MAMMARY DEVELOPMENT AND THE ROLE OF LEPTIN

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ABSTRACT

The control of mammary gland morphogenesis is very complex, involving autocrine, paracrine and endocrine factors. The obligate requirement for adipose tissue during early development has been well established but the reason(s) has yet to be resolved.

In the first part of our study, we investigated how this intimate association of the gland with adipose tissue affects development. Mammary cell proliferation and apoptosis were quantified in mice from weaning through until maturity. Cell proliferation was relatively constant in virgin mice, irrespective of age, whilst apoptosis was much lower in aged mice, and varied in a way which is deserving of further studies, as it could have significant implications for pathological mammary development and breast cancer. During pregnancy, proliferation increased from a barely-detectable level at the start of gestation to a maximum in mid-pregnancy. High levels were maintained through until day 1 of lactation and measurable proliferation continued throughout lactation. Apoptosis was absent until the very end of pregnancy, was high immediately post-partum but then decreased before reaching its maximum during post-lactational involution. Cell proliferation did not differ significantly between a first and second lactation, whereas the postpartum surge of apoptosis was only observed in a first lactation starting early in life. It was also demonstrated that lactational history affects the way in which the gland proliferates and undergoes apoptosis during oestrous cycles late in life. Proliferation was reduced by a previous lactation, but apoptosis

was not significantly affected. This also has implications for breast cancer incidence.

Next, we examined the hypothesis if leptin, an endocrine product of adipose tissue, alters mammary development, using a combination of *in vitro* mammary culture techniques and *in vivo* murine models of leptin deficiency. Caprine and murine mammosphere cultures were used to test the effects of leptin of murine and ovine origin on mammary cell differentiation and the proliferation of ovine mammary cell culture. There was no reproducible evidence of a direct effect of leptin on mammary differentiation, either using ovine or murine leptin in both caprine and murine mammospheres. Neither did leptin affect mammary cell proliferation in cell culture. Nonetheless, wholemount analysis showed that mammary development in leptin deficient *ob/ob* mice is impaired. These mice had reduced ductal growth and increase size of fat pad. To determine whether leptin was directly responsible or it is just the failure of these mice to reach puberty, oestrogen and progesterone were administered as subcutaneous implants. Treatment with mammogens stimulated mammary cell proliferation in both wildtype and *ob/ob* mice. Based on the *in vitro* and *in vivo* studies, we can conclude that leptin *per se* is not essential for mammary development and the defect observed was an indirect consequence of the fact that *ob/ob* mice are sterile

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LIST OF ABBREVIATIONS

APES	3-aminopropyltriethoxysilane
AgRP	agouti-related peptide
bFGF	basic fibroblast growth factor
BLG	β-lactoglobulin
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CART	cocaine- and amphetamine-regulated transcript
DAB	3,3-diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMM	dorsomedial hypothalamic
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EHS	Engelbreth-Holm-Swarm
ERK	externally regulated kinase
ERKO	estrogen receptor knockout
FGF	fibroblast growth factor
GAS	γ-interferon activation sequence
GH	growth hormone
HBSS	Hanks Balanced Salt Solution
HRP	horseradish peroxidase
ICE	interleukin-1β converting enzyme
IGF-1	insulin-like growth factor-1
JAK2	janus kinase 2
LH	latera hypothalamic
MAPK	mitogen-activated pathway kinase

MEAA	Minimum Essential Amino Acids
MEO	mammary epithelial organoids
MMPs	matrix metalloproteinase
MWCO	molecular weight cut-off
NP40	nonylphenoxy polyethoxy ethanol
NPY	neropeptide Y
Ob-R	leptin receptor
PBS	phosphate buffered saline
PBST	phosphate buffered saline/ 0.1% Tween 20
PI3	phosphatidyl inositol 3
PL	placental lactogen
POMC	proopiomelanocortin
PR	progesterone receptor
PRL	prolactin
PVDF	polyvinylidene fluoride
PVN	paraventricular nuclei
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
Stat	signal transducer and activator of transcription
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TEBs	terminal end buds
TER	transepithelial electrical resistance
TGFα	transforming growth factor alpha
TGF-β	transforming growth factor beta
uPA	urokinase-type plasminogen activator
VHM	ventromedial hypothalamic

PUBLICATION

The results published in this thesis have been published in the following journal:

Knight, C. H., Ong, E., Vernon, R.G. and Sorensen, A. (2002) Successful lactation in leptin-deficient obese (*ob/ob)* mice. Proceedings of the British Society of Animal Science 2002, 4.

Papers in preparation:

Ong, E., Knight, C. H., Vernon, R. G. and Wilde, C. J. Lack of effect of leptin on mammary cell proliferation and differentiation *in vitro*

Sorensen, A., Ong, E., Vernon, R.G. and Knight, C. H. Mammary development and lactation in leptin-deficient obese (*ob/ob*) mice.

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I would also like to take this opportunity to express my deepest gratitude to 친구, 余准模. Your friendship is more than words can say. Thank you for always being patient, tolerant, understanding, caring and inspirational to me. I am also very grateful to be given the privilege to try the tasty korean cuisine throughout my stay here! 대단히감사합니다大哥.

Last but not least, I would like to thank my parents for their constant love, encouragement and support.

DECLARATION

All data were collected by myself between 1998 and 2001. I declare that the work contained in this thesis is my own, undertaken under the supervision and guidance of Profs CH Knight, RG Vernon and Dr CJ Wilde. No part of this work has been submitted for consideration for any other degree or award.

Eddie Ong

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 OVERVIEW

The mammary gland is of great interest to biologists from different disciplines due to its unique developmental features and complex regulation by hormones and growth factors. Besides being influenced by many systemic endocrine factors, interactions between the different cell types and the extracellular matrix also play a role in the normal development and function of the gland. The fat pad of the gland, which previously was thought to be an inert matrix that supports epithelial growth, has been shown to be an essential component in normal epithelial growth, possibly through paracrine signalling (Hovey et al.,1999). Receptors for some of the key players involved in normal development of the gland have also been shown to be expressed in the fat pad, suggesting that the actions of key hormones and growth factors might be mediated through cell types other than the mammary epithelial cell.

The aim of this review is to discuss the roles of hormones and growth factors; the contribution of the mammary environment and the possible role of leptin in mammary gland development and function.

1.2 MAMMARY GLAND STRUCTURE

The mammary gland is an ectodermally-derived exocrine organ located within the subcutaneous tissue of the anterior thoracic wall (Sakakura,1987). It is made up of two main cellular components namely the parenchyma and adipose stroma. The parenchyma refers to the system of branching ducts

connected to the nipple by a single primary duct. The structure of the mammary ducts is made up of an inner layer of mammary epithelial ductal cells and an outer layer of myoepithelial cells. The other cellular component, adipose stroma, consisting of connective tissue and adipocytes, acts as a substratum within which the parenchyma grows and functions. The parenchyma and surrounding stroma is separated by the extracellular matrix (ECM). The ECM consists of a highly complex mixture of molecules including various collagens, fibronectin, laminin and glycosaminoglycans. In addition to the two main cellular components, the mammary gland is also composed of fibroblasts, endothelial cells and lymphatic vessels.

During late pregnancy and lactation, the mammary gland consists mostly of lobules of alveoli (Figure 1.1), each composed of a monolayer of specialised epithelial cells surrounding a central lumen. Milk is secreted into the lumen and removed through the ductal system. In ruminants (Figure 1.2), the ducts empty into a large cavity, the gland cistern, and milk is removed through a single aperture in the teat. By contrast, in humans and rabbits (Figure 1.2), each of the ducts draining separate lobules terminates at the nipple instead of being supplied by a common sinus.

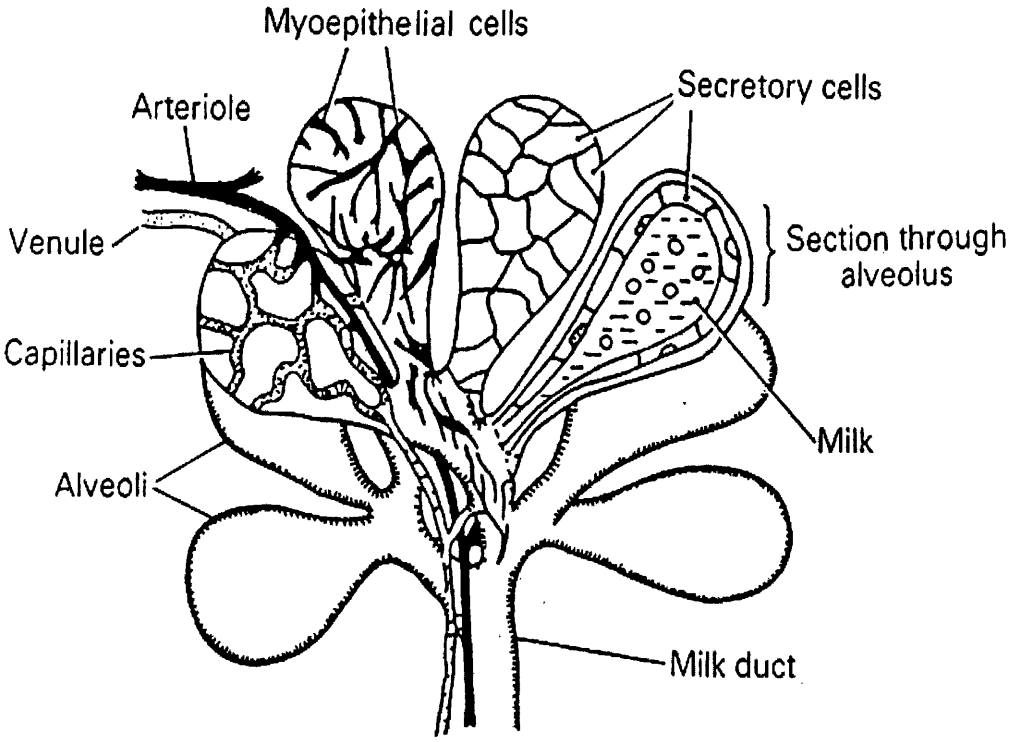

Figure 1.1 Diagrammatic representation of a cluster of mammary alveoli (Mepham,1987)

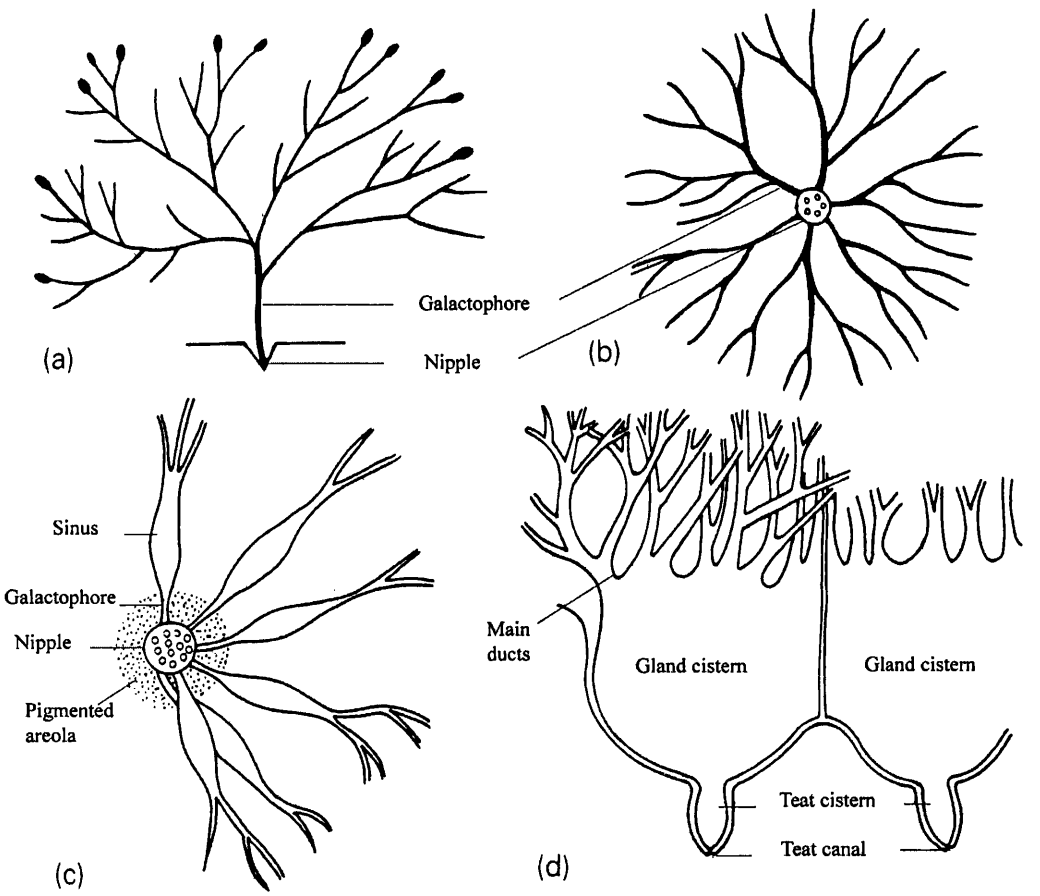

Figure 1.2 Arrangement of mammary duct systems in different species (Mepham,1987).

In rats (a), the ducts unite to form a single galactophore opening at the tip of nipple while in rabbits (b), several galactophores open at the nipple. For humans (c), each galactophore is dilated at the base of nipple (which is surrounded by a pigmented areola) to form a sinus. In cows (d), the main ducts open into a large gland cistern which communicates through a narrow aperture with the teat cistern. Milk is removed via the teat canal.

1.3 MAMMARY GLAND DEVELOPMENT

The development of the mammary gland is unusual in that the majority of the process occurs in the adult organism. Developmental stages are generally similar in all mammals and can be divided into: embryonic, pre-pubertal, pubertal, pregnancy, lactation and involution.

1.3.1 Embryonic

In the embryonic stage, very little development goes on in the gland. In mice, the mammary rudiment is observed around days 10-11 of life (Daniel and Smith,1999). These are seen as buds which appear on the mammary lines (bilateral, ectodermal thickenings on the ventrolateral body wall). After the mammary buds are fully formed, they undergo a resting phase until days 12-13. At this point, the surrounding mesenchyme, which has differentiated into mammary mesenchyme, stimulates outgrowth of the mammary bud. At days 15-16, a mammary sprout is observed and this sprout leads to the formation of the small ductal tree observed towards the end of gestation. The process has been reviewed by (Robinson et al.,1999).

1.3.2 Pre-Pubertal and Pubertal

At birth, the mammary duct system is still rudimentary. During the first 3 weeks of life, the mammary gland undergoes isometric growth, where the mammary ducts elongate and ramify at a rate not surpassing that of the animal (Knight and Peaker,1982). With the onset of puberty at around 4 weeks of age, ductal elongation and branching becomes allometric. Rapid proliferation occurs within the terminal end buds (TEBs), resulting in the invasion and filling of the fat pad by the duct system. The TEBs drive ductal morphogenesis

in the gland by producing a supply of differentiated ductal and myoepithelial cells (Daniel and Silberstein,1987). Once this is achieved, mammary growth becomes isometric. This growth is oestrous cycle associated and has been demonstrated in rodents and ruminants (Sinha and Tucker,1969; Grahame and Bertalanffy,1972). Sutton and Suhrbier (1967) showed highest mitotic activity in rats during metestrus which was consistent with Schedin et al.(2000) observations. However, in cows, oestrous instead was shown to have the highest rate of proliferation (Sinha and Tucker,1969). This might imply a species-dependent estrous associated proliferation in the mammary gland. The overall increase in cell number during the oestrous cycle is still small despite this mitotic activity. With each oestrous cycle, the mammary gland increases the complexity of its branching network. From having primary ducts, the formation of lower-order ducts, through the bifurcation of the end buds, is also observed. Besides that, formation of secondary ducts along differentiated ducts and in certain species lobular-alveolar development is also detected (Daniel et al.,1996).

1.3.3 Pregnancy

When the animal proceeds through pregnancy, the mammary gland undergoes extensive and rapid proliferation resulting in an exponential growth of the mammary gland. In fact, the great majority of mammary growth occurs during gestation. In hamsters, 94% of all mammary growth takes place during gestation (Sinha et al.,1970) while in mice 78% was observed during this period (Brookreson and Turner,1959). During this period, the lipid content of adipocytes in the gland stroma are displaced to allow maximal expansion of the mammary epithelial ductal network. Terminal end buds start forming

lobulo-alveolar structures responsible for milk secretion during lactation. Whilst during earlier stages of development proliferation of the mammary epithelial cells was mostly observed in the terminal end buds during gestation ductal cells are also capable of undergoing cell division (Bresciani,1971).

Besides undergoing allometric growth, the size of mammary epithelial cells also increases (Foster,1977). A possible explanation for this is that secretory capability is acquired as the animal gets nearer to parturition. In goats, there is an increase in udder volume due to an increased concentration of lactose, indicative of terminal differentiation, as the animal proceeds through pregnancy (Fleet et al.,1975). Besides observing an increase in lactose concentration, an increase in RNA content and the appearance of milk specific products were also detected (Cowie et al.,1980). In rats, casein messenger RNA could be detected as early as day 5 of pregnancy (Rosen et al.,1975).

1.3.4 Lactation

When lactation commences, there is a marked increase in the expression of the milk protein genes and the synthesis of the components of milk. After parturition, the secretion in the first few days is called colostrum. It differs from normal milk in the sense that there is a higher concentration of proteins, sodium, and chloride and a lower concentration of lactose and potassium. Besides that, colostrum also contains high amounts of immunoglobulins to provide immunity to the neonate. With successive milkings, the composition rapidly becomes that of whole milk which is attributed to the closure of tight junctions (Nguyen and Neville,1998).

Milk yield is a combination of the number of secreting cells and the activity of the cell. During lactation, the number of secretory cells continues

to increase in some species. In mice, an increase in cell proliferation *post partum* was observed for the first 5 days of lactation (Knight and Peaker,1982). In ruminants, in the case of goats (Anderson et al.,1981) a similar observation was observed but in sheep this was not true (Anderson,1975). This increase in the number of secretory cells due to proliferation occurring during lactation has been correlated with the subsequent increase in milk yield (Knight and Wilde,1987). Even though proliferation is observed during lactation, differentiation of the mammary epithelial cell is still more prevalent during this stage.

1.3.5 Involution

In rodents, significant changes occur in the mammary gland upon weaning. Secretory activities of the mammary epithelial cell decreases, the lobulo-alveolar structures observed during lactation collapse, the replacement of most of the epithelial cells with adipose tissue and extensive tissue remodelling occurs reverting the mammary gland to that of the mature virgin state. In the situation of ruminants, partial loss of the mammary cell population instead of extensive tissue remodelling is involved (Wilde et al.,1997). Unlike rodents, the involuting gland in ruminants does not regress to the virgin state, in addition to apoptosis occurring in some alveolar cells, the remainder of the cells have been shown to dedifferentiate (Wilde et al.,1997; Li et al.,1999).

The removal of secretory epithelial cells is achieved through apoptosis (Quarrie et al.,1996) and phagocytosis by surrounding macrophages. Apoptosis in the involuting gland can be divided into 2 stages namely proteinase-independent and proteinase-dependent pathways (Lund et al.,1996). The proteinase-independent pathway of apoptosis involves the expression of

genes for p53 and interleukin-1β converting enzyme (ICE), while in the proteinase-dependent pathway, expression of proteinases like matrix metalloproteinases (MMPs) and serine proteinase urokinase-type plasminogen activator (uPA), responsible for the degradation of the extracellular matrix (ECM) through tissue-remodelling, is involved.

1.4 ENDOCRINE CONTROL OF MAMMARY DEVELOPMENT AND FUNCTION

Classical studies have demonstrated that growth and differentiation of the mammary gland is regulated by systemic hormones (Lyons,1958; Cowie et al.,1965). These hormones include estrogen, progesterone, growth hormone, prolactin, insulin and glucocorticoids.

1.4.1 Estrogen

Estrogen is one of the essential hormones required for ductal growth during adolescence and lobuloalveolar growth during pregnancy. Mice lacking the estrogen receptor displayed a rudimentary ductal structure lacking any TEBs or alveolar development (Bocchinfuso and Korach,1997). In addition, when estrogen was administered to ovariectomised adult mice, there was an increase in DNA synthesis exclusively in the TEBs (Shyamala,1997). Estrogen receptors have been detected in the mouse mammary gland (Haslam and Shyamala,1981; Haslam,1989). Whether estrogen exerts its action locally on the mammary gland has been debated since *in vitro* studies failed to reproduce the mitogenic effect of estrogen on mammary epithelial cells (Richards et al.,1988). Nonetheless, studies conducted by Silberstein et al.(1994), which involved local implantation of anti-estrogen pellets in the mammary gland,

confirmed the action of estrogen was direct. However, Zeps et al.(1998) showed that in the proliferating mammary epithelial cells in pubertal mice, 50% are estrogen receptor negative suggesting that estrogen's effect might be mediated through paracrine signalling. This seems even more likely when both mammary epithelial and stromal cells express the estrogen receptor (Haslam and Nummy,1992). Based on all these observations, we can see that estrogen exerts its effect locally; whether it's through direct effect on mammary epithelial cells or cell-cell interactions remains to be determined.

1.4.2 Progesterone

Progesterone like estrogen has been shown to be mitogenic in the mammary gland (Haslam,1988). *In vitro* experiments have demonstrated that progesterone is capable of inducing side-branching of the ductal epithelium (Atwood et al.,2000) and generating multi-lobular ductal branching (Darcy et al.,1995). This effect of progesterone was further complemented by *in vivo* studies employing progesterone receptor (PR) null mice whereby PR-deficient mice exhibit impairment of pregnancy-associated phenotype (Humphreys et al.,1997).

Expression of the progesterone receptor is detected in virgin and pregnant mammary tissue but is down-regulated during lactation. In mature mice, the expression of the progesterone receptor is regulated by oestrogen (Haslam,1988). Intriguingly, this dependence was not detected in virgin mice, suggesting an estrogen-independent effect of progesterone (Haslam,1988). Besides regulating expression of PR, oestrogen also seems to have an additive effect on the actions of progesterone as when ovariectomised mice were treated with both oestrogen and progesterone, there was a significant increase

in cell proliferation when compared with either steroid alone (Shyamala,1997). This is understandable since in the presence of estrogen, progesterone not only stimulated DNA synthesis in the end buds; outgrowth from lateral walls of the ducts and branches were also observed.

Besides acting as a mitogen, progesterone has also been shown to inhibit the expression of prolactin (Mizoguchi et al.,1997) and accumulation of casein mRNA during pregnancy (Rosen et al.,1978). Thus we can see that progesterone, in concert with oestrogen, acts as a mitogen responsible for the formation of lobulo-alveolar structures responsible for milk secretion during lactation and at the same time it also act as an inhibitor of lactogenesis by preventing the expression of the lactogenic hormone, prolactin, during mammogenesis.

1.4.3 Prolactin

Early studies in 1928 demonstrated that administration of pituitary extracts to rabbits with a well-developed lobulo-alveolar mammary system initiated secretion of milk (Stricker and Grueter,1928). The active molecule responsible was later identified to be prolactin (PRL). Prolactin is produced in the anterior lobe of the pituitary gland. It influences the immune system, reproduction and also acts as an osmoregulator (Bole-Feysot et al.,1998). In the mammary gland, PRL exerts its effect by binding to receptors on the mammary epithelial cells or even adipocytes (Ling et al.,2000). Upon binding, the receptor dimerizes and activates Janus kinase 2 (JAK2). JAK2 in turn activates transcription factors belonging to the family of signal transducers and activators of transcription (STAT).

The relative importance of prolactin in mammary gland function varies between species. PRL is both a mitogen and a differentiation factor in the gland, however its mitogenic action is less well recognized. Early work by Lyons demonstrated that prolactin was one of the essential hormones in rats required to produce lobulo-alveolar growth (Lyons,1958). This dependence on PRL was again shown in C3H mice (Vonderhaar,1987). Recently, using PRL-deficient and PRL-receptor knockout mice, it was again shown that ductal branching during puberty and alveolar development during pregnancy was reduced (Horseman et al.,1997; Ormandy et al.,1997). *In vitro* studies carried out by Darcy et al.(1995) also showed that prolactin has a role in regulating lobulo-alveolar growth. In contrast, the mammogenic role of prolactin was not observed in ruminants. When pregnant sheep or cows were treated with bromocriptine, which inhibits prolactin secretion, there was no effect on mammary development (Schams et al.,1984).

The more established role of prolactin is at the initiation of lactation. In ruminants, both in cows (Schams et al.,1972) and goats (Davis et al.,1983), when prolactin was lowered around parturition, a delay in the onset of secretion of milk and reduction in milk yield *postpartum* was observed, an effect which could be reversed with exogenous prolactin (Akers et al.,1981). *In vitro* studies also demonstrated that prolactin alone was capable of inducing casein synthesis (Devinoy et al.,1978) and α-lactalbumin (Sankaran and Topper,1984) activity in rabbit explants. Whether prolactin is important for maintaining lactation requires more studies. In rodents, milk yield decreases with bromocriptine treatment (Knight et al.,1986) but in cows, there is only a slight correlation between serum prolactin and milk yield (Koprowski and

Tucker,1973). Furthermore, supplementary prolactin has no effect on bovine milk yield (Plaut et al.,1987). On the other hand, Knight and Wilde (1993) and Forsyth and Lee (1993) demonstrated that treating lactating goats with bromocriptine before milking led to a significant decrease in milk yield. In sheep treatment with bromcriptone, during established lactation, also leads to reduced milk yield (Kann et al., 1978).

From all the above observations, prolactin has proven to be a key factor, at least in some species, in mammary gland function. In ruminants, it is not a major player neither is its role trivial.

1.4.4 Growth Hormone

Increasing evidence suggests that growth hormone (GH) is important in normal mammary gland development. It has been demonstrated to be mitogenic in both rodents and pubertal heifers. GH stimulated end bud formation (Silberstein and Daniel,1987) and lobulo-alveolar development in mice (Plaut et al.,1993). In pubertal heifers, there was an increase in mammary parenchyma and a decrease in stromal tissue when treated with GH (Sejrsen et al.,1986).

The role of GH during lactogenesis remains unclear. It has been demonstrated to be lactogenic in mice (Nandi,1958) while in cows (Simmons et al.,1994), this was not the case. Nonetheless, when injected into lactating cows, there was a marked increase in milk production (Collier et al.,1984; Bauman and Eppard,1985; McCutcheon and Bauman,1986). This increase in milk yield is due to GH's effect on nutrient partitioning in the mammary gland (Bauman and Currie,1980). In lactating rats, when prolactin was deficient, anti-GH antiserum reduced milk yield by 50%, which could be restored by

injecting with GH (Madon et al.,1986). It should be noted that GH and prolactin have strong homology in their sequence and human GH is capable of binding to the human prolactin receptor (Kossiakoff et al.,1994; Somers et al.,1994). It is capable of activating transcriptional factors belonging to the Stat family and also inducing the binding of Stat5 to the GAS site in the β-casein promoter in vitro (Smith et al.,1997).

Whether growth hormone exerts its effect locally or through systemic interactions remain to be elucidated. When lactating cows were treated with somatotropin, there was an increase in IGF-1 both in the serum and the parenchyma of the mammary gland (Glimm et al.,1988), suggesting that GH might function through IGF-1. Recently, studies have demonstrated the expression of growth hormone receptors in bovine (Sinowatz et al.,2000; Plath-Gabler et al.,2001) and mouse mammary gland (Ilkbahar et al.,1999), suggesting that GH might have a more direct role in mammary growth and function. Paradoxically, it was observed that growth hormone did not bind to the receptor in the bovine mammary gland (Keys and Djiane,1988). Hence to elucidate whether GH exerts its effect locally or systemically needs further investigation.

1.4.5 Insulin

Insulin has little effect on mammogenesis *in vivo,* but at supraphysiological concentration has been demonstrated to be essential *in vitro* (Forsyth,1971). It is believed at these concentrations, insulin binds to the IGF-1 receptor (Kasuga et al.,1981). Tucker (2000) hypothesized that in this situation, insulin may substitute for growth hormone-induced secretion of IGF-1. Next, Hovey et al. (1998) showed in the mouse mammary epithelial cell line COMMA-1D that

treatment with insulin induced a mitogenic effect. Insulin has also been implicated to have a cell-survival role since it was shown that HC11 cells cultured only in prolactin and dexamethasone underwent apoptosis, which could be inhibited by the addition of insulin (Merlo et al.,1996). Besides that, insulin has also been shown to have a stimulatory effect in casein gene expression and induction of α-lactalbumin activity in mammary epithelial cells from mid-pregnant mice (Prosser et al.,1987).

During lactation, hypoinsulinaemia and insulin-resistance of adipocytes are a common feature of rats and domestic ruminants (Vernon and Pond,1997). These changes both support nutrient supply to the mammary tissue and away from body tissue. In lactating rats, insulin treatment caused an increase in glucose utilization and lipid uptake in the mammary gland (DaCosta and Williamson,1994). The main effect of insulin during lactation hence is likely to be on the partitioning of nutrients in the mammary gland.

1.4.6 Glucocorticoids

Early pituitary ablation and hormone replacement studies indicated that glucocorticoids are essential for the maintenance of lactation. Paterson and Linzell (1971) showed that at parturition there was a sharp increase in level of cortisol and this was accompanied by the formation of tight junctions between neighbouring mammary secretory cells. Hence it has been implicated in the regulation of lactogenesis through the induction of tight junction formation. Hydrocortisone was found to suppress ductal branching and induce alveolar morphogenesis in rat mammary organoid culture, and its removal leads to a loss of differentiated function in the cells (Darcy et al.,1995). Several *in vitro* studies, using transepithelial electrical resistance (TER) as a measure of tight

junction formation, have shown in mice that when the mammary epithelial cells were treated with glucocorticoids, there was enhanced tight junction formation (Zettl et al.,1992; Stelwagen et al.,1999). Furthermore, Thompson (1996) also demonstrated that local injection of cortisol into late pregnant mammary gland of the goat produced a decrease in sodium, and an increase in the potassium concentration of the mammary secretion product, indicating tight junction closure. When plasma cortisol was elevated in lactating cows, the permeability of mammary tight junctions was reduced (Stelwagen et al.,1998). All of this evidence clearly implicate glucocorticoids as a regulator of tight junction formation.

In addition to its role in tight junction formation, glucocorticoids have also been demonstrated to have an effect on milk protein synthesis. In rabbit mammary gland organ culture, cortisol enhances prolactin-induced casein synthesis (Devinoy et al.,1978) while in mouse mammary gland culture, cortisol is required for casein gene expression (Ganguly et al., 1980). Besides being a stimulator of milk protein synthesis, an anti-apoptotic role has also been suggested for glucocorticoids. Treatment with glucocorticoids during involution in mouse mammary gland reduced the extent of apoptosis (Feng et al., 1995) while in lactating rats, glucocorticoids prevented apoptosis (Berg et al., 2002).

