

**GROWTH FACTOR AND  
EXTRACELLULAR MATRIX REGULATION  
OF HEIFER MAMMARY DEVELOPMENT**

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# **Growth Factor and Extracellular Matrix Regulation of Heifer Mammary Development**

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## **(ABSTRACT)**

The overall objective of this project was to investigate the role of locally derived growth factors and extracellular matrix proteins in regulating prepubertal heifer mammary development. In the first experiment, short-term treatment of heifers with GH or E increased proliferation of mammary epithelial cells. Coinciding with increased epithelial cell proliferation, IGF-I protein increased and IGFBP-3 protein decreased within mammary tissues. Thus, proliferation was associated with an apparent net increase in the biological availability of IGF-I within the mammary gland. In the second experiment, decreased mammary development and epithelial cell proliferation in response to ovariectomy coincided with decreased mammary expression of IGF-I mRNA and decreased binding of IGF-I to mammary microsomes. Taken together, these results imply an important role for locally derived IGF-I in regulating heifer mammary development. However, in contrast to our hypothesis, IGF-I mRNA did not differ between cleared or intact mammary fat pad, suggesting that expression of IGF-I mRNA is not regulated by epithelial:stromal interactions. Neither ovariectomy or epithelial:stromal interactions influenced the mRNA expression of IGFBP-3 or IGFBP-5 within mammary tissues. Ovariectomy increased the proportion of ER $\alpha$  positive mammary epithelial cells. In contrast, GH administration to prepubertal heifers did not influence the proportion of ER $\alpha$ -positive epithelial cells. Interestingly, mammary development was more severely affected in heifers ovariectomized before six weeks of age than heifers ovariectomized at three months of age, implying a critical period of ovarian stimulation during the first six weeks of age. Localization of laminin, fibronectin, and collagen in mammary parenchyma suggested specific roles for extracellular matrix proteins in regulating mammary development and ductal morphogenesis. Laminin was decreased and fibronectin was increased by ovariectomy, suggesting a possible role for interactions between the ovary and extracellular matrix

proteins within the heifer mammary gland. Finally, the mitogenic capacities of mammary tissue extracts from control and ovariectomized heifers did not differ in their ability to stimulate in vitro proliferation of MAC-T cells. In conclusion, the overall results support the hypothesis that locally derived IGF-I regulates prepubertal heifer mammary development. However, ER $\alpha$  expression and extracellular matrix proteins also appear to be important regulators of heifer mammary development.

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## LIST OF ABBREVIATIONS

ADG	average daily gain
BCA	bicinchoninic acid
BrdU	bromodeoxyuridine
bST	bovine somatotropin
CFP	cleared fat pad
cpm	counts per minute
DAB	diaminobenzidine
DMEM	dulbeccos modified eagles medium
dpm	disintegrations per minute
E	estrogen
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER $\alpha$	estrogen receptor alpha
ER $\beta$	estrogen receptor beta
ERE	estrogen response element
ERK1	extracellular signal regulated kinase-1
FAK	focal adhesion kinase
FBS	fetal bovine serum
g	gravity
GH	growth hormone
GLM	general linear model
HRP	horse radish peroxidases
IGFBP	insulin like growth factor binding protein
IGF-I	insulin like growth factor one
IGF-IR	insulin like growth factor type one receptor
IRS-I	insulin receptor subunit one
kb	kilobases
kDa	kilodaltons
KGF	keratinocyte growth factor
MAPK	mitogen activated protein kinase
MEC	mammary epithelial cells
MFP	mammary fat pad
MMP	matrix metalloproteinases
OVX	ovariectomized
PAR	parenchyma
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PRL	prolactin
PTH	parathyroid hormone
PTH-1R	parathyroid hormone receptor one
PTHrP	parathyroid hormone related peptide
RDU	relative densitometric units
RIA	radioimmunoassay
SAGE	serial analysis of gene expression
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
SSC	salt sodium citrate
TCA	trichloric acid
TDU	terminal ductular unit

## Chapter 1: Literature Review

### 1.1 Overview of Heifer Mammary Development

The mammary gland is a dynamic organ that undergoes cyclic developmental changes during the reproductive cycle, and is unique in that the majority of its development occurs after birth. The mammary gland consists of two main tissue types: epithelial or parenchymal tissue, and the mammary fat pad or stromal tissue (Figure 1.1). Mammary epithelium derives from embryonic ectoderm under the influence of mammary mesenchyme, and proceeds through several developmental stages in the fetus (described in Akers 2002), so that at birth, the beginning of the ductal system exists (Cunha 1994). At this stage, the ruminant mammary fat pad is largely devoid of epithelial tissue. In ruminants, isometric growth of the gland continues after birth until about three months of age via extension of the ductal system. Beginning before, and continuing during the first few estrus cycles, ductal growth proceeds in an allometric manner (Sinha and Tucker 1968). Subsequently, growth returns to an isometric rate until gestation. During gestation, epithelial cells proliferate exponentially and form alveoli, which ultimately consist of fully differentiated secretory cells (Tucker 1981). By the end of gestation, epithelial tissue completely penetrates the fat pad, and in cattle, mammary development is essentially complete. During the periparturient period, differentiation of epithelial cells into active secretory cells occurs in response to hormonal signals. Secretory cells make up the alveoli and are responsible for synthesis and secretion of all milk components. At the end of lactation, the mammary gland undergoes involution, a period characterized by loss of secretory activity, decreased size of alveoli, and renewal of senescent epithelial cells (Capuco and Akers 1999). Involution is essential for proper re-development of the gland and for optimizing milk yield during the next lactation (Swanson 1965; Sorensen and Enevoldsen 1991). Figure 1.2 illustrates development of the bovine mammary gland from birth through to involution.

Hormones of the ovary and pituitary, together with an array of circulating and locally produced growth factors and mitogens drive mammary growth through each developmental stage (Tucker 1981; Akers *et al.* 2000). Interactions between such

components further increase the complexity of mammary growth regulation. The focus of this project is on the local regulation of prepubertal mammary development. Although prepubertal growth is quantitatively less important than gestational development, it should not be underestimated in its importance for determining future development and milk yield potential (Sejrsen and Purup 1997; Akers *et al.* 2000). A strong correlation ( $\sim 0.8$ ) exists between the number of secretory cells and milk yield (Tucker 1966); consequently optimal parenchymal proliferation and development is essential for maximizing future milk yield. Prepubertal and pubertal ductal proliferation provide a foundation for the later development of secretory alveoli during gestation (Akers 1990; Sejrsen 1994), and effects on mammary development during this time may later be translated into milk yield. For example, over-feeding of prepubertal calves results in reduced prepubertal mammary development and subsequently decreased milk yield (Sejrsen and Purup 1997). Therefore, there appears to be a degree of plasticity within the prepubertal mammary gland that may allow manipulation of mammary development and later milk yield. Identification of techniques for optimizing prepubertal mammary development may lead to enhanced milk yield.

## **1.2 Endocrine Control of Mammary Growth**

### **Ovarian Regulation of Heifer Mammary Development**

Classic experiments performed several decades ago convincingly demonstrated a critical role for the ovary in regulating rodent mammary development (Cowie 1949; Lyons 1958). Furthermore, exogenous estrogen stimulated ductal growth in intact, but not hypophysectomized rodents, suggesting that interactions between estrogen and pituitary hormones are important in regulating mammogenesis (Reece 1956). That the ovary is an essential requirement for proper mammary development in calves was shown by an early study in which ovariectomy abolished the typical period of prepubertal allometric mammary growth (Wallace 1953). Administration of exogenous estrogen restored mammary growth in the ovariectomized animals, suggesting that estrogen was the ovarian factor responsible for the observed effects. These observations were quantitatively confirmed in a more recent experiment in which ovariectomy of calves at

2.5 months of age reduced mammary parenchyma by approximately 90% (Purup *et al.* 1993). Interestingly, in these animals, the concentration of circulating estrogen decreased by only 0.1 pg/ml, suggesting that the role of the ovary in regulating mammary development may be more complex than initially imagined. Furthermore, administration of estrogen and progesterone to calves resulted in a 40% increase in mammary weight (Maple *et al.* 1998).

Although it is clear that the ovaries are essential for proper mammary development in calves, and that exogenous estrogen is potently mammogenic, the mechanisms by which ovarian secretion control mammogenesis are not established. Perhaps interactions with other endocrine and local factors are responsible for mediating ovarian effects on mammary development. Estrogen stimulates proliferation of ductal cells in pubertal heifers, as measured by <sup>3</sup>H-thymidine incorporation into DNA (Woodward *et al.* 1993). Although explants of bovine mammary tissue proliferate in response to estrogen (Akers *et al.* 2000), immortalized epithelial cells do not (Woodward *et al.* 1994), suggesting a requirement for interactions between stromal and epithelial tissues in mediating the full estrogen response. Recently, the ER $\alpha$  has been detected in bovine mammary epithelial tissue (Akers *et al.* 2000; Capuco *et al.* 2002) implying that estrogen acts directly on the mammary epithelium to stimulate cell proliferation. Possibly, interactions between mammary parenchyma and stroma regulate expression of ER (Akers *et al.* 2000).

The ER is a member of the steroid receptor super-family (Mangelsdorf *et al.* 1995) and is a ligand-activated transcription factor consisting of a hormone-binding domain, a DNA-binding domain and a transcriptional activation domain. Once activated by estrogen, the ER activates transcription by binding to the estrogen response element (ERE), and initiates transcription of specific genes. There are two distinct forms of the estrogen receptor, ER $\alpha$  and ER $\beta$ , which are each encoded by separate genes (Kuiper *et al.* 1996). Although mRNA for both ER $\alpha$  and ER $\beta$  have been detected in mammary tissue of mice (Couse and Korach 1999; Saji *et al.* 1999) and humans (Jarvinen *et al.* 2000), mRNA for ER $\beta$  is considerably less abundant than ER $\alpha$ . In agreement with mammary expression of the two receptor subtypes, disruption of ER $\alpha$  results in complete loss of postnatal

mammary development and function (Korach 1994), but disruption of ER $\beta$  does not affect development of mammary epithelial or stromal tissue. In the rodent mammary gland, ER $\alpha$  is distributed throughout epithelial and stromal tissues (Haslam 1989), both of which are required for mammary development (Mueller *et al.* 2002). Adding to the complexity of ER action is the observation that ER ligands produce different biological effects in different tissues (Paige *et al.* 1999) and that although similar in mechanism, signaling through ER $\alpha$  and ER $\beta$  leads to transcription of different estrogen-responsive genes and subsequently, distinct biological responses (Kuiper *et al.* 1996). To date, only ER $\alpha$ , and not ER $\beta$  have been detected in bovine mammary tissue. Traditionally, steroid receptors have thought to be activated solely by their steroid ligands, which diffuse into the cell to bind the receptor within the nucleus. However, interactions between steroid receptors and extra-cellular ligands do occur, as illustrated by activation of the progesterone receptor by dopamine (Power *et al.* 1991). Accumulating evidence suggests that ER is also activated by extracellular ligands, such as growth factors, including IGF-I. For example, IGF-I can activate ER $\alpha$  and induce subsequent transcription of estrogen-responsive genes in breast cancer cell lines (Lee *et al.* 1997). Anti-estrogens, such as tamoxifen, may also alter ER action by altering the conformation of the ER and consequently blocking its biological activity (Paige *et al.* 1999). Thus, the role of ER in modulating heifer mammary growth may be more complex than the simple ligand-activation of gene expression originally proposed.

### **Regulation of Heifer Mammary Development by the Somatotrophic Axis**

Growth hormone (GH) is produced in the pituitary gland and is released under the control of growth hormone releasing hormone (GRH) and growth hormone inhibiting hormone (GIH). GH influences the growth and metabolism of many tissues, although its major effects are in the development of bone and muscle (reviewed in Daughaday 2000). However, GH is an also important regulator of mammary development, as well as lactogenesis and galactopoiesis in many species. Early studies on hypophysectomized rodents demonstrated that pituitary hormones were essential for proper mammary development (Lyons 1958). Further investigation showed that GH was more important during pre- and peripubertal ductal growth than prolactin (PRL), which, along with

progesterone, stimulates alveoli development during gestation (Lyons 1958; Tucker 1981).

Administration of GH to pubertal heifers stimulates mammary growth, resulting in increased parenchymal mass (Sejrsen *et al.* 1986; Purup *et al.* 1993; Carstens *et al.* 1997), cell number and cell metabolism (Radcliff *et al.* 1997). Recent reports suggest that the GH receptor exists in ruminant mammary epithelial cells (Glimm *et al.* 1990; Sinowatz *et al.* 2000; Plath-Gabler *et al.* 2001). However, binding of GH to mammary epithelial cells has not been demonstrated (McFadden *et al.* 1990; Akers 1990), suggesting that the effects of GH on mammary development are indirect. Possibly, GH effects are mediated through the mammary fat pad, via paracrine production of IGF-I. Several lines of evidence from both in vivo and in vitro studies support the hypothesis that the IGF-I axis mediates the effects of GH in the mammary gland. For example, mammary growth and serum GH concentrations are correlated (Sejrsen *et al.* 1983), and GH stimulates IGF-I secretion in prepubertal heifers (Purup *et al.* 1993). Increased milk yield, in response to administration of GH, correlates with increased serum IGF-I concentrations (Cohick *et al.* 1989). GH also stimulates an increase in hepatic IGF-I mRNA in lactating cows (Sharma *et al.* 1994). In vitro, GH has no proliferative effect on explant or immortalized cell cultures but IGF-I is a potent mitogen (Akers 1990; Hovey *et al.* 1998b; Weber *et al.* 1999). Addition of extracts prepared from prepubertal heifer mammary tissue had a mitogenic effect on primary cell cultures that was more effective than serum or IGF-I alone (Weber *et al.* 1999). Addition of IGFBP-3 or antibodies against IGF-I completely abolished this effect, suggesting that IGF-I was a major contributor to the observed mitogenic activity. In addition, transgenic mice overexpressing IGF-I exhibit premature mammary parenchymal growth, further illustrating the influence of IGF-I on ductal development (Weber *et al.* 1998).

Several studies have demonstrated that mammary IGF-I expression is limited to the fat pad. In hypophysectomized rats, implants of GH increased IGF-I expression in the mammary fat pad (Walden *et al.* 1998). IGF-I expression was also localized primarily to the stromal portion of the gland in prepubertal ewes; furthermore, expression increased

during periods of rapid mammary development (Hovey *et al.* 1998a). Similarly, IGF-I mRNA in heifers was localized to the mammary stroma and increased following GH treatment (Weber *et al.* 2000b). However, the roles of GH and IGF-I in regulating mammary development are more complex because the GH-IGF-I axis is uncoupled by nutrient restriction. Heifers raised on a rapid-rearing program during the prepubertal period exhibit reduced mammary development and serum concentrations of GH, but elevated concentrations of serum IGF-I. Explants from overfed heifers were less responsive to IGF-I stimulated cell proliferation (Sejrsen *et al.* 1999) and mammary extracts from similarly treated heifers were less capable of stimulating mitogenesis of primary mammary epithelial cells (Weber *et al.* 2000a). Possibly, the reduction in mammary development caused by overfeeding may be explained by changes in local production of IGF binding proteins (IGFBP). For example, overfed heifers have a higher concentration of mammary IGFBP-3 (Sejrsen *et al.* 2000). GH may also interact with the ER pathway as expression of ER was increased in mice treated with GH (Feldman *et al.* 1999). Clearly, GH is an important regulator of mammogenesis in prepubertal heifers; however, the exact mechanisms by which this hormone acts remain undefined.

### **1.3 Local Control of Mammary Development**

#### **Influence of the Local IGF-I Axis**

As described in the earlier sections, IGF-I appears to be an important mammary mitogen. Increased expression of IGF-I mRNA in ovine mammary stroma coincides with periods of accelerated mammary growth (Hovey *et al.* 1998a), suggesting a role for local IGF-I in mediating mammary development. Overexpression of IGF-I in transgenic mice resulted in premature mammary development (Weber *et al.* 1998), reduced apoptosis, and delayed involution (Hadsell *et al.* 1996) suggesting roles for IGF-I in regulating mammary epithelial proliferation and survival. Mitogenic actions of IGF-I are mediated through the IGF-I receptor (IGF-IR) pathway. The IGF-IR is a receptor tyrosine kinase (RTK) and is similar in structure to the insulin receptor. Once activated, the receptor forms a homodimer or a heterodimer with an insulin receptor subunit, and tyrosine residues in the cytoplasmic domain are autophosphorylated (Adams *et al.* 2000). As a result, a number

of intracellular signaling molecules are activated, including insulin receptor substrate I (IRS-I) and MAP Kinase (MAPK), ultimately resulting in altered gene expression and subsequent cell proliferation.

Interaction of IGF-I with E may involve the ability of E to activate components of the IGF-I pathway. For example, treatment of human mammary epithelial cells (MCF-7) with E resulted in increased expression of the IGF-IR and IRS-I, resulting in increased IGF-I signaling through MAPK and increased proliferation (Lee *et al.* 1999). Loss of ER $\alpha$  from a similar cell line coincided with decreased IGF-IR and IRS-I mRNA expression, and loss of IGF-I stimulated cell proliferation (Oesterreich *et al.* 2001). Furthermore, E treatment of ovariectomized mice stimulated tyrosine phosphorylation of the IGF-IR and IRS-I, but not the EGF or PDGF receptors (Richards *et al.* 1996).

The IGF-I binding proteins (IGFBP) have several functions, which include transporting IGF-I through the circulation, increasing IGF-I half-life, sequestering, and localizing IGF-I to target cells. The mammary gland produces several forms of IGFBP as detected by northern blotting and ligand binding assays (Campbell *et al.* 1991; Gibson *et al.* 1999; Weber *et al.* 1999). Different IGFBP have distinct roles in mammary development. IGFBP-2 and -3 appear to be more important during development and lactation, while IGFBP-5 tends to be associated with involution (Tonner *et al.* 1997). IGF-I can induce expression of IGFBP; cells transfected with an IGF-I construct exhibit increased IGFBP-3 expression (Romagnolo *et al.* 1994b), and treatment of fibroblast (Conover 1991a) and bovine mammary epithelial (Cohick and Turner 1998) cell lines with exogenous IGF-I increased IGFBP expression.

In vivo, the majority of IGF-I is bound to IGFBP (Clemmons 1993); therefore, it is clearly important to include analysis of IGFBP when trying to further understand the biological effects of IGF-I. Whether IGFBP enhance or inhibit the effects of IGF-I within the mammary gland is not established. In MAC-T cells transfected with IGFBP-3, the ability of IGF-I to stimulate cell proliferation was enhanced (Cohick 1998), whereas the addition of IGFBP-3 to mammary extracts reduced the ability of the extracts to

stimulate proliferation of primary mammary epithelial cells (Weber *et al.* 1999). Part of the difference in responses to IGFBP-3 appears to be due to interaction with the extracellular matrix. Association of IGFBP-3 with the cell surface decreases its affinity for IGF-I (McCusker *et al.* 1990; Conover 1991b), possibly due to proteolytic processing (Conover and DeLeon 1994). Consequently, expression of different ECM proteins may alter the response of the cell to IGFBP, consequently altering the availability of IGF-I to stimulate cell proliferation.

IGFBP are not always dependent on IGF-I to exert a biological effect on cells. For example, IGFBP-3 induced apoptosis in an immortalized prostate cell line via IGF-I independent mechanisms (Rajah *et al.* 1997). These effects may be mediated through a specific IGFBP-3 receptor (Leal *et al.* 1997; Yang *et al.* 2002) followed by downstream signaling cascades involving activation of the orphan nuclear receptor TR3 and subsequent release of cytochrome c and subsequent apoptosis (Lee *et al.* 2002). Although ruminant mammary tissue produces IGFBP during the prepubertal period (Weber *et al.* 1999), the role in regulating GH and E mediated mammary growth remains to be fully elucidated. That mammary IGFBP-3 is more abundant in high-fed heifers than low-fed heifers, corresponding with reduced mammary growth, suggests that this protein may be inhibitory to mammary growth.

### **Effects of KGF, PTHrP and Leptin on Prepubertal Mammary Development**

Although evidence accumulated thus far suggests an integral role for IGF-I and related proteins in promoting prepubertal mammary development, mammary development is likely achieved through the actions of multiple locally derived growth factors. Although many growth factors are produced within the mammary gland, those considered here (KGF, leptin, and parathyroid hormone related peptide; PTHrP) have been selected because rodent and cell culture models suggest roles in mammary development, mammary epithelial cell proliferation or epithelial-stromal interactions.

KGF is a member of the fibroblast growth factor family and was originally identified as a growth factor that acted specifically on epithelial cells to stimulate proliferation (Rubin *et*

*al.* 1989). A role for KGF in promoting mammary development in species other than bovine has been previously suggested. In the mouse, KGF is expressed by mammary stromal cells, while its receptor is expressed by mammary epithelial cells (Finch *et al.* 1989). This implies a paracrine mechanism of action whereby stromal derived KGF may stimulate proliferation of epithelial cells. Similarly, within the ovine mammary gland, fibroblasts but not epithelial cells express KGF (Hovey *et al.* 2001). Furthermore, KGF caused proliferation of bovine mammary epithelial cells (MAC-T) but not for primary bovine mammary stromal cells. Administration of E to prepubertal mice increased the mammary expression of KGF (Pedchenko and Imagawa 2000). In contrast to the mouse data, expression of KGF within the ovine mammary gland was reduced by exogenous E and increased by ovariectomy (Hovey *et al.* 2001). Little is known about the importance of KGF in mediating bovine mammary growth and its expression within the bovine mammary gland has not been reported.

PTHrP was initially discovered as a tumor product that causes hypercalcemia in certain cancers (Burtis *et al.* 1987). Although it was named after its structural similarity to parathyroid hormone (PTH), the two proteins have distinct functions. While PTH is made solely by the parathyroid gland, and acts in the manner of a classic peptide hormone, PTHrP is made in a variety of tissues and does not circulate. Consequently, the actions of PTHrP are confined to local autocrine, paracrine, or intracrine mechanisms. PTH and PTHrP share a widely expressed G-protein coupled receptor (PTH-1R; Juppner *et al.* 1991). The mechanisms by which PTHrP may control development of the mammary epithelium appear to be paracrine, because, in mice, mammary epithelial cells express PTHrP and stromal cells express the PTH-1R (Dunbar *et al.* 1998). In mice, PTHrP mRNA is expressed by mammary epithelial cells during prepuberty, puberty, and pregnancy (Dunbar *et al.* 1998). In cows, PTHrP mRNA was expressed by mammary epithelial cells during late pregnancy and lactation (Wojcik *et al.* 1998). However, the exact mechanisms of PTHrP action within the mammary gland are unclear. Disruption of the gene for either PTHrP or PTH-1R in mice inhibits mammary epithelial development (Wysolmerski *et al.* 1998), and overexpression of PTHrP causes defects in ductal branching morphogenesis (Dunbar and Wysolmerski 1999). Furthermore, transient

overexpression of the PTHrP during embryogenesis results in impaired ductal branching during puberty, whereas overexpression during puberty impairs ductal elongation (Dunbar *et al.* 2001), suggesting distinct roles for PTHrP during different stages of mammary development. To date, hormonal regulation of expression of PTHrP and PTH-1R in prepubertal heifers has not been investigated.

Leptin is the recently discovered product of the Ob gene and acts via a specific cytokine receptor to maintain homeostasis of body weight and adiposity (Ahima and Flier 2000). The mouse Ob gene is expressed as a 4.5kb mRNA, which is subsequently translated into a 16kDa circulating protein, some of which is bound to plasma proteins (Ahima and Flier 2000). The majority of leptin is produced by white adipose tissue. Expression of leptin in adipose tissue is altered by feeding and obesity, as well as endocrine status, particularly insulin and glucocorticoid concentrations (Considine and Caro 1997). Recent evidence also suggests that GH may regulate leptin expression, for example, administration of bGH to castrated male cattle increased expression of leptin mRNA within subcutaneous adipose tissue (Houseknecht *et al.* 2000). The effect of GH differs for subcutaneous versus visceral adipose tissue and does not appear to be mediated by IGF-I (Isozaki *et al.* 1999). Leptin acts primarily on nerve cells of the hypothalamus, where the majority of the long-form leptin receptor is expressed (Isozaki *et al.* 1999); however, expression of a short-form receptor, and to a lesser extent, the long form receptor, has been detected in peripheral tissues, suggesting multiple functions for the leptin protein (Laud *et al.* 1999). With respect to the mammary gland, it is possible that leptin may act as a paracrine regulator of epithelial growth, proliferation or survival. Leptin receptor expression in ruminants is greatest during midpregnancy, coinciding with a period of rapid growth (Laud *et al.* 1999), and leptin is expressed in both mammary epithelial and mammary stromal cells of ruminants (Amstalden *et al.* 2000) and humans (Smith-Kirwin *et al.* 1998). Addition of leptin to MAC-T cells inhibited proliferation, but leptin had no effect on proliferation of primary cells from prepubertal heifers (Chilliard *et al.* 2001). Leptin may also act to mobilize adipose tissue for advancement of the mammary parenchyma into the mammary fat pad. Currently, there has been little

research, especially in ruminants, to allow formulation of more detailed hypotheses about the possible mammogenic roles of leptin.

### **Mammary Stroma is Integral for Optimal Mammary Development**

The fat pad of the ruminant mammary gland consists primarily of adipocytes and fibroblasts, as well as mast cells, endothelial cells, and lymphatic cells (Hovey *et al.* 1999). Although the fat pad provides structural support to the gland, it also plays an integral role in regulating mammary development. Transplantation studies in rodents demonstrated that the fat pad is required for complete parenchymal development (Hoshino and Gardner 1967). The mammary fat pad also produces numerous growth factors (e.g. IGF-I, KGF) and extra-cellular matrix components, which may directly stimulate mammary epithelial proliferation, mediate the effects of systemic hormones, interact with developing epithelium to modulate gene expression, or alter responsiveness to mammogenic stimuli (Hovey *et al.* 1999). For example, in mice, transplantation of epithelial tissue not responsive to estrogen onto mature mammary stroma induced premature estrogen responsiveness within the epithelial tissue (Haslam and Counterman 1991). Figure 1.3 (Hovey *et al.* 1999), illustrates a working model of regulation of mammary development, emphasizing the integral role played by the fat pad. Systemic hormones may act on the mammary fat pad (adipocytes or fibroblasts), altering expression of growth factors, such as IGF-I, which then act through specific epithelial cell receptors to have a positive or negative effect on epithelial cell proliferation. An important component of this model is the occurrence of ‘cross-talk’ between the developing epithelium and the fat pad. The epithelium may influence the response of the stroma to systemic hormones through positive or negative feedback interactions, which in turn alter the expression of growth factors and/or other genes. For example, the expression of IGF-I in pubertal ewes was lower in epithelium depleted fat pad compared with fat pad adjacent to developing epithelium (Hovey *et al.* 1998a).

### **Regulation of Mammary Development by the Extracellular Matrix**

The extracellular matrix (ECM) consists of secreted glycoproteins and polysaccharides, which have historically been thought to function as structural support for neighboring

cells. Recently, however, it has become clear that the ECM plays a much more integrated role in mediating cell growth, differentiation, motility, and survival (for review, see Boudreau and Jones 1999). Despite detailed knowledge of ECM biochemistry, literature regarding the importance of ECM proteins in regulating cell proliferation and mammary development in ruminants is disturbingly sparse.

