgenic mice should give an indication of the suitability of this system for transgene expression studies (data unavailable due to its commercial value).

The expression of these constructs and others in both primary mouse and ovine culture systems have failed (PPL Therapeutics pers. comm.). Clearly from this preliminary experiment KIM-2 cells can support the expression of these transgene constructs, however the basal levels of transcription in the uninduced cultures with pCorp6 and pCorp7 are high (aproximately 5 and 20 fold higher respectively compared to pCorp1 and pCorp8 uninduced). In addition pCorp6 and pCorp1 appear to have two different sizes of transcripts. At present it is unclear whether this is an artifact of the culture system or whether the temporal expression of these constructs are altered *in vivo*. However a high

TRANSIENT TRANSFECTION OF KIM-2 CULTURES USING $CaPO_4$

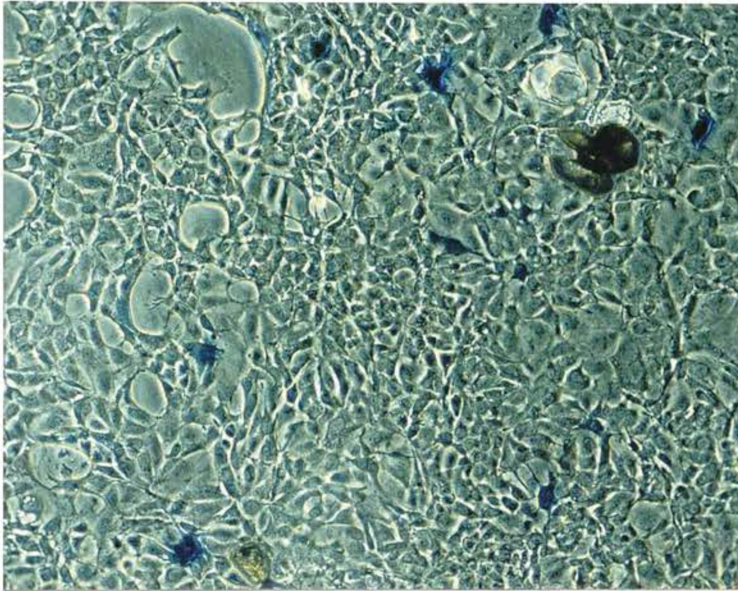


Figure 6.4 LacZ staining of KIM-2 cells transient transfected with a β-galactosidase expression construct (β-geo).

KIM-2 cells were transfected with the β-geo reporter construct fixed and stained 72hrs later for β-galactosidase activity

