GENETIC MANIPULATION OF RUMINANT MAMMARY EPITHELIAL CELLS IN PRIMARY CULTURE

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LIST OF ABBREVIATIONS

AAT	α_1-antitrypsin
αLA	α-lactalbumin
Bisbenzimide	2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole hydrochloride (Hoechst 33258)
βLG	ß-lactoglobulin
BSA	bovine serum albumin
CAS	casein
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (ß-aminoethyl ether) N, N, N′, N′-tetraacetic acid
EHS	Engelbreth-Holm-Swarm
FCS	foetal calf serum
FITC	fluoroscein isothiocyanate
GAS	γ-interferon activation sequence
GH	growth hormone
GHRH	growth hormone releasing hormone
HBSS	Hanks Basal Salt Solution
HPC	human protein C
HS	horse serum
JAK	just another kinase; Janus kinase
MAPK	mitogen-activated pathway kinase
MDGI	mammary derived growth inhibitor
MEAA	Minimum Essential Amino Acids
MGF	mammary gland factor
MWCO	molecular weight cut-off
PBS	phosphate buffered saline
PBST	phosphate buffered saline/0.1% Tween 20
PEI	polyethylimine
PMSF	phenylmethylsulphonyl fluoride
PVA	polyvinyl alcohol
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulphate
Stat	signal transducer and activator of transcription
TAE	40 mM Tris / 20 mM sodium acetate / 1 mM EDTA
TCA	trichloroacetic acid
TE	10 mM Tris / 1 mM EDTA
TRITC	tetramethylrhodamine isothiocyanate
WAP	whey acidic protein
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyronoside

LIST OF PUBLICATIONS

The results published in this thesis have been published in part in the following journals:

Finch LMB, Craig VA, Kind AJ, Schnieke A, Scott A, Wells M & Wilde CJ (1996) Genetic manipulation of mammary epithelial cells in primary culture. In *Animal Cell Technology: basic and applied aspects* (Matsushita T ed) Vol 8, pp 347-350 Kluwer Academic Press, Dordrecht, The Netherlands

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Signer EN, Dubrova YE, Jeffreys AJ, Wilde CJ, Finch LMB, Wells M & Peaker M (1998) DNA fingerprinting Dolly. *Nature* **394** 329-330

Papers in preparation:

Finch LMB, Bryson JM, Wells M & Wilde CJ (1999) Endocrine, autocrine and substratum-dependent control of milk protein secretion in goat mammary epithelial cells. *J Cell Physiol* in preparation

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(Phew! Worse than the Oscars.....)

DECLARATION

All data were collected by myself between 1995 and 1998, with the following exceptions; immunostaining of large cells (Figure 3.9) carried out by Ms V Craig; the fractional factorial experiment described in Chapter 5 carried out by myself in conjunction with Dr LMB Finch and Mrs I McCormick. With these exceptions, I declare that the work contained in this thesis is my own, undertaken under the supervision and guidance of Dr LMB Finch and Dr CJ Wilde. No part of this work has been submitted for consideration for any other degree or award.

Michelle Wells

ABSTRACT

Much work has been carried out into the control of milk protein secretion in rodents, but relatively little is known about ruminant species. Practical, financial and statistical constraints preclude studies *in vivo*, while no satisfactory *in vitro* model currently exists. In addition, the cloning of milk protein genes and the development of transgenic technology have opened up new opportunities for directing the expression of foreign proteins in milk. Gene constructs are traditionally tested in mice, but results cannot necessarily be extrapolated to other species. The aim of this project was to establish a ruminant cell culture system suitable for the study of mammary gland function, and the evaluation of transgenes prior to the generation of transgenic livestock.

Mammary epithelial cells were isolated from sheep or goats in the final trimester of pregnancy by enzymatic digestion with collagenase and hyaluronidase. Cells were fractionated by Percoll density gradient centrifugation. Cell typing of isolated cells showed that fraction 3 consisted predominantly of epithelial cells, with small numbers of myoepithelial and fibroblastic cells present. Culture of these cells on extracellular matrix derived from the Engelbreth-Holm-Swarm mouse sarcoma resulted in the formation of three-dimensional structures, termed mammospheres, and induction of milk protein secretion. Cells were found to be responsive both to substratum and to the lactogenic hormone prolactin, suggesting that this model may provide the most useful *in vitro* system to date. Cells were also successfully differentiated following recovery from liquid nitrogen, although synthetic and secretory activity was approximately 60% of that of freshly prepared cells.

Transfection of primary ruminant epithelial cells following optimisation, resulted in transfection efficiencies of ~0.3%. This low transfection rate necessitated extended passaging of cells. Investigation of differentiation following passaging showed that sheep cells did not secrete milk proteins after passage 2, while in goat cells β-lactoglobulin, α_{S1}-casein, and more importantly, α-lactalbumin were detected at late passage (passage 5-7), although in declining amounts. In addition, sheep cells were increasingly susceptible to the harvesting procedure, while this had no discernible detrimental effect on goat cells. Cell typing of passaged cells showed a loss of myoepithelial and fibroblastic cell types, with increasing passage, concomitant with the loss of secretory

function.

Expression of reference constructs in ruminant mammary epithelial cells resulted in the successful detection of only one foreign product, human α_1-antitrypsin (AAT). This construct was known to be expressed at very high levels (30 mg/ml) *in vivo*. Although post-translational processing was not investigated, resolution of proteins by SDS-PAGE and immunoblotting detected a single band running at the expected position of human AAT, when cells were cultured on EHS matrix in the presence of prolactin. Detection of other transgene products was unsuccessful.

This culture system provides a good model for the study of ruminant mammary gland function. In particular, the ability of cells to differentiate after cryopreservation increases feasibility and provides consistent starting material for large numbers of experiments. If the problems of loss of differentiation with passaging and/or low transfection efficiencies can be resolved, the manipulation or insertion of genes provides new opportunities to study mammary function or to evaluate transgenes prior to the generation of transgenic animals.

CHAPTER ONE
REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Mammary glands are a feature common to all mammals and are thought to have evolved from exocrine glands. They are situated on the anterior of the body, lying beneath the skin with an opening to the body surface, although their number and position vary greatly with species. Synthesis and secretion of milk components, primarily carbohydrate, fat and protein, is carried out by the mammary epithelial cells with, in addition, transfer of some milk constituents from the bloodstream. Milk composition can vary considerably with species.

1.1.1 Structure of the mammary gland

The secretory tissue consists of alveoli, each composed of a monolayer of specialised epithelial cells surrounding a central lumen. Milk is secreted into this lumen and removed via a ductal system. In ruminants, the ducts empty into a large cavity, the gland cistern, and milk removal is via a single opening in the teat. In other species (for example, humans and rabbits) the ducts draining various lobules of the mammary gland lead directly to the teat.

Overlying the secretory epithelial cells are contractile myoepithelial cells. These are spindle-shaped and arranged in a criss-cross fashion, giving a basket-like appearance. A capillary network associated with these cells supplies substrates and hormones. The alveoli are enclosed by a layer of basement membrane, separating them from the surrounding stroma, which consists of connective tissue and adipocytes.

1.1.2 Development of the mammary gland

A mammary bud arises from layers of ectodermal cells in the developing embryo. Even at this early stage, there is evidence that this tissue is influenced by hormones (Wasner *et al*, 1983) and interacts with the surrounding tissues (Sakakura *et al*, 1982). A rudimentary ductal system is present at birth, increasing extensively at puberty, when it invades the mammary fat pad (Knight & Peaker, 1982). Ductal elongation and branching occurs from highly proliferative regions, the terminal end buds, under the control of oestrogen and progesterone. During oestrus in mice,

cyclic periods of limited growth and regression occur, even some transient differentiation, resulting in low level milk gene expression, but terminal differentiation is dependent on subsequent pregnancy and lactation (Robinson *et al*, 1995).

1.1.3 Mammogenesis

Raised levels of oestrogen and progesterone during early pregnancy result in additional ductal branching and extensive proliferation of the terminal end buds into alveoli (Vondehaar & Ziska, 1989). As pregnancy continues, epithelial cell number and the size of the alveolar lobules increase, as does vascularisation of the tissue, concomitant with mammary fat pad regression.

1.1.4 Lactogenesis

During pregnancy there is a substantial increase in milk protein gene expression and synthesis of milk components, although onset of expression of individual milk proteins occurs at different times. This is commonly referred to as lactogenesis stage 1.

At parturition, a rapid drop occurs in circulating progesterone and oestrogen levels, accompanied by a dramatic increase in prolactin (Sinha *et al*, 1974) signalling the beginning of lactation. The secretion of copious amounts of milk at this time is termed lactogenesis stage 2. The fluid produced by the mammary gland immediately following parturition (colostrum) contains high amounts of immunoglobulins to provide immune protection to the neonate. Within a few days, however, the composition changes to that of mature milk and volume of production increases.

Milk production is a combination of both secretory cell number and activity. Secretory cell number continues to increase during lactation, although this varies with species, while alveolar cells become fully secretory. Milk is continuously secreted into the alveolar lumen where it is stored, although in ruminants, milk is also stored in the gland cistern. Suckling induces the milk let-down reflex mediated by oxytocin, resulting in the contraction of the myoepithelial cells and milk ejection.

1.1.5 Involution

After weaning, secretory activity declines, the lobulo-alveolar structures collapse and extensive tissue remodelling occurs. This process is characterised by a

proteolytic degradation of the extracellular matrix and a loss of secretory epithelial cells by apoptosis (Strange *et al*, 1992; Jaggi *et al*, 1996; Quarrie *et al*, 1996).

1.2 HORMONAL CONTROL OF MAMMARY GLAND FUNCTION

Mammary function is regulated by a number of different steroid and peptide hormones, although these mechanisms may vary with species. For example, in hypophysectomised animals the hormone requirements for the initiation or maintenance of lactation differ greatly. In rabbits, prolactin alone is sufficient to induce milk secretion, while rodents require adrenocorticotrophic hormone (ACTH) and sometimes growth hormone (GH) in addition. Ruminants require the more complex combination of prolactin, ACTH, GH and triiodothyronine (reviewed in Forsyth, 1986).

1.2.1 Prolactin

Prolactin, the most important of the lactogenic hormones, is a peptide produced in the anterior lobe of the pituitary gland. It plays an integral role in such diverse functions as osmoregulation, the immune system, reproduction and parental behaviour, while prolactin receptors or binding sites are widely found in many cells and tissues in a number of vertebrates (reviewed in Bole-Feysot *et al*, 1998).

As early as 1928, pituitary extracts were shown to elicit milk secretion when injected into pregnant rabbits (Stricker & Grueter, 1928). In pregnant rabbit explants, prolactin alone is sufficient to induce casein synthesis (Devinoy *et al*, 1978) and α-lactalbumin activity (Sankaran & Topper, 1984), while insulin and cortisol are necessary for prolactin induction of milk protein expression in rodent (Guyette *et al*, 1979; Ganguly *et al*, 1980) and ruminant cultures (Collier *et al*, 1977; Gertler *et al*, 1982; Goodman *et al*, 1983; Puissant *et al*, 1990; Osborne *et al*, 1995).

Prolactin increases casein mRNA transcription in rat organ cultures, but also stabilises the transcript, increasing mRNA half-life (Guyette *et al*, 1979). The action of prolactin on milk protein gene expression is mediated via a DNA binding protein, mammary gland factor (MGF) (Schmitt-Ney *et al*, 1991; Watson *et al*, 1991). This has been cloned in sheep (Wakao *et al*, 1994) and mouse (Liu *et al*, 1995) and shown to be a member of the family "signal transducers and activators of transcription" (STATs), being designated Stat 5. It occurs in two isoforms, 5a and 5b; although Stat 5a predominates in the mammary gland (Lui *et al*, 1995).

Prolactin acts through transmembrane receptors, which occur in long and short forms, differing in their cytoplasmic C-terminus (Kelly *et al*, 1989; Edery *et al*, 1989). The short form is unable to initiate milk protein gene transcription (Lesueur *et al*, 1991), and subsequently it has been demonstrated that the long form activates MGF, while the short form does not (Wakao *et al*, 1994). There is some evidence, however, that the short form may be involved in negative regulation of prolactin signalling (Berlanga *et al*, 1997). Long and short form receptors arise from alternative splicing, but these mechanisms differ between rodents and ruminants (Bignon *et al*, 1997).

Figure 1.1 shows a schematic representation of prolactin signalling. Association of the peptide with its receptor causes receptor dimerisation, and tyrosine phosphorylation by the receptor-associated Janus kinase, JAK2. Activated JAK2 also phosphorylates Stat5 factors at specific tyrosine residues. They then form either homo- or heterodimers and translocate to the nucleus where they bind a specific DNA motif, TTCnnnGAA, the *y*-interferon activation sequence ("GAS" site). These sites have been identified upstream of the rodent whey acidic protein (WAP; Li & Rosen, 1995) and ß-casein (Schmitt-Ney *et al*, 1991, 1992; Wakao *et al*, 1994; Liu *et al*, 1995) genes, and the sheep ß-lactoglobulin gene (Burdon *et al*, 1994). Stat 5a and Stat 5b phosphorylation correlates with the pattern of milk protein gene expression in mice (Liu *et al*, 1996) although only Stat 5a is mandatory since Stat 5a-null mice fail to lactate (Liu *et al*, 1997). There is some evidence, however, that control of lactation by Stat 5 may differ in ruminant species (Wheeler *et al*, 1997).

In addition to its important role in milk protein gene expression, prolactin is also implicated in the proliferation and differentiation of mammary cells. Prolactin increases DNA synthesis via both the long and short forms of the prolactin receptor (Das & Vonderhaar, 1995), while the terminal stage of mammary gland development, lobulo-alveolar growth, is directly regulated by prolactin (Darcy *et al*, 1995). Further evidence is provided by prolactin-deficient mice which exhibit impaired ductal elongation and branching (Horseman *et al*, 1997). It is also possible that locally produced prolactin acts in a paracrine fashion since prolactin mRNA has been detected in the secretory cells of rat mammary tissue (Kurtz *et al*, 1993; Steinmetz *et al*, 1993), and in human breast cancer cells (Ginsberg & Vonderhaar, 1995).

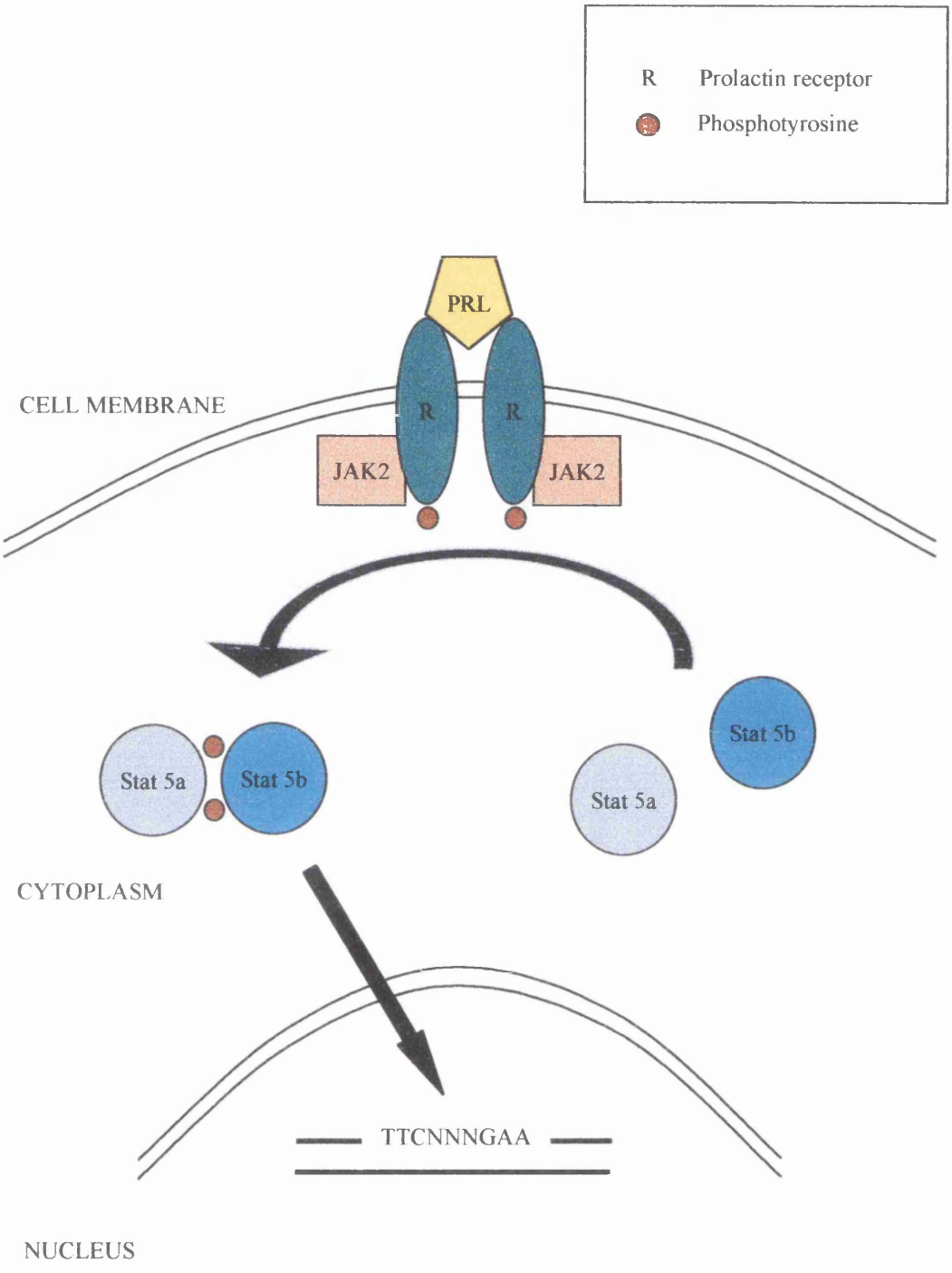


Figure 1.1 Schematic representation of initiation of milk protein secretion by prolactin

Prolactin binding results in receptor dimerisation and tyrosine phosphorylation by the receptor-associated Janus kinase, JAK2. Activated JAK2 also phosphorylates Stat 5 factors at specific tyrosine residues. They then form either homo- or heterodimers and translocate to the nucleus where they bind a specific DNA motif, TTCnnnGAA.

1.2.2 Glucocorticoids

Cortisol levels rise sharply at parturition (Paterson & Linzell, 1971) and are accompanied by the tightening of tight junctions between secretory cells of the mammary gland. The implication of cortisol in this event has been shown both *in vivo* (Thompson, 1996) and *in vitro* (Zettl *et al*, 1992; Woo *et al*, 1996). In rat mammary organoid culture, hydrocortisone is found to suppress ductal branching and induce alveolar morphogenesis while its removal leads to a loss of differentiated function of the cells without inducing apoptosis (Darcy *et al*, 1995b). It also increases expression of the prolactin receptor in the late pregnant mouse (Mizoguchi *et al*, 1997b)

In rabbit mammary gland organ culture, cortisol enhances prolactin-induced casein synthesis, but is inactive alone (Devinoy *et al*, 1978). In mouse mammary gland culture cortisol is required for casein gene expression (Ganguly *et al* 1980); similarly, in HC11 cells, optimal β-casein expression is achieved only in the presence of both prolactin and glucocorticoids (Doppler *et al*, 1989). Transcription of the WAP gene also depends on the synergistic effects of glucocorticoids with prolactin (Li & Rosen, 1994). Cortisol has a differential action, however, on the accumulation of α-lactalbumin in cultured mammary glands from virgin (Ona & Oka, 1980) and mid-pregnant (Nagamatsu & Oka, 1983) mice, with high concentrations inhibiting α-lactalbumin synthesis.

Chomczynski *et al* (1986) found that the presence of hydrocortisone in explant culture increases the half-life of the casein transcript and suggested that the hormone acts by decreasing nuclear degradation. It is now thought that glucocorticoids affect milk protein gene expression via the association of the receptor with Stat 5. This has been shown in both HC11 cells (Lechner *et al*, 1997) and in mice (Cella *et al*, 1998).

1.2.3 Insulin (and insulin-like growth factors)

Insulin and insulin-like growth factors (IGFs) are structurally related and may act through either the insulin receptor or the IGF-I receptor (Nissley & Rechler, 1984). *In vitro*, both insulin & IGF-I are mitogenic in COMMA-1D cultures (Hovey *et al*, 1998a), while HC11 cells, in the presence of prolactin and dexamethasone alone undergo apoptosis, but this is prevented by the addition of either insulin or IGF-I (Merlo *et al*, 1996). Insulin and IGF-I both have stimulatory, but not additive, effects

on casein gene expression and induction of α-lactalbumin activity in mammary epithelial cells from mid-pregnant mice (Prosser *et al*, 1987). Thus, insulin and IGF-I can apparently substitute for each other, although IGF-I may provide a local control mechanism in the mammary gland and is discussed further in this context in section 1.3.1.

1.2.4 Growth hormone

It is well established that significant increases in milk production in dairy cattle are achieved with the administration of exogenous bovine growth hormone (GH) (Bauman *et al*, 1985; McCutcheon & Bauman, 1986). Similarly, the infusion of GH-releasing hormone (GHRH) into dairy cows produces a dose-dependent rise in circulating growth hormone, IGF-I and in milk yield (Enright *et al*, 1987; Dahl *et al*, 1993). It is thought that growth hormone's main effect is on the partitioning of nutrients to the mammary gland (Bauman & Currie, 1980). Enhanced milk yields are maintained even after termination of GHRH treatment suggesting a mammogenic effect of growth hormone (Dahl *et al*, 1993). Tucker (1987) showed that the administration of GH to growing heifers increases mammary development, and that reduced mammary development is associated with decreased concentration of GH in blood serum. Intramammary infusion of GH in goats, however, is reported to have no effect, (Sjersen & Knight, 1993), but, in late pregnant cows, cell proliferation is stimulated (Collier *et al*, 1993) suggesting that there may be species differences in the action of growth hormone.

In rodents, the treatment of immature rats with mammary implants containing GH has been shown to cause local stimulation of growth and differentiation (Plaut *et al*, 1993), but GH activity varies considerably with species of origin (Caron *et al*, 1994). Injection of rats with anti-serum to growth hormone, leads to a decrease in weight gain of the pups, indicative of reduced milk yields, and a fall in levels of IGF-I and IGF-II (Flint *et al* 1992). In prolactin- and GH-deficient rats, milk production declines mainly via the loss of secretory cells. This effect was partially reversed by growth hormone administration, but not by IGF (Travers *et al*, 1996).

Growth hormone receptors have not been demonstrated in adult bovine tissue (Gertler *et al*, 1984; Akers *et al* 1984), although GH receptor mRNA has been detected (Glimm *et al*, 1990; Hauser *et al*, 1990) and recent work suggests their

presence in foetal mammary tissue (Knabel *et al*, 1998). In rats and rabbits, it has been demonstrated that growth hormone receptors are found localised in proliferating and lactating mammary epithelial cells (Lincoln *et al*, 1995).

It is hypothesised that the action of growth hormone is an indirect effect mediated by circulating insulin-like growth factors, since IGF-I has been shown to be present in substantial amounts in mammary secretory and stromal tissue after treatment with growth hormone (Prosser *et al*, 1991; Walden *et al*, 1998). IGF-I has also been shown to be produced locally in the mammary gland, and it is possible that it may be regulated locally by GH (Hoyt *et al*, 1988). It should be noted, however, that GH can bind to the prolactin receptor (Kossiakoff *et al*, 1994; Somers *et al*, 1994), can activate Jak2 tyrosine kinase and members of the Stat family of transcription factors, including Stat 5 (Xu *et al*, 1996), and has been shown to induce binding of Stat 5 to the GAS site in the β-casein promoter *in vitro* (Smits *et al*, 1997). In addition, GH stimulates β-casein secretion in mouse mammary epithelial cells cultured on floating collagen gels (Katiyar *et al*, 1978). This cumulative evidence suggests that a lactogenic role for GH via the prolactin signalling pathway cannot be ruled out.

1.2.5 Placental lactogen

Placental lactogen (PL) concentration *in vivo* correlates with the number of placentae in the mouse and the degree of mammary development (Knight & Peaker, 1982). Similarly, in sheep and goats, milk yields correlate with circulating PL and foetal number (Hayden *et al* 1979; Butler *et al* 1981). Milk yields of lactating cows are increased by treatment with recombinant bovine PL, although its action is less potent than GH (Byatt *et al*, 1992), and a mammogenic effect is also observed in dairy heifers (Byatt *et al*, 1997). Treatment with ovine PL, however, was found to have no mammogenic or galactopoietic effects in either pregnant (Currie *et al* 1996) or lactating ewes (Basset *et al*, 1997; Min *et al*, 1997). Species differences are also apparent in *in vitro* studies where PL is found to stimulate proliferation in mouse mammary epithelial cells (Dai *et al* 1996), but not in goat organ culture or bovine cells (Skarda *et al*, 1982; Collier *et al* 1993).

A number of *in vitro* studies also show effects of PL on milk protein gene expression. Although no effect of PL is observed in ewes *in vivo*, ovine and caprine PL is shown to stimulate β-casein synthesis in sheep and rabbit mammary explants

(Sakal *et al*, 1997a, 1998), while rat PL is capable of stimulating β-casein production in HC11 cells (Sakal *et al*, 1996). Mouse PL stimulates α-lactalbumin secretion in mouse mammary epithelial cell cultures in a dose responsive manner and is more potent than either mouse or ovine prolactin (Thordarson *et al*, 1986)

It is thought that GH's main effect is on the partitioning of nutrients, but bovine PL also binds to GH receptors with a similar affinity (Collier *et al*, 1995; Helman *et al*, 1998) and has been shown to decrease levels of circulating GH (Byatt *et al* 1992). This suggests that one possible mode of action of PL is to attenuate the effects of GH, by partitioning nutrients to the developing foetus during pregnancy and not into milk production. Conversely, however, recombinant bovine PL increases circulating levels of IGF-1 *in vivo,* therefore it is possible that PL stimulates mammary growth via locally acting growth factors (Collier *et al* 1993). In addition, ovine and caprine PL have been shown to bind prolactin receptors and cause receptor dimerisation (Sakal *et al*, 1997b, 1998), which may explain the stimulation of milk protein synthesis *in vitro*. It is possible that the effects of PL may vary with reproductive state, since it is known that persistency of lactation is lower in pregnant than non-pregnant cattle (Rook & Campling, 1965).

1.2.6 Progesterone

In early pregnancy, ductal branching and proliferation of the terminal end buds into alveoli, are brought about in response to increased levels of oestrogen and progesterone (Vondehaar & Ziska, 1989). In mice, oestrogen increases expression of the prolactin receptor gene but this is suppressed by progesterone during early pregnancy (Mizoguchi *et al*, 1997a). Progesterone also inhibits the accumulation of casein mRNA during pregnancy, even when administered along with prolactin (Rosen *et al*, 1978).

In primary culture of rat mammary epithelial cells, it has been shown that progesterone induces DNA synthesis (Taylor *et al*, 1996). Darcy *et al* (1995b) also demonstrated that progesterone induces cellular proliferation and ductal branching, but is not required for alveolar morphogenesis. Treatment with anti-progestins strongly inhibits epithelial DNA synthesis (Li *et al*, 1995; Taylor *et al*, 1996) and stimulates functional differentiation in whole mammary gland organ cultures of primed virgin mice (Li *et al*, 1995). Progesterone also inhibits the accumulation of casein mRNA in explant cultures, in the presence of lactogenic hormones (Rosen *et*

al, 1978; Guyette *et al* 1979). Thus, progesterone plays a dual role, both inducing mitogenesis and inhibiting milk protein synthesis.

Reciprocal transplantation experiments using progesterone receptor knockout mice suggest that progesterone receptors in the epithelium and stroma are involved in lobulo-alveolar or ductal development respectively (Humphreys *et al*, 1997). Progesterone receptor levels have been found to be regulated by oestrogen, but in addition, there is evidence that EGF, insulin, IGF-I, prolactin and progesterone itself also modulate progesterone receptor levels (reviewed in Darcy *et al*, 1995b).

1.3 LOCAL CONTROL OF MAMMARY GLAND FUNCTION

There are a number of local factors in the mammary gland that may complement or mediate hormonal control of mammary development and milk secretion.

1.3.1 Insulin-like growth factor

In sheep, IGF-I mRNA is highest during the prepubertal phase of mammary growth, and then low until late pregnancy (Hovey *et al* 1998b). Similarly, in mice IGF-I and IGF-I receptor mRNAs are expressed in terminal end buds during pubertal ductal growth and reinitiated in mammary epithelial cells in the differentiated alveoli at the end of pregnancy (Richert & Wood 1999). The effect of IGF-I on mammary development is enhanced by oestrogen (Ruan *et al*, 1995). It is also thought that IGF-I mediates the action of GH. The infusion of GH-releasing hormone (GHRH) into dairy cows produces a dose-dependent rise in both circulating growth hormone and IGF-I (Enright *et al*, 1987; Dahl *et al*, 1993) while GH stimulates IGF-1 mRNA in rats (Kleinberg *et al*, 1990).

In vitro, IGF-I stimulates proliferation in COMMA-1D cells (Hovey *et al*, 1998a), bovine mammary epithelial cells (McGrath *et al*, 1991) and promotes ductal growth in prepubertal mammary glands (Richert & Wood, 1999), but it appears to act as a cell survival signal in HC11 cells (Merlo *et al*, 1996). Further evidence for this role of IGF-I is provided by the inhibition of mammary gland involution in transgenic mice which overexpress IGF-I (Neuenschwander *et al*, 1996; Hadsell *et al*, 1997).

1.3.2 Epidermal growth factor

There is much evidence for a mitogenic effect for epidermal growth factor (EGF) in mammary epithelial cells, resulting in stimulation of ductal growth and

lobuloalveolar development in virgin mice *in vitro* (Tonelli & Sorof, 1980) and *in vivo* (Vondehaar, 1987). An increase in DNA synthesis is also observed in the pregnant mammary gland, with a greater effect seen in ductular epithelial cells than in alveolar epithelium (Spitzer *et al*, 1995), while local implants of EGF stimulate a normal pattern of ductal growth in regressed glands (Snedeker *et al*, 1991). EGF mRNA is detected in the mammary glands of virgin, pregnant and lactating mice (Snedeker *et al*, 1991) but during pregnancy there is an increasing abundance of EGF receptors. These then decline immediately before the onset of lactation (Edery *et al*, 1985).

As well as its mitogenic role, EGF has also been found to be closely associated with epithelial dedifferentiation. EGF treatment of mouse mammary epithelial cells or explant cultures results in a loss of presecretory phenotype, a loss of synthesis of milk components, and a decrease in milk protein mRNAs, especially WAP and ß-casein (Taketani & Oka, 1983; Spitzer *et al*, 1995). There is some evidence, however, that the effects of EGF on mitogensis and dedifferentiation are via differing pathways (Taketani & Oka, 1983; Spitzer *et al*, 1995).

EGF and EGF receptors are both regulated by a number of hormonal influences. Insulin is reported to augment the mitogenic action of EGF (Oka *et al*, 1991). In mouse mammary cells *in vitro,* oestrogen and EGF synergistically increase DNA synthesis, and EGF receptor numbers (Vanderboom & Sheffield, 1993), while Ankrapp *et al* (1998) have demonstrated EGF and oestrogen-dependent end-bud proliferation in the mouse mammary gland *in vivo.* Oestradiol and progesterone together have also been shown to enhance the ability of EGF to stimulate DNA synthesis in mammary tissue from midpregnant heifers. This is associated with increased EGF binding and with increased EGF-induced tyrosine kinase (Sheffield, 1998).

Lactogenic hormones have been shown to stimulate EGF mRNA accumulation in mouse mammary glands *in vivo* and in mouse mammary epithelial cells (Fenton & Sheffield, 1991). However, prolactin completely inhibits EGF-stimulated DNA synthesis *in vitro* (Fenton & Sheffield, 1993, Sheffield 1998). This was found to be associated with a loss of binding of EGF to its receptor, a decline in EGF receptor number and mRNA and decreased levels of receptor tyrosine phosphorylation (Fenton & Sheffield, 1993). More recently, prolactin has been demonstrated to inhibit the ability of EGF to activate the mitogenic MAPK pathway

(Johnson *et al*, 1996). EGF itself has also been shown to suppress Stat 5 expression *in vitro* (Petersen & Haldosen, 1998), thus EGF and prolactin may be mutually antagonistic.

It is suggested that the function of EGF *in vivo*, is to stimulate the final processing and maintenance of the lobulo-alveolar gland structure, while preventing premature milk production. However, EGF stimulates endogenous TGF-α production in explant culture, therefore it is possible that the effects of EGF are mediated partially though the induction of TGF-α (Spitzer *et al*, 1995).

1.3.3 Transforming growth factor α

Transforming growth factor-α (TGFα) is a mitogenic polypeptide that is structurally related to EGF (Derynck *et al*, 1984; Marquardt *et al*, 1984) and competes for binding to the same receptor (Bates *et al*, 1990). The effects of TGFα are very similar to those of EGF, but TGFα is a more potent mitogen (Vondehaar, 1987; Spitzer *et al*, 1995; Moorby *et al*, 1995). Local implants of TGFα also lead to stimulation of ductal growth and end bud formation (Vondehaar, 1987; Snedeker *et al*, 1991), although alveolar cells have a higher sensitivity to the mitogenic stimulus of TGFα (Spitzer *et al*, 1995).

In human epithelial breast cells the stimulatory effect of TGFα has been found to be enhanced by cortisol (Sakthivel *et al*, 1993), but, like EGF, it is inhibited by prolactin (Fenton & Sheffield, 1997). TGFα also induces cellular dedifferentiation, with a reduction in milk protein synthesis and MDGI expression (Spitzer *et al*, 1995).

TGFα is detected in mice in both virgin and pregnant glands, but unlike EGF it is not present in mouse lactating tissue (Snedeker *et al*, 1991). It is, however, found in the lactating rat suggesting species differences (Liscia *et al*, 1990).

Oestrogen induces TGFα mRNA in the rat mammary gland, and ovariectomy causes a rapid decline, suggesting that the effects of oestrogen on the mammary gland may be mediated by TGFα (Liu *et al*, 1987; Ahmed *et al*, 1991).

1.3.4 Transforming growth factor β

The polypeptide transforming growth factor-beta (TGFß) occurs as 3 isoforms, all of which are expressed in both lactating and non-lactating bovine mammary gland

(Maier *et al*, 1991). They are also abundant in the mammary gland of mice during pregnancy, but only present at very low levels during lactation (Robinson *et al*, 1991). Expression of TGFß is induced during post-lactational mammary gland involution in mice (Strange *et al*, 1992).

Local implants of TGFß reversibly inhibit mammary growth and morphogenesis in virgin mice (Silberstein & Daniel 1987), while in the pregnant animal they inhibit ductal elongation, but have no effect on alveolar proliferation (Daniel *et al*, 1989; Robinson *et al*, 1991). Conversely, transgenic mice overexpressing TGFß show little ductal impairment, but substantial inhibition of formation of lobuloalveolar structures and milk protein production (Jhappan *et al*, 1993). TGFß has also been shown to inhibit the expression of ß-casein in HC11 mouse mammary epithelial cells (Mieth *et al*, 1990) and mammary explants (Robinson *et al*, 1993), but had no effect on casein synthesis and secretion in lactating cells (Sudlow *et al*, 1994). Inhibition of casein production during pregnancy occurs post-transcriptionally since mRNA levels are not affected (Robinson *et al*, 1993). It is suggested that the role of TGFß *in vivo* is to suppress cellular differentiation and milk production during pregnancy, and is implicated in apoptotis during involution (Rosfjord & Dickson, 1999).

1.3.5 Fibroblast growth factors

Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) are members of a larger family of heparin-binding growth factors. *In vivo*, the bFGF content in the mouse mammary gland has been shown to increase with puberty and pregnancy but decrease considerably in lactation, with the number of receptors also changing in parallel (Lavandero *et al*, 1998). A similar pattern has been identified in the bovine mammary gland (Plath *et al*, 1998). In the developing gland, bFGF has a heterogeneous distribution, but during lactation, is predominantly localised to the basement membrane/myoepithelial region (Rudland *et al*, 1993, Plath *et al*, 1998). This suggests that fibroblast growth factors may be implicated in local regulation of the developing gland. In transgenic mice, fibroblast growth factor signalling was found to be necessary for lobuloalveolar development of the mammary gland during pregnancy (Jackson *et al*, 1997).

In vitro, fibroblast growth factors have been shown to stimulate DNA synthesis and proliferation in rat (Rudland *et al*, 1977; Smith *et al*, 1984), mouse

(Levay-Young *et al*, 1989; Riss & Sirbasku 1989) and human mammary cells (Takahashi *et al*, 1989) and in some cases, bFGF has been able to substitute for EGF (Chen *et al*, 1996; Merlo *et al*, 1996). In cells derived from mice, the greatest effect of bFGF was seen in cells from pregnant animals (Lavandero *et al*, 1998) consistent with the findings *in vivo*. Similarly, bFGF and its specific receptors have been found to be produced preferentially by rat myoepithelium-like cell lines (Barraclough *et al*, 1990, Fernig *et al*, 1990). Although very little work has been carried out in ruminant cells, aFGF and bFGF did not induce proliferation in caprine mammary epithelial cells (Pantshenko & Yang 1999). In addition to its effects on growth, FGF also inhibits the expression of milk protein genes in the presence of lactogenic hormones (Levay-Young *et al*, 1989; Oka *et al*, 1991; Lavandero *et al*, 1998). Thus, bFGF may have a physological role in stimulating growth and inhibiting functional differentiation of mammary epithelial cells, while some recent work also suggests that it may also act as a cell survival factor (Rosfjord & Dickson, 1999)

1.3.6 Heptaocyte growth factor/scatter factor

In vivo, hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, c-met, are expressed in the mesenchyme and regulated temporally during mouse mammary development. Levels progressively decline during pregnancy, are virtually undetectable during lactation, and rise again during involution (Niranjan *et al*, 1995; Pepper *et al*, 1995). In the virgin mouse HGF/SF is elevated during ductal branching (Yang *et al*, 1995a), while overexpression enhances ductal end bud size and number, and branching morphogenesis (Yant *et al*, 1998).

HGF/SF has been shown to be mitogenic in primary mouse mammary epithelial cells (Sasaki *et al*, 1994) and isolated luminal epithelial cells (Niranjan *et al*, 1995) but morphogenic for myoepithelial cells (Niranjan *et al*, 1995). In organ cultures of mouse mammary glands HGF/SF inhibited production of secretory proteins and promoted ductal branching (Yang *et al*, 1995a). Similarly, cells cultured on collagen gels (Brinkmann *et al*, 1995) or in extracellular matrix (Niemann *et al*, 1998) also formed branched tubules in the presence of HGF/SF. HGF/SF is reported to be secreted by human and mouse mammary fibroblasts (Sasaki *et al*, 1994; Niranjan *et al*, 1995) and by adipocytes (Rahimi *et al*, 1994) suggesting a paracrine regulatory role *in vivo*.

1.3.7 Mammary derived growth inhibitor

Mammary derived growth inhibitor (MDGI) is a 14.5 kDa protein which was first purified from lactating bovine mammary gland (Böhmer *et al*, 1987). Bovine and murine cDNAs have been cloned, showing a high sequence homology with fatty acid binding proteins (Kurtz *et al*, 1990; Binas *et al*, 1992). MDGI mRNA and protein is not expressed in the virgin gland (Kurtz *et al*, 1990) but is increasingly expressed during pregnancy, and maximal at the onset of lactation (Kurtz *et al*, 1990, Politis *et al*, 1992a,b; Politis *et al*, 1995). This, along with the observation that MDGI is predominantly detected in epithelial cells containing large fat droplets (Erdmann & Breter, 1993), suggests that the role of MDGI *in vivo* may be as a differentiation factor.

MDGI expression has been shown to be hormonally induced (Binas *et al*, 1992) and may also be modulated by a number of other local factors. EGF strongly suppresses MDGI mRNA expression and prevents differentiation in explants derived from virgin or pregnant mouse mammary gland (Binas *et al*, 1992; Spitzer *et al*, 1995), while MDGI can counteract the proliferative effects of EGF (Yang *et al*, 1994), thus EGF and MDGI are mutually antagonistic. MDGI is also downregulated by TGF-β in differentiated primary mouse mammary epithelial cells (Grosse, 1995), and administration of IGF-I or GH to mutant mice overexpressing MDGI suppresses MDGI mRNA levels in a dose-dependent manner (Huynh & Beamer 1998).

Treatment with MDGI inhibits growth in a bovine mammary epithelial cell line (Zavizion *et al*, 1993), and specifically inhibits growth of mammary epithelial, but not stromal, cells in primary mouse monolayer cultures (Yang *et al*, 1994). In organ culture DNA synthesis and ductal growth are inhibited, with associated alveolar differentiation (Yang *et al*, 1994). MDGI also inhibits proliferation in cancer cells (Lehmann *et al*, 1989; Huynh *et al*, 1995) and it is known that several breast cancer lines and most breast tumours fail to express MDGI. Addition of MDGI to cancer cells has been shown to reduce expression of the oncogenes c-fos, c-myc and c-ras (Lehmann *et al*, 1989), while transfection of the MDGI gene into breast cancer cells results in a differentiated morphology, demonstrating a possible function as a tumour suppressor gene (Huynh *et al*, 1995).

1.3.8 Feedback inhibitor of lactation

The feedback inhibitor of lactation (FIL) is a protein found in the whey fraction of milk. It is thought that its function is to match supply of milk to the demands of the young, since lactation constitutes a large investment of energy by the mother.

It has long been known that increasing milking frequency increases milk yield. Experiments utilising unilateral milking in goats (Linzell & Peaker, 1971; Henderson *et al*, 1983; Wilde *et al*, 1987b; Wilde & Knight, 1990) and cows (Hillerton *et al*, 1990) show that this is through a local, and not systemic, control mechanism. This was identified as a chemical inhibitor, since observations could not be accounted for by gland distension (Henderson & Peaker, 1984), and dilution of accumulated milk with an isotonic solution also increases secretion (Henderson & Peaker 1987). Intraductal injection of FIL into lactating mammary glands in rabbits (Wilde *et al*, 1987a) or goats (Wilde *et al*, 1988) decreases milk accumulation; this is both dose-dependent and reversible (Wilde *et al* 1988). Immunisation of lactating goats against FIL produces antibodies in milk, and delays the decline in milk yield following peak lactation (Wilde *et al*, 1996). The addition of FIL to goat explants results in casein degradation (Stewart *et al* 1988), and the same phenomenon is observed when explants from incompletely milked glands are cultured (Wilde *et al*, 1989). Rennison *et al* (1993) showed that while FIL partially inhibits protein synthesis, there is also blocking of the protein secretory pathway.

In addition to the acute effect on milk protein secretion, FIL may also have long-term effects. More frequent milking has been shown to result in an increase in cell number (Wilde *et al*, 1987b, Hillerton *et al*, 1990) while infrequent milking is associated with reduced differentiation of secretory cells (Wilde & Knight, 1990). Milk stasis leads to apoptosis (Quarrie *et al*, 1994; Quarrie *et al*, 1996), and the addition of FIL to cultured goat mammary epithelial cells also induces DNA laddering indicative of apoptosis (C J Wilde, personal communication). Milk accumulation *in vivo*, intraductal injection of FIL, and the addition of FIL to isolated cells, all result in a reduction in cell surface binding of prolactin (Bennett *et al*, 1990; 1992). Similarly, Kim *et al* (1997) found that the amount of mammary prolactin receptor mRNA decreases or increases following weaning or milk removal, respectively. In addition, prolactin has been shown to suppress apoptosis (Travers *et al*, 1996). Thus, *in vivo*, FIL may act to regulate milk secretion in the short term, but

following more prolonged milk stasis, may induce cell dedifferentiation followed by apoptosis, possibly mediated via a reduction in cell responsiveness to prolactin.

1.4 MAMMARY GLAND CULTURE SYSTEMS

The mammary gland is regulated by a number of factors acting in very complex inter-relationships. This means that research into the action of individual components of the system is very difficult, hence the importance of *in vitro* systems, which enable manipulation of the hormonal or structural environment.

1.4.1 Organ and explant cultures

Whole organ cultures from rodents, and explants from both rodents (Guyette *et al*, 1979; Ganguly *et al*, 1980) and ruminants (Collier *et al*, 1977; Gertler *et al*, 1982; Goodman *et al*, 1983; Puissant *et al*, 1990; Osborne *et al*, 1995) have been used for many years. These systems have the advantage that the architecture and cell composition of the mammary gland *in vivo* are maintained, however it is difficult to identify target cell types for exogenously applied factors, and cultures remain viable for only a short time.