1.4.7 Placental lactogen

Placental lactogen (PL) is secreted by placentas of primates, rodents and ruminants during pregnancy. It is structurally related to growth hormone and prolactin. It has been shown in mice that the degree of mammary development correlates with litter size and hence presumably with the concentration of PL

(Knight and Peaker,1982). Similarly, in ruminants a positive correlation is observed. The concentration of circulating PL in sheep and goats was directly related to litter size and milk yield (Hayden et al.,1979; Butler et al.,1981). PL has been demonstrated to increase milk yield in lactating cows (Byatt et al.,1992). In sheep, ovine PL when administered in an established lactation, did not increase milk yield (Min et al.,1997), whereas, when administered to ewes that were artificially induced to lactate, milk yield was significantly increased (Kann et al., 1997). Mouse PL was also shown to stimulate 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).

Whether PL is mitogenic remains plausible. *In vitro* studies showed that PL stimulated proliferation of mouse mammary epithelial cells (Dai et al.,1996) while in ovine and rabbit cultures, β-casein synthesis was stimulated by PL instead. PL has also been demonstrated to stimulate IGF-1 expression in lactating cows (Byatt et al.,1992). In sheep, PL stimulated IGF-1 production in ewes that were artifically induced to lactate (Kann et al., 1999) but not pregnant sheep (Currie et al.,1996). The role of PL as a mitogen in the mammary gland requires more studies to provide a clearer picture.

1.5 GROWTH FACTORS AND MAMMARY DEVELOPMENT

In addition to systemic control by circulating hormones, there are a number of local factors that have been suggested to either complement or mediate hormonal control of mammary development and function.

1.5.1 Insulin-like growth factors

The role of IGF-1 in mammary gland development is to mediate the actions of growth hormone (Kleinberg,1998). Studies demonstrated that IGF-1 can substitute for the pituitary gland in promoting mammary development and growth hormone increases the levels of IGF-1 mRNA in the mammary gland. Ruan et al. (1992) showed, in hypophysectomized, castrated and estradiol-treated prepubertal male rats, that when IGF-1 was implanted into the mammary gland, the development of terminal end buds and the formation of alveolar structures were induced. Next, using in-situ hybridisation, it was shown that IGF-1 and its receptor mRNA were expressed in the highly proliferative terminal end buds in pubertal murine mammary gland. Furthermore, when IGF-1 in combination with mammogenic hormones were added to the pubertal glands *in vitro*, ductal growth was again promoted. Other evidence includes the stimulation of bovine mammary epithelial cell proliferation *in vitro* (McGrath et al.,1991) and *in vivo* (Collier et al.,1993). Recently, using knockout models of the IGF system, the role of IGF-1 in the mammary gland was again demonstrated. When IGF-1 receptor null epithelium was transplanted into a wild-type cleared fat pad, there was limited growth of the epithelium (Hadsell and Bonnette,2000). On the other hand, during pregnancy, IGF-1 doesn't seem to have an essential role since overexpression of rat IGF-1 in mammary alveolar cells did not affect mammary development (Neuenschwander et al.,1996).

Whether IGF-1 has a role during lactation remains debatable. It has been shown to be capable of stimulating milk protein gene expression in primary cultures of mammary cells (Prosser et al.,1987). Local infusion of

IGF-1 in lactating goats was able to increase milk synthesis (Prosser et al.,1990) while systemic infusion did not obtain a similar response (Davis et al.,1989). In rats, treatment with exogenous IGF-1 did not increase milk synthesis (Flint et al.,1992) and in transgenic mice, overexpression of IGF-1 during lactation results in ductal hypertrophy (Hadsell et al.,1996). It seems that whether IGF-1 has a role in lactation is dependent on the species.

IGF-1 has also been shown to be a more potent inhibitor of apoptosis than insulin in cell culture models (Geier et al.,1992). This role of IGF-1 was also demonstrated in the mammary gland. In vitro studies showed that IGF-1 inhibits apoptosis (Farrelly et al.,1999). Neuenschwander et al.(1996) showed that when IGF-1 was over expressed in transgenic mice, mammary involution was inhibited.

1.5.2 Transforming growth factor α and β

Transforming growth factor α (TGFα) is a nonheparin-binding member of the epidermal growth factor receptor (EGFR) ligand family. TGFα overexpression is associated with some neoplastic cells and tissues and the fact that its mRNA is detected in the mammary gland at various stages of development raises interest as to what role TGFα might play in mammary development. The potential role of TGFα was demonstrated using organ cultures. When mammary tissue was cultured with TGFα in the presence of insulin, prolactin, aldosterone and hydrocortisone, it promoted the formation of full lobulo-alveolar structures which was not observed with the four mammogens alone (Vonderhaar,1988). Similarly, when pellets containing TGFα were implanted in the mammary gland *in vivo*, TGFα was able to stimulate local growth even in the absence of steroid supplement. Further studies carried out on TGFα

transgenic mice showed that when this growth factor was overexpressed, it led to the development of precocious alveolar development and a marked decrease in postlactational involution (Schroeder and Lee,1997). This led to the suggestion that TGFα might be involved in mammary gland tumorigenesis.

Transforming growth factor-β (TGF-β) belongs to a family of growth factors involved in regulating growth, differentiation and development in many organ systems (Roberts and Sporn,1990). It exist in three isoforms and is expressed in all stages of mammary gland development except lactation (Robinson et al.,1991). TGF-β, when implanted locally in the mammary gland, inhibited mammary growth in virgin mice (Silberstein and Daniel,1987) while in pregnant mice, ductal elongation was inhibited (Daniel et al.,1989). Other studies also showed that overexpressing TGF-β in pubertal mouse caused a reduction in the growth of the ductal tree and a simplification of the pattern of arborization. Besides that, this study also showed that when exogenous TGF-β was applied directly to the gland, not only was there an inhibition of DNA synthesis, extracellular matrix synthesis was strongly stimulated (Daniel et al.,1996). Despite all these observations, Soriano et al.(1998) showed that at low concentrations, TGF-β was capable of stimulating extensive elongation and branching of epithelial cords using an *in vitro* model. Nonetheless, at higher concentration TGF-β was still inhibitory. This biphasic effect of TGF-β might exist *in vivo*, however since a transformed mammary cell line was used in the Soriano experiment, it would be sensible to carry out further studies to establish this role of TGF-β.

Expression of TGF-β mRNA is elevated during mammary involution (Robinson et al.,1991), suggesting that TGF-β might have a role during that

period. Recently, it was demonstrated that one of the TGF-β isoforms, TGF-β3 is strongly expressed during the second phase of the mouse mammary gland involution (Faure et al.,2000). Based on this observation, TGF-β3 might be involved in mammary gland remodelling since TGF-β does stimulate synthesis of the extracellular matrix.

1.5.3 Epidermal Growth Factor

Epidermal growth factor (EGF) belongs to the same family of growth factors as TGFα. EGF's suggested role in the mammary gland is to act as a mitogen. By using slow-release pellets, EGF was demonstrated to stimulate local end bud growth in mammary glands of ovariectomised virgin mice in a dose-dependent manner (Daniel and Silberstein,1987). A similar study carried out by Vonderhaar (1988) reinforced this role of EGF. She showed that in ovariectomised virgin mice, primed with estrogen and progesterone, local implantation of EGF pellets induced both ductal elongation and lobulo-alveolar growth in the mammary gland, suggesting that EGF might act synergistically with estrogen and progesterone. Interestingly, treatment with estrogen and progesterone of pubertal mice resulted in an increase in EGF receptor (EGFR) concentration (Haslam et al.,1992). The expression of EGF and its receptor has been detected in the mammary gland at all stages of development. Immunoblot analysis carried out by Darcy et al. (1999) showed that high levels of EGFR are expressed during puberty, pregnancy, involution but not during lactation. This group also demonstrated that EGFR signalling was required for growth, differentiation and survival but not for maintaining functional differentiation of mammary epithelial cells. In fact, when mouse mammary epithelial cells were cultured in the presence of EGF, besides

stimulating proliferation, the synthesis of milk was also inhibited (Tonelli and Sorof,1980). EGF thus might have an antagonistic role during lactation. Indeed, prolactin was shown to inhibit EGF-stimulated DNA synthesis *in vitro* (Fenton and Sheffield,1993; Sheffield,1998). EGF was also shown to suppress Stat 5 expression *in vitro* (Petersen and Haldosen,1998). Based on all these observations, EGF might have an essential role during pregnancy, possibly to ensure that the mammary gland is well-developed before allowing the onset of lactation.

1.5.4 Fibroblast growth factors

Fibroblast growth factors (FGF) belong to the group of heparin-binding growth factors. Studies have shown that FGF is capable of stimulating growth of mammary cells. Bovine FGF stimulated the growth of stromal and myoepithelial-like cells in the rat mammary gland (Smith et al.,1984), and when added to human breast cancer cells, T47D, there was a significant increase in growth rate (Shiu,1981). Besides that, FGF and its receptors have also been detected in the mouse mammary gland (Coleman-Krnacik and Rosen,1994). Jackson et al. (1997) also showed that when dominant negative FGF receptors were created in mice, depressed lobulo-alveolar development was observed during pregnancy. In contrast, FGF did not stimulate proliferation in caprine mammary epithelial cells, suggesting a species-dependent response (Pantschenko and Yang,1999).

Besides having a growth promoting role, FGF has also been shown to inhibit the expression of milk proteins in the presence of lactogenic hormones (Levay-Young et al.,1989). With this observation, FGF might have a similar role as EGF during pregnancy, which is to prevent precocious differentiation.

1.6 PARACRINE SIGNALLING IN THE MAMMARY GLAND

Over the past two decades, evidence has increased indicating the involvement of paracrine regulation in mammary gland development.

1.6.1 Adipocyte-epithelial interactions

Pioneering studies by Kratochwil (1971&1977) and Sakakura et al.(1976) demonstrated that morphogenic development of the mammary epithelium is dependent on mesenchyme. When 16-day fetal mammary epithelium was recombined with mesenchyma of either mammary or salivary origin, a branching pattern characteristic of the source of mesenchyma was observed.

The major cell-cell interaction in the mammary gland after birth is between epithelium and adipocytes. The possibility that adipocytes exert a growth-promoting effect on mammary epithelium has been demonstrated through transplantation experiments (DeOme et al.,1959; Hoshino,1962). Besides that, Levine and Stockdale (1984) also showed that when mammary epithelial cells derived from mid-pregnant mice were co-cultured with the adipocyte cell line 3T3-L1, mammary cell growth was significantly higher than when those mammary cells were cultured on tissue culture plates. He also showed that when lactogenic hormones were added to the co-cultures, the mammary epithelial cells became differentiated and synthesized casein (Levine and Stockdale,1985). Recently, using mammary epithelial organoids (MEO) isolated from pubescent rats, adipocytes were again shown to enhance functional differentiation (Zangani et al.,1999). During ductal growth, migratory white blood cells, macrophages and eosinophils are recruited to the end buds and this has been indicated to be essential for development of the end buds (Gouon-Evans et al.,2000). Interestingly, DNA synthesis in the stroma is

detected during this activity, suggesting its involvement in the response (Berger and Daniel,1983).

Studies have indicated that the stromal component could exert its effect by being a necessary target for endocrine mammogens and a site for synthesis of stimulatory growth factors. In the mouse mammary gland, estrogen receptors are expressed both in the epithelial and stromal cells (Fendrick et al.,1998). Using tissue recombinations and estrogen-receptor knockout (ERKO) mice, Cunha et al.(1997) demonstrated that when ERKO mammary epithelium was grown in wild-type fat pad, the ERKO mammary epithelium underwent extensive ductal growth and branching morphogenesis. However, this was not observed in tissue recombinants containing wild-type mammary epithelium and ERKO fat pad. In addition, when adult mammary stroma was recombined with immature epithelium tissue, there was an induction of the progesterone receptor (Woodward et al.,1998), thus a role for the maintenance of oestrogen-responsiveness by the stroma is not dismissible. From these observations, there seems to be a critical role of stromal estrogen receptors in estrogen-dependent epithelial responses.

Evidence has also presented that progesterone might elicit a response within the mammary fat pad. Using local implants, progesterone induced stromal proliferation in mature female mice (Wang et al.,1990). Growth hormone (GH) actions have also been suggested to be mediated by stromal cells. Exogenous GH up-regulates IGF-1 expression in the fat pad (Kleinberg,1998), which was also detected in bovine mammary adipose tissue upon GH treatment *in vitro* (Akers et al.,2000).

Compelling evidence supports the mammary fat pad as a site for the synthesis of growth factors. Studies have demonstrated that IGF-1 is actually stroma-derived in the rodent, ruminant and human mammary gland (Hovey et al.,1998; Kleinberg,1998; Rasmussen and Cullen,1998). As for EGF receptors, besides being present on epithelial cells, they are also detected within the mouse mammary fat pad. Haslam et al.(1993) demonstrated that locally implanted EGF pellets in the mammary gland of ovariectomised mature female mice, not only stimulated DNA synthesis in the epithelial cells but also in the stromal cells. When the EGF receptors in the stromal cells were not present, normal ductal morphogenesis could not proceed (Sebastian et al.,1998). Stromal expression of the second member of the FGF family, basic FGF (bFGF), has been shown to be enhanced by the surrounding parenchyma (Coleman-Krnacik and Rosen,1994; Hovey et al.,1996), suggesting paracrine interactions.

Besides the regular growth factors mentioned above, the stroma is known to express other growth regulators. The *Wnt* gene family members are known to be expressed in the fat pad under the influence of ovarian hormones (Weber-Hall et al.,1994). Heregulin, the neu-differentiation factor has also been demonstrated to be expressed in the perialveolar stroma during pregnancy and is capable of inducing lobulo-alveolar growth and milk protein synthesis *in vitro* (Birchmeier and Birchmeier,1998).

From all the evidence gathered from these studies, we see that the mammary fat pad, rather than being an inert supporting matrix, is a key player in normal mammary gland development and function.

1.6.2 Extracellular matrix and cell interactions

The extracellular matrix (ECM) is composed of collagen, laminin, fibronectin, proteoglycan and elastin. The importance of ECM in mammary gland development and function has become increasingly evident, ECM proteins having been shown to be involved in the regulation of mammary epithelial cell proliferation, differentiation and apoptosis. Keely et al.(1995) demonstrated that collagen I, collagen IV and laminin mRNAs are expressed temporally in the mammary gland and this expression pattern corresponds with the stage of mammary development where active proliferation occurs. In another study, fibronectin and fibronectin-specific integrin, $\alpha_5\beta_1$, were shown to have the highest expression during active proliferation and lowest during non-proliferative phases and lactation in the mouse mammary gland (Woodward et al.,2001). In addition, this group also demonstrated that when the mice were ovariectomised, fibronectin expression was decreased by 70%, indicating that fibronectin might be an effector of hormone-dependent proliferation in the mouse mammary gland. Progesterone stimulated proliferation of mammary epithelial cells has also been demonstrated to be influenced by fibronectin and collagen IV (Xie and Haslam,1997). When mouse mammary epithelial cells were cultured in the presence of ECM proteins, these promoted a highly synergistic proliferative response to EGF plus IGF-1 (Woodward et al.,2000). In contrast, when human breast cancer cells were cultured on laminin, estrogen-induced proliferation was greatly reduced (Woodward et al.,2000), indicating that specific ECM proteins might be involved in stimulating proliferation in mammary epithelial cells.

ECM's role in differentiation of mammary epithelial cell was demonstrated by pioneering studies carried out in the 1970s (Emerman et al.,1977; Emerman and Pitelka,1977). These studies showed that mammary epithelial cells derived from pregnant mice, when cultured in the presence of lactogenic hormone, only synthesized milk proteins. However when these cells were cultured on a floating collagen gel, in addition to milk protein synthesis, they became polarized and demonstrated ultrastructures reminiscent of luminal mammary cells in lactating animals. This role of the basement membrane was further supported by studies carried out over the years (Blum et al.,1987; Aggeler et al.,1988; Barcellos-Hoff et al.,1989). The results from these studies consistently showed that when mammary epithelial cells were cultured on basement membrane, milk protein expression was significantly induced and if the cells were only cultured on plastic or non-released collagen gels, little or no milk proteins were detected even in the presence of lactogenic hormones. These studies also demonstrated that besides collagen gels, basement membrane matrix derived from Engelbreth-Holm-Swarm tumour (EHS matrix) is also capable of inducing such a response. When mammary epithelial cells are cultured on EHS matrix, in addition to high level milk production, the cells formed spherical structures that resemble the alveoli in the lactating gland. ECM exerts its effect on mammary epithelial cell differentiation through the induction of milk protein gene expression. Laminin-rich basement substratum was shown to induce β-casein expression via integrins since anti-integrin antibody severely diminished its synthesis (Streuli et al.,1991; Streuli and Edwards,1998).

In addition to its role in proliferation and differentiation of mammary epithelial cells, ECM has also been implicated as a regulator of apoptosis. Basement membrane extracellular matrix was shown to suppress apoptosis of mammary epithelial cells both *in vitro* and *in vivo* (Boudreau et al.,1995). They demonstrated that when ECM degradation was induced, apoptosis in these cells was initiated, and when β_1 integrins were blocked by antibodies, a similar effect was observed. Furthermore, one of the inducers of apoptosis, interleukin-1β converting enzyme (ICE) was also shown to be expressed during ECM- induced apoptosis. From this study we can say that ECM regulates apoptosis in mammary epithelial cells through an integrin-dependent negative regulation of ICE expression. Pullan et al.(1996) demonstrated that primary mammary epithelial cells cultured on laminin-rich membrane but not collagen I, did not apoptose. Recently, ECM role in mammary epithelial cell apoptosis was further demonstrated. Besides showing the requirement of integrins ($\alpha_6\beta_1$), insulin or insulin-like growth factors were also shown to be essential to suppress mammary cell apoptosis (Farrelly et al.,1999). This indicates that basement membrane and soluble factors coordinately regulate cell survival signals in mammary epithelial cells.

1.6.3 Fibroblast-epithelial cell interactions

The effect of estrogen on mammary epithelial cell proliferation *in vivo* has been well established. However, this effect could not be demonstrated *in vitro.* This led to the suggestion that estrogen's effect might be mediated through the stroma. Interestingly, mammary fibroblasts have been shown to express the estrogen receptor (Haslam and Levely,1985). Besides that, it was also shown that when mammary epithelial cells and mammary fibroblasts were cultured in

mixed cultures, estrogen was able to induce progesterone receptor concentration, which was not observed if mammary epithelial cells were cultured alone. In addition, Haslam (1986) also demonstrated that estrogen-dependent DNA synthesis in mammary epithelial cells only occurs in the presence of mammary fibroblasts which is consistent with earlier studies (McGrath,1983). Fibroblasts were later shown to stimulate proliferation of normal and neoplastic human mammary epithelial cells (Gache et al.,1998). Recently, Darcy et al.(2000) indicated that fibroblasts not only stimulated proliferation but also alveolar morphogenesis and functional differentiation in rat mammary epithelial cells.

1.7 LEPTIN

Leptin and its receptor were identified in parabiosis studies carried out in the 1970s using two strains of mutant mice, obese (*ob)* and diabetes (*db)* (Coleman,1973; Coleman,1978). Both strains of mice exhibit obesity, hyperglycemia and insulin resistance resembling type II diabetes when homozygous for these mutations. Parabiosis of an *ob/ob* mouse and a lean mouse resulted in partial normalization of body weight in the *ob/ob* mutant mice, leading to the suggestion that *ob/ob* mice were deficient in a circulating factor that is present in the blood of the lean animal. Using positional cloning, the *ob* gene and its product were later identified (Zhang et al.,1994). The ob gene product, leptin, is a 16KDa protein expressed mainly in adipose tissue.

1.7.1 Leptin receptor

The identification of the leptin receptor (OB-R) was achieved using an expression cloning strategy (Tartaglia et al.,1995). OB-R belongs to the class I

cytokine receptor family. Due to alternate splicing, at least five isoforms of the receptor exist (Lostao et al.,1998), which can be generally grouped into long and short forms of the receptor. The extracellular domains of the various isoforms of OB-R are identical throughout their entire length, differences arise in the intracellular domain, where the long form contains sequence motifs indicating intracellular signalling capabilities. Indeed, the long-form OB-R (Ob-Rb) has been demonstrated to activate Janus kinase 2, which in turn activates STAT proteins, in response to ligand binding (Tartaglia,1997; White et al.,1997). In addition, leptin was also able to stimulate insulin receptor substrate phosphorylation (Bjorbaek et al.,1997), PI3 kinase activity (Wang et al.,1997; Kim et al.,2000) and MAPK (ERK) activity (Takahashi et al.,1997). Even though the short-form OB-R does not possess potential signalling domains, it has been demonstrated to be capable of modulating signalling in the long-form receptor (White et al.,1997), raising the idea that the short-form receptor might have a regulatory role.

1.7.2 Leptin as a satiety factor

Mice deficient in leptin are severely obese while mice expressing a defective leptin receptor, in addition to being obese, are resistant to leptin (Chen et al.,1996; Iida et al.,1996; Marti et al.,1999). Moreover, leptin mRNA and protein expression have been demonstrated to be influenced by alteration in food-intake (reviewed in Tartaglia,1997). Fasting results in dramatic down-regulation of leptin expression whereas an increase in caloric intake results in up-regulation. The breakthrough came when it was shown that administration of leptin in rodents was capable of reducing food-intake and inducing weight

loss (Campfield et al.,1995; Halaas et al.,1995). All these observations led to the implication that leptin is a satiety factor.

In ruminants, in this case sheep, leptin gene expression in adipose tissue was also correlated with body fatness and food intake (reviewed by Chilliard, 2001). Leptin mRNA level in adipose tissue was higher in sheep from a fat selection line when compared with lean counterparts. In addition, level of adipose tissue leptin mRNA was also sharply decreased by food deprivation/undernutrition and increased by refeeding.

Expression of the long-form leptin receptor has been detected in the hypothalamus. Its expression has been localized in the arcuate (ARC) and paraventricular (PVN) nuclei as well as in ventromedial (VMH), lateral (LH) and dorsomedial (DMM) hypothalamic areas (Marti et al.,1999). Of particular interest is the PVN since it regulates body weight and/or food intake (Flier and Maratos-Flier,1998). The effects of leptin on body weight are thought to be mediated via the hypothalamus. Within the hypothalamus, leptin decreases expression of the orexegenic peptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP), and increases expression of the anorexigenic peptides, POMC and CART, resulting in a decrease in appetite (reviewed in Vernon et al.,2001).

In addtion to its central effects on food intake, leptin is also required for normal reproduction and immune function (Ahima and Flier,2000). However, we must note that leptin's role in reproduction was only observed in *ob/ob* mice and is not a limiting factor in most species.

Leptin has also been demonstrated to target peripheral tissues. Leptin has a lipolytic effect on adipocytes in rodents (Frühbeck et al.,1997; Frühbeck

et al.,1998; Kawaji et al.,2001; Marti et al.,2001) but not in sheep (Newby et al., 2001), it also alters the ability of preadipocytes to respond to lipogenic hormones (Bai et al.,1996) and induces proliferation, differentiation and functional activation of hemopoietic cells (Gainsford et al.,1996). As leptin seems to have diverse roles and is secreted by adipocytes, it would be interesting to investigate if leptin has a role in the mammary gland.

1.7.3 Leptin and the mammary gland

Besides being expressed in the hypothalamus, leptin and its receptor have also been detected in many other tissues including the mammary gland. Aoki et al. (1999) demonstrated that leptin is expressed in the mouse mammary gland. By using RT-PCR, they showed that expression of leptin is detected as early as in the virgin stage. This level of expression decreases as the animal goes into gestation and lactation. After lactation, the level of expression is restored to that of the virgin stage. In addition, this group showed that the mouse mammary cell line, COMMA-1D, also expresses leptin and this expression is reduced significantly when the cells are cultured in the presence of prolactin. A separate study carried out in ruminants showed that leptin (Bonnet et al.,2002), and both the long and short forms of its receptor (Laud et al.,1999) are expressed in the ovine mammary gland. In-situ hybridisation localized the expression of the receptor to the alveolar epithelial cells (Laud et al.,1999). Bonnet et al.(2002) using immunohisotchemistry showed that the cellular location of leptin in the ovine mammary gland changes during different phases of development. During early stages of development, leptin was exclusively located in mammary adipocytes and just before parturition, it was present in mammary epithelial cells. During lactation, leptin was located in

myoepithelial cells. This is an intriguing observation since it suggests that paracrine signalling might be occurring between different cell types during different stages of development. In both studies, expression of leptin and its receptor also decreases as the animal goes into lactation. Based on all of these observations, we can see that the expression of leptin and its receptor seems to be down-regulated during lactation. Besides rodents and ruminants, human mammary epithelial cells have also been shown to express leptin (Smith-Kirwin et al.,1998). Leptin was detected in human mammary tissue, cultured human mammary cells and secretory epithelial cells from breast milk. Interestingly, *ob* mice have been claimed to be incapable of lactating (Chehab et al.,1996). *Ob* mice are known to be sterile and in this particular study, it was shown that leptin treatment was able to restore fertilty, but these mice still lack the capability to secrete milk. Here, it seems like leptin might have a role in early mammary development. Indeed, it has been shown that mammary development in *ob* mice is seriously compromised (Knight and Sorensen,2001). In a different study, leptin was shown to partly inhibit IGF-1 and foetal bovine serum (FBS)-stimulated proliferation in the bovine mammary epithelial cell line, MAC-T (Silva and VandeHaar,1999).

1.8 AIM OF THE STUDY

This review has discussed the importance and contribution of both local and endocrine controls in mammary gland development and function. Besides that we have also established the fact that there is an obligatory requirement, especially during early development, for the mammary fat pad.

Since the fat pad influences ductal growth; the number of mammary epithelial cells is a major determinant of milk yield and it also influences the incidence of mammary gland lesions, our first objective is to examine factors such as proliferation and apoptosis in the mouse mammary gland during different stages of development.

As both leptin and its receptor are expressed in the mammary gland, our second objective is to examine the hypothesis that leptin is an obligate growth factor for mammary development. To address this, a combination of *in vitro* and *in vivo* models were employed

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Unless otherwise stated, laboratory chemicals and apparatus were supplied by Sigma-Aldrich Company Ltd., Poole, UK and Merck Ltd., Poole, UK. Cell culture consumables were obtained from Greiner Bio-One Ltd., Gloucestershire, UK while culture media was purchased from Life Technologies Ltd., Paisley, UK. Deionised water for general laboratory use was purified by reverse osmosis, ion/organic removal and photo-oxidation. Water used for tissue culture was ultrafiltered and further photo-oxidated to 18.2 mOhms resistance.

2.1.2 Radiochemicals

Radiochemicals were from Sigma-Aldrich Company Ltd., Poole, UK ; ICN Pharmaceuticals Ltd., Basingstoke, UK or NEN Dupont Ltd., Stevenage, UK. ^{14}C-, ^{3}H- and ^{35}S- activities were determined using a 1600TR liquid scintillation analyser with Emulsifier-Safe™ scintillation fluid (both from Canberra Packard, Berkshire, UK).

2.1.3 Animals

Mice (Tucks number one strain, A. Tuck and Son, Essex, UK) were housed individually in an animal house under a 12h light-dark cycle, and had diet CRMX (SDS, Manea, Cambridgeshire, UK) and tap water available *ad libitum.*

British Saanen goats in their third trimester of pregnancy (d107-d124) were obtained from A&K Wielkopolski, St. Helen's Farm, York, UK. They were

kept in the Institute's goat house and fed an 18% crude protein concentrate with hay and water available *ad libitum.*

2.2 HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

2.2.1 Coating of slides

Silane coated slides were prepared by first washing the slides in 5% (v/v) Decon for 30min, followed by rinsing thoroughly with tap water and deionised water. The slides were then incubated in absolute alcohol for 1min, 2% (v/v) 3-aminopropyltriethoxysilane (APES) in absolute alcohol for 30sec and then in absolute alcohol for 1min. Finally, the slides were washed in deionised water and left to dry overnight at room temperature.

2.2.2 Fixation of mammary tissue for immunohistochemistry

The inguinal glands were fixed overnight in 10% formalin and stored in 70% (v/v) ethanol until treated according to a standard dehydration/embedding protocol (Kiernan,1981). Tissues were first washed in absolute alcohol at room temperature for 6h with 3 changes of ethanol, followed by one 2h wash with absolute ethanol:chloroform (1:1,v/v) and finally washed in chloroform for 1h. After washing, the tissues were incubated in paraffin wax. To allow immunohistochemistry analysis, tissues were processed into 4μm thick sections on a microtome (Reichert-Jung, 2040 Autocut, Nussloch, Germany) using disposable steel blades (Leica model 819, Nussloch, Germany). Sections were floated onto a waterbath set at 50°C, then mounted onto silane-coated slides and air dried overnight.

2.2.3 Fixation of mammary gland for wholemount analysis

In this method the entire mammary gland was spread onto a silane coated slide and fixed in Carnoy's fixative (60% (v/v) absolute alcohol: 30% (v/v) chloroform: 10% (v/v) glacial acetic acid) for 2 to 4 h at room temperature. Next, the slide was subjected to a series of 15min washes starting with 70%(v/v) ethanol ; 50% (v/v) ethanol ; 25%(v/v) ethanol and finally with distilled water . The mammary gland was then stained overnight with carmine alum (0.2%(w/v) carmine and 0.5%(w/v) aluminium potassium sulphate). After staining, it was dehydrated by washing for 15min in 70% ethanol, 95% ethanol and absolute alcohol respectively. Finally, the mammary gland was cleared in histoclear and mounted with DPX mounting medium (Agar Scientific, Standsted, UK).

2.2.4 Fixation and sectioning of mammospheres

Culture medium was removed from cells on EHS matrix and ~300µl replaced. An equal volume of fixation solution (4% (w/v) paraformaldehyde in 0.1M phosphate buffer) was added for 30min. This was replaced twice more with fresh fixation solution. The cells were then washed three times for 10mins with rinse solution (0.1M phosphate buffer containing 2% (w/v) sucrose and 0.002% (w/v) $CaCl_2$). Cells were gently scraped from the plate and transferred to a cryotube containing ~100µl of OCT compound (Agar Scientific, Standsted, UK). The tube was left to stand for ~1hr to allow the cells to settle. Excess rinse buffer was removed and further OCT compound was added, into which the cells were gently mixed. The sample was frozen slowly (-1°C/min) at –70°C. 4µm sections were cut on a cryostat (Leica CM1800, Cambridge,

UK), transferred onto silane-coated microscope slides and allowed to air-dry overnight.