Studies in rodents (Keely *et al.* 1995b) have demonstrated that mammary expression of collagen I, collagen IV, and laminin is temporally and spatially regulated throughout mammary development, suggesting that these proteins may play an important role in mediating mammary development. In particular, greatest expression of these proteins prior to and during puberty corresponded to periods of rapid mammary growth. Interestingly, *in situ* hybridization studies of the same proteins revealed that stromal cells, not epithelial cells, were primarily responsible for synthesis of the basement membrane components collagen IV and laminin (Keely *et al.* 1995b). In another study, expression of integrins  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ , and  $\beta 4$  was detectable at puberty and increased during pregnancy (Huang and Ip 2001). While mRNA expression of  $\alpha 5$ ,  $\alpha 6$ , and  $\beta 1$  peaked during lactation, the mRNA for  $\beta 4$  decreased. Expression of each of the integrins decreased during involution to reach similar levels as during puberty. Integrin expression was also different between normal and cancerous mammary glands, suggesting a possible role for integrins in aberrant mammary growth. ECM proteins may also interact with ovarian steroids. For example, expression of mammary fibronectin in mice increased from three to ten weeks of age, and significantly decreased following ovariectomy (Woodward *et al.* 2001). Administration of E partially restored the fibronectin expression levels in ovariectomized mice. Expression of the fibronectin receptor,  $\alpha 5\beta 1$  integrin, increased between five and ten weeks of age and was down regulated during pregnancy and lactation, as well as following ovariectomy. In a separate study, collagen, fibronectin, and laminin synergized with IGF-I to stimulate proliferation of primary mammary epithelial cells (Woodward *et al.* 2000b). This synergy was the result of increased expression of IGF-I receptor, as well as a decrease in IGFBP-3, suggesting that ECM molecules may interact with growth factor pathways within the mammary gland. Moreover, (Woodward *et al.* 2000a) found that laminin inhibited E-stimulated

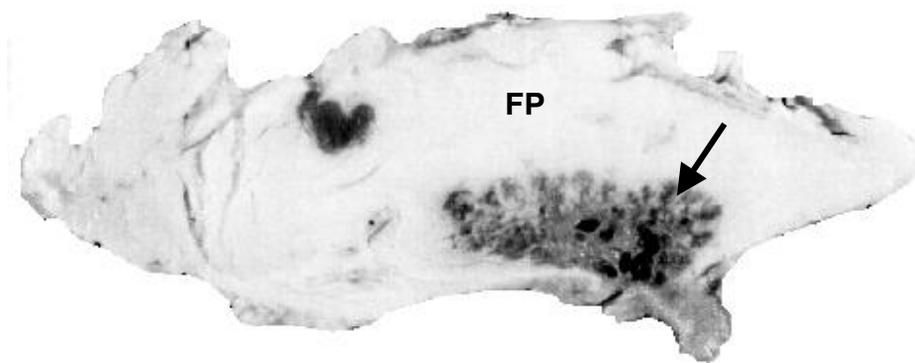
proliferation in MCF-7 breast cancer cells through decreased activity of the estrogen response element (ERE). Laminin had no effect on expression of the ER, IGF-IR, or EGFR.

Taken together, results from the above experiments suggest an important role for ECM proteins in mediating mammary development including IGF-I and E-stimulated mammary epithelial proliferation. Several other lines of evidence suggest a role for interactions between the ECM, growth factors, systemic hormones, and intracellular signaling pathways in mediating cellular processes. For example, mice expressing a dominant-negative mutant of the  $\beta 1$  integrin protein exhibited aberrant mammary development, including reduced epithelial proliferation and increased apoptosis (Faraldo *et al.* 2001). Phosphorylation of intracellular signaling components such as ERK1, JNK, and the p52 Shc isoform were also decreased, suggesting that the  $\beta 1$  integrin signals through the MAPK pathway to influence cell proliferation. In a separate study, mammary epithelial cells cultured on laminin were protected from apoptosis and showed enhanced phosphorylation of IRS-I (Farrelly *et al.* 1999), suggesting that ECM signals may be mediated by the IGF or insulin signaling pathways. ECM signals may also be mediated through the focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, which associates with focal adhesions (Schaller 2001). Although there is little information available to date regarding the role of ECM molecules in regulating heifer mammary development, evidence accumulated thus far from rodent models suggests a likely role.

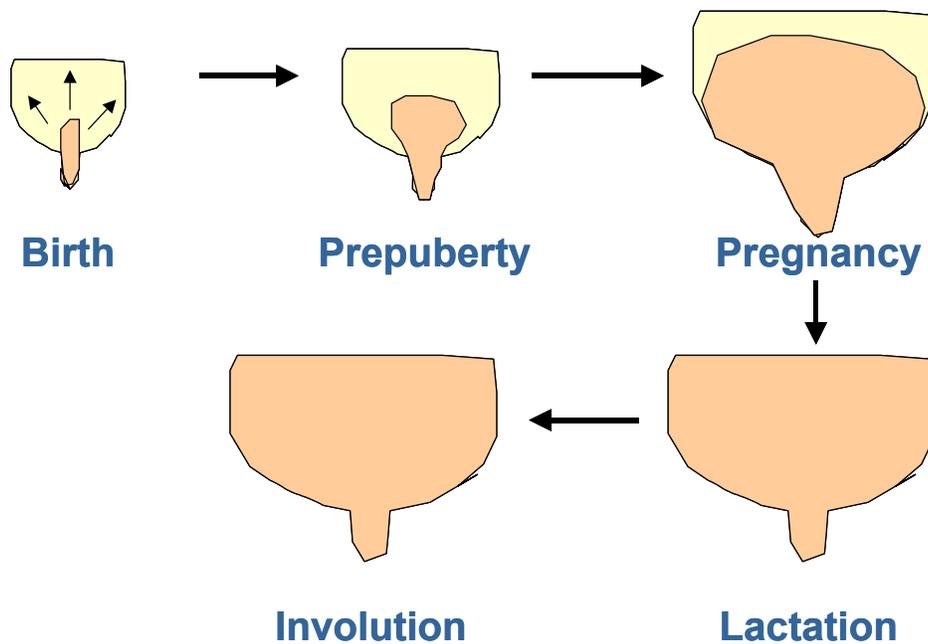
#### **1.4 Rationale and Significance**

The number of secretory cells present in the lactating gland directly influences milk production. Consequently, optimal development of the gland must be achieved to maximize milk yield. Although the mammary development that occurs during the pre- and peripubertal period is quantitatively less important than gestational growth, it does provide an important foundation for future development and milk yield. That overfeeding of pubertal heifers results in suboptimal pubertal mammary development, and subsequently, reduced milk yield (Sejrsen and Purup 1997), illustrates this concept.

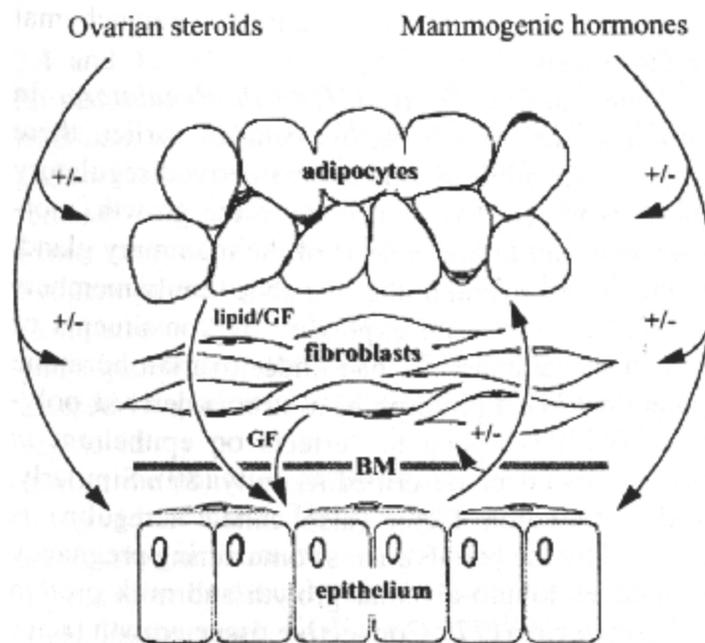
Currently, relatively little is known regarding the complex cellular and molecular mechanisms that regulate prepubertal mammary development. Elucidation of these processes and pathways will lead to new heifer management strategies that optimize mammary growth during the pre- and peripubertal period, thereby maximizing milk yield and lactation efficiency. The focus of this work is to further understand the importance of locally produced IGF-I and other growth factors and ECM proteins in regulating GH and E stimulated mammary development in prepubertal heifers.



**Figure 1.1:** Photograph of ewe mammary gland. Note two kinds of tissue within the mammary gland: fat pad (FP) and epithelium (indicated by arrow).



**Figure 1.2: Schematic of bovine mammary gland development from birth through to involution.** Note penetration of epithelial tissue (dark), into the mammary fat pad (light). Very little epithelial tissue is present at birth. During the prepubertal period, the epithelial tissue expands at an allometric rate, invading the mammary fat pad. Quantitatively, the majority of mammary development occurs during pregnancy and by the time lactation occurs, there is little to no fat pad remaining.



**Figure 1.3: A hypothesis for the role of the mammary fat pad in regulating mammary gland growth and development.** GF: growth factors, BM: basement membrane. Used with permission from Hovey *et al.* (1999)

## **Chapter 2: A Local Increase in the Mammary IGF-I: IGFBP-3 Ratio Mediates the Mammogenic Effects of Estrogen and Growth Hormone**

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### **2.1 Introduction**

The importance of ovarian and pituitary hormones in mammogenesis has long been recognized (Topper and Freeman 1980; Tucker 1981); however, the mechanisms by which these hormones regulate pre- and peripubertal mammary development are not fully elucidated. Accumulating evidence suggests that locally produced insulin-like growth factor (IGF)-I mediates the response of the mammary gland to growth hormone (GH) and perhaps estrogen (E). In addition, optimal production of growth factors by the mammary fat pad may require the presence of adjacent epithelium. The objectives of this experiment were 1) to determine the effects of short-term treatment with exogenous GH and E on mammary epithelial cell proliferation as well as local IGF-I and IGFBP production in heifers and 2) to determine the effects of epithelial-stromal interactions on IGF-I and IGFBP-3 production in mammary stroma.

### **2.2 Materials and Methods**

#### **Animals**

Twenty-five Friesian heifer calves were housed at the Ruakura Research Center, Hamilton, New Zealand. All animal-related procedures were conducted under approval of the Ruakura Research Station Animal Ethics Committee. At one month of age, surgery was performed on each animal to prepare an epithelium-free fat pad in one mammary gland as described previously for ewe lambs (Hovey *et al.* 2000). Briefly, the teat and epithelial tissue from one gland were removed by making an incision around the base of the teat and dissecting the parenchymal tissue from the fat pad, taking care not to leave any portions of parenchyma. Animals were sacrificed for tissue collection at approximately 18 months of age, 40 hours after hormone treatments were administered.

Bodyweight at slaughter averaged  $392.3 \pm 7.2$  kg. Prior to the beginning of the hormone treatments, two animals were removed from the trial for reasons unrelated to surgery. One additional animal was removed from the experiment because epithelium not removed during the surgery had re-grown into the fat pad. In total, 22 animals, each with a single epithelium-free mammary fat pad, were used in the experiment.

### **Experimental Design**

Animals were grouped into three blocks, according to treatment day. Within each block, hormone treatments were assigned randomly as control (C), growth hormone (GH), estrogen (E), or growth hormone + estrogen (GE). One injection of 500 mg recombinant bST (Posilac; Monsanto, St. Louis, MO) was administered i.m. to the GH and GE groups. Estrogen (0.1mg/kg BW) was administered s.c. daily for two days to the E and GE groups. Blood samples were taken at 0, 24, and 40 hours relative to the first injection. Animals were killed 40 hours after the treatments began. At slaughter, udders were sampled to provide tissues from the parenchyma (PAR), intact mammary fat pad (MFP), and cleared mammary fat pad (CFP) for analysis of cell proliferation, IGF-I and IGFBP-3 mRNA expression, and IGF-I and IGFBP protein content.

### **RNA Analysis**

RNA was isolated from PAR, MFP, and CFP using Tri-Reagent (MRC, Cincinnati, OH) and yield was determined from absorbance at 260 nm. Ten micrograms of RNA of each sample was applied to a 48-well Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA). Samples were mixed with 2.5 volumes of a MOPS-formamide loading buffer (65% formamide, 25% formaldehyde in 1X MOPS) and denatured at 65°C for 15 minutes. Samples were applied to the slot blot with 200µl 2.5% formaldehyde. Vacuum was applied to pull the samples through the wells onto Hybond N+ membrane (Amersham, Piscataway, NJ). Each well was rinsed with 200µl of 2.5% formaldehyde. Blots were UV cross-linked and stored pending Northern hybridization. Samples of liver RNA (5, 10, and 15 µg, in duplicate) were included on each blot as positive controls. An ovine 0.7-kb IGF-I cDNA probe (Ohlsen *et al.* 1993), a rat 0.44-kb IGFBP-3 cDNA probe (Smith *et al.* 1990), and an 18S cDNA probe were labeled with <sup>32</sup>P-α-dATP using

random prime labeling (Prime-A-Gene, Promega, Madison, WI). After 30 minutes of pre-hybridization, (68°C, in QuickHyb buffer; Stratagene, La Jolla, CA) the blots were incubated with denatured, labeled probe for 1 h. Post-hybridization washes to remove non-hybridized probe were carried out to a stringency of 0.1X SSC, 0.1% SDS. Blots were exposed to X-Ray film at -80°C for 24-72 hours. Following exposure, blots were stripped with two washes in 0.1 X SSC, 0.1% SDS at 95°C, and re-probed. Changes in gene expression were quantified using scanning densitometry. Samples were normalized for loading variations using data for 18S rRNA abundance.

### **Western Ligand Blotting**

Protein was extracted from each mammary sample using Tri-Reagent as described by manufacturer's instructions. The protein content of each sample was determined using the BCA total protein assay kit (Pierce, Rockford, IL). To prepare samples for western ligand analysis of IGFBP profiles in CFP, MFP, and PAR, four separate pools were made for each tissue, including equal protein from each sample within a treatment group. 200 µg protein from each pool was applied to the gel. Western ligand blotting was carried out as described (Hossenlopp *et al.* 1986). Briefly, samples were dissolved in non-reducing SDS-PAGE buffer and electrophoresed through a SDS-PAGE gel at 70 V for 18 hours. After electrophoresis, samples were transferred to nitrocellulose at 55 V for 5 hours. Blots were incubated in <sup>125</sup>I-IGF-I (1 x 10<sup>6</sup> cpm/ml) overnight and washed to remove unbound ligand. Plasma IGFBP profiles were analyzed similarly. Two separate pools of serum were made for each treatment group to represent pre- and post-treatment periods. 5 µl of each pool was loaded on the gel. Blots were exposed to Kodak X-Ray film for 48 hours at -80°C. Scanning densitometry was used to quantify changes in protein levels and purified IGFBP-3 was included as a positive control on each gel.

### **Radioimmunoassays**

Plasma concentrations of IGF-I and GH were determined by radioimmunoassays (RIA) as described previously (McFadden *et al.* 1990; Purup *et al.* 1993; Weber *et al.* 1999). To determine concentrations of IGF-I in mammary tissues, samples of CFP, MFP, and PAR were first homogenized in acid-ethanol (4:1 acid-ethanol:tissue) and centrifuged to

remove IGFBP. The resulting supernatant containing IGF-I was neutralized with 0.855 M tris base, stored at  $-20^{\circ}\text{C}$  for one hour and then centrifuged at  $4^{\circ}\text{C}$  for 20 minutes at 1500 x g. Supernatant was concentrated by speed-vac evaporation and reconstituted in IGF-I assay buffer for assay. Recombinant human IGF-I used for standards and iodination was purchased from GrowPrep (Adelaide, Australia). Mouse anti-human IGF-I antibody (1<sup>st</sup> antibody) was a gift of Dr. Bernard Laarveld (University of Saskatchewan). Goat anti-mouse antiserum (2<sup>nd</sup> antibody) was purchased from Sigma Chemical Company (St. Louis, MO, USA). IGF-I was radioiodinated as described previously for  $\alpha$ -lactalbumin (Akers *et al.* 1986) and the resulting specific activity averaged 38  $\mu\text{Ci}/\mu\text{g}$ . For assay, standards or unknown samples were suspended in RIA buffer (30 mM sodium phosphate, 10 mM EDTA, 0.02% protamine sulfate, 0.05% Tween-20, pH 8.0) to a final volume of 500  $\mu\text{l}$ . Subsequently, 100  $\mu\text{l}$  of radiolabeled IGF-I ( $\sim 30,000$  dpm) and 100  $\mu\text{l}$  of 1<sup>st</sup> antibody (1:70,000) were added to each tube. After 24 hours incubation at  $4^{\circ}\text{C}$ , 100  $\mu\text{l}$  of 2<sup>nd</sup> antibody (1:20) was added. Tubes were incubated for a further 72 hours at  $4^{\circ}\text{C}$ . 1.5 ml of PBS was added to each tube followed by centrifugation at 1500 x g for 30 min. The tubes were decanted and bound radioactivity was measured by gamma counting. Inter and intra assay variation averaged less than 10%. To determine the concentration at which IGFBP would cause significant interference in the assay, some samples were spiked with 0, 2, 5, 10 or 100 ng of purified IGFBP-3 prior to assay. For samples spiked with 10 or 100 ng of IGFBP-3, estimates of IGF-I concentration could not be determined. IGF-I concentrations of samples spiked with 0, 2, or 5 ng IGFBP-3 were accurately determined compared with controls. Samples were also subjected to western ligand analysis to ensure that remaining IGFBP were less than 5 ng.

### **<sup>3</sup>H-Thymidine Incorporation**

To determine the effect of hormone treatments on cell proliferation, the incorporation of <sup>3</sup>H-thymidine into DNA of tissue samples was determined as described (Woodward *et al.* 1993). Tissue samples (PAR, CFP, MFP; approximately 200 mg) were excised from the gland at slaughter, finely diced, and incubated with 3  $\mu\text{Ci}$  of <sup>3</sup>H-thymidine in 3 ml of DMEM (Life Technologies, Grand Island, NY). Incubations were carried out for 1 hour

at 37°C, and were followed with a wash of 3 ml of DMEM without tracer. Subsequently, the tissue pieces were blotted dry, weighed, and frozen for later analysis. To determine the incorporation of the <sup>3</sup>H-thymidine, samples were homogenized in 5 ml of PBS and then centrifuged to remove cell debris. The resulting supernatant was retained. One milliliter of supernatant was added to 3 ml of ice-cold 10% TCA. The samples were incubated on ice for 20 minutes and then centrifuged at 1500 x g for 20 minutes at 4°C. The supernatant was removed and a further 3 ml of ice-cold 10% TCA was added and the samples were washed and centrifuged again. After removal of the supernatant, 3 ml of ice-cold ethanol was added to each tube. The samples were centrifuged at 1800 x g for 20 min. The supernatant was aspirated and the tubes were allowed to dry. The resulting pellets were dissolved in 0.3 M NaOH. Samples were neutralized with HCl and radioactivity was quantified using a scintillation counter.

### **Statistics**

Data for <sup>3</sup>H-thymidine incorporation, IGF-I and IGFBP-3 mRNA expression, and IGF-I protein concentration were analyzed using the GLM procedure in SAS (SAS Inc. Cary, NC, 1999). Treatments were arranged in a factorial design. The model tested for the main effects of E, GH, block, and the E x GH interaction. Tissue type was included as a repeated measure. Levels of plasma IGF-I and plasma GH were analyzed using the mixed procedure in SAS. The model tested for main effects of E, GH, and time as well as interactions between these effects. Pearson correlations were calculated to determine the relationships between concentrations of IGF-I protein in mammary tissue, IGF-I concentration in plasma, and epithelial proliferation. Data are presented as least squares means ± standard error of mean. Differences were considered significant at  $p \leq 0.05$  and differences less than  $p \leq 0.1$  were considered a tendency.

## **2.3 Results**

### **Cell Proliferation**

Overall, both E ( $p < 0.01$ ) and GH ( $p < 0.05$ ) stimulated mammary epithelial cell proliferation as determined by <sup>3</sup>H-thymidine incorporation (Figure 2.1A). E ( $p < 0.02$ )

and GE ( $p < 0.001$ ) had the greatest effect on epithelial cell proliferation. The effect of GH alone on epithelial proliferation was not significant ( $p < 0.09$ ), although there was a tendency for cell proliferation to be increased by this treatment. The interaction of E x GH was also not significant, suggesting that the combined effect of these two hormones on mammary epithelial cell proliferation was additive. Cell proliferation in mammary stroma was minimal (Figure 2.1B). However, there was a significant main effect of GH on CFP proliferation, suggesting that GH may act on mammary stroma. Comparisons between individual means were non-significant for both CFP and MFP. DNA concentrations ranged between 1.0 and 3.0  $\mu\text{g}/\text{mg}$  tissue for PAR and between 0.1 and 0.4  $\mu\text{g}/\text{mg}$  tissue for stroma. Neither E nor GH had any effect on DNA concentrations (data not shown).

### **Plasma Hormone Concentrations**

Plasma concentrations of IGF-I were increased by administration of GH ( $p < 0.001$ ), but not E ( $p > 0.3$ , Figure 2.2A). Prior to hormone treatment, concentrations of IGF-I ranged from 205 – 238 ng/ml across the four treatment groups. Twenty-four hours after hormone treatments began, IGF-I levels were increased to between 430 and 455 ng/ml for the groups receiving GH. Plasma levels of GH did not differ between treatment groups at the beginning of the experiment (range 1.4 – 1.9 ng/ml; Figure 2.2B). Twenty-four and 40 hours after hormone treatments began, plasma GH levels were increased to between 103 and 133 ng/ml for the groups receiving GH ( $p < 0.001$ ). E had no effect on plasma GH levels ( $p > 0.4$ ).

### **IGF-I and IGFBP-3 mRNA in Mammary Tissue**

Overall, E tended to increase IGF-I mRNA expression in MFP (Table 2.1,  $p < 0.09$ ). E did not affect IGF-I mRNA expression in CFP or PAR, and GH did not affect IGF-I mRNA expression in any of the tissues. That IGF-I mRNA expression was increased by E in MFP but not CFP, indicates that the regulation of the IGF-I axis and the stromal response to E is modulated by adjacent epithelium.

In agreement with previous studies (Hovey *et al.* 1998a), the expression of IGF-I mRNA was, on average, greater in stroma (CFP and MFP) than in PAR (Table 2.1;  $p < 0.005$ ). The expression of IGFBP-3 mRNA in CFP or MFP was not affected by GH or E (Table 2.1). In PAR, GH tended to decrease IGFBP-3 mRNA ( $p < 0.07$ ) but E had no effect. Overall, IGFBP-3 mRNA was more abundant in CFP and MFP than in PAR ( $p < 0.001$ ).

### **IGF-I and IGFBP-3 Protein in Mammary Tissue**

E significantly increased IGF-I protein in CFP ( $p < 0.05$ ), MFP ( $p < 0.001$ ), and PAR ( $p < 0.01$ ; Figure 2.3). In agreement with the observed effect of E on IGF-I mRNA, the effect of E on IGF-I protein was more dramatic in MFP than CFP. GH did not affect mammary IGF-I protein in any of the tissues. Parenchymal IGF-I protein content was positively correlated with epithelial cell proliferation ( $r = 0.67$ ,  $p < 0.01$ ). Mammary IGF-I protein was not correlated to plasma IGF-I levels ( $r < 0.35$ ,  $p > 0.15$ ), suggesting that the IGF-I present in mammary tissue was at least in part, locally produced. Alternatively, IGF-I protein may be synthesized elsewhere and sequestered within the mammary tissues, perhaps by IGFBP. This is supported by the observation that within CFP and MFP, changes in mammary IGF-I protein were not consistently reflected by IGF-I mRNA levels. Overall, IGF-I protein was more abundant in PAR than in CFP or MFP, suggesting that IGF-I acts mainly within the mammary parenchyma.

Mammary IGFBP were analyzed by western ligand blotting (Figure 2.4). IGFBP profiles were distinctly different between parenchymal and stromal tissues. IGFBP-3 (identified as a doublet of approximately 40-43 kDa; Skaar and Baumrucker 1993; Weber *et al.* 2000b) was present mainly in the parenchymal tissue, whereas IGFBP-2 (34 kDa; Skaar and Baumrucker 1993; Donovan *et al.* 1994; Weber *et al.* 2000b), a 24 kDa IGFBP, and a 28-kDa protein (potentially IGFBP-1; Donovan *et al.* 1994) were more abundant in the stromal tissues. GH, E, and GE reduced IGFBP-3 levels in mammary parenchyma. The 24-kDa IGFBP present in CFP and MFP was increased by the hormone treatments (Figure 2.4). None of the remaining IGFBP were influenced by treatment.

IGFBP detected in plasma samples included IGFBP-3, IGFBP-2, the 24-kDa protein and the 28-kDa species that may be IGFBP-1. None of the plasma IGFBP were altered by hormone treatments (data not shown).

## **2.4 Discussion**

Several studies have previously demonstrated that continuous administration of GH over several weeks or months stimulates mammary growth, as evidenced by an increase in parenchymal weight (Sejrsen *et al.* 1986; Carstens *et al.* 1997; Radcliff *et al.* 1997). The increased weight coincided with increased DNA content (Radcliff *et al.* 1997), suggesting that one of the mechanisms by which GH acts in pubertal heifers is to stimulate proliferation of mammary epithelial cells. However, there has been no investigation of mammary responses to acute short-term GH treatment of heifers, and the mechanism by which GH may stimulate epithelial cell proliferation remains undefined. Furthermore, despite the essential role that E plays in regulating pubertal mammary development, the mechanisms for its action are unclear. The present results show that after 40 hours, E and GH stimulate proliferation of mammary parenchyma. Our observations that proliferation of mammary stroma was relatively low agree with previous studies (Woodward *et al.* 1993), where it was reported that 96 hours of E treatment caused proliferation of mammary epithelial cells and some fibroblasts adjacent to epithelial cells, but did not markedly affect stromal cell proliferation in prepubertal heifers.

Most current evidence suggests that the effects of GH are indirect (reviewed in Chapter 1). Consistent with previous studies in which exogenous GH was administered to dairy heifers (Cohick *et al.* 1989; Purup *et al.* 1993), serum levels of IGF-I in the present study were dramatically increased by administration of GH. However, changes in circulating IGF-I do not necessarily explain the observed changes in epithelial proliferation. Possibly, the proliferative response is more closely related to changes in mammary-derived IGF-I than serum IGF-I. Recent evidence supports this hypothesis. For example, mammary tissue extract was a more potent stimulator of cell proliferation in primary cultures of mammary epithelial cells than serum from the same heifer (Weber *et al.*

1999). Addition of IGFBP-3 or an antibody against IGF-I blocked the observed stimulatory effect, suggesting that IGF-I was the primary factor responsible for the mitogenic capacity of the extract. In the present study, increases in mammary epithelial cell proliferation coincided with changes in mammary IGF-I and IGFBP-3 levels, suggesting a role for these proteins in regulating E and GH induced mammary growth. E significantly increased levels of mammary IGF-I protein in CFP, MFP and PAR, and tended to increase IGF-I mRNA in MFP. In response to GH and E, IGFBP-3 was reduced, and the 24-kDa IGFBP was increased, suggesting that changes in the IGF-I:IGFBP ratio regulate mammary epithelial cell proliferation. Mammary tissue IGF-I protein was positively correlated to parenchymal proliferation ( $r = 0.67$ ,  $p < 0.001$ ). Furthermore, mammary tissue IGF-I protein was not correlated with serum IGF-I ( $r < 0.35$ ,  $p > 0.15$ ), suggesting that the mammary IGF-I protein was, at least in part, locally derived. In MFP, the increase in IGF-I protein in response to E was paralleled by an increase in IGF-I mRNA. However, in CFP and PAR, the changes in IGF-I protein were not consistently reflected by changes in IGF-I mRNA. This suggests that mammary IGF-I protein is derived from both local and systemic sources. Possibly, IGFBP enable mammary parenchyma to sequester non-mammary IGF-I within the gland, and more specifically, within the epithelium. Consequently, changes in IGFBP levels would alter the ability of the mammary gland and epithelium to accumulate IGF-I. Alternatively, more subtle changes in IGF-I mRNA levels in CFP and PAR may have been below the resolution of the assay.

As previously reported (Hovey *et al.* 1998a), IGF-I mRNA was more abundant in mammary stroma than parenchyma in prepubertal heifers and ewes, However detecting IGF-I mRNA expression in parenchyma is not surprising given that parenchymal tissue consists of a mixture of epithelial, connective tissue, and adipose cells (Weber *et al.* 2000b). IGF-I receptors are present on mammary epithelial cells (Purup *et al.* 1995), supporting the hypothesis that IGF-I may have an autocrine or paracrine role in stimulating mammary growth. In this experiment, IGF-I mRNA was highest in stroma, whereas the IGF-I protein was highest in parenchyma, suggesting the stromal-derived IGF-I predominantly acts within the parenchyma. Interactions between E and the GH –

IGF axis have also been reported previously. Ovariectomy decreased the expression of mammary IGF-I mRNA in prepubertal heifers (Akers *et al.* 2000), and E enhanced the GH induced expression of mammary IGF-I in rats (Ruan *et al.* 1995). In the present study, E increased mammary IGF-I protein and decreased IGFBP-3 protein. This increase in the IGF-I:IGFBP-3 ratio is positively correlated with proliferation of mammary parenchyma, indicating that heifer mammatogenesis is regulated by changes in the IGF-I axis molecules.