TRANSIENT TRANSFECTIONS IN KIM-2 CELLS

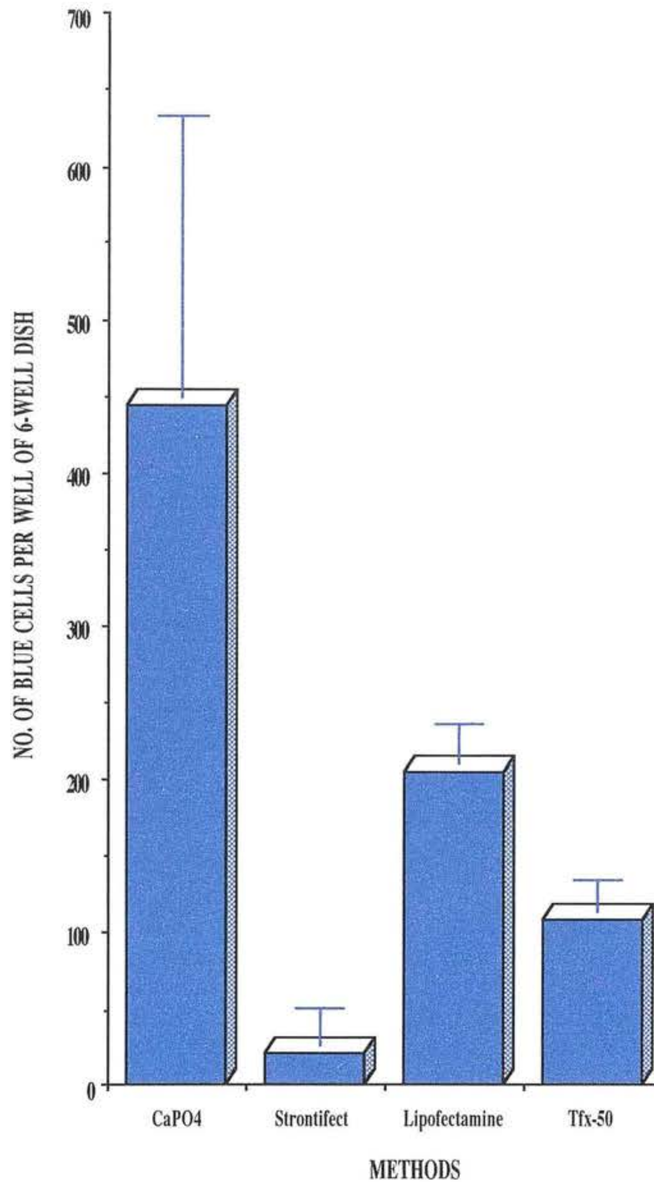


Figure 6.5 Comparison of transfection efficiencies obtained in KIM-2 cells with a variety of transfection methods

Each bar represents the average number of blue cells per well of a six-well dish obtained from 4 independent experiments.

RNA EXPRESSION OF A PROTEIN C TRANSGENE IN KIM-2 CELLS

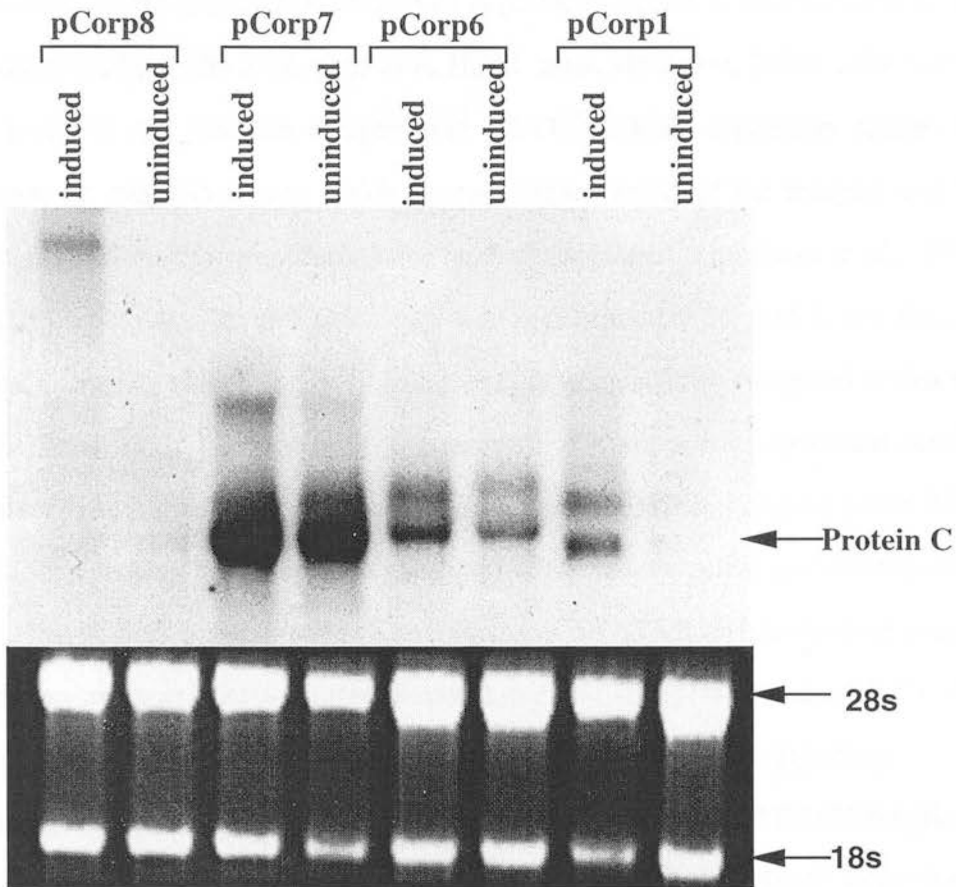


Figure 6.6 Northern blot showing Protein C transgene expression in KIM-2 cells A series of BLG-Protein C expression constructs were transfected into KIM-2 cultures and stable pools expanded. The cultures were either induced with lactogenic hormones (prolactin and dexamethasone) or uninduced (insulin) for 4 days. 20μg of total RNA was loaded on a formaldeyde gel. The Northern was hybridised with a Protein C cDNA probe.

