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thesis entitled

MECHANISMS REGULATING PARENCHYMAL DEVELOPMENT
IN THE BOVINE MAMMARY GLAND

presented by

Laurie Ellen Davis

has been accepted towards fulfillment
of the requirements for

M.S. degree in Animal Science

Major professor

Date June 6, 2002

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# MECHANISMS REGULATING PARENCHYMAL DEVELOPMENT IN THE BOVINE MAMMARY GLAND

By

Laurie Ellen Davis

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Animal Science

2002

ABSTRACT

MECHANISMS REGULATING PARENCHYMAL DEVELOPMENT IN THE BOVINE MAMMARY GLAND

By

Laurie Ellen Davis

The objective was to examine the proliferative response of mammary epithelial cells to extracts of parenchymal tissue from different regions of the mammary glands of dairy heifers. Mammary extracts were prepared from parenchyma collected from proximal and distal regions within the glands of prepubertal heifers (n=3, BW = 213 ± 16 kg). "Proximal" was defined as the 1/3 region closest to the teat and "distal" was defined as the 1/3 region farthest from the teat. MAC-T bovine mammary epithelial cells were cultured on collagen gels in basal medium with or without 1, 3 or 5% mammary extracts, using 3 wells per treatment in 3 separate assays. After 40 h incubation with treatments, total cellular DNA per well was measured. Mammary extracts of tissue from proximal regions stimulated 11% more cell proliferation than extracts of tissue from distal regions (P=0.02). Addition of mammary extracts stimulated a level of cell proliferation that was 108% greater than basal medium. Concentrations of IGF-I and leptin in extracts did not differ between regions (P=0.18; P=0.37, respectively). Distal regions tended to have greater abundance of IGFBP-3 than proximal regions (P=0.06). Abundance of IGFBP-2, a 28-kD and a 24-kD BP did not differ by region (P>0.1). In conclusion, extracts from proximal regions are more mitogenic than extracts from distal regions of the developing mammary parenchyma in prepubertal dairy heifers.

I dedicate this thesis to my family, who have given me the

love, support and courage to undergo any challenge...

Flora and Jim Williams, my mother and step-father;

Julie and Brad Smith, my sister and brother-in-law;

Crystal, Johnney, Mark, Sara and Beth Grove, my farm family.

ACKNOWLEDGEMENTS

I would first like to thank my major professor, Dr. Miriam Weber Nielsen, for the opportunity to come to Michigan State and for the care and guidance she has given me over many years. Your encouragement throughout the years, both in Virginia and Michigan, has allowed me to focus on reaching my goals and making plans for the future. Also, many thanks to my guidance committee, Dr. Mike VandeHaar, Dr. Cathy Ernst and Dr. Don Jump, for your many thought provoking questions and ideas to make this research project more productive and complete.

Special thanks to the lab group of 2270, especially to Luis Silva for your mentoring in the culture hood and sharing your knowledge of cell culture. Also, to Jim Liesman for all of your time and patience in helping me learn the in's and out's of ligand blots, RIAs and SAS!

Last, and certainly not least to my groups of insanity relief! A special thanks to Dr. Joe Domecq for your friendship, guidance and for allowing me to enter 'your' dairy judging world! To my office mates, Marci Charest, Christel Moons and Dave Mello, thanks for all of the talks in 1261 and for your life-long friendships. Also, thanks to Melissa and B. J. Macfarlane and Ashley Peterson for your friendship especially during that wonderful thesis writing phase!

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LIST OF ABBREVIATIONS

**ALS** = Acid labile subunit

**BM** = Basal medium

**BCA** = Bicinchroninic acid

**BSA** = Bovine serum albumin

**bST** = Bovine somatotrophin

**CFP** = Cleared fat pad

**CV** = Coefficient of variation

**CT** = Computed tomography

**CPM** = Counts per minute

**DMSO** = Dimethyl sulfoxide

**DPM** = Disintegrations per minute

**EGF** = Epidermal growth factor

**EDTA** = Ethylenediaminetetraacetic acid

**FBS** = Fetal bovine serum

**FCS** = Fetal calf serum

**FGF** = Fibroblast growth factor

**GLM** = General linear model

**GH** = Growth hormone

**GH-R** = Growth hormone receptor

**GHRF** = Growth hormone releasing factor

**GHRH** = Growth hormone releasing hormone

**HBSS** = Hanks' buffered salt solution

**HU** = Houndsfield units

**IGF** = Insulin-like growth factor

**IGFBP** = Insulin-like growth factor binding protein

**LAP** = Latency activated protein

**LS means** = Least square means

**MAC-T** = Mammary alveolar cell large T-antigen

**MFP** = Mammary fat pad

**PCA** = Perchloric acid

**PBS** = Phosphate buffered saline

**PMEC** = Primary mammary epithelial cells

**RIA** = Radioimmuno assay

**RT-PCR** = Reverse transcription polymerase chain reaction

**rpm** = revolutions per minute

**SV-40** = Simian virus 40

**SDS** = Sodium dodecyl sulfate

**SRIF** = Somatotrophin releasing inhibitory factor

**SEM** = Standard error of the mean

**TGF** = Transforming growth factor

**TCA** = Trichloroacetic acid

**TEMED** = Tetramethylethylenediamine

**TBS** = Tris buffered saline

# INTRODUCTION

Interactions between hormones and growth factors regulate mammary growth in prepubertal dairy heifers. Many reviews have focused on the importance of understanding mammary development in heifers and how this foundation for subsequent development and future milk yield can be affected by factors during early life (Akers, 1990; Knight, 2000; Tucker, 1981). A clearer understanding of the physiology and development of the mammary gland could lead to techniques that enhance mammary growth, thereby increasing milk yield. This could result in an increase in profitability for the dairy producer.

Since the 1950's, many scientists have studied factors affecting the number and dividing rate of mammary epithelial cells. A high correlation exists between milk yield and measurement of total mammary DNA (Sinha and Tucker, 1966). Milk yield is positively correlated with the number of mammary epithelial cells present in the gland and the activity of these secretory cells (Broster and Broster, 1984).

The mitogenic activity in the mammary gland is partially the result of interactions between growth factors and their binding proteins. These interactions can be synergistic and / or antagonistic in vivo and in vitro (Forsyth et al., 1998; Oka et al., 1991). Understanding mammary development and growth is the goal of numerous labs focused on many different species: murine (Beauloye et al., 1999; Robinson et al., 1996; Silberstein et al., 1990; Soriano et al., 1998), ovine (Hovey et al., 1998b; McFadden et al., 1990), caprine (Prosser et al., 1990; Prosser et al., 1994), porcine (Kraetzl et al., 1998), and human (Blat et al., 1994; Chen et al., 1994; Reiss and Barcellos-Hoff, 1997), in addition to the bovine (Akers, 1990).

Few studies have evaluated the relationship between mitogenic activity and location of epithelial cells within the mammary gland (review by Akers, 1990; Ellis et al., 2000; McFadden et al., 1990). Because these studies demonstrated that epithelial cells from the distal border of the growing parenchyma are more rapidly proliferating, it is logical that a "gradient" of mitogenic activity may exist within the mammary parenchyma. However, these studies have only focused on the mitogenic capacity of the epithelial cells from different regions within the mammary gland. Therefore, evaluation of the mitogenic activity of parenchyma tissue, which not only includes epithelial cells but also fibroblasts and adipocytes, should give more insight into the paracrine mechanism of mammary development. Furthermore, the mitogenic activity of parenchymal tissue isolated from different regions may differ due to the synthesis of factors by the mammary fat pad (Hovey et al., 1998a).

Insulin-like growth factor-I (IGF-I) has been shown in many studies to be a major mitogen in the mammary gland (Collier et al., 1993; Prosser et al., 1994; Shamay et al., 1988). IGF-I is produced in the stromal portion of the gland (Hauser et al., 1990) and IGF-I receptors were characterized on microsomes from bovine mammary tissue (Hadsell et al., 1990). Hence, a paracrine role for IGF-I action on the epithelium has been proposed (Hauser et al., 1990). Furthermore, to fully understand the mechanism of IGF-I growth stimulation in mammary development, the role of the IGF binding proteins must also be taken into consideration.

A novel protein, leptin, was recently shown to reduce the mitogenic activity of IGF-I when both were applied to bovine mammary epithelial cells in culture (Silva et al., 1999). Leptin is produced by adipocytes and MAC-T cells, a bovine mammary epithelial

cell line (Houseknecht et al., 1998; Smith and Sheffield, 2002). Receptors for leptin have been identified on bovine mammary epithelial cells (Silva et al., 2001).

Therefore, the objectives of this study focused on the mitogenic activity in extracts of tissue isolated from different parenchymal regions within the prepubertal heifer mammary gland. In addition, this project further characterized the mammary extracts by comparing the concentrations of IGF-I and leptin, and the abundance of IGFBP in extracts prepared from two different regions within the parenchymal tissue.

OBJECTIVES:

1. To compare the mitogenic activity in extracts of mammary tissue collected from proximal and distal regions of the parenchyma.

2. To compare the concentrations of IGF-I and leptin within mammary extracts.

3. To compare the abundance of IGFBPs in mammary extracts.

# LITERATURE REVIEW

## Physiology of Mammary Development:

During development of the embryo and after mammary gland differentiation, a mammary line or ridge is formed from the thickening of epithelial cells (Anderson, 1978). This ridge thickens to a hillock that differentiates into buds that form pairs of glands. The bovine has two pairs of glands that form in the inguinal area. The cells in this region differentiate into what will become the mammary parenchyma. Mammary buds determine the number of glands and teats an organism will have, corresponding to four in the bovine. These sprouts will later give rise to the gland cistern and major ducts of the mammary gland. The mesenchyme (fat pad) grows outwardly creating pressure that forms the shape of the teat. The fat pad provides the space for future development, as the parenchymal tissue grows into the fat pad in later stages during prepubertal development.

During prepubertal development, the mammary glands grow at a faster rate than the rest of the body (Sinha and Tucker, 1969). During this period of development, the allometric phase, there is rapid ductal growth into the fat pad in the rodent model. However, no alveoli are formed during this phase (Hovey, 1996). Puberty and the maturation of the ovaries signal the end of the allometric phase. At the end of the allometric period, weight of total mammary tissue is 2 to 3 kg, with 0.5 to 1 kg of this being parenchymal tissue. This parenchyma consists of 10-20% epithelium, 40-50% connective tissue and 30-40% adipose tissue (Sejrsen et al., 1982).

During the allometric phase, a high rate of gain in heifers leads to earlier ages at puberty but has negative effects on mammary development (Sejrsen et al., 1982). In this study, ad libitum feeding compared to restricted feeding (60%) during the prepubertal period reduced the wet weight of mammary secretory tissue by 23% and DNA content by 32%. Increases in growth rate from 400 to 850 g/d decreased the average age at first estrus from 16.6 to 8.4 months (Sejrsen, 1994). Thus, the onset of puberty is more closely related to nutrition and body development than actual chronological age (Daughaday and Rotwein, 1989; Schillo et al., 1992).

From approximately the third estrus until pregnancy, the growth of the mammary gland occurs at the same rate as body growth, which is referred to as isometric growth (Sinha and Tucker, 1969). During this time, high rates of gain do not negatively affect development of the gland (Sejrsen et al, 1982; Sejrsen et al., 2000).

This change from the allometric to the isometric phase is largely influenced by shifts in concentration of circulating hormones. As puberty approaches, luteinizing hormone (LH) pulse frequency increases, stimulating development of large dominant follicles and leading to an increase in estrogen production and secretion. After puberty, secretion of progesterone is also thought to contribute to the shift from the allometric to the isometric growth phase (Knight and Peaker, 1982).

Mammary secretory cell numbers increase rapidly during pregnancy until the onset of lactation (Tucker, 1981). These cells become fully differentiated between parturition and peak lactation. There is a gradual loss of mammary cells during the course of a lactation (Capuco et al., 2001). After peak lactation, the rate of cell atrophy

becomes greater than cell division and milk yield begins to decrease (Knight, 2000; Knight and Wilde, 1987).

Much research on mammary development focuses on either the bovine or murine model. However, large differences exist between murine and bovine mammary anatomy and development. Ducts are more widely dispersed with a rounded profile in the rodent (Akers, 1990). Ductal elongation involves terminal endbuds that elongate and grow to the distal borders of the murine fat pad (Hovey, 1996). The ruminant has a more complex tubular structure. The periphery of the duct in the bovine gland is surrounded by connective tissue. Therefore, those interested in the bovine should consider these differences even though there is a larger cost associated with using the bovine model.

Proximal and Distal Regions of Mammary Parenchyma:

During the allometric phase of development, the ductal portion of the gland grows rapidly into the fat pad. Recent work has been reported on the activity of cells isolated from different locations within the developing gland. "Distal" epithelial cells penetrate into the mammary fat pad and are those epithelial cells farthest from the teat cistern. The "proximal" cells are those that are closest to the teat cistern. Proximal epithelial cells are involved in local formation of ductal branches and later become part of the gland cistern and a branched ductal structure. Differences exist in the mitogenic capacity of epithelial cells from different regions. Ellis et al. (2000) defined these two regions as "peripheral" (referred to as "distal" in this thesis project) and "medial" (i.e., "proximal"). Primary epithelial cells were isolated from the distal and proximal sections of the bovine gland (Ellis et al., 2000). Distal cells, compared to proximal cells, were two to three times

more sensitive to the mitogenic actions of IGF-I or serum in cell culture. Ellis and co-workers attributed this difference to an increase in IGF-I receptor number in distal versus proximal cell regions. The same group (Akers, 1990; McFadden et al., 1990) observed tritiated thymidine labeled cells in cross sections of ovine mammary ducts and found more labeling on the distal groups of ducts compared with proximal portions, indicating a greater rate of cell division in the peripheral region.

Composition of parenchyma varies with location of tissue within the mammary gland. Sejrsen and co-workers (1982) used tissue slices stained for light microscopy for histological determination of individual cell types. Results showed that the area occupied by epithelial cells decreased as distance from the base of the gland ("proximal" region) increased. The percentage of fat cells in the parenchyma increased as distance from the base of the gland increased.

The fat pad is an important source of mitogenic activity within the mammary gland of mice (Hovey et al., 1998a). However, little research has been published on the role of the fat pad in ruminants. The distal cells are in closer vicinity to the fat pad than the proximal regions. Bovine mammary epithelial cells were growth inhibited when co-cultured with fat pad explants (McFadden and Cockrell, 1993). This inhibitory role may relate to the production of leptin within adipocytes (Houseknecht et al., 1998).

Quantitating Mammary Gland Development:

Many techniques have been used to measure mammary development in the bovine, including palpation scores, teat length (Lammers et al., 1999) and mammary biopsies. During the 1950's (Davidson and Leslie, 1950), quantifying DNA through

either determination of the phosphorus present or measuring intensity of light absorption due to purine and pyrimidine components became a popular way of measuring proliferation. Although inexpensive, these techniques do not allow direct assessment of epithelial: fat cell ratios and are not good estimates of total weight of the mammary parenchyma.

Size of the gland and the degree of development can be assessed through slaughter of the animal and collection of the udder. Proportion and weight of parenchyma and stroma can be measured in slices of the gland, along with differences in structure. Mammary gland growth is assessed by measurement of total DNA of mammary tissue. Immunohistochemistry staining of cells or histology staining of cross sections can determine organization of organelles, types of tissue and percentage of each cell type within the mammary gland. Growth factor receptor mRNA expression can be assessed by in situ hybridization. In addition, mammary tissue can be digested with enzymes to isolate a primary epithelial cell population. Measurement of DNA synthesis or accumulation of DNA in these primary cells can evaluate total epithelial cell number after in vitro treatment and the sensitivity of these cells to different mitogens. Homogenizing mammary tissue and preparation of extracts can be used to analyze the mitogenic activity of the tissue for mammary epithelial cell growth in vitro. This technique will be discussed further in the "Mammary Extracts" section.

Another technique is the use of computed tomography (CT) scanning which is measured in Houndsfield Units (HU). The amount of parenchyma and extraparenchymal tissue (fat) was estimated using this technique (Sejrsen et al., 1986; Sorensen et al., 1987) on thawed sections of bovine mammary glands. Tissue above 20 HU is considered

parenchymal tissue and tissue with values of 20 HU or below is labeled extra-parenchymal tissue. In these studies, the CT scanning technique gave results similar to the dissection technique. Ayadi and co-workers (2000) used a non-invasive, ultrasonographic procedure to estimate milk cistern volume to study the evolution of milk stored in the gland at different milking intervals. Ultrasound and CT scanning should be further investigated for possible measurement of mammary development by assessing the portion of gland that is fat pad versus parenchyma. These techniques may allow measurement of gland development in an animal over time without the need for slaughter.