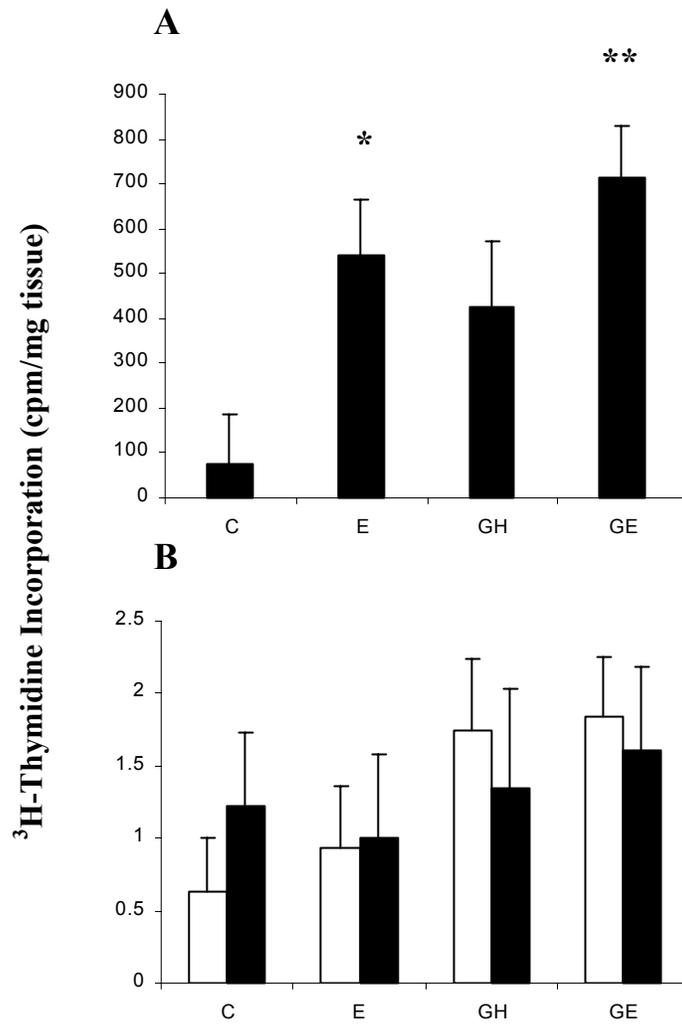
IGFBP-3 is the predominant IGFBP present in serum, and approximately 95% of circulating IGF-I is bound to this protein (Clemmons 1993). Bovine mammary epithelial cells also produce IGFBP-3, both in vivo (Weber *et al.* 2000b) and in immortalized cell culture systems (Cohick and Turner 1998). Effects of IGFBP-3 on modulating IGF-I activity have been shown to be either stimulatory or inhibitory, depending on experimental conditions (discussed in Chapter 1). Possibly, IGFBP are responsible for modulating mammary growth by regulating the amount of IGF-I available to stimulate cell proliferation. In support of this hypothesis, mammary IGFBP-3 tends to be higher in over-fed heifers, corresponding with reduced mammary growth (Sejrsen *et al.* 2000). In the present study, IGFBP-3 protein is present mainly in the parenchymal portion of the gland, and was reduced by GH and E. GH tended to decrease IGFBP-3 mRNA in parenchyma. Estrogen has previously been shown to reduce IGFBP-3 expression in MCF-7 cells, in which IGFBP-3 expression is negatively associated with cell proliferation (Huynh *et al.* 1996). The proliferative responses to GH and E in the present experiment may partly be explained by a release from inhibition through the reduction of IGFBP-3. At the same time, local IGF-I concentration is increased, resulting in a greater proportion of biologically available IGF-I. The mechanism by which IGFBP-3 acts to reduce mammary development is not clear. Possibly, IGFBP-3 binds to IGF-I, making it unavailable to the IGF-I receptor. IGF-I in solution has a higher affinity for IGFBP than for the IGF-I receptor, suggesting that the IGFBP could modulate IGF-I activity by providing competition for ligand binding (Cohick 1998). IGFBP may also aid to increase the local concentration of IGF-I by enabling the mammary gland to sequester IGF-I protein within mammary parenchyma. Different IGFBP may regulate IGF-I availability

in different ways. In the present study, E and GH reduced IGFBP-3 in parenchyma, whereas the 24-kDa protein in stroma was increased, implying that these proteins have different roles in regulating IGF-I availability and mammary growth.

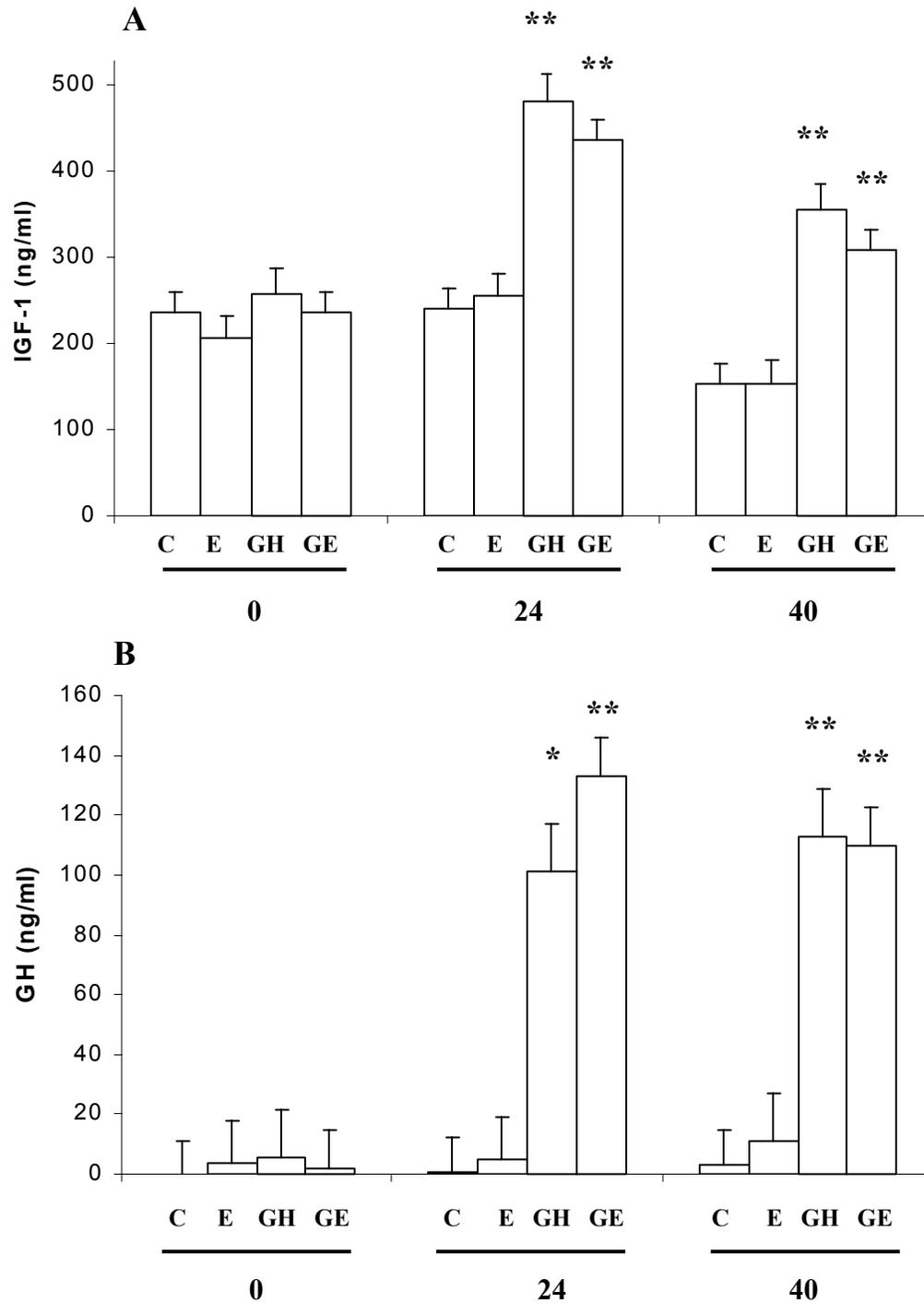
Along with the effects of GH and E on mammary growth, we sought to determine the importance of epithelial:stromal interactions in modulating the production of IGF-I and IGFBP-3 in mammary stroma. Previous studies have indicated that the expression of growth factor mRNA species by the mammary fat pad may be modulated by interactions between adjacent parenchyma and stroma. In ewe mammary glands, IGF-I mRNA abundance was lower in the cleared fat pad than in the intact fat pad (Hovey *et al.* 1998a), suggesting that local regulation of the IGF-I axis involves ‘cross-talk’ between different portions of the gland and may ensure optimal growth factor production for mammary development. In the present study, subtle differences between CFP and MFP were detected. IGF-I mRNA tended to be up regulated by E in MFP but not CFP. E increased IGF-I protein in both tissues; however, the response of MFP was much greater than that of CFP. These observations support the hypothesis that the presence of adjacent epithelium modulates the responsiveness of the fat pad to E and GH. An interaction with epithelium is also consistent with the presence of estrogen receptor in the mammary epithelium but not stroma of calves (Capuco *et al.* 2002). IGFBP protein content was similar between CFP and MFP. The importance of epithelial:stromal interactions may differ during different stages of development. In the study of ewes (Hovey *et al.* 1998a), the differences between the MFP and CFP in IGF-I mRNA expression varied substantially across various ontogenic stages, implying that the regulation of mammary development by the IGF-axis may vary with age or developmental stage.

In conclusion, the data presented demonstrate that short-term treatment with GH and E stimulates proliferation of mammary parenchyma. The proliferative responses coincided with increased mammary IGF-I protein, and decreased mammary IGFBP-3 protein. Thus, proliferation was associated with an apparent net increase in the biological availability of IGF-I in the mammary gland. Mammary stroma adjacent to epithelium was more responsive to E than epithelium-free stroma, suggesting that local regulation of

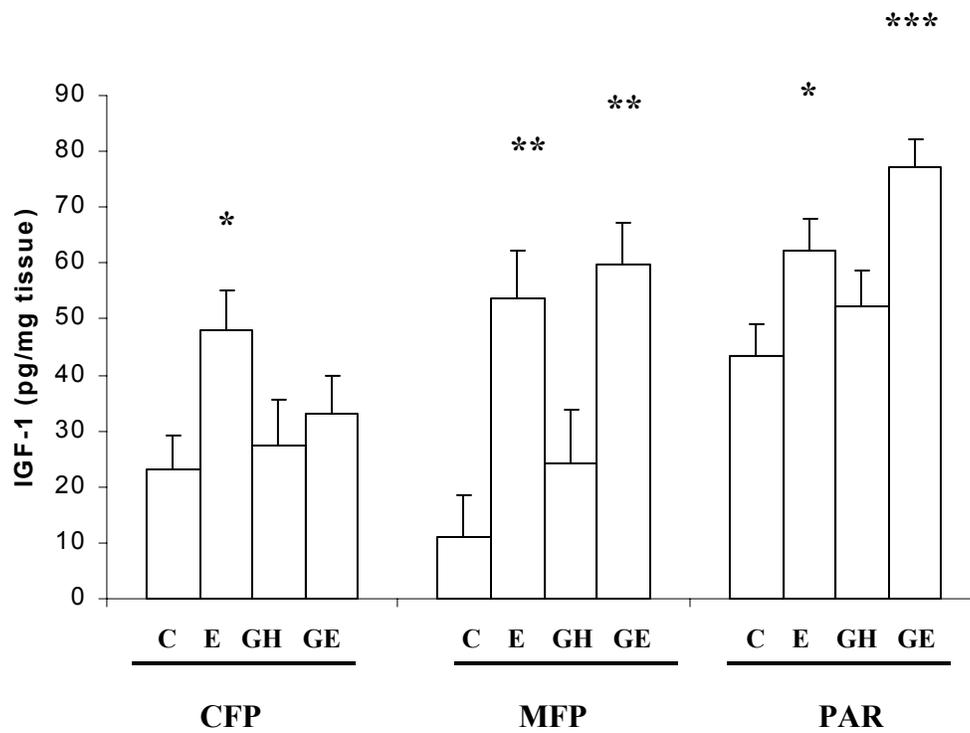
the IGF-I axis in heifers involves interactions between the epithelium and stroma. These findings support the hypothesis that local production or accumulation of components of the IGF-I axis may play an important role in mediating hormone-stimulated mammosgenesis in heifers.



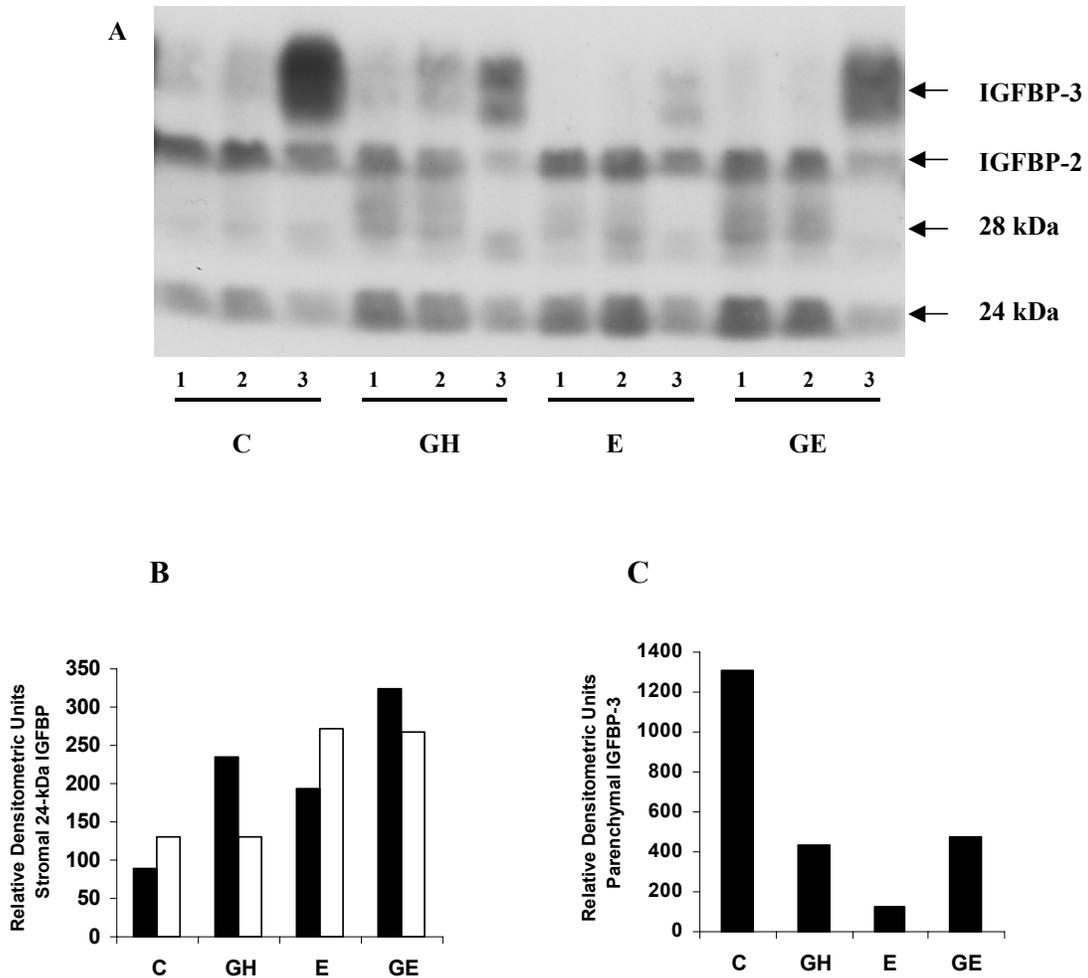
**Figure 2.1: Effect of GH and E on incorporation of  $^3\text{H}$ -thymidine into DNA of heifer mammary tissue.** Treatments are control (C), estrogen (E), growth hormone (GH), or growth hormone + estrogen (GE). Data are normalized Least squared means  $\pm$  S.E.M. for each treatment group. **A:** Parenchyma (PAR); **B:** Stroma (open bars: CFP, closed bars: MFP). Indicated data points are significantly different from controls: \*  $p < 0.02$ , \*\*  $p < 0.001$ . Note that the Y-axis differs in scale for A and B.



**Figure 2.2: Effect of GH and E on plasma IGF-I (A) and GH (B) concentrations in heifers.** Treatments are control (C), estrogen (E), growth hormone (GH), or growth hormone + estrogen (GE) for 0, 24, and 40 hours. Data are presented as Least squared means  $\pm$  S.E.M. \* $p < 0.005$ , \*\* $p < 0.001$  compared with the control group of the same time-period.



**Figure 2.3: Effect of GH and E on IGF-I concentration in heifer mammary tissues.** Data are presented as least squared means  $\pm$  S.E.M. Treatments are control (C); estrogen (E); growth hormone (GH) and growth hormone + estrogen (GE), for the mammary tissues CFP, MFP and PAR. Significance values for comparisons with control group of the same tissue type: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . For each of the tissues, there was a significant main effect of E ( $p < 0.05$ )



**Figure 2.4: Effect of GH and E on IGFBP profiles in heifer mammary tissues.** **A:** Ligand blot containing samples pooled from each treatment group. 1: CFP, 2: MFP, 3: PAR, for control (C), growth hormone (GH), estrogen (E) and growth hormone + estrogen (GE) treated heifers. IGFBP-3 and IGFBP-2 identified with MW markers (IGFBP-3: 40-43 kDa; IGFBP-2: 34 kDa). The 28-kDa band putatively contains IGFBP-1 but has not been immunologically identified. **B:** Densitometric quantification of the 24-kDa IGFBP in CFP (closed bars) and MFP (open bars). **C:** Densitometric quantification of IGFBP-3 in PAR tissue for each treatment group.

**Table 2.1:** Effect of GH and E on IGF-I and IGFBP-3 mRNA in heifer mammary tissues.

		Treatment				Significance <sup>b</sup>		Error <sup>c</sup>
		C	GH	E	GE	E	GH	
IGF-I <sup>d</sup>	MFP	31.1 <sup>a</sup>	38.2	47.7	36.6	< 0.09	NS	3.8 – 5.0
	CFP	38.3	38.2	36.7	42.7	NS	NS	4.8 – 6.3
	PAR	33.9	27.9	28.9	25.2	NS	NS	4.6 – 6.1
IGFBP-3 <sup>e</sup>	MFP	63.6	83.7	63.1	56.6	NS	NS	8.5 – 11.2
	CFP	54.2	68.3	82.6	68.8	NS	NS	8.5 – 11.3
	PAR	54.8	35.1	47.1	38.5	NS	<0.07	5.8 – 7.70

<sup>a</sup> Data are presented as Least squared means of densitometric units, normalized for RNA loading by comparison with 18S rRNA abundance in that sample.

<sup>b</sup> Significance of main effects for E and GH.

<sup>c</sup> Range for standard error of Least squared means for treatment effects within each tissue.

<sup>d</sup> Significant main effect of tissue  $p < 0.005$ .

<sup>e</sup> Significant main effect of tissue  $p < 0.001$ .

## **Chapter 3: Interactions Between the Ovary and Local IGF-I Axis Modulate Mammary Development in Prepubertal Heifers**

### **3.1 Introduction**

Interactions between the IGF-I axis and E within mammary epithelial cells have been previously described. IGF-I can activate ER $\alpha$  dependent gene transcription in human breast cancer cells (Lee *et al.* 1997) and E increased mRNA expression of IGF-IR and IRS-I in the same cell line (Lee *et al.* 1999). Furthermore, loss of ER $\alpha$  expression in a similar cell line resulted in decreased IGF-IR and IRS-I mRNA expression (Oesterreich *et al.* 2001). The objectives of this experiment were two-fold. First, we sought to determine the effects of ovariectomy on mammary development and epithelial proliferation, as well as IGF-I and IGFBP mRNA expression in prepubertal heifers. Second, we sought to examine the importance of epithelial-stromal interactions in regulating stromal expression of IGF-I and IGFBP mRNA during prepubertal mammary development.

### **3.2 Materials and Methods**

#### **Experimental Design and Sampling**

All experiments were conducted with the approval of the Virginia Tech Animal Care Committee (approval number 98-036-DASC). Fourteen newborn Holstein heifer calves were purchased for the experiment. The calves were housed at the Virginia Tech Dairy Center and raised according to standard calf rearing procedures. Animals were assigned to one of two treatments: control or ovariectomized, using a completely randomized design. Between one and three months of age, surgery was performed on each calf to remove the ovaries from animals in the ovariectomy group (n = 8), and to prepare an epithelium-free, (“cleared”) fat pad in each animal (n = 14). In total, eight animals were ovariectomized and six animals remained intact. All fourteen animals received a cleared fat pad in two out of four glands of the udder (left or right side, randomly determined). The remaining two glands were left intact to serve as internal controls. Blood samples were taken weekly for the duration of the experiment to enable determination of serum

IGF-I, GH, and PRL concentrations. Calves were purchased in groups and assigned randomly to treatments beginning in August 2000. The last group of calves was purchased in October 2000. Animals were sacrificed between January and April 2001, at six months of age, to provide mammary samples of parenchyma (PAR), intact mammary fat pad (MFP), and cleared mammary fat pad (CFP). Before samples were taken, we confirmed that cleared fat pads were completely free of epithelial tissues. Mammary tissue samples were frozen in liquid nitrogen for later analysis and udder and uterus weights were recorded.

### **Surgical Procedures**

Surgical preparation of epithelium-free cleared fat pads was conducted essentially as described previously (Hovey *et al.* 2000). Animals were sedated using a combination of intravenously administered xylazine HCl 0.1 mg/kg (The Butler Company, Dublin, OH) and butorphanol tartrate 0.1 mg/kg (Fort Dodge Animal Health, Fort Dodge, IA). Anesthesia was induced using thiopental sodium 25 mg/kg (Abbott Laboratories, Chicago, IL), i.v., and maintained by inhalation of halothane in oxygen. Following induction of anesthesia, the heifers were placed in dorsal recumbency and the caudo-ventral abdomen and inguinal regions were clipped and aseptically prepared for surgery.

Cleared fat pads were prepared by en bloc excision of ipsilateral teats and their associated epithelial tissue. An elliptical skin incision encompassing the base of both teats was made and the palpable nodules of epithelial tissue dissected free from the fat pad. Excised tissue and remaining fat pad were inspected to determine that excision of the epithelial tissue was complete. The subcutaneous tissues were apposed using 2-0 polyglycolic acid suture in a simple continuous pattern and the skin was closed using 2-0 nylon in a continuous horizontal mattress pattern. An ovariectomy was performed during the same anesthetic episode for those animals assigned to the ovariectomy group. Ovariectomies were performed via a 10-cm caudo-ventral midline celiotomy. The ovaries were isolated, and the ovarian pedicles were clamped and ligated using No. 0 polyglycolic acid suture. The ovaries were excised with scissors. The linea alba was closed using a simple continuous pattern of No. 1 polyglycolic acid. The subcutaneous

tissues were closed using 2-0 polyglycolic acid suture in a simple continuous pattern, and the skin was closed using 2-0 nylon in a continuous horizontal mattress pattern. Animals were kept in individual pens until they had fully recovered from anesthesia (approximately 1-2 hours). Procaine penicillin G (Pfizer, Exton, PA), at a dose of 22,000 IU/kg was administered twice daily for 2 days.

### **RNA Isolation and Northern Analysis of Gene Expression**

RNA was isolated from PAR, MFP, and CFP using Trizol (LifeTech, Rockville, MD) according to manufacturer's instructions. RNA yield was determined by absorption at 260 nm and integrity was determined by ethidium bromide staining after agarose-formaldehyde electrophoresis. Twenty micrograms of total RNA suspended in formaldehyde loading buffer (Ambion, Austin, TX) was electrophoresed through a 1% agarose-0.66 M formaldehyde gel. Samples were electrophoresed at 100 V for 2.5 hours before being photographed to confirm equal loading of RNA. Subsequently, RNA was transferred to Hybond nylon membrane (Amersham, Piscataway, NJ) by overnight capillary action. Blots were UV cross-linked and stored pending Northern analysis. Samples of liver RNA (10 µg) and bovine lactating mammary RNA (10 µg) were included as positive and negative controls respectively. An ovine 0.7-kb IGF-I cDNA probe (Ohlsen *et al.* 1993), a rat 0.44-kb IGFBP-3 cDNA probe (Smith *et al.* 1990), and a porcine 0.32-kb IGFBP-5 cDNA probe (White *et al.* 1996) were labeled with <sup>32</sup>P-α-dATP using random prime labeling (Prime-A-Gene, Promega, Madison, WI). After 30 minutes of pre-hybridization (68°C, in QuickHyb buffer; Stratagene, La Jolla, CA) the blots were incubated with denatured, labeled probe for 1 hour, at 68°C. High stringency post-hybridization washes to remove non-hybridized probe were performed using 0.1X SSC, 0.1% SDS. Blots were exposed to X-Ray film at -80°C for 24-72 hours. Following exposure, blots were stripped with 0.1X SSC, 0.1% SDS at 95°C (two washes of 15 minutes each) and re-probed. Changes in gene expression were quantified using scanning densitometry. RNA loading on the gel was made equal by adjusting for previous assessment of ethidium bromide staining.

### **Radioimmunoassays and IGF-I Receptor Binding Assay**

Plasma concentrations of IGF-I and GH were determined by radioimmunoassays (RIA) as described previously (Chapter 2, McFadden *et al.* 1990; Purup *et al.* 1993; Weber *et al.* 1999). To determine IGF-I receptor binding in mammary tissues, extracts of CFP, MFP and PAR were prepared as described (Weber *et al.* 1999). Briefly, samples were homogenized in physiological saline (4:1 saline:tissue). Mammary homogenates were shaken for 90 minutes at 4°C, and then centrifuged at 10 000 x g to remove cellular debris. Subsequently, the supernatant from each sample was ultra-centrifuged at 100 000 x g. The supernatant was retained for analysis of IGFBP. The pellets were resuspended in 1 ml of receptor buffer and stored for analysis of IGF-I receptor binding. To determine IGF-I receptor binding, 100 µg total protein (determined by BCA assay, data not shown) was incubated with <sup>125</sup>I-IGF-I or <sup>125</sup>I-IGF-I + excess IGF-I in a shaker overnight at 4°C as previously described (McFadden *et al.* 1990). Specific binding was determined by subtracting binding in the presence of excess IGF-I from binding in the presence of <sup>125</sup>I-IGF-I alone.

### **Western Ligand Blots**

Western ligand blots of mammary extracts (200 µg protein per lane) and serum samples (4µl serum per lane) to determine mammary IGFBP abundance was performed as previously described (Chapter 2, (Hossenlopp *et al.* 1986). Purified IGFBP-3 was included on each blot as an internal control.

### **Statistics**

Statistics were performed using the SAS statistical package version 8.0 (SAS Inc. Cary, NC, 1999). Data for uterus and mammary weights were analyzed using a t-test to compare the means from ovariectomized and control heifers. Data for mRNA expression, mammary IGFBP abundance, and IGF-I receptor binding were analyzed using the GLM procedure. The model tested for main effects of treatment (ovariectomized or control), tissue (PAR, MFP, or CFP) and the treatment x tissue interaction, with tissue included as a repeated measure. Data for serum IGFBP were also analyzed using the GLM procedure. The model tested for main effects of treatment

(ovariectomized or control), time (before and after surgery) and the treatment x time interaction. Serum hormone data was analyzed using the MIXED procedure. The model tested for main effects of treatment (ovariectomized or control), time (weekly time points for the duration of the experiment), and the treatment x time interaction. Time was included as a repeated measure. Differences of  $p < 0.05$  were considered significant and differences of  $p < 0.1$  were considered a tendency. Data are presented as Least squared means  $\pm$  standard error of the mean.

### **3.3 Results**

#### **Mammary and Uterus Development**

Both mammary ( $p < 0.001$ ) and uterus ( $p < 0.05$ ) weights were significantly reduced by ovariectomy (Figure 3.1A and 3.1B). Reduced mammary weight in ovariectomized animals corresponded with dramatically reduced mass of parenchymal tissue. In fact, for some animals, parenchymal growth had been completely inhibited and was essentially absent in comparison with parenchymal tissue development for glands examined at the time of surgery ( $3 - 5 \text{ mm}^2$ ; data not shown). Animals ovariectomized between four and six weeks of age appeared to be more dramatically affected by ovariectomy than animals ovariectomized between six and twelve weeks of age. Our observations were that animals ovariectomized before six weeks of age were essentially devoid of mammary parenchyma, whereas animals ovariectomized at three months of age had considerably more parenchyma than observed at the time of surgery.

#### **Systemic Concentrations of GH, IGF-I, and Prolactin**

Serum concentrations of IGF-I, GH, and Prolactin (PRL) are shown in Figure 3.2A-C. No significant effect of ovariectomy was observed for IGF-I, GH, or PRL. Furthermore, there was no significant interaction between treatment (ovariectomized vs. control) and time for any of the serum hormones. The only change in hormone concentration was that PRL concentration was significantly higher ( $p < 0.01$ ) in both ovariectomized and control animals during the last six weeks of the experiment, which is likely related to seasonal changes.

