The role of the mammary fat pad during mammogenesis

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Russell Charles Hovey

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ABSTRACT

Development of the female mammary gland involves the proliferation and morphogenesis of epithelial cells within a matrix of adipose and connective tissue which constitutes the mammary fat pad. The objective of this research was to investigate the mechanisms by which this stromal environment locally regulates postnatal mammogenesis.

Initial experiments showed that the mouse mammary fat pad liberates a diffusible activity in vitro which stimulates the growth of mouse mammary epithelial cells and enhances their proliferative response to insulin-like growth factor-I, epidermal growth factor and insulin. This effect was specific to these mitogens, and of a variety of cell lines tested was most pronounced for mouse mammary epithelial cells. Subsequent investigations indicated that these responses were likely induced by unsaturated fatty acids, particularly linoleic acid, from mammary adipocytes. Such responses may be effected by increased intracellular signalling via the actions of protein kinase C.

The mitogenic capacity of the mouse mammary fat pad was also evaluated across several physiological states. Mammary fat pad-stimulated proliferation during the estrus cycle was increased at estrus concomitant with a phase of ductal elongation in vivo. In certain medium treatments there was evidence for epithelial upregulation of the mitogenic effect of the mammary fat pad, where intact mammary tissue was more stimulatory than mammary fat pad cleared of endogenous epithelium. Further experiments demonstrated that while the mitogenic effect of the mammary fat pad was unaltered by ovariectomy, ovarian function was required for this effect to be increased by exogenous progesterone. The effect of estrogen was independent of ovarian function but was altered by the local epithelial-stromal interaction, where it increased the mitogenic effect of epithelium-free mammary fat pad and decreased that of intact mammary tissue. Mitogenic stimulation by mammary tissues also declined during virginal development to be least in mature virgin and mid-pregnant states. Stimulation by intact mammary tissue increased during lactation, while that from epithelium-free mammary fat pad remained constant in the presence of steroid hormones and increased in the presence of growth factors.

Further experiments investigated the stromal regulation of epithelial growth within the ruminant mammary gland. Differences between the ruminant and rodent mammary fat pad were emphasised in vitro where ovine mammary fat pad stimulated the growth of mouse mammary epithelial cells but did not markedly potentiate their growth factor-responsiveness. A subsequent study examined the expression of stroma-derived growth factors within the ruminant mammary gland during postnatal development, and their regulation by several physiological influences. The level of insulin-like growth factor (IGF)-I mRNA in the ovine mammary fat pad was elevated prior to puberty and during late gestation, while IGF-II mRNA was upregulated in mammary parenchyma of virgin ewes in a transcript-specific manner. Abundance of IGF-I mRNA in mammary tissues of prepubertal ewe lambs tended to be increased by exogenous estrogen whereas IGF-II mRNA levels were reduced. Messenger RNA for keratinocyte growth factor (KGF) was detected within the ovine mammary fat pad throughout development as 2.4 and 1.5 kb mRNA transcripts which were expressed by stromal adipocytes and fibroblasts, respectively. The level of KGF mRNA in mammary tissues of prepubertal lambs was increased by ovariectomy and decreased by estrogen, while KGF mRNA expression in cultures of mammary fibroblasts was suppressed by dexamethasone. Messenger RNA for hepatocyte growth factor, a paracrine mitogen and morphogen for mammary epithelial cells, was expressed in the ovine mammary fat pad and by cultured mammary fibroblasts. The abundance of basic fibroblast growth factor (bFGF) mRNA was highest within the ovine mammary fat pad, while in vitro results suggest bFGF may be a paracrine/autocrine mitogen for multiple cell types within the mammary gland. Basic FGF gene expression in mammary tissues of prepubertal ewes was reduced by estrogen treatment. For each of these growth factors there was evidence suggesting that their expression within the mammary fat pad was upregulated by the adjacent mammary epithelium.

In conclusion, these findings indicate that the mammary fat pad may stimulate the proliferation of mammary epithelial cells during postnatal mammogenesis by a variety of influences. Such mechanisms may involve the direct stimulation of epithelial growth or the modulation of epithelial responsiveness to other mitogens. These effects may function to mediate the actions of certain mammogenic hormones. Furthermore, strong

evidence indicates that mammary growth may be locally regulated by the interaction between epithelial and stromal cells.

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°C	degrees celsius
aFGF	acidic fibroblast growth factor
ANOVA	analysis of variance
BCA	bicinchoninic acid
b	bovine
bFGF	basic fibroblast growth factor
BM	basal medium
bp	base pairs
BP	binding protein
BSA	bovine serum albumin
BW	bodyweight
cAMP	3',5'-cyclic AMP
cDNA	complementary deoxyribonucleic acid
CFP	surgically-cleared mammary fat pad
CIDR	controlled intra-vaginal drug release
CM	conditioned medium
cpm	counts per minute
CTP	cytidine triphosphate
Da	daltons
DMBA	dimethylbenz [a] anthracene
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminesence
ECM	extracellular matrix
ED_{50}	median effective dose
EDTA	ethylenediamine tetra-acetate
EFA	essential fatty acids
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
FCS	foetal calf serum
FGF	fibroblast growth factor
FP	fat pad
FPLC	fast protein liquid chromatography
pg ng µg mg kg g	pico-, nano-, micro-, milli-, kilo-, gram
GH	growth hormone
GLM	general linear model
h	human
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethyl-1-piperazine-N'-2-ethane sulfonic acid
HGF	hepatocyte growth factor
HPLC	high-performance liquid chromatography
IGF	insulin-like growth factor
Ig	immunoglobulin

i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
KGF	keratinocyte growth factor
µl ml l	micro-, milli-, litre
LSD	least significant difference
nm µm mm cm m	nano-, micro-, milli-, centi-, metre
MFP	mammary fat pad
MMTV	mouse mammary tumour virus
pM nM µM mM M	pico-, nano-, micro-, milli-, mole
M_r	molecular weight
mRNA	messenger ribonucleic acid
NEFA	non-esterified fatty acids
NMU	*N*-nitroso-*N*-methylurea
n.s.	not significant
OHMG	1-*O*-hexadecyl-2-*O*-methyl glycerol
ovex	ovariectomised
PAR	parenchyma
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PG	prostaglandin
PgR	progesterone receptor
PKC	protein kinase C
PL	placental lactogen
PMA	12-myristate 13-acetate
Prl	prolactin
r	recombinant
REML	residual maximum likelihood
RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SSC	0.15M NaCl, 0.015M trisodium citrate
TBS	tris-buffered saline
TGF	transforming growth factor
Tris	Tris(hydroxymethyl)aminomethane
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1 ONTOGENY OF MAMMARY GLAND DEVELOPMENT

The mammary gland of the female mammal undergoes extensive development during embryonic and postnatal growth, its ultimate function realised at parturition when it synthesises and secretes milk to nourish the offspring. During this development, mammary epithelial cells undergo extensive proliferation and morphogenesis within the confines of a stromal matrix, the mammary fat pad. This unique pattern of organogenesis is regulated by an array of systemic and local influences, many of which are specific to certain reproductive states. An appreciation of this process is fundamental to more in depth investigation into specific areas of mammary developmental biology.

1.1.1 Embryonic development

The first structures to arise in the mammary gland are the mammary bands; bilateral zones of ectodermal thickening on the ventrolateral body wall which become apparent in the rodent around day 11 of embryonic life (reviewed by Topper and Freeman, 1980). This structural formation is associated with the local morphogenetic movement of cells rather than cell proliferation (Balinsky, 1950). The mammary bands subsequently divide into paired individual buds, the number and position of which correspond to the ultimate glands in the mature female (Knight and Peaker, 1982). Underlying these mammary buds are two distinguishable mesenchymal compartments. A dense mesenchyme comprised of several fibroblastic layers directly associates with the epithelial anlage while a second compartment develops separately from the mammary anlage and is the precursor tissue for the mammary fat pad (Kimata *et al.*, 1985). Lipid accumulation occurs in this latter tissue at day 16 of embryonic development (Sakakura *et al.*, 1982).

The rodent mammary bud grows very slowly to day 15.5 of embryonic development (resting phase), although recent evidence indicates that this period is associated with extensive DNA synthesis in the primary duct (Cunha and Hom, 1996). Rapid proliferation between days 16 and 21 of embryogenesis results in the formation of a mammary rudiment consisting of several branched, canalised cords that have penetrated the dense mesenchyme and which lie positioned within the fat pad precursor tissue at

birth (Balinsky, 1950). Such development is associated with the formation of one or more primary sprouts, the number of which dictates the number of galactophores per teat or nipple. Cows, goats, sheep, mice and rats have one opening while humans have 15-25 (Anderson, 1978).

A generally similar pattern of development is observed in the embryonic ovine and bovine mammary gland (Knight and Peaker, 1982; Raynaud, 1961). The mammary gland of male sheep grows at a constant rate of 2.8 times that of body weight while the female gland grows at 5 times that of body weight between days 44 and 70 of embryonic development (Martinet, 1962). By day 70, secondary ducts have developed, the teat cistern is evident, and parenchymal tissue is well developed. Mammary growth then declines to 1.7 times the rate of body growth until birth (Martinet, 1962). The four mammary buds are apparent in the bovine foetus at the 4 to 8 cm stage; at the 19 cm stage the mammary cord becomes canalised to form the streak canal and cistern, and at the 16 to 23 cm stage secondary branches arise from the dilated cistern (Raynaud, 1961). The mammary rudiment of the embryonic female bovine undergoes most rapid growth from the 7th month of gestation.

Within the mammary gland of the foetal human, a mass of epithelium lies embedded in mesenchyme at 5 weeks of gestational age, and by 13 weeks the mammary bud forms cords surrounded by a fibroblastic stroma (reviewed by Raynaud, 1961; Kellokumpu-Lehrinen *et al.*, 1987). Canalisation of ducts occurs at 20 weeks and epithelial cells develop a secretory appearance by the last trimester of pregnancy (Tobon and Salazar, 1974).

Sexual dimorphism in the embryonic mammary gland involves hormonal action via unique epithelial-mesenchymal interaction (Sakakura, 1991). Development of the mammary rudiment in male and female rodents is similar up until day 14 of embryonic development. The production of androgens by the foetal testis and the presence of epithelial-induced androgen receptors in the mammary mesenchyme at this time (Heuberger *et al.*, 1982) causes mesenchymal condensation around the mammary bud and rupture of the epithelial stalk (Kratochwil and Schwartz, 1976). The mammary rudiment in male mice undergoes extensive necrosis whereas in the male rat it remains within the mammary fat pad but is disconnected from the epidermis (Imagawa *et al.*,

1994a). Induction of the opposite sexual phenotype can be induced by male gonad-irradiation, or by treatment of females with testosterone (reviewed by Imagawa, 1994a).

1.1.2 Prepubertal development

A substantial degree of mammary growth occurs in rodents, and indeed in other species, prior to the onset of puberty. Ductal end buds on the mammary rudiment of neonatal rodents are probably formed in response to maternal hormones. These are transient and do not reappear until around 3-4 weeks of age (Imagawa *et al.*, 1994a). Correspondingly, the rate of mammary growth in rats and mice is isometric until the onset of positive allometric growth at around 3-4 weeks of age. This phase of growth involves the ramification of epithelium into the mammary fat pad to establish a highly-branched network of ducts lined with a single layer of luminal epithelial cells (Flux, 1954).

The perinatal human breast demonstrates epithelial differentiation and synthesis of "witches milk" in response to both maternal and endogenous hormones (Mayer and Klein, 1961). Morphological development within the prepubertal human breast is variable, where mammary parenchyma may range from being a collection of simple elongate ducts to a well developed set of branched ducts with terminal lobules (Anbazhagan *et al.*, 1991). The human breast then remains relatively dormant until puberty. Rat mammary tissue closely resembles that in the human gland due to the presence of terminal lobular units and an associated intralobular connective tissue (Sakakura, 1991). Mammary parenchyma in the prepubertal heifer consists of a gland cistern and a duct system lined with double-layered epithelium; associated with these ducts are terminal alveolar structures (Mayer and Klein, 1961). This parenchymal tissue as a whole expands and becomes less elongate during this development (Swett *et al.*, 1955).

Mammary gland area in rats (Sinha and Tucker, 1966) and mice (Flux, 1954) increases at 3.5 and 5 times that of metabolic body weight gain to 40 and 56 days of age, respectively. Positive allometric mammary growth commences in heifers at 2-3 months of age; thereafter the rate of increase in mammary DNA content is 3.5 times faster than that for metabolic liveweight until 9 months of age (Sinha and Tucker, 1969b). In ewe lambs, a period of rapid mammary parenchymal growth has been recorded between 8

and 12 (Wallace, 1953), and 12 and 16 weeks (Anderson, 1975) of age. Similarly, Johnsson and Hart (1985) recorded positive allometric mammary growth in ewe lambs between 4 and 20 weeks. This period of allometric mammary growth is particularly critical for subsequent mammogenesis and the milk yield potential of heifers and ewes (Sejrsen, 1994). Attempts to predict the milk yield potential of dairy heifers based on the extent of mammary development in the prepubertal mammary gland have proven unreliable (Elliott, 1957).

It is well established that this prepubertal phase of mammogenesis requires ovarian function in mice (Flux, 1954; Bresciani, 1968) and heifers (Wallace, 1953; Purup *et al.*, 1993b). This likely reflects a requirement for oestrogen, given its ability to restore ovariectomy-abrogated growth (Silver, 1953). Several reports similarly indicate that mammogenesis in prepubertal rats requires ovarian function (Cowie, 1949; Silver, 1953; Paape and Sinha, 1971). However, the reviews of Imagawa *et al.* (1990; 1994), quoting such studies as Astwood *et al.* (1937) and Reece and Leonard (1941), state that mammogenesis in prepubertal rats is ovary-independent. This discrepancy likely reflects the type of measurements made, and that development of the rat gland is probably suppressed, although not completely abrogated, by ovariectomy. Three separate studies (Wallace, 1953; Johnsson, 1984; Ellis *et al.*, 1996a) have indicated that prepubertal allometric mammary growth in sheep is ovary-independent. Using hypophysectomised, ovariectomised and adrenalectomised female mice it has been established that completely normal prepubertal mammary development requires oestrogen + adrenal corticoid + growth hormone, where progesterone can substitute for adrenal corticoids (reviewed by Imagawa *et al.*, 1990).

1.1.3 Peripubertal and postpubertal development

Allometric ductal growth in mice continues after the onset of puberty at 28-42 days of age, slows between 56 and 84 days, and plateaus by around 100 days (Flux, 1954). Likewise, there is only a small increase in total mammary DNA between 50-60 and 110 days of age in female rats (Tucker, 1969). Peripubertal mammogenesis in rodents involves the elongation and branching of mammary ducts until the ductal tree has extended to the bounds of the mammary fat pad. Ductal elongation is directed by highly mitotic terminal end bud structures which are able to penetrate the surrounding fatty

stroma (Daniel and Silberstein, 1987; Section 1.2.2). Some mice and rats may also display tertiary alveolar budding within the ductal network; the extent to which this occurs depends upon the strain (Imagawa *et al.*, 1994) and their stage of oestrous (Dulbecco *et al.*, 1982).

The onset of puberty not only initiates stromal and epithelial proliferation within the human breast, but also results in a conspicuous increase in its size due to the deposition of substantial amounts of fat in mammary adipocytes (Russo and Russo, 1987). Parenchymal growth is associated with the formation of terminal end buds and alveolar structures, where alveolar buds are clustered around a ductal termination to form terminal duct lobular units (Moffat and Going, 1996). Formation of lobules commences 1-2 years after the first menses; these lobules first appear at the periphery of the breast and extend centrally (Monaghan *et al.*, 1990).

Mammary gland growth in heifers continues to be allometric to around 9 months of age, thereafter slowing so that total mammary DNA content of 12- and 16-month old heifers is not different (Sinha and Tucker, 1969b). This development corresponds to the growth of mammary parenchyma toward the bounds of the mammary fat pad (Swett *et al.*, 1955). A similar pattern of parenchymal development has been observed in sheep (Wallace, 1953).

1.1.4 Oestrous cycle growth

The mammary epithelium undergoes cyclical patterns of cell proliferation and morphogenesis during early puberty in response to the changing hormonal profiles of the oestrous cycle as the ductal tree extends toward the bounds of the mammary fat pad. This leads to net cumulative increases in mammary gland size (Sinha and Tucker, 1969a; Vonderhaar, 1988). Total mammary DNA content in rats undergoes the greatest increases during the first and second cycles (Sinha and Tucker, 1969a). Within the oestrous cycle of the mature rat, the mitotic index of mammary epithelium is greatest at metoestrus and dioestrus while the duration of DNA synthesis is longest at metoestrus (Grahame and Bertalanffy, 1972; Purnell and Kopen, 1976). Using immature and pubertal rats, Dulbecco *et al.* (1982) examined DNA synthesis in epithelial cells of ducts and terminal end buds during the oestrous cycle. End bud labelling showed peaks at early oestrus and late oestrus-metoestrus, where the distribution of this label was

dependent upon the epithelial cell type (based on nuclear appearance) examined. Duct and ductule labelling was highest in late oestrus-metoestrus. The mammary gland is described as being most morphologically developed at oestrus and least developed at dioestrus (Lotz and Krause, 1978). It has not been determined whether morphology of the mammary gland and the type of epithelial cells that proliferate within it during the oestrous cycle at the onset of puberty (when the fat pad is not fully occupied) differs from that in a fully mature gland (when ductal elongation has ceased). It is conceivable that differences do exist; such temporal alteration may be present in the results of Sinha and Tucker (1969a), where elevated mammary DNA content at pro-oestrus and oestrus was maintained into metoestrus in the first and second cycles, while peak DNA content was only measured at oestrus in cycles 3-5. The significance of these changes within the mammary gland is emphasised by the fact that the stage of the oestrous cycle at which chemical carcinogen is administered to rats influences the extent and latency period of tumorigenesis (Lindsey *et al.*, 1981; Ratko and Beattie, 1985).

Epithelial DNA synthesis within the mammary gland of parous women is greater in the luteal than the follicular phase of the oestrous cycle (Masters *et al.*, 1977; reviewed by Laidlaw *et al.*, 1995). Again, it is possible that the human breast may proliferate more in the follicular phase during early puberty.

The DNA content of the heifer mammary gland is greatest at oestrus relative to other stages of the cycle (Sinha and Tucker, 1969b). This cyclical variation is accompanied by increased secretory activity at oestrus when epithelial cells assume a cuboidal appearance (Hammond, 1927). Levels of hydroxyproline, a measure of collagen, are also elevated in the heifer mammary gland at oestrus (Sinha and Tucker, 1969b).

1.1.5 Gestational development

The majority of postnatal mammogenesis occurs during pregnancy, although the relative contribution of such growth is species-dependent (reviewed by Cowie *et al.*, 1980). Likewise, there are substantial species differences in the relative contribution of maternal and foetal influences during this development (Thordarson and Talamantes, 1987). Gestational mammary development for a given species is exponential; DNA doubling time remains relatively constant and is primarily a function of gestation length (Sheffield and Anderson, 1985).

Gestational mammogenesis in rodents accounts for between 60 and 80% of mammary gland growth that occurs during pregnancy and early lactation (Munford, 1964). One exception is the hamster, in which the mammary gland is essentially fully developed at term (Sinha *et al.*, 1970). In the mouse, approximately 30% of total development occurs between days 6 and 12 of pregnancy, and approximately 50% occurs between day 12 and parturition (Brookreson and Turner, 1959). The first phase of gestational mammogenesis in rodents involves extensive side-branching of ducts and the budding of alveoli into the interductal spaces of the mammary fat pad. The autoradiographic studies of Traurig (1967) show peaks in DNA synthesis at days 4 and 12 of gestation, with similar findings for mitotic index reported by Grahame and Bertalanffy (1972). It is probable that the first peak is stimulated by maternal influences such as progesterone and prolactin, and that the second is due to the onset of placental stimulation. Studies with pseudopregnant rodents indicate that maternal factors are indeed the primary impetus for mammogenesis until days 10-12 of pregnancy, after which a placental influence is required to achieve the full development typical of a normal pregnancy (Wrenn *et al.*, 1966; Desjardins *et al.*, 1968). Associated with epithelial growth during pregnancy is an increased accumulation of collagen within the mammary parenchyma, while the number of cells within the mammary fat pad remains unchanged (Paape and Sinha, 1971). Another factor that may account for up to 50% of pregnancy-associated mammary growth in rats is self-licking (Roth and Rosenblatt, 1968), although the mechanism responsible for this dramatic effect remains unknown.

The changing hormonal environment during pregnancy also induces mammary epithelial cells to differentiate and assume their capacity to synthesise milk constituents. A small rise in mRNA levels for certain milk proteins can be detected in the mouse mammary gland as early as day 5 (reviewed by Rosen, 1987) although more substantial increases in the level of milk constituents such as α-lactalbumin occur around day 15. This corresponds to a time when epithelial cells also demonstrate a marked increase in cell volume (Foster, 1977). It is during the periparturient and early lactation periods, however, that maximal milk synthesis is initiated.

As for rodents, epithelial growth during pregnancy in the ruminant mammary gland is exponential, and in cows equates to a cell doubling time of 87 days (Sheffield and Anderson, 1985). However, development of the ruminant mammary gland differs from

that of rodents in that the ruminant gland is almost completely developed at term. It has been consistently reported that under normal conditions total mammary DNA content does not change after parturition in goats (Anderson *et al.*, 1981), heifers (Swanson and Poffenbarger, 1979; Baldwin, 1966), and sheep (Anderson, 1975b).

The mammary parenchyma of ruminants gradually replaces the interspersed adipose tissue as it undergoes extensive alveolar development during gestation (Tucker, 1969). This process involves extensive tissue remodelling and reduces the stromal connective tissue to narrow bands (Cowie *et al.*, 1980; Smith *et al.*, 1989a). As emphasised by Swanson and Poffenbarger (1979), these changes are unlikely to be reflected in total mammary weight. The total amount of mammary parenchyma inflects around 70-80 days in goats, 80-115 days in sheep, and 110-140 days in cows; a period when there is noticeable formation of alveoli-filled lobules. The highest percentage of epithelial tissue and [^{3}H]-thymidine-labelled epithelial cells in the mammary gland of ewes is around day 115 of gestation (Smith *et al.*, 1989a). This phase is also associated with an increase in epithelial cell size (Feldman, 1961), a general appearance of secretory activity within the mammary gland, and an elevation in lactose synthesis and the ratio of RNA to DNA (Swanson and Poffenbarger, 1979).

As in the rodent, development of the ruminant mammary gland is influenced by a variety of maternal and foetal factors which are further discussed in respective sections of this review. Davis *et al.* (1993) made the interesting observation that udder growth in hemimastectomised ewes remained at 50% of controls until day 144 of pregnancy, after which the single gland underwent compensatory growth to contain 70% of the DNA in control udders at term. Although the mechanisms which promote this compensatory growth are unknown, such a response illustrates the substantial growth potential of the mammary gland in the periparturient period.

Development of the human mammary gland during pregnancy has been summarised by Russo and Russo (1987). Early gestational mammogenesis involves both the proliferation of peripheral ducts and the formation of new lobules, where a range of morphologies is generally evident. By the end of the first half of pregnancy the ductal tree is essentially established. Thereafter, differentiated acini become increasingly apparent within some lobules and demonstrate an accumulation of secretion within the lumen. However, the development of individual lobules is quite heterogeneous, as some

may be fully differentiated whilst others may undergo extensive proliferation, even during lactation.

1.1.6 Lactational growth

As indicated earlier, the extent to which the mammary gland develops during lactation is largely species-dependent. The mechanisms which promote such growth are essentially unknown. A wide range of studies have investigated aspects of mammary growth during lactation (reviewed by Knight and Wilde, 1987).

A major proportion of mammary development in rats, mice and guinea pigs occurs during early lactation in order to yield maximum DNA content by about day 10 of lactation (reviewed by Munford, 1964). This growth corresponds to a peak of epithelial DNA synthesis on days 2 and 3 (Traurig, 1961) in association with increased cell proliferation in the surrounding connective tissue. It is generally assumed that the normal ruminant gland does not undergo such development during early lactation (Knight and Wilde, 1987), although one report (Knight and Peaker, 1984) suggests that goats display a small increment of growth in this time.

Growth of the mammary gland during lactation may be induced by various means. Several studies have demonstrated that increased lactation demand due to hemimastectomy (Knight, 1987) and increased milking frequency can stimulate mammary development (Wilde *et al.*, 1987). Likewise, increased suckling intensity stimulates mammary growth in a variety of species (reviewed by Tucker, 1987).

It is not known by what mechanisms lactational growth is regulated. Tucker (1987) points out that the results of various experiments indicate an unlikely involvement of ovarian hormones or prolactin. It also appears that the effect of growth hormone during lactation is to promote galactopoiesis more so than mammary development (Gluckman *et al.*, 1987). One possibility is that the lactating mammary epithelium requires adequate energy before it can proliferate (Goodwill *et al.*, 1996). The potential for lactational growth and increased milk yield make the underlying mechanisms for such a response particularly intriguing.

Embryonic and postnatal development of the mammary gland is a process which has fascinated researchers from a number of fields within the area of developmental biology.

The dramatic changes in epithelial proliferation and morphogenesis across several reproductive states in themselves provide valuable insight into the factors controlling mammary development. Growth within each of these stages is integral to the overall development of the gland before it can assume its ultimate role in milk synthesis and secretion. These changes also afford an excellent model for investigating the physiological mechanisms which regulate specific aspects of mammary gland function. Furthermore, an opportunity to study essentially the entire course of organogenesis in the postnatal animal is of particular utility to the biologist.

1.2 MAMMARY MORPHOGENESIS AND HISTOGENESIS

The mammary parenchyma displays a range of morphologies as it ramifies into the stromal tissues of the mammary fat pad. These are generally characteristic of different reproductive states and frequently represent responses to specific local and systemic influences. Such changes result in the mammary gland becoming an elaborate structure capable of providing copious quantities of milk to the offspring on demand. Epithelium with these various morphologies differs in its risk of progressing to a tumorous phenotype. Furthermore, the nature of epithelial histogenesis and morphogenesis differs substantially across a range of species. An appreciation of the histology and morphology of the mammary gland may enable a better understanding of the mechanisms which serve to regulate normal and neoplastic mammogenesis.

1.2.1 Cell heterogeneity

Mammary parenchyma comprises a heterogeneous population of epithelial cell types; 10 different types have been identified in the adult rat mammary gland (Dulbecco *et al.* 1983). Particular attention has focussed on the pluripotency of transplanted mammary epithelium and the possible existence of a stem cell population able to undergo complete mammary regeneration. Williams and Daniel (1983) proposed that the cap cells of the ductal end bud were one such population. A cytologically distinct, pale-staining cell type present throughout the developing mouse mammary gland may also represent a form of stem cell (Smith and Medina, 1988). The ability of the COMMA-1D mammary cell line to repopulate the mammary fat pad (Danielson *et al.*, 1984) led to its

subcloning to identify specific lineages that may represent stem cell types (Danielson *et al.*, 1989). The recent findings of Smith (1996) indicate that separate stem cell populations do exist within the mammary gland and that they serve as separate progenitors for ductal and lobular growth.

1.2.2 The ductal end bud

The end bud is a bulbous termination of the mammary duct that is found in the pre- and pubertal mammary gland of rodents and humans. Intriguingly, such a structure has not been identifiable in mammary tissues of ewe lambs and heifers by histological examination (Akers, 1990; Ellis *et al.*, 1995), although a full assessment based on several other parameters remains to be conducted.

The ductal end bud serves to provide a population of differentiated ductal and myoepithelial cells for ductal elongation, and to direct the path of ductal progression (Daniel and Silberstein, 1987). These structures range from 0.1 to 0.5 mm in diameter and are largest at the periphery of the ductal tree. The distal, basal layer of the rodent end bud is ensheathed by cap cells - cells which lack polarity and an organised cytoskeleton, and which are only loosely adherent with one another (Williams and Daniel, 1983; Figure 1.1). As cap cells progress along the periphery of the end bud they acquire structural and ultrastructural characteristics of myoepithelial cells. Some cap cells may also migrate towards the lumen of the end bud to become ductal cells. Hence, the cap cells may in fact represent a pluripotent cell type within the mammary gland (Dulbecco *et al.*, 1983). The apparent absence of end buds within the ruminant mammary gland raises the question as to how the mammary ducts progress into the mammary fat pad and the origin of the myoepithelial cell population in such species.

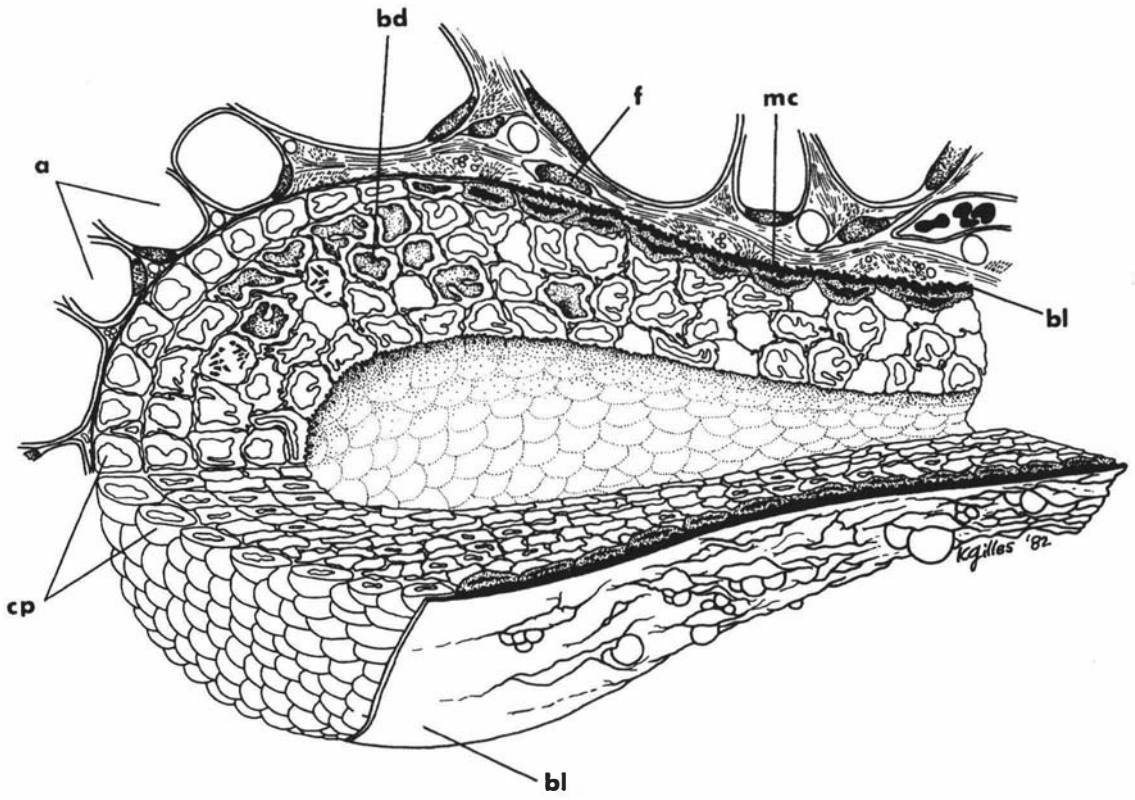


Figure 1.1 Composite drawing of the ductal terminal end bud made from light and electron microscope analyses. Adipocytes (a) abut against cap cells at the tip (left). Fibrous components and fibrocytes (f) comprise the connective tissue tunic around the neck region. The basal lamina (bl) is represented as a cutaway to expose the underlying cap cells (cp). Cap cells are cuboidal but become progressively flattened toward the midregion of the end bud, then differentiate into and are continuous with myoepithelial cells (mc) in the neck region. The basal lamina overlying myoepithelial cells in the midregion is 14 times thicker than that at the tip. Mitosis is seen in the cap and body cells (bd). Reproduced from Williams and Daniel (1983) with permission.

1.2.3 The mammary duct

The outer layer of the mammary duct subtending the terminal end bud consists of myoepithelial cells positioned on the basal lamina. These cells are arranged longitudinally along the ductal axis and form a collar of cells around the inner ductal epithelium (Warburton *et al.*, 1982; Emerman and Vogl, 1986). These inner layers of ductal cells may comprise several populations which are responsive to different stimuli (Sapino *et al.*, 1990). While primary and secondary ducts are lined with several layers of epithelium, terminal ducts are generally lined with a single layer of epithelial cells (Williams and Daniel, 1983). Luminal epithelial cells possess well developed intercellular junctions and have short, blunted microvilli.

1.2.4 Alveolar and lobuloalveolar development

Pregestational development of the mouse mammary gland involves the establishment of a sparse ductal tree within the confines of the mammary fat pad. In some strains, small amounts of alveolar budding may arise from ducts in response to hormonal changes during the oestrous cycle; these alveoli comprise a lumen surrounded by a single layer of epithelial cells (Vonderhaar, 1984) and may contain evidence of secretory activity. Extensive alveolar budding commences with the onset of pregnancy, and by 6 to 8 days post coitus these alveoli have begun to cluster to form true lobuloalveolar structures surrounding small lumina (Vonderhaar, 1988). By the end of pregnancy, the gland consists of lobules composed of many acini. As alveolar formation proceeds, myoepithelial cells alter their orientation to form a basket arrangement around each acinus (Emerman and Vogl, 1986).

The extent of alveolar development within the pubertal mammary gland varies across species. Mayer and Klein (1961) attributed this variation to the relative length of the luteal phase during the oestrous cycle. This association is not surprising given the well established influence of progesterone on alveolar growth (Haslam, 1988a; Lydon *et al.*, 1996). Consequently, in species such as rats and mice where the follicular phase predominates, only ductal morphogenesis is typically evident prior to the onset of pregnancy. In contrast, the mammary gland of species that have a long luteal phase (such as the bitch) may display lobuloalveolar development during puberty which is similar to that seen in pregnancy (Mayer and Klein, 1961).

The pubertal human breast displays several parenchymal morphologies which are distinct from those in the widely studied mouse mammary gland. These features have been previously classified in detail (Russo and Russo, 1987). As the ductal tree continues to elongate, the terminal end buds undergo lateral and dichotomous branching after the first menses, leading to the formation of small ductules or alveolar buds. These buds are arranged around a terminal duct to form a type 1 lobule composed of approximately 11 alveolar buds (Figure 1.2). With recurrent oestrous cycles, these type 1 lobules progress to type 2 lobules, and then to type 3. This progression is heterogeneous within the gland and involves ongoing alveolar budding such that type 2 and type 3 lobules contain approximately 47 and 80 alveolar buds, respectively.

Associated with this increase in number is a concomitant decrease in the size of each individual unit.

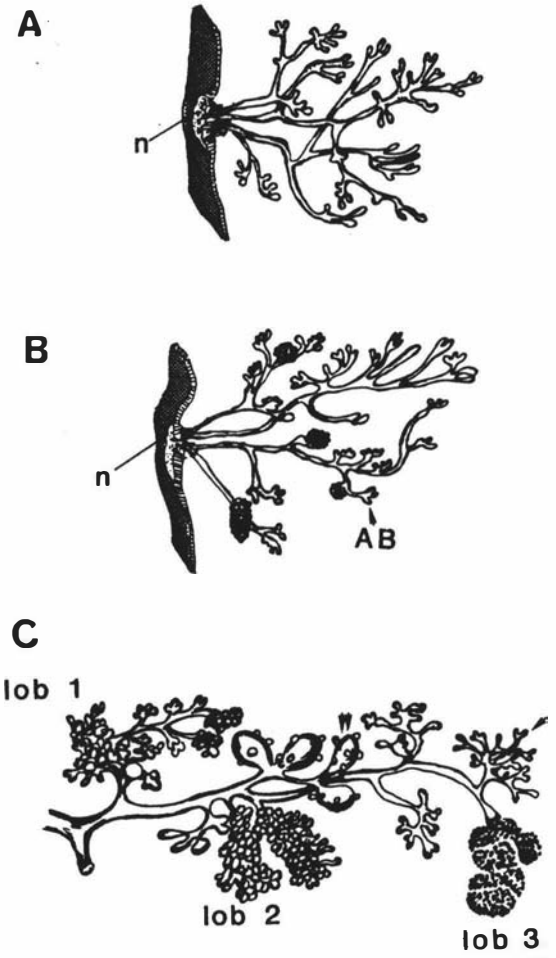


Figure 1.2 Schematic representation of breast development. (A) At puberty or during its onset, the ducts grow and divide in a dichotomous and sympodial basis ending in terminal end buds. (B) After the first menstruation, the first lobular structures appear (lobules type 1); they are composed of alveolar buds (AB). Some branches end in terminal end buds or terminal ducts. (C) The number of lobules increases with age and in the adult nulliparous female breast, three types of lobule may be found (lobules types 1, 2, and 3); n, nipple; lob, lobule. Reproduced from Russo and Russo (1987) with permission.

In studying the local regulation of mammary gland growth, it is important that species differences in parenchymal morphogenesis is acknowledged. This recognition may be of particular relevance when evaluating the regulation of processes such as branching morphogenesis, where it is conceivable that different factors influence the virgin human breast and the mouse mammary gland. There is essentially no information regarding parenchymal morphogenesis and histogenesis within the ruminant mammary gland,

although a realisation is slowly emerging that it differs substantially from that in the widely studied rodent gland (Sheffield, 1988b; Akers, 1990)

1.3 THE STROMAL ENVIRONMENT

Mammary epithelial cells within the embryonic and postnatal mammary gland demonstrate an extensive interaction with the constituents of the surrounding stroma. While early investigations considered this environment to be a relatively inert matrix in which epithelial cells grow, numerous lines of evidence now indicate that this is far from the case. There are also substantial differences in the relative proportions of various stromal tissues within the mammary gland across species, a fact which may warrant particular consideration in studies of human breast disease and ruminant mammogenesis.

1.3.1 The embryonic mesenchyme

Several studies have utilised tissue recombination strategies to demonstrate that the mesenchyme of the embryonic mammary gland exerts strong influence on the adjacent epithelium. Cunha *et al.* (1995) showed that mouse mammary mesenchyme induces the epidermis to adopt a mammary epithelial phenotype. Studies by Sakakura *et al.* (1976; 1982) also showed that the development of mammary epithelium depends on the type of mammary mesenchyme in which it grows. Various mesenchymes can induce mammary epithelium to assume a morphogenesis which is dependent upon the origin of the mesenchyme, while mammary epithelium still retains its milk synthetic potential. The precursor mammary fat pad best supports organotypic development of several foetal epithelial tissues at day 14 of embryogenesis (Sakakura *et al.*, 1987). Mammary fat pad precursor tissue transplanted into the adult mouse mammary gland or co-transplanted with epithelium under the renal capsule facilitates normal morphogenesis while the fibroblastic mesenchyme induces a nodular hyperplasia.

1.3.2 The mammary fat pad

The mammary fat pad is a vernacular term that refers to the subcutaneous depot of adipose tissue in which the mammary gland develops. This fat pad develops from a

specific population of precursor mesenchyme which is evident in the embryonic mouse mammary gland from day 14 (Sakakura, 1987). Transplantation of this precursor tissue to the kidney capsule of mature hosts results in its differentiation into adipose tissue after 2 weeks (Sakakura *et al.*, 1982). This tissue becomes less compact on days 15-16 of embryonic development, and on days 16-17 the preadipocytes proliferate, form lobular structures with a capillary network, and begin to accumulate lipid (Sakakura, 1987). The mammary fat pad in neonatal rodents is readily evident as a depot of white adipose tissue. The first sign of a definitive mammary fat pad in the bovine foetus is around day 80 of gestation (Sheffield, 1988b).

The mature mammary fat pad consists of several stromal elements including adipocytes, connective tissues, blood vessels, nerves and the lymphatic components. There are, however, species differences in the relative proportions of these tissues within the mammary fat pad, the pattern of development that they undergo, and the extent to which the stroma interacts with the developing epithelium.

As is evidenced histologically, the major proportion of the rodent mammary fat pad is comprised of adipocytes, with a thin sheath of connective tissue enveloping the established ducts. The mouse mammary fat pad is also serviced by a defined vascular network (Soemarwoto and Bern, 1958), and it is drained by lymphatics to one or more lymph nodes. The rat mammary gland demonstrates a net increase in the collagen content of both the fat pad and parenchymal fractions during puberty, while ovariectomy induces an increase in collagen and the total DNA content of the mammary fat pad (Paape and Sinha, 1971). The protein content of the mouse mammary fat pad remains relatively constant across mature virgin, pregnant and lactation stages, although it is unclear as to why the mammary fat pad demonstrates an increase in its total DNA content into lactation (Bandyopadhyay *et al.*, 1995).

The mammary fat pad of the human breast is somewhat different. At birth there is an already well developed network of connective and vascular tissue, and by prepuberty the developing glandular epithelium lies in veins of connective tissue that form conductive pathways for pubertal growth of the eventual duct system (Mayer and Klein, 1961; Anbazhagan *et al.*, 1991). After the onset of puberty, the human breast demonstrates a considerable increase in size due to deposition of substantial amounts of fat and the initiation of stromal proliferation (Knight and Peaker, 1982). Volume of the breast is

greatest around the time of menses (Cowie *et al.*, 1980) and can change by as much as 20% during the oestrous cycle due to changes in stromal volume (Rønnov-Jessen *et al.*, 1996). Epithelial growth is preceded by stromal proliferation and the deposition of inter- and intralobular fibroblastic connective tissue (Rønnov-Jessen *et al.*, 1996). As a result, and in contrast to the mouse mammary gland, human mammary epithelium develops while surrounded by a collagenous intralobular stroma (Rønnov-Jessen *et al.*, 1996). A similar although less pronounced tissue architecture is seen in the rat (Sakakura, 1991). There also exists a substantial proportion of interlobular collagenous stroma which consists of fibroblasts dispersed at a low density. The ratio of stroma to parenchyma during the course of development of the human mammary gland was reported by Russo and Russo (1987). During puberty only 10% of the breast is parenchyma; the remaining 90% is comprised of stroma, of which 17% is intralobular stroma. With age, the breast of nulliparous women comprises 30% parenchyma, and 28% intralobular stroma. By late pregnancy, parenchyma occupies 73% of gland area with concomitant declines in area of both the inter- and intralobular stroma.

Only subjective observations have been made regarding the nature of the mammary fat pad in ruminants. Mayer and Klein (1961) state that in the neonatal calf, "the non-glandular structures are almost mature in form, with already-established vascular and lymphatic systems. The adipose and connective tissues are also well organised. The early partitioning of the adipose tissue by the connective tissue system is remarkable, and the connective septa serve as paths for the future extension of the epithelial structures". Fat is deposited within the mammary fat pad during prepuberty and puberty, although the extent to which this occurs may be influenced by the animal's plane of nutrition. As the parenchymal elements progress into the mammary fat pad during prepuberty and puberty they apparently replace the adipose tissue and subsequently become enveloped by a zone of fibroblastic connective tissue similar to that seen in the human breast (Sheffield, 1988b).

1.3.3 Mammary transplantation studies

The ability to transplant normal and neoplastic mammary epithelium to various sites has facilitated investigations into several aspects of mammary biology (reviewed by Hoshino, 1978; Sheffield, 1988b; Medina, 1996), especially the interrelationships

between different tissue types within the gland. A large proportion of these studies in mice and rats have been aided by the cleared fat pad technique, a procedure described by DeOme *et al.* (1959) that involves ablating the mammary epithelial rudiment at around 3 weeks of age. This manipulation yields a mammary fat pad devoid of epithelium which can subsequently serve as a transplantation site. Several modifications of this original procedure have also been described (Hoshino, 1962).

A number of important observations have been made from transplantation studies in rodents. Firstly, repopulation experiments indicate that the amount of parenchyma which ultimately develops within the mammary gland reflects the volume of the mammary fat pad in which it grows rather than a characteristic of the epithelium itself (Hoshino, 1978).

Results from several transplantation studies also show that the mammary epithelial cell population possesses a great deal of pluripotency. Grafts from various sites of the mammary ductal tree, including the terminal end buds, can re-establish a normal ductal network when transplanted to a virgin host (Hoshino, 1964; Sakakura *et al.*, 1979; Ormerod and Rudland, 1986). The only region incapable of such growth is the nipple. Similar potential for regrowth is possessed by foetal (Sakakura *et al.*, 1979), neonatal (Currie *et al.*, 1977) and male (Blair and Moretti, 1970) mammary tissue. These recombinants undergo morphogenetic and functional differentiation when hosts are mated or hormonally treated (Sekhri *et al.*, 1967; Hoshino, 1983). Immediately after its transplantation, mammary tissue undergoes extensive remodelling and proliferation within the new stromal environment (Hoshino, 1978). Other recombination strategies have also been developed. One approach involves the isolation and *in vitro* genetic manipulation of mammary epithelial cells which are then inoculated into a cleared mammary fat pad or other sites, enabling the resultant phenotype to be examined (Edwards *et al.*, 1995; Edwards *et al.*, 1996). Other studies have used the mammary fat pad to demonstrate that mammary epithelial cell lines such as COMMA-1D (Danielson *et al.*, 1984) can undergo a complete and normal morphogenesis *in vivo*.

Transplantation studies have also revealed that normal parenchymal elements locally regulate their growth and spacing within the mammary gland (Faulkin and DeOme, 1960). Other experiments have shown that hyperplastic alveolar nodules are similar to normal epithelium in that they only grow within a depot of adipose tissue and,

furthermore, that their growth is inhibited by the presence of endogenous mammary epithelium relative to their growth in a cleared mammary fat pad (Miller *et al.*, 1981).

Transplantation approaches have also enabled investigation into the lifespan of mammary epithelium. The work of Daniel and co-workers (reviewed by Daniel and Silberstein, 1987) demonstrated that serially transplanted epithelium had a finite lifespan, and that growth rate declined by approximately 15% with each generation. This lifespan was a function of the number of cell divisions rather than actual cell age. Senescent epithelium did, however, retain an ability to respond to cholera toxin, leading to the suggestion that epithelial cells become refractory to *in vivo* mitogenic stimulation. On the other hand, cells from hyperplastic alveolar nodules were immortalised and did not demonstrate senescent tendencies (Daniel *et al.*, 1968). In contrast, the reports of Hoshino (reviewed by Hoshino, 1978) suggested that serially transplanted mammary epithelium does not suffer any reduction in its survival potential. A likely reason for these conflicting results is that the measurements made by Hoshino related to the success of transplant recovery, whereas a more valid parameter would be the outgrowth potential of epithelial transplants as measured by Daniel and co-workers.

The stromal requirements for normal mammary gland development have been investigated in the extensive studies of Hoshino (reviewed by Hoshino, 1978). When transplanted to subcutaneous sites, mammary grafts were maintained, but did not demonstrate any outgrowth beyond the adipose tissue associated with the original transplant. This limited outgrowth also occurred in transplants to the peritoneal cavity and the anterior chamber of the eye. In contrast, grafts to the cleared mammary fat pad, the perirenal fat pad or the mesometrial fat pad demonstrated extensive ductal outgrowth into the transplanted and host stroma. The extent of outgrowth was generally greatest in the cleared mammary fat pad, although substantial amounts of growth were also observed in the perirenal fat pad. A high rate of mammary graft recovery was also achieved in transplants to the interscapular depot of brown adipose tissue (Hoshino, 1967), although no measure was given as to the extent of the outgrowth. Nevertheless, the results of these studies indicate that mammary epithelium has an inherent requirement for a depot of adipose tissue in order for it to undergo normal growth and morphogenesis. The locality of the recipient fat pad also influences where metastatic tumours will arise; metastases from the mammary fat pad primarily arise in the lung

whilst metastases from ovarian and mesenteric adipose tissues generally occur in the liver, spleen and diaphragm (Elliott *et al.*, 1992).

The mammary fat pad of the immunologically-deficient athymic nude mouse and rat has been widely utilised as a site for heterologous mammary transplants (reviewed by Sheffield, 1988b). Interestingly, Welsch *et al.* (1987) showed that normal, but not carcinomatous, rat mammary epithelium can survive and grow within the mouse mammary fat pad. Attempts to transplant human mammary tissue have met with limited success. Although Outzen and Custer (1975) reported outgrowths from transplanted human mammary heterografts, the epithelium which they used was from abnormal mammary tissue. Transplantations conducted by Jensen and Wellings (1976) did not show any outgrowth of human epithelium into the host mammary fat pad; instead transplants appeared as rounded grey structures encapsulated by a glistening sheath of connective tissue. Similar results were reported by Sheffield and Welsch (1988) and Yang *et al.* (1995). DNA synthesis in these epithelial spheroids could be stimulated by hormones, yet their morphology remained unaltered. Such studies have been extended to the transplantation of bovine mammary tissue into the mammary fat pads of nude mice (Sheffield and Welsch, 1986). Similar to transplanted human cells, bovine cells do not outgrow but instead form hollow spheroid structures ensheathed by connective tissue. The same observations were reported by Ellis and Akers (1995) using the MAC-T bovine mammary cell line. Both studies reported increased DNA synthesis of spheroids in response to exogenous hormones without morphological change.

The reason for species differences in the ability of transplanted epithelium to outgrow into the mouse mammary fat pad is intriguing. It is unlikely that it reflects different hormonal conditions in host mice, as tissue slices of human and bovine mammary tissue are maintained, grow and differentiate when transplanted subcutaneously in nude mice (Welsch *et al.*, 1979; Sheffield and Welsch, 1986). One likely explanation is that the mouse mammary fat pad does not provide a stromal environment suitable for the outgrowth of human and bovine mammary epithelium. Sheffield (1988b) reported that the composition of the extracellular matrix that surrounds spheroids of heterografted bovine epithelium differs to that in normal bovine mammary tissue. Human and bovine epithelium normally grows within a matrix of connective tissue enriched with matrix components such as collagen and glycosaminoglycans. Furthermore, the mammary fat

pad in both of these species has an extensive, pre-established mesh of connective tissue. In contrast, the mouse mammary fat pad comprises predominantly adipocytes, and proliferating ductal epithelium abuts onto adipocytes rather than being enveloped by a fibroblastic connective tissue.

Transplantation studies have also shown that both intrinsic epithelial properties and the host environment can influence mammogenesis and tumorigenesis. For example, Alston-Mills and Rivera (1985) and Ethier and Cundiff (1987) have shown that the growth of transplanted DMBA-induced tumours is affected by properties such as growth factor independence. Others indicate that host factors such as age (Daniel *et al.*, 1968) and dietary fat (Ip and Sinha, 1981) can influence the growth of transplanted epithelium.

1.3.4 The epithelial-stromal reaction

The encroachment of parenchyma into the adjacent mammary fat pad leads to the formation of an intimate association between the mammary epithelium and the surrounding stromal elements. Within the mouse mammary gland, cap cells and the basal lamina of the advancing terminal end bud are in direct contact with adipocytes of the mammary fat pad (Daniel and Silberstein, 1987). This association induces DNA synthesis in stromal cells within 250 μm of the end bud, where [^{3}H]-thymidine labelling of stromal cells is greatest adjacent to the epithelium and decreases with distance (Berger and Daniel, 1983; Dulbecco *et al.*, 1982). Interestingly, this response distance of 250 μm is the same as that which separates ducts of the virgin mammary gland (Faulkin and DeOme, 1960). This DNA synthetic response by stromal cells can only be induced by epithelium that is undergoing proliferation, for senescent ductal transplants do not evoke this effect (Daniel and Silberstein, 1987). Proliferating epithelium in the ductal end bud also induces a local upregulation of stromal epidermal growth factor (EGF) receptors (Daniel and Silberstein, 1987). Furthermore, mammary parenchyma can locally influence lipolysis and lipogenesis in nearby adipocytes of the mammary fat pad (Elias *et al.*, 1973; Kidwell *et al.*, 1982; Bartley *et al.*, 1981) by liberating diffusible, soluble factors (Lucas *et al.*, 1976). This epithelial influence on adipocyte metabolism might also be mediated by local immune mast cells (Kidwell and Shaffer, 1984; Haslam, 1988b). It is not known to what extent this local influence on adipocyte metabolism subsequently regulates the growth of the mammary epithelium.

The constricted flank of the ductal end bud also demonstrates extensive stromal reaction. This region, encased by proliferating fibrocytes synthesising interstitial collagen (Williams and Daniel, 1983), is enriched with sulfated glycosaminoglycans such as chondroiton sulfate (Silberstein and Daniel, 1982). Fibrosis within this zone may be induced by the newly differentiated myoepithelium and/or the synthesis of the basal lamina (Wicha *et al.*, 1980). One question that does arise is whether this fibroblastic stroma originates from cells of the mammary fat pad, or from mesenchymal cells that have co-migrated with the advancing ductal epithelium. In the case of mammary epithelial heterografts which overexpress the *Wnt*-1 oncogene and induce extensive stromal fibrosis (Cunha and Hom, 1996), the resultant fibroblastic stroma has been shown to originate from constituents of the host's mammary fat pad (G.R. Cunha, personal communication). It remains to be determined whether the extensive fibrosis that occurs around parenchyma in the human and ruminant mammary gland similarly arises from the stromal cells of the mammary fat pad proper.

The myoepithelial cell population is closely associated with epithelial cells and forms a collar of longitudinally-oriented cells around the ductal epithelium in the nonpregnant female (Haslam, 1988b). The myoepithelium may therefore act to regulate exposure of epithelial cells to the underlying basement membrane.

The most pronounced example of stromal reaction is seen in the vicinity of breast tumours, where desmoplasia may account for greater than 90% of cells in tumours such as infiltrating ductal carcinomas (Rønnov-Jessen et al., 1996). This response may involve the upregulated expression of genes for certain mitogens such as IGF-II (Singer *et al.*, 1995). The importance of this stromal reaction may be emphasised by the finding that human adenocarcinoma cells transplanted to athymic nude mice have an increased tumour take and growth rate when they are co-inoculated with human fibroblasts (Noël *et al.*, 1994). In addition to markedly altering the composition of the extracellular matrix, desmoplasia may encourage the appearance of a modified fibroblast cell type, the myofibroblast, which is positive for α-smooth muscle actin. The stromal revelation of this cell type during tumour progression may further alter the composition of the extracellular matrix, or may allow contractile forces to be exerted during the growth of tumorous epithelium (Rønnov-Jessen *et al.*, 1996).

1.3.5 Modelling epithelial-stromal associations

With an increasing recognition that local influences play an important role during mammary development and tumorigenesis, numerous attempts have been made to model the interactions that take place between the epithelium and the adjacent stroma (reviewed by Ip and Darcy, 1996).

One such approach has been to "condition" cell culture medium by incubating it with different constituents of the mammary gland stroma. This conditioned medium can then be added to cultures of mammary epithelial cells to determine their response to soluble factors liberated by the stromal tissue or cells. Numerous studies have prepared conditioned medium from cultured monolayers of foetal mammary stroma (Grey *et al.*, 1989), and stroma of adult rodent (Sasaki *et al.*, 1994), human (Van Roozendaal *et al.*, 1996) and bovine (Woodward et al., 1992) mammary tissue, as well as mammary tumour cells (Cappelletti *et al.*, 1993). Other studies have conditioned medium using differentiated 3T3-L1 preadipocytes (Levine and Stockdale, 1984; Rahimi *et al.*, 1994), isolated mammary adipocytes, or explants of mammary fat pad tissue (Beck and Hosick, 1988). Almost all of these studies have demonstrated that the stromal constituents produce some form of biological activity (particularly polypeptide growth factors) capable of modifying such characteristics as anchorage-dependence, morphogenesis, proliferation, and milk protein synthesis.

Other studies have investigated physical and physico-chemical influences on epithelial growth and morphogenesis by co-culturing epithelial and stromal cells. These co-cultures may incorporate live cell populations (Wang and Haslam, 1994), stromal cells inhibited by glutaraldehyde or killed by irradiation (Levine and Stockdale, 1985; Haslam, 1986), or the matrix synthesised by stromal cells. Such conditions have been shown to stimulate epithelial DNA synthesis; likewise, stromal DNA synthesis is stimulated in live mixed cultures. Other characteristics such as epithelial morphology, milk protein expression (Wiens *et al.*, 1987) and progesterone receptor levels may also be altered. A major disadvantage of such an approach is that in many cases it is difficult to segregate the epithelial and stromal cell types for routine assay. A third, similar approach has been to culture epithelial and stromal populations that are physically separated from each other while still allowing bidirectional diffusion of soluble factors. This approach has included the use of culture well inserts and the co-casting of

mammary fat pad explants in collagen along with epithelial organoids (Carrington and Hosick, 1985).

1.3.6 The extracellular matrix

Literature concerning the role of the extracellular matrix in mammary development, differentiation, and lactogenesis is extensive and will not be reviewed in detail here. For comprehensive reviews of this subject the reader is referred to the reviews of Bissell and Hall (1987); Aggeler *et al.* (1988); Barcellos-Hoff and Bissell (1989) and Blaschke *et al.* (1994). It is pertinent, however, to briefly consider the involvement of the extracellular matrix in mammary gland development and morphogenesis, and the likely importance of the mammary fat pad in providing such a substratum.

The extracellular matrix on which the mammary epithelium is positioned consists of three different strata (Sakakura, 1991). Basal to the epithelial cells is a thin sheet referred to as the lamina densa that is separated from the epithelium by a narrow translucent space known as the lamina lucida. The lamina densa and the lamina lucida are collectively referred to as the basal lamina. Adjacent to the basal lamina and associated with the stromal components is a layer of variable thickness known as the reticular lamina. Epithelial cells in the virgin rodent mammary gland are actually separated from the basal lamina by a continuous collar of myoepithelial cells, whereas during pregnancy, epithelial cells become directly exposed to the basal lamina as the myoepithelial cells assume a basket-like arrangement (Haslam, 1988b). The basal lamina that ensheaths the distal region of the end bud and the subtending ducts in the virgin mammary gland is approximately 100 nm thick, whilst that along the constricted neck region of the end bud may be 13-20 times this thickness (Daniel and Silberstein, 1987). This latter region is a site of considerable sulfated glycosaminoglycan synthesis (Silberstein and Daniel, 1982).

Until recently it has been accepted that the epithelium produces components of the basal lamina such as laminin, type IV collagen and heparan sulfate proteoglycans (including nidogen and entactin), while cells of the stroma synthesise constituents of the reticular lamina such as type I and III collagens, fibronectin, and tenascin (Sakakura, 1991). These assumptions have been based on the results of immunolocalisation studies and *in vitro* experiments showing the presence of various matrix components in epithelial

cultures. However, more recent *in situ* hybridisation results indicate that only the stromal connective tissue cells and adipocytes, and not the epithelium, express mRNA for collagen I, collagen IV, and laminin in the mouse mammary gland (Keely *et al.*, 1995). Furthermore, this expression is developmentally regulated; collagen I mRNA levels are highest in prepuberty and early pregnancy and precede an upregulation of collagen IV and laminin expression. These findings emphasise the critical requirement for the mammary fat pad stroma during development and the importance of the epithelial-stromal reaction in regulating epithelial progression and morphogenesis. It remains unclear as to whether the detection of these matrix proteins in epithelial cultures *in vitro* is a function of cell adaptation to an artificial environment, or if it simply reflects contamination of cultures with stromal cells or their remnants.

While the extracellular matrix plays an integral role in regulating the development and function of mammary epithelium, the extent of its involvement and the specific roles of its numerous constituents remain to be fully characterised. *In vitro* studies frequently adopt the technique of embedding primary epithelial cells within collagen gels to achieve sustained growth and hormonal responsiveness (Yang *et al.*, 1980). However, collagen I alone does not fulfil the matrix requirements for normal morphogenesis as shown in an experiment conducted by Daniel and co-workers (Daniel *et al.*, 1984), where epithelium transplanted into collagen gel inside the mammary fat pad grew as radial spikes within the gel and only formed end buds when it reached the stroma of the mammary fat pad.

It is possible that specific components of the extracellular matrix are prerequisite for certain stages of mammogenesis. For example, tenascin is only expressed by the foetal mesenchyme in response to epithelial induction (Sakakura, 1991) while the stroma of the postnatal gland differentially expresses collagen I, collagen IV and laminin during virgin and gestational development (Keely *et al.*, 1995). Other studies have shown that the synthesis of extracellular matrix components is regulated by various hormones and growth factors (Blum *et al.*, 1989a), further supporting suggestions for their stage-specific roles. Furthermore, cell-surface receptors for these extracellular matrix proteins, the integrin subunits, are expressed by epithelial cells during specific stages of development (Anbazhagan *et al.*, 1995) and are markedly repressed during lactation (Keely *et al.*, 1995). Likewise, inhibition of type IV collagen synthesis *in vivo* using *cis*-

hydroxyproline leads to a reduction in epithelial growth and encourages the regression of epithelium to an involuted-like state (Wicha *et al.*, 1980). The most pronounced requirement of epithelium for an extracellular matrix is seen in studies of milk protein synthesis where epithelial differentiation and hormonal responsiveness is only achieved when cells are cultured on substratum such as collagen or a reconstituted membrane matrix such as Matrigel. Aside from having marked effects on epithelial characteristics such as morphogenesis and differentiation, the extracellular matrix can also regulate other cellular responses including the expression of hormone and growth factor receptors (Mohanam *et al.*, 1988; Haslam, 1986) and cell proliferation (Salomon *et al.*, 1981; Wicha *et al.*, 1982).

Taken together, this information strongly implicates the stroma as fulfilling a major role in regulating development of the mammary gland. This regulation may be effected both chemically and physically, and also likely depends upon the local reaction between epithelial cells and the surrounding stroma. Such information is of fundamental importance to our understanding of mammary gland biology. However, a great deal remains to be understood about the full extent of this regulation and its role during the course of normal and neoplastic mammogenesis. Furthermore, species differences remain to be explored, where it appears that there are several major distinctions between the stromal environment of the rodent mammary gland and that in other species such as humans and ruminants.

1.4 FACTORS INFLUENCING MAMMARY DEVELOPMENT

The postnatal proliferation of epithelial cells within the mammary fat pad is influenced by an array of systemically and locally derived factors. While it is well established which hormones primarily control mammogenesis, in several cases their mechanism of action on the mammary gland is unknown. Furthermore, an increasing body of information suggests that the local synthesis of other factors within the mammary gland may be critical to the growth and morphogenesis of mammary epithelium. A role for these factors has been studied in the normal mammary gland of various species as well as in the tumorous mammary gland of rodents and humans.

1.4.1 The ovarian steroids

1.4.1.1 Oestrogen

Numerous studies have demonstrated the important role of oestrogen during postnatal mammogenesis (reviewed by Haslam, 1987). Oestrogen is a potent stimulator of DNA synthesis in the mouse mammary gland (Traurig and Morgan, 1964), particularly within the terminal end buds (Bresciani, 1968). A requirement for the mammogenic effect of oestrogen is exemplified in prepubertal mice where ovariectomy-abrogated ductal growth can be restored by exogenous oestrogen (Flux, 1954). While development of the rat mammary gland is unaffected by prepubertal ovariectomy, administration of oestrogen to ovariectomised female rats promotes both ductal and lobuloalveolar development (reviewed by Nandi *et al.*, 1995). Oestrogen also stimulates DNA synthesis in human breast tissue transplanted to athymic nude mice (Laidlaw *et al.*, 1995).

Short-term administration of supraphysiological doses of oestrogen to prepubertal ewe lambs (Ellis *et al.*, 1996a) and heifers (Woodward *et al.*, 1993) increases DNA synthesis in the mammary epithelium. This oestrogen-induced proliferation in the mammary gland of heifers was followed by a phase of stromal DNA synthesis. It is also well established that heifers and ewes grazing pastures with a high phyto-oestrogen content frequently demonstrate precocious mammary gland development (Adams, 1995). Although ovariectomy abrogates prepubertal mammary development in heifers (Purup *et al.*, 1993b), the involvement of oestrogen in this effect is somewhat unclear as the serum oestrogen concentration was only reduced by 30% (0.1 pg/ml) following ovariectomy. In contrast, prepubertal mammary growth of ewe lambs is unaffected by ovariectomy (Wallace, 1953; Ellis *et al.*, 1996a). Others have shown that oestrogen, in combination with progesterone, can facilitate normal growth in ovariectomised heifers (Sud *et al.*, 1968); the combination of these two steroids is an essential requirement to adequately develop the mammary gland during hormonal induction of lactation (Sawyer *et al.*, 1986).

Responsiveness of the mouse mammary gland to oestrogen-induced proliferation also varies during postnatal development (Haslam, 1989). Specifically, oestrogen does not induce DNA synthesis in the mammary gland of 3-14 day old female mice, while by 3-4 weeks of age both stroma and epithelium proliferate in response to oestrogen. Along

these lines, it is unlikely that oestrogen serves a role during normal embryonic and perinatal mammary development given that the growth of the mammary rudiment is unaltered in female mice lacking a functional oestrogen receptor (Korach, 1994). On the other hand, administration of exogenous oestrogen to embryonic mice does result in malformed mammary gland (Raynaud, 1961).

The finding that ductal elongation in hypophysectomised mice was not promoted by exogenous oestrogen indicated that pituitary hormones were also required to elicit the mammogenic effect of oestrogen (Lyons *et al.*, 1958; Nandi, 1958). Subsequent studies in triply-operated mice and rats showed that the combination of oestrogen + corticoid + growth hormone could fully restore mammary development (reviewed by Imagawa, 1990). A synergistic response by ductal epithelium to oestrogen + prolactin has also been reported (Stoudemire *et al.*, 1975). However, it still remained unclear as to whether oestrogen initiated its effect by a direct action on the mammary gland, or via an indirect action through the pituitary. Using slow-release implants positioned within the immature mammary gland, Daniel *et al.* (1987) and Haslam (1988d) demonstrated that oestrogen acts directly on the mammary gland to stimulate ductal growth. This mode of action is further supported by the fact that ductal growth is suppressed in the presence of implants containing antiestrogens (Silberstein *et al.*, 1994). In contrast, oestrogen-stimulated proliferation in the mature gland is systemically mediated, while local oestrogen release can still induce an upregulation of epithelial progesterone receptors (Haslam, 1988d).

Even though a local action of oestrogen had been shown, there remained the paradox that cultured epithelial cells from various species were not stimulated to proliferate by oestrogen *in vitro* (Yang *et al.*, 1980; Richards *et al.*, 1988). Only occasional reports have demonstrated oestrogen responsiveness of cultured mammary epithelium (Gompel *et al.*, 1986). The establishment of an oestrogen-responsive breast cancer cell line (MCF-7) has led to its widespread adoption (Lippman *et al.*, 1976) as a model for hormone-dependent breast cancer. Yet while cultures of mammary epithelial cells do not proliferate in response to oestrogen, they do possess functional and prolactin-inducible oestrogen receptors (Haslam and Levely, 1985) and upregulate progesterone receptor levels in response to oestrogen (Edery *et al.*, 1984).

These findings have led several groups to propose that the mammogenic effect of oestrogen is mediated by the adjacent constituents of the mammary fat pad. In support of this proposal is the finding that a single injection of oestrogen stimulates DNA synthesis in mammary adipocytes and stromal cells prior to a phase of DNA synthesis in the adjacent epithelium (Shyamala and Ferenczy, 1984). McGrath (1983) also showed that oestrogen could only stimulate DNA synthesis in cultured epithelial cells after they came into contact with mammary fibroblasts. This effect requires live fibroblasts, and is associated with oestrogen-dependent stimulation of fibroblast proliferation (Haslam, 1986). An indirect action of oestrogen is further suggested by studies showing that explants of sheep (Forsyth, 1989) and heifer (Purup *et al.*, 1993a) mammary tissue demonstrate increased DNA synthesis in response to oestrogen *in vitro*, while cultured bovine mammary epithelial cells are unresponsive to oestrogen (Woodward *et al.*, 1994). This may reflect the local, oestrogen-induced synthesis of mitogens by stromal cells (Woodward *et al.*, 1992). That oestrogen stimulates the stromal expression of certain paracrine growth factors (Sirbasku, 1978) is considered in respective sections of this review.

Estrogen also increases progesterone receptor levels on epithelial cells when they are co-cultured with mammary fibroblasts, an effect that may be mediated by stromal synthesis of type I collagen (Haslam and Levely, 1985). This concurs with the findings of Sheffield and Anderson (1984a, 1984b) who showed that oestrogen stimulates the synthesis of collagen, DNA, and RNA by mammary fibroblasts. Such observations may be of direct physiological relevance to the developing mammary gland. Specifically, immature mammary epithelium which does not upregulate its progesterone receptor levels in response to oestrogen can prematurely acquire this type of oestrogen responsiveness upon transplantation to the mammary fat pad of a mature host (Haslam and Counterman, 1991). It is not known, however, by what mechanism(s) the mammary fat pad initiates this effect.

1.4.1.2 Local oestrogen biosynthesis

In evaluating the mammogenic effects of oestrogen, it must also be recognised that mammary stromal cells can synthesise oestrogens from C_{19} steroids via an aromatase enzyme complex (reviewed by Simpson *et al.*, 1989). This likely accounts for the

higher levels of oestrogen measured around breast tumours (Bulun *et al.*, 1993). Breast tumours may also exert a positive local feedback on stromal cells to induce an upregulation of aromatase activity (Schmidt and Löffler, 1994a,b). However, the full extent to which locally synthesised oestrogens contribute to tumorous and normal mammary gland growth remains to be shown.

1.4.1.3 The oestrogen receptor (ER)

The pronounced effects of oestrogen on mammary cells are initiated via the ER. Synthesised in the cytoplasm, the ER resides in the nucleus where it forms a receptor-ligand complex capable of interacting with oestrogen response elements in the vicinity of target gene promoters (Ciocca and Vargas Roig, 1995). The role of this receptor during mammary development is indicated by the fact that the mammary glands of mice lacking functional ERs are undeveloped except for the presence of vestigial ducts around the nipple (Korach, 1994). Meanwhile, the finding that mammary glands from these mice develop upon transplantation to normal hosts in the presence of isografted pituitaries (S. Nandi, personal communication) suggests that deficiencies in such mechanisms may be compensated for by other hormones such as prolactin.

By measuring ligand binding to cytoplasmic extracts, Haslam and Shyamala (1981) determined that approximately 50% of the high affinity ERs within the mouse mammary gland are present in stromal cells. Further investigations used steroid autoradiography (Daniel *et al.* 1987; Haslam, 1989) and immunocytochemistry (Haslam and Nummy, 1992) to localise the cellular expression of ERs within the mouse mammary gland. Only mesenchymal cells express ER within the foetal mammary gland. Within the virgin gland, cap cells, adipocytes and myoepithelial cells are consistently negative for ER while adjacent undifferentiated stromal fibroblasts are frequently positive. Luminal epithelium of the end bud and subtending ducts is ER positive. Within the ductal tree there is a gradient of ER level/binding capacity, where it is greatest in the nipple region and declines towards the end bud zones (Daniel *et al.*, 1987). The proportion of ER positive epithelial and stromal cells is least at 3 days of age (7.8 and 4.4%, respectively) and increases during puberty to be the highest in the epithelium at 7-10 weeks (20%), and at 6-7 weeks in the stroma (16.5%). However, further mechanisms must regulate the action of oestrogen as it only induces progesterone receptors in the mature gland

(Haslam, 1988c); this may be achieved at the local level of the mammary fat pad (Haslam and Counterman, 1991).

Multiple factors may regulate ER expression within the mammary gland. Treatment of ovariectomised mice with oestrogen generally results in down regulation of the number of ER positive epithelial and stromal cells, as does sialoadenectomy (Sheffield and Kotolski, 1993). Levels of ER within the dog mammary gland are increased with age and during the luteal phase of the oestrous cycle (Donnay *et al.*, 1995). *In vitro* studies have demonstrated that dissociated epithelial cells possess functional ER that can be upregulated by prolactin (Edery *et al.*, 1984; Edery *et al.*, 1985). ER status is also widely used as a determinant for breast cancer therapy. Approximately 7% of cells are ER positive in normal breast tissue while approximately 70% of primary breast cancers are ER positive (Nicholson *et al.*, 1995). Positive ER status favours a far greater response to therapeutic treatment. The involvement of variant forms of the ER (McGuire *et al.*, 1991) in normal and neoplastic mammary growth remains to be characterised.

Although a requirement for oestrogen during mammogenesis is unequivocal, a great deal remains to be understood about the specific nature of its effects on the mammary gland, and the means by which these are exerted. Several lines of strong, though indirect, evidence indicate that stromal constituents within the mammary fat pad may be important mediators of oestrogen action. Recent studies have shown that the mammary fat pad of ER negative mice does not support the growth of wild-type epithelium (G.R. Cunha, personal communication). This may be the first direct evidence that the mammary fat pad mediates oestrogen action on the mammary gland. Even once this requirement is confirmed, the downstream mode of action and the physiological implications of oestrogenic action on the mammary fat pad remain to be established.

1.4.1.4 Progesterone

Although progesterone exerts a major influence on mammary gland development, the means by which it elicits its effects at the level of the mammary gland are not fully defined. The primary response to progesterone during recurrent oestrous cycles and pregnancy is the initiation of ductal branching and lobuloalveolar morphogenesis. Exogenous progesterone stimulates DNA synthesis in the ductal epithelium of mature,

but not immature, ovariectomised mice to promote ductal side-branch formation and the differentiation of terminal buds into alveoli (Bresciani, 1968; Haslam, 1988a; Haslam, 1989). Associated with this growth is an increase in stromal DNA synthesis (Haslam, 1989). Although the immature gland is unresponsive to acute progesterone treatment, Vonderhaar and Plaut (1992) were able to increase DNA synthesis in the mammary epithelium of 4-week old mice after a prolonged 9-day treatment. The minimum requirement for full lobuloalveolar development in triply operated rodents is oestrogen + progesterone + adrenal corticoid + prolactin or growth hormone (Lyons *et al.*, 1958; reviewed by Imagawa *et al.*, 1994), where the presence of both pituitary hormones facilitates greater development than either alone.

Administration of progesterone to prepubertal heifers for 4 days did not stimulate DNA synthesis within mammary tissue (Woodward *et al.*, 1993). Similarly, treatment of nulliparous, post-pubertal ewes with progesterone had no apparent effect on mammary development or morphogenesis, where progression to lobuloalveolar development required oestrogen + progesterone in the presence of endogenous prolactin (Schams *et al.*, 1984). While the human breast displays maximal DNA synthesis during the luteal phase, it is unclear why breast tissue heterografted to mice is unresponsive to progesterone either alone or in the presence of oestrogen (Laidlaw *et al.*, 1995). Moreover, the growth of mammary cancer cells is inhibited by progesterone; the reasons for this distinction are also unclear (Lippman and Dickson, 1989; Elliott *et al.*, 1992). The role of progesterone in branching morphogenesis has recently been confirmed by the fact that the mammary glands of mice lacking functional progesterone receptors only develop a ductal tree (Lydon *et al.*, 1996). However, the full extent of the response by the mammary fat pad to progesterone and progesterone-stimulated epithelial growth remain to be examined.

What is clear from studies both *in vivo* and *in vitro* is that progesterone may largely act in concert with other mammogenic hormones. A primary example of this is the synergistic effect of oestrogen + progesterone on epithelial proliferation in the mature mouse mammary gland (Traurig and Morgan, 1964; Haslam, 1988c). It has been proposed that this effect is due to an upregulation of epithelial progesterone receptors by oestrogen. Such a response is not inducible in the immature gland where progesterone actually suppresses the DNA synthetic effect of oestrogen. Of various combinations of

oestrogen + progesterone administered to nulliparous heifers, a 1:250 combination induces mammary development equivalent to that seen at the 5th month of pregnancy (Sud *et al.*, 1968).

This interactive effect is also required to facilitate full lobuloalveolar development in mammary gland organ culture. Specifically, immature mice must be primed with oestrogen + progesterone for a minimum of 6 days before their mammary glands are cultured in medium supplemented with EGF, or for 9 days when EGF is absent from the medium (Vonderhaar, 1984). No development occurs *in vitro* when mice are primed with either oestrogen or progesterone alone, or when oestrogen and/or progesterone are instead added to the culture medium. This priming regimen may induce an upregulation of progesterone and EGF receptors and promote the local synthesis of transforming growth factor-α (Vonderhaar, 1988). In contrast to immature mice, older mice and immature rats may not need priming before their glands are cultured (Forsyth, 1971).

Progesterone also interacts with the mammogenic effect of prolactin to promote mammogenesis. Several reports have indicated that progesterone stimulates the growth (Edery *et al.*, 1984; Imagawa *et al.*, 1985) and morphogenesis (Darcy *et al.*, 1995) of primary mammary epithelial cells *in vitro*, where in all cases this responsiveness required the combined presence of prolactin. These hormones synergise to increase epithelial progesterone receptor levels (Edery *et al.*, 1985) and epithelial proliferation *in vitro* (Edery *et al.*, 1984) and *in vivo* (Stoudemire *et al.*, 1975). This co-stimulation by progesterone in the presence of other hormones is likely manifest within the oestrous cycle and during pregnancy when there are marked changes in the systemic temporal profiles of progesterone, oestrogen and prolactin.

1.4.1.5 The progesterone receptor (PgR)

The PgR is an intracellular receptor which can be transactivated by the ER, and consists of two ligand binding subunits which are rapidly translocated to the nucleus upon ligand binding (Haslam, 1987). Numerous studies have demonstrated that mammary epithelial cells express functional PgR both *in vitro* and *in vivo* (Edery *et al.*, 1985; Haslam and Shyamala, 1981), the levels of which can be substantially upregulated by oestrogen or the interactive effect of progesterone + prolactin (Edery *et al.*, 1985; Haslam and

Shyamala, 1979). In contrast, sialoadenectomy of mice suppresses the extent to which oestrogen induces the PgR (Sheffield and Kotolski, 1993).

By measuring cytoplasmic binding, Haslam and Shyamala (1981) found that approximately 20% of mammary PgR were present within the mammary fat pad and that, in contrast to epithelial PgR, levels of these receptors were unaffected by developmental state or exogenous oestrogen. Further studies demonstrated that the level of oestrogen-inducible PgR within the mouse mammary gland is age-dependent and increases markedly between 6 and 7 weeks of age (Haslam, 1988c). Acquisition of this oestrogen responsiveness corresponds to an increase in mammary gland growth. Autoradiographic receptor studies demonstrated a heterogeneous presence of PgR in epithelial cells at this time, where prior to this age only stromal cells bound ligand (Haslam, 1989). The significance of a distinct population of PgR in the mammary fat pad is not known, nor is their function during hormone-induced mammogenesis. There is also a subsequent decline in PgR levels in the mouse mammary gland with lactation (Haslam and Shyamala, 1981). The level of mammary PgR in pregnant ewes increases to a maximum at day 115 and declines by day 140 (Smith *et al.*, 1987).

The ability of the mammary stroma to modulate epithelial expression of PgR has been investigated by Haslam in several studies. Using a monolayer co-culture system, she demonstrated that mammary fibroblasts facilitate oestrogenic induction of PgR by fulfilling a physical requirement, possibly in the form of type I collagen (Haslam, 1988b). Further evidence for stromal regulation of PgR was provided by the finding that epithelium from immature donors acquired oestrogen-inducible PgR on transplantation to mammary fat pad tissue of mature hosts (Haslam and Counterman, 1991).

While it is likely that the mammogenic effect of progesterone is at least partly achieved by its direct action on the epithelial cell, it is also possible that progesterone acts on the mammary fat pad to elicit several other effects. A role for this type of pathway is suggested by findings from studies in other tissues which indicate that the expression of certain stroma-derived growth factors is promoted by progesterone (Koji *et al.*, 1994). Likewise, the local environment of the mammary fat pad can alter PgR status of epithelial cells and, potentially, their progesterone responsiveness (Haslam and

Counterman, 1991). The full physiological implications of this mechanism during the course of mammary gland development remains to be determined.

1.4.2 The pituitary hormones

1.4.2.1 Prolactin (Prl)

A wide body of evidence indicates a role for Prl during normal mammogenesis. Although a requirement for Prl during ductal development is unlikely, its involvement cannot be discounted. For example, ductal regression induced by hypophysectomy and ovariectomy of rats can be restricted by daily injections of Prl (Cole and Hopkins, 1962). When administered to doubly-operated mature virgin rats, Prl stimulates a small increase in ductal and alveolar DNA synthesis and markedly synergises with the effects of oestrogen or progesterone (Stoudemire *et al.*, 1975). The parenchymal morphology associated with these responses was not described. The results of more recent studies have been unable to clarify the importance of Prl during ductal development. Silberstein and Daniel (1987) reported that locally implanted homologous Prl stimulated end bud formation, whereas Kleinberg *et al.* (1990) reported only minimal growth stimulation by Prl.

The period of positive allometric mammary growth in prepubertal and pubertal heifers corresponds to increased pituitary Prl levels (Sinha and Tucker, 1969b). In contrast, other studies which have investigated the influence of liveweight gain on mammary gland development of prepubertal lambs (Johnsson *et al.*, 1985) and heifers (Sejrsen *et al.*, 1983) have reported a strong negative correlation between levels of serum Prl and parenchymal growth during this period. Reasons for this negative association are intriguing and unknown. Furthermore, administration of bromocriptine to prepubertal ewe lambs had no effect on their mammary development (Johnsson *et al.*, 1986). Although unresolved, it is likely that there is only a limited role for Prl during ductal development, particularly compared to the stimulatory effects of growth hormone.

A requirement for Prl during lobuloalveolar growth is well documented. Using triply-operated rats and local hormone implants, Lyons *et al.* (1958) established that the minimum requirement to initiate lobuloalveolar growth was oestrogen + progesterone + Prl; the inclusion of growth hormone facilitated maximum lobuloalveolar development. Prl alone cannot induce such growth, although Talwalker and Meites (1961) did

demonstrate some ductal branching in response to Prl in doubly-operated rats. Similar findings have been reported in mice, where Prl alone does not induce substantial development, but promotes development to at least the extent seen in mid-pregnancy when administered with oestrogen + progesterone (Nandi, 1958; Vonderhaar, 1984). It is also likely that the cyclic pattern of systemic Prl levels that occurs during the oestrous cycle is reflected in the development of the virgin mammary gland (Lotz and Krause, 1978). However, it has not been determined to what extent the interactive effect of Prl, oestrogen and progesterone is initiated at the local level of the mammary gland, and what proportion of this combined effect is due to feedback pathways between the pituitary gland and the ovary. Although the function of Prl in mammary cancer remains unknown, suppression of systemic Prl levels in mice markedly reduces tumorigenic risk (Welsch and Gribler, 1973) while the administration of Prl increases the incidence of mammary tumours. A further aspect that will require careful consideration is the recently demonstrated local synthesis of Prl by normal and tumorous mammary epithelium (Kurtz *et al.*, 1993; Fields *et al.*, 1993; Mershon *et al.*, 1995; Clevenger *et al.*, 1995). The physiological implications of this potentially important observation in the normal and cancerous mammary gland are yet to be detailed.

A variety of *in vitro* studies have demonstrated that Prl is required for the growth and morphogenesis of cultured epithelial cells (Ip and Darcy, 1996), and that its effects interact with those of progesterone (Imagawa *et al.*, 1985). In contrast, the growth of cultured guinea pig mammary fibroblasts is inhibited by Prl (Sheffield and Anderson, 1986). *In vitro* studies using whole gland organ culture have further indicated the requirement for Prl during mammogenesis. Such studies have shown that full lobuloalveolar development of mammary glands from steroid primed donors requires the presence of insulin + aldosterone + Prl either with or without growth hormone (Rivera, 1964; Ichinose and Nandi, 1966; Wood *et al.*, 1975). Neither ovarian or pituitary hormones can induce this response alone, and their combination requires medium supplemented with insulin. The essentiality of Prl for this development is indicated by the fact that if Prl is removed after the establishment of lobuloalveolar development, this lobuloalveolar development will fully regress (Banerjee *et al.*, 1983 cited by Vonderhaar, 1987).

The primary physiological function of Prl appears to be its role in promoting lobuloalveolar development during pregnancy. Coitus in rodents initiates twice-daily surges of systemic Prl, the levels of which remain elevated for 8-10 days (Forsyth, 1986), during which time the mammary gland undergoes extensive alveolar branching. Similar mammary development occurs in pseudopregnant rats during this period (Wrenn *et al.*, 1966). The importance of Prl in combination with oestrogen + progesterone is demonstrated by the fact that oestrogen + progesterone does not promote lobuloalveolar development when endogenous Prl is suppressed by bromocriptine (Delouis *et al.*, 1980; Schams *et al.*, 1984). In most species there is a mid-gestational increase in the level of systemic placental lactogens which may suppress Prl levels. Mammary development that occurs after this hormonal switching can be maintained by placental lactogens in these species, and to a lesser extent by Prl (Byatt *et al.*, 1994). The reduced importance of Prl for mammary growth in late pregnancy is indicated by the fact that the mammary gland of rats (Anderson, 1975a), goats (Buttle *et al.*, 1979), and sheep (Schams *et al.*, 1984) undergoes extensive development in the absence of Prl during this period, although a periparturient surge of Prl is required for full mammary differentiation and optimal milk synthesis (Akers *et al.*, 1981a, 1981b). Prl may play a more important role during this latter phase of gestational mammogenesis in those species which apparently lack a placental lactogen (Forsyth, 1986), except in pigs, where oestrogens may be of greater importance (DeHoff *et al.*, 1986).

1.4.2.2 The prolactin receptor

Prl initiates its mammogenic and lactogenic effects by binding to cell-surface Prl receptors. It is assumed that Prl receptors only exist on epithelial cells within the mammary gland as Prl does not bind to membrane preparations from cleared mammary fat pads (Bhattacharya and Vonderhaar, 1979), but does bind to isolated mammary epithelial cells (Sakai *et al.*, 1978). In another study, however, the growth of mammary fibroblasts *in vitro* was inhibited by Prl (Sheffield and Anderson, 1986), indicating that stromal cells may in fact possess Prl receptors.