1.4.2 Cell lines

The immortalised cell lines COMMA-1D (Danielson *et al*, 1984) and IM-2 (Reichmann *et al*, 1988) were both derived from mid-pregnant BALB/c mice, while subcloning of COMMA-1D has resulted in the HC11 (Ball *et al*, 1987) and CID9 (Schmidhauser *et al*, 1990) cell lines. All of these lines require the presence of the hormones insulin, hydrocortisone and prolactin for expression of β-casein. β-casein expression is not homogeneous, however, with only 8% of COMMA-1D, 10% of HC11 and 37% of CID9 cells expressing. HC11 cells only synthesise β-casein when confluent suggesting that cell-cell interactions are important, while IM-2 cells are found to consist of a mixture of fibroblastic and epithelial cell types, neither of which express β-casein independently of the other. β-casein expression can be reinstated on coculture of these two components, but also when the epithelial cell types are cultured on fixed fibroblast layers (Reichmann *et al*, 1989). This suggests that β-casein expression is mediated by extracellular matrix deposited by the fibroblasts. An epithelial sub-clone of IM-2, designated 31E, has been shown to secrete β-casein in the absence of fibroblasts, but also basally secretes laminin

(Strange *et al*, 1991). COMMA-1D and CID9 cells express β-casein when cultured on extracellular matrix (Medina *et al*, 1987; Schmidhauser *et al*, 1990) providing further evidence of the importance of substratum for β-casein expression in these cell lines.

Although it is widely held that the ability to express the milk protein β-casein is indicative of a differentiated mammary gland phenotype, it is notable that in all of the above cell lines the major whey protein WAP is expressed only poorly or not at all, with the exception of the CID9 clone (Dale *et al*, 1992). This raises the question of whether the majority of these immortalised cell lines provide a good model for the situation *in vivo*.

Very few cell lines have been derived from ruminant species, and until recently, these have all been of bovine origin. Neither BMEC+H (Schmid *et al*, 1983) or the spontaneously immortalised cell line HH2A (Huynh & Pollak, 1995) express β-casein, although the latter expresses MDGI in the presence of lactogenic hormones and extracellular matrix. The MAC-T cells (Huynh *et al*, 1991) have very varied casein expression, and are heterogeneous (Zavizion *et al*, 1991). In addition, they are unresponsive to prolactin or EGF (Huynh *et al*, 1991; Woodward *et al*, 1994). A fourth bovine cell line, BME-UV1, expresses very low levels of α_{S1}-casein and α-lactalbumin on collagen gels in the presence of prolactin, cortisol and insulin (Zavizion *et al*, 1996). More recently two spontaneously derived ovine cell lines have been reported. OMEC II cells, derived from non-pregnant, non-lactating cells do not secrete β-lactoglobulin in the presence of lactogenic hormones (Düchler *et al*, 1998), but the NISH cell line, derived from a mid-pregnant sheep, can secrete β-lactoglobulin in an extracellular matrix- and lactogenic hormone-dependent manner (Ilan *et al*, 1998).

1.4.3 Primary cell culture

The advantage of primary cell culture is that the morphological and physiological status of the cells relates most closely to that *in vivo*. However, the morphology and secretory function of the cells in culture is greatly influenced by substratum.

1.5 THE IMPORTANCE OF SUBSTRATUM IN PRIMARY CULTURE

1.5.1 Morphology

Mammary epithelial cells from pregnant or lactating mice lose their alveolar structure when cultured on plastic, even in the presence of lactogenic hormones (Emerman & Pitelka, 1977; Li *et al*, 1987). Scanning electron microscopy has shown that cells on plastic are flat and have few microvilli on the apical surfaces, which would usually be apparent in secretory cells, while transmission electron microscopy shows that they lack internal polarisation and secretory apparatus (Emerman & Pitelka, 1977).

Cells on type-I collagen gels are very similar in appearance to those on plastic (Emerman & Pitelka, 1977). If these collagen gels are floated into the medium, however, several morphological characteristics of functional epithelium are obtained, namely, the appearance of apical microvilli, the polarisation of organelles and the formation of a basal lamina (Emerman & Pitelka, 1977; Li *et al*, 1987).

Cell aggregates from mid-pregnant mice cultured on a reconstituted basement membrane derived from the Engelbreth-Holm-Swarm murine tumour (EHS matrix; Kleinman *et al*, 1986) form lumina and resemble secretory alveoli (Li *et al*, 1987; Barcellos-Hoff *et al*, 1989). The formation of these three-dimensional structures, termed "mammospheres", is very similar to the transition of cells from a pregnant to a lactating phenotype seen *in vivo* (Aggeler *et al*, 1991). As with floating collagen gels, electron microscopy reveals the presence of a basal lamina, numerous apical microvilli, well-developed rough ER and Golgi apparatus, and evidence of secretory activity.

1.5.2 Milk protein gene expression

Mammary epithelial cells isolated from pregnant or lactating mice secrete little or no milk protein when cultured on plastic, even in the presence of lactogenic hormones (Emerman *et al*, 1977; Lee *et al*, 1984; Li *et al*, 1987; Chen & Bissell, 1989). Lee *et al* (1985) found that only 10% of cells on plastic secrete caseins, and that this only occurs where cells exist in domes and not in a monolayer. Intracellular casein levels are also low on plastic and it was thought originally that casein was not being synthesised (Lee *et al*, 1984), but further study showed that newly synthesised caseins in cells on plastic undergo intracellular degradation (Lee *et al*, 1985). This is

possibly due to the production of extra- and intra-lysosomal proteases, which are expressed in poorly differentiated cells (Razooki Hasan *et al*, 1982), or may occur as a result of changes in post-translational modification, since caseins in cells on plastic are phosphorylated to a lesser degree than those produced in cells on floating collagen gels. (Lee *et al*, 1985)

Culturing cells on floating collagen gels increases mRNA levels over that obtained on plastic for caseins (Lee *et al*, 1985; Li *et al*, 1987) and transferrin (Li *et al*, 1987), although there is little or no WAP mRNA detected (Lee *et al*, 1984; Chen & Bissell, 1989). Protein synthesis and secretion is similarly found to increase for α, β and γ-caseins (Emerman *et al*, 1977; Lee *et al*, 1984) and transferrin (Li *et al*, 1987). Secretion of α-lactalbumin has been detected at low levels on floating collagen gels by some workers (Wicha *et al*, 1982) but not others (Lee *et al*, 1984).

Milk protein synthesis and secretion has been found to be maximal on complex substrata that closely resemble the extracellular matrix (ECM) *in vivo*. Cells cultured on a biomatrix extracted from rat mammary gland produce higher levels of α-lactalbumin than cells on plastic or floating collagen (Wicha *et al*, 1982). Similarly, on EHS matrix, α-lactalbumin and α-casein accumulation is considerably elevated (Blum *et al*, 1987). Most significantly, cells cultured on EHS matrix express WAP mRNA and secrete the protein vectorially into the luminal space (Chen & Bissell, 1989). Vectorial secretion has also been demonstrated for casein (Barcellos-Hoff *et al*, 1989; Neville *et al*, 1991; Seely & Aggeler, 1991; Hurley *et al*, 1994), while transferrin is secreted both apically and basally (Barcellos-Hoff *et al*, 1989; Seely & Aggeler, 1991; Hurley *et al*, 1994).

Li *et al* (1987) found that ß-casein is produced in 40% of cells from late pregnant mouse mammary gland, but is detectable in >90% of the same cells cultured on EHS indicating that EHS matrix induces casein gene expression. EHS may also act at the level of milk secretion: On collagen, swollen rough endoplasmic reticulum has been noted in mammary epithelial cells (Hurley *et al*, 1989). This was found to contain ß-casein, and it has been suggested that the swelling is caused by a block in the secretory pathway. On EHS however, this pathway is found to be intact and functioning properly, as evidenced by abundant casein micelles (Aggeler *et al*, 1991). Synthesis and secretion of β-casein are reportedly proportional to mRNA levels (Lee *et al*, 1984), while Blum *et al* (1987) show that increased α-casein and

α-lactalbumin synthesis and secretion are not accounted for purely by increasing message. This also suggests an effect of EHS at translational and/or post-translational levels.

Thus, the use of substrata affects morphological development and milk protein gene expression, although individual milk proteins are differentially regulated. Casein mRNA is detected in cells cultured on plastic and they initially synthesise significant amounts of protein, but secretion is impaired leading to intracellular breakdown. Secretion levels are enhanced on floating collagen gels, and maximal on EHS matrix. WAP mRNA and protein are detected in substantial amounts in cells cultured on EHS matrix only, possibly by ECM downregulation of TGFα production (Lin *et al*, 1995), while transferrin is secreted by cells under all conditions, although still in a substratum dependent manner. In addition to milk proteins, substratum also regulates other mammary proteins, for example MDGI cannot be induced in cells grown as monolayers or in single cells on exogenous basement membrane unless they form multicellular structures with luminal spaces (Grosse, 1995).

Substratum also mediates response to lactogenic hormones. Cells on plastic, and attached or floating collagen gels all retain the capability of responding to prolactin (Lee *et al*, 1985), but the magnitude of induction of both mRNA and protein is greatest on EHS matrix. In addition, on EHS small amounts of α_{S1}-casein are detectable in rabbit mammary epithelial cells cultured independently of hormones. This is not the case on collagen, suggesting that EHS matrix contributes to stabilise or induce this mRNA in the absence of hormones more efficiently than collagen gel (Puissant *et al*, 1994).

EHS matrix contains components that closely resemble that of ECM *in vivo*: laminin; type-IV collagen; entactin; heparin sulphate proteoglycans (HSPG); fibronectin (Warburton *et al*, 1982; Warburton *et al*,1984; Kleinman *et al*, 1986; Barcellos-Hoff *et al*, 1989), although no individual component can substitute for the complete matrix (Blum *et al* 1987; Li *et al* 1987; Medina *et al* 1987). Fibronectin or type IV collagen alone do not influence β-casein message but this is increased on HSPG and laminin (Li *et al*, 1987). Secretion of β-casein, however, is only found on laminin (Blum *et al*, 1987; Li *et al*, 1987).

1.5.3 Endogenous basal lamina production

Cells on plastic, or on attached collagen gels do not demonstrate an intact basement membrane, although this is apparent on released collagen gels (Emerman & Pitelka, 1977; Lee *et al*, 1984) or on EHS matrix (Li *et al*, 1987; Barcellos-Hoff *et al*, 1989). In an apparent contradiction to this, Streuli & Bissell (1990) showed that the highest mRNA levels of laminin, type-IV collagen, and fibronectin, are found in cells on tissue culture plastic, which also synthesise and secrete large quantities of ECM proteins. Cells on plastic, however, show little ECM deposition (Parry *et al*, 1985; Streuli & Bissell, 1990). Some components are detectable in the medium (Streuli & Bissell, 1990) and degradation may also occur (David & Bernfield, 1981). Other workers have shown that some collagen type IV is deposited on plastic, or on attached collagen gels, but in a discontinuous fashion (Ormerod *et al*, 1983; Warburton *et al*, 1988). Thus, additional factors are required for the assembly of ECM components into a continuous basal lamina.

On floating collagen gels ECM components are not degraded or secreted into the medium, but deposited as a basal lamina (Parry *et al*, 1985; Streuli & Bissell 1990). Expression of ECM mRNA and proteins, however, is low in these cultures (Streuli & Bissell, 1990). Although laminin expression is high at first, it declines after the deposition of ECM suggesting that there is an interaction between the cells and the basement membrane (Streuli & Bissell, 1990). Alternatively, since ECM deposition only occurs after release of the gel, it may be that the alterations in cell shape, which occur after flotation, exert a negative influence on the expression of the laminin mRNA (Streuli & Bissell, 1990). Thus, it appears that expression and secretion of ECM components are themselves regulated by substratum. It may be that the high levels of expression and synthesis of ECM proteins observed in cells on tissue culture plastic are a continuing attempt by the cells to reproduce their correct environment, in the absence of other factors required for the deposition and organisation of a basal lamina.

It is likely, therefore, that floating collagen gels allow cells to deposit an endogenous extracellular matrix whereas cells on attached gels or plastic do not, and that this is the reason for the induction of milk protein synthesis. Further evidence for this is that ß-casein mRNA levels on released collagen gels continue to increase with time in culture (Lee *et al*, 1984).

1.5.4 ECM-cell signalling

Laminin has been shown to induce synthesis of ß-casein (Streuli *et al*, 1991; Streuli *et al*, 1995b). This occurs via β_1-integrins, since antibodies to these receptors cause a dramatic reduction in ß-casein synthesis (Streuli *et al*, 1991). Integrin activation results in the increased tyrosine phosphorylation and activation of focal adhesion kinase (pp125FAK), possibly initiating any one of a number of signal transduction cascades, and ultimately regulating gene transcription (reviewed in Clark & Brugge, 1995). In fact, laminin induces a transient increase in the activity of AP1 family transcription factors just before the induction of β-casein expression (Roskelley *et al*, 1995). The ECM-induced expression of ß-casein may involve an 'ECM response element' in the promoter of the casein gene (Schmidhauser *et al*, 1990), while DNA binding of Stat 5 transcription factor is also dependent on cell interactions with both ECM and prolactin (Streuli *et al*, 1995a).

In addition to biochemical signalling, however, changes in cell shape and establishment of polarity are also required for full lactational differentiation. Cell-cell interactions may contribute to this, possibly by inducing the shape changes that lead to a polarised, secretory phenotype.

1.5.5 Cell-cell contact

Cell-cell contact is not sufficient in itself to provide differentiation signals for mammary cells, since milk protein secretion is not observed in monolayers. Cell-cell contact is necessary, however, for ß-casein expression in mouse cells on collagen gels, although this may be due to local accumulation of laminin (Streuli *et al*, 1991). Similarly, α_{S1}-casein synthesis is dependent on a minimum cell density in bovine cells on collagen (Talhouk *et al*, 1990), but single cells can be induced to synthesise (though not secrete) ß-casein when suspended in a laminin-enriched basement membrane (Streuli *et al*, 1991). Conversely though, MDGI cannot be induced in single cells on a reconstituted basement membrane (Grosse, 1995) again demonstrating differential regulation of mammary specific proteins.

Cell-cell contact is established via adhesion molecules (E-cadherins). These are distributed randomly over single cells, but localised at the lateral membranes in polarised cells (Nelson *et al*, 1990). This is apparent both in the mammary gland and in mammosphere cultures (Streuli *et al*, 1991). The association of E-cadherins with

the cytoskeleton induces cytoskeletal reorganisation, and may be a mechanism for the formation of cell polarity (Nelson *et al*, 1990).

1.5.6 Cell polarity

Polarised epithelial cells are characterised by having two functionally distinct regions, the apical and the basolateral plasma membranes, and a basally located nucleus. It is thought that cells need to change shape, becoming polarised, in order to deposit basement membrane. Evidence to support this has been found in MDCK cells, where laminin and heparin sulphate proteoglycans are secreted preferentially in a basal direction (Caplan *et al*, 1987).

A number of studies have shown that functional differentiation of mammary epithelial cells is related to cell shape and polarity (Shannon & Pitelka, 1981; Haeuptle *et al*, 1983; Parry *et al*, 1987). This is brought about by the flotation and contraction of collagen gels (Emerman & Pitelka, 1977) or as a result of the fluid-like nature of EHS matrix (Neville *et al*, 1991). Milk protein secretion is further enhanced by flotation of reconstituted basement membranes on top of collagen gels (Wicha *et al*, 1982; Li *et al*, 1987), while artificially rounding mammary epithelial cells prior to exposure to basement membrane, enhances expression of β-casein (Roskelley & Bissell, 1995). If floating collagen gels are cross-linked with gluteraldehyde so that they cannot be remodelled, milk protein secretion is reduced (Lee *et al*, 1984). Similarly WAP expression on EHS matrix is reduced to <10% of initial levels if EHS is fixed, although β-casein message is only slightly reduced (Chen & Bissell, 1989).

1.5.7 Signalling via the cytoskeleton

E-cadherins in lateral cell membranes, and integrins in basal membranes, both form adhesion complexes which associate with the cytoskeleton (Nelson *et al*, 1990; Clark & Brugge, 1995). Microfilaments form physical connections between the cell periphery and the nuclear matrix via nuclear lamins (Pienta & Coffey, 1992; Boudreau *et al*, 1995). The nuclear matrix consists of fibres that bind to DNA at matrix attachment regions along areas of active chromatin (Pienta & Coffey, 1992). In this way, cell shape or ECM-induced structural changes in the cytoskeleton, histone organisation and the nuclear matrix may contribute to tissue-specific gene expression.

There is some evidence that the cytoskeleton may also interact with polyribosomes: Seely & Aggler (1991) found that disruption of actin filaments in cells cultured on EHS matrix caused reduced association of mRNA with the cytoskeleton and inhibition of both total and milk protein secretion. Levels of mRNA remained unaffected, suggesting control at the level of translation. Yet other work, however, suggests that the cytoskeleton may be implicated in facilitating sorting and transport of proteins (Rennison *et al*, 1992; Mays *et al*, 1994). This may also account for Seely & Aggeler's (1991) finding that disruption of microfilaments affects vectorial milk protein secretion. Thus, changes in cytoskeletal organisation might influence functional differentiation of mammary epithelial cells at a number of levels.

In summary, cell-cell contact appears to be necessary to induce the correct organisation of the cytoskeleton, leading to cell polarisation. Polarised cells then secrete basement membrane components, which negatively regulate their own production, and control gene expression and protein synthesis via biochemical signals and interactions with the cytoskeleton.

Overall, mammary development and differentiation are influenced by a complex interplay of factors that include hormones, local factors and cell microenvironment. The development of a culture system that permits mammary gland epithelial cells to undergo structural and functional differentiation (EHS matrix) has provided an opportunity to explore the regulation of transcription, synthesis and secretion of milk protein genes, and to consider their manipulation.

1.6 MANIPULATION OF MILK COMPOSITION

Selective breeding for useful traits has been used for many years, but although milk yields have increased hugely, there has been little change in milk composition. The cloning of the milk protein genes, and greater understanding of their control mechanisms (reviewed in Mercier & Villotte, 1993; Bawden *et al*, 1994) has permitted their modification and provided new opportunities for manipulating endogenous milk proteins, or producing new products in milk.

1.6.1 Modification of milk for nutrition and processing

Human milk is very different in composition to bovine milk and yet the latter is used as the basis for infant formula. Genetic engineering could mimic human breast milk by replacing bovine milk proteins with human equivalents. Human milk does not contain β-lactoglobulin, which is the major whey protein in ruminants. This milk protein is thought to be one of the main causes of milk protein intolerance in humans. Although livestock species are limited to addition of genes by pronuclear injection (since no stem cell lines for genetic manipulation are yet readily available), anti-sense constructs could be used to reduce the presence of this protein.

In humans, lactoferrin is the major whey protein, but this is low in bovine milk. It is thought to have bacterocidal and bacteriostatic properties and may mediate iron transfer from mother to infant (Lonnerdal & Iyer, 1995). Human lactoferrin has already been expressed in the milk of transgenic mice (Platenburg *et al*, 1994). Active human lysozyme has also been produced in transgenic mouse milk (Maga *et al*, 1995) and is another protein that is likely to provide antibacterial activity in human milk. These proteins may also be protective against intramammary infection or mastitis, which costs the UK dairy industry over £90 million per year. Manipulation of other milk proteins could improve processing of dairy products, for example, manipulating caseins can enhance curd firmness, improve thermal stability and increase the rate of ripening in cheese making (reviewed in Wall *et al*, 1997).

1.6.2 Production of pharmaceutical proteins in milk

The mammary gland is ideally suited for the production of foreign proteins, since it has a high capacity for protein production, the products are stored separately from the rest of the body where they might cause adverse physiological reactions, and they can be removed easily in a non-invasive and non-stressful manner.

A number of important biomedical proteins have already been produced in livestock species: blood clotting factor IX (Clark *et al*, 1989); α_1-antitrypsin (Wright *et al*, 1991; Carver *et al*, 1993); human protein C (Velander *et al*, 1992); tissue plasminogen activator (Ebert *et al*, 1994); factor VIII (Niemann *et al*, 1996; Paleyanda *et al*, 1997) and anti-thrombin (Edmunds *et al*, 1998). A number of other useful products have been produced in rabbits: interleukin 2 (Bühler *et al*, 1990); human growth hormone (Limonta *et al*, 1995), or mice: urokinase (Meade *et al*,

1990); cystic fibrosis transmembrane conductance regulator (DiTullio *et al*, 1992) and serum albumin (Shani *et al*, 1992).

Many important therapeutic proteins are in short supply and are difficult or expensive to purify (summarised in Table 1.1). Growth hormone, for example, is species specific and the traditional source has been cadavers. Many of these proteins are currently derived from donated blood, where they are present only in tiny amounts, and are so difficult to produce that their expense precludes or severely limits their use as drugs. In addition, there is the added risk of transferring diseases such as HIV, hepatitis, Creuzfeld-Jacob or other, as yet unidentified, viruses.

Bacteria and yeast have commonly been used for expression of foreign proteins since they are easy to manipulate genetically and can be grown easily and cheaply in large amounts. Expression of mammalian genes in these hosts, however is often unsatisfactory because the proteins are incorrectly processed (Goeddel *et al*, 1979; King *et al*, 1988). This may result in an alteration or total loss of activity (Casolaro *et al*, 1987) and may also cause antigenicity. Mammalian cells are capable of specific post-translational modifications, such as glycosylation, but expression systems are expensive and technically demanding. In addition, for some products such as α_1-antitrypsin, yields are still insufficient to meet therapeutic demands (Archibald *et al*, 1990). Although the creation of transgenic livestock is time-consuming and expensive, once a founder flock had been established, this is expanded by traditional husbandry methods, making the large scale commercial production of pharmaceutical proteins in milk very attractive.

1.6.3 Generation of transgenic animals

Transgenesis has been defined as the stable incorporation, by artificial gene transfer, of exogenous or novel DNA into the genome of an animal (Moore & Mepham, 1995). Currently this is achieved by two methods:

Manipulation of embryonic stem cells (Evans & Kaufman, 1981).

Donor mice are superovulated, mated and the resulting blastocysts are collected and maintained in culture. Undifferentiated stem cells are isolated from the inner cell mass of the blastocyst, and transgenes are then introduced. This can be brought about by a number of methods, although electroporation is the most common. The insertion site of the DNA can be targeted by designing a construct with regions that

Table 1.1 Estimated annual US requirements and costs of some potential products
Source: Wall *et al*, (1997)

Item	Estimated need *(kg)*	Cost/g *($)*	Annual market *($ x 10^6)*
Factor VIII	0.3	2,900,000	882
Factor IX	4	40,000	160
Protein C	10	10,000	100
Antithrombin III	21	7,000	150
Fibrinogen	150	1000	150
Albumin	315×10^3	3.56	1,120

are homologous to that of endogenous DNA (Thomas & Capecchi, 1987). Similarly, the same approach may also be used to mutate or knock out endogenous genes. The modified cells undergo selection and are injected into the cavities of the blastocysts, where they become reintegrated into the inner cell mass, eventually contributing to development of the embryo; the blastocysts are then surgically transferred to pseudopregnant recipient mice. The resulting offspring will include some chimeric mice. If any of these are germline chimeras, the transgene may be passed on to their progeny. Currently, this method is only used routinely in mice, but pluripotent embryo-derived cell lines have been derived for the pig (Wheeler, 1994) and cow (Stice *et al*, 1996).

Pronuclear microinjection (Gordon & Ruddle, 1981; Hammer et al, 1985)

At present, this method is the only effective way of producing transgenic livestock species. Embryos are produced by mating or artificially inseminating (in the case of cattle) superovulated donors. They are collected by non-surgical flushing (cattle), by laparotomy, or by donor slaughter. Embryos have also been obtained using oocytes recovered from abattoirs, which are then matured and fertilised *in vitro*. Embryos are collected at the one-cell stage and maintained in culture until ready for injection (within 12 hours). About 1-2pl of DNA suspension is injected into the male pronucleus, following which the transgenes may then integrate into the chromosomal DNA. The insertion site is non-specific and may contain anything from one to 200 copies of the transgene, arranged head to tail. Microinjected embryos are cultured until the 6-7 cell stage, when they may be biopsied to test for integrations of the transgenes. They can then be transferred into synchronous recipient females.

1.6.4 Constraints to production of transgenic animals

The production of transgenic animals is extremely expensive, mainly because the process is so inefficient, whichever method is employed. Of the number of integrations that occur in embryonic stem cells, successful gene targeting is between 0.01% and 1%, although blastocysts that have been modified correctly can be selected before injection. About 70% of transferred blastocysts develop to term. Pronuclear microinjection in mice results in 1-10% of injected mouse embryos becoming transgenic animals, due to losses occurring at several stages: Losses of 15-

50% occur as a direct result of the microinjection procedure. Of the embryos that survive, only 10-30% develop to term, and of these, only 10-30% are transgenic.

The numbers of transgenic animals produced in livestock species is even lower (pigs 0.3-4.0%, sheep 0.1-4.4%, goats 1.0-1.7% and cattle 0.3-2.6%) in part due to technical difficulties in microinjecting eggs (Hammer *et al*, 1985). In mice, gene insertions can be targeted through homologous recombination, but in livestock transgene integration is a random event. This may produce greater embryonic or animal mortality resulting from disruption of physiologically important genes.

In producing transgenic animals as founders for a breeding programme, yet more difficulties arise, since approximately 20% of sheep and pigs do not transmit the transgene to their offspring, and 20-30% pass it on to less than half of their progeny (Hammer *et al*, 1985). Of the first generation animals resulting from these procedures, only germ-line chimeras are of interest, but these are greatly outnumbered by non-germ line transgenic, or non-transgenic animals which are culled. Thus there is also a great deal of animal "waste" in the procedures.

Transgenes are sometimes expressed aberrantly, or not at all, possibly through position effects, silencing by heterochromatin or the presence or absence of unidentified regulatory regions. Thus it is desirable to screen transgenes prior to generating transgenic livestock. Evaluation of transgenes currently takes place in lactating mice, but mice are not necessarily good predictors of expression in other species (Archibald *et al*, 1990; Wright *et al*, 1991). Testing of transgenes in the intended species is currently the only relevant method of determination, yet the time to first lactation (shown in Table 1.2), and associated husbandry costs make this unattractive.

Table 1.2 Times for generating and testing transgenic animals
Time (months) required to produce transgenic animals following microinjection of DNA into a one-cell embryo.

Species	Birth	Adult	First lactation (transgene expression)	Offspring lactates (germline transmission)
Mouse	0.75	2.25	3	5.25
Sheep or goat	5	13	18	31
Cow	9	23	32	55

1.7 AIMS OF THE STUDY

The cloning of milk protein genes and the development of transgenic technology have opened up new possibilities for directing the expression of foreign proteins in milk. This potentially would allow the large-scale production of a number of important therapeutic proteins, for which the current need cannot be met, either because of technical difficulties or expense. The ruminant mammary gland is particularly suited for the production of foreign proteins, since it has a high capacity for protein production, and these can be easily removed without detriment to the animal. In addition, once founder animals are established, stocks can be expanded by traditional breeding methods.

Prior to generating transgenic animals, gene constructs have traditionally been tested in mice, but results cannot necessarily be extrapolated to livestock species. Testing in the animals for which the gene construct is eventually intended is expensive and time-consuming. In addition, surgical techniques are frequently involved and large numbers of "waste" animals result. This is directly against current moves towards improving animal welfare, and the aims of recent legislation to reduce the use of animals in scientific research.

The overall aim of this project is to develop a ruminant cell culture system in which to evaluate gene constructs for expression and gene product secretion in lactating mammary tissue, and so determine their suitability for the generation of transgenic livestock. Currently, there are no ruminant cell lines capable of secreting the full range of milk proteins and attempts at primary culture have met with limited success. The specific aims of this project are:

i) to develop a primary ruminant mammary cell culture system;
ii) to investigate the performance of passaged cells;
iii) to determine conditions for transfection;
iv) to express reference constructs;
v) to investigate post-translational processing of proteins.

CHAPTER TWO
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

General laboratory chemicals were supplied by Sigma Chemical Co, Poole, UK; BDH, Poole, UK or Boehringer Mannheim Ltd, Lewes, UK unless stated otherwise. Cell culture media, chamber slides and plates were purchased from Life Technologies Ltd, Paisley, UK. All water for general laboratory use was purified by reverse osmosis, ion/organic removal and photo-oxidation. Tissue culture/molecular biology grade water was ultrafiltered and further photo-oxidated to 18.2 mOhms resistance.

2.1.2 Radiochemicals

Radiochemicals were from NEN DuPont Ltd, Stevenage, UK. Activity of [^{35}S] was determined using a 1600TR liquid scintillation analyser with Emulsifier-Safe^{TM} scintillation fluid (both from Canberra Packard, Meriden, USA).

2.1.3 Animals

Mammary tissue was obtained from Finn-Dorset ewes or British Saanen goats from the Hannah Research Institute herds, maintained under normal husbandry conditions. The transgenic sheep was provided by PPL Therapeutics, Roslin. UK. The animals were in the third trimester of pregnancy (d107-d124) at time of tissue collection.

2.2 ISOLATION OF RUMINANT MAMMARY EPITHELIAL CELLS

2.2.1 Preparation of cell culture media

The details of the media used in the isolation of mammary epithelial cells are given in Table 2.1. Basal media (pH 7.4) were sterilised through a 0.2 μm pore filter (Gelman Sciences, Southampton, UK) and stored at 4°C. Collagenase (type 3, Worthington Biochemical Corporation, New Jersey, USA), hyaluronidase, DNase I (both Boehringer Mannheim Ltd, Lewes, UK), trypsin inhibitor (Sigma Chemical Co, Poole, UK) and hormones were added to the basal media on the day of use.

2.2.2 Preparation of additional reagents

Reagents present in culture media were prepared in the following manner:

Insulin was prepared by dissolving 10 mg of insulin (from bovine pancreas, 27.4 USP units/mg) in 10 ml of water before addition of 100 µl of 0.34 M NaOH and dilution with water to 0.1 mg/ml. Stocks were stored in aliquots at -20°C.

Cortisol was prepared by dissolving 10 mg of hydrocortisone-21-acetate in 10 ml ethanol and dilution to 0.1 mg/ml with water. Stocks were stored in aliquots at -20°C.

Prolactin was prepared by dissolving 10 mg of prolactin (from sheep pituitary, 32 IU/mg) in 2.5 ml of 10 mM Hepes (pH 8.0) and diluting to 0.1 mg/ml with water. Stocks were stored in aliquots at -20°C.

Transferrin was prepared by dissolving 100 mg holo-transferrin (human) in water to 1 mg/ml. Stocks were stored in aliquots at -20°C.

Epidermal growth factor was prepared by dissolving 1 mg of epidermal growth factor (from mouse submaxillary gland) in 0.154 M NaCl to 0.01 mg/ml. The solution was blast frozen in liquid nitrogen in 100 µl aliquots and stored at -20°C.

2.2.3 Preparation of mammary epithelial cells

Sheep and goats were culled by captive bolt, followed by exsanguination. The mammary gland was scrubbed with Hibitane, and subsequently with 70% ethanol prior to excision. Once removed, it was trimmed of skin, cistern, ductal tissue and fat. The remaining tissue was cut into ~2 cm^3 pieces and immersed in sterile Hanks Basal Salt Solution (HBSS; pH 7.4, 37°C). The pieces of tissue were washed twice in sterile HBSS, prior to removal of residual fat and connective tissue.

The tissue was cut into smaller pieces (~0.5 cm^3) and injected with digestion medium (Table 2.1) using a 21g needle; this step was occasionally omitted depending on the texture of the recovered tissue. Tissue (80-100 g) was then incubated in digestion medium (4 ml/g) at 37°C in an orbital incubator (Gallenkamp, Loughborough, UK) shaking at 120 rpm. After approximately 1 h, the tissue was recovered and chopped finely with curved scissors, before being returned to the digestion medium.

After approximately 3-4 h, when the cells were predominantly in clumps of ~50 cells, the digest mixture was filtered (150 µm pore nylon mesh; Lockertex,

Table 2.1 Composition of media used in the isolation of mammary epithelial cells

	Digestion medium	Wash medium
Basal medium		
HBSS[1]	1x	1x
MEAA[2]	1x	1x
Glutamine (mM)	2	2
D-glucose (mM)	5.5	5.5
Bovine serum albumin (mg/ml)	40	-
Calcium chloride (μg/ml)	2.2	2.2
Magnesium sulphate (μg/ml)	2.5	2.5
Penicillin (μg/ml)	120	120
Kanamycin (μg/ml)	100	100
Fungizone (μg/ml)	2.5	2.5
Hepes buffer (mM)	-	5.4
Hormones		
Insulin (μg/ml)	5	5
Cortisol (μg/ml)	1	1
Enzymes		
Collagenase[3] (mg/ml)	1.2	-
Hyaluronidase (mg/ml)	0.5	-
DNAse I (μg/ml)	-	20
Trypsin inhibitor (μg/ml)	-	100

[1]Hanks Basal Salt Solution
[2]Minimum Essential Amino Acids
[3]Type 3, ~150u/mg

Warrington, UK). The cells were harvested by gentle centrifugation (80 *g*, 5 min), resuspended in wash medium and re-harvested. This wash step was repeated three more times. The final cell pellet was resuspended in HBSS. Undigested material was returned to the incubator for further digestion (1-2 h) after which cell harvesting was repeated.

Percoll fractions were prepared comprising Medium 199 containing 20 mM Hepes, 2% (w/v) BSA, 2% (w/v) Ficoll and Percoll to densities of 1.01-1.08 g/ml. A discontinuous gradient was prepared by gently layering 5 ml of each fraction into a 50 ml centrifuge tube. 5 ml of cell suspension was gently layered on top and the gradient was centrifuged (800 *g*, 20 min, Brake 0). Individual bands were removed sequentially. The resulting cell suspensions were diluted with HBSS and centrifuged (100 *g*, 10 min) to remove the Percoll. The cells were washed once again by resuspending in HBSS and centrifuging (80 *g*, 5 min). Finally, the cell pellet was resuspended in culture medium.

2.2.4 Estimation of cell yield

Cell yield was estimated by pelleting 100 µl of the final cell suspension in an elongated microtube (13,000 *g*, 30 s). The volume of the cell pellet was determined from the pellet dimensions, and cell number estimated with the aid of a standard plot determined by fluorometric measurement of DNA (Section 2.5.1).

2.2.5 Cryopreservation of mammary epithelial cells

Cells were pelleted (80 *g*, 5 min) and resuspended in foetal calf serum (37°C) containing 10% (v/v) DMSO at a cell density of 2 x 10^7 cells/ml. Cells were dispensed into cryovials and frozen slowly (-1°C/min). Once frozen, cells were transferred to liquid nitrogen for long term storage.

2.3 PREPARATION OF EHS MATRIX

EHS matrix was prepared from Engelbreth-Holm-Swarm sarcoma passaged in lathyritic C57/BL mice, by a method adapted from Kleinman *et al* (1986). Frozen tissue (-70°C) was washed by homogenisation (Ultra-Turrax T25; Janke & Kunkel, IKA Labortechnik) in 50 mM Tris HCl containing 4 mM EDTA and 3.4 M NaCl and centrifugation (45,000 *g*, 4°C, 30 min). This was repeated twice, before overnight extraction with 50 mM Tris HCl containing 4 mM EDTA, 2 M urea and 0.2 M NaCl.

The resulting extract was centrifuged (45,000 *g*, 4°C, 30min) and the supernatant was dialysed (molecular weight cut-off (MWCO) 12-14 kDa) first against 50 mM Tris HCl containing 4 mM EDTA and 0.15 M NaCl (48 h, 3 changes), and then for 16 h against Dulbecco's Modified Eagle's Medium (DMEM) containing 10 mM Hepes buffer and penicillin/streptomycin (200 U/ml and 200 μg/ml respectively). The dialysate was stored as aliquots at -20°C.

2.4 CELL CULTURE

2.4.1 Recovery of cells from liquid nitrogen

Cryovials were removed from liquid nitrogen and thawed quickly at 37°C. The cells were resuspended in culture medium and centrifuged (80 *g*, 5 min) in order to remove DMSO which is cytotoxic. The cell pellet was then resuspended in proliferation medium (Table 2.2) for plating.

2.4.2 Culture conditions

Cells were cultured in a humidified tissue-culture incubator (Jencons Nuair, Leighton Buzzard, UK) at 37°C and 5% (v/v) CO_2.

2.4.3 Proliferation of cells on plastic

Cells were plated in proliferation medium (Table 2.2) at a density of $3 \times 10^5/cm^2$ following recovery from liquid nitrogen, or $5 \times 10^4/cm^2$ after passaging. Medium was changed every two days. Cells were grown to 90% confluence before passaging.

2.4.4 Passaging of cells

In early experiments, cells were pre-treated for 5 min with Versene, which chelates Ca^{2+} ions, followed by treatment with trypsin until the majority of cells became detached from the surface. In later experiments, a solution of trypsin containing EGTA (0.4 mg/ml) and polyvinyl alcohol (0.1 mg/ml) was found to be less damaging to cells and was used routinely thereafter. In both methods, trypsin activity was neutralised by the addition of serum-containing medium. Cells were pelleted (80 *g*, 5 min) and resuspended in the appropriate culture medium for plating on either plastic or EHS matrix.

Table 2.2 Composition of media used in the culture of mammary epithelial cells

	Proliferation (on plastic)	Attachment (on EHS matrix)	Differentiation (on EHS matrix)
Basal medium			
Medium 199	1x	1x	1x
Hams F12	1x	1x	1x
Hepes buffer (mM)	20	20	20
$NaHCO_3$ (mM)	90	90	90
Sodium acetate (mM)	4	4	4
Penicillin (U/ml) / streptomycin[1] (μg/ml)	200; 200	200; 200	200; 200
Fungizone[2] (μg/ml)	2.5	2.5	2.5
Additions			
EGF (ng/ml)	10	-	-
Insulin (μg/ml)	5	5	5
Cortisol (μg/ml)	1	1	1
Prolactin (μg/ml)	-	3	3
Transferrin (μg/ml)	-	5	5
Horse serum (%)	20	20	-
Foetal calf serum (%)	5	5	-

[1] Omitted during transfection of cells.

[2] Included during cell preparation, or recovery from liquid nitrogen; subsequently omitted.

2.4.5 Differentiation of cells on EHS matrix

EHS matrix was thawed on ice and applied to ice-cold tissue culture plates (30 μl/cm^2). The matrix was then allowed to polymerise at room temperature for 30 min. Cells were plated very gently in attachment medium (Table 2.2) at a density of 3 x 10^5/cm^2. After 24 h, attachment medium was replaced with serum-free differentiation medium (Table 2.2). Subsequently, differentiation medium was changed daily.

2.5 MEASUREMENT OF CELL NUMBER AND PROLIFERATION

2.5.1 DNA assay

DNA content (as a measure of cell number) was measured by a fluorometric procedure (Labarca and Paigen, 1980). This assay is based on the binding of a fluorescent dye, bisbenzimide (Hoechst 33258), to DNA.

Pelleted cells were sonicated (15 s at setting 30; Kontes micro-ultrasonic cell disruptor, Burkard Scientific, Rickmansworth, UK) in 400 μl DNA assay buffer (0.1 M Na_2HPO_4 containing 2 M NaCl, pH 7.4) and an appropriate volume was removed for assay. DNA standards (calf thymus) were prepared (range 0-3 μg) and all assay volumes were made up to 1 ml with assay buffer before addition of 1 ml of fluorescent reagent (2 μg/ml). Assay samples were incubated for 15-30 min in the dark, after which fluorescence was measured on a fluorometer (Hoefer TK100, San Francisco, USA) with an excitation wavelength of 356 nm, an emission wavelength of 445 nm and range setting 5. 1 μg of DNA was estimated to be equivalent to 10^5 mammary epithelial cells, calculated from Lewin (1957).and Alberts *et al* (1983).

2.5.2 Methylene blue assay

This colorimetric assay (Oliver *et al*, 1989) is based upon the electrostatic binding of methylene blue dye to negatively charged groups of nucleic acids and proteins in fixed cell monolayers.

Following removal of culture medium, adherent cells were washed with PBS (pH 7.4) and fixed with 100% methanol for 5 min. Cells were stained with methylene blue (0.1% dye in 10 mM borate buffer, pH 8.4) for 30 min and washed 4 times in 10 mM borate buffer. The methylene blue was eluted from the cells by

addition of 0.1 M HCl containing 20% (v/v) ethanol and the absorbance was measured spectrophotometrically at a wavelength of 650 nm within 1 h.

2.6 IMMUNOSTAINING OF CELLS *in situ*

Cells were plated on 4- or 8-well chamber slides and allowed to attach and proliferate into small colonies. Following removal of medium, cells were washed with phosphate-buffered saline (PBS, pH 7.4) before fixation with methanol (5 min). Cells were washed again with PBS and pre-blocked (20 min) in PBS containing 0.1% (v/v) Tween 20, 0.1% (v/v) Triton X-100 and 0.3% (w/v) BSA. Non-antigenic sites were blocked with PBS containing 0.1% (v/v) Tween 20 and 25% (v/v) donkey non-immune serum for 30 min. Primary antibodies (Table 2.3) were diluted in PBS containing 0.1% (v/v) Tween 20 (PBST) and incubated with the cells (2 h, 37°C) in a humidified chamber. Cells were washed 3 times in PBST, followed by incubation with biotinylated anti-mouse immunoglobulin G (IgG; Sigma, Poole, UK) diluted 1:200 in PBST for 2 h at 37°C in a humidified chamber. Cells were washed as before and incubated with Extravidin-FITC (Sigma, Poole, UK) diluted 1:100 in PBST for 1.5 h at 37°C. A coverslip was mounted using Vectashield (Vector Laboratories, Peterborough, UK) containing DAPI (4,6-diamidino-2-phenylindole) nuclear stain (1.5 μg/ml) and the cells were visualised by fluorescence microscopy (Leitz DMRB, Leica, Cambridge, UK). Positive staining was identified by image analysis (Leica Quantimet 500). Photography was with Fujichrome Provia 400 film.

2.7 IMMUNOSTAINING OF SECTIONED MAMMOSPHERES

2.7.1 Preparation of subbed slides

Microscope slides were treated with 5% (v/v) Decon for 30 min, washed twice with distilled water, and immersed in ethanol. After draining, the slides were dipped in a 2% (v/v) solution of 3-aminopropyltriethoxysilane in ethanol, washed in ethanol followed by distilled water and air-dried overnight. Slides were wrapped in dust-free paper until required.

2.7.2 Fixation and sectioning of mammospheres

Culture medium was removed from cells on EHS matrix and ~250 μl replaced. To this was added an equal volume of fixation solution (4% (w/v) paraformaldehyde in 0.1 M phosphate buffer) for 30 min. This was replaced twice more with fresh

Table 2.3 Markers used in cell typing of ovine mammary epithelial cells

Marker and dilution	Reactive determinant	Cell type	Source
MAb LP34 1:50	Cytokeratins 5, 6, 18	Epithelial[1], Myoepithelial[1, 2]	Dako Ltd, Ely, UK
MAb CY-90 1:500	Cytokeratin 18	Epithelial[1]	Sigma, Poole, UK
MAb CKB1 1:100	Cytokeratin 14	Myoepithelial[2]	Sigma, Poole, UK
MAb 1A4 1:500	Smooth muscle α-actin	Myoepithelial[3]	Sigma, Poole, UK
MAb hSV-M 1:500	Smooth muscle myosin	Myoepithelial[3,4]	Sigma, Poole, UK
MAb V9 1:100	Vimentin	Myoepithelial[3], stromal	Sigma, Poole, UK

[1]Taylor-Papadimitriou *et al*, 1983
[2]Nagle *et al*, 1986
[3]Dulbecco, 1982
[4]Warburton *et al*, 1982

fixation solution. The cells were then washed (3 x 10 min) with rinse solution (0.1 M phosphate buffer containing 2% (w/v) sucrose and 0.002% (w/v) $CaCl_2$).

Cells in rinse solution were gently scraped from the plate and transferred to a cryotube containing a small amount of OCT freezing compound (Agar Scientific, Stansted, UK). The tube was left to stand for ~1 h to allow the cells to settle. Excess rinse buffer was removed and further OCT compound added, into which the cells were gently mixed. The sample was frozen slowly (-1°C/min) at -70°C. Sections (4 μm) were cut on a cryostat (Leica CM1800, Cambridge, UK), collected onto subbed microscope slides and allowed to air-dry overnight.

2.7.3 Immunostaining of sections for milk proteins

Sections were isolated using a PAP pen (Agar Scientific, Stansted, UK) and pre-blocked in PBST containing 0.1% (v/v) Triton and 0.3% (w/v) BSA for 20 min. Non-antigenic sites were blocked with PBST containing 25% (v/v) donkey non-immune serum for 30 min, after which sections were incubated with antisera to bovine α-lactalbumin (Universal Biologicals Ltd, London, UK) diluted 1:50, bovine β-lactoglobulin (prepared in-house) diluted 1:100 and goat α_{S1}-casein (prepared in-house) diluted 1:200 in PBST for 2 h at 37°C in a humidified chamber. Sections were washed 3 times in PBST, followed by incubation with mouse anti-rabbit IgG FITC (Sigma, Poole UK) diluted 1:500 for 2 h at 37°C in a humidified chamber. Cells were washed as before and sections were mounted using Vectashield (Vector Laboratories, Peterborough, UK) containing DAPI nuclear stain (1.5 μg/ml) and the cells were visualised by fluorescence microscopy (Leitz DMRB, Leica, Cambridge, UK). Photography was with Fujichrome Provia 400 film.

2.8 MEASUREMENT OF PROTEIN SYNTHESIS AND SECRETION

2.8 1 Labelling of synthesised proteins with [^{35}S]-methionine

Cells were washed, and pre-incubated for 1 h (37°C), in methionine-free culture medium containing 5 μg/ml insulin, 1 μg/ml cortisol and 3 μg/ml prolactin. This was subsequently replaced with fresh medium containing 150μCi [^{35}S]-methionine and supplemented with 30% (v/v) cold methionine. Cells were incubated for a further 4 h (37°C) after which the cells, culture medium and luminal secretion were collected for assay of [^{35}S]-labelled protein.