Mammary Extracts:

Waksman and colleagues (1991) conducted the first experiment using bovine mammary tissue extracts at a 3:1 saline to tissue ratio. Aqueous extracts of mammary tissue stimulated proliferation of undifferentiated bovine mammary epithelial cells in vitro in a dose-dependent manner. The mitogenic activity of extracts was synergistic with the addition of fetal calf serum (FCS), IGF-I or IGF-I plus epidermal growth factor (EGF). The addition of EGF to extracts did not stimulate epithelial cell proliferation significantly compared to extracts alone. Mean protein content of the extracts was 18 mg/ml and extracts were used at a 10% concentration in media in cell proliferation assays.

Weber and colleagues (1999; 2000b) used mammary extracts in two different studies. Addition of mammary extracts (5% concentration in medium) to bovine primary mammary epithelial cells in culture stimulated DNA synthesis ([$^3$H] thymidine

incorporation) 545 % compared to basal medium treatment. Addition of IGFBP-3 to mammary extracts inhibited extract-induced proliferation of primary cells. Further, addition of IGF-I antibodies to mammary extracts inhibited up to one half of the mitogenic activity of the mammary extracts (Weber et al., 1999). Mammary extracts were also prepared from mammary tissue of prepubertal heifers fed at a high or low feeding level and treated with growth hormone or placebo. A 5 % mammary extract treatment stimulated greater [$^3$H] thymidine incorporation into primary epithelial cells than fetal bovine serum (FBS, 10% of medium) or 100 μg IGF-I treatments. Extracts from heifers fed at a low feeding level stimulated significantly greater [$^3$H] thymidine incorporation than mammary extracts of heifers fed at a high level (Weber et al., 2000b). Mammary tissue extracts from heifers treated with growth hormone (GH) compared with placebo were higher in concentrations of IGF-I, higher in abundance of IGFBP-3, and lower in abundance of IGFBP-2 (Weber et al., 2000a). This mammary extract procedure could also be used to compare the mitogenic activity of tissue from heifers of the prepubertal and postpubertal stages or to compare tissue collected from different regions of the mammary parenchyma.

MAC-T: Mammary Alveolar Cell Large T Antigen:

The experiments in this study focused on evaluating the mitogenic activity in mammary parenchyma and how it influences proliferation of mammary epithelial cells. Use of mammary explants allows study of the interactions between different cell types and mitogens produced by each. However, this technique does not allow the

characterization of the response of an isolated cell type, in particular the epithelial cell, to mitogens in cell culture.

The MAC-T (Mammary Alveolar Cell large T antigen) cell line was chosen for use in this project. The MAC-T cell line has been used in numerous studies as a model for growth and morphological development of bovine mammary epithelial cells. Huynh and colleagues established this cell line using mammary epithelial cells from a lactating cow and transfecting these cells with the Simian Virus 40 (SV-40) large T antigen (Huynh et al., 1991). This cell line is immortalized, not transformed, and maintains many similar characteristics as primary cells in vitro and mammary tissue in vivo. This cell line has been well defined and has growth characteristics similar to mammary epithelial cells grown on both plastic and collagen gels. Zhao and co-workers (1992) showed that MAC-T cells are responsive to IGF-I as measured by both [$^3$H] thymidine incorporation and total DNA assay, and have high affinity IGF-I receptors. Also, Zhao used Scatchard analysis of an IGF-I binding assay to determine that IGF-I binds to MAC-T cell surface receptors (Zhao et al., 1992). MAC-T cells are able to synthesize lactose, lipid, and $\alpha$ and $\beta$ casein (Huynh et al., 1991). Also, MAC-T cells proliferate in response to insulin, IGF-I (including Des (1-3) and Long $R^3$ forms), IGF-II, and bovine serum albumin (BSA). This group further characterized this cell line, showing that MAC-T cells lack a mitogenic response to human and mouse EGF, bovine growth hormone (GH), bovine prolactin, estrogen, progesterone, and display a small response to transforming growth factor-$\alpha$ (TGF-$\alpha$) (Woodward et al., 1994, 1996). MAC-T cells, like primary bovine mammary epithelial cells, synthesize IGFBP-2,-3,-4 and -6 (Cohick, 1998). Thus, MAC-T cells are considered a useful model for in vitro studies of mammary cell proliferation.

Endocrine Regulation of Mammary Growth:

Older experiments have clearly demonstrated that growth hormone (GH) is required for normal mammary development (Forsyth, 1989; review by Sejrsen, 1994). Exogenous administration of GH increases milk production in cows (Peel and Bauman, 1987) and stimulates peri-pubertal mammary growth in sheep (McFadden et al., 1990) and dairy heifers (Sandles and Peel, 1987; Sejrsen et al., 1986). However, local infusion of GH into the mammary gland does not increase milk production in sheep (McDowell and Hart, 1984) indicating that GH does not act directly on mammary epithelial cells to stimulate production. Moreover, addition of GH does not stimulate cell proliferation in cultures of mammary epithelial cells (Sejrsen, 1994). Specific binding of GH to receptors in mammary tissue has not been detected by the use of GH-receptor (GH-R) binding studies (Akers, 1985; Purup et al., 1993). However, Glimm and colleagues (1990) used molecular hybridization analysis to detect GH-R mRNA (4.4 kb and 9.2 kb transcripts) in lactating bovine mammary tissue. The presence of GH-R mRNA suggests that the cells possess the GH-R. The receptor gene may be transcribed but it is not known whether the mRNA is translated. Further research is needed to understand these discrepancies.

GH / IGF-I Axis:

Much evidence suggests the effect of GH on mammary gland development is mediated indirectly through the insulin-like growth factor (IGF) system (Akers, 1985; Kleinberg, 1997; Sejrsen, 1994). The idea that IGF-I was important to mammary development and lactation originated from studies using exogenous somatotropin (GH) as

a treatment to increase milk production. Vander Kooi and co-workers (1995) observed increases in serum IGF-I concentrations, hepatic IGF-I mRNA abundance, and serum IGFBP-3 abundance after intravenous infusing lactating cows with either GH or GH Releasing Factor (GHRF). A subcutaneous injection of GH in lactating dairy cows and goats resulted in increases in milk yield (Bauman and Vernon, 1993; Faulkner, 1999). A greater increase of IGF-I in milk than plasma was noted and Faulkner suggested that after treatment with GH, changes in IGF-I concentrations within the mammary gland occur prior to the general circulation. IGF-I treatment stimulates the growth of primary bovine (Shamay et al., 1988) and ovine (Winder et al., 1989) mammary epithelial cells and a bovine mammary epithelial cell line (Zhao et al., 1992). This stimulation is caused by IGF-I binding to membrane receptors on secretory cells and is detected as an increase in DNA synthesis in myoepithelial cells, ductal and alveolar epithelial cells (Baumrucker and Stemberger, 1989; McGrath et al., 1991).

IGF-I also acts locally in vivo. Close arterial infusion of IGF-I or IGF-II into the external pudic artery of the goat mammary gland increased milk yield by increasing blood flow to the gland and by increasing the persistency of lactation (Prosser et al., 1990; Prosser et al., 1994). Infusion of IGF-I into the mammary gland of non-lactating beef cows (Collier et al., 1993) and dairy heifers (Silva et al., 2002) increased mammary growth. However, regulation of IGF-I synthesis within the mammary gland remains undefined. Walden and colleagues (1998) hypothesized that GH stimulates IGF-I production in the stromal compartments of the gland. Then, IGF-I acts on the ductal portion of the gland and stimulates formation of terminal end buds in mice. IGF-I $^{(-/-)}$ null mice had significantly less mammary development than same age wild-type controls

(Ruan & Kleinberg, 1999). Administration of IGF-I, but not GH or estrogen, had a stimulatory effect on mammary development in these null mice. Thus, even when GH is present, mammary development is reduced unless IGF-I is present.

In 1983, Sejrsen and others suggested that the negative influence of excess feeding on mammary growth in prepubertal heifers might be associated with a decrease in circulating GH concentrations (Sejrsen et al., 1983). Unlike GH, circulating IGF-I is greater in animals fed for high rates of gain compared to low rates of gain (Breier et al., 1986). Mammary growth and milk production are positively correlated with concentrations of GH (Bauman and Vernon, 1993; Sejrsen et al., 1983). During early lactation, plasma IGF-I concentrations are low and concentrations are inversely related to milk production. Ronge and others (1988) suggested that the relationship during early lactation could represent an uncoupling of the GH / IGF axis due to negative energy balance. These differences may also be due to the effect of IGF binding proteins (IGFBP) and altered synthesis of IGFBP in response to different feeding patterns, treatments, etc. More studies are now being done to investigate the effects of leptin (Silva et al., 1999), compensatory growth (Ford and Park, 2001) and additional growth factors (Woodward et al., 1995) and how these factors might influence growth of the mammary gland.

Another explanation for these differences between GH (also known as somatotropin, ST) and IGF-I concentrations could be a negative feedback mechanism. GH-releasing hormone (GHRH) is produced in the hypothalamus and regulates the amount of GH synthesized and secreted from the pituitary (review by Thissen et al., 1994) (see Figure 1). GH secretion is controlled by both the stimulatory influence of

GHRH and the inhibitory effect of somatostatin (SRIF, somatotrophin releasing inhibitory factor) (see Figure 1). The IGF-I peptide is primarily synthesized and secreted by the liver when GH binds to hepatic GH receptors (Gluckman et al., 1987). When pituitary cells were treated with GHRH, and with or without IGF-I (10 *mM*), IGF-I inhibited GHRH-elicited GH secreted by 67%. High levels of circulating IGF-I may provide a negative feedback mechanism by either decreasing the amount of GHRH secreted or increasing the amount of SRIF secreted and thus decreasing the amount of GH synthesized and secreted from the pituitary (Ceda et al., 1987).

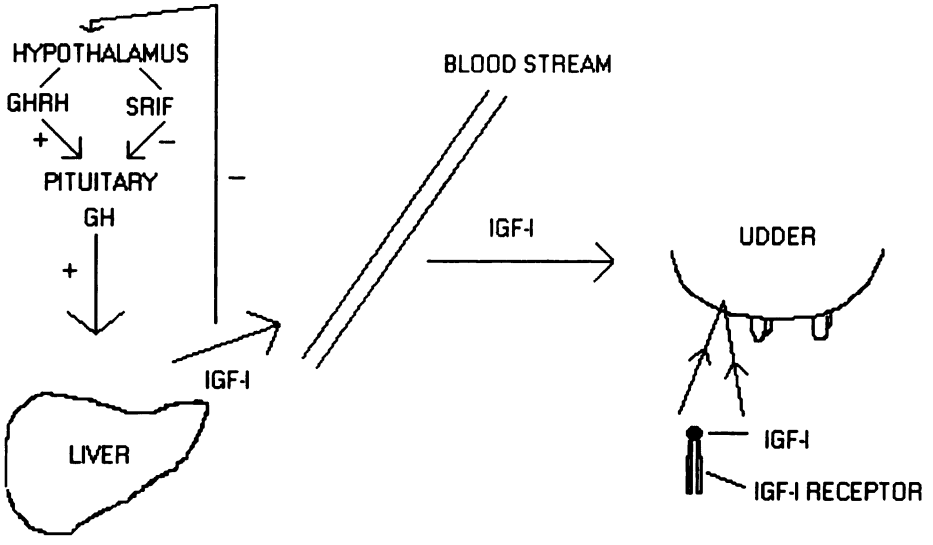


**Figure 1.** Overview of the GH / IGF-I axis. GH secretion from the anterior pituitary is regulated by the stimulatory influence of GH-releasing hormone (GHRH) and the inhibitory effect of somatostatin (SRIF), both produced in the hypothalamus. When GH binds to hepatic GH receptors, the IGF-I peptide is synthesized and secreted by the liver. High concentrations of circulating IGF-I may provide a negative feedback mechanism by working through SRIF and GHRH to decrease GH production from the pituitary. IGF-I found in the mammary gland is locally produced and / or travels to the gland via circulation. IGF-I receptors are found on mammary epithelial cells; however, this cell type does not produce IGF-I. IGF-I is produced in the stromal portion of the gland.

IGF Ligands, Receptor Structure and Localization:

IGF-I receptors are found on mammary epithelial cells as well as on pre-adipocytes, myoblasts, chondrocytes, mesenchymal cells, fibroblasts, endothelial cells, and hepatocytes (McCusker, 1998). Type I and II receptors have been found in the mammary glands of cows (DeHoff et al., 1988). Bovine mammary epithelial cells express IGF system receptors (IGF-I, -II, and insulin), do not produce IGF-I (Hadsell et al., 1990), and do secrete IGF-II (Baumrucker et al., 1993). IGF-I is produced in the stromal portion of the gland; therefore, a paracrine role for IGF-I action for the epithelium has been suggested (Hauser et al., 1990). However, data from Hodgkinson and co-workers (1991) suggests that blood is the major source of IGF-I in the mammary epithelium.

IGF-I has a molecular weight of 7.6-kD with approximately 70 amino acids (Wong et al., 1989). This ligand is composed of four domains on a single peptide chain (Thissen et al., 1994). The IGF-I molecule shares identical amino acid sequence in the bovine, human and porcine, and differs in the ovine by one amino acid and murine by five amino acids (Wong et al., 1989). The liver is the primary source of circulating IGF-I in the mouse (Sjogren et al., 1999) and other animals (Daughaday and Rotwein, 1989). Many tissues produce the IGF-I peptide including heart, lung, skeletal muscle and the gonads (D'Ercole et al., 1984). Weber and co-workers (1999) measured IGF-I concentrations in mammary extracts and serum of heifers, finding averages of 34 and 107 ng/ml, respectively. The abundance of IGF-I in many different organs has led to the suggestion that IGF-I may act in an endocrine, autocrine, and/or paracrine manner.

The type I IGF receptor is a heterotetramer and has high homology with the insulin receptor. The receptor consists of two extracellular α subunits that contain the ligand-binding domain where signal transduction is initiated by the ligand binding to the domain's cysteine-rich regions (review by Thissen et al., 1994). Each of these α subunits is linked via disulfide bonds to a β subunit that houses both the transmembrane domain and the intracellular tyrosine kinase catalytic domain. This tyrosine kinase activity is located within the cytoplasm. Ligand binding causes autophosphorylation on tyrosine residues and propagation of cytoplasmic signals. The Type I IGF-receptor binds IGF-I, IGF-II and insulin. The Type I receptor has a 10-fold higher affinity for IGF-I than IGF-II, and a 1000-fold lower affinity for insulin than IGF-I. The Kd for IGF-I is 1 n*M*. The IGF-I and insulin receptors are both $\alpha_2\beta_2$ heterotetramers and have a high degree of sequence homology. The insulin receptor binds insulin with highest affinity, IGF-I with 100-fold lower affinity, and IGF-II with an even lower affinity.

The Type II IGF receptor, also known as the mannose-6-phosphate receptor, is a 300-kD monomeric transmembrane glycoprotein (Thissen et al., 1994). This receptor binds IGF-I with a 500-fold lower affinity than IGF-II and does not bind insulin.

Des (1-3) IGF-I and Long $R^3$ IGF-I are two analogs of IGF-I that have a reduced affinity for IGFBP. Des (1-3) IGF-I lacks the N-terminal tripeptide Gly-Pro-Glu and shows similar or increased potency compared to normal IGF-I (Ross et al., 1989). The Long $R^3$ form of IGF-I has a substitution of an Arg for a Glu at position 3 and has a 13 amino acid extension peptide at the N terminus (Francis et al., 1992; Tomas et al., 1993).

The IGF-I ligand can be found in the circulation in three different forms. Little free IGF-I is found in the circulation. Approximately 80% of all circulating IGF-I is

bound to IGFBP. Eighty percent of this bound IGF-I is part of a 140-kD ternary complex [with the acid-labile subunit (ALS) and either IGFBP-3 or –5] and the other 20% is bound to only binding proteins. These binary complexes are small enough to cross the capillary endothelium, but the ternary complex is too large (Baxter, 1993). The half-life of free IGF-I is extended from 10 min to 30-90 min when in binary complexes, and to more than 12 hr when bound to the ternary complex (review by Boisclair et al., 2001).