### **Expression of Mammary IGF-I mRNA is Exclusive to Mammary Stroma**

Previous studies of IGF-I expression in mammary tissue from rodents (Kleinberg 1997) and humans (Yee *et al.* 1991; Cullen *et al.* 1992) have shown that IGF-I expression is limited to mammary stromal cells. To test whether this was true in prepubertal heifers, we isolated mammary epithelial cells from a prepubertal heifer (Weber *et al.* 1999). Figure 3.3 shows IGF-I expression in RNA isolated from purified bovine mammary epithelial cells, bovine prepubertal mammary stroma, bovine prepubertal mammary parenchyma, bovine lactating parenchyma, and bovine liver. As expected, a major IGF-I transcript of 7.5 kb was present in bovine liver, but not lactating mammary tissue. Expression of the 7.5 kb transcript was absent from the isolated epithelial cells, but present in both the prepubertal stroma and parenchyma. This observation confirms that IGF-I mRNA expression in bovine mammary parenchyma is derived from interspersed stromal cells such as fibroblasts.

### **Mammary Expression of IGF-I, IGFBP-3, and IGFBP-5 mRNA**

Northern analysis of mammary IGF-I mRNA expression revealed one major transcript of approximately 7.5 kb, which was present in both mammary parenchyma and mammary stroma (Figure 3.4A and 3.5A). Overall, expression of IGF-I mRNA was lower in ovariectomized animals than in controls (Figure 3.4A; significant main effect for treatment:  $p < 0.05$ ). In contrast to our hypothesis that IGF-I mRNA expression would be lower in cleared fat pad than intact fat pad, IGF-I mRNA levels did not differ between any of the tissue types ( $p > 0.05$ ). IGFBP-3 (Figure 3.4B and 3.5B) mRNA expression was present as one transcript of approximately 2.5 kb and did not differ between intact and ovariectomized animals, or between tissue types. IGFBP-5 mRNA expression was not influenced by ovariectomy, but was significantly higher in mammary stroma than mammary parenchyma (Figure 3.4C and 3.5C,  $p < 0.001$ ). This finding is in agreement with a previous study in which IGFBP-5 mRNA was localized to the stromal portion of the rat mammary gland (Manni *et al.* 1994).

### **Ovariectomy Reduces <sup>125</sup>I-IGF-I Binding to Mammary Microsomes**

To test whether ovariectomy reduced the ability of mammary tissue to bind IGF-I, we assayed binding of <sup>125</sup>I-IGF-I to mammary microsomes. Specific binding of <sup>125</sup>I-IGF-I to mammary parenchyma was significantly reduced from a mean of  $868 \pm 82$  cpm in intact animals to  $377 \pm 142$  cpm in ovariectomized animals (Figure 3.6;  $p < 0.01$ ). <sup>125</sup>I-IGF-I binding to microsomes from stromal tissue ranged from between 341 and  $486 \pm 82$  cpm and did not differ between ovariectomized and control animals, or between intact and cleared mammary fat pad.

### **Serum and Mammary Tissue IGFBP**

IGFBP were present in serum and mammary tissue as four major molecular weight bands ranging between approximately 24 and 46 kDa (Figure 3.7). The largest of the IGFBP (43-46 kDa) is likely IGFBP-3 as previously described in Chapter 2 and (Weber *et al.* 1999). IGFBP-2 is likely present in the 32-35 kDa band and the lower molecular weight proteins may consist of IGFBP-1 or IGFBP-4. More accurate identification of bovine IGFBP will require specific antibodies to each binding protein. None of the serum IGFBP were affected by ovariectomy ( $p > 0.05$ ). Although we hypothesized that ovariectomy would cause a reduction in mammary IGFBP-3 abundance, this protein was not significantly affected by ovariectomy ( $p > 0.1$ ; overall treatment means  $9205 \pm 1431$  vs.  $6340 \pm 1752$  relative densitometric units for control and ovariectomized animals respectively). Other IGFBP in mammary tissue were not influenced by ovariectomy except for the 28kDa protein, which tended to be lower in ovariectomized than control animals ( $p < 0.06$ ).

## **3.4 Discussion**

The complexity of interactions between systemic hormones and locally produced growth factors that regulate mammary development within the bovine mammary gland is only just beginning to be realized. IGF-I has received considerable attention in recent years for its potential role in mediating the effects of GH and E on mammary development. As well as reaching the mammary gland via systemic circulation, IGF-I is also produced

locally within the gland. However, the relative importance of local vs. systemic IGF-I in mediating mammary growth is still not fully known. Recently, a study of a conditional knockout mouse model suggested that local IGF-I may be more important than circulating IGF-I in mediating many aspects of growth and development (Liu *et al.* 2000). The mice lacked liver IGF-I and had dramatically reduced serum concentrations of IGF-I, but peripheral IGF-I expression was not affected. Interestingly, the knockout mice grew at a rate similar to wild-type controls. Furthermore, the pattern of mammary development in the knockout mice was not different from wild-type controls (DiAugustine and Richards 2002). Several lines of evidence from our laboratory also lend support to the hypothesis that local IGF-I is of greater importance in mediating bovine mammary growth. First, mammary extracts are more potent stimulators of mammary epithelial proliferation than serum from the same animal (Weber *et al.* 1999). Much of this mitogenic activity can be attributed to IGF-I as addition of anti-IGF-I antibodies or IGFBP-3 abolishes proliferation. Secondly, administration of E to heifers stimulates mammary epithelial cell proliferation (Chapter 2). In parallel with the increase in cell proliferation, mammary IGF-I protein was increased by estrogen, but systemic concentrations of IGF-I were unchanged. In the present experiment, reduced mammary development in ovariectomized animals coincides with reduced mammary IGF-I mRNA expression. In contrast, serum concentrations of IGF-I were unchanged implying that local expression of IGF-I mRNA may play a critical role in mediating E-stimulated mammary development in prepubertal heifers.

Previous studies have suggested a role for interactions between E and the IGF-I axis in mediating mammary growth. Possibly, E and IGF-I act in a synergistic manner to stimulate mammary epithelial proliferation. Several studies have previously shown that E can enhance transcription of the IGF-I gene in a variety of tissues and species (Umayahara *et al.* 1994). This supports our observations of decreased IGF-I mRNA expression in mammary tissue from ovariectomized animals. However, in the bovine mammary gland, it is unlikely that E directly stimulates IGF-I transcription through the estrogen receptor  $\alpha$  (ER $\alpha$ ), because IGF-I expression is exclusive to stromal cells and the ER $\alpha$  is found only in epithelial cells (Capuco *et al.* 2002, Chapter 4). Estrogen may

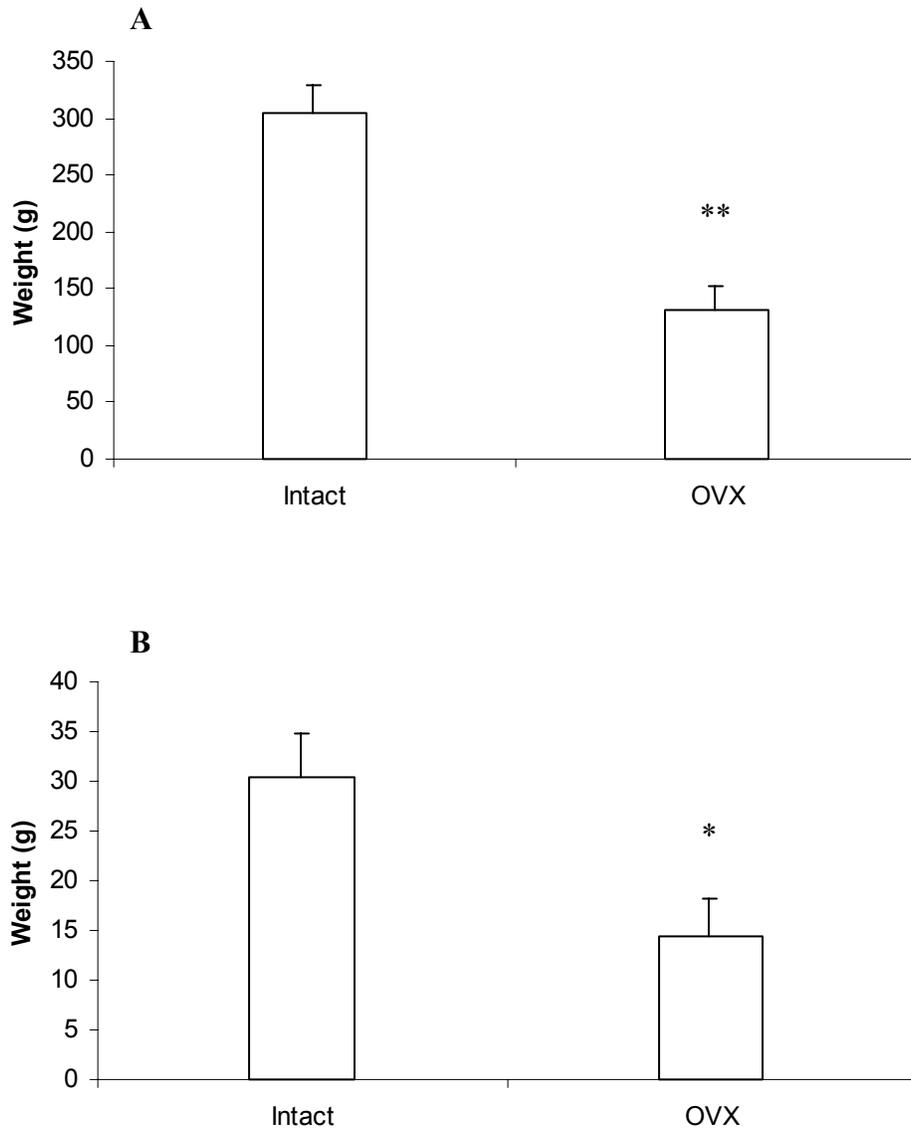
stimulate IGF-I mRNA expression through ER $\beta$ ; however, the role of ER $\beta$  in stimulating heifer mammary development has not yet been investigated. Estrogen may also increase expression and activation of the IGF-I receptor within mammary epithelium, resulting in increased sensitivity of mammary epithelial cells to IGF-I (Kahlert *et al.* 2000). In support of this hypothesis, we found that mammary parenchymal microsomes from OVX animals were significantly less able to bind <sup>125</sup>I-IGF-I than those from controls heifers. This may lead to reduced IGF-I receptor activation within mammary parenchyma from ovariectomized animals, resulting in reduced epithelial proliferation and mammary growth. Alternatively, E may interact with the downstream components of the IGF-I signaling pathway to enhance IGF-I signaling and IGF-I mediated proliferation. In the rat uterus, E stimulated tyrosine phosphorylation of the IGF-I receptor as well as the IRS-I protein, resulting in increased activation of the IGF-I pathway (Richards *et al.* 1996). Other growth factor pathways (EGF, PDGF) were not affected, demonstrating that the effects of E were specific to the IGF-I pathway. Similarly, estrogen was shown to stimulate phosphorylation of the IRS-I in human mammary epithelial cells (Lee *et al.* 1999), an effect that was abolished by the presence of anti-estrogens.

Although in the present experiment we did not measure systemic E concentrations, uterus weight in ovariectomized animals was reduced. This observation is indicative, but not proof of, reduced circulating estrogen (Hipkin 1969). In a previous study, E was significantly reduced in six-month-old ovariectomized heifers (Purup *et al.* 1993). However, the reduction was less than 0.1 pg/ml, thus raising the question of whether such a small reduction could be responsible for such large changes in mammary development. Possibly, the role of the ovary in prepubertal heifers is mediated through something other than circulating estrogen. Estrogen has also been shown to affect IGFBP expression and subsequent epithelial proliferation within mammary cells (Huynh *et al.* 1996; Chapter 2). In this experiment, serum concentrations of IGFBP were not changed by ovariectomy. For the most part, mammary IGFBP were also not influenced by ovariectomy, except for the 28-kDa protein, which tended to be lower in tissue from ovariectomized animals compared with controls.

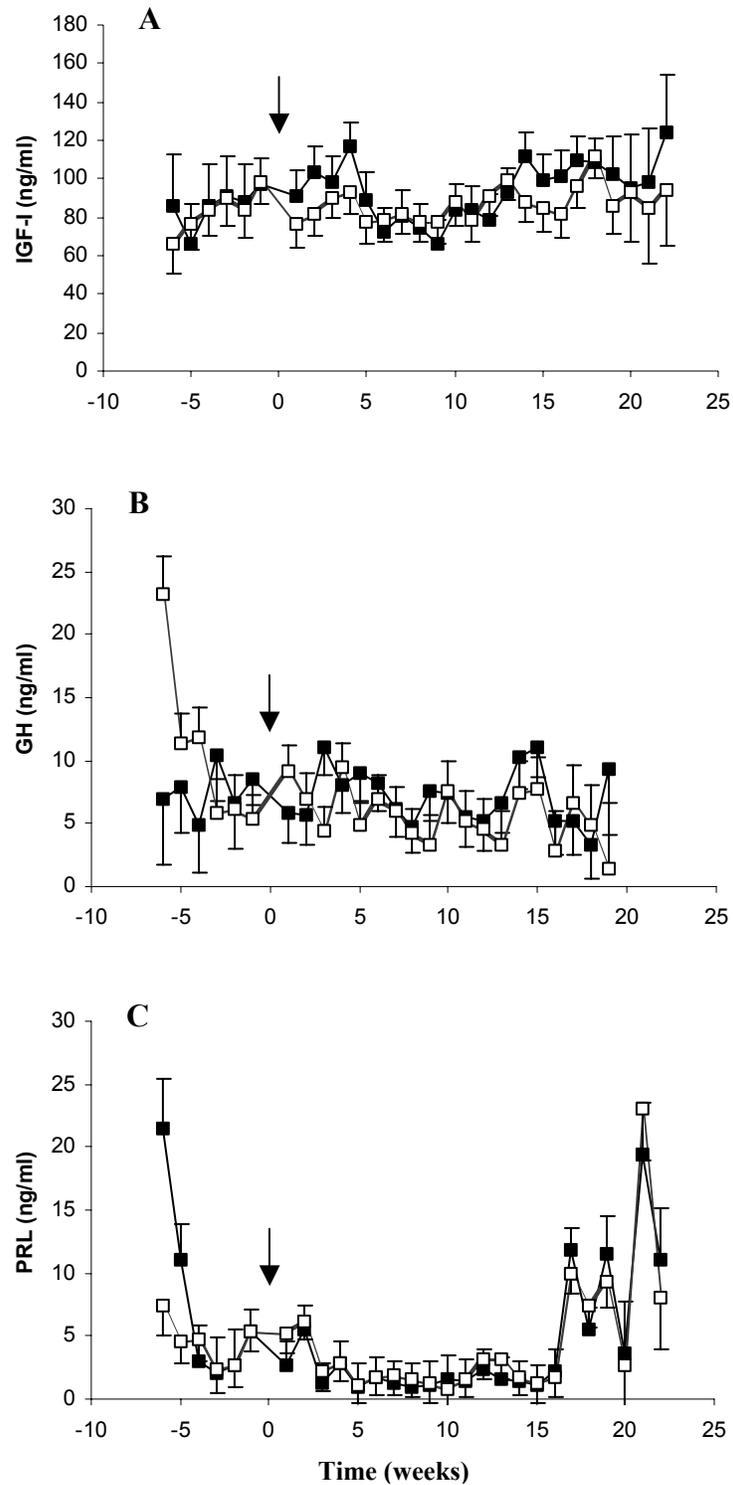
Although expression of IGF-I mRNA is observed within the parenchymal and stromal portions of the bovine mammary gland, previous studies of human breast tissue (Yee *et al.* 1991; Cullen *et al.* 1992) have suggested that mammary stromal cells exclusively produce IGF-I. Results shown here demonstrate that this is also true within the bovine mammary gland. IGF-I mRNA was not expressed by purified primary bovine mammary epithelial cells, but was expressed in both prepubertal stroma and parenchyma. Thus, the IGF-I mRNA observed within mammary parenchyma must be derived from surrounding stromal cells, such as fibroblasts, as previously hypothesized (Akers *et al.* 2000).

In addition to evaluating the effects of ovariectomy on local expression of IGF-I and IGFBP mRNA, we also sought to determine the effects of epithelial-stromal interactions on mRNA expression of the same molecules. Previous ovine work showed that expression of IGF-I mRNA within the mammary gland was influenced by the developing epithelium, suggesting a role for epithelial-stromal interactions in modulating expression of growth factors by the mammary fat pad (Hovey *et al.* 1998a). In the first experiment (Chapter 2), only subtle evidence of epithelial:stromal interactions in regulating mRNA expression of IGF-I and IGFBP-3 was found. Because the animals in the first experiment were approximately 18 months old at sacrifice, the CFP model was repeated in the current experiment to provide samples taken during the allometric growth period, which occurs between approximately three and nine months of age. This reasoning is supported by the previous observations (Hovey *et al.* 1998a) that epithelial:stromal interactions were more prominent during periods of rapid mammary growth. However, we have found no evidence for altered IGF-I or IGFBP mRNA expression in the cleared fat pad compared with the intact fat pad in the prepubertal heifers examined here. Possibly, in contrast to ewes, epithelial-stromal interactions are not important for regulating local expression of IGF-I axis components in the developing heifer mammary gland. Alternatively, interactions between the stroma and epithelium do regulate IGF-I or IGFBP mRNA expression, but only in the region near the epithelium. For example, it is possible that fibroblasts immediately adjacent to epithelial cells express a greater amount of IGF-I mRNA than fibroblasts several cell layers away from the epithelium.

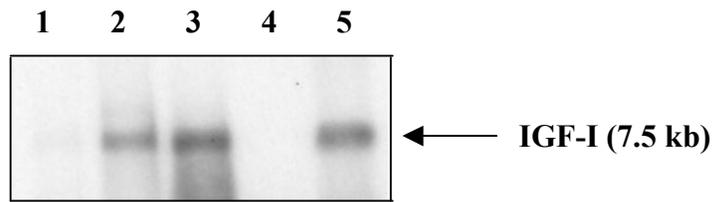
In conclusion, ovariectomy inhibits mammary development in prepubertal heifers and is associated with decreased IGF-I mRNA expression within the mammary gland, as well as decreased binding of IGF-I to mammary parenchymal microsomes. In contrast, no changes were observed in systemic concentrations of IGF-I, GH, PRL, or abundance of IGFBP, suggesting that the negative effects of ovariectomy were mediated primarily through changes in the local IGF-I axis. These results suggest a role for interactions between E and the local IGF-I axis in regulating mammary development in heifers. Continuing studies will attempt to elucidate mechanisms of such interactions in promoting mammary epithelial proliferation, IGF-I signaling, and growth factor expression within the prepubertal heifer mammary gland.



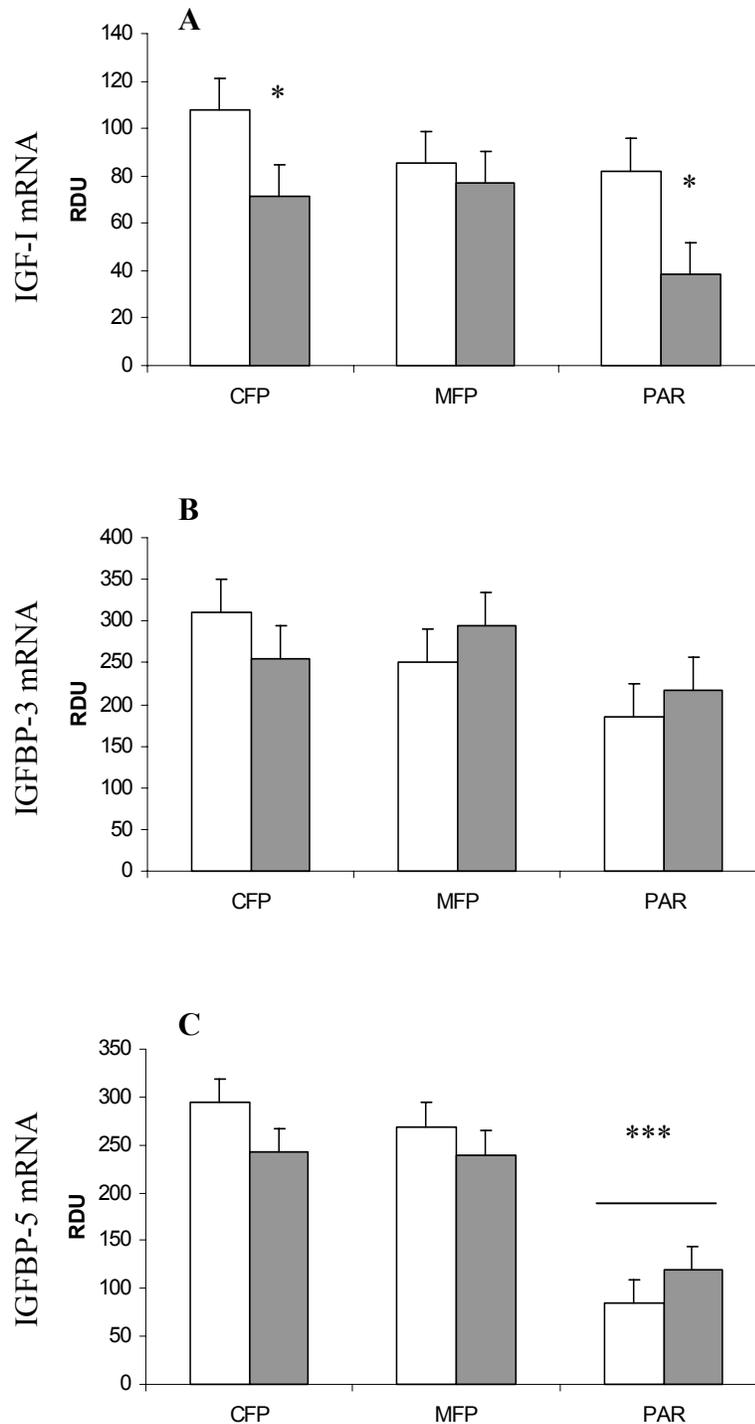
**Figure 3.1: Mammary gland (A) and uterus (B) weights of control and ovariectomized heifers.** Udders and uteri were removed at slaughter, trimmed of extra tissue such as skin and connective tissue, and weighed. \*\*  $p < 0.001$ ; \*  $p < 0.05$ ; compared with controls. Intact: controls; OVX: ovariectomized.



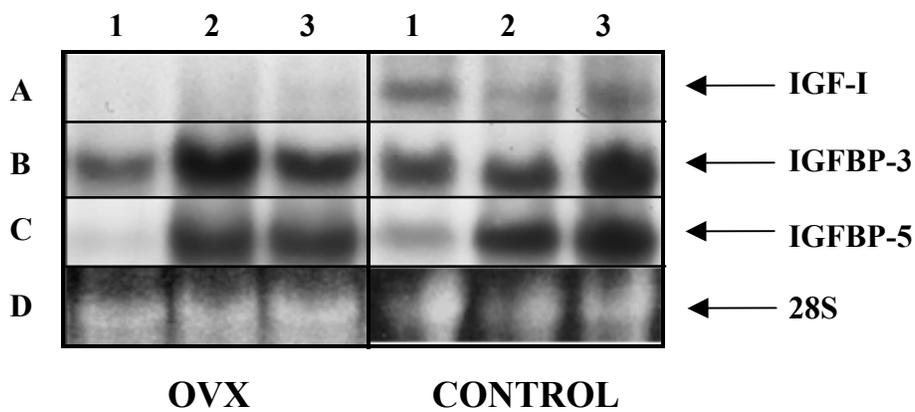
**Figure 3.2: Serum hormone concentrations in control and ovariectomized heifers.** Time in weeks is related to time of surgery: ovariectomies were performed at time zero (indicated by arrows) as described in materials and methods. Open squares: control heifers; shaded squares: ovariectomized heifers. **A)** IGF-I; **B)** Growth Hormone; **C)** Prolactin.



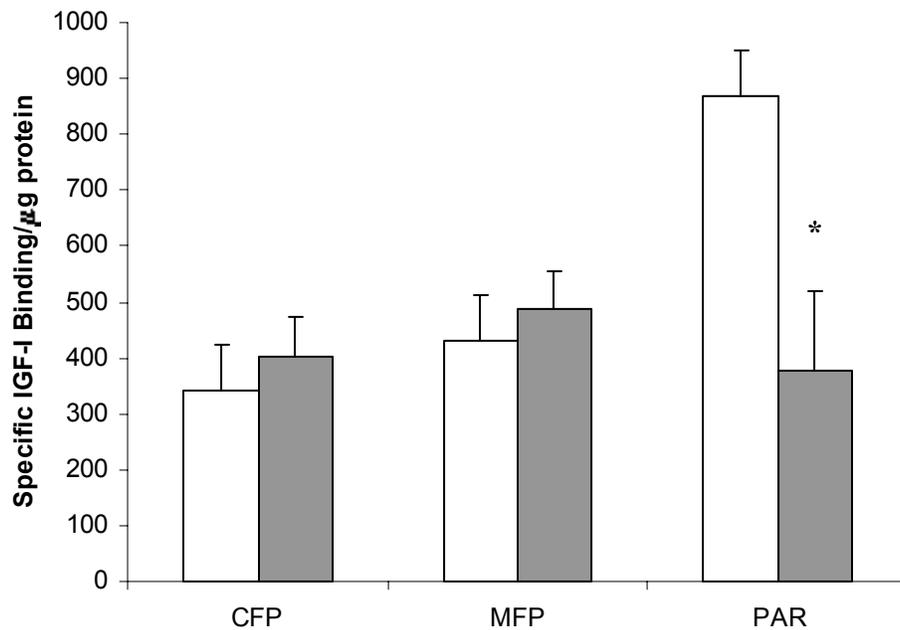
**Figure 3.3: Expression of IGF-I in bovine mammary tissue is exclusive to stromal cells.** Lane 1: purified bovine mammary epithelial cells from a prepubertal heifer; lane 2: bovine prepubertal mammary parenchyma; lane 3: bovine prepubertal mammary stroma; lane 4: bovine lactating parenchyma; lane 5: bovine liver. 15  $\mu$ g RNA per lane.



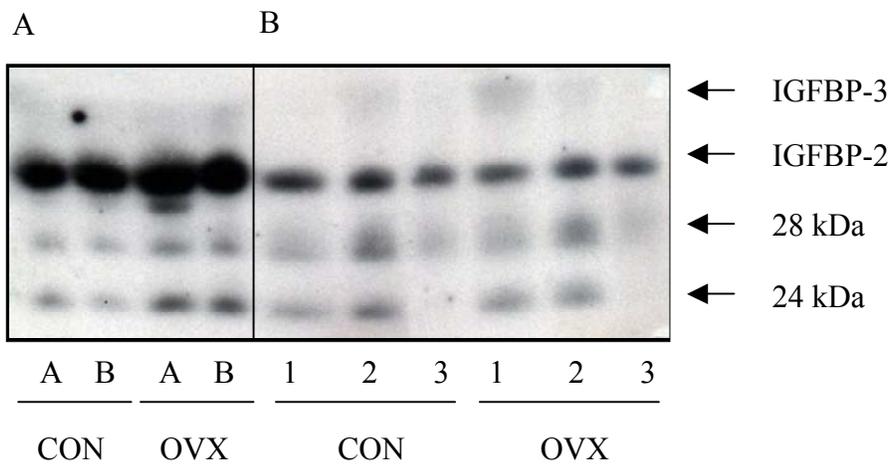
**Figure 3.4: Quantification of mRNA expression of IGF-I (A), IGFBP-3 (B), and IGFBP-5 (C) in mammary tissue from control and ovariectomized heifers.** Open bars: control animals; shaded bars: ovariectomized animals. Data are expressed as relative densitometric units (RDU). Total RNA was equal between lanes as determined by ethidium bromide staining. Least squared means and standard errors are presented, and significant main effects are described within the text. \*  $p < 0.05$  compared with control of same tissue type. \*\*\*  $p < 0.001$  main effect of PAR compared with CFP and MFP.



**Figure 3.5: Northern analysis of IGF-I, IGFBP-3, and IGFBP-5 expression in mammary tissue from ovariectomized (OVX) and control heifers.** 1: Mammary parenchyma; 2: Intact mammary fat pad; 3: Cleared mammary fat pad. Samples shown are one representative animal from each treatment group. Quantification and statistical analysis are shown in Figure 3.4A-C.



**Figure 3.6: Binding of  $^{125}\text{I}$ -IGF-I to mammary microsomes from control and ovariectomized heifers.** Specific binding was determined by calculating the difference between binding in the presence of excess IGF-I from binding in the presence of  $^{125}\text{I}$ -IGF-I alone. Open bars: control animals; shaded bars: ovariectomized animals. Results are presented as specific IGF-I binding (cpm) per 100  $\mu\text{g}$  protein. \*  $p < 0.05$  compared with control within same tissue type.



**Figure 3.7: IGFBP in serum and mammary tissue from control and ovariectomized heifers.** **A)** Serum IGFBP in two representative animals (control and OVX) before ovariectomy (A) and one week before sacrifice (B). **B)** Mammary IGFBP in two representative animals (control and OVX). 1) CFP; 2) MFP; 3) PAR.