2.2.5 Immunostaining for milk proteins

Sections were preblocked in PBS/0.1% Tween 20 (PBST) containing 0.1% (v/v) Triton and 0.3% (w/v) BSA for 20min. Non-antigenic sites were then blocked with PBST containing 25% (v/v) donkey non-immune serum for 30min. The sections were then incubated with sheep anti- bovine casein (Guildhay Ltd, Surrey, UK) diluted 1:250, and rabbit anti-bovine β-lactoglobulin (Bethyl Laboratories Inc., Texas, USA) diluted 1:250 in PBST for 2h at 37°C in a humified chamber. Sections were then washed three times in PBST, followed by incubation with donkey anti-sheep and anti-rabbit fluoroscein isothiocyanate (SAPU, Lanarkshire, UK) diluted 1:500 for 2h at 37°C in a humified chamber. The sections were washed as before and mounted with Vectashield (Vector Laboratories, Peterborough, UK) containing 4,6-diamidino-2-phenylindole (DAPI) nuclear stain (1.5μg/ml). The cells were visualised by fluorescence microscopy (Leitz DMRB, Leica, Cambridge, UK).

2.2.6 Counterstaining of paraffin-embedded tissue sections

Paraffin-embedded sections were dewaxed in histoclear and rehydrated through a graded series of ethanol and water (Stevens,1990). Rehydrated sections were placed in Mayers hematoxylin (Sigma, Poole, UK) for 5min and rinsed in water. To develop the dye, the sections were placed in a solution of $LiCl_2$ (Scott's tap water, Sigma, Poole, UK) until all the cell nuclei turned blue. Sections were washed in deionised water; dehydrated in 70% (v/v) ethanol, 90% (v/v) ethanol and absolute ethanol; cleared and mounted as described in section 2.2.3.

2.2.7 Detection of BrdU incorporation

BrdU incorporation was detected by immunohistochemistry using the indirect horseradish peroxidase (HRP) conjugated Streptavidin-biotin staining techniques as described and supplied by DAKO Ltd, Cambridge, UK. Paraffin-embedded sections were dewaxed and rehydrated as described in section 2.2.6. Sections were then washed in PBS for 5min followed by incubating in pronase prepared in PBS (50μg/ml) for 5min at 37°C and again washed in PBS for 5min. To quench any peroxidase activity that might be present, the sections were treated with 2% (v/v) H_2O_2 in methanol for 5min at room temperature. Sections were incubated with 2M HCl for 1h at 37°C to denature the DNA. They were then washed in PBS and treated with 0.2% Triton X-100 for 30min at RT. Sections were again washed in PBS for 5min and to prevent non-specific binding, they were incubated with normal rabbit serum (Scottish Antibody Production Unit (SAPU), Lanarkshire, UK) diluted 1:10 with Tris-HCl pH7.6 for 20min at RT and followed by 5min wash in PBS. After blocking, the sections were incubated with primary antibody, mouse anti-BrdU diluted 1:20 with BSA/PBS (0.5%(w/v) BSA in PBS), for 1h at RT in a humified chamber. Unbound antibodies were removed by washing the sections in PBS for 10min. Next the sections were incubated with biotinylated rabbit anti-mouse immunoglobulin diluted 1:1000 with 0.5% BSA/PBS, for 45min at RT in a humified chamber. The avidin-biotin complex was applied to sections for 45min followed by washing in PBS. The bound antibody was detected by incubating sections with DAB solution prepared as recommended by manufacturer (Sigma, Poole, UK). The colour reaction was monitored under the microscope and upon detection sections were washed in

deionised water. Finally, the sections were counterstained, developed, dehydrated, cleared and mounted as described in section 2.2.6.

2.2.8 In Situ Apoptosis detection

Apoptotic cells on tissue sections were detected using the ApopTag® Peroxidase apoptosis kit (Intergen Co., Oxford, UK) according to manufacturer's instructions. Histological sections were dewaxed and rehydrated as described in section 2.2.4. To remove DNA-binding protein, the sections were incubated with a protein digesting enzyme or Proteinase K (20μg/ml) for 15min at RT and then washed in two changes of ddH_2O for 4min. Next the sections were treated with 2% (v/v) H_2O_2 in PBS for 5min at RT so as to block endogenous peroxidase activity. Sections were then rinsed in PBS and immersed in an equilibration buffer before incubating with terminal deoxynucleotidyl transferase for 1h in a humified chamber at 37°C. To stop the reaction, the slides were transferred to stop/wash buffer (provided by kit), preheated to 37°C, for 10min at RT. Following this, the sections were rinsed in PBS and incubated with the peroxidase conjugated anti-dioxigenin antibody for 30min at RT. To detect apoptotic cells, the sections were again incubated with the substrate DAB. Finally, the sections were counterstained and mounted as described in 2.2.6.

2.2.9 Quantification of apoptosis and BrdU incorporation

Cells stained positive for apoptosis and BrdU incorporation were quantified using an image analysis system (Leica Q500MC, Nussloch, Germany). For each sample, three consecutive sections were prepared and mounted onto a slide. Cells from each section were counted by taking 12 separated views under the microscope. Results obtained were the mean of the three sections

and were expressed as a percentage of positive cells out of the total cells counted.

2.3 CULTURE OF CAPRINE MAMMARY CELLS

The approach comprised collection of mammary tissue from mid-pregnant goats followed by digestion to yield cell clumps suitable for cryo-preservation. Subsequently these cells were cultured on plastic (proliferation phase) and then on Engelbreth-Holm-Swarm (EHS) matrix (differentiation phase) to yield structures anatomically similar to alveoli, termed mammospheres.

2.3.1 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 kept at –70°C was washed by homogenising (Ultra-Turrax T25, Janke & Kunkel, IKA Labortechnik) in 50mM Tris HCl containing 4mM EDTA and 3.4M NaCl and centrifuged at 45,000 g for 30min at 4°C. This was repeated twice, before overnight extraction with 50mM Tris HCl containing 4mM EDTA, 2M urea and 0.2M NaCl. The resulting extract was centrifuged as before and the supernatant was dialysed [molecular weight cut-off (MWCO) 12-14 kDa] first against 50mM Tris HCl containing 4mM EDTA and 0.15M NaCl (48h, 3 changes), and then with Dulbecco's Modified Eagle's medium (DMEM) containing 10mM Hepes buffer and penicillin/streptomycin (200U/ml and 200μg/ml respectively) for 16h. The dialysate was stored in aliquots at -20°C.

2.3.2 Cell culture media

The composition of the basal medium is shown in Table 2.1. Basal media (pH 7.4) was sterilised through a 0.2μm pore filter (Gelman Sciences,

Southampton,UK) and stored at 4°C. To prepare digestion medium, trypsin inhibitor, collagenase (Type 2, Worthington Biochemical Corporation, New Jersey, USA), hyaluronidase, DNase I (both Boehringer Mannheim Ltd, UK) were added to the basal media on day of use. Insulin was prepared by dissolving 10mg of insulin (from bovine pancreas, 27.4 USP units/mg) in 10ml of deionised water followed by 100µl of 0.34M NaOH and finally diluted with deionised water to 100µg/ml. Cortisol was dissolved in absolute alcohol and again diluted with deionised water to 100µg/ml. Both stocks of insulin and cortisol were stored in aliquots at –20°C , and added to the basal medium on the day of use.

2.3.3 Preparation of mammary epithelial cells

Goats in their third trimester of pregnancy (d107-d124) were culled by captive bolt, followed by exsanguination. The mammary gland was scrubbed with Hibitane™ (Zeneca Ltd, Cheshire, UK) and subsequently with 70% ethanol before excision. Once removed, it was trimmed of skin, cistern, ductal tissue and fat. The remaining tissue was cut into ~2cm^3 pieces and immersed in sterile Hanks Balanced Salt Solution (HBSS; pH 7.4, 37°C). The tissue was washed twice in sterile HBSS, prior to removal of residual fat and connective tissue.

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

After approximately 3-4h, when the cells were predominately in clumps of ~50 cells, the digest mixture was filtered through a 150μm pore nylon mesh (Lockertex, Warrington, UK). To harvest the cells , they were centrifuged at 80 g for 5min, resuspended in wash medium and re-harvested. This wash step was repeated three more times and the final cell pellet was resuspended in HBSS. Undigested material was subjected to further digestion (1-2h) after which cell harvesting was repeated.

Percoll fractions were prepared comprising Medium 199 containing 20mM Hepes, 2%(w/v) Ficoll and Percoll to densities of 1.01-1.08 g/ml in increments of 0.01g/ml. A discontinous gradient was prepared by gently layering 5ml of each fraction into a 50ml centrifuge tube. 5ml of cell suspension was gently layered on top and the gradient was centrifuged (800 g, 20min, without braking). Individual bands were removed sequentially. The resulting cell suspensions were diluted with HBSS and centrifuged (100 g, 10min) to remove the Percoll. The cells were washed once again by resuspending in HBSS and centrifuging (80 g, 5min). Finally, the cell pellet was resuspended in culture medium.

Table 2.1 Composition of media used in the isolation of caprine 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	40	-
(mg/ml)	2.2	2.2
Calcium chloride (μg/ml)	2.5	2.5
Magnesium sulphate	120	120
(μg/ml)	100	100
Penicillin (μg/ml)	2.5	2.5
Kanamycin (μg/ml)	-	5.4
Fungizone (μg/ml)		
Hepes buffer (mM)		
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 II, ~ 330u/mg

2.3.4 Estimation of cell yield

Cell yield was estimated by pelleting 100µl of the final cell suspension in an elongated microtube (13,000 g, 30s). 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 2.7).

2.3.5 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 florescent dye, bisbenzimide (Hoechst 33258), to DNA. Pelleted cells were sonicated (15s at setting 30; Kontes micro-ultrasonic cell disruptor, Burkard Scientific, Rickmansworth, UK) in 400µl DNA assay buffer (0.1M Na_2HPO_4 containing 2M NaCl, pH 7.4) and an appropriate volume was removed for assay. DNA standards (calf thymus) were prepared (range 0-5µg) and all assay volumes were made up to 1ml with assay buffer before addition of 0.5ml of fluorescent reagent (3µg/ml). Assay samples were incubated for 1hr after which fluorescence was measured on a model LS-5 luminescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, UK) with an excitation wavelength of 360nm, an emission wavelength of 475nm.

2.3.6 Cryopreservation of mammary epithelial cells

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

2.3.7 Cell recovery from liquid nitrogen

Cryovials were removed from liquid nitrogen and thawed quickly at 37°C. The cells were resuspended in culture medium and centrifuged at 80g for 5min, so as to remove the DMSO present. The cell pellet was then resuspended in proliferation medium (Table 2.2) for plating prior to culture in a humified tissue-culture incubator (Jencons Nuair, Leighton Buzzard, UK) at 37°C and 5% (v/v) CO_2. Aseptic technique was used throughout this and the following three procedures.

2.3.8 Proliferation of cells on plastic

Cells were plated aseptically onto 6-well culture plates at a density of 1 x 10^5 cells/cm^2 and allowed to proliferate till they were about 90% confluent, determined using phase-contrast microscope, before passaging. Medium was changed every two days.

2.3.9 Passaging cells

Medium was removed from the wells and the cells were washed twice with 2ml of HBSS. To each well, 0.6ml of sterile trypsin solution containing EGTA (0.4mg/ml) and polyvinyl alcohol (0.1mg/ml), was added to the cells. The cells were incubated in the trypsin solution for at least 40min before sterile serum-containing medium was added to neutralise trypsin's activity. Cells were pelleted (80 g, 5min) and resuspended in the appropriate culture medium aseptically for plating on either plastic or EHS matrix.

Table 2.2 Composition of media used in the culture of caprine 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)	10	10	10
$NaHCO_3$ (mM)	27	27	27
Sodium acetate (mM)	2	2	2
Penicillin (U/ml)	200	200	200
Streptomycin (μg/ml)	200	200	200
Fungizone (μg/ml)	2.5	2.5	2.5
EGF[1] (ng/ml)	10	-	-
Insulin (μg/ml)	5	5	5
Cortisol (μg/ml)	1	1	1
Prolactin[2] (μg/ml)	-	3	3
Horse serum (%)	20	20	-
Foetal calf serum (%)	5	5	-

[1]EGF was prepared by dissolving 100μg in 10ml of 0.9% (w/v) saline

[2]Prolactin was prepared by dissolving 10mg in 2.5ml of 10mM Hepes (pH 8.0) and diluting to 0.1mg/ml with water.

2.3.10 Differentiation of cells on EHS matrix

EHS matrix was thawed on ice and applied to ice-cold tissue culture plates (50µl/cm^2). The matrix was then allowed to polymerise at RT for 30min. Cells were plated gently in attachment medium (Table 2.2) at a density of 3 x 10^5/cm^2. After 24h, attachment medium was replaced with serum-free differentiation medium (Table 2.2), allowing the mammary epithelial cell clumps to differentiate and form mammospheres. The medium was changed daily.

2.4 CULTURE OF MOUSE MAMMARY EPITHELIAL CELLS

Similarly, mammary tissue was collected from mid-pregnant mice and processed to yield mammary epithelial cell clumps, which when differentiated on EHS matrix, formed mammospheres.

2.4.1 Cell Culture media

Basal media (pH7.4) was prepared and stored at 4°C. All enzymes and hormones were added to the basal medium on the day of use. Table 2.3 gives a summary of the cell culture media used in the isolation of mouse mammary epithelial cells.

2.4.2 Preparation of mammary epithelial cells

Pregnant mice (d15-17) were sacrificed by cervical dislocation and both the inguinal glands were removed. Aseptic techinques was used from this point on. The mammary tissue was incubated in HBSS (pH 7.4, 37°C) and then finely chopped to aid digestion. The finely minced tissue was then transferred to the digestion medium (Table 2.3), and incubated at 37°C in a orbital incubator shaking at 120 rpm for 90min. The digest mixture was then filtered through a 150µm pore nylon mesh and the cells were harvested by

centrifugation (80 g, 5min). The cell pellet was resuspended in wash medium (Table 2.3) and re-harvested. The cells were subjected to three more washes before being filtered through a 50μm nylon mesh. The cells were harvested as before and resuspended in culture medium (Table 2.4). They were cultured for 1h at 37°C to allow fibroblast attachment. The non-adherent mammary epithelial cells were recovered and the cell yield was estimated as described in section 2.3.4.

2.4.3 Differentiation of cells on EHS matrix

EHS was applied to the tissue culture plates as described in section 2.3.9. Cells were plated at a density of 3 $x10^5/cm^2$ and allowed to attach for 24hr in attachment medium (Table2.4) before transferring to a serum-free differentiation medium (Table 2.4). The differentiation medium was replenished daily.

2.5 MEASUREMENT OF PROTEIN SYNTHESIS AND SECRETION

Protein synthesis and secretion in the caprine and mouse mammospheres were analysed using a radio-labelled precursor of protein synthesis.

2.5.1 Radio-labelling of synthesised proteins

The mammospheres were incubated in fresh differentiation medium, containing 30μCi/ml of ^{35}S-Methionine, for 24hr. The culture medium, luminal secretion and cells were collected for assay of [^{35}S]-labelled protein.

Table 2.3 Composition of media used in isolation of mouse mammary epithelial cells

	Wash Medium	Digestion medium
Basal medium		
HBSS	1x	1x
MEAA	1x	1x
D-glucose (mM)	5	5
Bovine serum albumin (mg/ml)	-	40
Calcium Chloride (ug/ml)	2.5	2.5
Magnesium Sulphate (ug/ml)	2.2	2.2
Hepes (mM)	5.7	-
Glutamine (mM)	2	2
Fungizone (ug/ml)	2.5	2.5
Penicillin (ug/ml)	120	120
Kanamycin (ug/ml)	100	100
Hormones		
Insulin (ug/ml)	5	5
Cortisol (ug/ml)	1	1
Enzymes		
Collagenase[1] (mg/ml)	-	1.2
Dnase I (ug/ml)	20	-
Trypsin inhibitor (ug/ml)	100	-

[1]Type 3, ~ 150u/mg

Table 2.4 Composition of media used in the culture of mouse mammary epithelial cells

	Attachment (on EHS matrix)	Differentiation (on EHS matrix)
Basal medium		
Medium 199	1x	1x
Hams F12	1x	1x
Sodium Bicarbonate (mM)	70	70
Hepes (mM)	20	20
Penicillin (U/ml)	120	120
Kanamycin (ug/ml)	100	100
Supplements		
Insulin (ug/ml)	5	5
Cortisol (ug/ml)	1	1
Prolactin (ug/ml)	3	3
Triiodothyronine (ng/ml)	0.65	0.65
Glucose (mM)	1	1
Hepes (mM)	1.5	2.5
Foetal calf serum (%)	10	-

2.5.2 Harvesting of secreted proteins and cells

The culture medium was removed from the cells and kept. To harvest the luminal proteins, cells were first rinsed in HBSS pH7.4 containing 10mM Hepes, followed by incubating in HBSS pH7.4 containing 10mM Hepes and 2.5mM EGTA, for 20min at 37°C. To detach the cells from the EHS matrix, they were treated with Dispase (Universal Biologicals Ltd, London, UK) and recovered by centrifugation (13,000g, 1min, 4°C). All samples collected were snap frozen in liquid nitrogen and stored at –20°C.

2.5.3 Precipitation of proteins

To all medium and luminal extracts, 0.01% (w/v) BSA was added. The cell pellets were sonicated (15s at setting 30; Kontes micro-ultrasonic cell disruptor, Burkard Scientific, Rickmansworth, UK) in 400µl of DNA assay buffer (Section 2.7), and then 100µl of the homogenate was used for precipitation. To all samples, an equal volume of ice-cold 20% (w/v) trichloroacetic acid was added and incubated on ice for 30min before being subjected to centrifugation (12,000 rpm, 5min, 4°C). The precipitated protein was washed twice in 1% (w/v) trichloroacetic acid (12,000 rpm, 4min) and dissolved in 100µl of 1M Tris-HCl (pH 7.6). Protein sample was transferred to a scintillation vial, containing 10ml of scintillation fluid, and [^{35}S] activity was determined.

2.5.4 Protein samples for gel electrophoresis

The remaining protein samples from culture medium and luminal extracts were re-precipitated as described in section 2.5.3, and after the final wash, the pellet was dissolved in 25µl of 50mM Tris-HCl (pH7.6). Samples were analysed based on an equal DNA concentration so appropriate volume of

samples were dissolved in NuPAGE® LDS sample buffer (Invitrogen, Groningen, The Netherlands). On the actual day of use, 0.1% (v/v) β-mercaptoethanol was added to the samples and heated at 70°C for 10min.

2.5.5 SDS-PAGE

Proteins were resolved by electrophoresis (Laemmli,1970) using the Xcell SureLock™ Mini-Cell kit (Invitrogen, Groningen, The Netherlands). Standards and samples were separated on NuPAGE® 12% Bis-Tris gel (Invitrogen) at 200 v for 45min in NuPAGE® MES SDS running buffer (Invitrogen, Groningen).

2.5.6 Staining and fixing of gels

The gel was first washed in deionised water (3 x 5min) before being stained with SimplyBlue™ Safestain (Invitrogen, Groningen, The Netherlands) for 1h. After staining, the gel was immersed in deionised water overnight. The gel was then fixed (50% (v/v) methanol ; 10% (v/v) acetic acid) for 15min and rinsed (7% methanol; 7% acetic acid and 1% glycerol) for 10min. Finally it was dried onto filter paper on a gel dryer (Bio-Rad Laboratories Ltd, Herts, UK) at 80°C for 1h using cycle 1.

2.5.7 Autoradiography

Gels were exposed to a phosphor-screen cassette for 4 days before being quantified using a Phosphoimager™ 445SI (Molecular Dynamics, Sunnyvale CA, USA).

2.5.8 Western blotting and ECL

Proteins were transferred (100 v, 90min) to PVDF membrane (Immobilon P, Millipore, Watford, UK) in NuPAGE® Transfer Buffer. The membrane was immediately blocked overnight by incubating in PBS containing 3% (w/v) BSA, 1% (v/v) fish gelatin and 0.05% (v/v) NP40 at pH 7.4. Antibodies were diluted in PBS containing 0.3% (w/v) BSA, 0.1% (v/v) fish gelatin and 0.05% (v/v) NP40, which also served as a wash buffer. The membrane was incubated with sheep anti-bovine casein (1:7000) or rabbit anti-bovine β-lactoglobulin (1:3500) for 1h and then washed three times for 15min in wash buffer. Biotinylated anti-sheep IgG (Vector Laboratories Inc., USA), diluted 1:5000, or anti-rabbit IgG (Amersham Pharmacia Biotech, Little Chalfont, UK), diluted 1:5000, was added to the membrane for 1h followed by washes as before. The membrane was incubated with avidin-biotin complex for 45min. Detection of protein bands was achieved by incubating the membrane with enhanced chemiluminescent reagent (Renaissance; NEN Dupont, Stevenage, UK) and exposing the membrane to x-ray film (Fuji RX) for 10 – 30s. Densitometry of blots was carried out using ImageQuant (Molecular Dynamics, Sunnyvale CA, USA).

2.6 PROTEIN ASSAY

Protein content was measured spectrophotometrically as described by (Bradford,1976), using BSA as the protein standard (0.03mg/ml). Standards were prepared in the range of 0 - 3ug. Both standards and samples were made up to 200µl before the addition of 140µl of threefold diluted Bradford reagent (Bio-rad Laboratories Ltd, Hempstead,UK). Coloured complex formation was

determined at a wavelength of 595nm using a Titertek multiskan plate reader (Labsystems Ltd., Basingstoke, UK).

2.7 PHOTOGRAPHY

Brightfield, phase contrast and fluorescence colour photographs were taken using Fujicrome Provia 400 or Fujichrome colour 64T 64 ASA tungsten film on a Leitz DMR microscope (Leica, Nussloch, Germany).

2.8 STATISTICS

Data was compared by ANOVA using Genstat 5 (Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station, Herts, UK) or Minitab (Release 11, Minitab Inc, State College, PA 16801, USA). The data from chapter 3 was first transformed using angular transformation and then analysed by Tukey's pairwise comparison while in Chapter 5, the data was normalized by log transformation.

CHAPTER THREE

PROLIFERATION AND APOPTOSIS IN MOUSE MAMMARY GLAND

3.1 INTRODUCTION

The mammary gland displays distinct morphological differences at various stages of development. The gland is composed mainly of adipocytes before puberty, while during pregnancy, the adipocytes are gradually displaced to allow development in preparation for lactation. At lactogensis, the adipocytes have virtually disappeared leaving secretory alveoli responsible for milk production. From this observation, we can see that the ratio of mammary epithelial cell to adipocytes, and possibly their interactions, changes depending on the stage of development.

Cell proliferation and apoptosis are two physiologically essential processes in normal mammary gland development and function. It is well established that proliferation in the mammary gland occurs mainly during the onset of puberty and especially during pregnancy while during weaning, apoptosis plays a central role in the remodelling of the gland. Throughout the years, studies have either examined proliferation (Korfsmeier,1979) or apoptosis (Strange et al.,2001) separately. Laboratories have measured mammary cell proliferation during pregnancy and lactation (Knight and Peaker,1982), using measurement of DNA content and incorporation of [^{3}H]-thymidine *in vitro* as a marker. Humphreys et al.(1996) demonstrated that in addition to its role during involution, apoptosis also occurs in TEB during ductal morphogenesis. With the exception of Capuco et al.(2001), where both proliferation and apoptosis during a bovine lactation was investigated, to date

no other study has ever simultaneously quantified proliferation and apoptosis during the whole of mammary gland development.

Inappropriate development in the mammary gland can have long term repercussions. Pre-pubertal heifers fed on high plane diets have less ductular outgrowth and reduced milk yield (Sejrsen and Purup,1997). Besides that, reproductive histories have also been demonstrated to influence mammary development in humans. Women with a lactational history are less prone to developing breast lesions. This beneficial effect of reproductive history has been suggested to be exerted through the control of mammary epithelial cell proliferation (Peris and Knight,1997).

The aims of the studies reported in this chapter were:

1. To examine mammary cell proliferation and apoptosis in the murine mammary gland at various stages of development using immunohistochemistry.
2. To determine if lactational history has a long-term effect on proliferation and apoptosis of mammary epithelial cells.

3.2 EXPERIMENTAL DESIGN

3.2.1 Comparison of proliferation and apoptosis during different stages of development

Mammary proliferation and apoptosis were measured in groups of 6 contemporaneous mice at various stages of development. Virgin mice were studied at 4wks, 8wks, 12wks and 24wks old, all measurements being made at oestrous, determined through virginal smears. Pregnant mice were sampled at d1, d6, d12 and d18 of pregnancy, which was established at 8wks of age. Lactating mice were studied at d1, d7, d14, d21 and d28, pups being left with the mothers throughout. For comparison with standard first lactation mice, an additional group was studied on d1 of a second lactation. Cell proliferation was detected by incorporation of bromodeoxyuridine (BrdU) *in vivo*. This was achieved by administrating 5 s.c. injections each of 150μg given at 1400 and 2000hr on the first day (pro-oestrus in virgins, determined from daily cervical smear, while in pregnant and lactating groups, 1 day before actual measurement i.e. day 5 for day 6 pregnant group) and at 0800, 1400 and 2000hr on the following day. Mice were sacrificed at 1000hr the next day and the fourth pair of mammary glands were removed. One of the glands was mounted onto slides for wholemount image analysis while the remaining gland was embedded in wax and later sectioned to measure proliferation and apoptosis. Procedures for detecting and counting proliferating and apoptotic cells were described in Chapter 2.

3.2.2 Effect of lactational history on mammary epithelial cell proliferation and apoptosis

Mammary cell proliferation and apoptosis were measured in 4 groups of contemporaneous mice at 24wks of age. The four treatment groups were: virgin (V); previously pregnant with no lactation (P); previously lactated (L) and previously unilaterally lactated (UL). For the last group, all 5 mammary glands on the right side of the mouse were teat-sealed using skin adhesive so as to prevent lactation. Adhesive was applied under general anaesthesia and then was checked twice daily and replaced when necessary. Litter size was adjusted to 5 pups for this group. Pregnancy commenced at 8 weeks of age. The four groups of mice were then left until 24wks of age before BrdU was administered at oestrous as described in section 3.2.1. The fourth mammary glands were removed as before for histological analysis.

3.3 RESULTS

3.3.1 Histological analysis of mouse mammary glands

Wholemount analysis of the mouse mammary gland was carried out at different stages of development. Figures 3.1 & 3.2 show examples of the mammary gland at the virgin stage. A simple branching network of mammary ducts is seen at 4wks of age (Figure 3.1A). The rudimentary ductal system originating from the nipple (N) is seen growing towards the lymph node (LN). Figure 3.1E at a higher magnification (168x) shows a primary ductal branch consisting of terminal end buds (TEBs). After puberty (Figure 3.1B), the fat pad in the mammary gland becomes completely infiltrated with the epithelial branching network. In addition, the formation of lower-order ducts and bifurcation of end buds has also started appearing (Figure 3.1F). At 12 & 24wks old (Figures 3.1C & D), the epithelial network has become more dense and the terminal end buds are more complex forming lobular structures (Figures 3.1G & H).

Figure 3.2 shows sections of the virgin mammary gland embedded in wax. In Figures 3.2A & B, which show the gland at 4wks old, we see the ducts and TEBs, stained blue, in cross-sections. At this stage, the mammary gland is still predominantly composed of adipocytes. As the gland matures, we see the formation of lobular structures (Figures 3.2C & D) and there is an indication that the ratio of epithelial cells to adipocytes is getting higher (Figure 3.1C & E). However, looking at the sections of aged mice (Figure 3.2F), there seem to be a lower ratio of epithelial cells to adipocytes when compared to early stages (Figures 3.2C & E).

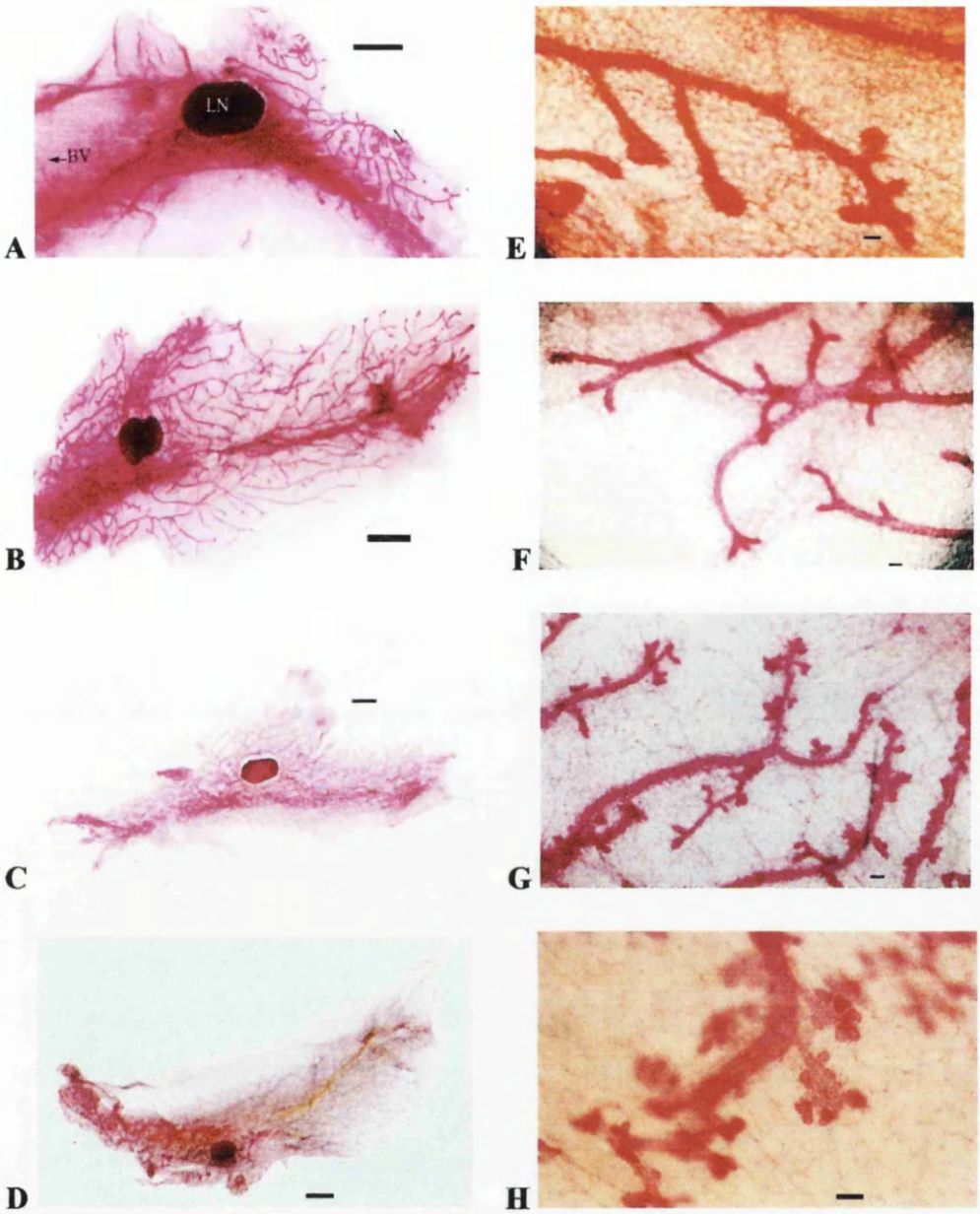

Figure 3.1 Wholemounts of virgin mouse mammary glands

Mammary glands from virgin mice were fixed and stained with carmine. A & E) 4wks old , B & F) 8wks old , C & G) 12wks old and D & H) 24wks old. Bar = 2mm (A, B, C & D) and Bar = 71.42μm (E, F & G at Magnification 168x). LN: Lymph node, BV: Blood vessel and N: Nipple.