2.8.2 Harvesting of secreted proteins and cells

The culture medium was aspirated from the cells and retained. Cells were washed twice in HBSS pH 7.4 containing 10 mM Hepes and treated with a solution of 2.5 mM EGTA in HBSS pH 7.4 containing 10 mM Hepes, for 20 min. The EGTA solution was harvested, and the cells were again washed twice. Cells were then detached by treatment with Dispase (Universal Biologicals Ltd, London, UK) and recovered by centrifugation (13,000 *g*, 1 min, 4°C). Cell pellets were snap frozen in liquid nitrogen. Culture medium and EGTA samples were stored at -20°C. This method was used for harvesting secreted milk proteins and cell pellets from either radio-labelled or non-radiolabelled cells.

2.8.3 TCA precipitation of proteins

Cell pellets were sonicated (15 s at setting 30; Kontes micro-ultrasonic cell disruptor, Burkard Scientific, Rickmansworth, UK) in 400 µl of DNA assay buffer (Section 2.5.1). Bovine serum albumin (100 µl; 0.1% (w/v)) was added to 50 µl of sonicated cell sample, or 100 µl of culture medium or EGTA extract. The sample was made up to a total volume of 400 µl with water, to which 400 µl of ice-cold trichloroacetic acid (20% (w/v)) was added. The sample was incubated on ice for 20 min and the resulting precipitate harvested by centrifugation (13,000 *g*, 5 min, 4°C). The tube was washed by the addition of 1 ml ice-cold 1% (w/v) trichloroacetic acid and centrifugation (13,000 *g*, 4 min 4°C); this wash step was repeated. The precipitate was dissolved in 400 µl of 50 mM Tris HCl (pH 8.0) and the procedure repeated. The final precipitate was re-dissolved in 400 µl of 50 mM Tris HCl (pH 8.0) and transferred to a scintillation vial. A further 400 µl Tris was used to wash the tube and also transferred to the scintillation vial. Scintillation fluid (10 ml) was added and [^{35}S] radioactivity was determined.

2.8.4 Processing of protein samples for gel electrophoresis

Culture medium and EGTA extracts

Culture medium or EGTA extracts from replicate wells were pooled, dialysed (MWCO 6-8 kDa) against 10 litres of water for 24 h at 4°C and lyophilised. Samples were resuspended in SDS sample buffer (0.625 M Tris HCl containing 2% (w/v)

SDS, 10% (v/v) glycerol, 1.2% (w/v) bromophenol blue and 0.1% (v/v) β-mercaptoethanol, pH 6.8).

Sheep and goat milk fractions

Protease inhibitors (15 mM ε-amino n-caproic acid and 2 mM PMSF) were added to whole milk, which was defatted by centrifugation at 500 *g* for 20 min and filtration through glass wool. A sample of the defatted milk was retained. The remainder was diluted with deionised water (1:1) and warmed to 37°C. Concentrated hydrochloric acid was added dropwise until coagulation of the caseins occurred (pH 4.5). Casein was pelleted by centrifugation (500 *g*, 20 min). The clear supernatant, which constitutes the whey fraction, was returned to neutral pH and filtered through glass-fibre filters, followed by a 0.45 μm pore filter (Gelman Sciences, Southampton, UK). The filtrate and the retained defatted milk were dialysed (MWCO 6-8 kDa) against 10 litres of water for 24 h at 4°C, lyophilised and stored at -20°C. The casein pellet was resuspended in water, lyophilised and stored at -20°C. Prior to freeze-drying, aliquots were removed for protein assay (Section 2.8.5). Lyophilised milk fractions were dissolved in SDS sample buffer to use as standards for electrophoresis.

2.8.5 Protein assay

The protein content of sheep and goat milk fractions was measured spectrophotometrically as described by Bradford (1976), using bovine serum albumin as the protein standard. Standards were prepared in the range 0-10 μg/ml. Standards and samples were assayed according to the manufacturer's instructions (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and coloured complex formation was determined at a wavelength of 595 nm.

2.8.6 SDS-polyacrylamide gel electrophoresis

Proteins were resolved by electrophoresis (Laemmli, 1970) using the Mini-Protean II gel kit (Bio-Rad Laboratories Ltd, Herts, UK). Standards and samples, dissolved in SDS sample buffer, were heated to >80°C to denature proteins and separated on 11% polyacrylamide gel (29% acrylamide:1% bis) at 200 v for 35 min, or 100 v for 95min. The running buffer comprised 0.025M Tris containing 0.192 M glycine and 0.1% (w/v) SDS.

2.8.7 Staining of protein gels

Gels were stained in Coomassie blue R-250 for 30 min and destained overnight or until clear. They were dried onto filter paper on a gel dryer (Bio-Rad Laboratories Ltd, Herts, UK) at 80°C for 2 h using cycle 3.

2.8.8 Fluorography

Gels were stained with Coomassie blue R-250 followed by immersion in Amplify (Amersham, Little Chalfont, UK) for 15 min. Gels were dried (Section 2.8.7) and exposed to x-ray film (Kodak X-OMAT AR) at -70°C for 2-3 weeks before developing.

2.8.9 Western blotting and chemiluminescence

Proteins were transferred (80 v, 2 h) to PVDF membrane (Immobilon P, Millipore, Watford, UK) in 0.025 M Tris containing 0.192 M glycine and 20% (v/v) methanol. The membrane was allowed to dry and blocked overnight by shaking in PBS containing 0.45% (v/v) fish gelatin and 0.05% (v/v) Igepal. Antibodies were diluted in PBS containing 0.045% (v/v) gelatin and 0.05% (v/v) Igepal; this was also used as a wash solution. The membrane was incubated with the primary antiserum (Table 2.4) for 1 h and then washed 5 times. Biotinylated donkey anti-rabbit IgG (Amersham, Little Chalfont, UK), diluted 1:7000, was incubated with the membrane for 1 h followed by washes as before. Avidin-biotin complex (Dako Ltd, Ely, UK) was prepared according to the manufacturer's instructions and incubated with the membrane for 45 min. Protein bands were visualised by addition of enhanced chemiluminescent reagent (Renaissance; NEN DuPont, Stevenage, UK) and exposure to x-ray film (Kodak X-OMAT AR) for 30 s-10 min. Densitometry of blots was carried out using ImageQuant (Molecular Dynamics, Sunnyvale CA, USA).

2.9 PREPARATION OF PLASMID DNA FOR TRANSFECTION

2.9.1 Preparation of agar plates

Agar (1.5% w/v) was dissolved by autoclaving, in Luria-Bertani broth (LB broth; 1% (w/v) tryptone / 0.5% (w/v) yeast extract / 1% (w/v) NaCl). The solution was allowed to cool to <50°C before adding ampicillin (100 μg/ml). Agar plates were poured using aseptic technique and allowed to set. Unused plates were stored at 4°C.

Table 2.4 Antisera used in western blotting

Antigen	Dilution	Source
Sheep whey proteins	1:7000	Sigma, Poole, UK
Bovine α-lactalbumin	1:5000	PPL Therapeutics Ltd
Bovine β-lactoglobulin	1:3500	Prepared in-house
Goat α_{S1}-casein	1:7000	Prepared in-house
Human α_1-antitrypsin	1:5000	Dako Ltd, Ely, UK
Human growth hormone	1:5000	Dako Ltd, Ely, UK
Human protein C	1:5000	Sigma, Poole, UK

2.9.2 Preparation of competent cells

A single bacterial colony (*E. coli,* strain DH5α) was inoculated into 5 ml LB broth and cultured overnight (37°C, 220 rpm). 1 ml of the starter culture was used to inoculate 400 ml LB which was incubated (37°C, 220 rpm) until an OD_{600} of 0.4 was reached. Cells were pelleted (3000 *g*, 15 min, 4°C) and resuspended in 40 ml of ice-cold calcium chloride solution (10 mM Pipes pH 7.0 containing 60 mM $CaCl_2$ and 15% (v/v) glycerol). Cells were pelleted again (800 *g*, 10 min, 4°C), resuspended in 40 ml of ice-cold calcium chloride solution once again and incubated on ice for 30 min. Finally, cells were pelleted (800 *g*, 10 min, 4°C) and resuspended in 10 ml of ice-cold calcium chloride solution. The cells were then dispensed into 200 µl aliquots in pre-chilled tubes, snap frozen and stored at -70°C.

2.9.3 Transformation of competent cells with plasmid DNA

Competent cells were thawed on ice prior to use. Plasmid DNA (100 ng) was incubated with 100 µl of cells on ice for 20 min, followed by heat shock at 42°C for 30 s. LB broth (800 µl) was added, and the mixture was incubated at 37°C for 1 h. Cells (10% and 90% of total volume) were spread onto agar plates, using aseptic technique and incubated overnight at 37°C. Transformed cells were selected through ampicillin resistance.

2.9.4 Small-scale preparation of plasmid DNA

Plasmid DNA was prepared from small-scale bacterial cultures by a method adapted from Boom *et al* (1990). A single bacterial colony was inoculated into 2 ml LB broth containing ampicillin (100 µg/ml) and the culture was incubated overnight (37°C, 220 rpm). 1.5 ml of culture was poured into an Eppendorf tube and centrifuged (12,000 *g*, 1 min) to pellet the cells. All traces of supernatant were removed and the pellet was resuspended in 150 µl of 50 mM Tris HCl pH 7.5 containing 10 mM EDTA. Cells were then lysed by the addition of 150 µl of 0.2 M NaOH containing 1% (w/v) SDS, and gentle mixing by inversion. The solution was neutralised by adding 150 µl of 2.55 M potassium acetate pH 4.8 and mixed by inverting several times. The mixture was centrifuged (12,000 *g*, 15 min) to remove protein, RNA and chromosomal DNA. The supernatant, containing plasmid DNA, was mixed with 900 µl of 80 mM Tris HCl pH 6.4 containing 35 mM EDTA, 0.1 M

guanidinium thiocyanate and 2% (v/v) Triton X-100, and 50 µl of acidified diatomaceous earth (20% (w/v)), vortexed briefly and allowed to stand for 2 min. The mixture was mixed briefly, added to a Wizard minicolumn (Promega, Southampton, UK) and a vacuum was applied to draw the mixture into the column. DNA bound to the diatomaceous earth was washed with 10 mM Tris HCl pH 7.5 containing 100 mM NaCl, 2.5 mM EDTA and 50% (v/v) ethanol. The column was removed from the vacuum and centrifuged (12,000 *g*, 1 min) to remove remaining wash solution. After transfer of the column to a clean Eppendorf tube, plasmid DNA was eluted by the addition of 50 µl TE buffer (10 mM Tris HCl pH 7.5 containing 1 mM EDTA) and centrifugation (12,000 *g*, 1 min).

2.9.5 Large-scale preparation of plasmid DNA

Plasmid DNA was prepared from large-scale bacterial cultures by the method of Birnboim and Doly (1979). A single bacterial colony was inoculated into 5 ml LB broth containing ampicillin (100 µg/ml) and cultured for 5-6 h (37°C, 220 rpm). Following this initial incubation period, 1 ml of the starter culture was used to inoculate 500 ml LB/ampicillin which was incubated overnight (37°C, 220 rpm). The cells were pelleted by centrifugation (3000 *g*, 10 min, 4°C). The pellet was resuspended in 10 ml of 25 mM Tris HCl pH 8.0 containing 10 mM EDTA and 50 mM glucose, and incubated on ice for 15 min. Cells were lysed with 20 ml of 0.2 M NaOH containing 1% (w/v) SDS with gentle mixing, neutralised with 15 ml ice-cold 3 M potassium acetate and incubated on ice for 15 min to precipitate chromosomal DNA and proteins. The mixture was centrifuged (3000 *g*, 10 min, 4°C) and the supernatant containing plasmid DNA was filtered through 2 layers of muslin and precipitated with 0.6 volumes of isopropanol. The plasmid DNA was pelleted by centrifugation (5000 *g*, 15 min), air-dried and resuspended in 5ml of TE buffer. Caesium chloride (1 mg/ml) and ethidium bromide (400 µg/ml) were added. The solution was transferred to Optiseal tubes (Beckman, High Wycombe, UK) and centrifuged (360,000 *g*, 2 h 40 min, brake 0, 20°C). After centrifugation, two discrete bands could be seen. The lower band, containing the closed circular and supercoiled DNA was removed to sterile Eppendorf tubes using a needle and syringe. An equal volume of iso-amyl alcohol was added and the tube inverted several times, to extract the ethidium bromide from the plasmid solution. The

mixture was centrifuged (12,000 *g*, 5 min), the upper pink organic phase was discarded and the extraction repeated until the aqueous layer was clear (usually 4 times). After dilution with water the DNA was precipitated overnight at -20°C with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The DNA was pelleted by centrifugation (12,000 *g*, 20 min) and resuspended in 200 µl of sterile water. A second precipitation was carried out at -80°C for >30min and the DNA was pelleted by centrifugation (12,000 *g*, 30 min). The pellet was washed with ice-cold 70% ethanol, air-dried, and resuspended in TE buffer. DNA yield and purity were measured using a Genequant DNA calculator (Pharmacia Biotech, Cambridge, UK).

2.9.6 Restriction digest

Plasmid DNA was confirmed by restriction endonuclease digestion with the enzyme EcoR1. DNA (0.5-1.0 µg) was digested with 1 unit of enzyme in the buffer supplied with the enzyme in a 20 µl reaction. Digestion was for 2 h at 37°C.

2.9.7 Agarose gel electrophoresis

Restricted DNA (0.5-1.0 µg) was subjected to electrophoresis in 1% (w/v) agarose gel prepared in TAE buffer (40 mM Tris pH 8.0 containing 20 mM sodium acetate and 1 mM EDTA) with the addition of 1 µg/ml ethidium bromide. Electrophoresis was carried out for ~40 min in TAE buffer at 80mA. The gel was photographed under UV illumination.

2.10 TRANSFECTION

Goat mammary epithelial cells were transfected using a variety of transfection methods according to the manufacturers' instructions. Initially, optimal conditions for transient transfection were established using reporter constructs pCH110 (Pharmacia, Cambridge, UK), pGK-ßgal and pGK-luc (both kindly provided by PPL Therapeutics, Roslin UK). The optimal transfection conditions were determined by counting the number of cells positively staining for β-galactosidase (Section 2.11.1) or luciferase (Section 2.11.2) activity.

Further transfections were performed with a reporter construct under the control of the β-lactoglobulin promoter (pBLUC) and constructs designed to express α_1-antitrypsin (pAATB) and human protein C (pCORP14 and pCORI69), all provided by PPL Therapeutics, Roslin, UK. Constructs to express human growth

hormone under the control of the WAP promoter (WAP2-hGH and ΔNRE-hGH) were provided by Andreas Kolb, Hannah Research Institute, Ayr, UK.

2.10.1 Plating of cells

Goat cells were recovered from liquid nitrogen and allowed to proliferate on tissue culture plastic until 90% confluent (~5 d). Cells were then passaged (1:3) the day before transfection was to take place. The following day, the cells were approximately 60% confluent. Transfections were carried out in 35 mm wells in duplicate or triplicate.

2.10.2 Calcium phosphate

Cells were given fresh medium 2 h before transfection. DNA (1-5 μg) was added without mixing to water to a volume of 87.5 μl for each well. 12.5 μl of 2 M $CaCl_2$ was carefully added (final concentration 0.125 M). Finally an equal volume of HBSP buffer (50 mM Hepes pH 7.0 containing 1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl and 12 mM glucose) was slowly released into the mixture. The solution was mixed very gently by blowing bubbles through the mixture with a pipette, to avoid the formation of large precipitates. The calcium phosphate precipitate was allowed to form for 15 min at room temperature. The precipitate was mixed gently by pipetting once, and then added dropwise into the culture medium covering the cells, gently rocking the plate to disperse the mixture. Cells were incubated for 4 h at 37°C, after which the transfection mixture was replaced with fresh medium. Cells were assayed after 48 h.

2.10.3 Lipofectamine (Life Technologies Ltd)

DNA (range tested 0.5-3.0 μg) was diluted into 100 μl of medium. Media tested were serum-containing, serum-free and Opti-MEM® 1 reduced serum medium (Life Technologies Ltd, Paisley, UK), while 2-16 μl of Lipofectamine was diluted into another 100 μl of medium. The two solutions were combined very gently and incubated at room temperature for 45 min to allow DNA-liposome complexes to form. The cells were rinsed once with medium. 800 μl of medium was added to the complex, mixed gently and overlaid onto the rinsed cells. The cells were incubated for 2-24 h at 37°C, after which 1 ml of serum-containing medium was added to the

transfection mixture. Alternatively the transfection mixture was removed and replaced with fresh medium. Cells were assayed 48 h after transfection.

2.10.4 Fugene (Boehringer Mannheim)

Fugene was allowed to reach room temperature before use and 2-12 µl was diluted into 100 µl of serum-free or Opti-MEM medium. The diluted Fugene reagent was incubated at room temperature for 5 min. DNA (0.5-3.0 µg) was added to a second sterile tube and the diluted Fugene reagent added dropwise. The solutions were mixed by gently tapping the tube, and incubated at room temperature for 15 min to allow complexes to form. The cells were rinsed with PBS and 2 ml of fresh medium (serum-containing, serum-free or Opti-MEM) was applied. The DNA complex was then added dropwise to the medium and dispersed by gently rocking the plate. The cells were incubated for 48 h until time of assay.

2.10.5 Superfect (Quiagen)

DNA (1.0-4.0 µg) was diluted in 100 µl serum-free or Opti-MEM medium. Superfect reagent (4-20 µl) was added to the diluted DNA and mixed by pipetting up and down. The mixture was incubated for 10 min at room temperature to allow complex formation. The cells were rinsed with PBS, after which 2 ml of serum-containing medium (or Opti-MEM) was added to the transfection complexes, mixed by pipetting up and down, and transferred to the cells. Initially, the cells were incubated with the complexes for 3 h, according to the manufacturer's instructions, after which the mixture was replaced with fresh medium, but in later experiments the cells were incubated for 48 h until time of assay.

2.11 SELECTION OF TRANSFECTED CELLS

2.11.1 G418 selection

Cells were transfected (as described in Section 2.10) using constructs containing the *neo* gene, which confers resistance to the antibiotic geneticin (G418). The cells were then passaged into selective medium containing G418 (range tested 0.1-1.0 mg/ml) 48 h after transfection.

2.11.2 Selection using Capture-Tec™ (Invitrogen)

This system utilises a specially designed vector (pHook™-1) that expresses and displays a single-chain antibody (sFv) against a specific hapten on the surface of transfected cells. Cells expressing the sFv can then be isolated from the culture by binding to hapten-coated magnetic beads.

Goat mammary epithelial cells were cotransfected with a 1:1 ratio of pHook and a β-galactosidase reporter construct (pcDNA 3.1, pCH110 or pGK-βGAL) or other construct of interest. The cells were harvested 2-48 h after transfection with PBS containing 3 mM EDTA, pelleted (80 *g*, 5 min) and resuspended in 1 ml culture medium. A 1 µl aliquot of magnetic beads (1.5 x 10^5 beads) was washed with 1 ml of culture medium by inversion three times. The beads were harvested using a magnetic stand and aspiration of the medium. The cell suspension was added to the beads and incubated for 30 minutes at 37°C on a slow rotator (5-10 revolutions per minute). To select the cells, the tubes containing the bead-cell mixture were placed in a magnetic stand and mixed for 1 minute with gentle end-over-end rotation. The non-selected cells were removed with a pipette. The tubes were removed from the magnetic stand and the bead-cell mixture resuspended in 1 ml of culture medium. Bound cells were then pelleted using the magnetic stand, and the supernatant removed. This wash step was repeated twice more. Selected and non-selected cells transfected with a reporter gene were resuspended in 100 µl of X-Gal staining solution (see Section 2.12.1) and incubated at 37°C overnight. Stained and total cell number were determined using a haemocytometer. Alternatively, selected and non-selected cells containing the gene of interest were resuspended in culture medium and plated onto tissue culture plastic or EHS matrix.

2.12 REPORTER GENE ASSAYS

2.12.1 ß-galactosidase

Transfected cells were washed twice with PBS and fixed in PBS containing 2% (w/v) paraformaldehyde for 20 min. The cells were rinsed three times with PBS to remove any residual fixative, which inhibits β-galactosidase activity. Fixed cells were incubated overnight (37°C) in staining solution (20 mM $K_3Fe(CN)_6$ / 20 mM $K_4Fe(CN)_6.3H_20$ / 2mM $MgCl_2$ / 1mg/ml X-Gal). Cells were rinsed in PBS and stored at 4°C under fresh PBS. The number of positive blue cells was counted in

>50 fields of view, and the number of blue cells/well was calculated using the dimensions of the counting frame. Total cell number was estimated using DNA assay, and transfection efficiency calculated as (no. blue cells/total cell number) x 100.

2.11.2 Luciferase

Transfected cells were washed twice with PBS and incubated with 100 µl cell lysis reagent (Promega) for 15 min at room temperature. Remaining adherent cells were scraped from the dish, and both cells and solution transferred to a sterile Eppendorf tube. Large debris was pelleted (12,000 *g*, 1 min) and known volumes of supernatant transferred to clean tubes for assay or storage at -70°C. Luciferase assay reagent (50 µl) was added to 20 µl of sample and the light emission read immediately (Cerenkov counts, 1 min) in a 1600TR liquid scintillation analyser (Canberra Packard, Meriden, USA).

2.13 STATISTICS

Results were compared by Student's t-test or ANOVA as appropriate, using Minitab (release 8). The fractional factorial experiment was designed and analysed using Minitab (release 10).

CHAPTER THREE
CHARACTERISATION OF CELLS ISOLATED FROM OVINE MAMMARY TISSUE

3.1 INTRODUCTION

There has been much investigation into the control of mammary function in the mouse both *in vivo* and *in vitro*, yet comparatively little is known about ruminants, despite their economic importance. The populations of milk proteins secreted, and their regulation, are very different in mice and dairy animals, making extrapolation of observations between species unsound. Studies *in vivo* in livestock species give rise to practical constraints in terms of animal manipulation and welfare, as well as financial constraints restricting animal numbers and making statistical significance difficult to demonstrate. This means that an *in vitro* system for the study of mammary gland function would be extremely useful. The specific aims of the work described in this chapter were:

i) to produce primary cultures of ovine mammary epithelial cells;
ii) to confirm the epithelial nature of the cells isolated;
iii) to determine the growth characteristics of isolated cells on tissue culture plastic.

3.2 RESULTS

3.2.1 Isolation of cells

Cells were isolated from ovine mammary tissue by enzymatic digestion with collagenase and hyaluronidase resulting in an average cell yield of 3.9 ± 0.7 x 10^7/g tissue (n = 11 ± SEM). Cell viability was found to be >90% by trypan blue exclusion. Density gradient centrifugation of the resulting cell suspension produced four discrete bands, which were designated fractions 1-4. Fraction 1 was harvested from the interface of densities 1.01- 1.02 g/ml and consisted of 1-2% of the total cell number, while fraction 2 was harvested at the interface of densities 1.02-1.03 g/ml and comprised 2-4% of total cell number. The largest band accounted for 85-90% of the total cell number and appeared at a density of 1.03-1.04 g/ml

(fraction 3). The remainder of the cells, comprising 6-8% of the total, sedimented at a density of 1.06 g/ml (fraction 4).

Fractions 1, 2 and 4 were discarded in later cell preparations. Occasionally, however, if fraction 2 was large, this was combined with fraction 3 to give a large, uniform batch of cells for cryopreservation and future experiments.

3.2.2 Microscopic observations of isolated cell fractions

Each of fractions 1-4 was plated onto tissue culture plastic, and photographed using phase contrast microscopy at plating (day 0) and following three days of proliferation (Figure 3.1). Fraction 1 consisted of debris and single cells, and proliferation was negligible. Fraction 2 contained single cells and small clumps, while fraction 3 contained mainly cell clumps of approximately 50 cells; both of these fractions grew well in culture. Fraction 4 contained larger cell clumps with some debris and residual Percoll, which may have been contributory factors to the slow growth of cells in this fraction.

In all but fraction 1, isolated cell clumps attached to tissue culture plastic within 24 h. Cells then proliferated around the perimeter of these clumps, forming small colonies, which continued to grow until confluency was reached. Although the majority of the cells showed the typical cuboidal appearance of epithelial cells, other elongated or stellate cells were evident suggesting a heterogeneous cell population (Figure 3.2).

3.2.3 Determination of ovine mammary cell types in primary culture

Freshly prepared cells from fractions 1-4 were plated onto chamber slides at equal cell densities, allowed to proliferate and fixed (described in section 2.6). Cell typing was carried out using the markers shown in Table 2.3, and visualised using fluorescence microscopy. Positive staining was identified by image analysis of cells *in situ* (10 fields of view) and this was expressed as a proportion of total cell number, determined by counterstaining with DAPI nuclear stain. Cell typing was performed on cells from three animals.

Results of immunocytochemical staining for all fractions are shown in Table 3.1. Cells in fraction 1 did not proliferate, and many of the single cells that had appeared to be attached were removed during the fixation procedure, thus, attempts to characterise the cells in this fraction were unsuccessful. Fractions 2, 3

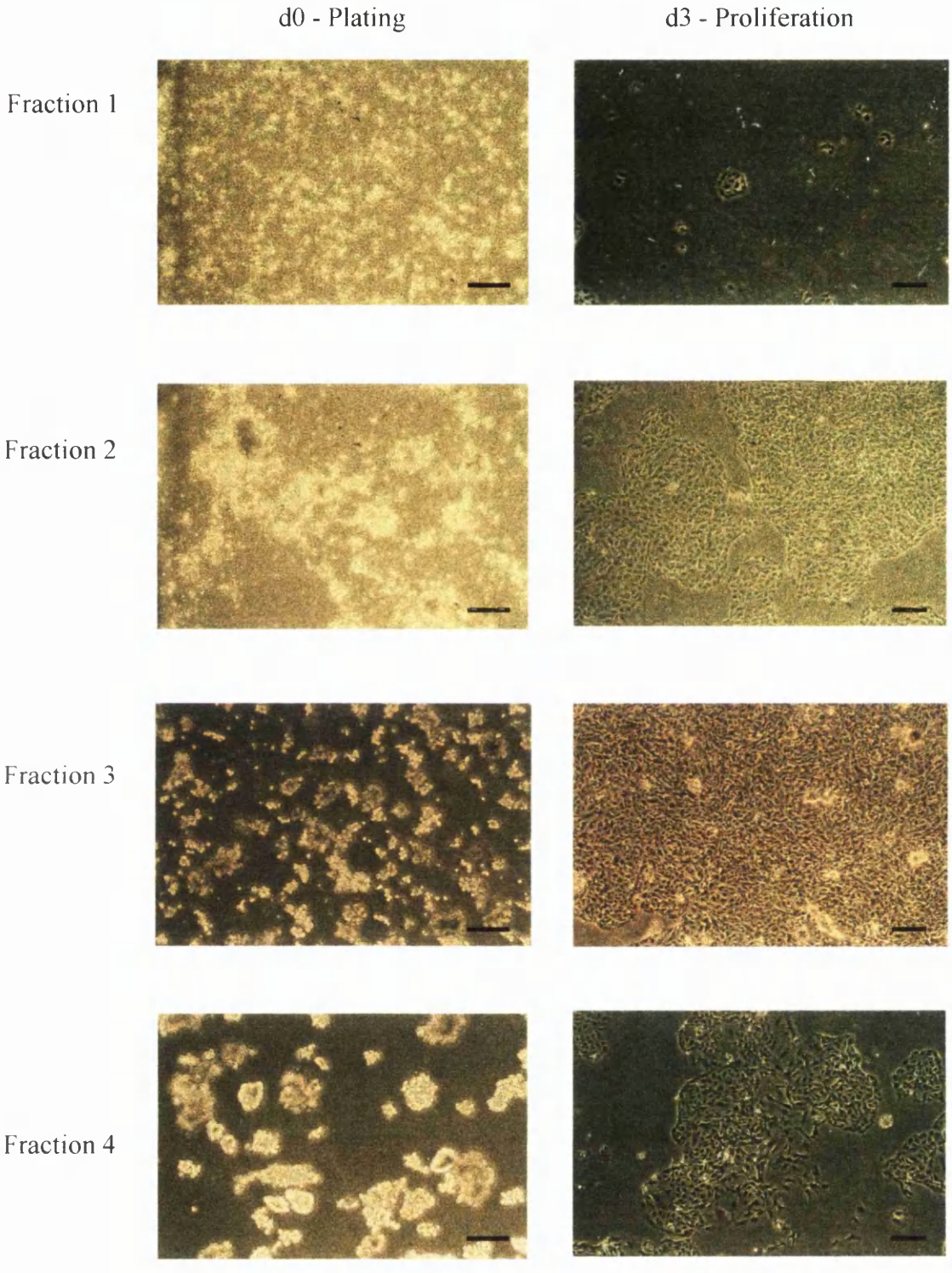


Figure 3.1 Microscopic appearance of isolated cell fractions
Isolated ovine mammary epithelial cells were subjected to density gradient centrifugation and each of the resulting bands, designated fractions 1-4, were plated onto tissue culture plastic. Cells were photographed at plating (d0) and following proliferation (d3) using phase contrast microscopy.
d0: Mag 61.5x, Bar = 100 μm; d3 Mag 24.6x, Bar = 200 μm

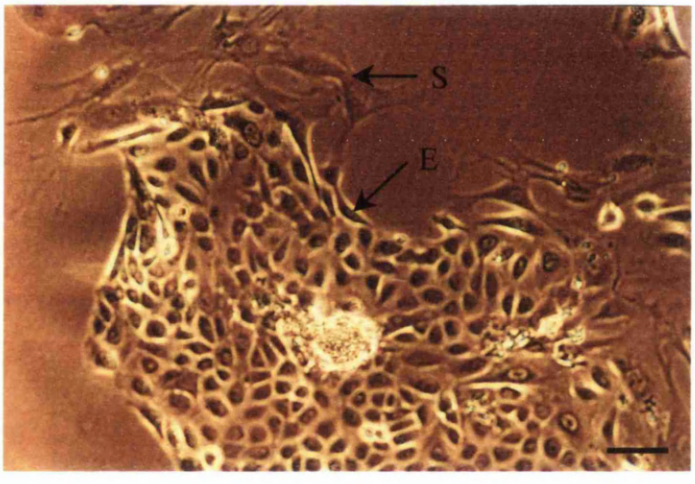


Figure 3.2 Proliferation of ovine mammary epithelial cells
Cells proliferated from a central attached clump. In addition to the typical cuboidal epithelial cells, elongated (E) and stellate (S) cells were evident.
Mag 75x, Bar = 100 μm

Table 3.1 Characterisation of cells isolated from ovine mammary tissue
Density gradient centrifugation of isolated ovine mammary cells resulted in 4 fractions. Each of these fractions was characterised by immunostaining for a number of markers. The number of positively staining cells was determined as a percentage of total cell number for 10 fields of view. Results are means ± SEM for 3 animals. nd = not determined

Fraction	Pan-CK	CK18	CK14	Actin	Myosin	Vimentin
1	nd	nd	nd	nd	nd	nd
2	96% ± 3	97% ± 2	12% ± 2	13% ± 3	8% ± 5	17% ± 6
3	98% ± 2	99% ± 1	15% ± 7	9% ± 4	12% ± 3	14% ± 6
4	90% ± 8	94% ± 4	12 % ± 4	13% ± 2	10% ± 2	22% ± 3

and 4 comprised similar cell type distributions, with >90% staining for pan-cytokeratin or cytokeratin 18, and approximately 12% staining for cytokeratin 14, actin and myosin. Staining for vimentin increased slightly in fraction 4 suggesting a greater proportion of fibroblasts in that fraction. Figures 3.3 and 3.4 show the staining patterns of fraction 3 cells for pan-cytokeratin and cytokeratin 14 respectively.

3.2.4 Cryopreservation and recovery of ovine mammary epithelial cells

Mammary epithelial cells were cryopreserved in foetal calf serum containing 10% (v/v) DMSO and stored in liquid nitrogen. Large stocks of cells ensured consistent starting material for a number of future experiments. On recovery, 30-50% cells remained viable as determined by trypan blue exclusion.

3.2.5 Proliferation of ovine mammary epithelial cells

Ovine mammary epithelial cells (fraction 3) were plated at an estimated density of 5×10^4/well (16 mm diameter) with a medium change each day. At 24 h intervals, cells were fixed in methanol and assayed spectrophotometrically, using the methylene blue method of Oliver *et al* (1989), at the end of the experimental period. Six replicates were assayed for each time point.

The growth curve obtained for these cells is shown in Figure 3.5. The generation time of these cells was 36 h and they attained confluence within 5-6 days. The final cell number was 6×10^5 cells per well.

3.2.6 Effect of serum concentration on cell attachment

Cell clumps attached to tissue culture plastic within 24 h when freshly prepared, but took 48 h or more after recovery from liquid nitrogen. After passaging, however, cell attachment was seen to be very rapid, and this was utilised to study the effect of serum concentration on cell attachment.

Passage 1 cells were resuspended and plated onto tissue culture plastic in serum-free culture medium. Different quantities of serum were subsequently added to final concentrations of 1%, 10% and 25% (v/v). The ratio of horse serum:foetal calf serum was 4:1 at all concentrations. The cells were fixed in methanol at various times from 20 min to 24 h after plating and cell number was determined using the methylene blue assay, at the end of the experimental period. Four replicates were assayed for each time point.

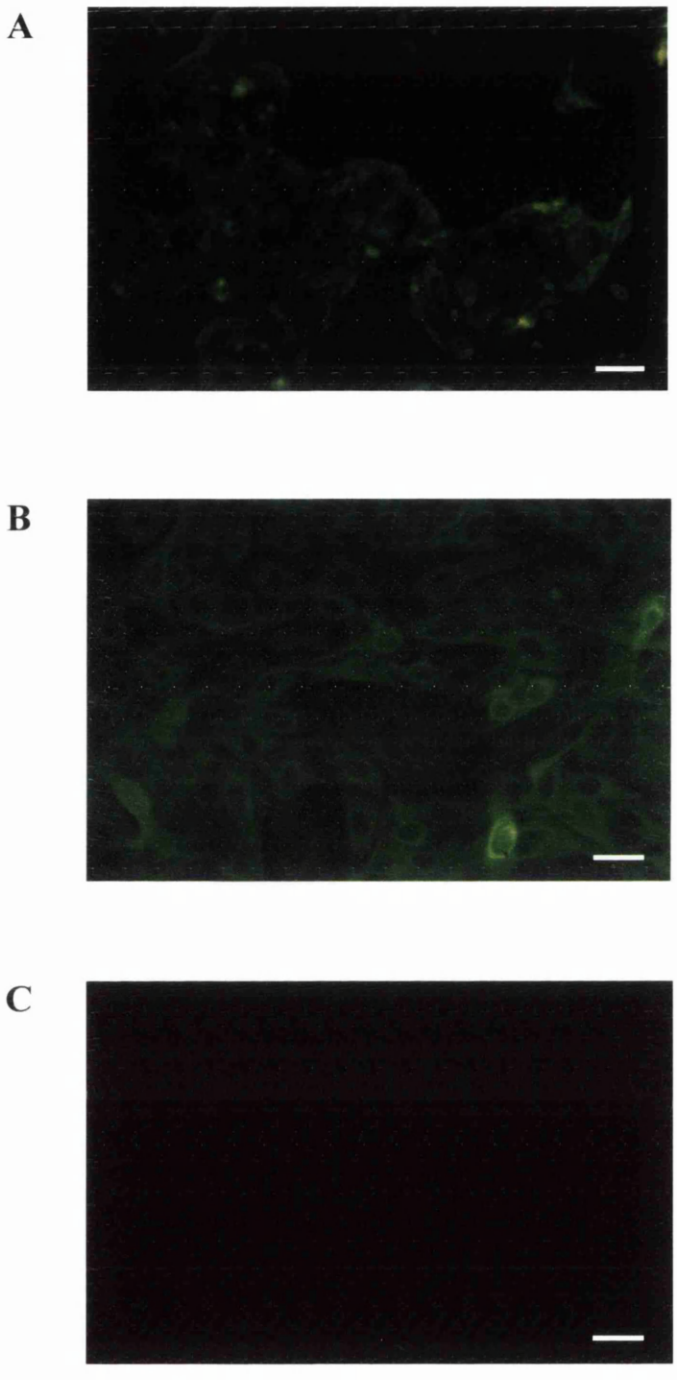


Figure 3.3 Immunostaining of ovine mammary cells for pan-cytokeratin
>98% of fraction 3 ovine mammary cells were epithelial in nature as shown by immunostaining for pan-cytokeratin (cytokeratins 5, 6 & 18). Cells were visualised by fluorescence microscopy at magnification of 66x (A) and 275x (B,C). Controls (C), without primary antibody, showed little staining. A: Bar = 100 μm; B,C: Bar = 25 μm

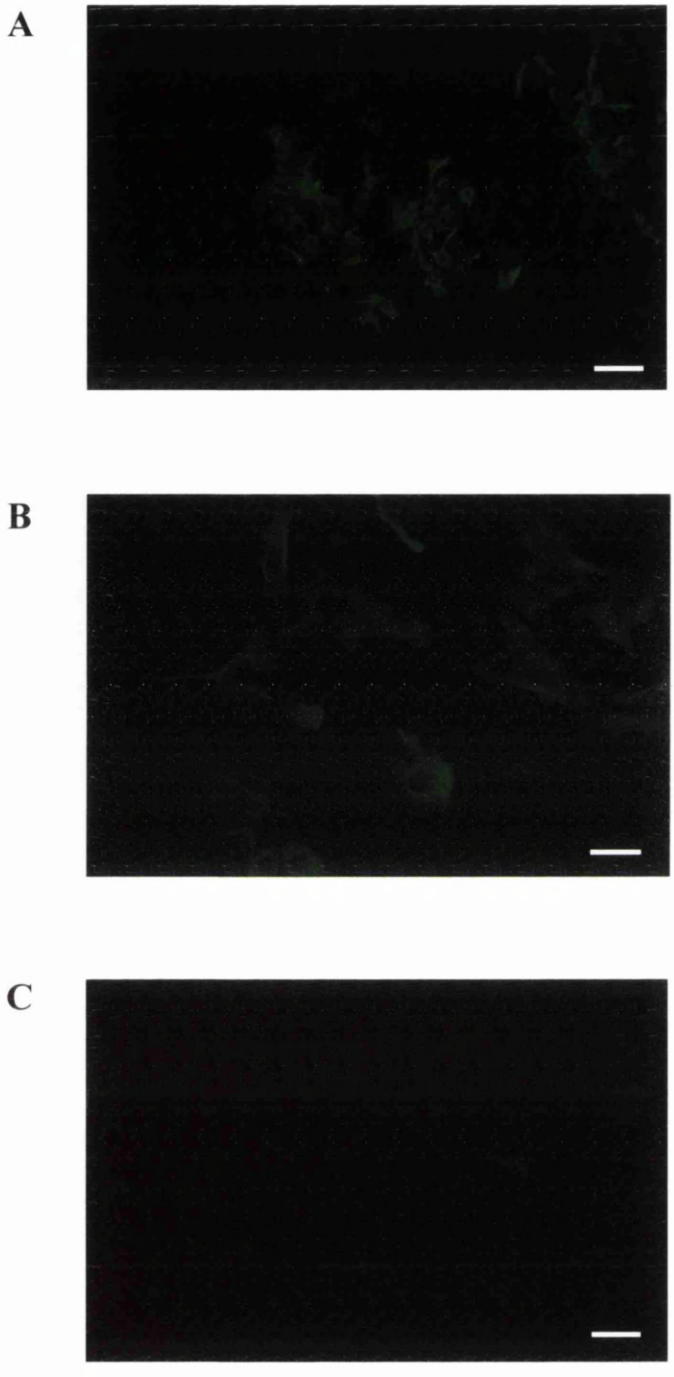


Figure 3.4 Immunostaining of ovine mammary cells for cytokeratin 14
Approximately 15% of ovine mammary cells were myoepithelial in nature as shown by immunostaining for cytokeratin 14. Cells were visualised by fluorescence microscopy at magnification of 66x (A, C) and 275x (B). Control (C), without primary antibody, showed little staining. A, C: Bar = 100 μm; B: Bar = 25 μm

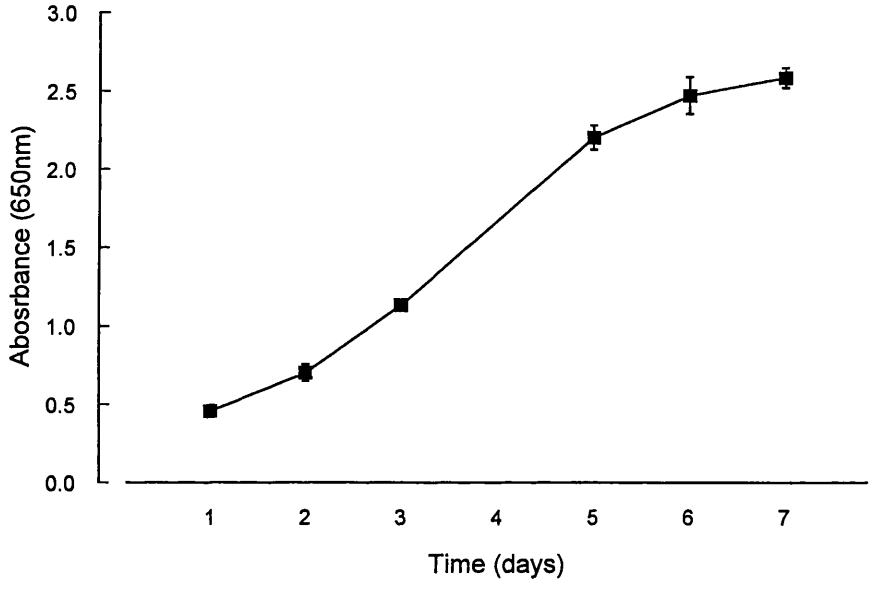


Figure 3.5 Growth curve for ovine mammary epithelial cells
Ovine mammary epithelial cells (fraction 3) were fixed in methanol on each day, stained using methylene blue and assayed spectrophotometrically. Results are mean ± SD for 6 replicates

After passaging, cell attachment began within 20 min at all serum concentrations and was maximal at 4-6 h, when the first signs of proliferation were observed. These results are illustrated in Figure 3.6. Statistical analysis (ANOVA) showed a significant difference between treatments ($p<0.05$).

3.2.7 Effect of serum concentration on cell proliferation

Ovine mammary epithelial cells were plated at equal cell density in medium containing 25% serum in order to allow attachment to take place (6 h). The medium was subsequently changed to test medium containing different concentrations of serum (1%, 10% or 25%). The proportions of HS:FCS were 4:1 at each concentration. The cells were fixed in methanol on day 2 and day 4 and assayed with methylene blue. Four replicates were assayed for each time point.

Analysis of variance showed significant treatment effects at both timepoints ($p<0.05$). At day 2 proliferation was significantly greater in 25% serum than in 1% serum ($p<0.05$, Student's t test). At day 4 there was no significant difference between the 10% serum and the 25% serum treatments, but proliferation was significantly less in 1% serum ($p<0.01$, Student's t test). These results are illustrated in Figure 3.7.

These results complemented experiments carried out by a colleague, which determined that the 4:1 combination of horse serum:foetal calf serum was optimal. In addition, there was shown to be a specific requirement for horse serum, except at very high levels of foetal calf serum (25%). Bovine serum albumin could not substitute for serum and higher concentrations had a deleterious effect on cell proliferation.

3.2.8 Passaging ability of cells

Confluent cells were harvested for passaging by pre-treatment for 5 min with Versene, which chelates Ca^{2+} ions, followed by treatment with trypsin until the majority of cells became detached from the surface. The cells were washed in culture medium and initially plated out at 2x, 4x and 8x dilutions. Microscopic observations over 4 days showed that cells plated at 2x and 4x dilutions became confluent within 24-48 h, while cells plated at an 8x dilution were sparsely distributed and grew poorly. This suggested that a 6x dilution may be optimal for

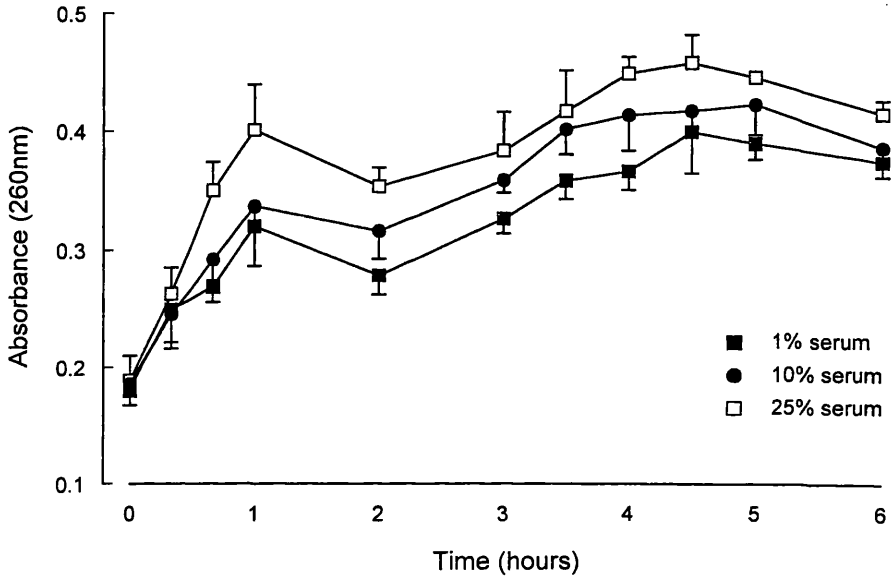


Figure 3.6 Effect of serum concentration on cell attachment
Cells were plated in proliferation medium at different concentrations of serum (HS:FCS = 4:1) and fixed in methanol at each time point. Cell number was assayed by staining with methylene blue and spectophotometry.
Results are means ± SD for 4 replicates at each time point.