Insulin-Like Growth Factor Binding Proteins (IGFBP):

To fully understand the mechanism of IGF-I growth stimulation in mammary development, the role of the IGF binding proteins must also be considered. The function of a labile pool of IGF-I in the circulation is to provide an available source of IGF-I for delivery to target tissues. Regulation of this labile pool is through the IGFBP. These multifunctional proteins have the capacity to both inhibit and enhance IGF actions. The IGFBP bind more than 80% of IGF in the circulation, either in binary or ternary complexes. The binding proteins assist in transporting IGF-I to target organs and tissues via the blood stream. Six high affinity BP (IGFBP 1-6) (Keifer et al., 1991) and more recently, nine IGFBP-related proteins (IGFBP rP1-9) were characterized that bind to IGFs with much lower affinity than the IGFBP (Hwa et al., 1999).

Bovine mammary epithelial cells from pregnant and lactating cows synthesize IGFBP-2 (34-kD), IGFBP-3 (46 and 42-kD), IGFBP-4 (24-kD) and IGFBP-5 (30-kD) (Gibson et al., 1999). All four of these binding proteins were detected in bovine blood, milk and cell culture-conditioned media using western ligand blots. Also, Gibson and others (1999) found that mRNA for IGFBP-3 is predominant in secretory epithelial cells

versus other cell types. Mammary tissue extracts from prepubertal heifers contained IGFBP-2 (32-kD), IGFBP-3 (40 to 43-kD), and IGFBP of 28-kD and 24-kD (putatively IGFBP-1 and -4) are present in the heifer mammary gland (Weber et al., 2000a).

Binding proteins function to extend the half-life of IGF-I in the circulation (mainly IGFBP-3 associating with the ALS), transport IGF-I from vasculature to tissues and localize IGF-I to specific target tissues and cells (Cohick, 1998). Binding proteins can have both stimulatory and inhibitory effects on the bioactivity of IGF-I (see Figure 2). Binding proteins may act by inhibiting the bioactivity of IGF-I through competition with receptors, enhancing IGF-I activity by concentrating the growth factor on the cell surface in receptor regions, prolonging the half-life of IGF-I in the circulation, interacting with other growth factors, or by acting independently of IGF-I (Baumrucker and Erondu, 2000; Oh, 1998; review by Rajah et al., 1997).

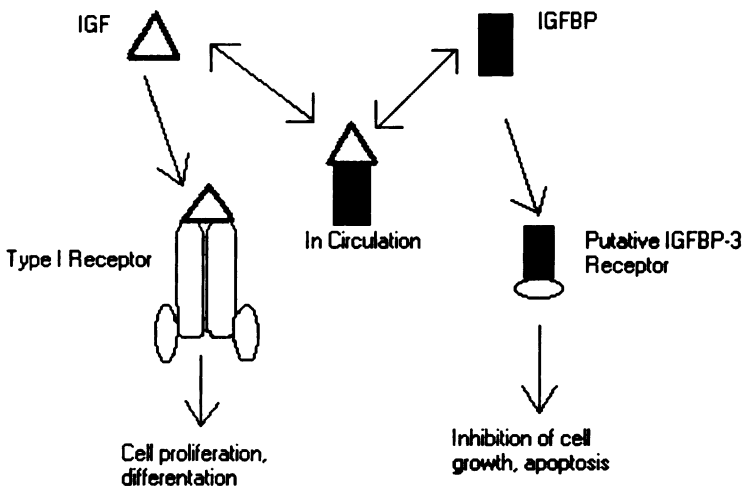


**Figure 2.** The role of IGFBP in the IGF-I system. Approximately 80% of circulating IGF-I is bound to IGFBP. IGFBP assist in transporting IGF-I to target tissues and in prolonging the half-life of IGF-I. Free IGF-I is able to bind to the Type I receptor and is involved in cell proliferation and cell differentiation. IGFBP can also inhibit IGF-I actions by competing for binding with the receptor. In particular, IGFBP-3 is thought to inhibit cell growth and may even cause apoptosis in certain cell types.

Both receptor numbers and circulating levels of IGF-I change over the lifetime of the bovine. Expression of IGF-I is regulated by developmental factors. IGF-I concentrations are low in the newborn calf and rise following birth (Skaar et al., 1994). Plasma concentrations of IGF-I increased from 50 to 450 ng/ml from 1 to 45 weeks of age. The postnatal rise in IGF-I is suggested to occur because of the appearance of receptors in the liver and the onset of GH-dependent IGF-I release.

Concentrations of IGF-I and GH and IGFBP abundance continue to fluctuate during adult life, depending on the physiological state of the cow. Growth hormone concentrations increase during early lactation when milk yield is high and increasing. Serum IGF-I concentrations and liver IGF-I mRNA abundance are low during early

lactation, increase as lactation persists and concentrations are high during the dry period (Sharma et al., 1994; Vicini et al., 1991). IGF-I blood concentrations are inversely related to milk production (Ronge et al., 1988; Vega et al., 1991). This is opposite of IGFBP-2, for which concentrations are higher in early versus late lactation (Cohick, 1998). During pregnancy, protease activity either appears or significantly increases, leading to degradation of IGFBP, especially IGFBP-3 (Hossenlopp et al., 1990).

During feed deprivation, concentrations of serum IGFBP-3 and IGF-I are generally decreased, while IGFBP-1 and IGFBP-2 are increased. These results are similar to the profile during early lactation (see above). The GH / IGF-I axis is said to be uncoupled during severe feed restriction and this restriction may even abolish the ability of GH to increase IGF-I (review by Bauman, 1999). In summary, many of these studies show a general positive relationship between IGF-I and IGFBP-3 and an inverse relationship between IGF-I and IGFBP-2.

IGFBP-3:

Insulin-like growth factor binding protein-3 constitutes the majority of IGFBP in the serum of heifers (McGrath et al., 1991) and in bovine mammary tissue extracts (Weber et al., 2000a). This binding protein has 6 to 24 times the circulating half-life of IGFBP-2 or IGFBP-4 (McGuire et al., 1998). IGFBP-3 serum levels are normally positively correlated with IGF-1. Because of the above information, much research has focused on the relationship between IGFBP-3 and IGF-I.

IGFBP-3 is identified as a doublet of proteins approximately 38 and 42-kD. In the circulation, IGFBP-3 forms a 140-kD ternary complex with the ALS and with either

IGF-I or IGF-II. In 1997, Erondu and colleagues cloned the bovine IGFBP-3 gene, a single copy gene. This gene is approximately 10 kb and is divided into 5 exons with the fifth containing a 3' untranslated region. The gene contains a TATA box, a transcription start site, a GC rich sequence element, and two overlapping AP-2 binding elements, and does not have a CAAT box. Using transfection techniques, 80% of the basal promoter activity was shown to be located in the first 130 bp of the 5' end and this region responded to IGF-I.

IGF-I is a potent regulator of IGFBP-3. Conditioned media from MAC-T cells (Cohick and Turner, 1998) and primary epithelial cells (McGrath et al., 1991) treated with IGF-I showed greater IGFBP-3 secretion into media compared to cells treated with basal medium (BM). Exposure of cells to IGF-I increased the half-life of IGFBP-3. IGF-I activates transcriptional and post-transcriptional mechanisms to stimulate IGFBP-3 synthesis in MAC-T cells (Cohick et al., 2000).

IGFBP-3 displays both a stimulatory and an inhibitory effect on proliferation of mammary epithelial cells. The ability of IGF-I to stimulate DNA synthesis is increased when MAC-T cells are transfected with IGFBP-3 (Cohick, 1998). However, in placental fibroblasts, IGFBP-3 is shown to inhibit IGF-I stimulation of DNA synthesis in a dose-dependent manner (Rodgers et al., 1996). When IGFBP-3 is added in culture, it is probable that it binds to IGF-I and reduces the chance for IGF-I to bind to IGF-I receptors on epithelial cells (Oh, 1998; Rajah et al., 1997). When IGF-I is allowed to bind to its own receptor, it causes a stimulation of cell proliferation or differentiation (see Figure 2, p. 19). If IGFBP-3 binds to its own putative receptor, it causes an inhibition of cell proliferation and in some systems, such as cancer cells, cell death can occur (Oh, 1998).

However, IGF-I binding to IGFBP-3 prevents these effects. Therefore, Rajah and co-workers (1997) imply that IGFBP-3 can mediate apoptosis by both IGF-I-independent and IGF-I-dependent mechanisms.

Limited evidence exists to support an IGF-I-independent effect of IGFBP-3 in bovine mammary tissue. When rhIGFBP-3 was added as a treatment to mammary epithelial cells in vitro, mitogenic activity of serum and IGF-I treatments was abolished. Addition of 40 and 800 ng/ml of IGFBP-3 to basal medium inhibited DNA synthesis of bovine primary mammary epithelial cells by approximately 25% and reduced mitogenic activity in mammary tissue extracts by 35% (Weber et al., 1999). Mammary epithelial cells do not secrete IGF-I (Hadsell et al., 1990), showing that this inhibitory effect was independent of IGF-I. Rodgers and co-workers (1996) transfected an IGFBP-3 gene into mouse embryo fibroblasts that lacked an IGF-I receptor, resulting in a decrease in the growth rate of the transfected cells. Grill and Cohick (2000) transfected MAC-T cells with IGFBP-3. Basal DNA synthesis was lower for the transfected cells but these cells responded more strongly to addition of IGF-I than did mock cells. Overexpression of IGFBP-3 inhibits epithelial cell proliferation (Cohen et al., 1993). These results suggest that the inhibitory effect of IGFBP-3 is independent of the IGF-I receptor dependent pathway.

IGFBP-2:

IGFBP-2 may have a greater function in transporting IGF-I than previously thought. Its role becomes important when IGFBP-3 is insufficient to bind all of the free IGF-I (Zapf, 1995). IGFBP-2 is the predominant form of IGFBP secreted by bovine

primary mammary epithelial cells cultured in serum-free media (McGrath et al., 1991). IGFBP-2 is secreted in a 34-kD form and is dimeric at 72-kD (Bourner et al., 1992). Serum IGFBP-2 concentrations are increased when ruminants enter a catabolic state such as early lactation or fasting (Sharma et al., 1994). IGFBP-2 concentrations are higher during early lactation compared to the dry period and a trend exists for concentrations to be higher during early versus late lactation. Administration of bST results in lower concentrations of serum IGFBP-2, in contrast to serum and mRNA levels of IGF-I (Sharma et al., 1994; Vicini et al., 1991). When IGF-I was upregulated by generating transgenic rabbits carrying fusion genes of synthetic DNA coding for human IGF-I, the increase in local hIGF-I peptide in milk also upregulated IGFBP-2 but not any other binding proteins (Wolf et al., 1997). Few studies have been published showing the effects of IGFBP-2 on bovine mammary epithelial cells. Thus, the role that IGFBP-2 has in mammary development and the IGF-I system is unclear.

Other Growth Factors and Hormones:

Many growth factors and their receptors have been identified within the bovine mammary gland. Many cell types are abundant in the mammary gland. Each of these cell types secrete and have receptors for different types of growth factors. This pool of growth factors, acting on epithelial cells in both paracrine and autocrine fashions, regulates mammary development. Some are thought to act synergistically with IGF-I, and others are thought to inhibit cell proliferation independently of IGF-I.

Transforming Growth Factor-β (TGF-β):

Transforming growth factor -β (TGF-β) mRNA has been identified in the mammary gland (Maier et al., 1991; Plaut, 1993). The TGF family can be sub-divided into three groups: TGF-βs, activins and bone morphogenetic proteins (BMP). TGF-β is a homodimer of two 12 to15-kD subunits linked by a single di-sulfide bond, or it is released as a biologically inactive precursor containing a dimer of the N-terminal pro-region / LAP (latency-activated) protein. There are three forms of TGF-β. The mature forms have 9 conserved cysteine residues.

Physiologically, TGF-$\beta_1$ has a biphasic response. That is, depending on the concentrations used in cell culture, TGF-$\beta_1$ can be either inhibitory or stimulatory to cell proliferation. When used as a treatment on primary bovine mammary cells (Purup et al., 2000), lower concentrations of TGF-$\beta_1$ (25, 50 pg/ml) stimulated proliferation of cells, while higher concentrations (>100 pg/ml) inhibited DNA synthesis. Addition of TGF-$\beta_1$ to cells inhibited the mitogenic response of IGF-I. Purup suggests that the bi-phasic response in vitro of TGF-$\beta_1$ may have a concentration-dependent role in mammary growth. TGF-$\beta_1$ was implanted into the mammary gland of mice (Daniel et al., 1989). In pregnant mice, TGF-$\beta_1$ inhibited ductal elongation and inhibited DNA synthesis in the epithelium, but not in the stroma.

Leptin:

Leptin is a 16-kD protein secreted by white adipocytes and MAC-T cells (Houseknecht et al., 1998; Smith and Sheffield, 2002). Leptin is involved with the regulation of food intake, energy expenditure and energy balance via interaction with

neurotransmitters (Houseknecht et al., 1998). Secretion of leptin is highly correlated with body fat mass and adipocyte size. Insulin and cortisol stimulate expression of leptin. Leptin is found in the circulation bound to proteins that may modulate the function of this hormone and its half-life. Leptin receptor mRNA expression has been shown in tissues such as kidney, lung, liver, and muscle (Jensen et al., 1999).

Leptin is of interest to scientists studying mammary development because of its suggested role in nutritional regulation, especially by dietary energy, of mammogenesis. Recently leptin receptor expression and protein were characterized in bovine mammary and other tissues using reverse-transcription PCR (RT-PCR) and immunohistochemical staining, respectively (Silva et al., 2001). Physiological concentrations of leptin (2 to 6 ng/ml) reduced the mitogenic effect of IGF-I on MAC-T cells (Silva et al., 1999). Leptin is suppressed during infusion of rhIGF-I in normal rats (Boni-Schnetzler et al., 1999). This suppression was due to a reduction in circulating insulin leading to increased fat mobilization. This study also showed that leptin was not suppressed by rhGH, suggesting that it is unlikely that GH regulates leptin in the rat model. However, when added with insulin or dexamethasone in vitro, GH attenuated the ability of insulin or dexamethasone to stimulate leptin expression in adipose tissue explants taken from young cattle (Houseknecht et al., 2000). This study also reported that young cattle treated with GH showed increased leptin concentrations in adipose tissue. Leptin abundance was positively correlated to adipose tissue IGF-I mRNA.

Fibroblast Growth Factor (FGF):

Fibroblast growth factor (FGF) is a heparin binding protein and was purified from prepartum bovine mammary gland secretion (Lametsch et al., 2000). The isolated cDNA is 1170 bp and the theoretical mass of the protein is 22.5-kD. The FGF family of growth factors is involved in tissue remodeling, angiogenesis and morphogenesis. This family has a dual-receptor system consisting of four high affinity tyrosine kinase receptors and a low affinity heparan sulfate proteoglycan (HSPG). FGF alone exhibited a dose-dependent mitogenic activity when used as treatment on bovine primary mammary undifferentiated cells (Sandowski et al., 1993). This mitogenic effect was synergistic when IGF-I was added. Also, mammary stromal cells have been shown to be stimulated by acidic FGF (aFGF), as well as by IGF-I (Hovey et al., 2001).

Keratinocyte Growth Factor (KGF):

KGF, produced by the stroma, modulates stromal-epithelial interactions (Rubin et al., 1989). Mammary epithelial cells respond differently to increasing doses of keratinocyte growth factor (KGF). Media supplemented with 10 ng/ml KGF increased DNA synthesis in MAC-T cells by 390% (Hovey et al., 2001). However, at higher concentrations of KGF (50 to 100 ng/ml KGF), proliferative responses were not different from those seen at 0 ng/ml KGF. Ovine stromal cells did not respond to KGF but did proliferate in response to IGF-I and IGF-II. KGF mRNA is detected in bovine mammary stromal cells but not in mammary epithelial cells (Hovey et al., 2001). In this same study, the mammary fat pad (MFP) had significantly higher levels of KGF mRNA than the epithelium-cleared fat pad.

The above results show the importance of the interactions between the stromal and epithelial portions of the gland, and the paracrine actions of many growth factors within the mammary gland.