## **Chapter 4: Mammary Epithelial Proliferation and Estrogen Receptor $\alpha$ Expression in Prepubertal Heifers: Effects of Ovariectomy and Growth Hormone**

### **4.1 Introduction**

Recent studies have shown that ER $\alpha$  expression in bovine mammary tissue is restricted to mammary epithelial cells (Capuco *et al.* 2002). Furthermore, the mechanism by which ER $\alpha$  stimulates proliferation of mammary epithelial cells appears to be more complex than initially imagined, because most proliferating cells do not express ER $\alpha$  (Zeps *et al.* 1998; Capuco *et al.* 2002), suggesting that estrogen does not directly stimulate cell proliferation. Hormonal regulation of ER $\alpha$  expression in the bovine mammary gland has not been investigated, but may be influenced by E or GH. Previous reports demonstrated that ovariectomy increased expression of uterine ER $\alpha$  mRNA in rats (Rosser *et al.* 1993; Mohamed and Abdel-Rahman 2000). Furthermore, mRNA expression levels in ovariectomized rats returned to that of control animals by administration of E. Administration of GH to virgin rats increased expression of ER mRNA in mammary tissue (Feldman *et al.* 1999) leading to the hypothesis that GH may act, in part, by enhancing the action of E via increased expression of ER $\alpha$ . To further elucidate the role of ER $\alpha$  in stimulating prepubertal heifer mammogenesis, we were interested in determining whether mammary expression of ER $\alpha$  was regulated by the ovary or by GH, and whether changes in ER $\alpha$  expression were related to changes in proliferation of mammary epithelial cells. Consequently, the objectives of this experiment were to determine the effects of ovariectomy and GH on mammary epithelial proliferation and corresponding ER $\alpha$  expression in prepubertal heifers.

### **4.2 Materials and Methods**

#### **Experimental Design and Sampling**

The experimental design for the ovariectomy experiment is described in section 3.2. To label proliferating cells, bromodeoxyuridine (BrdU; 5 mg/kg body weight; Sigma, St.

Louis, MO) was injected intravenously to each heifer two hours prior to sacrifice. At sacrifice, samples of mammary parenchyma were excised from the parenchymal:stromal interface and prepared for histological analysis as described below. To test the effect of GH on mammary epithelial proliferation and ER $\alpha$  expression, mammary tissues were obtained from a separate study. Five Holstein X Angus crossbred heifers, age six months, were used for the experiment and were assessed for epithelial proliferation and ER $\alpha$  expression before and after treatment with GH. Each heifer was administered with one injection given i.m. of 500 mg recombinant bST (Posilac; Monsanto, St. Louis, MO). Mammary biopsies were obtained immediately preceding and one week following the administration of bST injection to provide parenchymal tissue for assessment of mammary epithelial proliferation (by incorporation of  $^3\text{H}$ -thymidine to DNA) and ER $\alpha$  expression as described below.

### **Surgical Procedures**

Ovariectomies were performed as described previously (Chapter 3). Biopsies were performed as previously described (Woodward *et al.* 1993). Briefly, heifers were sedated with Xyalzine (100 mg/ml; Rompum, Mobay Corporation, KA), with 0.2 ml i.m. and 0.1 ml i.v. (jugular vein). The area around the udder was shaved and scrubbed with 70% ethanol. Skin was cut and blood vessels cauterized using an electroscapel, and an incision was made (approximately 4 cm) immediately dorsal and caudal to the teat. Subcutaneous connective tissue was separated using blunt dissection, a piece of mammary parenchymal tissue was removed (approximately 25 g), and skin was sutured. The subsequent biopsy was performed seven days later on one of the previously unsampled glands.

### **Detection of Estrogen Receptor $\alpha$**

Samples were fixed for 24 hours in 10% formalin in phosphate-buffered saline (PBS, pH 7.4) before they were embedded in paraffin using standard protocols. Five- $\mu\text{m}$  sections mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA) were deparaffinized in two changes of xylene and re-hydrated in a graded series of ethanol to water. Following rehydration, endogenous peroxidases were quenched in 3%  $\text{H}_2\text{O}_2$ .

Antigen sites were retrieved by microwaving the slides in 400 ml of 10 mM citrate buffer, pH 6.0, for three periods of five minutes each, with five minutes cooling between each period. Following the final microwaving burst, slides were allowed to cool for 30 minutes. The slides were then washed 3 x 2 minutes in PBS and blocked in 5% non-immune goat serum for 30 minutes. Mouse monoclonal anti-bovine ER $\alpha$  (C-311, Santa Cruz Biotechnology Inc, Santa Cruz, CA) was diluted in 1% non-immune goat serum to 2  $\mu$ g/ml. Sections were incubated with 100  $\mu$ l of the primary antibody overnight at 4°C. Subsequently, slides were washed in PBS (3 x 2 minutes) and detection of the primary antibody was performed using Histostain Kit (Zymed Laboratories Inc, San Francisco, CA). Sections were incubated with biotinylated secondary antibody (goat anti-mouse IgG) for 30 minutes, washed (3 x 2 minutes in PBS), and incubated with streptavidin-peroxidase (HRP) conjugate for 10 minutes. The sections were again washed (3 x 2 minutes in PBS) before the antibody-HRP complex was visualized by incubation with diaminobenzidine (DAB, Zymed Laboratories Inc., San Francisco, CA) for five minutes. Slides were counterstained in hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). ER $\alpha$ -positive cells were detected by dark brown staining of the cell nucleus. Negative controls were performed by omitting the primary antibody. No background staining was seen in any negative control slides.

### **Detection of BrdU Labeled Cells**

Tissues from the ovariectomy experiment were fixed in 10% formalin in PBS (pH 7.4) for 24 hours before being transferred to 70% ethanol. Samples were then dehydrated through a graded series of ethanol to 100% ethanol and embedded in Immunobed (Polysciences Inc., Warrington, PA) according to the manufacturer's directions. Sections were cut at 1  $\mu$ m thickness. BrdU labeled cells were detected as previously described (Ellis and Capuco 2002). Briefly, sections were hydrated, washed (PBS; 3 x 2 minutes), and blocked (1% non-immune goat serum + 1% BSA; 15 minutes). Mouse monoclonal anti-BrdU was diluted 1:100 to 2  $\mu$ g/ml in 1% non-immune goat serum + 1% BSA and incubated with sections for 1 hour. Following incubation, slides were washed (PBS; 3 x 2 minutes) and incubated with gold-conjugated goat anti-mouse IgG antibodies (Ted Pella Inc, Redding, CA) for 1 hour, followed by extensive washing in ddH<sub>2</sub>O and then

silver enhancement (Ted Pella Inc, Redding, CA) for 30 minutes. Sections were stained for 3 minutes in 0.5% azure II and 0.25% basic fuchsin in a 0.5% Na-borate solution, mounted (Bio-Mount, Ted Pella Inc, Redding, CA), and photographed. The number of BrdU-labeled cells was determined using digital photographs taken using a 100X oil-immersion objective lens.

### **Detection of <sup>3</sup>H-Thymidine Labeled Cells**

Incubations of tissues from the GH experiment in <sup>3</sup>H-thymidine was performed as previously described (Woodward *et al.* 1993). Briefly, parenchymal tissue from biopsies was finely diced into small explants (~2 - 3 mg each). Explants (~200 mg) were then incubated in Medium 199 (Sigma, St. Louis, MO) containing 2  $\mu$ Ci <sup>3</sup>H-thymidine/ml. Incubations were carried out for 1 hour at 37°C and were followed with a wash of 3 ml fresh media without tracer. Subsequently, explants were fixed overnight in 10% formalin in PBS (pH 7.4) before being transferred to 70% ethanol. Samples were then dehydrated through a graded series of ethanol to 100% ethanol and embedded in Immunobed (Polysciences Inc., Warrington, PA) according to the manufacturers directions. Sections were cut at 1  $\mu$ m thickness. Unstained slides were dipped in emulsion gel (Kodak NTB2, Eastman Kodak, Atlanta, GA), and subsequently developed (Kodak Developer D-19, Eastman Kodak, Atlanta, GA), fixed (Kodak Fixer, Eastman Kodak, Atlanta, GA) and stained in Azure II. Slides were exposed for two weeks before developing. Photomicrographs were made of sections using a 40X objective lens and the proportion of labeled epithelial cells was determined.

### **Statistics**

Statistics were performed using the SAS statistical package version 8.0 (SAS Inc. Cary, NC). Data obtained from the OVX experiment for the number of BrdU labeled and ER $\alpha$  positive cells were analyzed using a t-test to compare means between OVX and control groups. Data obtained from the GH experiment for <sup>3</sup>H-thymidine labeled and ER $\alpha$  positive cells were analyzed using a paired t-test to compare means for before and after GH treatment. Differences of  $p < 0.05$  were considered significant and differences of  $p <$

0.1 were considered a tendency. Data are reported as least squared means  $\pm$  standard error of the mean.

### **4.3 Results**

#### **Effect of Ovariectomy on Epithelial Cell Proliferation and ER $\alpha$ Expression**

Proliferating cells were distinguished by the presence of black granules in the nucleus, indicating incorporation of BrdU into DNA (Figure 4.1). Proliferation of mammary epithelial cells was decreased ten-fold in OVX heifers compared with control heifers (2.45% vs. 0.25%;  $p < 0.001$ ; Figure 4.2A) and corresponded to reduced total mammary mass ( $304 \pm 25\text{g}$  vs.  $130 \pm 21\text{ g}$ ,  $p < 0.001$ ). Reduced uterus weight in OVX heifers compared with control heifers ( $30.4 \pm 4.5\text{g}$  vs.  $14.5 \pm 3.8\text{g}$ ,  $p < 0.05$ ) suggests reduced circulating estrogen concentrations and implies that the effects of ovariectomy on mammary epithelial proliferation may be due to loss of estrogen.

In addition to changes in overall mammary development and epithelial proliferation, there were several differences in the histology and abundance of parenchymal tissue in control vs. OVX heifers (Figure 4.3). While control heifers routinely exhibited more complex branching of epithelial ducts (evidenced by multiple round structures in histological sections), tissue from most of the OVX animals was rudimentary and consisted of one or two major ducts with limited branching. The degree of ductular complexity appeared to be related to the abundance of parenchymal tissue present, which in turn appeared to be related to timing of the ovariectomy. Parenchyma from animals ovariectomized prior to six weeks of age was almost completely absent and consisted of one or two ductular structures with limited branching. Animals ovariectomized after approximately six weeks of age had substantially more parenchymal tissue, which consequently had more complex branching and many terminal ductular units (TDU) characteristic of actively growing ducts. The more arborescent structures observed in the mammary gland of control heifers appeared to reflect areas of active growth. Conversely, tissue from OVX heifers likely lacked any active growth centers and hence appeared as a rudimentary, less complex, ductular structure. Our perception was that the

mechanistic growth processes were the same in both groups of animals, but that active growth was inhibited in OVX animals, possibly because of reduced E concentration and subsequent loss of proliferative stimulus. In both OVX and control heifers, the mammary epithelial structures consisted of three cell layers: luminal (one surface of the cell in contact with the lumen), basal (one surface of the cell in contact with the basal membrane), and embedded (no contact with lumen or basal membrane).

As expected from previous observations (Capuco *et al.* 2002), expression of ER $\alpha$  was confined to mammary epithelial cells (Figure 4.4). The greatest number of ER $\alpha$ -positive cells was within the embedded layer of epithelial cells, although ER $\alpha$ -positive luminal and basal cells were also noted. The effect of ovariectomy on the proportion of ER $\alpha$  labeled cells is shown in Figure 4.2B. The proportion of ER $\alpha$  positive cells was significantly higher in OVX compared with intact animals ( $46.7\% \pm 2.4$  vs.  $36.1\% \pm 2.2$ ;  $p < 0.05$ ).

### **Effect of GH on Cell Proliferation and ER $\alpha$ Expression**

The effect of GH on proliferation of mammary epithelial cells is shown in Figure 4.5A. Treatment of heifers with GH resulted in a six-fold increase in epithelial cell proliferation ( $0.62\% \pm 0.02$  vs.  $3.92\% \pm 0.87$ ;  $p < 0.01$ ). In contrast to our hypothesis that GH would increase the proportion of ER $\alpha$  positive cells within the mammary gland, the proportion of ER $\alpha$  labeling was not affected by the GH treatment (Figure 4.5B;  $38.7 \pm 0.94\%$  vs.  $40.4 \pm 0.84\%$ ;  $p > 0.1$ ). As in the first experiment, ER $\alpha$  was confined to mammary epithelial cells and was most commonly present in the embedded layer, although some luminal and basal cells did express the ER $\alpha$ .

## **4.4 Discussion**

Although knockout mouse models have demonstrated that ER $\alpha$  is essential for proper postnatal mammary development in mice (Korach 1994), hormonal regulation of ER $\alpha$  expression, and the role of ER $\alpha$  in regulating mammary epithelial proliferation within the

bovine mammary gland is undefined. In this study, we determined the effects of ovariectomy and GH on proliferation of mammary epithelial cells and corresponding expression of ER $\alpha$  in prepubertal heifers. Ovariectomy before three months of age dramatically reduced mammary growth in prepubertal heifers at six months of age. The most obvious explanation for the decrease in mammary weight and epithelial proliferation is a loss of systemic estrogen concentrations. This is supported by a significant decrease in uterus weight from OVX animals. However, it is possible that the present observations are not related to changes in circulating estrogen. In a previous study, ovariectomy of prepubertal calves resulted in a small (0.1 pg/ml) decrease in systemic estrogen concentrations (Purup *et al.* 1993).

Whether the dramatic changes in mammary development and proliferation seen in the current experiment are due solely to a reduction in serum estrogen concentration is not clear. Interestingly, in the present experiment, the mammary glands of animals ovariectomized before approximately six weeks of age appeared to be more severely affected by the ovariectomy than animals ovariectomized between eight and twelve weeks of age. Animals ovariectomized before six weeks of age did not develop any additional epithelial tissue than what was present at time of surgery, and at six months of age, parenchymal tissue from an individual gland approximated an area of about 3 x 5 x 3 mm. On the other hand, animals ovariectomized after six weeks of age continued to develop epithelial tissue, so that at six months of age, mammary parenchyma was a substantially greater mass than it was at the time of ovariectomy. In other words, before six weeks of age, ovariectomy appeared to completely terminate mammary development, but after six weeks of age, ovariectomy appeared to hinder, but not completely inhibit, mammary development. This may be related to previous observations of mammary epithelial cell proliferation in prepubertal heifers (Ellis and Capuco 2002), in which proliferation was greater at two months of age than at five or eight months of age. Possibly, there is a critical period between birth and two months of age during which removal of ovarian stimulation of epithelial proliferation is more severe than after two months of age.

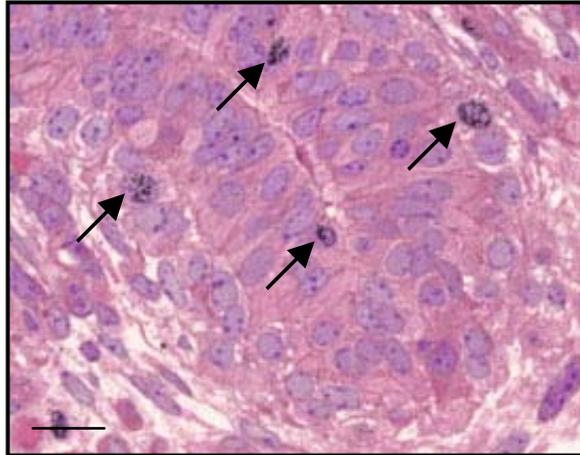
The pattern of ER $\alpha$  expression within the bovine mammary gland is similar to that of previous studies (Capuco *et al.* 2002). ER $\alpha$  was confined to mammary epithelial cells, and was present primarily in the embedded layer of epithelial cells. A smaller proportion of luminal and basal epithelial cells were also positive for ER $\alpha$ . Staining was mostly within the nucleus of epithelial cells, although cytoplasmic staining was occasionally evident in some cells. Interestingly, the increased ER $\alpha$  labeling observed in OVX heifers is similar to previous studies in which uterine ER $\alpha$  mRNA expression increased following ovariectomy. In the previously reported studies, ER $\alpha$  mRNA expression was returned to that of control animals by administration of E (Rosser *et al.* 1993; Mohamed and Abdel-Rahman 2000), suggesting that the effect of ovariectomy on ER $\alpha$  was via estrogen. The present observation of increased ER $\alpha$  expression in OVX heifers may be explained by two possibilities. First, systemic estrogen concentrations may be reduced (as implied by the decreased uterus weight for OVX heifers), thus leading to up-regulation of ER $\alpha$  expression due to loss of negative feedback mechanisms. Second, the change in ER $\alpha$  may be related to changes in epithelial cell populations. In a previous study (Ellis and Capuco 2002), the proportion of light staining cells (putatively mammary stem cells) decreased between two and five months of age, while the proportion of darkly staining cells increased. This observation implies that epithelial cells may undergo a transition from light to dark staining as the mammary epithelium matures. Possibly, epithelial cells also become ER $\alpha$  positive as they mature. This is supported by observations that proliferating cells are ER $\alpha$  negative (Capuco *et al.* 2002) and that lightly staining cells make up 90% of proliferating cells (Ellis and Capuco 2002). In contrast, dark staining cells comprise 30% of the total epithelial cell proliferation but only 7% of proliferating cells. Possibly, the increased proportion of ER $\alpha$ -positive cells in OVX animals is due to a smaller population of immature, proliferative, ER $\alpha$ -negative epithelial cells compared with intact controls.

Although accumulated evidence strongly suggests that locally derived IGF-I mediates the proliferative effects of GH within the mammary gland (Kleinberg 1997; Akers *et al.* 2000), recent reports demonstrating expression of GH receptor mRNA and protein in

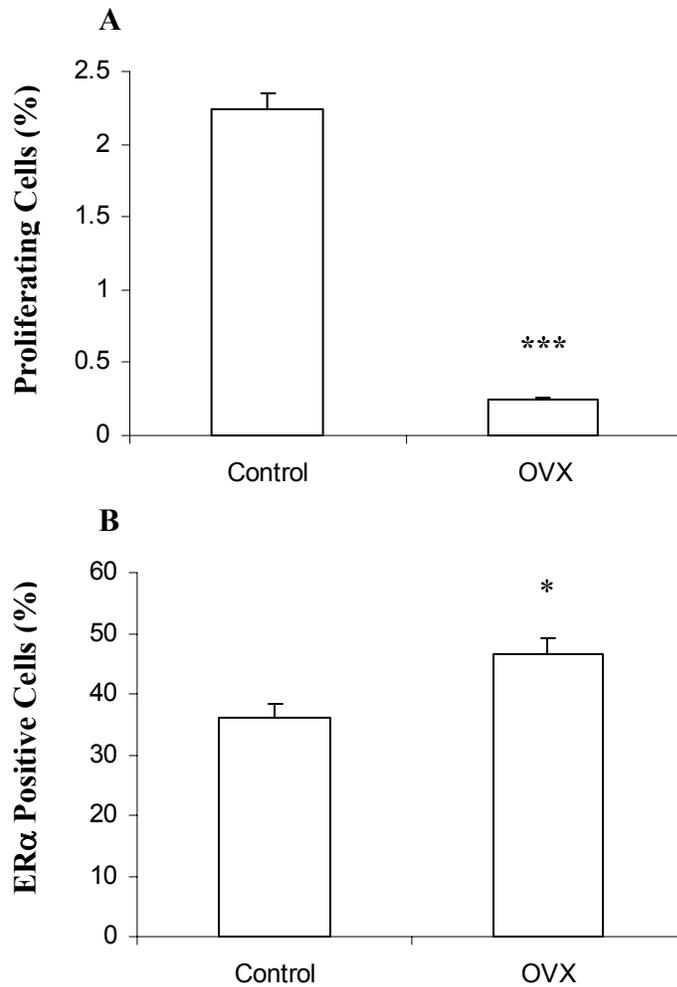
bovine mammary tissue (Sinowatz *et al.* 2000; Plath-Gabler *et al.* 2001) suggest that GH may have direct IGF-I independent effects on bovine mammary development. Reduced mammary development resulting from high feeding level is correlated with decreased serum concentrations of GH, but with increased serum concentrations of IGF-I. Changes in locally produced IGF-I or IGFBP also do not fully explain this effect, implying that the mechanism of GH action within the mammary gland is more complex than mere mediation by IGF-I and IGFBP. In rodents, administration of GH increased expression of ER $\alpha$  mRNA and protein within the mammary gland (Feldman *et al.* 1999). Possibly, one role of GH within the mammary gland is to enhance the effects of estrogen via increased expression of ER $\alpha$ . However, in contrast to these reports, we observed that GH had no effect on the proportion of ER $\alpha$ -positive cells within the mammary gland of prepubertal heifers, even though we observed a dramatic (~ six-fold) increase in epithelial cell proliferation. Therefore, in the present experiment, proportion of ER $\alpha$  labeling did not appear to be related to GH-induced proliferation within the mammary gland. The present results do not support a role for GH in enhancing E action through regulation of ER $\alpha$  expression. Possibly, the contrasting results between the present study and that reported by Feldman *et al.* are related to differences between species. While ER $\alpha$  is present in both epithelial and stromal compartments of the rodent mammary gland (Haslam and Counterman 1991), in the bovine ER $\alpha$  is confined to mammary epithelial cells. Interestingly, the capability of GH to increase ER $\alpha$  expression in the rodent mammary gland was confined almost entirely within the mammary fat pad. Consistent with the present findings, expression of ER $\alpha$  within epithelial tissue in the rodent mammary gland was not affected by GH treatment.

In summary, the results described here demonstrate that ovariectomy of prepubertal heifers decreased proliferation of mammary epithelial cells, but increased the proportion of ER $\alpha$ -positive cells. This may be attributed to decreased circulating E concentration and a removal of feedback inhibition, or to the previous observations that proliferating epithelial cells are negative for ER $\alpha$  (Capuco *et al.* 2002). GH stimulated mammary epithelial proliferation, but, in contrast to our hypothesis, no changes in the proportion of

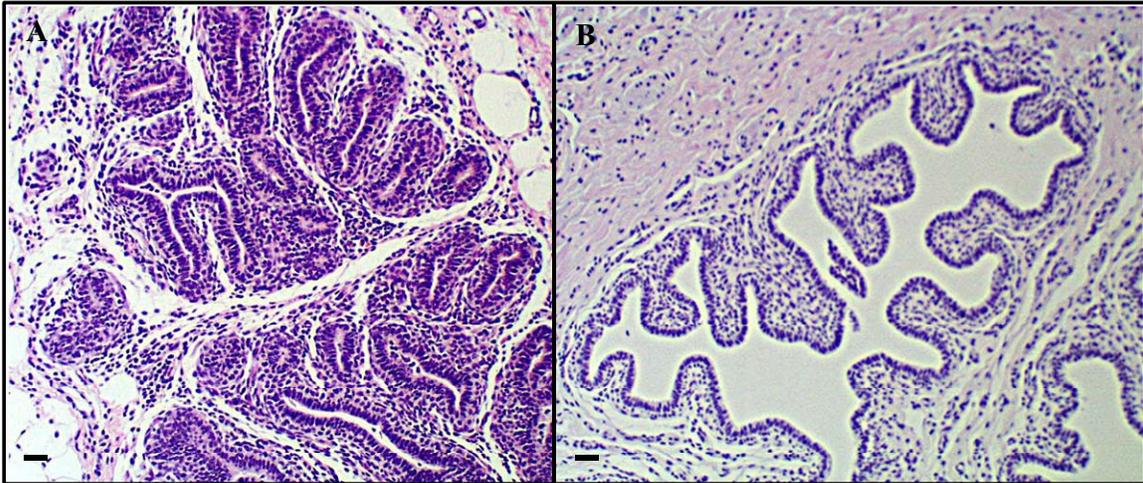
ER $\alpha$ -positive cells were found. It therefore appears unlikely that GH enhances the effects of E through up-regulation of ER $\alpha$  in prepubertal heifers. Furthermore, our observations that mammary development was more severely affected in heifers ovariectomized before six weeks of age imply that there is a critical period of ovarian stimulation during the first two months of age.



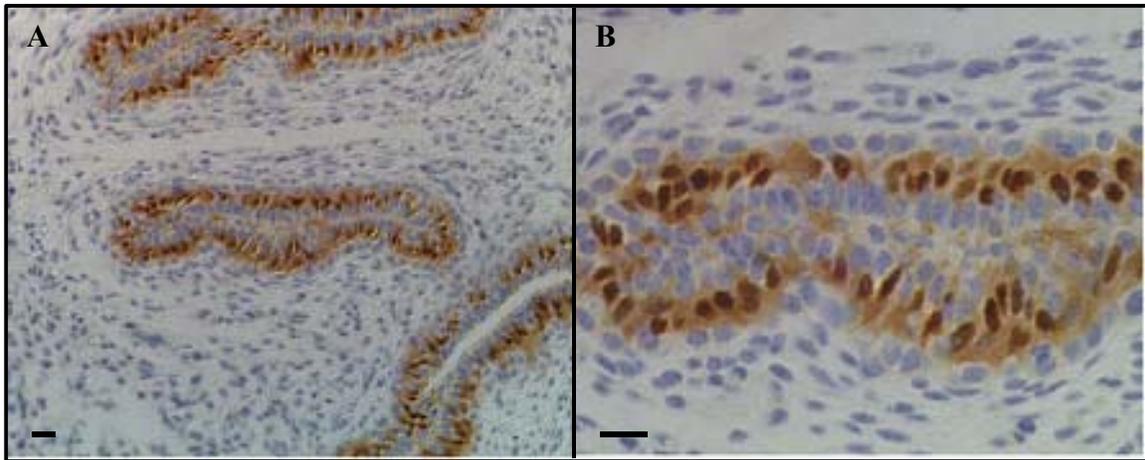
**Figure 4.1: Detection of DNA synthesis in mammary epithelial cells.** Arrows indicate BrdU-positive cells (detected by presence of black granules in nucleus). Bar represents 10  $\mu\text{m}$ .



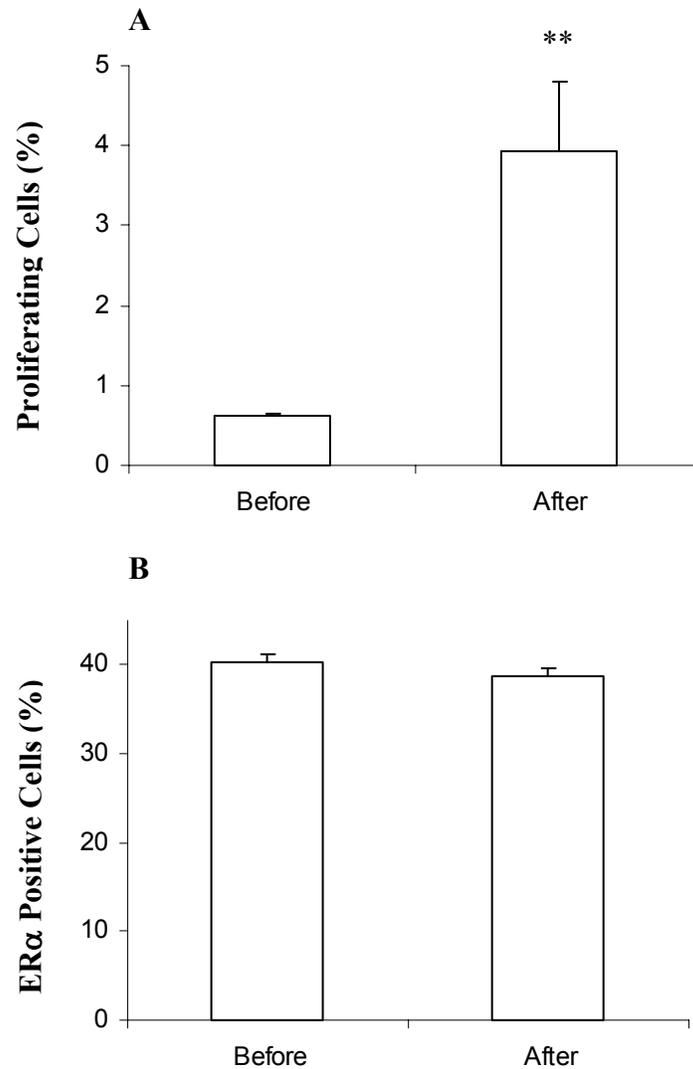
**Figure 4.2: Effect of ovariectomy on mammary epithelial cell proliferation and the proportion of ER $\alpha$ -positive cells. A)** Proliferation of mammary epithelial cells was measured by incorporation of BrdU into DNA. Number of proliferating cells is expressed as a percentage of total epithelial cells. **B)** Effect of ovariectomy on ER $\alpha$ . Positively labeled epithelial cells are expressed as percentage of total epithelial cells. \*\*\* $p < 0.001$ ; \*  $p < 0.05$  compared with controls.