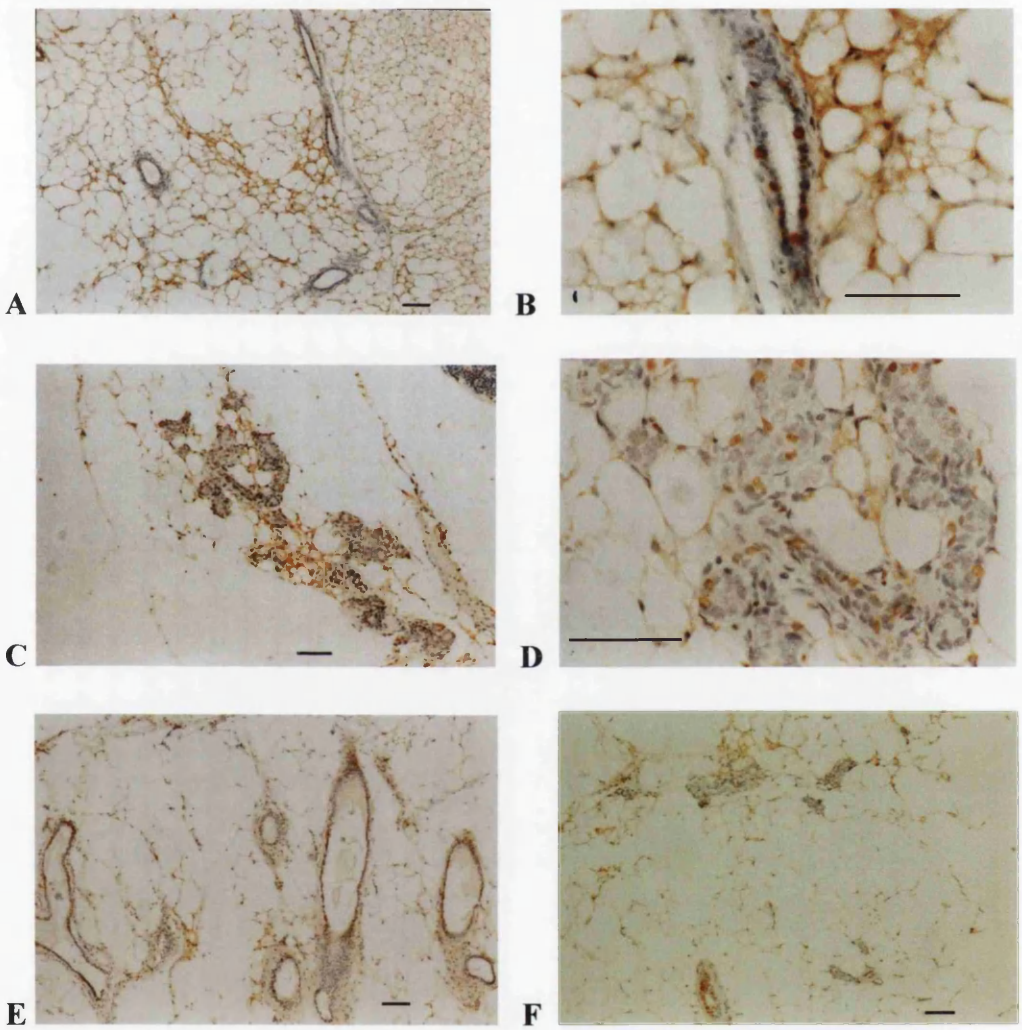

Figure 3.2 Wax-embedded sections of virgin mouse mammary gland

Mouse mammary glands were embedded in wax and sectioned at 4μm. A & B) 4wks old, C&D) 8wks old, E) 12wks old and F) 24wks old. A,C,E & F were taken at 420x magnification while B & D were at magnification 1680x

During pregnancy, the mammary gland at days 1 & 6 showed similar patterns of growth (Figures 3.3A & B). The ductal systems were growing towards the extreme end of the fat pad. Sections of the gland during these two periods showed that most of the gland was still predominantly occupied by the adipocytes (Figures 3.4A & B). This pattern of growth was not observed at day 12 of pregnancy; instead we observed an increase in complexity of the ductal system. In addition to occupying the fat pad (Figure 3.3C), there was also the formation of dense grape-like structures called alveoli (Figure 3.3D & E). Cross-sections of the gland at this stage, showed that it was no longer occupied predominantly by adipocytes, instead there were clusters of lobulo-alveolar structures (Figure 3.4C & D). By day 18 of pregnancy (Figure 3.3F), the whole gland was completely filled with alveoli-like structures. Histological sections of the gland at this stage showed that it is almost completely occupied by the alveoli (Figure 3.4E) which are secreting lipids and milk-like components (Figure 3.4F).

During lactation, the mammary gland is completely filled with lobulo-alveolar structures (Figure 3.5A-E). Sections confirmed that the mammary gland was composed almost entirely of milk-secreting alveoli [Figure 3.6a]. Adipocytes in the gland were no longer evident. Interestingly, at day 7 of lactation [Figure 3.6a (C&D)], the size of alveoli seems to be bigger compared to those during the onset of lactation [Figure 3.6a (A&B)]. At day 14 of lactation [Figure 3.6a (E&F)], there is an indication of the collapsing of the alveoli since the integrity of its structure has been reduced. By day 21, the gland has started to revert to that of the virgin stage [Figure 3.6b (A&B)]. Besides milk-secreting alveoli, mammary epithelial cells and adipocytes were

also observed. Wholemounts [Figure 3.5a (F)] and sections [Figure 3.6b (C&D)] at day 28, displayed a gland that has reverted back to a state similar to that of virgin mice. Adipocytes are once more the dominant feature.

The appearance of the gland was similar at the start of the second lactation compared to the first, although there did appear to be more adipocytes and, perhaps, larger alveoli (Figure 3.7).

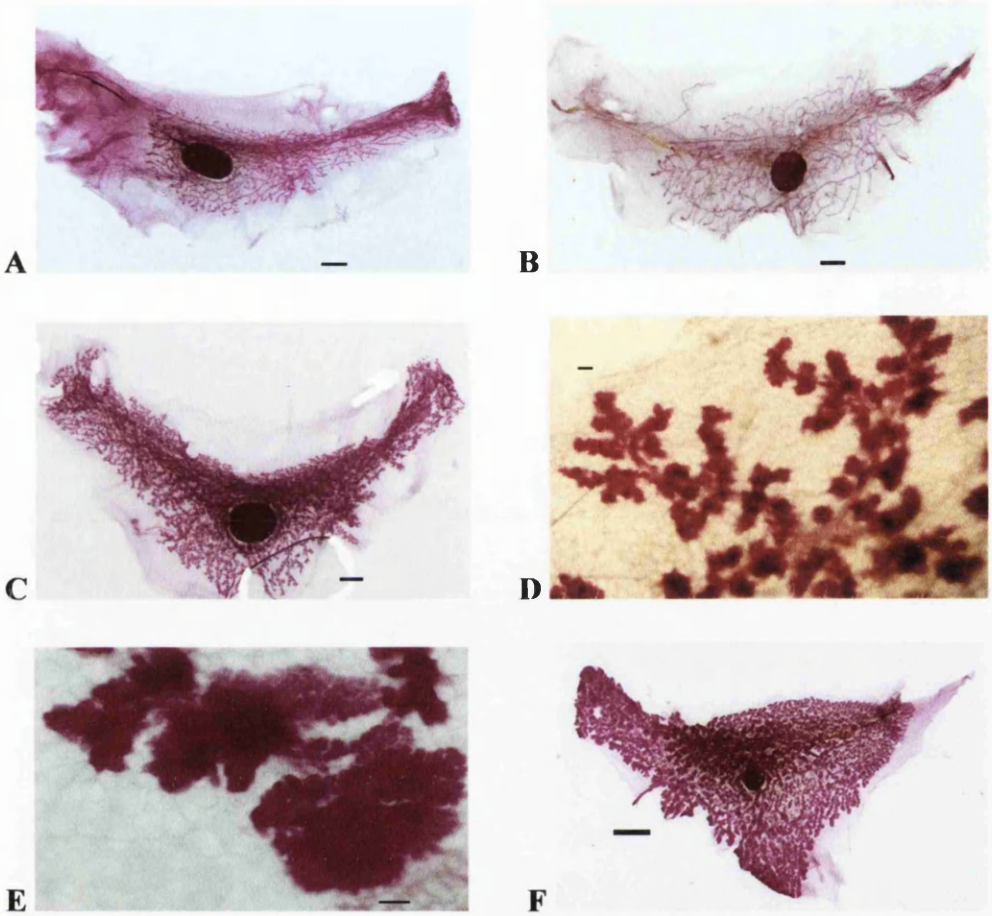

Figure 3.3 Wholemounts of pregnant mouse mammary gland

Mammary glands from pregnant mice were fixed and stained with carmine. A) day 1, B) day 6, C-E) day 12 and F) day 18. Bar = 2mm (A,B,C & F) while Bar = 71.2μm for D and E at magnification 168x and 420x respectively.

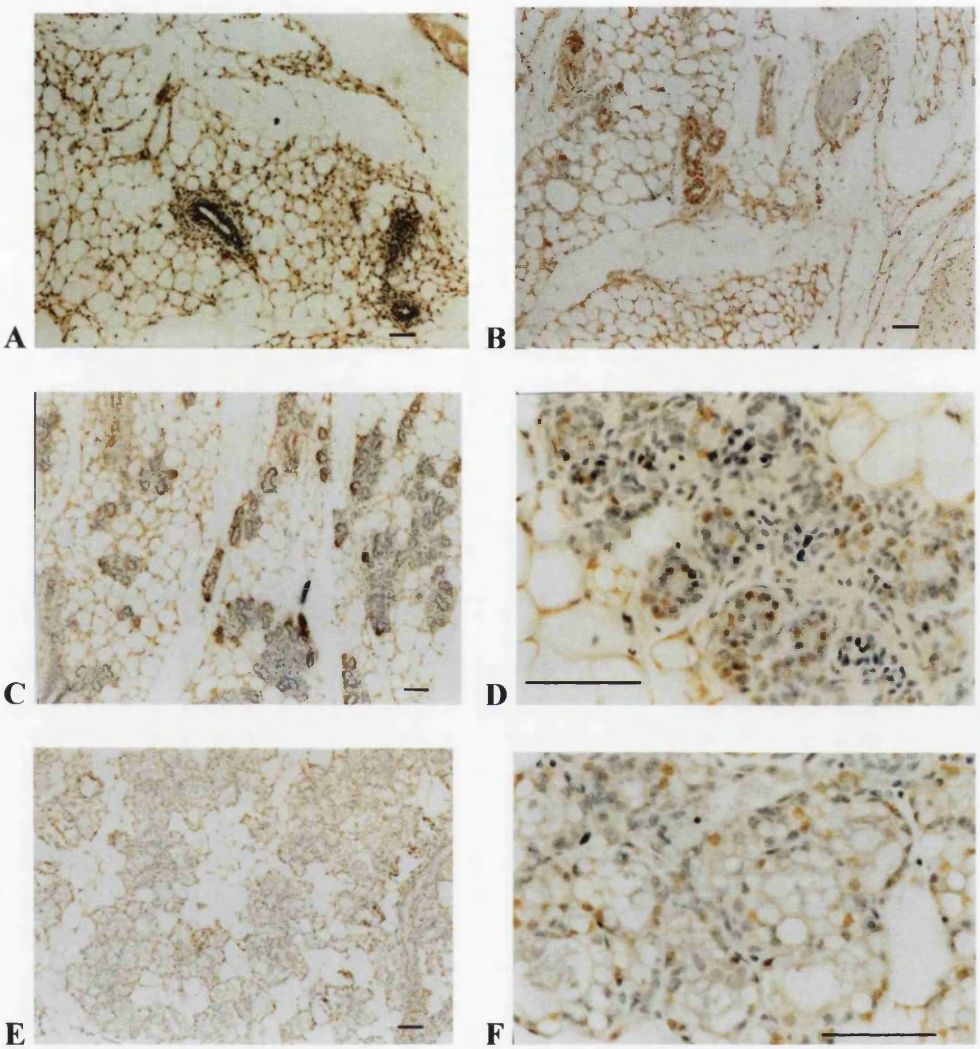

Figure 3.4 Wax-embedded sections of pregnant mice mammary gland

Mammary glands were embedded in wax and sectioned at 4μm. A) day1, B) day 6, C & D) day 12 and E&F) day 18. A, B, C, & E at magnification 420x while D & F were taken at magnification 1680x. Bar = 71.42μm

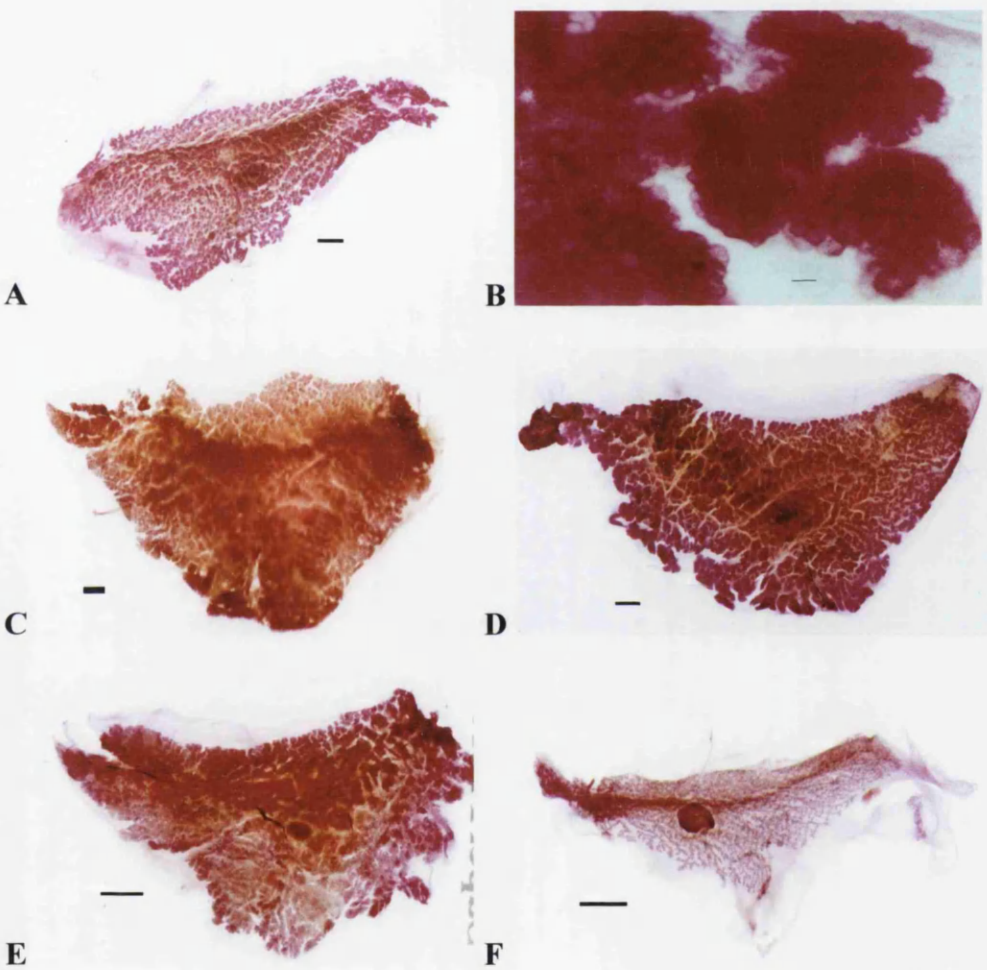

Figure 3.5 Wholemounts of mammary glands taken from lactating mice

Mammary glands from lactating mice were fixed and stained with carmine. A & B) day 1 , C) day 7, D) day 14, E) day 21 and F) day 28. Bar = 2mm (A, C, D, E & F) while B was taken at magnification 420x and Bar = 71.42μm.

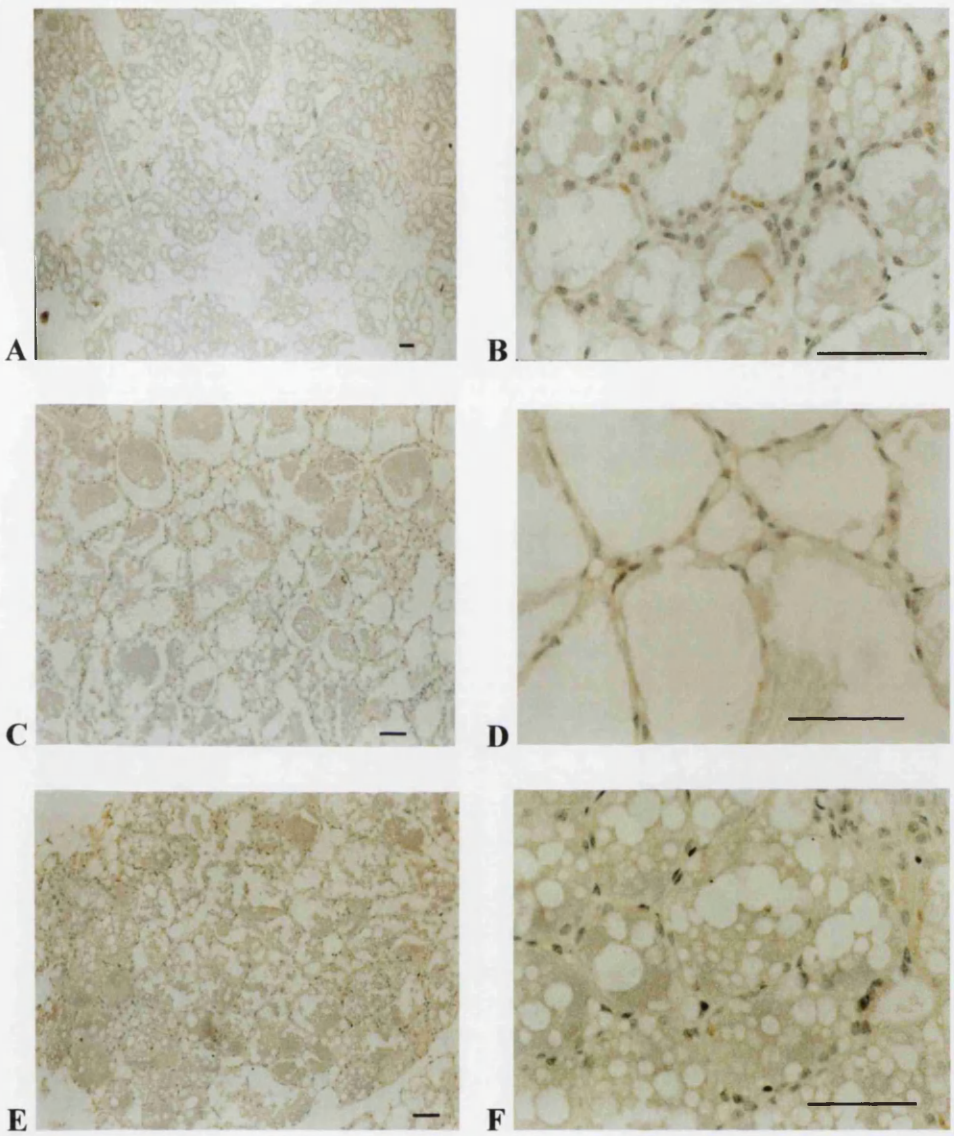

Figure 3.6a Wax-embedded sections of the lactating mouse mammary gland

Mammary glands were embedded in wax and sectioned at 4μm. A & B) day 1, C & D) day 7 and E & F) day 14. A, C & D at magnification 420x while B, E & F at magnification 1680x. Bar = 71.42μm

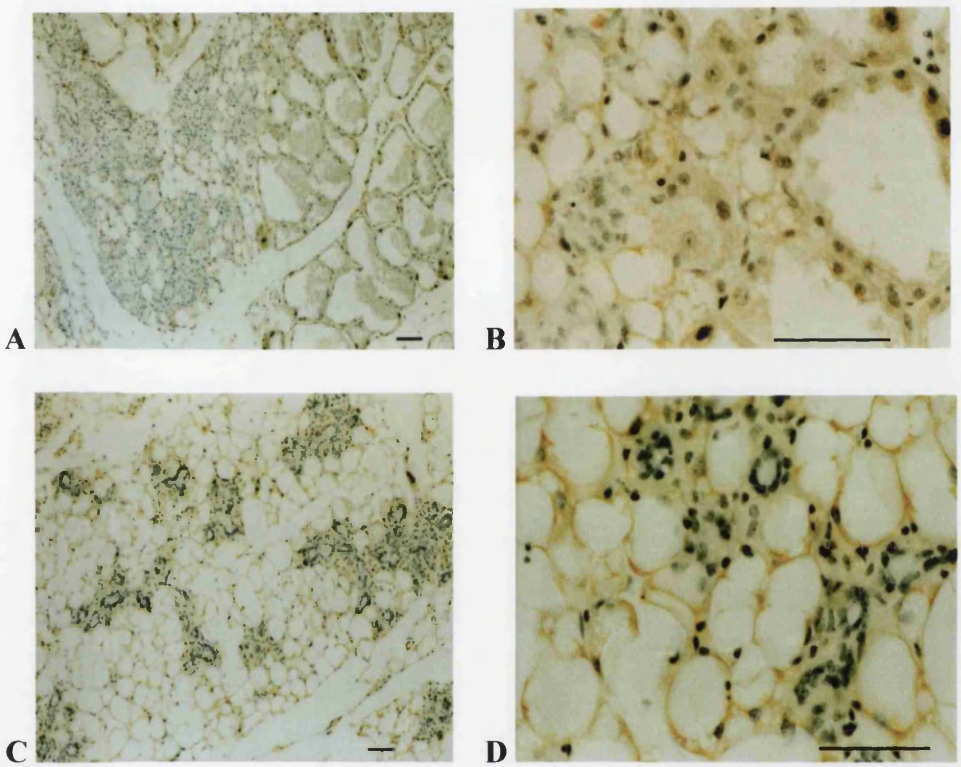

Figure 3.6b Wax-embedded sections of the lactating mouse mammary gland

Mammary glands were embedded in wax and sectioned at 4μm. A & B) day 21 and C & D) day 28. A & C at magnification 420x and B & D at magnification 1680x. Bar = 71.42μm.

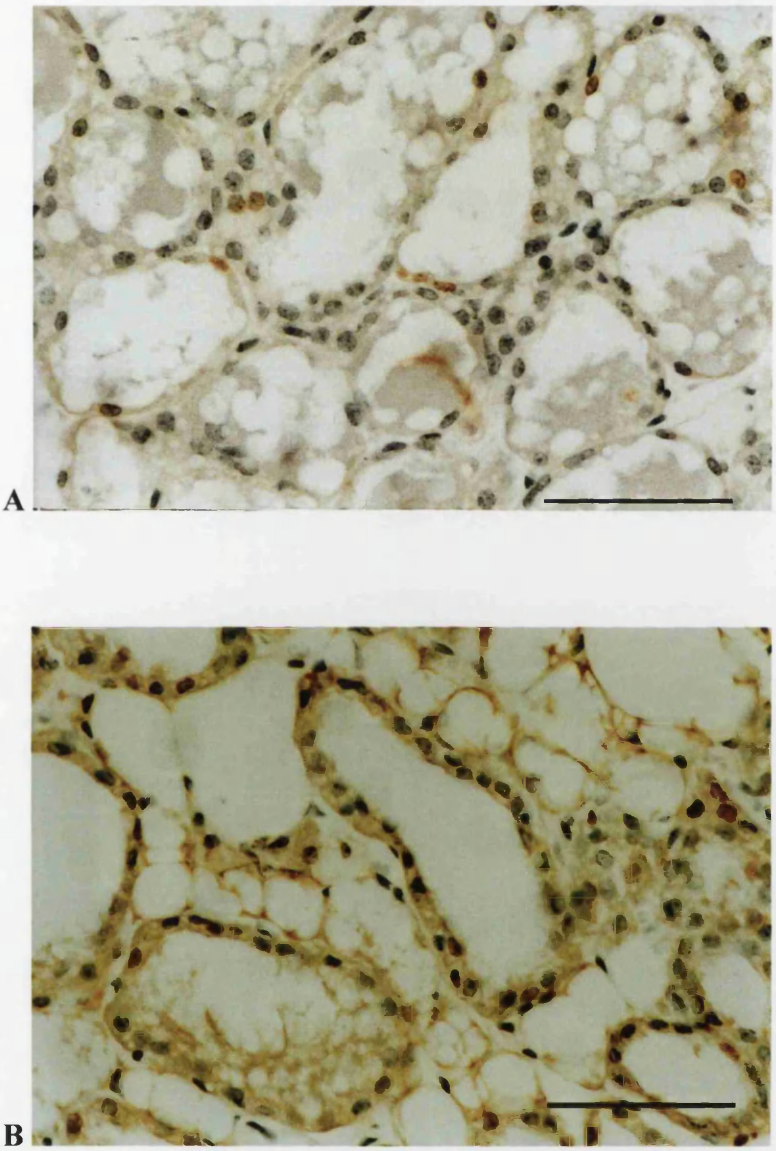

Figure 3.7 Comparison of 2nd lactation in mouse mammary glands

Mammary glands were embedded in wax and section at 4μm. A) 1ST lactation day 1 and B) 2nd lactation day 1. Magnification 1680x, Bar = 71.42μm.

3.3.2 Mammary epithelial cell proliferation and apoptosis during different stages of development

Figure 3.8 shows examples of proliferation and apoptosis at different stages of development. Since the two methodologies utilise the same detection system (DAB) proliferating and apoptotic cells both appear as brown-stained nuclei in the respective sections. Simple visual inspection shows that at day 6 of pregnancy, more proliferation (Figure 3.8A) was detected as compared to apoptosis (Figure 3.8B). On the other hand, at day 21 of lactation more apoptosis was detected instead (Figure 3.8D) then was proliferation (Figure 3.8C).

Proliferation and apoptosis were quantified in virgin, pregnant and lactating mice. Statistical analysis of the data is reported in Tables 3.1, 3.2 and 3.3, data is presented graphically in Figures 3.9 to 3.11. Data for virgin mice are in Figure 3.9. At 4wks old there seems to be more proliferation when compared to the other stages, however, this difference was not statistically significant ($P>0.05$). None of the virgin groups differed significantly in proliferation. Apoptosis, on the other hand, was higher at 4wks, 8wks and 12wks when compared to 24wks old, a difference that was statistically significant ($P<0.05$). The apparent increase from 4wks to 12wks was not statistically significant.When both processes were compared, there was more apoptosis than proliferation at 4wks, 8wks and 12wks old virgin mice ($P<0.01$), but not at 24wks (Table 3.3).

No proliferation could be detected on day 1 of pregnancy, but by day 6 approximately 10% of epithelial cells were proliferating (Figure 3.10). Values at day 6, 12 and 18 were significantly higher compared to day 1 ($P<0.05$). The

peak value was reached at day 12, higher than both day 6 (non-significantly) and day 18 ($P< 0.01$, Table 3.1) Apoptosis was detected in all stages of pregnancy and there was no significant difference between them ($P>0.05$). At day 6 and 12 of pregnancy, there was significantly more proliferation than apoptosis ($P<0.01$, Table 3.3). This was not the case at the end of the pregnancy.

Mammary epithelial cell proliferation was detected at modest levels throughout lactation (Figure 3.11) and did not differ significantly between days ($P>0.05$, Table 3.1). Proliferation on day 1 of a second lactation was not significantly different ($P>0.05$) from the same stage of the first lactation. Apoptosis was also detected throughout lactation. At day 1 of lactation there was more apoptosis detected compared to days 7, 14 and 21, however this was not statistically significant. At day 28 of lactation, there was significantly more apoptosis ($P<0.01$) compared to earlier stages of lactation (Table 3.2). Apoptosis on day 1 of a second lactation was not significantly different ($P>0.05$) from the same stage of the first lactation. At day 28 of lactation and day 1 of a second lactation, but not the other stages, there was more apoptosis occurring than proliferation ($P<0.01$, Table 3.3).