Estrogen:

For many years, estrogen has been thought to stimulate mammary ductal growth and progesterone to stimulate lobulo-alveolar development (Reece, 1956). In 1993, Purup and co-workers ovariectomized prepubertal heifers at 2.5 months of age and slaughtered at 9.5 months of age. Ovariectomized heifers had a greater percentage of stromal tissue and a lower percentage of epithelial tissue mass. Treatment of intact heifers with GH increased mammary tissue by 16%, but this treatment did not affect ovariectomized heifers. Therefore, this study suggested that exogenous GH had no effect in the absence of estrogen. In another ovariectomy study, ewe lambs given exogenous estrogen had higher levels of IGF-I mRNA expression in the mammary fat pad (Hovey et al., 1998b). Recent evidence has shown that parenchyma removed from heifers treated with estrogen and GH had increased incorporation of [$^3$H] thymidine into DNA than controls (Berry et al., 2001). This study also showed that estrogen treatments increased IGF-I concentrations in parenchyma and the mammary fat pad tissues. These studies confirm the importance of ovarian secretions in mammary development and growth. However, estrogen is incapable of stimulating growth of mammary epithelium in vitro (Oka et al., 1991) similar to progesterone, growth hormone, prolactin and thyroxine. These hormones have been shown to be involved in the growth of the gland during

pregnancy by using in vivo ablation and hormone replacement experiments (Nandi, 1958).

Summary:

Many growth factors and hormones regulate mammary development in heifers. As the mammary parenchyma extends into the surrounding fat pad, regional differences in its anatomy become evident. These regions later differentiate into distinct structures in the mature gland. Locally-acting growth factors contribute to parenchymal tissue development, although growth regulation is poorly understood. The objective of this study was to determine if different regions of the developing mammary parenchyma contained different amounts of mitogenic activity. The most potent mitogen known for bovine mammary epithelial cells in vitro is IGF-I. Both concentrations of IGF-I and leptin and a profile of the IGF-binding proteins were investigated to evaluate their role in the overall mitogenic activity within each region.

# MATERIALS AND METHODS

Preparation of Mammary Extracts:

Purchased Holstein heifers were weighed, stunned by captive bolt and slaughtered by exsanguination at the Michigan State University Meats Laboratory. Heifers (n=3) weighed 202, 205 and 232 kg at slaughter. Reproductive tracts were dissected to confirm that heifers were prepubertal. Blood samples were collected from two of the three heifers. After exsanguination, mammary glands were removed and transported to the laboratory for dissection. The exterior surface of mammary glands was cleaned with ethanol, and sterile blades were used to dissect the gland. Each glandular quarter was cut open to expose the teat cistern, parenchyma and fat pad. The parenchyma was visually separated into three sections: 'proximal', the third of the parenchyma closest to the teat cistern; 'distal', the third of the parenchyma closest to the fat pad; and 'intermediate', the parenchyma between the proximal and distal sections. Tissue was taken from each of these three sections, placed in WhirlPak bags and frozen at -80°C.

At a later date, 5 g of parenchymal tissue, each from proximal and distal regions, was thawed and diced into tared beakers containing 12 ml of saline (0.9% sodium chloride). Tissue was homogenized for 1 min using a Polytron (Switzerland, PT 10 20 350D). The tip of the Polytron was rinsed between samples with 3 ml of saline, which was then combined with the homogenate to yield a total of 15 ml, with a 3:1 saline to tissue ratio. Homogenate was placed into 50-ml tubes and shaken at 4°C for 90 min. Homogenate was filtered through a double layer of gauze into 35-ml Beckman tubes (Beckman Coulter, Fullerton, CA) and centrifuged (Sorvall Instruments, RC5C) at 10,000

x *g* and 4°C for 45 min. The supernatant was transferred to 10-ml Beckman tubes and ultracentrifuged (Beckman, L-80) at 105,000 x *g* and 4°C for 60 min. Supernatant was recovered, sterile filtered through a 0.22-micron filter and stored at -80°C until use in cell culture experiments. Approximately 7 ml of mammary extract was recovered after these centrifugation steps.

MAC-T Cell Culture Experiments:

Cells used for all cell culture experiments were MAC-T cells (Huynh et al., 1991), a transfected mammary epithelial cell line prepared from mammary tissue from a lactating dairy cow. MAC-T cells of a single passage were grown up for use in mammary extract experiments. Basal medium (Dulbecco's Modified Eagle Medium, DMEM/F12, Invitrogen) containing HEPES (Invitrogen, Carlsbad, CA, 7.4 g) and sodium bicarbonate (cell culture grade, Sigma, St. Louis, MO, 2.478 g), was titrated to a pH of 7.3 and sterile filtered. Basal medium (BM) also contained soybean trypsin inhibitor (Type I-S, Sigma, 1 mg/L), transferrin (apo-bovine, Sigma, 5 mg/L), glutathione (Sigma, 1 mg/L), insulin (bovine, 0.01 mg/L), sodium selenite (Sigma, 5 mg/L), antibiotic/ antimycotic (Invitrogen, 10 ml/L), gentamicin (Invitrogen, 2.5 ml/L), and bovine serum albumin (BSA) (Invitrogen, 2.625 g/L). MAC-T cells were plated at 1 X $10^4$ cells per well in a 24-well plate. Cells were plated on top of a collagen monolayer (Type I, Becton Dickinson, Bedford, MA) in a solution containing BM supplemented with 10% fetal bovine serum (FBS, Invitrogen). After 24 h in culture, medium was aspirated, cells were washed with BM, and medium was replaced with 1 ml of BM. Treatments were prepared and applied to the cells after 72 h in culture. All treatments

were done in triplicate allowing 8 different treatments per 24-well plate. Each plate contained control treatments of BM and FBS at 10% concentration in BM. Treatments included increasing amounts of IGF-I (0.25, 0.5, 1, 2, 4 and 8 ng/ml) and extracts of mammary tissue from each of three heifers. For each of these three heifers, extracts from proximal and distal regions were added at 1, 3 and 5% concentrations in BM. Concentrations at 1, 3 and 5% were chosen after analysis of effects on cell proliferation of a dose-response series of 1, 3, 5, 7 and 9% mammary extracts (see Results). Cell proliferation was evaluated at 18 h after treatment addition by [$^3$H] thymidine incorporation, at 18 h by total DNA assay and at 40 h by total DNA assay. An IGF-I dose-response series was included in this experimental model as a test for normal cell response to increasing doses of IGF-I. Basal medium was included as a control for the basal level of cell proliferation and was included in each assay. The three assays were each replicated on three different dates.

[$^3$H] Thymidine Incorporation Assay:

After 16 h of treatment, 1 μCi (2.2 x $10^6$ DPM) of [$^3$H] thymidine (ICN Pharmaceuticals, Irvine, CA) was added to each well in plates for analysis of [$^3$H] thymidine incorporation. After 2 h of incubation of cells with [$^3$H] thymidine, media was aspirated and cells were exposed to a series of washes: Hanks' Buffered Salt Solution (HBSS, Sigma), 90% ethanol, 10% trichloroacetic acid (TCA, Sigma), and an additional 90% ethanol wash. Then wells were allowed to dry for 5 min. Lysing buffer (250 μl / well, 0.5 *M* NaOH, 0.1% triton, Sigma) was added and plates were gently rocked for 30 min. A 100-μl sample was taken from each well and placed into a scintillation vial

(Research Products International (rpi), Mount Prospect, IL). Scintillation cocktail (5 ml, Safety-Solve, rpi) was placed into each vial and [$^3$H] was quantified on a beta counter (TM Analytical Mark V 32 ARI). Calculated counts per minute (CPM) were adjusted to disintegrations per minute (DPM) assuming 40% efficiency of the counter.

Total DNA Assay:

Eighteen hours after treatments were applied, media from each well was aspirated and cells were washed with 1 ml phosphate buffered saline (PBS, Invitrogen). Ethylenediaminetetraacetic acid (EDTA, 1.4 ml / well, 10 *nM* solution; J.T. Baker, Phillipsburg, NJ) was added and plates were incubated for 20 min. After this incubation period, $KH_2PO_4$ (100 µl / well, 1 *M* potassium phosphate; J.T. Baker) was added as a neutralizer and pH was measured. Plates were then kept on ice and cells were sonicated (Sonifier Converter, Model L, Branson Sonic Power, Danbury, CN). DNA standards (0, 0.624, 1.25, 2.5 and 5.0 µg/ml) and samples (160 µl) were pipetted into a black 96-microwell plate (Dynex Microfluor, Chantilly, VA). Hoechst No. 33258 dye was mixed with buffer (1 nM NaCl, 10 *nM* Tris) and added to each well (Labarca and Paigen, 1980). The fluorescence of this dye was measured (CytoFluor, PerSeptive Biosystems, Cambridge, England) as a quantification of total DNA per well. This procedure was repeated on different plates 40 h after treatment addition.

Total Protein Assay:

The bicinchoninic acid (BCA) protein assay kit (BCA-200, Pierce, Rockford, IL) was used to detect and quantitate total protein contained in the mammary extracts. A

dilution of 1:20 (mammary extract:deionized $H_2O$) was used. Standards (BSA: 0, 250, 500, 750, 1000 μg / ml) and mammary extracts were pipetted (25 μl) onto a clear 96-microwell plate. A working reagent (200 μl) was added that catalyzed a reduction of $Cu^{+2}$ to $Cu^{+1}$ by protein in the sample. A reagent containing bicinchoninic acid was used to colorimetrically detect the cuprous cation ($Cu^{+1}$) product formed by the chelation of two molecules of BCA with one cuprous ion ($Cu^{+1}$). Plates were incubated for 30 min at 37°C and the purple-colored product was colorimetrically quantitated using a plate reader (562 nm, SpectraMax 340, Molecular Devices, Sunnydale, CA) after cooling to room temperature.

IGF-I Radioimmunoassay (RIA):

IGF-I was measured in mammary extracts and blood serum. Binding proteins were separated from IGF-I by two different methods, acid-ethanol cryoprecipitation and size exclusion gel column chromatography. Acid-ethanol cryoprecipitation was similar to that described by Breier et al. (1991). A HCl / ethanol mixture (87.5% ethanol, 12.5% HCl (2 *M*), vol/vol) was added to each sample at a 4:1 ratio (1.2 ml HCl / ethanol at – 20°C and 300 μl of mammary extract). This mixture was vortexed, stored at -20°C for 1 h and then centrifuged at 4000 x *g* for 30 min at 4°C. A 750-μl aliquot of the supernatant (containing IGF-I) was transferred to a 2-ml eppendorf tube and neutralized using Tris base (300 μl at 0.855 *M*) at a 5:2 ratio. This mixture was incubated at -20°C for 1 h, centrifuged at 4000 x *g* for 30 min at 4°C, and the supernatant (300 μl) was recovered. The serum samples were reconstituted with 900 μl of deionized $H_2O$.

Extracted samples were assayed at 50 and 100 μl to check the parallelism of the

assay and of the extraction procedure. Samples were assayed in duplicates. Sample volumes were equalized to 100 µl with a Tris based neutralizing buffer [53.5% ethanol/HCL (87.5% ethanol, 12.5% 2 *M* HCl), 28.6% 0.855 *M* Tris, 17.9% deionized $H_2O$]. Samples were reconstituted to 200 µl with a PBS-based neutralizing buffer (0.1 *M* $NaPO_4$, 0.01 *M* EDTA, 0.003 *M* Na Azide, 0.005% Tween 20; pH to 7.5).

Controls included 100-µl serum samples that were high (H), low (L) and very low (LL) in IGF-I concentration. These serum samples were extracted to remove IGFBP similar to the mammary extract samples. Extracted serum samples were analyzed at 100 and 200 µl per tube. These serum samples were used to standardize the assay. Non-specific binding controls consisted of 200 µl of neutralizing buffer (1 part Tris neutralizing buffer : 1 part PBS neutralizing buffer) mixed with 250 µl assay buffer (0.03 *M* $NaH_2PO_4$, 0.01 *M* EDTA, 0.02% Na Azide, 0.005% Tween 20, 0.02% protamine sulfite; pH to 7.5). A standard curve was included in the assay, with IGF-I concentrations of 0, 25, 50, 100, 200, 400, 800, 1600, 3200 and 6400 pg/ml. These standards were assayed in duplicates.

The $^{125}$I-IGF-I isotope was diluted in assay buffer, so that 50 µl of the dilution would contain approximately 20,000 CPM. A rabbit anti-human IGF-I antisera (Gro-Pep, Adelaide, SA, Australia) was diluted with assay buffer 1:25,000 and 250 µl of diluted antiserum was added to each sample for a final dilution of antiserum at 1:50,000. This antibody was added to all tubes except the non-specific binding controls and total count tubes. Samples were then vortexed and incubated overnight at 4°C.

Protein A (Staphylococcus aureus, Roche, Switzerland; Zymed, San Francisco, CA) was added the next day at 0.001 g/tube and tubes were vortexed. After a 2 h

incubation at room temperature, 2 ml of assay buffer was added to each tube. Tubes were centrifuged for 30 min at 3070 x *g*, liquid was decanted and radioactivity of the pellet was quantified in a gamma counter (Cobra II Auto Gamma, Packard BioScience Co, Dowers Grove, IL).

The second separation procedure, size exclusion gel column chromatography, was similar to that used by Mohan and Baylink (1995). This method used a Sephadex G-50 (Pharmacia, Piscataway, NJ) gel column to separate proteins into different fractions based on their molecular weight. The G-50 gel column has a fractionation range of 1.5 to 30-kD, indicating that any proteins greater than 30-kD are excluded from the gel and pass through the gel in the liquid phase. Proteins of molecular weights within the fractionation range penetrate the gel particles and elute from the column in order of decreasing molecular size. Binding proteins predominant in the heifer mammary gland range from 24 to 43-kD in size. Therefore, most of these binding proteins should be excluded from the gel and elute first from the column.

Samples (200 µl) were acidified with 800 µl of 1.25 *M* acetic acid containing 0.125 *M* NaCl, incubated for 15 min and added to the column. The eluant solution contained 1 *M* acetic acid and 0.1 *M* NaCl. The volume of each fraction was determined after characterization of the column using samples of BSA (molecular weight = 60 kD; and $^{125}$I-IGF-I (molecular weight = 7 kD; IGF-I volume). The amounts of each fraction collected were as follows: 'A' (0-12 ml), 'B' (12-20 ml), 'C' (20-29 ml), 'D' (29-38 ml), 'E' (38-45 ml), and 'F' (45-54 ml).

A 1.8 ml sample from each collected fraction was dried (Heto Type VR-1 Speed Vacuum, Appropriate Technical Resources, Laurel, MD) to concentrate the sample and to

eliminate the acid. Samples were re-constituted in buffer (PBS neutralizing buffer) so that original mammary extracts and serum samples were diluted by a factor of 20 and 40, respectively. As a control for recovery of IGF-I from the column, 6 ng and 2 μg of IGF-I was added to 1 ml of mammary extract and BSA, respectively. Samples with added IGF-I were acidified and added to the column. In addition, recovery of IGF-I from the RIA was tested by adding 7.5 ng of IGF-I to 1 ml of serum just prior to assaying the sample. Mammary extracts were assayed at 50, 100 and 200 μl and serum samples were assayed at 25, 50 and 100 μl to confirm the parallelism of the assay. Volume of samples was equalized with buffer (PBS neutralizing buffer) to 200 μl. The remainder of the assay was similar to the RIA protocol as stated previously. Deviations from this assay included the use of only the PBS neutralizing buffer and not the Tris-based neutralizing buffer.

Western Ligand Blot:

Western ligand blotting was used to analyze the relative abundance of IGFBP in mammary extracts. This procedure was also used to confirm the absence of IGFBP in the IGF-I fractions that were collected using the Sephadex G-50 gel column. A 12.5% acrylamide running gel solution for four gels [24.9 ml monomer solution (30.8% acrylamide, 2.7% bisacrylamide), 15 ml 4X running gel buffer (1.5 *M* Tris-Cl, pH 8.8), 600 μl 10% SDS and 19.2 ml deionized $H_2O$] was prepared and degassed. Tetramethyl-ethylenediamine (TEMED, 20 μL, Eastman, Rochester, NY) and 10% ammonium persulfate (300 μl, Sigma) solution was added to the degassed running gel solution. Running gels were allowed to polymerize and the assembly was removed from the gel apparatus and moved to the loading apparatus.

A 4% acrylamide stacking gel solution for four gels [2.64 ml monomer, 4.98 ml 4X stacking gel buffer (0.5 *M* Tris-Cl, pH 6.8), 198 µl 10% SDS and 12.18 ml deionized $H_2O$] was prepared and degassed. Immediately after TEMED (10 µl) and a 10% ammonium persulfate solution (100 µl) was added to the stacking gel solution, the gel was pipetted on top of the separating gel and a comb was inserted into each stacking gel. The gel was then allowed to set up and the comb removed. Tank buffer (0.025 *M* Tris, 0.192 *M* glycine, 0.1% SDS, pH 8.3) was placed into each well and in the lower buffer chamber.