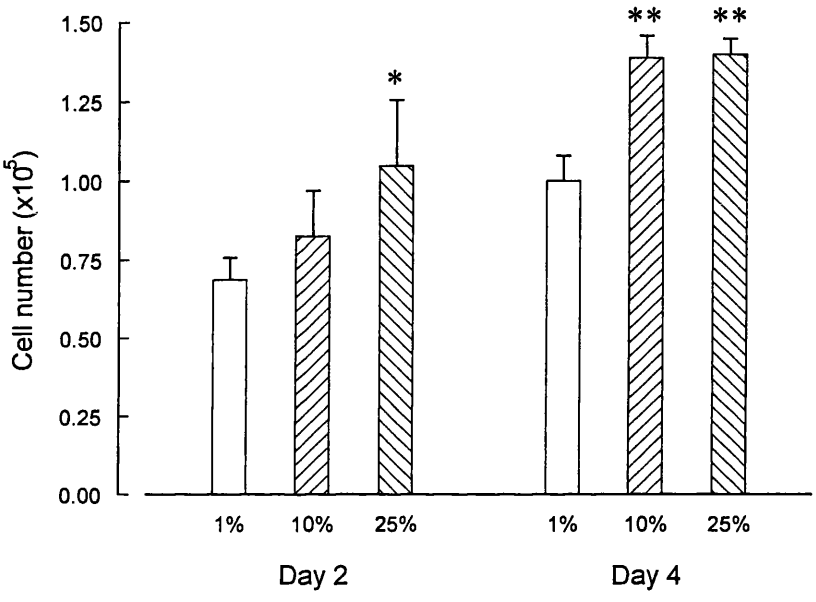


Figure 3.7 Effect of serum concentration on cell proliferation
Cells were plated at equal cell density and proliferated in medium containing different serum concentrations (HS:FCS = 4:1). Cells were fixed in methanol at day 2 and day 4 of culture, stained using methylene blue and assayed spectrophotometrically. Values are the mean ± SD for 4 determinations.
*$p<0.05$, **$p<0.01$ compared with 1% serum (ANOVA).

passaging of ovine mammary cells and this was routinely used in subsequent experiments.

A comparison of freshly prepared cells with cryopreserved cells (Table 3.2) showed that there was no significant difference in cell proliferation to passage 4. The difference in time to confluence before passaging was entirely contributable to the slower attachment time of cells recovered from liquid nitrogen. Time to confluence was significantly increased, however, in passages 5 and 6 ($p<0.05$, Student's t test). From passage 4, increasing amounts of very large cells were observed. These cells were often seen at the edges of a colony and appeared to inhibit proliferation.

3.2.9 Determination of ovine mammary cell types in passaged cells

Fraction 3 cells (both fresh and cryopreserved) were passaged 5 times and immunostaining was carried out for the markers pan-cytokeratin, cytokeratin 14, actin and vimentin. The results are shown in Table 3.3. After re-seeding on plastic, passage 1 cells showed a decrease in cell staining for cytokeratin 14, actin and vimentin. At passages 2 and 3, staining for these markers was further reduced, and no cells stained positive for actin. With passaging, an increasing number of large cells were unstained by any marker (Figure 3.8). All of the remainder showed positive staining for pan-cytokeratin indicating their epithelial nature, but intensity of fluorescence decreased with passage number.

3.3 DISCUSSION

Cells isolated from ovine mammary tissue were shown to be >90% viable, a value similar to those obtained previously for caprine (Hansen *et al*, 1986) and bovine (Zavizion *et al*, 1996; Delabarre *et al*, 1997) cells. Density gradient centrifugation of isolated cells gave a consistent pattern of bands, although the proportions of fractions 2 and 3 varied on rare occasions. Fraction 1 consisted mainly of cell debris resulting from the collagenase digestion, and a few single cells, which did not proliferate. Fraction 4 also contained debris in the form of residual Percoll granules, which may have been a contributory factor to the slower growth of this fraction. Fractions 2 and 3 both proliferated well and were indistinguishable microscopically, although fraction 3 usually predominated. In each of fractions 2-4, microscopic

Table 3.2 Passaging of fresh and cryopreserved ovine mammary cells
Cells were plated in proliferation medium at a density of 1 x $10^5/cm^2$ for freshly prepared cells, 3 x $10^5/cm^2$ following recovery from liquid nitrogen, or 5 x $10^4/cm^2$ after passaging. Results shown are time required (days) for cells to reach confluence.
Values are the mean ± SEM for 3-4 determinations.

	Passage number						
	0	1	2	3	4	5	6
Fresh cells	3.33 ± 0.33	3.33 ± 0.33	5.00 ± 1.00	5.00 ± 0.58	9.67 ± 1.76	9.00 ± 0.58	9.00 ± 0.00
Cryopreserved cells	5.00 ± 0.58	3.33 ± 0.33	5.25 ± 0.48	5.00 ± 0.63	6.33 ± 0.33	5.67 ± 0.33	6.00 ± 0.00

Table 3.3 Characterisation of cells after passaging

Fraction 3 cells were passaged 5 times, fixed *in situ* and characterised by immunostaining for the markers shown. The number of positively staining cells was determined as a percentage of total cell number for 10 fields of view. Results are means ± SEM for 5 animals.

Passage	Pan-CK	CK14	Actin	Vimentin
0	98% ± 2	15% ± 7	9% ± 4	14% ± 6
1	98% ± 2	7.4% ± 2.1	3.9% ± 1.6	3.5% ± 1.5
2	98% ± 2	1.2% ± 0.7	0%	7.3% ± 3.0
3	98% ± 2	1% ± 0.6	0%	5% ± 2.1
4	100%[1]	0%	0%	0%
5	100%[1]	0%	0%	0%

[1]Signal intensity declining although still greater than controls

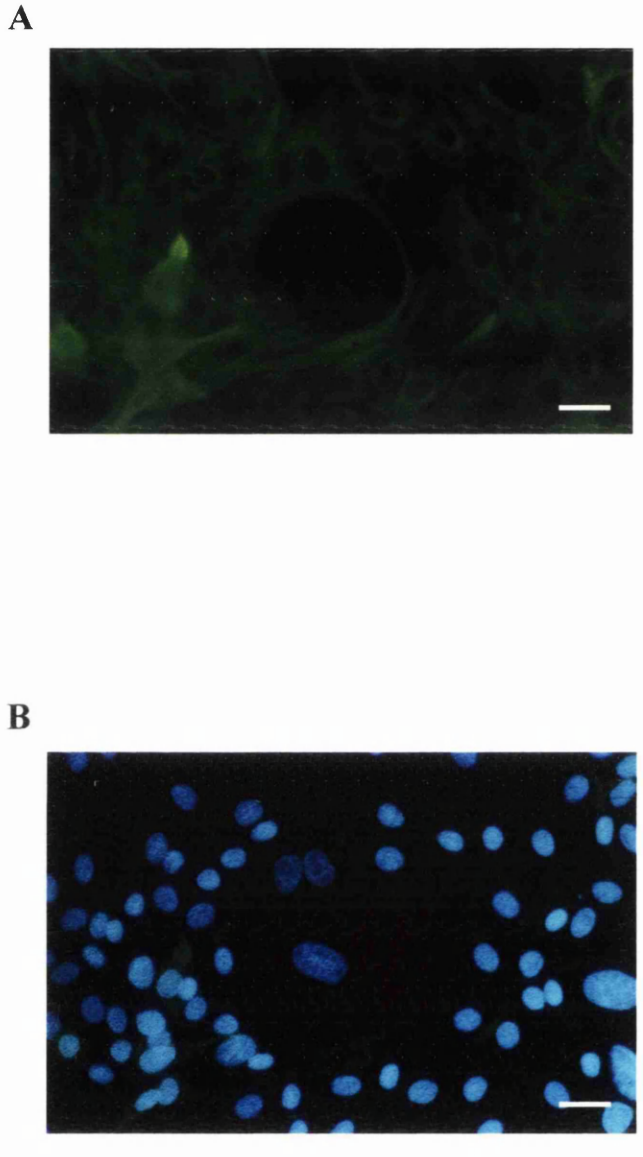


Figure 3.8 Large cytokeratin-negative cells
Immunostaining of ovine mammary epithelial cells for pan-cytokeratin (A) and counterstaining with DAPI nuclear stain (B) revealed occasional non-staining large cells, which increased in number with passage.
Mag 275x, Bar = 25 μm

observations suggested that a heterogeneous cell population was present and this was confirmed by immunocytochemical staining.

Cell typing was carried out on each of the fractions using markers that have previously been shown to stain mammary cells in rodent (Warburton *et al*, 1982), and human (Rudland & Hughes, 1989; Rudland 1991) mammary tissue. Subsequent work however, has demonstrated that the marker cytokeratin 18 also stains for myoepithelial cells in the goat, and that cytokeratin 7 is specific for epithelial cells (Li *et al*, 1999). The cell typing showed that fractions 2-4 comprised similar cell type distributions. The formation of discrete bands during gradient centrifugation, therefore, was probably due to the sizes of the cell clumps which increased with increasing fraction number. Since fraction 4 contained the largest clumps, it is likely that it contained proportionately higher amounts of connective tissue. This is suggested by the greater staining for vimentin, and the slightly reduced staining for pan-cytokeratin. Thus, in addition to the presence of debris, larger clump size may have contributed to the slower growth of this fraction. As a result of these observations, fractions 1 and 4 were discarded in later preparations. Fraction 2 usually only accounted for a very small percentage of the total cells isolated, but occasionally fractions 2 and 3 accounted for approximately 40-45% each, and in this instance these bands were combined, given that there were no discernible microscopic or immunocytochemical differences. The pattern of immunostaining determined for fraction 3 agrees with that obtained for isolated bovine cells (Zavizion *et al*, 1996).

Cryopreservation resulted in substantial cell losses (50-70%), as was also found with rabbit (Haeuptle *et al*, 1983) and goat (Hansen *et al*, 1986) cells. Compared with fresh cells, attachment time was longer by approximately 24 h following recovery. This agrees with the findings of Talhouk *et al* (1990), who reported that bovine cells plated after cryopreservation showed a 40-50% decrease in attachment. However, cryopreservation did not appear to be detrimental to proliferation of surviving cells on tissue culture plastic, and from the passaging information (Table 3.3), may even select for more viable cells.

Attachment and proliferation of ovine mammary epithelial cells were influenced by serum concentration in a dose dependent manner. No combination of horse serum and foetal calf serum tested was significantly better than the standard medium of 20% horse serum and 5% foetal calf serum, and cells were grown on

tissue culture plastic under these conditions for all future experiments. There was a specific requirement for horse serum, except at very high levels (25%) of foetal calf serum. It was hypothesised that one or more growth factors essential for the proliferation of ovine mammary epithelial cells may occur in the serum of the adult animal, but be absent in that of the foetus. Bovine serum albumin could not substitute for serum in promoting cell division.

Under these established conditions for cell attachment and growth, mammary epithelial cells were passaged up to 5 times. In all passages investigated, 100% of the cells stained for pan-cytokeratin indicating their epithelial nature, but intensity of fluorescence decreased with passage number, suggesting loss of cell antigen. At the same time, there was a decline in staining for the markers cytokeratin 14, actin and vimentin, suggesting the gradual loss of myoepithelial and fibroblastic cell types. This was also the experience of Ehmann *et al* (1984) who found that fibroblasts disappeared by passage 3 in cultures of mouse mammary epithelial cells. Cell proliferation rate of ovine mammary epithelial cells also decreased with passage number, and the appearance of large, cytokeratin-negative cells suggested that a proportion of cells were becoming senescent. These cells were frequently found at the periphery of a colony, particularly from passage 4 onwards, and these colonies failed to proliferate further. This phenomenon has also been reported for myoepithelial cells isolated from pregnant cows: Zavizion *et al* (1992) reported the appearance of non-dividing cells, 10-20 times larger than normal cells, after passage 3. It is well established that primary cells have a finite lifespan and it has been determined that bovine mammary epithelial cells normally undergo 20-25 population doublings before they enter crisis (Zavizion *et al*, 1996). Ovine mammary cells at passage 5 will have been cultured for approximately 30 days (Table 3.2), and with a doubling time of 36 h, will have undergone 20 cell doublings, supporting this finding.

3.4 CONCLUSIONS

Viable cells can be isolated from ovine mammary tissue by digestion with collagenase and hyaluronidase. Density gradient centrifugation of the resulting cell suspension produces a consistent pattern of bands. Of these, fractions 2, 3 and 4, are predominantly epithelial in nature, but contain small amounts of contaminating myopepithelial and fibroblastic cells, which are removed by passsaging. From

passage 4 onwards, there is an increasing number of large cytokeratin-negative cells, which do not divide.

Cells can be frozen and recovered from liquid nitrogen, although cell losses are high. Cell attachment in surviving cells is slower than in freshly prepared cells, but otherwise cryopreservation appears to have no adverse effects on cell proliferation, and may even select for more viable cells.

Cultured ovine mammary epithelial cells respond to increasing amounts of serum in a dose dependent manner. Horse serum is an essential component, and BSA can not substitute for serum.

CHAPTER FOUR

DIFFERENTIATION OF OVINE MAMMARY EPITHELIAL CELLS

4.1 INTRODUCTION

Results in Chapter 3 showed that cells could be isolated from ovine mammary tissue, enriched for an epithelial cell population, and grown successfully. However, an *in vitro* model of mammary gland function requires that cells can be induced to differentiate, and should be responsive to lactogenic hormones. It is well established that substratum is crucially important for the differentiation of mouse mammary epithelial cells (Emerman & Pitelka, 1977; Li *et al*, 1987; Barcellos-Hoff *et al*, 1989; Aggeler *et al*, 1991), and that milk protein synthesis and secretion are maximal on complex substrata that closely resemble the basement membrane *in vivo* (Wicha *et al*, 1982; Lee *et al*, 1984, 1985; Blum *et al*, 1987; Chen & Bissell, 1989; Hurley *et al*, 1994). Substratum also mediates the response to lactogenic hormones. Mouse cells on plastic or collagen retain the ability to respond to prolactin, but induction of both mRNA and protein is greatest on EHS matrix (Lee *et al*, 1985).

As this project began, studies of ruminant milk protein secretion *in vitro* had been limited to cells cultured on collagen substrata (Mackenzie *et al*, 1982, 1985; Talhouk *et al*, 1990; Hansen & Knudsen, 1991; Winder *et al*, 1992). Meanwhile, preliminary work carried out at the Hannah Research Institute, suggested that goat mammary epithelial cells cultured on EHS matrix behaved in a similar manner to murine cells (Finch *et al*, in preparation). The work in this chapter was intended to assess the potential of ovine mammary epithelial cells cultured under similar conditions and so define the limitations of the EHS culture system. Specifically, the aims of the research were:

i) to determine whether cells isolated from pregnant ovine mammary tissue can be induced to differentiate;

ii) to determine if the cells are prolactin and/or substratum dependent;

iii) to assess the effects of cryopreservation on cell performance;

iv) to investigate the effects of passaging on ability to differentiate;

v) to determine if an *in vitro* system is capable of secreting foreign proteins.

4.2 RESULTS

4.2.1 Formation of mammospheres by ovine mammary epithelial cells

Freshly-prepared cells were seeded directly onto EHS matrix (section 2.4.5) at a density of 3 x 10^6 cells per 35 mm well, and stimulated with lactogenic hormones (insulin, hydrocortisone, prolactin). Cryopreserved cells were transferred to EHS matrix after proliferation on tissue culture plastic to remove non-viable cells. Serum was present in the culture medium for the first 24 h on EHS matrix to aid cell attachment. Morphology was studied using phase contrast microscopy.

At plating on EHS matrix, the cells were evident as randomly distributed clumps of about 20-100 cells (Figure 4.1A). These cell clumps attached to the matrix, but did not flatten or proliferate to form a monolayer, as seen on tissue culture plastic (Figure 3.2). Within the first 24 h, the cells became interconnected by fine but prominent processes, and the cell clumps became more evenly spaced (Figure 4.1B). Occasionally, single cells were observed at points along these connections (Figure 4.1B, arrowed). After 72 hours of culture, the processes were usually no longer visible.

The EHS matrix itself appeared to undergo remoulding by the cells. At plating, it had an even, grainy, appearance, but following attachment of the cells, the graininess became much denser in the areas surrounding the cell clumps. By day 3, clear stress lines in the material were also evident (Figure 4.1C), while individual cells were indistinguishable where the clumps had become enshrouded. By day 4-6, the appearance of a dark area in the centre of the structure suggested the formation of a lumen (Figure 4.1D). At this stage, the mammospheres had a discrete, spherical, appearance, and were equidistantly spaced (Figure 4.1E). Large clumps occasionally formed double (Figure 4.1E, arrowed) or multiple lumina. Very few single cells were apparent on EHS matrix, the majority appearing to be recruited into mammosphere formation.

Collapse of the mammospheres was characterised by a flattening of the structure, and proliferating cells radiating out from it (Figure 4.1F). This event was the most variable in terms of timescale, occurring as early as day 8 in a few cultures, while in others, mammospheres remained intact until day 21. Collapse always began with the

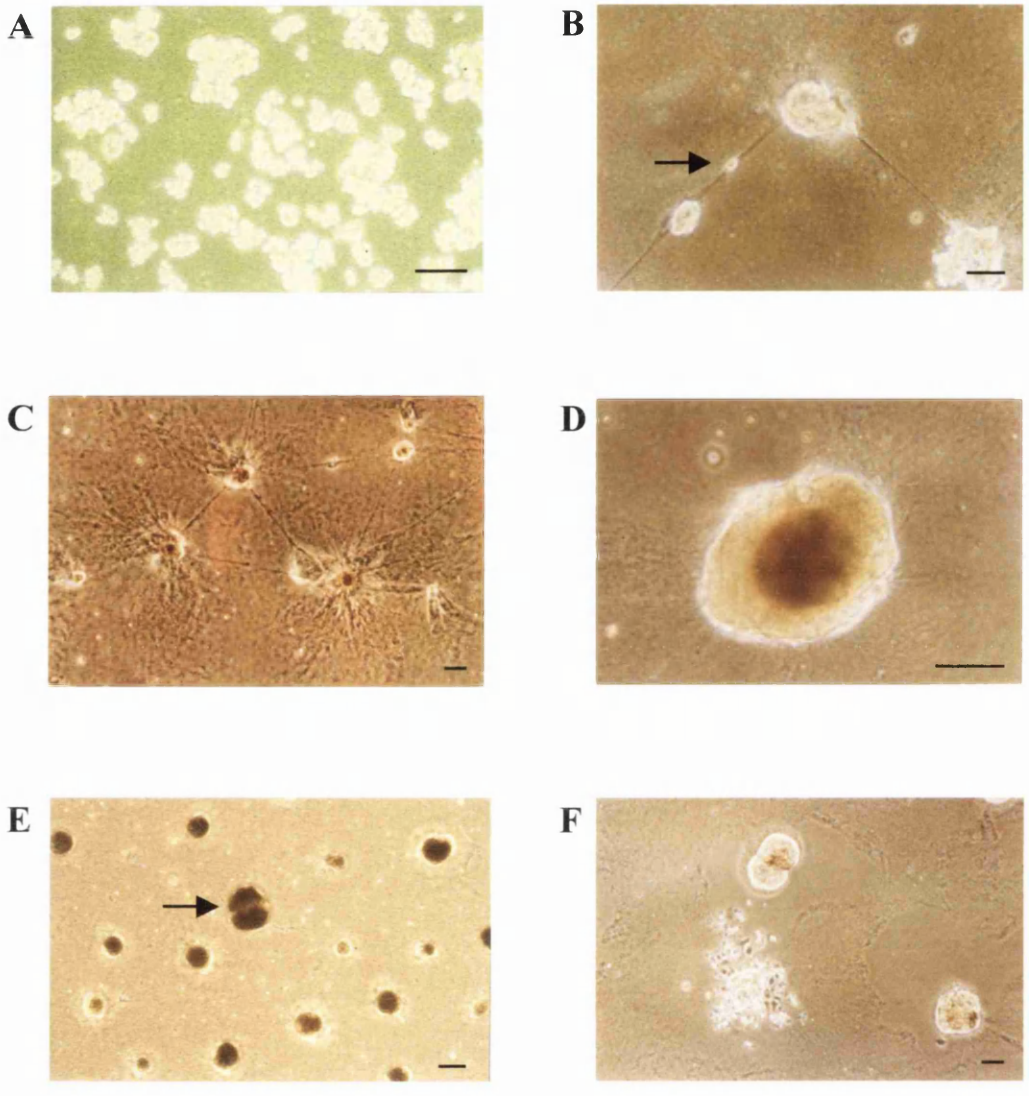


Figure 4.1 Mammosphere formation by ovine mammary epithelial cells
Cells were examined by phase contrast microscopy at various stages of culture: (A) at plating cells are apparent as random cell clumps; mag 45x; (B) clumps become interconnected by processes at d1-2, with what appear to be motile cells moving along them (arrowed); mag 33x; (C) at d3 remoulding of the matrix occurs resulting in prominent stress lines; mag 18x; (D) by d4-6 a central lumen is apparent; mag 61x; (E) at d 4-6 the mammospheres are regularly spaced and larger clumps may form a double lumen (arrowed); mag 23x; (F) mammospheres eventually collapse, with cells proliferating out onto exposed plastic; mag 18x. Bar = 150μm

disintegration of a few structures, followed in rapid succession by the remaining mammospheres.

4.2.2 Protein secretion in ovine mammospheres

Protein secretion was studied in ovine mammosphere cultures by immunohistochemical staining and western blotting for key milk proteins, and by [^{35}S]-methionine radiolabelling.

Immunohistochemistry

Sectioning of mammospheres, and staining with DAPI nuclear stain, showed that at day 2 on EHS matrix the cells were arranged in solid clumps, but that over time a central luminal space was formed (Figure 4.2; A, C, E, G). Staining with polyclonal antisera against α_{S1}-casein, ß-lactoglobulin and α-lactalbumin demonstrated the presence of cell associated milk proteins at all stages (Figure 4.2: B, D, F, H). By day 8, however, secreted proteins had accumulated in a central luminal space (Figure 4.2H). A diagrammatic representation of the fully formed mammosphere structure is shown in Figure 4.3. This closely resembles a single alveolus in mammary tissue. The lactating ruminant mammary gland possesses a very well organised alveolar structure *in vivo* (Figures 4.4A & B) and occasionally, a large clump of cells formed multiple lumina very reminiscent of this (Figure 4.4C).

Immunoblotting of secreted proteins

Immunoblotting for ß-lactogloblulin in culture medium and luminal extracts (Figure 4.5) detected a single band at 18 kDa, at the same position as the standard ß-lactoglobulin. The antiserum to α_{S1}-casein (Figure 4.6) detected a single band at ~26 kDa in the standard protein, and a very strong band at this position in the samples. The pattern of secretion was similar for both milk proteins. In these cells, luminal protein was negligible at day 2, rising in a linear fashion to a peak at day 8, before declining sharply. In contrast, protein secretion into the culture medium was evident at day 2, had increased ~3-fold by day 4, but then remained fairly constant to day 10. Immunoblots shown in Figures 4.5 and 4.6 are from one experiment only.

Densitometric comparison with protein standards indicated that between days 4 and 10 these cells secreted approximately 160 ng of ß-lactoglobulin into the culture medium in a 4-hour period, and α_{S1}-casein equivalent to that found in ~940 ng of

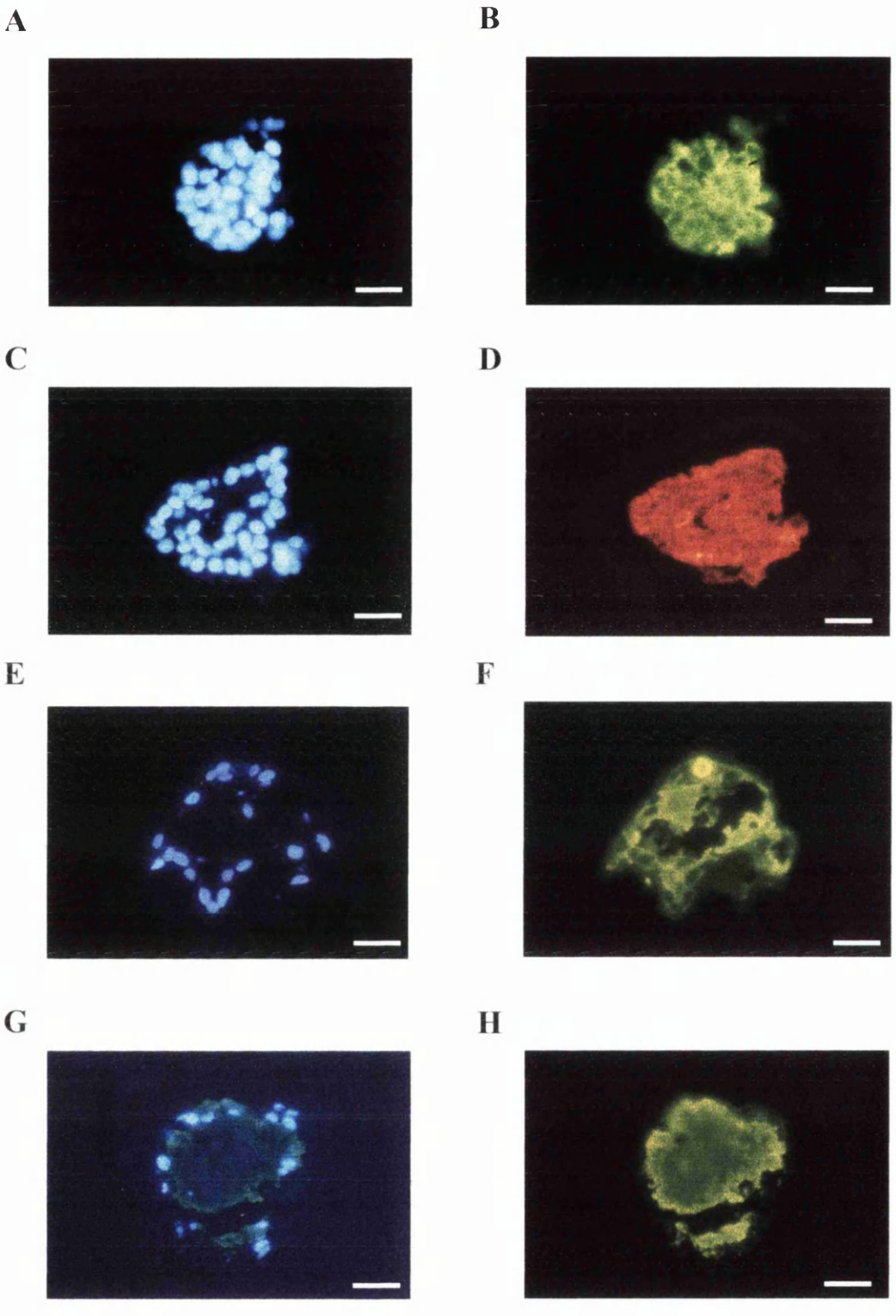


Figure 4.2 Mammosphere formation by ovine mammary epithelial cells
Cells were fixed and sectioned after 2 (A,B), 4 (C,D), 6 (E,F) and 8 (G,H) days of culture, and stained with DAPI (A, C, E, G) or immunostained for βLG (B, H), αLA (D) or α_{S1}-CAS (F). Mag 147x; Bar=50μm

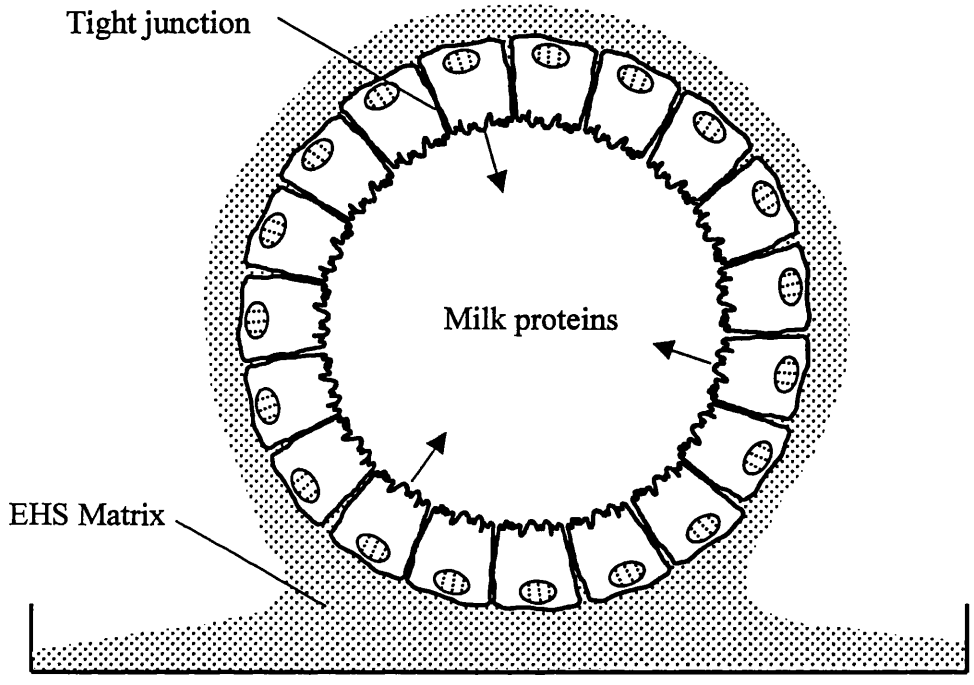


Figure 4.3 Diagrammatic representation of mammosphere structure
Mammary epithelial cells become enshrouded in EHS matrix, and are arranged around a central luminal space into which milk proteins are secreted.

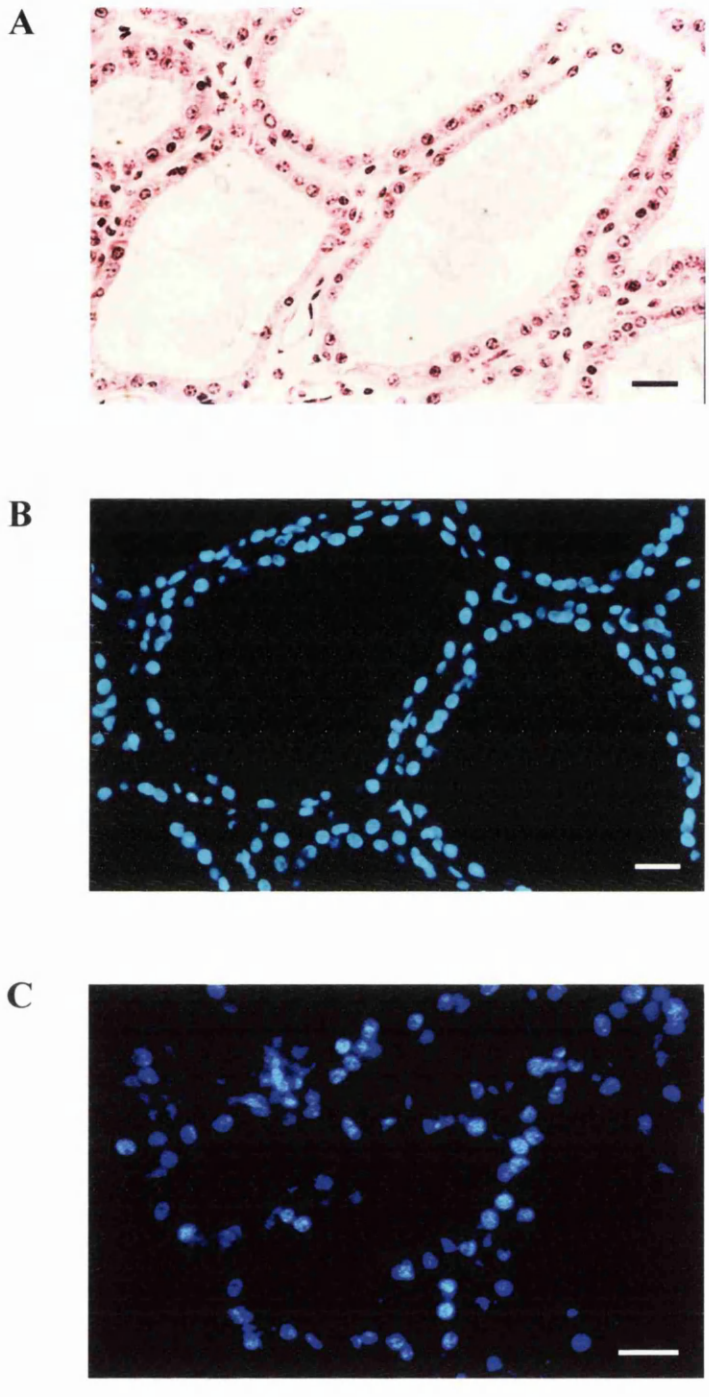


Figure 4.4 Arrangement of mammary epithelial cells *in vivo* and *in vitro*
Sectioned bovine lactating mammary tissue was stained with haematoxylin and eosin (A) or DAPI (B) to show the alveolar structure of secretory mammary tissue. Large clumps of isolated ovine mammary epithelial cells cultured on EHS matrix (C) were sectioned and stained with DAPI, showing the formation of "multi-lumen" mammospheres which are reminiscent of this.
A, B: Bar = 10μm, C: Bar = 100μm

Figures 4.4 A & B provided by CJ Wilde

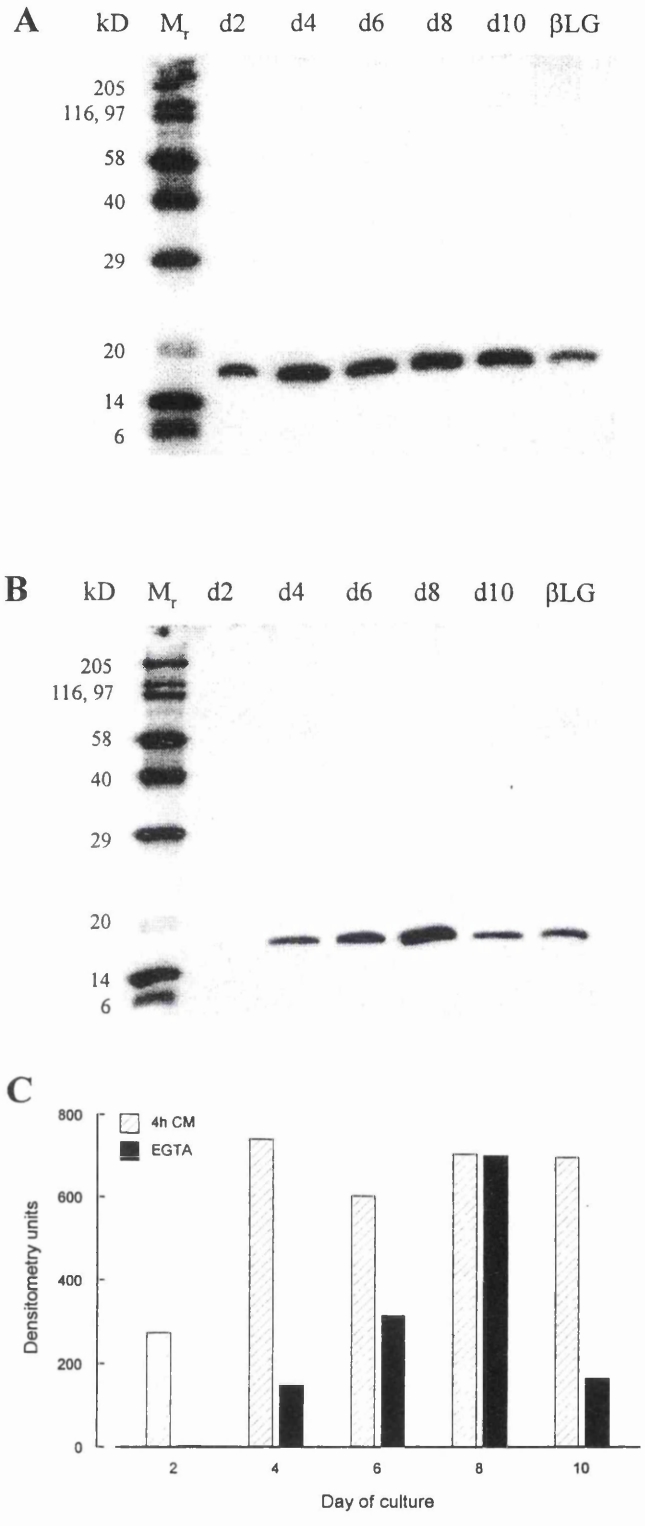


Figure 4.5 Secretion of βLG by ovine mammospheres from day 2 to day 10 of culture

A) Western blot of culture medium conditioned for 4h in ovine mammosphere cultures; B) Western blot of accumulated luminal contents extracted by EGTA treatment *in situ;* protein standard was 50 ng βLG; C) Densitometry of A and B.

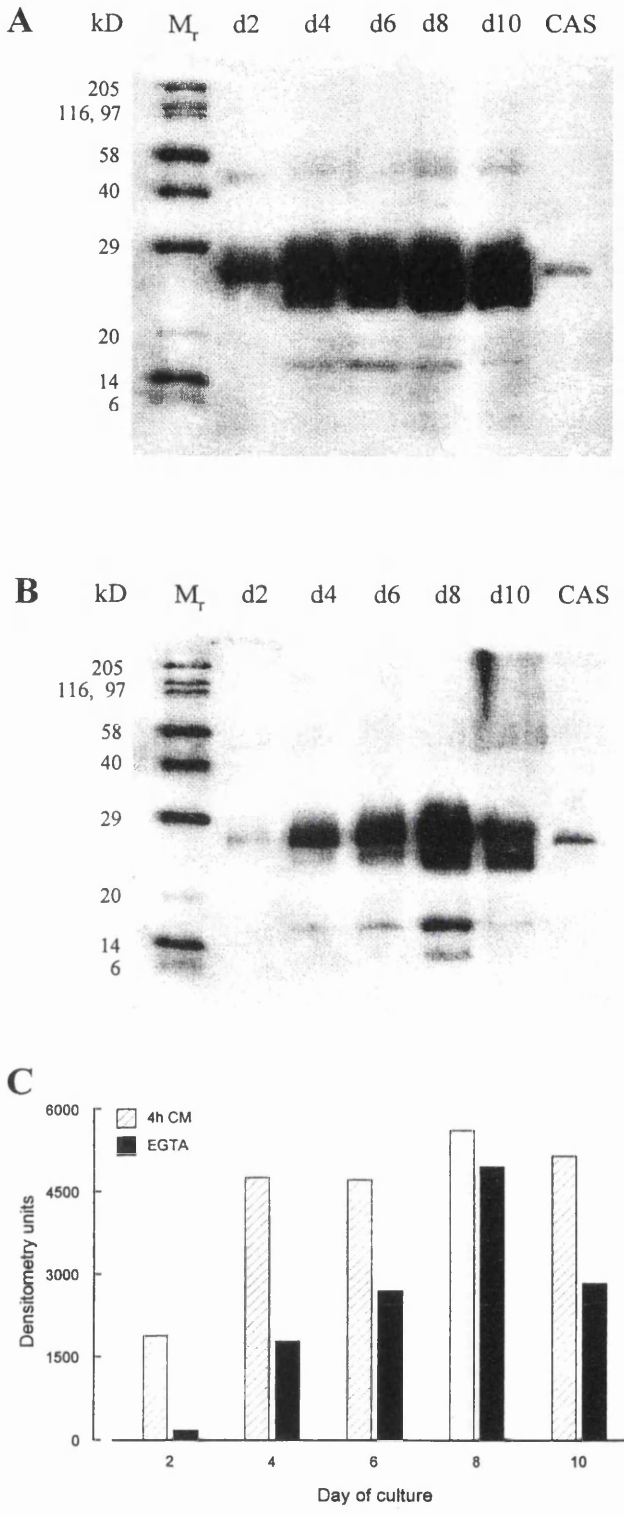


Figure 4.6 Secretion of α_{S1}–casein by ovine mammospheres from day 2 to day 10 of culture

A) Western blot of culture medium conditioned for 4h; B) Western blot of accumulated luminal contents extracted by EGTA treatment *in situ;* protein standard was 50ng total ovine casein (CAS); C) Densitometry of A and B.

whole casein (assuming that the proportions of the various caseins are the same *in vitro* and *in vivo*). Maximal luminal accumulation in these cells occurred on day 8 and was equivalent to ~210 ng ß-lactoglobulin and ~500 ng whole casein.

A major disadvantage of immunoblotting for milk proteins is that only the cumulative amount of luminal protein can be determined, while radiolabel incorporation over a limited period allows a truer determination of the partitioning of secreted milk proteins.

Radiolabelling

Radiolabelling of cells with [^{35}S]-methionine showed that total protein secretion increased to a maximum at days 5-7 in two experiments, (Figure 4.7A). The partitioning of radiolabelled protein into the lumen declined in early culture, but recovered at day 7 (Figure 4.7B).

4.2.3 Prolactin and substratum dependence of protein secretion

Freshly prepared cells were plated on tissue culture plastic (5 x 10^5 cells per 35 mm well) and on EHS matrix (3 x 10^6 cells per 35 mm well), in culture medium either with or without prolactin. On day 6, the conditioned culture medium and luminal contents (EGTA extract) were harvested. These were dialysed and resolved by SDS-PAGE, gels being loaded on an equal DNA basis. Protein was either visualised using Coomassie Blue stain, or immunoblotted to detect specific milk proteins.

Cells on tissue culture plastic required the presence of serum in the culture medium, but the high serum content caused the gel to run unsatisfactorily. In addition, immunoblotting for specific milk proteins was marred by the high non-specific binding of antibodies to serum proteins. These problems were avoided in later experiments by incubation of the cells for 4 hours with serum-free medium. Short-term culture in serum-free medium had no adverse effect on cell attachment or viability.

Staining of SDS-PAGE gels with Coomassie Blue (Figure 4.8) showed discrete bands, corresponding to casein in defatted sheep milk, in both culture medium and EGTA fractions from cells grown on EHS matrix in the presence of prolactin. Faint bands corresponding with β-lactoglobulin were also detected under these conditions. No α-lactalbumin was detected.

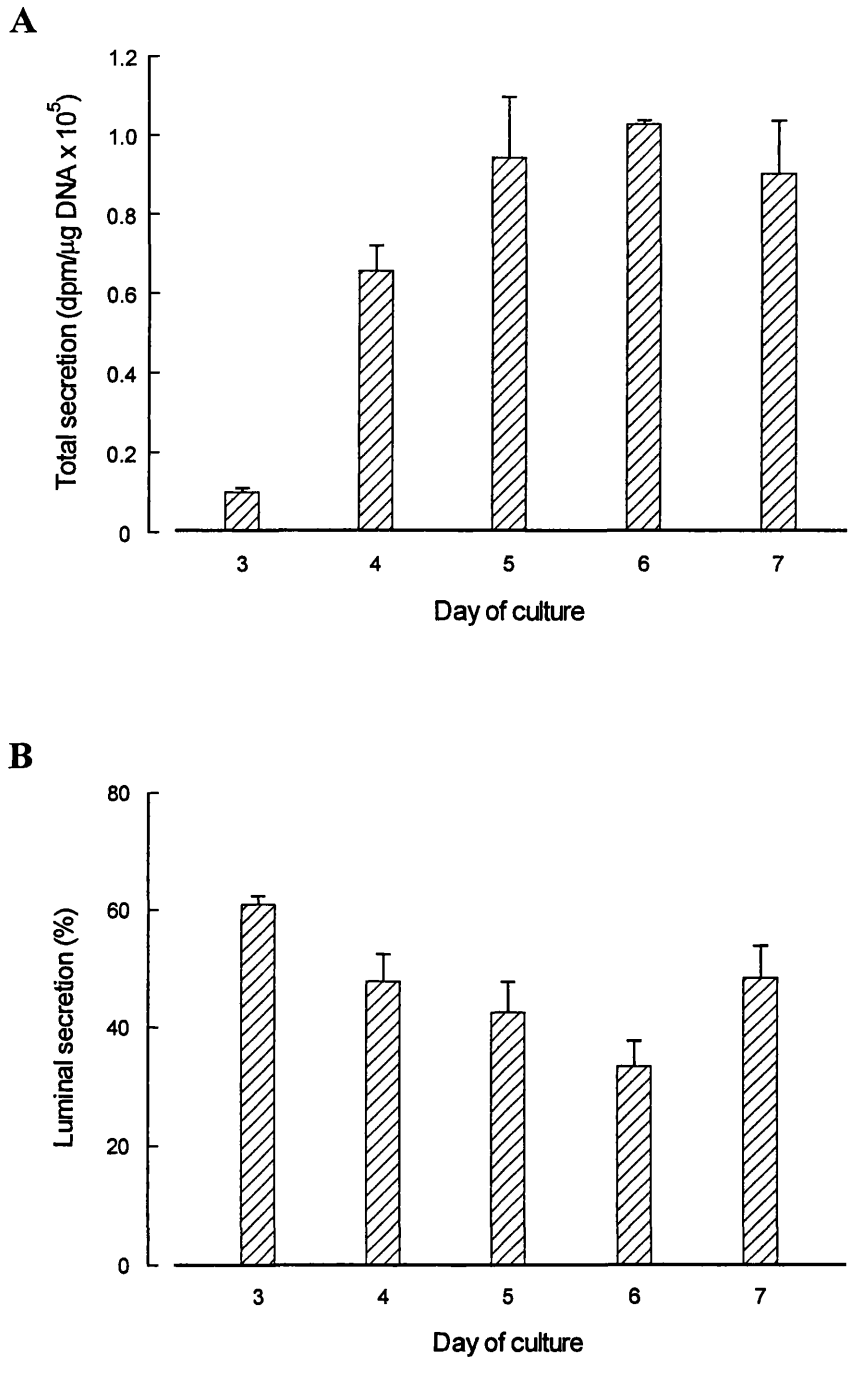


Figure 4.7 Protein secretion in sheep mammosphere cultures
[^{35}S]-methionine incorporation by ovine mammosphere cultures was used to determine total protein secretion (A) and proportion of secreted protein partitioned into the lumen (B). Results are means of 2 experiments (range shown) each performed in triplicate.