The Prl receptor exists in mouse mammary tissue as two distinct forms. One is classified as the short form, a 291 amino acid protein (approximate M_r 36 kDa) having a 57 amino acid cytoplasmic domain. The second is the long form, a 592 amino acid

structure (approximate M_r 87 kDa) with extracellular and transmembrane regions very similar to the short form, but having a larger 358 amino acid cytoplasmic domain (Ormandy and Sutherland, 1993). Rat mammary tissue contains approximately 70% short and 30% long forms of the Prl receptor (Kelly *et al.*, 1991). Smith *et al.* (1993) indicated that the short form was predominant in the ruminant mammary gland. A recent report has confirmed and extended previous studies concerning the biological signalling from these different Prl receptors. Using NIH 3T3 cells containing a Prl-responsive casein-reporter construct that were transfected with either the long or short form of the Prl receptor, Das and Vonderhaar (1995) demonstrated that both receptor types transmitted the mitogenic signal of Prl, while only the long form transmitted the lactogenic signal. This latter pathway may act to increase the transcription of milk protein genes via the family of STAT transcription factors.

A number of factors have been shown to regulate Prl receptor levels within the mammary gland. Of several developmental states, Sakai *et al.* (1978) recorded the highest number of Prl receptor sites on mature virgin mouse epithelial cells; these also possessed the highest ligand affinity. Levels remained relatively low during pregnancy and increased in the first week of lactation. Similar findings have been reported in the rat (Jahn *et al.*, 1991). Characterisation of mRNA levels for the short and long forms of the Prl receptor in the rat mammary gland indicated that the relative proportions of the two receptors did not change during this time (Jahn *et al.*, 1991). In contrast to these findings in rodents, several studies in ewes (Akers, 1985; N'Guema Emane *et al.*, 1986; Smith *et al.*, 1989a) have shown that mammary Prl receptor levels are markedly increased around 100-115 days of gestation. Other factors may also regulate Prl receptor levels in mammary tissue (reviewed by Vonderhaar, 1987b). For example, Prl induces upregulation of its receptor in mammary tissue while its levels are suppressed by bromocriptine. Progesterone and EGF have also been shown to decrease the number of Prl binding sites while ovariectomy increases the number of Prl receptors.

Although recognised as a critical influence during mammary gland development and lactogenesis, a great deal remains to be understood about the mechanisms by which Prl evokes its effects. For example, it has been proposed that Prl indirectly promotes the release of fatty acids from adipocytes (Kidwell and Shaffer, 1984); these fatty acids may in turn enhance the growth of mammary epithelium. Along these lines, it has been

shown that Prl upregulates levels of lipid-dependent protein kinase C within mammary epithelial cells (Banerjee and Vonderhaar, 1992), a signalling molecule which can enhance the responsiveness of epithelial cells to mitogens such as EGF (Bandyopadhyay *et al.*, 1993). The physiological relevance of these local growth regulatory pathways and the specific involvement of Prl remains to be elucidated. Extensive interest is also being directed towards the mechanism by which Prl elicits marked alterations in milk protein transcription within mammary epithelial cells.

1.4.2.3 Growth hormone (GH)

In addition to its well established galactopoietic properties which have been exploited in dairy production (reviewed by Burton *et al.*, 1994), GH is an important regulator of mammary gland development. Studies by Flux (1957) showed that ovariectomised mice treated with daily injections of GH had increased ductal development, and that this effect could be further potentiated by oestrogen. Using triply-operated rats, Lyons *et al.* (1958) demonstrated that the duct-promoting effects of GH required the presence of either oestrogen or adrenal corticoid, and that maximum ductal growth only occurred in the combined presence of GH + oestrogen + adrenal corticoid. More recently, Kleinberg *et al.* (1990) and Feldman *et al.* (1993) showed that the stimulation of ductal development by GH involved its direct, local action via the GH receptor. These findings, together with the demonstration that GH upregulates local IGF-I mRNA (Ruan *et al.*, 1995), and that locally released IGF-I can mimic the mammogenic effect of GH (Ruan *et al.*, 1992), have led to the proposal that GH stimulated mammogenesis is mediated by local IGF-I synthesis. However, the full physiological contribution of such a mechanism during the course of mammogenesis remains to be characterised.

Administration of GH to prepubertal and pubertal ewe lambs (Johnsson *et al.*, 1986; McFadden *et al.*, 1990a) and heifers (Sejrsen *et al.*, 1986; Purup *et al.*, 1993b) results in substantial stimulation of mammary parenchymal development, although the resultant parenchymal morphology has not been described. A report by Sandles *et al.* (1987) showed that this enhanced development did not translate to increased milk yield, although other trials have shown that prepubertal heifers treated with GH have increased liveweight gain and milk production (R.J. Collier, personal communication). Systemic levels of IGF-I in ruminants are elevated by the administration of GH, as well as by a

high plane of nutrition. This latter treatment frequently corresponds to a reduction in mammary gland development, suggesting that the mammogenic effect of GH in ruminants is realised via mechanisms other than systemic IGF-I. Likewise, a direct action of GH on mammary epithelium would appear unlikely as ruminant mammary epithelial cells apparently do not bind GH (Akers, 1985; McFadden *et al.*, 1990). The local synthesis of IGF-I in response to GH is unknown, although the expression of IGF-I mRNA in mammary parenchyma after GH treatment depends upon the rate of liveweight gain (Weber *et al.*, 1996). Further studies are needed to fully determine the local expression of IGF-I (insulin-like growth factor-I) within the mammary gland and its response to GH treatment.

The role of GH in lobuloalveolar development is not fully defined. Lyons *et al.* (1958) reported that the combination of oestrogen + progesterone + Prl promoted lobuloalveolar growth in rats, although maximal development was realised only when GH was also administered. An involvement of GH in regulating lobuloalveolar growth is also suggested by the finding that the mammary glands of mature virgin mice which overexpress hGH are developed to an extent typical of that seen at days 14-15 of pregnancy (Bchini *et al.*, 1991). In whole gland organ culture, Plaut *et al.* (1993) demonstrated that GH was able to substitute for Prl as a promoter of lobuloalveolar development, although at a somewhat higher concentration (1 μg/ml). Taken together, these findings suggest that GH is involved in promoting lobuloalveolar development. This may be further supported by the fact that the administration of GH to pregnant rats (Kumaresan and Turner, 1966) and prepartum ewes and heifers (Stelwagen *et al.*, 1992; Stelwagen *et al.*, 1993) increases mammary development. The relative importance of GH during gestational mammogenesis has not been widely investigated, probably because GH levels in the maternal circulation remain relatively constant until they increase in the periparturient period.

A further finding is that progesterone, also known to promote lobuloalveolar growth, induces a dramatic upregulation of GH expression in the mammary epithelium of dogs (Selman *et al.*, 1994; Mol *et al.*, 1995). It will be interesting to elucidate the physiological significance of such a finding.

1.4.2.4 The growth hormone receptor

The mechanism by which GH initiates its effect on the developing and lactating mammary gland and the role for a GH receptor has been the subject of extensive debate. Prompting this has been the inability of several groups to demonstrate the binding of radiolabelled GH to mammary parenchyma from cows (Akers, 1985) and sheep (McFadden *et al.*, 1990b). However, other studies utilising approaches such as immunocytochemistry, *in situ* hybridisation, and Northern analysis have indicated that rat (Lincoln *et al.*, 1990; Lincoln *et al.*, 1995), bovine (Glimm *et al.*, 1990; Hauser *et al.*, 1990), rabbit, ovine and porcine (Jammes *et al.*, 1991) mammary tissue does express GH receptor protein or mRNA which localises to mammary epithelial and stromal cells. Although these findings suggest the presence of GH receptors within the mammary gland, evidence to support their physiological function is lacking. The only evidence along these lines is that GH implants can locally stimulate mammogenesis via a mechanism independent of the Prl receptor (Feldman *et al.*, 1993). The likely cellular target for GH within the mammary gland is not known. Even if epithelial cells do not possess functional GH receptors, other cell types present within the mammary gland such as fibroblasts and adipocytes can bind GH (Kelly *et al.*, 1991). Hence, these constituents may mediate the actions of GH by the local synthesis of paracrine factors such as IGF-I (Ruan *et al.*, 1995). Along these lines, the proliferation of mammary fibroblasts is stimulated by GH *in vitro* (Sheffield and Anderson, 1986). Obviously further investigation needs to be conducted before the mechanism by which GH acts on the mammary gland is resolved.

1.4.3 The placental lactogens (PLs)

The mammogenic and lactogenic effects of the placenta by its synthesis of PLs has been the subject of considerable interest. Emerging evidence indicates that there may be substantial across-species variation in both the structure and physiological role of the PLs. For comprehensive reviews of this topic the reader is referred to articles by Bremel and Schuler (1987), Thordarson and Talamantes (1987), and Forsyth (1994).

A placental influence on gestational mammogenesis was suggested by the results of several early studies. Pregnant and pseudopregnant female rats displayed similar mammary development to day 12 of pregnancy, after which only pregnant females

demonstrated continued mammary growth (Wrenn *et al.*, 1966). Furthermore, mammary development in the latter part of pregnancy was dependant upon the presence of the placenta, but not the foetal units (Desjardins *et al.*, 1968). Anderson (1975a) made a similar observation in hypophysectomised rats and demonstrated that the placenta, but not the pituitary, was required for maximal mammogenesis in the second half of pregnancy. Similarly, mammogenesis in goats (Buttle *et al.*, 1979), ewes and heifers (Schams *et al.*, 1984) during the latter half of pregnancy is largely independent of Prl or pituitary hormones. Additional evidence for placental regulation of mammogenesis is provided by the fact that a positive relationship exists between the number of offspring and milk yield in goats and sheep (Forsyth, 1986). Furthermore, several different assay systems indicated that placental extracts contained substantial lactogenic activity (Forsyth, 1967).

The PLs are members of the GH/Prl family which were initially characterised by their ability to bind to both GH and Prl receptors. It has been shown more recently that there is species diversity within this family of hormones, where PL in humans bears a high homology to GH (85%) and a low homology to Prl (27%), while PL in ruminants has higher homology to Prl (49%) than GH (28%) (Forsyth, 1994). Furthermore, whereas bovine PL is a glycosylated protein of 32 kDa M_r, the primary PL in other species is a non-glycosylated molecule of approximately 22 kDa M_r.

Two distinctive PL activities have been recorded in rodents during pregnancy. The level of a larger glycosylated PL-I (29 to 42 kDa M_r) increases from day 6 to a maximum on day 10, and rapidly declines to remain at a basal level after day 13. In contrast, the level of the smaller non-glycosylated PL-II increases on day 9 and remains elevated throughout the remainder of gestation (Thordarson and Talamantes, 1987). Levels of PL-II are also increased in response to hypophysectomy in late gestation. Plasma PL levels in sheep begin to increase around day 50 of gestation and reach maximum levels just prior to parturition (Handwerger *et al.*, 1977). In contrast, only extremely low levels of bPL are found in the maternal serum of cows across the course of gestation (Bremel and Schuler, 1987). Across a range of species, maternal serum PL levels are correlated with the number of foetuses *in utero* (Thordarson and Talamantes, 1987; Byatt and Forsyth, 1983)

Although there is convincing evidence to support a role for PL during mammogenesis and lactogenesis, the extent of its effects and the means by which it initiates these are largely unknown. Administration of recombinant bPL to steroid-treated heifers stimulated substantial increases in mammary gland development (Byatt *et al.*, 1994). Collier *et al.* (1995) suggest that bPL stimulates mammary growth via an indirect mechanism given their finding that bPL does not stimulate the growth of cultured bovine mammary epithelial cells (Collier *et al.*, 1993). PL may initiate its mammogenic effect by a mechanism similar to GH, for oPL binds with high affinity to a somatogenic site in ovine mammary tissue that is competed for by various GHs but not Prl (N'guema Emane *et al.*, 1986). One potential mechanism may involve the local synthesis of IGFs by the stromal constituents of the mammary gland. It is obvious that a great deal more needs to be understood about the specific targets of PL within the mammary gland before its mechanism of action can receive thorough investigation. Likewise, several studies have shown that PL has lactogenic effects *in vitro* (Thordarson *et al.*, 1986; Forsyth, 1994) and *in vivo* (Byatt *et al.*, 1994), although once again, the mechanism by which this response is effected remains to be fully determined.

1.4.4 Plane of nutrition

1.4.4.1 Rodents

In contrast to that in ruminants, rodent mammogenesis is positively correlated with caloric intake. Female mice fed high calorie diets to 8 and 18 weeks of age demonstrated larger mammary fat pads and increased ductal development compared to controls, while mice fed a low calorie diet had smaller fat pads and less mammary development (McFadden *et al.*, 1988). Engelman *et al.* (1993; 1994) showed that caloric restriction suppressed the proliferation of mammary epithelium in virgin mice. Furthermore, 8 weeks of peripubertal caloric restriction afforded significant protection from spontaneous tumorigenesis while tumour risk was further reduced by sustained caloric restriction. Similar findings relating tumorigenic risk to caloric intake have also shown a positive correlation between caloric intake and serum Prl levels (Sarkar *et al.*, 1982; Engelman *et al.*, 1993). Further studies indicated that total caloric intake had a greater influence on mammary tumorigenesis than the proportion of fat which contributed to dietary energy (Engelman *et al.*, 1990).

1.4.4.2 Ruminants

Rearing heifers for dairy production seeks to develop a female capable of yielding large volumes of milk; economic considerations generally make it desirable for these animals to enter the herd at the earliest possible age. An early conception may be realised by promoting high rates of prepubertal and pubertal liveweight gain. However, such gains may impair mammogenesis and subsequent milk yield potential (reviewed by Sejrsen, 1994).

Using identical twins, Swanson (1960) demonstrated that the reduced lactational performance of heifers fed a high level of concentrate was associated with an impairment of mammogenesis. Since this study, numerous reports have shown that accelerated liveweight gain during prepuberty and puberty has a detrimental effect on the subsequent milk yield from dairy heifers (Plum and Harris, 1968; Little and Harrison, 1981; Valentine *et al.*, 1987; Peri *et al.*, 1993). This effect is essentially non-reversible and remains in subsequent lactations (Swanson, 1960; Little and Kay, 1979). The milk production potential of ewes (Gould and Whiteman, 1975; Umberger *et al.*, 1985; McCann *et al.*, 1989) and beef heifers (Johnsson and Obst, 1984) is similarly compromised by high rates of liveweight gain.

The resultant effect on mammogenesis is a reduced amount of total mammary parenchyma and parenchymal DNA in heifers (Sejrsen *et al.*, 1982; Harrison *et al.*, 1983; Petitclerc *et al.*, 1984; Capuco *et al.*, 1995), ewes (Johnsson and Hart, 1985) and goats (Bowden *et al.*, 1995). The mammary gland is larger and heavier due to extensive fat deposition in the mammary fat pad which leads to a far greater volume of the gland being occupied by adipocytes. Whether these dramatic changes within the mammary fat pad directly impair the growth of the mammary parenchyma is unknown. Some recent studies have been unable to demonstrate a negative effect of excessive nutrition on mammary development in heifers (Stelwagen and Grieve, 1990; Capuco *et al.*, 1996). Reasons for the absence of such an effect are not always obvious but may reflect the experimental design, or that heifers selected for high growth rates and milk production become refractory to the negative effects of high liveweight gain on mammary development.

The exact timing at which a high plane of nutrition affects mammary gland development remains uncertain. The findings of several studies strongly suggest that these negative effects are exerted during a phase of allometric growth prior to and during the first oestrous cycles; this concurs with results from other studies showing no effect of rapid gain during isometric mammary growth in puberty (Sejrsen *et al.*, 1982; Lacasse *et al.*, 1993) or during gestation (Valentine *et al.*, 1987). Emerging evidence indicates that this effect may actually be realised much earlier than puberty at a period around weaning (S. Purup, personal communication). This suggestion is not surprising as the mammary gland is likely to become sensitive to any adverse effects of excess nutrition at the same time that it commences positive allometric growth at around 2-3 months of age.

The actual rate of liveweight gain and the composition of the diet may also determine the impact that the plane of nutrition has on mammogenesis. Based on the results from a wide range of studies, Sejrsen (1994) concluded that virgin heifers fed to an average liveweight gain in excess of 600-700 g/day are susceptible to impaired mammary development. It is suggested that the primary cause of this effect is in fact excess energy intake, and that an adequate energy to protein ratio can permit higher gains without negative effects on the mammary gland (Capuco *et al.*, 1995). Such a relationship remains to be fully assessed. Recent studies indicate that a diet high in protein does not affect mammary development (Mantysaari *et al.*, 1995; Zhang *et al.*, 1995). At a restricted rate of gain there is no effect of dietary energy concentration on subsequent mammary growth (Sejrsen and Foldager, 1992).

The mechanism(s) underlying the adverse effect of a high plane of nutrition on mammogenesis is unknown. The most likely explanation appears to be a suppression of the somatotrophic axis by a high liveweight gain, where the amount of parenchyma in the mammary gland is positively correlated to GH levels (Sejrsen *et al.*, 1983; Sejrsen and Foldager, 1992). However, the mechanisms by which GH initiates its actions are not yet known. The proposal that GH acts via systemic IGF-I is disproven by the fact that plasma IGF-I levels are elevated in heifers with a high rate of liveweight gain (Peri *et al.*, 1993; Capuco *et al.*, 1995). However, this increase may be offset by the fact that mammary epithelium from heifers fed a high plane of nutrition is less responsive to the mitogenic effect of IGF-I (Wheatley *et al.*, 1988; Purup *et al.*, 1996). Likewise, the local synthesis of IGF-I and IGF-BPs may be important considerations in this effect

(Weber *et al.*, 1996) although the actual levels of IGF-I within the mammary gland have not been reported. Furthermore, the involvement of other mammogens such as Prl and insulin, the profiles of which are also altered by plane of nutrition (Johnsson *et al.*, 1985; Sejrsen *et al.*, 1983), and growth factors locally synthesised within the mammary gland, remains to be investigated.

1.4.5 Dietary fat

1.4.5.1 In vivo studies

Dietary fat can substantially influence mammary gland development, where both the level and composition of dietary fat is reflected in the lipid composition of the mammary gland. A requirement for essential fatty acids (EFA) during mammogenesis was indicated by the results of Knazek *et al.* (1980) and Miyamoto-Tiaven *et al.* (1981). Mice fed an EFA-deficient diet from birth had impaired ductal and alveolar development, while already-established alveolar growth underwent regression if this treatment was initiated after weaning. This effect could be prevented if mice were administered s.c. injections of linoleic acid. Using epithelium transplanted to hosts fed EFA deficient diets, Faulkin *et al.* (1986) indicated that mammary growth was only suppressed when the EFA-deficient diet was initiated prepubertally and that this negative effect may be at least partly due to an impairment of ovarian function.

The level of dietary fat influences mammary development and tumorigenesis in laboratory rodents (reviewed by Welsch, 1985; 1992), and is positively correlated with the incidence of human breast cancer. Numerous studies have shown that the growth of spontaneous, chemically-induced, transplantable and metastatic mammary tumours is increased with the quantity of fat ingested and the length of time that a high fat diet is consumed. The stimulatory effect of excess dietary fat is most apparent after tumour induction, suggesting that this response is initiated at the promotional stage of tumorigenesis (Ip and Sinha, 1981). There does not appear to be a particular age relative to puberty at which the cancer-promoting effect of high fat diets is increased (Silverman *et al.*, 1989). In contrast, the response of tumours to the suppressive effect of dietary fat restriction differs in pubertal and mature mice (Ip and Ip, 1980). In mice, Welsch and O'Connor (1989) showed that normal mammary development *in vivo* and *in vitro* was increased as the fat content of the diet increased from 0 to 5 to 20%. This did

not, however, correspond to any appreciable effect of dietary fat concentration on epithelial responsiveness to the mammogenic effects of oestrogen + progesterone.

Dietary fat composition also substantially alters mammary development. Immature mice fed a diet supplemented with menhaden oil (rich in long chain n-3 fatty acids) have suppressed ductal development while a diet supplemented with corn oil (high levels of 18:1 and 18:2) promotes mammary gland growth (Welsch and O'Connor, 1989). Similar findings have been reported in studies of experimental tumorigenesis, where high levels of polyunsaturated fats enhance, and long chain n-3 fatty acids restrict, mammary tumour development (Karmali *et al.*, 1984; Abou-El-Ala *et al.*, 1988). Similar results have been recorded in sheep, where high levels of protected polyunsaturated fats fed to prepubertal ewe lambs increases the amount of mammary parenchymal tissue (McFadden *et al.*, 1990a).

1.4.5.2 Mechanisms of dietary fat influence

The means by which dietary fat influences mammary cell proliferation has been the subject of extensive investigation and has led to the proposed involvement of several different pathways at the cellular level (reviewed by Welsch, 1985; Welsch, 1987). In addition, high fat diets may act systemically to stimulate the secretion of thyroid hormone and Prl which might subsequently promote mammogenesis.

Several studies have demonstrated that the *in vitro* proliferation of normal and neoplastic mammary epithelial cells is stimulated by unsaturated fatty acids while saturated fats are inhibitory or without effect (Kidwell *et al.*, 1978; Wicha *et al.*, 1979; Beck *et al.*, 1989). Epithelial cells within the mouse mammary gland are able to utilise fatty acids sequestered from adipocytes of the mammary fat pad (Bandyopadhyay *et al.*, 1995). This corresponds to the finding that the proportions of unsaturated fatty acids in the rat mammary gland correspond to the concentration optimums required for these fatty acids to induce epithelial growth *in vitro* (Wicha *et al.*, 1979; Kidwell *et al.*, 1982). Furthermore, the proliferative effect of medium conditioned by mammary fat pad tissue on mammary epithelial cells can be simulated by unsaturated fatty acids (Beck and Hosick, 1988; Beck *et al.*, 1989). In addition, epithelial cells can influence lipid metabolism within mammary adipocytes (Elias *et al.*, 1973; Lucas *et al.*, 1976; Bartley *et al.*, 1981). Kidwell and Shaffer (1984) proposed a mechanism whereby Prl-

stimulated epithelial cells could induce the release of histamine from adjacent mast cells to act on mammary adipocytes and stimulate their liberation of free fatty acids. This is consistent with the recent immunocytochemical demonstration of histamine expression by mast cells within the mammary gland (Maslinski *et al.*, 1993). While these suggestions propose that unsaturated fatty acids from mammary adipocytes can influence the growth of the adjacent mammary epithelium, no direct evidence exists in support of such involvement within the mammary gland. Likewise, the relative importance of such a mechanism during various stages of development and its interrelationship with other growth regulatory pathways has not been widely investigated.

There are several possible mechanisms by which fatty acids may influence cell growth. One such pathway involves local prostaglandin synthesis. It has been demonstrated that mammary prostaglandin levels are elevated in rodents fed high fat diets, and that prostaglandin levels and tumour growth can be reduced by feeding the cyclooxygenase inhibitor, indomethacin (Carter *et al.*, 1983; Kollmorgen *et al.*, 1983). Likewise, rats fed diets high in n-3 fatty acids, which competitively inhibit cyclooxygenase, have lower tumour prostaglandin levels and tumorigenic risk (Karmali *et al.*, 1984). Furthermore, it has been shown that cultured rat mammary preadipocytes synthesise high levels of PGE_2 which is mitogenic for cultured mammary epithelial cells (Rudland *et al.*, 1984). Mammary epithelial cells can also process 18:2 and 20:4 fatty acids into prostaglandins (mainly PGE_2) and other eicosanoid derivatives such as hydroxyeicosatetraenoic acids. These products can enhance the proliferation of mammary cells in response to EGF *in vitro*, possibly by upregulating the cAMP-dependent protein kinase A (Bandyopadhyay *et al.*, 1987; Bandyopadhyay *et al.*, 1988; Imagawa *et al.*, 1988).

Another mechanism by which unsaturated fatty acids may promote cell proliferation is by upregulating levels of protein kinase C, a lipid dependent second messenger involved in growth factor receptor signalling (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994). Along these lines, it has been demonstrated that levels of protein kinase C within the mammary gland are developmentally and hormonally regulated (Caulfield and Bolander, 1986; Holladay and Bolander, 1986). As well as stimulating mitogenesis, unsaturated fatty acids can also modulate other aspects of mammary cell function including cell

migration (Connolly and Rose, 1993), ER function (Borras and Leclercq, 1992) and casein synthesis (Levay-Young *et al.*, 1987).

Obviously there exists the potential for adipocytes of the mammary fat pad to modulate the function of the neighbouring epithelial cells by modifying their lipid environment. The means by which lipid metabolism in the mammary fat pad is regulated, and the extent to which this subsequently modifies the response of epithelial cells to other growth regulatory influences, remains to be determined.

1.4.6 Peptide growth factors

1.4.6.1 The insulin-like growth factors (IGFs)

Structurally related to insulin, the insulin-like growth factors IGF-I and -II are single chain, 70 and 67 amino acid polypeptides of M_r 7650 and 7470 Da, respectively (Sara and Hall, 1990). While the liver is the primary source of circulating IGFs, a wide range of studies indicate that local IGF synthesis may be particularly important for the growth and differentiation of a variety of tissues (Cohick and Clemmons, 1993; Jones and Clemmons, 1995). That the expression and synthesis of IGF-I in both hepatic and local sites is upregulated by GH has led to the general belief that the actions of GH are mediated by IGF-I (Jones and Clemmons, 1995).

1.4.6.1.1 IGF-I

Numerous *in vitro* studies have demonstrated that IGF-I is a potent mitogen for primary and cell line epithelial cells derived from normal rodent (Imagawa *et al.*, 1986), ovine (Winder *et al.*, 1989) and bovine (Baumrucker and Stemberger, 1989) mammary tissue, as well as for human mammary tumour cells (Ellis *et al.*, 1994). Also, it is likely that the mitogenic effect of supraphysiological concentrations of insulin is initiated via the IGF-I receptor (Imagawa *et al.*, 1986). The ability of IGF-I to stimulate epithelial growth *in vivo* is indicated by the fact that infusions and local implants of IGF-I and des-IGF-I promote extensive ductal and alveolar development in hypophysectomised, castrated, oestradiol-treated prepubertal male rats (Ruan *et al.*, 1992) while intramammary infusion of IGF-I stimulates mammary development in pregnant cows (Collier *et al.*, 1995).

Only a limited number of reports provide information regarding the physiological role of IGF-I during mammogenesis. *In situ* hybridisation and immunocytochemistry studies have revealed that stromal cells express IGF-I in the human breast (Ellis *et al.*, 1994) and in the foetal ovine (Morgan *et al.*, 1996) and lactating bovine (Glimm *et al.*, 1988) mammary gland. These findings correspond to demonstrations that stromal cells isolated from human breast (Singer *et al.*, 1995) and pregnant bovine mammary gland (Hauser *et al.*, 1990) express IGF-I mRNA. Intriguingly, Marcotty *et al.* (1994) immunolocalised IGF-I to myoepithelial cells of pregnant and lactating rat mammary tissue. Whereas rat (Lavandero *et al.*, 1991) and bovine mammary explants synthesise and secrete IGF-I, isolated acini cultures only secrete IGF-I (Campbell *et al.*, 1991), a difference that may represent the stromal synthesis of IGF-I. Stromal IGF-I mRNA expression within the human breast is locally upregulated by normal epithelium and downregulated by tumorous epithelium (Singer *et al.*, 1995; Ellis *et al.*, 1994).

Levels of IGF-I mRNA in the rat mammary gland decrease during gestation (Marcotty *et al.*, 1994). This decline may partly reflect RNA dilution by milk protein and epithelial RNA, as IGF-I secretion by mammary explants is elevated in late pregnancy and lactation (Lavandero *et al.*, 1991). A similar pattern of IGF-I mRNA and protein expression has been shown in the pregnant pig mammary gland (Lee *et al.*, 1993). There have been no detailed reports of IGF-I mRNA or protein levels in the virgin mammary gland during the course of development.

Studies of impaired mammogenesis in ruminants fed a high plane of nutrition have found no correlation between mammary development and circulating levels of IGF-I (Capuco *et al.*, 1995). This effect may instead represent a reduced sensitivity of mammary tissue to the mitogenic effect of IGF-I (Wheatley *et al.*, 1988; Purup *et al.*, 1996). Abundance of IGF-I mRNA within the mammary parenchyma is reduced by a high plane of nutrition, an effect which interacts with that of exogenous GH (Weber *et al.*, 1996). The associated expression of IGF-I mRNA within the adjacent mammary fat pad remains to be determined. Likewise, it is not known whether the responsiveness of mammary epithelial cells to IGF-I is modulated by other locally- or systemically-derived factors.

Evidence does exist to support the proposal that locally synthesised IGF-I mediates the mammogenic effect of GH. Kleinberg *et al.* (1990) demonstrated that GH, but not Prl,

increases local IGF-I mRNA expression and mammary development in hypophysectomised, castrate male rats. In a further study using hypophysectomised, ovariectomised prepubertal female rats, Ruan *et al.* (1995) showed that oestrogen upregulates GH induced IGF-I mRNA expression within the mammary gland, and also enhances the mammogenic effects of IGF-I and des-IGF-I. This latter effect may coincide with oestrogen-induced upregulation of the IGF-I receptor (Daws *et al.*, 1996). In contrast, IGF-I mRNA expression in lactating bovine mammary parenchyma is either unaltered (Sharma *et al.*, 1994) or decreased (Glimm *et al.*, 1992) in response to exogenous GH. GH induces the expression of an immunoreactive IGF-I within the alveolar cells of lactating tissue (Glimm *et al.*, 1988), although the full nature of this response has not been examined. Overexpression of a des-hIGF-I construct in the mammary glands of lactating mice led to ductal hypertrophy, increased collagen deposition and impaired involution in the mammary gland (Hadsell *et al.*, 1996). In another study, overexpression of oIGF-I driven by the MMTV promoter stimulated mouse mammary gland development and IGF-I expression in milk (Weber *et al.*, 1996). The findings of such overexpression studies need to be carefully evaluated, however, as the mammary gland consequently becomes exposed to an extremely high level of IGF-I which is being atypically expressed by the mammary epithelium.

1.4.6.1.2 The IGF-I receptor

The IGF-I receptor is a heterotetramer which consists of two ligand-binding α subunits and two β subunits with a tyrosine kinase activity which stimulates receptor autophosphorylation (Pessin, 1994). The IGF-I receptor bears structural similarity to the insulin receptor. Post-receptor signalling may utilise several pathways including MAP kinases and the formation of phosphatidylinositol-3 phosphate (Jones and Clemmons, 1995). IGF-I has a 2- to 15-fold greater affinity for the IGF-I receptor than does IGF-II. While a truncated form of IGF-I, des-IGF-I, has a reduced affinity for the IGF-I receptor, it stimulates a greater proliferative response by mammary epithelial cells (Collier *et al.*, 1989).

Studies investigating the role of the IGF-I receptor within the mammary gland of various species show that normal and tumorous mammary epithelial cells express mRNA for the IGF-I receptor (Cullen *et al.*, 1990; Glimm *et al.*, 1992) and possess high affinity ligand

binding sites (Lavandero *et al.*, 1991; Zhao *et al.*, 1992; Collier *et al.*, 1989). A positive correlation exists between the levels of receptors for IGF-I, oestrogen and progesterone in cancerous breast tissue (Pekonen *et al.*, 1988). IGF-I receptor mRNA localises to epithelial cells in the foetal and prepubertal ovine mammary gland, its expression being greatest at 4 weeks of postnatal age (Morgan *et al.*, 1996). Specific binding of IGF-I to mammary parenchymal tissue of heifers is unaltered by exogenous GH and is slightly increased by ovariectomy (Purup *et al.*, 1995). In the rat (Collier *et al.*, 1989), ovine (Disenhaus *et al.*, 1988; Winder *et al.*, 1993) and bovine (DeHoff *et al.*, 1988) mammary gland there is an overall decrease in the number of IGF-I binding sites across pregnancy and into lactation along with a concomitant rise in IGF-I binding affinity.

1.4.6.1.3 IGF-II

The role of IGF-II during postnatal mammogenesis is even less well-defined than that of IGF-I. The primary mitogenic effect of IGF-II is likely initiated through the IGF-I receptor. Although an initial report by Imagawa *et al.* (1986) indicated that IGF-II did not stimulate the growth of cultured mouse mammary epithelial cells, subsequent studies have shown that IGF-II does, to a lesser extent than IGF-I, promote the proliferation of mouse (Riss and Sirbasku, 1987), ovine (Winder *et al.*, 1989) and bovine (McGrath *et al.*, 1991) mammary epithelium and human breast cancer cells (Ellis *et al.*, 1996b). Overexpression of IGF-II in MCF-7 cells results in an altered growth morphology, a loss of contact inhibition and their attainment of an anchorage-independent growth characteristic (Cullen *et al.*, 1992). This may correspond to the recent demonstration that overexpression of IGF-II in the mouse mammary gland induces the formation of mammary tumours (Bates *et al.*, 1995).

It is likely that IGF-II locally synthesised within the mammary gland exerts its effects via paracrine/autocrine action. Morgan *et al.* (1996) localised IGF-II mRNA to the stroma of foetal and postnatal ovine mammary tissue. A low level of IGF-II mRNA is expressed in the stroma of the human breast, where its expression is markedly upregulated in the presence of invasive breast tumours (Ellis *et al.*, 1994). This response is probably elicited by soluble factors secreted by the cancer cells (Singer *et al.*, 1995). IGF-II may also be expressed at low levels by the neoplastic breast epithelium itself (Osborne *et al.*, 1989).

Essentially nothing is known about the influence of IGF-II on the developing mammary gland. IGF-II mRNA expression in NMU-induced rat mammary tumours is reduced by ovariectomy and can be restored by hormone repletion (Manni *et al.*, 1994). Oestrogen also increases the expression of IGF-II by breast cancer cells (Westley and May, 1994). The abundance of IGF-II mRNA in foetal ovine mammary tissue increases with embryonic age and is reduced in 4-week postnatal and adult mammary tissue (Morgan *et al.*, 1996). The level of IGF-II mRNA in the pig mammary gland generally declines during gestation (Lee *et al.*, 1993).

While little is known about the function of locally expressed IGF-II during mammogenesis, it is tempting to speculate from these few reports that the local synthesis of IGF-II by the mammary stroma may be an important factor in controlling normal and neoplastic mammary development.

1.4.6.1.4 The IGF-II receptor

The IGF-II receptor is the mannose-6-phosphate receptor which is also involved in lysosomal transport (Braulke *et al.*, 1994). This receptor has a high affinity for IGF-II, a 500-fold lower affinity for IGF-I, and does not bind insulin (Jones and Clemmons, 1995). The full physiological role of the IGF-II receptor is not known although it may serve an important function in the uptake and degradation of IGF-II. This function may represent a local regulatory mechanism in breast cancer whereby the availability of extracellular IGF-II to the IGF-I receptor is controlled (Ellis *et al.*, 1996b). Expression of the IGF-II receptor in mammary tissue has only been investigated during pregnancy and lactation. Level of the IGF-II receptor within rat mammary tissue is markedly increased with pregnancy (Collier *et al.*, 1989) and is elevated in the pig mammary gland during early- and mid-pregnancy (Lee *et al.*, 1993). The number of IGF-II receptors in the pregnant bovine mammary gland is greater than for IGF-I (DeHoff *et al.*, 1988; Collier *et al.*, 1989), where the level of IGF-II receptors declines into lactation (Disenhaus *et al.*, 1988; Collier *et al.*, 1989).

1.4.6.1.5 The IGF binding proteins (IGF-BPs)

An extensive body of literature exists regarding the nature and candidate roles of the IGF-BPs (for reviews see Sara and Hall, 1990; Jones and Clemmons, 1995). Several

functions have been proposed for the IGF-BPs which include the transport of IGFs, the regulation of IGF half-life, induction of tissue specificity, and alteration of cell responsiveness to the IGFs. Given the potent mitogenic effect of the IGFs on mammary epithelial cells and the ability of the IGF-BPs to modulate this (Blum *et al.*, 1989; McCusker *et al.*, 1991), the IGF-BPs may have a role in regulating mammary gland development.

Experiments using the MCF-7 breast carcinoma cell line showed that IGF-BP2 and -3 enhanced their mitogenic response to IGF-I, an effect also evident when these cells overexpressed IGF-BP3 (Chen *et al.*, 1994). In contrast, IGF-BP4 and -5 were without effect. Manni *et al.* (1992) demonstrated that chemically-induced mammary tumours expressed mRNA for IGF-BP2, -3 and -4. *In situ* hybridisation revealed that IGF-BP2 mRNA was expressed by epithelial cells whereas IGF-BP5 and -6 was expressed in the stroma (Manni *et al.*, 1994). Expression of these IGF-BPs was also differentially regulated by ovariectomy and hormonal treatment. Oestrogen upregulates the expression of IGF-BP2, -4 and -5 by breast cancer cells while their expression of IGF-BP3 is concomitantly downregulated (Lee and Yee, 1995).

Studies by Campbell *et al.* (1991) and McGrath *et al.* (1991) demonstrated that bovine mammary epithelial cells express several IGF-BPs, two of which were identified as IGF-BP2 and -3. Furthermore, the expression of IGF-BP2 and -3 is upregulated in the presence of IGF-I (McGrath *et al.*, 1991). Upregulation of a 32 kDa IGF-BP in milk has also been reported in mice which overexpress des-IGF-I in the mammary gland (Hadsell *et al.*, 1996). The level of IGF-BP2 mRNA in the pig mammary gland doubles in late pregnancy whereas IGF-BP3 levels are depressed from early pregnancy (Lee et al., 1993).

To date, the very function of the IGF-BPs remains largely unknown. Differential expression of the various IGF-BPs by epithelial and stromal constituents suggests they may serve an important role in modulating IGF action on the mammary gland during growth and lactogenesis.

1.4.6.2 Epidermal growth factor (EGF)-like polypeptides

Epidermal growth factor (EGF) is a 53-amino acid polypeptide with a M_r of 6045 Da. This mature form is proteolytically cleaved from a 9 kDa pro-EGF derived originally from

a 1217-amino acid prepro-EGF molecule of M_r 130 kDa. Structurally related to EGF is transforming growth factor-α (TGF-α), a 5.6 kDa, 50-amino acid polypeptide that is derived from a 160-amino acid precursor. Both EGF and TGF-α initiate their biological effects through the EGF receptor (reviewed by Plaut, 1993). Two recently identified members of the EGF family that are also mitogenic for normal and neoplastic mammary cells are CRIPTO-I (Brandt *et al.*, 1994) and amphiregulin (Li *et al.*, 1992; Kenney et al., 1993; Normanno *et al.*, 1994).

The highest levels of prepro-EGF mRNA in mice are found in the submandibular glands. Relatively high levels of prepro-EGF are also expressed in the kidney and lactating mammary gland, although the translated product in these tissues either remains as a cell membrane component (Rall *et al.*, 1985), or is enzymatically degraded and excreted into the urine or milk (Brown *et al.*, 1986).

The circulating concentration of EGF derived from the submandibular gland is influenced by a variety of factors. Concentrations in the rat increase from 4 to 8 weeks of age, increasing markedly thereafter to plateau by 12 weeks (Hiramatsu *et al.*, 1994). Submandibular and plasma levels of EGF in the female mouse increase approximately fivefold during pregnancy and remain high throughout lactation (Oka *et al.*, 1991). The concentration of EGF in the submandibular gland of female mice is increased after ovariectomy and is suppressed by exogenous oestrogen. Several progestins also induce an increase in the glandular concentration of EGF. Hypophysectomised, hypothyroid and diabetic mice all possess reduced levels of submandibular EGF which can be reversed by hormonal repletion (Oka *et al.*, 1991; Hiramatsu *et al.*, 1994).

1.4.6.2.1 In vitro studies

Since their discovery, EGF and TGF-α have been widely adopted as mitogens for cultured rodent mammary epithelial cells. EGF enhances the growth of primary cells and cell lines cultured in collagen or on plastic (Imagawa *et al.*, 1985; Riss and Sirbasku, 1987) and can induce an altered cell morphology (McGrath *et al.*, 1985). The mitogenic effect also applies to neoplastic mammary epithelial cells (Stoker *et al.*, 1976) and mammary stromal cells (Smith *et al.*, 1984).

Taketani and Oka (1983) found that while EGF increased DNA synthesis by lactating mammary epithelial cells, it also induced a concomitant decrease in casein and α-

lactalbumin synthesis. This likely represents a suppressed lactogenic response to Prl (Vonderhaar and Nakhasi, 1986). On the other hand, EGF may be a specific requirement for lobuloalveolar morphogenesis, as mammary glands in organ culture can only undergo a second round of development in the presence of EGF (Tonelli and Sorof, 1980). This may represent an association of EGF action with the mammogenic effect of Prl (Plaut, 1993; Darcy *et al.*, 1995), where Prl upregulates epithelial EGF mRNA expression (Fenton and Sheffield, 1994). The requirement for female mice to be steroid primed before their mammary glands are cultured *in vitro* may serve to increase the level of TGF-α within the mammary gland for transferral to the culture environment (Vonderhaar, 1988).

1.4.6.2.2 In vivo studies

Numerous findings indicate a role for EGF during growth of the normal and neoplastic mammary gland (Plaut, 1993). Sialoadenectomised female mice exhibit less pregnancy-associated mammary development and have lower subsequent milk yields; effects which can be recovered by the daily administration of exogenous EGF (Okamoto and Oka, 1984). Sheffield and co-workers (Sheffield and Welsch, 1987; Sheffield and Kotolski, 1993) extended these observations and demonstrated that sialoadenectomy of virgin female mice abrogated ductal development and epithelial responsiveness to oestrogen + progesterone, and oestrogen + progesterone + cholera toxin. The former effect could be partially overcome by subcutaneous salivary grafts or treatment with EGF. Sialoadenectomy of females from a tumour-susceptible strain of mice markedly reduces mammary tumour incidence and increases the latency period of tumour development (Kurachi *et al.*, 1985). A recent report also indicates that neonatal administration of TGF-α has extended effects on mammary development (Hilakivi-Clarke *et al.*, 1995). Mice overexpressing TGF-α (Sakai *et al.*, 1994) or lacking a functional EGF receptor (van Haaster *et al.*, 1995) have apparently normal development, while a subsequent inability to lactate may represent a disruption in signalling from mammary Prl receptors (Sakai *et al.*, 1994).

The local effect of EGF and TGF-α within the mammary gland has been examined using slow release implants. Vonderhaar (1987a) found TGF-α to be a more potent stimulator of lobuloalveolar development than EGF in virgin mice treated with oestrogen + progesterone. In contrast to EGF, the effects of TGF-α were independent of a requirement

for ovarian steroids. A similar response to an EGF-like growth factor derived from mammary tissue of steroid-primed mice was also likely due to TGF-α (Vonderhaar, 1988). EGF implants stimulated DNA synthesis in ducts and end buds of ovariectomised females and restored their morphology (Coleman *et al.*, 1988). Similar studies by Haslam et al. (1993) demonstrated EGF-induced DNA synthesis in epithelial and stromal cells of immature glands that was independent of a requirement for ovarian steroids. In mature glands, EGF further promoted ductal side-branching compared to that stimulated by oestrogen + progesterone. In contrast, the proliferation of actively growing ducts can be directly inhibited by locally-released EGF (Coleman and Daniel, 1990).

The expression of EGF and TGF-α within the mammary gland has been examined in several studies. Messenger RNA for both EGF and TGF-α is expressed in the virgin and pregnant mammary gland, whereas only EGF mRNA is expressed in lactating tissue (Snedeker *et al.*, 1991). EGF immunolocalises to the epithelium of the inner terminal end bud and ducts, while TGF-α mRNA is expressed by the cap cells of the ductal end bud and the stromal fibroblasts around the flank of the end bud (Snedeker *et al.*, 1991; Harigaya *et al.*, 1994a). *In situ* hybridisation revealed that the expression of TGF-α mRNA in ductal and alveolar mammary epithelium in rat and human mammary tissue is increased during pregnancy and that TGF-α mRNA is also expressed by the mammary gland stroma (Liscia *et al.*, 1990). Amphiregulin and CRIPTO-I are also developmentally expressed by the stroma and epithelium of the normal mouse mammary gland (Kenney *et al.*, 1995), where higher levels in breast carcinomas localise to the tumour epithelium (Kenney *et al.*, 1996). TGF-α in tumorous breast tissue localises to both the neoplastic epithelium and the surrounding stromal tissue (Castellani *et al.*, 1994; Harigaya *et al.*, 1994b). This coincides with the demonstration that mammary epithelial, myoepithelial and tumour cells all express TGF-α *in vitro* (Smith *et al.*, 1989b; Salomon *et al.*, 1989; Cappelletti *et al.*, 1993). Both the *in vivo* and *in vitro* expression of TGF-α by mammary tumour cells is upregulated by oestrogen (Dickson *et al.*, 1986; Liu *et al.*, 1987; Bates *et al.*, 1988), indicating that TGF-α may function as an oestrogen-inducible paracrine or autocrine growth factor in the mammary gland. Lactating mammary tissue and milk also contain significant levels of EGF and TGF-α (Brown *et al.*, 1989; Smith *et al.*, 1989b); their appearance in milk is associated with the processing of EGF precursor molecules in alveolar epithelium (Jahnke *et al.*, 1994).

Only limited evidence exists concerning a role for EGF and TGF-α in ruminant mammogenesis. Several reports have demonstrated mild proliferative effects of EGF and TGF-α on bovine and ovine mammary epithelial cells *in vitro*, where TGF-α is more potent than EGF (Tou *et al.*, 1990; Winder *et al.*, 1989; Woodward *et al.*, 1994; Moorby *et al.*, 1995; Collier *et al.*, 1993). A role for these growth factors *in vivo* is suggested by the fact that intramammary infusion of EGF into heifers and ewes increases mammary gland development (McGrath and Collier, 1988; Collier *et al.*, 1993). TGF-α mRNA has been detected by RT-PCR in bovine mammary tissue (Collier *et al.*, 1993), and exists as several developmentally-regulated mRNA transcripts (Paquin Platts *et al.*, 1993; Koff and Plaut, 1995). In contrast, EGF mRNA was undetectable by Northern analysis. Correspondingly, whereas TGF-α is detectable in bovine serum by RIA, EGF is not (Paquin Platts *et al.*, 1993). Eckert *et al.* (1985) detected an EGF-like factor in extracts of bovine mammary tissue that may be distinct from TGF-α (Byatt *et al.*, 1990). Taken together, these results suggest that TGF-α and other EGF-like molecules, but not EGF, may be involved in the regulation of ruminant mammary gland development. The site of TGF-α synthesis within the ruminant mammary gland remains to be determined. A recent report by Forsyth (1996) indicates that locally synthesised amphiregulin may also play a role in regulating ruminant mammogenesis, although further studies are required to fully establish the role of this mitogen.

1.4.6.2.3 The EGF receptor (EGFR)

The mitogenic effects of both EGF and TGF-α are initiated via the transmembrane tyrosine-kinase EGFR (Kienhaus *et al.*, 1993). This receptor undergoes ligand-induced autophosphorylation; the extent to which this occurs is influenced by the proliferative state of the epithelial cell (McIntyre *et al.*, 1995). EGFRs within the virgin mouse mammary gland are present on the end bud cap cells, the ductal myoepithelium, and stromal cells adjacent to end bud and ductal epithelium (Coleman *et al.*, 1988). The EGFR is also expressed by a variety of mammary tumours and serves as a prognostic marker in breast cancer (Sainsbury *et al.*, 1987).

Expression of the EGFR and its capacity to bind EGF is regulated by a number of factors. EGFR are present on ovine and bovine mammary epithelial cells, the expression of which is elevated in pregnancy, reduced during lactation and down-regulated by GH treatment

(Spitzer and Grosse, 1987; Moorby *et al.*, 1995; Glimm *et al.*, 1992). During the course of murine development, mammary EGFR levels are greatest in mid-pregnancy and decline to low levels throughout lactation (Edery *et al.*, 1985b). Oestrogen increases the number of EGFR levels on mammary cells *in vitro* (Vanderboom and Sheffield, 1993) while oestrogen + progesterone interact to increase EGFR levels in mammary epithelium, but not stroma, derived from mature, but not immature, female mice (Haslam *et al.*, 1992; Vonderhaar, 1984; Sheffield, 1988a). Hypothyroidism is associated with a reduction in mammary EGFR levels (Vonderhaar *et al.*, 1986) while local cholesterol implants induce an upregulation of EGFR sites (Vonderhaar, 1993). Bandyopadhyay *et al.* (1993) demonstrated that cells cultured in the presence of linoleic acid had a 15% reduction in EGFR number and affinity. Furthermore, the extracellular matrix can alter the ability of EGF to down-regulate its own receptor on mammary epithelium (Mohanam *et al.*, 1988; Coleman and Daniel, 1990). Daniel and Silberstein (1987) reported that EGFR number is increased 5-fold in stromal cells adjacent to end buds compared to distal stromal cells. The progression of mammary tumours to hormone independence is associated with an increase in EGFR levels (Kienhuis *et al.*, 1993). Hence there appears a strong association between stromal influences and the local expression of EGFR. How this translates to mammary gland development in different states and in different stromal environments remains to be shown.

A substantial body of evidence indicates that EGF-like molecules are potent stimulators of mammary gland development, at least in rodent species. Their full biological function in the ruminant mammary gland remains to be characterised. It also remains to be established how EGF-like polypeptides interact with other hormonal and local influences during the course of mammogenesis. In particular, recent *in vitro* studies show that unsaturated fatty acids can substantially modulate the mitogenic actions of EGF. The relationship of this finding to mammary gland growth and the responsiveness of mammary epithelial cells to EGF at different dietary fat levels and compositions has not been investigated.

1.4.6.3 Hepatocyte growth factor (HGF)

HGF was originally purified from serum and rat platelets as a mitogen for isolated hepatocytes (Nakamura *et al.*, 1987). Identical to HGF is scatter factor which was first

identified as a mesenchymal cell-derived mitogen able to dissociate epithelial colonies (Gherardi *et al*, 1989; Rubin *et al.*, 1991). HGF is an 82 kDa heparin-binding, heterodimeric glycoprotein consisting of a 69 kDa α-subunit and a 34-kDa β-subunit (Nakamura *et al.*, 1987); it shares a 38% amino acid sequence identity to plasminogen, but lacks proteolytic activity (Nakamura *et al.*, 1989). This growth factor has subsequently been shown to increase the motility and proliferation of several epithelial cell types and is primarily synthesised by mesenchymal cells *in vitro* (Rosen *et al.*, 1994b). HGF has a potent tubulogenic effect on kidney epithelial cells (Barros *et al.*, 1995), and mRNA for HGF has been detected in a number of tissues including liver, kidney, lung, brain, and ovarian theca (Tashiro *et al.*, 1990; Maher, 1993; Parrott *et al.*, 1994). The receptor for HGF is the *c-met* oncogene product which is a transmembrane tyrosine kinase primarily expressed by epithelial cells (Rosen *et al.*, 1994a).

Unknowingly, Enami *et al.* (1983) first showed HGF to be a paracrine mitogen for mammary epithelium when they demonstrated epithelial proliferation in response to medium conditioned by mammary fibroblasts. This mitogenic activity was recently identified as HGF (Sasaki *et al*, 1994). In a further study, increasing concentrations of fibroblast CM suppressed casein synthesis by epithelial cells concomitant with increased DNA synthesis (Taga *et al.*, 1989). It was recently demonstrated that 3T3-L1 adipocytes secrete HGF which can stimulate the growth of SP1 mammary carcinoma cells (Rahimi *et al*, 1994), whereas Johnston *et al.* (1992) reported that the growth of MCF-7 cells was inhibited by a similar factor.

Several findings indicate that HGF may serve to promote mammary ductal morphogenesis. Subcloned NMuMG mammary cells grown in collagen gels formed elaborate branched ductal structures with a lumen following exposure to HGF (Soriano *et al*, 1995). HGF-induced ductal development and lumen formation was further enhanced by dexamethasone in association with increased expression of the *c-met*/HGF receptor (Soriano *et al.*, 1995). Brinkmann *et al.* (1995) found that only some of several mammary epithelial cell lines surveyed were responsive to the potent morphogenic effects of HGF. This finding may correspond to the demonstration that HGF is morphogenic for myoepithelial cells, yet only stimulates the proliferation of luminal epithelial cells (Niranjan *et al.*, 1995).

Messenger RNA for HGF is expressed as 3, 3.6 and 6 kb transcripts by mammary fibroblasts (Sasaki *et al.*, 1994) and is present in mouse mammary tissue (Soriano *et al.*, 1995). In a separate study, Northern analysis showed HGF gene expression as a single mRNA in the virgin mouse mammary fat pad, with the highest expression levels in mammary tissue of mature virgin and early pregnant females (Niranjan *et al.*, 1995). HGF mRNA has also been detected in samples of normal and tumorous human breast and is expressed by cultures of human mammary stroma (Wilson *et al.*, 1994). In further support of a paracrine action of HGF is its presence in human breast cancer tissue extracts, but not in medium conditioned by human breast cancer cells (Yamashita *et al.*, 1993). Compared to other factors, the level of HGF in breast tumour extracts is the single best predictor of relapse-free breast cancer and overall patient survival (Yamashita *et al.*, 1994). Recent findings suggest, however, that HGF may not solely function as a stroma-derived paracrine mitogen/morphogen. Two separate studies have reported that in addition to its expression by the stroma, HGF mRNA also localises to tumorous epithelium in the human breast, and occurs at a higher intensity around regions of ductal hyperplasia (Wang *et al.*, 1994; Tuck *et al.*, 1996).

Messenger RNA for the HGF receptor has been detected in both stromal and epithelial cell cultures of human mammary tissue (Wilson *et al.*, 1994), while in a separate study HGF receptors were only localised to ductal epithelium of human mammary tissue, especially in cells bordering the lumen (Tsarfaty *et al.*, 1992). *In situ* hybridisation by Tuck *et al.* (1996) confirmed that HGF receptor mRNA only localises to mammary epithelial cells. The level of mRNA for the HGF receptor within the rodent mammary gland is highest in mature virgin and early pregnant females (Niranjan *et al.*, 1995; Soriano *et al.*, 1995).

Although the physiological regulation of mammary HGF expression has not been described, there is strong evidence to suggest that such regulation occurs. Direct and indirect co-culture with epithelial cells down regulates fibroblast HGF expression (Seslar *et al.*, 1993). Furthermore, tumour cells and fibroblasts both produce soluble factors capable of either stimulating (Rosen *et al.*, 1994b) or suppressing fibroblast HGF production (Seslar *et al.*, 1995). Other peptide growth factors such as TGF-α and EGF have been shown to downregulate stromal HGF mRNA levels, but not protein synthesis (Seslar *et al.*, 1993). Expression of HGF is also upregulated by the direct interaction of

the ER complex with oestrogen response elements in the HGF gene (Liu *et al.*, 1994). Induction of kidney epithelial tubulogenesis by HGF can also be influenced by the composition of the extracellular matrix (Santos and Nigam, 1993).

Compelling evidence therefore suggests an important physiological role for HGF as a stroma-derived polypeptide able to influence normal and neoplastic mammary development. The extent to which this growth factor regulates growth and ductal morphogenesis remains to be fully understood, as does the involvement of HGF in mediating the effects of specific mammogenic hormones such as oestrogen, and the local influences of epithelial-stromal associations.

1.4.6.4 The fibroblast growth factors (FGFs)

The fibroblast growth factor family contains at least 9 identified polypeptides that are classified as members on the basis of a conserved coding sequence. The FGFs bind and interact to various degrees with high affinity tyrosine kinase receptors encoded by four separate genes; they also bind with a lower affinity to heparan sulfate receptors (reviewed by Johnson and Williams, 1993). This section will outline knowledge relating to three FGFs which have been studied within the normal mammary gland: acidic FGF, basic FGF, and keratinocyte growth factor. Other members such as FGF-3 (*int*-2) and FGF-4 will not be considered here, although their activation by viral insertion into some mouse mammary tumours has demonstrated their ability to induce substantial proliferation.

1.4.6.4.1 acidic fibroblast growth factor (aFGF)

Bovine aFGF has a molecular mass of 16 kDa and shares a 53% amino acid homology with bovine bFGF (Esch *et al.*, 1985), and a 27% amino acid homology with interleukin-1 (Gimenez-Gallego *et al.*, 1985). Like bFGF, aFGF lacks a classic signal peptide sequence typically required for secretion, leading to suggestions for an as yet unknown secretion pathway (Abraham *et al.*, 1986).

Acidic FGF stimulates the growth of collagen-embedded mouse primary epithelial cells (Imagawa *et al.*, 1994b) and cell line cultures (Riss and Sirbasku, 1987; Kotolski and Sheffield, 1992), an effect which is enhanced by supplementing cultures with heparin (Imagawa *et al.*, 1994b). No data exists regarding the mitogenic effect of aFGF on mammary epithelial cells from ruminants.

Only a few studies have investigated the regulation of aFGF expression within the rodent mammary gland. Barraclough *et al.* (1990) detected a 3.9 kb mRNA transcript for aFGF in cultured rat mammary fibroblasts and lesser expression in epithelial and myoepithelial cells. Evaluation of aFGF mRNA and protein in mouse mammary tissue revealed that levels were similar in both epithelium-free and intact mammary fat pad tissue (Chakravorti and Sheffield, 1996a), indicating that aFGF is probably stroma-derived. The abundance of aFGF mRNA increases during postnatal development and is associated with 4 immunoreactive proteins of 30, 48, 52 and 55 kDa which are differentially expressed across these states. In addition, aFGF mRNA expression in ovariectomised female mice is upregulated by treatment with oestradiol + progesterone (Chakravorti and Sheffield, 1996b). In another study, however, Coleman-Krnacik and Rosen (1994) reported highest abundance of aFGF mRNA in the immature mouse mammary gland and decreased levels in mature and pregnant tissues. The reason for these conflicting results is unclear, although the developmental pattern of aFGF expression needs to be clarified before a physiological role can be ascribed to this growth factor. The demonstration that aFGF is stroma-derived and hormonally regulated prompts the suggestion that this FGF may function as a local mediator of hormonal action within the mammary gland. Although messenger RNA for aFGF is present at high levels in cancerous breast tissues (Penault-Llorca *et al.*, 1995), there are also contrasting reports as to how this level compares to the normal state (Renaud *et al.*, 1996).

1.4.6.4.2 basic fibroblast growth factor (bFGF)

Basic FGF stimulates the growth of mammary epithelial cells from virgin and midpregnant mice within collagen gels, although they subsequently have a reduced ability to synthesise casein (Levay-Young *et al.*, 1989). The growth of bovine mammary epithelium can also be stimulated by bFGF (Sandowski *et al.*, 1993). In contrast, Smith *et al.* (1984) used an array of rat mammary cell types to demonstrate that bFGF is mitogenic for mammary fibroblasts and myoepithelial cells, but not epithelial cells. This cell-specificity correlated with the expression of cell surface receptors for bFGF (Fernig *et al.*, 1990). The cellular target for bFGF within the mammary gland is therefore not immediately obvious. *In situ* hybridisation in normal and tumorous breast

tissue has localised the FGF type 1 receptor (which binds bFGF with high affinity) to the epithelial compartment (Jacquemier *et al.*, 1994), although no distinction was made between epithelial and myoepithelial cells.

It is myoepithelial cells from rat mammary tissue that express the greatest abundance of a 5.7 kb bFGF mRNA *in vitro*; this expression was 2.5 times that in fibroblasts, and 4 to 7 times that in epithelial cells. A large proportion of the secreted bFGF was found to be associated with the extracellular matrix (Barraclough *et al.*, 1990). Gomm *et al.* (1991) immunolocalised bFGF to the myoepithelial cells of the benign human breast and to the perimeter of enlarged hyperplastic ducts, but did not detect bFGF in malignant tissue. A more extensive study in the rat by Rudland *et al.* (1993) showed that bFGF is primarily associated with myoepithelial cells and the extracellular matrix of the basement membrane in resting states, whereas in growing end buds and alveolar buds bFGF becomes associated with the epithelial cells. Basic FGF in the lactating bovine mammary gland immunolocalises to the myoepithelium and endothelium (Schams *et al.*, 1995).

Expression of bFGF mRNA in both the intact mouse mammary gland and the epithelium-free mammary fat pad is elevated during pregnancy and declines after parturition (Chakravorti and Sheffield, 1994a). This expression is associated with immunoreactive proteins of 30 and 55 kDa, the levels of which decline during late pregnancy. As for aFGF, Coleman-Krnacik and Rosen (1994) reported a different ontogenic pattern of bFGF mRNA expression within the mammary gland, where highest levels were recorded in immature virgins but with levels decreased during pregnancy. Expression of bFGF mRNA is also increased in response to treatment of ovariectomised mice with oestrogen, or oestrogen + progesterone + Prl + GH (Chakravorti and Sheffield, 1994b).

Taken together, these results suggest that the stroma and myoepithelium are principle sites of bFGF synthesis within the mammary gland, while the cellular targets of this mitogen are uncertain. Likewise, components of the extracellular matrix may be particularly important in modulating the local availability of bFGF within the vicinity of proliferating epithelium. Whether this availability is influenced by the association of epithelial cells with the surrounding stroma is unknown.

1.4.6.4.3 Keratinocyte growth factor (KGF)

KGF is a 28 kDa FGF originally isolated from embryonic lung fibroblast conditioned medium as a mitogen for keratinocytes (Rubin *et al.*, 1989). The KGF cDNA exhibits a 37 and 39% sequence identity to aFGF and bFGF cDNA, respectively (Finch *et al.*, 1989). KGF acts as a true paracrine mitogen in that it is only synthesised by mesenchymal cells (Aaronson *et al.*, 1991) and stimulates the proliferation of epithelial cells, but not fibroblasts or endothelium (Finch *et al.*, 1989). The KGF receptor (KGFR) is an alternative transcript of the FGF type 2 receptor (Miki *et al.*, 1991) that binds aFGF with high affinity, and to a lesser extent, bFGF (Bottaro *et al.*, 1990).

KGF may function as a paracrine mitogen for mammary epithelium during normal and neoplastic mammogenesis. Mouse mammary epithelial organoids and terminal end buds are responsive to the mitogenic effects of KGF *in vitro* (Imagawa *et al.*, 1994b), as are cultures of human mammary epithelium (Wilson *et al.*, 1994). Tesfayohannes *et al.* (1992) reported that KGF was mitogenic for bovine mammary epithelial cells; these results, however, must be disregarded as the cell line used in these experiments (PS-BME) was later demonstrated to be of murine origin (Woodward *et al.*, 1994). In contrast to aFGF, heparin does not modulate the mitogenic effect of KGF (Imagawa *et al.*, 1994b). KGF-induced proliferation is also negatively regulated by the lipid-activated signalling molecule, protein kinase C (LePanse *et al.*, 1994). This finding may be particularly relevant to the role for lipids in modulating epithelial responsiveness to certain mitogens within the mammary gland.

Intravenous administration of KGF to female rats induces mammary ductal neogenesis, intraductal hyperplasia and alveolar hyperplasia (Ulich *et al.*, 1994). This effect was developmentally specific as KGF did not affect the epithelium of lactating rats. KGF administered to intact and ovariectomised mice had similar hyperplastic effects (Yi *et al.*, 1994). Furthermore, KGF administered with oestrogen and progesterone induces a dilated ductal hyperplasia and produces a hyperplastic fibrosis in the periductal adipose tissue. The epithelial hyperplasia is reversed following cessation of KGF treatment while the stromal fibrosis remains (Yi *et al.*, 1994).

Finch *et al.* (1995) localised KGF mRNA expression to mesenchymal cells within the foetal mammary gland and correspondingly showed expression of KGFR mRNA in the epithelial anlage. Messenger RNA for KGF and the KGFR has also been detected in

samples of normal and tumorous human breast stroma and epithelium, respectively (Wilson *et al.*, 1994; Penault-Llorca *et al.*, 1995).

There is little information regarding the physiological regulation of KGF within the developing mammary gland. Within the rat mammary gland, expression of KGF mRNA was elevated at 6 days postpartum compared to day 20 of gestation while there was no difference in the level of KGF receptor mRNA between these states (Ulich *et al.*, 1994). Examination of mouse mammary tissues revealed that KGF mRNA was present as different transcripts across the course of postnatal development (Coleman-Krnacik and Rosen, 1994). Surprisingly these researchers did not detect any KGF mRNA in the mammary fat pad itself. Recombinant mammary glands containing KGF receptor-negative epithelium have sparse ductal outgrowths with an extremely fine architecture (G.R. Cunha, personal communication).

From the evidence at hand it appears that KGF may be an important stroma-derived paracrine mitogen involved in the regulation of mammogenesis, although the full extent of its involvement and the physiological regulation of its expression await investigation. In particular, it will be interesting to evaluate whether this growth factor is of greater relevance in the human or ruminant mammary gland where there is an increased presence of fibroblastic stroma. Along these lines, findings from studies in other tissues such as skin (Guo *et al.*, 1993; Werner *et al.*, 1994), ovary (Parrott *et al.*, 1994), seminal vesicle (Alarid *et al.*, 1994) and uterus (Koji *et al.*, 1994) indicate that KGF expression is altered in response to epithelial-stromal interaction. Furthermore, KGF is markedly up-regulated by cytokines such as interleukin-1α, and to a lesser extent by PDGF, interleukin-6 and TGF-α (Chedid *et al.*, 1994), all of which may influence mammary gland growth. Expression of KGF in the endometrial stroma of the uterus is markedly increased by progesterone, but not oestrogen (Koji *et al.*, 1994), indicating that KGF may serve as a paracrine mediator of progesterone action.

An expanse of information indicates that the growth and morphogenesis of mammary epithelium is influenced by a range of systemically- and locally-derived factors. These factors may also act together to potentiate their mammogenic effects. However, in many cases the mechanism(s) of their individual actions remains unknown. In other cases their physiological function(s) remains to be fully established before the relative

importance of their effect on the mammary gland can be assessed. Another aspect to emerge is the ability of the mammary fat pad to provide factors which either directly stimulate epithelial growth or modulate the actions of other mammogenic agents. The full implications of these actions have received limited attention.

1.5 PURPOSE AND SCOPE OF THE INVESTIGATION

The mammary gland is rather unique in that the majority of its development occurs postnatally as a process which spans several physiological states, each state being characterised by a specific pattern of cell proliferation and morphogenesis. Cell types within the mammary gland of species including humans may also be at risk of progressing to a tumorigenic phenotype during this development.

On reviewing the present understanding of mammary gland biology it becomes obvious that the mammogenic process is directed by an array of growth regulators. Historical studies identified that several key endocrine hormones were required to facilitate full development of the mammary gland. Subsequent investigations have shown that in many cases these hormones do not act directly on the epithelial cell but that their effects are instead mediated by other factors within the mammary gland.

Central to this local mediation is the matrix of the mammary gland fat pad. Even following the revelations of Hoshino that such an environment was prerequisite for epithelial development, little attention was directed to its growth regulating capacity. More recently it has been recognised that the constituents of the mammary fat pad may be key components of several different mechanisms involved in regulating normal and neoplastic mammogenesis. Such mechanisms include the mediation of systemic hormone action, the local synthesis of growth-regulating polypeptides, provision of a suitable physical matrix and modification of the lipid environment of the epithelial cell. These processes might also be influenced by the interaction between the developing epithelium and the adjacent stromal cells. The relative contributions of these mechanisms may vary across species which have a different mammary gland anatomy.

Although the integral role of the mammary fat pad is now widely appreciated, the biological significance of the aforementioned pathways is largely unknown, and has, in many cases, been difficult to elucidate. An understanding of these is of fundamental

importance to the quest to modify certain aspects of mammary gland function; a quest which may result in practical applications ranging from increased milk production by dairy animals to the prevention of breast disease in humans.

The objective of the research reported herein was to investigate the means by which the mammary fat pad regulates the process of postnatal development in the normal mammary gland. These investigations have been conducted in rodents and ruminants which differ substantially in their mammary tissue composition and parenchymal architecture, and in which the mammary fat pad may utilise different mechanisms to regulate mammary development. Common to these studies has been an examination of the mitogenic effects of the mammary fat pad in different developmental states. Analysis of these effects in relation to ontogenic state, ovarian steroid hormones and the epithelial-stromal interaction has allowed a biological significance to be ascribed to the findings of this research.

CHAPTER 2

MAMMARY FAT PAD MODULATES THE PROLIFERATIVE RESPONSE OF MOUSE MAMMARY EPITHELIAL CELLS TO SPECIFIC MITOGENS *IN VITRO*

2.1 ABSTRACT

The ability of the murine mammary fat pad to directly stimulate the growth of mammary epithelial cells and to modulate the effects of various mammogenic agents has been investigated in a newly described, hormone- and serum-free co-culture system. COMMA-1D mouse mammary epithelial cells were cultured for 5 or 7 days with various supplements in the absence or presence of explants of epithelium-free mammary fat pad from virgin female BALB/c mice. Co-cultured fat pad stimulated a 2- to 3-fold, and 6- to 8-fold increase in the amount of DNA in COMMA-1D cultures after 5 and 7 days, respectively. This effect was additive to that of 10% foetal calf serum (FCS). The effect of co-cultured mammary fat pad could not be attributed to the release of prostaglandin E_2 (PGE_2) or the synthesis of prostaglandins by epithelial cells. In addition, bovine serum albumin (BSA) attenuated ($P<0.05$) the mitogenic effect of co-cultured mammary fat pad. Added alone, insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) and insulin increased ($P<0.05$) total DNA of COMMA-1D cultures by 2.5-, 3.7-, and 2.3-fold, respectively. Co-cultured mammary fat pad markedly interacted ($P<0.01$) with these mitogens to yield final DNA values that were 21.2-, 13.3-, and 22.1-fold greater than in basal medium only. Associated with this proliferation was the formation of numerous domes above the COMMA-1D cell monolayer. There was no proliferative response to growth hormone or prolactin in the absence or presence of co-cultured fat pad ($P>0.05$). Whereas hydrocortisone alone did not alter cell number, it attenuated ($P<0.05$) the mitogenic effect of co-cultured mammary fat pad. These results indicate that the murine mammary fat pad is not only a direct source of mitogenic activity, but that a factor(s) from the mammary fat pad modulates the response of mammary epithelial cells to certain mammogens.

2.2 INTRODUCTION

Normal mammary gland development encompasses several distinct phases of epithelial cell proliferation which occur within the confines of the adipose and connective tissue matrix which comprises the mammary fat pad. Prior to and during puberty, epithelial cells advance from the mammary rudiment and ramify into the fat pad to establish a

ductal network (Imagawa *et al.*, 1994). The onset of gestation initiates the formation of lobuloalveolar structures so that by parturition the mammary fat pad is occupied by epithelial cells able to synthesise and secrete milk. This course of development is regulated by several key hormones (Topper and Freeman, 1980).

Increasing evidence suggests that the local environment of the mammary fat pad plays an integral role in controlling the proliferation of both normal and neoplastic mammary epithelial cells *in vivo*. Transplantation experiments have demonstrated that a depot of white adipose tissue such as the mammary fat pad is essential for normal mammary development (Hoshino, 1978). However, the relative importance of local growth regulatory mechanisms during mammogenesis is not well defined.

Stromal cells within the mammary gland synthesise components of the extracellular matrix to provide a physical environment for epithelial proliferation and morphogenesis (Levine and Stockdale, 1984; Keely *et al.*, 1995). The mammary fat pad is also the source of paracrine mitogens including fibroblast growth factors (Wilson *et al.*, 1994; Chakravorti and Sheffield, 1996a), hepatocyte growth factor (Sasaki *et al.*, 1994; Rahimi *et al.*, 1994), transforming growth factor-α (Snedeker *et al.*, 1991), insulin-like growth factors (Singer *et al.*, 1995), *Wnt* proteins (Weber-Hall *et al.*, 1994), and unsaturated fatty acids (Wicha *et al.*, 1979). Furthermore, the results of both *in vivo* (Shyamala and Ferenczy, 1984) and *in vitro* (Haslam, 1986) studies indicate that the proliferative effect of oestrogen on mammary epithelium may be mediated by the stromal cells of the mammary gland.

Attempts to unravel these local growth regulatory mechanisms and their biological function are hindered by the intimate association of epithelial cells with constituents of the mammary fat pad. Several investigators have utilised *in vitro* co-culture and conditioned medium approaches to recombine these components, and have demonstrated a mitogenic response by mammary epithelium to diffusible factors from the mammary fat pad (Carrington and Hosick, 1985; Beck and Hosick, 1988). These studies, however, utilised a medium that included additives such as serum, EGF and supraphysiological levels of insulin, all of which can stimulate cellular proliferation under appropriate conditions. Consequently, it is not possible to delineate whether the observed mitogenic response was directly attributable to factors from the mammary fat

pad, or whether such factors modified the responsiveness of epithelial cells to mitogens already present in the medium.

As mammary epithelial cells are less responsive to mammogenic agents *in vitro* than *in vivo* (Yang *et al.*, 1980), it was hypothesised that a diffusible factor(s) from the mammary fat pad modulates the responsiveness of epithelial cells to various mitogenic stimuli. To test this hypothesis a system of co-culturing COMMA-1D normal mouse mammary epithelial cells with mammary fat pad tissue in a hormone- and serum-free basal medium (BM) was developed. Findings indicate not only that the mammary fat pad is directly mitogenic for mammary epithelial cells, but that it also markedly potentiates the effects of specific mammogenic agents.

2.3 MATERIALS AND METHODS

2.3.1 Reagents and chemicals

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle's/Ham's nutrient mixture F12 (DME/F12, 1:1) and FCS were purchased from Gibco (Grand Island, NY and Auckland, New Zealand); Dispase and EGF were from Boehringer Mannheim (Germany). Aquasil was from Pierce (Rockford, IL). Recombinant human IGF-I was from Genentech (San Francisco, CA) and recombinant bovine growth hormone (rbGH) was from Elanco (Indianapolis, IN). All other reagents were from Sigma (St Louis, MO). Bovine serum albumin was Fraction V, fatty acid content 0.003%.

2.3.2 Cell culture

COMMA-1D mouse mammary epithelial cells were used in these experiments (Danielson *et al.*, 1984; passages 28-34). Stock cultures were propagated in growth medium comprising DME/F12 with phenol red and supplemented with sodium bicarbonate (44 mM), FCS (2%), insulin (6 μg/ml), EGF (5 ng/ml), BSA (0.3 mg/ml), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). All cultures were maintained at 37°C in a humidified 5% CO_2:95% air atmosphere.

COMMA-1D cells for proliferation experiments were harvested using Dispase and plated into 24-well culture plates (Falcon) in growth medium at either $5x10^4$ or $6x10^4$

cells/well. After 24 h for attachment, monolayers were rinsed with PBS and quiesced for 48 h in 0.5 ml defined, hormone-free BM which comprised DMEM supplemented with sodium bicarbonate (44 mM), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Preliminary studies indicated that DNA synthesis (as measured by [^{3}H]-thymidine incorporation) reached a baseline level after this period (Appendix 1).

Hydrocortisone, PGE_2 and indomethacin were prepared as ethanolic stocks. All treatments within an experiment contained the same concentration of ethanol which never exceeded 0.15%.

2.3.3 Co-cultures

All animal procedures and manipulations described in this thesis were conducted in accordance with the guidelines of the Ruakura Animal Ethics Committee. Virgin female BALB/c mice were from the Ruakura small animal colony and were maintained under standard housing and lighting conditions with *ad libitum* access to food (Diet 86, 17.8% protein, 4.1% oil, NRM New Zealand Ltd) and water. Mice were weaned at 21-23 days of age and sacrificed by cervical dislocation. The 4th abdominal mammary glands were exposed and the mammary fat pad dorsal to the supramammary lymph node aseptically excised to provide an epithelium-free mammary fat pad (DeOme *et al.*, 1959). Mammary fat pad tissue was then sectioned into 5-10 mg explants (approximately 8 mm^3) which were rinsed briefly in BM.

Co-cultures of COMMA-1D cells with epithelium-free mammary fat pad were prepared by placing an explant of fat pad tissue on a $1cm^2$ raft of sterile, siliconised lens paper (Topper *et al.*, 1975). Loaded rafts were then floated on 0.5 ml of the appropriate treatment medium added to quiescent monolayers of COMMA-1D cells (time 0). A further 0.5 ml of treatment medium was added on day 3 for both 5 and 7 day cultures. For 7 day cultures, 0.5 ml medium was replaced with 0.5 ml fresh treatment medium on day 5. Within an experiment, each replicate well represented a separate mouse, and tissue from each mouse was assigned across all co-culture treatments.

At the end of the culture period, monolayers were rinsed with PBS, trypsinised and sonicated (3 x 6 sec, Sonic Instruments). Final cell number was quantified as total DNA by a fluorometric assay (Labarca and Paigen, 1980) using calf thymus DNA as a standard.

2.3.4 Statistical analyses

Multiple treatment comparisons were conducted using the one-way ANOVA and GLM procedures of SAS (SAS, 1994). Interactions between main effects in factorial experiments were tested using the GLM procedure of SAS. Comparison of individual treatment means to time 0 values was by Student's t-test.

2.4 RESULTS

In developing this co-culture model, certain culture conditions were initially optimised. When cultured in the hormone-free BM for 5 to 7 days, COMMA-1D cells remained quiescent and did not alter in DNA yield ($P>0.05$) relative to time 0 (Figures 2.1 and 2.2). Substantial growth of COMMA-1D cells resulted ($P<0.05$) when cells were co-cultured with an explant of mammary fat pad (Figures 2.1 and 2.2). Preliminary experiments indicated that a 5-10 mg explant of mammary fat pad elicited a maximal growth response by COMMA-1D cells (Appendix 2).

The growth of COMMA-1D cells in response to co-cultured fat pad was also influenced by the medium changing routine (Figure 2.1). Maximal growth resulted after 5 days when explants were initially added to 0.5 ml BM and a further 0.5 ml BM was added on day 3. Cell growth was relatively less when fat pad explants were initially added to 1 ml BM, or when the medium conditioned by the fat pad tissue was fully replaced after 3 days. The medium changing routine adopted in subsequent experiments was as described in Materials and Methods.

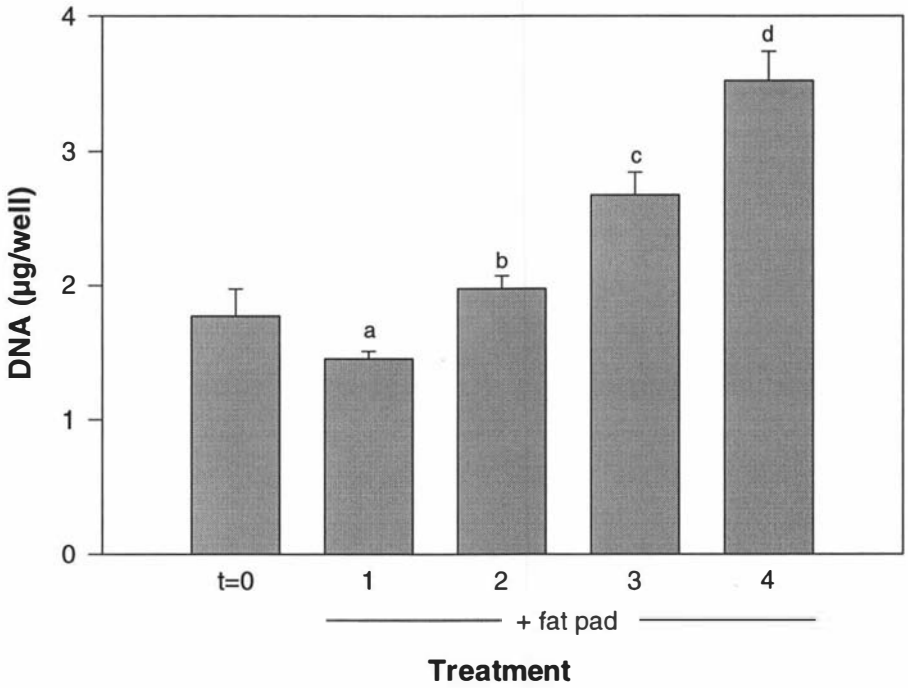


Figure 2.1 Effect of medium changing routine on the growth response of COMMA-1D cells to co-cultured mammary fat pad. The treatments were initiated at t=0 (n=4). Cells were cultured for 5 days, and medium changed as follows: 1) BM only, 0.5 ml BM with a further 0.5 ml BM added at day 3 (n=5); 2) co-culture with fat pad, 1 ml BM replaced completely on day 3 (n=4); 3) co-culture with fat pad in 1 ml BM, 0.5 ml of which was replaced at day 3 (n=3); 4) co-culture with fat pad, medium changed as per treatment 1 (n=4). Data are means ± SEM. Final DNA yield for treatment 1 was not significantly different to t=0 ($P>0.05$). ^{a,b,c,d} Means with different superscripts are significantly different ($P<0.05$).

The growth-promoting effect of co-cultured fat pad was compared to that of FCS, a supplement frequently used to maximise cell growth. DNA yield following 7 days co-culture with mammary fat pad was 6.1-fold greater than that in BM alone ($P<0.05$), and 57% of that in 10% FCS (Figure 2.2). The mitogenic effect of co-cultured fat pad was additive to, and not masked by, that of 10% FCS.

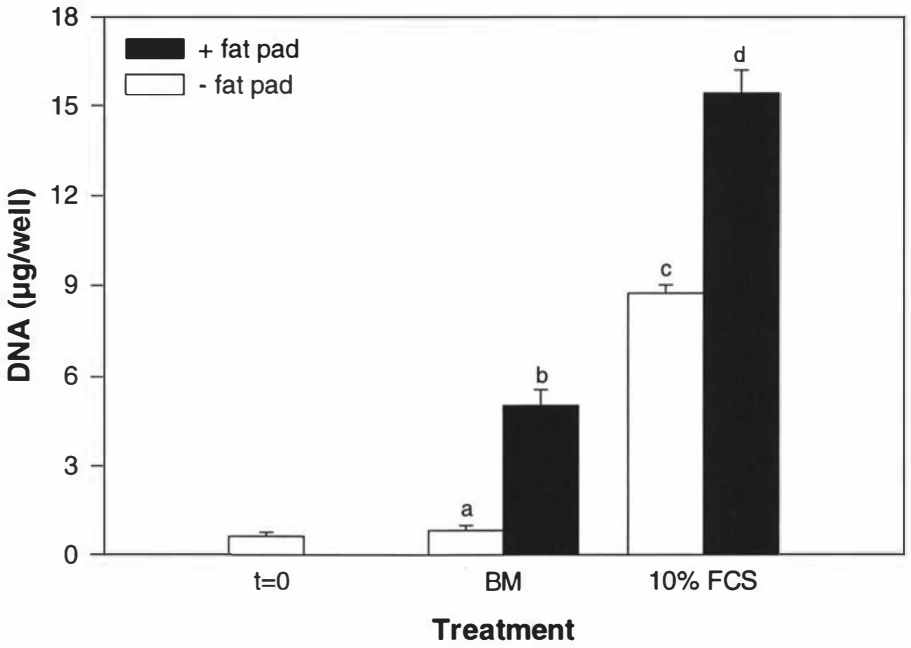


Figure 2.2 Growth of COMMA-1D cells in response to co-cultured mammary fat pad and FCS. Cells were cultured in BM or 10% FCS for 7 days in the absence or presence of an explant of mammary fat pad. t=0 represents cell number at the commencement of treatments. Data are means ± SEM (n=6). ^{a,b,c} Means with different superscripts are significantly different ($P<0.05$). Similar responses were obtained in 3 separate experiments.

As the growth promoting effect of mammary fat pad *in vitro* may be due to the release of unsaturated fatty acids (Beck *et al.*, 1989), an attempt was made to enhance this effect by including BSA as a lipid carrier. Added alone, BSA at concentrations of 50 and 100 µg/ml induced a small but non-significant (P>0.05) increase in DNA yield (Figure 2.3). In contrast, the growth promoting effect of co-cultured mammary fat pad was increasingly attenuated (P<0.05) as the concentration of BSA was raised.

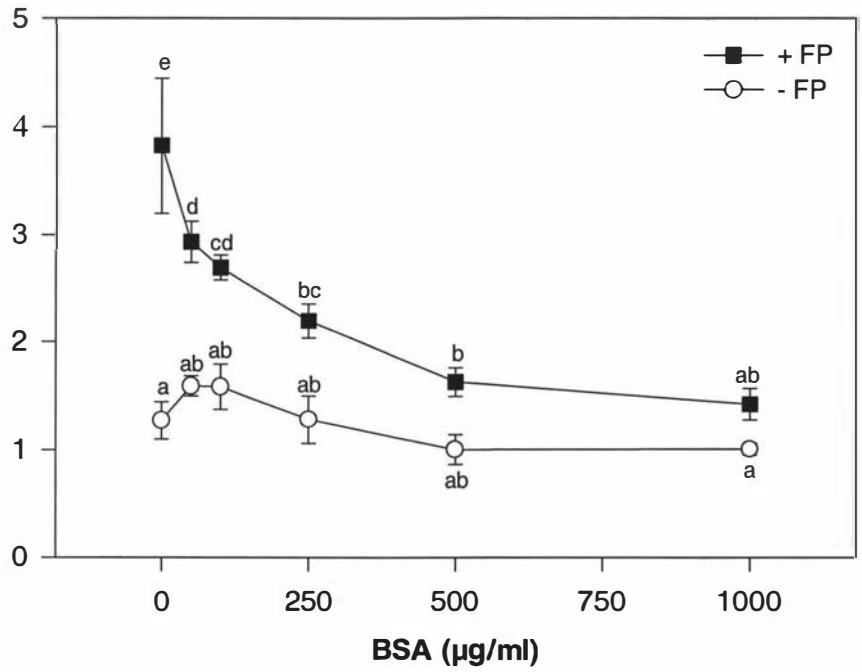


Figure 2.3 Effect of BSA and co-cultured mammary fat pad on COMMA-1D cell proliferation. Cells were cultured in the absence (○) or presence (■) of mammary fat pad tissue with various concentrations of BSA for 5 days. Data points are means ± SEM (n=3 without fat pad, n=4 with fat pad). Similar responses were obtained in 2 separate experiments. [a,b,c] Means with different superscripts are significantly different (P<0.05).

It was tested whether the proliferative effect of co-cultured fat pad could be attributed to the presence or synthesis of prostaglandins. Addition of 0.5 μg/ml PGE_2 did not mimic the effect of co-cultured mammary fat pad, nor was there a response to PGE_2 in the presence of co-cultured fat pad (Figure 2.4). Indomethacin (5 μg/ml), an inhibitor of the cyclooxygenase pathway of prostaglandin synthesis, did not attenuate ($P>0.05$) the growth promoting effect of mammary fat pad.