basal level of expression of the BLG promoter was observed in HC11 cells (Burdon *et al.*, 1994b)

6.3 DISCUSSION

The time course of activation of STAT5 in prolactin stimulated KIM-2 cells is similar to that observed by Welte *et al.*, (1994) in HC11 cells. However, HC11 cells only express low levels of the prolactin receptor and STAT5 making expression studies difficult. Attempts to establish clones which express higher levels of the receptor and STAT5, through gene transfer procedures have been unsuccessful (Gouilleux *et al.*, 1994). The STAT5 binding activity of KIM-2 cells was approximately 50 fold lower than lactating mouse mammary gland. This is 10 fold higher binding activity compared to that observed for HC11 cells (Dr. C. Watson pers. comm.). The competition experiment confirms that the binding activity observed is specific and antibodies which recognise either STAT5a or STAT5b supershift the complex suggesting both forms are active in differentiated KIM-2 cells. This mimics the activation of both forms of STAT5 in the developing gland during pregnancy as the epithelium differentiates (Lui *et al.*, 1996; Philp *et al.*, 1996). Therefore KIM-2 cells could be valuable in the *in vitro* analysis of prolactin signalling.

The recently published distinct phenotypes of the STAT5a and STAT5b-deficient mice makes this prospect even more attractive. The STAT5a "knockout" mice (Lui *et al.*, 1997b) displayed impaired alveolar development and failed to lactate during their first pregnancy. Although both β-casein and WAP genes contain STAT5 binding sites only WAP expression was reduced in these mice. This suggests that STAT5a is not the primary transcription factor involved in the control of β-casein expression. Since KIM-2 cells can be induced to express both β-casein and WAP genes this observation can be investigated further in this culture system. STAT5a binding could be "knockout" *in vitro* using a specific STAT5a neutralising antibody or dominant negative construct and induced KIM-2 cells examined for the expression of both milk genes. The absence of WAP expression and the presence of β-casein expression would reinforce the *in vivo* data. If this observation is confirmed then KIM-2 cells may also prove useful in

unravelling which transcription factor(s) are primarily important in the activation of β-casein and WAP expression.

STAT5b "knockout " mice (Davey *et al.*, 1997) display growth retardation in the males and abnormality in the reproductive organs of both sexes which resulted in fertility problems. Despite the similarity (96%) between STAT5a and 5b (Liu *et al.*, 1995) and the superimposable expression pattern during mammary development, STAT5b does not compensate for the absence of STAT5a or vice versa in these animals. It has been suggested that the unique C-termini of both forms of STAT5 may be responsible for the non-overlapping activities (Liu *et al.*, 1995) observed. Again this could be examined *in vitro* by transfecting hybrid constructs composed of the STAT5b gene with the C-terminal domain of STAT5a and vice versa into KIM-2 cells. Alternatively it may be that STAT5a/b heterodimers are important. This could be investigated *in vitro* by swapping the dimerization domains.

Genetic manipulation of KIM-2 cells can be achieved through transfection. Calcium phopshate was determined to be the most efficient and most variable (ranging from 0.09%-0.29%) transfection method as assessed by transient transfections of a LacZ reporter construct. Although this level of efficiency may be too low for transient expression studies it is sufficient when selectable systems are used.

The expression data generated from the stable transfection of 4 BLG-Protein C hybrid constructs (kindly provided by PPL Therapeutics) into KIM-2 cells demonstrates that this culture system can support the expression of milk protein promoter driven transgenes. The variation in expression levels of the different constructs is consistent with the expression levels observed in transgenic lines of mice carrying these constructs (PPL Therapeutics pers. comm.). However, the high level of basal expression in pCorp6 and pCorp7 and was unexpected. At present it is unclear whether this is an artifact of the system or whether it is an inherent property of these particular constructs. This particular question is being investigated by PPL Therapeutics with this series and other transgene expression constructs. Once sufficient data is available, *in vitro* expression results can be compared with *in vivo* data from transgenic mice and the predictive value of KIM-2 cells assessed.