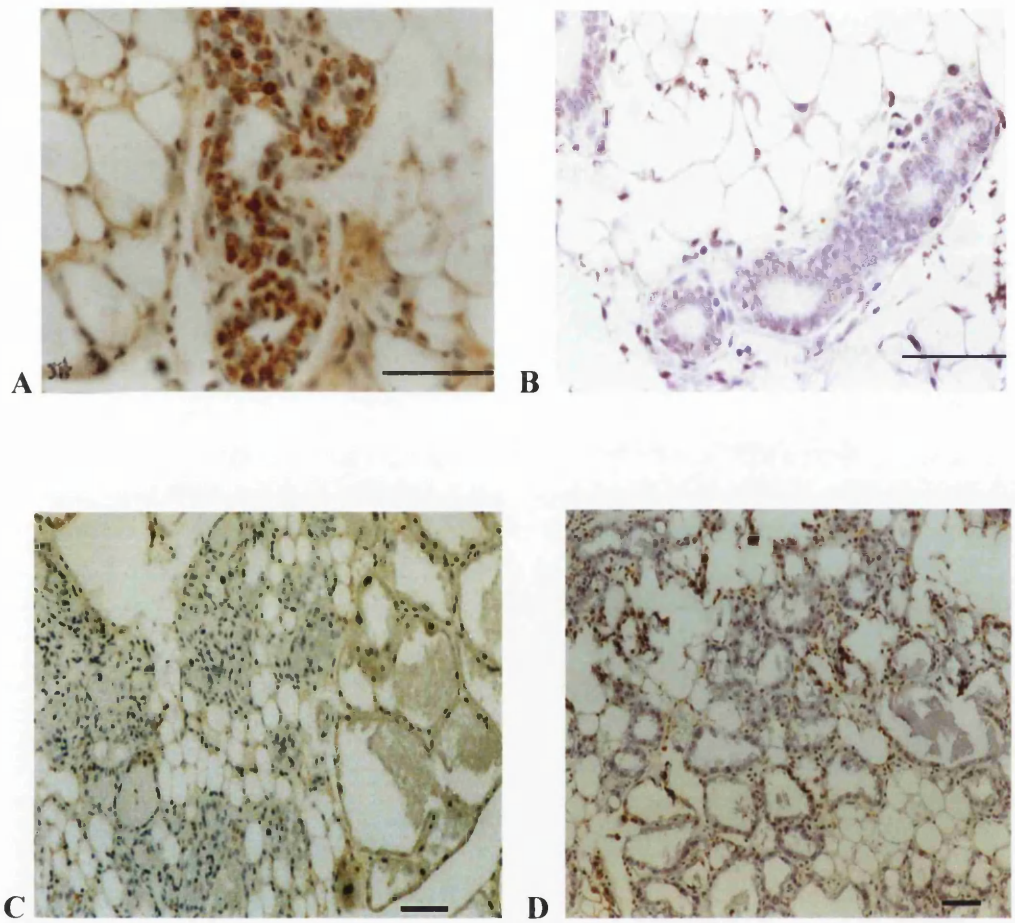

Figure 3.8 Mammary proliferation and apoptosis at different stages of development

Figures A & B shows sections of the mouse mammary gland at day 6 pregnancy while Figures C & D shows sections of the gland at day 21 of lactation. At day 6 of pregnancy, there is more proliferation (A) as compared to apoptosis (B). In contrary, more apoptosis (D) is detected during day 21 of lactation than proliferation (C). Figures A & B were taken at magnification 1680x while Figures C & D were at magnification 420x. Bar = 71.42μm

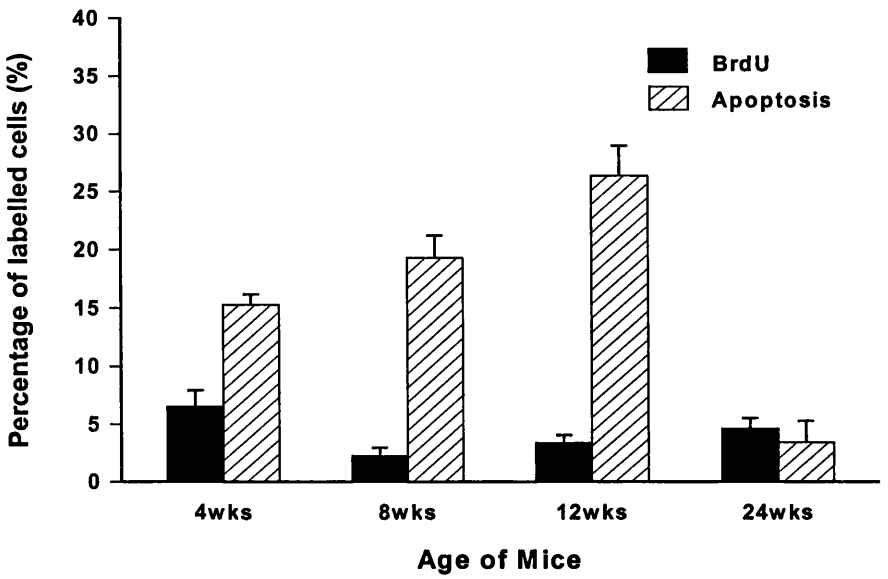

Figure 3.9 Mammary epithelial cell apoptosis and proliferation in virgin mice

Mammary epithelial cell proliferation and apoptosis were compared at various time points in virgin mice. Values are the mean ± SE for 6 animals and expressed as a percentage of positive over total cells counted. At 4, 8 & 12wks, there were more apoptosis as compared to proliferation in the virgin mammary gland ($P < 0.01$)

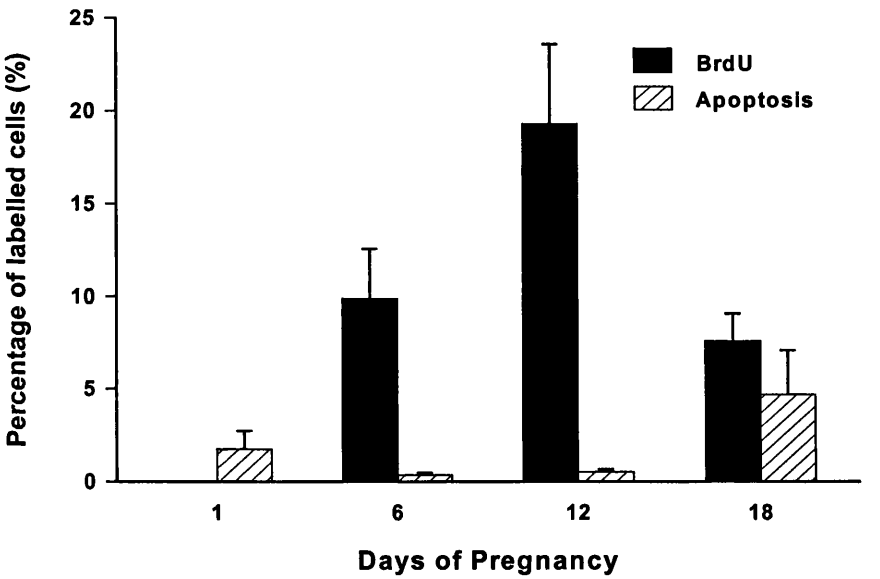

Figure 3.10 Mammary epithelial cell apoptosis and proliferation in pregnant mice

Mammary epithelial cell proliferation and apoptosis were compared at various time points in pregnant mice. Values are the mean ± SE for 6 animals and expressed as a percentage of positive over total cells counted. At day 6 & 12 of pregnancy, there was an increase in proliferation compared to apoptosis ($P < 0.01$).

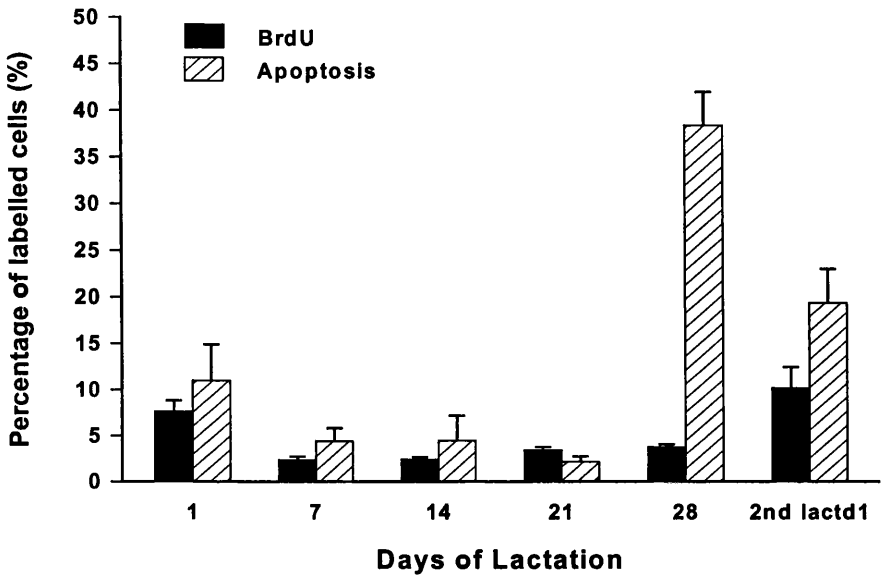

Figure 3.11 Mammary epithelial cell apoptosis and proliferation in lactating mice

Mammary epithelial cell proliferation and apoptosis were compared at various time points in lactating mice. Values are the mean ± SE for 6 animals and expressed as a percentage of positive over total cells counted. At day 28 and 2nd lactation day 1, there was more apoptosis compared to proliferation ($P<0.01$).

	V4	V8	V12	V24	P1	P6	P12	P18	L1	L7	L14	L21	L28	2nd Lact d1
V4														
V8														
V12														
V24														
P1	< 0.01		< 0.01	< 0.01										
P6		<0.05			< 0.01									
P12	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01									
P18					< 0.01		< 0.01							
L1					< 0.01									
L7					< 0.05		0.01							
L14					< 0.05		< 0.01							
L21					< 0.01		< 0.01							
L28					< 0.01		< 0.01							
2nd Lact d1		< 0.01			< 0.01					< 0.01	< 0.01			

Table 3.1 Statistical significance in proliferation between different stages of mouse mammary development

Data from virgin (V), pregnant (P) and lactating (L) mice were angular transformed and p-values were generated using Tukey's analysis. Comparisons that were statistically insignificant have been left blank.

	V4	V8	V12	V24	P1	P6	P12	P18	L1	L7	L14	L21	L28	2nd Lact d1
V4														
V8														
V12														
V24	< 0.01	< 0.01	< 0.05											
P1	< 0.01	< 0.01	< 0.01											
P6	< 0.01	< 0.01	< 0.01											
P12	< 0.01	< 0.01	< 0.01											
P18	< 0.01	< 0.01	< 0.01											
L1			< 0.01		< 0.05	< 0.01								
L7	< 0.01	< 0.01	< 0.01											
L14	< 0.01	< 0.01	< 0.01											
L21	< 0.01	< 0.01	< 0.01											
L28					< 0.01		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
2nd Lact d1				< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		< 0.01	< 0.01	< 0.01	< 0.01	

Table 3.2 Statistical significance in apoptosis between different stages of mouse mammary development

Data from virgin (V), pregnant (P) and lactating (L) mice were angular transformed and p-values were generated using Tukey's analysis. Comparisons that were statistically insignificant have been left blank.

Stage of development	Ratio of proliferation to apoptosis	P-value
Virgin 4wks	0.42	< 0.01
Virgin 8wks	0.12	< 0.01
Virgin 12wks	0.13	< 0.01
Virgin 24wks	1.33	0.37
Pregnant D1	0.00	< 0.05
Pregnant D6	27.41	< 0.01
Pregnant D12	37.09	< 0.01
Pregnant D18	1.62	0.06
Lactation D1	0.69	0.47
Lactation D7	0.53	0.38
Lactation D14	0.54	0.59
Lactation D21	1.59	0.36
Lactation D28	0.09	< 0.01
2^{nd} Lactaction d1	0.52	0.01

Table 3.3 Comparison between proliferation and apoptosis within each developmental group in the mouse mammary gland.

Data from virgin (V), pregnant (P) and lactating (L) groups were angular transformed and the p-values were generated using post-hoc test.

3.3.3 Effect of lactational history on mammary epithelial cell proliferation and apoptosis

Cell proliferation and apoptosis were measured at estrous in aged mice of differing reproductive histories. Proliferation of mammary epithelial cells in mice that have previously lactated (Figure 3.12), was significantly lower compared to virgins ($P<0.01$). On the other hand, in mice that had gestational but not lactational history, proliferation was reduced marginally ($P= 0.056$). Between ex-pregnant and ex-lactated groups, there was no significant difference in mammary epithelial cell proliferation ($P> 0.05$). The effect of lactational history on mammary epithelial cell proliferation was further demonstrated in Figure 3.15. Within mice, when one side of the gland was prevented from lactating, this gland was shown to have more proliferation occurring when compared with the gland that was allowed to lactate ($P<0.01$).

Apoptosis data for mice of different reproductive histories is shown in Figures 3.13-3.15. A significant difference was detected between groups ($P< 0.05$, Figure 3.13). Apoptosis was significantly reduced by a previous pregnancy ($P< 0.05$, Figure 3.13) but this was partially reversed in mice which had then gone on to lactate normally. However, the unilateral lactation model did not detect any difference between individuals glands which had been suckled or not (Figure 3.15). When apoptosis, between the two individual fourth glands was compared within animal apoptosis in the left gland of virgins and ex-pregnant groups was significantly higher than in the right gland ($P< 0.01$, Figure 3.14).

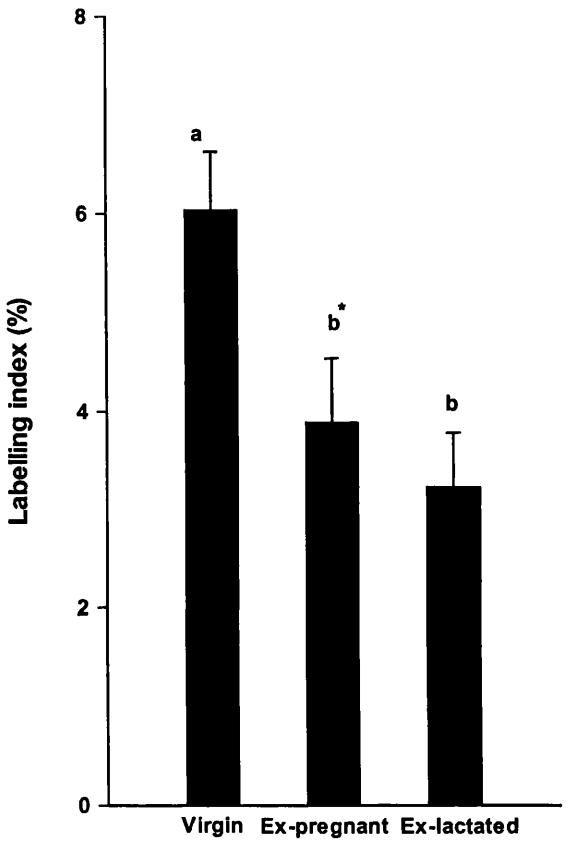

Figure 3.12 Mammary epithelial cell proliferation in mice of different reproductive histories.

Mammary epithelial cell proliferation was measured at oestrous in virgin, ex-pregnant and ex-lactating mice using BrdU incorporation. Values are the mean ± SD for 6 animals and are expressed as a percentage of positive over total cells counted. Data with different superscripts differ significantly ($P < 0.01$). * denotes $p = 0.056$ when compared with virgin group.

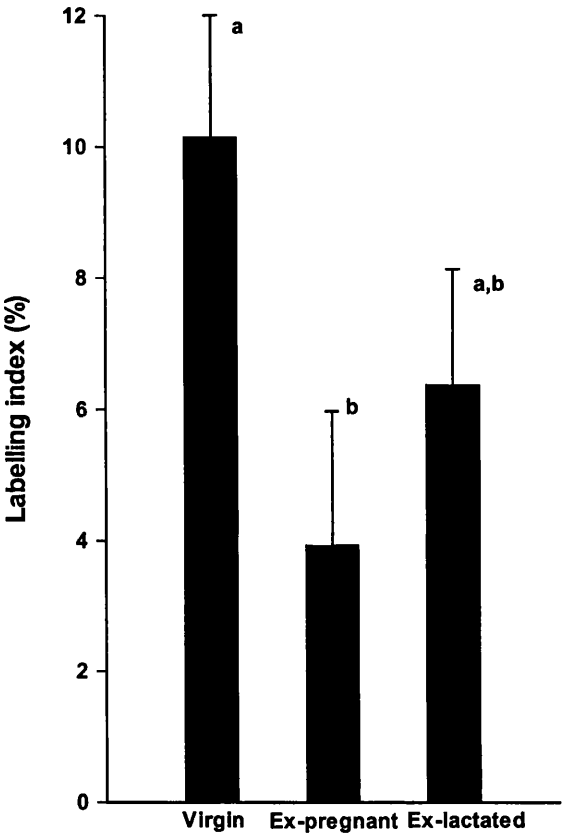

Figure 3.13 Mammary epithelial cell apoptosis in mice of different reproductive histories

Mammary epithelial cell apoptosis was measured at oestrous in virgin, ex-pregnant and ex-lactating mice. Values are mean ± SD for 6 animals and are expressed as a percentage of positive over total cells counted. Data with different superscripts differ significantly ($P < 0.05$)

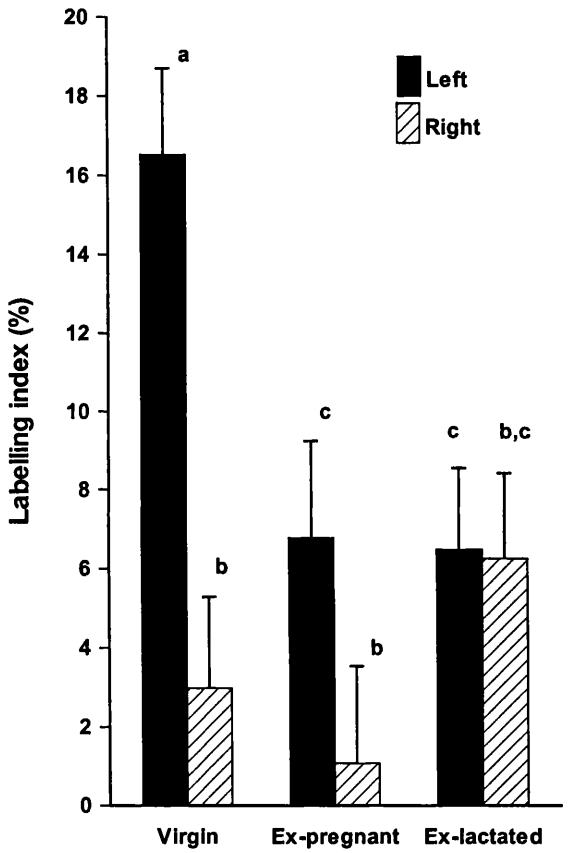

Figure 3.14 Mammary epithelial apoptosis in the left and right inguinal glands of mice with different reproductive histories

Mammary epithelial apoptosis was investigated in mice of different reproductive histories. Values are the mean ± SD from both fourth glands within each group for 6 animals and are expressed as a percentage of positive over total cells counted. Data with different superscripts differ significantly ($P < 0.01$).

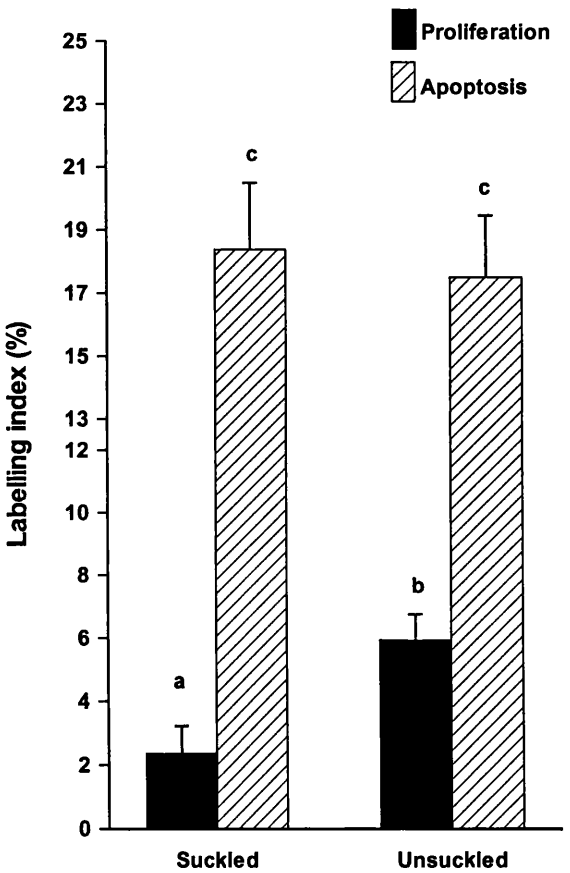

Figure 3.15 Effect of unilateral suckling on mammary epithelial cell proliferation and apoptosis

The effect of unilateral suckling on mammary epithelial cell proliferation and apoptosis. Values are the mean ± SD for 6 animals and are expressed as a percentage of positive over total cells counted. Data with different superscripts differ significantly ($P < 0.01$)

3.4 DISCUSSION

Wholemount analysis and histological studies carried out in Section 3.3.1 provided a detailed picture of mammary development in the mouse. Development of the gland in prepubertal mice is rudimentary. At this stage, adipocytes are the predominant cell type in the gland. With the onset of puberty, the ductal branching network infiltrates the fat pad. These observations are consistent with previous studies (Richert et al.,2000). As the mouse ages, this ductal system becomes more dense and complex. At 24wks old (Figure 3.1H), these mice displayed alveoli-like structures containing a hollow centre resembling the alveoli during lactation. Such structures have also been previously demonstrated (Richert et al.,2000; Schedin et al.,2000) and have been attributed to the cyclic secretion of ovarian hormones during the estrous cycle (Andres and Strange,1999).

During pregnancy, mammary epithelial cell number increases as a consequence of increased proliferation. At day 12 (Figure 3.4 C&D), alveolar structures have appeared and the size of these alveoli increases as the animal approaches parturition (Figure 3.4 E&F). In fact, from early pregnancy to day 18 of lactation, there is a 21-fold increase in cell volume (Foster,1977). During lactation, this increase in cell volume is due to the hypertrophy of secretory alveolar cells [Figure 3.6a (B&D)]. The onset of lactogenesis is associated with an increase in amounts of milk specific products such as lactose, caseins, α-lactalbumin and β-lactoglobulin (in mice), hence the hypertrophy during early lactation. On the other hand, during established lactation, hypertrophy [Figure 3.6a (D)] in these secretory alveolar cells could be the consequence of milk

accumulation. The rate of milk secretion gradually increases post-partum which leads to more milk in the lumen and hence hypertrophy of secretory alveolar cells.

Sections obtained from the lactating gland (day1) in a second lactation (Figure 3.7), demonstrated the presence of adipocytes which were previously absent during the first lactation (day1). The observation of adipocytes during early lactation has previously been reported (Richert et al.,2000). That study showed that at day 2 of lactation, 30% of the mammary gland was still filled with adipocytes.

Besides observing adipocytes during early lactation, under the same magnification, in addition to less alveoli present, the alveolar size on day 1 of a second lactation seems to be bigger than those observed on day 1 during a first lactation (Figure 3.7). Does this mean a higher capacity to cope with expected demand thus a reduction in alveoli number during a subsequent lactation? This remains plausible since it has been shown that as lactation becomes more established, the adipocytes would be displaced completely to allow expansion of alveoli to fill the gland (Richert et al.,2000). This is further supported by the fact that proliferation still occurs in early lactation (Knight and Peaker,1982; Tucker,1987).

One of the original objectives had been to accurately quantify the relative proportions of stromal adipose tissue and epithelial parenchymal tissue at different stages of development. The intimate association of mammary epithelial cells with adipocytes has been discussed in Chapter one, however no study to date has actually quantified the relative proportions and distributions of adipose and

alveolar tissue. By doing so, we hoped to provide insight about the relationship between the two cell types in the mammary gland.

Manual quantification of specific features within histological sections is time-consuming and labour-intensive. However, image-analysis techniques, which allow quantification and classification of images and objects of interest within images, are available to generate accurate data rapidly. Image-analysis algorithms suitable for mammary tissue are being developed as part of a collaboration between our laboratory, Biological Statistics Scotland and Strathclyde University. Because this work is ongoing it was decided not to proceed with quantitative analysis of parenchymal and stromal areas, pending availability of the new technique.

In order to ensure normal development of the mammary gland, a balance between proliferation and apoptosis is required. Most studies have only focused on apoptosis during involution; nonetheless, detecting apoptosis in virgin mice is not unusual (Strange et al.,2001). Cap cells, which form the outermost layer of the end bud, have been demonstrated to have the highest rate of proliferation and concurrently body cells, which fill the interior of the end bud, are undergoing apoptosis so as to form the lumen of the duct (Richert et al.,2000). Apoptosis and tissue remodelling are thus essential to prepare the gland morphologically for future roles such as formation of more complex and differentiated structures.

Hence, the second objective of this chapter was to measure both proliferation and apoptosis simultaneously in the mouse mammary gland at different stages of development. Both proliferation and apoptosis were detected in

the virgin mice at all stages examined (Figure 3.9). Proliferation, in the presence of ovarian hormones, was highest at 4wks old, when compared to 8, 12 and 24wks old, even though it was not statistically significant ($P>0.05$). This observation is consistent with previous studies (Sinha and Tucker,1966; Korfsmeier,1979). Since highest proliferation is detected during that stage, this would also explain why we see a decrease in mammary epithelial cell proliferation at 8wks old (Figure 3.9). At 12wks and 24wks old, there was no significant difference in the rate of proliferation. Proliferation of mammary epithelial cells detected at these stages is probably involved in the establishment of secondary and tertiary structures in the mammary gland.

Unlike proliferation, apoptosis in virgin mice increased with age up to 12wks old. As the mice aged further (24wks old), apoptosis occurring in the virgin gland had decreased significantly. Why apoptosis increases earlier in life and then decreases as the animal ages remains to be determined.

When both apoptosis and proliferation were compared in these mice, we can see that at 4, 8 and 12 wks old, there was a higher rate of apoptosis occurring in the gland. This is an interesting observation as one would expect proliferation rather than apoptosis to be the predominant process occurring in the virgin gland. Proliferation and apoptosis in these virgin mice were measured at proestrous/estrous and estrous respectively. Schedin et al.(2000) demonstrated that in cycling rats, the highest proliferative index was achieved between metestrus and diestrus-1. If this is also true in mice, that could explain why there was lower proliferation detected compared to apoptosis. Schedin et al.(2000) also

showed that, even though there was no significant difference between the stages in estrous cycle, at estrous, apoptosis was at one of it's highest (~ 7%). This could be why a higher rate of apoptosis was detected in the present study.

We have raised the possibility that the stage of estrous cycle could have contributed to the low proliferation and high apoptosis detected in our study. Nonetheless, other factors might be involved as well. One possible consideration is the methodology that was employed for the detection of apoptosis. Apoptosis was detected through terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labelling (TUNEL) assay. However, the fidelity of this assay is in doubt since in some analyses it failed to fully discriminate between apoptosis and necrosis (Grasl-Kraupp et al.,1995; Frankfurt et al.,1996). To eradicate this doubt, a sensible precaution would be to complement TUNEL assay with other specific markers of apoptosis such as DNA-laddering or caspase activation.

During pregnancy and lactation (Figures 3.10 & 3.11), proliferation of mammary epithelial cells was highest at day 12 of pregnancy. This observation is consistent with Knight and Peaker (1982). who showed that incorporation of ^{3}H-thymidine into mammary tissue was highest on day 12 of pregnancy. They also showed that after day 12, there was a decrease in proliferation followed by a second peak at day 2 of lactation. This is again rather similar to our observation, with the exception that our data did not indicate a higher peak at day 1 of lactation. Apoptosis was highest at day 18 of pregnancy and since peak mammary differentiation occurs during day 19-21 of pregnancy (Nandi,1958), some cell

death might have to occur to allow the formation of alveolar cells. Studies have shown that formation of ducts in the early developing mammary gland involves selective death of a population of terminal end bud epithelial cells (Humphreys et al.,1996). The same process might also be involved during peak mammary differentiation. From day 7 to day 21 of lactation (Figure 3.11), minimal cell turnover was detected. The huge increase in apoptosis at day 28 of lactation ($P<0.01$) is due to involution of the gland (Lund et al.,1996; Strange et al.,2001). Mammary apoptosis has been studied primarily in mouse mammary tissue by litter removal at peak lactation (Strange et al.,1992; Richert et al.,2000). In our experiments, natural weaning, leaving pups till day 28 of lactation, was employed instead. This could have resulted in the increase in apoptosis at day 28 instead of earlier stages as previously reported (Quarrie et al.,1996).

At day 1 of a second lactation there was significantly more apoptosis occurring when compared to proliferation (Figure 3.11). Why this is so remains to be determined. However, Pitkow et al.(1972) did demonstrate that in rats a high percentage of alveolar cells are carried over to the next lactation. If applied to our experiment, it might explain why a higher rate of apoptosis is required to replace the spent alveolar cells from the previous lactation. However, the experimental design used by Pitkow et al.(1972) was different from ours. In their study animals were mated during the first parturition and hence had a short interval between lactations, whereas for ours, the animals were remated after the pups had been weaned. As our animals were allowed to involute, less alveolar cells might be carried over to the next lactation and hence there would be less need to replace

spent alveolar cells. If the high apoptosis detected in our study was to replace spent alveolar cells, one would also expect to observe more proliferation to produce new alveolar cells. However, this was not observed in our study (Figure 3.11). The reason for the increase in apoptosis at day 1 of a second lactation remains to be determined.

The third objective of this chapter was to investigate the effect of previous reproductive histories on mammary epithelial cell proliferation and apoptosis. The effects of pregnancy and lactation on the incidence of breast cancer has been well documented (Ing et al.,1977; Newcomb,1997; Yang et al.,1999). Previously, a recent lactation has been shown to decrease oestrous-associated cell proliferation in the mouse mammary gland (Peris and Knight,1997). The current objective was to determine whether this effect persists in the long-term. In addition, the effect of lactational history on apoptosis in mammary epithelial cells was studied. The effect of a previous lactation on mammary epithelial cell proliferation observed in this study was consistent with the earlier report (Peris and Knight,1997). At 24wks old, mice that had undergone lactation had lower oestrus-associated cell proliferation compared to the virgins (Figure 3.12). However, in contrast to the earlier data, a previous pregnancy without lactation had reduced cell proliferation (Figure 3.12). Although pregnancy and lactation appear to have similar effects, within-animal comparison of individual glands that had or had not lactated, revealed a reduction in cell proliferation only in ex-lactated glands (Figure 3.15). From these observations, we can see that proliferation of the mammary epithelial cells has been altered as a result of previous lactational history. At least part of

this effect is exerted locally, since lactated glands showed the greatest reduction in mammary epithelial cell proliferation.

Before considering the apoptosis data it is necessary to reiterate a potential methodological problem with the data. When apoptosis within animals between groups were compared, we see that virgin and ex-pregnant mice exhibited higher apoptosis on the left gland than the right, which was not observed in ex-lactated mice (Figure 3.14). The difference in apoptosis between both glands could be a methodological consequence. When harvesting the mammary glands, the left gland was always collected and fixed before the right gland. This time-gap could have resulted in more apoptosis (or necrosis) occurring after the animal was sacrificed. However this does not account for the fact that the ex-lactators did not exhibit such differences between the glands.

Not withstanding this problem, apoptosis in mammary epithelial cells was more prevalent in virgin than in ex-pregnant mice (Figure 3.13). Ex-lactating mice had an intermediate value, not significantly different from virgins.

In the unilateral milking experiment (Figure 3.15), there was no significant difference in apoptosis between glands that had or had not lactated. It appears that the reduction in apoptosis caused by a previous pregnancy is subsequently reversed if the mouse lactates. Apoptosis, unlike proliferation, is subject to systemic rather than local influences since the unilateral milking showed that when one side of the gland has lactated, it reverse the reduction in apoptosis, observed in the ex-pregnant group, on the other gland that did not lactate.