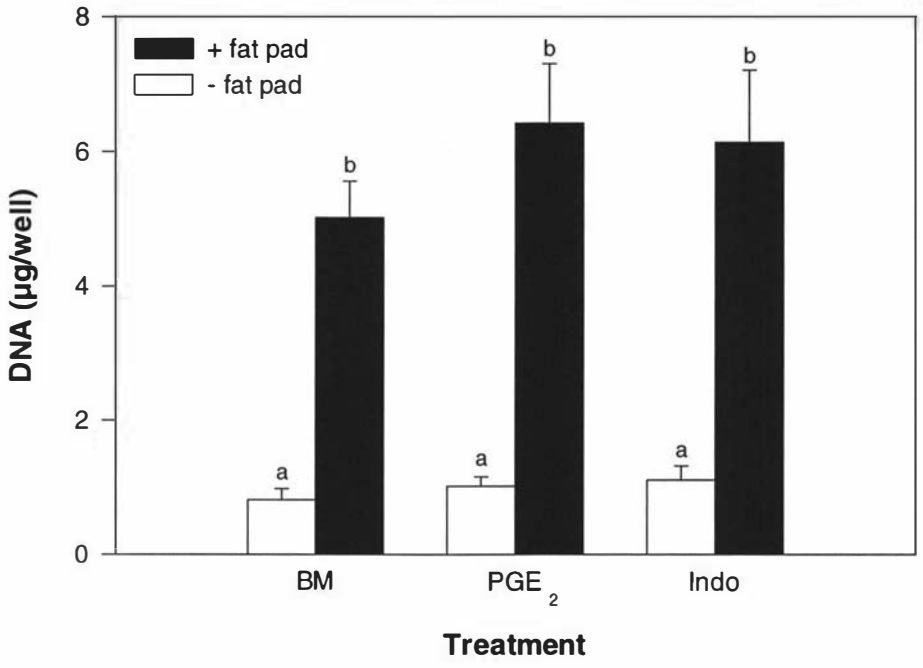


Figure 2.4 Effect of PGE_2 (0.5 μg/ml) and indomethacin (Indo; 5 μg/ml) on the growth of COMMA-1D cells. Cells were cultured for 7 days in the absence or presence of mammary fat pad explants. Data are means ± SEM (n=6). ^{a,b} Means with different superscripts are significantly different ($P<0.05$).

Having demonstrated a mitogenic effect of co-cultured fat pad on COMMA-1D cells, the ability of this activity to modulate the effects of several other mitogens was investigated. Supplementation of BM with insulin (10 μg/ml) increased ($P<0.05$) cell number by 2.3-fold while there was no response ($P>0.05$) to either prolactin or hydrocortisone (Figure 2.5). There was a significant interaction ($P<0.001$) between the effects of co-cultured fat pad and insulin, increasing final DNA yield to 22.1-fold that in BM only. There was no effect ($P>0.05$) of prolactin in the presence of co-cultured mammary fat pad in any treatment combination. In contrast, hydrocortisone significantly ($P<0.05$) attenuated the growth of COMMA-1D cells in response to co-cultured mammary fat pad in all treatments.

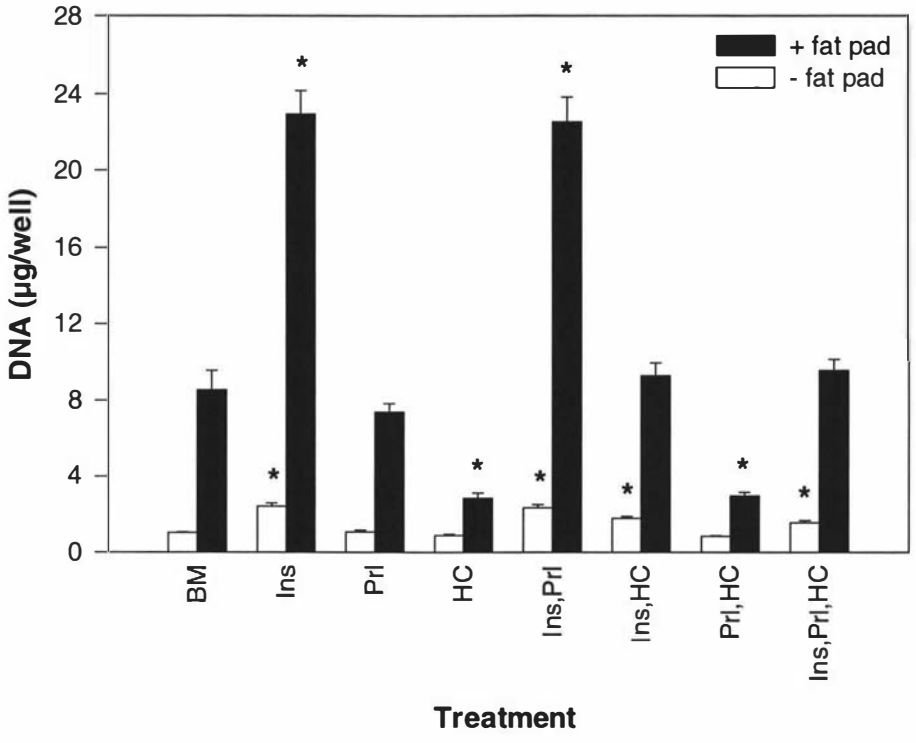


Figure 2.5 Response of COMMA-1D cells to mammogenic hormones and co-cultured mammary fat pad. Cells were cultured for 7 days in various combinations of insulin (Ins; 10 μg/ml), prolactin (Prl; 2.5 μg/ml), and hydrocortisone (HC; 2.5 μg/ml) in the absence or presence of mammary fat pad. Data are means ± SEM (n=5). *$P<0.05$ compared to the respective BM treatment.

Although growth hormone is a potent mammogen *in vivo* and in organ culture *in vitro* (Plaut et al., 1993), the final DNA level in COMMA-1D cultures was unaltered ($P>0.05$) by rbGH (1 μg/ml) in either the absence or presence of co-cultured mouse mammary fat pad (Figure 2.6). This concentration of rbGH has been previously shown to stimulate the proliferation of murine mammary epithelium in organ culture (Plaut *et al.*, 1993).

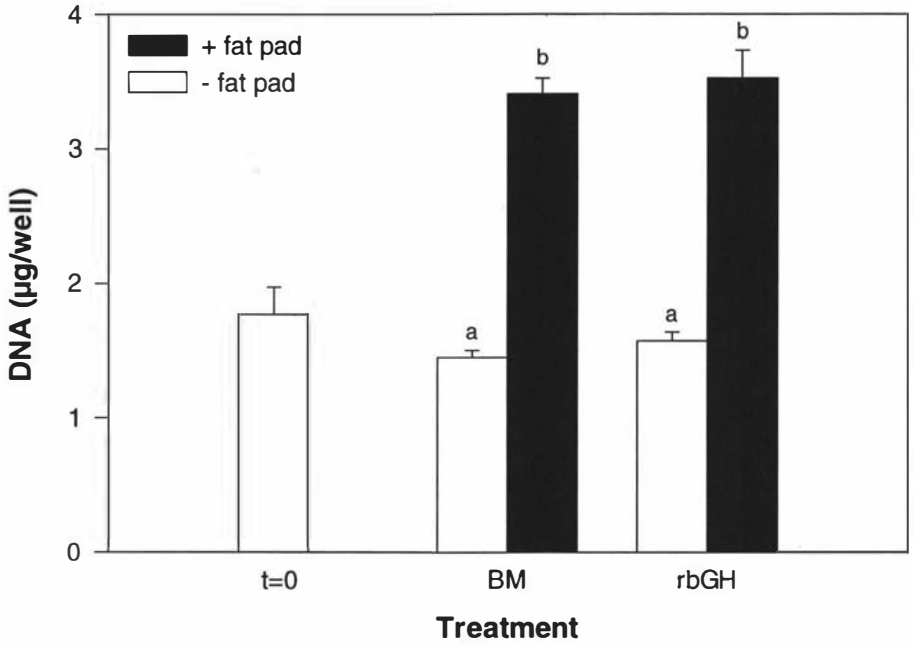


Figure 2.6 Effect of rbGH (1 μg/ml) on COMMA-1D cell growth in the absence or presence of co-cultured mammary fat pad. Cells were cultured for 5 days in BM alone or BM supplemented with rbGH. Data are means ± SEM (n=5). Results are representative of those obtained in 2 separate experiments. t=0 represents cell number at the initiation of treatments (n=4). ^{a,b} Means with different superscripts are significantly different ($P<0.05$).

A number of reports have demonstrated that the *in vivo* and *in vitro* proliferation of mammary epithelial cells is stimulated by IGF-I and EGF. Supplementation of BM with IGF-I, EGF, or IGF-I + EGF increased ($P<0.05$) final cell number by 2.5-, 3.7-, and 4.6-fold, respectively (Figure 2.7). Co-culture of COMMA-1D cells with mammary fat pad increased DNA yield by 6.8-fold. There was a significant interaction ($P<0.001$) between the effects of mammary fat pad and both IGF-I and EGF whereby final DNA yields were 21.2- and 13.3-fold that in BM alone. Final cell number in the combined presence of

IGF-I + EGF and mammary fat pad was the additive response to the individual growth factor interactions.

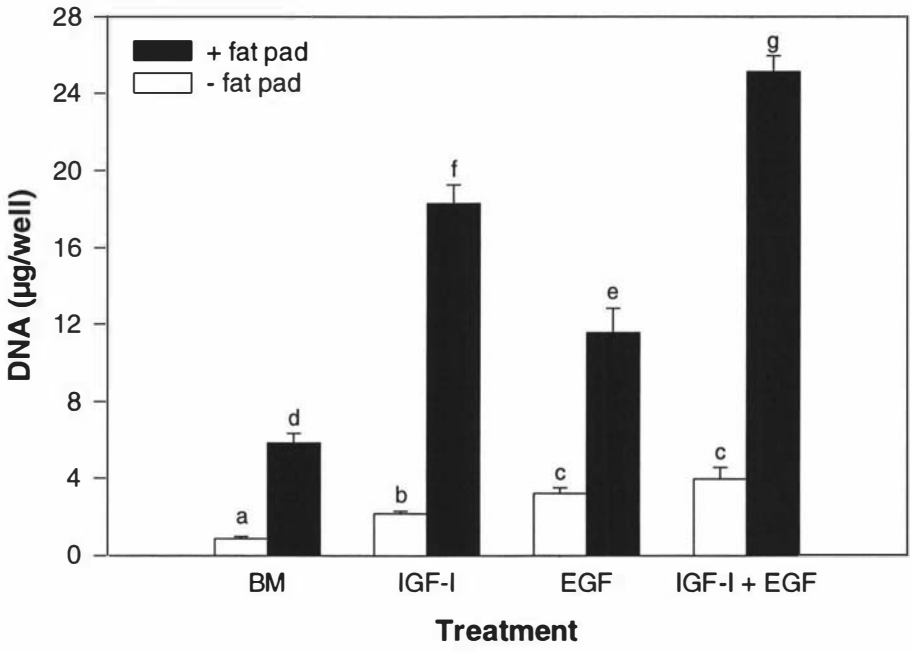


Figure 2.7 Response of COMMA-1D cells to growth factors and co-cultured mammary fat pad. Cells were cultured for 7 days in BM, IGF-I (75 ng/ml) and/or EGF (25 ng/ml) in the absence or presence of mammary fat pad. Data are means ± SEM (n=5). Results are representative of those obtained in 4 separate experiments. [a,b,c] Means with different superscripts are significantly different ($P<0.05$).

Figure 2.8 depicts photomicrographs of COMMA-1D cells cultured for 5 days in these treatments. Cells cultured in BM either alone or supplemented with IGF-I + EGF displayed similar morphology at their respective final density (Figures 2.8a and 2.8b). Co-culture with mammary fat pad alone induced subconfluent cells to assume a more diffuse morphology (Figure 2.8c). The marked proliferative response to IGF-I + EGF in co-culture was associated with a high final density of cells with a typical, cobblestone-like morphology (Figures 2.8d and 2.8e). These cultures were also characterised by the formation of numerous multicellular domes above the confluent monolayer (Figures 2.8d and 2.8e). The growth of cells in this treatment may not be contact-inhibited as it has been observed that cells overgrow onto zones of already confluent cells after longer culture periods. The raised domes were not evident on monolayers cultured in 10% FCS for the same period (Figure 2.8f).

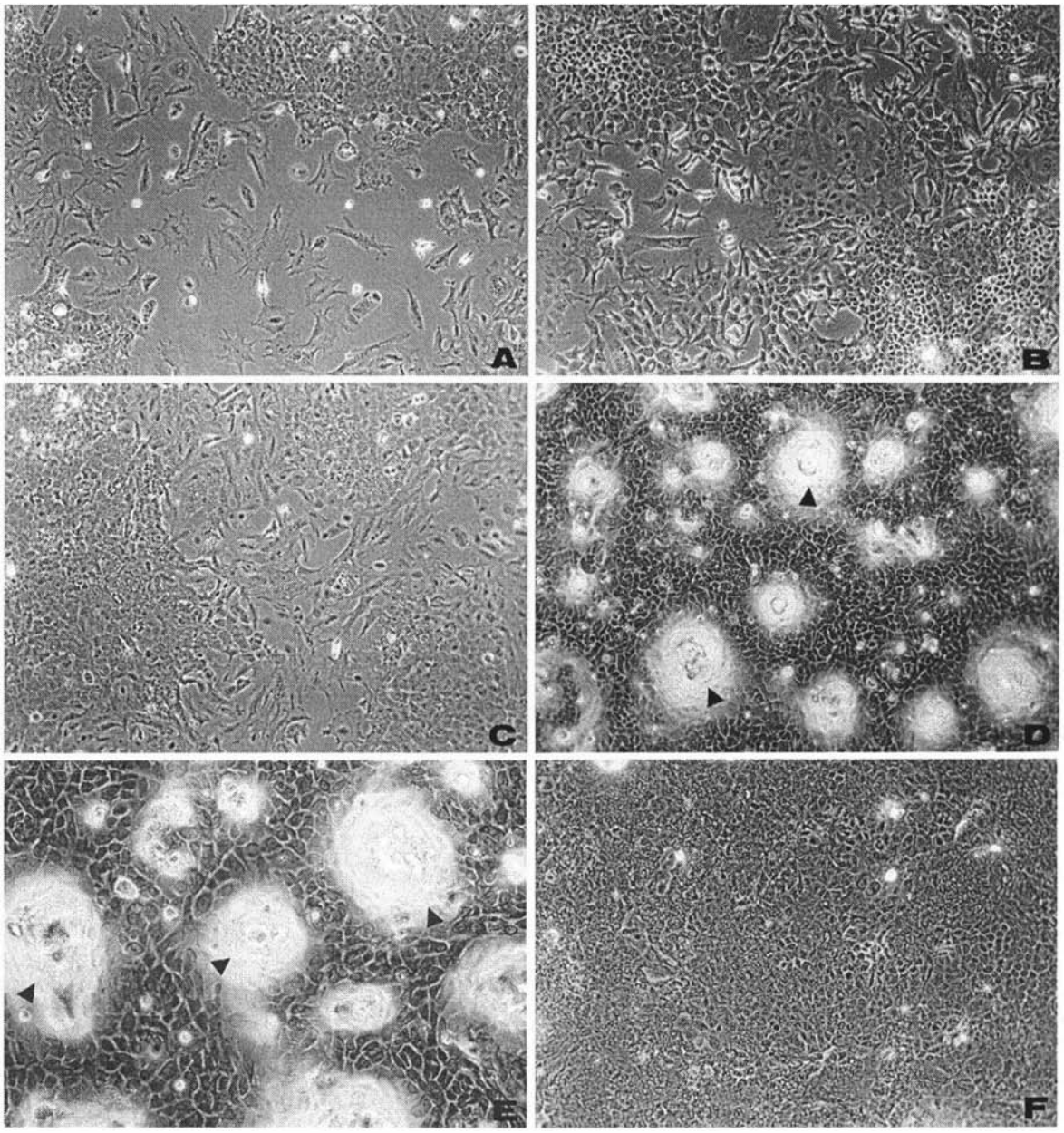


Figure 2.8 Phase contrast photomicrographs of COMMA-1D cells after 5 days culture in various treatments. Cells were cultured in BM alone (A, 250x); BM supplemented with IGF-I (75 ng/ml) + EGF (25 ng/ml) (B, 250x); BM in the presence of co-cultured fat pad (C, 250x); BM plus IGF-I + EGF in the presence of co-cultured fat pad (D, 250x and E, 500x); or BM supplemented with 10% FCS (F, 250x). Arrows indicate domes in co-cultures supplemented with IGF-I + EGF (D and E).

2.5 DISCUSSION

Previous *in vitro* studies using explants of mammary fat pad embedded in collagen (Carrington and Hosick, 1985) and medium conditioned by mammary adipose tissue (Beck *et al.*, 1989) have demonstrated that the mouse mammary fat pad is the source of a diffusible mitogenic factor(s). However, the requirement for the medium used in those studies to be supplemented with either serum or EGF and high concentrations of insulin left the question as to whether the mammary fat pad was the source of a direct-acting mitogen, or whether a factor(s) from the mammary fat pad was modulating the action of other mitogenic supplements. Here it has been demonstrated that a factor(s) from the mammary fat pad is both directly mitogenic for mammary epithelial cells and also markedly enhances the proliferative effect of specific mammogens.

The nature of the mitogenic activity derived from the murine mammary fat pad, and of that which modulates the effect of other specific mitogens, remains to be elucidated. Beck *et al.* (1989) ascribed the growth promoting effect of mammary adipose conditioned medium to adipocyte-derived unsaturated fatty acids. Unsaturated fatty acids have been shown to stimulate the proliferation of mammary epithelial cells *in vitro*, while saturated fatty acids inhibit cell growth (Wicha *et al.*, 1979). In these experiments a 2- to 8-fold increase in cell number in response to co-cultured mammary fat pad was consistently recorded. The additivity of this effect and that of FCS suggests that the mitogenic activity from the mammary fat pad is either absent from FCS or that it is only present in FCS at suboptimal concentrations. That the effect of co-cultured fat pad may be due to its liberation of unsaturated fatty acids coincides with the finding that relative to rat serum, FCS-supplemented medium does not support the growth of mammary tumour cells *in vitro* due to a deficiency of linoleic acid (Kidwell *et al.*, 1978).

In an attempt to increase the mitogenic effect of mammary fat pad, co-cultures were supplemented with BSA as a carrier of free fatty acids (Nilausen, 1978). Surprisingly, increasing concentrations of BSA attenuated the response to co-cultured fat pad in a dose-dependent manner. One explanation for this response may be that excess BSA competed for free unsaturated fatty acids to make them unavailable for cellular utilisation (Spector, 1986). Likewise, there may be discrete differences in the way that

BSA modulates the effects of certain mitogens, as the growth of COMMA-1D cells in response to EGF differs in the presence of ovalbumin and BSA (Riss and Sirbasku, 1987).

Unsaturated fatty acids may be further processed into eicosanoid derivatives such as prostaglandins to subsequently stimulate mammary cell proliferation (Rudland *et al.*, 1984). As PGE_2 did not simulate the effect of co-cultured mammary fat pad, and the cyclooxygenase inhibitor indomethacin did not abolish the effect of mammary fat pad in these experiments, the influence of prostaglandin derivatives in this response is unlikely.

The growth of COMMA-1D cells in response to BM supplemented with IGF-I, EGF and insulin was similar to that previously reported for both primary and cell line mammary epithelial cells *in vitro* (Imagawa *et al.*, 1982; Riss and Sirbasku, 1987). It is likely that the concentration of insulin used here mimicked the effects of IGF-I (Imagawa *et al.*, 1986). Of specific interest, however, was the marked interaction that occurred between the proliferative effects of these mitogens and a factor(s) from co-cultured mammary fat pad.

Recent findings indicate that unsaturated fatty acids and their hydroxy fatty acid derivatives enhance the mitogenic effect of EGF on mammary epithelial cells (Bandyopadhyay *et al.*, 1987; Bandyopadhyay *et al.*, 1988) and 3T3 fibroblasts (Eling and Glasgow, 1994), an effect which involves increased intracellular signalling through an upregulation of protein kinase C (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994). It has not been reported whether unsaturated fatty acids can amplify the intracellular signals initiated by IGF-I in mammary epithelial cells. Given that the insulin and IGF-I receptors, similar to the EGFR, possess protein-tyrosine kinase activity (Pessin, 1994), it is possible that exogenous unsaturated fatty acids incorporated by the mammary epithelial cell could also enhance the mitogenic actions of other growth factors. The observation that hydrocortisone suppressed the effect of mammary fat pad and its interaction with insulin may further implicate unsaturated fatty acids in these responses as another glucocorticoid, dexamethasone, suppresses cellular protein kinase C activity (Zor *et al.*, 1990) and also inhibits EGF-induced proliferation and prostaglandin formation in BALB/c 3T3 fibroblasts (Nolan *et al.*, 1988).

In contrast, co-culture with mammary fat pad did not facilitate a response to other recognised mammogenic factors such as prolactin, rbGH or PGE_2. The reason

underlying this specificity is unclear. It may be that the modulatory effect of the mammary fat pad is restricted to certain mitogenic pathways, or that response to the latter agents in co-culture requires additional influences such as an appropriate extracellular matrix (Salomon *et al.*, 1981).

However, it is also possible that the stimulatory effect of co-cultured mammary fat pad recorded in these experiments is due to other paracrine mitogens derived from the mammary fat pad. Hepatocyte growth factor is mitogenic for mammary epithelial cells and is secreted by cultured mammary fibroblasts (Sasaki *et al.*, 1994) and adipocytes (Rahimi *et al.*, 1994). The mouse mammary fat pad is also a source of acidic- and basic-fibroblast growth factor (Chakravorti and Sheffield, 1996), both of which are mitogenic for COMMA-1D cells (Riss and Sirbasku, 1987). The stimulatory effect of these mitogens, however, is typically additive to, and not interactive with, the effects of IGF-I and EGF (Rahimi *et al.*, 1994; Enami *et al.*, 1987; Riss and Sirbasku, 1987).

This investigation of the mitogenic capacity of the mammary fat pad has been facilitated by the use of the COMMA-1D cell line cultured in a hormone- and serum-free medium. COMMA-1D cells cultured under similar conditions have been used to bioassay the mitogenic effect of tissue extracts as well as that of various hormones and growth factors (Riss and Sirbasku, 1987). This cell line exhibits characteristics of normal mammary epithelium including an ability to synthesise casein *in vitro* (Eisenstein and Rosen, 1988) and to form both ductal and alveolar structures when inoculated into cleared mouse mammary fat pads (Danielson *et al.*, 1984). It has also been observed that NMuMG mammary epithelial cells demonstrate similar synergistic responses to co-cultured mammary fat pad and growth factors (Chapter 3).

Utilising COMMA-1D cells, a simple and inexpensive co-culture system has been developed for studying epithelial-stromal interactions whereby explants of tissue such as mammary fat pad are floated at the gas:medium interface above a cell monolayer cultured on plastic or other substrata. Siliconised lens paper has been widely used in whole mammary gland organ culture (Plaut *et al.*, 1993) to permit the bi-directional diffusion of soluble factors between culture medium and the floated tissue. An alternative system incorporates a wire grid to support explants at the gas:medium interface (Levay-Young *et al.*, 1987). However, the potential sedimentation of non-adherent cells from tissue explants onto the monolayer under study would likely

confound cell growth assays. No such sedimentation onto bare plastic has been observed when using siliconised lens paper. This approach also allows the cellular components of the mammary fat pad to remain arranged in an *in situ* form and may more accurately represent intercellular associations that occur within the mammary fat pad *in vivo*. This contrasts to other systems where isolated epithelial and fibroblast cells are co-cultured directly together (Wang and Haslam, 1994) or in compartments separated by a permeable membrane (Ip and Darcy, 1996).

The present findings demonstrate that the murine mammary fat pad is the source of a diffusible mitogenic activity which markedly enhances the proliferation of mammary epithelial cells in response to certain mitogens. Given that growth factors such as IGF-I and EGF have been implicated in stimulating both normal mammary development and mammary tumorigenesis (Ruan *et al.*, 1995; Coleman *et al.*, 1988; Dickson and Lippman, 1995), the regulation of their mitogenic effect by the mammary fat pad may represent an important local growth regulatory mechanism within the mammary gland. The nature of this activity and the role it serves during mammogenesis will be the focus of future investigation.

CHAPTER 3

MOUSE MAMMARY FAT PAD SPECIFICALLY REGULATES GROWTH FACTOR-INDUCED PROLIFERATION OF MAMMARY EPITHELIAL CELLS *IN VITRO*

3.1 ABSTRACT

The objective of this study was to characterise the ability of the mouse mammary fat pad to stimulate the growth of mammary epithelial cells and to modulate their responsiveness to polypeptide growth factors. Treatment of COMMA-1D cells with various concentrations of insulin-like growth factor (IGF)-I, epidermal growth factor (EGF) or insulin in a defined, hormone-free basal medium (BM) stimulated small increments of growth. In contrast, these mitogens stimulated marked proliferation in the presence of co-cultured mammary fat pad. Final DNA levels increased asymptotically in response to increasing concentrations of IGF-I, EGF and insulin to realise maximum values that were 45-, 29-, and 24-fold that in BM only, respectively. Using medium conditioned by mammary fat pad tissue (conditioned medium; CM) it was shown that the mitogenic and growth factor-modulating effects of the mammary fat pad were due to a diffusible factor(s), the mitogenic effect of which was additive to that of 10% FCS. The enhanced growth of COMMA-1D cells in response to IGF-I and EGF in CM could not be ascribed to an increase in the number of cell-surface receptors for these ligands; cells cultured in CM plus various mitogens consistently bound less IGF-I while EGF binding was unaltered or reduced by CM in these same treatments. Furthermore, potentiation of mitogenic stimulation by the mammary fat pad is apparently specific for certain growth factors as COMMA-1D responsiveness to fibroblast growth factors (FGFs) and IGF-II was essentially unaltered in the presence of mammary fat pad-derived activity. Whereas several different cell types grew in response to co-cultured mammary fat pad alone, only some cell lines, in particular normal mammary epithelial cell lines, demonstrated additional interactive growth responses to IGF-I and EGF. These findings indicate that the mammary fat pad may be an important regulator of mammary epithelial cell growth, particularly by its provision of a diffusible activity that markedly enhances the mitogenic effects of certain growth factors.

3.2 INTRODUCTION

The postnatal proliferation of mammary epithelial cells within the stromal matrix of the mammary fat pad is influenced by a variety of systemic and local factors (Imagawa *et*

al., 1994). Classical experiments firmly established that several key hormones are required to achieve normal and fully functional mammary gland development (Nandi, 1958). More recent findings suggest that polypeptide growth factors, particularly those of local derivation, may also function to regulate this development (Imagawa *et al.*, 1994). Specific attention has been given to the role that these growth factors may serve in locally mediating the actions of systemic hormones during normal and neoplastic mammary gland growth (Dickson and Lippman, 1995).

Growth factors from several families have been implicated as paracrine and/or autocrine mitogens for mammary epithelial cells *in vivo.* The IGFs (IGF-I and -II) are potent mitogens for normal and neoplastic mammary epithelial cells *in vitro* (Lee and Yee, 1995) and are expressed by constituents of the mammary stroma (Ellis *et al.*, 1994; Chapter 9). Furthermore, the mammogenic effect of both growth hormone and oestrogen may be locally mediated by IGF-I (Ruan *et al.*, 1995), while the stromal expression of IGF-II may be particularly responsive to local epithelial influence (Singer *et al.*, 1995).

Members of the EGF family such as EGF and transforming growth factor-α (TGF-α), have a recognised mitogenic effect on mammary epithelium *in vitro* (Plaut, 1993) and exert pronounced effects on the normal and tumorous mammary gland *in vivo* (Kurachi *et al.*, 1985; Vonderhaar, 1987). The cellular expression of EGF and EGF-like factors within the mammary gland (Liscia *et al.*, 1990; Snedeker *et al.*, 1991) suggests they may act via both paracrine and autocrine pathways. Furthermore, an upregulation of TGF-α within the mammary gland may account for the combined mammogenic effect of the ovarian steroids, oestrogen and progesterone (Vonderhaar and Plaut, 1992).

Several reports have demonstrated that the FGFs are mitogens for mammary epithelial cells *in vitro* (Levay-Young *et al.*, 1989; Imagawa *et al.*, 1994b) and are expressed within the mammary gland (Rudland *et al.*, 1993; Chakravorti and Sheffield, 1996a). Although their precise physiological role has not been established, FGF expression within the mammary gland is regulated by several mammogenic hormones (Chakravorti and Sheffield, 1996b; Chapters 10 and 11) and is altered during mammary tumorigenesis (Penault-Llorca *et al.*, 1995).

While these growth factors are potent mitogens for mammary epithelial cells *in vitro*, evidence suggests that their effects *in vivo* may be modulated by other soluble and

physical factors within the mammary gland. For example, the mitogenic effect of basic FGF is regulated by heparan sulfate proteoglycans that form part of the extracellular matrix (Blum *et al.*, 1989a; Barraclough *et al.*, 1990). Furthermore, mouse mammary epithelial cells grow in close association with adipocytes of the mammary fat pad and are exposed to a local enrichment of fatty acids (Bandyopadhyay *et al.*, 1995) which may increase the responsiveness of mammary epithelial cells to the proliferative effect of EGF (Bandyopadhyay *et al.*, 1988; Sylvester *et al.*, 1994).

Further evidence for such growth modulation within the mammary gland was recently obtained whereby the proliferation of mouse mammary epithelial cells in response to insulin, IGF-I and EGF was markedly enhanced in the presence of co-cultured mammary fat pad (Chapter 2). Given this finding and the likely role for these and other growth factors during mammogenesis, the objective of this study was to further characterise the specificity and mechanisms of this *in vitro* effect of the mammary fat pad. Here it is reported that the mammary fat pad enhances the responsiveness of mammary epithelial cells to specific growth factors via a diffusible factor, and that this mechanism of growth regulation may be particularly relevant to epithelial proliferation within the mammary gland.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle's/Ham's F12 (DME/F12, 1:1) and foetal calf serum (FCS) were from Gibco (Grand Island, NY). Dispase was from Boehringer Mannheim (Germany). Na-^{125}I was obtained from NEN (Wilmington, DE). Aquasil and Iodo-Gen were from Pierce (Rockford, IL). Recombinant human IGF-I was from Genentech (San Francisco, CA), recombinant human IGF-II was from Eli Lilly (Indianapolis, IN) and recombinant human keratinocyte growth factor (KGF) was from R and D Systems (Minneapolis, MN). All other reagents were purchased from Sigma (St Louis, MO). Acidic- and basic-FGF were from bovine brain and mouse EGF was tissue culture grade.

3.3.2 Cell culture

COMMA-1D normal mouse mammary epithelial cells used in these experiments have been characterised previously (Danielson *et al.*, 1984). Cells were routinely maintained in growth medium comprising DME/F12 supplemented with FCS (2%), EGF (5 ng/ml), insulin (6 μg/ml), bovine serum albumin (BSA; 0.3 mg/ml), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Other cell lines used were L6 myoblasts, HeLa human cervical carcinoma, Wi-38 human embryonic lung fibroblasts, C127 mouse mammary tumour, NLFK feline kidney epithelium, and NMuMG normal murine mammary epithelial cells. All cell lines were maintained in the same growth medium as COMMA-1D cells and were passaged weekly.

Cells for proliferation experiments were seeded into 24-well plates (Falcon) for 24 h after which they were rinsed with PBS and quiesced for a further 48 h in 0.5 ml of defined, hormone-free BM comprised of DMEM supplemented with sodium bicarbonate (44 mM), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Treatments were then applied to the appropriate cultures. At the end of the culture period cell monolayers were rinsed with PBS and trypsinised. Cell suspensions were sonicated and DNA content measured by fluorimetry (Labarca and Paigen, 1980) using calf thymus DNA as a standard.

3.3.3 Co-cultures and conditioned medium

Virgin female BALB/c mice were weaned at 21 days of age and then sacrificed by cervical dislocation. Mammary fat pad tissue devoid of endogenous epithelium (DeOme *et al.*, 1959) was excised from the fourth abdominal mammary glands and diced into 5-10 mg explants (approximately $8mm^3$). COMMA-1D cells were co-cultured with mammary fat pad tissue by floating an explant on lens paper above COMMA-1D monolayers as described (Chapter 2). Co-cultures were incubated in 0.5 ml of treatment medium for the first 3 days with a further 0.5 ml treatment medium added for the final 2 days.

To prepare CM, mammary fat pad tissue was sectioned into explants (5-10 mg) which were rinsed in BM and then incubated in BM (7.5 mg tissue/ml) for 48 h. Control BM was incubated for 48 h in the absence of mammary fat pad tissue. CM and BM were

collected, filtered (0.2 μm) and added to cultures (1 ml/well) for 3 days. All supplements were added to CM and BM immediately prior to their use in cultures.

3.3.4 Ligand binding assays

IGF-I (2 μg) and EGF (2 μg) were radiolabelled with 500 μCi Na-^{125}I using the Iodo-Gen procedure (Fraker and Speck, 1978). COMMA-1D cells which had been plated (1 x 10^5 cells/well) and quiesced as above were cultured for 3 days in either CM or BM (1 ml) supplemented with various treatments. Treatment media were then aspirated and monolayers rinsed 3 times with ice-cold assay buffer (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, 10 mg/ml BSA). Monolayers were incubated with radiolabelled IGF-I (7.2 x 10^4 cpm/well for 20 h at 4°C) or EGF (1.25 x 10^5 cpm/well for 8 h at 4°C) in 1 ml assay buffer. These conditions had been previously established to yield maximal binding (Appendices 3 and 4). Non-specific binding was determined in the presence of unlabelled ligand (500 ng/ml). Monolayers were then rinsed 3 times with ice-cold assay buffer and trypsinised. The cell suspension was sonicated and the bound radioactivity measured in a γ-counter (Wallac, Finland). Binding of radiolabelled ligand in each well was adjusted for the respective DNA level.

3.3.5 Statistical analyses

Data were analysed by one- and two-way ANOVA using the GLM procedure of SAS, where individual means comparisons were performed by LSD. DNA yields of the various cell lines were transformed ($\log_{10} + 1$) prior to analysis.

3.4 RESULTS

3.4.1 Co-cultured mammary fat pad potentiates mitogenic stimulation

Given previous results showing that the mouse mammary fat pad markedly potentiates the *in vitro* mitogenic effect of insulin, IGF-I and EGF (Chapter 2), the response of COMMA-1D cells to various concentrations of these mitogens in the absence and presence of co-cultured mammary fat pad was determined. The greatest response to IGF-I alone was a 1.9-fold increase in final DNA yield at an IGF-I concentration of 150 ng/ml (Figure 3.1a).

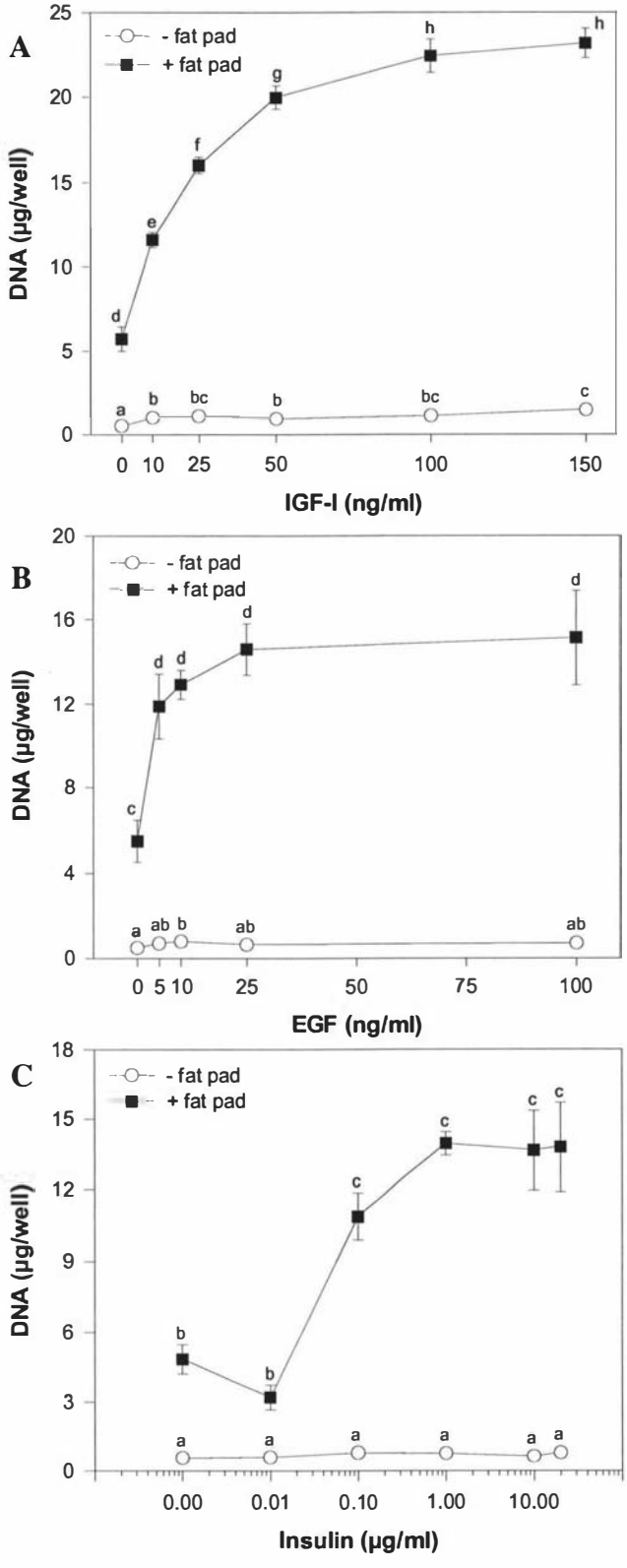


Figure 3.1 Concentration-response curves for mitogens added to cultures of COMMA-1D cells in the absence (○) or presence (■) of co-cultured mammary fat pad. Cells were cultured for 5 days with various concentrations of (A) IGF-I, (B) EGF, or (C) insulin. Data are means ± SEM (n=5). [a,b,c] Means with different superscripts are significantly different ($P<0.05$).

Co-cultured mammary fat pad in BM stimulated a 10.1-fold increase ($P<0.05$) in total DNA. Increasing concentrations of IGF-I in the presence of co-cultured mammary fat pad asymptotically raised final DNA levels to a maximum response at an IGF-I concentration of 150 ng/ml that was 45-fold that in BM alone.

Supplementation of BM with EGF induced small increases in total DNA, where the maximum response was realised at an EGF concentration of 10 ng/ml (Figure 3.1b). This mitogenic effect was markedly potentiated by co-culture with mammary fat pad whereby final DNA yield increased asymptotically to a maximum 29.2-fold elevation at an EGF concentration of 100 ng/ml.

Insulin added alone at concentrations up to 20 μg/ml induced small, but non-significant ($P>0.05$), amounts of cell growth (Figure 3.1c). When added in combination with co-cultured fat pad, insulin initiated substantial growth responses by cultures of COMMA-1D cells. The maximum response to insulin in co-culture was a 24.2-fold increase in DNA yield at an insulin concentration of 1 μg/ml.

3.4.2 *In vitro* effects of the mammary fat pad are due to a diffusible activity

The co-culture approach demonstrated not only that the mammary fat pad promotes a mitogenic response by mammary epithelial cells, but that it also enhances their proliferative response to growth factors. Within this system, however, it was not possible to determine whether these growth factors elicited their effect by acting directly on epithelial cells, or if they induced co-cultured mammary fat pad to release a secondary mitogenic stimulus. To resolve this issue CM was prepared to which specific mitogens were subsequently added.

Increasing proportions of CM induced a linear increase in final DNA yield after 3 days culture (Figure 3.2a). When medium supplemented with 10% FCS was similarly conditioned, an asymptotic response to the mitogenic stimulation from the mammary fat pad was evident (Figure 3.2a). The growth of mammary epithelial cells in response to growth factors added to increasing levels of CM was then examined. EGF-stimulated growth was increased with the proportion of CM, where a maximal 2-fold increase in total DNA was realised in 100% CM plus EGF (Figure 3.2b). Increasing levels of CM produced an asymptotic growth response up to 100 ng/ml IGF-I; the maximum response was in 75% CM where final DNA was 4.2 times that in BM alone. The proliferation of

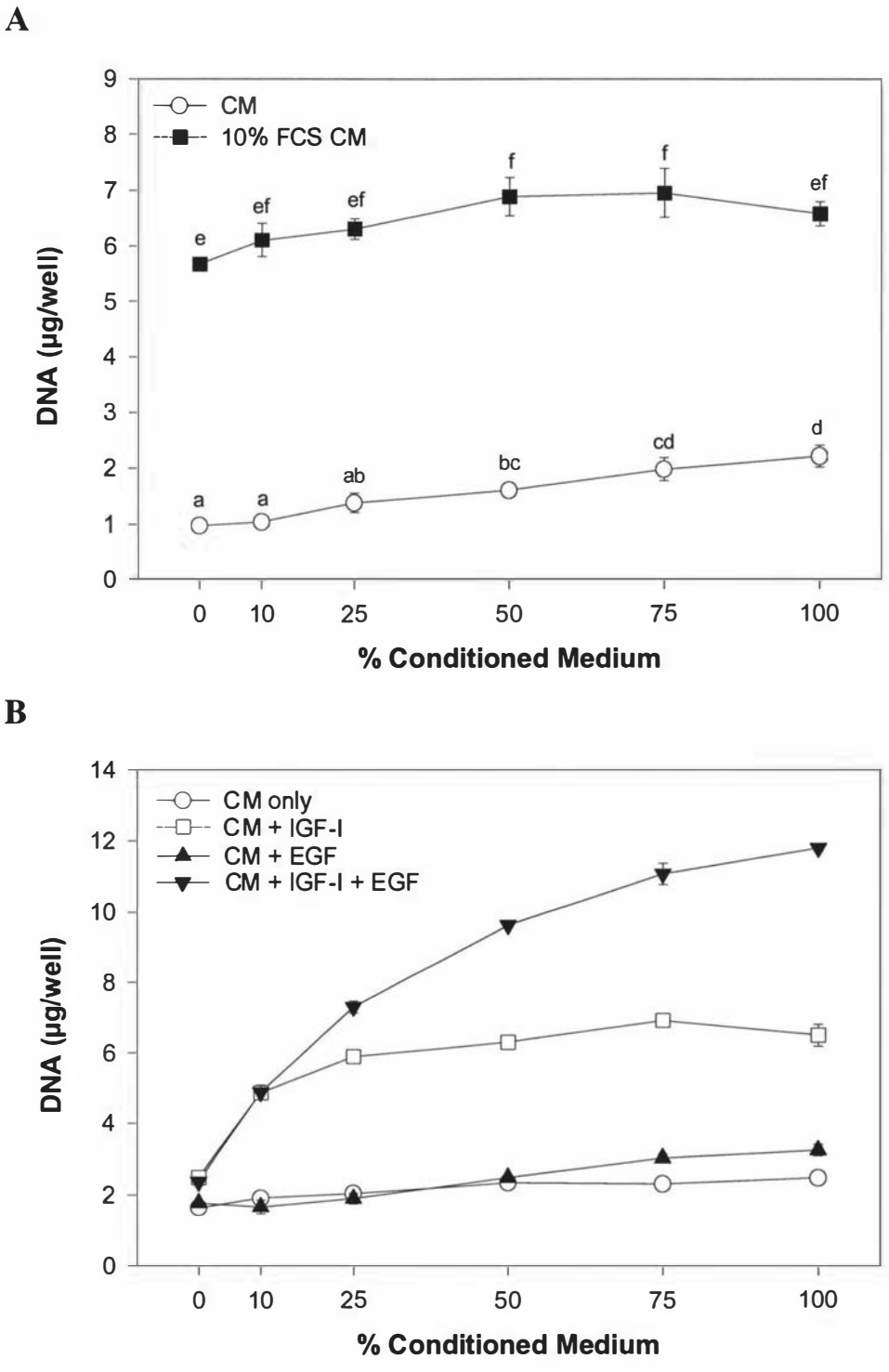


Figure 3.2 Response of COMMA-1D cells to increasing concentrations of CM supplemented with mitogens. (A) COMMA-1D cells were cultured for 3 days in 1 ml of medium containing various proportions of CM (○) or 10% FCS similarly conditioned by mammary fat pad tissue (■). Dilutions were made using BM and 10% FCS, respectively. [a,b,c] Means with different superscripts are significantly different ($P<0.05$). (B) COMMA-1D cells were cultured for 3 days in various proportions of CM (final volume 1 ml) without supplement (○), or in the presence of 25 ng/ml EGF (▲), 100 ng/ml IGF-I (□) or IGF-I + EGF (▼). Data are means ± SEM (n=3).

COMMA-1D cells in response to IGF-I + EGF was also increased asymptotically to reach a maximum 6.1-fold increase in final DNA in 100% CM.

3.4.3 Mitogenic potentiation by the mammary fat pad is growth factor-specific

It was of interest to determine whether a diffusible activity from the mammary fat pad could enhance the responsiveness of COMMA-1D cells to other growth factors. Acidic FGF added to BM induced a small but non-significant ($P>0.05$) increase in final DNA after 3 days (Figure 3.3a), a tendency which was also evident in the presence of IGF-I or EGF. Whereas the mitogenic effect of CM interacted with that of both IGF-I and EGF, no such interaction occurred between CM and aFGF. Acidic FGF enhanced the mitogenic effect of IGF-I in both BM and CM but attenuated the response to EGF and IGF-I + EGF in CM.

While the supplementation of BM with bFGF did not alter the final amount of DNA, it tended ($P<0.1$) to attenuate the mitogenic effects of IGF-I and IGF-I + EGF in BM (Figure 3.3b). Similar to aFGF, bFGF did not interact ($P>0.05$) with the effect of CM. Furthermore, addition of bFGF to CM suppressed the interaction between CM and IGF-I or EGF, and reduced cell proliferation induced by their combination.

The effect of KGF on COMMA-1D cell growth was tested in a separate experiment. Supplementation of BM with KGF did not effect an increase in final DNA yield (Figure 3.3c). KGF stimulated increased growth in the presence of IGF-I ($P<0.05$), but not in the presence of EGF or IGF-I + EGF. There was no additional growth in response to KGF in the presence of co-cultured fat pad either alone or with the various combinations of IGF-I and EGF.

The growth of COMMA-1D cells in response to IGF-II in the absence and presence of co-cultured mammary fat pad was also assessed. Addition of IGF-II to BM stimulated a small increase ($P<0.05$) in final DNA yield at an IGF-II concentration of 250 ng/ml (Figure 3.3d). Co-cultured mammary fat pad alone increased ($P<0.05$) final DNA levels and facilitated a small increment of growth in the presence of increasing concentrations of IGF-II, but not to the extent of that promoted by IGF-I.

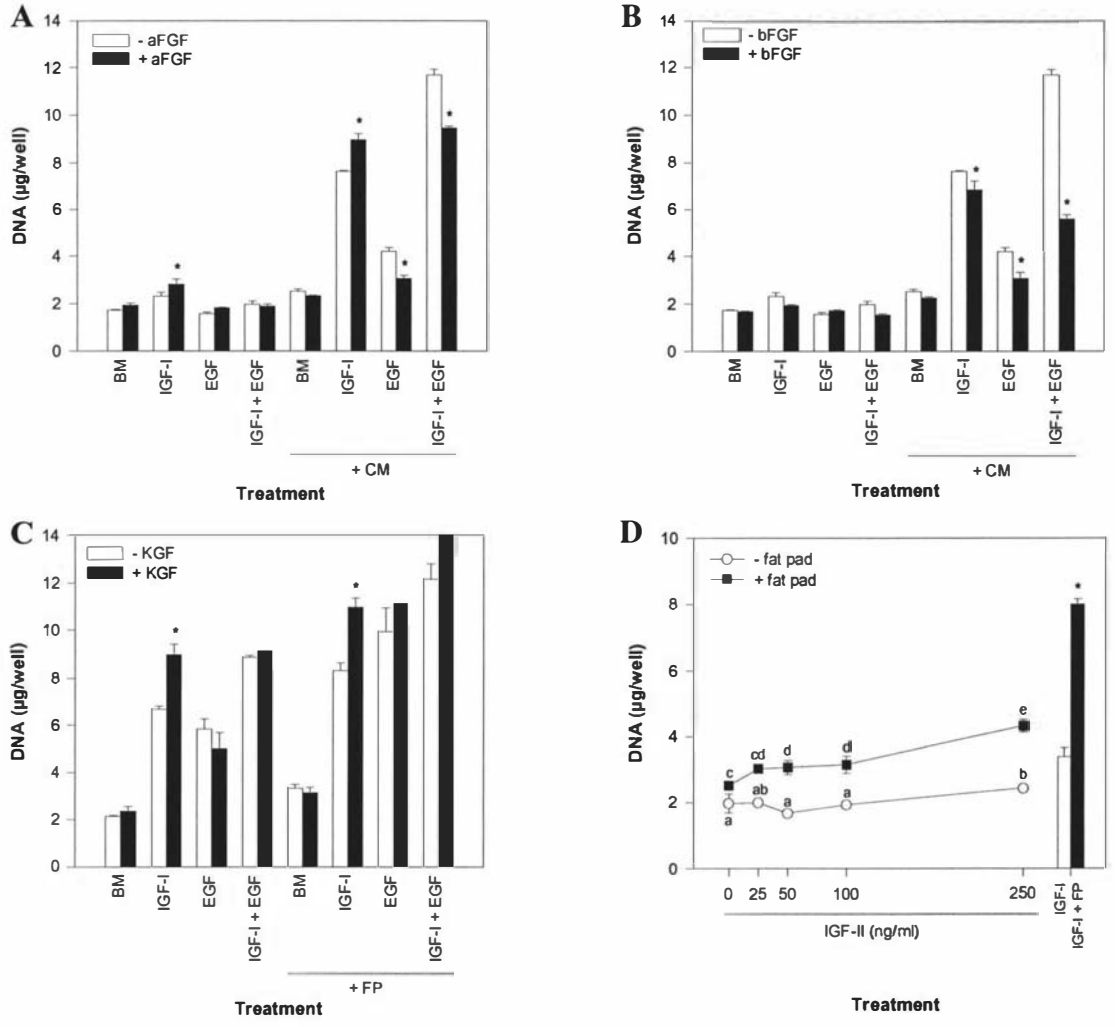


Figure 3.3 Growth of COMMA-1D cells in response to growth factors in CM or in co-cultures. (A and B) Cells were cultured for 3 days in BM or CM supplemented with various combinations of IGF-I (100 ng/ml), EGF (25 ng/ml) and either (A) acidic-FGF (5 ng/ml) or (B) basic FGF (5 ng/ml). (C) COMMA-1D cells were cultured for 5 days in combinations of IGF-I, EGF and KGF (25 ng/ml) in the presence or absence of co-cultured mammary fat pad. *$P<0.05$ vs the respective mean without FGF. (D) In a separate experiment, COMMA-1D cells were cultured for 4 days in various concentrations of IGF-II in the absence (○) or presence (■) of co-cultured mammary fat pad. Bars indicate cell growth responses to IGF-I (100 ng/ml) in the absence or presence of co-cultured mammary fat pad. Data are means ± SEM (n=3). ^{a,b,c} Means with different superscripts are significantly different ($P<0.05$).

3.4.4 Effect of mammary fat pad on IGF-I and EGF receptors

To begin investigating how the mitogenic effect of IGF-I and EGF might be modulated by an activity from the mammary fat pad, binding of these ligands to COMMA-1D cells was examined. Relative to that in BM, IGF-I binding was reduced ($P<0.06$) after 3 days culture in CM, and CM tended ($P<0.15$) to exert a similar effect in the presence of EGF (Figure 3.4a). Cells cultured in the presence of IGF-I or IGF-I + EGF alone demonstrated marked reductions in IGF-I binding; in the latter treatment binding was further suppressed ($P<0.01$) in the presence of CM. Whereas IGF-I binding was unaltered ($P>0.4$) by insulin alone, the amount of IGF-I bound was reduced by 70% when cells were cultured in CM plus insulin. IGF-I binding was also reduced ($P<0.01$) when cells were cultured in 10% FCS, and was further reduced when it was added to CM ($P<0.1$).

Binding of EGF to COMMA-1D cells was unaltered after 3 days culture in CM alone (Figure 3.4b). When cells were cultured in BM plus IGF-I, the level of EGF bound doubled, although this response was partly suppressed ($P<0.01$) in CM. Similar responses were recorded for cultures supplemented with insulin. EGF binding was substantially downregulated ($P<0.001$) when cells were cultured in medium supplemented with EGF. The reduction in EGF binding to cells after culture in 10% FCS was unchanged by culture in CM.

3.4.5 Responses to mammary fat pad are cell type-specific

It was examined whether the mitogenic and modulatory effects of co-cultured fat pad were specific to mammary epithelial cells. For all of the cell lines tested, co-cultured mammary fat pad increased final DNA levels to between 1.8- and 5.2-fold that in BM alone (Table 3.1). The normal mammary epithelial cell lines, COMMA-1D and NMuMG, demonstrated a marked proliferative response to the various mitogenic supplements in the presence of co-cultured fat pad. HeLa epithelioid carcinoma cells, and to a lesser extent Wi-38 foetal lung fibroblasts, also displayed increased growth in response to certain mitogens when co-cultured with mammary fat pad, while other cell types did not respond further. The various cell lines also differed in their ability to undergo further growth in response to the mammary fat pad above that stimulated by 10% FCS.

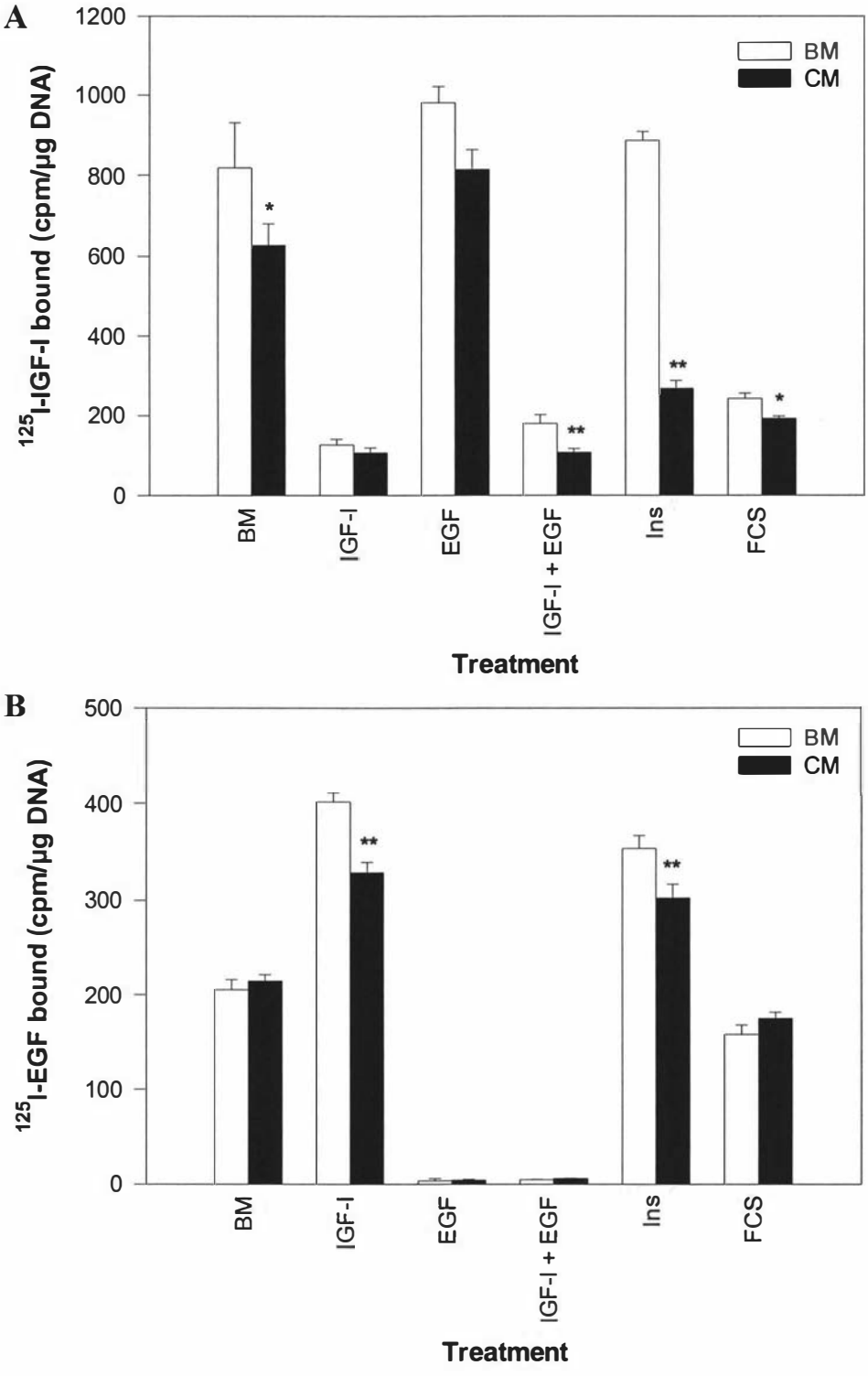


Figure 3.4 Specific binding of (A) IGF-I and (B) EGF to COMMA-1D cells cultured in BM or CM supplemented with various treatments. Cultures which had been grown for 3 days in 1 ml of BM or CM supplemented with IGF-I (100 ng/ml), EGF (25 ng/ml), IGF-I + EGF, insulin (10 µg/ml) or 10% FCS were then incubated with radiolabelled ligand. Non-specific binding was determined in the presence of 500 ng/ml unlabelled ligand. Specific binding values are adjusted for the DNA content of cultures. Data are means ± SEM (n=3). *P<0.08 **P<0.01 compared to the respective BM treatment.

Table 3.1 Growth* of various cell lines in response to mitogens in the absence and presence of co-cultured mammary fat pad.

	Treatment	L6	NLFK	HeLa	C127	Wi-38	NMuMG	COMMA-1D
No FP	BM	1.0^{c}	1.0^{abc}	1.0^{a}	1.0^{a}	1.0^{bc}	1.0^{a}	1.0^{a}
	IGF-I	0.75^{ab}	1.09^{abc}	0.79^{b}	0.74^{a}	0.34^{a}	1.33^{b}	3.18^{c}
	EGF	0.81^{abc}	1.40^{abc}	1.06^{b}	0.83^{a}	0.56^{ab}	1.65^{bc}	1.94^{b}
	IGF + EGF	0.65^{a}	0.73^{a}	0.84^{b}	0.95^{a}	0.38^{a}	2.04^{c}	5.22^{d}
	Insulin	0.96^{bc}	0.88^{ab}	0.96^{b}	1.06^{a}	0.62^{ab}	1.39^{b}	3.11^{c}
	FCS	62.0^{i}	1.35^{abc}	11.84^{f}	16.28^{c}	0.33^{a}	21.84^{g}	8.74^{ef}
+ FP	BM	4.05^{g}	2.51^{c}	3.15^{d}	2.7^{b}	1.36^{cd}	3.03^{d}	2.62^{abc}
	IGF-I	2.41^{de}	1.41^{abc}	4.84^{de}	2.72^{b}	1.11^{cd}	9.0^{f}	16.75^{fg}
	EGF	2.59^{ef}	1.72^{bc}	6.35^{ef}	1.96^{b}	3.74^{e}	5.07^{e}	6.16^{de}
	IGF + EGF	1.92^{d}	1.59^{bc}	7.18^{ef}	2.52^{b}	2.14^{de}	9.06^{f}	23.57^{g}
	Insulin	3.36^{fg}	1.91^{c}	8.78^{f}	2.6^{b}	2.08^{de}	7.25^{f}	12.48^{f}
	FCS	43.4^{h}	4.09^{e}	9.81^{f}	19.54^{d}	1.20^{cd}	23.96^{g}	19.8^{g}
	pooled SE	*0.04*	*0.1*	*0.08*	*0.06*	*0.11*	*0.03*	*0.07*

Cells seeded at 5 x 10^4 (COMMA-1D and NMuMg) or 1 x 10^4 cells/well were quiesced and then cultured for 5 days in BM alone or in the presence of either IGF-I (100 ng/ml), EGF (25 ng/ml), IGF-I + EGF, insulin (10 μg/ml) or 10% FCS in the absence or presence of co-cultured mammary fat pad. * Final DNA values (n=3) are expressed as a proportion of that in the respective BM only treatment. ^{a,b,c} Means within a column with different superscripts are significantly different ($P<0.05$).

3.5 DISCUSSION

It was demonstrated in a previous study (Chapter 2) that co-cultured mammary fat pad markedly enhances the growth of mouse mammary epithelial cells in response to IGF-I, EGF, and a supraphysiological concentration of insulin. Given the magnitude of this response and the likely roles of these polypeptide mitogens during mammary development and tumorigenesis (Plaut, 1993; Lee and Yee, 1995; Panico *et al.*, 1996), the ability of the mammary fat pad to regulate the *in vitro* growth of mammary epithelial cells was further evaluated.

As reported by others (Imagawa *et al.*, 1982; Riss and Sirbasku, 1987), mammary epithelial cells in the hormone-free BM displayed small increments of growth in response to a range of IGF-I and EGF concentrations. Over this same range, epithelial responsiveness to these mitogens was markedly upregulated by the presence of co-cultured mammary fat pad. From their respective concentration-response curves, the ED_{50} for IGF-I and EGF in the presence of co-cultured mammary fat pad was approximately 20 and 5 ng/ml, respectively. Similar low levels of IGF-I (Ruan *et al.*, 1992; M. Weber, personal communication) and EGF (Vonderhaar, 1984) within the mammary gland are associated with extensive epithelial proliferation. This leads to the suggestion that diffusible factors released from the mammary fat pad are an important component of IGF-I- and EGF-induced mitogenesis *in vivo*. Substantial mitogenic stimulation by supraphysiological levels of insulin was also recorded in co-culture. This effect was likely initiated via the type I IGF receptor (Imagawa *et al.*, 1986), consistent with results showing that the responses to IGF-I and insulin in the presence of co-cultured mammary fat pad are not additive (Appendix 5).

It was considered that the effect of IGF-I and EGF on COMMA-1D cell growth in co-cultures may have occurred indirectly through IGF-I and EGF receptors present in the mammary stroma (Edery *et al.*, 1985b; Daniel and Silberstein, 1987; Hodgkinson *et al.*, 1991). Likewise, COMMA-1D cells may have liberated soluble factors to induce the synthesis of paracrine growth factors by the co-cultured mammary fat pad (Singer *et al.*, 1995; Coleman-Krnacik and Rosen, 1994). Using CM it was established that the observed effects of the mammary fat pad are due to a diffusible activity liberated independent of any influence by IGF-I, EGF or COMMA-1D cells. This activity

initiated its effects in a dose-dependent manner, and its mitogenic effect was additive to that of 10% FCS as observed previously (Chapter 2).

Subsequent experiments indicated that potentiation of IGF-I- and EGF-stimulated growth by a diffusible factor(s) from the mammary fat pad could not be ascribed to an increase in the number of cell-surface receptors for these ligands. In fact, culture in CM suppressed IGF-I binding in all treatments and reduced EGF binding in the presence of IGF-I and insulin. That IGF-I and EGF substantially down-regulated the number of their own receptors is consistent with the findings of others (Carpenter and Cohen, 1979; De Vroede *et al.*, 1984). One possibility is that the mitogen-potentiating effect of CM was associated with a corresponding increase in receptor affinity for these ligands. However, it is also conceivable that CM amplified intracellular signalling after formation of the ligand-receptor complex. This notion is supported by the finding that the synergistic response to CM and IGF-I + EGF is abrogated upon inhibition of the lipid-activated signalling molecule, protein kinase C (Chapter 4).

While a mitogenic factor(s) from the mammary fat pad enhanced the proliferative response to both IGF-I and EGF, this effect was specific in that no substantial interaction was observed with other growth factors including three FGFs (aFGF, bFGF and KGF), and IGF-II. That there was no increase in cell growth in response to these FGFs may reflect the culture conditions of these experiments, for separate experiments (Appendix 6) and others (Riss and Sirbasku, 1987) have shown that they do induce COMMA-1D cells to increase their DNA synthesis (as [^{3}H]-thymidine incorporation). Likewise, others have shown that these FGFs are mitogenic for mammary epithelial cells *in vitro* (Levay-Young *et al.*, 1989; Imagawa *et al.*, 1994) and *in vivo* (Ulich *et al.*, 1994).

The ability of the mammary fat pad to potentiate the mitogenic effect of specific growth factors may represent a local growth regulatory mechanism within the mammary gland. Specifically, the release of a diffusible activity by the mammary fat pad might allow maximum epithelial responsiveness to mitogens such as IGF-I and EGF at certain stages of development (Plaut, 1993; Chapter 9), even when other growth factors may be present (Niranjan *et al.*, 1995; Chakravorti and Sheffield, 1996a). The specificity of growth factor potentiation by CM also corresponds to results which suggest that CM achieves its effect via protein kinase C (Chapter 4) for, whereas EGF and IGF-I

stimulate mitogenesis via protein kinase C dependent pathways, FGF-induced proliferation occurs independently of protein kinase C (Neri *et al.*, 1994). It will be of interest to determine whether the proliferation of mammary epithelial cells in response to other growth factors involved in mammary development such as hepatocyte growth factor (Niranjan *et al.*, 1995) and the transforming growth factors-α and -β (Plaut, 1993; Pierce *et al.*, 1993) can also be modulated by the mammary fat pad *in vitro*.

Although the various cell types differed in their responsiveness to mitogens in BM, co-cultured mammary fat pad stimulated the growth of all lines to DNA yields that were 2 to 5-fold that in BM alone. This finding suggests that the mitogenic activity liberated by the mammary fat pad is either a broad spectrum mitogen or a generic requirement for cell growth. In contrast, only certain cell types, in particular normal mammary epithelial cells, utilised this activity to markedly enhance their proliferative response to IGF-I, EGF, and high concentrations of insulin. This may reflect the fact that mammary epithelium is rather unique in its requirement for a fatty stroma in which to develop (Hoshino, 1978), and that its development is markedly influenced by IGF-I and EGF (Ruan *et al.*, 1992; Plaut, 1993). Similar cell-specific growth regulation by the mammary fat pad has also been recorded *in vivo*, where only mammary and hair follicle epithelium develops when transplanted into the mammary fat pad (Sakakura *et al.*, 1979). It is likely that adipocytes of the mammary fat pad play an important role in this effect as the growth of transplanted mammary epithelium is supported in several adipose depots but not in other sites (reviewed by Hoshino, 1978). Therefore, the mammary fat pad, particularly the mammary adipocytes, may liberate a diffusible factor(s) that meets specific and important requirements for the growth of mammary epithelium, particularly by increasing its responsiveness to certain growth factors.

In conclusion, these findings demonstrate that a diffusible activity from the mouse mammary fat pad markedly potentiates the mitogenic actions of certain growth factors, an effect that may be particularly relevant to the proliferation of mammary epithelium. Current studies are focussed on identifying the nature of this factor(s) and the physiological implications of this potentially important growth regulatory mechanism.

CHAPTER 4

ROLE FOR MAMMARY FAT PAD-DERIVED UNSATURATED FATTY ACIDS IN POTENTIATING GROWTH FACTOR-STIMULATED GROWTH OF MAMMARY EPITHELIAL CELLS *IN VITRO*

4.1 ABSTRACT

The stromal environment of the mammary fat pad plays a pivotal role in regulating the growth and morphogenesis of mammary epithelium. Mouse mammary fat pad tissue liberates an activity *in vitro* that stimulates the growth of mammary epithelial cells and markedly enhances their proliferative response to insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF). The present studies were undertaken to identify this activity and the mechanism by which its effects are achieved. The growth-stimulating effects of medium conditioned by mouse mammary fat pad (CM) were not trypsin-labile but were heat-sensitive and were reduced by pH-dependent charcoal stripping. That these effects may be due to a bioactive lipid constituent coincides with the presence of unsaturated fatty acids in CM. Of several unsaturated fatty acids tested, linoleic acid and, to a lesser extent, arachidonic acid stimulated the growth of COMMA-1D cells and enhanced their proliferative response to the mitogenic effects of IGF-I + EGF. Furthermore, linoleic acid, similar to CM, potentiated the individual mitogenic effects of IGF-I and EGF, and was maximally effective at a concentration of 5-10 μg/ml. Consistent with a ability to stimulate cell growth via enhanced intracellular signalling, CM, both alone and in the presence of IGF-I or EGF, stimulated tyrosine phosphorylation on several proteins in COMMA-1D cells. The mitogenic response to CM either alone or in the combined presence of IGF-I + EGF was abolished by inhibiting the lipid-activated signalling mediator, protein kinase C (PKC). Taken together, these findings indicate that the mammary fat pad may fulfil an important role during mammogenesis by liberating unsaturated fatty acids to stimulate the growth of mammary epithelial cells and their responsiveness to specific growth factors via PKC.

4.2 INTRODUCTION

Postnatal development of the rodent mammary gland involves the ramification of mammary epithelium into the fatty stroma of the mammary fat pad (Imagawa *et al.*, 1994). During this proliferation, epithelial cells are continually in close proximity to adipocytes (Imagawa *et al.*, 1994) with rapidly dividing cells in the terminal end bud abutting onto adjacent adipocytes (Williams and Daniel, 1983). The importance of this

adipose environment is emphasised by the fact that mammary epithelium can only undergo normal growth and morphogenesis when transplanted to a depot of adipose tissue (reviewed by Hoshino, 1978).

What is unclear is the precise role that this fatty stroma serves in the local regulation of epithelial growth. Several studies have shown that the mammary fat pad is a source of paracrine growth factors (Chakravorti and Sheffield, 1996a; Niranjan *et al.*, 1995; Chapters 9-11) which may mediate the actions of several hormones on the mammary gland (Chakravorti and Sheffield, 1996b; Chapters 9-11). Fatty acids released from mammary adipocytes and transported across the basement membrane may also influence epithelial proliferation (Bandyopadhyay *et al.*, 1995). Specifically, unsaturated, but not saturated, fatty acids stimulate the *in vitro* growth of normal (Wicha *et al.*, 1979) and neoplastic (Kidwell *et al.*, 1978; Wicha *et al.*, 1979) mammary epithelial cells. Other investigations have demonstrated that unsaturated fatty acids such as linoleic and arachidonic acid enhance the mitogenic effect of epidermal growth factor (EGF) on mammary epithelial cells (Bandyopadhyay *et al.*, 1987; Sylvester *et al.*, 1994), possibly by amplifying post-receptor signalling through PKC (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994). However, it has not been determined whether the mammary fat pad liberates unsaturated fatty acids to induce similar effects.

It was shown in previous studies that the mouse mammary fat pad liberates a diffusible mitogenic factor(s) that markedly and specifically enhances the growth of mammary epithelial cells in response to IGF-I and EGF (Chapters 2 and 3). The objective of this study was to determine the nature of this mammary fat pad-derived stimulation and its mechanism of action. The results suggest that the mammary fat pad releases unsaturated fatty acids to markedly enhance the proliferative effects of both IGF-I and EGF on mammary epithelium. This response is likely associated with enhanced post-receptor signalling via PKC.

4.3 MATERIALS AND METHODS

4.3.1 Cell cultures

The COMMA-1D mouse mammary epithelial cell line (Danielson *et al.*, 1984) used in these experiments was maintained under previously described conditions (Chapter 2). The defined, hormone-free basal medium (BM) comprised DMEM supplemented with sodium bicarbonate (44 mM), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml).

4.3.2 Conditioned medium

Medium conditioned by mouse mammary fat pad tissue (CM) was prepared as described (Chapter 3). Briefly, abdominal mammary fat pads devoid of endogenous epithelium were aseptically excised from weanling female BALB/c mice and then diced into 5-10 mg explants. CM was prepared by incubating mammary fat pad explants in BM (7.5 mg/ml) for 48 h and collecting the resultant CM. Control BM was similarly prepared in the absence of mammary fat pad tissue. All supplements were added to CM and BM immediately prior to their use in culture.

4.3.3 Conditioned medium analyses

Proteolysis was performed by incubating CM with trypsin (50 μg/ml, type XI, Sigma) for 4 h at 37°C. Trypsin was subsequently inactivated by adding a combination of soybean trypsin inhibitor (500 μg/ml; Sigma), leupeptin (1 μg/ml; Boehringer Mannheim) and aprotinin (2 μg/ml; Boehringer Mannheim) for a further 2 h. Trypsin that had been pre-neutralised with the same mixture of inhibitors served as a negative control. Proteolysis was confirmed by the ability of trypsin to abolish the mitogenic effect of IGF-I + EGF in the presence of CM (Appendix 7).

Charcoal stripping of media was performed according to the method of Chen (1967). Briefly, CM and BM were incubated with activated charcoal (BDH; 2 mg/ml) for 1 h at 4°C with continual swirling. Suspensions were centrifuged at 20 000 g for 20 mins, the supernatant was then collected, filtered (0.2 μm) and added to cultures. Stripping was conducted at both pH 7.4 and 2.5; the latter pH increases the efficiency of fatty acid removal (Chen, 1967). Acidified media was readjusted to pH 7.4 with 3 M NaOH before its addition to cultures.

CM and BM were also heated for 5 minutes at either 60°C or 100°C, filtered (0.2 μm), and added to COMMA-1D cultures.

4.3.4 Cell growth experiments

COMMA-1D cells were plated into 24-well plates at 1 x 10^5 and 5 x 10^4 cells/well for 3 and 5 day cultures, respectively. Treatments were added in 0.5 ml BM for the first 3 days with treatment media for 5 day cultures replaced on day 3. Prostaglandin E_2 (PGE_2; Sigma) and unsaturated fatty acids as sodium salts (Sigma) were prepared as ethanolic stocks; all medium treatments within an experiment contained the same concentration of ethanol. Linoleic acid-BSA (Sigma; 0.5 to 1.0% linoleic acid) was dissolved in BM prior to use. Recombinant human IGF-I was from Genentech, and mouse EGF was from Sigma. At the end of the culture period cell monolayers were trypsinised and the amount of DNA determined by a fluorometric assay (Labarca and Paigen, 1980).

4.3.5 Western blotting

Monolayers of COMMA-1D cells which had been cultured in various medium treatments were rinsed with PBS plus orthovanadate and scraped in 100 μl loading buffer (Laemmli, 1970). Samples were electrophoresed through a 10% polyacrylamide gel and transferred to nitrocellulose. Membranes were blocked for 1 h with 4% BSA in TBS-tween (0.1%) and then incubated overnight with a rabbit polyclonal anti-phosphotyrosine antibody (Transduction Laboratories) in TBS-tween at a dilution of 1:500. Membranes were incubated with an anti-rabbit secondary antibody (Sigma, 1:10 000), washed 4 times in TBS-tween, and immunocomplexes detected using an ECL kit (Amersham).

4.3.6 Fatty acid analyses

Total lipids were extracted from CM (135 mls) according to the method of Bligh and Dyer (1959). The chloroform extract was evaporated under vacuum and the sample was then similarly re-extracted. Esterified and free fatty acids were derivatised to nitrophenylhydrazides as described by Miwa and Yamamoto (1990) and separated

through a C-8 column by reversed-phase HPLC. Blank values were determined for BM that had been similarly extracted.

4.3.7 Statistical analyses

Data was analysed by one- and two-way analysis of variance using the GLM procedure of SAS, where individual means comparisons were by LSD.

4.4 RESULTS

4.4.1 Analysis of the mitogenic activity in CM

The first experiments sought to identify the mitogenic and growth factor-potentiating activity present in mammary fat pad CM. An initial experiment revealed that these responses were unaffected by preincubating CM with trypsin (Table 4.1). In a second experiment, cell growth induced by CM alone was reduced after CM was incubated with activated charcoal at pH 2.5 (Table 4.1). The growth of COMMA-1D cells in response to CM supplemented with IGF-I + EGF was reduced by preincubating CM with activated charcoal at pH 7.4, and was essentially abolished when it was similarly incubated at a pH of 2.5 (Table 4.1). These mitogenic responses were also suppressed when CM was heated at 60°C, and were further reduced on heating to 100°C (Table 4.1). Other experiments have demonstrated that the mitogenic activity in CM is not depleted by incubating CM with heparin sepharose (Appendix 8), a treatment which removes a variety of heparin-binding polypeptide growth factors (Gospodarowicz *et al.*, 1984).

Table 4.1 Effects of treatments applied to CM on the subsequent growth of COMMA-1D cells.

	Medium treatment	alone (μg DNA/well)	+ IGF-I + EGF† (μg DNA/well)
Expt. 1	BM‡	1.8 ± 0.1^{a}	4.1 ± 0.5^{a}
	CM‡	2.7 ± 0.2^{a}	13.4 ± 0.8^{b}
	CM + trypsin	2.4 ± 0.3^{a}	15.7 ± 0.7^{c}
Expt. 2	BM	3.0 ± 0.1^{a}	6.5 ± 0.4^{a}
	CM	4.1 ± 0.1^{c}	18.9 ± 0.4^{d}
	CM + charcoal, pH 7.4	4.1 ± 0.1^{c}	11.8 ± 0.2^{c}
	CM + charcoal, pH 2.5	3.6 ± 0.1^{b}	7.5 ± 0.3^{b}
Expt. 3	BM	3.6 ± 0.2^{a}	8.2 ± 0.2^{b}
	CM	6.1 ± 0.2^{d}	22.5 ± 0.4^{d}
	CM, 60°C	5.2 ± 0.1^{c}	18.2 ± 0.4^{c}
	CM, 100°C	4.1 ± 0.1^{b}	4.5 ± 0.2^{a}

COMMA-1D cells (1×10^5 cells/well) were cultured for 3 days in 1 ml of treatment media. Data are means ± SEM (n=3) for final DNA yields. Each experiment was performed 3 times and the data is from one representative experiment.

† Media were supplemented with IGF-I (100 ng/ml) and EGF (25 ng/ml).

‡ Included pre-neutralised trypsin.

[a,b,c] Within an experiment, means in a column with different superscripts are significantly different ($P<0.05$).

That the effects of CM were insensitive to trypsin and could be removed by pH-dependent charcoal stripping suggested that the mitogenic factor(s) present in CM was lipid in nature. Analysis of fatty acids in CM revealed that approximately two thirds of those present were in the free form, predominantly as palmitic and stearic acids (Table 4.2). Relative to free fatty acids, triglycerides contained a much lower proportion of stearic acid and a far greater level of oleic acid.

Table 4.2 Concentrations[a] of major fatty acids in CM.

Fatty acid	Free fatty acid	Triglyceride
	Area %	*Area %*
Myristic	4.8	0.8
Palmitic	46.0	33.8
Palmitoleic	5.3	1.9
Stearic	22.1	12.5
Oleic	9.6	35.2
Linoleic	6.0	8.0
Arachidonic	2.0	1.2
Others	4.2	6.6
Total	***100***	***100***
Concentration[a]	4.91 μM	2.45 μM

[a] Concentrations of fatty acids as free fatty acids and as triglycerides were determined from an internal standard after HPLC analysis as described in Methods.

4.4.2 Fatty acid effects on COMMA-1D cell growth

Given that unsaturated fatty acids can stimulate the growth of mammary epithelial cells (Wicha *et al.*, 1979) and enhance their proliferative response to EGF (Bandyopadhyay *et al.*, 1987; Sylvester *et al.*, 1994), the growth of COMMA-1D cells in response to several unsaturated fatty acids was determined. When added to BM, linoleic acid and, to a lesser extent, arachidonic acid stimulated the growth of COMMA-1D cells (Table 4.3). Prostaglandin E_2, which may account for the effect of linoleic acid on mammary epithelial cell growth (Bandyopadhyay *et al.*, 1988), induced an increase in the final yield of DNA. Linoleic acid also potentiated the mitogenic response to IGF-I + EGF, an effect achieved to a lesser extent by arachidonic acid. In contrast, the response to these growth factors was unaltered in the presence of PGE_2.

Table 4.3 Effect of unsaturated fatty acids and prostaglandin E_2 on the growth of COMMA-1D cells.

Treatment	Conc.	alone (μg DNA/well)	+ IGF-I + EGF[†]
BM only		2.8 ± 0.1^{b}	5.7 ± 0.2^{b}
Oleic acid[‡]	5 μg/ml	2.5 ± 0.1^{a}	4.3 ± 0.2^{a}
Linoleic acid[‡]	5 μg/ml	3.2 ± 0.1^{c}	9.1 ± 0.7^{d}
Linoleic-BSA[¥]	1 mg/ml	3.8 ± 0.1^{d}	9.9 ± 0.2^{d}
Arachidonic acid[‡]	7.5 μg/ml	3.2 ± 0.1^{c}	7.3 ± 0.4^{c}
Prostaglandin E_2	0.5 μg/ml	3.6 ± 0.1^{d}	5.2 ± 0.5^{ab}

COMMA-1D cells (1×10^5 cells/well) were cultured for 3 days in 1 ml of BM supplemented with various treatments. Data are means ± SEM (n=3) for final DNA yields.

[a,b,c] Means within a column with different superscripts are significantly different ($P<0.05$).

[†] Medium was supplemented with IGF-I (100 ng/ml) and EGF (25 ng/ml).

[‡] Added as the sodium salt.

[¥] Linoleic-BSA at 1 mg/ml provides approximately 5-10 μg linoleic acid/ml.

The mitogenic effects of various concentrations of CM and linoleic acid-BSA were compared both alone and in the presence of IGF-I + EGF. As observed previously (Chapter 3), CM stimulated COMMA-1D cell growth in a dose-dependent manner (Figure 4.1a). The maximum growth response was in 100% CM where the final DNA yield in the absence and presence of IGF-I + EGF was increased by 2.3- and 11.2-fold, respectively. Supplementation of BM with linoleic acid also increased final DNA levels in a dose-dependent fashion (Figure 4.1b); the maximum response in both the absence and presence of IGF-I + EGF was realised at a linoleic-acid BSA concentration of 1 mg/ml (equivalent to 5-10 μg/ml linoleic acid). The respective DNA yields were 2.1- and 7.4- times that in BM alone. Separate experiments demonstrated that BSA alone had no effect on the mitogenic responsiveness of cells to these growth factors (Appendix 9).

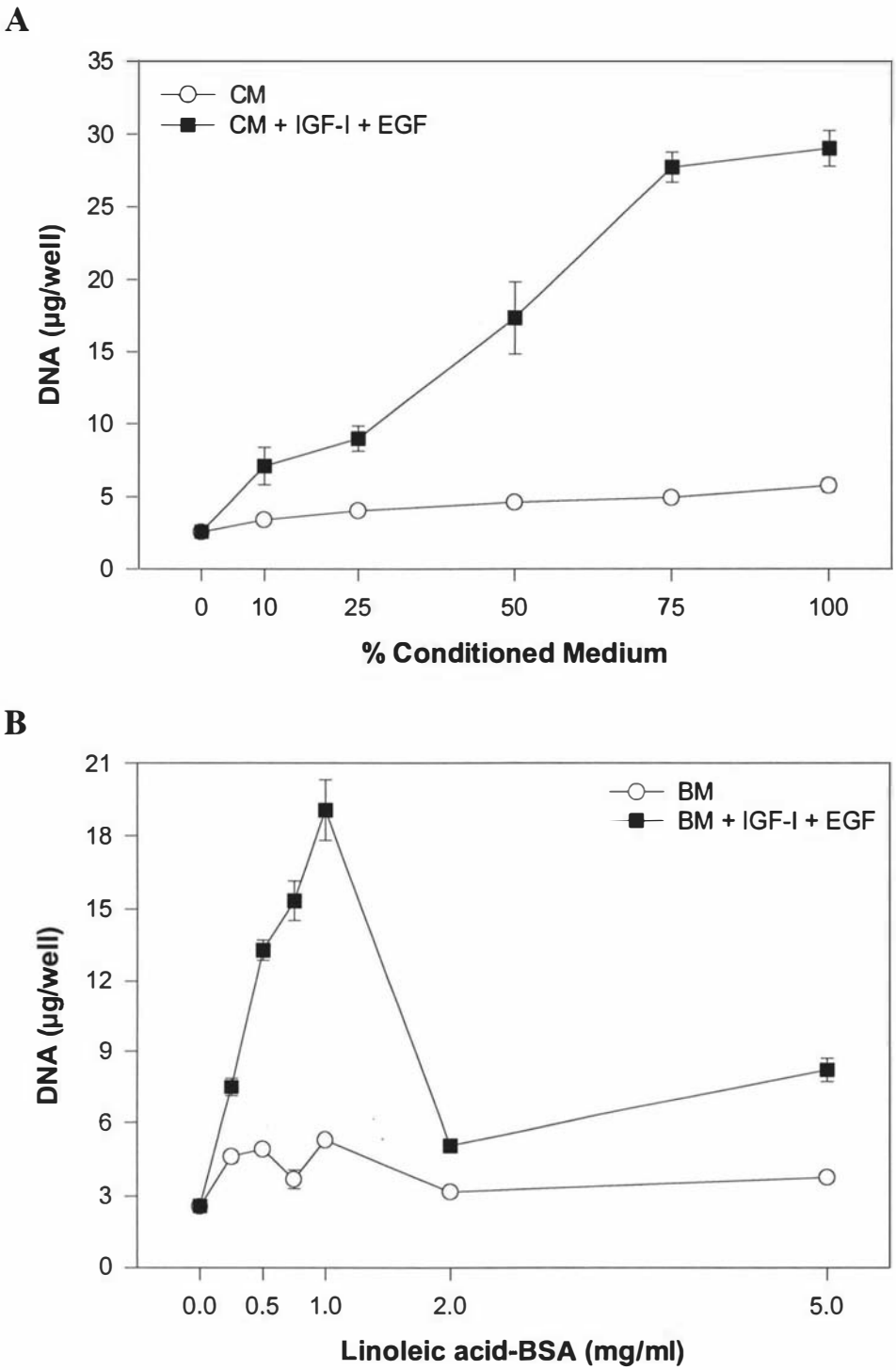


Figure 4.1 Growth of COMMA-1D cells in response to various concentrations of (A) CM and (B) linoleic acid-BSA added to cultures either alone (○) or in the presence of IGF-I + EGF (■). CM dilutions were made using control BM. Linoleic acid-BSA contained between 0.5 and 1% linoleic acid. Cultures were for 5 days and were incubated in 1 ml treatment medium which was replaced on day 3. Data are means ± SEM (n=3).