The samples were prepared using equal parts of protein sample and 2X treatment buffer (0.125 *M* Tris-Cl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, pH 6.8). The sample with treatment buffer was placed in a 70°C waterbath for 20 min to denature the protein within the sample. The sample was cooled and loaded at a concentration of 200 µg protein per lane. A molecular ladder (BenchMark Prestained Protein Ladder, Invitrogen) containing proteins from 10 to 190-kD was used as a marker for binding protein sizes. Samples of IGFBP-2 (bovine, GroPep, Adelaide SA, Australia) and IGFBP-3 (human recombinant, Upstate Biotechnology, Lake Placid, NY) were included in the gels as positive controls for binding of [$^{125}$I] IGF-I. The voltage remained constant at 180 V throughout electrophoresis (75 min) and was stopped when the bromophenol blue dye reached the bottom of the gel.

Gels and filter paper (BioRad, Hercules, CA) were placed into transfer buffer (0.025 *M* Tris, 0.192 *M* glycine, 20% methanol, pH 8.3). Polyvinylidene difluoride (PVDF, BioRad) membranes were cut to fit the size of the gels and pre-wetted in 100% methanol before rinsing in transfer buffer. On the semi-dry transfer cell (BioRad

TransBlot SD), items were layered in the following order: filter paper, PVDF membrane, gel and filter paper. Voltage was set constant at 25 volts for 160 min (BioRad PowerPac 200). Membranes were removed and placed in a Tris buffer [TBS with 1% BSA (RIA grade, Sigma) and 0.1% Tween 20] for 1 h, incubated 12 h with 700,000 CPM [$^{125}$I]-IGF-I per ml of buffer, washed 3X with TBS + 0.1% Tween 20 and 3X with TBS, and then placed in a x-ray cassette (Autoradiography Cassette, Fisher Scientific, Pittsburgh, PA) with two pieces of film (Kodak BioMax MS Film, Eastman). After approximately 24 and 48 h of exposure, each sheet of film was developed. Film was then scanned with a densitometer (Fluor-S MultiImager, BioRad; Quantity One v4.1, BioRad) to quantify differences in the density of the bands corresponding to various IGFBP.

Leptin Radioimmunoassay:

Serum and mammary extract samples were sent to Dr. Duane Keisler's laboratory at the University of Missouri. Leptin concentrations were determined as in Delavaud et al. (2000). A standard curve (0.08 to 4 ng) was included in the assay prepared from recombinant ovine leptin. For samples, triplicate aliquots of 200 µl were assayed. Both sample and standard tubes were incubated for 24 h at 4°C with 50 µl of a 1:1,500 dilution of rabbit anti-ovine leptin antisera. After this incubation, tubes were incubated an additional 20 hours after 20,000 CPM $^{125}$I-ovine leptin was added to each tube. Final dilution of leptin antisera was 1:15,000. Bound and free leptin were separated by addition of 100 µl of sheep anti-rabbit plasma and 2 ml of 4.4% polyethylene glycol, followed by centrifugation. Radioactivity of the pellet was quantified with a gamma counter (Cobra II, Packard).

Statistical Analysis:

Statistical analysis of cell culture experiments was performed using the Mixed procedure of SAS (SAS, Version 8.1, Cary, NC). The statistical model tested for the main effect of parenchymal region and concentration. Random effects included heifer, heifer*region interaction, and well (experimental date*heifer*treatment interaction). Statistical differences were based on least square means (LS means) of concentration*region. Linear and quadratic effects of mammary extract treatments were analyzed across and within region. Coefficients of orthogonal polynomials for equally spaced levels were used. Linear and quadratic effects of IGF-I treatments were analyzed across and within treatments. Coefficients for non-equally spaced levels were determined using Proc IML. For ligand blot analysis, contrast statements were used to test differences in IGFBP density between parenchymal regions. Differences in protein content were evaluated using the Tukey - multiple comparison test in Proc GLM. Pearson correlations were calculated to determine the relationships between protein content and mitogenic activity. Differences in IGF-I and leptin concentrations in mammary extracts from different regions were analyzed using the Mixed procedure. Statistical differences were based on LSmeans of region. Data are presented as LS means ± standard error of the means (SEM). Differences were considered significant at $P \leq 0.05$.

# RESULTS

Cell Culture Experiments:

The main objective of the cell culture experiments was to evaluate the mitogenic activity of mammary extracts of tissue collected from proximal and distal regions of the mammary gland. The mitogenic capacity of mammary extracts for MAC-T cells plated on collagen was analyzed using two different cell proliferation assays: [$^3$H] thymidine incorporation assay and measurement of total DNA. An IGF-I dose response curve and FBS were also included in assays to confirm that the MAC-T cells would respond to known mitogens. Also, a dose response curve of mammary extracts was done in initial experiments to determine which concentrations of extracts would be optimal for testing the main objective.

MAC-T cells were treated with increasing doses of IGF-I (0.25, 0.5, 1, 2, 4 and 8 ng/ml) to test the cells' growth response to a known mitogen. This dose range stimulated cell proliferation in a dose-dependent manner (Figures 3, 4 and 5). Linear and quadratic effects of IGF-I doses were significant in both total DNA assays ($P<0.0001$) and the linear effect was significant in the [$^3$H] thymidine incorporation assay ($P<0.0001$). In all three assays, IGF-I stimulated cell proliferation ($P<0.01$) at concentrations $\geq$ 0.5 ng/ml, compared with BM. Addition of 8 ng IGF-I/ml stimulated cell proliferation 224%, 64% and 78% in the [$^3$H] thymidine incorporation assay, total DNA assay at 18 h, and total DNA assay at 40 h, respectively. A 10% concentration of FBS stimulated cell proliferation 134%, 50% and 199% in the [$^3$H] thymidine incorporation assay, total DNA assay at 18 h, and total DNA assay at 40 h, respectively.

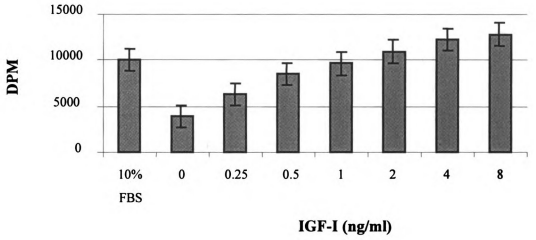


**Figure 3.** Effect of increasing doses of IGF-I (0.25, 0.5, 1, 2, 4 and 8 ng/ml of media) on [$^3$H] thymidine incorporation into DNA of MAC-T cells. Results are reported as DPM (disintegrations per minute). Data are presented as LS means ± SEM from three different assays. Basal medium (BM) and 10% fetal bovine serum (FBS) were included as controls. IGF-I treatments are different ($P$<0.001) from BM (0) at doses ≥ 0.5 ng/ml.

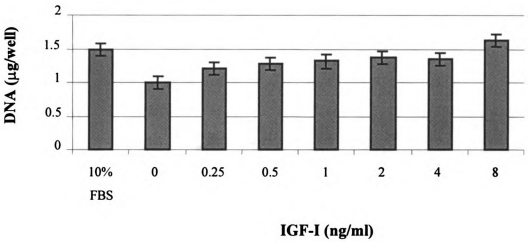


**Figure 4.** Effect of increasing doses of IGF-I (0.25, 0.5, 1, 2, 4 and 8 ng/ml of media) on total DNA of MAC-T cells after 18 h. Results are reported as micrograms of DNA per well. Data are presented as LS means ± SEM from three different assays. Basal medium (BM) and 10% fetal bovine serum (FBS) were included as controls. IGF-I treatments are different ($P<0.01$) from BM (0) at doses ≥ 0.5 ng/ml.

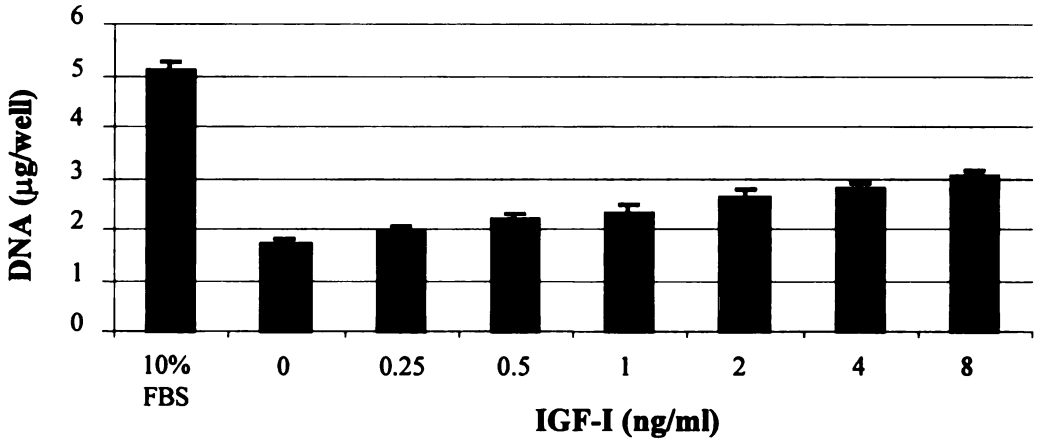


**Figure 5.** Effect of increasing doses of IGF-I (0.25, 0.5, 1, 2, 4 and 8 ng/ml of media) on total DNA of MAC-T cells after 40 h. Results are reported as micrograms of DNA per well. Data are presented as LS means ± SEM from three different assays. Basal medium (BM) and 10% fetal bovine serum (FBS) were included as controls. IGF-I treatments are different ($P<0.001$) from BM at doses ≥ 0.5 ng/ml.

Cells in all three assays were treated with increasing concentrations of mammary extracts (1, 3, 5, 7 and 9% of BM) (Figures 6, 7 and 8). The purpose of this experiment was to determine the concentrations of extracts that would be used in future experiments. Equal amounts of mammary extracts processed from two different heifers and from both proximal and distal regions were pooled for this experiment. Concentrations of 1, 3 and 5% were chosen for use in later experiments. A dose of 1% mammary extract was mitogenic in all three assays ($P<0.001$). Compared to BM, concentrations of 1, 3 and 5% mammary extracts increased total DNA at 40 h by 38%, 122% and 159%, respectively. The mitogenic activity of 5% extract after 40 h was not different from 7% ($P=0.45$) and 9% ($P=0.12$), showing a plateau effect (Figure 8). Therefore, 1% mammary extracts gave a detectable mitogenic response and 5% mammary extracts showed a maximal response after 40 h of treatment.

Subsequent assays focused on the treatment effects of mammary extracts at 3% of BM because this concentration gave a response in the middle of the growth curve in the total DNA assay at 40 h. Linear and quadratic effects of concentration (1, 3, 5, 7 and 9%) were both significant ($P<0.0001$), thereby showing that there was a dose response and also a plateau effect in this range of doses.

Both [$^3$H] thymidine incorporation and total DNA assays were performed for all subsequent assays. However, the total DNA assay was used for further analysis because the total DNA assay measures accumulation of DNA over 18 or 40 h, whereas the [$^3$H] thymidine incorporation assay measures a snapshot of DNA synthesis during 2 h. Because of this, the total DNA assay results are likely a more reliable indicator of cell proliferation in response to treatment. The 18 h DNA assay was used to provide results

on DNA accumulation to complement the results of the [$^3$H] thymidine assay. MAC-T cells responded in a dose-dependent manner to treatments of IGF-I but not to increasing concentrations of mammary extracts in the [$^3$H] thymidine assay. A linear increase in DNA synthesis (using a [$^3$H] thymidine assay) in response to increasing concentrations of mammary extracts was noted using primary cells in a previous study (Weber et al., 1999), however, it is not known why the MAC-T cells did not respond in a linear fashion. Thus, conclusions of this study were based on the results of the 40 h DNA assay, to allow measurement of treatment effects over a longer period of time.

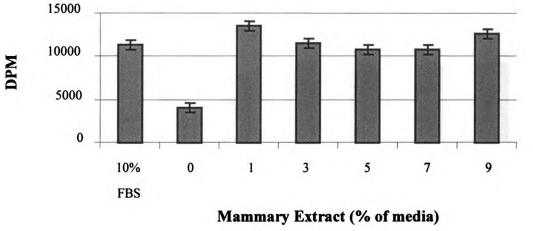


**Figure 6.** Effect of mammary extracts [1, 3, 5, 7 and 9% of basal medium (BM)] on [$^3$H] thymidine incorporation into DNA of MAC-T cells. Results are reported as DPM (disintegrations per minute). Data are presented as LS means ± SEM. BM (0) and 10% fetal bovine serum (FBS) were included as controls. Mammary extracts are different ($P$<0.0001) from BM at concentrations of ≥ 1% of BM.

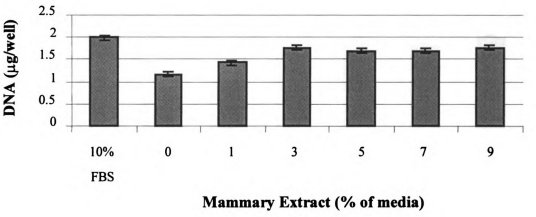


**Figure 7.** Effect of mammary extracts [1, 3, 5, 7 and 9% of basal medium (BM)] on total DNA of MAC-T cells after 18 h. Results are reported as micrograms of DNA per well. Data are presented as LS means ± SEM. BM (0) and 10% fetal bovine serum (FBS) were included as controls. Mammary extracts are different ($P<0.0001$) from BM at concentrations of ≥ 1% of BM.

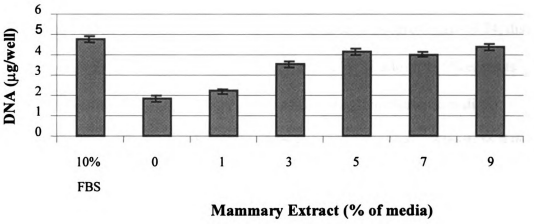


**Figure 8.** Effect of mammary extracts [1, 3, 5, 7 and 9% of basal medium (BM)] on total DNA of MAC-T cells after 40 h. Results are reported as micrograms of DNA per well. Data are presented as LS means ± SEM. BM (0) and 10% fetal bovine serum (FBS) were included as controls. Mammary extracts are different ($P<0.001$) from BM at concentrations of ≥ 1% of BM.

Results for extracts of tissue collected from three different heifers, each assayed in three separate experiments, were pooled for statistical analysis. Mammary extracts at 1, 3 and 5% stimulated proliferation in a dose-dependent manner in the total DNA assay at 40 h of treatment (Figure 9). Mammary extracts at 3% and 5% increased total DNA 108% and 122% respectively, compared to BM. Mammary extracts at 3% from proximal regions increased total DNA more than those from distal regions (proximal = 3.74, distal = 3.38 μg DNA/well; SEM = 0.11; $P$=0.02) (Figures 9, 10). In addition, 5% extracts from proximal tissue tended ($P$=0.07) to be more mitogenic than those from distal regions (Figure 9). In comparing the mitogenic response of mammary extracts to IGF-I, mammary extracts at 3% stimulated cell proliferation 17% more than 8 ng of IGF-I/ml.

Significance existed for both linear ($P$=0.0001) and quadratic ($P$=0.0002) effects at 40 h for both concentration of mammary extract and the interaction between concentration and the parenchymal tissue region from which extracts were made. The linear significance shows that there is a linear increase in mitogenic activity from 1, 3 and 5% mammary extracts. A quadratic effect indicates that the response is starting to plateau, i.e. differences between 3 and 5% were smaller than between 1 and 3% extracts (Figure 9).

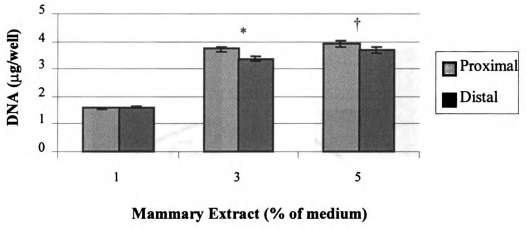


**Figure 9.** Effect of mammary extracts [1, 3 and 5% of basal medium (BM)] from proximal and distal regions of the mammary gland on total DNA of MAC-T cells after 40 h. Results are reported as micrograms of DNA per well. Data are presented as LS means ± SEM from three different assays. Asterisks indicate treatment with extracts from proximal regions increased DNA more than those from distal regions (at 3% of BM : * $P$=0.02; at 5% of BM : † $P$=0.07).