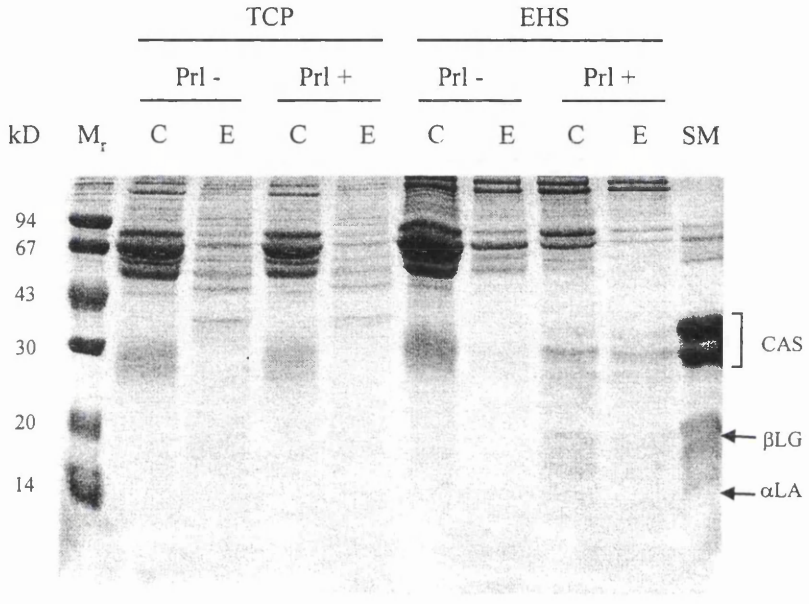


Figure 4.8 Effect of substratum and prolactin on milk protein secretion *in vitro*

Culture medium conditioned for 4 h (C) and EGTA extracts of cells *in situ* (E) were resolved on a 12% SDS-PAGE gel on an equal DNA basis and stained with Coomassie Blue. Cells were grown on tissue culture plastic (TCP) or EHS matrix (EHS) in the presence or absence of prolactin (Prl). The protein standard used was 20 μg defatted sheep milk (SM).

In the absence of prolactin or EHS matrix, a blurred and indistinct band was observed in the culture medium at the position of sheep caseins, but no bands were detected at 14 kDa or 18 kDa, the expected positions for α-lactalbumin or ß-lactoglobulin respectively.

Immunoblotting with antisera to sheep whey proteins or individual milk proteins confirmed that milk protein secretion occurred predominantly on EHS matrix in the presence of prolactin (Figures 4.9-10). Densitometry of the blot shown in Figure 4.9 demonstrated that ß-lactoglobulin secretion was induced ~12-fold by prolactin, in cells grown on EHS matrix. In Figure 4.10 the ß-lactoglobulin band was below the level of detection by densitometry, but prolactin induced α_{S1}-casein secretion ~50-fold.

On tissue culture plastic, low levels of milk proteins were detected in the culture medium harvested from cells grown on tissue culture plastic, irrespective of the presence or absence of prolactin, in cells derived from 2 animals (Figure 4.9). In another cell preparation, however, milk-protein specific antibodies failed to detect ß-lactoglobulin (Figure 4.10A) or α_{S1}-casein (Figure 4.10B) on tissue culture plastic, even in the presence of prolactin. No α-lactalbumin was detected under any culture conditions.

4.2.4 Effect of cryopreservation on cell performance

Cells recovered from liquid nitrogen were not plated directly onto EHS matrix, but allowed to expand on plastic to remove non-viable cells. Once transferred to EHS matrix, mammosphere formation was indistinguishable from that of freshly prepared cells. No discernible differences were observed between fresh and cryopreserved cells after immunostaining of sections, and cryopreserved cells demonstrated a similar response to prolactin and substratum. Fluorography of culture medium and luminal extract obtained from [^{35}S]-methionine labelled fresh and cryopreserved cells showed no differences in the populations of proteins secreted (Figure 4.11).

4.2.5 Differentiation of ovine mammary epithelial cells after passaging

Freshly prepared cells were plated directly onto EHS matrix (designated P0) at a density of 3 x 10^6 cells/35 mm well. Cells were simultaneously plated onto tissue culture plastic at a density of 5 x 10^5 cells/35 mm well. The latter were grown until

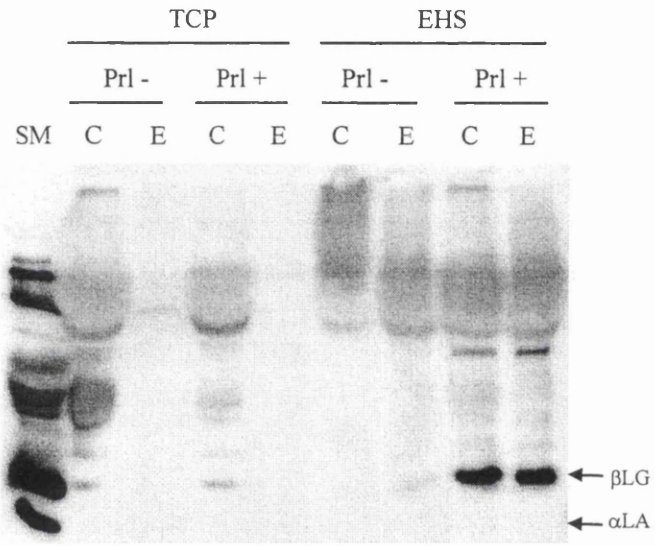


Figure 4.9 Effect of substratum and prolactin on secretion of sheep whey proteins *in vitro*

Culture medium conditioned for 4 h (C) and EGTA extracts of cells *in situ* (E) on d6 of culture were resolved on a 12% SDS-PAGE gel and immunoblotted for sheep whey proteins. Cells were grown on tissue culture plastic (TCP) or EHS matrix (EHS) in the presence or absence of prolactin (Prl). The protein standard used was 20 μg of defatted sheep milk (SM).

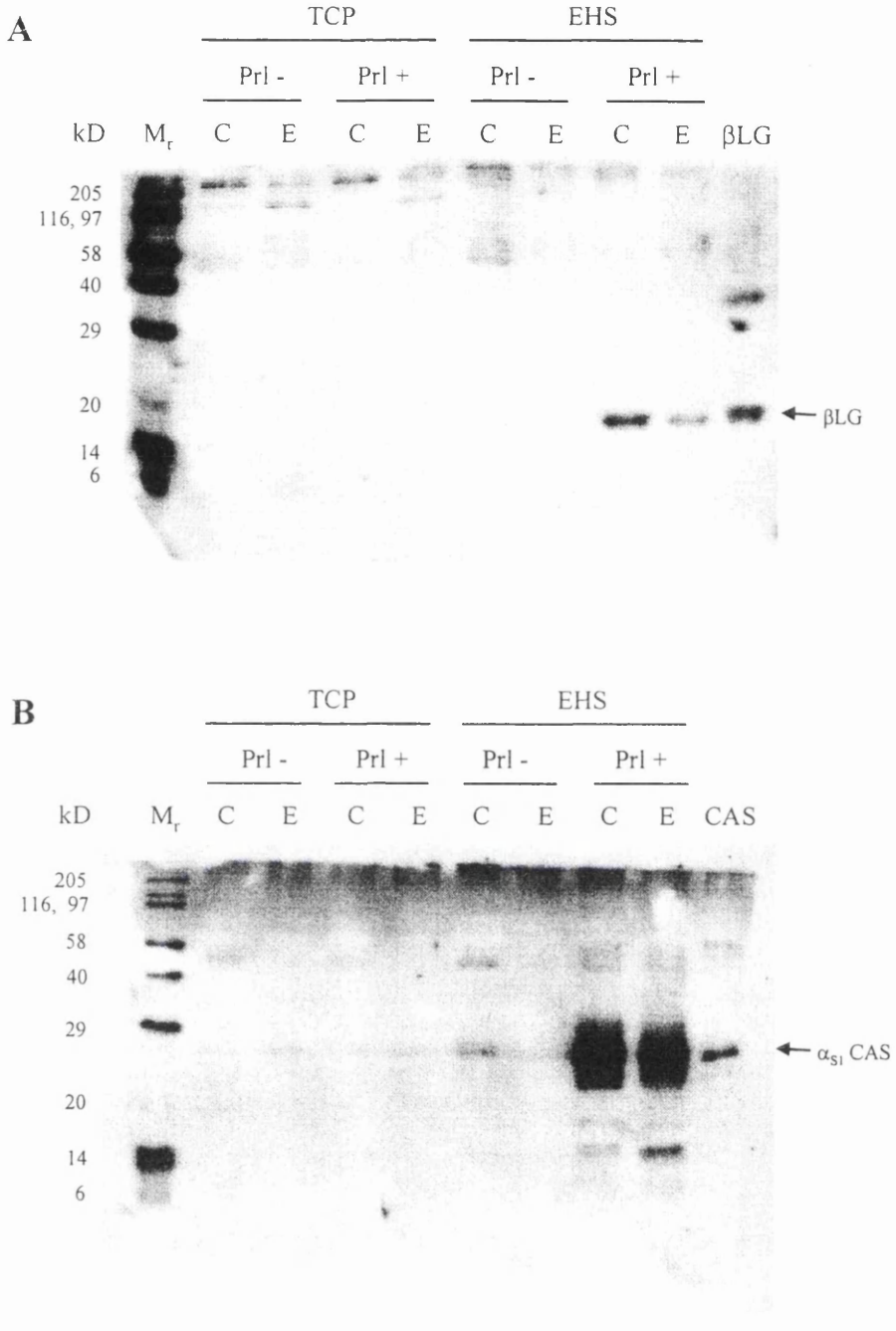


Figure 4.10 Effect of substratum and prolactin on secretion of β-lactoglobulin and α_{S1}-casein.

Culture medium conditioned for 4 h (C) and EGTA extracts of cells *in situ* (E) on d6 of culture were resolved on a 12% SDS-PAGE gel and immunoblotted for the milk proteins β-lactoglobulin (A) and α_{S1}-casein (B). Cells were grown on tissue culture plastic (TCP) or EHS matrix (EHS) in the presence or absence of prolactin (Prl). The protein standards used were 50 ng of bovine β-lactoglobulin (βLG) or 50 ng of whole ovine casein (CAS).

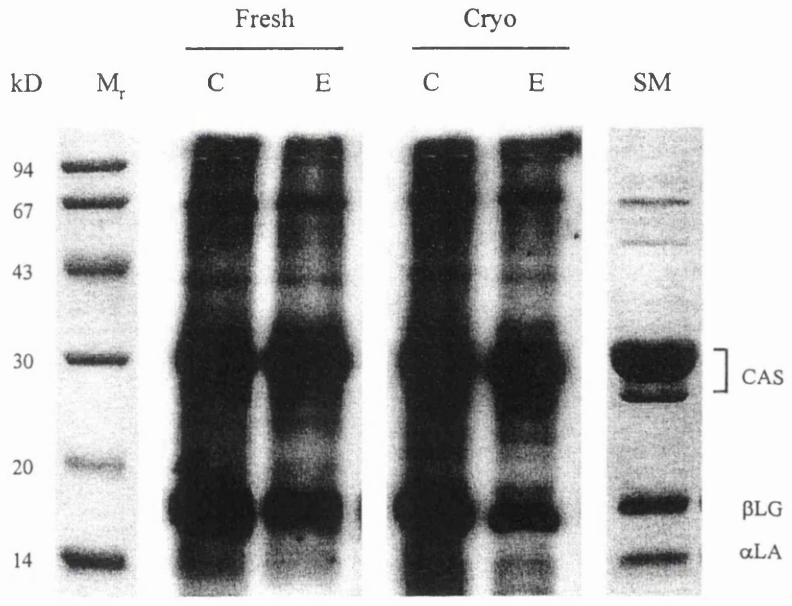


Figure 4.11 Protein secretion in fresh and cryopreserved cells
Fresh and cryopreserved cells were radiolabelled on d6 of culture on EHS matrix. Culture medium (C) and EGTA extracts of cells *in situ* (E) were loaded on an equal DNA basis and subjected to fluorography. Coomassie blue-stained protein standard, was 20 μg of defatted sheep milk (SM).

~95% confluent, harvested with trypsin, and transferred to EHS matrix (1 well tissue culture plastic to 1 well EHS matrix). Some cells were diluted 1:6 and replated onto tissue culture plastic for continued proliferation. This procedure was carried out successively until cells had been passaged up to 3 times before transfer to matrix. At each passage, cells were labelled with [^{35}S]-methionine on day 6 of culture on EHS matrix, and culture medium and luminal EGTA extracts were collected as described previously. Cryopreserved cells were cultured and radiolabelled in the same manner, except that cells were not plated directly onto EHS matrix on recovery from liquid nitrogen.

Freshly prepared cells, plated directly onto EHS matrix, secreted 54% ± 1.8% of total protein synthesised. Of this, 38% ± 2.8% was contained in the luminal fraction. When cells were passaged up to 3 times before transfer onto EHS matrix, total protein synthesis and secretion (expressed on a unit DNA basis) remained similar to that of freshly prepared cells (Figure 4.12A). The proportion of radiolabelled protein secreted into the luminal space was similar in fresh and passage 1 cells, but decreased in passage 2 and was only 13.3 ± 4.7% of total secreted protein after 3 passages (Figure 4.12B). By this stage, mammospheres were smaller, and DNA content per well was ~10% of that for freshly prepared cells.

Cryopreserved cells at passage 1 synthesised protein at approximately 60% of that obtained in fresh cells, but unlike the fresh cells, cryopreserved cells showed a progressive decline in total protein synthesis per unit DNA with increasing passage (Figure 4.13). In addition, cryopreserved cells showed a significant reduction in the proportion of newly-synthesised protein that was secreted (Figure 4.14).

Fluorography of [^{35}S]-labelled protein detected intense bands at the expected positions for ß-lactoglobulin and ß-casein in fresh cells and those passaged once on tissue culture plastic (Figure 4.15). Other bands were detected at the migration positions of transferrin and α-lactalbumin. Differences were observed in the distribution of the major protein bands. ß-lactoglobulin was secreted predominantly in the culture medium in fresh cells, but distributed evenly between medium and luminal extract in passage 1. Casein was evenly distributed between medium and mammosphere lumina in fresh cells, but was secreted preferentially into the lumen in passage 1 cells. Protein secretion in passages 2 and 3 appeared to be negligible,

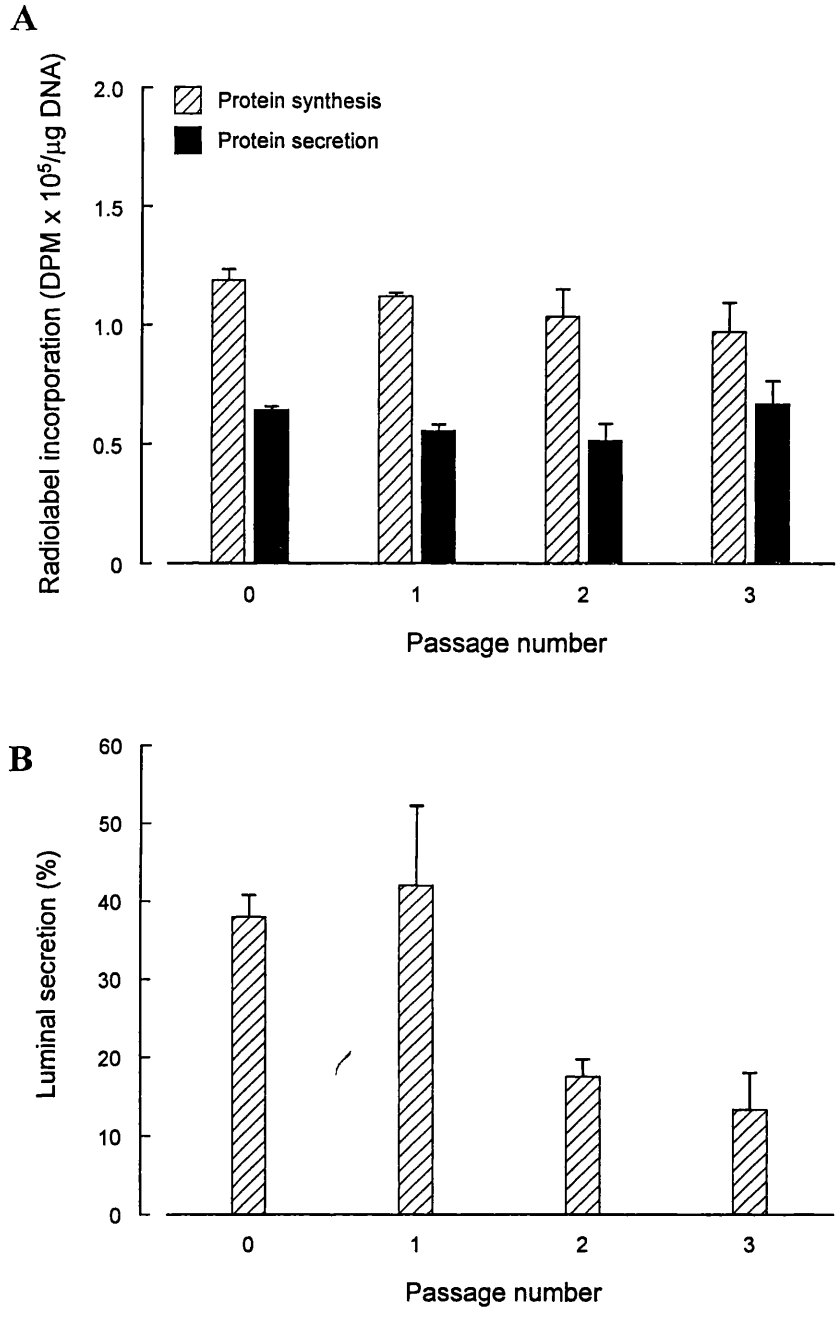


Figure 4.12 Protein synthesis and secretion in fresh and passaged ovine mammary epithelial cells

Sheep mammary epithelial cells radiolabelled with [^{35}S]-methionine on day 6 of culture on EHS matrix at each stage. Total protein synthesis and secretion (A) and the proportion of secreted protein partitioned into the mammosphere lumen (B) are shown for freshly prepared cells, and those passaged up to three times. The results are means of 2 experiments (range shown) each performed in triplicate.

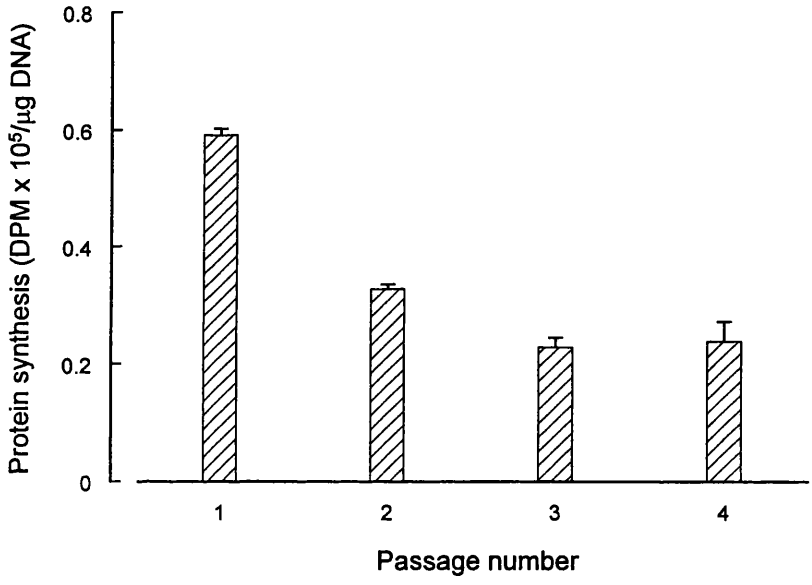


Figure 4.13 Effect of passaging on protein synthesis in cryopreserved cells Cells were radiolabelled on d6 of culture on EHS matrix and total protein synthesis was determined by [^{35}S]-methionine incorporation. Results are means of 2 experiments (range shown) each performed in triplicate.

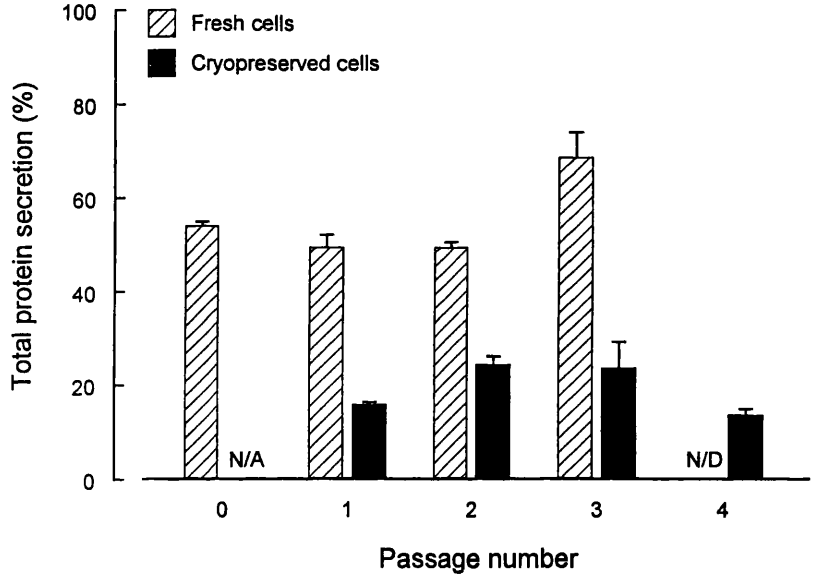


Figure 4.14 Effect of cryopreservation on protein secretion
Cells were radiolabelled on day 6 of culture on EHS matrix and total protein secretion, expressed as a percentage of protein synthesis was determined by [^{35}S]-methionine incorporation. Results are means of 2 experiments (range shown) each performed in triplicate.
N/A = Not applicable; N/D = Not determined

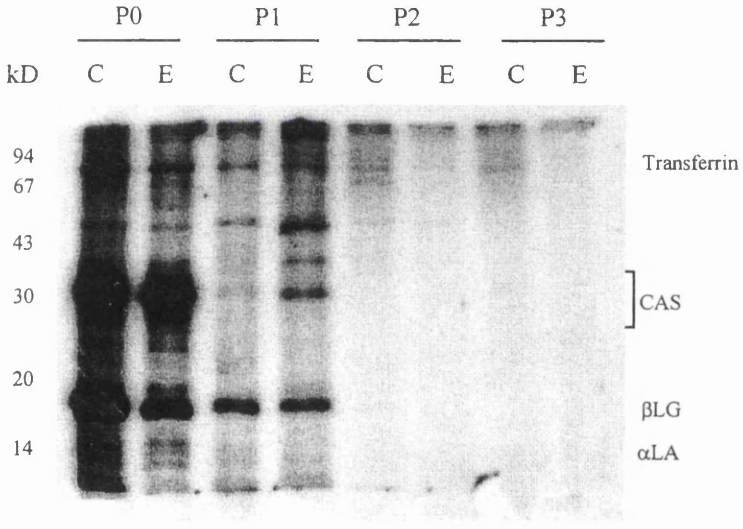


Figure 4.15 Effect of passaging on protein secretion in fresh cells
Freshly prepared cells (P0), and those passaged up to 3 times, were radiolabelled on d6 of culture on EHS matrix. Culture medium (C) and EGTA extracts of cells *in situ* (E) were loaded on an equal volume basis and subjected to fluorography. The positions of the major milk proteins are indicated.

apparently in contradiction to Figure 4.12A, but this was due to loading of the gel on an equal volume of well contents, rather than an equal DNA basis. Thus, results may have been compromised due to the declining DNA content per well, mentioned previously. A similar loss of milk protein secretion from passage 2 onwards was observed with cryopreserved cells, although in one instance, vectorial secretion of casein was detected by fluorography (Figure 4.16).

4.2.6 Effect of serum concentration during proliferation on differentiation

All of the experiments discussed had been performed with cells grown in medium containing 25% serum. It was possible that the presence of growth factors present in the serum may have adversely affected cell differentiation, therefore, protein synthesis and secretion was investigated in reduced serum medium.

Cryopreserved cells were passaged up to 3 times and transferred to EHS matrix at each stage as described in Section 4.2.5. The proliferation phase, on tissue culture plastic, was carried out using medium containing 10% or 25% serum, The ratio of horse serum:foetal calf serum was 4:1 at both concentrations. No significant differences were observed between the two treatments in protein synthesis or secretion (Figure 4.17).

4.2.7 Secretion of a foreign protein *in vitro*

Mammary epithelial cells were prepared from a sheep transgenic for human α_1-antitrypsin and cultured on tissue culture plastic or EHS matrix. Culture medium and EGTA fractions were immunoblotted for human α_1-antitrypsin (Figure 4.18). On EHS matrix a single prominent band was detected at 54 kDa, a similar position to that of the α_1-antitrypsin standards, in both the culture medium and luminal extracts. On tissue culture plastic, two closely spaced bands were detected in the culture medium. Newly synthesised and secreted proteins were also radiolabelled with [^{35}S]-methionine and culture medium and EGTA extract were subjected to fluorography (Figure 4.19). A single predominant band at the expected position was observed in both fractions in fresh cells and those passaged once or twice. Densitometry showed that this band accounted for approximately 33% of total protein. Interestingly, in fresh cells labelled on d13 of culture, there was a strong band visible at the expected position of α-lactalbumin.

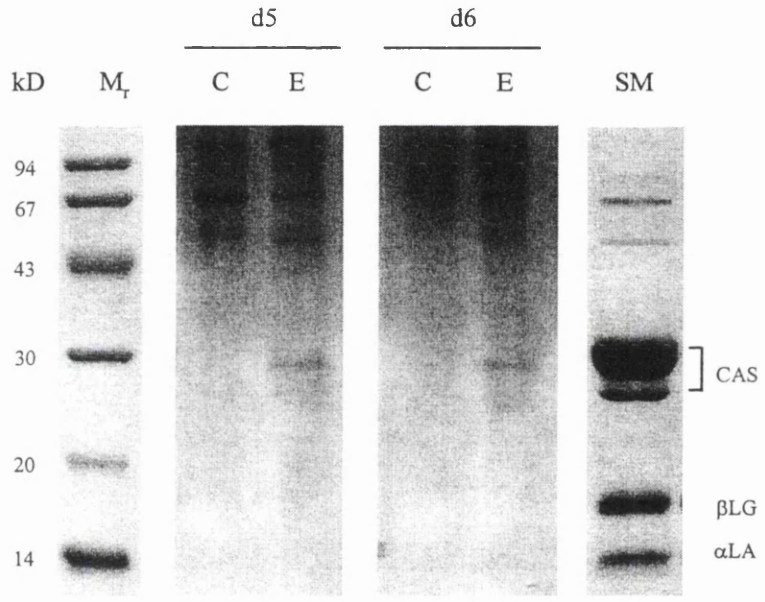


Figure 4.16 Protein secretion in cryopreserved passage 2 cells
Cryopreserved passage 2 cells were radiolabelled on d5 and d6 of culture on EHS matrix. Culture medium (C) and EGTA extracts of cells *in situ* (E) were loaded on an equal DNA basis and subjected to fluorgraphy. Coomassie blue-stained protein standard was 20μg of defatted sheep milk (SM).

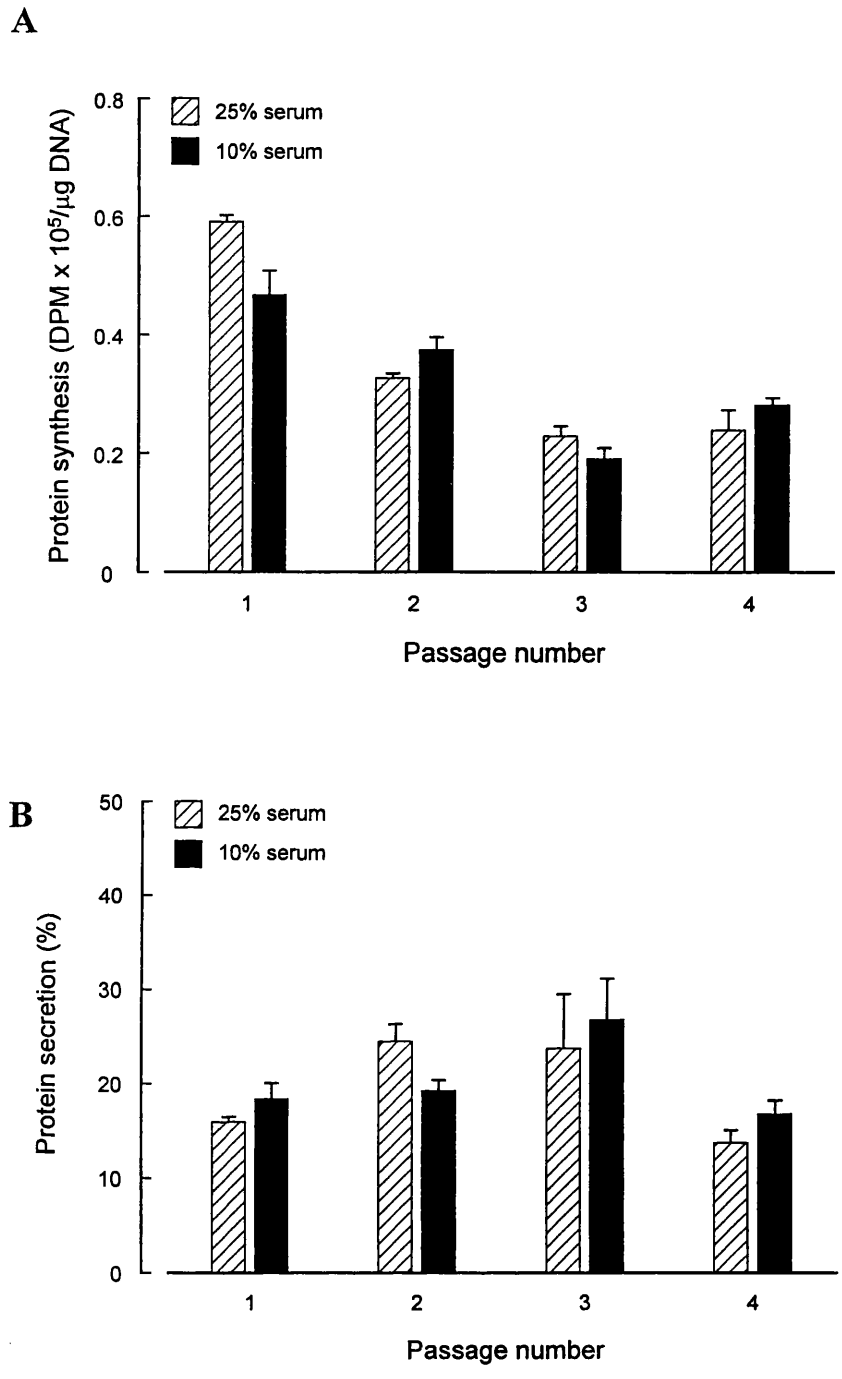


Figure 4.17 Effect of serum on subsequent differentiation of ovine mammary epithelial cells

Cells were cultured on tissue culture plastic in either 10% or 25% serum before transfer to EHS matrix. Total protein synthesis (A) and percentage of synthesised protein secreted (B) were measured by [^{35}S]-methionine incorporation on d6 of culture on EHS matrix. Results are means of 2 experiments each performed in triplicate ± SEM.

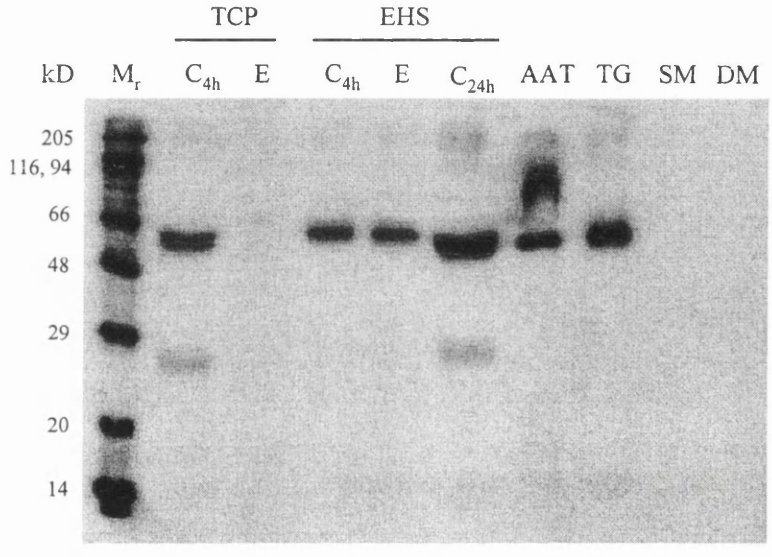


Figure 4.18 Effect of substratum on secretion of α_1-antitrypsin *in vitro*
Cells were prepared from a sheep transgenic for human α_1-antitrypsin and cultured on tissue culture plastic (TCP) or EHS matrix (EHS). Culture medium (C), conditioned for 4 h or 24 h, and EGTA (E) fractions were resolved by SDS-PAGE (12%) and immunoblotted for human α_1-antitrypsin. Protein standard used was 50 ng human α_1-antitrypsin (AAT); TG = defatted milk from sheep transgenic for human α_1-antitrypsin; SM = 20μg defatted sheep milk; DM = unconditioned differentiation medium

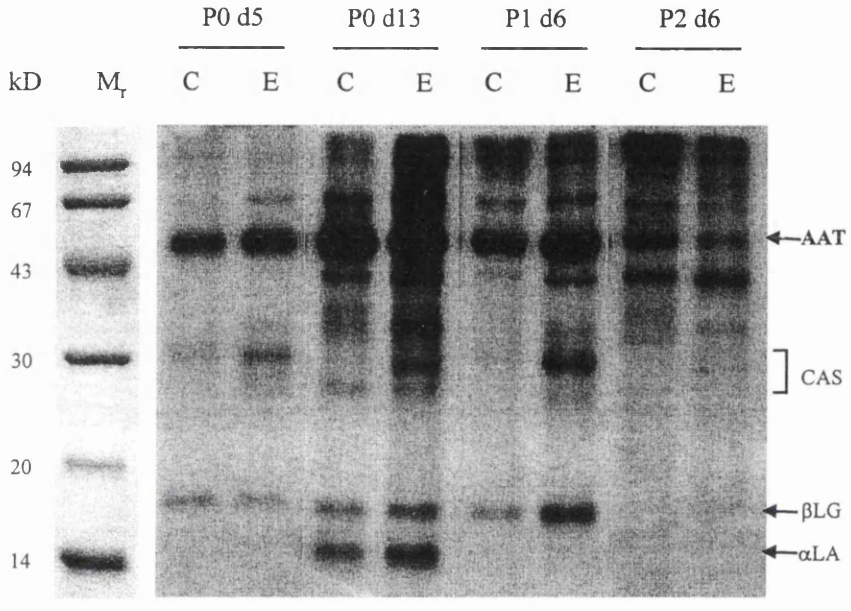


Figure 4.19 Effect of passaging on secretion of α_1-antitrypsin *in vitro*
Fluorogram of [^{35}S]-labelled protein: Cells were prepared from a sheep transgenic for human α_1-antitrypsin and radiolabelled for a 4 h period. Culture medium (C) and EGTA extracts (E) were loaded on an equal DNA basis.

4.3 DISCUSSION

Sheep cells plated onto EHS matrix formed three-dimensional structures termed "mammospheres", similar to those reported for murine (Li *et al*, 1987; Barcellos-Hoff *et al*, 1989) and caprine cells (Wilde *et al*, 1995). Almost all cells were recruited into these structures, and from microscopic observations it is suggested that this occurs via translocation along the processes interconnecting the cell aggregates. From days 4-6 the formation of a central lumen was observed by phase contrast microscopy, and the peripheral arrangement of cells circumscribing the mammosphere was confirmed by sectioning and staining with DAPI. A series of sections taken over a period of days showed that the lumen was formed by the progressive loss of cells from the centre of the structure. This has recently been shown to occur via apoptosis (Blatchford *et al*, 1999).

Cell-associated milk protein secretion was detectable in sections taken on day 2, while immunoblotting at this stage also demonstrated secretion into the culture medium. This may be attributable to induction of protein synthesis by EHS matrix. Alternatively it may be due to the partial differentiation of cells at the time of isolation, since copious amounts of milky fluid were observed in the mammary tissue during dissection on a number of occasions (also noted by Li *et al*, 1999 in goat tissue). Harris *et al* (1991) have shown that β-casein is expressed at low levels in the mammary gland of virgin mice, and is rapidly induced at day 10 of pregnancy, while WAP gene expression increases sharply between day 14 and day 16 of pregnancy (Pittius et al, 1988). Similarly, in sheep, low level β-lactoglobulin expression is detected in virgin animals and elevated during the second half of pregnancy (Harris et al, 1991), thus some milk protein expression in the third trimester of pregnancy would not be unexpected. Radiolabelling with [^{35}S]-methionine, however, demonstrated that total protein synthesis and secretion were induced when ovine mammary epithelial cells were cultured on EHS matrix in the presence of lactogenic hormones. Similarly immunoblotting of culture medium showed that both β-lactoglobulin and α_{S1}-casein levels increased 3-fold between days 2 and 4 and remained at similar levels until at least day 10 in culture. This demonstrates that under these culture conditions, ovine mammary epithelial cells were well-differentiated.

Mouse mammospheres possess intercellular tight junctions when cultured on EHS matrix, and demonstrate vectorial secretion of milk proteins (Barcellos-Hoff *et al*, 1989; Hurley *et al*, 1994; Blatchford *et al*, 1998). Immunohistochemistry of ovine cells also showed luminal accumulation of milk proteins. Treatment of cultures with EGTA chelates calcium ions, causing disruption of intercellular tight junctions and permitting recovery of the luminal contents. Immunoblotting of this fraction showed that β-lactoglobulin and α_{S1}-casein accumulated in the lumen, reaching a maximum at ~d8 of culture. Milk proteins were also detected in the culture medium, but it was not determined whether this was due to basolateral secretion or whether the mammosphere structures were consistently "leaky". This could be investigated by radiolabelling for various periods and measuring the luminal [^{35}S]-labelled protein.

α-lactalbumin was only rarely detected by fluorography or immunoblotting of culture medium, even from freshly prepared cells, and then only at low levels. It was, however, detectable in immunostained sections. This may suggest that α-lactalbumin remained intracellular at that particular stage of culture. Alternatively, it may simply be due to technical reasons, such as sub-optimal transfer conditions, or disruption of the antigenic site resulting from the denaturing conditions in SDS-PAGE. Interestingly, though, fluorography of the proteins produced by the transgenic sheep shows a prominent band at the expected position for α-lactalbumin (14 kDa) on day 13 of culture, while labelling/immunoblotting experiments were routinely performed at days 6-8 of culture. It is possible, therefore, that optimal secretion of α-lactalbumin occurs much later in culture than other milk proteins. This may reflect the situation *in vivo*, where α-lactalbumin is only expressed around the time of parturition, unlike the other milk proteins, which begin to be expressed during pregnancy (Burdon *et al*, 1991, Harris *et al*, 1991). This result suggests that the degree of differentiation of ovine mammary epithelial cells may vary with length of culture on EHS matrix.

Prolactin had no discernible effect on milk protein secretion when ovine mammary epithelial cells were cultured on tissue culture plastic, but this may be due to inhibition of gene expression (Guyette *et al*, 1979), intracellular degradation (Razooki Hasan *et al*, 1982; Lee *et al*, 1985) or changes in post-translational modification (Lee *et al*, 1985). On EHS matrix, in the absence of prolactin,

immunoblotting detected small amounts of β-lactoglobulin or α_{S1}-casein in the culture medium. In the presence of prolactin, however, there was a marked induction of milk protein secretion and also secretion into the EGTA fraction. Milk protein induction and accumulation has been shown to be prolactin-dependent in explants from rabbit (Devinoy *et al*, 1978; Sankaran & Topper, 1984), rat (Guyette *et al*, 1979;) and mouse (Darcy *et al*, 1995a) and there is considerable evidence that its action is mediated via the Jak-Stat pathway (Schmitt-Ney *et al*, 1991; Watson *et al*, 1991). Stat5 phosphorylation correlates with the pattern of milk protein gene expression in mice (Liu *et al*, 1996, 1997) but this relationship has not been observed in ruminant species (Wheeler *et al*, 1997). A prolactin-responsive cell culture system would therefore be invaluable for investigating regulatory mechanisms of milk protein secretion in ruminants.

Ovine mammary epithelial cells were also found to be responsive to substratum. Protein secretion on tissue culture plastic was detected at very low levels in freshly prepared cells, if at all, even in the presence of prolactin, in agreement with findings in mice (Emerman *et al*, 1977; Lee *et al*, 1984; Li *et al*, 1987; Chen & Bissell, 1989). Milk proteins were not detected in culture medium from cryopreserved or passaged cells, suggesting that this capability is lost by continued culture on plastic. Nevertheless, milk protein secretion is reinstated on transfer to EHS matrix, in agreement with findings in mice (Emerman *et al*, 1977; Streuli *et al*, 1991). This suggests that cells are able to dedifferentiate and redifferentiate. The presence of small amounts of β-lactoglobulin or α_{S1}-casein in the culture medium on EHS matrix in the absence of prolactin, suggested that the matrix itself may promote mRNA expression or stabilisation. Evidence for a transcriptional/post-transcriptional role of EHS has been demonstrated in rabbit (Puissant *et al*, 1994) and rodent cultures (Wicha *et al*, 1982; Blum *et al*, 1987; Chen & Bissell, 1989).

EHS and prolactin influence gene expression and mRNA stability (Guyette et al, 1979; Puissant et al, 1994) and post-translational events (Lee *et al*, 1985; Hurley *et al*, 1989; Aggeler *et al*, 1991). In cells prepared from a sheep transgenic for AAT, an aberrant product was detected in the absence of prolactin and EHS matrix. Similarly, the presence of an indistinct band at the expected position for casein in the absence of one or both of these factors also suggested the secretion of an aberrant or

degraded product, but in both instances, these proteins were expressed correctly under permissive conditions.

Cells that had been cryopreserved demonstrated lower levels of synthetic activity and total secretion in comparison to freshly prepared cells, similar to the findings of Hansen *et al* (1986) with lactating goat cells. Populations of secreted proteins were indistinguishable between freshly prepared and cryopreserved cells, however, and readily detectable by fluorography or immunoblotting. This suggests that the cryopreservation and recovery of ovine mammary epithelial cells, and subsequent transfer to EHS matrix, provides a practical model for the study of milk protein secretion in ruminant cells.

Protein secretion during passaging was initially determined biochemically by assay of [^{35}S]-methionine incorporation. Passaging three times had no adverse effect on total protein synthesis and secretion. This did not reflect milk protein secretion, however, which declined with increasing passage, rarely being detected beyond passage 2. A reduction in serum concentration did not improve differentiative ability. The loss of milk protein secretion may be as a result of phenotypic changes in the cells. Another possibility, however, is that the observed decline in cell number, resulting in lower plating densities, may have resulted in poor mammosphere formation. Further evidence for this is the change in distribution of milk protein secretion from the EGTA fraction to the culture medium. This suggests that a low plating density may have resulted in a loss of mammosphere lumen integrity.

Cells derived from a sheep transgenic for human AAT were isolated and cultured to determine whether a foreign protein, produced *in vivo*, could also be expressed *in vitro*. Large amounts of a protein migrating at the expected position for human AAT (54 kDa) were detected in both culture medium and EGTA extracts, in a substratum/prolactin dependent manner. Densitometry indicated that this was the principal secreted protein. Immunoblotting, performed at PPL Therapeutics confirmed that the protein was detected easily in cells up to passage 3, but the signal decreased markedly in passage 4. These experiments demonstrate that a foreign protein can be expressed *in vitro*, in a similar manner to *in vivo*. In addition, mammary epithelial cells have been prepared from mice transgenic for the pCORP12 construct, which expresses an aberrant form of human protein C. The protein C variants detected in culture medium and EGTA fractions were similar to those

detected in milk of lactating pCORP12 transgenic mice (V Craig, unpublished data), further supporting this finding.