The ability of CM and linoleic acid-BSA to enhance the response of COMMA-1D cells to the individual mitogenic effects of IGF-I and EGF was also assessed. Cultures supplemented with IGF-I and/or EGF demonstrated small increases in their final DNA yield (Figure 4.2). Both CM and linoleic acid-BSA stimulated cell growth alone and similarly potentiated the individual mitogenic effects of IGF-I and EGF.

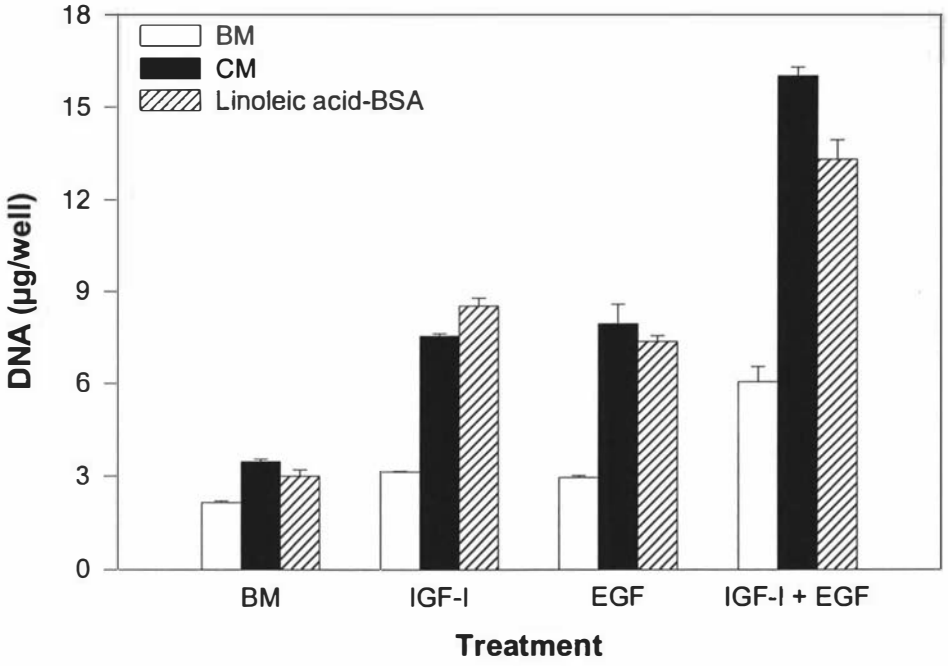


Figure 4.2 Comparison of the mitogenic effects of CM and linoleic acid-BSA alone and in the presence of IGF-I and/or EGF. Cultures were grown for 3 days in 1 ml of CM or BM plus 1 mg/ml linoleic acid-BSA (0.5-1% linoleic acid) either alone or in the presence of IGF-I (100 ng/ml) and/or EGF (25 ng/ml). Data are means ± SEM (n=3).

4.4.3 Effects of CM on intracellular signalling

The phosphorylation of tyrosine residues on COMMA-1D cell proteins was assessed to begin evaluating the intracellular responses to CM (Figure 4.3). Cells cultured for 30 mins in CM + IGF-I demonstrated a pattern of tyrosine phosphorylation similar to that for cells cultured in BM supplemented with phorbol 12-myristate 13-acetate (PMA; Sigma), a tumour promoter and inducer of PKC in mammary epithelial cells (Wada *et al.*, 1994). After 6 h culture in CM either alone or plus IGF-I or EGF, several proteins with sizes of approximately 47, 42, 39, 34-36, 25 and 18 kDa demonstrated increased tyrosine phosphorylation compared to those in cells exposed to these treatments in BM.

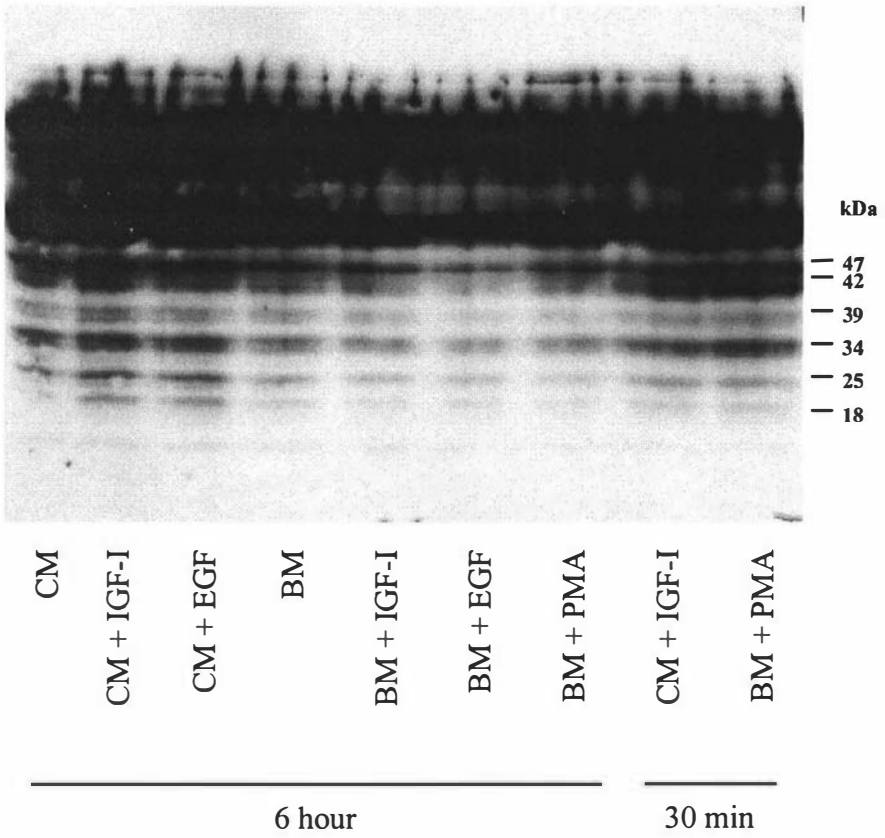


Figure 4.3 Western blotting of protein tyrosine phosphorylation in COMMA-1D cells following their incubation for 30 mins or 6 h in various medium treatments. Cells (2 x 10^5/well) were cultured for the indicated time in BM or CM supplemented with IGF-I (100 ng/ml), EGF (25 ng/ml) or PMA (100 ng/ml). The approximate molecular size of several tyrosine-phosphorylated proteins is indicated.

Given that unsaturated fatty acids may enhance EGF-induced growth via PKC (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994), it was examined whether CM-induced growth was influenced by 1-*O*-hexadecyl-2-*O*-methyl glycerol (OHMG; Sapphire Biosciences), a specific inhibitor of PKC (Daniel *et al.*, 1988; Kramer *et al.*, 1989). While OHMG at concentrations above 1 μM suppressed cell growth in BM and in the presence of IGF-I + EGF (Figure 4.4a), lower concentrations of OHMG substantially reduced the growth responses that were induced by CM alone or by the combined presence of CM and IGF-I + EGF (Figure 4.4b).

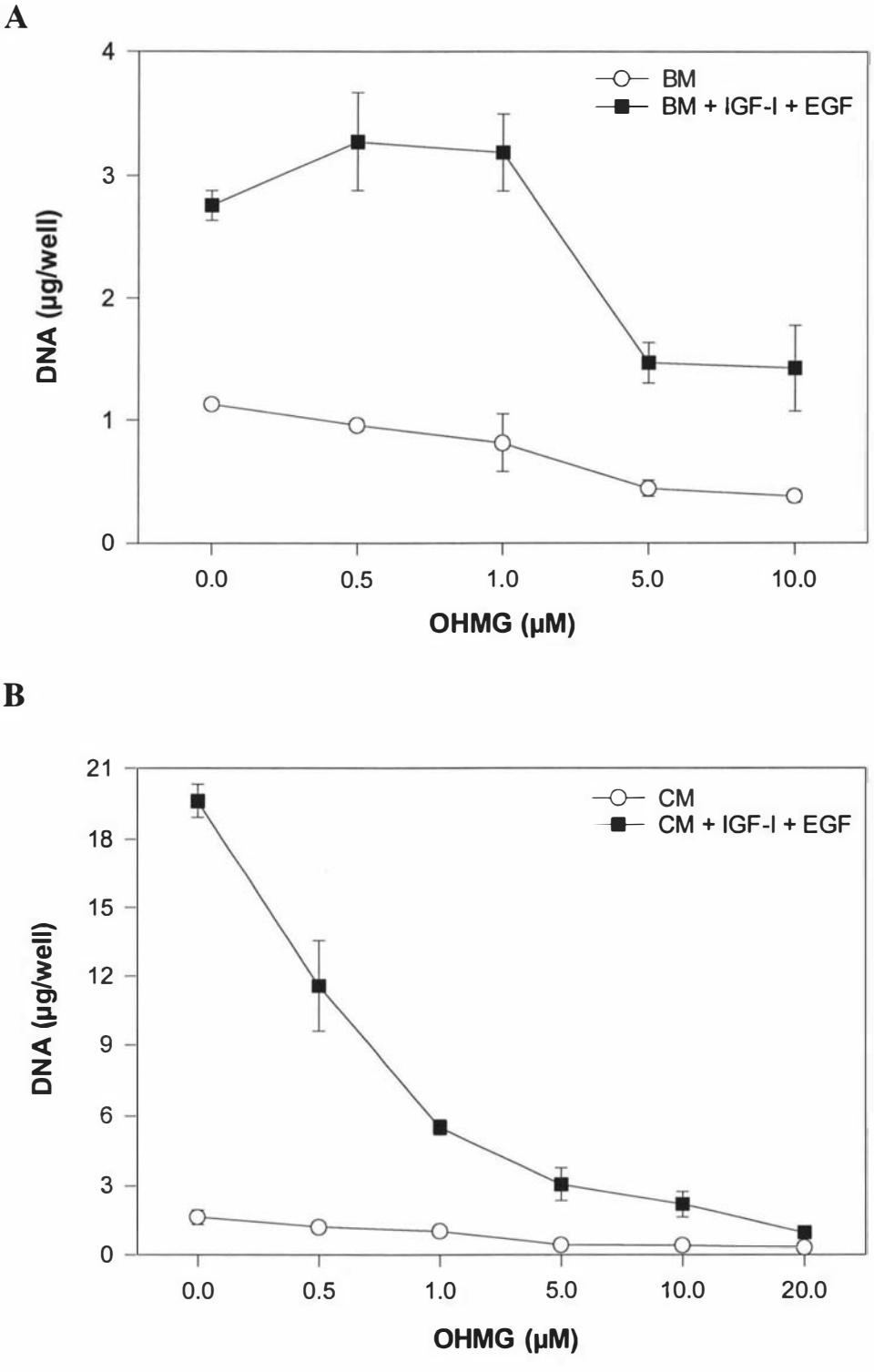


Figure 4.4 Effect of various concentrations of the PKC inhibitor, OHMG, on the growth of COMMA-1D cells in (A) BM or (B) CM, either alone (○) or in the presence of IGF-I (100 ng/ml) + EGF (25 ng/ml) (■). Cells were cultured for 3 days in 1 ml of treatment medium. Data are means ± SEM (n=3).

4.5 DISCUSSION

In previous studies it was identified that the mouse mammary fat pad releases a diffusible factor(s) *in vitro* that markedly stimulates the growth of mammary epithelial cells (Chapters 2 and 3). Several treatments within the present study, including proteolysis and charcoal stripping, strongly suggested that these effects were due to the presence of a bioactive lipid fraction. This suggestion was supported by the fact that cultured mammary fat pad tissue released unsaturated fatty acids into the incubation medium. The proportions of the various fatty acids in CM were similar to those reported by Beck *et al.* (1989), although the total level of non-esterified fatty acids was somewhat lower than that recorded by others under similar conditions (Kidwell *et al.*, 1982; Beck *et al.*, 1989). This discrepancy is probably due to an inefficiency of the lipid extraction procedure used and it is likely that CM did in fact contain higher levels of the various fatty acids.

The results of subsequent experiments indicated that linoleic acid, one of the unsaturated fatty acids in CM and the rodent mammary gland (Wicha *et al.*, 1979), likely accounts for a large proportion of the mitogenic effects induced by CM. This finding is consistent with an earlier report suggesting that the increased growth of preneoplastic mammary epithelial cells in response to a diffusible activity from the mammary fat pad was effected by unsaturated fatty acids (Beck *et al.*, 1989). Likewise, several other studies have demonstrated that unsaturated fatty acids can directly stimulate the *in vitro* growth of normal (Wicha *et al.*, 1979; Sylvester *et al.*, 1994) and neoplastic (Kidwell *et al.*, 1978; Beck *et al.*, 1989) mammary epithelial cells, while saturated fatty acids are generally inhibitory (Wicha *et al.*, 1979; Sylvester *et al.*, 1994). Of the various unsaturated fatty acids, linoleic acid typically promotes the greatest independent stimulation of mammary epithelial cell growth (Kidwell *et al.*, 1978; Wicha *et al.*, 1979; Beck *et al.*, 1989).

As well as its direct mitogenic effect, the diffusible factor from the mammary fat pad enhances the responsiveness of mammary epithelial cells to mitogenic stimulation by IGF-I, EGF and supraphysiological concentrations of insulin (Chapters 2 and 3). It was conceivable that these effects were also due to unsaturated fatty acids present in CM, given that EGF-induced proliferation of mammary epithelial cells is potentiated by

linoleic (Bandyopadhyay *et al.*, 1987; Bandyopadhyay *et al.*, 1993) and arachidonic (Sylvester *et al.*, 1994) acids. No link to date, however, has been established between this effect of unsaturated fatty acids and that due to the mammary fat pad; nor has the modulation of IGF-I induced mitogenesis by unsaturated fatty acids been demonstrated. These results indicate that linoleic acid, similar to an activity released from the mammary fat pad, potentiates the mitogenic effect of both IGF-I and EGF on mammary epithelial cells. This, to my knowledge, is the first demonstration that polyunsaturated fatty acids can enhance the responsiveness of mammary epithelial cells to the established proliferative effect of IGF-I (Imagawa *et al.*, 1994). This has probably not been identified previously because of the intrinsic requirement for primary mammary epithelial cells to be maintained in a basal medium supplemented with a high concentration of insulin (Imagawa *et al.*, 1982) which simulates IGF-I (Imagawa *et al.*, 1986).

As well as showing that linoleic acid mimics the effects of CM, several other findings further the notion that unsaturated fatty acids from the mammary fat pad account for its *in vitro* effects on mammary epithelial cell growth. For example, COMMA-1D cells co-cultured with mammary fat pad in the presence of IGF-I + EGF form numerous dome structures (Chapter 2), as occurs in medium supplemented with linoleic acid and IGF-I + EGF (personal observation). Furthermore, supplementation of mammary epithelial cultures with either linoleic acid (Bandyopadhyay *et al.*, 1993) or CM (Chapter 3) in the presence of insulin similarly reduces the binding of EGF to cell surface receptors. Also, the finding that BSA suppressed the mitogenic effect of co-cultured mammary fat pad on mammary epithelial cells (Chapter 2) may reflect a reduced availability of free unsaturated fatty acids (Spector, 1986). COMMA-1D cells co-cultured with ovarian adipose tissue demonstrate responses similar to those seen in co-culture with mammary fat pad (Appendix 10), strongly suggesting that this activity is liberated by adipocytes, and strengthening the likelihood that these effects are due to unsaturated fatty acids.

The potentiation of IGF-I and EGF-induced mitogenesis by CM prompted an investigation into the mechanism(s) underlying this response. As this effect is not associated with increased ligand binding to the cell-surface receptor (Chapter 3), it was investigated whether it is achieved through enhanced intracellular signalling. Consistent with this suggestion, CM upregulated tyrosine phosphorylation of numerous proteins,

several of which may correspond to those phosphorylated in primary mammary epithelial cells grown in the presence of linoleic acid (Bandyopadhyay *et al.*, 1993). A similar pattern of protein tyrosine phosphorylation was observed in COMMA-1D cells treated with the tumour promoter and PKC activator, PMA. Furthermore, the ability of OHMG to suppress the effects of CM suggests that a diffusible activity from the mammary fat pad potentiates IGF-I- and EGF-induced mitogenesis via the lipid activated signalling molecule, PKC. This coincides with the fact that both of these growth factors induce specific, rapid translocation of PKC-α to the nucleus (Neri *et al.*, 1994), where PKC-α is the major PKC isoform present in mouse mammary epithelial cells (Sylvester *et al.*, 1994).

Further consistent with this suggestion is the demonstration that unsaturated fatty acids enhance the mitogenic effect of EGF on mammary epithelial cells in association with an increased activity of PKC (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994). In contrast, the mitogenic effect of several fibroblast growth factors is not potentiated by CM (Chapter 3), corresponding to the fact that FGF stimulates cell proliferation through a PKC-independent pathway (Magnaldo *et al.*, 1986; Mohammadi *et al.*, 1992; Virdee *et al.*, 1994) and does not induce the nuclear translocation of PKC (Neri *et al.*, 1994). It will be interesting to determine whether unsaturated fatty acids can enhance the proliferation of mammary epithelial cells in response to platelet-derived growth factor, a mitogen for mammary tumour cells (Miller and Bukowski, 1994) that also induces the nuclear translocation of PKC (Neri *et al.*, 1994).

These findings lead to a suggestion that adipocytes within the mammary fat pad liberate unsaturated fatty acids, particularly linoleic acid, which subsequently modify the fatty acid composition of mammary epithelial cell membranes (Sylvester *et al.*, 1994). This may elevate PKC levels and allow maximal cell proliferation in response to IGF-I and EGF. Such a mechanism is of potential physiological importance given that both IGF-I and EGF are potent mitogens for normal and tumorous mammary epithelium *in vivo* (Imagawa *et al.*, 1994; Dickson and Lippman, 1995). Furthermore, the expression of IGF-I and EGF-like factors within the mammary gland is upregulated by oestrogen and/or growth hormone (Liu *et al.*, 1987; Vonderhaar, 1988; Ruan *et al.*, 1995), hormones which also stimulate lipolysis in adipocytes (Hansen *et al.*, 1980; Rebuffé-Scrive, 1987). Therefore, these hormones may realise their mammogenic effect by

inducing the local synthesis of polypeptide growth factors and potentiating the growth factor-induced proliferation of mammary epithelial cells via a local release of unsaturated fatty acids. Mammary epithelium may also use this combinatory mechanism to regulate its growth given it can stimulate lipolysis in mammary adipocytes (Bartley *et al.*, 1981) and growth factor expression in the adjacent stroma (Coleman-Kmacik and Rosen, 1994; Kenney *et al.*, 1995; Singer *et al.*, 1995). Likewise, the mammogenic effect of prolactin may involve its action through the mammary epithelium (Kidwell and Shaffer, 1984) to initiate the local release of unsaturated fatty acids from mammary adipocytes (Kidwell *et al.*, 1982).

Along these lines, the *in vitro* mitogenic capacity of the mammary fat pad is altered with hormonal changes during the oestrous cycle, and by the presence of endogenous mammary epithelium (Chapter 5). This mitogenic capacity also varies throughout the course of mammary gland development (Chapter 6) when there are substantial changes in lipid metabolism within the mammary gland (Bandyopadhyay *et al.*, 1995). Furthermore, it is well established that rodents fed a diet containing a high proportion of fat or unsaturated fatty acids have increased mammary gland growth and tumorigenic risk (Welsch and O'Connor, 1989) whereas normal and neoplastic mammogenesis is impaired by a diet deficient in essential fatty acids (Knazek *et al.*, 1980; Miyamoto-Tiaven *et al.*, 1981), or which contains a high proportion of saturated fats (Karmali *et al.*, 1984). Given that the fatty acid composition of mammary epithelial cell membranes reflects that of the diet and the surrounding mammary fat pad (Welsch, 1987), it is conceivable that dietary fat composition may regulate the development of normal and neoplastic mammary epithelium, at least in part, by influencing its ability to proliferate in response to specific growth factors.

In conclusion, these results show that unsaturated fatty acids are liberated from the mammary fat pad and may subsequently enhance the proliferation of mammary epithelial cells in response to IGF-I and EGF. This growth potentiating effect and the potential for its hormonal and dietary regulation warrants further investigation as it may fulfil an important role in the developing normal and neoplastic mammary gland.

CHAPTER 5

MODULATION OF STEROID- AND GROWTH FACTOR-STIMULATED GROWTH OF MAMMARY EPITHELIAL CELLS BY THE MAMMARY FAT PAD AND EPITHELIAL-STROMAL INTERACTIONS DURING THE OESTROUS CYCLE

5.1 ABSTRACT

Ovarian steroid hormones exert pronounced effects on mammary epithelial growth and morphogenesis which may be mediated by the mammary gland stroma and its interaction with the mammary epithelium. COMMA-1D mouse mammary epithelial cells have been used in a co-culture system to determine whether the mitogenic capacity of the mouse mammary fat pad is altered during the oestrous cycle. The influence of epithelial-stromal interaction was evaluated by comparing cell growth responses to mammary fat pad cleared of endogenous epithelium (CFP) and intact mammary tissue (MFP). When co-cultured in the presence of either oestrogen, progesterone, epidermal growth factor (EGF), or insulin-like growth factor-I (IGF-I) + EGF, there was a significantly ($P<0.05$) greater *in vitro* mitogenic response to mammary tissue removed at oestrus relative to the other stages of the oestrous cycle. In addition to this systemic regulation, mitogenic stimulation afforded by the mammary fat pad was influenced by the endogenous epithelium. There was a significantly ($P<0.01$) greater effect of MFP compared to CFP across the oestrous cycle in medium supplemented with either progesterone, IGF-I, EGF, or IGF-I + EGF. In contrast, there was no difference between the response to CFP and MFP in the presence of oestrogen either alone or in combination with progesterone ($P>0.75$). Furthermore, the growth of COMMA-1D cells in response to oestrogen tended ($P<0.08$) to be increased by co-cultured mammary tissue while progesterone suppressed ($P<0.05$) the mitogenic effect of CFP. These effects were similarly evident in medium conditioned (CM) by CFP. Further investigation of the oestrogenic response revealed that CM increased the growth factor-induced expression of a ~55 kDa oestrogen receptor (ER) protein by COMMA-1D cells. Taken together, these findings demonstrate that a growth-stimulatory activity is released from the mammary fat pad under hormonal and local regulation during the oestrous cycle; an activity that also differentially modulates the responsiveness of mammary epithelial cells to oestrogen, progesterone and growth factors. Such findings are discussed in terms of the diverse roles proposed for mammary adipocyte-derived unsaturated fatty acids.

5.2 INTRODUCTION

Accumulating evidence indicates that the growth and morphogenesis of normal and neoplastic mammary epithelium may be substantially influenced by its associations with the surrounding adipose and connective tissue matrix of the mammary fat pad (Sakakura, 1991; Cunha and Hom, 1996). The proliferation of epithelial cells within the mammary fat pad during this development also initiates an extensive reaction by the surrounding stroma (Rønnov-Jessen *et al.*, 1996). This reaction results in an array of stromal responses including increased DNA synthesis (Berger and Daniel, 1983) and growth factor expression (Ellis *et al.*, 1994), altered levels of growth factor receptors (Daniel and Silberstein, 1987) and lipolysis in adjacent adipocytes (Elias *et al.*, 1973).

Interaction between the epithelium and stroma may fulfil a crucial role during ovarian steroid-induced mammogenesis (Haslam, 1988b). While oestrogen stimulates extensive proliferation by epithelial cells within the mammary gland and promotes their morphogenesis as ducts (reviewed by Haslam, 1987), mammary epithelial cells are generally unresponsive to oestrogen *in vitro* (Richards *et al.*, 1988). This paradox has led several groups to propose that the mammogenic effects of oestrogen are mediated by the stromal elements of the mammary gland (McGrath, 1983; Shyamala and Ferenczy, 1984; Haslam and Counterman, 1991). Consistent with this, receptors for oestrogen exist within the mammary gland stroma as well as on mammary epithelium (Haslam and Shyamala, 1981). Furthermore, oestrogen stimulates DNA synthesis in stromal fibroblasts prior to the proliferation of proximal mammary epithelium (Shyamala and Ferenczy, 1984). This response may coincide with the local expression of paracrine and autocrine growth factors (Dickson and Lippman, 1987; Ruan *et al.*, 1995; Chakravorti and Sheffield, 1996b). While progesterone directly stimulates the *in vitro* proliferation of primary mammary epithelial cells (Edery *et al.*, 1984; Imagawa *et al.*, 1985), progesterone receptors also exist within the stroma (Haslam and Shyamala, 1981), raising the possibility that progesterone acts on several mammary cell types to initiate a variety of responses (Haslam, 1987; Selman *et al.*, 1994).

Alterations in the systemic profile of ovarian steroid and pituitary hormones during the oestrous cycle induce marked developmental changes within the mammary gland (Vonderhaar, 1988). The rodent mammary gland is morphologically most developed at

oestrus and least developed at dioestrus (Lotz and Krause, 1978). This corresponds to the formation of ductal end buds during the follicular phase (Laguchev, 1962) while the formation of alveolar buds may be initiated during the luteal phase (Dulbecco *et al.*, 1982; Vonderhaar, 1988). DNA synthesis in mammary epithelial cells within ductal end buds peaks in pro-oestrus/early oestrus (Sutton and Suhrbier, 1967; Dulbecco *et al.*, 1982) while DNA synthesis and mitotic index in mammary epithelial cells of ductules is greatest at late oestrus-metoestrus (Dulbecco *et al.*, 1982) and dioestrus (Purnell and Kopen, 1976), respectively. These cycles of ductal elongation and side branching in recurrent oestrous cycles result in an incremental increase in mammary gland development as the female approaches maturity (Lotz and Krause, 1978; Vonderhaar, 1988). The significance of these acute cyclical changes is emphasised by the fact that stage of the oestrous cycle modifies the susceptibility of the mammary gland to chemically-induced carcinogenesis (Lindsey *et al.*, 1981; Ratko and Beattie, 1985).

Recent investigations into the stromal regulation of mammary gland development have demonstrated that the mammary fat pad stimulates the *in vitro* growth of normal mammary epithelial cells and markedly enhances their responsiveness to the mitogenic effects of insulin, IGF-I and EGF (Chapters 2 and 3). The present study has investigated whether the ability of the mammary fat pad to modulate the growth of mammary epithelial cells in response to growth factors and ovarian steroid hormones is altered by hormonal changes during the oestrous cycle and by the endogenous mammary epithelium. Results show that the *in vitro* modulatory effects of the mammary fat pad vary during the oestrous cycle correspondent with marked changes in mammary gland development *in vivo*. Furthermore, these effects are regulated by the interaction of epithelium with the surrounding stroma, an association that may modulate epithelial responsiveness to ovarian steroid hormones.

5.3 MATERIALS AND METHODS

5.3.1 Animals

BALB/c mice were maintained under standard conditions in the Ruakura Small Animal Colony with ad libitum access to food and water. Nulliparous females were mated to provide mice for the stage-of-oestrous cycle experiment, where the resultant litters were

standardised to 8 pups. Around the time of weaning at 21-23 days of age, female pups were anaesthetised with Avertin (2.5%, 16 μl/g BWT i.p.) and the epithelial mammary rudiment was excised from one abdominal #4 mammary gland to leave a mammary fat pad cleared of endogenous epithelium (CFP; Faulkin and DeOme, 1960). The contralateral mammary gland remained intact (MFP) to allow normal mammary development. Operated females were subsequently housed at four per box.

On the morning of sacrifice (at approximately 56 days of age), their stage of oestrous was determined from vaginal smears (Rugh, 1967) as confirmed by vaginal appearance (Champlin *et al.*, 1973). By this age the mammary ductal tree had filled approximately 80% of the abdominal mammary fat pad. Four mice at each stage of the oestrous cycle [dioestrus (DI); pro-oestrus (PRO); oestrus (EST); and metoestrus (MET)] were selected to be the source of mammary tissue for *in vitro* co-culture experiments.

5.3.2 Cell cultures

The COMMA-1D mouse mammary epithelial cell line (Danielson *et al.*, 1984) was maintained under previously described conditions (Chapter 2). Cells were plated into 24-well plates (5 x 10^4 cells/well) in 0.5 ml hormone-supplemented growth medium. After 24 h attachment, monolayers were rinsed with PBS and quiesced in DMEM basal medium (BM) for a further 48 h.

CFP and MFP tissue was aseptically excised from mice after sacrifice by cervical dislocation. These tissues were sectioned into explants (~8mm^3, ~7.5 mg) which were rinsed in BM prior to use in co-culture. Co-cultures of COMMA-1D cells and mammary tissue were prepared as previously described (Chapter 2). Briefly, an explant of CFP or MFP was placed on a raft of siliconised lens paper and floated at the gas:medium interface of appropriate cultures. Explants were added to an initial 0.5 ml treatment medium, with a further 0.5 ml treatment medium added on day 3. Cultures were terminated after 5 days. Monolayers were trypsinised and their DNA content measured using a fluorometric assay (Labarca and Paigen, 1980).

Conditioned medium (CM) was prepared by incubating epithelium-free mammary fat pad tissue in BM as described (Chapter 3) and was stored at -80°C without any adverse affect on its activity (Appendix 11).

5.3.3 Assessment of mammary development

Mammary gland development during the oestrous cycle was determined for mice which provided mammary tissues for co-cultures. Thoracic mammary glands were removed and spread on slides, fixed in Carnoy's fixative and defatted in acetone. Preparations were then stained with alum carmine, dehydrated through graded alcohols, clarified in toluene and photographed using a dissecting microscope. Photomicrographs were subjectively scored on a scale of 0 to 5 for both end bud development (0 = no end buds, 5 = numerous large end buds) and alveolar development (0 = no alveolar ductules, 5 = numerous alveolar ductules). Whole mounts were then embedded in paraffin and sectioned (5 μm) for histological examination.

5.3.4 Estrogen receptor (ER) immunoblotting

COMMA-1D cells plated at 2 x 10^5 cells/well were cultured for 48 h in either BM or CM treated with various supplements. Monolayers were rinsed with PBS and scraped in 50 μl loading buffer (Laemmli, 1970). Samples of cell lysate (2 μg DNA) were electrophoresed through a 10% polyacrylamide gel under reducing conditions and transferred to nitrocellulose. After blocking in 4% BSA in TBS-tween, the membrane was incubated with a rabbit anti-rat ER antibody (Furlow *et al.*, 1990) diluted 1:1000 in TBS-tween-0.1% BSA. The membrane was then washed in TBS-tween-0.1% BSA and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma, 1:10 000 dilution in TBS-tween-0.1% BSA). After the membranes had been washed four times in TBS-tween-0.1% BSA, immunoreactive proteins were detected using enhanced chemiluminesence (Amersham).

5.3.5 Statistical analyses

All statistical analyses were performed using SAS. Scores for mammary gland development during the oestrous cycle were analysed by one way ANOVA. Final co-culture DNA levels in each medium treatment were analysed for effects of stage-of-cycle and mammary tissue type and their interaction using the GLM procedure. Response to the individual medium treatments added to BM was analysed by Student's t-test. Cell growth responses to CM, oestrogen, progesterone and their interactions were compared by the GLM procedure.

5.4 RESULTS

5.4.1 Mammary gland development

Morphology of the mammary gland parenchyma was markedly altered during the oestrous cycle. At dioestrus, mammary ducts demonstrated moderate amounts of side-branching and alveolar budding (Figures 5.1a and 5.1b). The regression of alveolar structures at pro-oestrus coincided with the formation of end bud structures at the tips of mammary ducts (Figures 5.1c and 5.1d). By oestrus, bulbous end buds were evident at the tips of most ducts along with a general absence of alveolar budding (Figures 5.1e and 5.1f). End buds had begun to regress at metoestrus coincident with some signs of alveolar branching (Figures 5.1g and 5.1h). These substantial changes were also evidenced histologically, where the ductal end bud structures observed at oestrus (Figure 5.2a) differed substantially from the ductal alveolar buds seen at dioestrus (Figure 5.2b). Parenchymal morphology during the oestrous cycle was subjectively scored for end bud- and alveolar development. Mean end bud score increased to a maximum at oestrus and was least at metoestrus (Table 5.1). Conversely, the degree of alveolar budding peaked at dioestrus and declined to a minimum at oestrus.

5.4.2 Co-cultures

The growth of COMMA-1D cells in response to co-cultured mammary tissue in various medium treatments was used as a measure of the mitogenic and modulatory capacity of the mouse mammary fat pad during the oestrous cycle. Comparison of the response to CFP and MFP allowed an evaluation of whether epithelial-stromal interactions influenced these effects.

Co-cultured mammary fat pad stimulated substantial growth by COMMA-1D cells in the hormone-free BM (Figure 5.3a) as recorded previously (Chapters 2 and 3). MFP obtained at oestrus stimulated the greatest mitogenic response by COMMA-1D cells. In contrast, the ability of CFP to stimulate cell growth was significantly ($P<0.05$) increased at metoestrus relative to other stages.

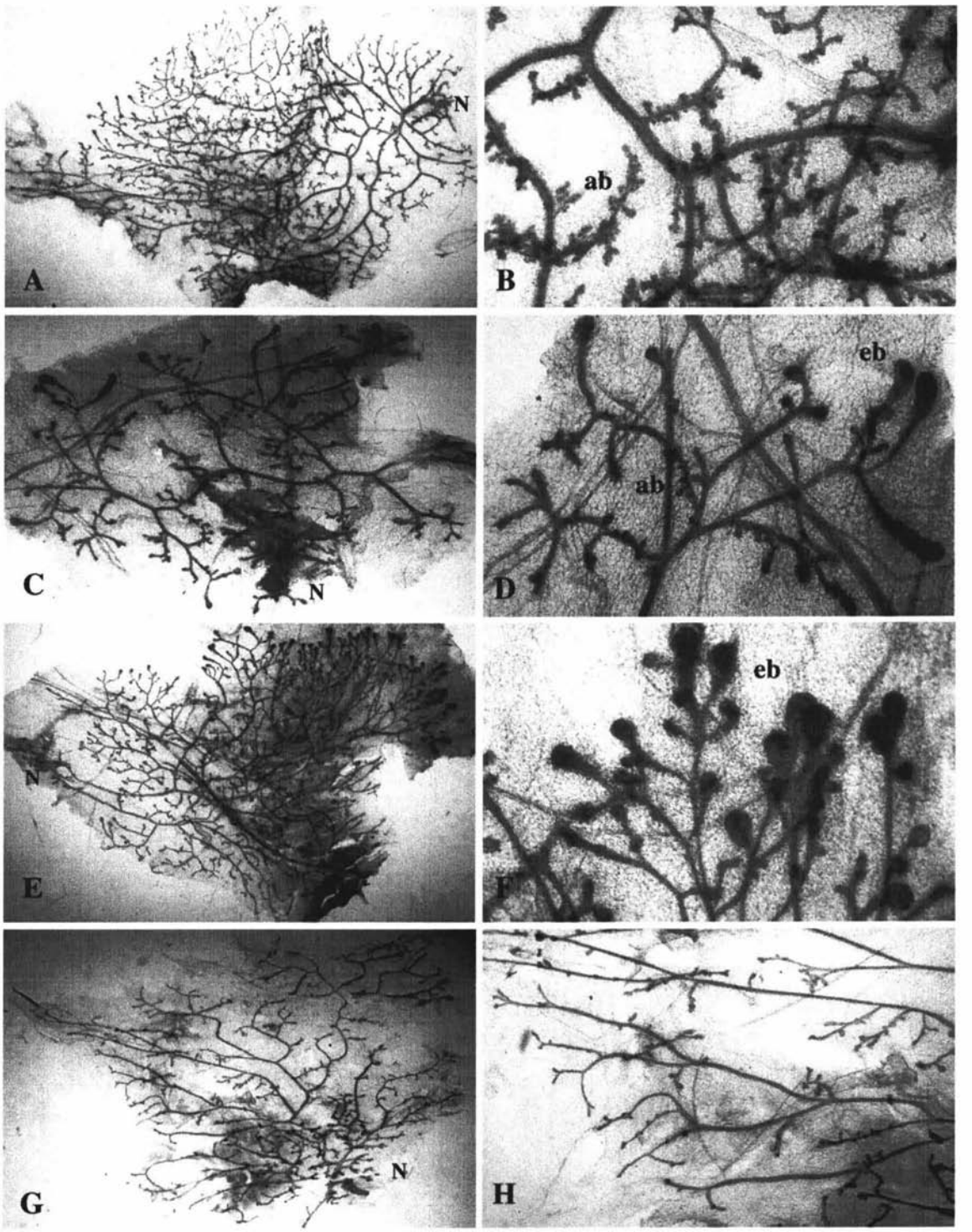


Figure 5.1 Mammary gland morphology in virgin female BALB/c mice during the oestrous cycle. Thoracic mammary glands were from females at dioestrus (A and B), pro-oestrus (C and D), oestrus (E and F), and metoestrus (G and H) were prepared as whole mounts and stained with alum carmine. N, nipple; ab, alveolar buds; eb, end buds. Magnifications: A, E and G, 11x; B and F, 55x; C, 17x; D and H, 33x.

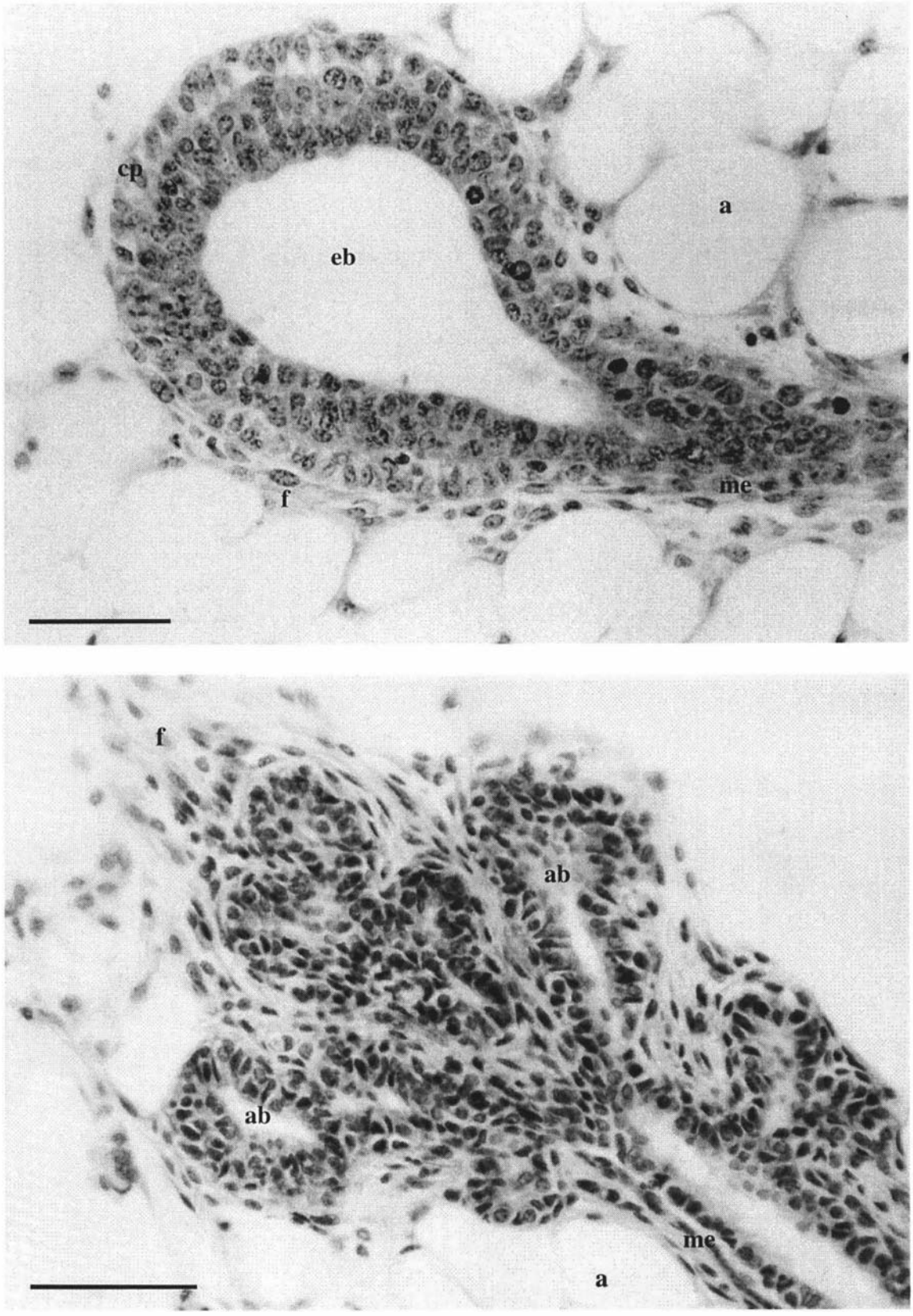


Figure 5.2 Histomorphology of mammary parenchyma at (A) oestrus, and (B) dioestrus. Mammary tissues were sectioned (5 μm) and stained with Gomori's trichrome. At oestrus, epithelial cells at the tips of ducts form into bulbous end buds (eb). At dioestrus, ductal epithelium is organised into clusters of alveolar buds (ab). cp, cap cells; a, adipocytes; me, myoepithelial cells; f, fibroblasts. Scale bar = 10 μm.

Table 5.1 Assessment of mammary gland development in virgin female BALB/c mice during the oestrous cycle.

Stage of cycle	End bud score	Alveolar score
Dioestrus	0.9 ± 0.7^{a}	3.4 ± 1.0^{b}
Pro-oestrus	2.4 ± 0.7^{ab}	1.9 ± 0.8^{ab}
Oestrus	3.6 ± 1.0^{b}	0.6 ± 0.4^{a}
Metoestrus	0.8 ± 0.3^{a}	1.8 ± 0.8^{ab}

Mammary gland whole mounts from mice at each stage of the oestrous cycle were subjectively scored on a scale of 0 to 5 for end bud development (0 = no end buds, 5 = numerous, large end buds) and alveolar development (0 = no alveolar ductules, 5 = numerous alveolar ductules). Data are means ± SEM (n=4).
[a,b] Means within a column with different superscripts are significantly different ($P<0.05$).

Supplementation of BM with 17β-oestradiol at 1 ng/ml did not alter final DNA levels in cultures of COMMA-1D cells ($P>0.05$; Figure 5.3b). The overall effects of MFP and CFP throughout the oestrous cycle in the presence of oestrogen were not different ($P>0.05$). There was a highly significant stage-of-cycle effect ($P<0.001$) whereby both MFP and CFP induced greater ($P<0.05$) cell growth at oestrus relative to other stages of the cycle.

Supplementation of BM with progesterone had no effect on the final DNA level in cultures of COMMA-1D cells ($P>0.05$; Figure 5.3c). There was a highly significant effect of tissue type ($P<0.001$) in the presence of progesterone where the mitogenic effect of MFP was greater than that of CFP across the oestrous cycle. Furthermore, there was a highly significant stage-of-cycle effect ($P<0.001$) whereby cell growth stimulated by co-cultured CFP and MFP was greatest at oestrus and had subsequently decreased ($P<0.05$) at metoestrus.

Addition of oestrogen and progesterone to BM had no effect on final DNA yield ($P>0.05$; Figure 5.3d). In this medium there was no difference ($P>0.05$) between the effects of MFP and CFP during the oestrous cycle. Response to MFP was unaltered during the cycle ($P>0.05$) while there was more ($P<0.05$) growth in response to CFP at dioestrus and oestrus.

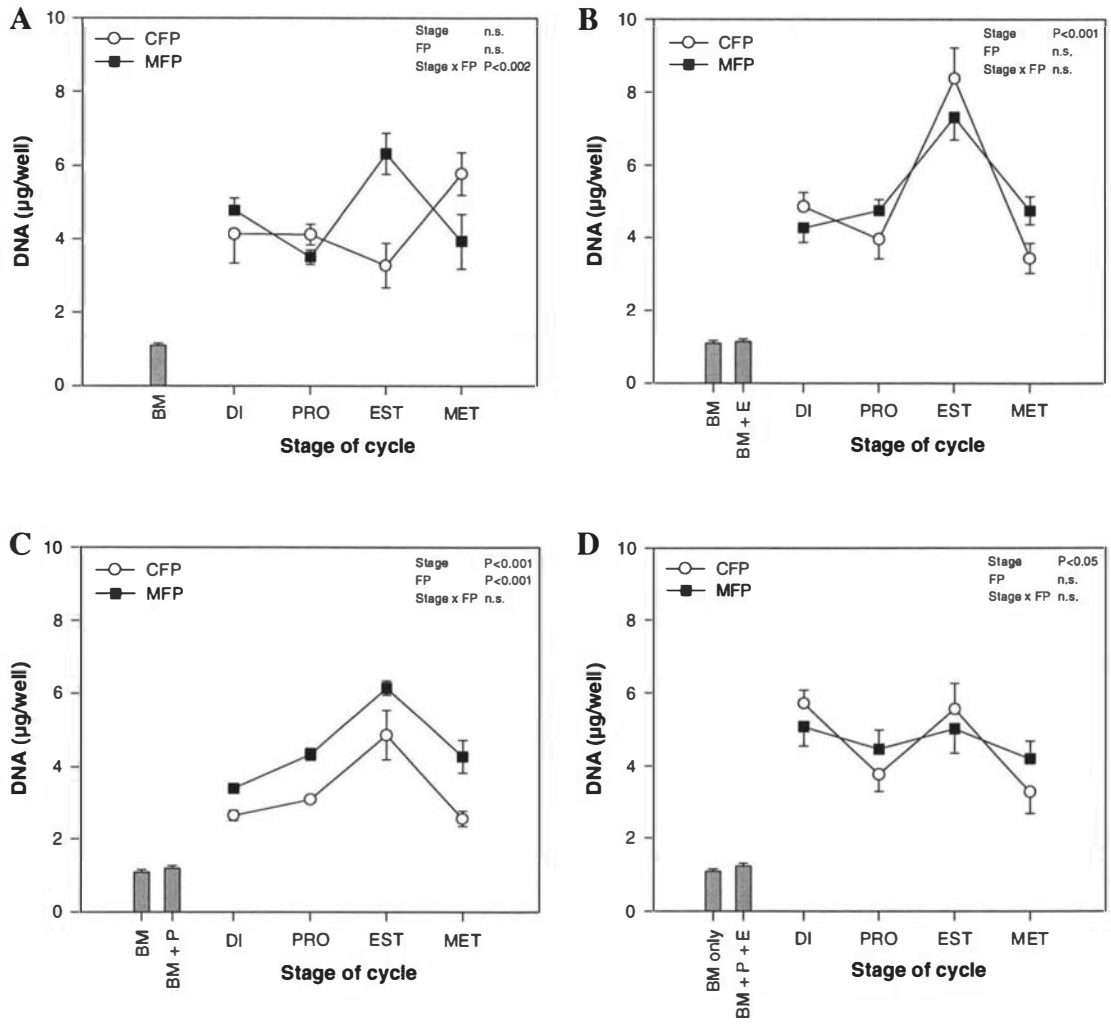


Figure 5.3 *In vitro* mitogenic stimulation by virgin mouse CFP and MFP during the oestrous cycle in (A) hormone-free BM, or BM supplemented with (B) 17β-oestradiol (E; 1 ng/ml); (C) progesterone (P; 1 µg/ml); or (D) 17β-oestradiol + progesterone. COMMA-1D cells were co-cultured for 5 days with mammary tissues from females at the indicated stages of the oestrous cycle and the resultant DNA yields measured. Values are means ± SEM (n=4). The significance of main effects and their interaction are indicated.

Analysis of the overall responses to co-cultured mammary tissue in media supplemented with oestrogen and/or progesterone revealed that growth stimulated by CFP was suppressed ($P<0.05$) in the presence of progesterone and was increased ($P<0.05$) by oestrogen, while it remained unchanged in their combination (Table 5.2). DNA levels in response to MFP tended ($P<0.08$) to be increased in the presence of oestrogen while responses were unaltered by including progesterone either alone or with oestrogen ($P>0.05$).

Table 5.2 Least squares mean COMMA-1D growth responses to co-cultured mammary tissue in medium supplemented with ovarian steroid hormones.

Medium treatment	CFP‡	MFP†
BM	4.32^{b}	4.63^{a}
17β-oestradiol	5.16^{c}	5.27^{b}
progesterone	3.29^{a}	4.54^{a}
17β oestradiol + progesterone	4.57^{b}	4.69^{a}

^{a,b,c} Least squares means within a column with different superscripts are significantly different by LSD. ‡($P<0.05$) †($P<0.08$). Pooled SE = 0.25

Previous investigations have shown that co-cultured fat pad markedly potentiates the mitogenic effect of IGF-I and EGF on COMMA-1D cells (Chapters 2 and 3). Cultures showed a slight but non-significant ($P>0.15$) response to IGF-I added to BM (Figure 5.4a). In the presence of IGF-I there was a significantly ($P<0.01$) greater response to MFP than CFP. There was no change ($P>0.05$) in the proliferative effect of MFP during the oestrous cycle while there was a significantly greater response to CFP at metoestrus ($P<0.05$).

EGF added to BM increased ($P<0.05$) the final DNA level in COMMA-1D cultures by 21% (Figure 5.4b). The mean response to CFP across the oestrous cycle in the presence of EGF was 58% of that stimulated by MFP ($P<0.001$). There was a significant stage-of-cycle effect ($P<0.05$) whereby the mean response to co-cultured mammary tissue was maximal at oestrus and subsequently declined ($P<0.05$) at metoestrus. The maximal response to MFP was recorded at pro-oestrus while CFP stimulation was greatest at oestrus and least at pro-oestrus and metoestrus.

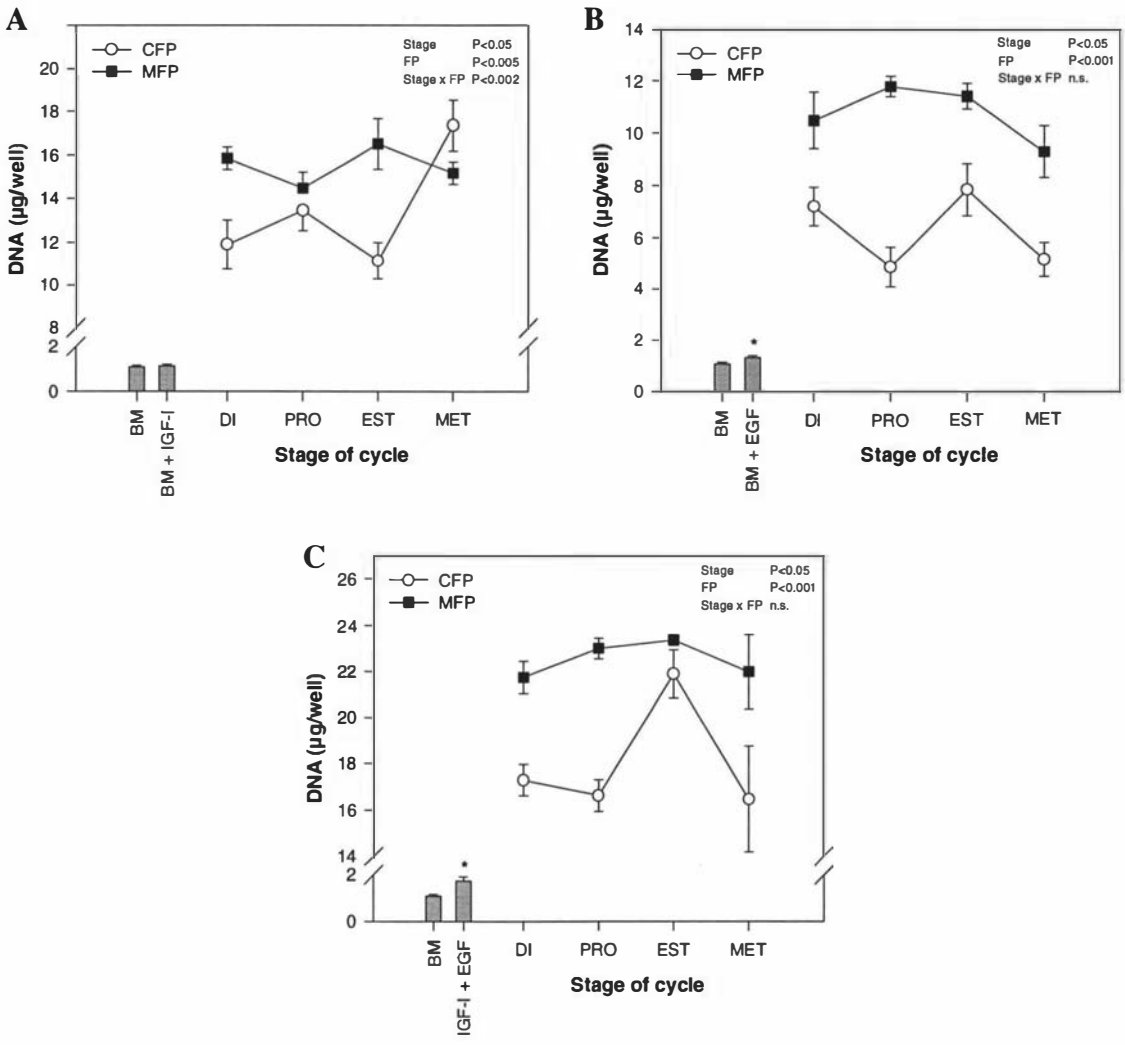


Figure 5.4 *In vitro* mitogenic stimulation by virgin mouse CFP and MFP during the oestrous cycle in BM supplemented with (A) IGF-I (100 ng/ml); (B) EGF (25 ng/ml); or (C) IGF-I + EGF. COMMA-1D cells were co-cultured for 5 days with mammary tissues from females at the indicated stages of the oestrous cycle and the resultant DNA yields measured. Values are means ± SEM (n=4). *P<0.05 compared to BM only. The significance of main effects and their interaction are indicated.

Cell growth was significantly (P<0.05) increased by supplementing BM with IGF-I + EGF (Figure 5.4c). There was a greater (P<0.001) overall effect of MFP compared to CFP in the presence of IGF-I + EGF. In this medium treatment there was a significant (P<0.05) stage-of-cycle effect whereby cell growth in response to co-cultured tissue was greater at oestrus compared to other stages of the cycle.

5.4.3 CM modulation of steroid action

The finding that co-cultured mammary fat pad facilitated the growth of COMMA-1D cells in response to oestrogen while its mitogenic effect was suppressed by progesterone was further investigated. CM was utilised to delineate whether these effects involved hormonal action on the mammary tissue, or if an activity was liberated independently from MFP and CFP to subsequently modulate epithelial responsiveness to these steroids. Cultures grown in CM alone showed a small but non-significant increase in final DNA yield. Supplementation of BM with oestrogen stimulated an increase in cell growth ($P<0.05$) which was further increased in CM ($P<0.05$). This effect did not occur in the presence of progesterone. Furthermore, progesterone suppressed the CM-facilitated growth response to oestrogen.

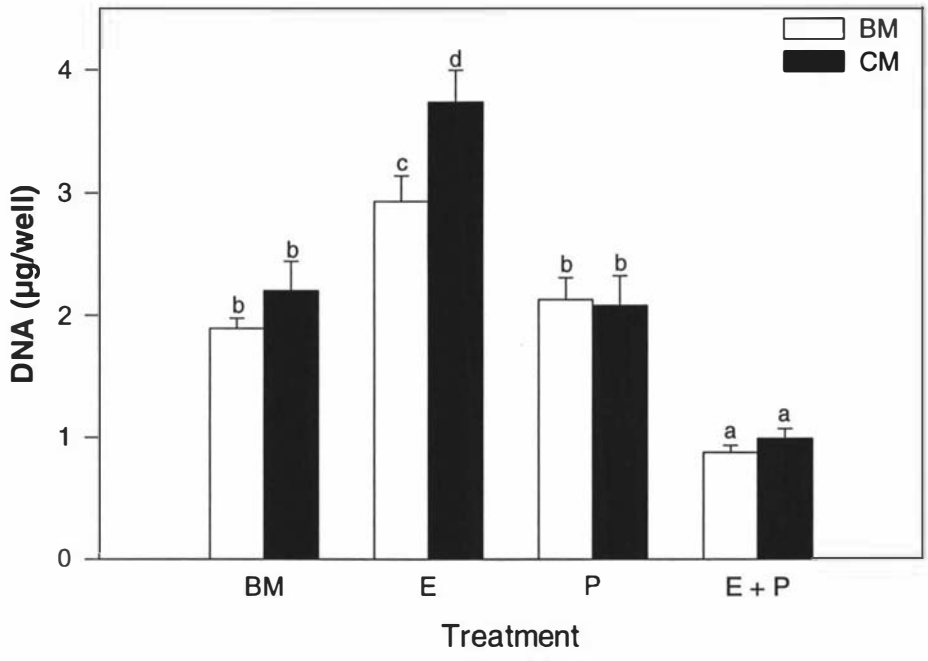


Figure 5.5 *In vitro* proliferation of COMMA-1D cells in BM or CM supplemented with ovarian steroid hormones. COMMA-1D cells were cultured for 3 days in the presence of 17β-oestradiol (E; 1 ng/ml) and/or progesterone (P; 1 µg/ml). Data are means ± SEM (n=3). ^{a,b,c} Means with different superscripts are significantly different.

The ability of CM to modulate the responsiveness of COMMA-1D cells to oestrogen was examined at the level of the oestrogen receptor (ER). Western blotting detected the ~66 kDa ER in an extract of mouse uterus but not in cultures of COMMA-1D cells. However, an immunoreactive ER protein of ~55 kDa was expressed by cells cultured for 2 days in BM supplemented with IGF-I + EGF, but not oestrogen or progesterone. This protein was not detected in COMMA-1D cells cultured in CM alone or with oestrogen or progesterone, but CM markedly increased the level of its expression induced by IGF-I + EGF.

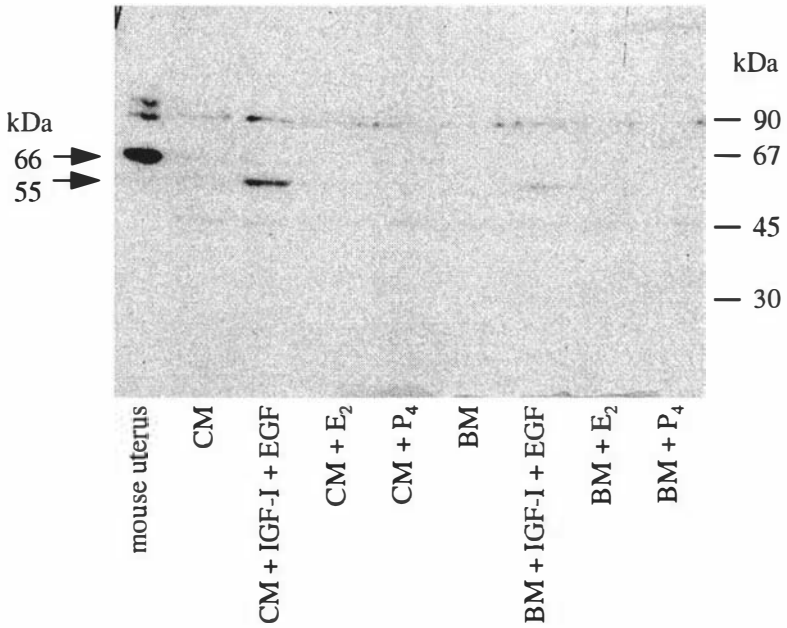


Figure 5.6 Western blotting of ER proteins in COMMA-1D cells after their culture in various medium treatments. COMMA-1D cells were cultured for 2 days in either BM or CM alone or supplemented with IGF-I (100 ng/ml) + EGF (25 ng/ml), 17β-oestradiol (E_2; 1 ng/ml), progesterone (P_4; 1 μg/ml) or E_2 + P_4. Each lane contained an equivalent amount of DNA (2 μg). An extract of virgin mouse uterus (80 μg protein) served as a positive control. The position of molecular weight markers is indicated.

5.5 DISCUSSION

Mammary parenchyma responded to the changing hormonal profiles of the oestrous cycle by demonstrating marked differences in its morphology as described by others (Dulbecco *et al.*, 1982; Vonderhaar, 1988). The appearance of highly mitotic end buds

at oestrus is presumably in response to an increased circulating oestrogen concentration (Daniel *et al.*, 1987; Haslam, 1988). As these hormonally-induced responses may be mediated by the mammary gland stroma (Shyamala and Ferenczy, 1984; Haslam and Counterman, 1991), a co-culture system (Chapter 2) was utilised to determine whether the *in vitro* mitogenic stimulation afforded by the mammary fat pad was concomitantly altered during the oestrous cycle.

The present findings show that the mitogenic effect of the mammary fat pad is increased at oestrus relative to other stages of the cycle in the presence of certain medium supplements, particularly oestrogen and progesterone. That this stage-of-cycle effect occurred in response to both CFP and MFP indicates that it was initiated by a stroma-derived activity. Results of previous studies (Chapter 4) and those of others (Beck *et al.*, 1989) suggest that the mouse mammary fat pad stimulates the growth of mammary epithelial cells *in vitro* by its liberation of unsaturated fatty acids. Given the present findings, hormonal changes during the oestrous cycle may modulate the release of unsaturated fatty acids from adipocytes within the mammary fat pad. Consistent with this notion are reports that oestrogen stimulates lipolysis (Rebuffé-Scrive, 1987; Steingrimsdottir *et al.*, 1980) and inhibits lipogenesis in adipocytes (Steingrimsdottir *et al.*, 1980; Ramirez, 1981), while progesterone promotes lipogenesis (Krotkiewski and Björntorp, 1976; Shirling *et al.*, 1981). Furthermore, lipolysis in rat adipocytes is least at dioestrus and increases to a maximum at oestrus (Hansen *et al.*, 1980), a pattern which parallels the mitogenic stimulation afforded by the co-cultured mammary tissues during the oestrous cycle in this study. The level of systemic prolactin also peaks at pro-oestrus-oestrus (Lotz and Krause, 1978), during which time it may indirectly stimulate lipolysis within mammary adipocytes (Kidwell and Shaffer, 1984). However, it is unlikely that prolactin is involved in the present results as receptors for prolactin do not occur within the CFP (Bhattacharya and Vonderhaar, 1979).

This stage-specific pattern of mitogenic stimulation also aligns with the proliferation of mammary epithelium *in vivo*, where DNA synthesis in ductal epithelium increases to a maximum at oestrus (Sutton and Suhrbier, 1967) coincident with the formation of ductal end buds (Dulbecco *et al.*, 1982). The growth of ductal end buds *in vivo* is also stimulated by locally implanted IGF-I (Ruan *et al.*, 1992) and EGF (Coleman *et al.*, 1988). While it has not been reported, it is conceivable that the local expression of

these growth factors within the mammary gland is elevated at oestrus in response to oestrogen (Carlsson *et al.*, 1989; Stevenson *et al.*, 1994; Dickson and Lippman, 1987). It was shown in Chapter 4 and by others (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994) that unsaturated fatty acids potentiate the mitogenic effects of these growth factors on mammary epithelial cells. Together, these findings raise the possibility that ductal proliferation at oestrus is stimulated by oestrogen-inducible growth factors whose mitogenic effects are enhanced by a concomitant local release of unsaturated fatty acids. Consistent with this suggestion is the fact that ductal growth in immature mice is impaired by a diet deficient in essential fatty acids (Miyamoto-Tiaven *et al.*, 1981). Furthermore, unsaturated fatty acids may enhance growth factor-induced mitogenesis via the lipid-inducible signalling messenger, protein kinase C (PKC; Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994; Chapter 4), where oestrogen elevates, and progesterone suppresses, mammary epithelial PKC levels within the developing mammary gland (Holladay and Bolander, 1986). These changes may reflect alterations in the local availability of lipids. Hormonal regulation of mammary lipid metabolism may also account for the fact that the growth of mammary tumour cells within various adipose depots is stimulated by oestrogen and suppressed by progesterone (Elliott *et al.*, 1992). Such results may be manifest in the demonstration that mammary glands of rats treated with carcinogen at pro-oestrus and oestrus have a higher tumour incidence and a shorter latency period compared to rats treated at dioestrus (Lindsey *et al.*, 1981; Ratko and Beattie, 1985).

As well as its potential for endocrine regulation, mitogenic stimulation afforded by the mammary fat pad may be locally influenced by the mammary epithelium. Such an influence has been implicated in various aspects of stromal activity including lipid metabolism (Bartley *et al.*, 1981) and growth factor expression (Singer *et al.*, 1995). Comparison of the response to co-cultured epithelium-free CFP and MFP which contains endogenous epithelium suggests that mammary epithelium can promote the release of a growth-promoting factor(s) from the mammary fat pad. This effect was specific to medium supplemented with progesterone or growth factors and was antagonised by the presence of oestrogen.

The mechanisms underlying these responses remain to be established. Epithelial upregulation of stromal stimulation may reflect its ability to induce the expression of

stroma-derived growth factors (Singer *et al.*, 1995; Chapters 9-11). In keeping with an earlier suggestion however, it is proposed that this effect more likely represents epithelial modulation of lipid metabolism within proximal mammary adipocytes. CFP from prepubertal mice markedly potentiates the mitogenic effects of IGF-I and EGF on COMMA-1D cells, as in the present study, by liberating unsaturated fatty acids into the culture medium (Chapter 4). The finding that MFP was more stimulatory than CFP in combination with these growth factors may represent epithelial-induced liberation of fatty acids, particularly unsaturated fatty acids, from mammary adipocytes. This concurs with other reports showing that mammary epithelium induces lipolysis in adjacent mammary adipocytes (Elias *et al.*, 1973), perhaps via the local synthesis of histamine (Kidwell and Shaffer, 1984) or prostaglandin $F_{2\alpha}$ (Kidwell *et al.*, 1982). Furthermore, co-cultured MFP stimulates higher levels of casein synthesis by mammary epithelium *in vitro* than CFP, possibly by liberating more unsaturated fatty acids such as linoleic acid (Levay-Young *et al.*, 1987). Kidwell *et al.* (1982) reported, however, that MFP released less linoleic acid than CFP tissue *in vitro*, an inconsistency probably associated with increased sequestration of unsaturated fatty acids by endogenous epithelium in the presence of prolactin (Kidwell *et al.*, 1982).

Differential modulation of the mitogenic effects of CFP and MFP *in vitro* by progesterone and oestrogen is intriguing, particularly given the pivotal roles of these steroids during mammogenesis. It is presently unclear as to whether this difference involved epithelial modulation of hormone action on the co-cultured mammary stroma, or whether CFP and MFP differentially liberated certain factors to regulate COMMA-1D responsiveness to oestrogen and progesterone. Such results do indicate, however, that there may be an important relationship between the local interaction of mammary epithelial and stromal cells and the actions of ovarian steroid hormones on epithelial proliferation.

Co-cultured mammary fat pad also promoted the stimulation of COMMA-1D cell growth by oestrogen. This oestrogenic stimulation, as well as a corresponding negative effect of progesterone, was similarly evident in CM, a finding which suggests that these effects were independent of steroid action on the co-cultured mammary tissue. A possible effector of such responses are unsaturated fatty acids which likely account for the growth-promoting effects of CM (Beck *et al.*, 1989; Chapter 4). That unsaturated

fatty acids modulate ovarian steroid-induced mammary growth may relate to the fact that both tumour growth within adipose tissue (Elliott *et al.*, 1992) and mammary epithelial levels of lipid-activated PKC (Holladay and Bolander, 1986) are increased by oestrogen and decreased by progesterone.

The ability of CM to facilitate oestrogen-induced growth was examined at the level of the ER. Although the mature ~66 kDa ER was not expressed by COMMA-1D cells, treatment with IGF-I + EGF did induce the expression of a ~55 kDa ER protein. This form of the ER is a proteolytic cleavage product (Horigome *et al.*, 1988) which binds oestrogen with the same affinity as the mature ER (Horigome *et al.*, 1987), and which has an increased affinity for DNA (Baskevitch and Rochefort, 1985). A physiological role for this ER is suggested by the fact that its expression in the uterus is increased at oestrus (Faye *et al.*, 1986) and by treatment with exogenous oestrogen (Horigome *et al.*, 1988). Upregulation of its expression in response to these growth factors in the present study may correspond to the finding that EGF increases levels of both the ~66 kDa and ~55 kDa ER in the uterus (Ignar-Trowbridge *et al.*, 1992). The abundance of the ~55 kDa protein as induced by IGF-I + EGF was further increased by CM. This may be in response to unsaturated fatty acids present in CM as supplementation of MCF-7 cells with unsaturated, but not saturated, fatty acids leads to cleavage of the mature ER protein (Borras and Leclercq, 1992). Such an effect may involve ER mRNA destabilisation via PKC (Saceda *et al.*, 1991). While the mechanisms underlying these responses are unclear, such findings could relate to the interrelationship between steroid receptor- and growth factor-signalling through PKC (Cho and Katzenellenbogen, 1993; Katzenellenbogen, 1996; Martínez-Lacaci and Dickson, 1996).

In conclusion, these results indicate an important role for the mammary fat pad in mediating the mammogenic effects of the ovarian steroids during the oestrous cycle. Systemic hormonal changes may alter the liberation of mitogenic activity from the mammary stroma to subsequently modulate the responsiveness of mammary epithelium to growth factors and ovarian steroids. Such effects are also likely regulated at the local level of the mammary gland by the interaction of epithelial cells with the surrounding stroma.

CHAPTER 6

INFLUENCE OF ONTOGENIC STATE, OVARIECTOMY, AND OVARIAN STEROID HORMONES ON THE *IN VITRO* PROLIFERATIVE EFFECT OF THE MAMMARY FAT PAD

6.1 ABSTRACT

The present experiments were conducted to investigate whether the *in vitro* mitogenic effects of the mouse mammary fat pad are modulated by stage of postnatal development, ovarian steroids and epithelial-stromal interaction. These effects were bioassayed using COMMA-1D mouse mammary epithelial cells co-cultured with mammary fat pad tissue cleared of epithelium (CFP) or intact mammary tissue (MFP) from females at various stages of postnatal development, and from 8-week old intact and ovariectomised females treated for 5 days with oestrogen and/or progesterone.

Mitogenic stimulation by CFP and MFP was substantial at 3 weeks of age coincident with a period of rapid ductal growth *in vivo*. This stimulation subsequently declined ($P<0.05$) to lowest levels in mature virgin and mid-pregnant mice. During this period MFP was more mitogenic than CFP in specific medium treatments, possibly reflecting a local influence of endogenous epithelium. MFP-induced cell growth in various growth factor and steroid medium treatments was increased ($P<0.05$) by 2-3 fold during lactation. This effect in the basal medium (BM) either alone or in the presence of ovarian steroids subsequently declined ($P<0.05$) after weaning. Stimulation of cell growth by CFP during this period was dependant upon the medium treatment; it remained low in the basal medium (BM) or in the presence of ovarian steroids but increased ($P<0.05$) in late lactation and post-weaning in the presence of IGF-I and/or EGF.

Prepubertal ovariectomy abrogated mammary development but did not reduce ($P>0.05$) the mitogenic effect of co-cultured MFP or CFP on COMMA-1D cells. Conversely, ovarian function was necessary for progesterone treatment to increase ($P<0.05$) the mitogenic effect of both MFP and CFP. Furthermore, co-cultured MFP from saline-treated mice was more stimulatory than contralateral CFP tissue ($P<0.05$), indicating that endogenous epithelium may promote the release of a mitogenic activity from the mammary fat pad. Oestrogen modulated this response in that the mitogenic effect of CFP was increased ($P<0.05$) when mice were treated with oestrogen while that of the contralateral MFP was suppressed. Taken together, these findings indicate that mitogenic stimulation afforded by the mammary fat pad may be involved in regulating postnatal development of the mammary gland. This stimulation may be locally induced

by the mammary epithelium, an influence which specifically modulates hormonal effects on the mammary fat pad.

6.2 INTRODUCTION

Epithelium within the rodent mammary gland demonstrates marked changes in its proliferation and morphology as the female passes through a series of postnatal reproductive states (reviewed by Imagawa *et al.*, 1994). Parenchyma of the neonatal gland constitutes little more than a branched ductal rudiment around the nipple. Epithelial cells within the pre- and peripubertal mammary gland then undergo rapid proliferation to establish a ductal network within the bounds of the mammary fat pad. Alveolar growth commences with the onset of pregnancy so that by parturition the mammary gland is comprised of lobuloalveolar structures capable of active milk secretion. Stage of development also influences the tumorigenic susceptibility of the mammary gland (Grubbs *et al.*, 1983) with age being one of the main risk factors in human breast cancer (Kelsey and Berkowitz, 1988).

This course of postnatal mammogenesis is directed by the actions of several endocrine hormones (Topper and Freeman, 1980). Of these, the ovarian steroids oestrogen and progesterone fulfil pivotal roles (Lyons *et al.*, 1958; Nandi, 1958). While oestrogen stimulates epithelial proliferation and its ductal morphogenesis, progesterone promotes tertiary branching and alveolar budding (Bresciani, 1968).

Emerging evidence also suggests that the adipose and connective tissue of the mammary fat pad mediates the mammogenic effects of ovarian steroids. While oestrogen acts directly on the mammary gland (Daniel *et al.*, 1987; Haslam, 1988d), mammary epithelial cells are unresponsive to oestrogen *in vitro* (Yang *et al.*, 1980; Richards *et al.*, 1988), leading to suggestions of an indirect action via the mammary stroma (Shyamala and Ferenczy, 1984). Consistent with such proposals, receptors for both oestrogen and progesterone are present within the mammary fat pad as well as on mammary epithelial cells (Haslam and Shyamala, 1981; Haslam, 1989; Haslam and Nummy, 1992). Developmental changes in the effects of oestrogen and progesterone on the mammary epithelium (Haslam, 1989) may reflect an altered stromal expression of steroid receptors

(Haslam, 1988c; Haslam and Nummy, 1992), or a differing ability of the stroma to modulate epithelial responsiveness to ovarian steroids (Haslam and Counterman, 1991). What remains unclear is how these developmental changes and the local effects of ovarian steroids relate to the requirement of mammary epithelium for a depot of adipose tissue in which to develop *in vivo* (reviewed by Hoshino, 1978). Adipose tissue stimulates the metastatic growth of mammary tumour cells and facilitates their oestrogen-responsiveness while progesterone suppresses their growth (Elliott *et al.*, 1992). It was recently shown that the *in vitro* mitogenic effect of the mammary fat pad is altered during the oestrous cycle and that this activity can modulate the responsiveness of mammary epithelial cells to ovarian steroids *in vitro* (Chapter 5). These results suggest that adipocyte-derived factors, under the influence of endocrine signals, may directly or indirectly control mammary epithelial cell proliferation. Such effects may also be regulated at the local level of the mammary gland by the epithelium (Cunha and Hom, 1996) which can influence various aspects of lipid metabolism (Bartley *et al.*, 1981) and growth factor expression (Coleman-Krnacik and Rosen, 1994). Given the marked effects of developmental state and ovarian hormones on epithelial growth and the candidate role for the mammary fat pad in modulating these, the objective of these experiments was to investigate whether such responses reflect changes in the mitogenic capacity of the mammary fat pad. A co-culture system was used to bioassay this capacity at various stages of development and after ovariectomy and ovarian steroid treatment. The ability of endogenous epithelium to modulate these effects was examined by comparing the responses to epithelium-free and intact mammary tissue. Results indicate that the mitogenic capacity of the mammary fat pad and its ability to modulate epithelial responsiveness to other factors are altered during ontogeny and are influenced by the effects of ovariectomy, oestrogen and progesterone. Importantly, these effects may be locally regulated by the endogenous epithelium.

6.3 MATERIALS AND METHODS

6.3.1 Animals

BALB/c mice were housed in the Ruakura Small Animal Colony under standard conditions with free access to food (Diet 86, NRM Stockfeeds, NZ) and water.

The effect of ontogenic state on the mitogenic capacity of the mammary fat pad was studied in a group of virgin females weaned together from standardised litters. The epithelial component of one abdominal mammary gland of these mice was surgically excised at 21-23 days of age according to the procedure of DeOme *et al.* (1959) to leave a mammary fat pad cleared of endogenous epithelium (CFP). The contralateral gland was left intact to allow normal epithelial development within the mammary fat pad (MFP). Mice were subsequently housed in groups of 4.

Females were subsequently sacrificed at the following stages of development to provide mammary tissues for co-culture experiments (n=4 per group): virgins at 21, 35 and 80 days of age; day 9 of pregnancy (80 days of age); early (days 1-2) and late (day 18) lactation; and 2 days post-weaning (18 days post-partum). For those females sacrificed in pregnancy, day of vaginal plug detection was deemed day 0 of gestation; pregnancy was confirmed at sacrifice. Litters of lactating females were standardised to 8 pups within 24 hours of parturition.

In a separate study a group of virgin females was used to investigate the influence of ovariectomy and ovarian steroids on the mitogenic capacity of the mammary fat pad. At the time that one abdominal mammary fat pad was surgically cleared of epithelium as described above, half of the females were bi-laterally ovariectomised while the remainder were sham-operated. From 50 days of age, groups of ovariectomised and sham-operated mice were treated for 5 days with daily injections (0.1 ml, s.c.) of saline, 17β-oestradiol (Sigma; 1 μg), progesterone (Sigma; 1 mg), or 17β-oestradiol + progesterone (n=5 per group). Steroids were prepared as ethanolic stocks diluted into saline; all treatments contained an ethanol concentration of 6%. The final injection was given on the morning of sacrifice. Stage of oestrous for saline-treated sham-operated females was determined on the morning of sacrifice by vaginal lavage (Rugh, 1967) and confirmed by vaginal appearance (Champlin *et al.*, 1973), and 4 females in pro-oestrus

were selected to represent this group. Completeness of ovariectomy was determined at sacrifice.

6.3.2 Co-cultures

An *in vitro* co-culture system (Chapter 2) was used to measure the mitogenic and growth modulatory effects of mammary tissues from mice in the various treatment groups. COMMA-1D cells seeded into 24-well plates (5 x 10^4 cells/well) were then quiesced for 48 h in hormone-free DMEM basal medium (BM). CFP tissue or the epithelial region of MFP was sectioned into explants (5-10 mg) which were weighed and rinsed in BM. Explants were placed on rafts of siliconised lens paper and floated at the gas:medium interface of appropriate cultures. All cultures were incubated in 0.5 ml treatment medium for the first 3 days, with a further 0.5 ml of fresh treatment medium added on day 3. Cultures were terminated after 5 days and the final level of DNA in the trypsinised monolayers was measured using a fluorometric assay (Labarca and Paigen, 1980). CFP and MFP explants were blotted and weighed at the end of the co-culture period.

17β-oestradiol and progesterone were added to BM from ethanolic stocks and all media contained the same concentration of ethanol. Recombinant human IGF-I was from Genentech (San Francisco, CA), and mouse EGF was from Sigma.

6.3.3 Mammary gland whole mounts

Thoracic mammary glands were spread on slides and fixed in Carnoy's fixative for 24 h. Preparations were defatted in acetone and stained with alum carmine as described (Chapter 5). Whole mounts were photographed using a dissecting microscope.

6.3.4 Statistical analyses

Groups of females within the ontogeny experiment were sacrificed on separate occasions, except the mature virgin and day 9 pregnancy groups which were sacrificed together at 80 days of age, and the late lactation and post-weaning groups which were sacrificed together at 110 days of age. To directly compare the *in vitro* responses to co-cultured mammary tissues at these different times, culture DNA yields on each occasion were normalised against DNA values in the respective BM only and 10% FCS

treatments using the formula $^{(x-BM)}/_{(FCS-BM)}$, where the final DNA level in BM and 10% FCS treatments equalled 0 and 1, respectively. Data expressed in this way were analysed using REML analysis and the MIXED procedure of SAS to test the main effects of stage of development, *in vitro* medium treatment and fat pad type and their interactions, where mouse was included as a random term within stage of development.

Results from the ovariectomy and steroid priming experiment were subjected to analysis of variance using the GLM procedure of SAS to test the main effects and interactions of *in vivo* oestrogen treatment, *in vivo* progesterone treatment, ovariectomy, fat pad type and the *in vitro* medium. Mammary tissue weights and change in the weight of co-cultured mammary tissues were compared by the GLM procedure. These data were expressed as the net change in weight, where similar results were obtained when weight change was expressed as a percentage of their initial weight.

6.4 RESULTS

6.4.1 Effect of ontogenic state

6.4.1.1 Mammary gland development

The marked changes in parenchymal growth and morphogenesis which occurred during postnatal development are depicted in Figure 6.1 (a-g). The mammary parenchyma of 3-week old females comprised a simple rudiment about the nipple. This subsequently underwent extensive proliferation prior to and during puberty to establish a ductal tree within the bounds of the mammary fat pad in mature virgin females. Ductal alveolar budding was evident by day 9 of pregnancy and lobules of alveoli occupied virtually the entire gland during lactation. Post-weaning involution was associated with parenchymal regression and a concomitant repletion of adipocyte lipids. The CFP comprised a depot of adipose tissue devoid of mammary parenchyma (Figure 6.1h).

This development was reflected by changes in the weight of the mammary tissues (Figure 6.2). Change in the weight of CFP up until mid-pregnancy closely paralleled that recorded for MFP. CFP weight during lactation was significantly ($P<0.05$) reduced relative to that in mid-pregnancy. In contrast, MFP weight was markedly increased ($P<0.05$) with the onset of lactation and declined abruptly after weaning.

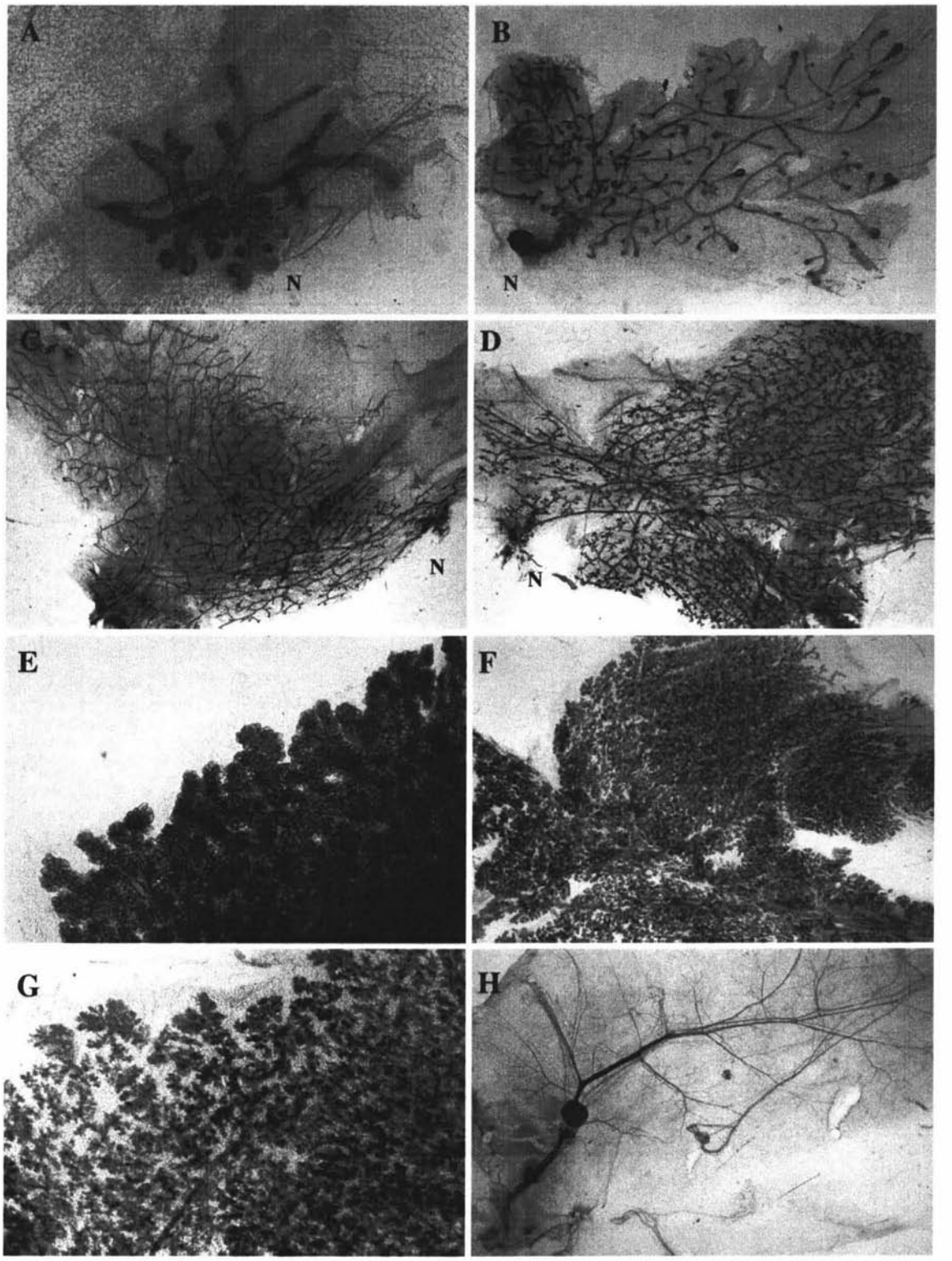


Figure 6.1 Ontogeny of postnatal mammary gland development and morphogenesis in female BALB/c mice. Thoracic mammary glands were from females at various stages of postnatal development (A) 21 days; (B) 35 days; (C) 80 days; (D) day 9 of pregnancy; (E) early lactation; and (F and G) involution. (H) #4 abdominal CFP devoid of endogenous epithelium from a 90-day old virgin female. N, nipple. Magnifications: A, 70x; B, 19x; C-H, 10x.