The studies in this chapter have provided novel insights in relation to breast cancer. From our proliferation and apoptosis studies, it is apparent that apoptosis in aged virgin mice was significantly reduced when compared to early stages. This is an interesting observation since breast cancers in women increase dramatically with age (Ries et al.,1996). These results suggest that this increase could be a consequence of decreased apoptosis. Furthermore, even though not statistically significant, a slight increase in proliferation is detected at this stage (Figure 3.9). With a lower rate of apoptosis (than usual) occurring, it means the probability of removing 'cancer-causing' mammary epithelial cells would be reduced This remains to be confirmed. The lactational history studies reveal a long-term beneficial effect of lactation. The decrease in proliferation in the face of maintained apoptosis produced by having lactated offers a potential mechanistic explanation for the protective effect of lactation against mammary tumours.

Since the number of mammary epithelial cells is a major determinant of milk production, an understanding of the cell kinetics in the mammary gland would also allow us to manage successful lactation more efficiently.

The wholemount analysis on mammary development was executed to determine the relationship between adipocytes and mammary epithelial cells. It is well established that the mammary gland has an obligate requirement for the fat pad. However, excessive obesity can compromise mammary development and lactation both in dairy species (Sejrsen and Purup,1997) and humans (Rasmussen et al.,2001). Indeed obese mice, *ob/ob*, have been suggested to have impaired mammary development (Knight and Sorensen,2001) and fail to lactate (Chehab et

al.,1996). Nonetheless, caution still needs to be taken when associating observations obtained from heifers with those in *ob/ob* mice since different mechanism(s) might be involved in both situations. The observations of epithelial-adipocyte interactions made in the wholemount analyses form the starting point for a more detailed study, initially based around the *ob/ob* mouse. The interaction(s) between adipocytes and mammary epithelial cells could be investigated using in vivo models employing either nutritional or surgical manipulations so as to modify the adipocyte/epithelial ratio. This modification could be install at various stages of development to elucidate the potential interactions invovled.

CHAPTER FOUR

THE EFFECT OF STEROID HORMONES ON MAMMARY DEVELOPMENT IN LEPTIN-DEFICIENT, PRE-PUBERTAL MICE

4.1 INTRODUCTION

Interactions between the mammary fat pad and epithelial cells are obligatory for mammary development. The mammary gland requires a fat pad in which to develop. On the other hand, excessive obesity can compromise mammary development both in dairy species (Sejrsen and Purup,1997) and human (Rasmussen et al.,2001).

One of the potential mediators of adipocyte:epithelial interactions is leptin. In addition to its central role in the regulation of body energy homeostasis, leptin and its receptors have also been demonstrated to be expressed in the mammary gland (Smith-Kirwin et al.,1998; Aoki et al.,1999; Laud et al.,1999; Bonnet et al.,2002; Smith and Sheffield,2002). Mice lacking a functional leptin gene (*ob/ob*) are obese and sterile. Even though these mice exhibit normal early sexual development, they remain prepubertal and ovulation never occurs. This sterility has been associated with the insufficiency of hormones at the hypothalamic-pituitary level (Swerdloff et al.,1976). Chehab et al.(1996) demonstrated that fertility could be restored by administration of exogenous leptin. However, these mice still lack the capability to lactate upon parturition. Preliminary data showed that in young *ob/ob* mice, mammary development is depressed (Knight and Sorensen,2001). Ductal branching was restricted to the nipple region and so was

greatly compromised when compared to lean counterparts in which an extensive network of ducts occupied much of the fat pad. Leptin might thus have an important stimulatory role in mammary development. However, since these mice fail to attain puberty, this compromised development in the *ob/ob* mammary gland could also be due to the deficiency of sexual hormones. Confirmation of this observation would allow us to determine whether leptin is obligatory for ductal growth.

The objectives of this chapter were first to confirm that mammary development was indeed impaired in the young *ob/ob* mouse and then to determine if this compromised development was a direct consequence of leptin deficiency. A cholesterol-based-slow-release pellet containing known mammogens, estrogen and progesterone in this instance, would be implanted into pre-pubertal lean and *ob/ob* mice. By determining whether TEBs in ob/ob mice are stimulated in the presence of estrogen and progesterone, we can establish whether lack of steroid mammogens or absence of leptin is responsible for the *ob/ob* phenotype.

4.2 EXPERIMENTAL DESIGN

Female C57BL/6OlaHsd-Lep^{ob} (*ob/ob*) and –lean (lean) mice were purchased from Harlan (Harlan UK, OX6 0TP U.K.), housed at a constant 17^{0}C on a 12/12h light/dark cycle and fed a standard laboratory chow (CRMX:BS&S, EH7 6UL, U.K.). Four groups of mice were established. The first and second groups, comprising of 6 *ob/ob* mice each, were treated with cholesterol mixed with oestrogen and progesterone in the ratio 2002:1:1001 (OB-T) or cholesterol alone (OB-C) as a solid 10mg pellet implanted s.c. under Halothane anaesthesia on d22 of life. The remaining two groups, 6 lean mice each (lean-T and lean-C), were treated identically. Fourteen days post-implantation, all mice were sacrificed and inguinal mammary glands were dissected. Wholemounts were prepared as described in section 2.2.3 except that an additional step of incubating the glands in acetone for 2 days before staining with carmine was included to remove as much fat as possible. Terminal end-buds (TEBs) were identified in the wholemounts and counted. The area of the fat pad and area occupied by epithelial component were also quantified by tracing the outline onto graph paper and counting the number of squares occupied.

4.3 STATISTICAL ANALYSIS

Results were interpreted by General Linear Model using Mintab (release 11.21). Data for area occupied by ducts in the fat pad and ductal area were log transformed before being interpreted.

4.4 RESULTS

Figures 4.1 & 4.2 illustrate wholemounts of the mammary gland, which in this instance are the inguinal, of a lean (A) and *ob/ob* (B) mice implanted with pellets containing either only cholesterol or estrogen, progesterone and cholesterol. Development of the ductal branching network in the *ob/ob* mice has been seriously compromised when compared to that in the lean mice. The branching network in the lean mice is infiltrating the fat pad while in the *ob/ob* mice, it is restricted to the area around the nipple. The control and steroid treated mice appear qualitatively similar

Visual inspection of the wholemounts suggested that the *ob/ob* mice had larger fat pads than the lean. This was quantified by tracing the outline of the fat pad onto graph paper and counting the number of squares. The data are in Figure 4.3. Fat pads were, indeed, significantly larger in *ob/ob* mice, by 118% ($P< 0.001$). Treatment with oestrogen and progesterone, on the other hand, had no effect on fat pad size in either genotype.

The extent of the duct system differed greatly between *ob/ob* and lean mice (Figures 4.1 & 4.2, quantified in Figure 4.4). In obese mice the duct system occupied 2.8% of the fat pad, whereas in lean it occupied 49.3%. This difference was significant ($P< 0.01$). In addition, treatment with oestrogen and progesterone stimulated an increase in the percentage of ducts in the fat pad in both genotypes ($P= 0.01$). The extent of the increase did not differ between lean and *ob/ob*.

The difference in ductal percentage occupancy was partly a consequence of the enlarged fat pad of obese mice, but mostly due to an absolute difference in

the size of the duct system (Figure 4.5). This was more than 7-fold greater in lean mice compared to *ob/ob* mice ($P< 0.01$) and was increased by 18.3% by treatment with oestrogen and progesterone ($P< 0.05$). The increments due to steroid treatment were 15.8 mm^2 and 3.5 mm^2 for lean and *ob/ob*, respectively. Although this suggests a greater response in lean, statistical analysis revealed no significant interaction between genotype and treatment, indicating that the treatment effect did not differ significantly between lean and *ob/ob*.

In addition to quantifying the area of fat pad and ducts occupying it, terminal end buds (TEBs) in the mammary gland were also scored (Figure 4.6). In lean mice, there was significantly more TEBs than in *ob/ob* mice ($P<0.001$). Treatment with oestrogen and progesterone increased TEBs in both genotypes ($P<0.05$). There was no significant interaction between treatment and genotype ($P>0.05$).

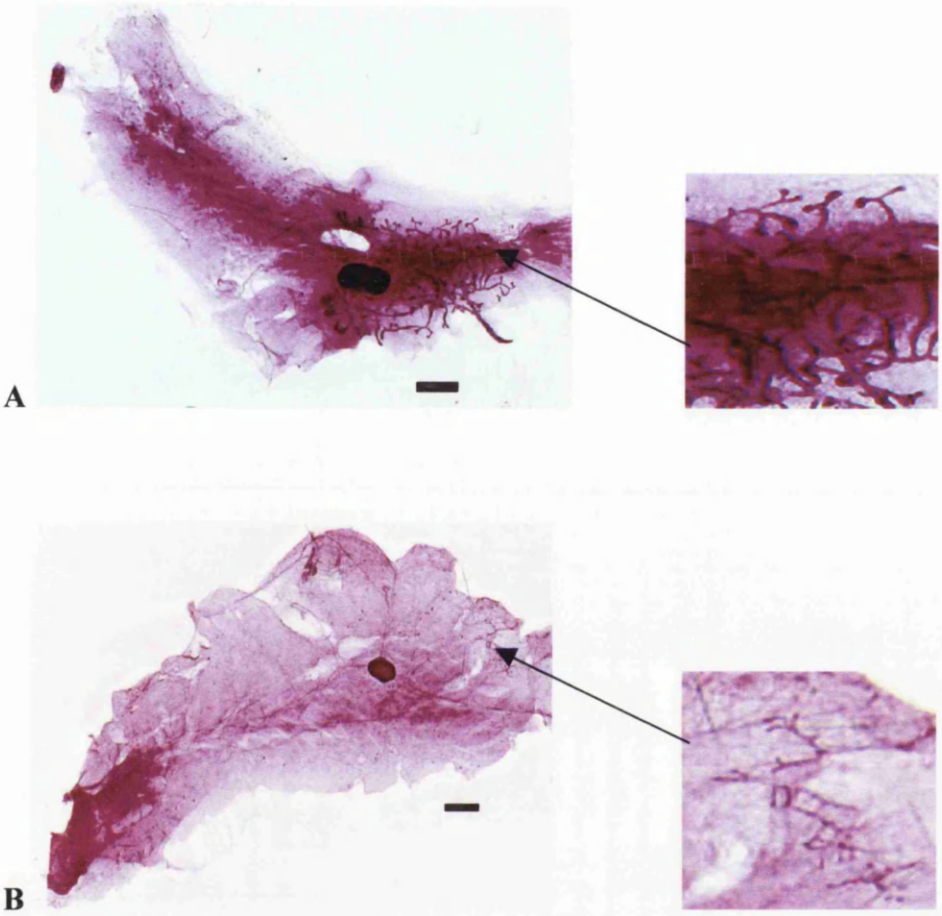

Figure 4.1 Wholemounts of mammary gland from wildtype and *ob/ob* mice implanted with cholesterol pellets

Inguinal glands from both wildtype and *ob/ob* prebubertal mice were fixed, defatted and stained with carmine. Panel A shows the mammary gland derived from a wild type mouse while Panel B shows that of a *ob/ob* mousee. Bar = 2mm. The magnified areas showed that in the normal mouse the ductal system is well developed, but in the obese mouse the fat pad contains a limited number of ducts.

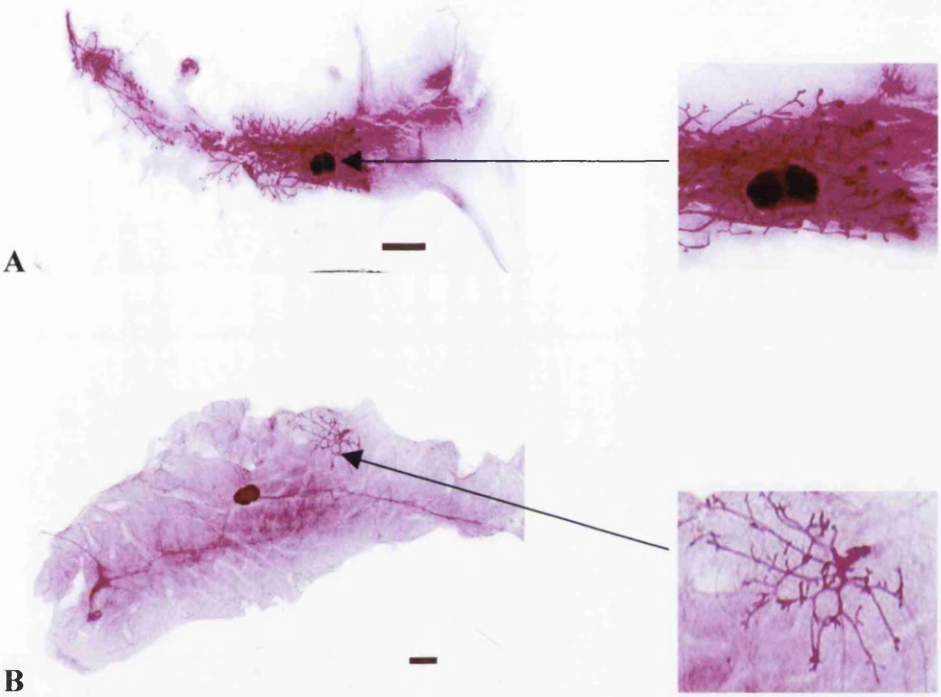

Figure 4.2 Wholemounts of mammary gland from wildtype and *ob/ob* mice implanted with pellets containing estrogen, progesterone and cholesterol.
Inguinal glands from both wildtype and *ob/ob* prebubertal mice were fixed, defatted and stained with carmine. Panel A shows the mammary gland derived from a wild type mouse while Panel B shows that of a *ob/ob* mouse. Bar = 2mm. The magnified areas showed that in the normal mouse the ductal system is well developed, but in the obese mouse the fat pad contains a limited number of ducts. In each case, the appearance of the gland is qualitatively similar to that of the untreated equivalent.

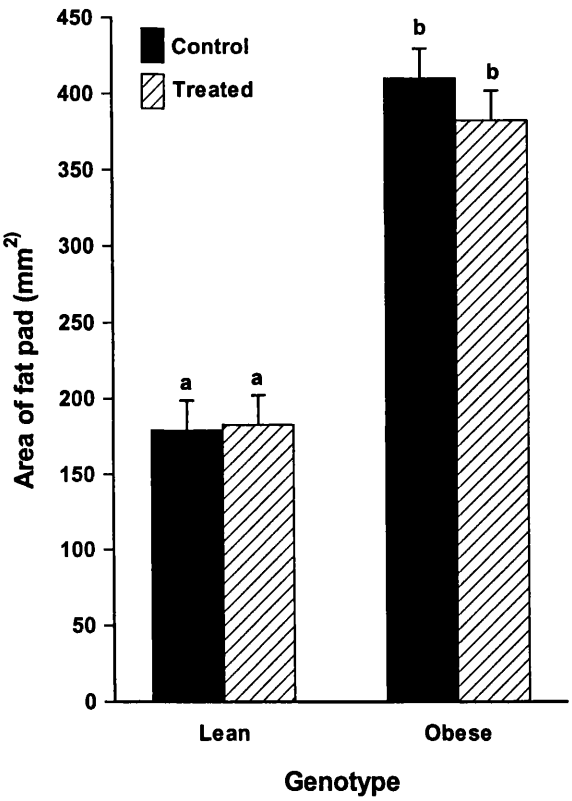

Figure 4.3 Effect of genotype and steroid treatment on fat pad area

Lean and *ob/ob* mice were implanted with either cholesterol (control) or cholesterol mixed with oestrogen and progesterone in the ratio 2002:1:1001 (treated). Mammary fat pad from both genotypes were quantified using standard grid square graph paper. Results are means ± SD for 6 animals and superscripts that differ are statistically significant ($P < 0.01$)

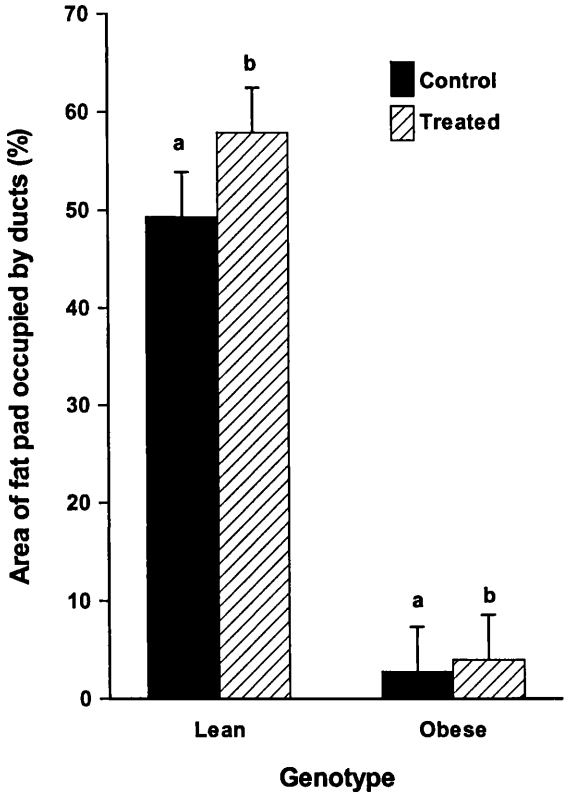

Figure 4.4 Effect of genotype and steroid treatment on the area of the fat pad occupied by ducts

Lean and *ob/ob* mice were implanted with either cholesterol (control) or cholesterol mixed with oestrogen and progesterone in the ratio 2002:1:1001 (treated). Area of fat pad occupied by ducts, from both lean and obese mice, was quantified using standard grid square graph paper. Results are means ± SD for 6 animals and superscripts that differ are statistically significant. Lean vs *ob/ob* mice ($P< 0.01$) while control vs treated ($P= 0.01$)

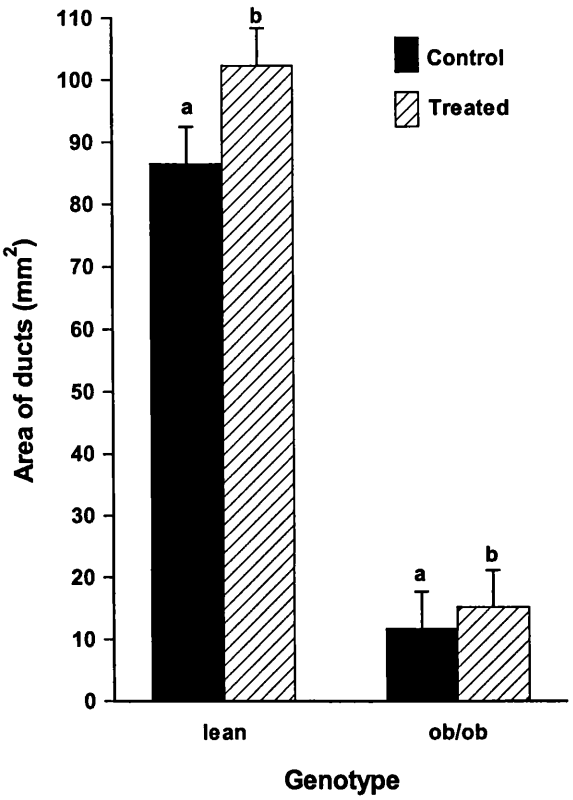

Figure 4.5 Effect of genotype and steroid treatment on ductal area

Wildtype and *ob/ob* mice were implanted with either cholesterol (control) or cholesterol mixed with oestrogen and progesterone in the ratio 2002:1:1001 (treated). Ductal area from both lean and obese mice were quantified using standard grid square graph paper. Results are means ± SD for 6 animals and superscripts that differ are statistically significant. Lean vs *ob/ob* mice ($P < 0.01$) while control vs treated ($P < 0.05$)

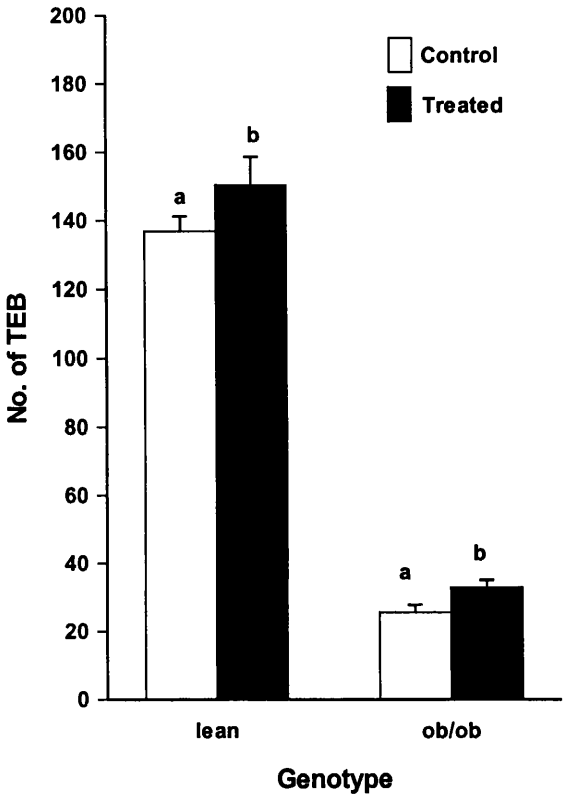

Figure 4.6 Effect of genotype and steroid treatment on the number of terminal end buds

Wildtype and *ob/ob* mice were implanted with either cholesterol (control) or cholesterol mixed with oestrogen and progesterone in the ratio 2002:1:1001 (treated). After 2wks, wholemounts were prepared from these mice and the terminal end buds were quantified using histological counting. Results are means ± SE for 6 animals. Superscripts that differ are statistically different. Lean vs *ob/ob* mice ($P < 0.01$) while control vs treated ($P = 0.01$)

4.5 DISCUSSION

The first objective of this chapter was to establish if mammary development is compromised in the *ob/ob* mice. As shown in Figure 4.1 and 4.2, mammary development has definitely been impaired. In the lean mice, the ducts were observed to be infiltrating the fat pad whereas in the *ob/ob*, there was no such indication. The percentage of the fat pad occupied by ducts in the untreated *ob/ob* mice was 2.8% while the equivalent lean mice had 49.3% occupancy (Figure 4.4). Besides that, the size of the fat pad in the *ob/ob* mice was larger than lean mice [lean (44.78); *ob/ob* (102.47)] (Figure 4.3). Leptin when deficient results in over-feeding which leads to an increase in body fat store (Coleman,1978). When leptin was administered in ob/ob mice, it resulted in weight loss which was restricted to adipose tissue (Halaas et al.,1995). Lean mice when given chronic infusions of leptin lost adipose mass in a dose-dependent manner (Halaas et al.,1997). Besides that it has also been shown that the lipid content and corresponding size of individual adipocytes correlates with the amount of ob gene expression (Maffei et al.,1995).

Both leptin and its receptor have been demonstrated to be expressed in mammary adipocytes and adipose tissue respectively (Lee et al.,1996; Bonnet et al.,2002). Thus the increase in size of fat pad in *ob/ob* mice could be due to the absence of possible autocrine control. Further studies are required to validate this claim.

Besides observing an increase in the size of fat pad, as mentioned earlier, the *ob/ob* mice also have reduced ductal growth in the mammary gland. This

reduction in ductal growth could also be due to the direct consequence of leptin deficiency. Mammary epithelial cells have been shown to express the leptin receptor (Laud et al.,1999) and since mammary adipocytes produce leptin, potential paracrine signalling might be involved. The absence of leptin in *ob/ob* mice could have prevented cross-talk between the two cell types and hence a reduction in ductal growth. Recently, a paper showed that leptin is also produced in the bovine mammary epithelial cells (Smith and Sheffield,2002). This is an interesting observation since it indicates that leptin signalling in the mammary gland might be subjected to both autocrine and paracrine controls. This possibility seems even more likely when a recent paper demonstrated that leptin actually stimulated proliferation in the human breast cancer cell line T47-D (Laud et al.,2002). However, this observation remains contradictory as it did not agree with previous studies. Both Silva and VandeHaar (1999) and Purup and Sejrsen (2000) showed that leptin reduced the mitogenic effect of IGF-1 instead of stimulating proliferation in mammary epithelial cells. An important point to note is that, since proliferation was stimulated in a breast cancer cell line, it would be wise to determine if a similar effect is observed in normal human mammary epithelial cells when treated with leptin.

The second objective of this chapter was to determine if the mammary gland in *ob/ob* mice would respond to known mammogens since these mice are deficient of hormones at the hypothalamic-pituitary level (Swerdloff et al.,1976). The number of terminal end buds (TEBs) were quantified to assess the response to a combination of estrogen and progesterone. When the TEBs were quantified in

both genotypes, there was significantly less TEBs in the *ob/ob* compared to the lean (Figure 4.6). When both lean and *ob/ob* mice have been treated with estrogen and progesterone, a significant increase in the number of TEBs was detected (Figure 4.6). Besides that, there was also an increase in the ductal area in both genotypes (Figures 4.4 & 4.5). From all these observations, we can see that the mammary gland is capable of responding to steroid-induced proliferation even in the absence of leptin. Since the mammary gland of *ob/ob* mice was capable of responding to steroid treatment, the mammary phenotype of these mice is at least partly a consequence of defective steroid secretion rather than solely a direct effect of leptin deficiency *per se*. However, since the individual variation was large (Figures 4.4-4.6) and the number of animals used in this experiment was small, the results obtained should be interpreted with caution.

Previous studies have shown that in heifers, increasing food intake during pre-pubertal stage results in a reduction in the ratio of ductular parenchyma:fatty stroma in the pubertal mammary gland (Sejrsen,1994). It has been suggested that the reduced mammary growth is due to a reduction in growth hormone (GH) (Weber et al.,1999). This is an interesting observation since *ob/ob* mice do have reduced GH (Sinha et al.,1975; Larson et al.,1976). Besides having reduced GH, prolactin was also reduced in these mice. GH and prolactin are known to be mitogenic in the mouse mammary gland so they might be responsible for the impaired development in the *ob/ob* mammary gland. To confirm this, further studies need to be conducted.

Besides local and endocrine factors, the microenvironment in the mammary gland of the ob/ob mice could have also contributed to the reduced ductal growth. The increase in the area of fat pad in *ob/ob* mice could have had an adverse effect on mammary development since transplantation experiments have shown that the ultimate number of epithelial cells within the mammary gland is dictated by the amount of adipose tissue in the mammary fat pad (Hoshino,1964). The fat pad could be releasing inhibitory factors to reduce normal ductal development. A potential inhibitory factor is TGF-β. It is secreted by the fat pad (Barcellos-Hoff,1996) and has been demonstrated to inhibit ductal elongation and branching in the mouse mammary gland (Daniel et al.,1989).

So far, we have seen that leptin is not directly involved in the pre-pubertal development of the mouse mammary gland since treatment with oestrogen and progesterone stimulated proliferation in the TEBs. In this instance, it is rather the lack of steroid hormones. However, as the effect of steroid implantation on mammary development in *ob/ob* mice were small and since the effect of leptin and its possible interaction with steroid hormones was not examined, a role for leptin at this stage could not be ruled out. Furthermore, since the mammary gland expresses leptin and its receptor, the involvement of leptin in mammary development is still possible. Besides that we have also discussed the possibility of other factors like GH, prolactin and TGF-β contributing to the reduced mammary development in these mice. Our studies on the role of leptin in mammary development even though still at its infancy, has nevertheless provided us insight for devising strategies for future work.

CHAPTER FIVE

EFFECT OF LEPTIN ON PROLIFERATION AND DIFFERENTIATION OF MAMMARY EPITHELIAL CELLS

5.1 INTRODUCTION

Leptin when deficient in mice results in obesity, sterility and compromised development of the mammary gland (Coleman,1973; Knight et al.,2002). In addition to having compromised development, these mice also fail to lactate upon parturition (Chehab et al.,1996). Leptin and its receptor are expressed in mammary tissue but this expression is down-regulated during lactation (Aoki et al.,1999; Laud et al.,1999). This has prompted the suggestion of a mammary developmental role for leptin. The *in vivo* experiments described in Chapter 4 suggested that the failure of mammary development in *ob/ob* mice was at least partly due to the absence of steroid mammogenic stimuli, rather than lack of leptin. However, steroid treatment did not totally restore mammary growth, and so a role for leptin could not be ruled out.

The disadvantage of *in vivo* models is that one cannot easily distinguish between direct and indirect effects. Tissue culture circumvents this problem and allows for more rapid evaluation of putative bioactive factors. However, not all systems properly replicate the *in vitro* situation. The establishment of primary mammary epithelial cultures paved the way towards the elucidating of *in vivo* mammary function *in vitro*. Mouse (Barcellos-Hoff et al.,1989; Blatchford et al.,1995) and ovine (Wheeler et al.,1995; Finch et al.,1996) mammary epithelial

cells, cultured in the presence of lactogenic hormones on Engelbreth-Holm-Swarm (EHS) matrix, formed alveoli-like structures (mammospheres) capable of synthesizing and secreting milk proteins. With a model such as this at hand, there is the possibility to confirm our *in vivo* observation on the role of leptin as a mammogen and also establish the possible involvement of leptin during lactation.

The aims of this chapter were

1. To determine the effect of leptin on proliferation of caprine mammary epithelial cells.
2. To determine the effect of leptin on differentiation of murine and caprine mammary epithelial cells.

5.2 EXPERIMENTAL DESIGN

5.2.1 Effect of leptin on proliferation of caprine mammary epithelial cells

Caprine mammary epithelial cells were recovered from liquid nitrogen and plated at a density of 1 x $10^5/cm^2$ with a medium change each day. Cells were allowed 24hr to attach before treatment. Ovine leptin, reconstituted in BSA (10mg/ml), was added to individual culture wells at a final concentration of 10nM, 20nM and 50nM. Triplicate wells were used for controls and each concentration of leptin tested. To assess proliferation, ^{3}H-thymidine at 5μCi/ml was added to the medium for 24hr at day 2, 3 and 4 of treatment. After ^{3}H-thymidine incorporation, the medium from control and treated wells were first aspirated followed by harvesting of cells (Section 2.5.4). Cells were washed twice with PBS and then pelleted (13,000g, 1 min). The cell pellet was then resuspended in DNA assay buffer before being sonicated (Section 2.7). 10μl of homogenate was used to determine the incorporation of ^{3}H-thymidine using liquid scintillation analyser while the remaining homogenate was used for DNA quantification.