4.4 CONCLUSIONS

These results compare favourably with attempts by other workers to establish functional ruminant mammary cultures. Hansen & Knudsen (1991), failed to detect α-lactalbumin or β-casein secretion in culture medium from lactating goat cells on collagen, and cells were unresponsive to prolactin. Attempts to culture lactating sheep cells on EHS matrix by other workers have resulted in a rapid decline in levels of synthesis and secretion of the major milk proteins (Wheeler *et al*, 1995).

This research has shown that ovine mammary epithelial cells isolated from pregnant animals form mammosphere structures on EHS matrix which closely resemble the alveoli *in vivo*. The cells are induced to differentiate in culture, secreting milk proteins in a prolactin and substratum dependent manner. Cryopreservation does not appear to be prohibitively detrimental to the performance of the cells, and provides a practical means of investigating milk protein secretion in ruminants with relatively little expense, in comparison to *in vivo* studies. Milk protein secretion decreases with passage, but this may be a consequence of declining cell density and subsequent poor mammosphere formation. The preparation of mammosphere cultures from transgenic animals suggests that foreign proteins are capable of being produced *in vitro*.

CHAPTER FIVE
EXTENDED PASSAGING IN RUMINANT MAMMARY EPITHELIAL CELLS

5.1 INTRODUCTION

Results in Chapter 4 showed that sheep mammary epithelial cells could be induced to differentiate in culture, in a prolactin and substratum dependent manner, in contrast to the findings of other workers (Wheeler *et al*, 1995). This provides a good model for the study of milk protein secretion in the ruminant mammary gland, but further insights into the molecular biology of the secretory mechanisms could be provided by the manipulation of endogenous genes, or inserting foreign genes. Preliminary transfection experiments carried out at PPL Therapeutics Ltd, (Roslin, UK) suggested that transfection efficiencies were low, necessitating selection of transfected cells during passaging on tissue culture plastic, prior to differentiation on EHS matrix. However, results in Chapter 4 showed that there was a decline in secretory activity of ovine mammary epithelial cells after 2 or 3 passages. Cell loss during passaging, and the resultant decline in plating density may have been a contributory factor to this, leading to poor mammosphere formation. Since harvesting of cells was always carried out at 90-100% confluency it was likely that cell losses were occurring during the trypsinisation procedure. Cells had been routinely harvested using trypsin, and it was decided to compare a number of different treatments. The optimum harvesting method was then used for further passaging experiments, in conjunction with DNA assays to ensure that equal cell densities were plated at successive stages. In addition, anecdotal information from colleagues suggested that the high cell mortality seen during passaging of sheep cells was not experienced with goat cells, so this too was investigated. Specifically, the aims of the work described in this chapter were:

i) to reduce cell death during the harvesting of ovine cells;

ii) to investigate differentiation of ovine cells, when cell number is maintained;

iii) to compare the performance of goat cells with those of sheep cells;

iv) to optimise culture conditions for differentiation after extended passaging.

5.2 RESULTS

5.2.1 Optimisation of cell harvesting

Ovine mammary epithelial cells were plated onto tissue culture plastic at equal densities and grown to 95% confluence. Cells were washed twice with HBSS prior to detachment using one of six harvesting methods, detailed in Table 5.1. Each treatment was performed in duplicate wells. Treatments were assessed on the basis of a number of criteria, namely: time taken for detachment; cell number recovered; microscopic appearance of cells; cell viability (measured by trypan blue exclusion) and appearance of the cells following transfer to EHS matrix.

Detachment times, and numbers of cells recovered are shown in Figures 5.1A and 5.1B respectively. The non-enzymatic Cell Dissociation Solution (Sigma) failed to detach any cells within 45 minutes. Dispase detached the cells in the shortest time (15 min) and produced the second highest yield, but the cells failed to re-attach on transfer to EHS matrix. In fact, the matrix itself was digested by residual Dispase, even though the cells were washed twice with culture medium before plating. Cells were removed in 25-30 min with trypsin or trypsin-EDTA, but ~50% of the cells recovered were non-viable, as determined by trypan blue exclusion. The remaining viable cells attached on EHS matrix and formed small mammospheres. Trypsin containing EGTA and PVA took longest to detach the cells (39 min) but resulted in the highest cell yields (2 and 4 fold greater than with trypsin alone or trypsin EDTA, respectively). Almost all cells were viable, and they formed mammosphere structures on EHS matrix larger than those of the other trypsin-based treatments. This method was used for cell detachment in subsequent passaging experiments.

5.2.2 Passaging of sheep mammary epithelial cells

Sheep mammary epithelial cells were plated onto tissue culture plastic at a density of 5×10^5 cells per 35 mm well (6 well plate) and grown until 95% confluent. Cells were harvested and replated onto tissue culture plastic for further passaging as above, or transferred to EHS matrix at a density of 2×10^6 cells/well. Cell number was estimated by DNA assay prior to plating, to ensure that cell density remained constant at successive passages.

Despite the change in harvesting conditions, the number of cells recovered still declined with increasing passage number (Figure 5.2). By passages 5-6 the

Table 5.1 Methods tested for optimisation of recovery of ovine epithelial cells

Treatment	Source
Trypsin	Life Technologies, Paisley, UK
Trypsin (detached cells harvested and neutralised every 5 min)	Life Technologies, Paisley, UK
Trypsin-EDTA	Life Technologies, Paisley, UK
Trypsin with EGTA (0.4 mg/ml) and polyvinyl alcohol (0.1 mg/ml)	Life Technologies, Paisley, UK (EGTA & PVA, Sigma)
Dispase	Universal Biologicals Ltd, London, UK
Cell Dissociation Solution (non-enzymatic)	Sigma Chemical Co, Poole, UK

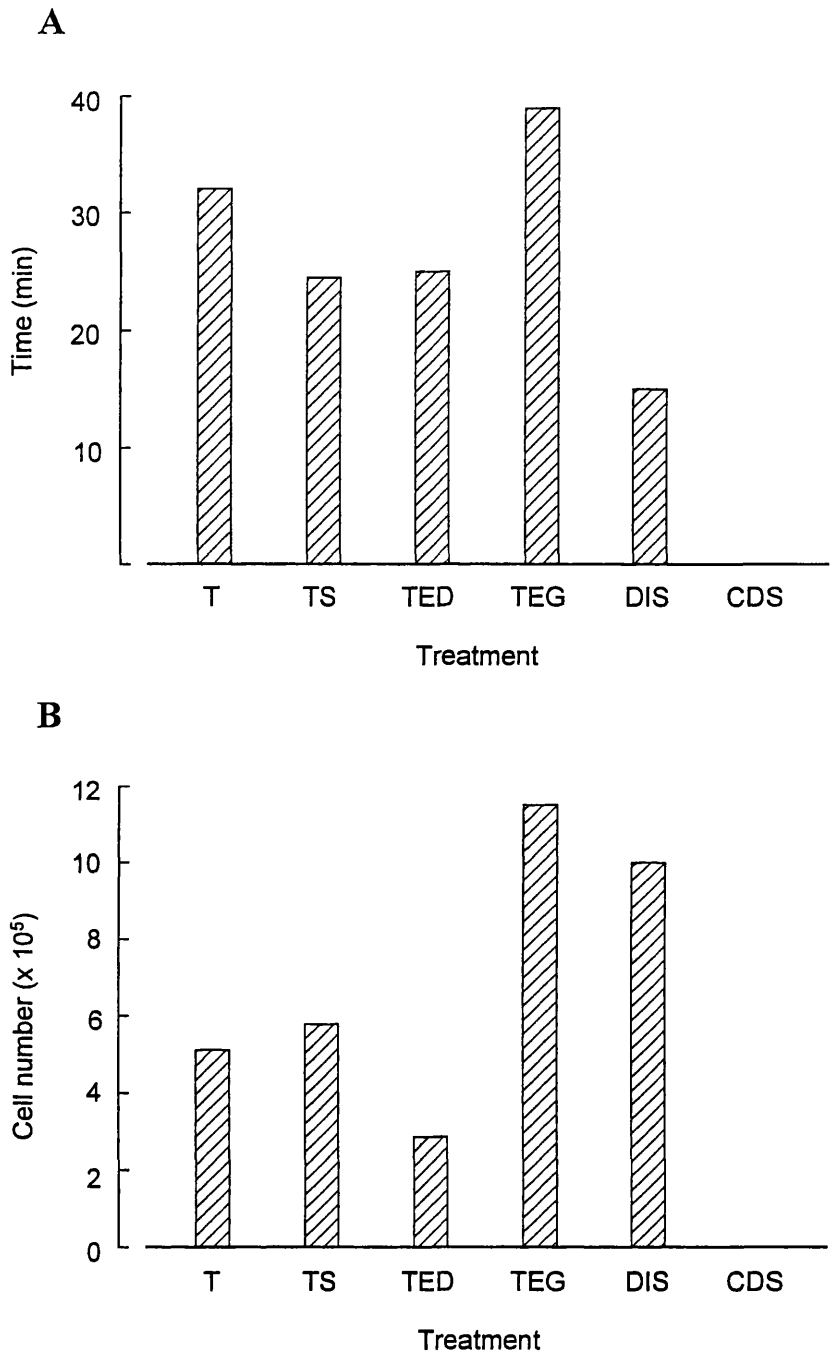


Figure 5.1 Optimisation of cell harvesting
Cells were plated at equal densities, grown to 95% confluence and recovered from tissue culture plastic using trypsin (T), tryspin with sequential harvesting of cells (TS), trypsin-EDTA (TED), trypsin-EGTA-polyvinyl alcohol (TEG), Dispase (DIS) or Cell dissociation solution (CDS). Detachment times (A) and number of cells harvested (B) are shown. Results are means of duplicate wells for a single experiment.

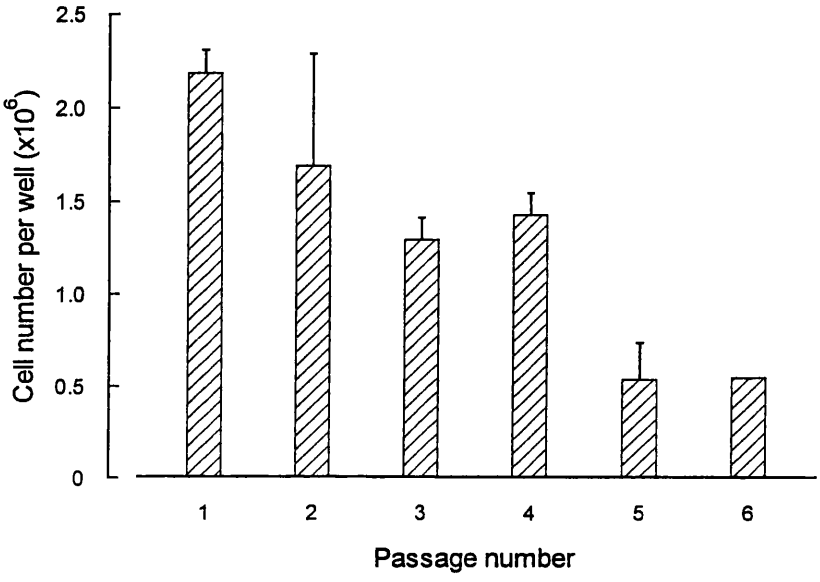


Figure 5.2 Yields of sheep mammary epithelial cells after passaging
Sheep cells were plated onto tissue culture plastic at a density of 5 x 10^5 per well, grown to 95% of confluency and harvested with trypsin containing 0.4 mg/ml EGTA and 0.1 mg/ml PVA. Cell yield was calculated by DNA assay. Results shown are means ± SEM for cell preparations derived from 3 animals.

number of cells recovered was equivalent to that originally plated, resulting in insufficient cells for transfer to EHS matrix. Further passaging could not amplify cell number, necessitating the end of the experiment. The experiment was attempted 4 times with cells derived from 3 different animals, with similar results each time.

Milk protein secretion by ovine mammary cells was investigated by immunoblotting culture medium and luminal extracts harvested at d6-7. No α-lactalbumin was detected in any fraction, while β-lactoglobulin and α_{S1}-casein were detected predominantly in the culture medium (Figure 5.3). It was also apparent that there were differences in protein secretion between animals. Cells prepared from two sheep (designated OP5 and OP11) did not secrete β-lactoglobulin at passage 2, while cells prepared from a third animal (designated OP2) secreted into the culture medium ~6-14 ng/μg DNA/h (based on densitometry of immunoblots against a known standard). The amount of protein secreted also varied with the EHS batch used. The latter was investigated further but measurements of total protein or laminin content in different batches of EHS showed no correlation with cell performance. All animals secreted α_{S1}-casein at passage 2, but no milk proteins were detected from passage 3 onwards. It appeared, therefore, that sheep mammary epithelial cells were adversely affected by passaging and unattractive for transfection.

5.2.3 Passaging of goat mammary epithelial cells

Goat mammary epithelial cells had been characterised prior to this study (Finch *et al*, in preparation) and shown to differentiate effectively when cultured on EHS matrix (Wilde *et al*, 1995). Observations that protein synthesis and secretion were maintained with extended passage had formed the basis of the current project, and it was decided to perform direct comparisons with sheep mammary epithelial cells. The goat mammary epithelial cells used in these experiments were prepared in the same way as sheep cells and similarly shown to be prolactin responsive (Figure 5.4).

Goat cells were passaged in exactly the same manner as sheep cells, and plated at the same cell density, based on DNA assay. Following trypsinisation, the number of goat cells recovered (from 3 animals in a total of 5 experiments) was consistently higher than that of sheep cells, and did not decline with passaging (Figure 5.5).

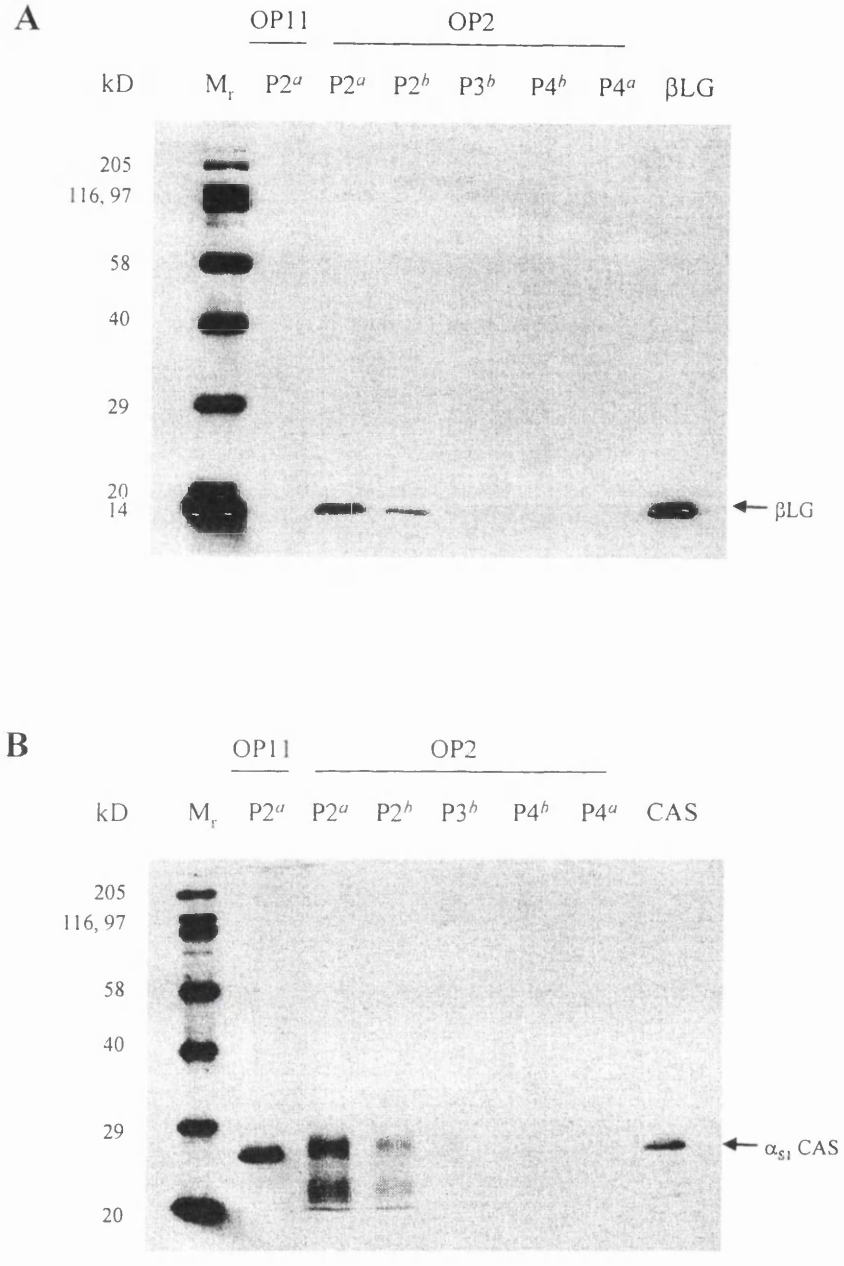


Figure 5.3 Protein secretion in passaged sheep cells
Cells were passaged up to 4 times and plated onto EHS matrix at equal density. Culture medium conditioned for 4 h on d6 of EHS culture was immunoblotted for βLG (A) and α_{S1}-casein (B). Results shown are for 2 animals (OP11 and OP2) and performed using 2 different batches of EHS matrix (*a* and *b*). Gels were loaded on an equal DNA basis. Protein standards used were 50 ng βLG and 50 ng whole ovine casein (CAS).

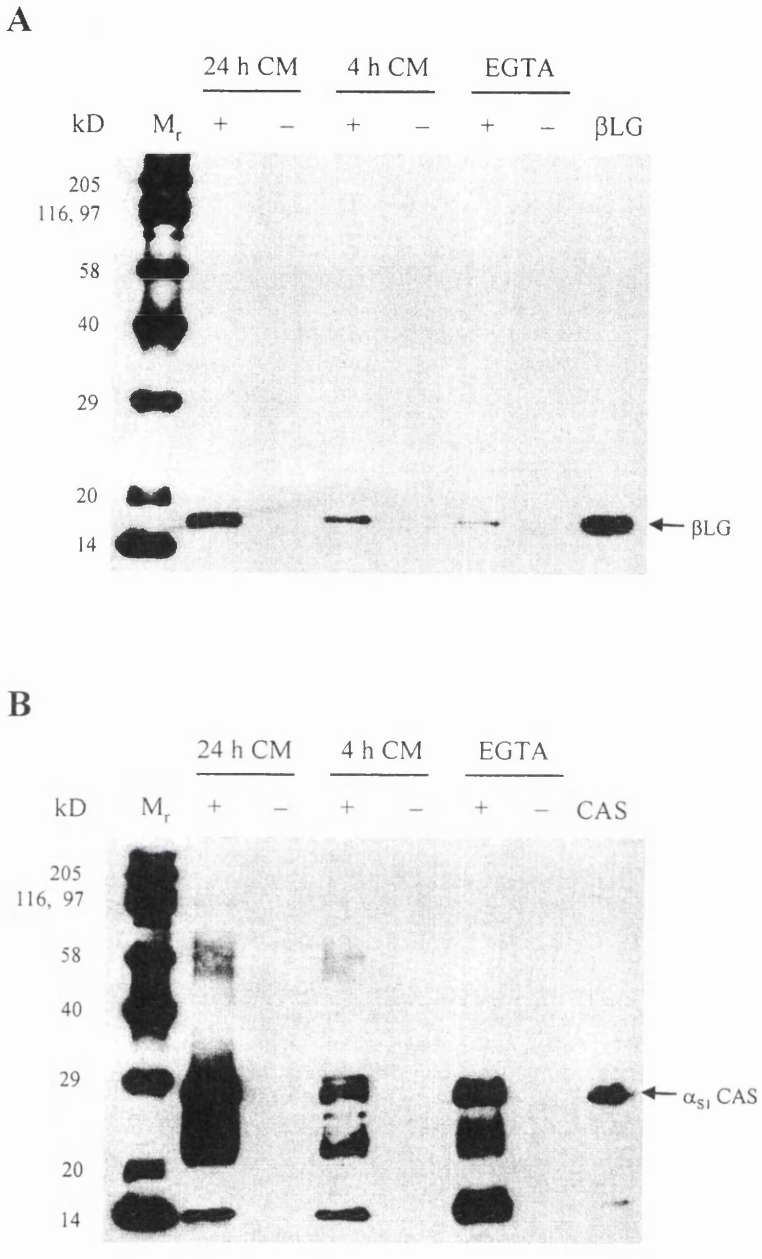


Figure 5.4 Prolactin responsiveness of goat mammary epithelial cells
Culture medium (CM) conditioned for 24 h or 4 h and EGTA extracts of cells *in situ* were resolved on a 12% SDS-PAGE gel and immunoblotted for the milk proteins β-lactoglobulin (A) and α_{S1}-casein (B). Cells were grown on EHS matrix in the presence (+) or absence (–) of prolactin. The protein standards used were 50 ng of bovine β-lactoglobulin (βLG) or 50 ng of whole goat casein (CAS).

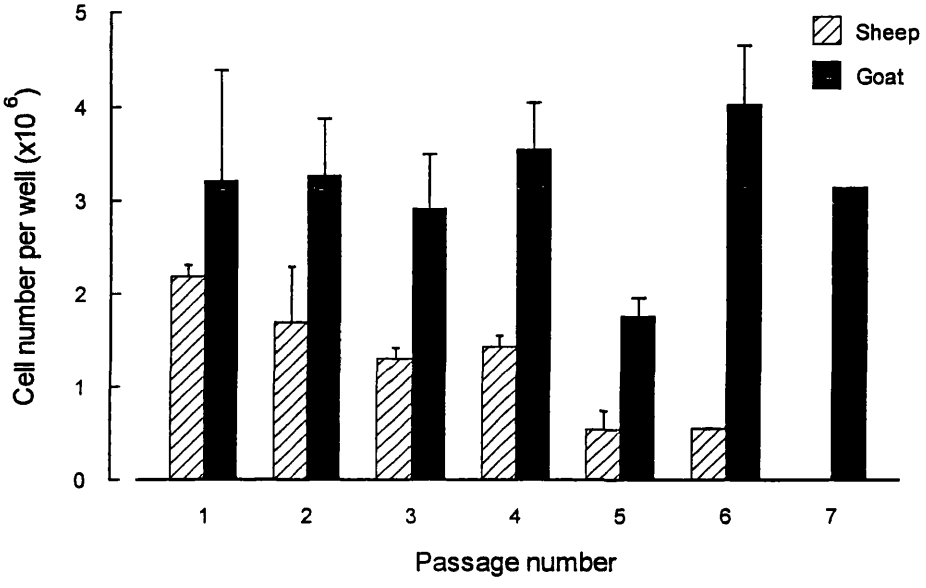


Figure 5.5 Yields of sheep and goat mammary epithelial cells after passaging

Sheep and goat cells were plated onto tissue culture plastic at a density of 5 x 10^5 per well, grown to 95% of confluency and harvested with trypsin containing 0.4 mg/ml EGTA and 0.1 mg/ml PVA. Cell yield was calculated by DNA assay. Results shown are means ± SEM for cell preparations derived from 3 animals.

In one experiment, goat cells were passaged up to 7 times and transferred to EHS matrix at each stage. Radiolabelling with [^{35}S]-methionine showed that levels of protein synthesis and secretion were maintained with passage (Figure 5.6A), and that the partitioning of secreted protein into the lumen was also maintained (Figure 5.6B). Immunoblotting of culture medium and EGTA extracts for β-lactoglobulin (Figure 5.7), α_{S1}-casein (Figure 5.8) and α-lactalbumin (Figure 5.9) showed that they were secreted in broadly similar patterns. Total secretion was highest after three passages, when β-lactoglobulin, α_{S1}-casein and α-lactalbumin were respectively 3-fold, 1.9-fold and 1.7-fold higher than passage 1 cells. Passage 4 cells performed poorly, but secretion recovered in passage 5 and then declined again in passages 6 and 7. This change in secretion levels was also accompanied by changes in partitioning of milk proteins. At passages 1-3, and passage 5, the highest proportion of milk protein detected was in the luminal fraction, while at passages 4, 6 and 7, secretion was predominantly into the culture medium. Densitometry of immunoblots using known standards estimated that these goat cells were secreting ~4 ng β-lactoglobulin/μg DNA/h into the culture medium at passage 1 and ~16 ng/μg DNA/h at passage 3. In addition, ~80 ng β-lactoglobulin/μg DNA was harvested from the EGTA fraction of passage 1 cells on d6 of culture, and ~160 ng from passage 3 cells.

A second experiment with cells from the same animal confirmed that milk proteins were secreted at passages 2, 5 and 6, and that levels of secretion of milk proteins were similar to those obtained in the first experiment.

These results suggested that goat cells were preferable to sheep cells for extended passaging and eventually, expression of foreign proteins. It was desirable, however, for protein secretion levels to be maintained until passage 6. To this end, a further experiment was carried out to determine if manipulation of culture conditions could improve differentiated function in goat mammary epithelial cells.

5.2.4 Optimisation of culture conditions for differentiation at late passage in goat mammary epithelial cells

Investigation of the effects of culture conditions on cell performance is complicated by the fact that they may not act in isolation. Statistical advice suggested the use of a fractional factorial design for this experiment. A full factorial design contains all possible combinations of the treatments, thus for a number of treatments, *n*, with 2

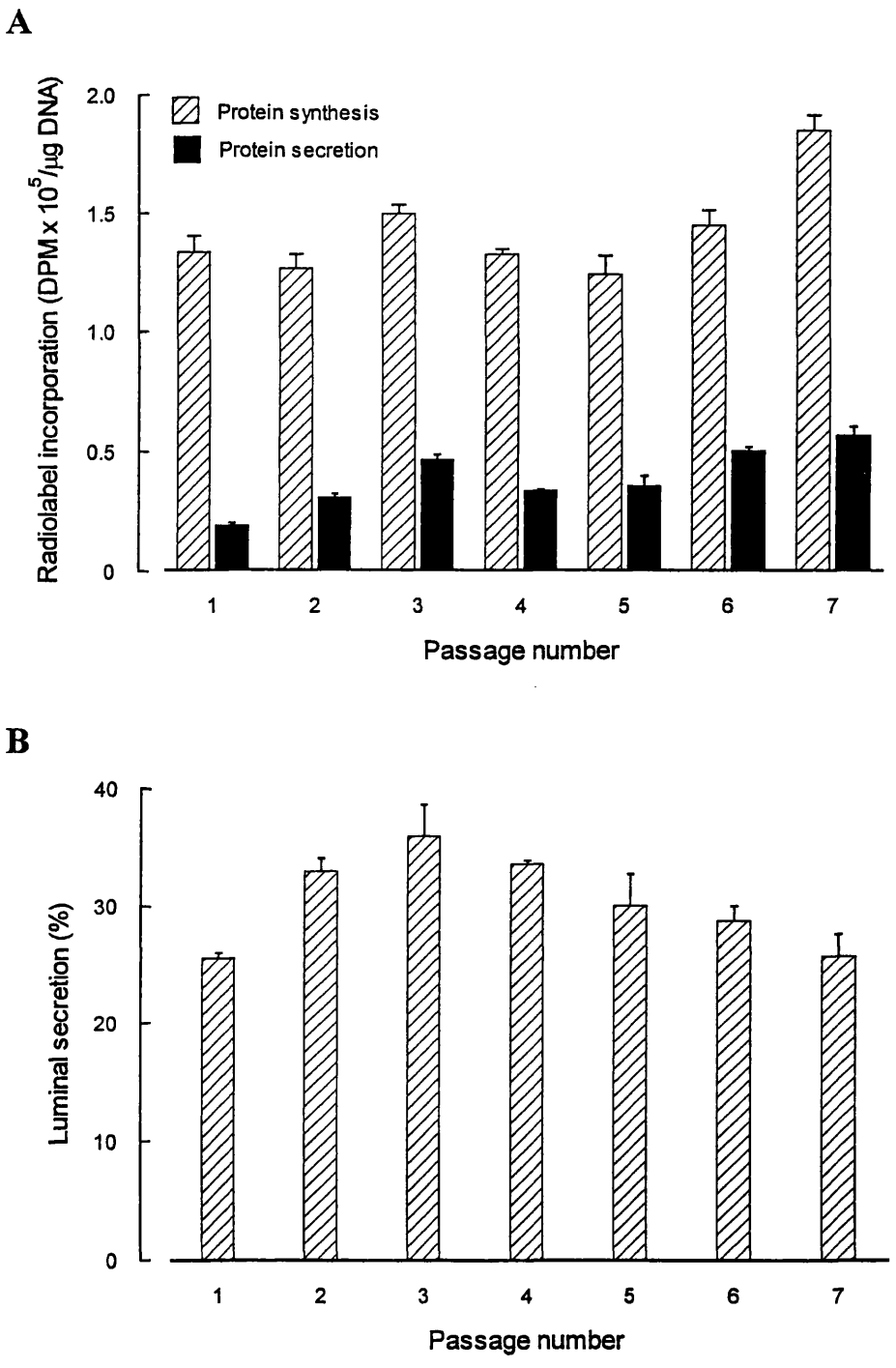


Figure 5.6 Protein synthesis and secretion in passaged goat mammary epithelial cells

Total protein synthesis and secretion (A) and partitioning into the lumen (B) were measured on d6 of culture on EHS at successive passages by radiolabel incorporation. Luminal secretion is expressed as a percentage of total secretion. Results are means of 6 wells ± SEM for a single experiment.

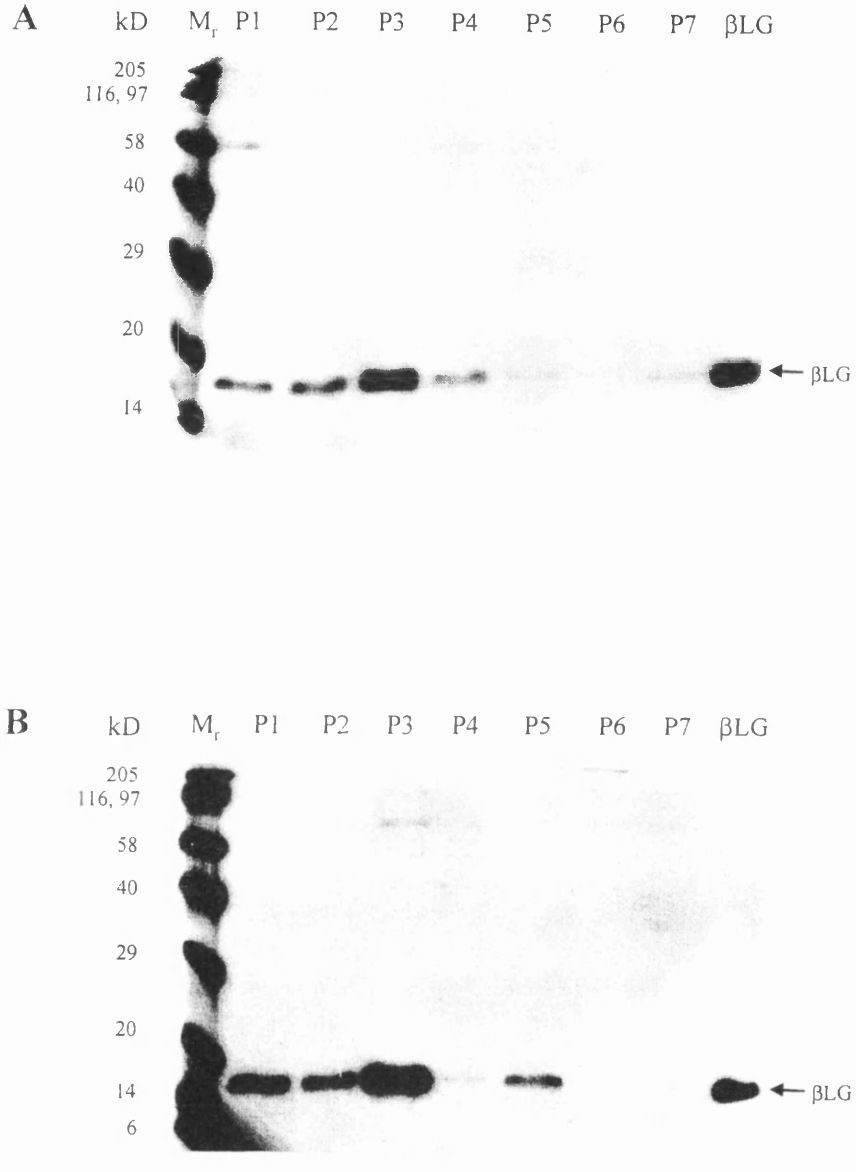


Figure 5.7 Secretion of β-lactoglobulin by goat mammospheres after passaging
Goat cells were plated onto EHS matrix at equal density at successive passages. Culture medium conditioned for 4 h (A) and EGTA extract (B) were immunoblotted for βLG. Protein standard was 50 ng bovine βLG.

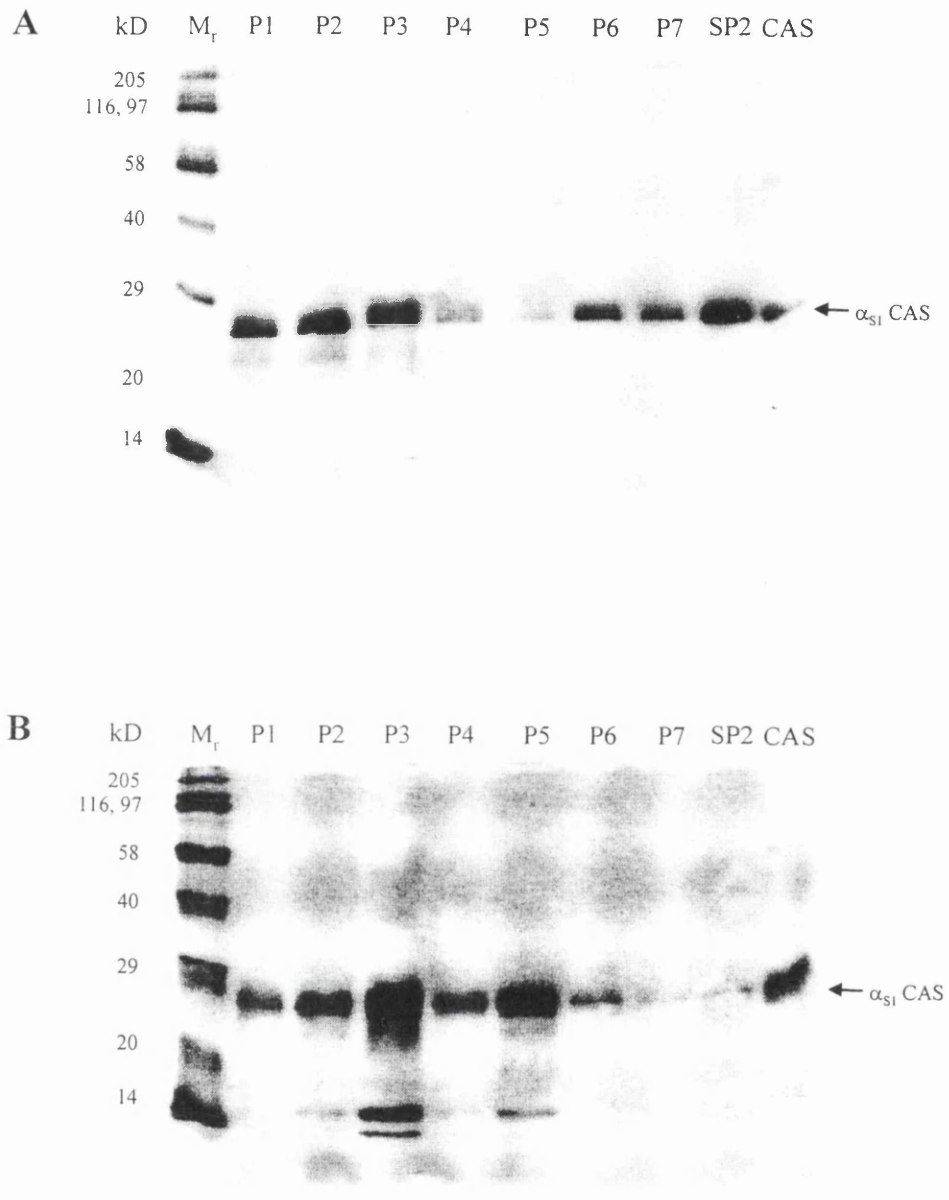


Figure 5.8 Secretion of α_{S1}-casein by goat and sheep mammospheres after passaging

Goat cells were plated onto EHS matrix at equal density at successive passages. Culture medium conditioned for 4 h (A) and EGTA extract (B) were immunoblotted for α_{S1}-casein. One lane of sheep cells at passage 2 (SP2) is run for comparison. Protein standard was 50 ng goat whole casein.

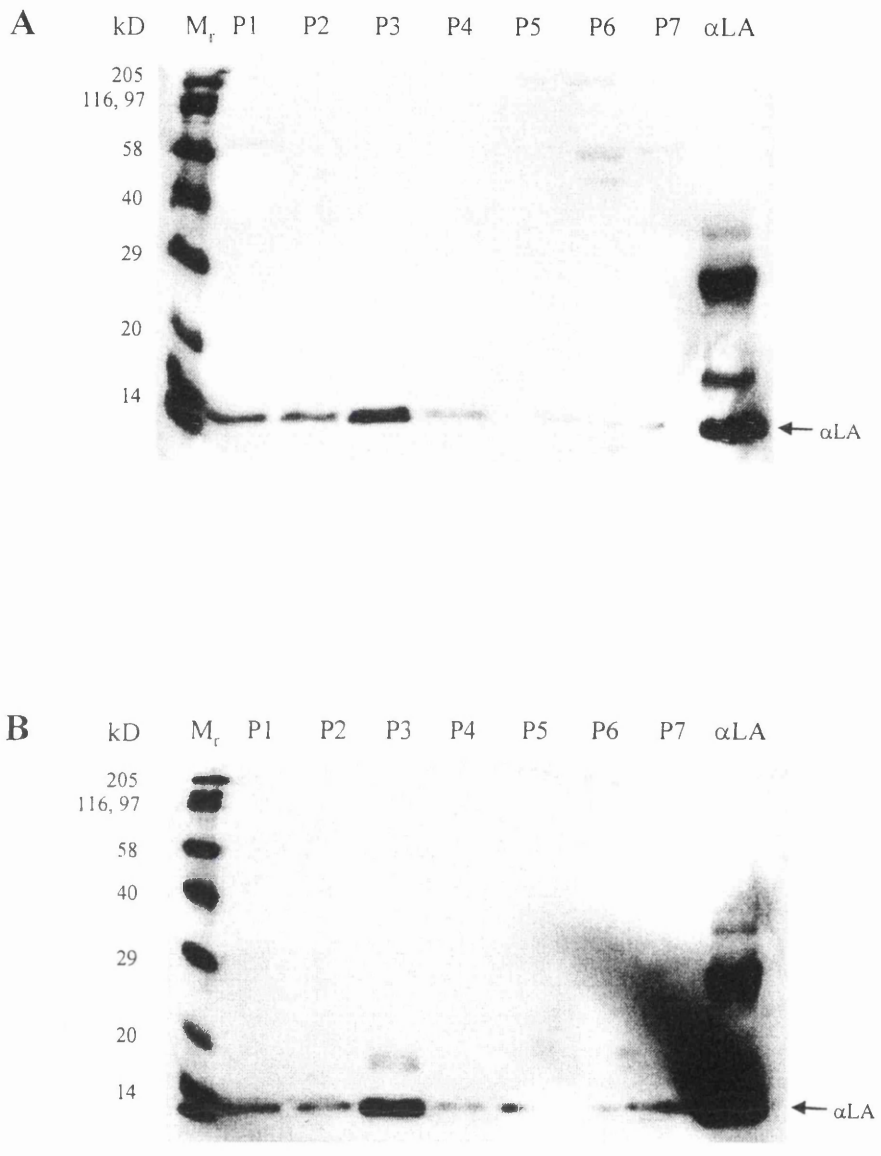


Figure 5.9 Secretion of α-lactalbumin by goat mammospheres after passaging
Goat cells were plated onto EHS matrix at equal density at successive passages. Culture medium conditioned for 4 h (A) and EGTA extract (B) were immunoblotted for αLA. Protein standard was 100 ng bovine αLA.

levels, there are 2^n combinations. Factorial experimental designs measure the main effects of a treatment and the interactions between treatments, where main effects are extracted before 2 way interactions, and 2 way interactions before 3 way interactions etc. When a large number of variables ($\geq$ 5) are introduced into the design, higher order interactions tend to become negligible and can be disregarded. Therefore, in order to obtain the essential information (single effects and double interactions) only half the number of runs are required, since there is sufficient hidden replication. Five factors were investigated and these are detailed in Table 5.2. The 16 treatments testing permutations of these conditions were randomly generated using Minitab 10.02, and are shown in Table 5.3.

The cells' ability to differentiate was assessed at passage 4 and passage 6, on d6 of culture on EHS matrix, by immunoblotting for the milk proteins β-lactoglobulin, α_{S1}-casein and α-lactalbumin. To study possible within-treatment variability, culture medium and luminal extracts were analysed from individual culture wells. Insufficient material prevented analysis of all milk proteins in all wells, but each well was analysed for one or more milk proteins.

In this experiment, milk protein secretion was not detected at passage 6, nor in EGTA extracts at passage 4 (results not shown), but representative immunoblots of culture medium at passage 4 are shown in Figures 5.10-12. Densitometry of immunoblots, expressed relative to the standard conditions (treatment N), is shown in Figures 5.13-15. Statistical analysis of these data showed that the secretory response under different culture conditions is similar for each milk protein, irrespective of the culture well analysed (Figure 5.16). One exceptional set of data for β-lactoglobulin is primarily due to anomalous responses to treatments H and M (Figure 5.13).

Compared with the standard conditions used in previous experiments, secretion of milk proteins was improved only by reducing the concentration of serum from 25% to 10% during the proliferation phase and reducing the concentration of prolactin during differentiation (Treatment M). In the case of β-lactoglobulin and α_{S1}-casein the increase was only marginal; only α-lactalbumin showed a more marked increase of 29%.

Table 5.2 Variables tested for optimisation of differentiation at late passage in goat mammary epithelial cells

	Standard Conditions	Test Conditions
Collagen Type I	Uncoated (plastic)	Coated (collagen)
Cortisol	1 μg/ml	0.1 μg/ml
Serum (HS 4: FCS 1)	25% (v/v)	10% (v/v)
Epidermal growth factor	10 ng/ml	0 ng/ml
Prolactin	3 μg/ml	0.5 μg/ml

Table 5.3 Computer-generated randomised treatment matrix

	Proliferation conditions				Differentiation
Treatment	Collagen	Serum (%)	Cortisol (μg/ml)	EGF (10 ng/ml)	Prolactin (μg/ml)
A	-	10	0.1	-	0.5
B	+	25	1.0	-	3.0
C	+	10	1.0	+	3.0
D	-	10	0.1	+	3.0
E	-	10	1.0	-	3.0
F	+	25	0.1	-	0.5
G	+	25	1.0	+	0.5
H	-	25	1.0	-	0.5
I	+	10	0.1	+	0.5
J	-	25	0.1	+	0.5
K	+	10	0.1	-	3.0
L	+	25	0.1	+	3.0
M	-	10	1.0	+	0.5
N	-	25	1.0	+	3.0
O	+	10	1.0	-	0.5
P	-	25	0.1	-	3.0

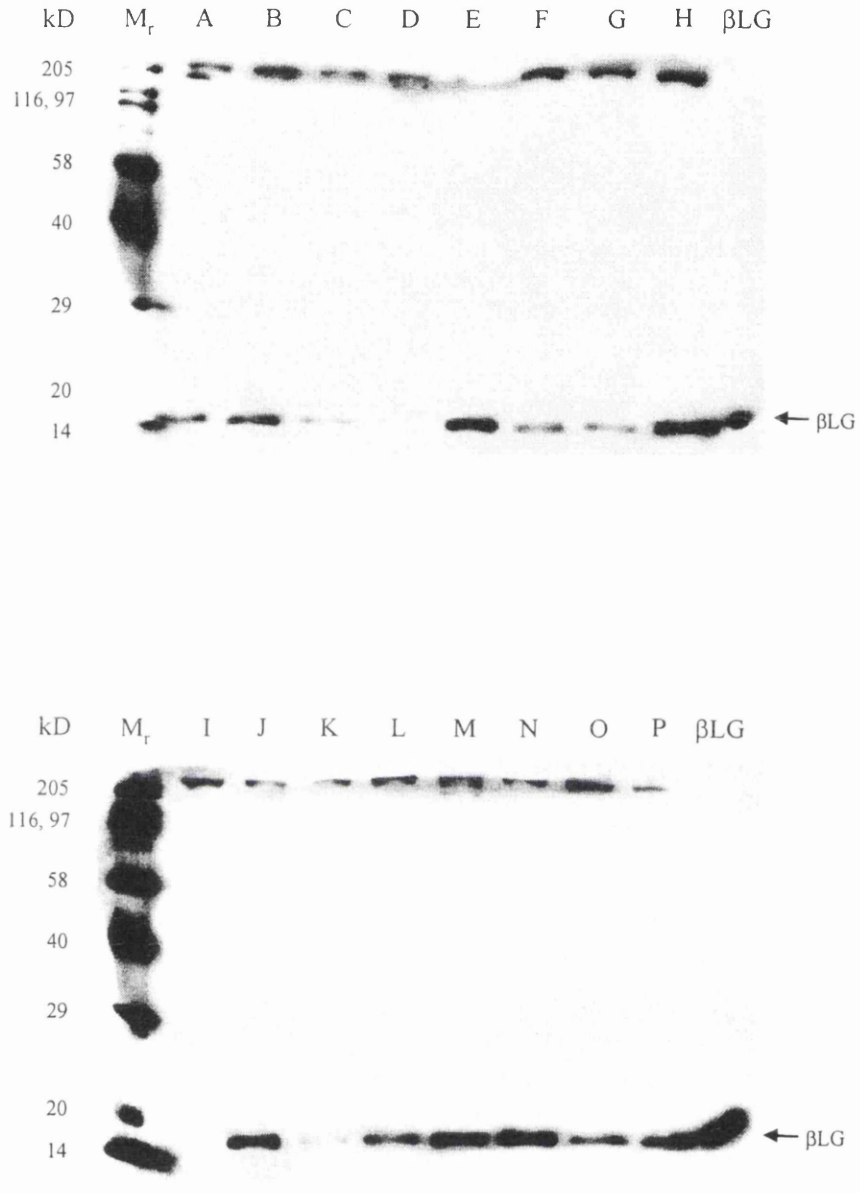


Figure 5.10 Secretion of β-lactoglobulin by goat mammary epithelial cells passaged and differentiated under various conditions

Goat cells were cultured under the conditions described in Table 5.2, being plated onto EHS matrix at equal density at passage 4. Culture medium conditioned for 4 h on d6 of EHS culture was immunoblotted for βLG. Protein standard was 50 ng bovine βLG.