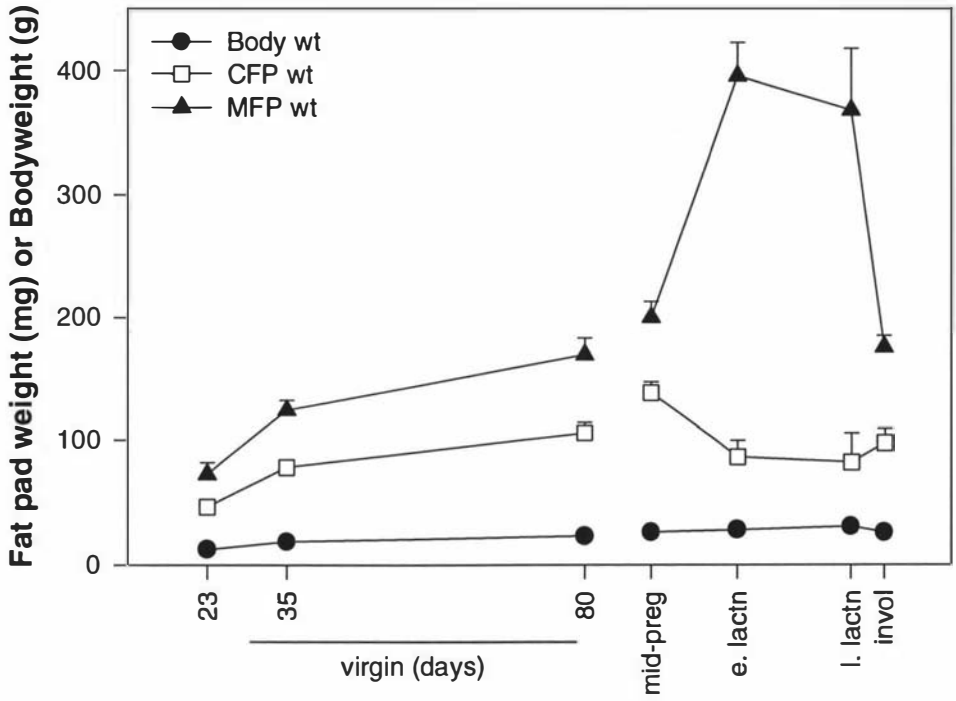


Figure 6.2 Body and mammary tissue weights of female BALB/c mice during postnatal development. Females were sacrificed at the stages indicated and the weights of the epithelium-free CFP and contralateral MFP were determined prior to their use in co-culture experiments. Each point is mean ± SEM (n=5).

6.4.1.2 Co-cultures

The growth of COMMA-1D cells in several medium treatments was used to bioassay the mitogenic effects of CFP and MFP from mice across various physiological states and stages of mammary gland development.

Cell growth in the hormone-free BM was markedly stimulated by CFP or MFP taken at 3 weeks of age; this then declined ($P<0.05$) to low levels for tissue from mature virgin (day 80) and mid-pregnant females (Figure 6.3a). The mitogenic effect of CFP did not differ ($P>0.05$) across the remaining developmental states. In contrast, cell growth in response to MFP was significantly ($P<0.001$) increased in early and late lactation relative to mid-pregnancy, a response which subsequently declined ($P<0.05$) after weaning.

As reported previously (Chapter 5), co-cultured virgin MFP and CFP markedly potentiated the mitogenic effect of IGF-I on COMMA-1D cells. As in BM, growth stimulated by both CFP and MFP in the presence of IGF-I declined ($P<0.05$) from 3 weeks of age to lowest levels in mature virgin and mid-pregnancy states (Figure 6.3b).

In contrast to the responses in BM, mitogenic stimulation afforded by CFP increased ($P<0.001$) in late lactation and remained elevated post-weaning in a pattern similar to that induced by MFP. The response to MFP at 5 weeks of age, in early and late lactation, and post-weaning was significantly greater ($P<0.05$) than that to CFP.

The mitogenic effect of EGF was also potentiated by co-cultured mammary tissue. Cell growth stimulated by MFP and CFP declined to lowest levels in mature virgin, mid-pregnancy, and early lactation states; the response to CFP increased ($P<0.05$) in late lactation in parallel with that to MFP (Figure 6.3c). There was a consistently greater ($P<0.01$) overall effect of MFP compared to CFP in the presence of EGF.

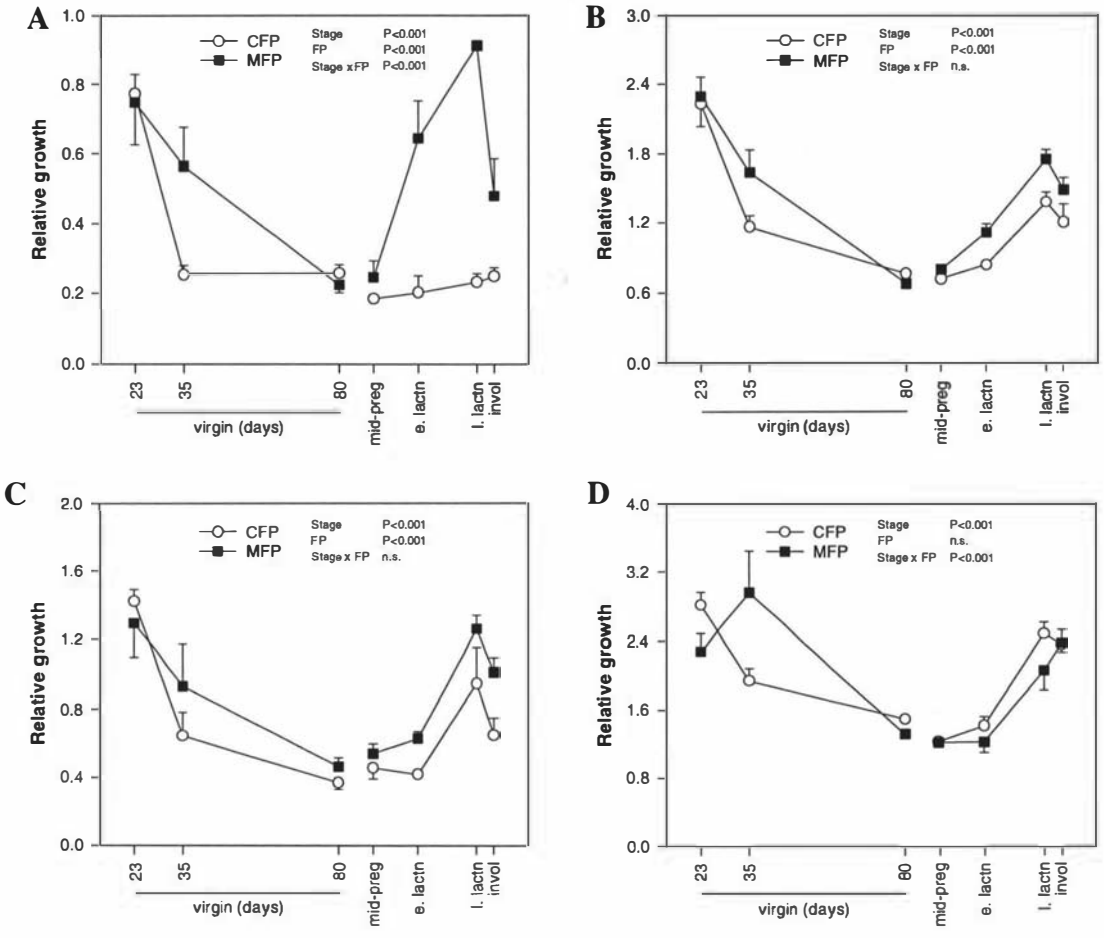


Figure 6.3 Ontogeny of mitogenic stimulation by mouse CFP and MFP in (A) hormone-free BM, or BM supplemented with (B) IGF-I (100 ng/ml); (C) EGF (25 ng/ml); or (D) IGF-I + EGF. COMMA-1D cells were co-cultured for 5 days with mammary tissues from female mice at the indicated stages of postnatal development and the resultant DNA yields measured. Data are corrected for DNA values in BM alone (equals 0) and are expressed as a proportion of the response to 10% FCS (equals 1) as detailed in the Methods. Values are means ± SEM (n=4).

Responses to co-cultured mammary tissue in the combined presence of IGF-I and EGF generally represented the additive result of the individual growth factor interactions. Both MFP and CFP were least ($P<0.05$) stimulatory in mature virgin, mid-pregnant, and early lactation states, while mitogenic stimulation afforded by MFP and CFP was increased ($P<0.001$) in late lactation and involution relative to that in early lactation (Figure 6.3d). There was no difference between the overall effect of MFP and CFP across development in this growth factor combination ($P>0.5$).

The ontogeny of growth stimulation by MFP and CFP in the presence of BM supplemented with 17β-oestradiol (Figure 6.4a) was similar to that in BM alone (refer Figure 6.3a). The presence of oestrogen tended to reduce the mitogenic effect of both MFP and CFP at 3 weeks of age, and that of MFP during lactation. Furthermore, the different growth stimulation by MFP and CFP observed in the presence of progesterone at 35 days of age and in mid-pregnancy (Figure 6.4b) was not evident in medium supplemented with oestrogen.

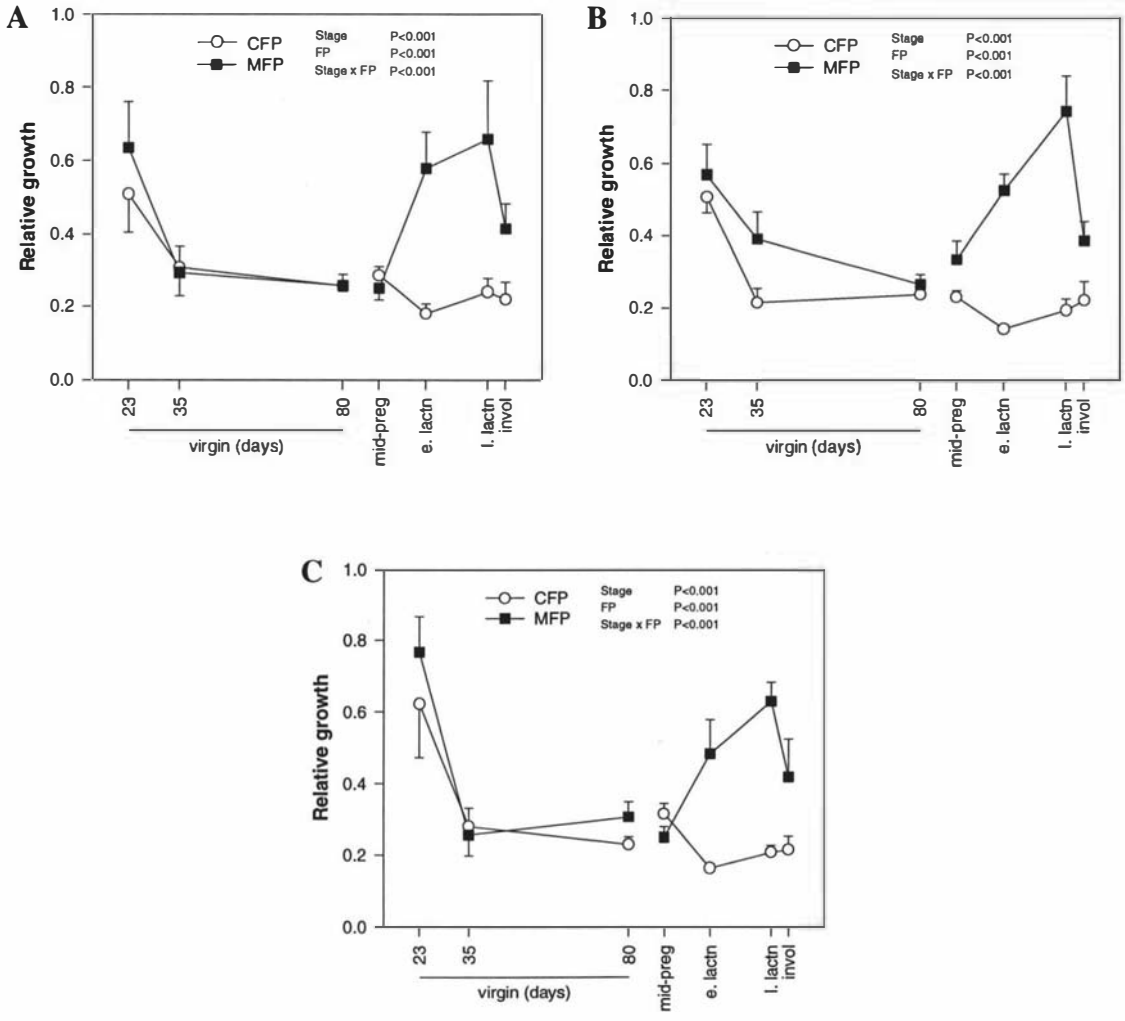


Figure 6.4 Ontogeny of mitogenic stimulation by mouse CFP and MFP in BM supplemented with (A) 17β-oestradiol (1 ng/ml); (B) progesterone (1 μg/ml); or (C) 17β-oestradiol + progesterone. COMMA-1D cells were co-cultured for 5 days with mammary tissues from females at the indicated stages of postnatal development and the resultant DNA yields measured. Data are corrected for DNA values in BM alone (equals 0) and are expressed as a proportion of the response to 10% FCS (equals 1) as detailed in the Methods. Values are means ± SEM (n=4).

The patterns of response to MFP and CFP in the presence of progesterone (Figure 6.4b) were similar to those observed in BM (refer Figure 6.3a). Supplementation of medium with progesterone tended to reduce the proliferative responses to MFP and CFP relative to those in BM.

The combination of 17β-oestradiol and progesterone in BM restored the mitogenic effect of MFP and CFP at 3 weeks while the response to MFP at 35 days was still reduced relative to that in BM alone (compare Figure 6.4c and Figure 6.3a).

Change in the weight of co-cultured CFP and MFP explants was recorded as a possible indicator of metabolic differences in these tissues (Figure 6.5). The weight of CFP and MFP explants from 3-week females decreased by 35 and 30% during the co-culture period. Weight reductions were generally much less in subsequent states. However, from mid-pregnancy onwards MFP explants lost significantly ($P<0.001$) more weight than CFP. Part of the increased weight loss from MFP in early lactation presumably represents milk lost from this tissue.

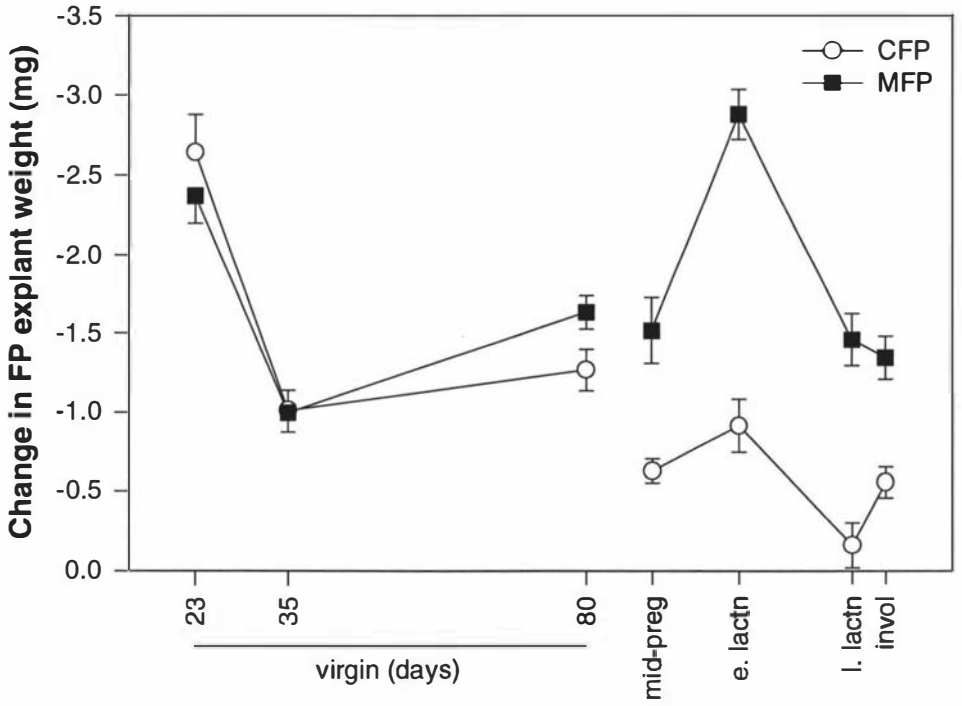


Figure 6.5 Change in the weight of co-cultured MFP and CFP explants from mice at various stages of development. Explants of tissue were weighed prior to co-culture and then re-weighed at the end of the culture period 5 days later. Values are means ± SEM for explants co-cultured in the 7 different medium treatments (n=28).

6.4.2 Effects of ovariectomy and ovarian steroids

6.4.2.1 Mammary development

Whole mounts illustrate the response of mammary parenchyma to ovariectomy and exogenous ovarian steroids (Figure 6.6, a-h). Sham-operated females developed an expansive ductal tree within the mammary fat pad by 55 days of age. In contrast, prepubertal ovariectomy completely abrogated ductal elongation to leave a quiescent, simply branched rudiment. Short-term treatment of sham-operated and ovariectomised females with oestrogen promoted end bud formation at the tips of ducts both within the ductal tree and at its periphery. Progesterone had little obvious effect on the parenchyma of ovariectomised females while it induced the formation of numerous alveolar buds on the ductal epithelium of sham-operated mice. Administration of oestrogen plus progesterone to ovariectomised females resulted in small, variable degrees of end bud and alveolar formation, where similar but more pronounced effects were observed in sham-operated mice.

Whereas mean bodyweight tended ($P<0.1$) to be increased across all ovariectomised groups relative to sham-operated females, it was unaffected ($P>0.05$) by the different steroid treatments (Table 6.1). Weight of the MFP and CFP from saline-treated mice was unaltered ($P>0.05$) by ovariectomy. Relative to the saline group, weight of the MFP and CFP was significantly increased by administering oestrogen and/or progesterone to ovariectomised mice, while oestrogen tended to increase the weight of MFP and CFP from intact females.

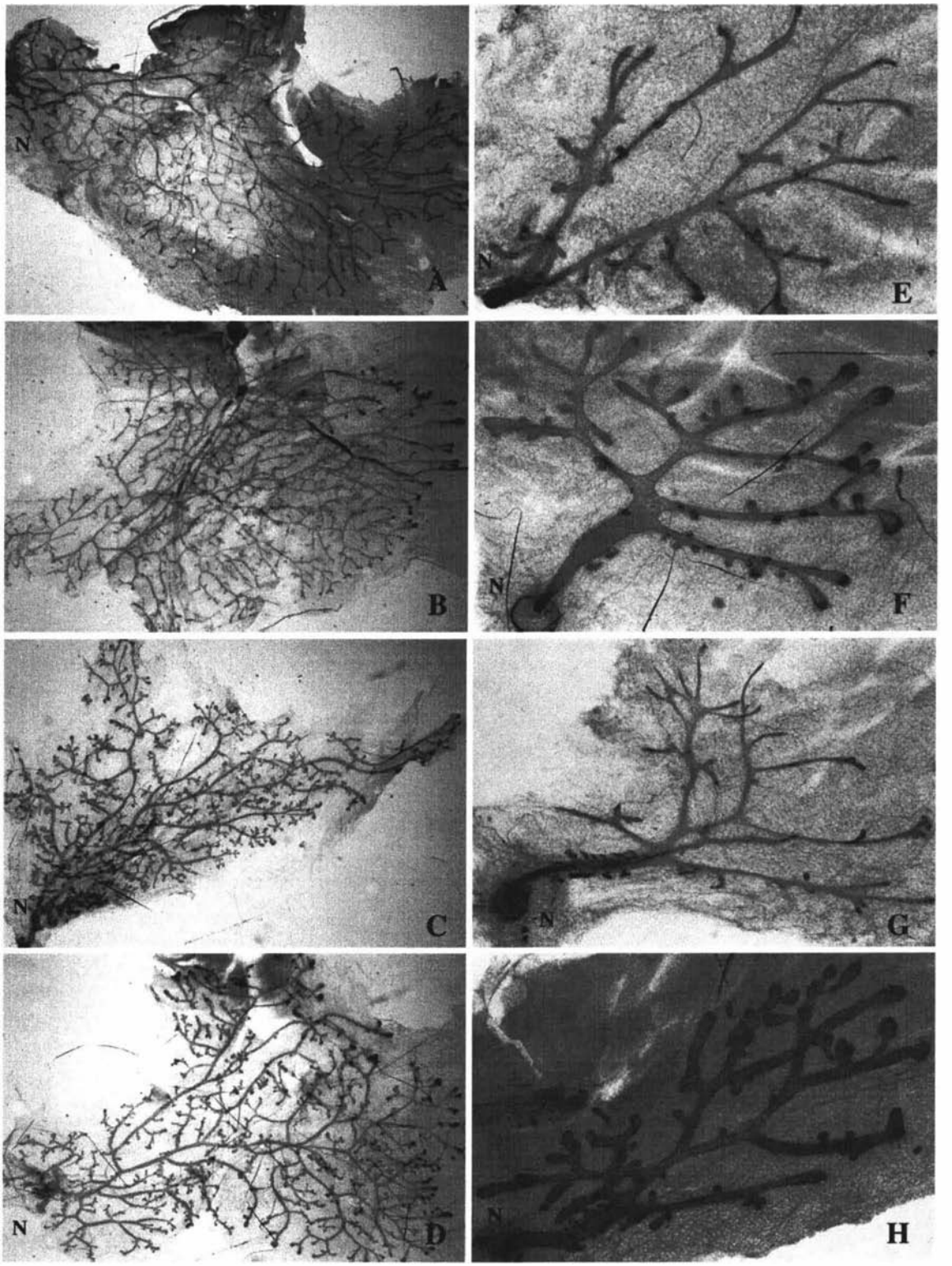


Figure 6.6 Mammary gland development in sham-operated (A-D) and ovariectomised (E-H) female mice treated for 5 days with excipient (A and E), 17β-oestradiol (B and F), progesterone (C and G) or 17β-oestradiol + progesterone (D and H). Females were ovariectomised or sham-operated at 3 weeks of age and were administered the various treatments from 50 days of age. N, nipple. Magnifications: A-D, 13x; E, 66x; F-H, 50x.

Table 6.1 Mean (± SEM) body and mammary tissue weights of sham-operated and ovariectomised (ovex) female mice treated for 5 days with saline, progesterone, oestrogen, or progesterone + oestrogen.

	Treatment	n	Body wt (g)	CFP wt (mg)	MFP wt (mg)
sham	saline[†]	4	19.6 ± 0.4^{ab}	71.7 ± 7^{ab}	125.8 ± 13^{ab}
	oestrogen	5	19.3 ± 0.6^{a}	87.0 ± 6^{bc}	146.9 ± 7^{bc}
	progesterone	5	19.6 ± 1.0^{ab}	70.4 ± 7^{ab}	133.8 ± 12^{abc}
	prog. + oestrogen	5	20.3 ± 0.2^{ab}	71.5 ± 4^{ab}	128.2 ± 11^{ab}
ovex	saline	5	20.0 ± 0.3^{ab}	61.6 ± 5^{a}	113.0 ± 6^{a}
	oestrogen	5	21.7 ± 1.5^{b}	95.6 ± 10^{c}	181.4 ± 10^{d}
	progesterone	5	20.4 ± 1.0^{ab}	83.6 ± 7^{bc}	162.4 ± 15^{cd}
	prog. + oestrogen	5	20.7 ± 0.6^{ab}	82.8 ± 5^{bc}	163.0 ± 5^{cd}

Body weights and weights of MFP and CFP of female mice which had been ovariectomised or sham-operated at approximately 21 days of age and were then treated for 5 days from 50 days of age.

[†] Saline-treated, sham-operated females were at pro-oestrus.

[a,b,c] Means within a column with different superscripts are significantly different ($P<0.05$).

6.4.2.2 Co-cultures

COMMA-1D cells did not demonstrate any increase ($P>0.05$) in final DNA yields when BM was supplemented with IGF-I, EGF, IGF-I + EGF, oestrogen, progesterone or oestrogen + progesterone (Figure 6.7). In contrast, 10% FCS stimulated a 4.6-fold increase ($P<0.001$) in final DNA yield. Consistent with results presented in Chapters 2 and 5, co-cultured mouse mammary fat pad directly stimulated the growth of COMMA-1D cells and potentiated the mitogenic effects of IGF-I and EGF. Figure 6.7 depicts an example of these responses when COMMA-1D cells were co-cultured with CFP and MFP tissue from saline-treated, sham-operated mice.

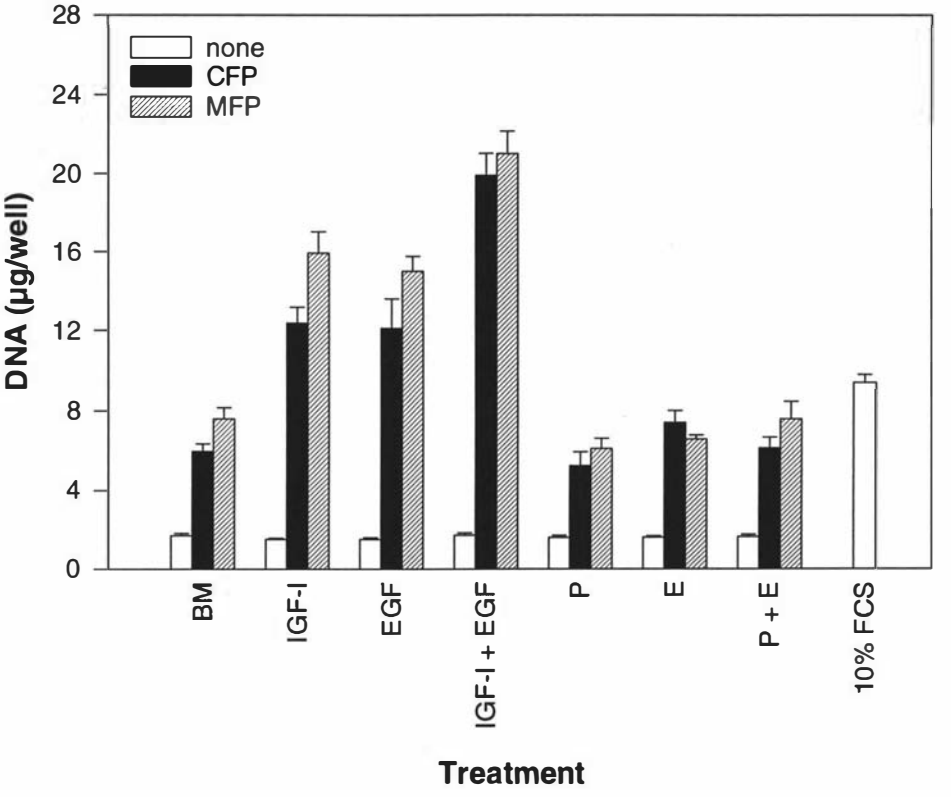


Figure 6.7 Growth of COMMA-1D cells in response to various medium supplements either alone, or when co-cultured with explants of CFP or MFP. Cells were cultured for 5 days in BM either alone, or supplemented with IGF-I (100 ng/ml), EGF (25 ng/ml), IGF-I + EGF, progesterone (P; 1 µg/ml), 17β-oestradiol (E; 1 ng/ml), P + E, or 10% FCS. Co-cultures were with explants of either CFP or MFP from saline-treated, sham-operated female mice. Data are means ± SEM (n=5).

Analysis of growth responses by COMMA-1D cells indicated that there were no interactions between the various *in vitro* medium treatments and those *in vivo* (ovariectomy, oestrogen, progesterone and fat pad type). Results in Table 6.2 are least squares means for the various *in vivo* treatments averaged across the seven *in vitro* medium treatments.

Table 6.2 Least squares means of COMMA-1D cell growth responses to co-cultured CFP and MFP tissue from sham-operated or ovariectomised (ovex) female mice treated for 5 days with saline, progesterone, oestrogen, or progesterone + oestrogen.

Treatment	sham			ovex		
	CFP (μg DNA)	MFP (μg DNA)	CFP vs MFP (P<)	CFP (μg DNA)	MFP (μg DNA)	CFP vs MFP (P<)
saline	9.44^{a}	11.27^{b}	0.001	10.16^{a}	11.24^{b}	0.02
oestrogen	10.78^{b}	10.33^{a}	0.33	11.48^{b}	10.1^{a}	0.003
progesterone	11.46^{b}	12.6^{c}	0.02	10.05^{a}	10.91^{ab}	0.06
prog. + oestrogen	11.67^{b}	11.2^{b}	0.32	11.55^{b}	10.53^{ab}	0.03

Data are least squares means for DNA yield/well averaged across 7 different *in vitro* treatments (n=5 replicates per treatment except for saline-treated, sham-operated females where n=4).
Pooled SE = 0.33 except for saline-treated, sham-operated CFP and MFP LS means where pooled SE = 0.37.
^{a,b,c} Least squares means within a column with different superscripts are significantly different (P<0.05).

There was a significantly (P<0.001) greater mitogenic effect of MFP compared to the contralateral CFP from saline-treated, sham-operated mice. These effects were unaffected by ovariectomy (P>0.1). Progesterone treatment for 5 days significantly increased the mitogenic effect of both the CFP and MFP from intact (P<0.01), but not ovariectomised (P>0.4) females. In contrast, administration of oestrogen increased (P<0.01) the mitogenic effect of the CFP from both intact and ovariectomised females while that of the contralateral MFP from both groups was correspondingly decreased (P<0.06). The response to CFP and MFP from females treated with oestrogen and progesterone generally represented the combined effect of the individual hormones.
Measurement of weight changes in co-cultured explants indicated that MFP from all groups decreased in weight while that of CFP explants from saline-treated ovariectomised and intact females was increased (Table 6.3). Progesterone treatment tended (P<0.1) to reduce the weight of MFP and CFP explants from intact, but not ovariectomised, females. Exogenous oestrogen reduced (P<0.05) the weight of MFP explants from both groups, but only the weight of CFP from ovariectomised females. The combination of oestrogen + progesterone stimulated a significant (P<0.05) decrease in the weight of explants from all groups.

Table 6.3 Change in the weight of co-cultured CFP and MFP explants from sham-operated and ovariectomised (ovex) female mice treated for 5 days with saline, progesterone, oestrogen, or progesterone + oestrogen.

Treatment	CFP		MFP	
	sham (mg)	ovex (mg)	sham (mg)	ovex (mg)
saline	0.21^{a}	0.42^{a}	-0.18^{a}	-0.2^{a}
oestrogen	0.06^{ab}	-0.19^{b}	-0.95^{b}	-1.24^{c}
progesterone	-0.34^{b}	0.08^{ab}	-1.22^{b}	-0.46^{ab}
prog. + oestrogen	-0.48^{b}	-0.4^{b}	-1.23^{b}	-1.1^{bc}

Data are least squares means for changes in explant weight across 7 different *in vitro* treatments (n=5 replicates per treatment except for saline-treated, sham-operated females where n=4).
Pooled SE = 0.22 except for saline-treated, sham-operated CFP and MFP least squares means where pooled SE = 0.25.
^{a,b,c} Least squares means within a column with different superscripts are significantly different ($P<0.06$).

6.5 DISCUSSION

Postnatal development of the rodent mammary gland is influenced by a variety of systemic and local factors. The present findings lend strong support to suggestions that the mammary fat pad plays a pivotal role in promoting the growth of the mammary gland epithelium and in mediating the mammogenic actions of ovarian steroids.

The ontogeny of mitogenic stimulation by co-cultured mouse mammary tissue *in vitro* was such that it declined from 3 weeks of age to reach basal levels in mature virgin and mid-gestation females. That similar responses were elicited by CFP and MFP tissue indicates that this activity was derived from the stromal constituents of the mammary gland. This pattern of fat pad-derived stimulation reflects the ontogeny of ductal elongation *in vivo*, where epithelial growth becomes allometric around 3 weeks of age (Silver, 1953) and essentially ceases once ducts reach the bounds of the mammary fat pad by around 8-9 weeks of age (Nandi, 1958). DNA synthesis in the terminal end bud epithelium concomitantly declines from a maximum at 3 weeks of age (Haslam, 1989). Similarly, mitogenic stimulation by the mammary fat pad during the oestrous cycle is greatest at oestrus during a phase of ductal proliferation (Chapter 5). This developmental pattern of mitogenic stimulation by the mammary fat pad parallels the

susceptibility of the mammary gland to chemical-induced carcinogenesis (Haslam, 1979; Grubbs *et al.*, 1983).

Such findings suggest that postnatal change in the mitogenic environment of the mammary fat pad regulates ductal proliferation within the mammary gland. Data presented in Chapter 4 suggests that the *in vitro* mitogenic stimulation afforded by the prepubertal CFP is due to its liberation of unsaturated fatty acids. These results are consistent with the demonstration that a diet deficient in essential fatty acids impairs ductal development in immature mice (Miyamoto-Tiaven *et al.*, 1981). While the metabolism of lipids by mammary gland adipocytes during this period has not been characterised, the substantial changes in the weight of co-cultured mammary tissues *in vitro* suggests that their ability to liberate lipids may progressively decline. In addition, ductal proliferation in the virgin mammary gland is promoted by IGF-I (Ruan *et al.*, 1992) and EGF (Coleman *et al.*, 1988), the mitogenic effects of which are potentiated by unsaturated fatty acids (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994; Chapter 4). Furthermore, IGF-I mRNA expression is elevated within the mammary fat pad of lambs prior to puberty (Chapter 9). It is therefore tenable that prepubertal ductal growth is a function of growth factor-induced proliferation which is modulated by locally-derived fatty acids.

While mammary tissues were highly mitogenic during the prepubertal phase of ductal proliferation, those from the period of rapid alveolar proliferation in mid-pregnancy (Traurig, 1967) were not. This raises the possibility that in contrast to ductal growth, alveolar growth occurs independently of local stimulation from the mammary fat pad. Rather, it may be directly governed by the mammogenic effects of systemic hormones such as progesterone, prolactin and placental lactogen (Cowie *et al.*, 1980; Vonderhaar, 1987b; Forsyth, 1994). Consistent with this notion and the aforementioned potential role for unsaturated fatty acids is the demonstration that alveolar growth is unaffected by dietary fat composition (Faulkin *et al.*, 1986). Furthermore, elevated lipoprotein lipase activity within mammary adipocytes during pregnancy (Jensen *et al.*, 1991) may suppress the liberation of unsaturated fatty acids from mammary adipocytes for the stimulation of epithelial growth.

These results also indicate that the mitogenic capacity of mammary tissues is differentially altered during lactation. While stimulation by CFP remained constant in

BM, responses to MFP during this period were markedly increased. This may relate to the substantial proliferation of epithelium which occurs within the rodent mammary gland during lactation (Traurig, 1961; Munford, 1964). This increased stimulation was presumably derived from, or induced by, the epithelium within MFP tissue.

One possible explanation for the enhanced effect of MFP during lactation is an increased contribution of unsaturated fatty acids to co-cultures. Lactating epithelium stimulates lipolysis in adjacent mammary adipocytes (Elias *et al.*, 1973), and MFP has a higher lipid content than CFP during lactation (Bandyopadhyay *et al.*, 1995) in association with increased lipoprotein lipase activity (Hamosh *et al.*, 1970; Jensen *et al.*, 1994). Furthermore, lactating MFP contains raised levels of 18:2ω6 and 20:4ω6, elevation of the latter being associated with an increased activity of epithelial Δ5 desaturase (Bandyopadhyay *et al.*, 1995). In addition, the effect of MFP declined after weaning when the metabolism of lipids by mammary epithelium is markedly decreased (Hamosh *et al.*, 1970).

In contrast to the MFP, proportions of individual fatty acids in CFP do not change during lactation and the CFP demonstrates relatively low levels of lipolysis during this period (Elias *et al.*, 1973; Bandyopadhyay *et al.*, 1995). The latter may explain the lesser weight change of CFP explants in co-culture at these stages. While the stimulatory effect of CFP remained relatively constant during this period in BM and steroid treatments, it further potentiated the mitogenic effect of growth factors on COMMA-1D cells. This may have been due to low levels of lipolysis within the CFP during lactation (Elias *et al.*, 1973; Bandyopadhyay *et al.*, 1995), given that only low levels of unsaturated fatty acids can markedly potentiate the mitogenic effect of these growth factors on COMMA-1D cells (Chapter 4).

Results from the second experiment, prompted by earlier findings of stage-specific regulation of mitogenic stimulation by the mammary fat pad during the oestrous cycle (Chapter 5), indicate that the ovary and its steroid hormones have substantial effects on both mammary gland development and the mitogenic capacity of the mammary fat pad.

As anticipated (Imagawa *et al.*, 1994), prepubertal ovariectomy completely abrogated subsequent mammogenesis. This response was not associated with a reduction in mitogenic stimulation from the mammary fat pad, suggesting that its basal mitogenic effect *in vitro* is independent of ovarian function. Such a finding concurs with

attribution of the mitogenic effect of co-cultured CFP to unsaturated fatty acids (Chapter 4) in that lipolysis in subcutaneous adipocytes is unaltered by ovariectomy (Lacasa *et al.*, 1991), and lipogenesis is maintained at a level similar to that in pro-oestrus (Hansen *et al.*, 1980). Also, change in the weight of co-cultured mammary fat pad explants, which may reflect a change in lipid mass given other findings *in vitro* (Shirling *et al.*, 1981; Benoit *et al.*, 1982; Rebuffé-Scrive, 1987), was unaffected by ovariectomy.

Progesterone increased the weight of mammary tissues in ovariectomised, but not intact, females. This response contrasts with the effect of progesterone on other adipose depots where fat deposition and adipose LPL activity is increased in intact (Steingrimsdottir *et al.*, 1980), but not ovariectomised (Hamosh and Hamosh, 1975; Gray and Wade, 1981) females. This inconsistency may represent differential regulation of lipid metabolism within adipocytes from mammary and subcutaneous depots relative to other sites (Krotkiewski and Björntorp, 1976; Steingrimsdottir *et al.*, 1980; Rebuffé-Scrive *et al.*, 1986; Bandyopadhyay *et al.*, 1995).

Progesterone also increased the *in vitro* mitogenic effect of MFP and CFP from intact females, an effect which was dependent on ovarian function. Such a response presumably occurred within the stroma of mammary tissue. Consistent with the likelihood that progesterone altered lipid metabolism in mammary adipocytes was the finding that the weight of CFP and MFP explants from progesterone-treated intact females decreased in co-culture while that of explants from ovariectomised females did not. This suggests that exogenous progesterone promoted lipolysis in mammary adipocytes *in vitro*. That these responses did not occur in mammary tissues from ovariectomised females coincides with the fact that progesterone only influences lipid metabolism in adipocytes of intact females (Steingrimsdottir *et al.*, 1980; Gray and Wade, 1981), possibly due to a requirement for oestrogen to increase PgR levels (Gray and Wade, 1981; Haslam, 1988c).

In contrast, oestrogen acted independently of ovarian function and increased mammary tissue weight in both intact and ovariectomised females. While oestrogen typically decreases adipose tissue weight by increasing lipolysis and suppressing lipogenesis (Steingrimsdottir *et al.*, 1980; Ramirez, 1981), the results of Kim and Kalkhoff (1975) show, in contrast, that oestrogen induces a specific increase in LPL activity within the

mammary gland. Such results further emphasise that ovarian steroids may affect lipid metabolism within mammary adipocytes differently to that in adipocytes at other sites.

Exogenous oestrogen increased the mitogenic effect of CFP, perhaps by increasing lipolysis *in vitro* through elevated levels of hormone sensitive lipase (Benoit *et al.*, 1982). This corresponds with the earlier finding that mammary tissue is significantly more mitogenic at oestrus relative to other stages of the oestrous cycle (Chapter 5). However, this effect was reversed by the presence of endogenous epithelium in MFP. The reason behind this is presently unclear although it may relate to the observation that the increased mitogenic effect of MFP relative to CFP does not occur in medium supplemented with oestrogen (Chapter 5). Such a response was also associated with a substantial decline in the weight of co-cultured MFP tissue. One possibility along the lines of a report by Kidwell *et al.* (1982) is that oestrogen stimulated mammary adipocytes to liberate unsaturated fatty acids which were preferentially sequestered by endogenous MFP epithelium to make them unavailable for co-cultured COMMA-1D cells.

As observed previously (Chapter 5), co-cultured MFP was often more stimulatory than contralateral CFP devoid of endogenous epithelium. This result suggests that mammary epithelium acts on the surrounding mammary fat pad to upregulate its mitogenic effect. Consistent with the hypothesis that these responses are induced by unsaturated fatty acids from mammary adipocytes are reports that mammary epithelium exerts a lipolytic effect on adjacent adipocytes (Elias *et al.*, 1973; Kidwell and Shaffer, 1984). Likewise, however, it is possible that endogenous epithelium upregulated the stromal expression of particular paracrine growth factors (Coleman-Krnacik and Rosen, 1994; Ellis *et al.*, 1994). As well as this direct effect, the present results also indicate that this interaction may modulate the action of oestrogen on the mammary gland, consistent with the results of earlier *in vitro* studies (McGrath, 1983; Haslam and Levely, 1985; Haslam, 1986). While the mechanisms associated with this are presently unknown, such findings emphasise the potentially important role of the interaction between epithelial and stromal cells within the mammary gland (Cunha and Hom, 1996).

In conclusion, the present findings lend strong support to a role for the mammary fat pad in regulating postnatal mammogenesis. Such regulation likely represents a complex

inter-relationship between the effects of stage of development, ovarian hormones and epithelial-stromal associations.

CHAPTER 7

DIFFERENTIAL MODULATORY EFFECTS OF MURINE AND OVINE MAMMARY FAT PAD ON THE PROLIFERATION OF MAMMARY EPITHELIAL CELLS *IN VITRO*

7.1 ABSTRACT

Several lines of evidence suggest that different mechanisms locally regulate epithelial growth within the rodent and ruminant mammary glands. The objective of these experiments was to compare the *in vitro* mitogenic effects of murine and ovine mammary fat pad (FP) tissue on murine (COMMA-1D) and bovine (MAC-T) mammary epithelial cells. The growth of COMMA-1D cells was increased ($P<0.05$) by approximately 2-fold in the presence of co-cultured FP from prepubertal ewe lambs, with a similar response to lamb FP conditioned medium (CM). This stimulation was typically additive to the mitogenic effects of 10% FCS, insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF). Subsequent comparisons indicated that the growth of COMMA-1D cells in a hormone-free basal medium (BM) was equally stimulated by co-cultured ovine FP and murine FP, while ovine FP was less stimulatory than murine FP for MAC-T cells. Evidence for a local influence of endogenous epithelium on the surrounding stroma was provided by the result that ovine mammary parenchyma (PAR) was more mitogenic ($P<0.05$) than ovine FP for both cell lines. Comparison of the ability of these tissues to modulate the responsiveness of mammary epithelial cells to various medium treatments revealed several distinctions. Murine FP substantially potentiated the mitogenic effects of IGF-I, EGF, and insulin for COMMA-1D cells, more so than ovine FP or PAR. There was no response by COMMA-1D cells to other factors such as prolactin, oestrogen or progesterone in co-culture with any of these tissues. All co-cultured tissues similarly interacted with IGF-I to stimulate the growth of MAC-T cells while only ovine PAR facilitated a response to EGF. MAC-T cells were unresponsive to acidic FGF or basic FGF either alone or in the presence of murine or ovine FP, while ovine PAR tended to potentiate their effect. MAC-T cells were unresponsive to prolactin, oestrogen, or progesterone in any of the conditions tested. These results indicate differences in the mitogenic effect of murine and ovine mammary fat pads and their ability to modulate the growth responsiveness of murine and bovine mammary epithelial cells. Furthermore, interactions between the epithelial and stromal constituents in the ovine mammary gland may modulate the effects of certain mammogenic factors.

7.2 INTRODUCTION

The mammary fat pad comprises a matrix of adipose and connective tissue which supports the extensive proliferation of epithelial cells during development of the female mammary gland (Sheffield, 1988b). The size of the mammary fat pad determines the extent of parenchymal growth (Hoshino, 1978) which in turn dictates the ultimate milk yield potential of the mammary gland (Tucker, 1981).

The mammary fat pad may regulate the growth of mammary epithelial cells by several different mechanisms. Various constituents of the mammary stroma express polypeptide growth factors (Hauser *et al.*, 1990; Niranjan *et al.*, 1995; Chakravorti and Sheffield, 1996a) which exert potent mitogenic and morphogenic effects on mammary epithelium. These effects may be further modulated by fatty acids locally released from mammary adipocytes (Bandyopadhyay *et al.*, 1993; Chapter 4). Stromal cells also provide a physical substratum (Keely *et al.*, 1995) capable of modifying both the morphogenesis (Wiens *et al.*, 1987) and hormonal responsiveness (Salomon *et al.*, 1981) of mammary epithelium. Such mechanisms might locally mediate the mammogenic effects of hormones such as oestrogen (Shyamala and Ferenczy, 1984) and growth hormone (Hauser *et al.*, 1990). Within this cellular interaction, epithelial cells can exert reciprocal effects on stromal activities including DNA synthesis (Dulbecco *et al.*, 1982; Berger and Daniel, 1983), lipolysis (Kidwell and Shaffer, 1984) and growth factor expression (Coleman-Krnacik and Rosen, 1994).

Earlier observations (Chapter 8) and those of others (Sheffield, 1988b; Akers, 1990) emphasise that the ruminant mammary gland undergoes a histogenesis and morphogenesis distinct from that of the widely studied rodent mammary gland (Imagawa *et al.*, 1994). As well as being more dichotomously branched, ruminant mammary epithelium is continually enveloped by a fibroblastic connective tissue (Chapter 8). This connective tissue, which is also interspersed among the adipocytes of the ruminant mammary fat pad, is much less abundant within the rodent mammary gland. In several of these respects the ruminant gland is similar to the human breast which also undergoes a morphogenesis distinct from that of the rodent mammary gland (Sakakura, 1991; Rønnov-Jessen *et al.*, 1996).

Such differences may be manifest in the findings of transplantation experiments, where mouse (reviewed by Hoshino, 1978) and rat (Daniel *et al.*, 1983; Welsch *et al.*, 1987)

mammary epithelium develops normally within the mammary fat pads of mice while bovine (Sheffield and Welsch, 1986; Ellis and Akers, 1995) and human (Sheffield and Welsch, 1988; Yang *et al.*, 1995) mammary epithelium does not outgrow but instead forms cystic structures. These findings have prompted the suggestion that regulatory influences exerted by the stromal elements of the ruminant and human mammary gland are distinct from those within the rodent gland (Sheffield, 1988b).

It has been recently shown that murine FP releases an activity *in vitro* which stimulates the growth of mouse mammary epithelial cells and markedly enhances their proliferative response to certain mitogens (Chapters 2 and 3). Furthermore, these effects of the mammary fat pad are altered at various physiological stages of mammary development (Chapters 5 and 6). It was hypothesised that differences between the rodent and ruminant mammary fat pads would be reflected in this co-culture system. The experiments herein have compared the ability of murine and ovine FP to modulate the response of murine and bovine mammary epithelial cells to various mitogens. The influence of epithelial-stromal interactions on the mitogenic effect of ovine mammary stroma *in vitro* has also been investigated.

7.3 MATERIALS AND METHODS

7.3.1 Cell cultures

Stocks of COMMA-1D mouse mammary epithelial cells (Danielson *et al.*, 1984) were propagated in DME/F12 (Gibco, New Zealand) supplemented with 2% foetal calf serum (FCS; Gibco, New Zealand), 6 μg/ml insulin (Sigma), 5 ng/ml mouse EGF (Sigma) and 0.3 mg/ml bovine serum albumin (Sigma). MAC-T bovine mammary epithelial cells (Huynh *et al.*, 1991) were cultured in DMEM (Gibco, New Zealand) plus 10% FCS, 1 μg/ml hydrocortisone (Sigma) and 5 μg/ml insulin. COMMA-1D and MAC-T stocks were passaged weekly using Dispase and trypsin/EDTA, respectively. COMMA-1D and MAC-T cells for co-culture experiments were plated in hormone-supplemented media at 5×10^4 and 3.5×10^4 cells/well. COMMA-1D cells for conditioned medium experiments were seeded at 1×10^5 cells/well. After 24 h for attachment, cultures were quiesced for 48 h in DMEM basal medium (BM) before treatments were applied.

7.3.2 Animals and tissues

Coopworth x Dorset ewes maintained under standard grazing conditions in the Ruakura flock were the source of mammary tissues for these experiments. Mammary tissues for initial experiments conducted on three separate occasions were from one 1-week old and two 5-week old ewe lambs. These lambs were kept with their dam until they were sacrificed. Mammary tissues for the second set of experiments were from two 35-week old virgin ewes. Sheep were euthanased by captive bolt and exsanguination. The udder was removed and immediately transported to the tissue culture facility. Pieces of mammary fat pad (FP) tissue were aseptically sampled from the extra-parenchymal region of each gland as delineated by parenchymal pigmentation. In the second series of experiments parenchymal tissue (PAR) was also sampled. Tissues were briefly rinsed in BM before use in co-culture experiments.

In the second set of experiments extra-parenchymal FP tissue from the abdominal mammary fat pads of 3-week old female BALB/c mice was also used as previously described (Chapter 2).

7.3.3 Co-cultures and conditioned medium

Mammary tissue was co-cultured with monolayers of mammary epithelial cells as described in Chapter 2. Briefly, tissues were sectioned into explants of approximately 5-10 mg which were rinsed in the hormone-free BM. Explants were then placed on rafts of siliconised lens paper and floated at the gas:medium interface of appropriate cultures. Mammary explants were added to an initial 0.5 ml treatment medium, and a further 0.5 ml of medium was added on day 3. Co-cultures were for 5 days.

Medium conditioned by mammary fat pad tissue (CM) was prepared as described in Chapter 3. Explanted ovine FP was incubated in BM (7.5 mg/ml) for 48 h at 37°C in a humidified 5% CO_2:95% air atmosphere. The resultant CM and control BM (incubated in the absence of FP tissue) were filtered (0.2 μm) and supplemented with various treatments before being added to cultures for 3 days.

Other medium supplements included recombinant bovine growth hormone (rbGH; Elanco, Indianapolis, IN), acidic and basic FGF (Sigma), prolactin (Sigma) and recombinant human IGF-I (Genentech, San Francisco, CA). 17β-oestradiol (Sigma), progesterone (Sigma) and hydrocortisone (Sigma) were added from ethanolic stocks, where all treatments within an experiment contained the same concentration of ethanol.

At the end of the culture period monolayers were trypsinised and the final DNA content measured by a fluorometric assay (Labarca and Paigen, 1980) using calf thymus DNA as a standard.

7.3.4 Statistical analyses

Cell growth results were analysed for the main effects of *in vitro* culture treatment, co-cultured mammary tissue and their interactions using the GLM procedure of SAS (1994). Where appropriate, data were natural log transformed to address heterogeneity of variance. Individual means comparisons were by LSD.

7.4 RESULTS

The first set of experiments determined the mitogenic effect of co-cultured ovine FP on COMMA-1D mouse mammary epithelial cells, the proliferation of which is markedly stimulated by murine FP (Chapter 2). Final DNA yield in the presence of co-cultured ovine FP was 2.2-fold that in BM only ($P<0.05$; Figure 7.1). Supplementation of BM with 10% FCS increased ($P<0.05$) the final DNA yield by 7.9-fold while ovine FP furthered this response to a 10.5-fold increase.

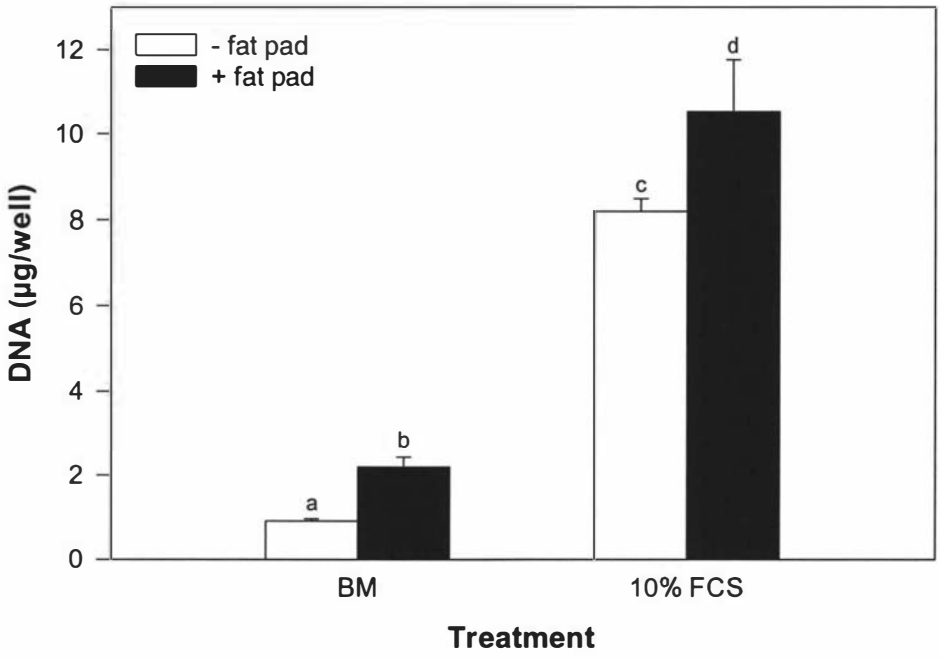


Figure 7.1 Growth response of COMMA-1D cells to co-cultured ovine FP and 10% FCS. COMMA-1D cells were cultured for 5 days in either BM alone or BM supplemented with 10% FCS in the absence or presence of co-cultured FP from a 5-week old ewe lamb. Data are means ± SEM (n=4). [a,b,c] Means with different superscripts are significantly different ($P<0.05$).

In Chapters 2 and 3 it was shown that co-cultured murine FP markedly potentiates the effect of IGF-I and EGF on COMMA-1D cell growth (Chapters 2 and 3). It was tested whether co-cultured ovine FP could similarly modulate these effects. The final level of DNA in the presence of co-cultured ovine FP was 2.5-times that in BM alone (Figure 7.2; $P<0.001$). Supplementation of cultures with IGF-I, EGF, or their combination increased ($P<0.05$) final cell number by 85, 78, and 135%, respectively. While there was a slight ($P<0.05$) interactive response between the effects of co-cultured ovine FP and IGF-I, the effects of EGF and IGF-I + EGF were additive to that of ovine FP. Similar results were obtained for FP from 1- and 5-week old ewe lambs.

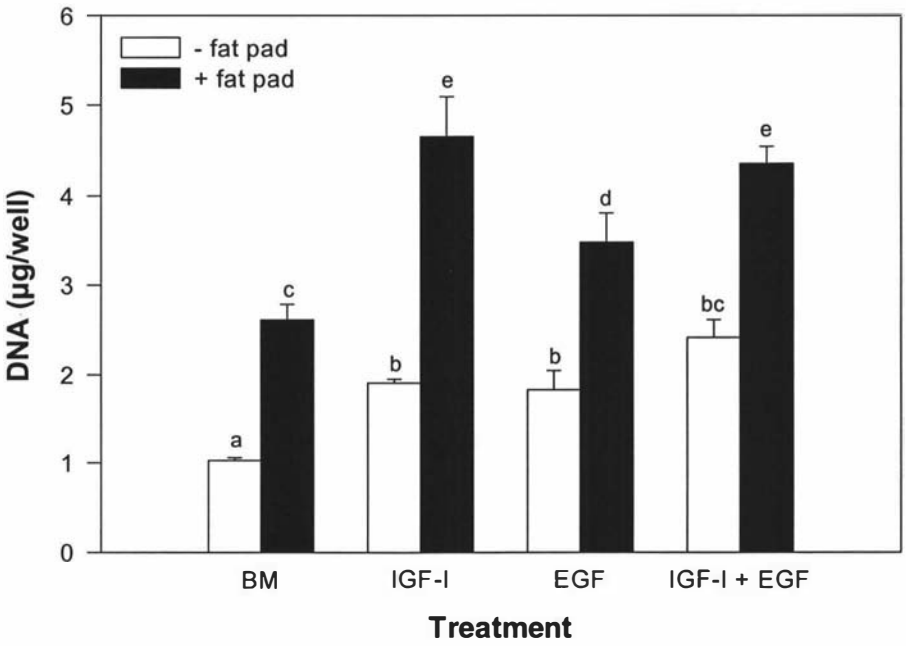


Figure 7.2 Growth of COMMA-1D cells in response to co-cultured ovine FP, IGF-I and EGF. COMMA-1D cells were cultured for 5 days in BM either alone or supplemented with IGF-I (100 ng/ml) and/or EGF (25 ng/ml) in the absence or presence of co-cultured FP from a 5-week old ewe lamb. Data are means ± SEM (n=4). [a,b,c]Means with different superscripts are significantly different ($P<0.05$).

Determination of the response to CM prepared using ovine FP revealed that final DNA values increased ($P<0.05$) with the proportion of CM alone, and that parallel responses occurred in the presence of IGF-I, EGF, or IGF-I + EGF (Figure 7.3). Final DNA yield in 100% CM either alone or in the presence of IGF-I, EGF or IGF-I + EGF was increased ($P<0.05$) by 22, 39, 35 and 59%, respectively relative to DNA yield in BM only. Inclusion of at least 25% CM in medium supplemented with 10% FCS realised the maximum additional response to CM (Figure 7.3).

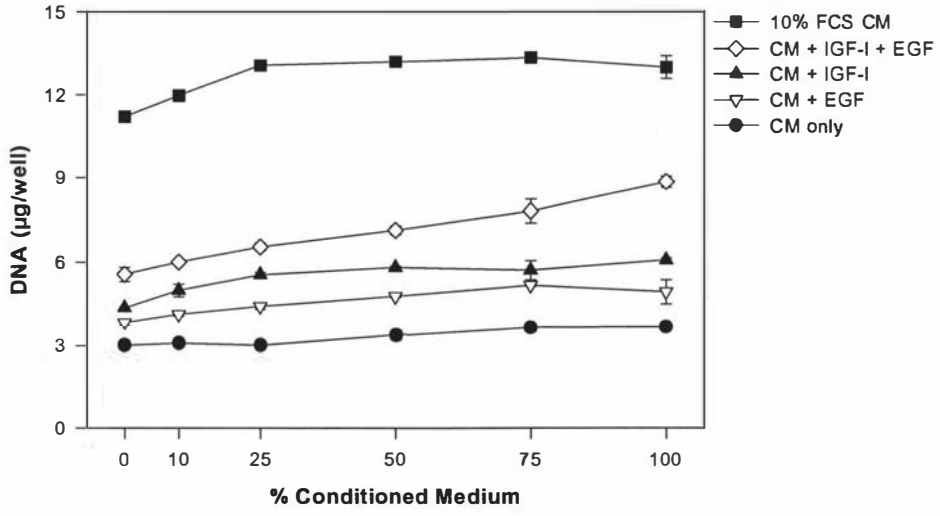


Figure 7.3 Response of COMMA-1D cells to a range of concentrations of CM prepared using ovine FP, both alone and in the presence of various mitogenic supplements. COMMA-1D cells were cultured for 3 days in the indicated concentrations of CM (1 ml) which was also supplemented with IGF-I (100 ng/ml) and/or EGF (25 ng/ml), or 10% FCS. CM was prepared using FP tissue from a 5-week old ewe lamb. Data are means ± SEM (n=3).

Several groups have proposed that the mammogenic effect of GH in ruminants may be mediated by the mammary fat pad (Hauser *et al.*, 1990; Chapter 9). While IGF-I stimulated the growth of COMMA-1D cells alone and in the presence of ovine FP, rbGH had no effect on the growth of COMMA-1D cells either alone or in the presence of FP, IGF-I, or their combination (Figure 7.4).

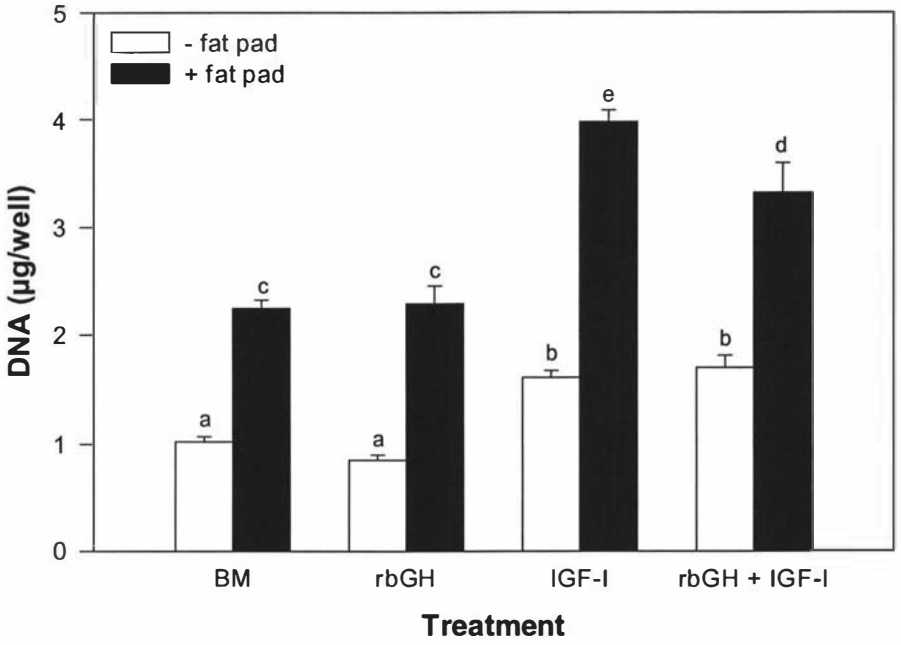


Figure 7.4 Effect of co-cultured ovine FP, IGF-I and rbGH on the growth of COMMA-1D mouse mammary epithelial cells. Cells were cultured for 5 days in the absence or presence of co-cultured ovine FP in BM either alone or supplemented with IGF-I (100 ng/ml) and/or rbGH (1 µg/ml). FP tissue was from a 5-week old ewe lamb. Data are means ± SEM (n=4). ^{a,b,c} Means with different superscripts are significantly different ($P<0.05$).

In a second series of experiments the mitogenic effect of murine FP was compared to that of FP from 35-week old ewes, and the influence of epithelial-stromal interactions examined by determining the response to ovine PAR. These tissues were co-cultured with COMMA-1D and MAC-T cells in various medium treatments.

COMMA-1D cells demonstrated similar responses to co-cultured murine and ovine FP in that the respective final DNA yields were 2.2- and 2.5-fold that in BM only (Figure 7.5a). Ovine PAR stimulated a further ($P<0.05$) increment of growth whereby the final DNA level was increased by 3.3-fold. A similar pattern of responses was observed above the proliferative effect of 10% FCS.

Ovine FP was less ($P<0.05$) stimulatory for MAC-T cells than either murine FP or ovine PAR, where murine FP, ovine FP and ovine PAR stimulated growth to final DNA values that were 2.5-, 1.7- and 3.2-fold that in BM only (Figure 7.5b). While murine FP did not alter the growth of MAC-T cells in 10% FCS ($P>0.05$), ovine FP and PAR suppressed ($P<0.05$) this response by 17 and 32%, respectively.

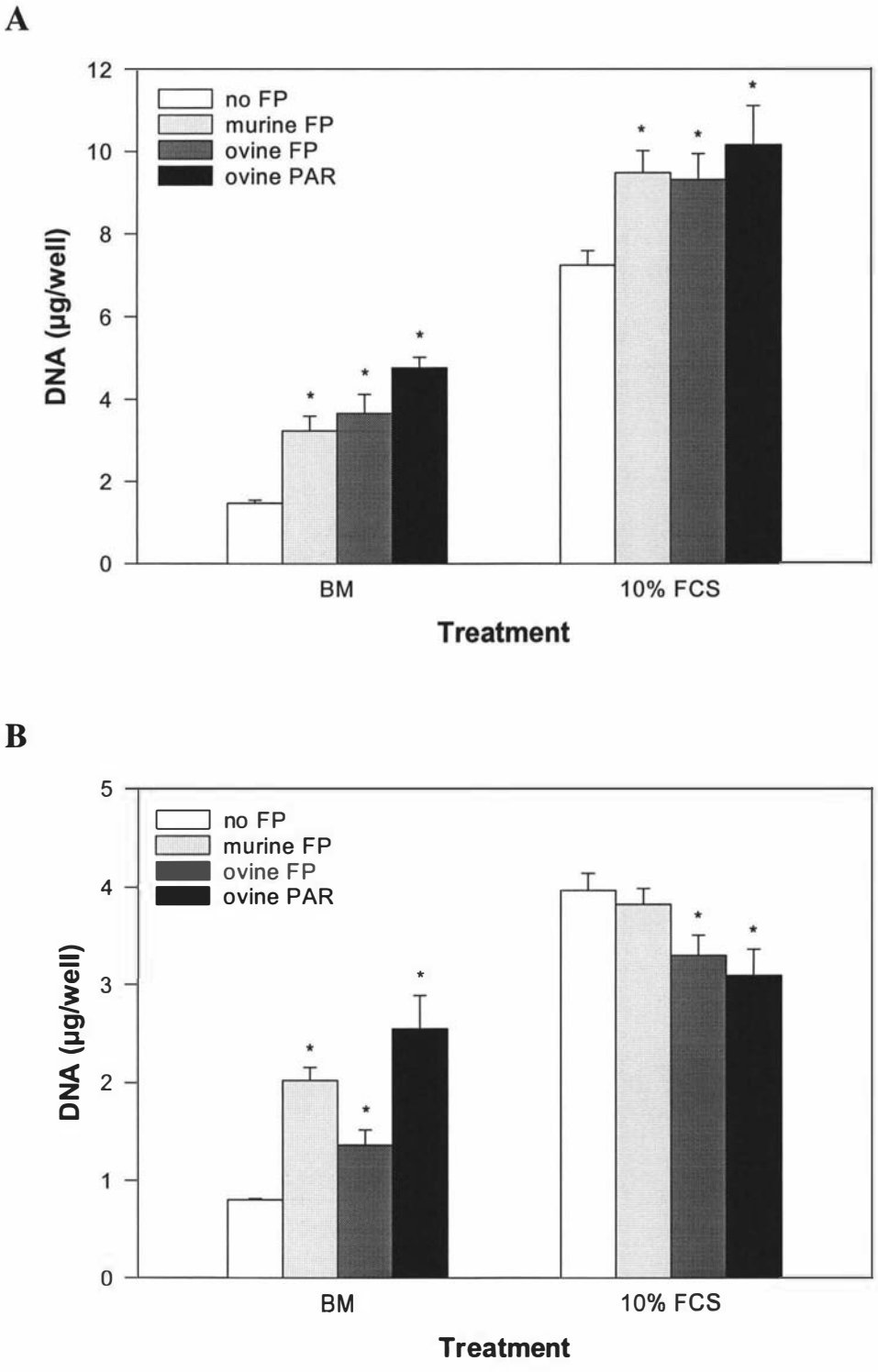


Figure 7.5 Growth of (A) COMMA-1D, and (B) MAC-T cells in response to co-cultured mammary tissues and 10% FCS. Cells were cultured for 5 days in BM or 10% FCS either alone or in the presence of co-cultured murine FP, or ovine FP or PAR from a 35-week old ewe. Data are means ± SEM (n=4). *P<0.05 compared to the respective no FP response.

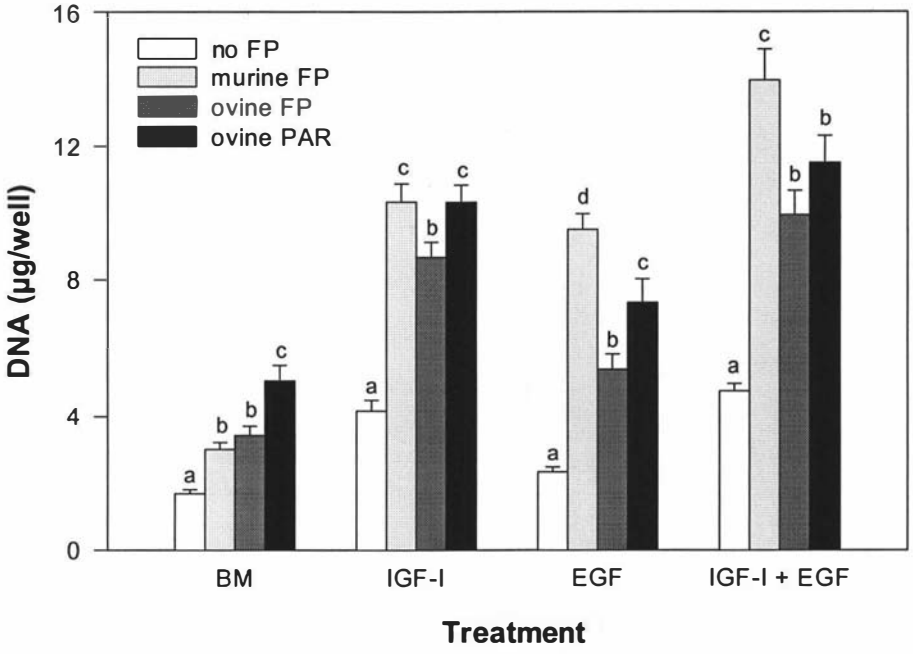
The relative mitogenic effect of these tissues on COMMA-1D cells was altered in the presence of IGF-I and/or EGF (Figure 7.6a). The most marked interaction was in co-culture with murine FP where growth responses in all treatments were equal to or greater than that due to ovine PAR, which in turn was equal to or greater than the response to ovine FP.

For MAC-T cells there were differential effects of these tissues in the presence of IGF-I and/or EGF. All co-cultured tissues interacted ($P<0.05$) with IGF-I to yield a distribution of responses similar to that in BM (Figure 7.6b). EGF alone did not alter the yield of DNA ($P>0.05$) from MAC-T cultures, and no response was effected by EGF in the presence of co-cultured murine FP or ovine FP. In contrast, there was a significant ($P<0.05$) interaction between the effects of EGF and ovine PAR that was reduced in the presence of IGF-I.

Supplementation of COMMA-1D cultures with aFGF and bFGF increased ($P<0.05$) final cell number by 43 and 47%, respectively (Figure 7.7a). While there were modest interactions between the effects of co-cultured ovine FP and PAR with aFGF and bFGF, the most substantial response was in the presence of murine FP where final cell number in the presence of aFGF and bFGF was 3.3- and 3.8-times that in BM only.

Acidic FGF and bFGF alone did not stimulate any increase ($P>0.05$) in the final DNA level of MAC-T cultures (Figure 7.7b). There was no additional effect of these FGFs in the presence of murine FP ($P>0.05$), with slight but non-significant effects in co-culture with ovine FP. In the presence of ovine PAR there was a tendency ($P<0.15$) for both aFGF and bFGF to evoke an additional response over that due to PAR alone.

A



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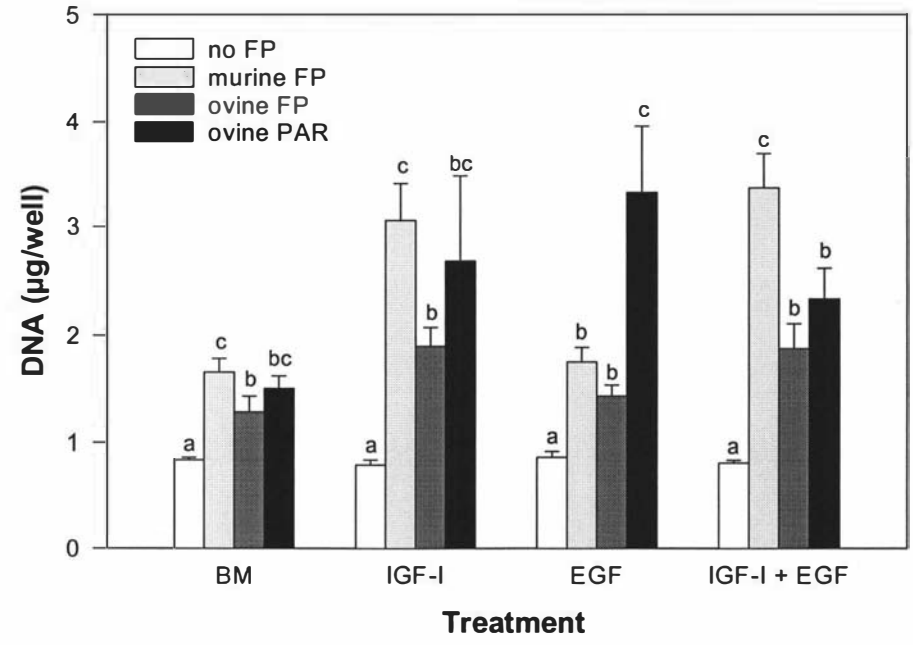


Figure 7.6 Effect of IGF-I, EGF, and co-cultured mammary tissues on the growth of (A) COMMA-1D, and (B) MAC-T cells. Cultures were for 5 days in BM alone or supplemented with IGF-I (100 ng/ml) and/or EGF (25 ng/ml) in the presence of co-cultured murine FP, or ovine FP or PAR from a 35-week old ewe. Data are means ± SEM (n=4). [a,b,c] Means with different superscripts within a treatment group are significantly different ($P<0.05$).

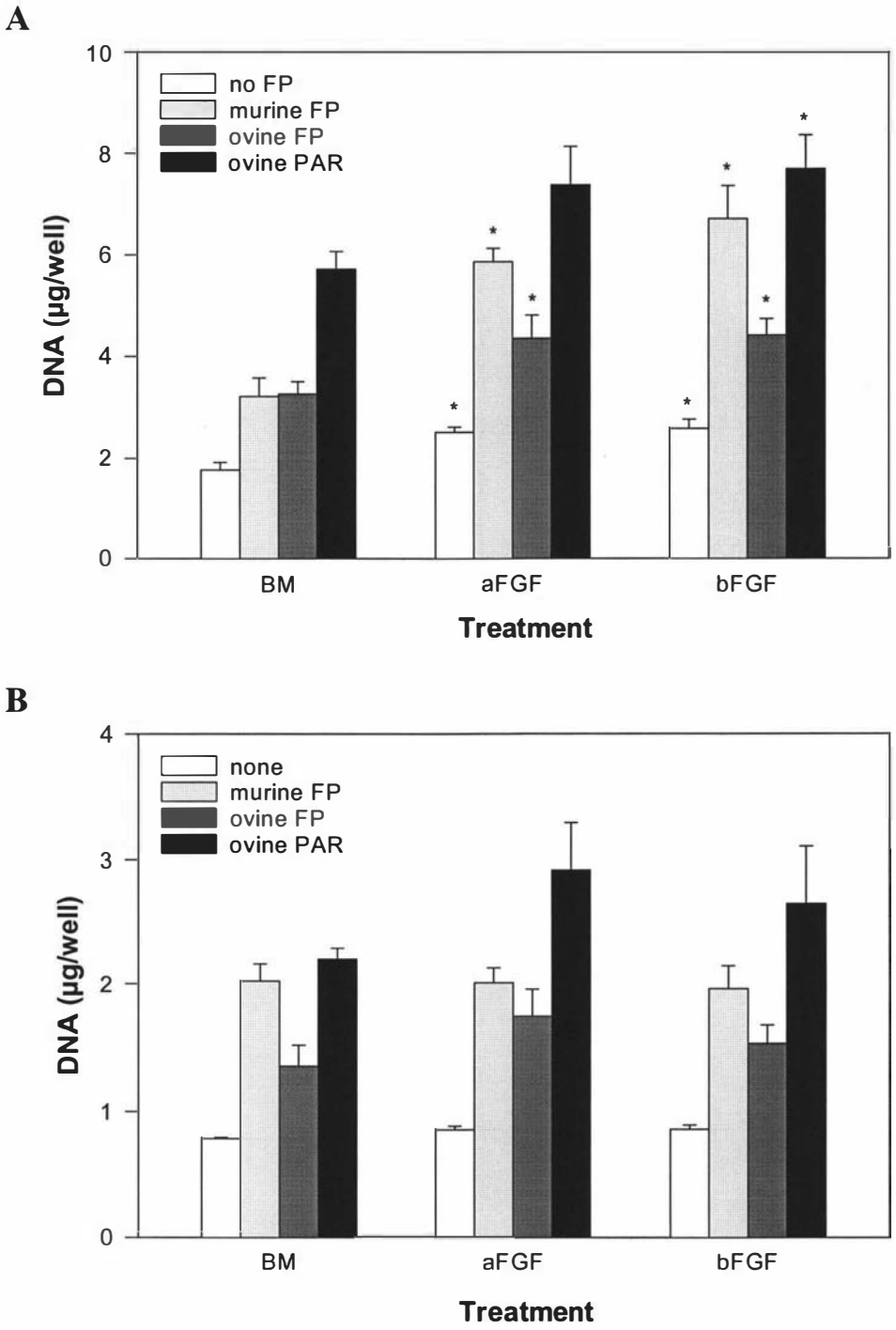


Figure 7.7 Proliferative response of (A) COMMA-1D, and (B) MAC-T cells to aFGF, bFGF, and co-cultured mammary tissues. Cells were cultured for 5 days in BM either alone or supplemented with aFGF (5 ng/ml) or bFGF (5 ng/ml) in the presence of co-cultured murine FP, or ovine FP or PAR from a 35-week old ewe. Data are means ± SEM (n=4). *P<0.05 compared to respective BM value.

Supplementation of BM with prolactin did not alter ($P>0.05$) the response of either COMMA-1D (Figure 7.8a) or MAC-T (Figure 7.8b) cells to co-cultured mammary tissues. Insulin alone stimulated ($P<0.05$) the growth of COMMA-1D cells but did not affect that of MAC-T cultures ($P>0.05$). For COMMA-1D cells, this effect was additive to that of co-cultured ovine FP and PAR while it significantly ($P<0.05$) interacted with that of co-cultured murine FP. For MAC-T cells, insulin promoted small but non-significant increases in final DNA values above the effects of ovine FP and PAR, while it significantly ($P<0.05$) interacted with the effect of murine FP. Hydrocortisone did not alter the response of COMMA-1D or MAC-T cells to murine FP but suppressed or tended to suppress the effect of ovine FP and PAR on both cell lines.

Oestrogen and progesterone did not affect ($P>0.05$) final DNA levels in COMMA-1D (Figure 7.9a) or MAC-T (Figure 7.9b) cultures, although their combination reduced ($P<0.05$) MAC-T cell growth. Neither cell line demonstrated any additional response to these steroids or their combination above that elicited by co-cultured mammary tissue alone.

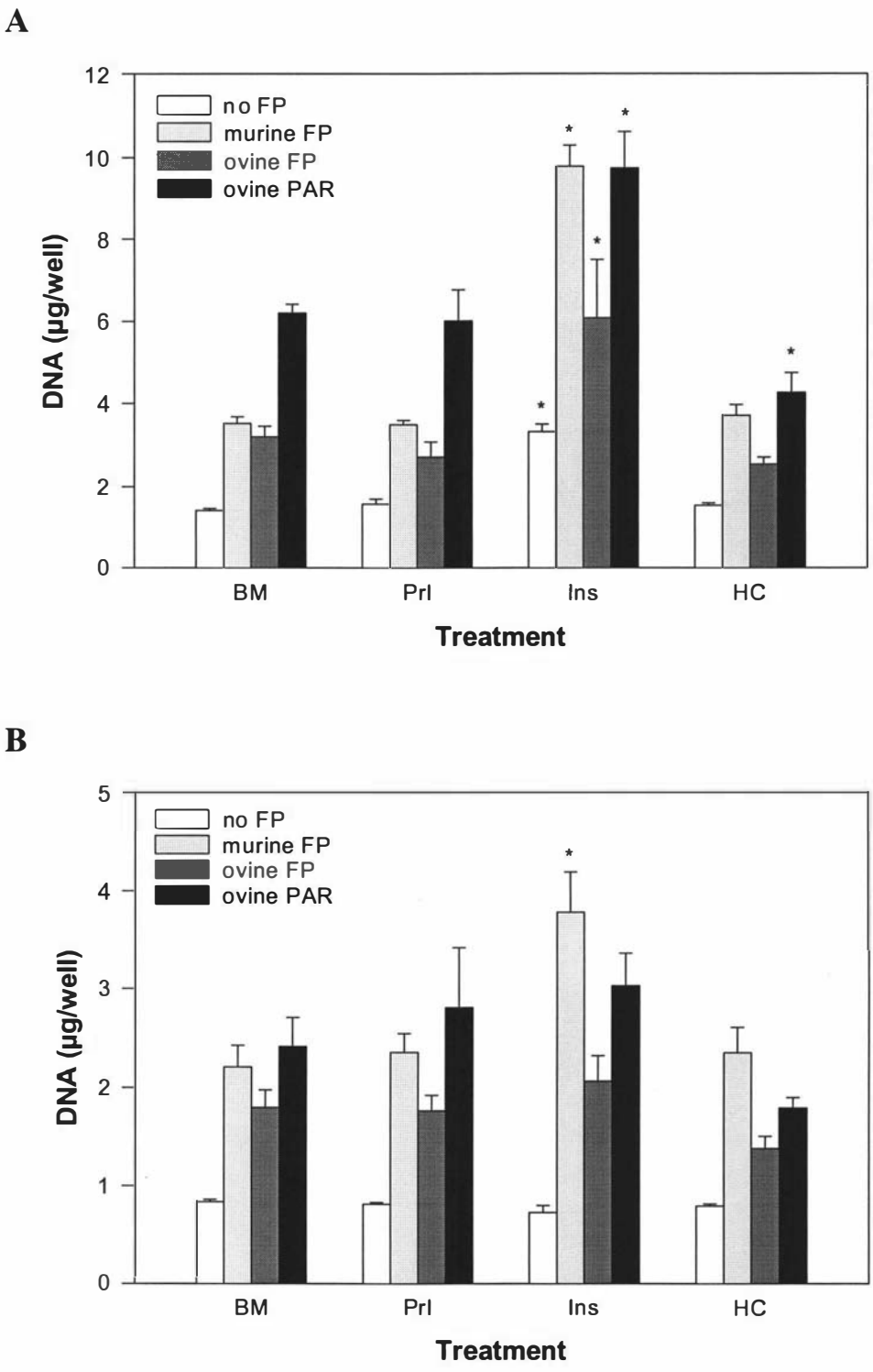


Figure 7.8 Growth of (A) COMMA-1D, and (B) MAC-T cells in response to mammogenic hormones and co-cultured mammary tissues. Cells were cultured for 5 days in BM either alone or supplemented with prolactin (2.5 µg/ml), insulin (10 µg/ml), or hydrocortisone (2.5 µg/ml) in the presence of co-cultured murine FP, or ovine FP or PAR from a 35-week old ewe. Data are means ± SEM (n=4). *P<0.05 compared to respective BM value.

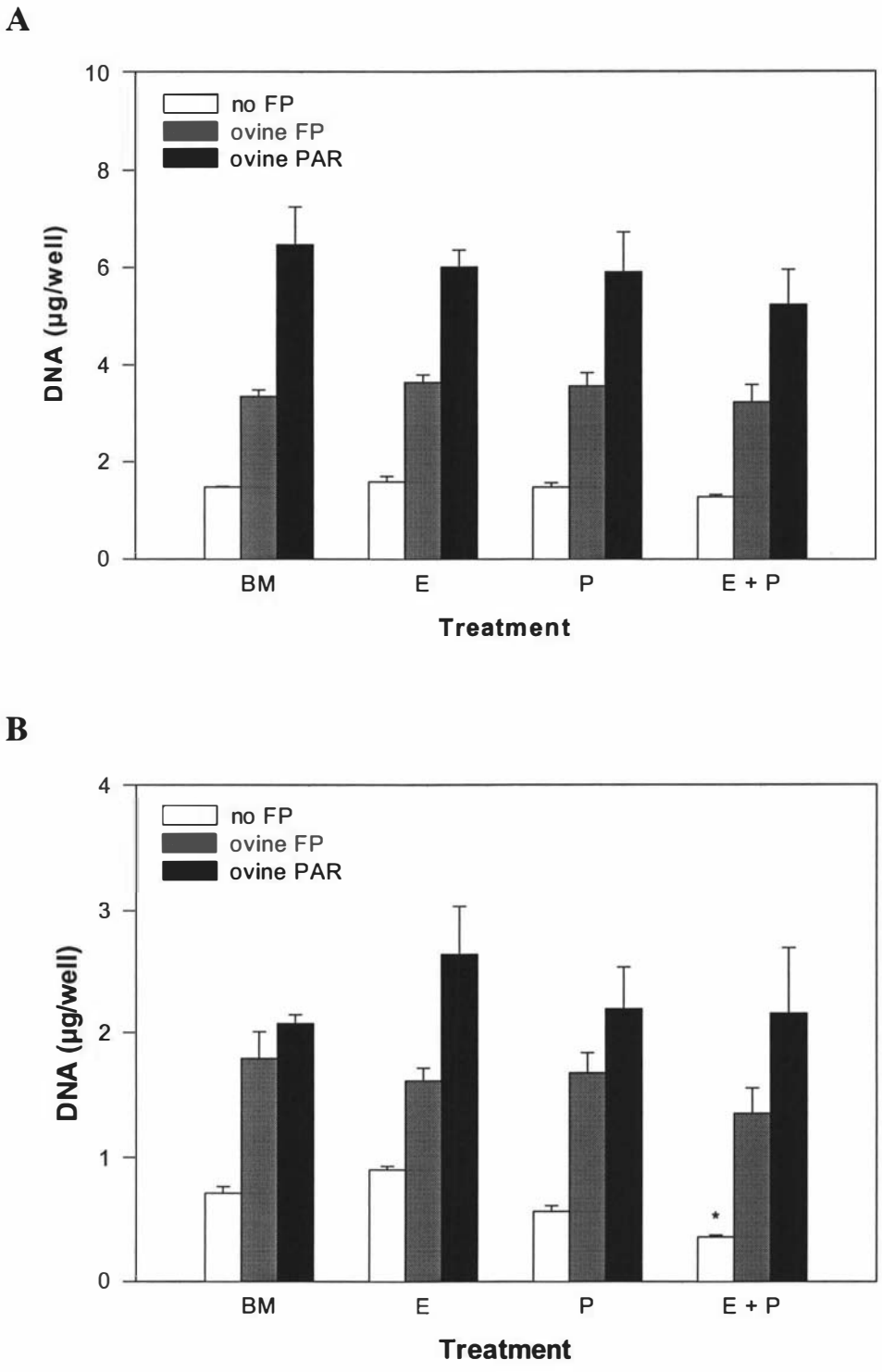


Figure 7.9 Effect of ovarian steroids and co-cultured mammary tissues on the growth of (A) COMMA-1D, and (B) MAC-T cells. Cultures were for 5 days in BM either alone or supplemented with oestrogen (1 ng/ml) and/or progesterone (1 µg/ml) in the presence of co-cultured murine FP, or ovine FP or PAR from a 35-week old ewe. Data are means ± SEM (n=4). *P<0.05 compared to respective BM value.