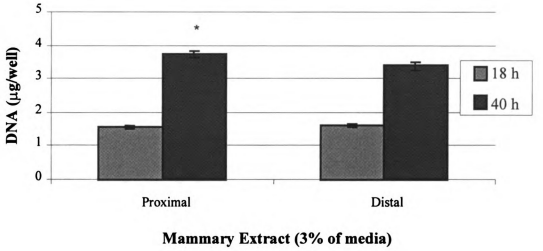


**Figure 10.** Effect of mammary extracts [3% of basal medium (BM)] from proximal and distal regions of the mammary gland on total DNA of MAC-T cells after 18 and 40 h. Results are reported as micrograms of DNA per well. Data are presented as LS means ± SEM from three different assays. Asterisk indicates treatment with extracts from proximal regions increased DNA more than those from distal regions (at 40 h: * $P$=0.02).

Coefficients of variation (CV) were calculated to test the variation within the cell culture experiments. The average inter-assay variation was 5.2, 10.7 and 5.8% for [$^3$H] thymidine incorporation assay, total DNA assay at 18 h and total DNA assay at 40 h, respectively. Intra-assay variation was 14.3, 20.4 and 17.6% for [$^3$H] thymidine incorporation assay, total DNA assay at 18 h and total DNA assay at 40 h, respectively.

Protein Content:

Protein content did not differ between mammary extracts (LS means = 12.6 mg/ml, SEM = 0.17; $P$=0.15). Basal medium contained bovine serum albumin (BSA) at 2.6 mg protein/ml of media. Addition of FBS to BM increased protein content an additional 2.9 mg/ml of media. Addition of 1, 3 and 5% extracts to BM increased protein content by 0.13 ± 0.02, 0.38 ± 0.04 and 0.63 ± 0.07 mg/ml, respectively. Correlations, using Pearson coefficients, were done between the protein concentration of extracts and the mitogenic capacity of mammary extracts from different regions within each heifer. This mitogenic activity was determined using data from total DNA assays at 40 h after treatment addition, comparing extracts from different regions at 3% of media. Pearson correlation coefficients did not show a strong correlation of extract protein concentration with total DNA per well ($r$=0.05, $P$=0.55).

Abundance of IGFBP in Mammary Extracts:

Ligand blots were performed to determine the relative abundance of IGFBP in each mammary extract (proximal and distal regions of three heifers). IGFBP-2 and IGFBP-3 served as positive controls for [$^{125}$I]-IGF-I binding. The IGFBP-2 was clearly

visible on the autoradiograph (Figure 11); however, IGFBP-3 was not detected, possibly because of degradation during storage. For statistical analysis, heifers were pooled so that the main contrast compared extracts from proximal versus distal regions of the mammary gland. A tendency existed for IGFBP-3 to be greater in distal than proximal regions ($P<0.06$). Abundance of IGFBP-2, a 28-kD BP and a 24-kD BP did not differ by region ($P>0.1$).


**Figure 11.** Autoradiograph of a western ligand blot showing relative IGFBP abundance in mammary tissue extracts prepared from proximal ('P') and distal ('D') regions of the mammary parenchyma. 'BP-3' = IGFBP-3 standard, 'BP-2' = IGFBP-2 standard; '444', '452' and '510' indicate three different heifers. Extracts (200 μg protein/lane) were fractionated on a gel, transferred to a membrane and hybridized with [$^{125}$I]-IGF-I. Mr = relative molecular mass.

**Table 1.** The effect of proximal ('P') and distal ('D') region of the mammary parenchyma on the abundance of IGF-binding proteins (IGFBP) in mammary extracts. Values are based on the densitometric units (Odu/mm$^2$) recorded on the autoradiograph. Data represent means for each region for three heifers.

| IGFBP | Mean P | Mean D | SE | Probability |
|---|---|---|---|---|
| 43-kD | 54.9 | 70.6 | 5.2 | 0.06 |
| 32-kD | 27.9 | 24.6 | 1.4 | 0.14 |
| 28-kD | 28.0 | 24.3 | 1.8 | 0.17 |
| 24-kD | 11.1 | 9.6 | 1.5 | 0.53 |

IGF-I Concentrations in Mammary Extracts:

Assays of samples that were separated by acid-ethanol cryoprecipitation did not yield valid results due to problems with linearity of the assay over concentrations tested and large coefficients of variation (>10%). However, RIAs using samples of fractions from column chromatography yielded valid results. The RIA was linear when different amounts of sample were assayed. Average inter-assay variation was 4.5 and 5.1%. Average intra-assay variation was 9.4 and 8.0%.

The volumes of each fraction collected from the column were as follows: 'A' (0-12 ml), 'B' (12-20 ml), 'C' (20-29 ml), 'D' (29-38 ml), 'E' (38-45 ml), and 'F' (45-54 ml). The 'B' fraction contained the proteins that were greater than the upper limit and those of greatest molecular weight within the fractionation range (1.5 to 30-kD). The 'B' fraction was identified by measuring the protein content of fractions collected after BSA was applied to the Sephadex G-50 gel column (Figure 12). To capture the binding proteins less than 30-kD, 2 to 3 ml additional were collected than the volume in the BSA peak. The 'C' fraction contained the majority of $^{125}$I-IGF-I, as determined by quantification using a gamma counter, and was used to characterize the fraction that would include the 7.6-kD protein, IGF-I. Two other peaks were noted during the characterization of the column with the labeled ligand. The first peak ('B') likely contained degraded $^{125}$I-IGF-I, while the third peak ('E') likely included free iodine.

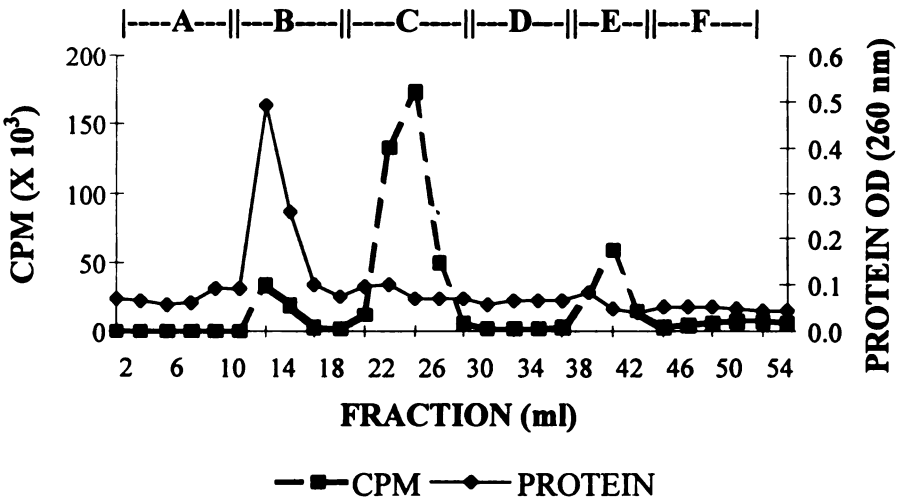


**Figure 12.** Characterization of protein flow from Sephadex G-50 size exclusion gel chromatography column using BSA and $^{125}$I-IGF-I. The BSA contains proteins (albumin) greater than the upper limit of the fractionation range (1.5-kD to 30-kD) and is measured as "protein" in optical density (OD) as shown on the z-axis. The $^{125}$I-IGF-I penetrates the gel particles, is eluted within the fractionation range, and was quantified using a gamma counter where CPM = counts per minute on the y-axis. Each tick mark represents a 4-ml fraction on the x-axis. Letters 'A' through 'F' represent the fractions that were collected for all samples.

Concentrations of IGF-I in mammary extracts did not differ between proximal and distal regions of the parenchyma ($P$=0.18). Mean IGF-I concentrations were 30.5 ng/ml for the proximal region and 27.9 ng/ml for the distal region (SEM = 2.2) of mammary extract or 116.8 ng/g of mammary parenchymal tissue. This calculation is based on the 3:1 ratio of saline:tissue used in preparation of mammary extracts. Serum samples contained 231.9 ± 17.3 ng/ml of serum.

Samples containing added IGF-I were used for validation of column chromatography and the RIA. Thirty nanograms of IGF-I were added to a mammary extract sample before addition to the Sephadex column, and 108% (± 7) was recovered. Also, 2 ng of IGF-I was added to a sample of 5% BSA and 113% (± 16) was recovered. Recovery for 6 ng of IGF-I added to a serum sample just before the RIA was 97% (± 6).

The effectiveness of the IGFBP separation by column chromatography also was evaluated by ligand blotting of fractions collected from the column. Fractions 'A', 'D' and 'E' did not contain IGF-I as quantitated in the RIA. The 'B' fraction containing proteins greater than the upper limit and those of greatest molecular weight within the fractionation range, should have contained the majority of the IGFBP. Assay of IGF-I concentrations in the 'B' fraction by RIA did not yield linear results (i.e., increasing amounts of sample decreased amount of bound [$^{125}$I]-IGF-I in a manner that was not parallel to the standard curve) over the range of amounts assayed. The 'C' fraction that penetrated the gel particles contained IGF-I, similar to previous results in characterization of the column using $^{125}$I-IGF-I, and yielded linear results in the RIA. Both 'B' and 'C' fractions were further characterized using ligand blots (Figure 13). The 'B' fraction for both mammary extracts and serum samples contained four IGFBP, similar to results for

ligand blotting of mammary extracts. These included IGFBP-3, IGFBP-2 and lower molecular weight IGFBP (28 and 24-kD). However, as expected, the subsequent 'C' fraction collected from the column for either mammary extracts or serum samples did not contain detectable binding proteins. As positive controls for [$^{125}$I]-IGF-I binding, IGFBP-2 and IGFBP-3 were included on all blots. Also, a molecular weight ladder and a non-fractionated serum sample were included on each gel.

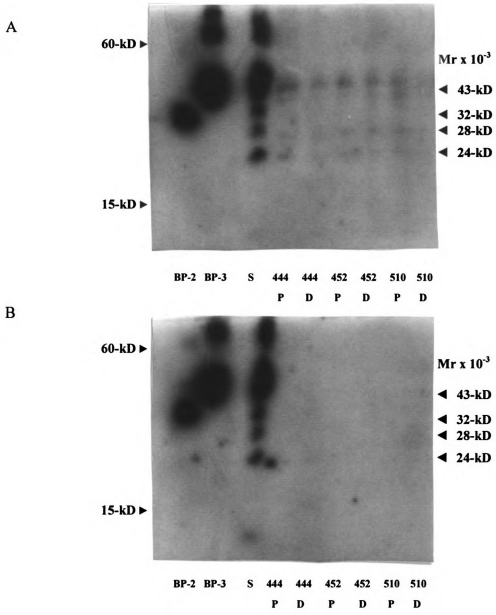


**Figure 13.** Autoradiograph of a western ligand blot showing relative IGFBP abundance in mammary tissue extracts from two different fractions collected from a Sephadex G-50 gel column. Panel A shows proteins from fraction 'B' and Panel B shows proteins from fraction 'C'. 'L' = molecular weight ladder; 'BP-3' = IGFBP-3 standard; 'BP-2' = IGFBP-2 standard; '444', '452'and '510' indicate three different heifers; 'P' and 'D' refer to proximal and distal regions of the mammary parenchyma, respectively; 'S' = serum. Mr = relative molecular weight. Extracts (200 µg protein/lane) were fractionated on a gel, transferred to a membrane and hybridized with [$^{125}$I]-IGF-I.

Leptin Concentrations in Mammary Extracts:

Leptin concentrations did not differ between proximal and distal regions of the mammary parenchyma ($P = 0.37$). Mean leptin concentrations were 3.9 ng/ml for the proximal region and 3.6 ng/ml for the distal region (SEM = 0.3) of mammary extract or 15.2 ng/g of parenchymal tissue. Serum samples for two of the three heifers contained 3.6 (± 1.2) ng leptin/ml of serum.

**Table 2.** Summary table of overall results.

| | Proximal | Distal | % Change | P value |
|---|---|---|---|---|
| **MAC-T Assay (3%)** | Higher | Lower | 11.1 | 0.02 |
| **IGF-I Concentrations** | Higher | Lower | 10.9 | 0.18 |
| **IGFBP-3 Abundance** | Lower | Higher | 12.9 | 0.06 |
| **IGFBP-2 Abundance** | Higher | Lower | 11.3 | 0.14 |
| **28-kD IGFBP Abundance** | Higher | Lower | 11.5 | 0.17 |
| **24-kD IGFBP Abundance** | Higher | Lower | 11.6 | 0.53 |
| **Leptin Concentrations** | Higher | Lower | 10.8 | 0.37 |

# DISCUSSION

A clearer understanding of prepubertal heifer mammogenesis could lead to techniques that enhance mammary growth, resulting in increased milk production and profitability for the dairy producer. Future milk yield is positively correlated with the number of mammary epithelial cells present and the activity of these cells (Broster and Broster, 1984). Many stimulatory and inhibitory factors that modulate mammary epithelial cell proliferation are produced within the mammary gland. Thus, greater knowledge of the regulation of synthesis and action of these factors could enable researchers to increase mammary cell proliferation. This study focuses on the mitogenic activity within the mammary parenchyma and how this activity differs by region of the heifer mammary gland.

To study the mitogenic activity of specific regions of the parenchyma within the mammary gland, mammary extracts were prepared from tissue collected from proximal and distal regions of the parenchyma. Mammary extracts of tissue from the proximal region stimulated proliferation of MAC-T cells 11% more than extracts from distal regions, as measured by total DNA content per culture well. Protein content of extracts was not correlated to the amount of mitogenic activity in mammary extracts used as treatments on MAC-T cells. The amount of increase in protein concentration in basal medium with the addition of extracts was minimal. Thus, the greater mitogenic activity in parenchyma from proximal compared with distal regions is not explained by differences in total protein content.

One of the factors known to stimulate mammary development is IGF-I. IGF-I mRNA has been detected in stromal cells (Hauser et al., 1990). IGF-I is a potent mitogen for the bovine MAC-T mammary epithelial cell line (Zhao et al., 1992, Woodward et al., 1994) and for primary bovine (Shamay et al., 1988) and ovine (Winder et al., 1989) mammary epithelial cells. MAC-T cells (Huynh et al., 1991) were chosen for this thesis project because of the problems associated with harvesting a pure mammary epithelial cell population from prepubertal heifers as described by Shamay et al. (1988) (see Appendix). MAC-T cells proliferated in a dose-dependent manner in response to increasing doses of IGF-I, similar to what has been shown previously (Zhao et al., 1992). Mammary epithelial cells have receptors for both IGF-I and IGF-II (McCusker, 1998). However, little is known about the mechanisms required for growth and differentiation of tissue within the developing mammary gland.

The mammary extracts in this experiment ranged in IGF-I concentrations from 27.5 to 45.5 ng/ml of extract, which is within the range of those determined in a previous experiment (Weber et al., 2000a). Concentrations of IGF-I did not differ within mammary extracts from different parenchymal regions. These results are similar to another study that focused on the mitogenic response of mammary epithelial cells to mammary tissue extracts. Mammary extracts of tissue collected from heifers fed at a high compared to a low rate of gain stimulated less [$^3$H] thymidine incorporation (Weber et al., 2000b). Extracts of mammary tissue from these two groups of heifers did not differ in concentrations of IGF-I (Weber et al., 2000a). In both our experiment and the Weber study, IGF-I concentrations did not explain the differences in mitogenic activity of the mammary extracts.

Separation and removal of IGFBP from IGF-I is essential for samples to be assayed accurately without interference by the IGFBP in the radioimmunoassay. Initially an IGFBP extraction procedure, similar to the acid-ethanol cryoprecipitation used by Breier et al. (1991) was used. Results of this extraction procedure indicated that this was not a valid extraction procedure for mammary extract samples. This is in agreement with studies that have compared different separation procedures (Breier et al., 1991; Frey et al., 1994; Gutierrez et al., 1997; Mohan and Baylink, 1995). Size exclusion gel chromatography was noted to be an efficient method for removal of IGFBPs from IGF-I in all four references. Thus, IGFBP were separated from IGF-I in the mammary extract and serum samples by applying acidified samples to a Sephadex G-50 gel column. RIA results showed that two different fractions gave readings in the IGF-I assay. The 'B' fraction should have contained proteins that were greater than the upper limit and those of greatest molecular weight within the fractionation range (1.5 to 30-kD). This fraction yielded IGFBP when tested on a ligand blot and results of increasing concentrations of the 'B' fraction in a RIA showed non-linearity and high coefficients of variation. This indicates that the IGFBP did interfere by cross-reacting with the antigen and competing for binding to the antibody. The 'C' fraction contained IGF-I and no IGFBP were detected by western ligand blotting. This separation procedure yielded a valid RIA of samples, showing linearity over the concentrations tested with low inter- and intra-assay variation. These results further confirm that if binding proteins are not removed successfully, these proteins will interfere with an IGF-I RIA.