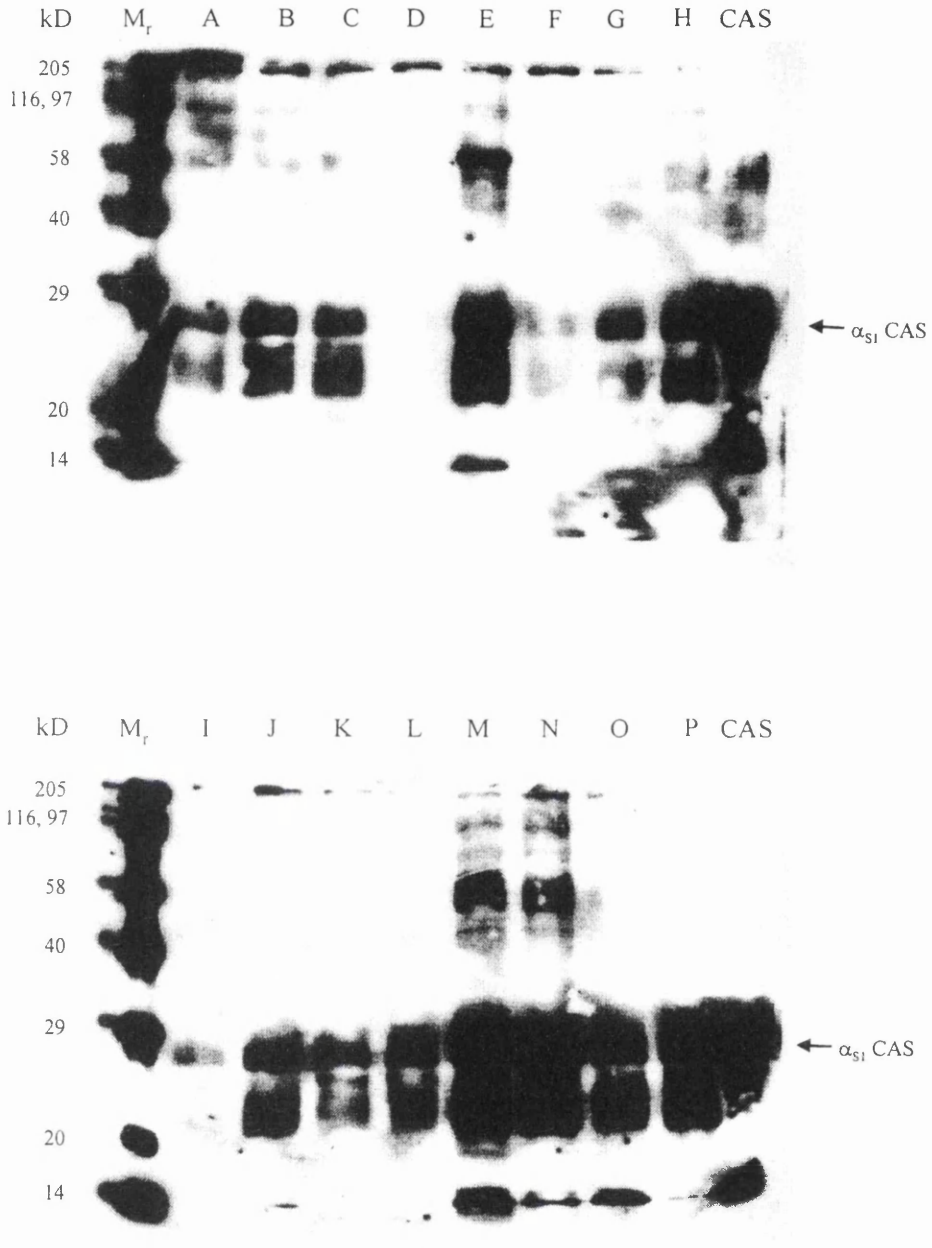


Figure 5.11 Secretion of α_{S1}-casein by goat mammary epithelial cells passaged and differentiated under various conditions

Goat cells were cultured under the conditions described in Table 5.2, being plated onto EHS matrix at equal density at passage 4. Culture medium conditioned for 4 h was immunoblotted for a_{S1}-casein. Protein standard was 50 ng goat whole casein.

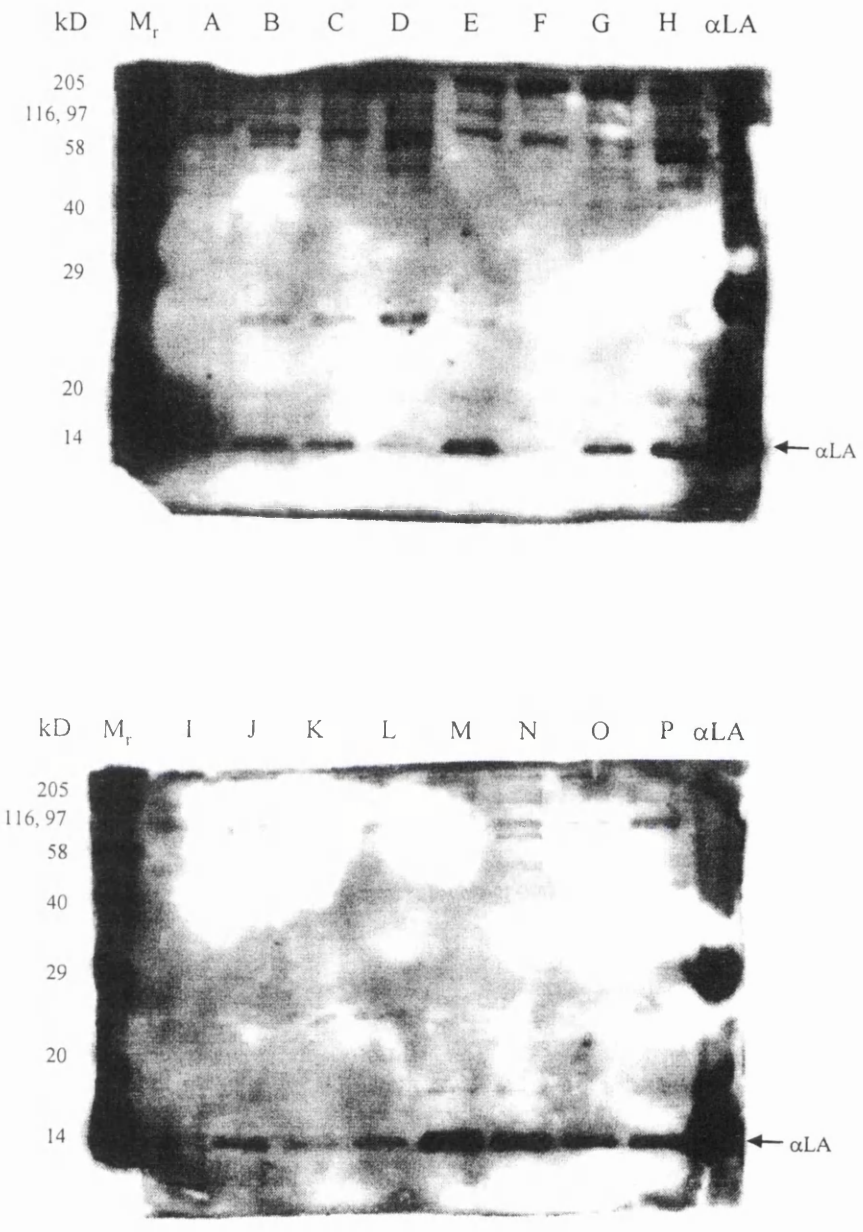


Figure 5.12 Secretion of α-lactalbumin by goat mammary epithelial cells passaged and differentiated under various conditions

Goat cells were cultured under the conditions described in Table 5.2, being plated onto EHS matrix at equal density at passage 4. Culture medium conditioned for 4 h was immunoblotted for α-lactalbumin. Protein standard was 25 ng α-lactalbumin.

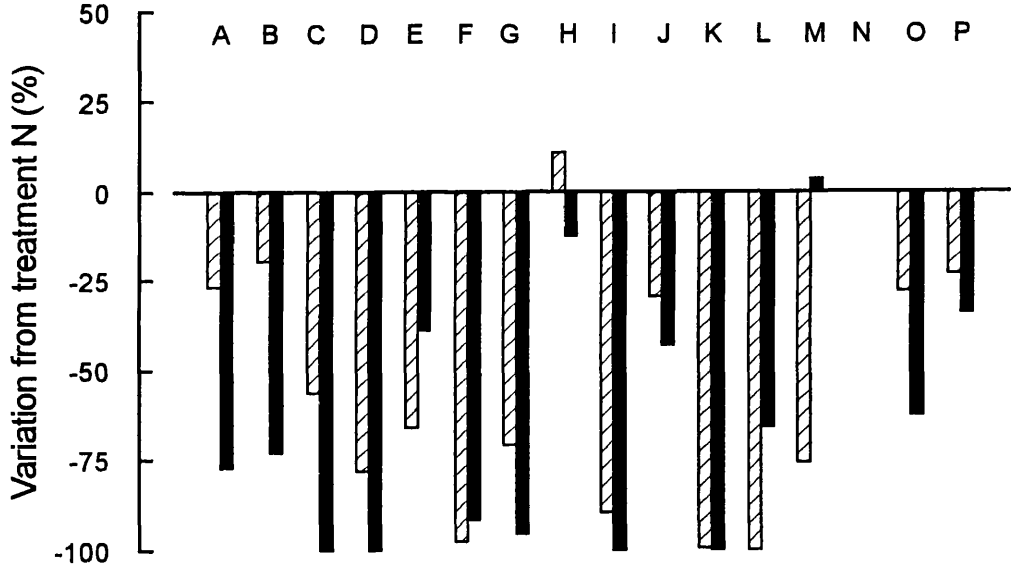


Figure 5.13 Effect of culture conditions on secretion of βLG
Secretion of βLG was measured by densitometry of immunoblots. Results are expressed relative to standard conditions, treatment N. Results are shown for 2 wells, indicated by hatched and solid bars.

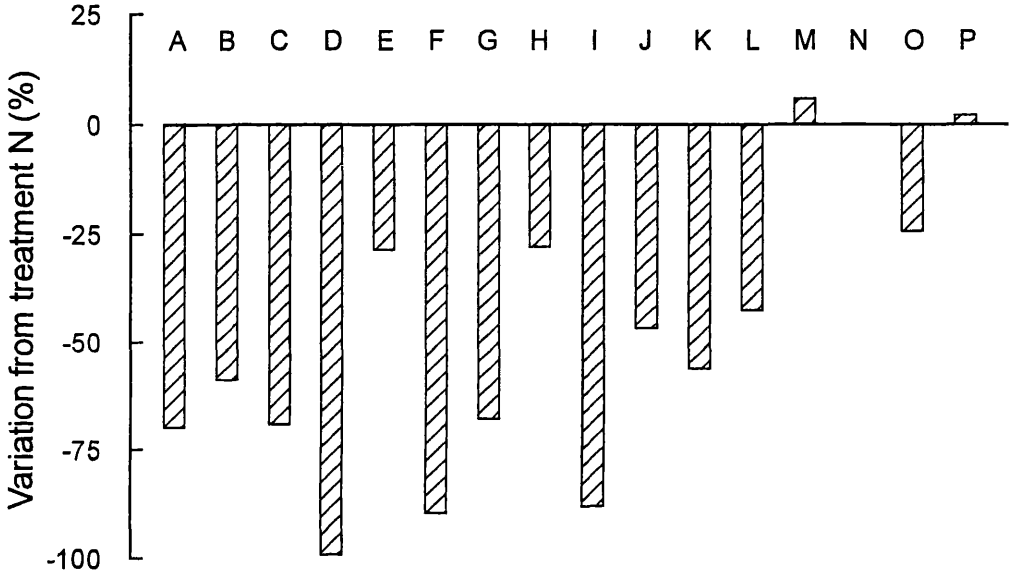


Figure 5.14 Effect of culture conditions on secretion of α_{S1}-casein
Secretion of α_{S1}-casein was measured by densitometry of immunoblots. Results are expressed relative to standard conditions, treatment N.

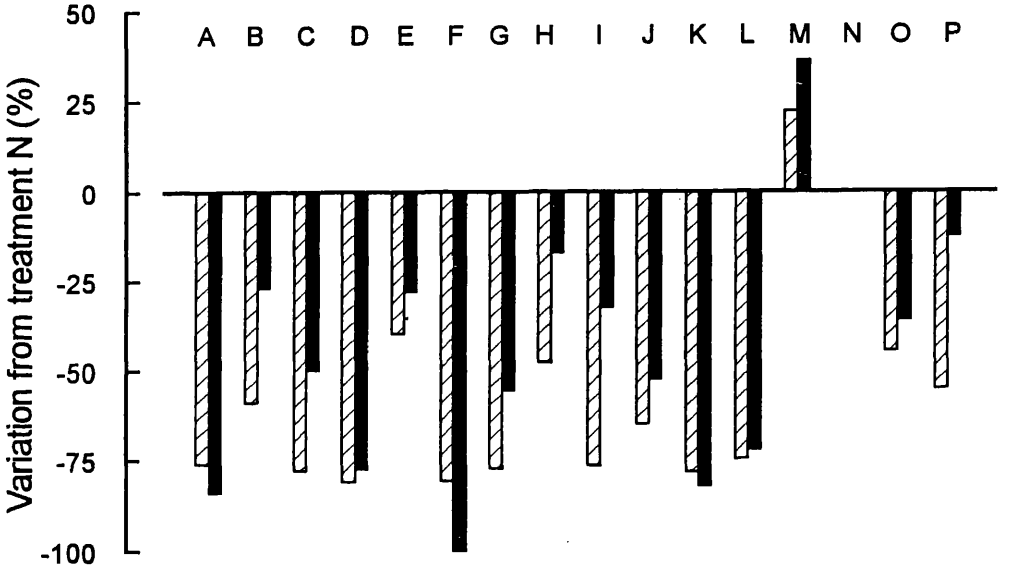


Figure 5.15 Effect of culture conditions on secretion of αLA
Secretion of αLA was measured by densitometry of immunoblots. Results are expressed relative to standard conditions, treatment N. Results are shown for 2 wells, indicated by hatched and solid bars.

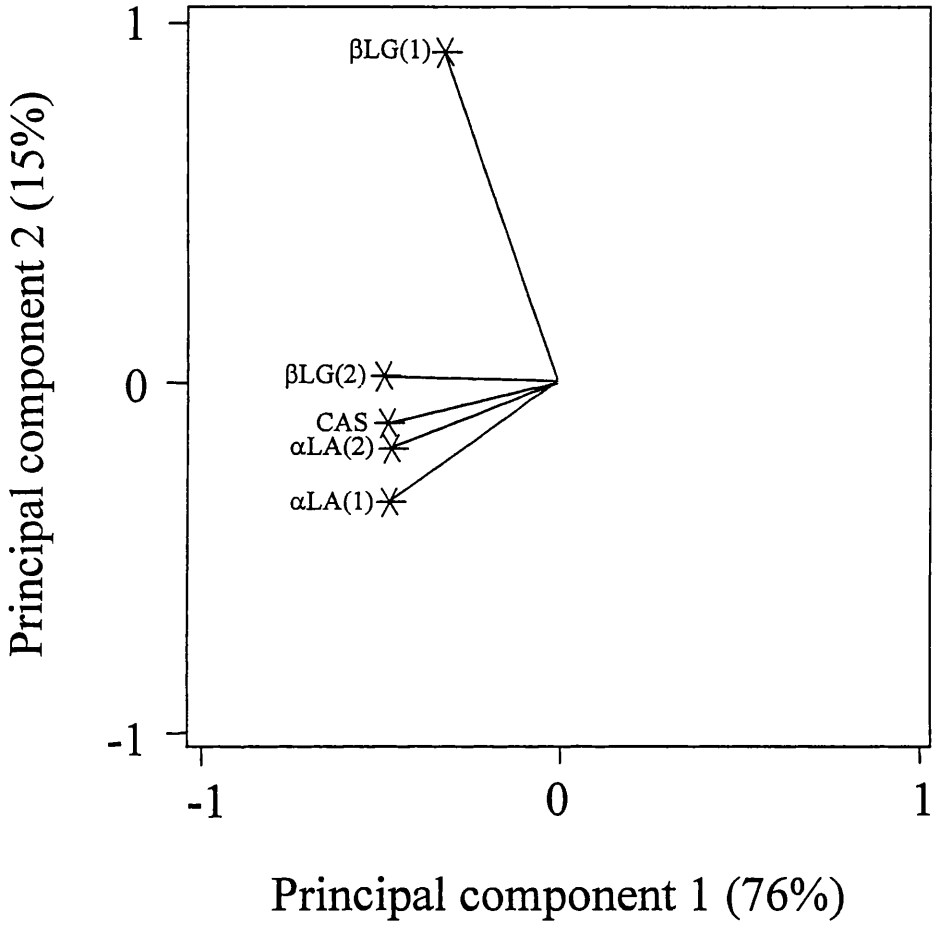


Figure 5.16 Reproducibility of treatment effects on protein secretion
The secretion of 3 milk proteins, βLG, α_{S1}-casein and αLA were analysed from quadruplicate sets of culture wells.

A normal probability plot (Figure 5.17), illustrates the magnitude of the different effects (but not whether inhibitory or stimulatory). The most significant individual factors influencing secretory performance were (i) the presence of collagen during cell proliferation, which adversely affected differentiated function and (ii) cortisol concentration, the lower concentration resulting in lower secretory activity. Statistical analysis also revealed a weak interaction between serum concentration during proliferation, and prolactin concentration during differentiation on EHS matrix. This interaction indicates that with low serum concentration during proliferative phases, a lower prolactin concentration does not adversely affect protein secretion. Conversely, a high serum concentration during cell growth requires a higher prolactin concentration thereafter to achieve optimal protein secretion. All other treatments did not deviate from a normal distribution (Figure 5.17).

5.2.5 Persistence of secretion in goat mammary epithelial cell culture

Two experiments, each using cells from a different animal, were performed to determine the persistence of milk protein secretion in goat mammosphere cultures. Cells were plated onto EHS matrix at passages 3 and 4 respectively, and culture medium was harvested each day, until collapse of the mammospheres occurred. Analysis was by immunoblotting.

In passage 3 cells, secretion of both β-lactoglobulin and α_{S1}-casein was induced on d4 of culture on EHS matrix, was maximal on d6, and was maintained until day 11 or day 15 of culture respectively (Figure 5.18). In passage 4 cells from a different animal, β-lactoglobulin was not tested, but α_{S1}-casein was evident from d6 to d21, with maximal secretion at d11-13 (Figure 5.19).

In general, milk protein secretion was sustained for longer, and easier to detect, in the goat cells. It was hypothesised that the reason for this may be related to age or parity of the animal, since all sheep cells were derived from multiparous animals aged between 3 and 6 years, while goat cells were derived from 2 year old animals in their first pregnancy. To address this, cells were prepared from a primiparous ewe, but cell recovery data did not differ from that obtained with other sheep cell preparations and milk protein secretion was not detected, although cells grew normally.

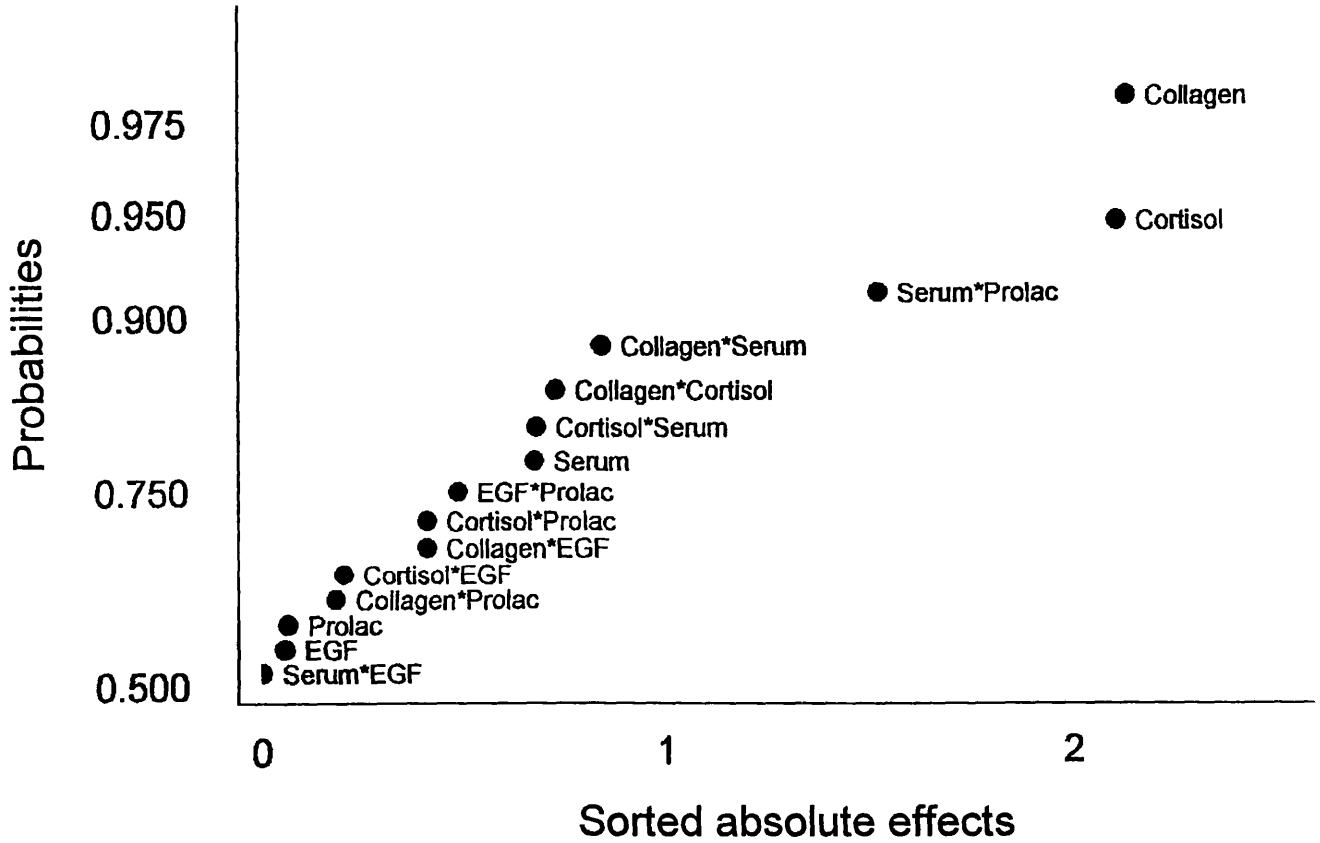


Figure 5.17 Normal probability plot for principal component 1 (76%)
Graph shows the magnitude of effects and deviation from normal distribution.

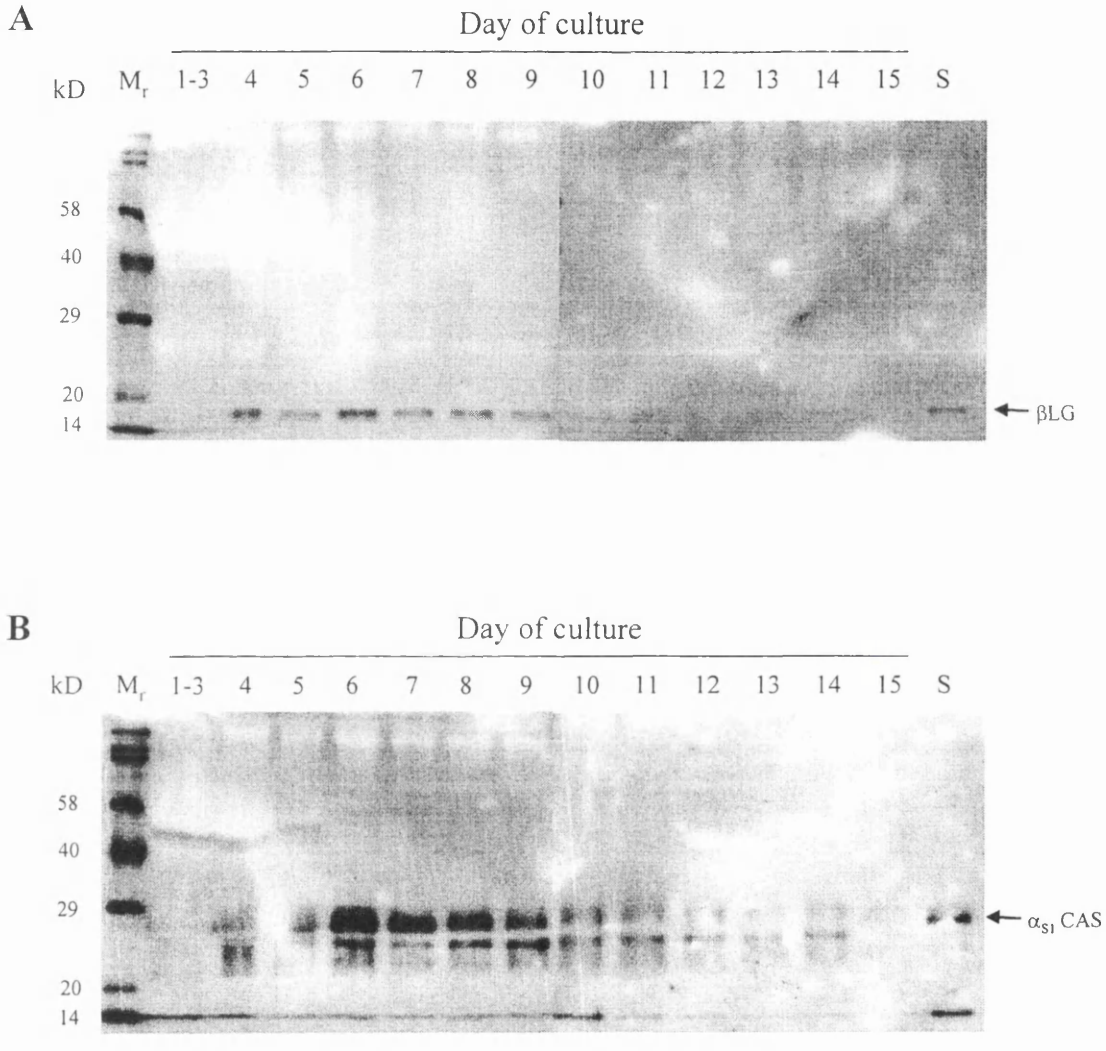


Figure 5.18 Sequential harvesting of milk proteins from goat mammosphere cultures

Passage 3 goat cells (animal GP25) were plated onto EHS matrix with a medium change each day. Retained medium was immunoblotted for β-lactoglobulin (A) and α_{S1}-casein (B). Protein standard was 50 ng bovine βLG or 50 ng goat whole casein (S).

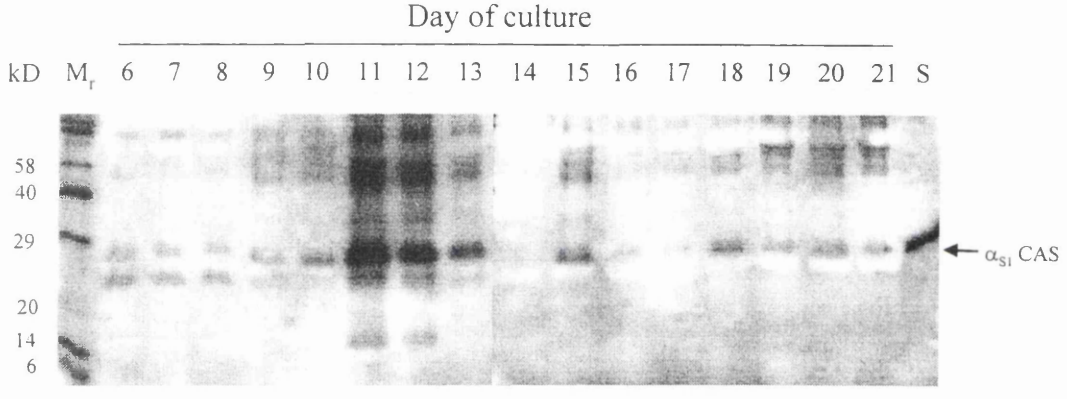


Figure 5.19 Persistence of milk protein secretion by goat mammospheres
Passage 4 goat cells (animal GP28) were plated onto EHS matrix with a medium change each day. Retained medium was immunoblotted for α_{S1}-casein. Protein standard was 50 ng goat whole casein (S).

5.3 DISCUSSION

Sheep cell passaging experiments (Chapter 4) had shown that substantial cell losses occurred during harvesting. Removal of sheep cells from tissue culture plastic routinely took 20-30 min, in comparison to the figure of 10-15 min for mouse cells reported by Streuli *et al* (1991). It is possible that this long exposure to trypsin was detrimental to the cells. Trypsin has been shown to cause accumulation of lysophospholipids in the cell surface, which diminishes adhesion, but this is reportedly reversible during incubation with serum under warm conditions (Curtis & Hill, 1979). Another possibility is that a ligand molecule may be damaged by trypsinisation. In addition, there is evidence to suggest that cells have different sensitivities to trypsin digestion, which may be related to cell cycle (Sharpe *et al*, 1986). The inclusion of EGTA and polyvinylalcohol in the formulation did improve cell viability in the short term. EGTA is present as a chelating agent, while PVA is reported to act as a protectant for cells against injury in suspension (Michaels & Papoutsakis, 1991). With extended passaging, however, cell recovery continued to decline. This phenomenon was not observed for the goat cells. This may be purely a species difference, since all culture conditions were the same, and many passaging experiments with sheep and goat cells were carried out simultaneously. Alternatively, while culture conditions have been shown to be near optimal for goat cells, this may not have been the case for sheep cells.

The results of the sheep passaging experiments described in this chapter confirmed that milk proteins were only detected up to passage 2 in culture. In addition, β-lactoglobulin and α_{S1}-casein were detected predominantly in the culture medium fraction, suggesting poor mammosphere integrity. This was not caused by low plating density, but appeared to be an inherent property of the cells. Milk protein secretion showed variation between animals, but also with EHS batch.

In contrast to sheep cells, the numbers of goat cells recovered were consistently higher, and yields were maintained throughout successive passages. Although protein secretion declined with increasing passage, milk proteins were readily detectable up to passage 6 or 7 in duplicate cultures from one animal. In a third experiment with the same cells, protein failed to be detected at passage 6, but this was probably due to a change in sampling method, resulting in increased sample losses during processing. Milk proteins were also detected up to passage 4 in cells

derived from a second animal. Milk protein secretion was not detected in cells from a third animal, but these cells grew abnormally, and their use was discontinued. On two occasions milk protein secretion was detected for a number of days in culture on EHS, raising the possibility of harvesting transgene products in this manner, for further analysis. Interestingly, at passage 4 maximal protein secretion was seen to occur between days 11 and 13, yet milk protein measurements were routinely taken at days 6-8 of culture. It is possible that with increasing passage number, cell differentiation is delayed.

5.4 CONCLUSIONS

Passaging experiments were designed to generate sufficient cells for analysis of transgene expression on transfer to EHS matrix, based on a transfection efficiency of ~0.5%. Three experiments showed that goat cells from one animal would secrete milk proteins until passages 4-7, although secretion levels declined after passage 3. This was confirmed by replication of the initial passaging experiments with cells from another animal. In addition, the fractional factorial experiment suggested that culture conditions for differentiation at late passage were near optimal. This suggested that an alternative strategy for transgene expression might be to generate a smaller number of more differentiated cells by passaging only three times, and to harvest secreted protein from the culture medium over the lifespan of the mammosphere culture.

In addition to providing a means of testing transgenes, these culture systems provide further opportunities to study the regulation of milk protein gene expression. Both goat mammary cells and, more markedly, sheep mammary cells lost differentiative ability with increasing passage. It would be interesting to determine whether there are species differences in mechanisms controlling milk protein expression, for example in signal transduction, or whether differentiation could be "rescued" perhaps by transfection of exogenous genes.

CHAPTER SIX

TRANSFECTION OF RUMINANT MAMMARY EPITHELIAL CELLS

6.1 INTRODUCTION

The introduction of DNA into isolated mammary epithelial cells would provide a powerful means of studying the regulation of milk protein secretion in ruminants, about which relatively little is known, as well as allowing the evaluation of transgenes in culture prior to making transgenic animals. This strategy is dependent, however, on the selection and differentiation of stable transfectants. Results in Chapters 4 and 5 suggested that sheep cells are incapable of sustaining milk protein secretion after the extended passaging required for the selection of stable transfectants, but that this might be achievable with some preparations of goat cells. It was therefore decided to investigate transfection of goat mammary epithelial cells.

Transfection conditions differ for every cell type, and are influenced by a number of factors, including cell density, DNA and reagent concentration, serum concentration and incubation time. These factors were investigated using a range of transfection methods selected on the basis of their different strategies for getting DNA into the cell. Four transfection reagents were tested: Lipofectamine (Life Technologies, Paisley, UK), a formulation of liposomal lipids; Fugene (Boehringer-Mannheim Ltd, Lewes, UK), a blend of non-liposomal lipids; Superfect (Quiagen, Crawley, UK), a synthetic polycationic dendrimer; which assembles the DNA into compact structures that resemble histones; and calcium phosphate, which appears to facilitate entry of the DNA into the cell via binding to cell membranes and endocytosis (Graham & van der Eb, 1973). The aims of the work in this chapter were to:

i) optimise conditions for transient transfection of goat mammary epithelial cells using plasmids containing a constitutive reporter gene;

ii) optimise conditions for selection of stable transfectants;

iii) look for differentiation-specific expression of a reporter gene linked to a milk protein gene promoter (ß-lactoglobulin);

iv) look for expression of a foreign gene linked to the ß-lactoglobulin promoter in differentiated cells.

6.2 RESULTS

6.2.1 Optimisation of transient transfection

All optimisation of transfection conditions was carried out using reporter gene construct pCH110 (SV40 promoter), with an absorbance ratio (A_{260}:A_{280}) of 1.7-1.9 (pure DNA is 1.8).

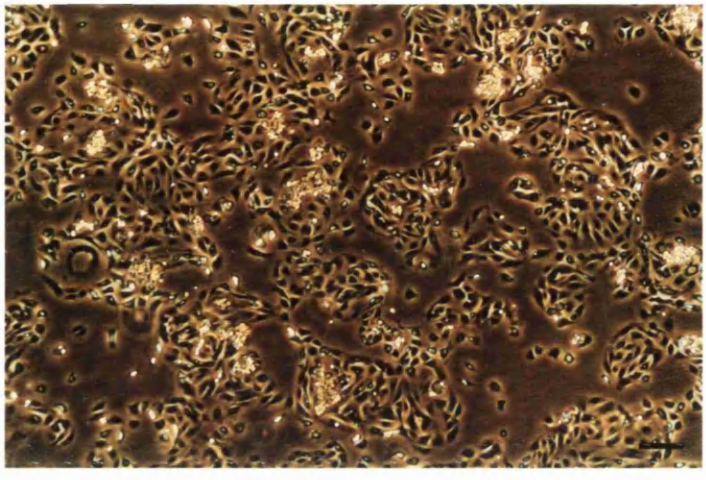
Lipofectamine

Goat mammary epithelial cells and HCll cells, which were used as a positive control, were transfected with 2 μg DNA /35mm well in Opti-MEM medium (a commercial reduced-serum preparation recommended for this purpose) with varying amounts of Lipofectamine reagent (range 0-16 μl). Cells were incubated with the DNA/reagent complex for a 5 h period.

Microscopic observations at 2 h after transfection showed the presence of DNA/reagent complexes, which appeared as faint particles scattered over the cell monolayer. Some cytotoxicity was also apparent, where Lipofectamine concentration exceeded 4 μl/well in HC11 cells, or 8 μl/well in goat cells (Figure 6.1). This cytotoxicity was more pronounced after the 5 h incubation was completed. At 48 h after transfection, positively staining cells (illustrated in Figure 6.2) were counted in duplicate wells, and additional wells were harvested for DNA assay. These results are shown in Figure 6.3. Cell losses occurred with increasing Lipofectamine concentration in a dose dependent manner (regression coefficient –0.99, $p<0.001$). Transfection of goat epithelial cells and HC11 cells was most effective at 8 μl Lipofectamine per well. The transfection efficiency (number of positively staining cells from total surviving) at this concentration was ~0.5% in goat epithelial cells, but was ~8% in HC11 cells.

A second experiment showed that the use of Opti-MEM resulted in a greater number of positive cells than transfection in standard culture medium with or without serum. Transfection was further improved by adding serum-containing medium to the transfected cells after 5 h, instead of replacing it with fresh medium (Figure 6.4). Initial experiments used a DNA concentration of 2 μg/well, but subsequent tests indicated that 3 μg DNA/well produced the greatest number of positive blue cells (Figure 6.5). Further transfections, maintaining the ratio of 3 μg DNA:8 μl Lipofectamine,

A



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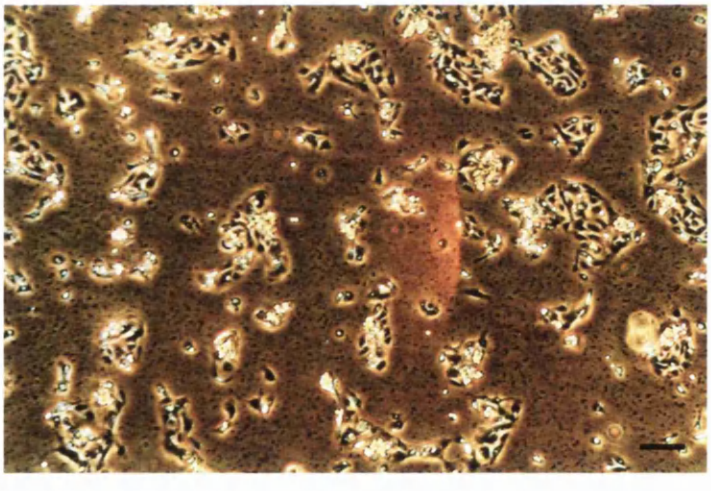

Figure 6.1 Effect of Lipofectamine on goat mammary epithelial cells
Goat mammary epithelial cells were examined by phase contrast microscopy 2h after transfection with 2 μg DNA and 0 μl (A) or 16 μl (B) Lipofectamine. (Mag 30x; Bar = 200 μm)

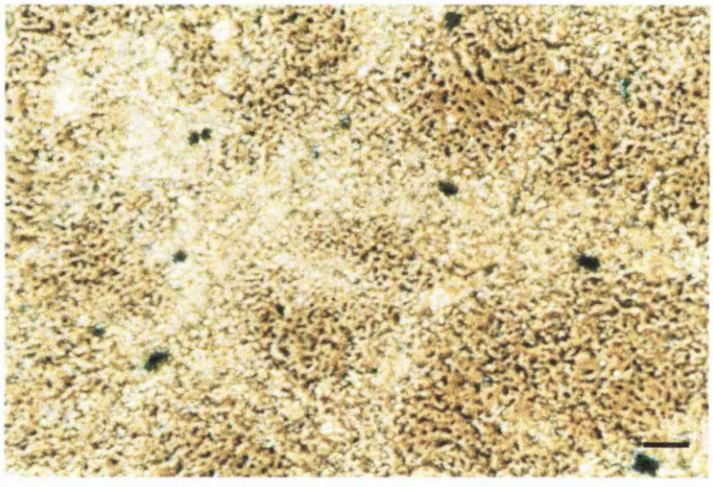

Figure 6.2 Transfected goat mammary epithelial cells stained for β-galactosidase activity

Goat mammary epithelial cells were transfected with a reporter construct pCH110, which expresses the Lac Z gene, using Lipofectamine. Cells were assayed for β-galactosidase activity (indicated by blue staining) 48 h after transfection and examined by phase contrast microscopy.
(Mag 30x; Bar = 200μm).

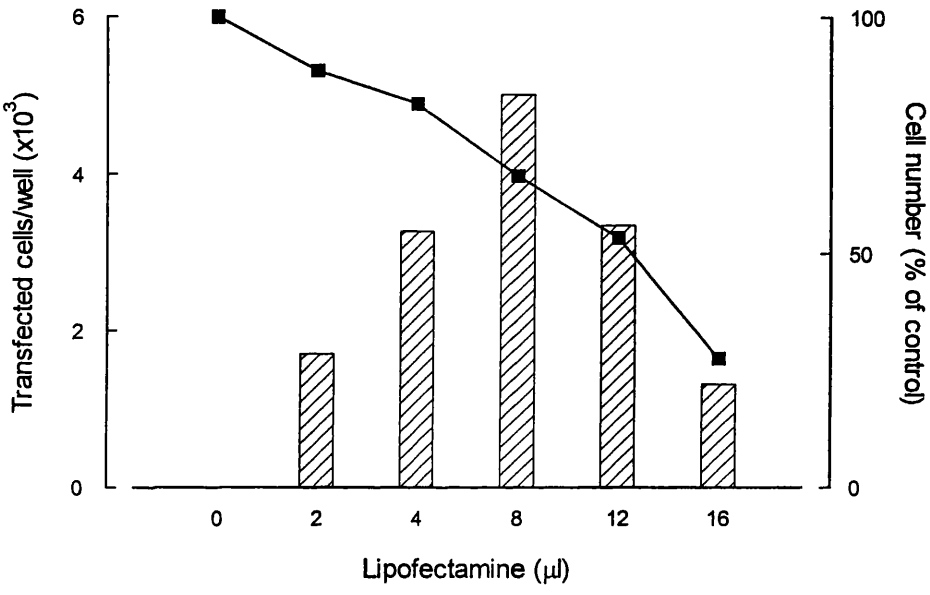

Figure 6.3 Effect of Lipofectamine on transfection and cell toxicity
Goat mammary epithelial cells were transfected with 2 μg DNA for 5 h in Opti-MEM. 48 h after transfection, cells were assayed for positively staining transfected cells (bars), and cell number determined by DNA assay (line graph). Results are means of duplicate determinations.

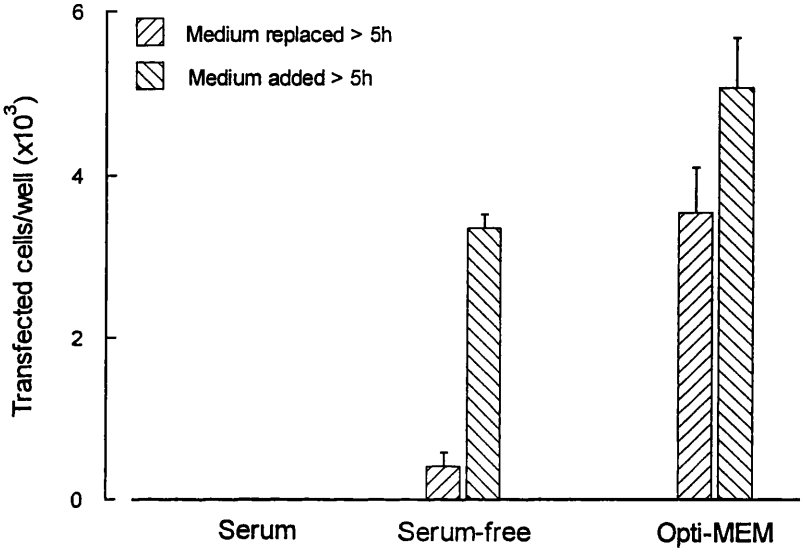

Figure 6.4 Effect of medium composition on Lipofectamine transfection
Goat mammary cells were transfected with 8 μl of Lipofectamine and 2 μg of DNA for 5 h. Cells were assayed for positively staining transfected cells 48 h after transfection. Results are means of triplicate wells ± SEM.