7.5 DISCUSSION

Transplantation studies have demonstrated that COMMA-1D (Danielson *et al.*, 1984) and primary mouse mammary epithelial cells (DeOme *et al.*, 1959) undergo normal growth and morphogenesis within the murine mammary fat pad whereas MAC-T (Ellis and Akers, 1995) and primary bovine mammary epithelial cells (Sheffield and Welsch, 1986) do not. Human mammary epithelium is also unable to outgrow into the murine mammary fat pad (Sheffield and Welsch, 1988; Yang *et al.*, 1995). One factor which may account for these differences is the environment of the endogenous mammary fat pad. The present findings indicate that the mouse and ruminant mammary fat pads differ in their mitogenic effects *in vitro*, effects which result in differential growth responses by murine COMMA-1D and bovine MAC-T mammary epithelial cells.

Co-cultured ovine FP consistently stimulated an approximate doubling in DNA yields of COMMA-1D and MAC-T cultures in the hormone-free BM. It was recently shown that co-cultured murine FP releases unsaturated fatty acids which can stimulate the growth of COMMA-1D cells (Chapter 3). Unsaturated fatty acids also promote the growth of MAC-T cells (Cockrell *et al.*, 1992) while saturated fatty acids inhibit the growth of rodent (Wicha *et al.*, 1979) and bovine (Cockrell *et al.*, 1992) mammary epithelial cells. The rodent mammary fat pad contains a high proportion of unsaturated fatty acids, particularly linoleic acid (Wicha *et al.*, 1979; Bandyopadhyay *et al.*, 1995), while ovine mammary tissue contains a high proportion of saturated fatty acids and low amounts of linoleic acid (McFadden *et al.*, 1990b).

Consistent with the predicted fatty acid composition of the tissues, co-cultured murine FP was more stimulatory for MAC-T cells than ovine FP while, interestingly, co-cultured ovine FP stimulated COMMA-1D cell growth to an extent similar to murine FP. This apparent inconsistency may reflect the demonstration that ovine FP expresses a relatively high abundance of mRNA for several growth factors which are mitogenic for mammary epithelial cells (Chapters 9-11). Furthermore, ovine mammary tissue contains a high proportion of fibroblasts (Akers, 1990; Chapter 8) which secrete a soluble activity that is mitogenic for COMMA-1D cells (Chapter 11). Although the relative contributions of such factors remain unclear, it is evident that several means exist by which the ovine FP may elicit its growth promoting effect; means which may exert differential effects on murine and bovine mammary epithelial cells.

The ability of murine and ovine FP to modulate the proliferative effect of various factors on mammary epithelial cells was also compared. While the mitogenic effect of co-cultured tissues for COMMA-1D cells was additive to that of 10% FCS, the response of MAC-T cells to FCS was suppressed by co-cultured ovine mammary tissues. These contrasting responses may reflect different sensitivity of murine and bovine mammary epithelial cells to the effects of high fatty acid concentrations (Wicha *et al.*, 1979; Cockrell *et al.*, 1992) or growth inhibitory factors such as transforming growth factor-β (Woodward *et al.*, 1995; Soriano *et al.*, 1996).

A finding that may be of particular significance is the different ability of murine and ovine mammary fat pads to modulate the responsiveness of murine and bovine epithelial cells to certain mitogens. While murine FP substantially potentiates the effects of IGF-I, EGF and insulin on COMMA-1D cells, ovine FP only adds to or slightly interacts with these effects. In contrast, MAC-T responsiveness to IGF-I and insulin is similarly enhanced by murine and ovine FP.

In these experiments the most pronounced cell growth responses occurred when COMMA-1D cells were co-cultured with murine MP. It was recently reported that murine FP likely elicits such responses by its liberation of unsaturated fatty acids to enhance intracellular signalling via protein kinase C (Chapter 4). Such a mechanism may be of increased importance within the developing mouse mammary gland where epithelium grows in close association with adipocytes (Daniel and Silberstein, 1987) which can transport free fatty acids directly across the basement membrane (Bandyopadhyay *et al.*, 1995). While the growth of ruminant mammary epithelial cells is stimulated by unsaturated fatty acids *in vitro* (Cockrell *et al.*, 1992) and *in vivo* (McFadden *et al.*, 1990a), these cells may have a reduced ability to utilise unsaturated fatty acids to enhance their responsiveness to certain growth factors, a suggestion which could be reflective of the fatty acid composition of the ruminant mammary fat pad.

Such findings, however, do not fully resolve the question of why ruminant mammary epithelium does not outgrow into the mouse mammary fat pad. It is possible that the endogenous mammary fat pad provides other factors to satisfy requirements for such growth. Sheffield (1988b) indicated that the extracellular matrix in the rodent mammary gland differs substantially from that in ruminants and humans by virtue of differences in the proportion of connective tissue surrounding the mammary epithelium. Additionally, the ovine mammary gland stroma expresses a relatively high abundance of

mRNA for certain growth factors (Chapters 9-11) which may fulfil specific requirements for the growth and morphogenesis of ruminant mammary epithelium.

Co-cultured ovine PAR was consistently more mitogenic than ovine FP, suggesting that ovine mammary epithelium locally upregulates mitogenic stimulation by the adjacent stromal constituents. A similar effect was recently recorded within mouse mammary tissue at different stages of postnatal development (Chapter 6) and during the oestrous cycle (Chapter 5), an effect which may represent epithelium-induced lipolysis within adjacent mammary adipocytes (Levay-Young *et al.*, 1987; Kidwell *et al.*, 1982). However, the mechanism underlying this effect in ovine mammary tissue is unknown. It is possible that endogenous epithelium induced the increased or specific release of unsaturated fatty acids in co-culture, perhaps through an epithelial desaturase activity (Bandyopadhyay *et al.*, 1995) or by preferential sequestration of unsaturated fatty acids from mammary adipocytes (Kidwell *et al.*, 1982). It is also possible that epithelium increased the mitogenic effect of PAR by increasing the stromal synthesis of paracrine growth factors (Chapters 9-11). While the cause(s) underlying this increased stimulation remains to be established, such findings support the concept that mammary epithelium can signal the adjacent stroma to locally promote its growth and morphogenesis.

This cellular interaction might also modulate epithelial responsiveness to certain mammogenic factors. While intact mouse mammary tissue is significantly more mitogenic than murine FP *in vitro* (Chapter 5), this difference does not exist in the presence of oestrogen (Chapters 5 and 6). That this effect was not apparent in co-culture with ovine FP and PAR may relate to the uncertain role of the ovary during ovine mammogenesis (Ellis *et al.*, 1996a). However, a similar effect was observed where MAC-T cells only responded to EGF in the presence of PAR, even though an earlier report established that MAC-T cells are unresponsive to the mitogenic effect of EGF (Woodward *et al.*, 1994). One means by which this response may have occurred is through an indirect action of EGF via EGF receptors within the parenchymal stroma, the expression of which is induced by the adjacent epithelium (Daniel and Silberstein, 1987). Certainly other factors resulting from the epithelial-stromal interaction within PAR may have also accounted for, or contributed to, this response. It is possible that such effects may be required to elicit a local mammogenic effect of GH, although such a mechanism remains to be investigated.

In conclusion, these findings demonstrate that the murine and ovine mammary fat pads exert distinctive mitogenic effects on murine and bovine mammary epithelial cells and differentially modulate their responsiveness to certain mammogenic factors. As in the mouse mammary gland, epithelial-stromal interactions within the ovine mammary gland may serve to locally regulate epithelial proliferation.

CHAPTER 8

PREPARATION OF A PARENCHYMA-FREE MAMMARY FAT PAD AND SUBSEQUENT MAMMARY GLAND DEVELOPMENT IN SHEEP

8.1 ABSTRACT

A surgical procedure was developed for the preparation of an epithelium-free mammary gland fat pad (cleared mammary fat pad; CFP) in ewes. At 7-10 days of age, ewe lambs (n=43, mean BW 9.2 ± 0.2 kg at 14 days) were sedated, the udder aseptically prepared, and one gland locally anaesthetised. Palpation indicated the extent and location of epithelial parenchyma to be removed from this gland. An incision was made that circumscribed the base of the teat. Blunt dissection was then performed through the extraneous mammary fat pad tissue to enable the parenchyma and teat to be wholly removed. A preliminary study indicated that any residual epithelium could regenerate into the mammary fat pad. Mean diameter of the parenchymal rudiment was 8.9 ± 0.5 mm (range 5-16 mm). The site of excision was closed with wound clips, and recovered lambs were returned to their dam. The contralateral mammary gland remained intact, allowing it to undergo normal development.

Liveweight gain was unaffected by this surgical procedure. Groups of ewes were subsequently slaughtered at various stages of prepuberty, puberty, gestation and lactation. Of 39 operated glands recovered, only one demonstrated epithelial growth into the mammary fat pad. The contralateral, intact gland underwent phases of rapid growth in prepuberty, puberty, and late gestation, and was capable of milk synthesis after steroid hormone induction or parturition. Weight change by the CFP paralleled that of the intact mammary gland to 100 days of pregnancy. Sham CFP surgery was performed on an additional 4 ewes wherein parenchyma was fully excised and immediately replaced. Sham-operated epithelium populated the mammary fat pad and synthesised milk that could be expressed via the teat. A CFP in sheep will be a useful model for future investigations into the local growth regulatory mechanisms associated with postnatal ruminant mammogenesis.

8.2 INTRODUCTION

The mammary gland undergoes a series of proliferative and morphological changes as it develops postnatally (Imagawa *et al.*, 1994). By far the majority of studies which have investigated the processes underlying such development have been conducted in rodents as a model for human breast cancer (Nandi *et al.*, 1995).

An aspect of mammary biology that has received increased attention is the local growth regulatory capacity of the mammary fat pad. This stromal matrix may direct epithelial development via the local synthesis of growth factors (Chakravorti and Sheffield, 1996a; Chapters 9-11) and extracellular matrix components (Keely *et al.*, 1995), and by locally mediating hormone action (Shyamala and Ferenczy, 1984). Investigations of such mechanisms in rodents have been greatly facilitated by the "cleared mammary fat pad" technique. Originally described by DeOme *et al.* (1959), this relatively simple procedure involves ablating the epithelial rudiment to yield a mammary fat pad devoid of endogenous epithelium. A wide range of studies have utilised the CFP to study various aspects of mammary fat pad function including lipid metabolism (Bandyopadhyay *et al.*, 1995) and growth factor expression (Coleman-Krnacik and Rosen, 1994; Chakravorti and Sheffield, 1996a). The CFP has also been extensively used as a transplantation site for murine (Hoshino, 1978), bovine (Sheffield and Welsch, 1986), tumorous (Miller *et al.*, 1981), and genetically transformed (Edwards *et al.*, 1996) mammary epithelium.

Although the results of some rodent experiments are directly relevant to aspects of ruminant mammogenesis, several distinctions between mammary gland development in the two orders strongly discourage an extensive extrapolation of results (Akers, 1990). For example, whereas mouse mammary epithelium ramifies into the mammary fat pad as a sparse network of ducts guided by peripheral end buds (Imagawa *et al.*, 1994), ruminant mammary epithelium progresses into the fat pad as a more dichotomously branched parenchyma (Hammond, 1927) with no apparent end bud structures (Ellis *et al.*, 1995). This latter morphogenesis is associated with the epithelium being encompassed by a much greater proportion of connective tissue (Sheffield, 1988b; Akers, 1990), somewhat similar to that seen in the human breast (Rønnov-Jessen *et al.*, 1996). Additionally, whereas rodents fed a diet high in energy have increased mammary

development and tumorigenic risk (Engelman *et al.*, 1994), ruminants fed a high plane of nutrition have impaired mammogenesis and lactogenic potential (Johnsson and Hart, 1985; Little and Kay, 1979). Furthermore, adipocytes of the rodent mammary fat pad contain a high proportion of unsaturated fatty acids (Bandyopadhyay *et al.*, 1995) which influence mammary epithelial development and tumorigenesis (Wicha *et al.*, 1979; Welsch, 1992) whereas ruminant mammary adipocytes contain a high proportion of saturated fatty acids (McFadden *et al.*, 1990a).

The objective of this study was to prepare a CFP in sheep as a model for studies into the local regulation of ruminant mammogenesis. The successful development of such a procedure is described herein, and some aspects of subsequent mammary gland development in these ewes are reported.

8.3 MATERIALS AND METHODS

8.3.1 Animals

A total of 53 Dorset x Coopworth ewe lambs from the Ruakura flock were used in this study. Of these, 40 were singles and the remainder twins. A total of 47 ewe lambs were surgically prepared; the remaining six served as unoperated controls. Lambs were weaned at 11 weeks of age. All sheep grazed ryegrass pastures as a single flock and were subjected to standard management practices.

8.3.2 Surgical procedure

All manipulations were approved by the Ruakura Animal Ethics Committee. Surgery was conducted over 3 consecutive days on 43 ewe lambs that were 7-10 days of age. Lambs were separated from their dam on the morning of surgery and were held in pens. The mean bodyweight of these lambs 4 days post-surgery was 9.2 $\pm$ 0.2 kg. Each lamb was sedated (Rompun, 2%; 0.15 ml i.v.), placed in a dorsally recumbent position, and the posterior abdomen, udder region and inside hind legs shaved and aseptically prepared. One mammary gland of each lamb was subsequently prepared as a CFP. The region at the base of the appropriate teat was palpated to ascertain the size and location of the underlying parenchymal tissue to be ablated. The periphery of this gland was locally anaesthetised with a field block (Xylocaine, 1 ml). A concavo-convex incision

circumscribing the base of the teat was then made. Blunt dissection was performed subcutaneously to beyond the bounds of the parenchymal tissue, and then through extraneous adipose tissue of the mammary fat pad such that the teat and associated parenchyma could be wholly removed. The site of excision was closed with stainless steel clips and topically treated with Terramycin powder. A systemic antibiotic was also administered (Depomycin, 0.5 ml, i.m.). Once fully recovered (approximately 2 h), lambs were returned to their dam and the establishment of ewe-lamb bonding confirmed.

On a separate occasion a sham CFP was prepared in four 10-day old lambs. In these instances the parenchymal tissue and teat were removed according to the above procedure and were then replaced into the excision site which was closed with wound clips.

A total of 6 lambs died throughout the course of this study (5 operated, 1 control), although in no case was death attributed to the surgical manipulation.

8.3.3 Experimental design

Ewe lambs were weighed weekly to 16 weeks of age, fortnightly to 31 weeks, and at varying intervals thereafter. The onset of oestrus in ewes under similar conditions is typically around 30-38 weeks of age (Smith *et al.*, 1993a). At 32 weeks, all ewes were administered an intravaginal CIDR™ for 14 days to synchronise oestrus (Paton *et al.*, 1993). Ewes to be mated were pastured with rams fitted with a marking harness for two subsequent oestrous cycles, and marked ewes were recorded each morning. Pregnancies were confirmed at approximately 40 days post coitus by ultrasound scanning. Standard gestation length in these ewes is 145-150 days.

Ewes with CFPs were sacrificed in groups at various stages of development, specifically at 6 weeks (n=4), 10 weeks (n=4), 15 weeks (n=4), 22 weeks (n=5), 35 weeks (n=4), 53-57 weeks (n=5), 50 days gestation (n=4), 100 days gestation (n=2), 140 days gestation (n=2) and 1 day post-partum (n=1). One sham CFP ewe was sacrificed at 53 weeks of age, and another at 5 days post-partum. One control ewe was sacrificed at 100 days gestation, and another at 140 days gestation. The remaining virgin ewes were hormonally induced into lactation as described below and were sacrificed thereafter at 65 weeks of age.

8.3.4 Mammary sampling

Ewes were killed by captive bolt and exsanguination. The udder was removed and separated along the medial suspensory ligament into the intact and CFP halves which were then weighed. Both udder halves were sliced sagitally in the dorso-ventral plane into slices of approximately 8 mm thickness. The first cut through the intact gland bisected the parenchymal mass.

To obtain a non-destructive measure of mammary development within the intact gland, the face of each slice was traced onto transparency film along the bounds of the skin, parenchyma (on the basis of colour) and mammary fat pad. Each tracing was then photocopied onto paper, the parenchymal and mammary fat pad regions cut out, and their area determined with an LI-3000 portable area meter (Li-Cor, USA). Values presented are the maximal parenchymal cross-sectional area measured within each gland along with the corresponding area of mammary fat pad.

A gross inspection was conducted on dissection of the CFP to confirm the absence of any epithelium within the CFP. In addition, any sites that possibly contained epithelium were collected for histological evaluation.

8.3.5 Hormonal induction of lactation and milking

Virgin ewes (3 CFP, 2 sham CFP, 3 controls) were induced into lactation by steroid treatment at 64 weeks of age (Smith and Schanbacher, 1974). Daily s.c. injections of 17β-oestradiol (0.1 mg/kg; Sigma) and progesterone (0.25 mg/kg; US Biochemical) were administered for 7 days; on days 8 and 9 ewes were treated with 1 mg dexamethasone s.c. (Dexadreson, Intervet). Ewes were hand milked once daily on days 9, 10, and 11, and milk yields recorded. These ewes were sacrificed on day 11.

For the one parturient sham CFP ewe, the lamb was removed 48 h after birth and the hand-milked yields from each gland were recorded twice-daily following oxytocin treatment (0.2 ml, i.v.).

8.3.6 Chemical analyses

The protein level in milk from hormonally-treated ewes was determined by the BCA assay (Pierce, IL) and the lipid content measured following chloroform/methanol (2:1) extraction (Bligh and Dyer, 1959). Lactose concentration was determined

fluorometrically (McFadden *et al.*, 1995). Composition of milk (fat, protein, lactose) from the sham CFP ewe was determined by Milkoscan analysis.

8.3.7 Histology and whole mounts

Tissues for histological examination were fixed in phosphate-buffered 10% formalin for 24-48 h at 4°C. Samples were prepared for paraffin microtomy and sectioned at 5-7 µm. Sections were mounted onto poly-lysine subbed slides and processed for routine light microscopy.

Mammary tissue for whole mounts was cut into 1-2 mm thick slices using a Stadie-Riggs hand microtome. Slices were spread on slides and fixed in Carnoy's fixative for 24 h. Preparations were defatted in acetone, stained with alum carmine, dehydrated through graded alcohols, and clarified in xylene. Whole mounts were photographed using a dissecting microscope.

8.3.8 Statistical analyses

Data were analysed by Student's t-test or one-way ANOVA using SAS.

8.4 RESULTS

8.4.1 Mammary CFP technique

In establishing this procedure, a preliminary study was conducted whereby 6 mammary glands of four 10-day old lambs were surgically prepared using an approach similar to that described in Materials and Methods. On this occasion an attempt was made to closely dissect the denser parenchyma from the surrounding stroma of the mammary fat pad. Examination of these glands 3 months later revealed that epithelium had extensively populated the mammary fat pad in 3 of the 6 glands. This parenchymal regrowth was grossly evident within the mammary fat pad and consisted of terminally-branched ducts (Figures 8.1a and 8.1b). Such an outcome emphasised the need to fully encapsulate the parenchymal elements while performing blunt dissection for the main study.

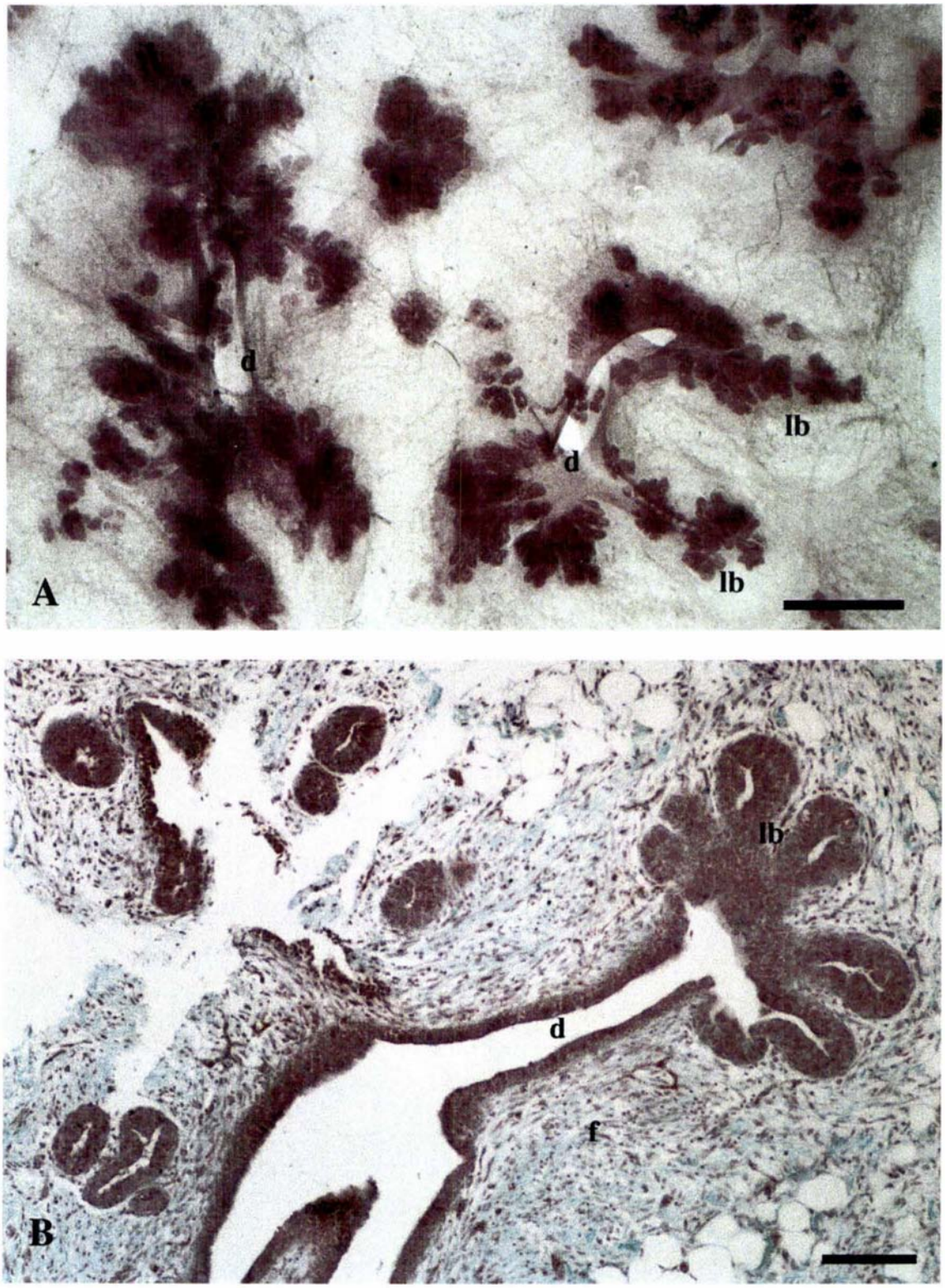


Figure 8.1 Histomorphogenesis of parenchymal regrowth within the mammary fat pad of virgin ewe lambs. The preliminary CFP procedure was performed at 1 week of age as described in 8.4.1, and ewe lambs were sacrificed at 3 months of age. (A) Whole mount of regrown mammary parenchyma. Scale bar = 1 mm. (B) Histology of regrown mammary parenchyma depicting ductal epithelium encapsulated by fibroblastic stromal tissue. Stained with Gomori's trichrome. Scale bar = 100 μm. lb, lobular bud; d, duct; f, fibroblasts.

Tissue excised from the neonatal mammary gland included a discrete node of parenchyma at the base of the teat (Figure 8.2a) that consisted of numerous branched ducts encapsulated by stromal connective tissue (Figure 8.2b). Where epithelial regrowth was subsequently recorded in the preliminary study, the site from where the parenchyma had not been completely removed was sometimes evident (Figure 8.2c). In the main study the excised tissue was bisected and the diameter of the parenchymal node measured. The mean diameter (± SEM) of this region was 8.9 ± 0.5 mm (n=37) with a range of 5-16 mm.

Of the 39 CFPs that were recovered at slaughter or necropsy in the main study, only one displayed epithelial regrowth. Variable evidence of scarring was also recorded within the CFPs. This ranged from no detectable scarring to a thin band of scar tissue at the site of the excision (Figure 8.2d).

Figure 8.3 illustrates the udder of mature ewes which had been prepared as either a CFP or a sham-operated CFP.

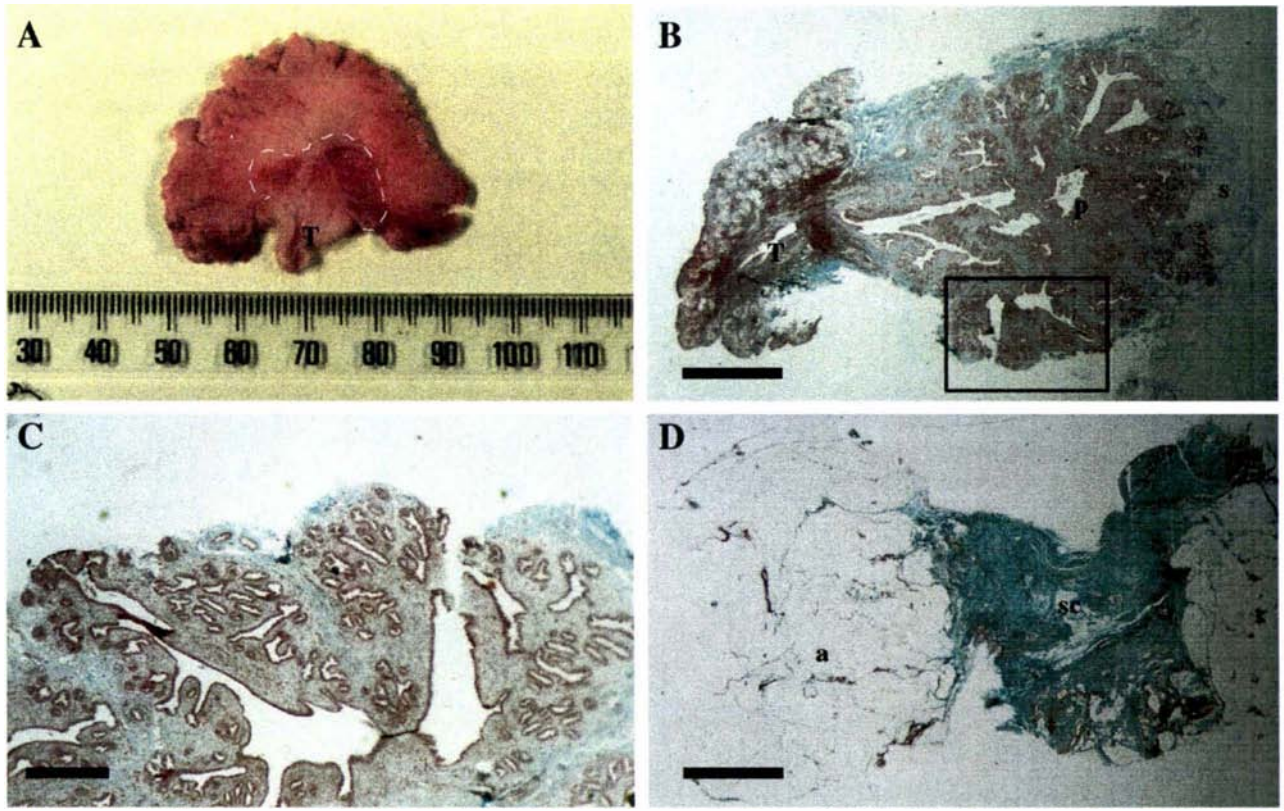


Figure 8.2 Mammary tissues from the CFP procedure. (A) Mammary tissue excised from neonatal ewe lambs by the CFP procedure in the main experiment was bisected and fixed. T, teat. The dashed line delineates the parenchymal tissue. (B) Mammary tissue excised from neonatal ewe lambs in the preliminary CFP procedure described in Section 8.4.1 was sectioned (7 μm) and stained with Gomori's trichrome. T, teat; p, parenchyma; s, stroma. Scale bar = 3 mm. (C) Higher magnification of the boxed area in (B) showing the peripheral region of parenchymal tissue which had not been completely removed during the CFP procedure. Scale bar = 1 mm. (D) Section of scar tissue within the CFP of a 3-month old ewe lamb. Scale bar = 3 mm. sc, scar tissue; a, adipocyte.

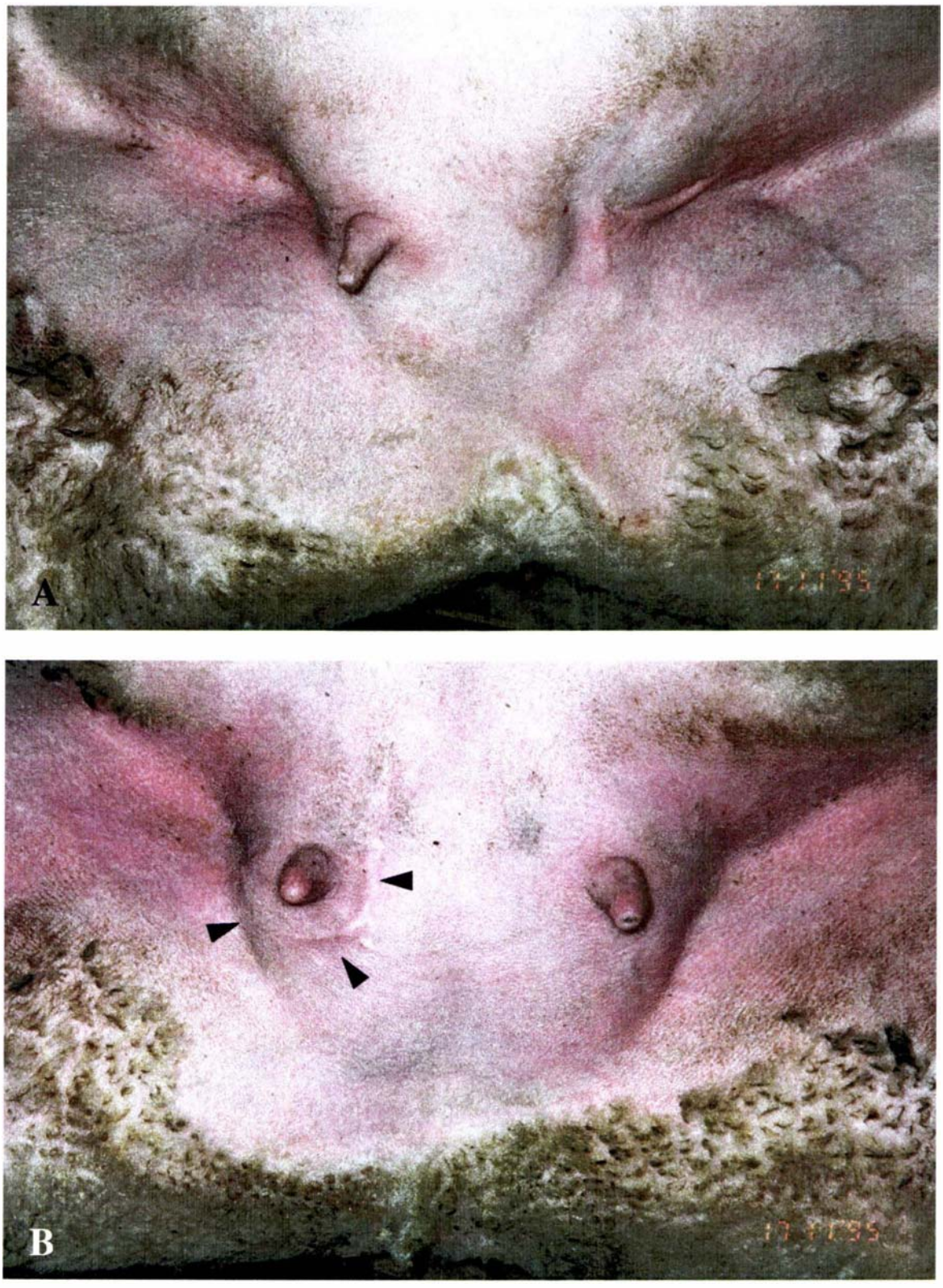


Figure 8.3 Photograph of the udder of a ewe in which one gland had been prepared as (A) a CFP or (B) a sham-operated CFP. Both ewes had been hormonally-induced to lactate and had been hand milked for 3 days. Arrows in (B) indicate the location of the scar which resulted from the surgical procedure.

8.4.2 Mammary gland development

Liveweight gain for CFP ewe lambs to 23 weeks of age was not different (P>0.05) to that for control lambs (171 vs 169 g/day, Figure 8.4), reaching a maximum average daily gain of 255 g/day. The lower initial bodyweight of the control group reflected that these lambs were from twin sets.

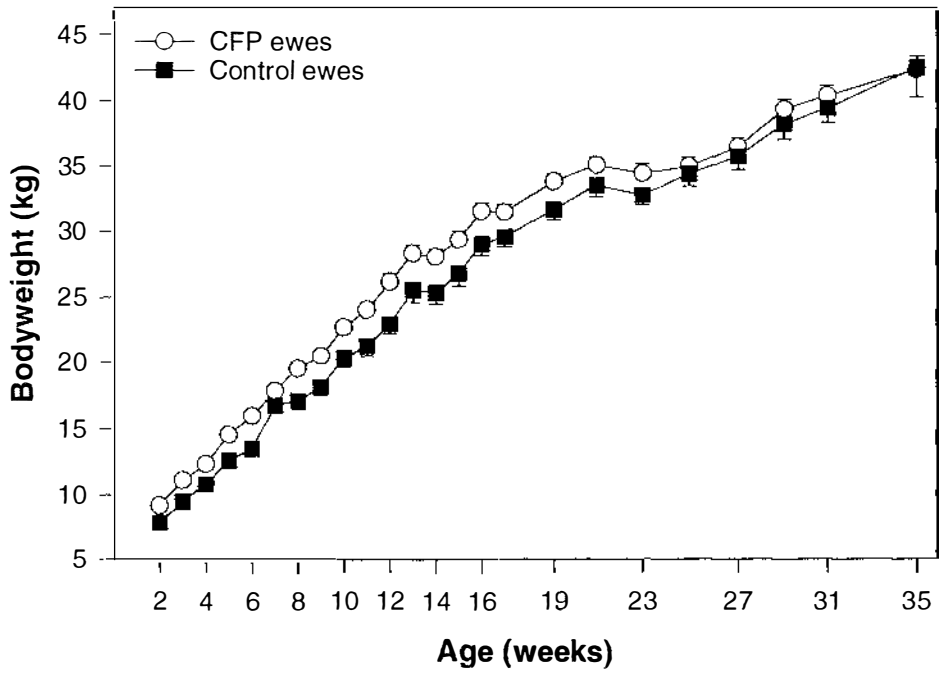


Figure 8.4 Liveweights of virgin CFP (O) and control (■) ewe lambs. Data are means ± SEM for the entire group at the indicated ages.

Weights of the CFP and the contralateral intact mammary gland underwent similar changes during the course of development, and only diverged after day 100 of pregnancy (Figure 8.5). The weight of intact glands and CFPs from virgin ewes increased significantly (P<0.05) between 6 and 10, and 23 and 35 weeks of age. Likewise, intact glands increased in weight substantially during the periparturient period while CFP weight remained relatively static over the same time.

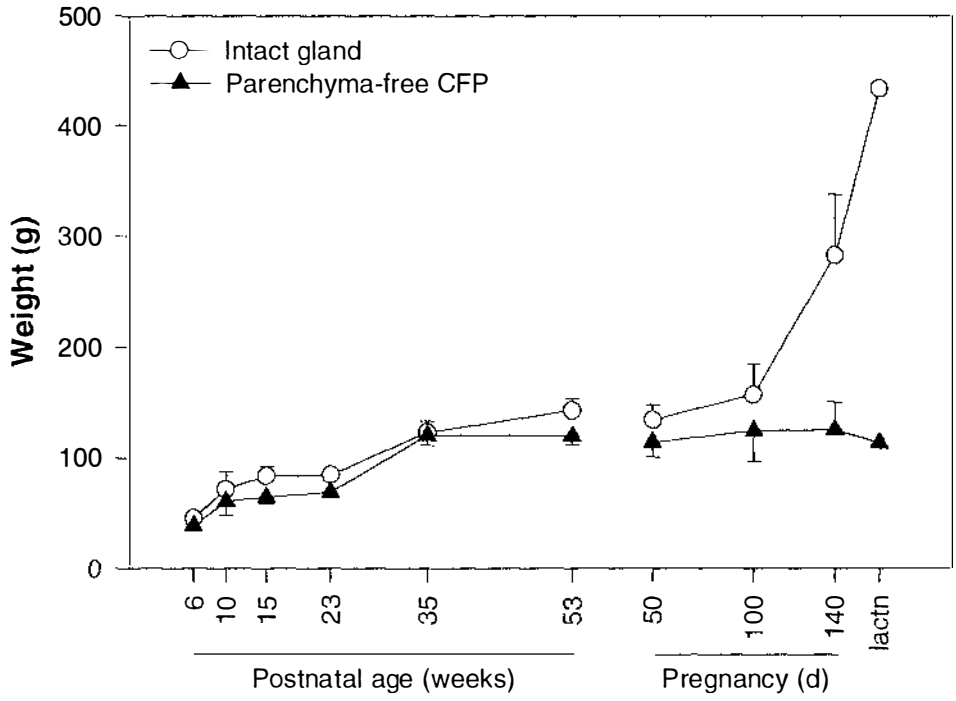


Figure 8.5 Weight of CFP (▲) and intact (O) mammary glands from ewes sacrificed at various stages of postnatal development. Data are means ± SEM. The number of ewes in each age group is detailed in Section 8.3.3.

The cross sectional area of parenchymal tissue within mammary glands was measured as an assessment of mammogenesis. Between 6 and 10, and 10 and 15 weeks of age, the rate of parenchymal area increase was 44.3 and 79.4 mm^2/week, respectively (Figure 8.6a). This subsequently declined to 24 mm^2/week between 15 and 23 weeks of age. Rate of parenchymal area change increased to 54 mm^2/week between 23 and 35 weeks and remained around this level until day 100 of pregnancy when it increased immediately prior to parturition.

To evaluate the dynamics of parenchymal growth within the mammary fat pad, parenchymal areas were expressed as a percentage of the respective total gland area. The pattern of parenchymal occupation was generally similar to that recorded for parenchymal area, particularly in relation to the increased growth between 10 and 15 weeks, and after 23 weeks of age (Figure 8.6b). One distinction was that in contrast to parenchymal area, percentage of the gland occupied did not change between days 100 and 140 of pregnancy.

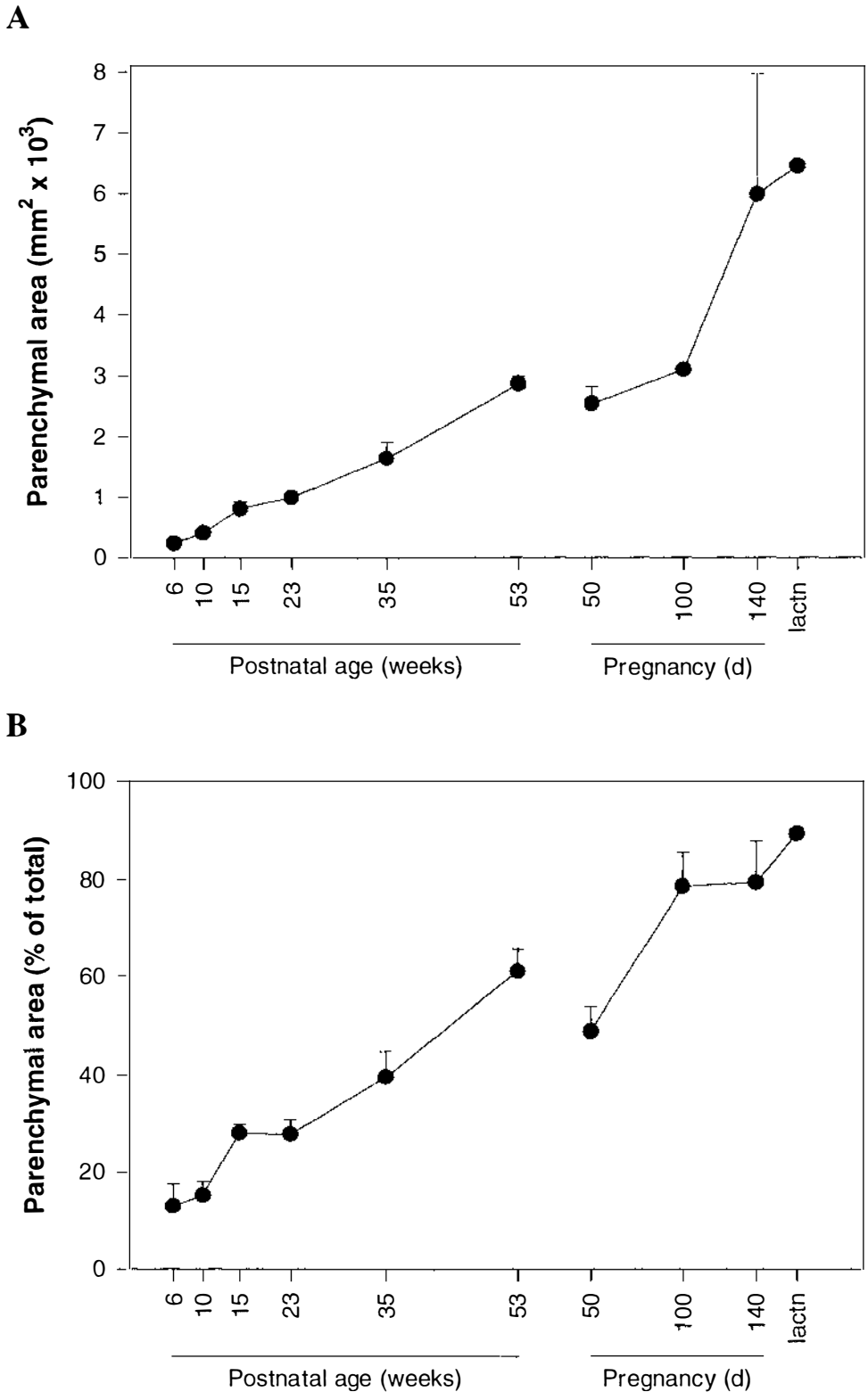


Figure 8.6 Development of parenchyma within the intact mammary gland of CFP ewes sacrificed at various stages of postnatal development. (A) Parenchymal cross-sectional area. (B) Proportion of mammary cross-sectional area occupied by parenchyma. Data are means ± SEM. The number of ewes in each age group is detailed in Section 8.3.3.

The growth and lactogenic responses by mammary glands of CFP and sham CFP ewes to hormonal induction of lactation or parturition were also examined. While there were only limited numbers in each group, glands contralateral to a CFP produced milk yields comparable to those from control glands, and having a similar composition (Table 8.1). Even after neonatal disruption, parenchyma in sham-operated ewes grew into the mammary fat pad and produced milk of a normal composition that could be expressed from a fully functional teat. The extent of this regeneration and the subsequent milk yield varied; parenchyma in sham-operated glands was frequently interspersed with various amounts of scar tissue. It was possible, based on the measurement of parenchymal area, that the intact gland of udders in hormonally-treated, sham-operated ewes demonstrated some degree of compensatory growth.

8.5 DISCUSSION

This report describes the novel preparation of a parenchyma-free mammary fat pad in sheep, a technique that has been widely utilised in rodents to investigate various aspects of mammary gland biology (Sheffield, 1988b; Medina, 1996). Such a procedure should prove particularly useful for studies into the local regulation of mammary gland development in ruminants. Ongoing investigations have utilised this approach to examine the local expression of growth factors by the mammary fat pad and the growth regulatory influence of epithelial-stromal interactions (Chapters 9-11).

Consistent with findings from rodent studies is the demonstration that ovine mammary epithelium has a substantial capacity to proliferate and regenerate within the mammary fat pad. This was illustrated by two separate observations. First, remnants of mammary epithelium that were not ablated grew into the mammary fat pad and assumed an apparently normal morphology. A similar response has been documented in rodents (Hoshino, 1978). Furthermore, this regenerative capacity suggests the existence of a pluripotent population of epithelial cells within the ruminant mammary gland (Ellis *et al*., 1995), as may exist within the rodent gland (Smith and Medina, 1988). Second, substantial development occurred in sham-operated glands wherein the parenchyma had been excised and then immediately replaced, despite the presence of moderate amounts of scarring. This epithelium subsequently synthesised and secreted normal milk. Such a finding is consistent with rodent studies showing that ductal segments and dispersed

Table 8.1 Lactogenic and mammogenic responses in mammary glands of surgically modified ewes.

			Induced		Parturient	
	Control ewes	CFP ewes	Sham ewes			
Gland (n)	(6)	(3)	Control (2)	Sham (2)	Control (1)	Sham (1)
Yield (g)[†]	53.3 ± 12	41.5 ± 5	70.7 ± 32	46.4 ± 3	607.9	90.2
Fat (%)[‡]	6.0 ± 1.2	7.9 ±1.2	6.8 ± 2.2	4.5 ± 1.0	8.5	8.1
Protein (%)[¥]	4.4 ± 0.5	4.5 ± 0.7	3.9 ± 0.6	3.9 ± 0.8	6.1	6.4
Lactose (%)[‡]	4.0 ± 0.1	4.0 ± 0.2	3.7 ± 0.7	3.7 ± 0.3	4.7	4.1
Parenchymal DNA (mg)	127 ± 29	158 ± 44	161.3[❖]	111 ± 16	nd	nd
Parenchymal area (mm^2)	3166 ± 427	3284 ± 485	7040 ± 1253	2820 ± 454	6740	2441
Parenchyma (% gland)	61 ± 4	62 ± 7	83 ± 13	72 ± 2	100	41.9

This part of the trial was conducted in collaboration with Danielle Auldist, Massey University.
nd = not determined
[†]Cumulative milk yield; 3 once-daily milkings for induced ewes, 3 half-daily milkings for parturient ewe.
[‡]Fat and lactose content is average for all milkings.
[¥]Protein content for induced ewes is average for milkings 2 and 3.
[❖]n=1

epithelial cells undergo normal morphogenesis, differentiation and milk protein expression when inoculated into a CFP (Hoshino, 1983; Ormerod and Rudland, 1986).

The ontogeny of mammary gland development in these sheep, as measured by parenchymal area and mammary weight, was characterised by phases of rapid parenchymal growth prior to and during puberty, and immediately prior to parturition. Other studies have similarly reported stage-specific development of the ovine (Anderson, 1975b; Johnsson and Hart, 1985; Smith *et al.*, 1989a) and bovine (Sinha and Tucker, 1969b; Tucker, 1969) mammary gland. The prepubertal phase of positive allometric growth is especially critical for subsequent mammogenesis in ruminants, as indicated by its susceptibility to negative effects due to a high plane of nutrition (reviewed by Sejrsen, 1994). As measured by changes in parenchymal area, mammary glands of these ewe lambs underwent the most rapid development between 10 and 15 weeks of age. The timing of this rapid growth corresponds to positive allometric growth in previous studies (Wallace, 1953; Anderson, 1975b; Johnsson and Hart, 1985). Utilising the ovine CFP, it has been demonstrated that the local expression of paracrine mitogens such as the insulin-like growth factors may be particularly important in regulating this phase of development (Chapter 9).

The ruminant mammary gland also undergoes extensive tissue remodelling as epithelium progresses into the stromal matrix of the mammary fat pad. Specifically, parenchymal tissue within the virgin mammary gland "replaces" a large volume of the mammary fat pad as it progresses *en bloc*. This development contrasts to that in the virgin rodent mammary gland where epithelium forms a sparse network of elongate ducts within an expansive mammary fat pad (Imagawa *et al.*, 1994). Parenchyma in the virgin ruminant mammary gland consequently consists of epithelial ducts surrounded by an extensive fibroblastic stroma, and only a limited number of adipocytes (Figure 8.7a). Within this study, parenchymal replacement of the mammary fat pad was evidenced by the fact that weight of the intact mammary glands paralleled that of the contralateral CFP, even though the amount of parenchymal tissue within the mammary gland increased substantially during this time. Furthermore, the proportion of mammary gland area occupied by parenchyma closely followed the profile for parenchymal area during development, indicating that the major changes in tissue area occurred within the parenchymal compartment. An exception was during late gestation when parenchymal

area increased dramatically while the proportion of gland area occupied by parenchyma remained unchanged. This difference likely reflects epithelial cell hypertrophy and the rapid division of epithelial cells during this period (Foster, 1977; Smith *et al.*, 1989a).

Histological examination of ovine mammary tissue emphasises several distinctions between the mammary gland of ruminants and rodents, and reiterates the need for separate investigations into their development (Akers, 1990). Specifically, ruminant mammary tissue contains a substantial proportion of fibroblastic connective tissue, both in the intralobular and interductal regions of parenchyma (Figure 8.7a), and as a pre-established network throughout the mammary fat pad (Figure 8.7b). The histology and morphology of nulliparous ovine mammary parenchyma and stroma strongly resembles that described for the normal, nulliparous human breast (Russo and Russo, 1987; Monaghan *et al.*, 1990; Moffat and Going, 1996; Rønnov-Jessen *et al.*, 1996). This contrasts with the rodent mammary gland in which actively dividing cells, particularly those within the ductal end bud, are in close or direct contact with adipocytes of the mammary fat pad (Williams and Daniel, 1983).

The interspersement of connective tissue throughout the ruminant mammary fat pad and parenchyma likely provides an essential internal support for the pendulous lactating udder. By virtue of its position, the human breast is also supported by such a framework. This network of connective tissue might serve to regulate the ultimate location and distribution of lobules within the mammary gland (Mayer and Klein, 1961). Species differences in the proportion of connective tissue within the mammary gland may be embodied by the findings of xenotransplantation experiments showing that whereas rat and mouse mammary epithelium proliferates extensively within the CFP of mice (Hoshino, 1978; Daniel *et al.*, 1983), epithelial cells from bovine (Sheffield and Welsch, 1986; Ellis and Akers, 1995) and human (Sheffield and Welsch, 1988; Yang *et al.*, 1995) mammary tissue do not outgrow, but instead form hollow spheroids encapsulated by connective tissue. These species-specific responses may reflect epithelial requirements for particular components of the extracellular matrix as provided by the appropriate mammary fat pad (Sheffield, 1988b). It will be interesting to evaluate the ability of ruminant, human, and rodent mammary epithelial cells to develop within the ruminant CFP once immunological tolerance can be addressed.

In conclusion, these present findings emphasise that development of the ruminant mammary gland involves dynamic changes in tissue proliferation, morphogenesis and remodelling. Preparation of an epithelium-free CFP in sheep should prove particularly useful for investigating the mechanisms underlying these changes. Resultant findings may not only be relevant to attempts at increasing milk yield of dairy animals, but may also be directly applicable to studies of human breast cancer.

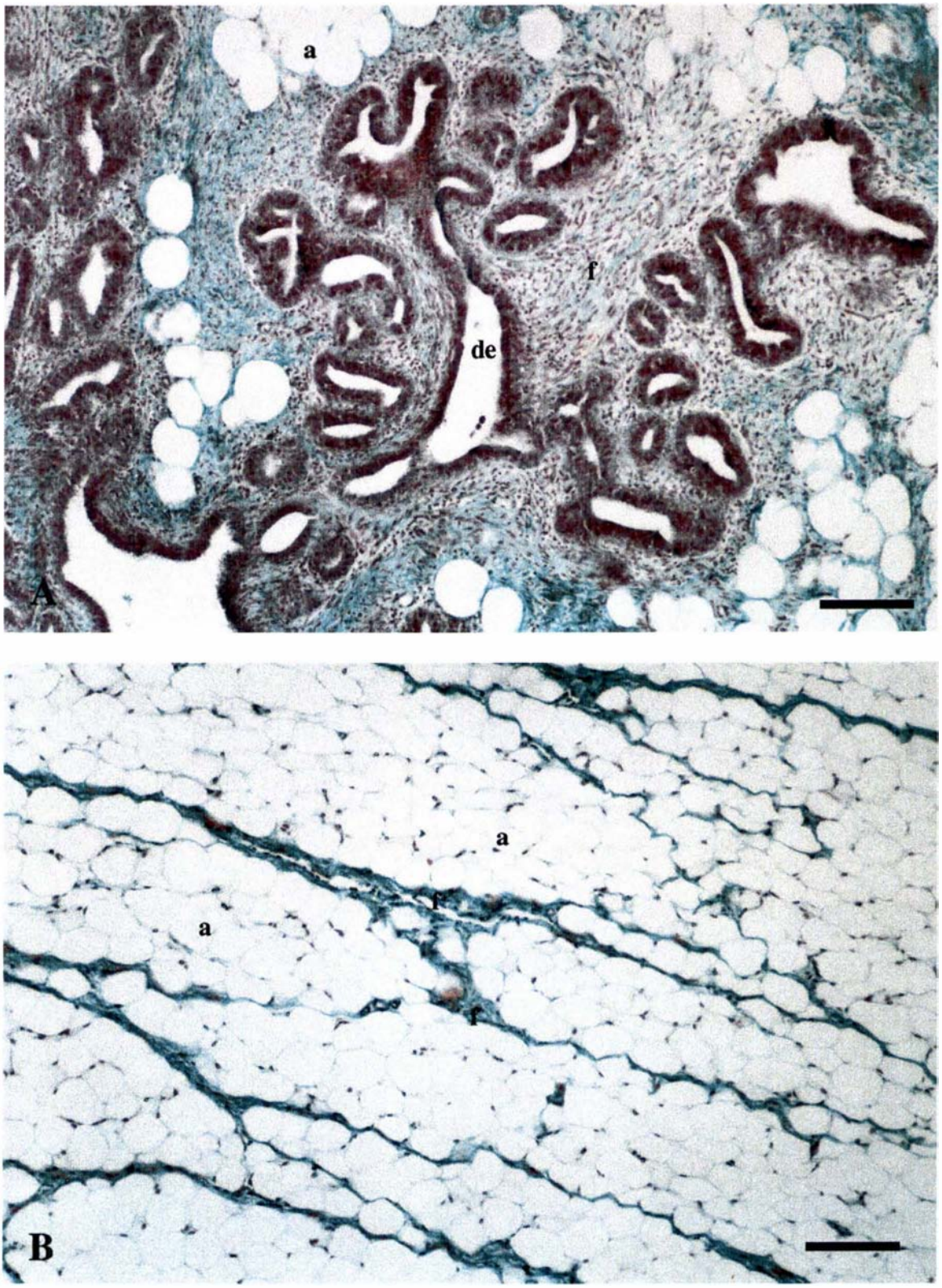


Figure 8.7 Histology of mammary parenchyma (A) and fat pad (B) tissue from a 3-month old virgin ewe lamb (6 μm). Scale bar = 100 μm. de, ductal epithelium; f, fibroblasts; a, adipocytes. Stained with Gomori's trichrome.

CHAPTER 9

DEVELOPMENTAL EXPRESSION SUGGESTS ROLES FOR LOCALLY-DERIVED INSULIN-LIKE GROWTH FACTORS-I AND -II DURING OVINE MAMMOGENESIS

9.1 ABSTRACT

Locally-derived insulin-like growth factors (IGF-I and -II) have been implicated in the stimulation of mammogenesis although evidence supporting such a role is limited. In the present study the expression of IGF-I and -II mRNA in the mammary parenchyma and supporting mammary fat pad of ewes during postnatal development was assessed. A single 7.5 kb IGF-I mRNA was most abundant in mammary fat pad tissue adjacent to parenchyma (MFP), while its expression was less in the contralateral fat pad that had been surgically cleared of epithelium (CFP). The lowest levels of IGF-I mRNA were in the mammary parenchyma. Expression of IGF-I mRNA was elevated at 6 and 10 weeks of age, had declined by 15 weeks, and remained low until it increased in late pregnancy. The level of IGF-II mRNA in mammary parenchyma was elevated from 1 to 23 weeks correspondent with the upregulated expression of specific mRNA transcripts. The abundance of IGF-II mRNA in the mammary fat pads was initially low but increased with age; between 1 and 23 weeks of age levels were consistently higher within the MFP than in the CFP. IGF-I mRNA expression was unaffected by ovariectomy while oestrogen treatment increased the level of IGF-I mRNA in the mammary fat pad of ovariectomised ewes and the parenchyma of intact ewes. Ovariectomy increased the expression of IGF-II mRNA in mammary parenchyma whereas exogenous oestrogen suppressed levels in both the parenchyma and mammary fat pad. These findings indicate a role for locally expressed IGF-I and -II during ovine mammary development and demonstrate their regulation by stage of ontogeny, ovarian hormones, and epithelial-stromal interaction.

9.2 INTRODUCTION

A number of *in vitro* studies have demonstrated that IGF-I and -II are potent mitogens for normal murine, ovine and bovine mammary epithelial cells (Imagawa *et al.*, 1986; Winder *et al.*, 1989; Collier *et al.*, 1993), as well as for mammary tumours (Lee and Yee, 1995). A direct effect of IGF-I on mammary epithelium has been shown *in vivo*, where rats (Ruan *et al.*, 1995) and cows (Collier *et al.*, 1993) display increased parenchymal growth in response to locally administered IGF-I. In addition,

overexpression of IGF-I in the mammary glands of mice leads to ductal hypertrophy and incomplete involution (Hadsell *et al.*, 1996), while overexpression of IGF-II increases the incidence of mammary adenocarcinoma (Bates *et al.*, 1995).

IGFs produced within the mammary gland likely function as paracrine mitogens. Messenger RNA for IGF-I and -II has been detected in stromal fractions from the human (Singer *et al.*, 1995) and bovine (Hauser *et al.*, 1990) mammary gland. *In situ* hybridisation has confirmed the stromal origin of IGF-I and -II expression in normal and neoplastic mammary tissues from rats (Manni *et al.*, 1994) and humans (Singer *et al.*, 1995; Ellis *et al.*, 1994), and in foetal and neonatal ovine mammary tissue (Morgan *et al.*, 1996). IGF-I also immunolocalises to the stromal cells of the lactating mammary gland (Glimm *et al.*, 1988).

Despite this information, little is known about the physiological role of IGFs synthesised within the mammary gland. Locally-expressed IGF-I may mediate the mammogenic effect of growth hormone (GH; Ruan *et al.*, 1995) as IGF-I expression is upregulated by GH, but not prolactin (Kleinberg *et al.*, 1990). Furthermore, oestrogen upregulates GH-induced IGF-I expression and epithelial responsiveness to IGF-I (Ruan *et al.*, 1995) while ovariectomy suppresses IGF-II mRNA levels in mammary tumours (Manni *et al.*, 1994). Expression of IGF-I and -II mRNA in the pig mammary gland is highest in early pregnancy (Lee *et al.*, 1993). Taken together, these findings indicate that several factors which regulate mammary gland development may also direct the local expression and function of paracrine IGFs.

Mammary epithelium undergoes extensive proliferation and morphogenesis postnatally as it penetrates the adipose and connective tissues of the mammary fat pad. Although the above evidence suggests a role for locally-derived IGFs during this development, regulation of IGF-I and -II expression within the postnatal mammary gland has not been reported. The influence of developmental state, ovarian hormones and epithelial-stromal association on IGF-I and -II mRNA expression in the fat pad and parenchyma of the ovine mammary gland has been investigated in this study. Candidate roles for these IGFs during mammogenesis are discussed.

9.3 MATERIALS AND METHODS

9.3.1 Animals and tissues

Details of sheep used in the ontogeny study have been reported elsewhere (Chapter 8). Briefly, one mammary gland of ewe lambs was surgically cleared of parenchyma at 1 week of age to leave an epithelium-free mammary fat pad (CFP). The contralateral gland remained intact to allow the normal development of parenchyma into the surrounding mammary fat pad (MFP). Virgin ewes were sacrificed at 6, 10, 15, 23, 35 and 53 weeks of age (n=4/group), or at days 50 (n=4), 100 (n=2), and 140 (n=2) of pregnancy. One ewe was sacrificed at 1 day post-partum. Ewes were stunned and immediately exsanguinated. The udder was removed and tissue samples obtained from the parenchyma and extra-parenchymal MFP, and the contralateral CFP. Parenchyma surgically excised at 1 week of age was also used. All tissues were immediately frozen in liquid nitrogen and stored at -80^{o}C.

The effects of ovariectomy and oestrogen were studied using tissues from lambs in a separate trial (Ellis *et al.*, 1996a). Lambs were either intact or had been ovariectomised at 10 days of age; these lambs received daily s.c. injections of either 17β-oestradiol (0.1 mg/kg liveweight) or excipient for 7 days prior to sacrifice at 12 weeks of age (n=3 lambs/group). Samples of mammary parenchyma and MFP tissue were collected at slaughter.

9.3.2 Probes and labelling

IGF-I and IGF-II cDNA probes (Wong *et al.*, 1989; Ohlsen *et al.*, 1994) were labelled by random priming (Rediprime, Amersham) with [^{32}P]-dCTP (3000 Ci/mM, Amersham). For normalisation of loading, a 26-mer oligonucleotide probe corresponding to human 28S rRNA (Barbu and Dautry, 1989) was labelled with [^{32}P]-dCTP (3000 Ci/mM, Amersham) using terminal transferase.

9.3.3 Northern analysis

Tissues were homogenised in 4 M guanidinium isothiocyanate and RNA extracted with phenol and chloroform (Chomczynski and Sacchi, 1987). RNA samples (20 μg) were electrophoresed through 1% agarose/formaldehyde gels and transferred to Hybond N^{+}

membrane (Amersham). For ontogeny samples, each of four blots had RNA from the three tissues of a single ewe at each stage of development. Membranes were hybridised with the IGF-I cDNA probe for 16 h at 60 °C in 0.5M $NaHPO_4$, 1 mM EDTA, 7% SDS buffer (Church and Gilbert, 1984), and were then washed at 60°C to 1x SSC (10 minutes each with 0.5% SDS). After autoradiography, membranes were stripped in 0.1x SSC, 0.5% SDS for 1 h at 85°C and reprobed for IGF-II and 28S rRNA. Autoradiograms were quantified by scanning densitometry (Image Quant, Molecular Dynamics). Values for IGF-II mRNA are reported as the sum total of multiple transcripts.

9.3.4 Statistical analyses

Ontogeny data were analysed for the effects of stage of development and tissue type, and their interaction, using a mixed model within the REML procedure of SAS. Ewe was included as a random term across development and tissue type was analysed within ewe. Individual means for the ovariectomy and oestrogen treatments were compared by Student's t-test to address variance heterogeneity and the significance of main effects was tested using the GLM procedure of SAS.

9.4 RESULTS

Northern analysis revealed a single 7.5 kb IGF-I mRNA transcript in ovine mammary tissues which was most abundant in the mammary fat pads (Figures 9.1a and 9.1b). Lower levels were detected in parenchymal tissue, likely reflecting mRNA expression by the interspersed stroma (Morgan *et al.*, 1996). Expression in both the extra-parenchymal MFP and the CFP was highest at 10 weeks of age, declining ($P<0.05$) by 15 weeks to low levels through puberty and into early pregnancy (Figure 9.1b). Levels increased ($P<0.05$) into late pregnancy and remained high in early lactation. Prior to 23 weeks of age the level of IGF-I mRNA in the extra-parenchymal MFP was greater than in the CFP ($P<0.08$).

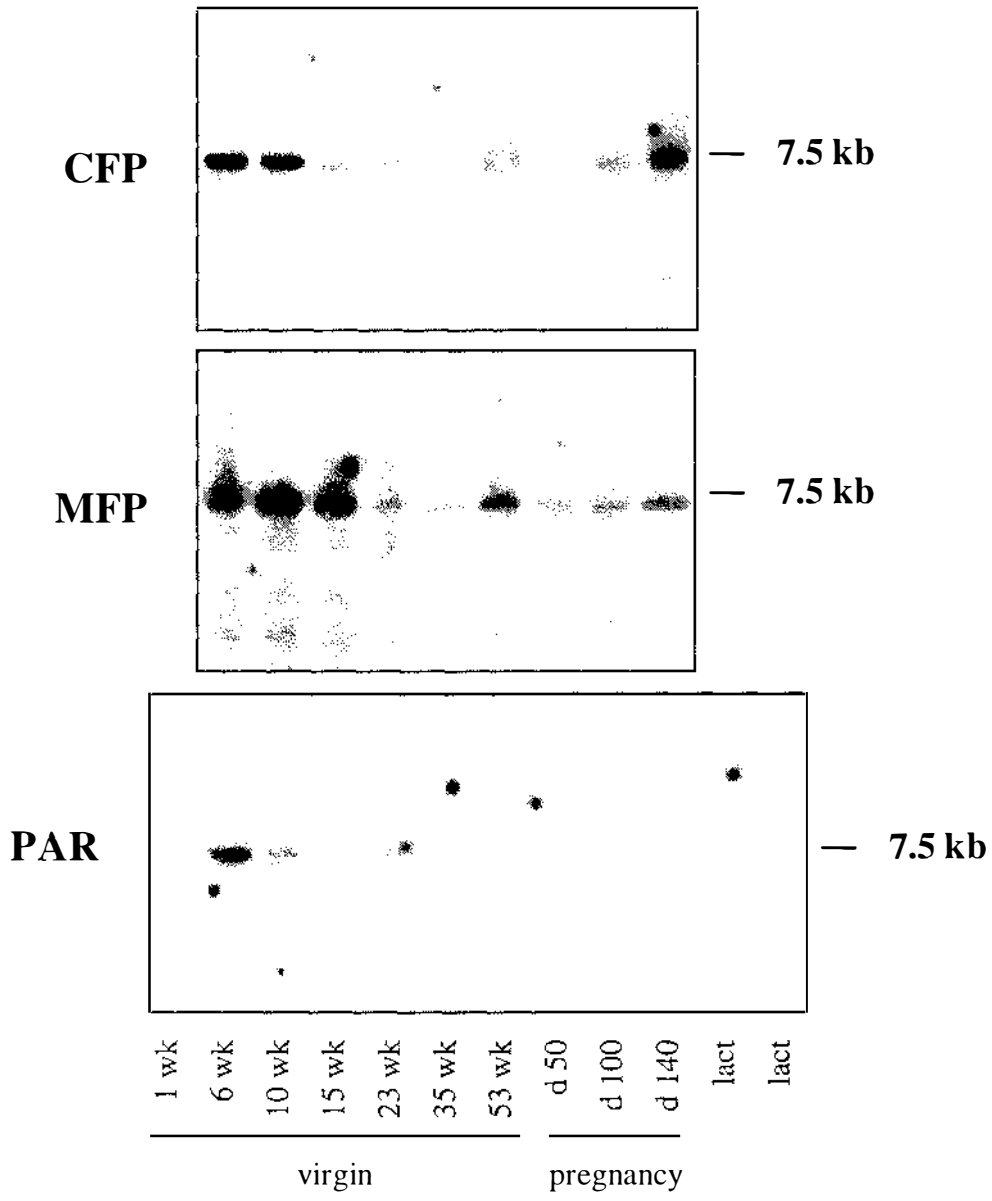


Figure 9.1 Northern analysis of mammary IGF-I mRNA in postnatal ovine mammary tissues. (A) Total RNA (20 μg) from the extra-parenchymal MFP, CFP, and mammary parenchyma (PAR) at various stages of postnatal development was hybridised with an ovine IGF-I cDNA (3 day exposure).

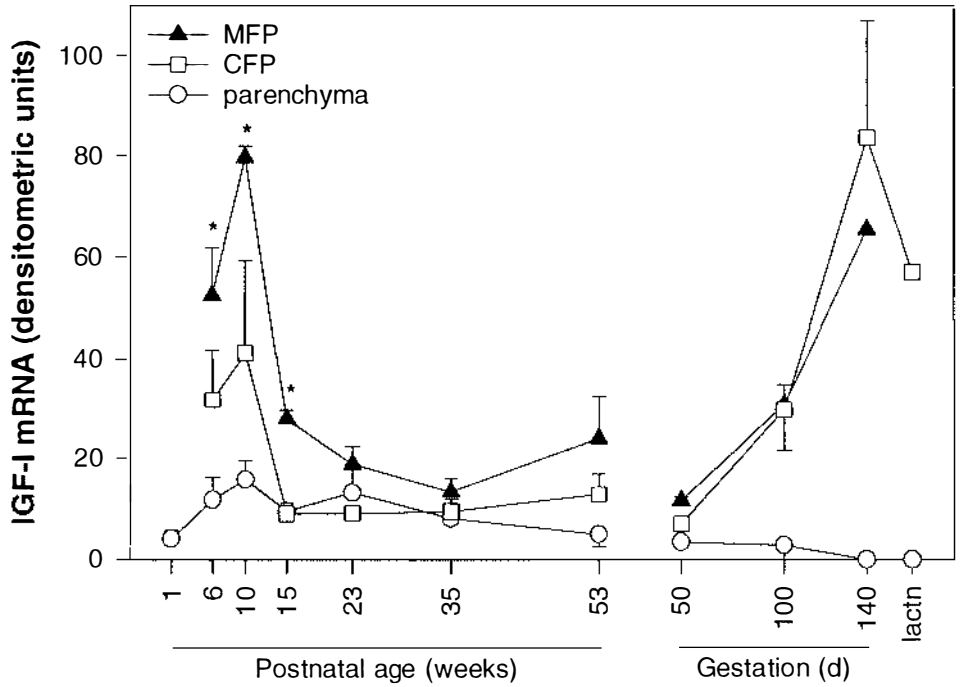


Figure 9.1 Northern analysis of mammary IGF-I mRNA in postnatal ovine mammary tissues. (B) Densitometric quantification of IGF-I mRNA in MFP (▲), CFP (□) and parenchyma (○), normalised for loading against 28S rRNA levels. Data points are means ± SEM. Number of replicates are n=4 for virgins and 50 days gestation, n=2 for 100 and 140 days gestation, and n=1 in lactation. *P<0.08 vs respective CFP value.

IGF-II mRNA was detected in both mammary fat pads as transcripts of approximately 5.7, 4.7 and 0.9 kb (Figure 9.2a). Total expression in the extra-parenchymal MFP was generally higher than in the CFP to 23 weeks of age, after which levels were similar (Figure 9.2b). The total level of IGF-II mRNA in mammary parenchyma was significantly (P<0.05) greater than in either of the fat pads between 1 and 23 weeks of age (Figure 9.2b). This corresponded to increased expression of the 5.7 and 0.9 kb mRNAs and the detection of three additional transcripts of estimated sizes 4.0, 3.2, and 2.5 kb (Figure 9.2a). In contrast, parenchymal expression of the 4.7 kb mRNA remained relatively constant across development at a level similar to that in the mammary fat pads.

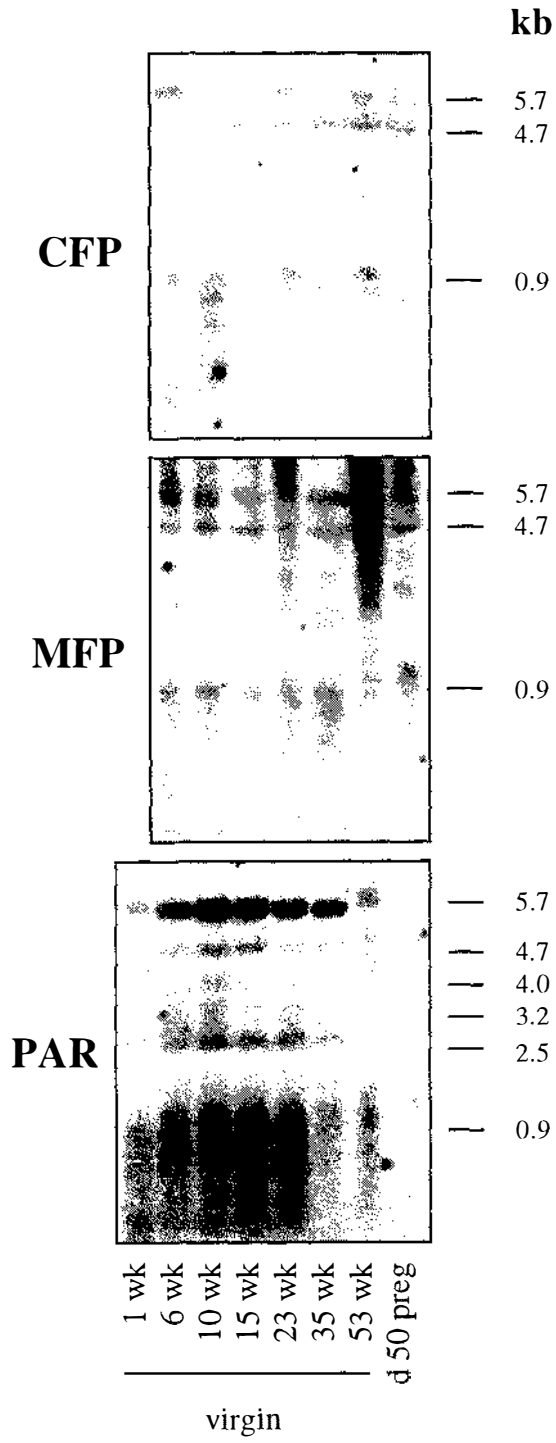


Figure 9.2 Northern analysis of IGF-II mRNA expression in ovine mammary tissues during postnatal development. (A) Membranes similar to that in Figure 9.1a were stripped and reprobed for ovine IGF-II (9 day exposure).

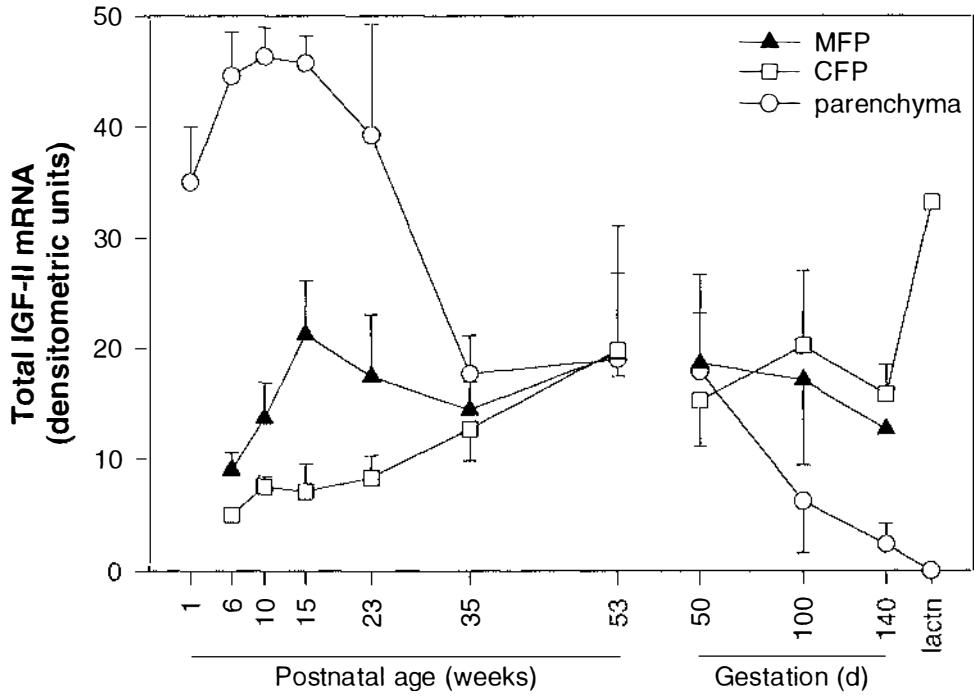


Figure 9.2 Northern analysis of IGF-II mRNA expression in ovine mammary tissues during postnatal development. (B) Densitometric quantification of IGF-II mRNA in MFP (▲), CFP (□) and parenchyma (○). Data points (means ± SEM) are the sum expression of IGF-II mRNA as multiple transcripts which have been normalised for loading against 28S rRNA levels. Number of replicates are n=4 for virgins and 50 days gestation, n=2 for 100 and 140 days gestation, and n=1 in lactation.

IGF-I mRNA expression in the parenchyma and extra-parenchymal MFP of prepubertal ewe lambs was unaltered by ovariectomy (Table 9.1). Exogenous oestrogen increased IGF-I mRNA levels in the parenchyma of intact ewes and in the extra-parenchymal MFP of ovariectomised ewes. Ovariectomy did not alter IGF-II mRNA levels in the extra-parenchymal MFP ($P>0.6$), but significantly increased levels in parenchyma ($P<0.002$). Administration of oestrogen suppressed IGF-II expression in all mammary tissues; the overall main effect of this treatment was highly significant ($P<0.001$).

Table 9.1 Effects of ovariectomy (ovex) and oestrogen on IGF-I and -II mRNA levels[a] in prepubertal ovine mammary tissues.

			excipient	oestrogen	P[b]
IGF-I	fat pad	intact	11.3 ± 3.1	8.4 ± 2.5	n.s.
		ovex	7.3 ± 0.2	12.3 ± 0.6	0.002
	parenchyma	intact	2.5 ± 0.5	4.1 ± 0.7	0.13
		ovex	5.1 ± 2.9	4.5 ± 2.1	n.s.
IGF-II	fat pad	intact	28.7 ± 6.0	12.4 ± 2.7	0.07
		ovex	25.9 ± 9.3	10.7 ± 4.1	n.s.
	parenchyma	intact	36.4 ± 4.8	26.3 ± 3.5	n.s.
		ovex	63.4 ± 5.1*	39.8 ± 4.9*	0.03

[a]Data are mean densitometric units ± SEM, normalised to 28S rRNA levels (n=3).
[b]Comparison of two means by Student's t-test.
*P<0.1 vs respective intact value.

9.5 DISCUSSION

IGF-I and -II are potent mitogens for mammary epithelial cells *in vitro* although their precise role during mammary gland development has not been defined. Our finding that mRNA for both IGF-I and -II mRNA is expressed by the mammary fat pads of ewes upholds previous suggestions that IGFs produced within the mammary gland function as paracrine mitogens for mammary epithelium (Hauser *et al.*, 1990; Manni *et al.*, 1994; Ellis *et al.*, 1994; Glimm *et al.*, 1988).

The interaction of epithelial and stromal constituents within the mammary gland has marked effects on cell growth and morphogenesis (Cunha and Hom, 1996). In this study, expression of mRNA for both IGF-I and -II prior to 35 weeks of age was higher in the MFP adjacent to rapidly growing parenchyma than in the contralateral CFP devoid of endogenous epithelium. It is also likely that the increased expression of specific IGF-II mRNAs within mammary parenchyma during this period was in the interspersed stroma, given that three of these transcripts were detected in the mammary fat pads, and that IGF-II mRNA localises to the stroma of mammary parenchyma in 4-

week old ewe lambs (Morgan *et al.*, 1996). Other recent reports have also demonstrated epithelial induction of stromal IGF expression in normal and malignant mammary tissue (Singer *et al.*, 1995; Manni *et al.*, 1994; Ellis *et al.*, 1994). Taken together, these findings strongly suggest that proliferating epithelium exerts a positive feedback on the surrounding stroma, possibly via the local release of a diffusible factor (Singer *et al.*, 1995), to upregulate the stromal expression of IGF-I and -II.

The developmental changes in IGF-I and -II mRNA levels within the ovine mammary gland support growth stimulatory roles for locally expressed IGFs during postnatal mammogenesis. Detection of IGF-I mRNA as a single 7.5 kb transcript may be particularly relevant to this proposal, as this unstable message undergoes post-translational modification (Lund, 1994), and may encode IGF-I with a paracrine function (LeRoith and Roberts, 1991). Similarly, the multiple ovine IGF-II mRNAs detected in mammary tissue are transcribed from different promoters (Ohlsen *et al.*, 1994) and may be differentially translated.

Elevation of IGF-I mRNA within the mammary fat pads at 6 and 10 weeks corresponds to the onset of prepubertal allometric mammary growth in these (Chapter 8) and other lambs (Johnsson and Hart, 1985). Parenchymal expression of IGF-II mRNA was also markedly increased at these ages. Whereas IGF-I mRNA levels in mammary fat pads declined by 15 weeks, parenchymal expression of IGF-II mRNA remained elevated until 35 weeks, during which time mammary epithelium continues to proliferate (Johnsson and Hart, 1985; Chapter 8). Furthermore, IGF-I mRNA levels in virgin mammary fat pads declined with age while IGF-II mRNA levels concurrently increased. These results indicate that the expression of IGF-I and -II at the local level of the mammary gland is differentially regulated. Given these findings, it is tempting to speculate that paracrine IGF-I stimulates the onset of rapid epithelial proliferation, and that this proliferation subsequently induces paracrine IGF-II expression to maintain ongoing parenchymal growth. As in prepuberty, IGF-I mRNA levels in the mammary fat pads were upregulated in late pregnancy when parenchyma undergoes substantial growth (Smith *et al.*, 1989a; Chapter 8). The fact that parenchymal IGF-II expression was not subsequently increased may reflect differences in the morphology of epithelial growth between the two states.

Previous investigations into prepubertal ruminant mammogenesis have shown a positive correlation between mammary growth and systemic GH levels (Johnsson *et al.*, 1985; Sejrsen *et al.*, 1983; Capuco *et al.*, 1995). Ruminant mammary epithelium, however, does not bind GH (McFadden *et al.*, 1990b), and suggestions of an indirect action of GH via systemic IGF-I (Purup *et al.*, 1995) are countered by the fact that GH levels and mammary development do not always correlate with serum IGF-I levels (Capuco *et al.*, 1995). The present results support the suggestion that GH acts via the mammary fat pad and upregulates the paracrine expression of IGF-I (Hauser *et al.*, 1990). Adipocytes and fibroblasts, both of which exist within the mammary fat pad, have been shown to bind GH (Kelly *et al.*, 1991), and GH is known to upregulate IGF-I expression in various peripheral tissues (Jones and Clemmons, 1995). The pattern of IGF-I mRNA expression in the mammary fat pads during prepuberty was similar to that for serum GH levels recorded in prepubertal lambs (Johnsson *et al.*, 1985). In particular, the significant decline in IGF-I mRNA levels between 10 and 15 weeks coincides with a 55% reduction in serum GH levels between 10 and 14 weeks as recorded by Johnsson *et al.* (1985). Furthermore, increased IGF-I expression in the mammary fat pad during late pregnancy corresponds to a gradual increase in serum GH levels of ewes during this time (Smith *et al.*, 1989a). This increased expression might also be in response to a more substantial rise in placental lactogen (PL) levels (Handwerger *et al.*, 1977). This possibility is supported by the likelihood that PL stimulates mammary development via an indirect mechanism (Collier *et al.*, 1995), and the knowledge that PL displays a high affinity for somatogenic sites (N'Guema Emane *et al.*, 1986) and mainly targets adipose tissues (Delouis *et al.*, 1980).

Consistent with findings from rodent studies (Ruan *et al.*, 1995), exogenous oestrogen could upregulate IGF-I expression in mammary tissues. In contrast, IGF-II mRNA expression was significantly down-regulated by oestrogen and was increased in parenchyma by ovariectomy, even though ovariectomy did not effect mammary gland development (Ellis *et al.*, 1996a). A negative effect of oestrogen on IGF-II expression has also been reported in the mammary gland of pseudopregnant pigs (Lee *et al.*, 1993) whereas IGF-II expression in rat mammary tumours is reduced by ovariectomy (Manni *et al.*, 1994). These results demonstrate ovarian regulation of IGF-II expression within

the mammary gland, although the physiological significance of this regulation remains unknown.

In conclusion, these findings suggest that IGF-I and -II expressed by the mammary gland stroma may fulfil an important role in the stimulation of postnatal ovine mammogenesis. Factors which influence mammary development such as epithelial-stromal association, stage of ontogeny and ovarian hormones also influence expression of IGFs within the mammary gland. Several lines of evidence are consistent with suggestions that the mammogenic action of GH is mediated via paracrine IGF-I.

CHAPTER 10

MULTIPLE FACTORS REGULATE THE PARACRINE EXPRESSION OF KERATINOCYTE GROWTH FACTOR (KGF) IN THE DEVELOPING RUMINANT MAMMARY GLAND

10.1 ABSTRACT

Keratinocyte growth factor (KGF) is a stroma-derived mitogen implicated in the mediation of epithelial-stromal interactions. These experiments have investigated the role and regulation of KGF in the ruminant mammary gland in which epithelium develops within an extensive stromal matrix. The target-cell specificity of KGF was demonstrated by the finding that KGF stimulated the *in vitro* proliferation of mammary epithelial cells, but not mammary fibroblasts. Consistent with a paracrine action, cultured mammary fibroblasts, but not epithelial cells, expressed KGF mRNA as transcripts of approximately 4.6, 2.4, 1.5 and 0.9 kb. This expression was reduced by 80% in the presence of dexamethasone. KGF mRNA was present in the ovine mammary fat pad as two transcripts of 2.4 and 1.5 kb while only the 2.4 kb mRNA was detected in mammary parenchyma. Levels of KGF mRNA in the extra-parenchymal mammary fat pad were higher than in the contralateral fat pad cleared of endogenous epithelium prior to puberty; levels were lowest in mammary parenchyma throughout development. Ovariectomy of prepubertal ewe lambs tended to increase the abundance of KGF mRNA within the mammary gland whereas its expression was significantly reduced by exogenous oestrogen. Of several ovine tissues examined, a 2.4 kb mRNA transcript was only detected in mammary parenchyma while adipose tissues expressed a single 1.5 kb KGF mRNA. This suggests differential transcription of KGF by adipocytes and fibroblasts within the mammary fat pad. These findings support a role for paracrine KGF in development of the ruminant mammary gland and indicate that local and systemic factors may regulate its tissue-specific expression.

10.2 INTRODUCTION

Investigations into the embryonic and postnatal development of numerous tissues, including the mammary gland, have demonstrated a vital role for the stromal environment in regulating the proliferation and morphogenesis of both normal and neoplastic epithelium (Sakakura, 1991; Birchmeier *et al.*, 1995; Cunha and Hom, 1996). This epithelial-stromal association may be of particular importance during development of the ruminant mammary gland where, in contrast to the rodent mammary gland, there

is a marked abundance of connective tissue in which the glandular epithelium proliferates (Sheffield, 1988b; Chapter 8).