The experiments described here suggest that KIM-2 cells are good model system to study prolactin signalling *in vitro* and preliminary evidence indicates they may provide an assay system to evaluate the suitablity of expression constructs before generating transgenic animals.

OVERVIEW

7.1 INTRODUCTION

The challenge driving this project was the desire to develop a mammary epithelial cell culture system that could more accurately mimic mammary development *in vivo*.

The mammary gland is one of the few tissues that develops fully only in the adult. During pregnancy, the epithelial ductal network that fills the fat pad in a virgin mouse undergoes rapid proliferation with the development of branching structures which differentiate to form lobuloalveolar structures. After birth of the young, the gland enters a secretory phase, producing copious amounts of milk. Following the cessation of lactation, involution of the lobuloalveolar structures occurs and these secretory epithelial cells die by apoptosis. This results in the remodelling of the gland to a structure very similar to that in the virgin animal.

The mammary gland therefore provides a very interesting experimental system in which proliferation, differentiation and apoptosis can all be studied. In addition, the interactions between stromal components and epithelial cells can be investigated. For example, the role of extracellular matrix and the inductive interaction of mesenchyme on epithelial cell development are interesting questions which can be addressed in the mammary gland.

As a result of these complex interactions and developmental processes, it has, to date, proven difficult to mimic even some of these processes in mammary epithelial cell culture. A model of the entire spectrum of mammary development is not available. The goal of this project, to develop such a cell culture model, was a daunting prospect. However, a novel approach to generating immortal cell lines provided the impetus to undertake this challenge.

The immortomouse was developed by Jat *et al.*, in 1991. This line of transgenic mice globally express a SV40 T-antigen mutant under the control of the $H2K^b$ gene promoter. The novel aspect of these mice is that the T-antigen mutant is temperature sensitive. Thus, at the permissive temperature of 33°C, the T-antigen is fully functional and will immortalise any cells in which it is expressed. However, at 39°C this T-antigen mutant is unable to bind p53 and pRb, the cells are 'normal' and can therefore presumably proliferate and differentiate in response to their usual signals.

Since expression of the temperature-sensitive T-antigen occurs in a wide range of cells and tissues of the immortomouse, this approach was modified by restricting expression of the T-antigen mutant to the secretory epithelial cells of the mammary gland. The choice of promoter for this purpose was straightforward. Since milk protein genes are expressed exclusively in the secretory epithelial cells of the mammary gland from mid-pregnancy onwards, a milk protein gene promoter was the ideal choice. The ovine β-lactoglobulin promoter (BLG) has been extensively used in this laboratory for directing expression to the mammary glands of transgenic mice. The promoter region required for tissue-specific expression is thus clearly defined and was used in this project to create a hybrid transgene with the SV40 tsA58 mutant of T-antigen.

7.2 EXPRESSION OF THE TRANSGENE

The high degree of ectopic expression observed in the transgenic lines of mice carrying the BLG-tsA58 construct was not anticipated. However, the onset of tumour development and the types of tumour observed are similar to those found in the p53 null mice and in p53-/-; pRb-/+ double mutants. These lines of mice could potentially be valuable in investigating tumour progression and interactions of p53 and pRb *in vivo*. In particular the eye phenotype observed in the SV40-13 line would be worth investigating further as a potential animal model for retinoblastoma. The tumours observed in both males and females developed beween 4-6 weeks of age and grew from retinal tissue or the unpigmented epithelium of the ciliary body and progressed to the lens and vitreous layer. The tumours appeared to be undifferentiated and resembled retinoblastomas or primitive neuroectodermal tumours (PNET). Developmental studies are being carried out by Dr. R. Ali (Dept. of Molecular Genetics, University College London) to further investigate this phenotype.

Although the degree of ectopic expression observed in the transgenic lines of mice correlated with the copy number of the transgene mammary specific expression did not. The lowest copy line (SV40-2) which did not display ectopic expression of the trangene, showed higher levels of T-antigen protein in the mammary gland than the highest copy line which displayed a high degree of ectopic expression (SV40-13).