**Figure 4.3: Representative images of hematoxylin and eosin staining of parenchymal tissue from control (A) and ovariectomized (B) heifers. Note presence of multiple structures in the intact heifer compared with a singular ductular structure in the ovariectomized heifer. Bars represent 20 µm.**



**Figure 4.4: Expression of ER $\alpha$  in mammary epithelial cells.** **A)** low magnification of three ductular structures from a prepubertal heifer. Note presence of ER $\alpha$  in epithelial cells only (brown stained nuclei). **B)** higher magnification of duct similar to that shown in A. ER $\alpha$  is present primarily in the embedded layer of epithelial cells. Bars represent 10  $\mu$ m.



**Figure 4.5: Effect of GH on mammary epithelial cell proliferation and the proportion of ER $\alpha$ -positive cells.** **A)** Proliferation of mammary epithelial cells as measured by incorporation of  $^3\text{H}$ -thymidine into DNA. Number of proliferating cells is expressed as a percentage of total epithelial cells. **B)** Effect of GH on ER $\alpha$ . Positively labeled epithelial cells are expressed as percentage of total epithelial cells. \*\*  $p < 0.01$  compared with controls.

## **Chapter 5: Mammary Localization and Abundance of Laminin, Fibronectin, and Collagen IV in Prepubertal Heifers.**

### **5.1 Introduction**

As the mammary epithelium advances into the mammary fat pad, it is surrounded by connective tissue (stroma) that consists of fibroblasts, adipocytes, capillaries, and extracellular matrix (ECM) components. Consequently, stromal tissue adjacent to developing epithelium must be remodeled or degraded to allow penetration of mammary epithelial structures. One aspect of heifer mammary gland development that has not been studied is the role of the ECM.

The objectives of this study were to describe the localization and abundance of collagen, laminin, and fibronectin proteins in ovariectomized and intact prepubertal heifers to provide a foundation for future studies of the mechanisms for ECM regulation of bovine mammary development. To enable comparison of different physiological states, we also examined the localization of each protein in parenchyma from post pubertal heifers (age 18 months) and one lactating cow.

### **5.2 Materials and Methods**

#### **Animals, Surgery, and Experimental Design**

The experimental design for the ovariectomy experiment is described in section 3.2. Samples were taken from the parenchymal:stromal interface and prepared for histological analysis as described below. Samples of mammary parenchyma (PAR), intact mammary fat pad (MFP), and cleared mammary fat pad (CFP) were obtained for western blotting analysis of fibronectin and laminin. Mammary tissues were also obtained from previous experiments (control heifers from the experiment in Chapter 2) to provide different developmental stages (post-pubertal heifer and lactation) for comparison with the prepubertal heifer tissue obtained from the current experiment.

## **General Histological Procedures**

Mammary tissue samples were fixed in phosphate-buffered formalin (4%, pH 7.4) overnight before being transferred to 70% ethanol. Subsequently, tissues were dehydrated through a graded series of ethanol and embedded in paraffin. Sections were cut at 5  $\mu\text{m}$  and placed on positively charged microscope slides (Fisher Scientific, Pittsburgh, PA). One slide from each block was stained with hematoxylin and eosin as follows to allow analysis of general histological structures. Slides were de-paraffinized in two changes of xylene and subsequently rehydrated through a graded series of alcohol to water. Sections were then stained by incubating in Ehrlich's Hematoxylin and Eosin (Sigma, St. Louis, MO) for ten minutes. Following staining, slides were washed in water, dehydrated through a series of ethanol, cleared in xylene, and cover slipped using Permount mounting media (Fisher Scientific, Pittsburgh, PA). To determine abundance and distribution of collagen localization, sections were stained for 1 hour in 0.1% Sirius Red / 0.1% Fast Green (counter-stain) in saturated picric acid (Sigma, St. Louis, MO).

## **Immunohistochemistry: Collagen IV, Fibronectin, and Laminin**

Sections were deparaffinized in two changes of xylene and re-hydrated in a graded series of ethanol to water. Following rehydration, endogenous peroxidases were quenched in 3%  $\text{H}_2\text{O}_2$  (15 minutes, room temperature). Antigen sites were retrieved by microwaving the slides in 400 ml 10 mM citrate buffer, pH 6.0, for three periods of five minutes each, with 5 minutes cooling between each period. Following the final microwaving burst, slides were allowed to cool for 30 minutes. The slides were then washed 3x 2 minutes in phosphate-buffered saline (PBS) and blocked in 5% non-immune goat serum for 30 minutes. Sections were incubated with 100  $\mu\text{l}$  of the primary antibody overnight at 4°C. Primary antibodies were mouse monoclonal anti-fibronectin, 1:500 (clone FBN11, NeoMarkers, Fremont, CA); rabbit polyclonal anti-laminin, 1:100 (NeoMarkers, Fremont, CA); and mouse monoclonal anti-collagen IV, 1:10 (Clone CIV 22; Oncogene, Boston, MA). Subsequently, slides were washed in PBS (3 x 2 minutes) and detection of the primary antibody was performed using the Broad Spectrum Histostain Kit (Zymed Laboratories Inc., San Francisco, CA). Sections were incubated with biotinylated secondary antibody (an equimolar mixture of goat anti-mouse, rat, rabbit, and guinea pig

IgG) for 30 minutes, washed (3 x 2 minutes in PBS), and incubated with streptavidin-peroxidase (HRP) conjugate for 10 minutes. The sections were again washed (3 x 2 minutes in PBS) before the antibody-HRP complex was visualized by incubation with diaminobenzidine (DAB, Zymed Laboratories Inc, San Francisco, CA ) for 5 minutes. Slides were briefly counterstained in hematoxylin, dehydrated, and mounted. Negative controls were performed by omitting the primary antibody. Little to no background staining was observed in all negative control sections. To analyze distribution of each protein, between five and ten photomicrographs at various magnifications (objective lenses 20X, 40X and 100X) were made of each section. The photomicrographs were then compared for differences in localization patterns and staining intensity.

### **Western Blotting**

To determine the relative amounts of fibronectin and laminin in mammary tissue from intact and ovariectomized calves, mammary extracts were prepared from each tissue as previously described (Berry *et al.* 2001). Fifty µg of protein was applied to each lane, electrophoresed through a 7.5% SDS-PAGE gel, and transferred to nitrocellulose. Membranes were blocked in 5% BSA and subsequently incubated in either mouse monoclonal anti-fibronectin, 1:400 (clone FBN11, NeoMarkers, Fremont, CA) or rabbit polyclonal anti-laminin, 1:400 (NeoMarkers, Fremont, CA) for two hours at room temperature. Following incubation, membranes were washed (3 x 15 minutes, TBS-NP40) and incubated with goat anti-mouse IgG (Sigma, St. Louis, MO) or donkey anti-rabbit IgG (Amersham, Piscataway, NJ) at 1:1000 for 1.5 hours at room temperature. Detection was by ECL, using the Supersignal Chemiluminescent Substrate Kit (Pierce, Rockford, IL) for 5 minutes followed by exposure to Kodak Biomax ML film (Sigma, St. Louis, MO) for 1 to 5 minutes. The laminin antibody resulted in a doublet of approximately 200 kDa and the fibronectin a band at approximately 200 kDa. Negative controls were performed by omitting the primary antibody and resulted in no signal for either of the secondary antibodies. Western blots were quantified using scanning densitometry.

## **Statistics**

Statistics for the western blot data were performed using the GLM procedure in the SAS statistical package version 8.0 (SAS Inc. Cary, NC, 1999). The model tested for main effects of treatment (ovariectomized or control), tissue (PAR, MFP, or CFP), and the treatment x tissue interaction. When the treatment x tissue interaction was significant, t-tests were used to make comparisons between individual means. Differences of  $p < 0.05$  were considered significant. Data are presented as Least squared means  $\pm$  standard error of the mean.

## **5.3 Results**

### **Tissue Structure and Collagen Distribution**

Representative image of sections stained with Sirius Red/Fast Green are shown in Figure 5.1A-D. Within mammary parenchyma, collagen staining was most intense in the interlobular stroma and within intra-lobular stroma (interspersed throughout groups of terminal ductular units [TDU]), collagen staining was diffuse and less intense (Figure 5.1A). All epithelial structures were surrounded with a distinct, continuous structure characteristic of a basement membrane (Figure 5.1B). Larger, more mature ductular structures were surrounded with densely packed collagen fibers whereas the smaller TDU were surrounded with loosely arranged, pale staining collagen (Figure 5.1B and 5.1C). Ovariectomized and control animals could be distinguished on the basis of Sirius Red staining, because OVX animals had abundant areas of interlobular stroma with intensely stained, densely packed collagen and few TDU surrounded with pale staining intralobular stroma. On the other hand, control animals had abundant TDU and intra-lobular stroma and fewer areas of inter-lobular stroma. Parenchyma from postpubertal heifers consisted of TDU that ranged from immature ductular structures to the rounded structures characteristic of secretory alveoli. Secretion from epithelial cells was evident in some sections. Sirius Red staining of sections from postpubertal heifers revealed a similar pattern of collagen distribution as for control prepubertal heifers (Figure 5.1D). TDU were surrounded with a continuous collagen structure and there was little collagen

staining within intralobular stroma, whereas interlobular stroma consisted of intensely stained, tightly packed collagen fibers.

### **Localization of Fibronectin**

Western analysis of fibronectin revealed a band at approximately 200 kDa (Figure 5.2A). Fibronectin protein was significantly more abundant in PAR than MFP or CFP (478 vs. 92 vs. 89 relative densitometric units/mg tissue, respectively;  $p < 0.001$ ; Figure 5.2B). A significant treatment x tissue interaction revealed that fibronectin was more abundant in parenchyma from OVX than control heifers. (572 vs. 384 relative densitometric units/mg tissue;  $p < 0.05$ , Figure 5.2B). Immunohistochemistry showed that fibronectin was loosely arranged around TDU of prepubertal mammary parenchyma (Figure 5.3A) and more densely arranged immediately next to larger ducts (Figure 5.3C) and within interlobular stroma. In prepubertal heifers, there was significant nuclear staining of epithelial and stromal cells. Interestingly, fibronectin was arranged differently in postpubertal heifers (Figure 5.3B). In the older heifers, fibronectin formed a distinct, continuous structure around individual TDU. Overall, fibronectin fibers within the intralobular stroma of postpubertal heifers took on a more organized appearance. Nuclear staining was absent from epithelial and stromal cells. Fibronectin arrangement next to large ducts and in interlobular stroma was similar in older heifers and prepubertal heifers. Parenchyma from a lactating animal showed that fibronectin formed a continuous structure around the basal side of alveoli (Figure 5.3D).

### **Expression of Laminin**

Western analysis of laminin revealed a doublet of approximately 200 kDa (Fig 5.4A). A significant treatment x tissue interaction revealed that the lower molecular weight band was significantly more abundant (per mg tissue) in control vs. OVX parenchyma (30 vs. 17 densitometric units/mg tissue;  $p < 0.05$ ; figure 5.4C) but did not differ between parenchyma and mammary fat pad. There were no significant main effects of treatment or tissue for the higher molecular weight band (Figure 5.4B). The observation that laminin was less abundant in parenchyma from OVX animals was supported by immunohistochemistry (Figure 5.5A-D). In control animals, laminin staining in

parenchymal stroma was abundant and consisted of many laminin “fibers” that were most abundant adjacent to TDU and were arranged to follow the shape of the epithelial structures (Figure 5.5A). Laminin was also present in the basement membrane of capillaries. In OVX animals, laminin staining was less prominent and consisted mainly of capillaries (Figure 5.5B). Within the epithelial structures, laminin staining was observed within the cytoplasm (but not nuclei) of epithelial cells, as well as along the basement membrane of epithelial structures (Figure 5.5C). Laminin staining was similar in postpubertal heifers compared with the control prepubertal heifers. Parenchymal from lactating animals showed that laminin formed a continuous basement membrane around each alveoli (Figure 5.5D).

### **Expression of Collagen IV**

Type IV collagen was present in the basement membrane of capillaries and along the basal surface of ductal epithelial structures. Collagen IV was more abundant in the basement membrane around larger ducts (Figure 5.6A-C) than TDU structures. In the structure shown in Figure 5.6A-C, collagen IV is prominent along the edge of the larger duct (large arrow in Figure 5.6A, enlarged in Figure 5.6B), but disappears along the edge of the smaller TDU (small arrow in Figure 5.6A, enlarged in Figure 5.6C). Collagen IV localization did not differ between OVX and control heifers. In lactating mammary tissue, collagen IV formed a continuous membrane around the base of each alveolus and of capillaries in between alveoli (Figure 5.6D).

## **5.4 Discussion**

Despite detailed knowledge of extracellular matrix biochemistry, surprisingly little information is available regarding the *in vivo* functions of ECM proteins in regulating mammary epithelial cell proliferation, survival, morphogenesis, or differentiation. Consequently, the aim of our experiment was to describe the localization of collagen, laminin, and fibronectin with respect to developing epithelial structures in actively growing (control) and growth impaired (OVX) prepubertal heifers mammary tissue. Ovariectomy provided an excellent model of impaired mammary development for two

reasons. First, the treatment was applied for an extended period (approximately four months), potentially allowing significant remodeling of the mammary fat pad and intralobular stroma. Secondly, the treatment was very effective, allowing for a dramatic contrast between the two groups. In OVX controls, total udder weight was reduced by more than 50% and epithelial proliferation was reduced by ten-fold (chapters 2 and 3, respectively). The results described here provide initial evidence for the involvement of laminin, fibronectin, and collagen IV in regulating heifer mammary development.

Our observations of collagen localization are in general agreement with the findings of Keely *et al.* (1995). Using Sirius Red, a stain with affinity for all collagen proteins, we found that collagen was densely arranged around large ducts and in interlobular stroma within the bovine mammary gland. Conversely, groups of TDU (which represent areas of proliferation and ductular branching; Capuco *et al.* 2002) were surrounded with intralobular stroma, which contained much less collagen than what was observed adjacent to major ducts. Collagen I is the most ubiquitous fibrillar collagen of connective tissue (Linsenmayer 1981), so it is likely that much of the staining we observed in the connective tissue stroma is due primarily to collagen I. In the previously reported experiment (Keely *et al.* 1995b), collagen I was most abundantly expressed around major ductular structures in the virgin mouse mammary gland, and was sparse around actively growing endbuds, implying a role for collagen I in regulating formation of mammary ducts. Our observations suggest a similar role for collagen proteins in prepubertal heifer mammary development. Similarly, local implants of TGF $\beta$  inhibited epithelial development in rodent mammary glands and induced deposition of collagen around terminal end buds (Silberstein *et al.* 1990). Conversely, in control animals, collagen was abundant around ductal structures but terminal end buds were relatively free of collagen fibers. That collagen I may act to direct branching morphogenesis or ductal development rather than epithelial cell proliferation within the mammary gland is supported by several *in vitro* observations. Human-derived mammary epithelial cells (T47D) formed duct-like structures on collagen I but not on matrigel (Keely *et al.* 1995a). Growth of mammary epithelial cells on collagen I does not promote cell survival, but mammary epithelial cells cultured on laminin or collagen IV are protected from apoptosis (Farrelly *et al.* 1999).

Ovine granulosa cells also grew rapidly on laminin or fibronectin but not on collagen I (Huet *et al.* 2001). In ruminant mammary development, the epithelial structure advances from the gland cistern as a fairly solid, highly branched mass of tissue into the mammary fat pad. As the tissue advances, the mammary fat pad must be remodeled to allow epithelial penetration. This is in contrast to rodent mammary development in which the ductal system penetrates the entire mammary fat pad before significant branching occurs. In comparison to interlobular stroma, intralobular stroma was relatively devoid of collagen. It is tempting to speculate that, as mammary ducts branch into smaller TDU, collagen is reorganized to allow penetration of epithelial structures into the mammary fat pad. Several distinct metalloproteinases (MMP) are secreted by mammary fibroblasts. For example, in rat primary mammary epithelial cells, MMP-2 and MMP-9 had maximum activity during periods of rapid proliferation and ductal branching (Lee *et al.* 2001). Whether MMP specific for collagen are developmentally regulated in the heifer mammary gland is not known. In OVX heifers, mammary epithelium was characterized by major ductal structures with little to no branching and very few TDU. Consistent with a role in duct formation but not epithelial proliferation, parenchyma from OVX heifers had very little of the pale-staining intralobular stroma. In contrast, mammary tissue from control heifers had abundant TDU and consequently abundant areas of the pale-staining intralobular stroma. In other words, actively growing mammary epithelium was associated with weak collagen staining, whereas inactively growing mammary tissue was associated with intense collagen staining.

Our observations that laminin was down regulated in parenchyma OVX heifers suggests an important role for this protein in regulating ovarian stimulated mammary development. A role for laminin in active mammary growth was also implied in a previous experiment (Keely *et al.* 1995b), who reported that laminin was localized primarily around actively growing end buds. Previous *in vitro* studies have demonstrated that laminin is a survival factor for mammary epithelial cells (Farrelly *et al.* 1999). However, the exact role of laminin in mammary epithelial development is undefined. MCF-7 cells cultured on laminin prevented estrogen-induced cell proliferation via reduced activity of the estrogen response element (Woodward *et al.* 2000a). However,

laminin did not impair the proliferative response of MCF-7 cells to other mitogens such as IGF-I or EGF. In another study, laminin and estrogen synergized to increase tissue plasminogen activator activity of MCF-7 cells, potentially enhancing ECM remodeling (Sonohara *et al.* 1998). Clearly, the mechanisms of laminin action within the mammary gland are complex and involve regulation at multiple levels.

Consistent with previous immunohistochemistry studies (Warburton *et al.* 1982; Ferguson *et al.* 1992), fibronectin was abundant throughout mammary stroma. Around TDU fibronectin formed fibrous structures that followed the shape of the epithelial structure, and fibronectin was densely arranged adjacent to major ducts. It was interesting to note that fibronectin appeared to become more organized with each developmental state (prepubertal, postpubertal, and lactating animals). In lactating mammary tissue, fibronectin was present primarily as a distinct basement membrane around individual alveoli. This is consistent with previous observations in which fibronectin was observed to be in the basement membrane of lactating, but not resting, rat mammary tissue (Warburton *et al.* 1982). In contrast to previous observations (Woodward *et al.* 2001) and to our hypothesis that fibronectin would be reduced in OVX heifers, we found that fibronectin was more abundant (per mg tissue) in parenchyma of OVX compared with control heifers. The changes in fibronectin localization through developmental stages imply a role for this protein in mammary development.

The localization of collagen IV also suggested a role for this protein in heifer mammary development. Interestingly, collagen IV was more prominent in the basement membrane of major ducts compared with TDU, perhaps implying a role for collagen IV in duct formation or branching morphogenesis.

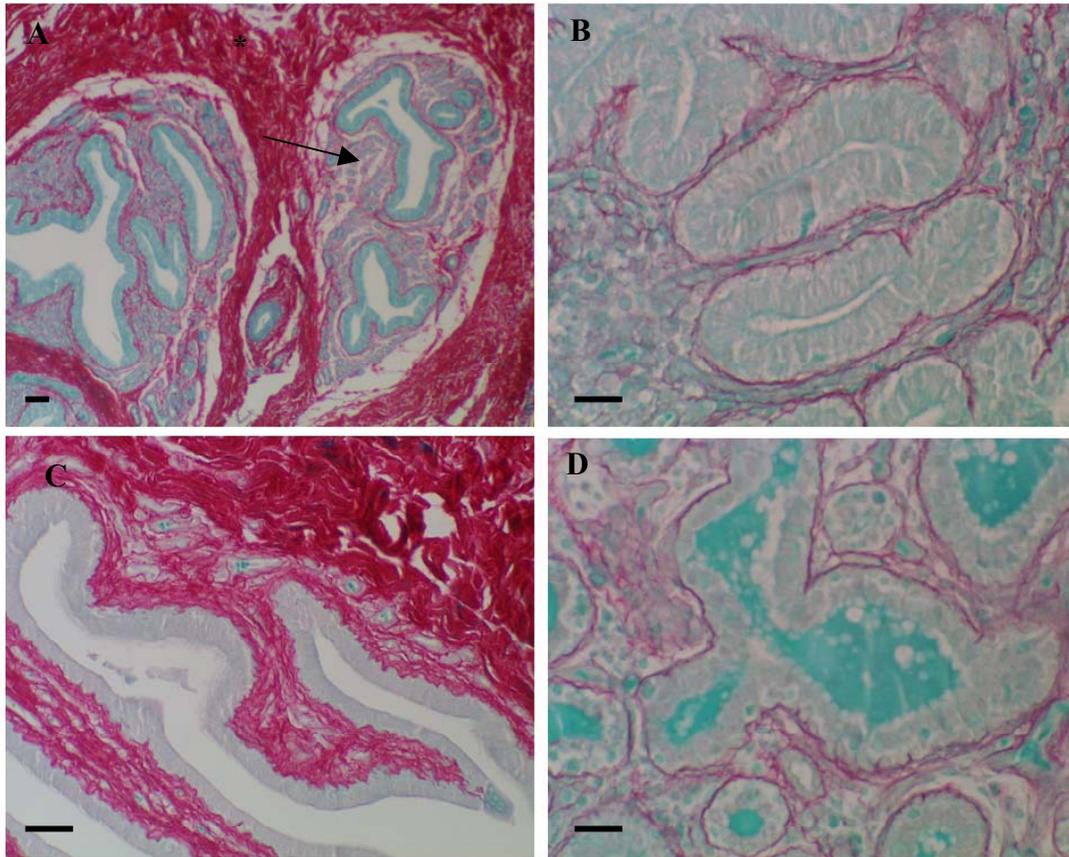
Taken together, the results presented here provide initial evidence that laminin, fibronectin, and collagen are involved in regulation of prepubertal heifer mammary development. There are a number of possible ways in which ECM proteins could influence development of mammary epithelium. First, ECM proteins may provide physical attachments for epithelial and stromal cells, as well as physical barriers that

prevent invasion of the mammary fat pad. This is supported by evidence that MMPs are differently regulated throughout mammary development (Witty *et al.* 1995). MMPs cleave ECM proteins allowing remodeling or degradation of the ECM to allow epithelial penetration of surrounding stroma. In fact, previous studies have shown that the presence of ECM alone is not sufficient for epithelial development and branching morphogenesis. Local remodeling of ECM by MMP action is also required (Witty *et al.* 1995; Lee *et al.* 2001; Simian *et al.* 2001). Possibly, cleavage of ECM proteins may also enhance mammary development through release of stored growth factors or through the direct actions of proteolytic peptides produced from MMP enzymatic reactions (Schedin *et al.* 2000). Second, ECM proteins may mediate the effects of systemic hormones such as GH or E. This hypothesis is supported by observations that fibronectin was reduced in mammary tissue of ovariectomized mice (Woodward *et al.* 2001), suggesting that this protein may act to mediate the effects of estrogen within the mammary gland. Interestingly, the epithelium was required for this effect because no change was observed within the cleared fat pad. Furthermore, ECM expression within human breast tissue was regulated throughout the menstrual cycle, with periods of low proliferation corresponding to increased expression of ECM components (Ferguson *et al.* 1992).

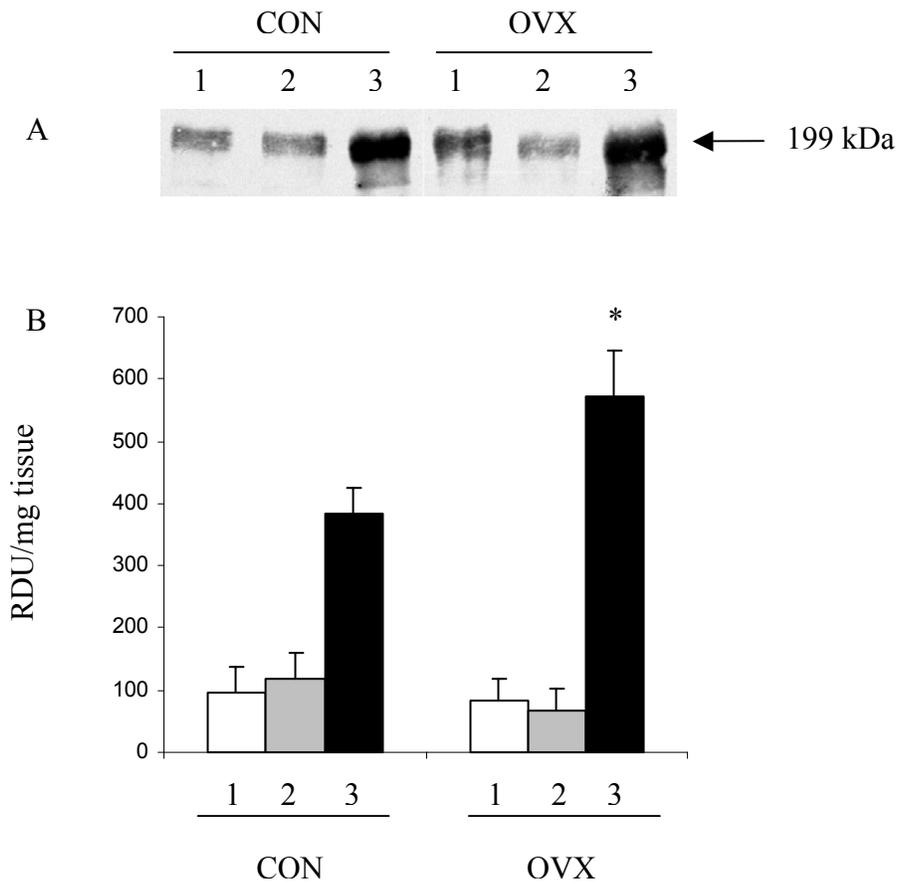
A possible role for ECM proteins in mediating the effects of mammogenic hormones in heifer mammary development is suggested by our observations of fibronectin and laminin abundance in OVX heifers. Third, ECM proteins may influence cell proliferation by interacting with growth factor signaling pathways. ECM proteins promoted synergy between IGF-I and EGF in MCF-7 cells via increased binding to IGF-I and EGF receptors as well as decreased expression of IGF binding proteins (Woodward *et al.* 2000b). Furthermore, the cell survival effects of laminin appear to be mediated through the  $\alpha 6\beta 1$  integrin and through enhanced phosphorylation of IRS-I as well as (in the presence of insulin) PI-3-kinase association with IRS-I (Farrelly *et al.* 1999).

In conclusion, the results presented here describe the localization of collagen, fibronectin and laminin proteins within the prepubertal heifer mammary gland. That each of these proteins was localized to distinct portions of the mammary epithelium and stroma

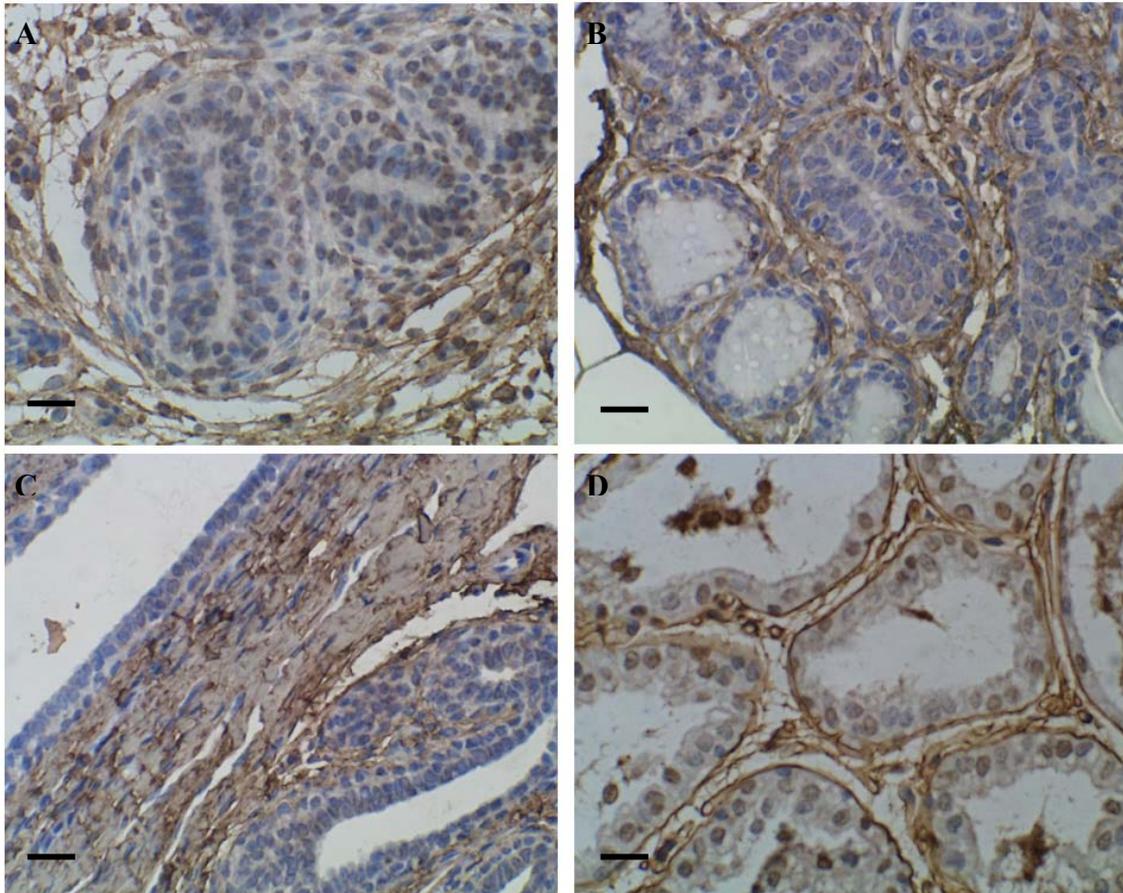
suggests specific roles in regulating development of mammary epithelium. Furthermore, laminin was significantly less abundant and fibronectin significantly more abundant in mammary parenchyma from OVX heifers, implying regulation by, or interactions with, ovarian secretion.