The majority of IGF-I in the circulation is bound to IGFBP (Baxter, 1993). Gene expression of IGFBP by specific tissues may regulate their local effects on IGF-I, while

the availability of serum IGF-I may be regulated by alterations in serum IGFBP levels (Lemozy et al., 1994). Binding protein -2, -3, and the 28-kD and 24-kD binding proteins (putatively IGFBP-1 and -4) are present in the heifer mammary gland (Weber et al., 2000a). In this study, ligand blots of mammary extracts displayed IGFBP with relative molecular masses of 40 to 43 (IGFBP-3), 32 (IGFBP-2), 28-kD and 24-kD, as seen previously (Weber et al., 2000a). Abundance of IGFBP-2, a 24-kD and a 28-kD IGFBP within the mammary extracts did not differ with parenchymal tissue region. Abundance of IGFBP-3 showed a strong tendency to be greater in distal compared to proximal regions. Hence, parenchymal tissue region does not influence IGF-I concentrations in mammary extracts but does influence local abundance of IGFBP-3 in the mammary gland. These observations agree with the above suggestion by Lemozy and others (1994) that IGFBP gene expression by the mammary gland regulates the local effects of IGF-I.

The IGFBP-3 is of major importance because it constitutes the majority of IGFBP in serum of heifers (McGrath et al., 1991) and in bovine mammary tissue extracts (Weber et al., 2000a). In addition, mRNA for IGFBP-3 is predominant in the secretory epithelial cell compared to other tissue cell types within the mammary gland (Gibson et al., 1999). IGFBP-3 may act independently of IGF-I in certain environments or under specific conditions. Purup et al. (2000) suggested that IGFBP-3 in mammary tissue inhibits IGF-I activity while IGFBP-3 in serum potentiates IGF-I activity. When acting independently of IGF-I, IGFBP-3 usually has an inhibitory effect on epithelial cell proliferation (Cohen et al., 1993; Rodgers et al., 1996). Little evidence is available for the role of IGFBP-2 in mammary development. IGFBP-1 is thought to be an inhibitor of IGF-I action (Lewitt et al., 1991). IGFBP-3 was more abundant in mammary extracts of tissue from the distal

region that inhibited total DNA by 11% compared to extracts of tissue from the proximal region of the mammary parenchyma. This indicates that IGFBP-3 may have had an inhibitory effect on MAC-T epithelial cell proliferation.

This study was the first to analyze the mitogenic activity of mammary extracts of parenchymal tissue collected from different regions within the bovine mammary gland. Two previous studies reported the activity of cells from different regions within the developing gland. Explants of mammary tissue taken from prepubertal lambs were incubated with [$^3$H] thymidine (Akers, 1990). The majority of labeled cells were located at the periphery of groups of ducts. Microsomes from the distal parenchyma region of a prepubertal heifer bound more radiolabeled IGF-I than microsomes from the proximal parenchyma (Ellis et al., 2000). In addition, epithelial cells from the peripheral (distal) region were more responsive to both IGF-I and TGF-β (Ellis et al., 2000). Results of these two studies suggest that epithelial cells were proliferating faster on the distal regions of the parenchyma. In the present study the mitogenic activity within the parenchyma was evaluated, which includes the products of multiple cell types. Parenchymal tissue consists of fibroblasts, epithelial and adipocyte cells, which produce different mitogens and have receptors for different growth factors. Overall, mitogenic activity is a measure of the difference between the effects of stimulatory factors minus those that are inhibitory to cell proliferation.

Three major morphological differences between the distal and proximal regions are 1) the vicinity of the region to the fat pad, 2) the abundance of different cell types within the parenchyma of the two regions and 3) the regional structural differences that become evident later in development. First, tissue in the distal region is closer in vicinity

to the fat pad than the proximal region. Therefore, the distal parenchyma is also closer to possible stimulatory and inhibitory factors produced by adipocytes within the fat pad. Second, the proportion of cell types differs between different regions within the mammary gland (Sejrsen et al., 1982). Using tissue slices stained for light microscopy for histological determination of individual cell types, this group determined that the epithelial cells decreased from 11.9% to 9.7% ($P<0.01$) as distance from the base of the gland or teat (i.e., proximal region) increased. The percentage of fat cells in the parenchyma increased from 25.4% to 45.4% ($P<0.001$) as distance from the base of the gland increased. Lastly, later in development, the parenchyma in the proximal region likely differentiates into the primary ductal structures, whereas the distal region differentiates into pre-alveolar structures (Ellis et al., 2000). Different factors that influence gene expression must be present in the two regions to allow the parenchymal tissue to differentiate into different structures.

Leptin, a factor shown to inhibit the stimulatory action of IGF-I, is produced by adipocytes, mammary tissue, and MAC-T cells (Houseknecht et al., 1998; Smith and Sheffield, 2002). Bovine mammary epithelial cells from prepubertal heifers display receptors for leptin (Silva et al., 2001). Partly because of recent interest in leptin, much research has focused on the function of the mammary fat pad in mammary development. The fat pad in mice is crucial to normal growth and development of the mammary gland (Hoshino & Martin, 1974). Mouse mammary epithelial cells (COMMA-1D cells) grew when co-cultured with and without floating murine mammary fat pad explants (Hovey et al., 1998a). Hovey et al. suggested that the murine mammary fat pad is a direct source of mitogenic activity and that it is involved in modulating the response of epithelial cells to

certain mitogens. However, McGrath's (1983) data showed that primary mouse mammary epithelial cell growth, measured by [$^3$H] thymidine labeling, was inhibited when in close contact with stromal cells.

The role of the fat pad in bovine mammary development is not clear. Woodward et al. (1993) suggested that adipocytes have different roles in the induction of mammary epithelial cell proliferation in rodents compared with ruminants. For example, the cow has a lack of close association of epithelial cells and mammary adipocytes compared with the rodent. Because of morphological differences, the results from rodent studies should not be assumed to be the same for the bovine (Akers, 1990). Few studies have been published on the role of the bovine fat pad in mammary development. Interestingly, fat pad explants from both beef and dairy heifers significantly inhibited primary bovine mammary epithelial cell growth in vitro (McFadden and Cockrell, 1993).

The importance of interactions between the mammary fat pad, stroma and the developing epithelium has been demonstrated. Two studies have compared the intact mammary fat pad (MFP) to the epithelium cleared fat pad (CFP) of heifers (Berry et al., 2001) and ewe lambs (Hovey et al., 1998b). Berry and co-workers cleared epithelium from a single fat pad in heifers at 1 mo of age; at 18 mo, heifers were treated with estrogen before slaughter. Estrogen treatment increased the amount of IGF-I protein in MFP and parenchyma but to a lesser extent in CFP. Estrogen tended to increase abundance of IGF-I mRNA in MFP, but not CFP, implying that the regulation of IGF-I expression is modulated by adjacent epithelium. IGF-I mRNA was more abundant in MFP than CFP in ewe lambs, further indicating that IGF-I mRNA expression was regulated by epithelial-stromal interactions (Hovey et al., 1998b). Hovey suggested that

proliferating epithelium might exert a positive feedback on surrounding stroma, causing an increase in mRNA expression of IGF-I. Sheffield noted the importance of species differences in stromal requirements for mammary epithelial growth and that this area requires further investigation (Sheffield, 1988). By transplanting mammary epithelium into gland free fat pads of mice, Sheffield showed that the mouse mammary epithelia were capable of organizing into ductal structures if grown with the appropriate stroma. However, bovine mammary epithelia were unable to proliferate when injected into gland free mammary fat pads of athymic nude mice (Sheffield and Welsh, 1987). Thus, existing data from the bovine indicates substantial differences in function compared with the fat pad in mice. More research is needed to explain differences seen in these studies. The evidence from this thesis indicates that bovine parenchyma tissue in closer vicinity to the fat pad has less mitogenic activity than tissue more distant from the fat pad.

One potential explanation for the difference in mitogenic activity is the role of leptin in mammary development. Leptin is produced by adipocytes (Houseknecht et al., 1998). Parenchymal tissue in the distal region, which is closer to the fat pad than the proximal region, is closer in vicinity to an area of leptin production. In addition, Sejrsen and co-workers (1982) found that the percentage of fat cells in the parenchyma increased as the distance from the teat increased. Therefore, a higher percentage of fat cells would be located in the distal region than the proximal region of the parenchymal tissue. Leptin, when used as a treatment in cell culture, is shown to inhibit bovine mammary epithelial cell proliferation (Silva et al., 1999). Therefore, it would seem logical that mammary extracts prepared from the proximal region would have lower concentrations of leptin and would stimulate more DNA synthesis than extracts from the distal region.

However, when mammary tissue was homogenized to make mammary extracts, it was noted that a large quantity of fat and also connective tissue remained on top of the gauze when the homogenate was poured through a double layer of gauze. Data from this experiment did show greater mitogenic activity of extracts prepared from parenchyma in the proximal region but region did not influence leptin concentrations in mammary extracts. Evidence from this research supports the idea of leptin as a potential local regulator of mammary development, as leptin concentrations were easily detectable in mammary tissue. Linearity of the leptin RIA for serum samples has been tested but not for the mammary extract samples. Therefore, a question remains as to whether these leptin concentrations are accurate for the mammary extract samples.

The observation that IGF-I and leptin concentrations did not differ between regions suggests that there must be another stimulator or inhibitor that influences mammary development. Further research is needed in this area to determine if there are other regulators of mammary development that contributed to observed differences in mitogenic activity. The use of antibodies against both stimulatory and inhibitory growth factors such as IGF-I, IGFBP, leptin, FGF, KGF, and TGF-$\beta_1$, would be one technique that could be used to quantitate the proliferative activity that each factor is contributing to the overall mitogenic effect of these mammary extracts.

# SUMMARY AND CONCLUSIONS

Mammary extracts of tissue from proximal regions of the parenchyma were 11 % more mitogenic than extracts of tissue from the distal region. Two main differences between these two regions, known prior to this study, included vicinity of the region to the fat pad and the abundance of different cell types (epithelial and fat cells) within the parenchyma of the tissue. In addition, as the parenchyma extends into the fat pad during development, regional differences in its anatomy become apparent. This study further characterized differences in region of the mammary parenchyma by measuring the concentration of IGF-I and leptin and the abundance of IGFBP within mammary extracts of tissue from these different regions. Concentrations of IGF-I were measured in extracts because it is a known potent mitogen for mammary epithelial cells in vitro. Furthermore, the role of the IGFBP must be considered to fully understand the mechanism of IGF-I growth stimulation in mammary development. Concentrations of leptin were analyzed because of its inhibitory effect in vitro on mammary epithelial cell proliferation. The parenchymal tissue region did not influence IGF-I or leptin concentrations in mammary extracts but did affect the local abundance of IGFBP-3 in the mammary gland. These findings indicate that IGFBP-3 may have had an inhibitory role in MAC-T epithelial cell proliferation. Because IGF-I and leptin concentrations did not explain the difference in mitogenic capacity of the mammary extracts, differences in IGFBP-3 or another local regulator likely contribute to regional differences in the mammary gland during development.

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# APPENDIX

Introduction:

The initial research proposal for this master's project is outlined in this appendix. Primary bovine mammary epithelial cell populations were harvested from prepubertal heifers, however contamination of fibroblasts was observed and verified using immunohistochemistry. Changes were made in the primary cell isolation to prevent or lower fibroblast cell contamination, but these modifications proved unsuccessful. Therefore, this project was amended using the MAC-T cell line and mammary tissue extracts (see Thesis). The initial project consisted of two objectives, with each objective tested on both the primary epithelial cell population and the MAC-T cell line. Much of the in vitro studies of the role of IGF-I on bovine mammary development involve either primary cells or the MAC-T cell line. In addition, another experiment was performed which focused on the differences in mitogenic activity of mammary tissue extracts prepared from a prepubertal heifer and a postpubertal heifer.

Insulin-like growth factor binding protein-3 is a potent regulator of the biological actions of IGF-I in mammary tissue (see p. 21-23). The regulation of IGFBP-3 synthesis in epithelial cells in the developing bovine mammary gland and the effect of IGFBP-3 on cell proliferation remain unclear. TGF-$\beta_1$ stimulates IGFBP-3 production in various cell systems such as breast and prostate cancer cell lines (Oh, 1998). Many studies suggest that TGF-$\beta_1$ and IGFPB-3 may be interrelated with respect to how they affect cell proliferation (Oh et al., 1995; Purup et al., 2000). TGF-$\beta$ receptors have been characterized on bovine mammary epithelial cells (Woodward et al., 1995) and TGF-$\beta_1$

mRNA has been identified in the bovine mammary gland (Maier et al., 1991; Plaut, 1993). Levels of 25 to 50 pg/ml of TGF-$\beta_1$ stimulated DNA synthesis, whereas levels of greater than 100 pg/ml inhibited DNA synthesis (Ellis et al., 2000). TGF-$\beta_1$ was also effective in inhibiting proliferation of MAC-T cells (Woodward et al., 1995). Research involving the role of TGF-$\beta_1$ on bovine mammary development has only recently surfaced.

The purpose of this project was to define under what conditions is the role of IGFBP-3 stimulatory or inhibitory to the development of the mammary gland and possible regulatory factors affecting this role. The first objective of this project was to determine if IGFBP-3 regulates proliferation of primary bovine mammary epithelial cells independently of IGF-I. The second objective was to investigate the regulation of IGFBP-3 synthesis by TGF-$\beta_1$ in mammary epithelial cells.

Primary Bovine Mammary Epithelial Cell (PMEC) Isolation:

Primary bovine mammary epithelial cells (PMEC) were isolated and cultured similar to Shamay et al. (1988). Mammary parenchymal tissue from five prepubertal heifers (184.5 ± 29.5 kg body weight) was collected immediately after slaughter. Reproductive tracts were dissected to confirm that heifers were prepubertal. Tissue was placed in medium 199 with 2 g $NaH_2CO_3$ (M-199, HEPES modified, Sigma, pH 7.4, sterilized using 0.22-μm filters). Basal medium also contained soybean trypsin inhibitor (Type I-S, Sigma, 1 mg/L), transferrin (apo-bovine, Sigma, 5 mg/L), glutathione (Sigma, 1 mg/L), insulin (bovine, 0.01 mg/L), sodium selenite (Sigma, 5 mg/L), antibiotic/ antimycotic (Invitrogen, 10 ml/L), gentamicin (Invitrogen, 2.5 ml/L), and bovine serum

albumin (BSA) (Invitrogen, 2.625 g/L). In a sterile culture hood (Biological Safety Cabinet, Forma Scientific, Marietta, OH), parenchymal tissue was diced into small pieces using sterile scissors and placed into a petri dish containing medium. Medium was aspirated and tissue was separated into 15 g quantities using tared petri dishes. Each 15 g quantity of tissue was placed into a sterile bottle containing 150 ml of medium (M-199 with 5X antibiotics) with 1 ml of DNase (Sigma, 4 mg/ml) and 100 µl insulin (Sigma, 1 mg/ml). DNase digests nucleic acids, which leak into the medium as a result of cell damage, without damage to the intact cell (Freshney, 1994). Bottles were placed on a shaker (Brinkmann OrbiMix 1010, Incubator 1000, Germany) for 10 min at 37°C and 180 rpm. Tissue was allowed to settle for 5 min and medium in the middle phase was aspirated. Tissue was transferred to 50-ml tubes and again was allowed to settle before medium was aspirated. An enzyme digestion mixture [150 mg collagenase (Worthington Biochemical, Type II, CLS-2, Lakewood, NJ), 150 mg hyaluronidase (Sigma), 150 ml of medium; for 15 g of tissue] was made and sterile filtered. Collagenase degrades the triple-helical collagen fibrils and disperses stroma, leaving epithelial cells in small clusters. Hyaluronidase, a polysaccharidase, breaks bonds in hyaluronic acid and chondroitin sulfate that are both found in high concentrations in connective tissue (Freshney, 1994). The enzyme mixture was transferred to an Erlenmeyer flask and 15 g of tissue was added. Also, 1 ml of DNase and 100 µl of insulin were added to each flask. During the first digestion, flasks were incubated at 37°C and shaken at different times and speeds depending on the isolation (see Table). The floating fatty layer was removed. The remaining mixture was poured through Nitex filters (75 and 200 µm) so that organoids between 75 and 200 µm in size were harvested. Organoids greater than 200

μm were transferred to the digestion solution and placed on the shaker for additional time periods depending on the isolation (see Table, second digestion). For isolations #1, #2 and #3, only a 200-μm filter was used. The 75-μm Nitex filter was added into the protocol for the last two isolations to filter out most of the single-cell suspension that resulted from the digestion procedure.