A common problem with transgenesis is variegated expression of the transgene. This phenomenon results in patchy expression, frequently observed as clusters of expressing cells next to clusters of non-expressing cells. The reason for this is not clear but it has been suggested that the chromosomal site of transgene integration is responsible. Integration close to the centromere may cause random inactivation of the transgene (Dobie *et al.*, 1996). This has been observed with many transgenes and it would appear that the mammary gland is particularly prone to variegated expression. For example, the same transgene exhibits variegated expression in the mammary gland whilst in the salivary gland, almost uniform expression is observed (Ewald *et al.*, 1996)). Pehaps this reflects expression of endogenous genes *in vivo*. WAP is expressed in a patchy manner during late gestation (Robinson *et al.*, 1995).

In this study only primary cultures isolated from SV40-2 grown at the semi-permissive temperature (37°C) retained a cobblestone morphology. This cannot easily be explained in terms of expression levels of T-antigen *in vivo*. However, future studies examining the level of wild-type T-antigen compared to the mutant *in vivo* and *in vitro* from the different transgenic lines of mice and subsequent cell lines may provide a clearer picture.

7.3 POTENTIAL USES OF KIM-2 CELLS

The KIM-2 cell line generated and characterised in this study could potentially be a valuable *in vitro* culture system in many areas of mammary gland research.

Functionally the cells respond to lactogenic hormones and induce milk protein gene expression. The level of differentiation observed in KIM-2 cultures exceeds that observed in other isolated cell lines with both an early (β-casein) and a late differentiation marker (WAP) being expressed on tissue culture plastic. This functional property could be exploited and used to decipher the intracellular signalling mechanisms involved in the specific activation of both early and late milk protein genes. The functional significance of different transcription factor binding sites in the WAP promoter has been addressed previously using transgenic mice (Rosen. MCB or Mol endo). It should now be possible to carry out such analysis in KIM-2 cells. This is currently being tested in collaboration with Dr Jeff Rosen, Baylor, Texas who has provided us with his WAP promoter constructs.

Mechanistic questions concerning the key intracellular components which are activated in response to regulatory signals at the epithelial cell surface epithelial cells could be addressed. For example, prolactin signalling *in vitro* has been examined in HC11 and Nb-2 pre-T lymphoma cells (prolactin dependant for growth). The HC11 cells have mainly been used in studies on transcriptional regulation exerted by prolactin and Nb-2 cells have been used to examine prolactin receptor-associated kinases (Rui *et al.*, 1992). However both cell lines are limited in their usefulness. HC11 cells only express low levels of prolactin receptor and STAT5 making such studies difficult and attempts to isolate clones which express higher levels of the receptor have failed (Gouilleux *et al.*, 1994). The level of STAT5 activation in KIM-2 cells in response to prolactin shown here suggested that these cells could provide an alternative model mammary culture system to study the prolactin signalling pathway.

In this thesis the cell surface receptors expressed by KIM-2 cells has not been examined however this could provide some useful information. The prolactin receptor, for example, exists in 3 forms. The long (90kDa) and short form (40kDa) which are generated by differential splicing of the same gene (Arden *et al.*, 1990; Shirota *et al.*, 1990) and differ only in their cytoplasmic domains (Boutin *et al.*, 1988, Boutin *et al.*, 1989; Davis and Linzer 1989). The intermediate form of the receptor is a deletion mutant of the long form and lacks 198 amino acids in its cytoplasmic region. Both the long and intermediate forms of the receptor have been shown to induce β-casein expression (Lesueur *et al.*, 1991) however the short form does not (Gouilleux *et al.*, 1994). Although the different forms of the receptor bind prolactin they appear to have different functions. By examining the prolactin receptor status of KIM-2 cells it may be possible to assign a role to the short form of the prolactin receptor and determine whether the different forms have overlapping or distinct functions.

The KIM-2 cells may also provide a useful *in vitro* tool to examine other signalling pathways which are thought to be activated by prolactin such as the mitogen-activated protein (MAP) kinase cascades.