5.2.2 Effect of leptin on differentiation of caprine mammary epithelial cells

Caprine mammary epithelial cells were recovered from liquid nitrogen and plated at a density of 1 x $10^5/cm^2$ with a medium change each day. The cells were allowed to proliferate until ~90% confluent before transferring to the EHS matrix. When the cells had attached (after 24hr), they were treated with ovine leptin, at final concentrations of 10nM, 20nM or 50nM, for a period of 6 days. Triplicate wells were set up for both control and treated cells. At day 2, 4 and 6 of treatment, ^{35}S-methionine (30μCi/ml) was added to the medium for 24hr. At days

2, 4 & 6, medium, luminal extracts and cells were collected after incubating with ^{35}S-methionine. Protein synthesis and secretion were analyzed using TCA precipitation and a liquid scintillation analyser. Milk protein expression was assessed using western blotting for casein and β-lactoglobulin. Methods are described in detail in Chapter 2.

5.2.3 Effect of leptin on differentiation of mouse mammary epithelial cells

Mouse mammary epithelial cell clumps were differentiated on EHS matrix. After attachment of the cell clumps, recombinant murine leptin prepared as recommended by manufacturer (R&D Systems, Abingdon, UK), at final concentrations of 10nM, 20nM or 50nM was added to the medium of treated wells. Triplicate wells were set up for both control and treated cells. Fresh medium with or without leptin was replenished daily. At day 2, 4 and 6 of treatment, ^{35}S-methionine (30μCi/ml) was added to the medium for 24hr. Medium, luminal extracts and cells were collected after incubating with ^{35}S-methionine. Protein synthesis and secretion were measured using TCA precipitation and a liquid scintillation analyser. Milk protein secretion was analysed using SDS-PAGE and autoradiography for casein.

5.2.4 Effect of ovine leptin on differentiation of mouse mammary epithelial cells

Mouse mammary epithelial cell clumps were differentiated on EHS matrix. After attachment of the cell clumps, ovine leptin (50nM final concentration), was added to the medium of treated wells. Triplicate wells were set up for both control and treated cells. Fresh medium with or without leptin was replenished daily. At day

2, 4 and 6 of treatment, ^{35}S-methionine (30μCi/ml) was added to medium for 24hr. Medium, luminal extracts and cells were collected after incubating with ^{35}S-methionine. Protein synthesis and secretion were analyzed using TCA precipitation and a liquid scintillation analyser. Milk protein expression was assessed using SDS-PAGE and autoradiography for casein.

5.3 Statistical analysis

Results were first normalised by log transformation before being compared by ANOVA using Genstat Genstat 5 (Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station, Herts, UK). For all experiments, ANOVA was fitted with concentration of leptin and days of treatment as fixed factors and experiment number as a random factor.

5.4 RESULTS

5.4.1 Biological activity of ovine leptin

The ovine leptin used was a gift from Professor Arieh Gertler (The Hebrew University of Jerusalem, Israel). The bioactivity of ovine leptin was confirmed by its administration into an *ob/ob* mouse (Fig 5.1). Bovine serum albumin (BSA), used to reconstitute ovine leptin, was injected into a second *ob/ob* mouse as a control. The mice were injected twice daily either with ovine leptin (24μg) or BSA (1.5mg). The mouse treated with ovine leptin displayed rapid weight loss (~1g/day) whereas the mouse treated with BSA did not show any weight loss and on the contrary gained weight from days 2 - 3 of treatment.

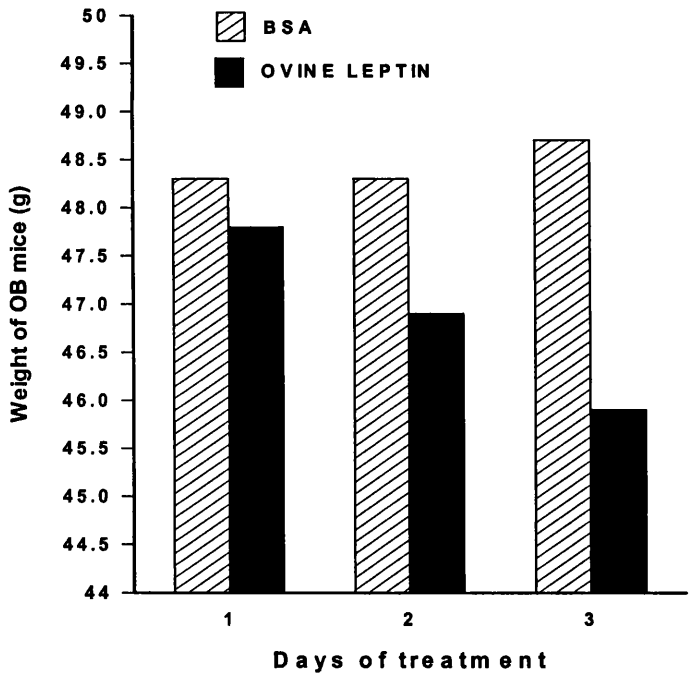

Figure 5.1 Administration of ovine leptin to OB mice

Ob mice were injected twice daily with ovine leptin (24μg) and BSA (1.5mg) as a control for a period of 3 days. The weight of these mice was recorded daily.

5.4.2 The effect of ovine leptin on proliferation of caprine mammary epithelial cells

To determine the mitogenic effect of leptin, caprine mammary epithelial cells were plated at equal cell density onto culture wells (triplicates) and treated with ovine leptin. The concentrations of ovine leptin tested were 10, 20 and 50nM. Data presented in Figure 5.2 are the mean of 3 experiments, each with cells from a different goat, and analysis was carried out with experiment numbers as a random factor. There was a significant day effect ($P< 0.01$) on the proliferation of these cells in both control and treated wells, proliferation increasing with days of culture (Figure 5.2). However, ovine leptin, at all concentrations tested, did not stimulate or inhibit proliferation in caprine mammary epithelial cells since incorporation of ^{3}H-thymidine did not differ significantly between control and treated cells ($P> 0.05$, Figure 5.2).

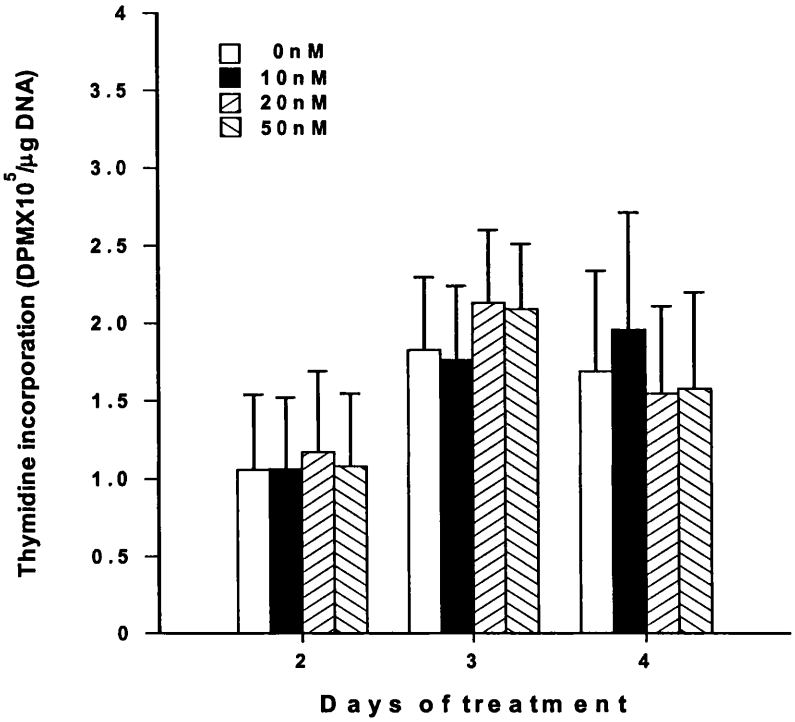

Figure 5.2 Effect of leptin on proliferation of caprine mammary epithelial cells

Caprine mammary epithelial cells were plated at a density of 1x $10^5/cm^2$ and treated with ovine leptin for 4 days. Cell proliferation was determined using ^3H-thymidine incorporation. Results shown are means ± SE for 3 experiments.

5.4.3 Characterization of caprine mammosphere cultures

Before leptin's effects on differentiation of caprine mammary epithelial cells could be assessed, a method had to be developed for culture of caprine mammospheres. In our laboratory, we have demonstrated that when murine and ovine mammary epithelial cells were cultured on EHS matrix, in the presence of lactogenic hormones (insulin, hydrocortisone, prolactin), they were capable of differentiation. Furthermore, instead of forming a monolayer, they formed clumps called 'mammospheres'.

Figure 5.3 demonstrates caprine mammary epithelial cells forming mammospheres. Using phase-contrast microscopy (Figure 5.3B) and also DAPI nuclear-staining (Figure 5.3C), these mammospheres were shown to contain a luminal area which is morphologically reminiscent of the alveoli *in vivo*.

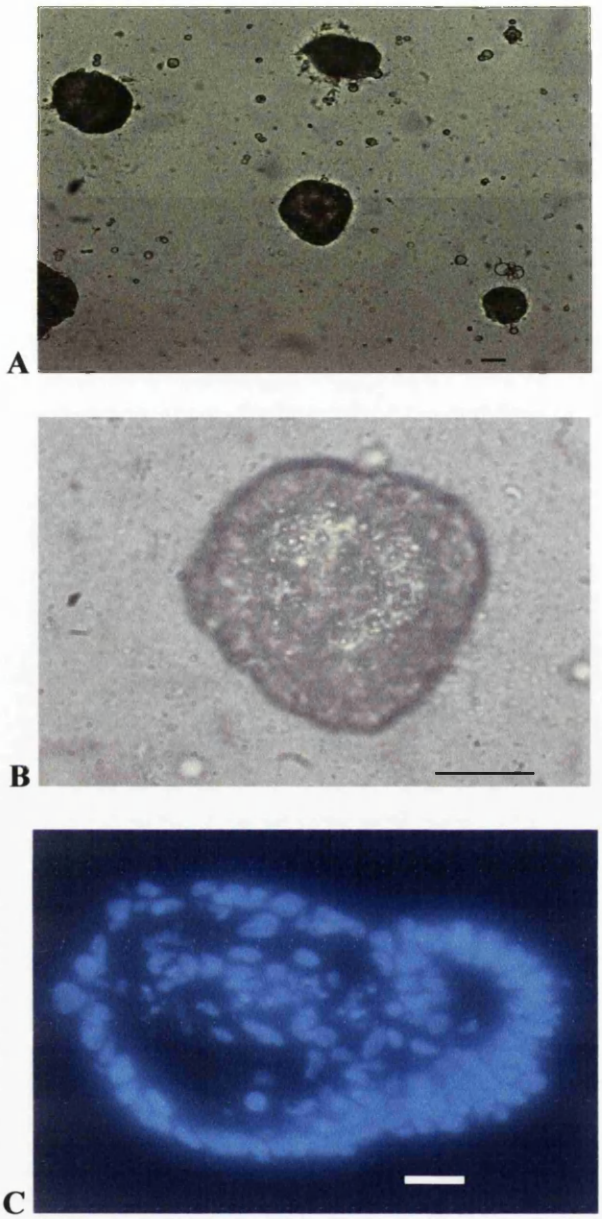

Figure 5.3 Caprine mammary epithelial cells forming mammospheres

Caprine mammary epithelial cells when cultured on EHS in the presence of lactogenic hormones formed 'mammospheres'. All three panels represented mammospheres from Day 2 of culture. Panels A & B were taken using a phase-contrast microscope under the magnification of X10 and X40 respectively. Bar = 5μm. Panel C is a 4μm thick cryo-section of a mammosphere stained with the nuclear stain, DAPI, and photographed using brightfield microscope under X40 magnification. Bar = 20μm.

Besides forming structures resembling a lactating mammary gland, the luminal cells were demonstrated to be actively secreting proteins. Figure 5.4 shows luminal secretion of proteins in the caprine mammospheres. Data presented are the means of 3 experiments. Protein secretion in the lumen increased with days of culture.

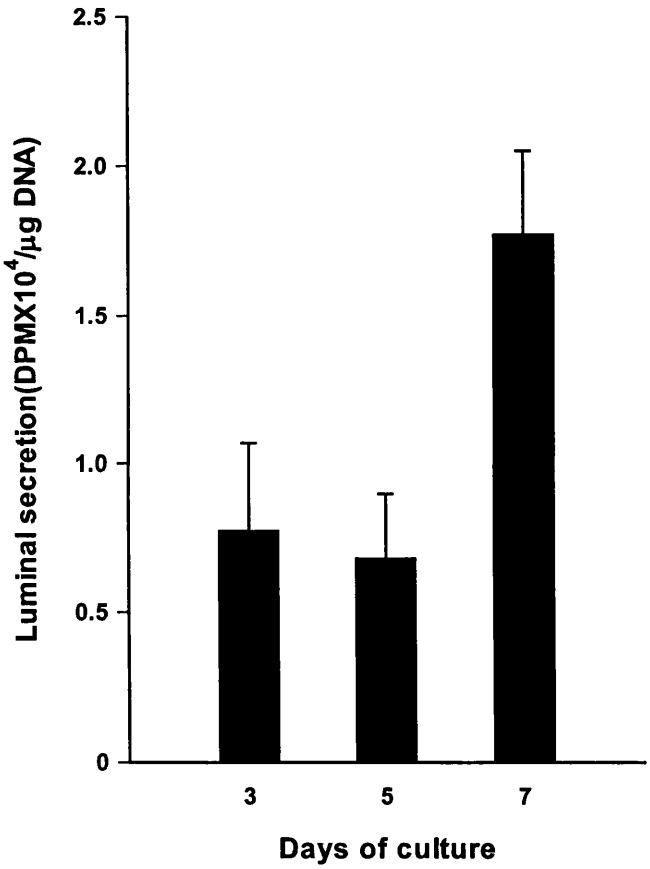

Figure 5.4 Luminal secretion of proteins in the caprine mammospheres
Caprine mammospheres were cultured on EHS matrix, in the presence of lactogenic hormones, for 7 days. ^{35}S-methionine incorporation was used to determine luminal secretion of protein. Luminal proteins were harvested using EGTA. Results are means ± SE of 3 experiments.

To determine if milk proteins were secreted by the caprine mammospheres, western blotting for casein and β-lactoglobulin was carried out. Figure 5.5 shows that both milk proteins were being secreted by the caprine mammospheres. Casein secretion was highest at day 7 of culture while β-lactoglobulin secretion reached its maximum at day 5 of culture.

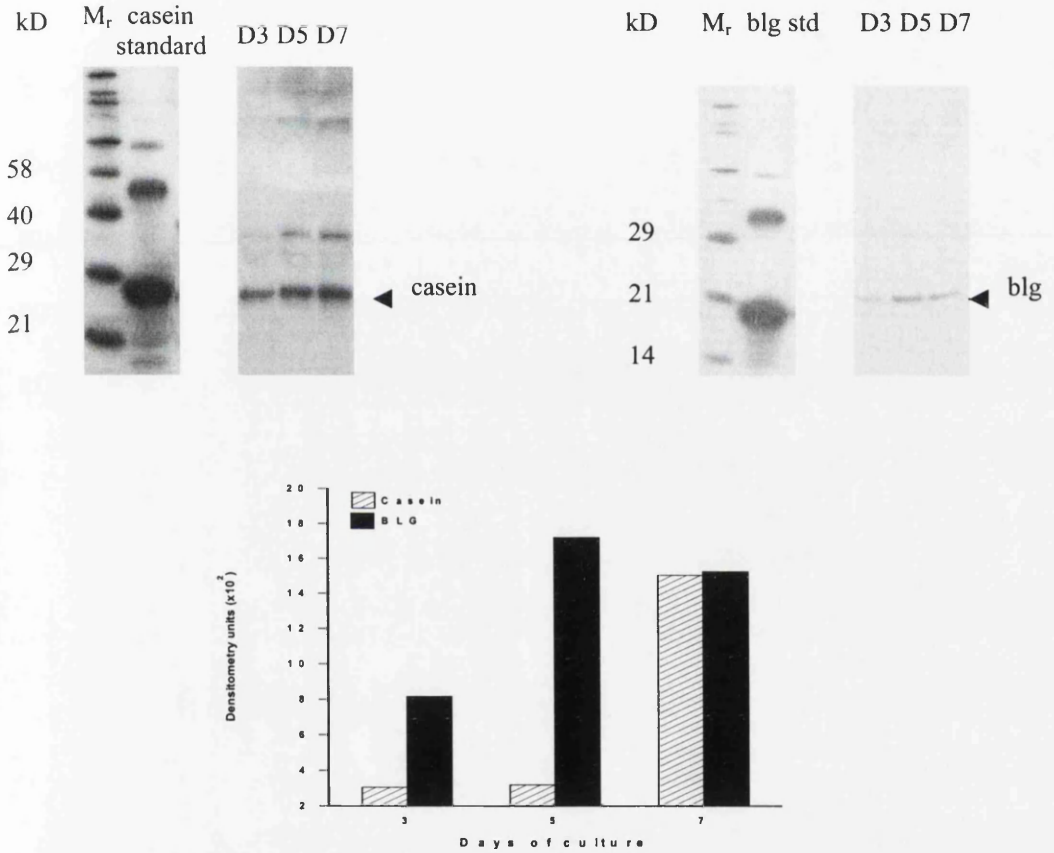

Figure 5.5 Total secretion of casein and beta-lactoglobulin in caprine mammospheres

Culture medium and luminal extracts were first resolved on a 12% Bis-Tris gel on an equal DNA basis followed by western blotting with primary antibodies for bovine casein and β-lactoglobulin. Both proteins were quantified using a densitometer.

5.4.4 The effect of ovine leptin on differentiation of caprine mammospheres

To evaluate the role of ovine leptin on the differentiation of caprine mammospheres, protein synthesis and secretion, in particular casein and β-lactoglobulin secretion, were analyzed. Data presented in this section are the means of 3 experiments, each with cells from a different goat, and analysis was carried out with number of experiment as a random factor.

Figure 5.6 shows the effect of ovine leptin on total (luminal & basolateral) secretion of proteins by caprine mammospheres. Protein secretion in the mammospheres increased with days of culture in both control and treated mammospheres ($P<0.01$). However, treatment with ovine leptin, at all concentrations tested, of caprine mammospheres did not have any significant effect on secretion of proteins ($P> 0.05$).

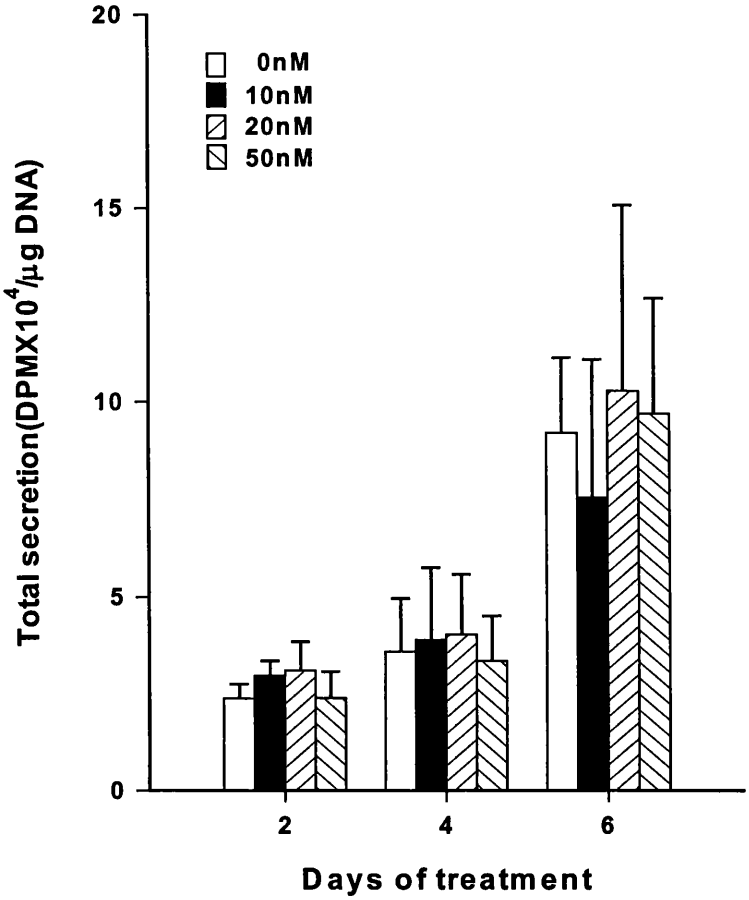

Figure 5.6 Total protein secretion by caprine mammospheres treated with ovine leptin

Total protein secretion (luminal & basolateral) by caprine mammospheres treated with ovine leptin was determined by ^{35}S-methionine incorporation. Luminal secretion was harvested using EGTA while basolateral secretion was obtained from conditioned medium. Results are means ± SE of 3 experiments.

Since leptin did not affect protein secretion in the caprine mammospheres, the next step was to determine if protein synthesis in these mammospheres was affected by leptin. Data presented is the means of 3 experiments, and analysis was carried out with number of experiment as a random factor.

Figure 5.7 shows protein synthesis in caprine mammospheres when treated with ovine leptin. Protein synthesis in both control and treated mammospheres were constant with days of culture. At all concentrations of leptin tested, there was no significant effect on protein synthesis ($P > 0.05$).

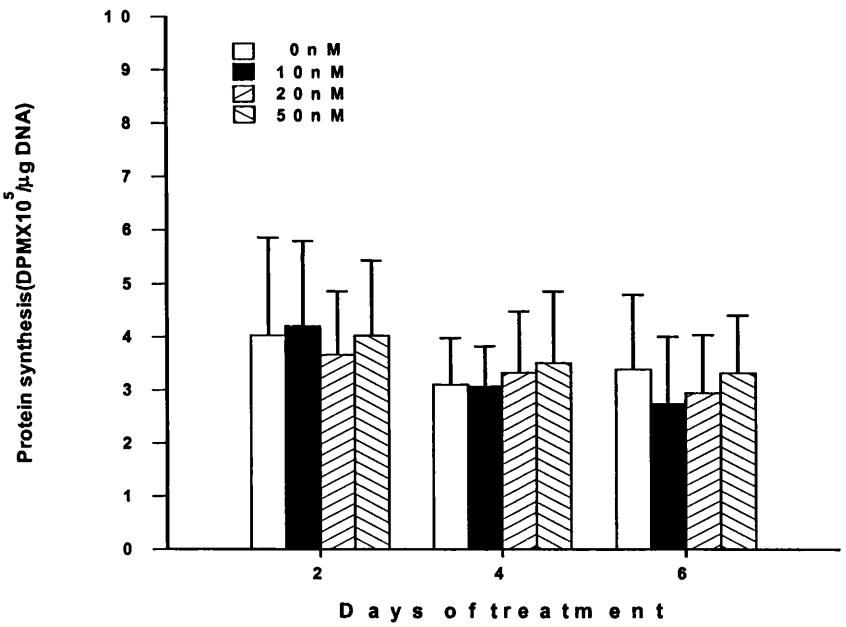

Figure 5.7 Effect of ovine leptin on protein synthesis by caprine mammospheres

Caprine mammospheres were treated with ovine leptin for 6 days. Intracellular protein synthesis on days 2, 4 and 6 of treatment as determined by ^{35}S-methionine incorporation. Cell pellets derived from mammospheres were first sonicated and then analysed using a liquid scintillation analyser. Results are means ± SE of 3 experiments.

In addition to measuring protein synthesis and secretion in the caprine mammospheres, the secretion of milk proteins was also studied. The two milk proteins analyzed in this experiment were casein and β-lactoglobulin (BLG). Figure 5.8 shows total secretion of casein in caprine mammospheres when treated with ovine leptin. The secretion of casein in these mammospheres increased with days of culture ($P< 0.01$). At day 6 of treatment, casein secretion had increased remarkably when compared to day 2 and 4 of treatment. Despite this, treatment of ovine leptin, did not stimulate nor inhibit casein secretion by caprine mammospheres ($P> 0.05$).

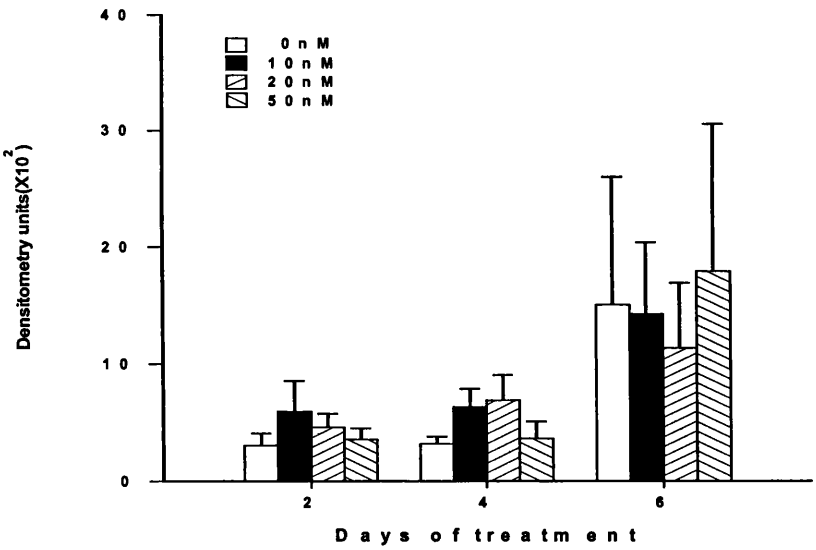

Figure 5.8 Total casein secretion of caprine mammospheres treated with ovine leptin

Culture medium and luminal extracts from days 2, 4, and 6 of treatment were first resolved on a 12% Bis-Tris gel on an equal DNA basis, followed by western blotting with primary antibodies for bovine casein. Casein was quantified using a densitometer. Total casein (luminal and basolateral) secretion shown is the mean ± SE of 3 experiments.

After examining the effect of leptin on the secretion of casein, the secretion of whey protein, BLG, was analysed. Figure 5.9 shows total secretion of BLG when the caprine mammospheres were treated with ovine leptin. Treatment of leptin did not affect total secretion of BLG by caprine mammospheres ($P > 0.05$).

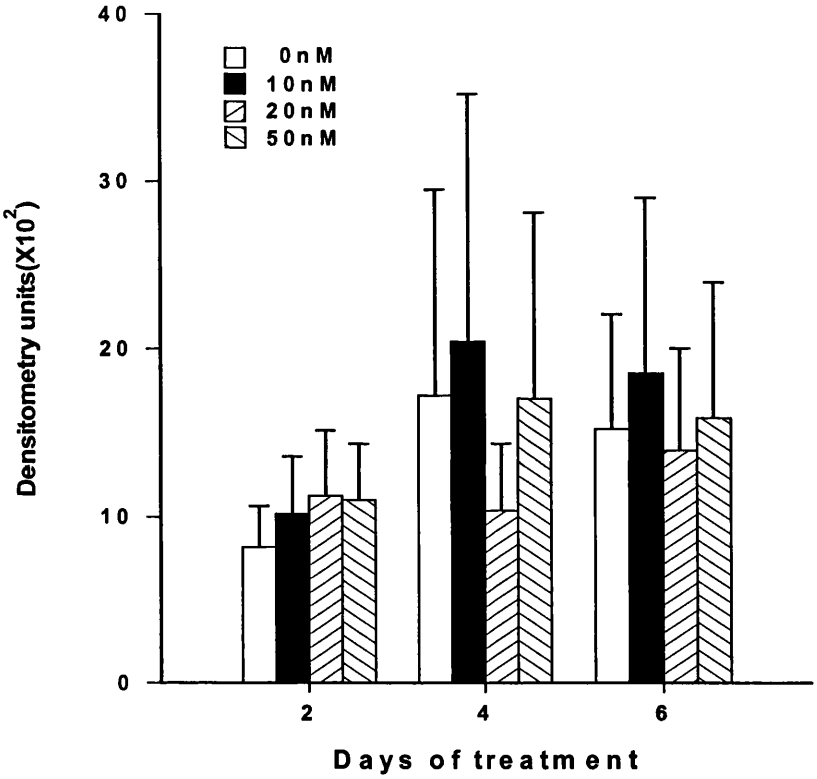

Figure 5.9 Total BLG secretion by caprine mammospheres treated with ovine leptin

Culture medium and luminal extracts from days 2, 4 and 6 of treatment were first resolved on a 12% Bis-Tris gel on an equal DNA basis followed by western blotting with primary antibodies for bovine β-lactoglobulin (BLG). BLG was quantified using a densitometer. Total BLG (luminal and basolateral) secretion shown is the mean ± SE of 3 experiments.

5.4.5 The effect of murine leptin on differentiation of mouse mammospheres

To evaluate the role of murine leptin on the differentiation of mouse mammospheres, they were treated with recombinant mouse leptin for a period of 6 days. As previously, protein synthesis, secretion and milk protein expression were examined to determine the effect of leptin on differentiation. Data presented in this section are the means of 3 experiments and analysis was carried out with experiment number as a random factor.

Figure 5.10 shows total protein secretion by the mouse mammospheres when treated with mouse leptin. Leptin at 50nM seemed to have an inhibitory effect on protein secretion on days 4 and 6 of treatment but this was not statistically significant ($P > 0.05$).

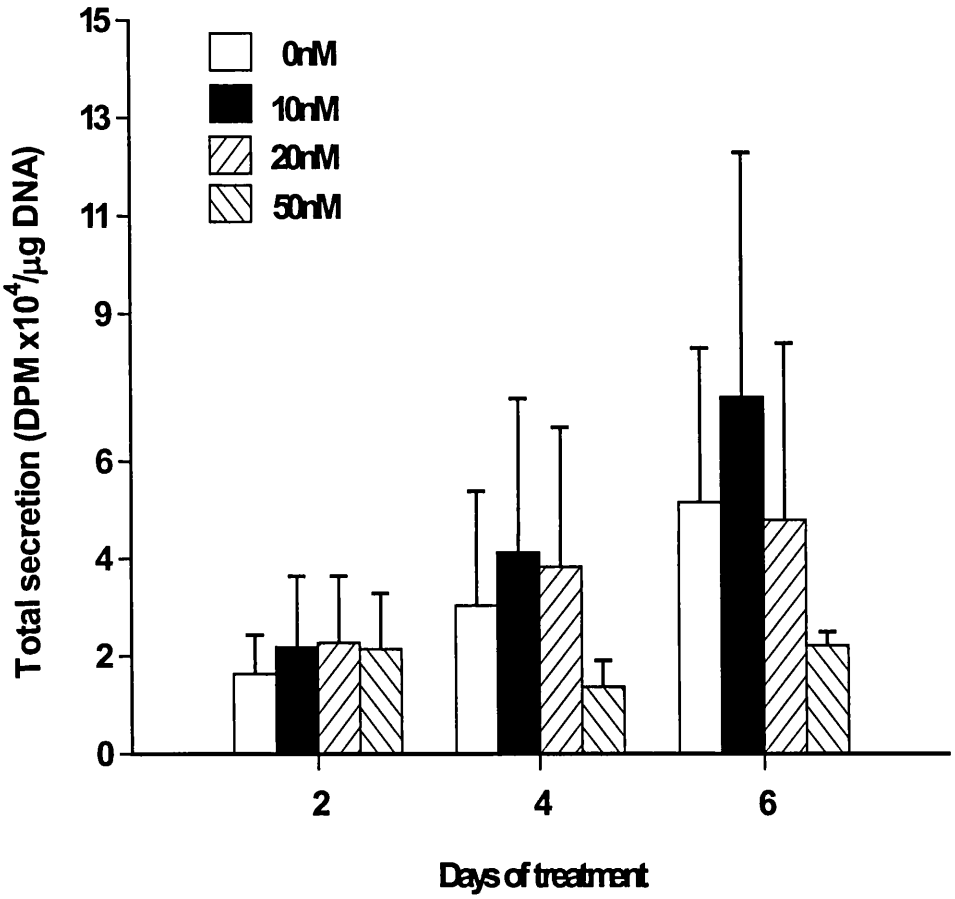

Figure 5.10 Total protein secretion by mouse mammospheres treated with mouse leptin

Total protein secretion (luminal & basolateral) by mouse mammospheres treated with mouse leptin was determined by ^{35}S-methionine incorporation. Luminal secretion was harvested using EGTA while basolateral secretion was obtained from conditioned medium. Results are means ± SE of 3 experiments.