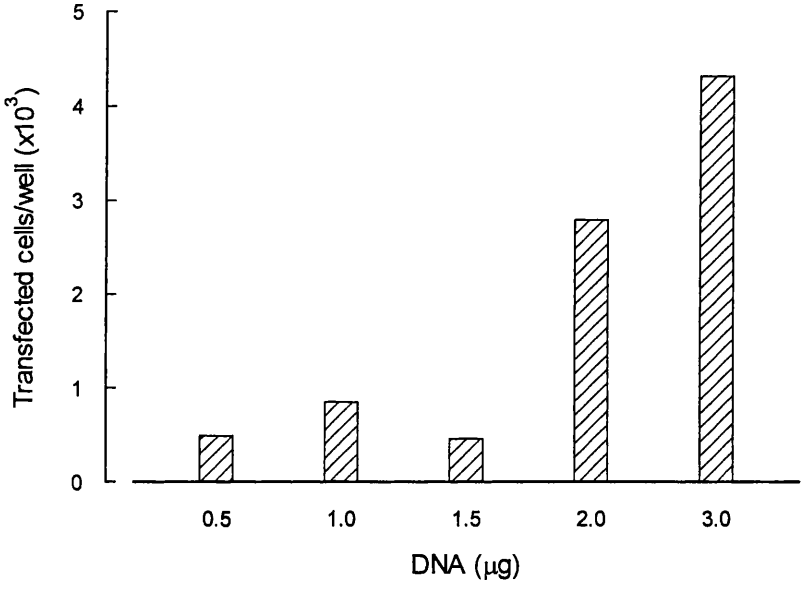

Figure 6.5 Effect of DNA concentration on transfection with Lipofectamine Cells were transfected with 8 μl Lipofectamine for 5 h in Opti-MEM, and assayed for positively staining cells 48 h after transfection. Results are means of duplicate determinations.

suggested that short-term exposure and a high Lipofectamine concentration was more effective than transfection with a lower concentration for longer, before serum addition (Figure 6.6) although results were not statistically significant (ANOVA).

Fugene

DNA-Fugene complexes were formed in serum-free medium, and transfection took place in Opti-MEM or normal serum-containing medium. Results from two experiments showed that transfection was maximal using a ratio of 3 μl Fugene:1 μg DNA per well (Figures 6.7 and 6.8), and 50% greater in normal culture medium than Opti-MEM (results not shown). The transfection efficiency of goat mammary epithelial cells was ~0.35%. Fugene had no discernible toxic effect, and was routinely left in contact with the cells until assay.

Superfect

Transfection using Superfect was most successful when a 10 μl reagent:2 μg DNA ratio was used (Figure 6.9). Cytotoxicity was only evident at the lowest concentration of reagent tested, possibly resulting from the formation of very large complexes. Transfection rate was higher in normal medium than in Opti-MEM (results not shown). Initially, the manufacturer's recommended incubation time for primary cells of 3 h was adopted (Figure 6.9A). In a second experiment, however, incubation of cells and DNA complex until assay (48 h) increased transfection ten-fold (Figure 6.9B).

Calcium phosphate

The results of transfection using calcium phosphate and a 4 h incubation time are shown in Figure 6.10. Initial results showed very low levels of transfection in comparison to the other methods already tested (Figure 6.11), and no further optimisation was carried out.

The three transfection methods used under optimal conditions produced a small and roughly similar number of positive cells (Figure 6.11), but Fugene was the method selected for future transfections owing to its lack of cytotoxicity, and ease of use.

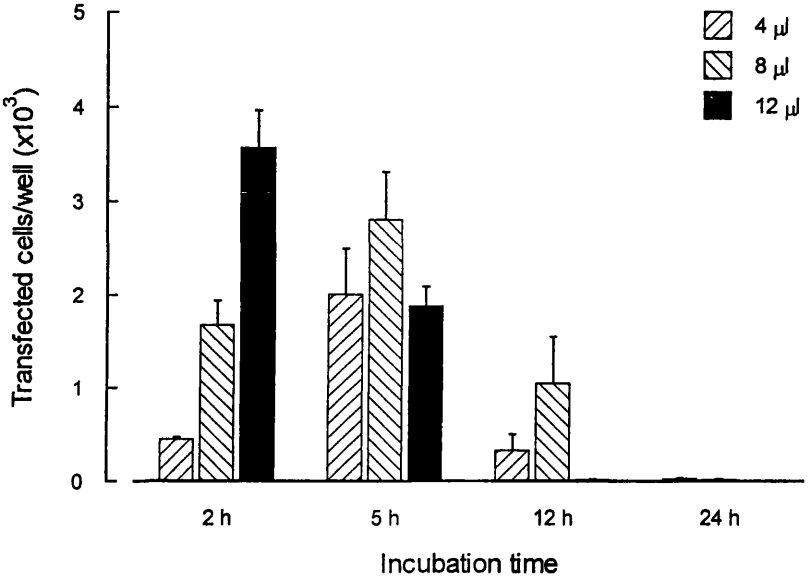

Figure 6.6 Effect of incubation time on Lipofectamine transfection
Cells were transfected in Opti-MEM with varying amounts of Lipofectamine over a range of incubation times. The ratio of Lipofectamine:DNA was maintained at 8 µl:3 µg. Results shown are means of triplicate wells ± SEM.

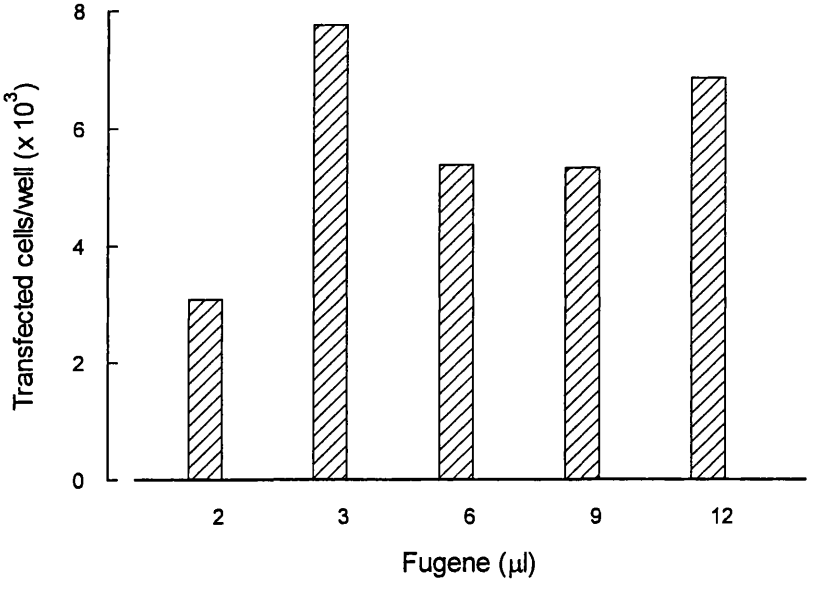

Figure 6.7 Effect of Fugene on transfection of goat mammary epithelial cells Goat mammary epithelial cells were transfected in normal proliferation medium with 1 µg DNA for a 48 h period, before being assayed for positively staining cells. Results are means of duplicate determinates.

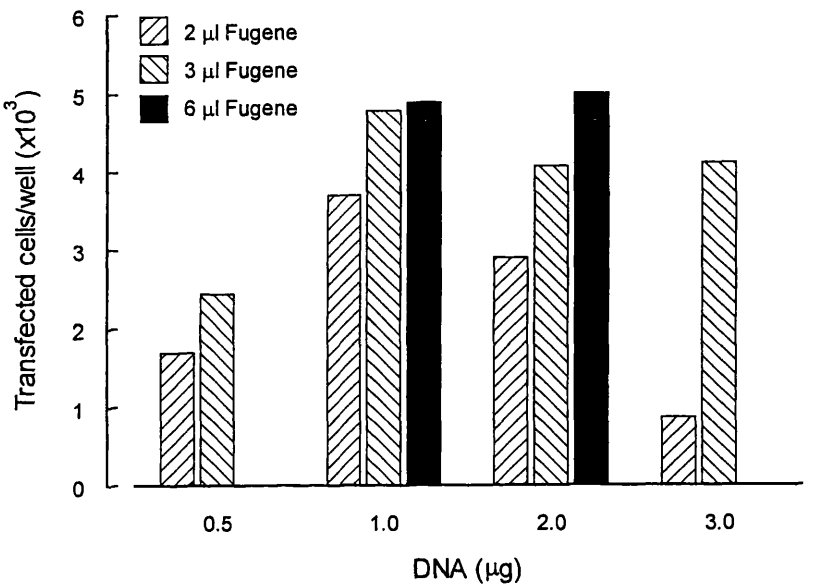

Figure 6.8 Effect of Fugene and DNA concentration on transfection of goat mammary epithelial cells

Goat mammary epithelial cells were transfected in normal proliferation medium for a 48 h period with various combinations of reagent and DNA per well. Results are means of duplicate determinations.

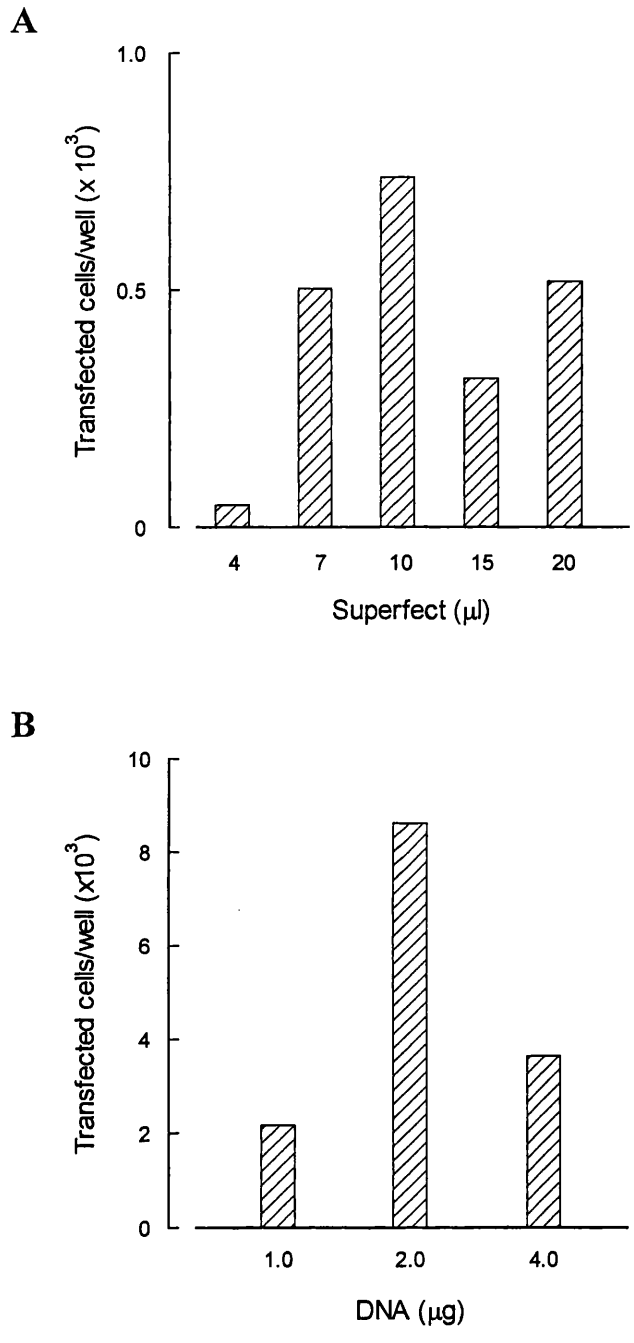

Figure 6.9 Effect of Superfect and DNA concentration on transfection
Goat mammary epithelial cells were transfected in normal proliferation medium with Superfect (range 4-20 µl) and 2 µg DNA for a 3 h period (A) or with 10 µl Superfect for 48 h (B). Results are means of duplicate determinations.

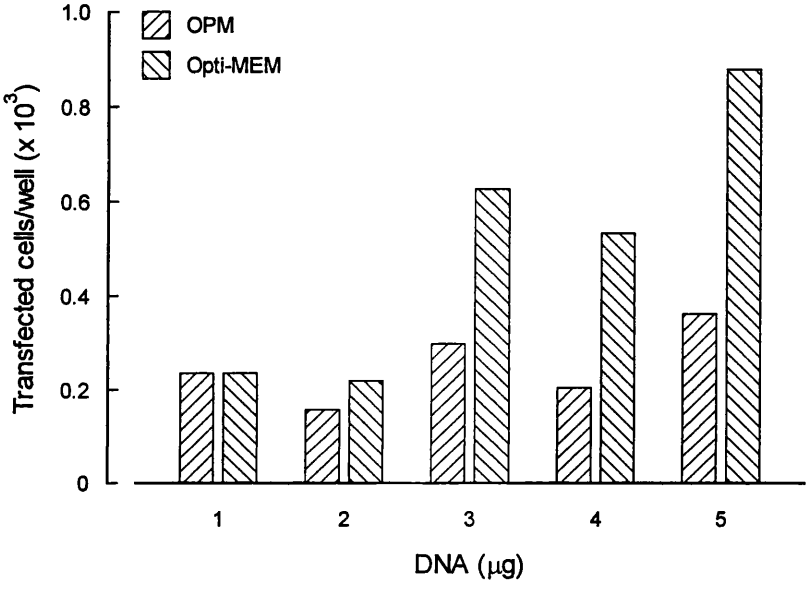

Figure 6.10 Effect of DNA concentration and medium composition on transfection of goat mammary epithelial cells with calcium phosphate
Goat mammary epithelial cells were transfected with calcium phosphate for a 4 h period in normal proliferation medium (OPM) or Opti-MEM. Positively staining cells were assayed 48 h after transfection. Results are means of duplicate determinates.

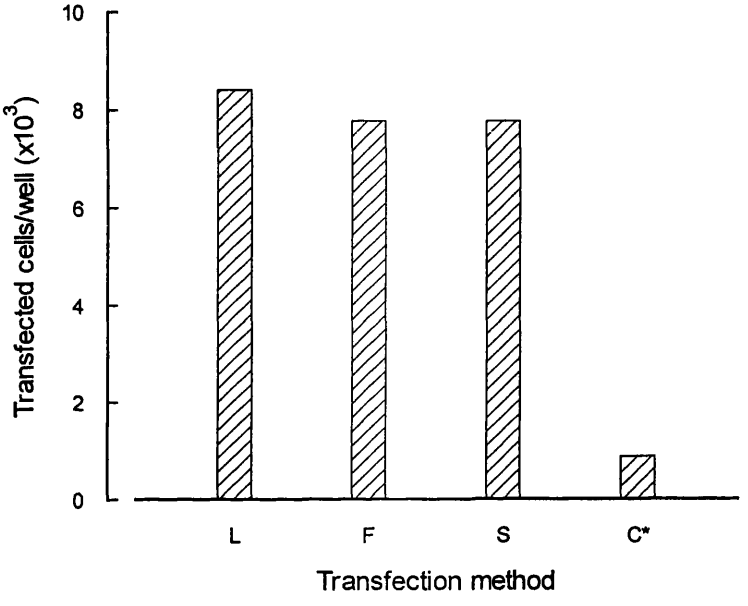

Figure 6.11 Comparison of transfection methods
Transfection methods were used at optimal conditions* with same cell batch and standard plating conditions. L, Lipofectamine; F, Fugene; S, Superfect; C, calcium phosphate; *not optimised.

6.2.2 Evaluation of other reporter constructs

It was decided to evaluate the performance of other reporter constructs in goat mammary epithelial cells, firstly to examine whether expression levels of the reporter gene could be improved, and secondly, because the plasmid pCH110 does not contain a selectable marker gene, for selection of stable transfectants. To this end, two constitutively expressed constructs (pGK–βGAL and pGK-LUC) and one lactation-specific construct (pβLG-LUC), all of which contain the selectable marker *neo*, were transfected into goat mammary epithelial cells using Fugene under optimal conditions. Positive staining for β-galactosidase in the cells transfected with pGK-βGAL indicated a very low transfection efficiency (~0.1%). In two experiments, luciferase activity in cells transfected with the other two constructs was at background levels. Overall, the results indicated that efficiency of transfection was uniformly low, irrespective of the construct tested.

6.2.3 Selection of transfected cells

Selection with G418

Transfection of cells with a plasmid containing the bacterial gene *neo* for amino-glycoside phosphotransferase allows selection for stable transformants in the presence of the drugs neomycin and G418 (geneticin). Mammalian cells vary greatly, however, in their tolerance of G418. In order to identify optimum conditions for selection, therefore, untransfected goat mammary cells were grown on tissue culture plastic at concentrations in the range 0-1.2 mg G418/ml culture medium.

Cells were passaged 3 times before becoming susceptible to G418 at a concentration of 0.8-1.0 mg/ml (a value similar to that determined previously at PPL Therapeutics Ltd for sheep mammary cells). G418 selection was also studied in cells transfected with the constitutively expressed construct pGK-βGAL, which contains the neo gene, conferring G418 resistance. Transfected cells were grown on tissue culture plastic in the presence of 1 mg/ml G418. In two experiments passage 1 and 2 cells grew to confluence without apparent inhibition, whereas at passage 3 virtually no cells were recovered.

Selection with Capture-Tec (Invitrogen, Groningen, Netherlands)

This system allows the preferential selection of transiently transfected cells. Co-transfection of the plasmid pHook-1 results in expression of a single chain antibody on the cell membrane. Transfected cells are then selected using magnetic beads coated with the hapten. This technique was tested in two experiments in which pHook-1 was co-transfected (1:1) with either a pGK-βGAL or pcDNA3.1 (Invitrogen) constitutive reporter gene construct. Preliminary experiments showed that pHook was expressed within 5 h and optimal after 18-24 h, and that β-galactosidase expression was also detectable after 24 h. Cells were therefore selected for pHook expression 24 h after transfection and β-galactosidase expression was examined immediately thereafter. The Capture-Tec magnetic beads were used at 10% of the manufacturer's recommended concentration, to take account of the expected low transfection efficiency. Cells selected by pHook constituted ~0.3% of the total cell population, consistent with previous determinations of mammary cell transfection efficiency. It was not possible, however, to determine if selected cells also expressed β-galactosidase, since coating with the magnetic beads obscured the selected cells, preventing detection of any positively staining, blue, cells.

6.2.4 Transfection of constructs into goat mammary epithelial cells

In two experiments, goat mammary epithelial cells from two different animals were co-transfected with pHook and the construct AATB, provided by PPL Therapeutics Ltd, Roslin UK. This construct expresses human α_1-antitrypsin under the control of the β-lactoglobulin promoter. Selected and non-selected cells were cultured separately on tissue culture plastic or EHS matrix, and transgene expression was measured by immunoblotting for the protein product.

Secretion of α_1-antitrypsin was detected in the pooled, conditioned medium of both selected and non-selected cell populations, collected 3-7 days after transfection (Figure 6.12A). AAT was present in d3-7 culture medium irrespective of whether cells were cultured on tissue culture plastic or EHS matrix (Figure 6.12A). In pooled medium collected 7-11 days after transfection, AAT secretion was maintained in cells cultured on EHS matrix, but appeared to be lost in both selected and unselected cells cultured on plastic (Figure 6.12B). Immunoblotting of d5-7 culture medium from a second animal suggested that secretion occurred predominantly on EHS matrix, and a

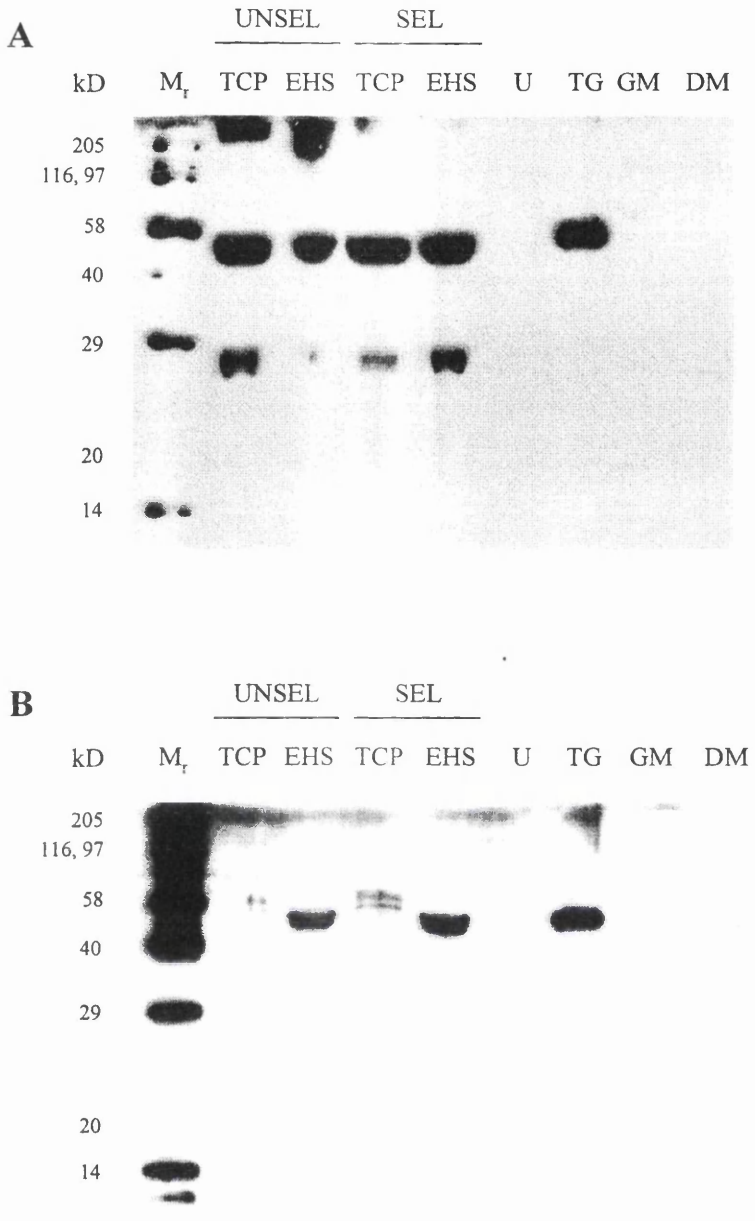

Figure 6.12 Secretion of α_1-antitrypsin in goat cells (animal GP25)
Goat mammary epithelial cells were co-transfected with pHook and construct AATB. Cells selected by pHook (SEL) and those remaining (UNSEL) were cultured separately on tissue culture plastic (TCP) or EHS matrix (EHS). Culture medium collected on days 3-7 (A) or days 7-11 (B) was immunoblotted for human α_1-antitrypsin. U = medium from untransfected cells on EHS matrix; TG = defatted milk from a sheep transgenic for human α_1-antitrypsin; GM = 20 μg defatted goat milk; DM = unconditioned differentiation medium.

double protein band was evident on tissue culture plastic. (Figure 6.13). Although no protein bands are visible for selected cells in Figure 6.13, this was due to technical problems. Further exposure of immunoblots to film demonstrated similar amounts of protein detected in both selected and unselected cells, but also very high background, preventing reproduction of the image. Densitometry suggested that α_1-antitrypsin (AAT) was only 19% or 27% higher in selected cells in comparison to unselected cells, for the two experiments. Substratum dependence of AAT secretion was also observed in a third experiment in which no selection was applied after cell transfection (Figure 6.14).

Two further experiments were performed with goat mammary epithelial cells. The first experiment tested 2 different constructs expressing human growth hormone, driven by the mouse whey acidic protein promoter (WAP2-hGH and ΔNRE-hGH, provided by Andreas Kolb, Hannah Research Institute, UK). The same GH sequence expressed in BHK cells under a metallothionine promoter was used as a positive control. No GH expression was detected in transfected goat mammary epithelial cells, while a band at the expected size for hGH (22 kD) was detected in the BHK cell control (results not shown).

In a second experiment, two constructs driven by the β-lactoglobulin promoter and encoding native and mutated forms of human protein C (pCORP14 and pCORI69, provided by PPL Therapeutics Ltd, Roslin, UK) were tested. In neither case was a transgene detected, although human protein C standards were detected under non-reducing conditions at the expected size of ~62 kD (results not shown).

6.2.5 Transfection of constructs into sheep mammary epithelial cells

The construct AATB was transfected into sheep mammary epithelial cells under the same conditions as those used for goat cells. Pooled culture medium for days 4-7 in culture on tissue culture plastic or EHS matrix was immunoblotted. The results showed a single, subtratum-dependent band at the same position as that of protein standards (Figure 6.15).

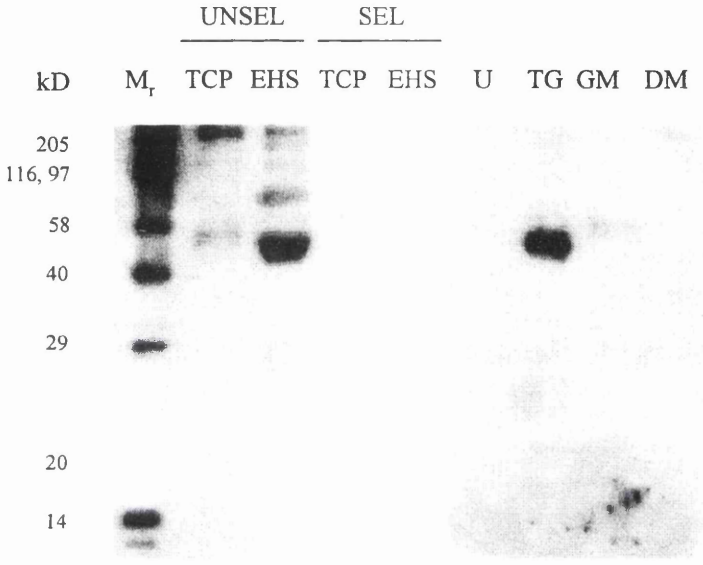

Figure 6.13 Secretion of α_1-antitrypsin in goat cells (animal GP28)
Goat mammary epithelial cells from a second animal were co-transfected with pHook and construct AATB. Cells selected by pHook (SEL) and those remaining (UNSEL) were cultured separately on tissue culture plastic (TCP) or EHS matrix (EHS) cell. Culture medium collected on days 5-7 was immunoblotted for human α_1-antitrypsin. U = medium from untransfected cells on EHS matrix; TG = defatted milk from a sheep transgenic for human α_1-antitrypsin; GM = 20 μg defatted goat milk; DM = unconditioned differentiation medium.

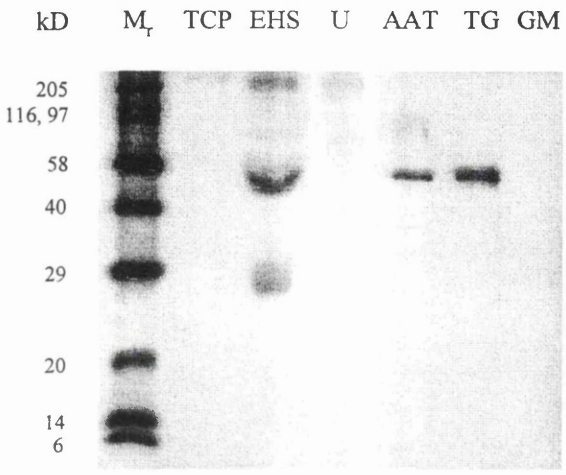

Figure 6.14 Secretion of α_1-antitrypsin in unselected goat cells
Goat mammary epithelial cells were transfected with construct AATB and cultured on tissue culture plastic (TCP) or EHS matrix (EHS). Culture medium collected on days 4-7 was immunoblotted for human α_1-antitrypsin. U = medium from untransfected cells on EHS matrix; AAT = 50 ng human α_1-antitrypsin; TG = defatted milk from a sheep transgenic for human α_1-antitrypsin; GM = 20 μg defatted goat milk.

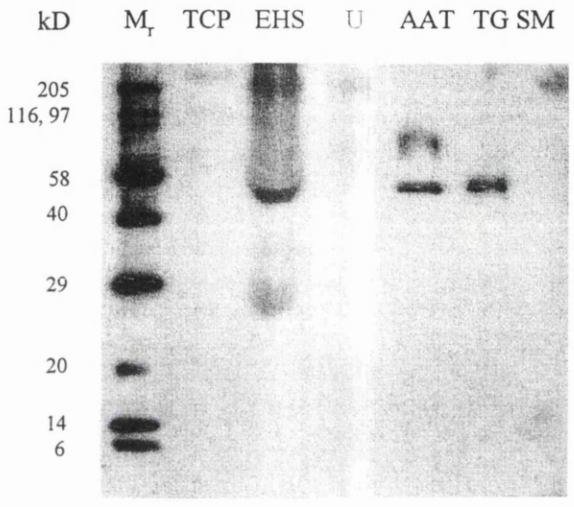

Figure 6.15 Secretion of α_1-antitrypsin in sheep mammary epithelial cells
Sheep mammary epithelial cells were transfected with construct AATB and cultured on tissue culture plastic (TCP) or EHS matrix (EHS). Culture medium collected on days 4-7 was immunoblotted for human α_1-antitrypsin. U = medium from untransfected cells on EHS matrix; AAT = 50 ng human α_1-antitrypsin; TG = defatted milk from a sheep transgenic for human α_1-antitrypsin; SM = 20 μg defatted sheep milk.

6.3 DISCUSSION

Several methods have been developed to introduce DNA into cultured cells. However, certain cell types and cell lines are intrinsically easier to transfect than others, although the exact reason for these differences is currently unknown. For every cell type, therefore, a considerable degree of optimisation of transfection conditions is necessary. HC11 cells were initially used as a positive control for transfection, since they are known to be easily transfectable using calcium phosphate, with efficiencies of ~10%. Transfection with Lipofectamine resulted in comparable results (~8%) in HC11 cells, but much lower efficiencies in goat mammary epithelial cells (~0.5%). Transfection with Fugene or Superfect produced similar results after optimisation. Of these three products, Lipofectamine resulted in considerable cytotoxicity in a dose dependent manner. This did not result from the use of Opti-MEM, since culture in standard medium, either with or without serum, did not prevent cell death, and resulted in less effective transfection. Cytotoxicity was evident at the lowest concentration of Superfect tested, possibly resulting from the formation of very large complexes. Only Fugene showed no cytotoxicity and was therefore used routinely.

Initial attempts at transfection with calcium phosphate resulted in low transfection efficiencies. This method has been used to transfect bovine primary mammary epithelial cells in culture. In this case, the use of glycerol osmotic shock following transfection improved transfection 4-8 fold (Ahn *et al*, 1995). Similarly Basolo *et al*, (1990) found that glycerol shock enhanced calcium phosphate transfection of human breast epithelial cells by 3-fold. The exact mechanism of action is unknown, but it may modify cell membrane structure. Increases of this magnitude, however, if achieved in goat cells, would still not have enhanced transfection beyond that obtained with the commercial preparations. In addition, transfection with calcium phosphate is extremely variable in reproducibility. It is known that this method is pH critical (Wilson *et al*, 1995; Yang & Yang, 1997) and that formation of the precipitate can proceed too far (Jordan *et al,* 1996). For these reasons, further optimisation of this procedure was not pursued.

Optimisation experiments were initially performed using the β-galactosidase reporter construct pCH110, and postively staining blue cells were easily identifiable (Figure 6.2). Cell counts were taken across the entire diameter of the well, to account

for the differing degree of cell confluency in the centre of the well, from that at the periphery. Detection of luciferase resulting from two other reporter constructs was only detected at, or marginally above, background levels. This may have been due to technical difficulties of measuring samples on a scintillation counter, rather than a luminometer. Transfection with the constitutive reporter pGK-βGAL construct confirmed the low transfection efficiencies obtained with pCH110.

Selection for transfected goat cells was very difficult. Untransfected goat cells, exposed to high concentrations of G418 remained unaffected until passage 3, after which few cells remained. Since the cells had already been passaged once, prior to transfection, those cells had already reached the stage of culture where milk protein secretion was compromised. An alternative method, using magnetic beads was also unsuccessful. Expression of β-galactosidase was obscured by large numbers of beads adhering to the cells. This may be because the beads were present in excess, despite reducing the concentration from that suggested by the manufacturer's instructions. Therefore, it was decided to co-transfect with a milk-specific gene that was already known to secrete high amounts of α_1-antitrypsin *in vivo* (30 mg/ml; A Kind, personal communication) and *in vitro* (Chapter 4). On this occasion, α_1-antitrypsin was detected in the culture medium, although selection was only partially effective, with selected cells secreting ~23% greater than unselected. Further experiments showed that secretion of α_1-antitrypsin was reproducible. Culture medium was collected at approximately 3-4 day intervals to allow accumulation of secreted product.

The products of other constructs failed to be detected. The human protein C constructs had been successfully expressed *in vivo*, but only at maximum levels of 200 μg/ml, approximately 100-fold lower than that of AAT. It is possible, therefore, that in combination with low transfection rates, product secretion might be below the level of detection. Similarly, no human growth hormone was detected when this gene was transfected under the control of the WAP promoter. This product has been readily detectable in culture medium conditioned by CK cells transfected with the WAP2-hGH construct (Kolb, 1993) and the WAP promoter has been shown to be effective in directing transgene expression in transgenic mice (Devinoy *et al*, 1994; Wei *et al*, 1995), pigs (Wall *et al*, 1991) and sheep (Wall *et al*, 1996). It appears likely, therefore, that the main constraint to the expression of transfected genes in this culture system

was the extremely low transfection efficiency. It is possible that these problems could be addressed through the use of other transfection methods. Particle bombardment has been found to be five times more effective in transfecting primary rat mammary epithelial cells than lipofection, calcium phosphate or electroporation (Thomspson *et al*, 1993). Transfection of mouse and human primary mammary epithelial cells using an adenoviral vector resulted in 70-90% of cells expressing a β-galactosidase reporter gene (Yang *et al*, 1995b), and transfection has recently been performed by receptor-mediated endocytosis in sheep (Sobolev *et al*, 1998). Other work suggests that polymers such as polyethylimine (PEI) promote gene delivery from the cytoplasm to the nucleus and enhance transgene expression, but that this is prevented by cationic lipids (Pollard *et al*, 1998). More recently a combination of adenovirus and PEI has been used to transfect polarised mammary epithelial cells successfully (Bischof *et al*, 1999).

Although factors affecting transfection are little known, it is possible that stage of cell cycle or the differentiative state of cells may be implicated. For all the transfection procedures tested in this chapter it was recommended that cells were ~50% confluent and actively proliferating. In addition, the goat mammary epithelial cells that grew abnormally and could not be induced to secrete milk proteins, were capable of transfection efficiencies of ~10%, comparable to that of HC11 cells (A Kolb, personal communication). Thus, future work might investigate whether differentiation capability is inversely related to transfection capability.

6.4 CONCLUSIONS

Ruminant mammary epithelial cells cultured on EHS matrix can provide a good model for the mammary gland *in vivo* and transfection of these cells would provide further opportunities for the study of regulatory mechanisms. The transfection methods tested in this project, however, were unsuccessful in providing reliable expression of foreign proteins, except in the case of an unusually high-expressing construct (pAATB). The reasons for poor transfection capability in primary cells are not known and further work in this area may provide important insights into cellular function, as well as overcoming transfection difficulties. Alternatively, more recent transfection methods may allow the manipulation of already differentiated cells, and the use of polymers such as PEI may enhance transgene expression. If the difficulties of transfection can be

resolved, this system has great potential in the study of mammary gland function. In addition, it would also provide an attractive alternative to transgenic mice for the evaluation of transgenes, since the same constructs may be spliced and expressed differently in various species (Archibald *et al*, 1990; Wright *et al*, 1991; Aigner *et al*, 1999).

CHAPTER SEVEN
SUMMARY AND DISCUSSION

7.1 Primary culture of ruminant epithelial cells

Much of the work in the field of mammary gland biology has been performed in mice, yet there are considerable differences between species (Forsyth, 1986). *In vivo* studies in ruminants, however, are subject to practical, financial and statistical constraints, making the use of *in vitro* systems particularly attractive. A very small number of cell lines have been derived from ruminant species, but many of these express milk proteins poorly (Huynh *et al*, 1991; Zavizion *et al*, 1996) or not at all (Schmid *et al*, 1983; Huynh & Pollak, 1995; Düchler *et al*, 1998). Similarly, previous attempts at culturing ruminant primary cells have also been unsuccessful (Hansen & Knudsen, 1991; Wheeler *et al*, 1995). In contrast, primary cells derived from mice, when cultured on EHS matrix, demonstrate a morphology similar to that *in vivo* (Li *et al*, 1987; Barcellos-Hoff *et al*, 1989; Aggeler *et al*, 1991), and secrete milk proteins in a prolactin dependent manner (Emerman & Pitelka, 1977; Emerman *et al*, 1977).

The initial aim of the work in this thesis was to establish a ruminant cell culture system. Cells isolated from sheep during the third trimester of pregnancy were shown to be predominantly epithelial in nature, while containing smaller proportions of myoepithelial and fibroblastic cells. Freshly prepared sheep cells cultured on EHS matrix formed structures with cells peripherally arranged around a central luminal space. These mammospheres were similar to those observed with mouse cells (Li *et al*, 1987; Barcellos-Hoff *et al*, 1989; Aggeler *et al*, 1991). β-lactoglobulin and α_{S1}-casein were detected in both culture medium and luminal fractions, although it was not possible to determine whether this was due to disruption of the tight intercellular junctions, or to basolateral secretion. These milk proteins were secreted optimally at days 6-8 of culture on EHS matrix, yet at this stage α-lactalbumin was rarely detected and then only at low levels. On one occasion, however, a strong band was detected by fluorography at the expected position of α-lactalbumin, this in fresh cells at day 13 of culture on EHS matrix. It is possible that the optimal conditions for secretion of individual milk proteins may

vary and may represent differing degrees of cell differentiation. Differential control of milk protein gene expression is also observed *in vivo*, where α-lactalbumin is the latest to be expressed (Harris *et al*, 1991). In addition, milk protein expression has been shown to be heterogeneous in the mammary glands of sheep and cattle suggesting cyclical variation in expression, or the presence of secretory and non-secretory subpopulations of cells (Molenaar *et al*, 1992).

Most established ruminant cell lines (Huynh *et al*, 1991; Woodward *et al*, 1994; Düchler *et al*, 1998) and cultured primary cells are unresponsive to prolactin (Hansen & Knudsen, 1991; Wheeler *et al*, 1995). In contrast to these findings, sheep and goat mammary epithelial cells cultured under the conditions described in this thesis were prolactin responsive on transfer to EHS matrix. *In vivo* studies in goats have demonstrated that an increase in blood prolactin is essential for the successful induction of mammary growth and lactation (Hart & Morant, 1980), while other work suggests that, once established, lactation in goats is independent of prolactin (Forsyth & Lee, 1993). This is in direct contrast to findings in mice, where prolactin implants prevent a decline in milk yield (Flint *et al*, 1984). Prolactin has been shown to induce Stat5 signalling, and in mice Stat5 phosphorylation corresponds with milk protein gene expression during pregnancy and lactation (Liu *et al*, 1996). However, a similar relationship is not found in ruminants (Wheeler *et al*, 1997) providing further evidence for differential prolactin signalling between species. These findings suggest that much work still remains to be done in establishing the factors responsible for maintainance of milk protein secretion in ruminants, and that this culture system may facilitate this.

Cryopreservation of cells resulted in considerable cell losses, but surviving cells also demonstrated substratum and prolactin dependence. The mammosphere structures were slightly smaller, and a greater proportion of secreted milk proteins was partitioned into the culture medium (50% in comparison to 30% in fresh cells), but the populations of proteins secreted were identical to those of freshly prepared cells. Since the average yield per preparation was ~10^{10} cells, large stocks can be frozen to provide a uniform starting material for large numbers of experiments.

This work may have important implications. The mammary gland is a complex organ, and is unique in that it undergoes cyclical development and regression in the adult animal. It is controlled by a complex interplay of systemic and

locally acting factors, and a greater understanding of these mechanisms could provide insights into developmental and regulatory processes, and the causes and consequences of abnormal function. For example, mammary tumours are relatively common in mice and humans, but rare in ruminants. Identification of control mechanisms that differ between species may provide the potential for new therapies. At the very least, this cell culture system provides the best model to date in which to study mammary gland function in ruminants, particularly as the levels of milk protein mRNAs are as high as that obtained from lactating tissue (LMB Finch, personal communication).

7.2 Passaging of ruminant mammary epithelial cells

The overall aim of this project was to develop a ruminant cell culture system which could be used to evaluate gene constructs for the expression of foreign genes, prior to generating transgenic livestock. This approach would also have further benefits. The use of transgenic technology in mice has vastly increased the scope of research into mammary gland function, allowing the investigation of knock-out or over-expression of genes. The time and expense involved in creating transgenic livestock, however, precludes their use purely for research in this way. The transfection and differentiation of cells *in vitro* could permit over-expression and knock-out (by using anti-sense constructs) of genes in order to manipulate function in a similar manner to that achieved in transgenic mice. The selection of transfected cells prior to differentiation, however, would necessitate passaging, and it was important to establish whether this would have any adverse effects on milk protein secretion.

In sheep cells, milk protein secretion declined with passage, and was not detected after passage 2 on EHS matrix. Simultaneously, the proportions of other cell types declined until all cells stained as epithelial at passage 3. It is possible that the presence of other cell types is essential for milk protein secretion *in vitro*. Mammary tissue in the developing embryo interacts with surrounding tissues (Sakakura *et al*, 1982), while studies *in vitro* demonstrate an involvement of adipocytes (Levine & Stockdale 1984; Wiens *et al*, 1987; Beck & Hosick, 1988) or fibroblasts (Reichman *et al*, 1989; Gache *et al*, 1998) in the local control of mammary function. Myoepithelial cells may also provide local signals to epithelial cells (Barraclough *et al*, 1990; McAndrew *et al*, 1994a; 1994b). The effects of these

cell types, either individually or collectively could be explored by adding back other cell types at each passage and assessing milk protein secretion.

Another possibility for the loss of secretion could be the selection of a sub-population of non-differentiating stem cells. Passaging of sheep mammary epithelial cells resulted in a progressive cell loss, with surviving cells staining less intensely with epithelial markers. The removal of cells by apoptosis (Strange *et al*, 1992; Quarrie *et al*, 1996; Jaggi *et al*, 1996) and repopulation of secretory material at the onset of a subsequent lactation, necessitates the existence of a stem cell population. Further evidence of this is provided by transplantation of mammary ductal segments into a cleared fat pad, which results in the regeneration of a functional ductal tree (Ormerod & Rudland, 1986; Smith & Medina, 1988; Rudland, 1991). Recent work to characterise these stem cells in mammary tissue from rodents suggests that differentiated cells arise as a result of the division of two further progenitor cells (Chepko & Smith, 1997). It is possible that substratum and lactogenic hormones induce differentiation in the final stages of this progression, while cells appearing early in this sequence of events require further factors for differentiation.

Cells isolated from a 6 year old ewe as part of this project were subsequently used in nuclear transfer experiments (Wilmut *et al*, 1997) resulting in the birth of a live lamb (Dolly). DNA fingerprinting has since confirmed that Dolly is indeed derived from this cell preparation (Signer *et al*, 1998). The cells used for the nuclear transfer experiments however, had been passaged 3-6 times. At this stage in culture, cells could not be sufficiently differentiated to be capable of milk protein synthesis. In addition, although Wilmut *et al* (1997) do not specify culture conditions, it is assumed that cells are passaged on tissue culture plastic, a substratum that does not support milk protein secretion even in freshly prepared cells. Thus, it is likely that Dolly is derived from a stem cell (ie a progenitor of other cell types) but not from a terminally differentiated secretory cell.

In contrast to sheep cells, milk protein secretion was detected following extended passaging in some goat cells. This was not related to age or parity of the animals but appeared to be a true species difference. Species differences in regulation of milk protein secretion are well-established (Forsyth 1986; Wheeler *et al*, 1997). This difference *in vitro* could be exploited, however, in determining the

causes of loss of differentiation and in providing a greater understanding of possible mechanisms *in vivo*.

7.3 Transfection of ruminant epithelial cells

Transfection of cells met with only limited success. α_1-antitrypsin was detected in both sheep and goat cells, while other products were undetected. It is possible that all of the transfected constructs were expressed, but in only a very small proportion of cells. In this case, the exceptional expression levels of AAT permitted detection, while more modestly expressed constructs were below detectable limits. Extremely low transfection efficiencies resulted in the need for extended passaging which ultimately compromised cell differentiation and milk protein secretion. If the problem of low transfection efficiencies could be overcome, perhaps through ongoing work into new techniques (Sobolev *et al*, 1998; Bischof *et al*, 1999), then this system might still provide an attractive model for transgenic ruminants.

7.4 Post-translational processing

Low transfection efficiencies and difficulties in selecting transfected cells prevented production of sufficient material to investigate post-translational processing *in vitro*. SDS-PAGE of the product α_1-antitryspin, however, suggested that the product secreted by cells ran at the expected molecular weight of purified AAT. Again, further work in this area would be possible if loss of differentiation or low transfection efficiencies could be resolved.

7.5 Conclusions

Despite the technical difficulties in the latter stages, this project was successful in establishing a primary cell culture system which mimics the situation *in vivo*. Cells were shown to be both prolactin and substratum dependent. These novel findings provide many opportunities for the study of ruminant mammary gland biology. Resolving difficulties with transfection may permit manipulation of gene expression and the evaluation of transgenes *in vitro*, prior to producing transgenic animals, either by traditional technologies, or conceivably, using the same cells for nuclear transfer.

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