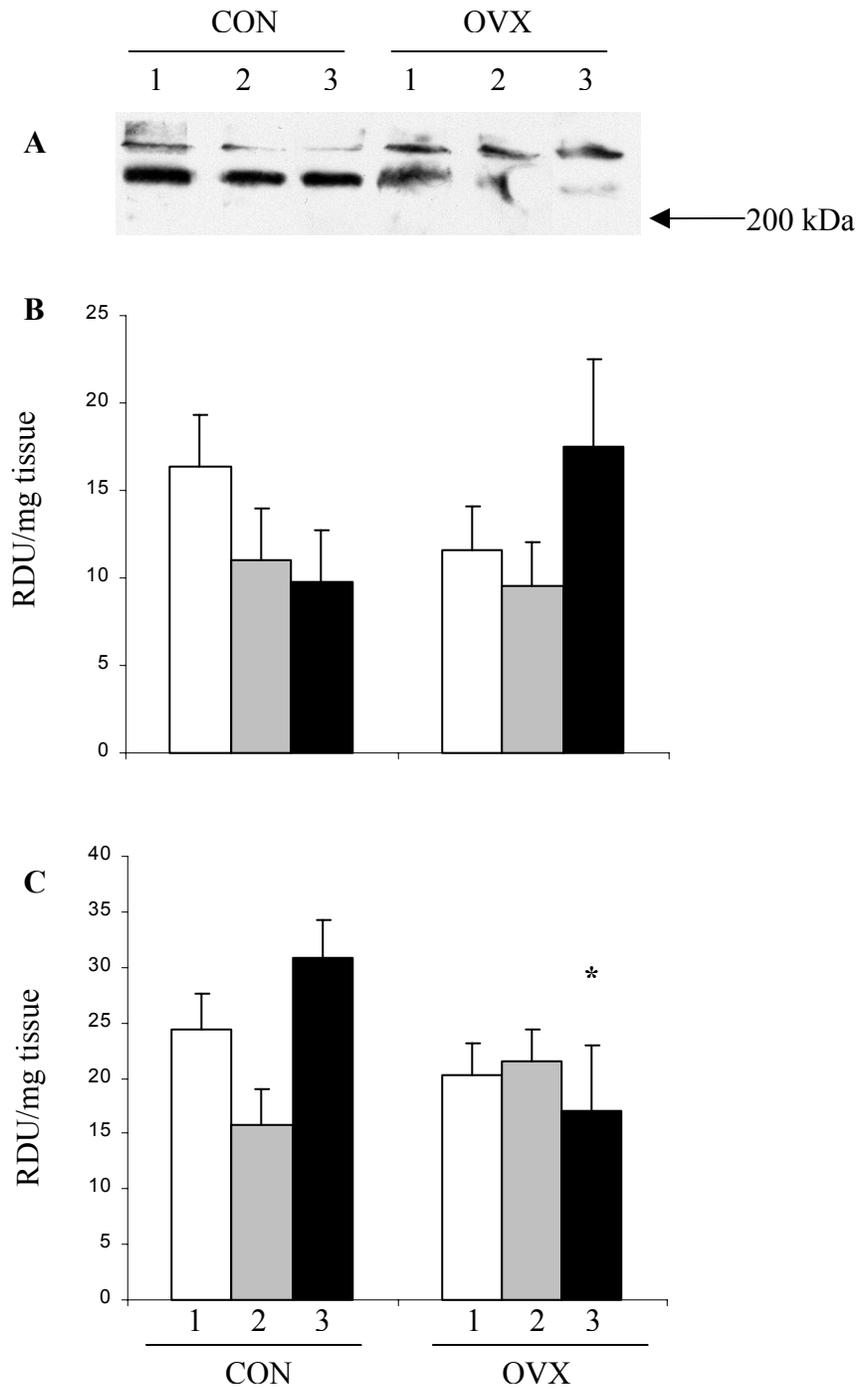
**Figure 5.1: Localization of collagen within the heifer mammary gland.** A) Parenchyma from OVX heifer. Note large quantities of intensely stained interlobular stroma (asterisk), compared with paler-staining intralobular stroma (arrow). B) Higher magnification of TDU and surrounding intralobular stroma from a control heifer. Note distinct BM and little intralobular staining. C) Large duct from prepubertal heifer with abundant, densely arranged, collagen adjacent. D) Parenchyma from postpubertal heifer. Bars represent 10  $\mu$ m.



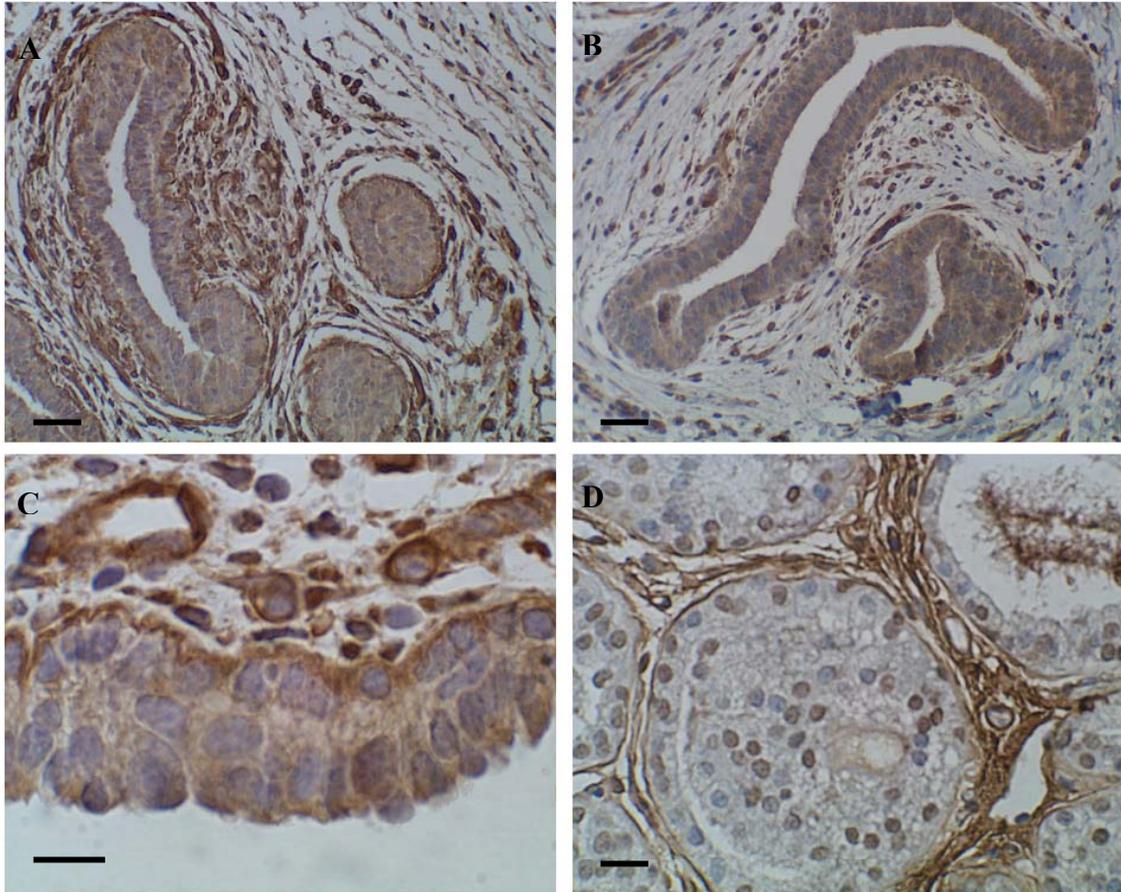
**Figure 5.2: Western analysis of fibronectin in prepubertal heifer mammary tissue.** **A)** Representative western of one control (CON) and one ovariectomized (OVX) heifer. 1 = CFP; 2 = MFP; 3 = PAR. **B)** Quantification of western blots. There was a significant main effect of tissue ( $p < 0.001$ ; described within text). \* $p < 0.05$  compared with CON PAR.



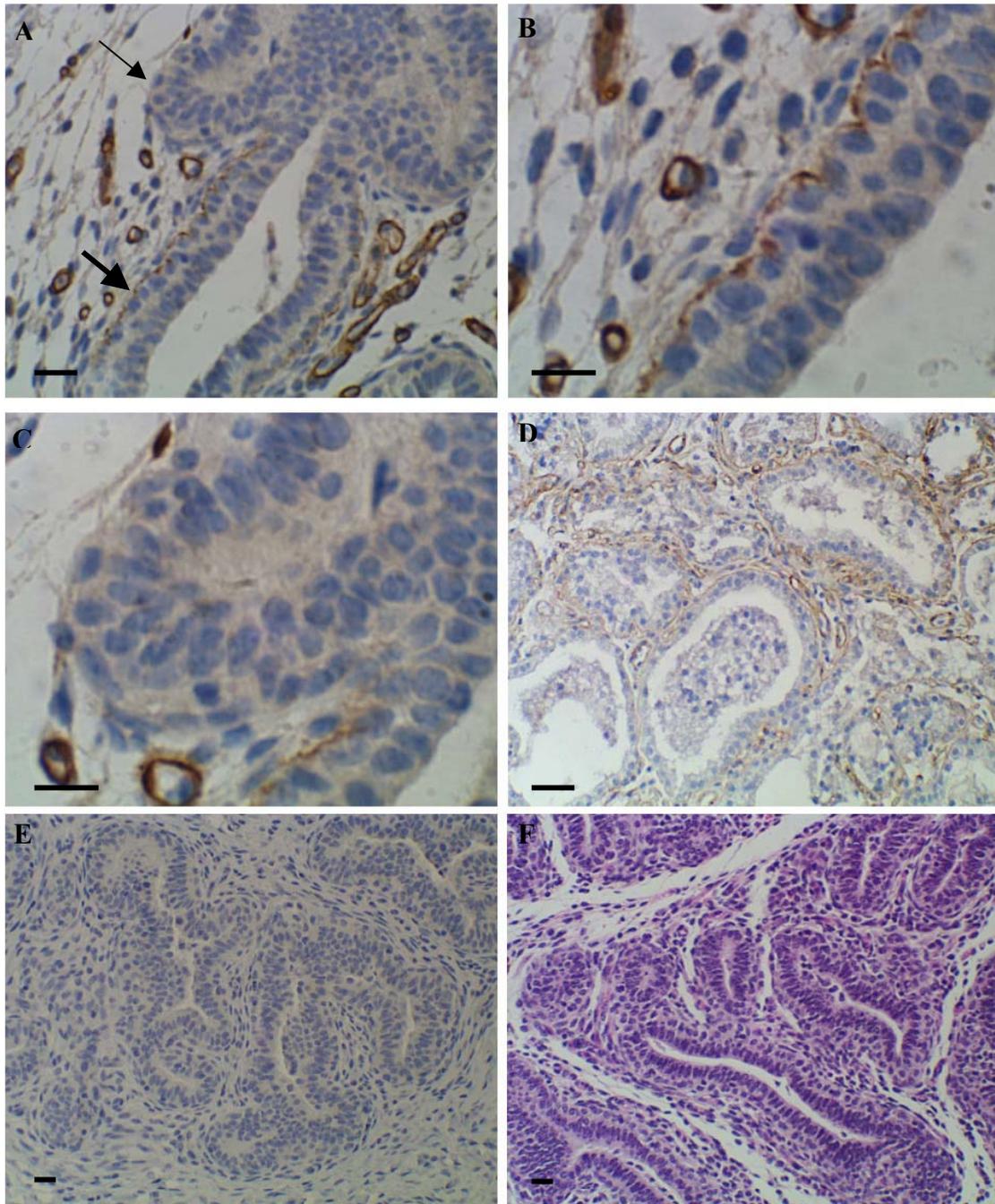
**Figure 5.3:** Examples of fibronectin (brown) localization in heifer mammary tissue. **A)** Prepubertal parenchyma. **B)** Postpubertal parenchyma; note appearance of distinct basement membrane around epithelial structures. **C)** Example of a large duct with a dense arrangement of fibronectin adjacent to it. **D)** Lactating parenchyma. Note distinct basement membrane and little intralobular stromal staining. Bars represent 10  $\mu$ m.



**Figure 5.4: Western analysis of laminin in prepubertal heifer mammary tissue.** **A)** Representative western of one control (CON) and one ovariectomized (OVX) heifer. 1 = CFP; 2 = MFP; 3 = PAR. **B)** Quantification of higher molecular weight band. There were no significant main effects of treatment or tissue. **C)** Quantification of lower molecular weight band. \* p < 0.05 compared with CON PAR.



**Figure 5.5:** Examples of laminin localization in heifer mammary tissue. **A)** Parenchyma from a control heifer. Note abundant stromal staining near TDU structures. **B)** Parenchyma from OVX heifers with less apparent staining in surrounding stroma. **C)** High magnification of an epithelial structure to show presence of laminin in the basement membrane. **D)** Parenchyma from a lactating cow. Note distinct basement membrane around individual alveoli. Bars represent 10  $\mu\text{m}$ .



**Figure 5.6:** Examples of collagen IV localization in heifer mammary tissue. **A)** Epithelial structure containing large duct (large arrow) and smaller TDU (small arrow). Note the presence of a prominent basement membrane on the large duct and its absence on TDU. **B)** Higher magnification of the edge of the large duct shown in A. **C)** Higher magnification of the TDU shown in A. **D)** Parenchyma from a lactating cow. Note presence of basement membrane around individual alveoli. **E)** Negative control for immunostaining showed little to no background. **F)** Hematoxylin and Eosin staining of heifer mammary tissue. Bars represent 10  $\mu$ m.

## **Chapter 6: Use of an Immortalized Bovine Mammary Epithelial Cell Line (MAC-T) to Measure the Mitogenic Capacity of Tissues from Heifer Mammary Tissue: Effects of Nutrition and Ovariectomy**

### **6.1 Introduction**

Previous studies from our lab have shown that nutrition-induced changes in heifer mammary growth are reflected by the proliferative responses of bovine primary mammary epithelial cells (MEC) to mammary tissue extracts (Weber *et al.* 2000a). Heifers raised on a high rate of gain had reduced mammary development compared with heifers raised on a low rate of gain. Accordingly, mammary tissue extracts from heifers raised on a high rate of gain were less capable of stimulating proliferation of primary MEC than tissue extracts from heifers raised on a low rate of gain. Because the use of primary MEC in culture is technically more difficult and time consuming than the use of immortalized cell cultures, as well as expensive, we were interested in determining whether MAC-T cells would also provide a suitable test cell. MAC-T cells are an immortalized epithelial cell line isolated from bovine mammary tissue (Huynh *et al.* 1991) and provide a useful *in vitro* model for mammary epithelial cell proliferation, because they retain a number of biochemical and morphological characteristics typical of mammary epithelial cells *in vivo*. In addition, MAC-T cells proliferate in response to IGF-I and secrete several forms of IGFBP into media (Romagnolo *et al.* 1994a).

As well as determining the usefulness of MAC-T cells as a test cell for mitogenesis assays, we were also interested in determining the mitogenic capacities of mammary extracts obtained from ovariectomized (OVX) and control heifers. In OVX heifers, mammary gland development, as well as mammary epithelial proliferation, was dramatically reduced (Chapters 3 and 4, respectively). Furthermore, IGF-I mRNA and <sup>125</sup>I-IGF-I binding to parenchymal microsomes were also decreased in OVX heifers (Chapter 3), suggesting that reduced proliferation was at least partly regulated by changes in the local IGF-I axis.

Therefore, the objectives of this experiment were two-fold. First, we aimed to determine whether MAC-T cells would accurately mimic the previously observed responses of primary MEC to mammary tissue extracts obtained from high- and low-fed heifers. Second, we aimed to examine whether mammary tissue extracts from OVX heifers would result in impaired mitogenic capacity compared with intact control heifers.

## **6.2 Materials and Methods**

### **Experimental Design**

Mammary tissue extracts from heifers raised on a high or low rate of gain were obtained from a previous experiment (Weber *et al.* 2000a). Briefly, heifers were randomly assigned to either high (ADG of 1.1 kg/day) or low (ADG of 0.55 kg/day) feeding levels. Treatments were continued for four weeks before heifers were sacrificed and samples of mammary parenchyma were obtained. Heifers were approximately nine months of age at slaughter. Mammary tissue extracts from OVX and control heifers were also obtained from a previous experiment (Chapter 3).

### **Preparation of Mammary Extracts**

Extracts of mammary tissues were prepared as previously described (Weber *et al.* 2000a). Briefly, samples were homogenized in physiological saline (4:1 saline:tissue). Mammary homogenates were shaken for 90 minutes at 4°C, and then centrifuged at 10 000 x g to remove cellular debris. Subsequently, the supernatant from each sample was ultra-centrifuged at 100 000 x g. The resulting supernatant was retained and sterilized through 0.2 µm syringe filters (Acrodisc, Fisher Scientific, Pittsburgh, PA) and stored at –80°C until assay. Serum samples taken from animals on the OVX trial were taken before sacrifice and were also filter sterilized and stored at –80°C until assay.

### **Cell Cultures**

MAC-T cells were plated in 24-well plastic dishes (Falcon, Fisher Scientific, Pittsburgh, PA) at  $2 \times 10^4$  in DMEM (GibCo, Rockville, MD) supplemented with 10% FBS (GibCo, Rockville, MD). Following overnight attachment, cells were washed with 1 ml DPBS

(GibCo, Rockville, MD) and 1 ml of fresh DMEM (no FBS) was added. After 48 hours serum starvation, medium was removed and fresh medium was added along with mammary tissue extracts at varying concentrations (1 – 8%). Cells were incubated with the extracts for 16 hours, after which time  $^3\text{H}$ -thymidine was added to each well at a concentration of 1  $\mu\text{Ci/ml}$ . Results for  $^3\text{H}$ -thymidine incorporation were determined using a liquid scintillation counter. 5% FBS was included as a positive control on each plate.

### **Statistics**

Data presented are representative experiments, which were repeated a minimum of three times with similar results. Within each experiment, samples were replicated in four wells to provide estimates of intra-assay variation (less than 10%). Data were analyzed using the GLM procedure of SAS. For the dose response curves, a Bonferroni test was used to determine significant differences between each dose. Data from the feeding trial were analyzed with a model that tested for the effect of treatment. Data from stromal tissues obtained from the OVX trial were analyzed with a model that tested for the effects of treatment (OVX or control) and tissue (CFP or MFP). Data from serum and the parenchymal tissues obtained from the OVX trial were analyzed with a model that tested for the effects of treatment. Data are presented as least squared means  $\pm$  S. E. M. Differences were considered significant at  $p < 0.05$ .

## **6.3 Results**

### **MAC-T Cells Mimic the Response of Primary Bovine Mammary Epithelial Cells to Mammary Tissue Extracts**

Addition of a pool of mammary tissue extract at concentrations ranging from 1 - 8% stimulated a dose dependent increase in proliferation of MAC-T cells cultured on plastic (Figure 6.1A). A similar dose-dependent increase in cell proliferation in response to mammary tissue extracts was observed for MAC-T cells cultured on collagen (Figure 6.1B). Mammary extracts from low-fed heifers stimulated a greater proliferative response than mammary extracts from high-fed heifers ( $40.6$  vs.  $21.9 \pm 1.8$  cpm  $\times 10^3$ ,  $p$

< 0.001; Figure 6.2). These results are similar to what we previously observed for primary mammary epithelial cells (Weber *et al.* 2000a). Therefore, we determined that MAC-T cells would be a good alternative to primary MEC for studying the mitogenic capacities of heifer mammary tissues.

### **The Mitogenic Capacities of Heifer Mammary Tissue Extracts was not Influenced by Ovariectomy or by Epithelial-Stromal Interactions**

Addition of pooled extracts derived from parenchyma of control or OVX heifers to MAC-T cells at concentrations ranging from 1 – 8% resulted in dose-dependent increases in cell proliferation (Figure 6.3). Addition of pooled extracts from either CFP or MFP also resulted in dose-dependent increases in cell proliferation (data not shown). To test the mitogenic capacities of individual extract samples, we chose a concentration of 4%, which fell in the middle of the dose-response curve for each tissue type. Ovariectomy did not influence the mitogenic capacities of mammary extract tissues for PAR (Figure 6.4), CFP or MFP (Figure 6.5). Furthermore, there was no overall difference in mitogenic capacities for CFP or MFP (Figure 6.5). In addition to mammary tissue extracts, we also tested the capacity of serum to stimulate MAC-T proliferation and found that there was no difference in mitogenic responses to serum from control or OVX heifers (Figure 6.6).

## **6.4 Discussion**

Because *in vivo* heifer experiments are time-consuming and expensive, good *in vitro* models of bovine mammary epithelial cell proliferation are essential supplemental tools for studies of heifer mammary development. Previous experiments from our lab have demonstrated that responses of primary MEC to mammary tissue extracts reflect changes in overall mammary development (Weber *et al.* 2000a). However, primary cell cultures are technically more demanding than immortalized cell cultures, and more difficult to maintain as long-term model systems. Consequently, we were interested in using immortalized mammary epithelial cells as test cells for studies of the mitogenic capacities of heifer mammary tissues. MAC-T cells represent a useful model for studies of bovine mammary epithelial proliferation because these cells proliferate in response to IGF-I, a

known mammary mitogen (Cohick 1998), and also retain several characteristics of *in vivo* differentiated mammary epithelial cells (Huynh *et al.* 1991).

Our data demonstrate that MAC-T cells do mimic the responses of primary MEC to mammary tissue extracts from high- or low-fed heifers. Mammary glands from heifers raised on a high rate of gain (1.1 kg/day) have reduced development compared with mammary glands from heifers raised on a low rate of gain (0.55 kg/day). These differences in overall mammary development were reflected by the mitogenic capacities of the mammary tissue extracts. Mammary tissue extracts from high-fed heifers were less capable than mammary tissue extracts from low-fed heifers in stimulating proliferation of MAC-T cells. These data suggested that MAC-T cells would indeed provide a useful and accurate model of mammary tissue extract-stimulated proliferation. However, in contrast to our hypothesis that reduced mammary development in OVX heifers would be reflected by impaired capacity to stimulate proliferation of MAC-T cells, no difference between mammary tissue extracts obtained from OVX or control heifers was observed. This was surprising because the differences in overall mammary growth as well as *in vivo* mammary epithelial proliferation (measured at sacrifice by incorporation of BrdU into DNA) were dramatically (ten-fold) reduced in OVX heifers. Furthermore, no evidence was found for a reduction in the mitogenic capacity of CFP, compared with MFP.

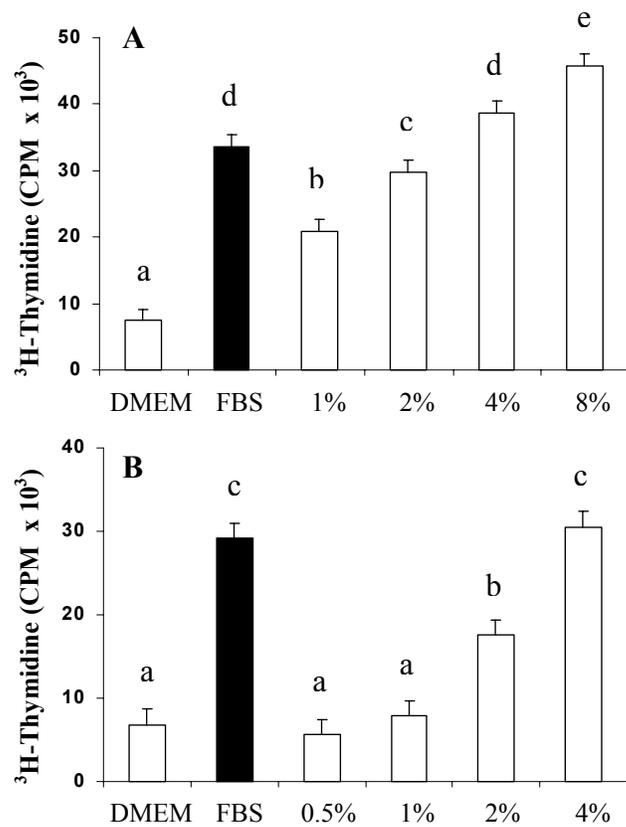
There are several possible explanations for the observations reported here. First, MAC-T cells may be a good model for nutrition-induced changes in mammary growth, but not ovarian-induced changes. This hypothesis is supported by the fact that MAC-T cells do not proliferate in response to estrogen. If the changes in mammary development in OVX heifers are due primarily to reduced circulating estrogen concentrations and subsequently decreased estrogen-stimulated mammary epithelial proliferation, then it is feasible that the two treatment groups would have the same capacities to stimulate proliferation of the (estrogen receptor negative) MAC-T cells. Uterus weight in OVX heifers was significantly lower compared with control heifers (Chapter 3), implying decreased serum estrogen concentrations. However, the relevance of circulating estrogen in such young

heifers is questionable. Furthermore, recent experiments related to mammary expression of ER $\alpha$  within prepubertal heifers suggest that the proliferative effects of estrogen are indirect. More than 90% of proliferating cells are ER $\alpha$  negative, implying that the proliferative effects of estrogen are via production of a paracrine factor which then stimulates proliferation of neighboring cells (Capuco *et al.* 2002). Extending the hypothesis, OVX heifers would lack estrogen-stimulated production of the putative paracrine growth factor, whereas control heifers would continue to produce the paracrine growth factor. So even though MAC-T cells do not respond to estrogen, differences in the mitogenic capacity of the tissue extracts should still be reflected in the proliferative response of MAC-T cells.

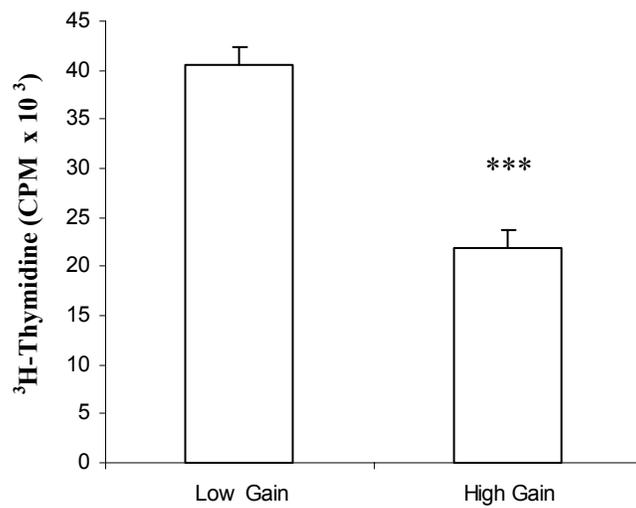
Second, that mammary tissue extracts from OVX and control heifers have the same capacity to stimulate proliferation of MEC implies that the tissue composition of the two treatment groups was the same, despite major differences in overall mammary development. Histological studies of OVX and control parenchymal tissue (Chapter 4) showed that the overall structural composition of the tissue was similar in OVX and control heifers, but OVX heifers had dramatically less parenchymal tissue and lacked terminal ductular units that represent active growth centers. Possibly, the observations that nutrition-induced but not ovarian-induced changes in mammary development are reflected by the mitogenic capacities of mammary tissue extracts suggest relative differences the mechanisms of growth inhibition.

Third, the lack of difference between mammary tissue extracts from OVX and control heifers is in contrast to our observations of locally produced IGF-I mRNA. We observed significantly less IGF-I mRNA in mammary tissue of OVX heifers compared with control heifers (Chapter 3). Because MAC-T cells respond to exogenous IGF-I in terms of increased proliferation (Cohick 1998), we would expect to see that extracts from OVX heifers stimulate less proliferation of MAC-T cells than extracts from intact heifers, in reflection of local IGF-I mRNA expression.