Protein synthesis was measured in these mammospheres (Figure 5.11). There was no significant difference between treated and controls (P> 0.05).

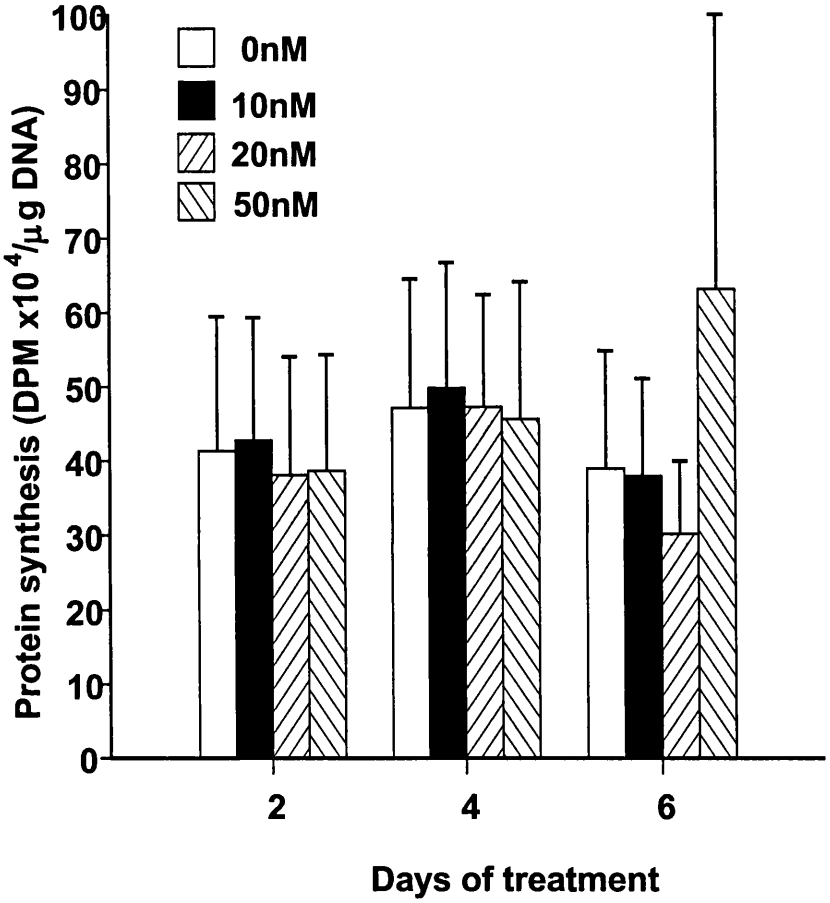

Figure 5.11 Intracellular protein synthesis by mouse mammospheres treated with mouse leptin

Intracellular protein synthesis in mouse mammospheres treated with mouse leptin was determined by ^{35}S-methionine incorporation. Cell pellets derived from mammospheres were first sonicated and then analysed using a liquid scintillation analyser. Results are means ± SE of 3 experiments.

When casein secretion was analyzed, leptin again had no significant effect on total secretion of casein (Figure 5.12). Casein secretion in both control and treated wells were highest on day 4 when compared with days 2 and 6 of treatment ($P< 0.01$). As previously, casein secretion seems to be inhibited, especially at days 4 and 6 of treatment, when the mouse mammospheres were treated with 50nM leptin. However, this was not statistically significant when compared to the controls ($P> 0.05$). All the results from the mouse mammospheres showed that leptin did not affect differentiation of mouse mammospheres.

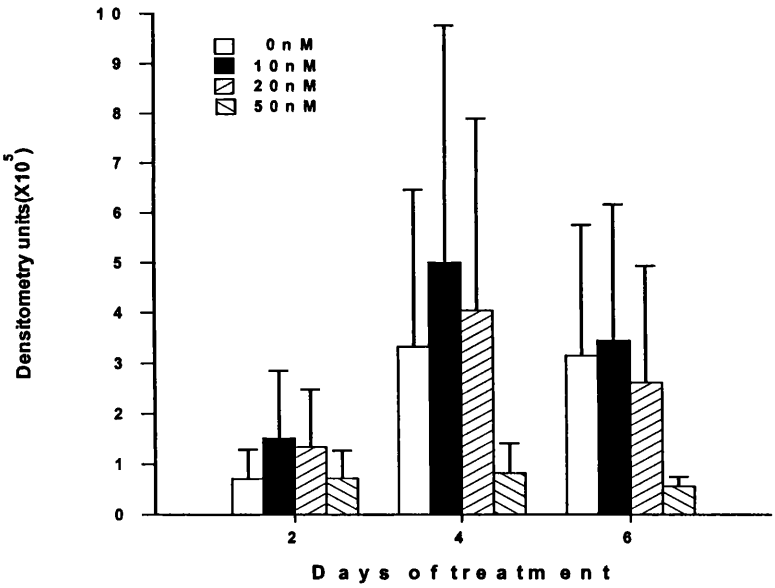

Figure 5.12 Total secretion of casein by mouse mammosphere treated with mouse leptin

Culture medium and luminal extracts from days 2, 4 and 6 of treatment were first resolved on a 12% Bis-Tris gel on an equal DNA basis followed by autoradiography. Total casein secretion (luminal and basolateral) was quantified using a densitometer. Results are means ± SE of 3 experiments.

5.4.6 The effect of ovine leptin on differentiation of mouse mammospheres

Data presented in this section are the means of 4 experiments and analysis was carried out with experiment number as a random factor. When mouse mammospheres were treated with ovine leptin (50nM), total secretion of proteins (Figure 5.13), on day 6 of treatment, was marginally higher than the controls (P= 0.069). In addition there was also a day effect on treated mammospheres (P<0.05). However, when data were transformed, by taking a natural logarithm, this significant effect of leptin on total protein secretion was no longer observed

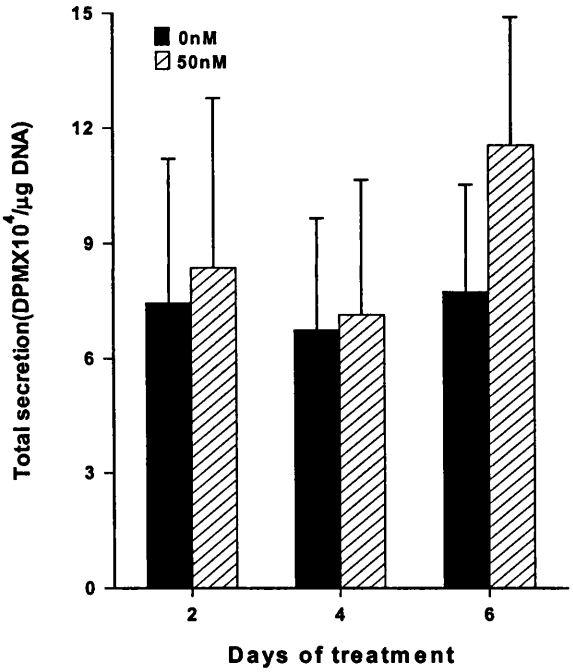

Figure 5.13 Total secretion of proteins by mouse mammospheres treated with ovine leptin

Total protein secretion (luminal & basolateral) by mouse mammospheres treated with ovine leptin for 6 days was determined by ^{35}S-methionine incorporation. Results are means ± SE of 4 experiments.

Whether this supposingly increased secretion in protein as a consequence of leptin treatment was due to an increase in intracellular protein synthesis was addressed in Figure 5.14. Leptin treatment did not stimulate more protein synthesis in the mouse mammospheres when compared with the controls ($P > 0.05$).

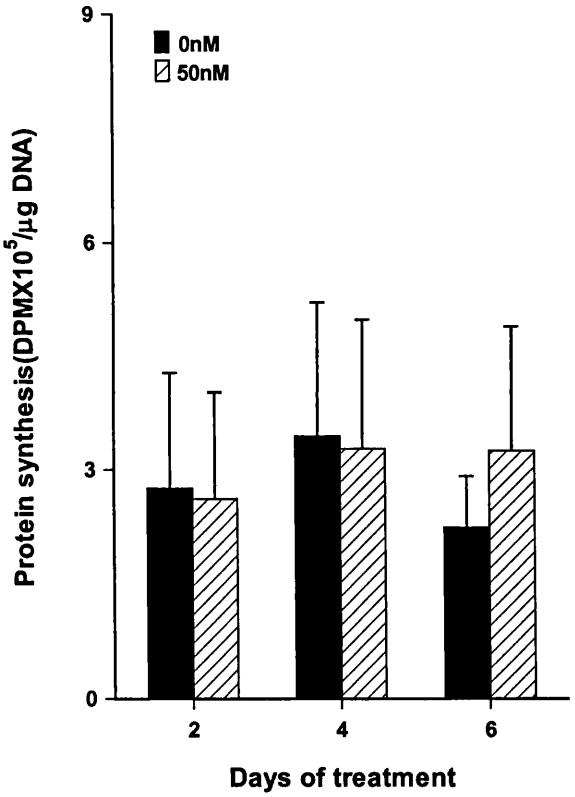

Figure 5.14 Intracellular protein synthesis by mouse mammospheres treated with ovine leptin

Intracellular protein synthesis in mouse mammospheres treated with ovine leptin was determined by ^{35}S-methionine incorporation. Cell pellets derived from mammospheres were first sonicated and then analysed using a liquid scintillation analyser. Results are means ± SE of 4 experiments.

The next stage of analysis was to determine whether treatment with ovine leptin affected casein secretion in the mouse mammospheres. Figure 5.15 shows total secretion of casein by mouse mammospheres when treated with ovine leptin. Treatment with leptin seems to be stimulating secretion of casein especially from day 4 to 6 of treatment, however this was not statistically significant ($P>0.05$). In this experiment, ovine leptin did not have any significant effect on the synthesis and secretion of proteins, and differentiation of the mouse mammospheres.

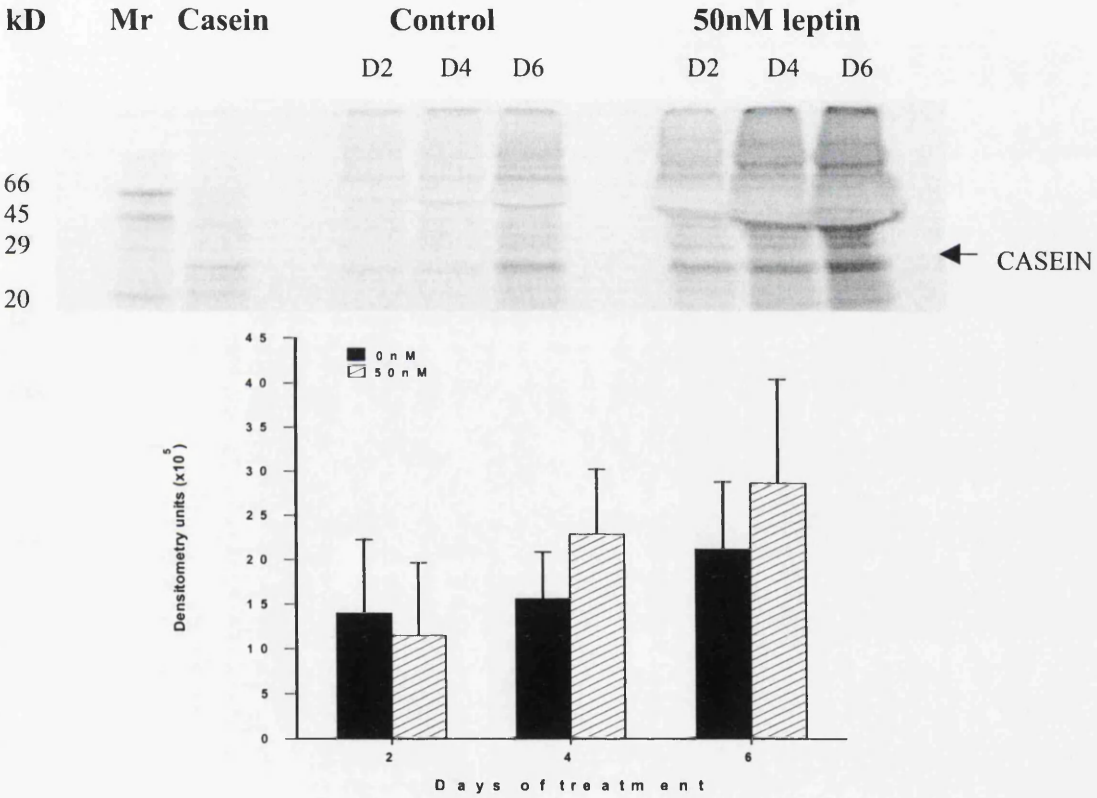

Figure 5.15 Total secretion of casein by mouse mammospheres treated with ovine leptin

Culture medium and luminal extracts were first resolved on a 12% Bis-Tris gel on an equal DNA basis followed by autoradiography. Total casein secretion was quantified using a densitometer. Results are means ± SE of 4 experiments

5.5 DISCUSSION

Leptin, in addition to its role as a satiety factor has been shown to stimulate proliferation in various cell types. It has been shown to stimulate proliferation of the mouse embryonic cell line, C3H10T1/2, as a consequence of increased mitogen-activated protein kinase activity (Takahashi et al.,1997). Other studies have also shown that leptin is capable of stimulating proliferation of hemopoietic cells (Gainsford et al.,1996) through activation of janus kinase 2 (Ghilardi and Skoda,1997). Previously, Laud et al.(1999) suggested a mitogenic role for leptin in the ovine mammary gland, since the highest expression of its receptor was detected at the stage where initiation of mammary epithelial cell proliferation occurs during pregnancy. From our current studies, we demonstrated that ovine leptin does not stimulate or inhibit proliferation in caprine mammary epithelial cells (Figure 5.2). The recombinant ovine leptin used is biologically active (Figure 5.1) thus the lack of effect observed here can be considered genuine. However, as late pregnant goat mammary epithelial cells were used in our study and highest expression of leptin receptor in the ovine mammary gland was detected in early pregnancy (Laud et al.,1999), it could have had an impact on the response of mammary epithelial cells to leptin. Nevertheless, our observation generally agrees with previous studies. Leptin, on its own, has been shown not to have any effect on proliferation of primary bovine mammary epithelial cells cultured on collagen (Purup and Sejrsen,2000), and bovine mammary epithelial cell line, MAC-T (Silva and VandeHaar,1999). Conversely, these studies showed that leptin, in the presence of IGF-1, tend to be inhibitory to

mammary epithelial cell proliferation, suggesting an antagonistic effect on IGF-1 mediated signalling. Contradictory to all these observations, a recent paper showed that leptin actually stimulates proliferation in the breast cancer cell line T47-D (Laud et al.,2002). The applicability of this observation to normal mammary epithelial cells is debatable, since breast cancer cells are 'transformed' so they might exhibit properties or responses that might not be observed in normal cells.

Differentiation of mammary epithelial cells in culture has been established in both rodents and ruminants. It has been shown that when mammary epithelial cells are cultured on EHS matrix, in the presence of lactogenic hormones, they form structures called mammospheres which resemble the alveoli in the lactating gland (Hurley et al.,1994; Wilde et al.,1995; Finch et al.,1996). Besides being structurally similar, these mammospheres exhibited polarized epithelial ultrastructure to allow vectorial secretion of milk proteins. Here, mammosphere culture was successfully applied to caprine mammary epithelial cells. Mammosphere formation and milk protein synthesis and secretion were achieved (Figures 5.3-5.5).

In this study, leptin has been demonstrated not to have any significant effect on the differentiation of mouse and caprine mammary epithelial cells (Section 5.4.4- 5.4.6). In both species, protein synthesis, secretion and the expression and secretion of milk proteins were not significantly affected by leptin treatment. However, this study did show that when mouse mammospheres were treated with ovine leptin, total secretion of proteins showed a marginal significant

increase. When the raw data were normalised by log transformation, this effect was removed.

It is perhaps not surprising to see this effect of ovine leptin on mouse mammospheres, even though not significant, since ovine leptin has been shown to have 84% sequence identity to mouse leptin (Dyer et al.,1997), indicating that the leptin is highly conserved between species. Furthermore, various studies have also demonstrated that using leptin from a different species does activate leptin signalling. Ovine leptin was able to stimulate DNA synthesis in cells expressing the human leptin receptor (Gertler et al.,1998). Human leptin, when injected into *ob/ob* mice was able to induce weight loss and restore fertilty (Chehab et al.,1996; Mounzih et al.,1998). This was further supported by our own studies which also showed that ovine leptin was able to induce weight loss in *ob/ob* mice (Figure 5.1).

Expression of leptin (mouse and ovine) and its receptor (ovine) has been detected in the mammary gland (Aoki et al.,1999; Laud et al.,1999; Bonnet et al.,2002) and this expression is down-regulated during lactation. It has been shown in rats that levels of serum leptin are suppressed during lactation due to the metabolic drain of milk production (Pickavance et al., 1998; Brogan et al.,1999). This suppression of serum leptin has been suggested to be due to an increase in energy demand and hence the need to increase appetite. On the other hand, prolactin has been demonstrated to reduce leptin expression in the mouse mammary epithelial cell line, COMMA-1D (Aoki et al.,1999) and insulin-induced leptin secretion in mouse adipocytes (Ling and Billig,2001). This is interesting as

it might suggest an antagonistic role for prolactin since we know that expression of leptin and its receptor is lowest during lactation. On the other hand, this might not be the case since in sheep, short-days and melatonin treatment which decreases prolactin, did not effect plasma leptin or adipose tissue leptin mRNA (reviewed in Chilliard et al., 2001). What needs to be established is whether prolactin reduces the expression of leptin receptor in the mammary gland.

The ovine mammary gland expresses the short-form receptor, which has been localized to the mammary alveolar epithelial cells (Laud et al.,1999). However, whether mouse and caprine mammary epithelial cells and mammospheres express the leptin receptor *in vitro* remains to be determined. This lack of effect by leptin observed in the present study could be due to the absence of leptin receptor.

If cultured mammary epithelial cells express the leptin receptor, it would be worthwhile determining whether it is the long or short form receptor since the two isoforms have different signalling capabilities. The short form receptor, unlike the long form, lacks the intracellular domain that is responsible for leptin signalling in the hypothalamus. On the other hand, short form receptors have been shown to mediate leptin-dependent tyrosine phosphorylation of JAK2 and IRS-1 and activation of MAPK activity in transient transfection models (Bjorbaek et al.,1997).

The short form receptors of leptin have also been shown to be capable of modulating signalling in the long form receptor (White et al.,1997). This indicates a potential regulatory role for the short form receptor. If mammary epithelial cells

expresses the short-form receptor in vitro, this could also explain why a local effect was not observed since the short form receptor might be acting as a feedback mechanism that eventually relates a signal to the long-form receptors in the hypothalamus to instruct. This remains to be determined

A very recent study showed that leptin expression changes in cellular location during different stages of mammary gland development (Bonnet et al.,2002). During early pregnancy, leptin was located in adipocytes and just before parturition, it was localised to the mammary epithelial cells. During lactation, expression of leptin was detected in the myoepithelial cells. This observation provided evidence that leptin could act as a paracrine factor rather than autocrine in the mammary gland.

From all the observations we have obtained so far, leptin clearly has no effect on proliferation of caprine and differentiation of both caprine and mouse mammary epithelial cells *in vitro*. Nonetheless, leptin might still have a role in the mammary gland. Since supraphysiological concentrations of leptin were employed in this study, it is possible that the putative physiological effect could have been masked. In addition, there is the possibilty that the exogenous leptin could have been subjected to significant proteolysis *in vitro*. Besides that, various studies have demonstrated the lipolytic effect of leptin on adipocytes (Frühbeck et al.,1997; Frühbeck et al.,1998; Kawaji et al.,2001; Marti et al.,2001). Even though this effect has not been demonstrated in the sheep mammary gland (Newby et al., 2001), it still raises the possibility that leptin might exert its effect by acting as a

controller of adiposity and since leptin expression in the gland varies with stage of development, paracrine rather than autocrine signalling might be involved.

CHAPTER SIX

GENERAL DISCUSSION

Mammary gland development and function is a complex process involving autocrine, paracrine and endocrine control. The gland, at birth, consists of a rudimentary branching network, which upon puberty, infiltrates the fat pad. Previously, the fat pad has been regarded as an inert matrix solely acting as a support for the growth of the mammary epithelial branching networks. However, this has been shown not to be the case; instead the fat pad is obligatory for normal mammary development (Neville et al.,1998; Hovey et al.,1999). One way the fat pad exerts its effect is by acting as a site for the synthesis and secretion of biologically active substances. Some of the substances secreted by the fat pad include IGF-1, TGF-β, FGF, *Wnt* gene family members, ECM components and leptin, the cytokine involved in satiety (Maffei et al.,1995; Barcellos-Hoff,1996; Hovey et al.,1999). From earlier results and discussion, it is apparent that adipose-epithelial interactions play a pivotal role in mammary development and function. Hence the elucidation of the reasons for this association and the understanding of how paracrine factors contribute to normal development, would provide a better understanding of the control of mammary development and prevention of breast cancers.

6.1 Mammary epithelial cell apoptosis and proliferation in the mouse mammary gland during different stages of development

The initial objective of this thesis was to examine mammary development in relation to mammary epithelial cell proliferation and apoptosis occurring in the gland during different stages of development. Since the number of mammary epithelial cells is a major determinant of milk production and a shift from the equilibrium between these two processes can lead to lesions in the mammary gland, a better understanding of both proliferation and apoptosis is beneficial. The first experiment comprised a wholemount analysis of murine mammary gland development during different stages of development with the aim of providing a detailed picture of mammary gland morphogenesis and to measure the relative proportions and distributions of adipose and alveolar tissue. The former study generated observations that were consistent with those of Richert et al.(2000) and proved to be an efficient and reliable protocol for assessing development of the gland as a whole.

In studying proliferation and apoptosis of mammary epithelial cells during the different stages of development, several intriguing observations were noted even though the absolute values of the ratio of proliferation/apoptosis were not related to the true rate of mammary development in the experimental animals. Proliferation in the virgin mice, as expected, was consistent regardless of age. In contrast, apoptosis tended initially to increase with age but this increase was no longer observed as the animal aged further. Rather, there was a marked subsequent decrease in apoptosis later in life, which is intriguing since it has been

shown in women that incidence of breast cancer increases with age. The reduction of apoptosis may lead to the preservation of genetically aberrant cells hence favouring neoplastic development. This is one area of research that deserves further study. Proliferation of mammary epithelial cells during pregnancy and lactation generated expected findings, with highest proliferation occurring at mid pregnancy.

The next part of this study was to examine the effect of lactational history on apoptosis and estrous-associated proliferation of mammary epithelial cells in aged mice. Again, the study generated some significant observations. The first observation showed clear evidence of decreased mammary apoptosis in later life in mice that had undergone pregnancies, when compared to virgins, and this decrease was partly reversed by lactation. Secondly, lactational history was also demonstrated to have a long-term effect on oestrous-associated proliferation of mammary epithelial cells. Cell proliferation was significantly lower in mice that had been pregnant than it was in virgin, but the lowest rate was recorded in mice that had been pregnant and had also lactated. Beneficial effects of pregnancy and lactation on the incidence of breast cancer have been established (Ing et al.,1977; Newcomb,1997; Yang et al.,1999). Based on our observations, we could postulate that this 'protective' effect of lactational history is modulated through the control of mammary epithelial cell apoptosis and oestrous-associated proliferation. It is known that in some breast cancers, the increase in cell proliferation (Christov et al.,1994) and also a decrease in apoptosis (Hassan and Walker,1998) are contributing factors to the lesions. In rats, the neoplastic transformation of

mammary epithelial cells in TEBs have also been associated with decreased apoptotic cell death (Shilkaitis et al.,2000).

6.2 Role of leptin in mammary gland

Obesity is becoming more prevalent, especially in western countries, and there is increasing evidence linking obesity to lactation failure in humans as well (Donath and Amir,2000; Rasmussen et al.,2001). It is well known that obesity affects milk yield in both rats and cows. Overfeeding of dairy cows leads to the development of the fat cow syndrome resulting in lactation failure (Morrow,1975). When pre-pubertal heifers were fed on high planes of nutrition, they had less secretory tissue in their mammary gland (Sejrsen et al.,1982) resulting in reduced milk yield potential (Sejrsen and Purup,1997; Sejrsen et al.,2000). In rats, over-nutrition also produced similar observations (Rasmussen,1998).

The second objective of the study was to examine the role of leptin in mammary gland development and function, since both leptin and its receptor are expressed in the mammary gland (Aoki et al.,1999; Laud et al.,1999; Bonnet et al.,2002). Studies so far have indicated highest expression of leptin in virgin and pregnant animals (Aoki et al.,1999; Laud et al.,1999). Furthermore, prepubertal mice deficient in leptin have also been demonstrated to have impaired mammary development (Knight et al.,2002). For these reasons we decided to determine if leptin has a mammogenic role in the mammary gland.

Using both *in vitro* and *in vivo* models, data were obtained to indicate that leptin does not have such a role. Steroid implantation in prepubertal *ob/ob* mice

concluded that the impaired development of the mammary gland was not a direct effect of leptin but a consequence of the inability to attain puberty since proliferation of TEBs was stimulated in the absence of leptin. Furthermore, when mid-late pregnant caprine mammary epithelial cells were exposed to leptin *in vitro*, proliferation was neither stimulated nor inhibited.

Since the expression of leptin and its receptor in the mammary gland is down-regulated during lactation (Aoki et al.,1999; Laud et al.,1999) and mice deficient in leptin fail to lactate in the first instance (Chehab et al.,1996), we decided to examine if leptin influences lactation in relation to differentiation of mammary epithelial cells. Leptin at all concentrations tested did not affect the differentiation of either murine or caprine mammary epithelial cells *in vitro*. We concluded that leptin is not directly responsible for the failure to lactate in *ob/ob* mice.

This failure to lactate in *ob/ob* mice is more likely a consequence of impaired development in the mammary gland induced by factors other than leptin deficiency. Moreover, it was evident that this impairment could be overcome since in subsequent pregnancies, this failure to lactate was partially removed in some of the *ob/ob* mice (Chehab et al.,1996). Besides that, our current study also demonstrated that after the initial failure of lactation (first 3 days) by *ob/ob* mice, the pups which were litter-swapped for the first 3 days, gradually gained weight as milk secretion was fully established. By day 10 of lactation weight gain was indistinguishable from the lean mice (Knight et al.,2002). To verify the 'slower-development' hypothesis, wholemounts and wax-embedded sections of the

mammary gland from *ob/ob* mice, undergoing their first and subsequent rounds of pregnancy and lactation, could be prepared and compared. If a delayed development was detected during first pregnancy, that would explain why these mice were unable to lactate in the first instance.

6.3 Conclusion

The studies described in this thesis were to address two key points in relation to mammary gland development and function. Studies on mammary epithelial cell proliferation and apoptosis showed that the two processes varied during different stages of development, especially between different reproductive histories. This has provided a novel approach towards improved understanding and prevention of pathological mammary development and breast cancer.

The second objective was to evaluate the role of leptin in the mammary gland. Leptin *per se* is very unlikely to have a functional role in mammogenesis and lactogenesis. The impaired mammary development and failure to lactate displayed by leptin-deficient *ob/ob* mice is rather the consequence of a lack of steroid mammogenic hormones. Thus, while the fat pad plays an important role in mammary gland development, leptin is not primarily involved.

Nonetheless, recent studies have shown increasing evidence linking obesity and breast cancer (Stoll,2000; La-Guardia and Giammanco,2001; McCann,2001; Wolk et al.,2001). Our studies when taken together provided us an insight towards forming strategies for future investigations to elucidate the relationship between adipose and mammary tissues.

Future studies could employ primary cultures, as used in the present study, to determine the contribution(s) of the fat pad towards mammary gland development, function and breast cancer. For instance, factors known to be secreted by the fat pad could be introduced into mammary epithelial cells *in vitro.* However, this method is effective only if the factor(s) have a direct effect on mammary epithelial cells.

As previous studies have suggested that some growth factors might exert their effects in the mammary gland through cross-talk, conditioned medium derived from pre-adipocytes and adipocytes, either of mammary origin or from other fat depots, could also be introduced into primary mammary epithelial cell cultures to study putative paracrine interactions. Medium from pre-adipocyte and mature adipocyte would allow us to determine if such interactions are developmentally regulated. Media that affected epithelial cell proliferation or differentiation could then be analysed for growth factors or other active constituents, which could subsequently be identified through standard techniques such as chromatography. In addition to these recommendations, another promising area of research is to establish primary cultures of adipocytes derived from obese humans/animals. Conditioned medium would facilitate the elucidation of unknown factors that might only be secreted in obese but not lean counterparts.

Putative paracrine interactions between mammary adipocytes and epithelial cells could also be studied using co-cultures. Models as such allows one to mimick the tissue's organisation *in vivo* and also determine whether synthesis/secretion of respective factors is subject to paracrine influences.

Mammary epithelial cells derived from different stages of development could be co-cultured with pre-adipocytes/adipocytes to determine if such interactions are developmentally regulated. In addition, adipocytes from fat depots other than those of mammary origin could also be co-cultured with mammary epithelial cells to establish whether interactions as such are mammary-specific. Mammary epithelial cells and adipocytes, when separated by a basement membrane like EHS matrix, would also allow one to determine the influence of basement membrane on paracrine signalling as binding of growth factors and other molecules may modify the epithelial response to adipocyte-generated signals.

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