A recently identified mitogen implicated as a mediator of epithelial-stromal associations is KGF (Rubin *et al.*, 1989), a member of the fibroblast growth factor family (Finch *et al.*, 1989). KGF likely functions as a paracrine mitogen for, whereas stromal cells synthesise and secrete KGF, only epithelial cells are responsive to its mitogenic effect (Rubin *et al.*, 1989). Results from other studies suggest that stroma-derived KGF stimulates epithelial development in a wide range of foetal (Finch *et al.*, 1995) and postnatal (Guo *et al.*, 1993; Alarid *et al.*, 1994; Siddiqi *et al.*, 1995) tissues. Several lines of evidence also indicate that KGF may act locally to effect the mitogenic and morphogenic actions of specific steroid hormones (Yan *et al.*, 1992; Koji *et al.*, 1994; Fasciana *et al.*, 1996).

A role for KGF during mammogenesis is emerging from the results of recent rodent and human studies. As in other tissues, it is the stromal constituents of the mammary gland which express KGF mRNA (Wilson *et al.*, 1994; Finch *et al.*, 1995). KGF directly stimulates the growth of mouse mammary epithelial cells and ductal end buds *in vitro* (Imagawa *et al.*, 1994b). This proliferative effect is consistent with the finding that systemically administered KGF induces extensive epithelial hyperplasia within the mammary glands of virgin and pregnant mice (Ulich *et al.*, 1994; Yi *et al.*, 1994).

Although these results indicate a role for KGF within the mammary gland, little is known of its regulation and physiological function. Information is limited to observations showing that mRNA for KGF is expressed and differentially transcribed in the mouse mammary gland during postnatal development (Coleman-Krnacik and Rosen, 1994), and that KGF mRNA is present in a high proportion of breast tumour samples (Koos *et al.*, 1993).

Given the candidate role for KGF in the developing rodent and human mammary gland, and the extensive epithelial-stromal association that occurs within the ruminant mammary gland, it was hypothesised that KGF would be mitogenic for ruminant mammary epithelium, and that it would be expressed by the stroma of the mammary fat pad. The regulation of local KGF gene expression by factors which influence mammary gland development has also been investigated. The findings reported herein demonstrate that KGF is a paracrine mitogen for ruminant mammary epithelial cells,

and that constituents of the mammary fat pad differentially transcribe KGF mRNA. Furthermore, KGF mRNA expression is regulated by several mechanisms that are of physiological relevance to normal mammogenesis.

10.3 MATERIALS AND METHODS

10.3.1 Cell culture

MAC-T bovine mammary epithelial cells (Huynh *et al.*, 1991) were maintained in DMEM (Gibco) plus 10% FCS (Gibco), 5 µg/ml insulin (Sigma), 1 µg/ml hydrocortisone (Sigma), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml; Sigma).

Bovine mammary epithelial and fibroblast cells were isolated from parenchymal tissue of a non-pregnant Friesian-cross heifer following a procedure essentially as described (McGrath, 1987). Tissue was digested in HBSS (Gibco) which contained collagenase (0.2% type II; Sigma), hyaluronidase (0.1%; Sigma) and DNAse (0.02%; Boehringer Mannheim) for 3 h at 37°C. The filtered (150 µm) cell suspension was then treated with trypsin (0.15%; Gibco) and type II collagenase in HBSS for 20 min. Epithelial and fibroblast cells were separated using a Percoll gradient (McGrath, 1987) and were cultured on plastic in DMEM plus 10% FCS. Epithelial and fibroblast cultures were further purified by selective trypsinisation of loosely adherent fibroblasts from the firmly attached epithelial cells (Danielson *et al.*, 1984). Bovine epithelial and fibroblast cells used in these experiments were passage 2.

Ovine mammary epithelial organoids were prepared from mammary parenchyma of a 12-week old ewe. Tissue was digested with type V collagenase (0.4%) in medium 199 plus 10% FCS for 2 h at 37°C. The resultant cell suspension was pelleted and washed in medium 199 plus 1% FCS and the epithelial organoids collected by differential centrifugation (45 g, 2 min). Ovine mammary fibroblasts were obtained by similarly digesting extra-parenchymal mammary fat pad (MFP) tissue from the same ewe and plating the pelleted cell fraction. All primary epithelial and fibroblast cultures were maintained in DMEM supplemented with 10% FCS.

10.3.2 Proliferation studies

MAC-T cells and ovine mammary fibroblasts were seeded into 24-well plates in DMEM plus 10% FCS at 2.5 x 10^4 and 5 x 10^4 cells/well, respectively. After 24 h, monolayers were rinsed with PBS and quiesced for 48 h in DMEM. Various growth factor treatments were then added in fresh DMEM (0.5 ml). Recombinant human KGF was from R and D Systems (Minneapolis, MN), recombinant human insulin-like growth factor-I (IGF-I) was from Genentech (San Francisco, CA) and recombinant human IGF-II was from Eli-Lilly (Indianapolis, IN). Acidic fibroblast growth factor (aFGF; from bovine brain) was from Sigma and was added with heparin (Leo Lab, 3.5 IU/ml).

After 24 h treatment, cultures were labelled for 2 h with [^{3}H]-methyl thymidine (1 μCi/ml, 85 Ci/mM, Amersham). Monolayers were then rinsed 3 times with 0.5 ml ice-cold PBS, twice with 0.5 ml ice-cold 10% TCA and fixed in 100% ethanol. Monolayers were solubilised with 3 M NaOH and then neutralised with 1 M HCl to a final volume of 1 ml. Samples were added to Ready-Safe scintillation cocktail (Beckman) and radioactivity measured in a scintillation counter (Wallac, Finland).

10.3.3 Animals and tissues

All animal experimentation was conducted with the approval of the Ruakura Animal Ethics Committee. Samples of mammary tissue from ewes at various stages of development were from a trial reported elsewhere (Chapter 8). Briefly, one mammary gland of neonatal ewe lambs was surgically prepared whereby the epithelial component was excised to leave a mammary fat pad devoid of endogenous epithelium (cleared fat pad, CFP; Chapter 8). The contralateral gland remained intact to allow epithelial growth into the surrounding extra-parenchymal mammary fat pad (MFP). Ewes were reared under standard grazing practices and groups were sacrificed at the following physiological states and ages: virgins at 6, 10, 15, 22, 35 and 53 weeks (n=4 per group); days 50 (n=4), 100 (n=2) and 140 (n=2) of pregnancy; and 1 day post-partum (n=1). Ewes were euthanased by captive bolt and exsanguination. The udder was removed, and tissue samples were collected from the parenchyma and extra-parenchymal MFP of the intact gland, and from the contralateral CFP. The CFP was always sampled at a site free from any scarring resultant from the surgical procedure. Tissue samples were snap frozen in liquid nitrogen and stored at -80°C.

The effect of ovariectomy and oestrogen on mammary KGF expression in prepubertal ewe lambs was determined in samples from a separate study (Ellis *et al.*, 1996a). Ovariectomy was performed at approximately 10 days of age. Prior to sacrifice at 12 weeks of age, groups of intact and ovariectomised lambs were treated for 7 days with daily s.c. injections of either oestrogen (17β-oestradiol; 0.1 mg/kg bodyweight) or vehicle (n=3/group). Samples of mammary parenchyma and extra-parenchymal MFP collected at slaughter were immediately frozen in liquid nitrogen and stored at -80°C.

10.3.4 Probes and labelling

A 622 bp cDNA spanning the ovine KGF coding sequence (GenBank accession number Z46236) was kindly provided by Dr Jane Mitchell, Moredun Research Institute. This cDNA was labelled with [^{32}P-α]dCTP (3000 Ci/mM, Amersham) by random priming (Rediprime, Amersham). RNA loading was standardised using a human 28S ribosomal RNA 26-mer oligonucleotide probe (Barbu and Dautry, 1989) labelled with [^{32}P-α]dCTP (3000 Ci/mM) using terminal transferase.

10.3.5 RNA isolation and Northern analysis

Frozen tissues were homogenised in 4 M guanidinium isothiocyanate; cell monolayers in 100 mm dishes were scraped in this solution. RNA was extracted with phenol/chloroform and precipitated with isopropanol (Chomczynski and Sacchi, 1987). Total RNA (20 μg) was electrophoresed through 1% agarose/formaldehyde gels. Integrity of RNA was confirmed by ethidium bromide staining and RNA was then transferred to Hybond N$^+$(Amersham). For analysis of KGF mRNA expression across ontogenic states, each of four replicate blots carried RNA from the three tissues of one ewe at each stage of development. Membranes were prehybridised for 2 h and then hybridised with the KGF cDNA probe for 16 h at 60°C in 0.5 M $NaHPO_4$, 1 mM EDTA, 7% SDS buffer (Church and Gilbert, 1984). Post hybridisation washes (to 0.5x SSC, all plus 0.5% SDS) were at 60°C. Membranes were exposed to x-ray film against intensifying screens and then stripped and reprobed for 28S rRNA. Autoradiograms were quantified by scanning densitometry using Image Quant software (Molecular Dynamics). RNA from cultures of bovine embryonic lung fibroblasts (BeLu) served as a positive control for KGF mRNA and gave a result similar to that for RNA from Wi-38

embryonic fibroblasts (Finch *et al.*, 1989; Appendix 12). No KGF mRNA was detected in several epithelial cell lines (C127 mammary carcinoma, NLFK feline kidney epithelial cells; MDCK canine kidney epithelial cells; COMMA-1D mouse mammary epithelial; Appendix 12).

10.3.6 Statistical analyses

All statistical analyses were conducted using SAS (SAS, 1994). Data were log transformed where appropriate. Cell proliferation results were analysed by one-way ANOVA. The ontogeny of KGF expression data was analysed using a mixed model within the REML procedure to test the effects of stage of development and tissue type, and their interaction. Ewe was included as a random term across stage of development and tissue type was analysed within ewe. The effects of ovariectomy, oestrogen treatment and tissue type were compared using the GLM procedure.

10.4 RESULTS

10.4.1 KGF is specifically mitogenic for mammary epithelium

When MAC-T bovine mammary epithelial cells were cultured in medium supplemented with human KGF, DNA synthesis was increased by 390% at a KGF concentration of 10 ng/ml; above this level their proliferative response was suppressed (Figure 10.1a). DNA synthesis by MAC-T cells was also stimulated by IGF-I and IGF-II. In contrast, DNA synthesis by ovine mammary fibroblasts was unaffected by a range of KGF concentrations ($P>0.05$; Figure 10.1b). Their proliferation was, however, stimulated by other fibroblast growth factors, aFGF and bFGF, as well as by IGF-I.

10.4.2 Mammary stroma expresses KGF mRNA *in vitro*

To determine the cellular origin of KGF within the mammary gland, epithelial and fibroblast cells isolated from bovine mammary tissue were cultured as a source of RNA for Northern analysis. Mammary fibroblasts, but not epithelial cells, expressed abundant KGF mRNA as transcripts of approximately 4.6, 2.4, 1.5, and 0.9 kb (Figure 10.2a). No expression of KGF mRNA was detected in virgin mouse mammary tissue after this exposure. It was also tested as to whether various treatments known to influence mammary growth could regulate KGF gene expression by bovine mammary cells *in vitro*. When bovine mammary fibroblasts were cultured in the presence of dexamethasone or dexamethasone plus insulin, total KGF mRNA was reduced by 80% compared to that in DMEM alone (Figure 10.2b). A smaller reduction of approximately 30% was also apparent in cultures supplemented with progesterone or progesterone plus oestrogen.

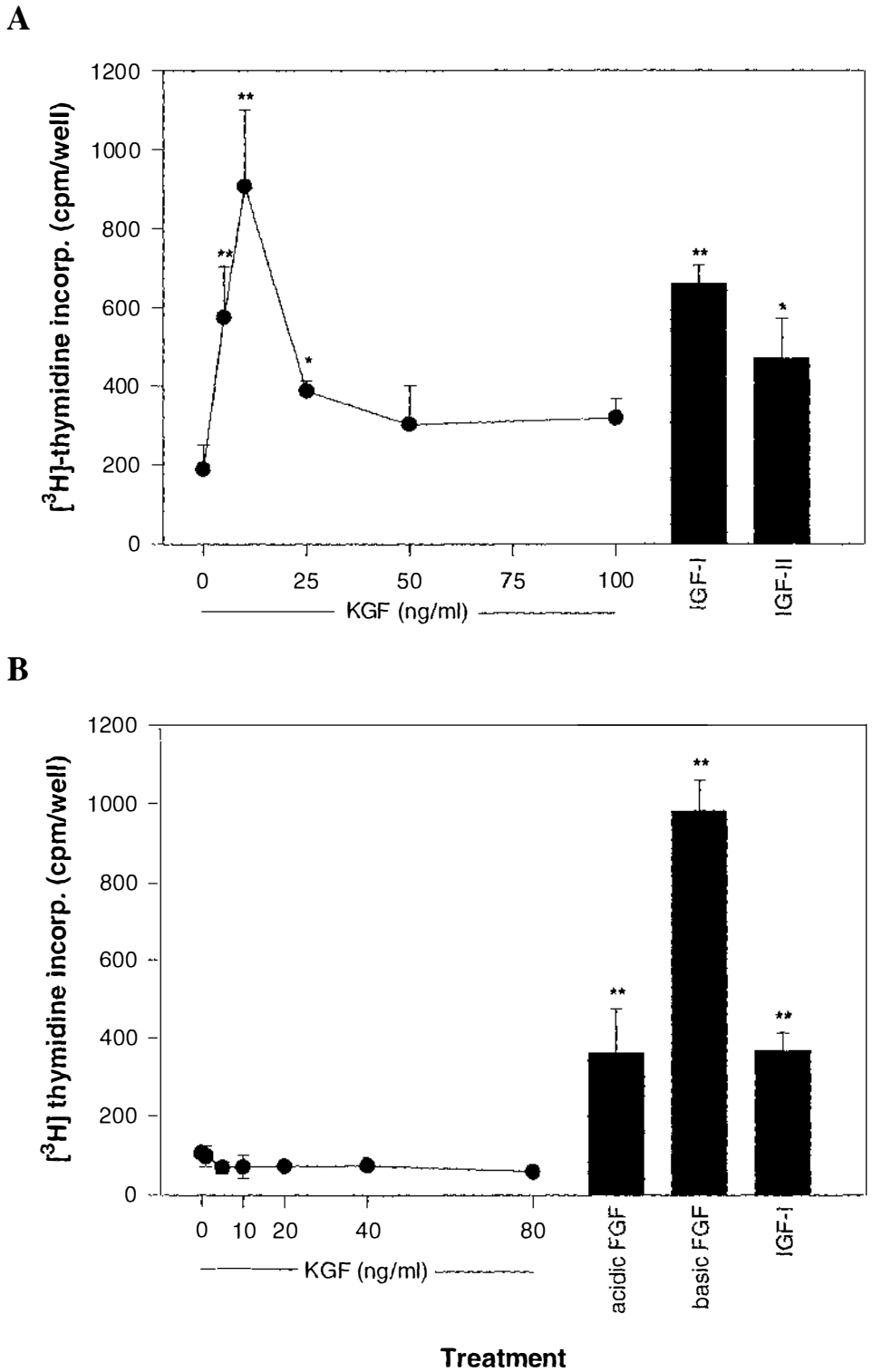


Figure 10.1 Effect of various concentrations of recombinant human KGF on the proliferation of (A) MAC-T bovine mammary epithelial cells, and (B) ovine mammary fibroblasts. Cells were cultured in DMEM supplemented with various treatments for 24 h, after which DNA synthesis was measured as [^{3}H]-thymidine incorporation. Acidic FGF was added at 5 ng/ml in the presence of heparin (3.5 IU/ml); IGF-I and -II were added at 100 ng/ml. Data are means ± SEM (n=3). *P<0.02, **P<0.001 vs 0 ng/ml KGF.

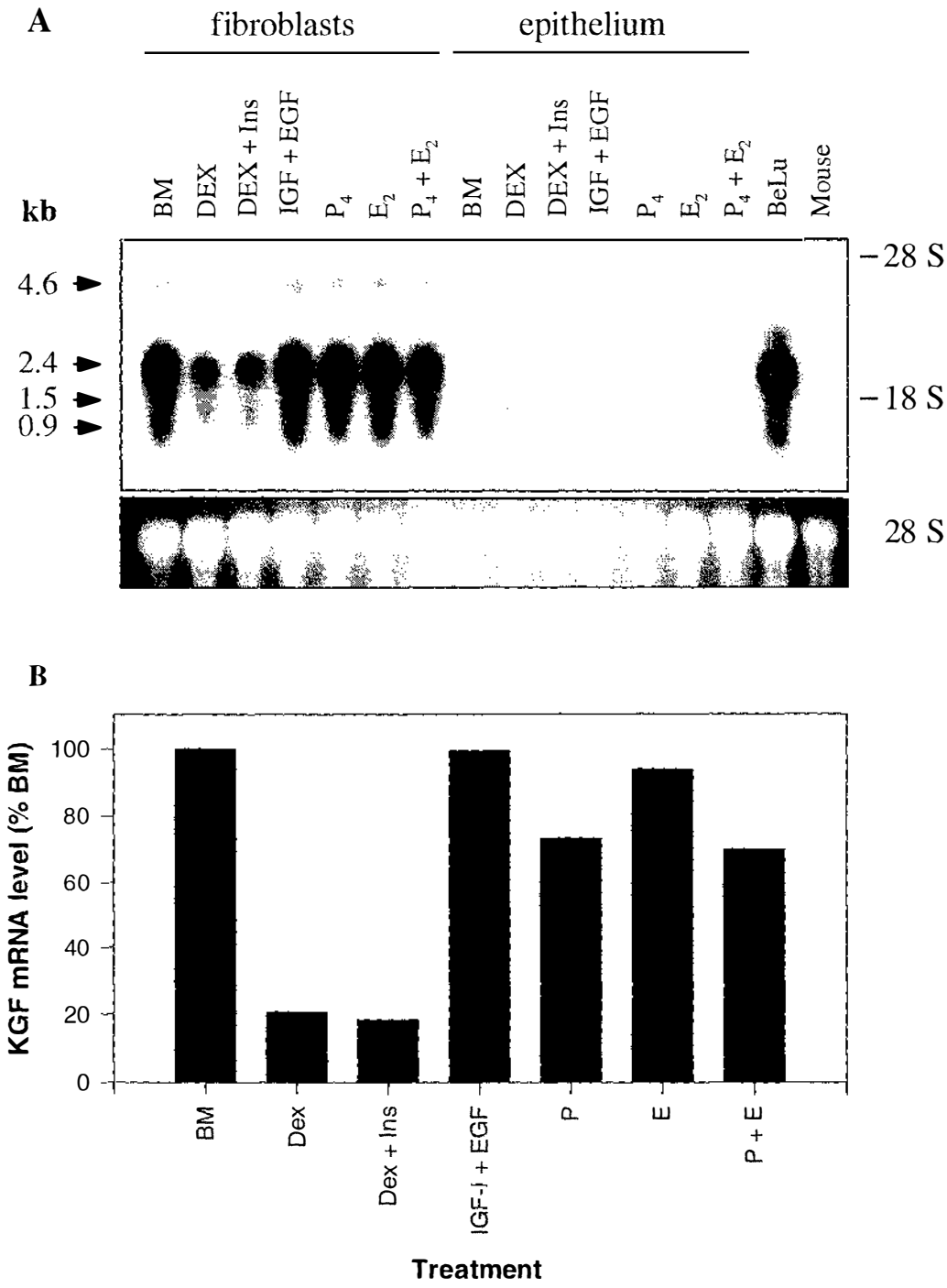


Figure 10.2 (A) Northern analysis of KGF gene expression in primary cultures of bovine mammary epithelial and fibroblast cells (20 h exposure). Confluent monolayers were cultured for 24 h in DMEM supplemented with various treatments prior to RNA extraction. Treatments were dexamethasone (Dex; 250 nM), insulin (Ins; 10 μg/ml), IGF-I (100 ng/ml), EGF (25 ng/ml), progesterone (P_4; 1 μg/ml), and 17β-oestradiol (E_2; 1 ng/ml). Other RNA was from BeLu cells (positive control) and mature virgin mouse mammary tissue. The position of the 28S and 18S rRNA is shown. (B) Densitometric quantification of total KGF mRNA expressed by mammary fibroblasts. Levels are normalised to 28S rRNA levels and are a percentage of abundance in DMEM only.

10.4.3 The KGF gene is differentially transcribed in the ovine mammary gland

Northern analysis revealed abundant expression of KGF mRNA in the mammary fat pads and parenchyma of the ovine mammary gland during ontogenesis (Figure 10.3). While two transcripts of 2.4 and 1.5 kb were detected in the extra-parenchymal MFP and CFP at all stages, only the 2.4 kb mRNA was consistently detected in parenchyma. Abundance of these transcripts was unaltered by high stringency washing to 0.1x SSC, 0.1% SDS.

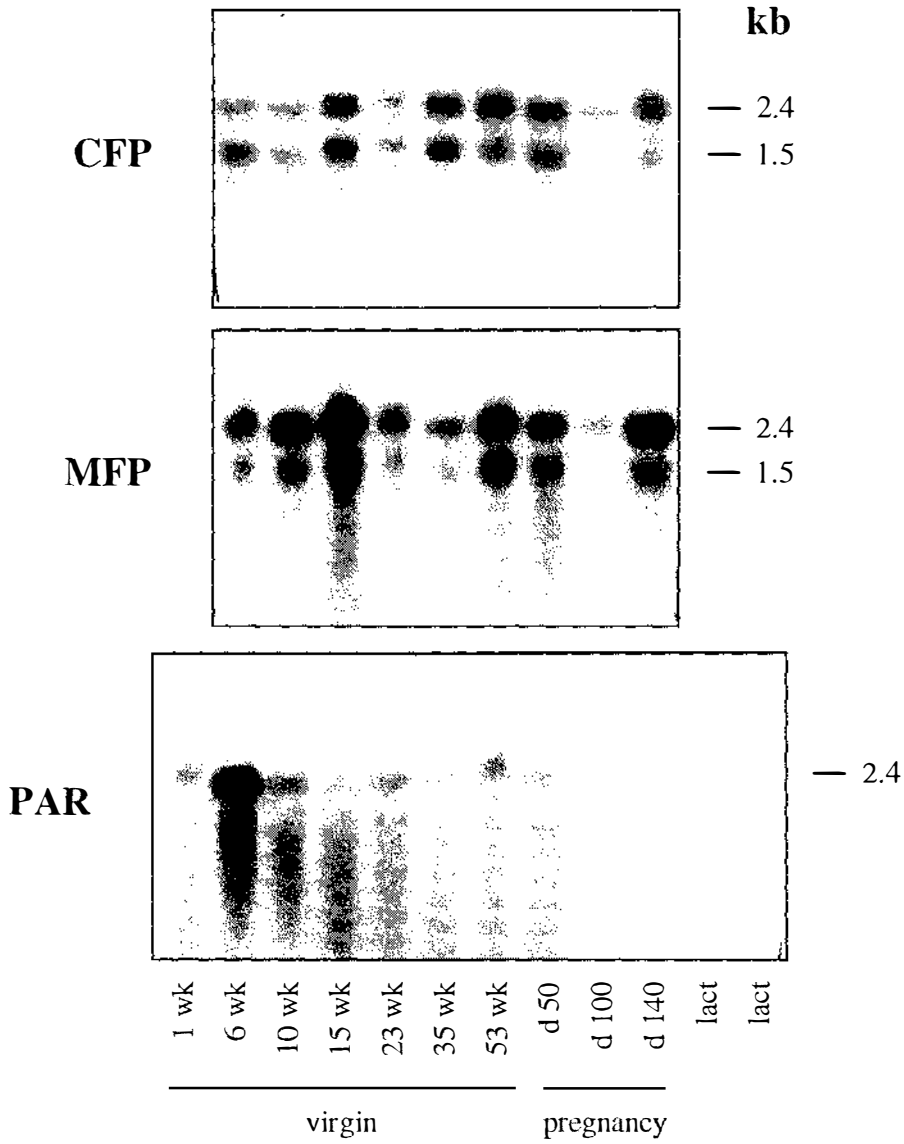


Figure 10.3 Northern analysis of KGF mRNA in ovine mammary tissues throughout development. Total RNA (20 μg) from the extra-parenchymal MFP, CFP, and mammary parenchyma (PAR) at the indicated stages of development was hybridised with an ovine KGF cDNA (20 h exposure).

To determine whether absence of the 1.5 kb mRNA in mammary parenchyma reflected epithelial regulation of KGF gene expression by the interspersed stroma, ovine mammary fibroblasts were cultured in medium conditioned by ovine mammary epithelial cells, or were directly co-cultured with epithelial cells. Neither of these treatments had any effect on the KGF mRNA transcripts expressed by ovine mammary fibroblasts (Figure 10.4). The reduced abundance of KGF mRNA in co-cultures was attributed to dilution of fibroblast RNA by that from epithelial cells.

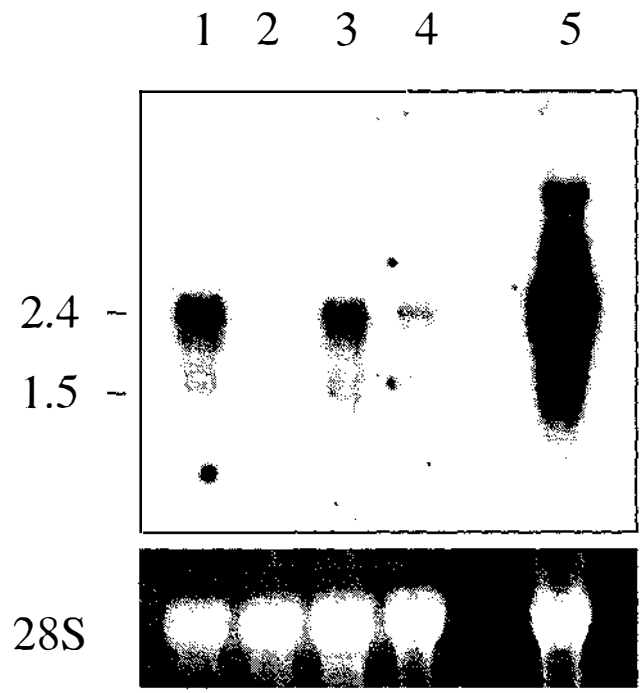


Figure 10.4 KGF gene expression by ovine mammary cells *in vitro*. RNA for Northern analysis was from cultures of ovine mammary fibroblasts (lane 1), ovine mammary epithelial cells (lane 2), ovine mammary fibroblasts cultured for 24 h in ovine epithelial cell conditioned medium (lane 3), and co-cultures of mammary fibroblast and epithelial cells (lane 4; plated at 1:1). Lane 5 is BeLu positive control (3 day exposure). Ethidium bromide-stained 28S rRNA is displayed in the lower panel to demonstrate similar loading.

Further experiments examined whether differential transcription of the KGF gene occurred in other ovine tissues. Of those tested, the 2.4 kb transcript was detected only in mammary parenchyma from virgin ewes, presumably due to expression by the interspersed stromal cells (Figure 10.5). No KGF mRNA was detected in mammary parenchyma in late pregnancy, likely due to mass dilution by epithelial and milk protein

RNA. Interestingly, adipose tissue from two separate depots only expressed the smaller 1.5 kb KGF mRNA transcript. No KGF mRNA was detected in RNA samples from the other tissues after this exposure.

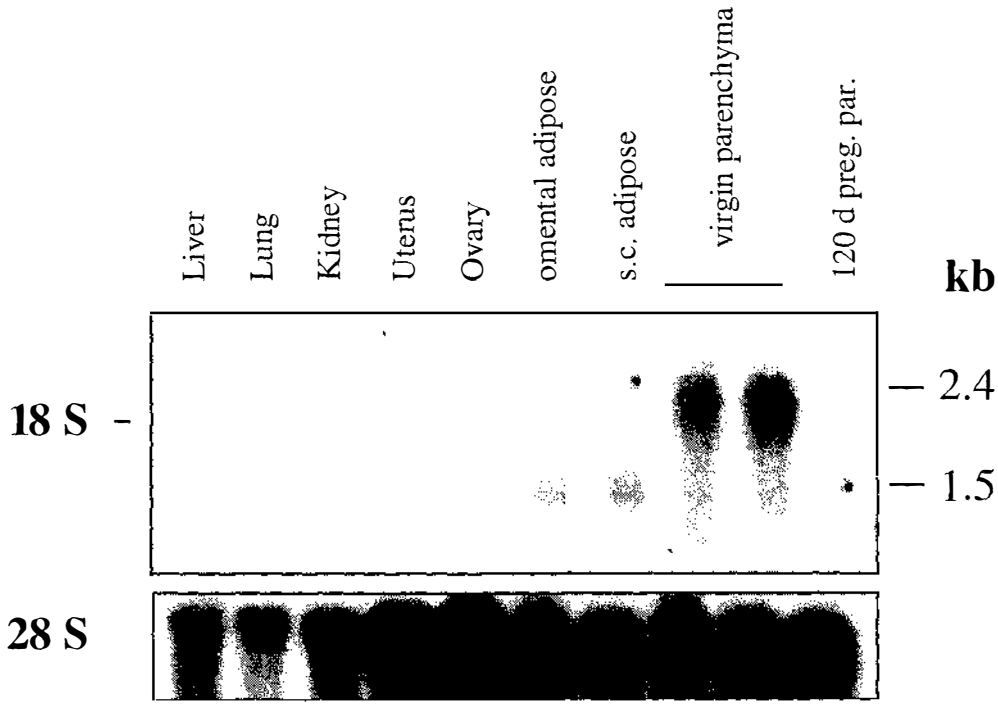


Figure 10.5 Expression of KGF mRNA in various ovine tissues. Samples were from the indicated tissues of a 35-week old ewe (mammary parenchyma was from 2 different ewes). Mammary parenchyma was also obtained from a ewe sacrificed at day 120 of gestation. Total RNA (20 μg) was analysed for KGF mRNA by Northern analysis (16 h exposure). The lower panel displays corresponding 28S rRNA levels.

10.4.4 Mammary KGF expression is physiologically regulated

Quantification of KGF mRNA expression as detected by Northern analysis revealed developmental changes in KGF gene expression within the ovine mammary gland (Figure 10.6). Parenchymal expression remained relatively low and constant throughout development and declined in late pregnancy. KGF mRNA levels in the extra-parenchymal MFP of prepubertal ewes at 6, 10 and 15 weeks of age were significantly ($P<0.06$) higher than in the contralateral CFP. The level of KGF mRNA in the CFP also progressively increased up until 35 weeks of age; thereafter it remained similar to that in the extra-parenchymal MFP.

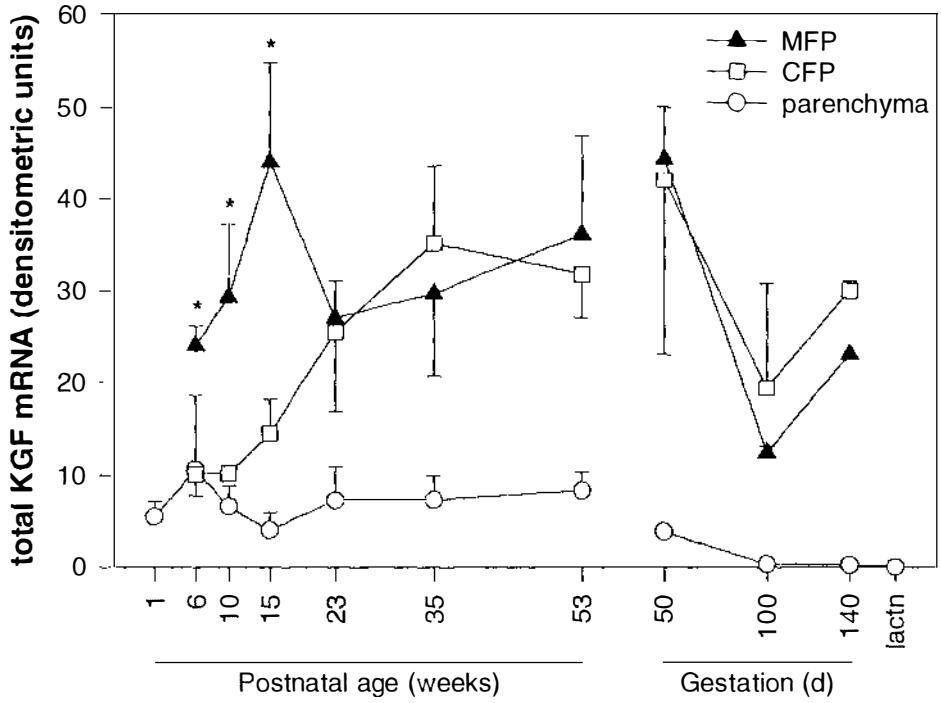


Figure 10.6 Quantification of KGF mRNA in ovine mammary tissues throughout postnatal development. Total mRNA expression in parenchymal, extra-parenchymal MFP and CFP tissues, detected by Northern analysis, was quantified by scanning densitometry and normalised to respective 28S rRNA values. Data are means ± SEM. Number of replicates at each stage are n=4 for virgins and day 50 gestation, n=2 for 100 and 140 days gestation, and n=1 for lactation. *P<0.06 vs respective CFP value.

The effect of ovariectomy and exogenous oestrogen on mammary KGF expression was examined in prepubertal ewe lambs. Ovariectomy increased the level of KGF mRNA in parenchymal tissue (P<0.05), but not in the extra-parenchymal MFP (Figure 10.7). The overall effect of ovariectomy tended to increase mammary KGF expression (P=0.1). Exogenous oestrogen suppressed KGF mRNA levels in both the parenchyma and extra-parenchymal MFP; the effect of oestrogen was highly significant (P<0.001). The increased relative expression of KGF mRNA in parenchyma was due, at least in part, to the detection of the 1.5 kb KGF mRNA in association with a high proportion of adipose tissue in these samples.

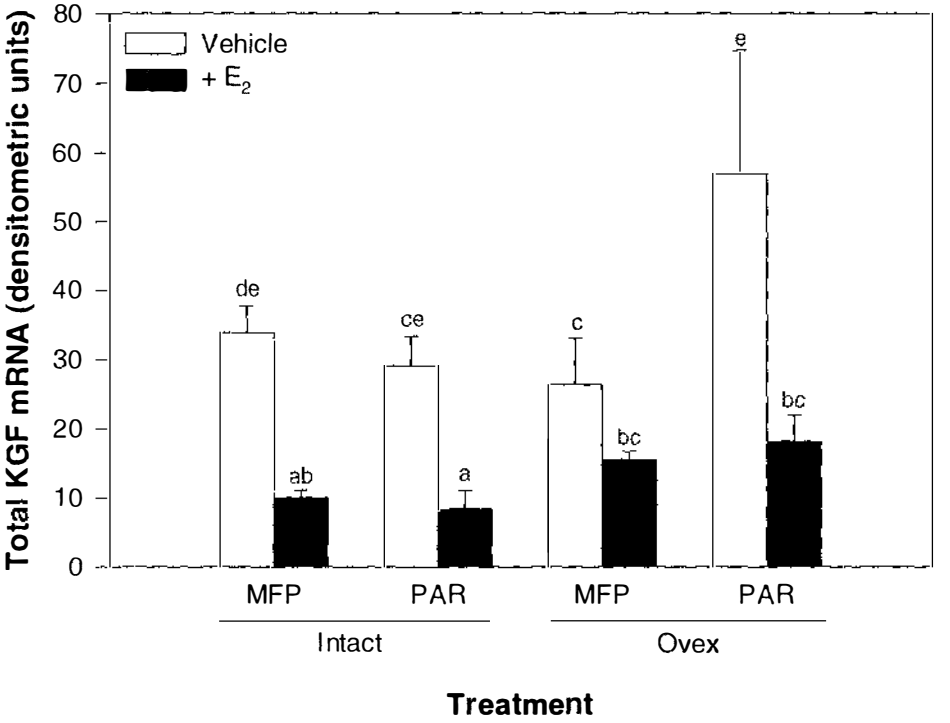


Figure 10.7 Effect of ovariectomy (ovex) and oestrogen (E_2) on KGF expression in mammary parenchyma (PAR) and extra-parenchymal MFP of prepubertal ewe lambs. Total KGF mRNA, detected by Northern analysis, was quantified and normalised to respective 28S rRNA levels. Data are means ± SEM (n=3). ^{a,b,c} Means with different superscripts are significantly different ($P<0.05$). Main effect of ovariectomy, $P=0.1$; main effect of oestrogen, $P<0.001$.

10.5 DISCUSSION

The developing ruminant mammary gland, similar to the human breast and somewhat distinct from the rodent mammary gland, is characterised by the proliferation and morphogenesis of epithelium within a matrix of connective tissue interspersed throughout the mammary fat pad (Sheffield, 1988b). Given this extensive epithelial-stromal interaction, a role for the stroma-derived paracrine mitogen, KGF, in the local regulation of ruminant mammogenesis was investigated.

Increased DNA synthesis by MAC-T cells establishes that bovine mammary epithelial cells are responsive to the mitogenic effect of KGF. It was necessary to demonstrate this effect on ruminant mammary epithelium since another polypeptide growth factor, EGF, stimulates the proliferation of mammary cells from humans and rodents, but not ruminants (Woodward *et al.*, 1994; Moorby *et al.*, 1995). A previous report of KGF-

induced proliferation in PS BME-1 bovine mammary cells (Tesfayohannes *et al.*, 1992) must be disregarded as this cell line was subsequently shown to be of murine origin (Woodward *et al.*, 1994).

A paracrine action of KGF within the ruminant mammary gland is strongly suggested by several findings of this study. First, bovine and ovine mammary fibroblasts (Figures 10.2a and 10.4), but not the respective epithelium, express KGF mRNA *in vitro*. Second, stromal constituents of the ovine mammary fat pad express mRNA for KGF *in vivo*. Third, KGF stimulates the proliferation of ruminant mammary epithelial cells, but not mammary fibroblasts. The target-cell specificity for the mitogenic effect of KGF is emphasised by the fact that the proliferation of mammary fibroblasts is stimulated by other fibroblast growth factors such as aFGF and basic FGF (Chapter 11).

Both *in vivo* and *in vitro* studies have generally reported KGF mRNA as a predominant transcript of 2.4 kb (Finch *et al.*, 1989; Kelley *et al.*, 1992; Chedid *et al.*, 1994; Koji *et al.*, 1994; Chedid *et al.*, 1996). This transcript was present at relatively high abundance in both the parenchymal and fat pad tissues of the ovine mammary gland. *In vitro*, the KGF gene was transcribed in mammary fibroblasts as four mRNAs of approximately 0.9, 1.5, 2.4, and 4.6 kb. The absence of the 4.6 kb mRNA *in vivo* may reflect its correspondence to an unstable 5.0 kb transcript (Finch *et al.*, 1989). Of particular interest was a 1.5 kb KGF transcript that was expressed within the mammary fat pad at a level similar to the 2.4 kb mRNA, but that was generally not detected in mammary parenchyma. This transcript may correspond to a 2.0 kb splice variant of KGF mRNA present in certain cell lines (Kelley *et al.*, 1992). Differential transcription of KGF mRNA has also been reported in the mouse mammary gland where the expression of a 1.1 kb KGF mRNA is altered during development (Coleman-Krnacik and Rosen, 1994).

Given that epithelial cells can regulate the stromal expression of certain paracrine growth factors (Rosen et al., 1994b; Singer *et al.*, 1995), including KGF (Smola *et al.*, 1993), it was considered that differential transcription of KGF mRNA in mammary parenchyma may reflect a local influence from the epithelium. Subsequent analysis of other tissues revealed that the two KGF mRNAs within the mammary fat pad probably represent a 1.5 kb mRNA expressed by adipocytes and a 2.4 kb transcript expressed by the interspersed connective tissue fibroblasts. Therefore, transcription of the KGF gene within different tissues probably reflects their cellular composition; whereas the

mammary fat pad comprises adipocytes with a greater degree of connective tissue (Figure 10.8a) relative to other adipose depots (Figure 10.8b), mammary parenchyma is characterised by epithelium enveloped by a predominantly fibroblastic stroma (Figure 10.8c). It remains to be established whether the 1.5 kb mRNA expressed by adipose tissue bears any significance to the finding that KGF increases the level of circulating fatty acids in mice and rats (Nonogaki *et al.*, 1995).

These investigations also indicate that KGF expression is regulated by several factors which are physiologically relevant to the course of mammogenesis. To date, hormonal regulation of KGF expression within the mammary gland has not been investigated. In this study, the level of KGF mRNA in mammary tissues of prepubertal lambs was markedly suppressed by exogenous oestrogen. Interestingly, mRNA expression for another stroma-derived mitogen, IGF-II, is similarly affected (Chapter 9). This response for KGF mRNA contrasts to that for acidic FGF mRNA, the expression of which is increased by oestrogen within the mouse mammary gland (Chakravorti and Sheffield, 1996b). Conversely, studies in other tissues indicate that KGF expression is increased by another ovarian steroid, progesterone (Koji *et al.*, 1994; Taga *et al.*, 1996). Suggestion for opposing effects of oestrogen and progesterone on mammary KGF expression is consistent with their distinctive roles during mammogenesis in rodents; whereas oestrogen promotes ductal elongation, progesterone stimulates ductal alveolar branching (Imagawa *et al.*, 1994). KGF has also been implicated as a promoter of epithelial branching morphogenesis (Alarid *et al.*, 1994; Peters *et al.*, 1994; Post *et al.*, 1996). Hence, a specific function of KGF within the mammary gland may be to locally mediate progesterone-induced ductal branching, particularly during pregnancy. A morphogenic effect of KGF might also be an ongoing requirement for ruminant mammary epithelium which demonstrates a dichotomous, branched morphogenesis throughout development. This may account for the abundance of KGF mRNA that was detected in the ovine mammary fat pad relative to other ovine tissues, and to the mouse mammary gland in which there is minimal ductal branching prior to pregnancy (Imagawa *et al.*, 1994).

Dexamethasone acutely suppressed the expression of KGF mRNA by mammary fibroblasts *in vitro*. Glucocorticoids have recently been shown to suppress KGF expression by dermal fibroblasts *in vitro* and *in vivo* (Brauchle *et al.*, 1995; Chedid *et*

al., 1996), an effect which is associated with reductions in both transcription rate and mRNA stability (Chedid *et al*., 1996). The negative effect of oestrogen and glucocorticoids on KGF expression in the ruminant mammary gland may be manifest together immediately prior to parturition when both hormones display a preparturient surge (Cowie *et al*., 1980). Suppression of local KGF expression at this time may contribute to the fact that ruminant mammary epithelium undergoes extensive lobuloalveolar proliferation in late pregnancy, but not into lactation (Cowie *et al*., 1980). The possibility that KGF-induced mammary growth ceases at parturition is further supported by the finding that mammary epithelium of lactating rats is unresponsive to the proliferative effect of KGF (Ulich *et al*., 1994).

During a phase of rapid prepubertal mammary growth in these ewes (Chapter 8), the level of KGF mRNA in the extra-parenchymal MFP was significantly higher than in the contralateral CFP. This difference may represent the local induction of KGF expression in the MFP by the adjacent mammary epithelium, for others have shown that epithelial cells can upregulate stromal expression of KGF *in vitro* (Smola *et al*., 1993). Similarly, epithelial influence has been shown to increase the expression of other stroma-derived paracrine growth factors within the mammary gland (Singer *et al*., 1995; Chapters 9 and 11). Such findings emphasise the potentially important role of epithelial-stromal interactions in the local regulation of cell growth.

In conclusion, these findings support a role for KGF as a paracrine mitogen during the course of mammary gland development in ruminants. It is evident that a number of local and systemic factors regulate the tissue-specific gene expression of KGF. By determining the precise function of KGF and the mechanisms which underlie its regulation, it may be possible to unravel some of the complexities associated with epithelial-stromal interactions and their involvement in mammary gland development and tumorigenesis.

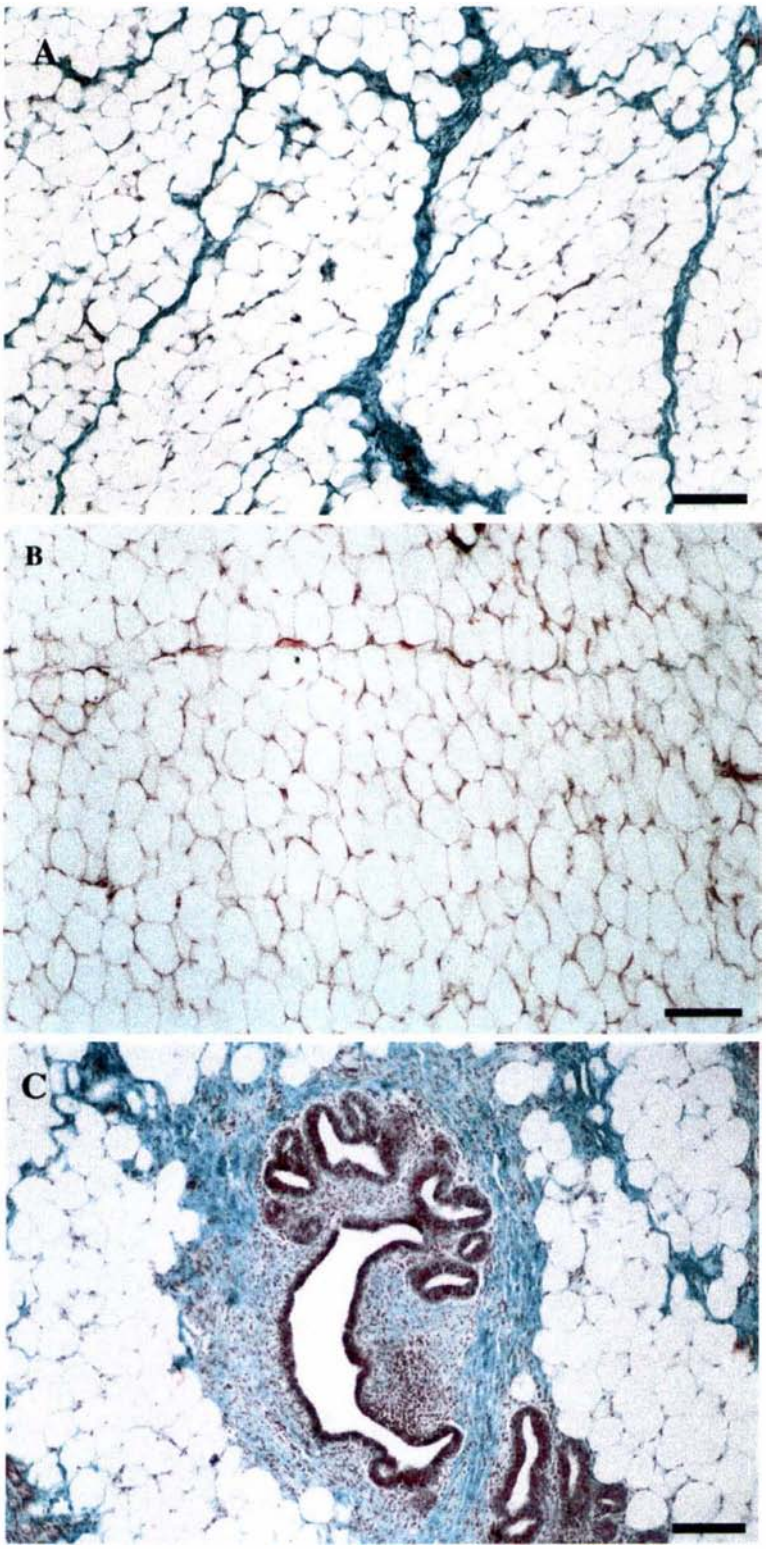


Figure 10.8 Histology of mammary and adipose tissues from a prepubertal ewe lamb. (A) mammary fat pad comprised of adipocytes and interspersed connective tissue, (B) subcutaneous adipose tissue primarily comprised of adipocytes, and (C) mammary parenchyma consisting of ductal epithelium growing within the connective and adipose tissues of the mammary fat pad. Gomori's trichrome. Scale bar = 100 μm.

CHAPTER 11

EXPRESSION AND ROLE OF PARACRINE GROWTH FACTORS DURING DEVELOPMENT OF THE RUMINANT MAMMARY GLAND

11.1 ABSTRACT

The objective of these experiments was to investigate the role of two heparin-binding paracrine mitogens, hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF), during postnatal development of the ruminant mammary gland. Northern analysis revealed that bFGF mRNA was expressed in ovine mammary tissues as two transcripts of approximately 6.9 and 4.5 kb. The level of bFGF mRNA within the extra-parenchymal mammary fat pad (MFP) of prepubertal ewe lambs was significantly higher than in the contralateral epithelium-free mammary fat pad (cleared fat pad, CFP), possibly due to a local influence of the mammary epithelium. Expression of bFGF mRNA in these mammary fat pads throughout development was greater than that in mammary parenchyma. In contrast, cultured bovine mammary epithelial cells expressed a greater abundance of bFGF mRNA than bovine mammary fibroblasts. The level of bFGF mRNA in mammary tissues of prepubertal ewe lambs was increased following ovariectomy whereas it was decreased within the mammary fat pad by exogenous oestrogen.

Low levels of HGF mRNA were detected in ovine mammary fat pads, but not parenchyma, as four transcripts of approximately 5.7, 3.5, 2.0, and 1.2 kb. The developmental pattern of HGF mRNA expression within mammary fat pads was similar to that for bFGF mRNA. Only bovine mammary fibroblasts expressed HGF mRNA *in vitro*; the level of this expression was markedly reduced in the presence of dexamethasone.

Medium conditioned by ovine mammary fibroblasts contained a mitogenic activity which also induced ovine mammary epithelial organoids to form branched cords within collagen gels, responses which may be due to the secretion of HGF by mammary fibroblasts. These results suggest that the local synthesis of bFGF and HGF within the ruminant mammary gland may have an important physiological role during postnatal mammogenesis.

11.2 INTRODUCTION

Interactions between the epithelial and stromal elements of the mammary gland likely serve a critical role in regulating the growth, morphogenesis and differentiation of both normal and tumorous epithelium (Sakakura, 1991; Cunha and Hom, 1996). Epithelial cells within the rodent mammary gland grow in close proximity to adipocytes of the mammary fat pad (Williams and Daniel, 1983; Rønnov-Jessen *et al.*, 1996). The importance of this association is demonstrated by the fact that mouse mammary epithelium can undergo normal development only when it is transplanted to a depot of adipose tissue (reviewed by Hoshino, 1978). In contrast, epithelium within the ruminant and human mammary gland is continually enveloped by a fibroblastic connective tissue (Sheffield, 1988b; Akers, 1990). Findings from numerous studies suggest that an important function of this type of stromal tissue is the synthesis of polypeptide growth factors which influence the adjacent epithelium by paracrine action (Birchmeier *et al.*, 1995).

Two heparin-binding growth factors that may fulfil such a role are hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF). Originally purified from platelets as a mitogen for hepatocytes (Nakamura *et al.*, 1987), HGF stimulates the proliferation of epithelial cells from several tissues (Rubin *et al.*, 1991). The synthesis of HGF by mouse mammary fibroblasts (Sasaki *et al.*, 1994) and its mitogenic effect on mammary epithelial cells (Niranjan *et al.*, 1995) strongly implicate HGF as a paracrine mitogen within the mammary gland. HGF may also fulfil an important morphogenic role as it induces mammary epithelial cells within collagen gels to form extensively branched cords with a central lumen (Brinkmann *et al.*, 1995; Niranjan *et al.*, 1995; Soriano *et al.*, 1995). These findings coincide with the presence of the HGF receptor, c-*met*, on luminal epithelial cells in human breast tissue (Tsarfaty *et al.*, 1992). In terms of its physiological role, the expression of mRNA for HGF and c-*met* in the mouse mammary gland is altered with stage of development (Niranjan *et al.*, 1995) while the level of immunoreactive HGF in breast tumour extracts is the single best predictor of relapse-free breast cancer and overall patient survival (Yamashita *et al.*, 1994). Stromal HGF expression is also influenced by the mammary epithelium which can exert both a

positive (Rosen *et al.*, 1994b) and negative influence (Johnston *et al.*, 1992; Seslar *et al.*, 1993).

Basic FGF belongs to the family of fibroblast growth factors, members of which have been widely implicated as mediators of epithelial-stromal interactions (Gospodarowicz, 1991). Basic FGF stimulates the proliferation of rodent (Levay-Young *et al.*, 1989) and ruminant (Sandowski *et al.*, 1993) mammary epithelial cells *in vitro*. Findings from several studies indicate that multiple cell types may express bFGF within the mammary gland. Two reports show that the majority of bFGF mRNA within the mammary gland is expressed by the extra-parenchymal stroma (Coleman-Krnacik and Rosen, 1994; Chakravorti and Sheffield, 1996a), while others report that bFGF is primarily expressed by the myoepithelium, and to a lesser extent by stromal fibroblasts (Barraclough *et al.*, 1990). Although bFGF immunolocalises to myoepithelial cells in the rat (Rudland *et al.*, 1993), human (Gomm *et al.*, 1991), and bovine (Schams *et al.*, 1995) mammary gland, the high affinity of bFGF for components of the extracellular matrix (Barraclough *et al.*, 1990) may confound the interpretation of such results. A role for locally-derived bFGF during postnatal mammogenesis is indicated by the fact that bFGF mRNA expression within the mammary gland is regulated by stage of development (Coleman-Krnacik and Rosen, 1994; Chakravorti and Sheffield, 1996a) and mammogenic hormones (Chakravorti and Sheffield, 1996b). Furthermore, stromal expression of bFGF may be influenced locally by the adjacent mammary epithelium (Coleman-Krnacik and Rosen, 1994).

The objective of these experiments was to evaluate a role for the local expression of these growth factors within the ruminant mammary gland. Results show that the HGF and bFGF genes are transcribed by tissues of the ruminant mammary gland *in vivo* and *in vitro*, and that their products may act via paracrine mechanisms to exert pronounced mitogenic and morphogenic effects during ruminant mammogenesis.

11.3 MATERIALS AND METHODS

11.3.1 Tissues

Mammary tissue samples were from surgically modified ewes sacrificed at various stages of development in a previous study (Chapter 8). Briefly, the epithelial

parenchyma was excised from one mammary gland of these ewes when they were neonates to leave a mammary fat pad that was devoid of endogenous epithelium (cleared fat pad, CFP). The contralateral gland remained intact to allow parenchymal growth into the surrounding extra-parenchymal mammary fat pad (MFP). Ewes were subsequently sacrificed in groups at several stages of prepuberty (6, 10, 15, 23 weeks), puberty (35 and 53 weeks) and gestation (days 50, 100 and 140); one ewe was also killed 1 day postpartum. The udder was removed and tissue was sampled from the mammary parenchyma, the extra-parenchymal MFP, and the contralateral CFP, and was snap frozen.

The effects of ovariectomy and exogenous oestrogen were investigated using mammary tissue samples from a trial reported elsewhere (Ellis *et al.*, 1996a). Ewe lambs that were either intact or had been neonatally ovariectomised were treated with daily s.c. injections of 17β-oestradiol (Sigma; 0.1 mg/kg liveweight) or excipient for 7 days prior to slaughter at 12 weeks of age. Samples of mammary parenchyma and extra-parenchymal MFP were collected at slaughter and snap frozen.

11.3.2 Cell cultures

Cultures of bovine mammary fibroblasts and epithelial cells were prepared by gradient separation of enzymatically-digested mammary parenchyma as described previously (Chapter 10). The procedure used to prepare epithelial organoids from mammary parenchyma obtained from prepubertal ewe lambs has been detailed elsewhere (Chapter 10). These organoids were cast into gels of rat tail collagen (McGrath, 1987) and were maintained in 10% FCS (Gibco) for 24 h prior to the application of treatments.

DNA synthesis by COMMA-1D mouse mammary epithelial cells was used as a bioassay in these experiments, as described earlier (Chapter 2) and by others (Riss and Sirbasku, 1987). Cells were seeded into 24- or 96-well plates at 1 x 10^5 and 1.5 x 10^4 cells/well, respectively. Monolayers were quiesced in DMEM (Gibco) basal medium (BM) for 48 h before treatments were applied for a further 24 h. DNA synthesis was measured as the incorporation of radioactivity into the TCA precipitable fraction after 2 h incubation with [^{3}H]-thymidine (1 μCi/ml; specific activity 85 Ci/mM; Amersham). Cultures of MAC-T bovine mammary epithelial cells (Huynh *et al.*, 1991) were maintained in BM supplemented with 10% FCS, 5 μg/ml insulin (Sigma), and 1 μg/ml

hydrocortisone (Sigma), and were plated at 5 x 10^4 cells/well for similar assay of DNA synthesis.

11.3.3 Probes and Northern analysis

RNA was extracted from tissues and cell monolayers using guanidinium isothiocyanate and phenol/chloroform (Chomczynski and Sacchi, 1987). Samples of total RNA (20 μg) were electrophoresed and transferred to Hybond N^+ (Amersham). Membranes were hybridised with [^{32}P]-labelled cDNA probes for 16 h at 60°C in 0.5 M $NaHPO_4$, 1 mM EDTA, 7% SDS buffer (Church and Gilbert, 1984). The bFGF probe was a 1.4 kb fragment of the bovine bFGF cDNA (Abraham *et al.*, 1986) kindly provided by Dr Judith Abraham. The bovine HGF probe was a 300 bp cDNA (Parrott *et al.*, 1994) kindly provided by Dr Jeff Parrott. Membranes were washed to 1x SSC at 60°C, exposed to x-ray film, and reprobed for 28S rRNA using a 26-mer oligonucleotide. Abundance of autoradiographed mRNA was quantified by scanning densitometry (Image Quant, Molecular Dynamics) where appropriate.

11.3.4 Conditioned medium

Ovine mammary fibroblasts were grown to confluence in 75 cm^2 flasks in DMEM supplemented with 10% FCS. Fibroblast-conditioned medium (CM) was prepared according to previously described procedures (Enami *et al.*, 1983; Rubin *et al.*, 1989). Fibroblast monolayers were rinsed twice with BM (5 mls) and incubated in 20 ml BM for 48 h. The resultant CM was collected, filtered (0.2 μm) and stored at -80°C. Control BM was similarly prepared in the absence of mammary fibroblasts.

11.3.5 Fractionation of CM

CM (450 ml) was concentrated 50-fold by ultrafiltration (10 kDa molecular weight cutoff, Amicon), and the retentate resuspended in PBS. Protein concentration was determined using the BCA reaction (Pierce, IL). Approximately 200 μl of concentrated CM was applied to a Superose 12 column for size-fractionation by FPLC (Pharmacia). The column had been equilibrated in 0.01 M NH_4HCO_3 (pH 7.3), and the sample was eluted in this buffer at a flow rate of 0.5 ml/min. Fractions were dessicated under vacuum and stored at -20°C prior to their use in culture.

11.3.6 Statistical analyses

All statistical analyses were performed using SAS. Means for the ontogeny of bFGF expression were compared using a mixed model within the REML procedure to test the effects of stage of development and tissue type, and their interaction. Ewe was included as a random term across stage of development, and tissue type was analysed within ewe. The significance of ovariectomy, oestrogen and mammary tissue effects and their interactions were analysed using the GLM procedure. The results of cell growth experiments were analysed by one way ANOVA.

11.4 RESULTS

11.4.1 Expression of growth factor mRNA in the ovine mammary gland

Northern analysis detected two bFGF mRNA transcripts of approximately 6.9 and 4.5 kb in postnatal ovine mammary tissues (Figure 11.1a). Densitometry indicated that the total level of this expression in the extra-parenchymal MFP and the CFP was typically greater ($P<0.05$) than in the parenchyma throughout development (Figure 11.1b). Prior to 35 weeks of age, abundance of bFGF mRNA was significantly ($P<0.08$) higher in the extra-parenchymal MFP than in the contralateral CFP. In addition, gene expression for bFGF declined significantly in both fat pads between 6 and 10 weeks of age ($P<0.05$), and then increased by 15 weeks. Thereafter bFGF mRNA levels in the CFP increased to a peak at 100 days of pregnancy while expression in the extra-parenchymal MFP was greatest at day 50 of pregnancy. Expression of bFGF mRNA in mammary parenchyma was maximal in 53-week old virgins and at day 50 of pregnancy.

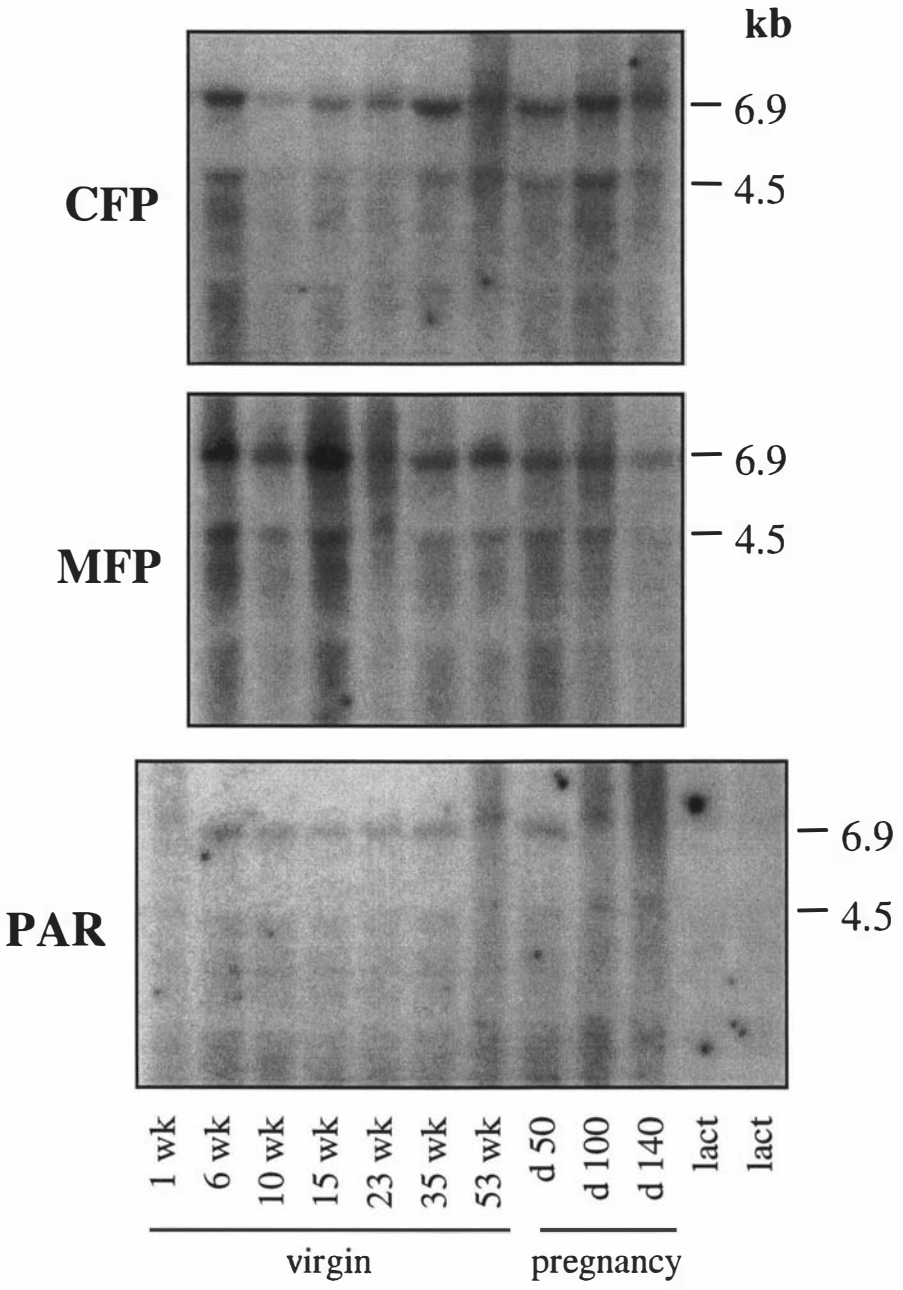


Figure 11.1 (A) Northern analysis of bFGF mRNA in postnatal ovine mammary tissues. Total RNA (20 μg) from the CFP, extra-parenchymal MFP and mammary parenchyma (PAR) at the indicated stages of development was hybridised with a bovine bFGF cDNA probe (18 day exposure).

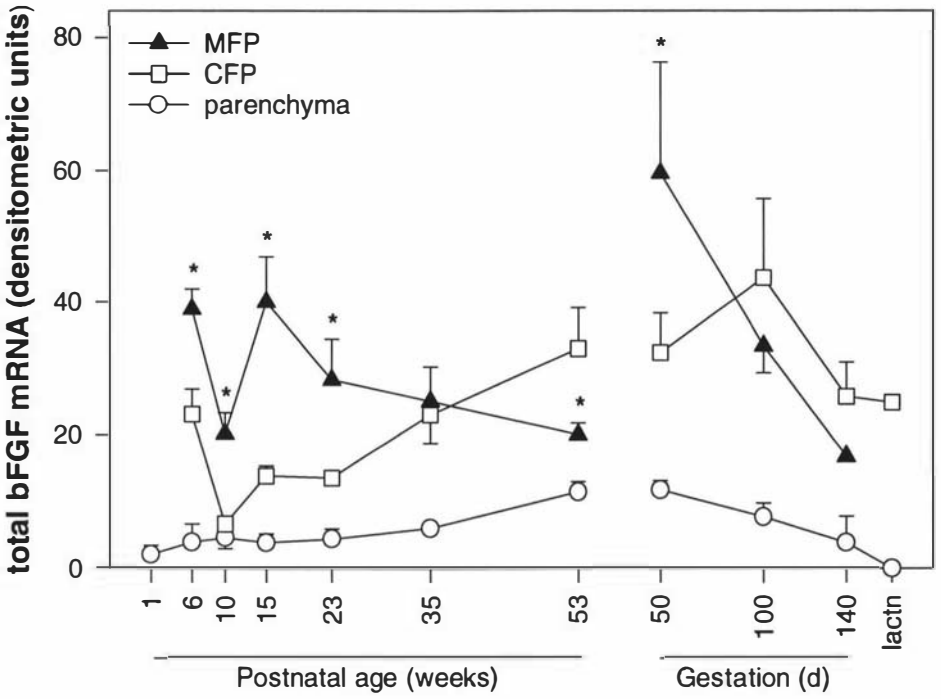


Figure 11.1 (B) Densitometric quantification of bFGF mRNA in ovine mammary tissues. Each data point represents the total amount of bFGF mRNA normalised to 28S rRNA levels. Data are means ± SEM. Number of replicates are n=4 for virgins and day 50 gestation, n=2 for days 100 and 140 of gestation, and n=1 in lactation. *P<0.08 vs respective CFP value.

Expression of HGF mRNA was detected at low levels in mammary fat pads, but not in parenchyma, as four transcripts of approximately 5.7, 3.5, 2.0 and 1.2 kb (Figure 11.2). Although low levels precluded densitometric quantification, it was apparent from replicate blots that the abundance of HGF mRNA within the CFP increased with developmental age and was highest during pregnancy. In contrast, expression of HGF mRNA in the contralateral extra-parenchymal MFP appeared to be higher prior to puberty and slightly reduced into pregnancy.

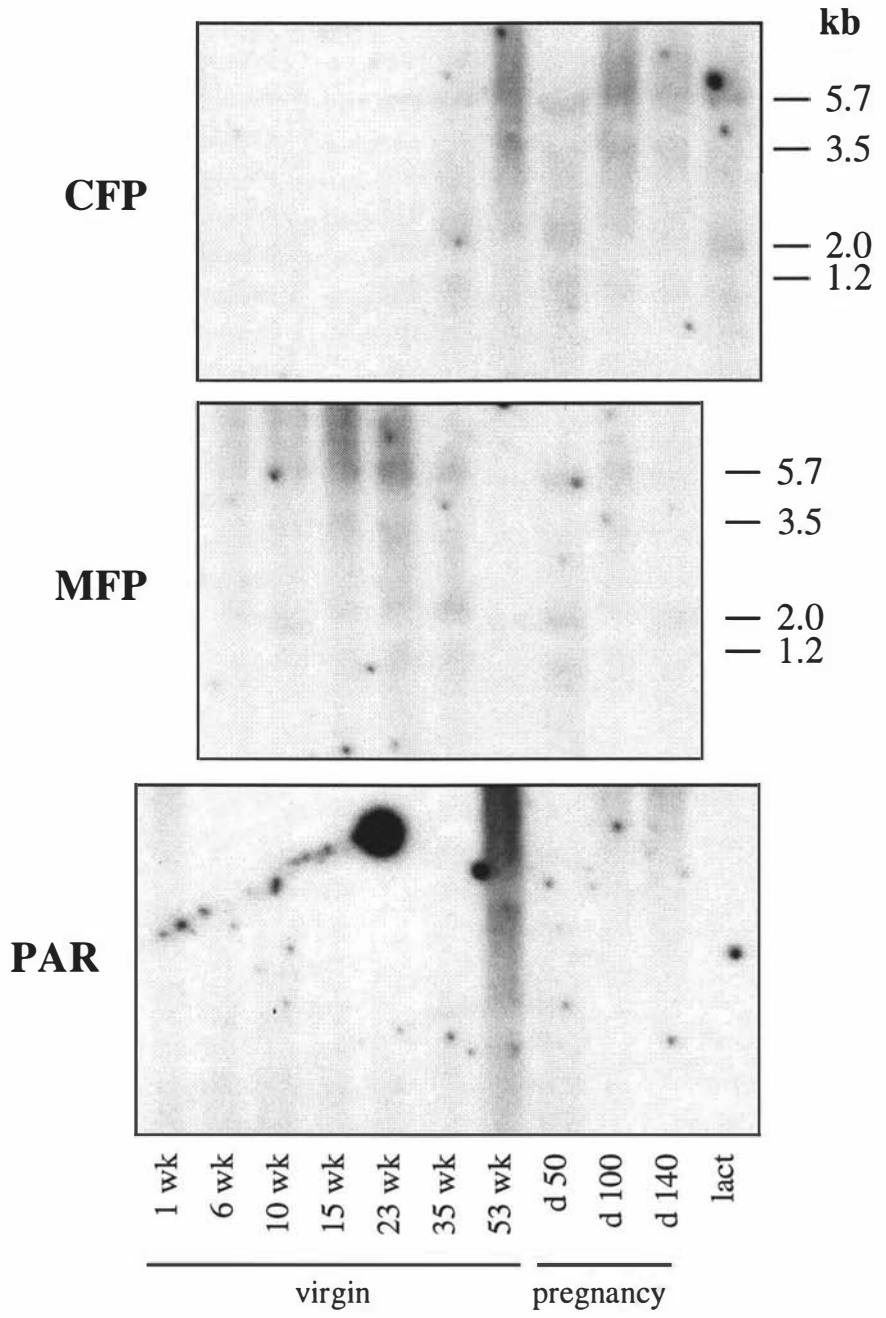


Figure 11.2 Northern analysis of HGF mRNA expression in ovine mammary tissues during postnatal development. Membranes described in Figure 11.1a were stripped and hybridised with a bovine HGF cDNA probe (7 day exposure).

Quantification of bFGF mRNA expression in mammary tissues from ovariectomised and/or oestrogen-treated ewe lambs revealed substantial effects of these treatments. Oestrogen markedly suppressed ($P<0.01$) bFGF mRNA levels within the extra-parenchymal MFP and tended ($P<0.15$) to reduce its expression in parenchyma (Figure 11.3). Ovariectomy increased ($P<0.05$) bFGF mRNA abundance in both tissues, although this effect was abrogated when lambs were also treated with oestrogen. No appreciable levels of HGF mRNA were detected in these samples by Northern analysis, even after extensive exposures.

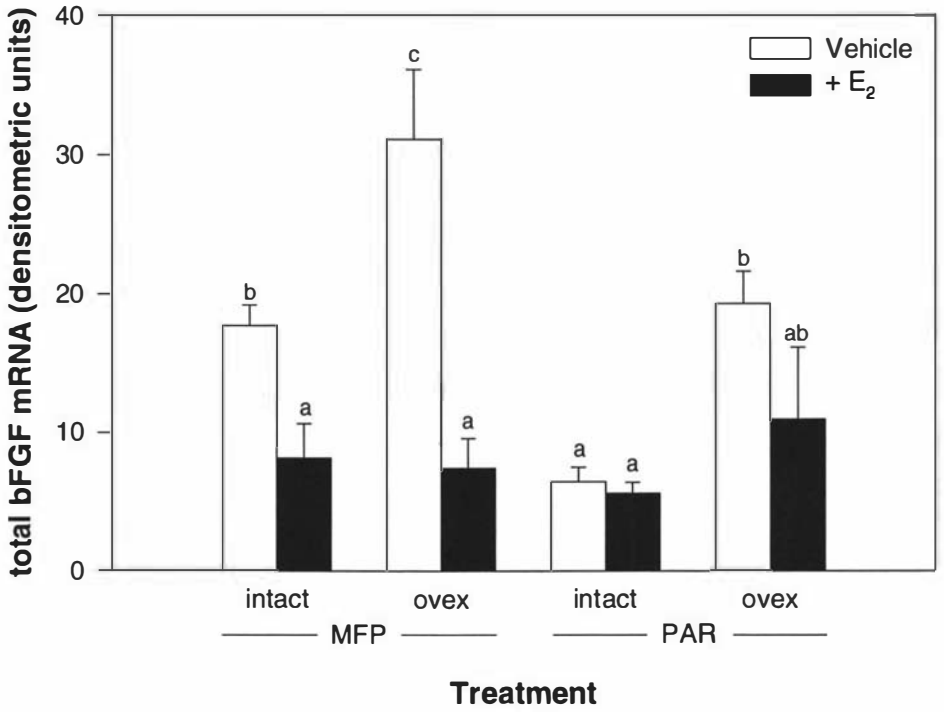


Figure 11.3 Effect of ovariectomy (ovex) and oestrogen (E_2) on bFGF mRNA levels in mammary parenchyma and extra-parenchymal MFP of prepubertal ewe lambs. Total expression of bFGF mRNA was quantified by densitometry and normalised for loading against 28S rRNA levels. Data are means ± SEM (n=3). ^{a,b,c} Means with different superscripts are significantly different ($P<0.05$).

11.4.2 *In vitro* expression of growth factor mRNA by mammary cells

To determine the cellular expression of growth factor mRNA within the ruminant mammary gland, RNA isolated from cultures of bovine mammary epithelial and fibroblast cells was subjected to Northern analysis. HGF mRNA was expressed only by fibroblasts as four transcripts of approximately 5.7, 3.5, 2.0 and 1.1 kb (Figure 11.4a). HGF mRNA was also detected in virgin mouse mammary tissue as a predominant 5.7 kb mRNA, and as a less abundant 2.0 kb mRNA. The abundance of bFGF mRNA was greatest in cultures of mammary epithelium, whereas mammary fibroblasts expressed approximately 20% of this level (Figure 11.4b). bFGF mRNA was predominant in both cell types as transcripts of approximately 6.9 and 4.5 kb, and to a lesser extent as 2.5 and 1.1 kb transcripts. No expression of bFGF mRNA was detected in mouse mammary tissue after this exposure.

This experiment also investigated whether various supplements could influence the expression of mRNA for these growth factors by mammary cells. Of those tested, only dexamethasone altered levels of HGF mRNA when it was added alone or with insulin, down-regulating mRNA expression by 60% (Figure 11.4a). In contrast, none of the treatments altered bFGF mRNA expression in either cell type (Figure 11.4b).

11.4.3 Growth stimulation by mammary fibroblasts

Given the stromal expression of mRNA for these growth factors both *in vivo* and *in vitro*, and the fact that ruminant mammary epithelium grows within a fibroblastic stroma *in vivo*, it was investigated whether mammary fibroblasts secrete a mitogenic activity *in vitro*. Ovine mammary fibroblast CM stimulated the proliferation of COMMA-1D cells in a dose-dependent manner to maximally increase their DNA synthesis by 380% (Figure 11.5a). This response was double that induced by 25 ng/ml EGF. A mitogenic activity was retained in the greater than 10 kDa fraction which also stimulated a concentration-dependent increase in DNA synthesis (Figure 11.5b). Chromatographic fractionation of this preparation eluted a major peak of mitogenic activity with a molecular size of approximately 76 kDa, and a second, smaller peak with an approximate molecular size of 23 kDa (Figure 11.6).

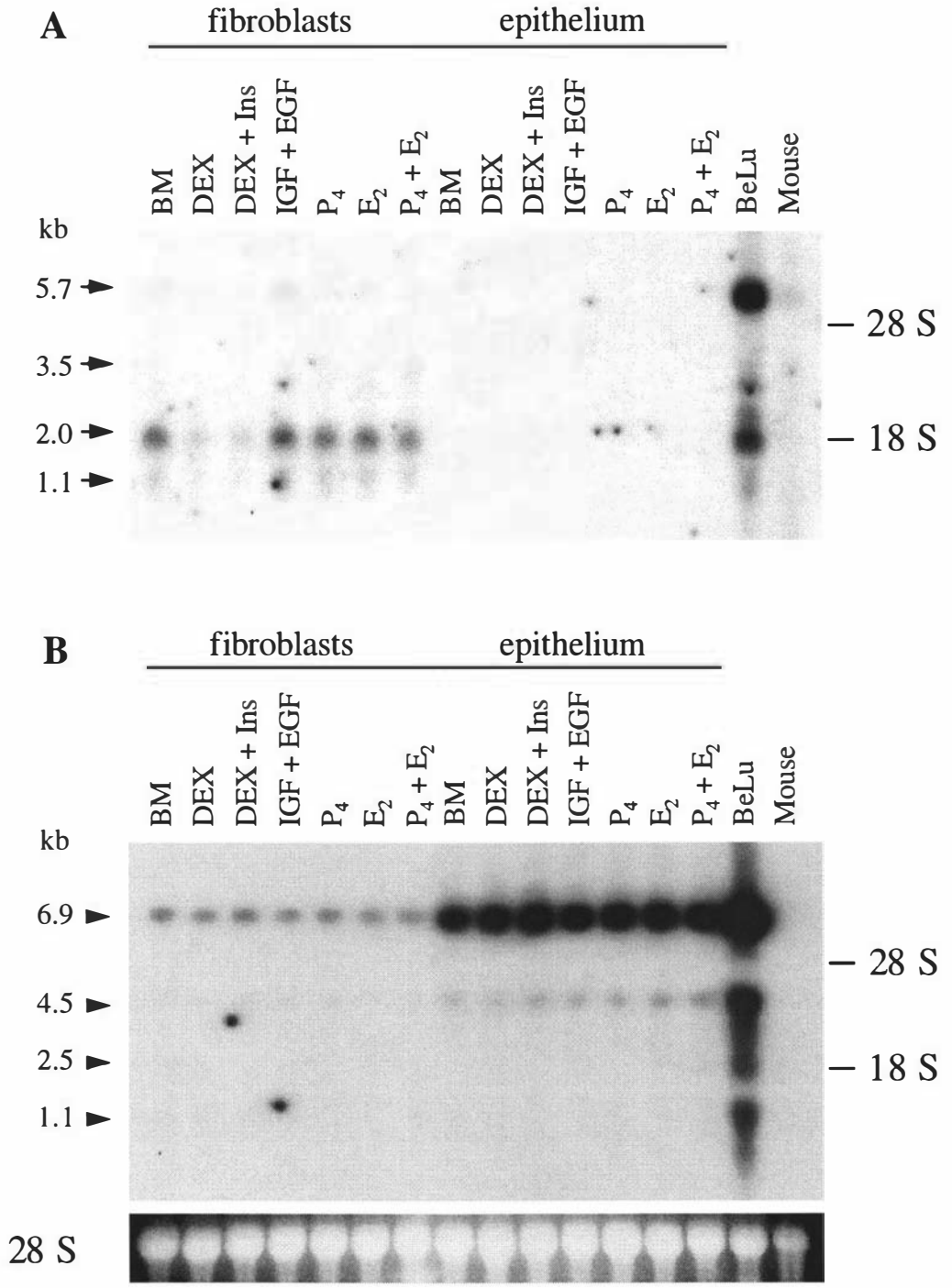


Figure 11.4 *In vitro* expression of mRNA for (A) HGF and (B) bFGF by cultures of bovine mammary epithelial and fibroblast cells. Confluent monolayers were cultured in various medium treatments for 24 h prior to RNA extraction for Northern analysis. Treatments were dexamethasone (Dex; 250 nm), insulin (Ins; 10 μg/ml), IGF-I (100 ng/ml), EGF (25 ng/ml), progesterone (P_4; 1 μg/ml), and 17β-oestradiol (E_2; 1 ng/ml). RNA from cultured bovine embryonic lung fibroblasts (BeLu) and mature virgin mouse mammary tissue was also used. The position of the 18S and 28S rRNA is indicated. Ethidium bromide-stained 28S rRNA shows similar loading of RNA.

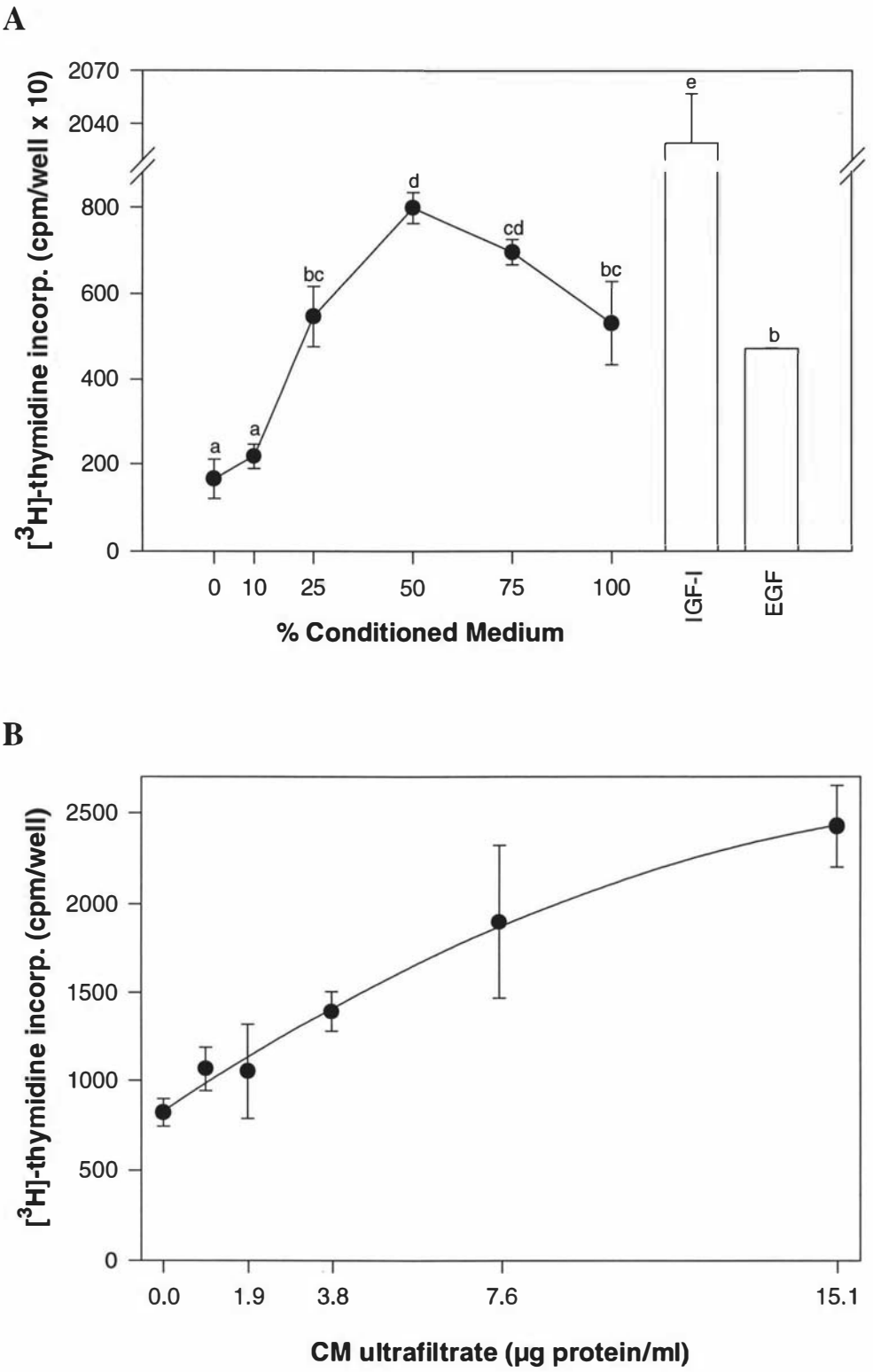


Figure 11.5 (A) Dose-dependent response of COMMA-1D cell proliferation to ovine mammary fibroblast CM. Cells were cultured in various treatments for 24 h prior to pulse labelling with [^{3}H]-thymidine. Data are means ± SEM (n=3). ^{a,b,c} Means with different superscripts are significantly different ($P<0.05$). (B) DNA synthetic response by COMMA-1D cells cultured with increasing concentrations of the >10 kDa fraction of CM. Data are means ± SEM (n=3).

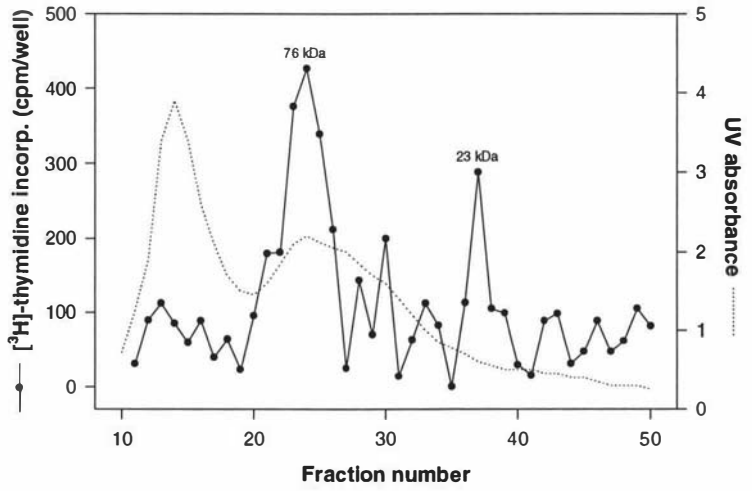


Figure 11.6 DNA synthesis by COMMA-1D cells in response to size-fractionated CM from cultures of ovine mammary fibroblasts. Concentrated CM was applied to an FPLC Superose 12 column and the eluted fractions added to cultures of COMMA-1D cells in DMEM for 24 h. DNA synthesis was determined after 2 h labelling with [^{3}H]-thymidine.