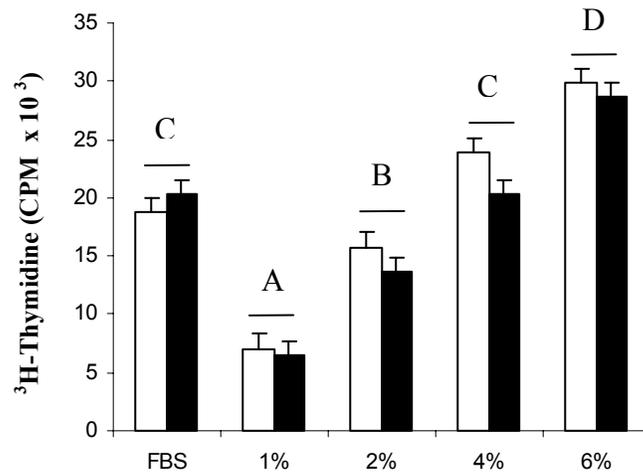
In conclusion, the data presented show that the proliferative responses of MAC-T cells to mammary tissue extracts from high- and low-fed prepubertal heifers mimic the response of primary MEC and reflect changes in overall mammary development. In contrast, the proliferative capacities of mammary tissue extracts from OVX vs. intact prepubertal heifers did not differ, despite dramatic changes in overall mammary development. This observation may be related to limitations of the MAC-T cell culture model for testing mitogenic capacity of mammary tissue extract, or to differences in mechanisms between nutrition-induced and ovarian-induced changes in mammary growth. The data also raise the question of the relative importance of systemic vs. local growth regulation in the heifer mammary gland.



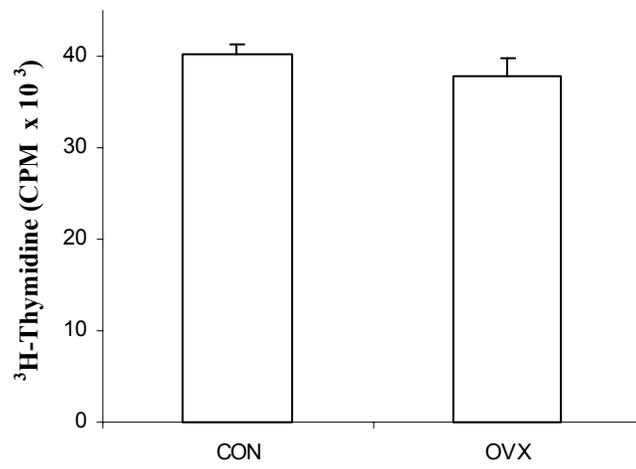
**Figure 6.1: Dose-dependent response of MAC-T cells to mammary tissue extracts.** **A)** MAC-T cells plated on plastic. **B)** MAC-T cells plated on collagen. Bars with different subscripts are significantly different. Representative experiment of three replicates.



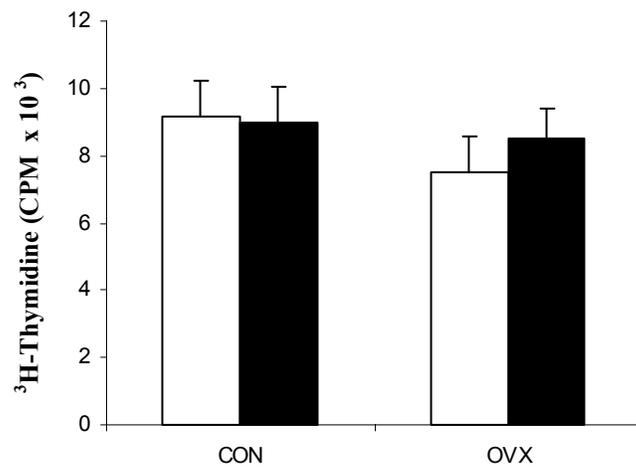
**Figure 6.2:** Response of MAC-T cells to mammary extracts from heifers raised on a high or low rate of gain reflects overall mammary development and mimics response previously observed from primary bovine mammary epithelial cells (Weber *et al.* 2000a). \*\*\*  $p < 0.001$  compared with low gain.



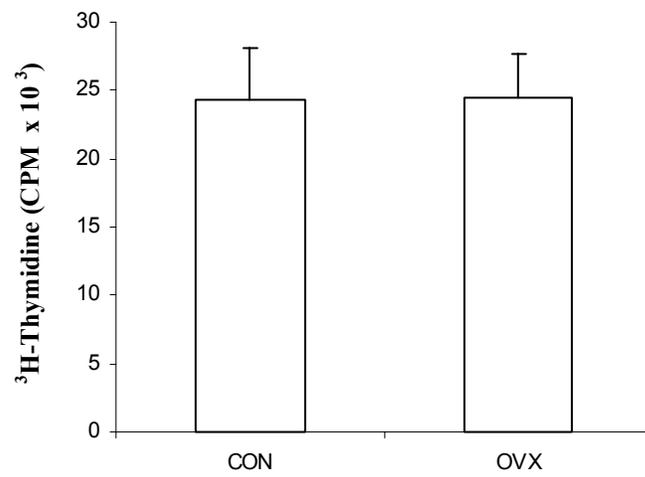
**Figure 6.3:** Dose-dependent response of MAC-T cells to mammary tissue extracts from control (open bars) and ovariectomized (shaded bars) heifers. Bars with different subscripts are significantly different. Representative experiment of three replicates.



**Figure 6.4: Response of MAC-T cells to mammary tissue extracts (4%) from control (CON) and ovariectomized (OVX) heifers.**



**Figure 6.5: Response of MAC-T cells to mammary stromal extracts (4%) from control (CON) and ovariectomized (OVX) heifers.** Extracts were prepared from epithelium-free (cleared) fat pad (CFP; open bars) or from intact fat pad (MFP; shaded bars).



**Figure 6.6: Response of MAC-T cells to serum (4%) from control (CON) and ovariectomized (OVX) heifers.**

## Conclusions, Implications, and Future Perspectives

The importance of heifer mammary development to future lactation is illustrated by experiments in which over-feeding prepubertal heifers decreases milk yield in the subsequent lactation (Lammers *et al.* 1999; Sejrsen *et al.* 2000). Such examples demonstrate that there exists a degree of plasticity within the prepubertal mammary gland that could be exploited to improve milk yield. Alternatively, increasing prepubertal development could lead to reduced age at first calving. Consequently, new technologies and heifer management strategies that optimize prepubertal mammary development would be of enormous benefit to the dairy industry. However, to achieve this ultimate goal, a detailed understanding of the biological mechanisms controlling cell proliferation and epithelial morphogenesis within the mammary gland is required. Data presented in this thesis provide evidence that locally derived IGF-I and related molecules, ER $\alpha$ , and the ECM are important components of mammary gland development in prepubertal heifers.

Although the overall hormone requirements for proper mammary development have long been identified (Topper and Freeman 1980; Tucker 1981), the complexity of interactions between systemic hormones, locally derived growth factors, extracellular matrix proteins and intracellular signaling cascades that regulate mammary epithelial cell proliferation is only just starting to be realized. A central focus of the research presented in this dissertation is the role of IGF-I in regulating heifer mammary gland development. Several lines of evidence from our laboratory support the idea that locally derived IGF-I is critical for heifer mammary growth. For example, IGF-I stimulates proliferation of mammary epithelial cells in culture, and much of the proliferative activities of mammary tissue extracts can be attributed to IGF-I (Weber *et al.*, 1999). Changes in the expression of local IGF-I axis molecules also relate to changes in mammary development induced by high- or low-feeding of prepubertal heifers (Weber *et al.*, 2000). However, recent advances in gene sequencing and gene discovery technologies (e.g., microarray, SAGE) have revealed a plethora of genes that are expressed within the mammary gland during various stages of development. Many of these genes may regulate mammary epithelial cell proliferation, survival, or apoptosis, or ductal formation and branching

morphogenesis. Thus, the question that has to be raised is whether IGF-I is responsible for the majority of local regulation of heifer mammary growth, or whether other local factors have equally important (but as yet unidentified) roles.

Initially, the focus of this project was to examine the role of mammary derived IGF-I in regulating prepubertal heifer mammary development, by examining IGF-I expression within mammary tissues. Overall, our results support previous observations from our laboratory (see previous paragraph) and the hypothesis that IGF-I is an important regulator of mammary epithelial cell proliferation and development. Coinciding with GH and E stimulated mammary epithelial cell proliferation, IGF-I protein and mRNA were increased within mammary tissues. In addition, expression of IGF-I mRNA was decreased in mammary tissues of ovariectomized heifers, coinciding with decreased mammary epithelial cell proliferation. <sup>125</sup>I-IGF-I binding to mammary microsomes was also decreased in parenchyma of ovariectomized heifers, implying decreased sensitivity of mammary tissues to IGF-I. In both experiments, changes in cell proliferation were more closely paralleled with local changes in IGF-I than with changes in systemic IGF-I or mammogenic hormones, suggesting that the local IGF-I axis is more important for heifer mammary development than systemic IGF-I. Surprisingly, there was no effect of GH or E on in vitro expression of IGF-I mRNA in mammary explants derived from control or ovariectomized heifers (Appendix B). This is in contrast to our previous observations in which we reported that E increased expression of IGF-I mRNA in explants from prepubertal heifer mammary tissue (Jobst *et al.* 2001).

To expand our view of local regulation of heifer mammary development, and to investigate the relative importance of IGF-I, we also examined the mRNA expression of several other potential growth factors within the heifer mammary gland of control and ovariectomized heifers (Appendix A). Ovariectomy did not influence the expression of KGF, PTHrP or PTH-1R, however, overall expression of leptin appeared to be up regulated in ovariectomized heifers. Furthermore, there was a tendency for KGF to be more highly expressed in parenchymal rather than stromal tissues. The finding that KGF mRNA was not changed by ovariectomy was surprising because previous reports have

shown this growth factor to be estrogen responsive. For example, ovariectomy increased mRNA expression within the ovine mammary gland (Hovey *et al.* 2001) whereas estrogen increased KGF expression within the rodent mammary gland (Pedchenko and Imagawa 2000). Furthermore, although previous reports have shown KGF to be expressed from stromal cells and not epithelial cells, we found significant amounts of KGF in bovine primary epithelial cells (Appendix A). The discrepancies between the current observations and the previously mentioned reports may be explained by the relatively large species differences in mammary gland development (Hovey *et al.* 1999). While ovariectomy clearly abolishes mammary development in heifers, ovariectomy does not influence mammary development in ewes (Ellis *et al.* 1998). Consequently, it is difficult to extrapolate observations from ovine or rodent studies to the processes of heifer mammary development. The lack of changes in expression for these growth factors in response to ovariectomy lend support to the idea that the role of IGF-I within the prepubertal heifer mammary gland cannot be ignored, and that the IGF-I axis may play a more significant role in regulating heifer mammary development than other locally derived growth factors.

An interesting aspect of this project was the observation that ovariectomy before six weeks of age affected mammary development at six months of age more dramatically than ovariectomy at three months of age. This finding perhaps suggests that a critical period exists between birth and six weeks of age during which time exposure to ovarian secretion permanently influences mammary development. This hypothesis is supported by the observation that proliferation of mammary epithelial cells is greater at two months of age than at five or eight months of age (Ellis *et al.*, 2002). Also, between birth and three months of age, the mammary parenchymal tissue grows from approximately 50 – 100 mg to more than 50 g of tissue mass (approximately 1000-fold increase; personal observations). In terms of cell proliferation, this must represent an enormous increase in cell number, and perhaps represents a foundation of mammary epithelium that closely determines future mammary development. To examine the observations related to timing of ovariectomy more closely, we separated the ovariectomy treatment group into two further groups: early ovariectomy (before six weeks of age) and late ovariectomy (after

six weeks of age). Analysis of our data for mRNA expression of IGF-I closely mirrored the observed developmental changes and demonstrated that IGF-I was lowest in PAR from early OVX heifers and highest in PAR from control heifers (main effect of treatment;  $p < 0.05$ ). Expression of IGF-I in late OVX heifers was mid-way between the control and early OVX groups (least squared means 82 vs. 44 vs.  $27 \pm 13$  densitometric units, for control, late OVX and early OVX, respectively). These observations provide further evidence that changes in the local IGF-I axis regulate heifer mammary development. Unfortunately, we could not repeat this analysis for  $^{125}\text{I}$ -IGF-I binding, westerns, or cell culture experiments because of a lack of tissue from animals in the early OVX group. However, the observation that IGF-I mRNA expression mirrors the developmental state of early and late OVX certainly supports both a role for IGF-I in regulating heifer mammary development as well as the idea that there is a critical period of ovarian stimulation for mammary growth before six weeks of age. We have previously focused on the allometric period of mammary development (approximately three to nine months of age) but the observations discussed above suggest that another critical period of mammary development occurs earlier in life. An interesting extension of the data presented here would be to examine the effects of ovariectomy at one vs. three months of age on mammary epithelial cell proliferation and  $\text{ER}\alpha$  expression.

Although the evidence presented here, combined with previous evidence from our laboratory suggest a critical role for locally derived IGF-I in regulating heifer mammary growth, it seems that a more complete picture of the heifer mammary gland must include steroid receptor signaling pathways and ECM proteins. The changes we observed in the proportion of  $\text{ER}\alpha$ -positive cells suggest an important role for the  $\text{ER}\alpha$  signaling pathway within the prepubertal heifer mammary gland. It would be interesting to investigate the expression of downstream signaling components as well as the genes transcribed as a result of  $\text{ER}\alpha$  activation. Based on our results, it would also be interesting to study the details of  $\text{ER}\alpha$  and IGF-I interactions within bovine mammary epithelial cells. Furthermore, based on our findings of increased fibronectin and decreased laminin in ovariectomized heifers, it is tempting to hypothesize that these proteins also interact with the  $\text{ER}\alpha$  or IGF-I pathways. Further studies of ECM proteins

within the heifer gland might start with in situ hybridization and northern analysis to determine which mammary cells are responsible for ECM synthesis. Extending this, it would be interesting to examine changes in mRNA expression of ECM proteins in response to E and GH.

In conclusion, our results suggest an important role for locally derived IGF-I in regulating heifer mammary development. Furthermore, interactions between the local IGF-I axis and the ovary appear to regulate proliferation of mammary epithelial cells. The results suggest that locally derived IGF-I has a critical role within the heifer mammary gland in stimulating prepubertal development. In contrast, expression of other locally derived growth factors (KGF, Leptin and PTHrP), were not influenced by ovariectomy, suggesting a less important role for these molecules in ovarian stimulated mammary development. As well as the importance of IGF-I in heifer mammary development, our results suggest that a more complete view of local regulation of heifer mammary development should include the roles of the estrogen receptor signaling pathways, the extracellular matrix, and interactions between each of these components.

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# APPENDICES

## APPENDIX A

### mRNA EXPRESSION OF KGF, LEPTIN, PTHrP AND PTH-1R IN HEIFER MAMMARY TISSUE

#### Introduction

Northern analyses of mammary tissue from ovariectomized and control heifers was conducted to determine changes in mRNA expression of KGF, Leptin, PTHrP and the PTHrP receptor (PTH-1R).

#### Methods

RNA was prepared and Northern blots were conducted as described in section 3.2. Probes were gratefully received from the following sources:

1. KGF: Ovine KGF cDNA (622 bp) was obtained from Dr. Jane Mitchell (Moredun Institute); via Dr. Russell Hovey (Hovey *et al.* 2001).
2. Leptin: Bovine leptin cDNA (504 bp) was obtained from Dr. Yves Boisclair, Cornell University
3. PTHrP and PTHrP-1R: Mouse PTHrP (467 bp) and mouse PTH-1R (283 bp) cDNAs were obtained from Dr. John Wysolmerski, Yale University.

#### Results

##### 1. KGF

KGF was present in bovine epithelial cells and heifer mammary tissue as an approximately 2.4kb sized transcript (Figure A1). No KGF transcript was detected in bovine lactating mammary tissue, bovine liver, mouse virgin mammary tissue or mouse lactating mammary tissue. Ovariectomy did not influence KGF expression ( $p > 0.3$ ; Figure A2 and A3). However there was a tendency for an overall main effect of tissue ( $p < 0.08$ ), with parenchymal tissue having a higher level of mRNA expression than stromal tissues.

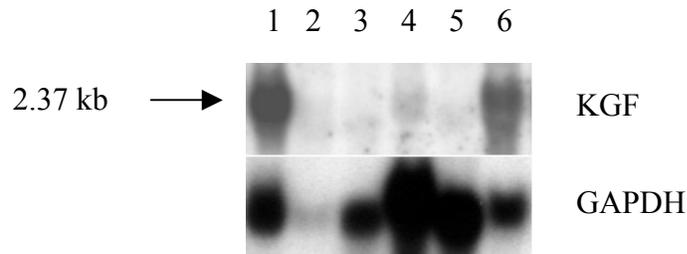
##### 2. Leptin

Leptin was present as two main transcripts of approximately 2.5 kb and 7.5 kb. The 2.5 kb transcript was most prominent in mammary stromal tissues, while the 7.5 kb transcript

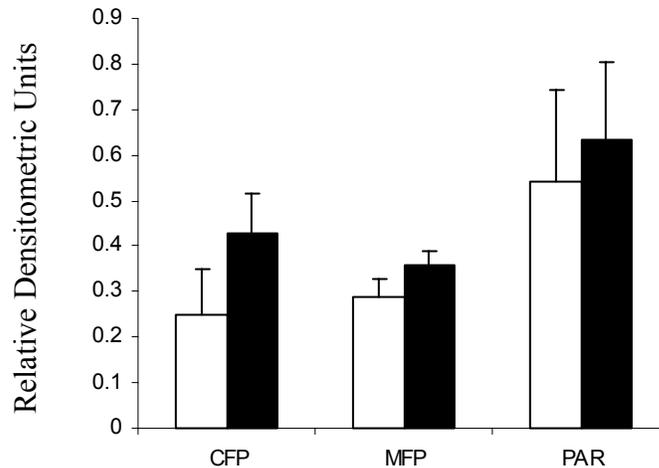
was most prominent in mammary parenchymal tissues ( $p < 0.05$ ; Figure A3). There was a significant main effect of treatment for the 2.5 kb transcript, which was evident as increased expression in ovariectomized heifers ( $p < 0.05$ ; Figure A4). There was no significant effect of treatment for the 7.5 kb transcript.

#### PTHrP and PTH-1R.

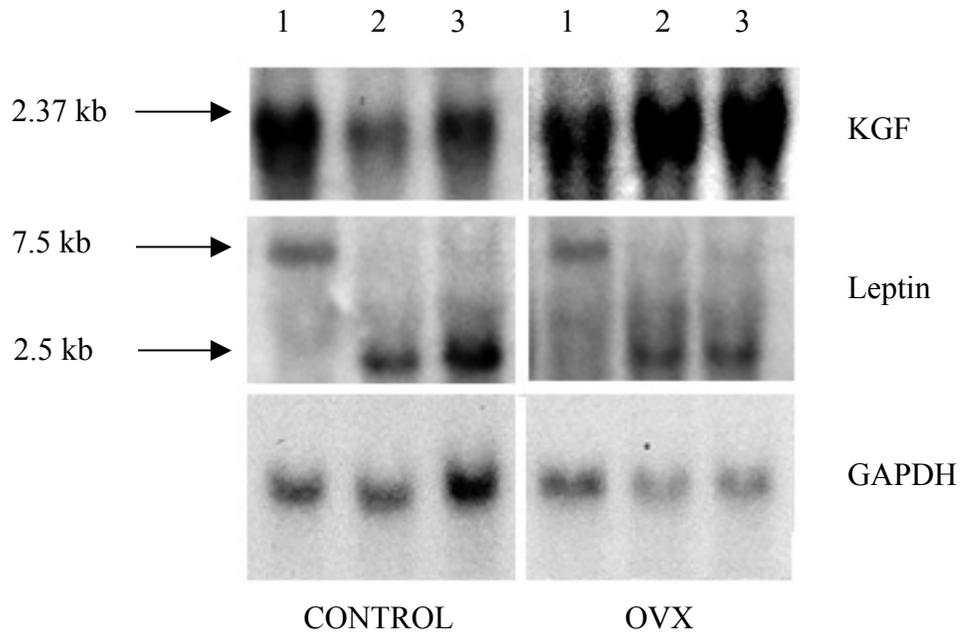
PTHrP mRNA was expressed in lactating bovine mammary tissue as three main transcripts approximately 6kb, 4kb and 1.35 kb in size (data not shown). Only the 4kb and 1.35 kb transcripts were present in heifer mammary tissue (Figure A5). PTHrP was not affected by treatment or by tissue type. PTH-1R was present as a 4kb sized transcript that was not affected by treatment or tissue type (Figure A6).



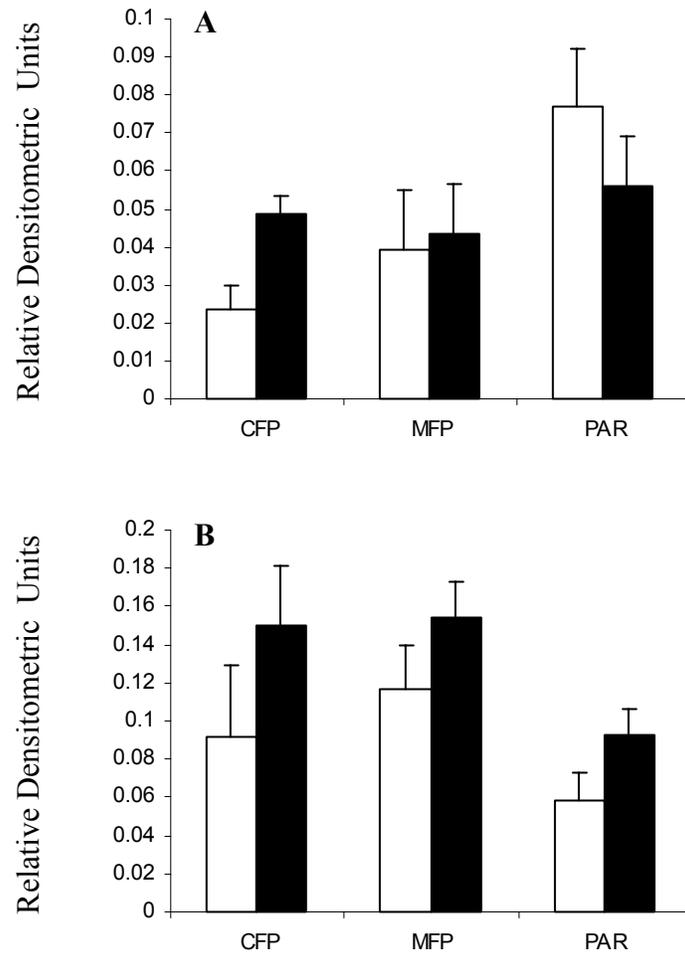
**Figure A1: KGF expression in mouse and bovine mammary tissues.** 1: bovine primary mammary epithelial cells; 2: bovine lactating mammary tissue; 3: bovine liver; 4: mouse virgin mammary tissue; 5: mouse lactating mammary tissue; 6: prepubertal heifer mammary tissue.



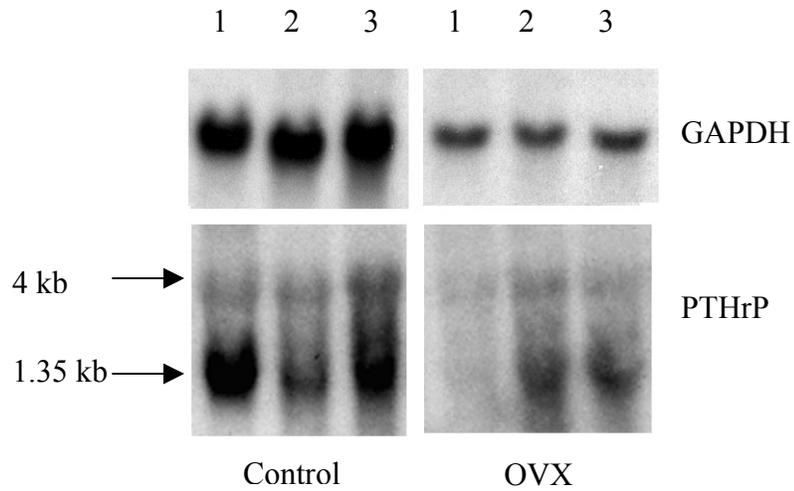
**Figure A2: Quantification of KGF mRNA expression in prepubertal heifer mammary tissues.** Open bars: control heifers, closed bars: ovariectomized heifers.



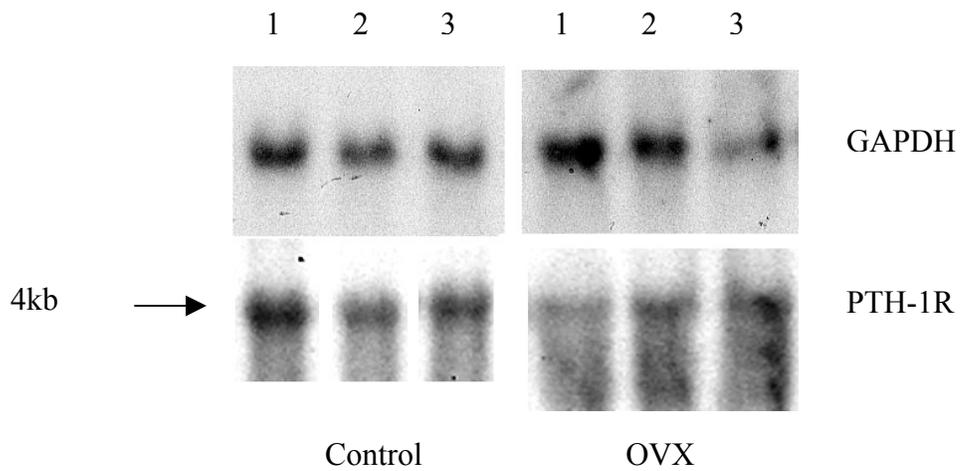
**Figure A3: KGF and Leptin expression in mammary tissues from control and ovariectomized heifers. 1: PAR, 2: MFP, 3: CFP.**



**Figure A4: Quantification of Leptin mRNA expression in mammary tissues from control and ovariectomized heifers.** Open bars: control heifers; shaded bars: ovariectomized heifers. A) Quantification of 7.5 kb transcript. B) Quantification of 2.5 kb transcript.



**Figure A5:** Expression of PTHrP in mammary tissues from control and ovariectomized heifers. 1: PAR; 2: MFP; 3: CFP.



**Figure A6:** Expression of PTH-1R in mammary tissues from control and ovariectomized heifers. 1: PAR; 2: MFP; 3: CFP.

## APPENDIX B

### IGF-I and IGFBP-3 mRNA expression in explants of heifer mammary tissue

#### Methods:

Explants were prepared from mammary tissue from control and ovariectomized heifers (experiment described in section 3.2). Explants were prepared by finely dicing samples of CFP, MFP and PAR into tissue pieces approximately 3-5 mm<sup>2</sup>. Approximately 200mg explants were incubated in 1 ml DMEM media containing either media alone (no additions), GH (1 µg /ml), E ( 100 pg/ml) or GH + E. The explants were incubated for 30 hrs with the treatments, after which time tissue was removed from media and frozen in liquid nitrogen for later analysis of mRNA expression. RNA was prepared as described in section 3.2. mRNA expression of IGF-I and IGFBP-3 was determined using slot blot analysis, as described in section 2.2. Data were corrected for 18S rRNA and for expression in basal media. Statistics were analyzed using the proc mixed procedure of SAS.

#### Results:

Neither IGF-I or IGFBP-3 mRNA expression was significantly affected by GH, E or GH + E, in CFP, MFP or PAR from ovariectomized or control heifers. The results are shown in Table A1, as a percentage of mRNA expression in basal media.

**Table A1: Expression of IGF-I and IGFBP-3 mRNA in explant tissues from control and ovariectomized heifers.**

	<i>Animal Treatment</i>	<i>Tissue</i>	<i>GH<sup>a</sup></i>	<i>E</i>	<i>GH+E</i>	<i>Error</i>
IGF-I	Control	CFP	146	123	141	21.7
		MFP	114	133	115	22.7
		PAR	92	96	115	21.2
	OVX	CFP	139	119	141	18.4
		MFP	107	119	91	18.4
		PAR	120	99	121	24.1
IGFBP-3	Control	CFP	143	109	142	19.6
		MFP	108	123	108	21.1
		PAR	96.7	107	117	19.6
	OVX	CFP	120	113	90	16.9
		MFP	112	138	96	16.9
		PAR	147	123	153	22.8

A: data are Least squared means of densitometric units, expressed as percent change over basal media and corrected for 18S rRNA abundance.

## VITA

Sarah Dianne Knowles Berry was born in Tauranga, New Zealand on May 26, 1974. She attended the University of Waikato, graduating with a Bachelor of Science in Biology in 1996. Subsequently, she began her Masters of Science degree in conjunction with University of Waikato and the Dairy Science Group at AgResearch, Ruakura, New Zealand and graduated with a Masters of Science in Biology (First Class Honors), in 1998. In 1999 she began her doctoral studies in the Department of Dairy Science at Virginia Polytechnic and State University. While at Virginia Tech, she was a delegate to the graduate student assembly, a member of the American Society for Dairy Science, and a member of the Gamma Sigma Delta Society.