The morphological differences observed in KIM-2 cells cultured at the fully permissive (33°C) and semi-permissive temperatures (37°C) are also potentially intriguing. Both morphologically and immunohistochemically the cultures established and maintained under these conditions are quite strikingly different. This transdifferentiation has been observed in a

number of tissues *in vivo*. It is of developmental importance in the lung and the eye where mesenchymal/epithelial conversions occur (Danto *et al.*, 1995; Kodama *et al.*, 1994). An understanding of this process at the molecular level is therefore of clinical importance. It is possible that KIM-2 cells will transdifferentiate to provide the correct cellular environment for survival and that these cells constitute the small proportion (around 5%) of the non-epithelial cells in the KIM-2 cultures. Further characterisation of this phenomenon may provide a useful insight into cell commitment. In particular establishing whether this conversion is reversible (epithelial to mesenchymal as well as mesenchymal to epithelial) and if there becomes a stage when the cells are commited to a particular lineage could be addressed. At the molecular level examination of growth factors and their receptors which have been implicated in transdifferentiation could be carried out. For example, TGFβ alters the phenotype of the mammary epithelial cell line NMuMG causing a reversible epithelial to mesenchynal conversion. However in genetically engineered NMuMG cells which overexpress a truncated type I TGFβ receptor (Tsk7L) the transfected cells no longer underwent this converison (Miettinen *et al.*, 1994). Over expression of the Met proto-oncogene in NIH3T3 fibroblasts, on the other hand, resulted in a mesenchymal to epithelial cell conversion (Tsarfaty *et al.*, 1994).

During involution of the mammary gland, the secretory epithelial cells are removed by apoptosis. The signals which induce apoptosis in these cells are not known but a reduction in the levels of prolactin and the breakdown of the extracellular matrix are required for involution to occur. It is not surprising therefore that it has been observed that levels of activated STAT5 drop drammatically after the initiation of involution following forced weaning (Philp *et al.*, 1996). Previously, it has been shown that apoptosis can be induced in the mammary epithelial CID-9 cell line only when these cells have become fully differentiated by plating on exogenously added extracellular matrix (Boudreau *et al.*, 1996). However, KIM-2 cultures which contain 3-dimensional dome structures will undergo apoptosis upon lactogenic hormone withdrawal (Dr R. Chapman pers comm.) with up to 30% of the cells undergoing apoptosis within 24 hours. The induction of apoptosis via a number of pathways can therefore be investigated with differentiated KIM-2 cultures.

The ability to genetically manipulate KIM-2 cells by transfection of foreign DNA constructs may also provide an *in vitro* system in which the expression level and fidelity of milk protein promoter driven constructs can be assessed. This could prove to be particularly valuable to pharmaceutical companies which express therapeutic proteins in the milk of transgenic animals. It is envisaged that a variety of constructs could be screened *in vitro* to determine which constructs gave the highest expression of the foreign protein either at the RNA or protein level. It is anticipated that the success of this screening method would drastically reduce the number of transgenic mice presently used for screening purposes. This would be cost effective and valuable under the present ethical climate towards animal experimentation.

The ability to generate stably transfected KIM-2 cells will also be of value in studies of signalling pathways. Transdominant negative mutants of signalling molecules and transcription factors can be stably expressed in KIM-2 cells following transfection and the isolation of pools of stably transfected cells. Recently developed binary systems such as the tetracycline inducible system (Grossen and Bujard) will add an extra dimension of control, allowing expression of the mutant proteins to be induced at specific developmental points. For example, the role of a particular factor in apoptosis can be determined by inducing expression of this factor only after the cells have become fully differentiated.

The focus of this project has been the derivation and functional characterisation of the KIM-2 cell line. The full potential of KIM-2 cells as an *in vitro* model of the mammary gland is still to be determined. A collaboration has been initiated with Dr Jesus Soriano in Basel to assess the performance of KIM-2 cells embedded within 3-dimensional collagen gels. However, work carried out to date clearly demonstrates that this culture system is superior to others currently available in its morphology, uniformity, capacity to differentiate, and ability to undergo apoptosis.

The principal caveat of this cell culture system is that these cells express an oncogene. Whilst this is inactive at 39°C, there are a number of consequences including an abnormal and unstable karyotype and unknown effects from T-antigen complexing with other cellular proteins such as protein tyrosine phosphatases. However, culture models of the mammary gland are an essential complement to studies *in vivo* using genetically modified mice and the KIM-2 cell line described in this thesis is a promising addition to those cell lines currently in

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