The effect of ovine mammary fibroblast CM on the growth of ovine mammary epithelial organoids cultured within collagen gels was also examined. Organoids grown for 7 days in medium supplemented with 10% FCS displayed small outgrowths as broadened spikes (Figure 11.7a). In contrast, epithelial organoids cultured for the same period in 100% CM demonstrated extensive outgrowth, often occupying large proportions of the collagen gel and inducing its contraction (personal observation). This proliferation was associated with the formation of elongate, extensively branched cords (Figure 11.7b). A similar proliferative and morphological response is promoted by the >10 kDa fraction of CM (personal observation).

Given that different mammary cell types expressed bFGF mRNA *in vitro* (Figure 11.4b), a comparison was made of the mitogenic effect of bFGF on MAC-T bovine mammary epithelial cells and ovine mammary fibroblasts. MAC-T cells demonstrated a slight but non-significant ($P>0.05$) mitogenic response to 5 ng/ml bFGF whereas substantial DNA synthesis was promoted by IGF-I (Figure 11.8a). In contrast, bFGF markedly stimulated the proliferation of mammary fibroblasts, more so at a concentration of 5 ng/ml than at 20 ng/ml (Figure 11.8b). This effect was significantly ($P<0.05$) greater than that induced by IGF-I.

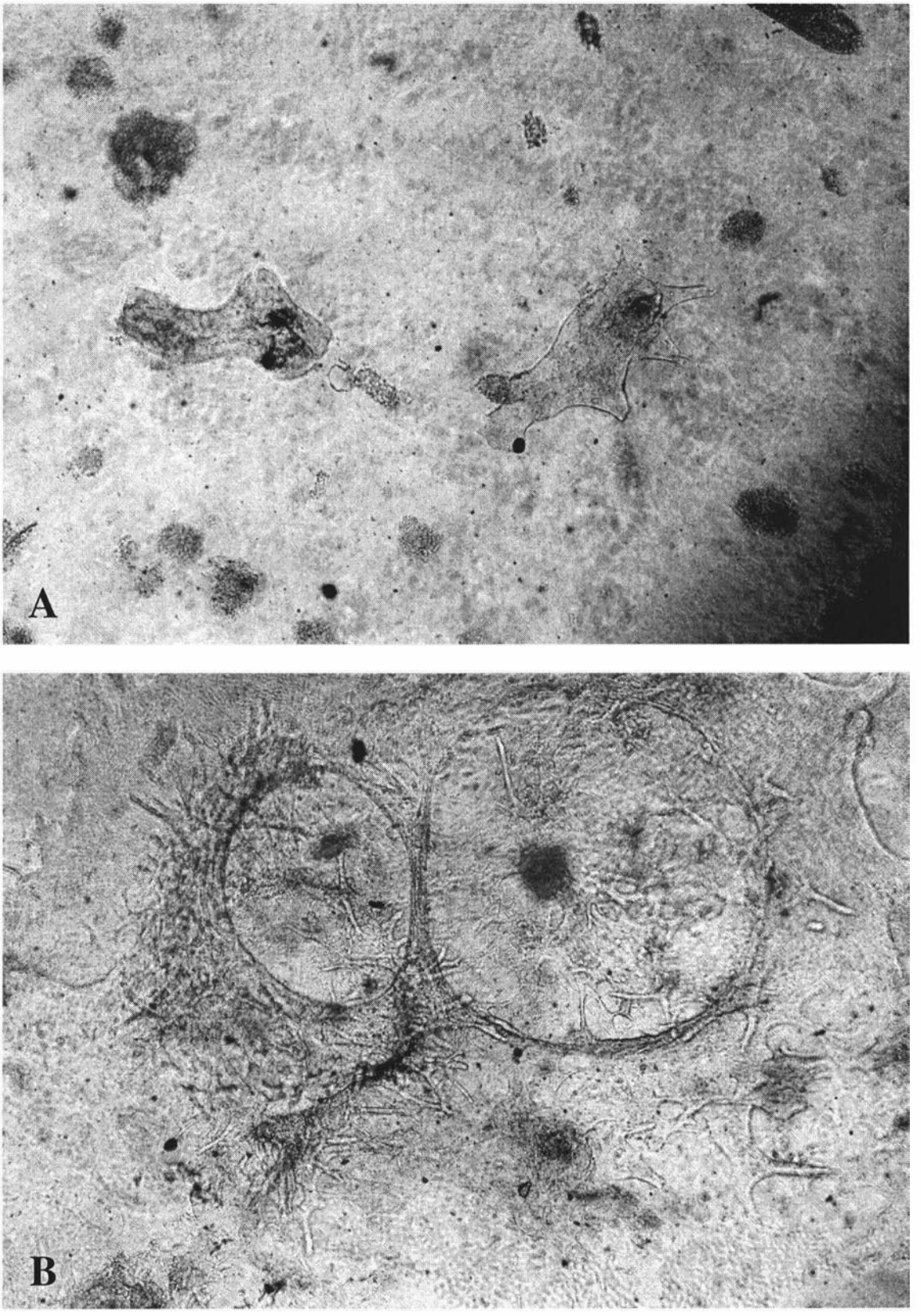


Figure 11.7 Morphological characteristics of ovine mammary epithelial organoids cultured within collagen gels for 7 days in the presence of either (A) 10% FCS or (B) ovine mammary fibroblast CM. 100 x magnification.

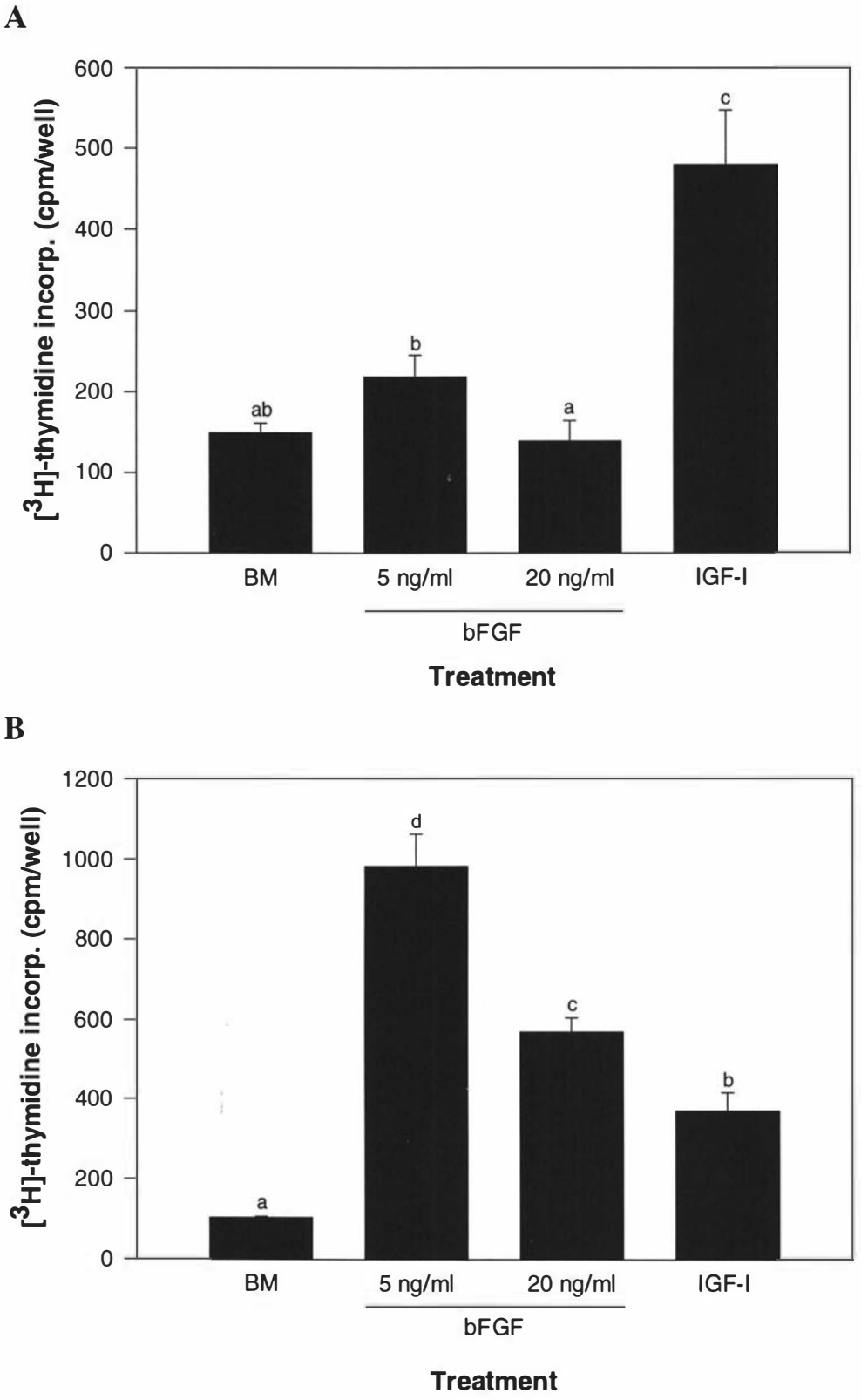


Figure 11.8 Proliferation of (A) MAC-T bovine mammary epithelial cells and (B) ovine mammary fibroblasts in response to bFGF (5 and 20 ng/ml) and IGF-I (100 ng/ml). MAC-T and mammary fibroblast cells (5 x 10^4 cells/well) were cultured for 24 h in 0.5 ml of DMEM supplemented with various treatments before labelling with [^{3}H]-thymidine. Data are means ± SEM (n=3). ^{a,b,c} Means with different superscripts are significantly different ($P<0.05$).

11.5 DISCUSSION

Interspersed through both the parenchyma and fat pad of the ruminant mammary gland is an abundance of fibroblastic connective tissue which may synthesise various polypeptide growth factors capable of regulating epithelial growth and morphogenesis. It was hypothesised that HGF and bFGF are two such growth factors involved in mediating this epithelial-stromal association.

The present findings, consistent with those from rodent studies (Sasaki *et al.*, 1994; Niranjan *et al.*, 1995; Soriano *et al.*, 1995), suggest that HGF may function as a paracrine mitogen and morphogen within the mammary gland. Northern analysis revealed relatively low levels of HGF mRNA within the CFP and extra-parenchymal MFP while no expression was readily apparent in mammary parenchyma. The presence of HGF mRNA in the ovine mammary fat pad corresponds to its expression in the CFP of virgin female mice (Niranjan *et al.*, 1995). Based on other reports, it is likely that HGF mRNA detected within the ovine mammary fat pad was expressed by adipocytes (Rahimi *et al.*, 1994) and/or connective tissue fibroblasts (Sasaki *et al.*, 1994). The detection of HGF mRNA in the stromal fraction of ovine mammary tissue also coincides with the finding that bovine mammary fibroblasts, but not epithelial cells, express multiple mRNA transcripts for HGF *in vitro.* Similar cell-specific expression of the HGF gene has been reported in cultures of human mammary cells (Wilson *et al.*, 1994). Consistent with a paracrine action of HGF, mammary epithelial cells, but not fibroblasts, express the receptor for HGF, c-*met* (Wilson *et al.*, 1994; Niranjan *et al.*, 1995).

Ovine mammary fibroblasts secrete an activity that promotes a substantial mitogenic and morphogenic response by mammary epithelial cells. Several lines of evidence suggest that these responses were effected by HGF. First, similar experiments have shown that CM from mouse mammary fibroblasts stimulates mammary epithelial proliferation (Enami *et al.*, 1983) due to the presence of HGF (Sasaki *et al.*, 1994). Second, size fractionation of CM revealed a major peak of mitogenic activity with a molecular size of approximately 76 kDa. A similar elution profile has been reported for HGF purified from embryonic lung fibroblast CM (Rubin *et al.*, 1991). Third, CM not only stimulated the growth of ovine epithelial organoids within collagen gels, but also

induced them to form elongate, branched cords. Purified HGF and HGF in fibroblast CM induce a similar morphogenesis by mouse mammary (Brinkmann *et al.*, 1995; Niranjan *et al.*, 1995; Soriano *et al.*, 1995) and kidney epithelial cells (Rosen *et al.*, 1994a; Barros *et al.*, 1995) cultured within collagen gels. Taken together, these results suggest that stromal fibroblasts within the ruminant mammary gland may induce epithelial proliferation and morphogenesis by secreting HGF.

The expression of HGF mRNA within the ovine mammary CFP was evidently increased in mature virgin and gestational states, a developmental profile similar to that reported for the mouse mammary gland (Niranjan *et al.*, 1995). Also, levels of HGF mRNA in the extra-parenchymal MFP prior to puberty were apparently greater than in the contralateral CFP, possibly due to an inductive effect by the adjacent epithelium as it underwent rapid proliferation (Chapter 8). This suggestion is consistent with the demonstration that HGF synthesis is increased by soluble factors secreted by mammary epithelial cells (Rosen *et al.*, 1994b). Along these lines, separate evidence suggests that the stromal expression of other growth factors is similarly upregulated by the epithelium during this period of prepubertal mammogenesis (Chapters 9 and 10).

HGF may also function as a local mediator of hormonal action on the mammary gland. Dexamethasone acutely down-regulated the expression of HGF mRNA by mammary fibroblasts *in vitro*, a response that has also been reported for other types of fibroblasts (Gohda *et al.*, 1992; Matsumoto *et al.*, 1992). It has also been shown that mammary fibroblast expression of another paracrine mitogen, keratinocyte growth factor, is similarly down-regulated by this treatment (Chapter 10). This effect of dexamethasone does, however, bear some degree of specificity in that the level of bFGF mRNA in epithelial and fibroblast cultures was unaffected. The basis for this glucocorticoid-induced response remains to be established, although it may be particularly relevant to growth in the peri-parturient ruminant mammary gland where epithelial proliferation ceases coincident with a pre-parturient rise in serum glucocorticoid levels (Cowie *et al.*, 1980).

As only negligible levels of HGF mRNA were detected in mammary tissues from ovariectomised and oestrogen-treated ewe lambs, the effects of these treatments on HGF expression remain unknown. However, it is conceivable that the mitogenic and morphogenic effects of oestrogen on mammary epithelium (Imagawa *et al.*, 1994) are

mediated by HGF. Specifically, oestrogen acts upon the stromal constituents of the mammary gland (Shyamala and Ferenczy, 1984) to promote ductal elongation (Imagawa *et al.*, 1994) similar to that induced by HGF *in vitro* (Soriano *et al.*, 1995). Along these lines, the HGF gene is preceded by multiple oestrogen response elements (Liu *et al.*, 1994) and oestrogen acutely upregulates HGF mRNA expression in the ovary (Liu *et al.*, 1994). Based on these reports, further investigation into the role of HGF as a mediator of oestrogenic action on the mammary gland is warranted.

The precise function of bFGF in the ruminant mammary gland remains unresolved. *In vivo*, mammary fat pads expressed higher levels of bFGF mRNA than parenchyma, while *in vitro*, the level of bFGF mRNA was substantially higher in cultured bovine mammary epithelial cells than in mammary fibroblasts. Indeed, it may be that multiple cell types express bFGF within the ruminant mammary gland. It has also been considered that the cultures of bovine mammary epithelium in these experiments may have contained myoepithelial-like cells expressing high levels of bFGF mRNA; such a proposal would be in keeping with other reports showing that rodent myoepithelial-like cells, but not mammary epithelial cells, express mRNA for bFGF (Barraclough *et al.*, 1990). A further possibility is that as myoepithelial cells only constitute a small proportion of cells within the mammary gland (Warburton *et al.*, 1982), the low levels of bFGF mRNA detected in ovine mammary parenchyma may simply be due to a dilution of myoepithelial RNA. Further analyses are required to confirm which cell types synthesise bFGF within the mammary gland.

Whereas epithelial cultures expressed the greatest amount of bFGF mRNA, mammary fibroblasts were most responsive to mitogenic stimulation by bFGF. This finding leads to a proposal that bFGF functions as a paracrine/autocrine mitogen for mammary fibroblasts in addition to its previously proposed role as a paracrine/autocrine mitogen for myoepithelial cells (Smith *et al.*, 1984; Barraclough *et al.*, 1990). This action may also serve to regulate the stromal expression of HGF, as bFGF increases the synthesis and secretion of HGF by fibroblasts (Roletto *et al.*, 1996). Myoepithelial and/or epithelial cells could therefore secrete bFGF to regulate HGF-induced mammary epithelial proliferation and morphogenesis. This proposal may correspond to the observation that the expression of mRNA for both of these growth factors displayed

similar developmental profiles, particularly prior to puberty when their abundance was greater in the extra-parenchymal MFP than in the contralateral CFP.

The level of bFGF mRNA in mammary tissues of prepubertal ewe lambs was suppressed by exogenous oestrogen and increased by ovariectomy. Likewise, it has been found that the stromal expression of other growth factors such as IGF-II and KGF is similarly altered (Chapters 9 and 10), prompting a suggestion that the effects of these growth factors may be incompatible with the type of growth and morphogenesis promoted by oestrogen. Interestingly, the expression of bFGF mRNA in the mammary glands of mature virgin mice is unaffected by oestrogen (Chakravorti and Sheffield, 1996b), possibly reflecting a developmental state-specific or species-specific effect.

In conclusion, the results of these experiments suggest that HGF and bFGF may serve as paracrine mediators of epithelial-stromal interactions within the developing ruminant mammary gland. Furthermore, these growth factors likely function in a physiological role given that their expression is regulated by several influences which also direct epithelial growth and morphogenesis. Ongoing investigation is required to determine the full extent to which locally-derived bFGF and HGF function during postnatal ruminant mammogenesis.

CHAPTER 12

GENERAL DISCUSSION AND CONCLUSIONS

12.1 GENERAL DISCUSSION

The mammary gland must undergo a unique and elaborate course of postnatal development before it can assume its ultimate function of synthesising and secreting copious volumes of milk to nourish the offspring. Such development involves the establishment of epithelial cells as a ductal network within the virgin gland; these cells subsequently undergo lobuloalveolar growth and differentiation during pregnancy. This fascinating process of epithelial proliferation and morphogenesis occurs within the confines of a matrix of adipose and connective tissue collectively referred to as the "mammary fat pad".

It has long been established that specific endocrine hormones serve pivotal roles in regulating the growth of epithelium within the normal and neoplastic mammary gland. The first evidence for the local regulation of mammary development was provided from the transplantation studies of DeOme *et al.* (1959) and Hoshino (reviewed by Hoshino, 1978) which showed that a stromal environment such as the mammary fat pad was required for normal epithelial growth. While this fact remained, relatively little attention was focussed towards the ability of the mammary fat pad to regulate normal and neoplastic development. Only in more recent times has it been recognised that the mammary fat pad may fulfil a critical role during mammogenesis, both by its ability to locally regulate growth (reviewed by Imagawa *et al.*, 1994), and to mediate the actions of systemic hormones (Shyamala and Ferenczy, 1984; Haslam and Counterman, 1991). However, information regarding the specific nature of these mechanisms is limited. The objective of this research, therefore, was to investigate some of the pathways by which the mammary fat pad may regulate epithelial growth within the normal mammary gland.

12.1.1 Mitogenic effects of the mammary fat pad

In seeking to evaluate the net mitogenic capacity of the mouse mammary fat pad, co-culture and conditioned medium approaches were used to bioassay its effect on mammary epithelial growth. Consistent with a previous report (Beck *et al.*, 1989), these experiments showed that the mouse mammary fat pad liberates a diffusible factor(s) *in vitro* which stimulates the proliferation of mammary epithelial cells (Chapter 2). Furthermore, this factor(s) markedly potentiates the mitogenic response of mammary

epithelial cells to IGF-I and EGF (Chapters 2 and 3). This modulatory effect may be important in regulating epithelial growth within the mouse mammary gland, a proposal given credence by subsequent results which showed that this effect was altered during the oestrous cycle (Chapter 5), and by stage of development (Chapter 6), ovarian steroid hormones (Chapter 6), and the recognised (Cunha and Hom, 1996) influence of epithelial-stromal interactions (Chapters 5 and 6). These alterations were associated with substantial changes in mammary gland development *in vivo*. The present findings also indicate that this effect may satisfy particular requirements for the growth of mammary epithelial cells (Chapter 3) which have a unique requirement to grow within a depot of adipose tissue (reviewed by Hoshino, 1978).

Further investigations revealed that the proliferative responses of COMMA-1D cells in co-culture are probably due to the liberation of unsaturated fatty acids by the mouse mammary fat pad (Chapter 4). Several previous studies have demonstrated that unsaturated fatty acids stimulate the growth of mammary epithelial cells *in vitro* (Kidwell *et al.*, 1978; Wicha *et al.*, 1979; Beck *et al.*, 1989). Two laboratories have also shown that EGF-induced proliferation of mammary epithelial cells is potentiated by unsaturated fatty acids (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994). The research reported in Chapter 4 is, to my knowledge, the first to show that linoleic acid also markedly enhances the proliferative effect of IGF-I on mammary epithelial cells. Consistent with reports by others (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994; Neri *et al.*, 1994), these studies suggest that the growth factor-potentiating effects of the mammary fat pad are likely initiated via the lipid-activated second messenger, protein kinase C (PKC; Chapter 4).

That unsaturated fatty acids modulate the growth of mouse mammary epithelial cells is not surprising given the close proximity of these cells to adipocytes within the mammary fat pad. These findings have important potential implications within the developing normal and neoplastic mammary gland. The level and composition of dietary fat is known to alter mammary gland development and tumorigenic risk in rodents (reviewed by Welsch, 1985; 1992), while fat intake is an important factor in the determining the risk of human breast cancer (Kelsey and Berkowitz, 1988). Likewise, both IGF-I (Lee and Yee, 1995; Forsyth, 1996) and EGF (Plaut, 1993) have substantial effects on the growth of normal and tumorous mammary epithelium *in vivo* and *in vitro*.

The combined response to these influences could therefore substantially increase the proliferation of mammary epithelium. Obviously a great deal remains to be understood about the way in which lipids influence mammary gland growth, and in particular, how they interact with the effects of specific growth factors.

In seeking to elucidate the mechanisms by which the mammary fat pad regulates ruminant mammary development, attention was directed toward the role of stroma-derived, paracrine growth factors. It was previously shown that growth factors such as IGF-I and EGF, which are expressed locally within the mouse mammary gland (Snedeker *et al.*, 1991; Ruan *et al.*, 1995), can make an important contribution to fat pad-induced proliferation (Chapters 2-6). However, the main impetus for examining the function of stroma-derived growth factors within the ruminant mammary gland was an appreciation that the ruminant mammary epithelium grows whilst continually enveloped by a substantial proportion of fibroblastic connective tissue (Sheffield, 1988b; Akers, 1990; Chapter 8). This type of stroma has been shown to be a source of mitogenic and morphogenic growth factors in various tissues (Koji *et al.*, 1994; Rosen *et al.*, 1994a; Birchmeier *et al.*, 1995), leading to the consideration that it may represent a significant source of these polypeptides within the ruminant mammary gland. While a full comparative study remains to be conducted, the ability to readily detect mRNA for several growth factors in ovine mammary stroma, but not mouse mammary tissue (Chapters 9-11), would appear to support this proposal. Such a notion does not preclude an involvement of lipids in the regulation of ruminant mammogenesis; in fact, dietary unsaturated fatty acids have been shown to stimulate ovine mammogenesis (McFadden *et al.*, 1990a). Rather, it is suggested that the relative contributions of these two influences may differ to those within the mouse mammary gland.

In several cases the results presented are the first demonstration of mRNA expression for these growth factors in the mammary stroma of any species. A finding of particular significance is that IGF-I mRNA is expressed within the mammary fat pad of the postnatal ovine mammary gland (Chapter 9). This result, and those from recent *in situ* hybridisation analysis of ovine (Morgan *et al.*, 1996) and human (Singer *et al.*, 1995) mammary parenchyma, unequivocally indicates that IGF-I is expressed by the stromal tissues within the developing mammary gland. This finding has a number of far-reaching implications which extend from the mechanism by which GH and plane of

nutrition may influence ruminant mammogenesis, to the local regulation of mammary tumorigenesis. Establishment of this fact emphasises the important function of the mammary fat pad during postnatal mammogenesis and will enable more precise investigations into the mechanism by which IGF-I regulates mammogenesis.

These results also indicate that IGF-II is locally expressed by the fat pad tissues of the ovine mammary gland. Of particular significance may be the observation that IGF-II mRNA was transcriptionally upregulated within the mammary parenchyma of prepubertal ewe lambs. Based on the results of *in situ* hybridisation studies by Morgan *et al.* (1996), it is likely that this upregulation occurred within the fibroblastic stroma. A similar observation has been reported in mammary tumours where malignant epithelium induces the stromal expression of IGF-II mRNA (Singer *et al.*, 1995). These findings suggest the existence of a positive feedback loop on the stromal expression of IGF-II. The full significance of this transcriptional modulation is not immediately obvious although it may allow for the specification of certain physiological functions. A great deal remains to be understood about the role of IGF-II during mammogenesis, where its paracrine action may be of particular importance during mammary tumorigenesis (Ellis *et al.*, 1994; Manni *et al.*, 1994).

Several laboratories have recently focussed a great deal of attention toward the role of KGF within the developing mammary gland (Coleman-Krnacik and Rosen, 1994; Imagawa *et al.*, 1994b; Wilson *et al.*, 1994; Cunha and Hom, 1996). Results presented in Chapter 10 are the first demonstration that KGF truly functions as a stroma-derived, paracrine mitogen within the mammary gland. Furthermore, it was found that the KGF gene is differentially transcribed in mammary adipocytes and connective tissue fibroblasts (Chapter 10). The implications of this result are presently unknown, although it has been suggested that KGF may fulfil roles in branching morphogenesis (Alarid *et al.*, 1994; Post *et al.*, 1996) and lipid metabolism (Nonogaki *et al.*, 1995), as well as being a mitogen for epithelial cells (Imagawa *et al.*, 1994b). It will be interesting to determine whether these 1.5 and 2.4 kb KGF mRNAs are translated into different proteins.

Experimental results indicate that HGF is expressed by the stromal constituents of the ruminant mammary gland (Chapter 11) consistent with recent findings in mice (Niranjan *et al.*, 1995). While only circumstantial, preliminary evidence indicates that ruminant

mammary fibroblasts may secrete HGF to influence the morphogenesis of ovine mammary epithelial cells (Chapter 11). Further experiments are required to measure the *in vitro* morphogenic response of ruminant epithelial cells to purified HGF, as well as to measure the level of HGF in ovine mammary fibroblast-conditioned medium.

The expression of bFGF mRNA within the ovine mammary gland indicates that this growth factor may also be involved in regulating postnatal mammogenesis. While bFGF mRNA was most abundantly expressed within the ovine mammary fat pad (Chapter 11), consistent with recent findings in the mouse mammary gland (Chakravorti and Sheffield, 1996a), both bovine epithelial and fibroblast cells express mRNA for bFGF *in vitro*. These contrasting results, as well as the finding that bFGF stimulates the proliferation of both epithelial and fibroblast cells, suggest that bFGF acts on multiple cell types within the mammary gland. The specific cellular expression of bFGF mRNA within the mammary gland of any species remains to be determined by *in situ* hybridisation. Personal attempts along these lines have, to date, proven unsuccessful, possibly due to the expression of an antisense bFGF mRNA (Li *et al.*, 1996).

The demonstration that the ovine mammary stroma expresses mRNA for several growth factors provides important information regarding the local regulation of mammary development. Future experiments promise to reveal further exciting information in this area. It will be important to determine the level of these growth factors in mammary tissue, and to relate these levels to *in vitro* studies which have determined the concentration range over which they are mitogenic for mammary epithelial cells (Collier *et al.*, 1993; Chapter 10). Other studies are required to localise the cellular expression of these growth factors, either by immunohistochemistry or *in situ* hybridisation.

Combined together, these results demonstrate that the stromal environment of the mammary fat pad can provide a variety of factors capable of directly stimulating the growth and morphogenesis of mammary epithelial cells. Likewise, there are probably numerous other factors, either identified or unidentified, which are produced within the mammary fat pad and which can subsequently modulate the actions of other factors on mammary epithelial cells. While in many cases the full physiological role of these factors is unknown or is only just being realised, the diversity of their effects illustrates the important function that the mammary fat pad undoubtedly fulfils during mammogenesis.

12.1.2 Influence of epithelial-stromal interactions on the mitogenic capacity of the mammary fat pad

An aspect of local growth regulation which may be particularly important within the mammary gland (Sakakura, 1991; Cunha and Hom, 1996), and indeed other tissues (Cunha *et al.*, 1980), is the means by which the interaction between epithelial and stromal cells subsequently regulates epithelial growth and morphogenesis. A number of findings from this research emphasise the importance of such a mechanism in regulating the proliferation of mammary epithelial cells.

The results of co-culture experiments indicate that endogenous epithelium may locally stimulate the release of mitogenic factors from the virgin mouse mammary fat pad (Chapters 5 and 6). Based on the findings of others (Kidwell and Shaffer, 1984; Levay-Young *et al.*, 1987) and the results presented in Chapter 4, this probably reflects the increased liberation of unsaturated fatty acids from mammary adipocytes. Measurement of the level of fatty acids released into the culture media is needed to confirm this suggestion. It is presently unclear as to how mammary epithelial cells might stimulate the release of unsaturated fatty acids from the mammary fat pad, although several molecules including prostaglandin $F_{2\alpha}$ (Kidwell *et al.*, 1982), mast cell-derived histamine (Kidwell and Shaffer, 1984), and an adipocyte lipase-activating factor (Masuno *et al.*, 1981), are potential candidates. In several ways it is not surprising that proliferating mammary epithelial cells induce lipolysis within surrounding adipocytes, given that epithelial cells are responsive to unsaturated fatty acids, that there exists an intimate relationship between epithelial cells and adipocytes, and that an extensive reaction occurs as mammary parenchyma ramifies into the fatty stroma. The importance of this mechanism during mammary tumorigenesis, particularly within the human breast, has not been adequately addressed within the literature.

The present findings also indicate that the interaction between epithelial cells and the mammary stroma may function to modulate the actions of ovarian steroids on the mouse mammary gland. This was evidenced in two ways. First, the mitogenic effect of co-cultured CFP was frequently less than that of MFP in medium supplemented with progesterone, but not oestrogen (Chapters 5). Second, treatment with exogenous oestrogen *in vivo* increased the *in vitro* mitogenic effect of CFP and decreased that of MFP, while the effect of progesterone was independent of the mammary tissue type

(Chapter 6). At this stage it is unclear as to how these differential effects were manifest in the presence of oestrogen and progesterone. However, such findings further underscore the importance of the interaction between epithelial and stromal cells within the mammary gland, an importance emphasised by the pivotal roles of oestrogen and progesterone during postnatal mammogenesis.

The presence of endogenous epithelium in ovine mammary stroma also increased its proliferative effect on mouse and bovine mammary epithelial cells in co-culture (Chapter 7). While further tests are required to determine the cause of this effect, one possibility is that endogenous epithelium promoted an increased availability of stimulatory fatty acids. If this was in fact the case, the mechanism by which it occurred may differ to that within mouse mammary tissue as ruminant epithelium is enveloped by an extensive amount of connective tissue and does not abut onto mammary adipocytes. However, parenchymal encroachment into the ruminant mammary fat pad is associated with a concomitant depletion of lipids from mammary adipocytes and an extensive fibrosis (Sheffield, 1988b; personal observation). Thus, one possibility may be that fibroblasts, rather than the epithelial cells, stimulate lipolysis in ruminant mammary adipocytes.

Alternatively, increased mitogenic stimulation from ovine mammary tissue containing endogenous epithelium may reflect epithelium-induced growth factor expression within the surrounding stroma. This suggestion is supported by the demonstration that the level of mRNA for several stroma-derived growth factors (IGF-I and -II, KGF, HGF, and bFGF) is greater in the extra-parenchymal MFP than the contralateral CFP of prepubertal ewe lambs (Chapters 9-11). Furthermore, the expression of IGF-II mRNA in mammary parenchyma was increased during this period in a transcript-specific manner (Chapter 9). That mammary parenchyma expressed the least mRNA for other growth factors would appear, at first, to refute this proposal. However, mammary parenchyma comprises a large proportion of epithelial tissue which would lead to the substantial dilution of stromal RNA used during Northern analysis. The actual level of growth factor expression by stromal cells within the ovine mammary parenchyma needs to be assessed by techniques such as *in situ* hybridisation or immunocytochemistry. Certainly results from other studies, both within the mammary gland (Coleman-Krnacik and Rosen, 1994; Singer *et al.*, 1995) and in other tissues (Smola *et al.*, 1993), indicate

that epithelial cells can induce the surrounding stromal cells to upregulate their expression of paracrine growth factors. This growth-regulatory mechanism may be of increased importance within the ruminant mammary gland because of the substantial presence of fibroblastic connective tissue.

Viewed together, these findings suggest an important feedback mechanism by which mammary epithelium may regulate its growth within the mammary fat pad. Proliferating epithelial cells may stimulate lipolysis within mammary adipocytes, either directly or indirectly, to increase the local availability of non-esterified unsaturated fatty acids. Likewise, epithelial signalling may increase the local expression of specific paracrine growth factors. These factors may then act in concert, perhaps via the effects of PKC (Bandyopadhyay *et al.*, 1993; Sylvester *et al.*, 1994; Chapter 4), to stimulate maximum cell proliferation. The relative contribution of lipids and growth factors may depend upon the species in question and the cellular composition of the mammary tissue (Chapter 7). This effect may be further regulated by the actions of ovarian steroids on both stromal and epithelial cells, perhaps also via PKC (Cho and Katzenellenbogen, 1993; Katzenellenbogen, 1996; Martínez-Lacaci and Dickson, 1996). The physiological significance of this combinatory mechanism remains to be established. It is also conceivable that this mechanism functions to regulate other aspects of mammogenesis including parenchymal morphogenesis and epithelial differentiation.

12.1.3 The hormonal regulation of mitogenic stimulation by the mammary fat pad

Several researchers have proposed that stromal constituents within the mammary gland serve to mediate the mammogenic effects of various hormones (Kidwell and Shaffer, 1984; Shyamala and Ferenczy, 1984; Hauser *et al.*, 1990; Haslam and Counterman, 1991), although information relevant to this suggestion is scant. The present findings provide strong evidence to support an important role for the mammary fat pad during hormone-stimulated mammogenesis.

The ovarian steroids exert pronounced effects on mouse mammary development. Specifically, oestrogen stimulates ductal elongation (Bresciani, 1968) while progesterone promotes alveolar budding (Bresciani, 1968). Within the oestrous cycle, ductal growth at oestrus corresponds to an increased mitogenic effect of the mammary fat pad (Chapter 5). Consistent with this stimulation being due to unsaturated fatty acids

(Chapter 4), the pattern of fat pad-induced proliferation parallels that of lipolysis within rat adipose tissue during the oestrous cycle (Hansen *et al.*, 1980). Further experiments indicated that oestrogen and progesterone do indeed have significant effects on the mitogenic capacity of co-cultured mammary tissue. This may represent modification in the metabolism of lipids by mammary adipocytes. Such results lead to a new proposal that the action of ovarian steroids on the developing mouse mammary gland involves their influence on the metabolism of lipids by mammary adipocytes. Specifically, oestrogen may stimulate lipolysis to increase the local availability of unsaturated fatty acids while progesterone may encourage lipogenesis. Further measurements of *in vitro* lipid metabolism by mammary adipocytes are required to confirm the nature and extent of these effects. In addition, the effects of ovarian steroids on the mammary gland apparently involve a great degree of interaction with other factors such as the ovary and epithelial-stromal associations (Chapter 6) which remain to be understood. Such effects may also encompass the actions of other regulatory pathways including those of the pituitary and adrenal gland. Furthermore, these interrelationships may be involved in modulating the mitogenic effect of the mammary fat pad during postnatal development (Chapter 6).

Further to this proposed mechanism, a diffusible factor from the mammary fat pad enhances the responsiveness of mammary epithelial cells to oestrogen while its effect is suppressed by progesterone (Chapters 5 and 6). Several lines of evidence suggest that unsaturated fatty acids account for these responses, possibly through PKC. Oestrogen and progesterone may therefore act on the mammary gland through at least two distinct mechanisms; one whereby they regulate the liberation of a diffusible activity from the mammary fat pad, and a second in which this activity differentially modulates epithelial cell responsiveness to oestrogen and progesterone. It is also conceivable that the latter pathway converges with those utilised by other mammogenic agents such as polypeptide growth factors (Cho and Katzenellenbogen, 1993; Katzenellenbogen, 1996; Martínez-Lacaci and Dickson, 1996). These mechanisms warrant particular attention given the paramount role that ovarian steroids play in normal and neoplastic mammogenesis.

These studies of ruminant mammary development indicate that the stromal expression of paracrine growth factors is also regulated by mammogenic hormones. To date there has been limited investigation of such regulation within the mammary gland of any

species. Levels of IGF-I mRNA expression within the mammary fat pads of prepubertal ewe lambs (Chapter 9) followed a profile of serum GH levels reported for ewe lambs over this period (Johnsson *et al.*, 1985), indicating that paracrine IGF-I may mediate the mammogenic action of GH. Other tissues (Daughaday and Rotwein, 1989), including the rodent mammary gland (Kleinberg *et al.*, 1990), also increase their expression of paracrine IGF-I in response to GH. Furthermore, IGF-I mRNA levels within mammary tissues of prepubertal ewe lambs could be increased by exogenous oestrogen (Chapter 9), consistent with a recent finding in rodents (Ruan *et al.*, 1995).

In contrast, the expression of KGF, IGF-II, and bFGF mRNA within the prepubertal ovine mammary gland was substantially down-regulated by exogenous oestrogen and was increased following ovariectomy (Chapters 9-11). Previous to this study, hormonal regulation of KGF expression within the mammary gland had not been reported. It will be interesting to determine whether progesterone upregulates the stromal expression of KGF within the mammary gland as in other tissues (Koji *et al.*, 1994), and whether KGF subsequently functions as a paracrine branching morphogen (Alarid *et al.*, 1994; Post *et al.*, 1996) for mammary epithelium.

A recent report showed that the expression of bFGF mRNA within the mouse mammary fat pad is unaltered by oestrogen while it is increased by progesterone (Chakravorti and Sheffield, 1996b). Likewise, oestrogen increases, and ovariectomy decreases, the expression of IGF-II mRNA in rat mammary tumours (Manni *et al.*, 1994). The disparity between these findings and the effect of exogenous oestrogen and ovariectomy in the present experiments (Chapter 9) may reflect differences in the species studied or the developmental state of the females examined.

The combined results of these experiments suggest that the mammary fat pad, through its mitogenic effects, has the potential to mediate a substantial amount of hormone-stimulated mammogenesis. The ultimate response to hormone-induced growth may represent a combined response to several different effects. It is suggested that oestrogen stimulates lipolysis within mouse mammary adipocytes to increase the availability of non-esterified unsaturated fatty acids to the mammary epithelium. Growth hormone may exert a similar lipolytic effect within the mammary fat pad as in other adipose depots (Barber *et al.*, 1992). At the same time, both of these hormones upregulate the expression of IGF-I within the mammary stroma (Kleinberg *et al.*, 1990; Ruan *et al.*,

1995; Chapter 9), while oestrogen increases the local synthesis of EGF-like factors (Vonderhaar, 1984; Liu *et al.*, 1987). It was demonstrated in Chapter 4 that unsaturated fatty acids markedly potentiate the proliferative effect of these growth factors on mouse mammary epithelial cells. Therefore, oestrogen- and GH-induced growth within the mammary gland may represent a combined response to the ability of these hormones to stimulate the release of unsaturated fatty acids from mammary adipocytes and to increase the local synthesis of certain growth factors. This mechanism may be particularly relevant to ductal proliferation within the virgin mammary gland, given that ductal growth is stimulated by the individual effects of oestrogen (Daniel *et al.*, 1987), GH (Feldman, 1993), IGF-I (Ruan *et al.*, 1992), EGF (Coleman *et al.*, 1988) and unsaturated fatty acids (Miyamoto-Tiaven *et al.*, 1981). Furthermore, this proposal corresponds to the recent finding that the major synergistic effect of oestrogen with IGF-I occurs after IGF-I is produced (Ruan *et al.*, 1995). At the same time, oestrogen down-regulates the expression of other growth factors such as IGF-II, bFGF and KGF, consistent with the demonstration that the mitogenic effect of these growth factors is not markedly enhanced by unsaturated fatty acids present in CM (Chapters 3 and 4). This is consistent with the suggestion that these particular growth factors may stimulate other aspects of parenchymal development such as alveolar proliferation and morphogenesis.

12.1.4 Effect of ontogenic state on the mitogenic capacity of the mammary fat pad

The ontogeny of postnatal mammogenesis provides an extremely useful model for comparing and contrasting the growth regulatory-effect of the mammary fat pad across several physiological states.

The present findings indicate that the mitogenic capacity of the mouse mammary fat pad changes during the course of postnatal mammogenesis (Chapter 6). In particular, mitogenic stimulation from the mammary fat pad may promote ductal elongation within the virgin mammary gland, consistent with findings during the oestrous cycle (Chapter 5). This may reflect postnatal changes in the influence of unsaturated fatty acids, a suggestion that is consistent with reports that unsaturated fatty acids are an important requirement for ductal (Miyamoto-Tiaven *et al.*, 1981), but not alveolar (Faulkin *et al.*, 1986) development. Further experiments are required to confirm this suggestion. In particular, information is required about changes in mammary adipocyte lipid

metabolism which might occur during this period; information which would complement that reported during later development (Bandyopadhyay *et al.*, 1995). Such information could be gleaned from *in vitro* studies of mammary adipocyte lipogenesis and lipolysis (Hansen *et al.*, 1994) and from determinations of the fatty acid composition of mammary adipose tissue and the local expression of factors such as lipoprotein lipase and hormone-sensitive lipase during this time.

Another finding of interest was that intact mouse mammary tissue provided increased mitogenic stimulation during lactation. The physiological relevance of this observation is not, however, immediately obvious. While the rodent mammary gland undergoes substantial growth in early lactation (reviewed by Munford, 1964), maximal mitogenic stimulation from this tissue was generally recorded in late lactation (Chapter 6). Another possibility is that the active factor is secreted by lactating epithelial cells for a purpose such as the stimulation of gut development in the neonate (Koldovsky, 1996). Alternatively, this result may reflect the responsiveness of this *in vitro* system to supplemental lipids (Chapter 4) provided by the milk fat from the lactating tissue.

Findings from these studies of ovine mammogenesis demonstrate that the stromal expression of paracrine growth factors is also altered across the course of development. These changes were particularly evident within the virgin mammary gland. An elevation in the local expression of IGF-I prior to puberty, possibly in response to systemic GH, may be an important finding with regard to the onset of allometric mammary growth and the susceptibility of the ruminant mammary gland to the negative effects of a high plane of nutrition. In contrast, IGF-II may fulfil a different requirement as its expression by mammary parenchyma remained elevated after IGF-I expression had decreased. Furthermore, the expression of bFGF, HGF and KGF mRNA within the epithelium-free mammary fat pads of ewe lambs generally increased with age (Chapters 10 and 11), raising the possibility that they function in roles other than as direct mitogens for epithelial cells. Along these lines, both HGF and KGF are implicated as regulators of branching morphogenesis (Peters *et al.*, 1994; Soriano *et al.*, 1995; Post *et al.*, 1996) and may satisfy an important requirement for the extensively-branched mammary parenchyma of the virgin ruminant mammary gland.

The combined findings of these studies in mice and sheep indicate that the mammary fat pad may be particularly important in stimulating a phase of prepubertal allometric

mammary growth. This effect in mice probably occurred due to an increase in the availability of unsaturated fatty acids, while in ewe lambs there was increased stromal expression of IGF-I which can have a pronounced proliferative effect on mammary epithelial cells. While the level of growth factors such as IGF-I within the mammary gland of prepubertal mice has not been reported, it is certainly possible that rapid prepubertal mammary growth represents a combined response to an increased local availability of both lipids and growth factors such as IGF-I, and perhaps EGF. This proposal is consistent with the demonstration that the prepubertal mouse fat pad, probably via the release of unsaturated fatty acids, markedly potentiates the proliferative effect of IGF-I and EGF *in vitro* (Chapters 2-4). Furthermore, this proposal may be of particular significance to the tumorigenic susceptibility of the mammary gland which is greatest in immature females and subsequently declines with age (Haslam, 1979).

12.1.5 A combined model for fat pad stimulation of mammogenesis

The findings of this research and their implications for local growth regulation by the mammary fat pad are best assimilated from a depiction of the cellular and chemical interrelationships which exist within the mammary gland (Figure 12.1). Mammary epithelial cells are surrounded by stromal fibroblasts, the abundance of which depends upon the species under consideration. While ovarian steroids and other mammogenic hormones (such as GH and prolactin) may directly target epithelial cells, they may also act on the adjacent stromal constituents. This latter action may alter lipid metabolism in mammary adipocytes as well as regulate the expression of paracrine growth factors by adipocytes and/or fibroblasts. While not shown, myoepithelial and epithelial cells may synthesise paracrine/autocrine mitogens. Where an upregulation in local growth factor expression is accompanied by hormone-induced lipolysis, an increased local availability of unsaturated fatty acids can potentiate growth factor-stimulated proliferation of epithelial cells via the actions of PKC. Unsaturated fatty acids and PKC may also modulate the responsiveness of epithelial cells to ovarian steroids. Furthermore, proliferating epithelial cells may locally signal to the adjacent stroma to effect an increase in stromal growth factor synthesis and fatty acid mobilisation.

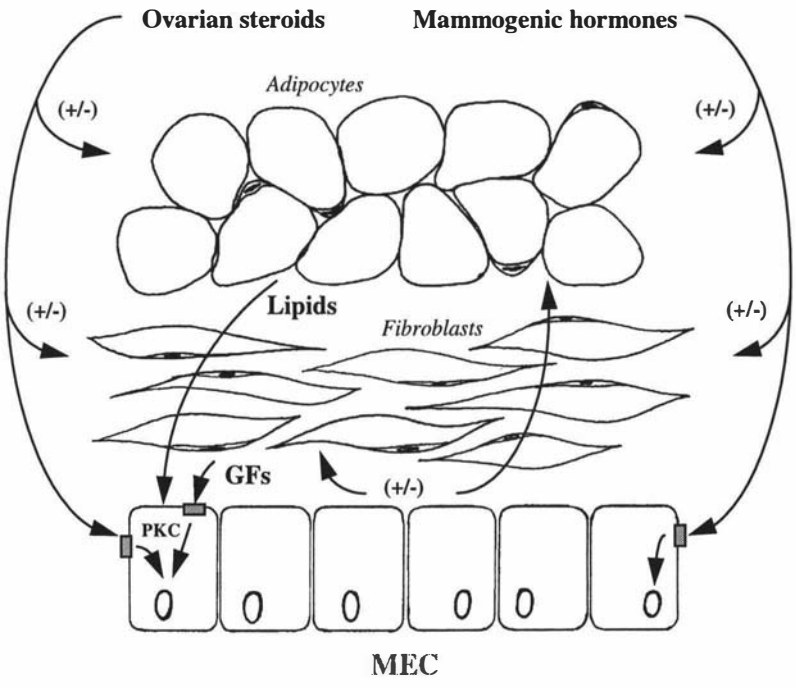


Figure 12.1 Diagrammatic representation of potential growth regulatory mechanisms within the mammary gland. Not to scale.

12.2 CONCLUSIONS

Several important conclusions can be drawn from this study which has investigated the role of the mammary fat pad during mammogenesis:

1. The mouse mammary fat pad, probably through its liberation of unsaturated fatty acids, provides a mitogenic activity which also markedly and specifically potentiates the mitogenic effect of IGF-I and EGF on mammary epithelial cells.

2. Constituents of the ruminant mammary fat pad express mRNA for several growth factors which can exert pronounced effects on the proliferation and morphogenesis of mammary epithelial cells.

3. The mitogenic capacity of the mouse mammary fat pad is regulated by systemic hormones such as oestrogen and progesterone. Such regulation may be manifest at certain stages of the oestrous cycle and ontogenesis.

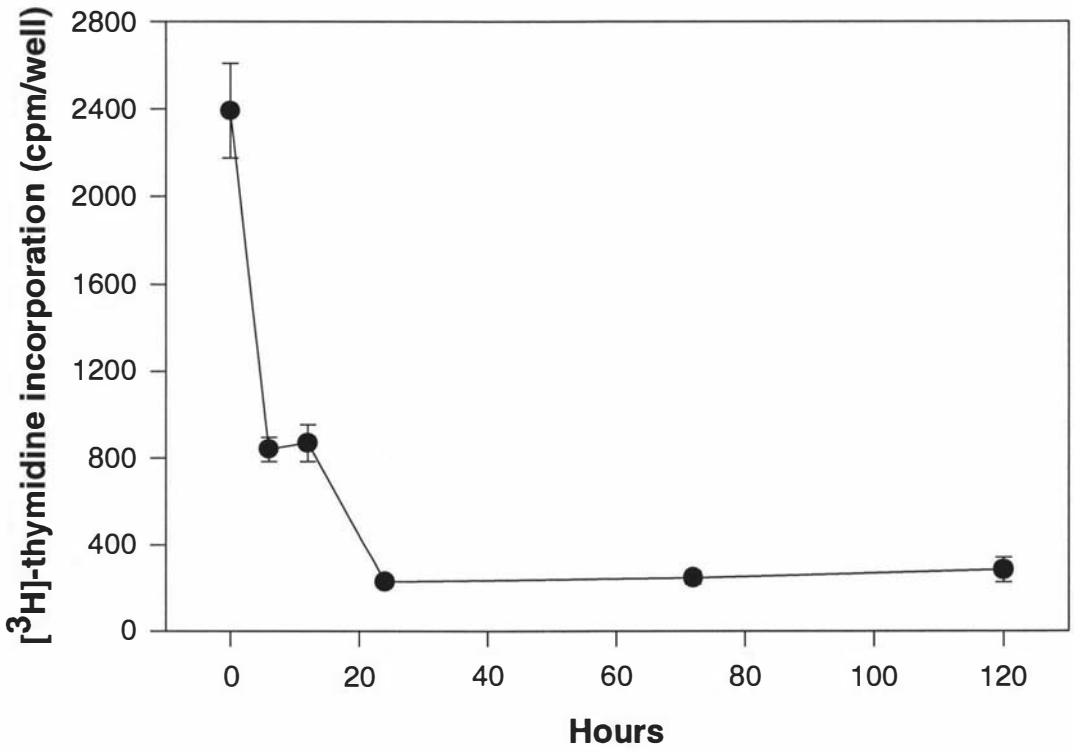
4. The stromal expression of paracrine mitogens within the ovine mammary gland is regulated by systemic influences such as oestrogen, and potentially by other hormones such as GH. Such influences may direct local growth factor expression during the course of postnatal mammogenesis.

5. Endogenous epithelium may regulate the local provision of mitogenic stimulation by the mouse mammary fat pad.

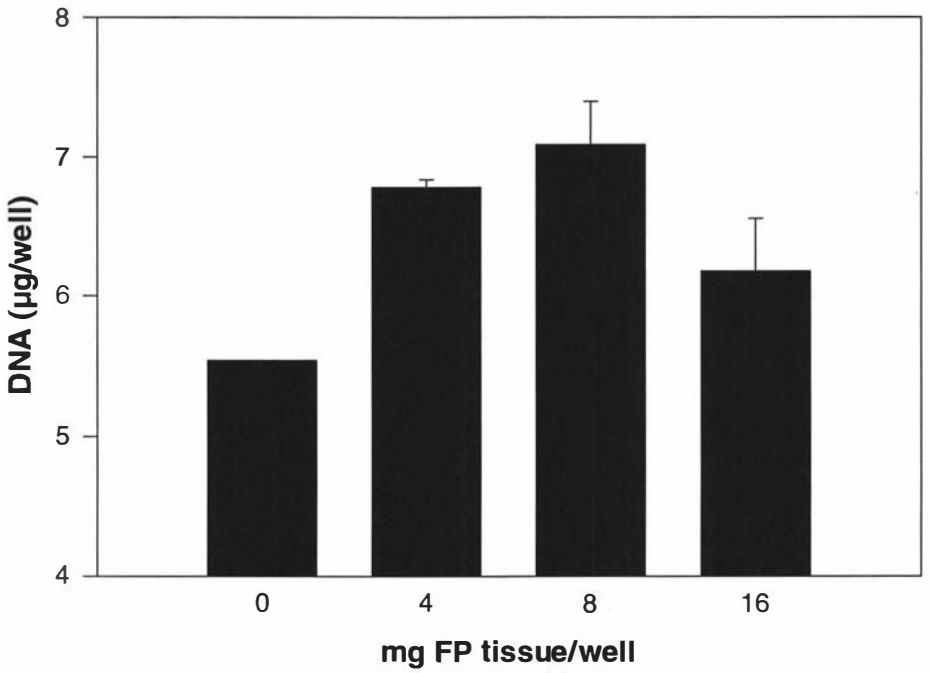
6. Endogenous epithelium may modulate the expression of stroma-derived paracrine growth factors within the ovine mammary gland.

Together, these findings indicate that the stromal environment of the mammary fat pad serves a crucial role in regulating postnatal mammogenesis.

APPENDICES

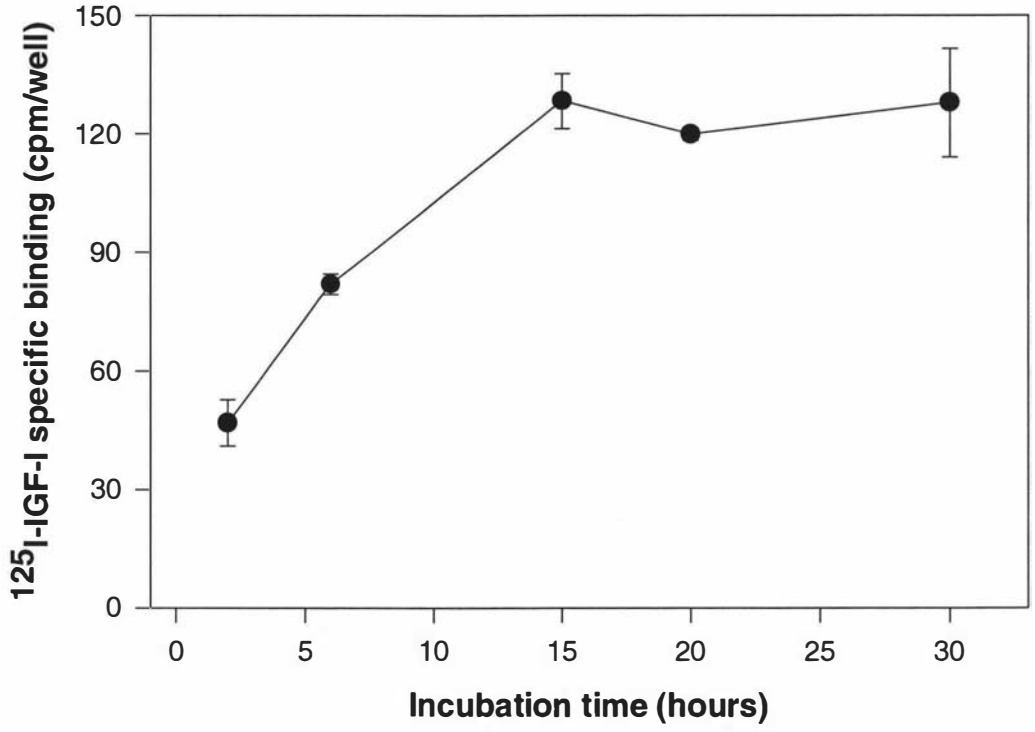


Appendix 1. Time course of DNA synthesis by COMMA-1D cells cultured in hormone-free BM.

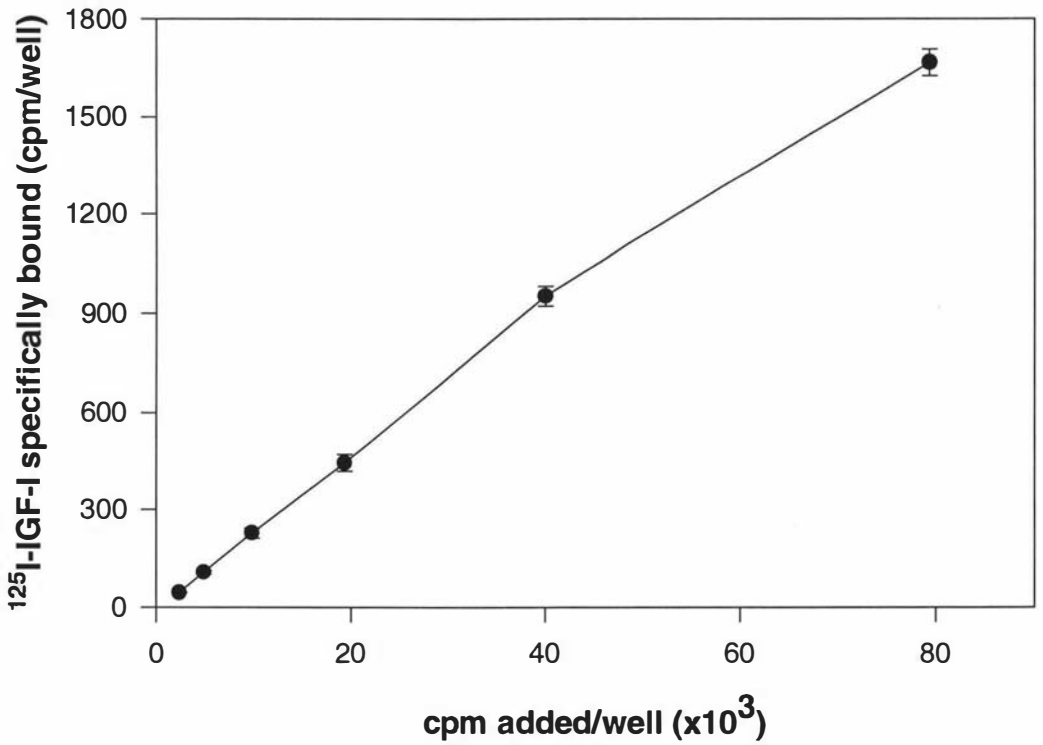


Appendix 2. Growth of COMMA-1D cells in response to medium conditioned with various amounts of mouse mammary fat pad tissue. Cultures were for 3 days.

A

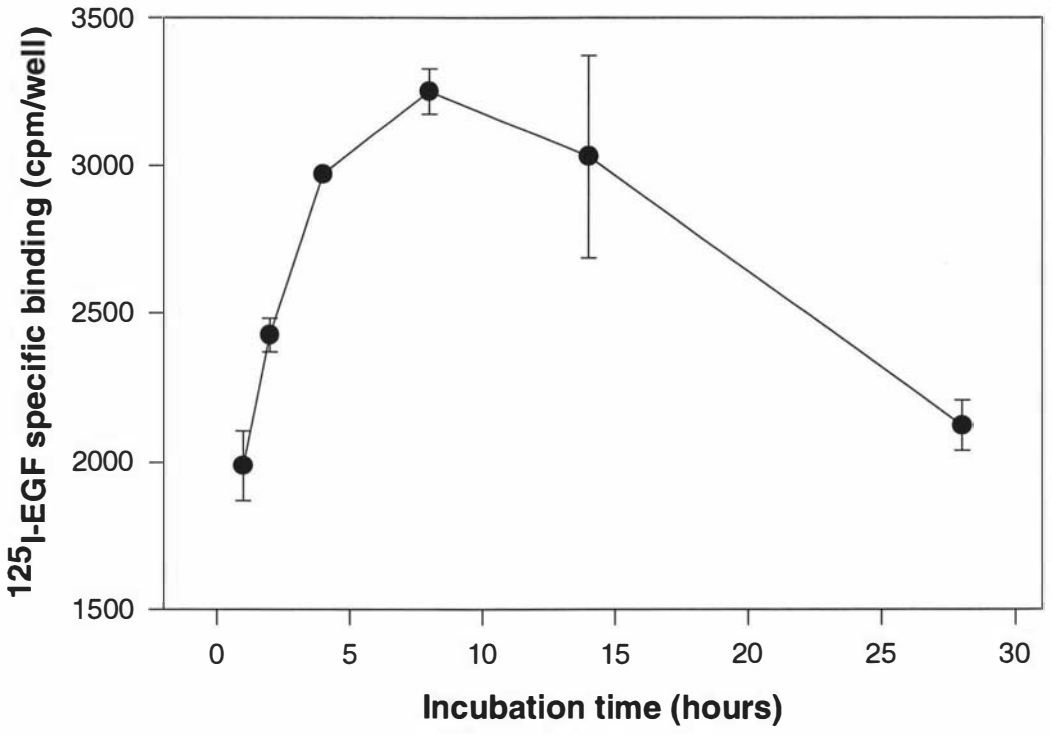


B

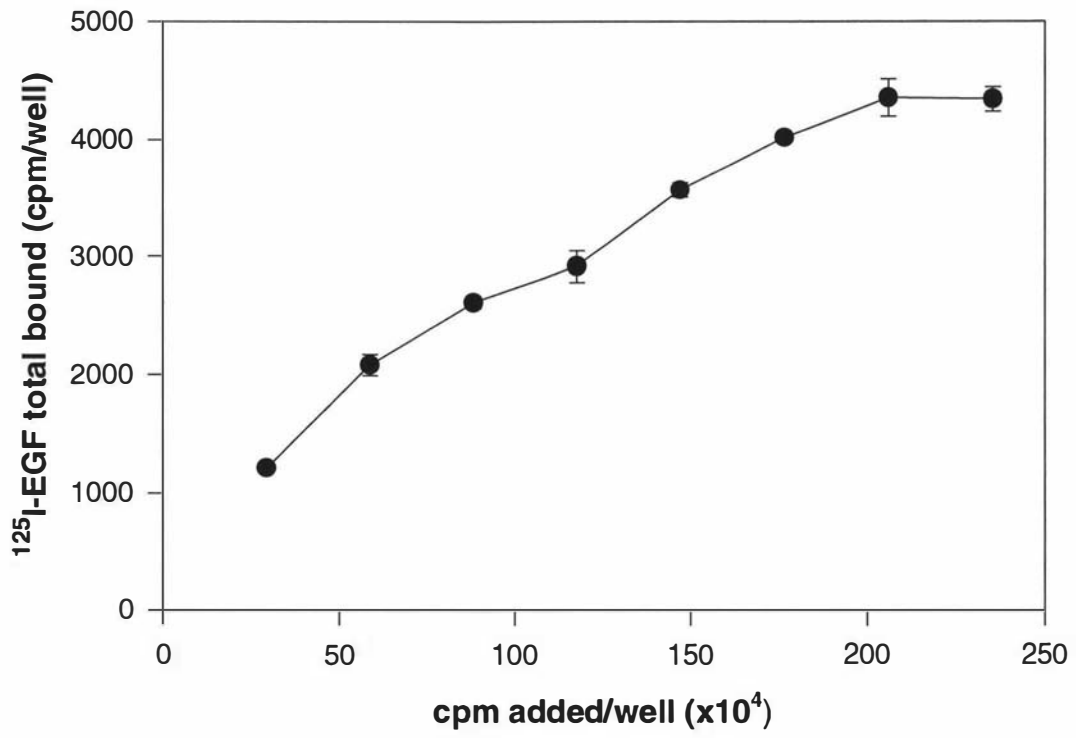


Appendix 3. IGF-I ligand binding assay parameters. (A) Effect of incubation time at 4°C on subsequent binding of ^{125}I-IGF-I to monolayers of COMMA-1D cells. Cells were incubated with approximately 3800 cpm/well. (B) Specific binding of increasing concentrations of ^{125}I-IGF-I to COMMA-1D cells. Non-specific binding was determined in the presence of 500 ng/ml IGF-I. Incubation was for 20 hours.

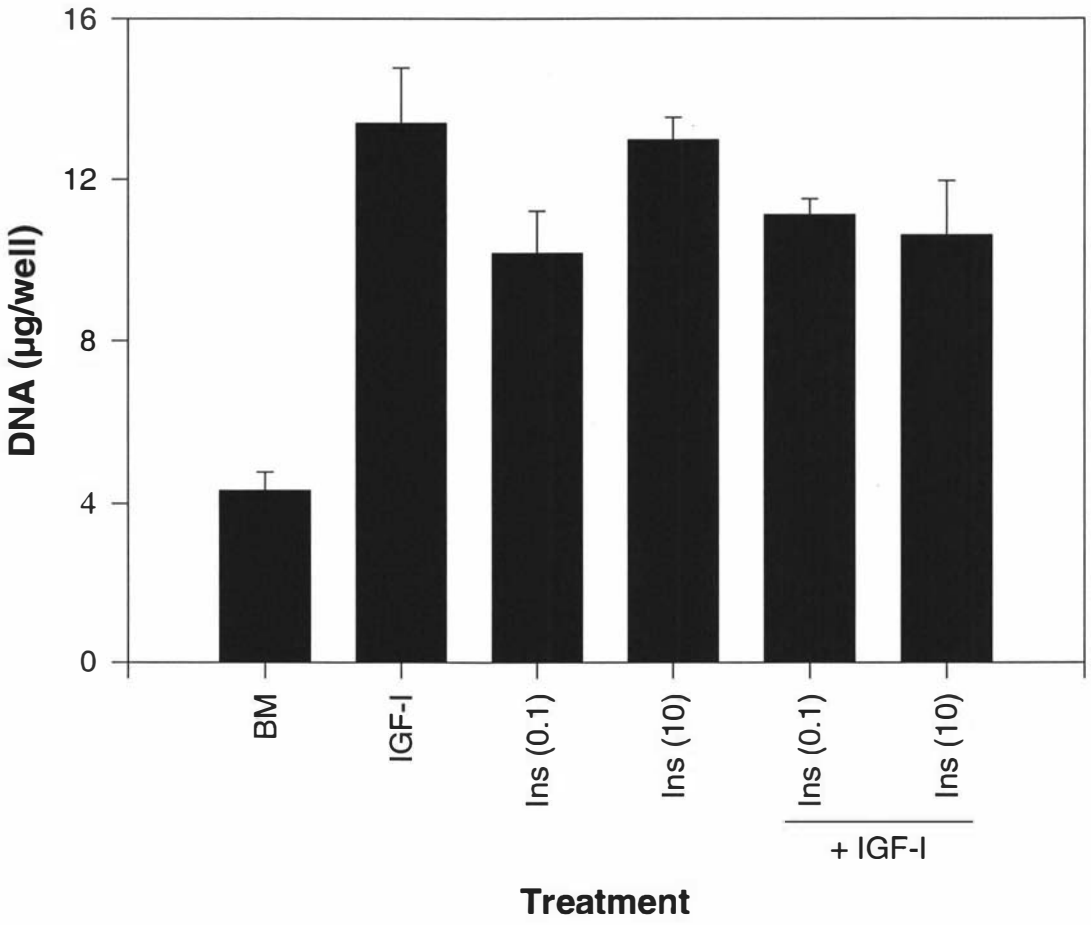
A



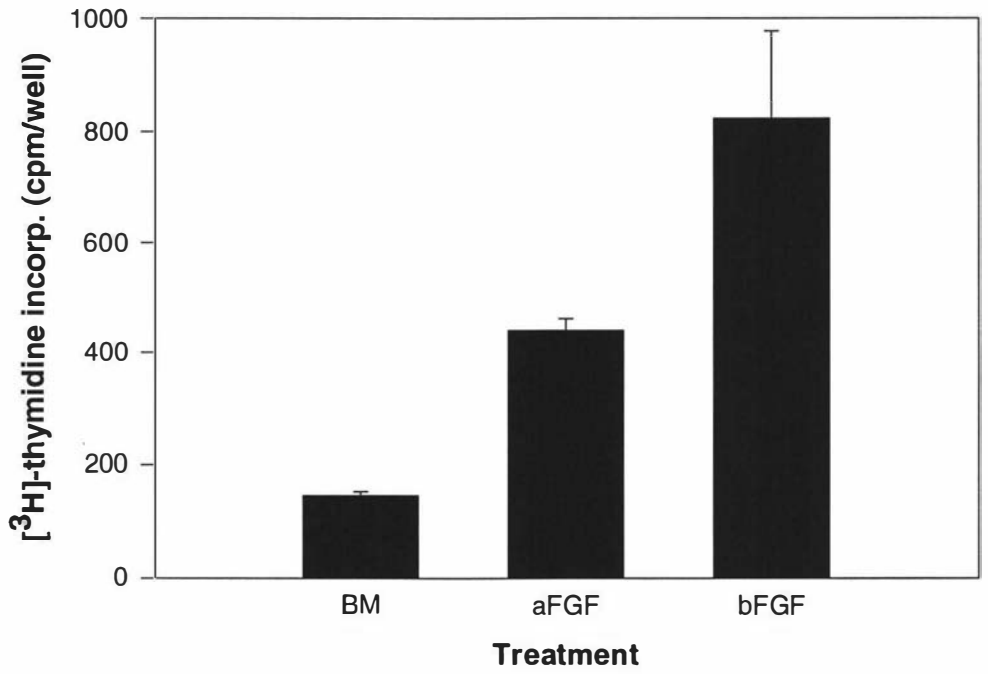
B



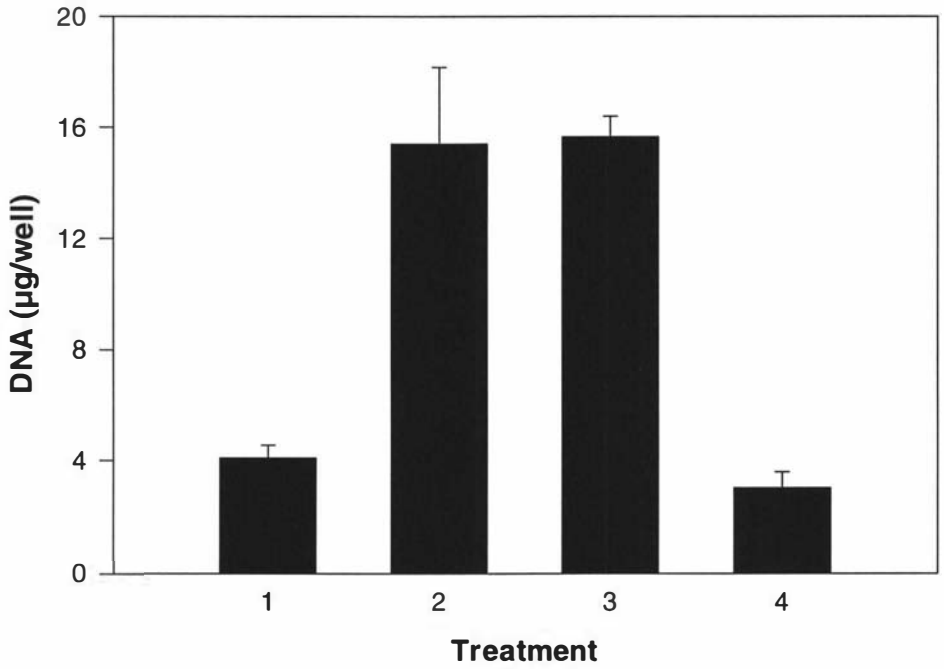
Appendix 4. EGF ligand binding assay parameters. (A) Effect of incubation time at 4°C on subsequent binding of ^{125}I-EGF to monolayers of COMMA-1D cells. Cells were incubated with approximately 1.1 x 10^5 cpm/well. (B) Specific binding of increasing concentrations of ^{125}I-EGF to COMMA-1D cells. Non-specific binding was determined in the presence of 500 ng/ml EGF. Incubation was for 5 hours.



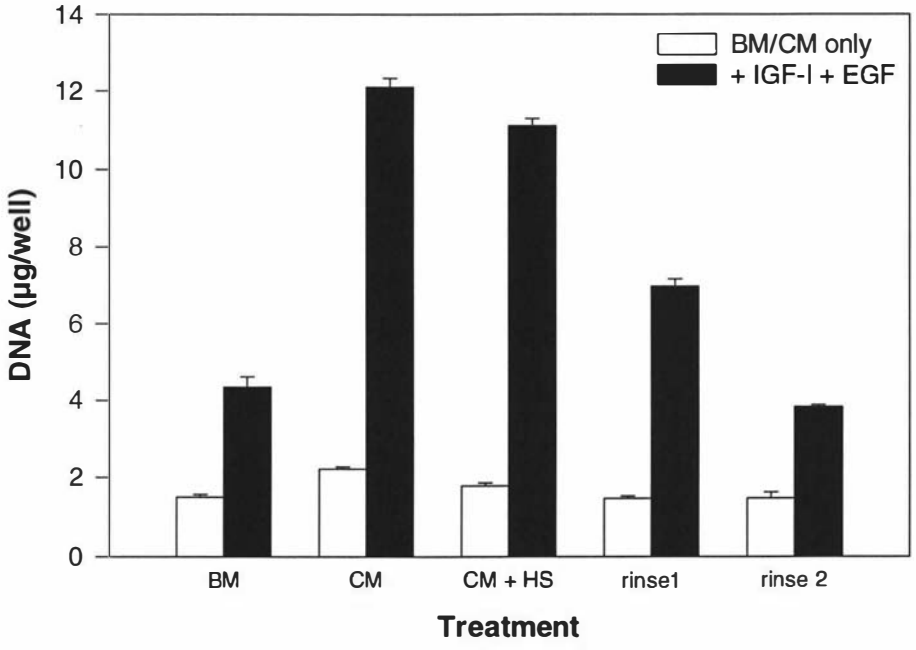
Appendix 5. Growth of COMMA-1D cells in the presence of co-cultured mouse mammary fat pad and various combinations of IGF-I (100 ng/ml) and insulin (0.1 and 10 µg/ml). Cultures were for 5 days. Data are means ± SEM (n=3).



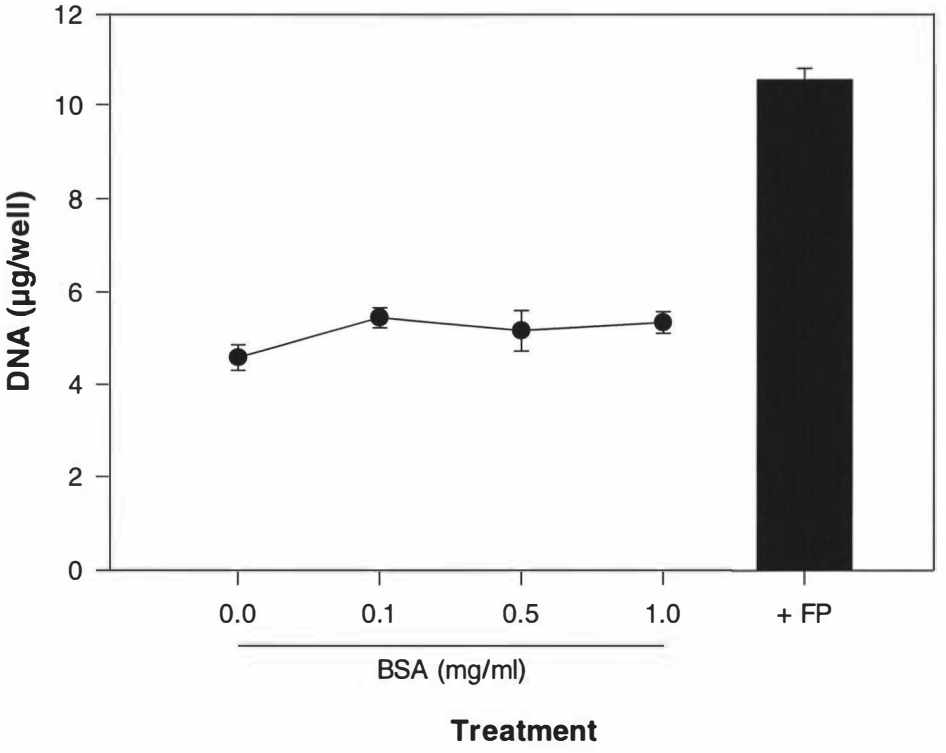
Appendix 6. DNA synthesis by COMMA-1D cells in response to 5 ng/ml acidic- and basic-FGF after 24 h culture.



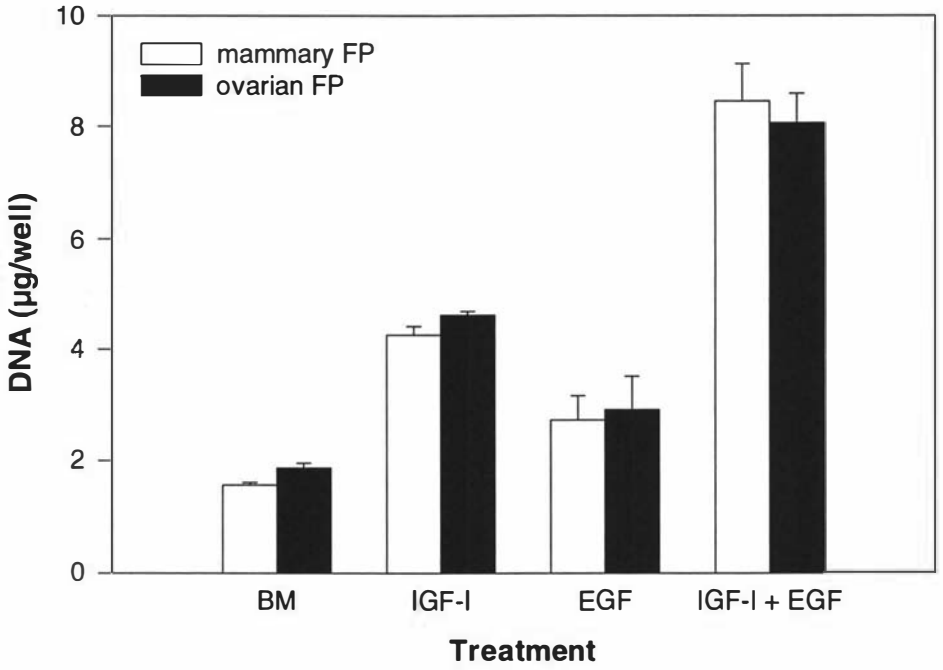
Appendix 7. Effect of trypsin on the mitogenic effect of IGF-I + EGF. COMMA-1D cells were cultured for 3 days in the following treated media. (1) BM + IGF-I + EGF treated with pre-neutralised trypsin, (2) CM + IGF-I + EGF treated with pre-neutralised trypsin, (3) CM treated with trypsin, and subsequently neutralised with protease inhibitors prior to supplementation with IGF-I + EGF, (4) CM + IGF-I + EGF which was treated with trypsin, and subsequently neutralised with protease inhibitors.



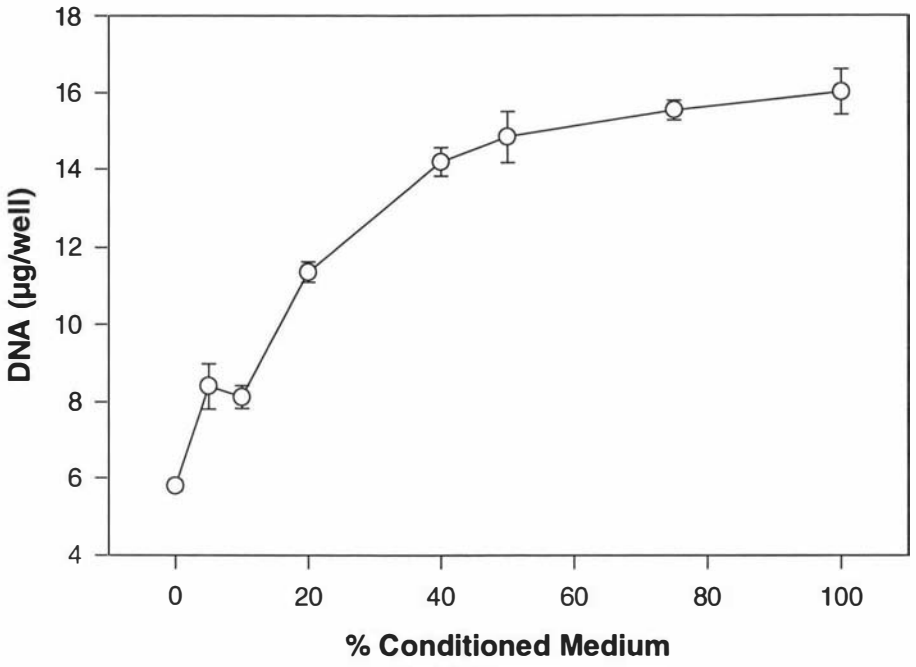
Appendix 8. Effect of treating CM with heparin sepharose (HS). Four mls of CM were passed through 1 ml of heparin sepharose, which was subsequently rinsed twice with the same volumes of BM. The various media were then added to cultures of COMMA-1D cells for 3 days in the absence or presence of IGF-I (100 ng/ml) + EGF (25 ng/ml).



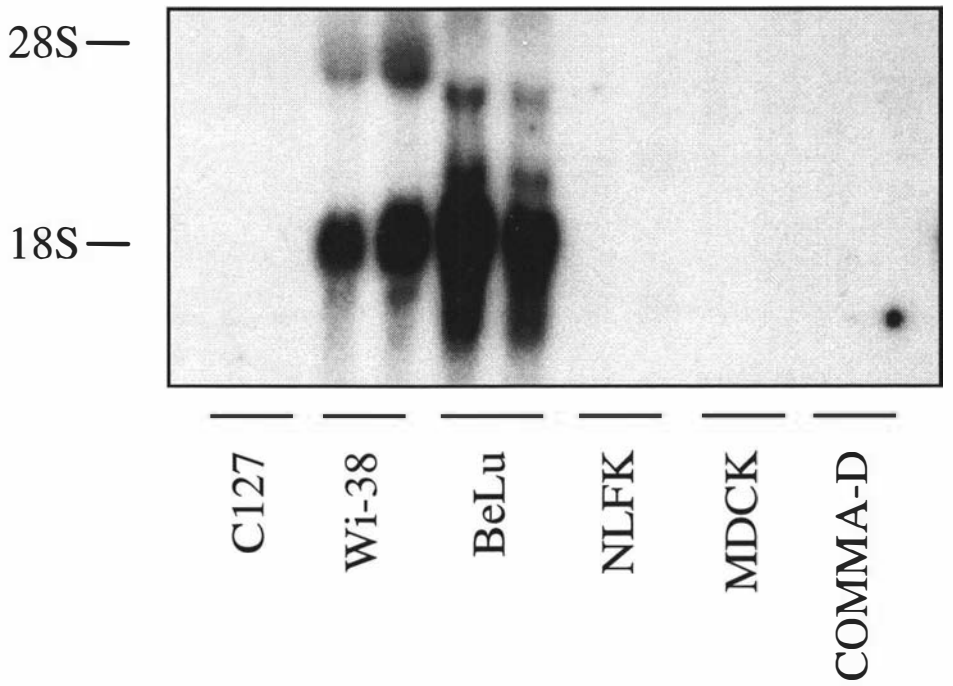
Appendix 9. Growth of COMMA-1D cells in medium supplemented with IGF-I (100 ng/ml) + EGF (25 ng/ml) and various concentrations of BSA, or in the presence of co-cultured mammary fat pad (FP). Cultures were for 3 days.



Appendix 10. Growth of COMMA-1D cells in the presence of co-cultured mammary or ovarian fat pad tissue. Cells were cultured for 5 days in BM alone, or supplemented with IGF-I (100 ng/ml) and/or EGF (25 ng/ml).



Appendix 11. Growth of COMMA-1D cells in various concentrations of CM stored for 6 months at -80°C. Cultures were supplemented with IGF-I (100 ng/ml) + EGF (25 ng/ml), and were for 3 days.



Appendix 12. Northern analysis of KGF mRNA expression in various cell lines.

Appendix 13. List of publications.

The following publications have been derived from work presented in this thesis.

Hovey, R.C., McFadden, T.B. and Mackenzie, D.D.S. (1994) Mouse mammary fat pad interacts with mitogens to stimulate epithelial growth *in vitro*. Proceedings of the New Zealand Society of Animal Production 54: 125-129.

Hovey, R.C., McFadden, T.B. and Mackenzie, D.D.S. (1994) Response of mammary epithelial cells to ovarian steroids is modulated by the mammary fat pad during the estrous cycle. Journal of Dairy Science (Supplement 1) 77: 68.

Hovey, R.C., McFadden, T.B. and Mackenzie, D.D.S. (1994) Diffusible factor(s) from the mouse mammary fat pad enhances growth of mammary epithelial cells in response to growth factors. Journal of Dairy Science (Supplement 1) 77: 117.

Hovey, R.C., McFadden, T.B. and Mackenzie, D.D.S. (1995) *3 Posters presented to the Gordon Conference for Mammary Biology.*

Hovey, R.C., McFadden, T.B. and Mackenzie, D.D.S. (1995) Mitogenic activity of lamb mammary fat pad *in vitro* is distinct from that of mouse mammary fat pad (*invited abstract*). Journal of Dairy Science 78 (Supplement 1): 153.

Hovey, R.C., McFadden, T.B., Davey, H.W. and Mackenzie, D.D.S. (1996) Keratinocyte growth factor as a paracrine regulator of ruminant mammary development. Journal of Dairy Science 79 (Supplement 1): 130.

Hovey, R.C., McFadden, T.B., Davey, H.W. and Mackenzie, D.D.S. (1996) Expression of growth factors during development of the ruminant mammary gland. Journal of Dairy Science 79 (Supplement 1): 146.

Hovey, R.C., McFadden, T.B. and Mackenzie, D.D.S. (1996) A procedure for the preparation of a parenchyma-free mammary fat pad in sheep. Journal of Dairy Science 79 (Supplement 1): 146.

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