Filtered cell organoids were transferred to 50-ml tubes and centrifuged at 110 x *g* for 10 min. The supernatant was aspirated, cells were transferred into clean 50-ml tubes with 45 ml of media, and tubes were centrifuged at 110 x *g* for 10 min. The supernatant was aspirated and cells were transferred to clean 15-ml tubes with 10 ml of medium. Cells were mixed with medium and then cells were allowed to settle for 6 minutes. The supernatant was aspirated and medium was replaced to total 10 ml. This washing process was repeated 10 times. Freezing solution [50% FBS, 44% basal medium, 6% DMSO (dimethyl sulfoxide, J.T. Baker)] was prepared with 1 ml of a cell pellet equaling 10 vials of cells plus media (1.8 ml/vial). The 2-ml cryogenic vials were immediately placed in freezing containers (Nalgene Cryo 1°C, Rochester, NY) in an -80°C freezer and stored overnight. The following day, cryogenic vials were transferred to liquid nitrogen until use in cell culture experiments.

**Table 3.** Differences between primary bovine mammary epithelial cell isolation procedures from five different heifers. Different times and shaker speeds were used for each isolation. A 75-μm filter was used in two of the isolations and the enzyme, pronase, was used in one isolation. During the last two isolations, collection of tissue near the parenchyma/fat pad interface was less aggressive.

| Heifer | 75-um Nitex | 1st Digest | 2nd Digest | 3rd Digest | Pronase | Total Tissue |
|---|---|---|---|---|---|---|
| 1 | | 4.5 h @ 180 rpm | 14 h @ 90 rpm | | | 75 g |
| 2 | | 3 h @ 180 rpm | 14 h @ 90 rpm | | | 53 g |
| 3 | | ½: 1 h @ 165 rpm | 15 min @ 90 rpm | | | 85 g |
| | | ½: 1.5 h @ 165 rpm, 1 h at 90 rpm | 45 min @ 180 rpm | | | |
| 4 | X | 1 h @ 165 rpm | 20 min @ 90 rpm | 30 min @ 90 rpm | X | 28 g |
| 5 | X | 4.75 h @ 90 rpm | 14 h @ 90 rpm | | | 59 g |

PMEC Observations and Controlling Fibroblast Contamination:

Our laboratory and other groups have dealt with problems associated with fibroblast cell contamination in primary cell populations (Freshney, 1994). Primary cells, harvested from tissue isolated from five heifers, were observed while growing on both plastic and in a collagen gel matrix. On both surfaces, fibroblast cells were present along with epithelial cell organoids. The presence of fibroblasts was verified by immunohistochemistry using antibodies against proteins specific for different cell types (see below).

Changes were made in the primary cell isolation to prevent or lower the amount of fibroblast cell contamination. One of these modifications was the inclusion of a 75-μm Nitex filter, so that organoids would be between 75- and 200-μm in size. Most fibroblast cells were observed to be in a single-cell suspension; therefore, it was thought that adding a smaller size filter to the protocol would eliminate a majority of the fibroblast cells. However, addition of this step did not prevent fibroblast contamination.

Baffled shake flasks (Kimax, Kimble Glass Inc., Vineland, NJ) were used during tissue digestion for the second and third isolations. The baffled flasks have indents at the bottom to provide greater turbulance so that the tissue is more agitated during the digestion process. However, using these baffled flasks decreases the length of the digestion process. After personal communication with Dr. Sandy Haslam (Michigan State University), it was decided to use Erlenmeyer flasks instead to allow the tissue to be in contact with the digestive enzymes for a longer period of time.

The use of pronase (Protease, Streptomyces griseus, Calbiochem) was included in one of the PMEC isolations. Half of the tissue in this isolation was subjected to pronase

(15 mg / 150 ml of enzyme digestion mixture) while the other half was not; therefore, the use of pronase could be compared against normal conditions. Observations of cells showed that fibroblasts were still evident in the isolation using pronase. Cells did not seem affected by the use of pronase as cells grew in a dose-dependent manner in response to increasing amounts of IGF-I.

Manipulating the culture conditions by using selective medium is a standard method in isolating specific cell types (Freshney, 1994). Sordillo et al. (1988) replaced L-valine in the culture medium with D-valine and demonstrated that contamination of bovine mammary epithelial cell cultures with fibroblasts could be prevented by use of the D-valine medium. Epithelial cells, but not fibroblasts, possess the D-amino acid oxidase and therefore grew normally. Minimum Essential Medium (MEM) D-valine (Gibco) was chosen to use as a selective medium in our experiments. Both MEM D-valine and M-199 medium were used and cells were treated with basal medium, FBS at 10% concentration of medium and an IGF-I dose-response series. Observations of cells grown in MEM D-valine showed no response for IGF-I at all doses and only a minimal response to FBS compared to M-199. The cells grown in D-valine medium yielded little proliferation and had fewer fibroblasts compared to cells grown in M-199. To test the MEM media, both L-valine and D-valine MEM medium were tested on PMEC. Observations of plates yielded little proliferation of cells grown in both L-valine and D-valine. More fibroblasts were apparent when grown in L-valine than with D-valine. In conclusion, the PMEC isolations did not grow normally in the MEM medium and therefore this medium must lack an essential nutrient needed for this cell type to grow. In addition, use of D-valine

did decrease the number of fibroblasts present but this procedure was discontinued due to the lack of normal cell growth and cell response to a known mitogen.

Cell isolations (#1 and #3) were re-digested to increase the time that the epithelial and fibroblast cells were in contact with the digestive enzymes, hyaluronidase and collagenase. Vials of cells were thawed and washed in medium. Cells were added to a flask containing medium, 1 ml DNase and 100 µl insulin. The flask was shaken for 10 min at 37°C and 180 rpm. A digestive enzyme solution (200 mg hyaluronidase, 200 mg collagenase; 100 ml medium) was made and added to the flask containing cells. Cells were digested with enzymes for 30 min at 37°C and 90 rpm. Cells were then filtered through the two sizes of Nitex, washed and frozen as previously stated. When grown in culture, re-digested cell isolations had approximately 5 to 10% fibroblasts.

The use of a Percoll gradient was not used as a cell dissociation method in this project because the fibroblasts seemed to be embedded within the organoid. However, this gradient has been used to separate mammary epithelial components from stromal cells and blood elements (Hauser et al., 1990). Epithelial fragments sediment to 1.06-1.07 g/ml density region, while stromal and blood elements can be collected at 1.03-1.04 g/ml density region (McGrath, 1987). The use of a Percoll gradient should be investigated more to see if this technique could separate the epithelial cell organoids from the fibroblast cells in these cell populations.

Immunohistochemistry:

The amount of fibroblast contamination within the primary mammary epithelial cell population was quantified using immunohistochemistry. The Vectastain ABC kit

(Vector Labs, Burlingame, CA) and monoclonal antibodies against vimentin, Cytokeratin Peptide 18 and Cytokeratin Peptide 17 (Sigma) specific to fibroblast, epithelial and myoepithelial cells, respectively were used to identify cell types.

PMEC were grown overnight in a 10% FBS medium on top of round plastic cover slips placed into 24-well plates. After 24 h in culture, the 10% FBS was aspirated, cells were washed and medium was changed to basal medium. Medium was aspirated, cells were washed with Dulbecco's phosphate buffered saline (PBS, Gibco), PBS was aspirated and cells were then air-dried, after three days in culture. Methanol (750 µl, 100%) at 4°C was added to each well, incubated for 5 min and aspirated. Hydrogen peroxide (0.3% $H_2O_2$, Sigma, 750 µl) diluted with methanol was added, incubated for 30 min, and aspirated. Cells were rinsed with PBS, gently rocked for 5 min, and PBS was aspirated. The Vectastain ABC universal kit (Vector Laboratories, Inc., Burlingame, CA) contained the blocking serum, a secondary antibody and the ABC complex. Normal blocking serum (15 ml PBS, 3 drops serum; 750 µl) was added, incubated for 20 min, and aspirated. Cells were rinsed with PBS, gently rocked for 5 min, and PBS was aspirated. Primary antibody (750 µl) was added, incubated 30 min, and aspirated. Primary antibodies included Cytokeratin Peptide 18 (1:100), Cytokeratin Peptide 17 (1:400) and vimentin (1:100) and were diluted using a buffer solution (100 ml PBS, 2 drops serum). Cells were rinsed with PBS, gently rocked for 5 min, and PBS was aspirated. Secondary antibody (10 ml PBS, 4 drops serum, 4 drops secondary antibody; 750 µl) was added, incubated 30 min, and aspirated. Cells were rinsed with PBS, gently rocked for 5 min, and PBS was aspirated. The ABC complex (15 ml PBS, 6 drops reagent 'A', 6 drops reagent 'B'; 750 µl) was added, incubated 30 min, and aspirated. Cells were rinsed with

PBS, gently rocked for 5 min, and PBS was aspirated. The PBS wash was repeated three times. Vector NovaRed peroxidase solution (15 ml water, 9 drops reagent '1', 6 drops reagent '2', 6 drops reagent '3', 6 drops $H_2O_2$; 750 µl) was added, incubated for 1.5 to 2 min and aspirated. Binding of the peroxidase solution is indicated by the red appearance of the cells in the figures (Figures 14, 15 and 16). Some of the slides were counterstained with hematoxylin solution (Gill No. 2, Sigma, blue appearance) by addition of stain (750-µl), incubated for 30 sec, and aspirated. Cells were then rinsed with water and dried using ethanol at 95 and 100%. Cover slips were fixed to glass slides. Immunohistochemistry images in this thesis are presented in color.

After immunohistochemical staining, it was apparent that the cultures of the primary cell population harvested contained approximately 10% fibroblast cells (Figure 15). Primary cells were highly stained for Peptide 18 (Figure 14), and both organoids and single scattered cells stained for vimentin. Fib-C cells, an immortalized cell line with fibroblast-like properties, (Woodward et al., 1995) and the MAC-T cell line were also used to test the antibodies on other bovine cell types. MAC-T cells stained positive for Cytokeratin Peptide 18, were lightly stained for Cytokeratin Peptide 17, and were negative for vimentin. Fib-C cells stained positive for both vimentin and Cytokeratin Peptide 18. The Fib-C cells had fibroblast-like morphology. One reason for why the Fib-C cells stained for Peptide 18 could be because the cell line was isolated while the cells were differentiating.

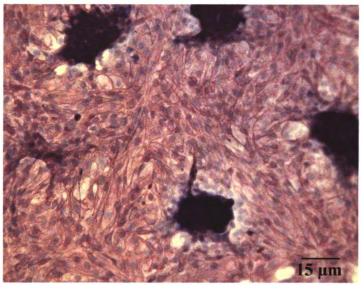


**Figure 14.** Illustration of primary bovine mammary epithelial cells stained red using immunohistochemistry: Vectastain ABC kit and monoclonal antibody against Cytokeratin Peptide 18. This antibody is specific for epithelial cells. Hematoxylin (blue) was used as a counterstain. Image is presented in color.

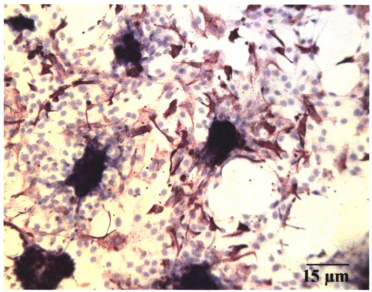


**Figure 15.** Illustration of primary bovine mammary epithelial cells stained red using immunohistochemistry: Vectastain ABC kit and monoclonal antibody against Vimentin. This antibody is specific for fibroblast cells. Hematoxylin (blue) was used as a counterstain. Image is presented in color.

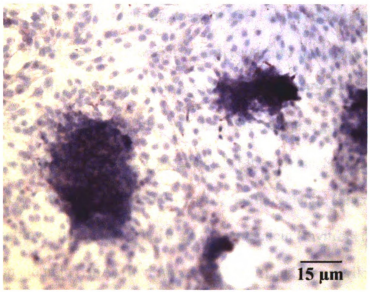


**Figure 16.** Illustration of primary bovine mammary epithelial cells stained red using immunohistochemistry: Vectastain ABC kit and monoclonal antibody against Cytokeratin Peptide 17. This antibody is specific for myoepithelial cells. Hematoxylin (blue) was used as a counterstain. Image is presented in color.

PMEC Culture Experiments:

PMEC were grown in a collagen matrix to achieve the following: 1) observe the proliferation and morphology of epithelial organoids in culture, 2) estimate the percentage of fibroblast cells contaminating the primary epithelial cell population, 3) test primary cells' response to increasing doses of IGF-I and 4) determine if IGFBP-3 regulates the proliferation of epithelial cells dependently and independently of IGF-I.

Basal medium was prepared using Medium 199 (HEPES Modification, Sigma) with 2 g $NaH_2CO_3$ added and pH to 7.4. Basal medium also contained soybean trypsin inhibitor (Type I-S, Sigma, 1 mg/L), transferrin (apo-bovine, Sigma, 5 mg/L), glutathione (Sigma, 1 mg/L), insulin (bovine, 0.01 mg/L), sodium selenite (Sigma, 5 mg/L), antibiotic/ antimycotic (Invitrogen, 10 ml/L), gentamicin (Invitrogen, 2.5 ml/L), and bovine serum albumin (BSA) (Invitrogen, 2.625 g/L) and was sterile filtered. Collagen solution was prepared [10% 10X M-199 medium, 10% collagen (Type 1, Becton Dickinson), 0.23% 1 *N* NaOH, 79.8% deionized $H_2O$] and 500 µl of solution was pipetted into each well. This was allowed to incubate for 30 min to allow collagen to gel. One vial of cells was thawed for each 24-well plate. Cells were washed three times in basal medium, added to the remaining collagen solution and 500 µl of the collagen/cell mixture was pipetted into each well on top of initial collagen gel. Basal medium (1 ml/well) was added and plates were stored 24 h in an incubator (Nuaire, US Autoflow) at 5% $CO_2$ and 37°C.

Treatments were prepared and applied to cells after 24 h in culture. All experiments included basal medium, FBS at 10% concentration of medium and increasing doses of IGF-I (3.125, 6.25, 12.5, 25, 50 and 100 ng IGF-I/ml of medium).

Increasing doses of IGFBP-3 (1, 10, 50, 100, 250 and 500 ng/ml of medium) were included to test the PMEC response to IGFBP-3 independently of IGF-I. Also, equimolar concentrations of IGFBP-3 (300 ng/ml) and IGF-I (50 ng/ml) were included to evaluate the effects of IGFBP-3 and IGF-I addition concurrently. After 48 h, treatments were aspirated and replaced with fresh treatment media. Forty-eight hours later, 1 μCi (2.2 x $10^6$ DPM) of [$^3$H] thymidine was added to each well and incubated for 2 h. Media was aspirated and collagen gels were transferred to eppendorf tubes. Acetic acid (150-μl, 25%) was added to each tube and incubated for 30 min at 37°C to dissolve the gel. Eppendorf tubes were centrifuged at 500 x *g* for 10 min and the supernatant was aspirated. Perchloric acid (750 μl, 4%) was added to the cell pellet, tube was centrifuged for 10 min at 500 x *g* and the supernatant was aspirated. This step was repeated with 750 μl of 80% ethanol, 750 μl of 100% ethanol and 750 μl of 4% perchloric acid. Next, 500 μl of 6% perchloric acid was added to the cell pellet. Cells were hydrolyzed in an 80°C waterbath for 1 h, cooled to room temperature and centrifuged for 15 sec at 500 x *g*. The supernatant was transferred to a scintillation vial and 5 ml of scintillation fluid was added. Tritiated thymidine was quantified on a beta counter (TM Analytical Mark V 32 ARI). Calculated counts per minute (CPM) were adjusted to disintegrations per minute (DPM) assuming 40% efficiency of the counter.

Results showed that increasing doses of IGFBP-3 did not inhibit proliferation of PMEC (Figure 16). However, this effect may have not been independent of IGF-I, since fibroblasts were present in the cell population and stroma is known to produce IGF-I (Hauser et al., 1990). A dose of 10 ng of IGFBP-3/ml of media stimulated cell proliferation when compared to IGFBP-3 doses of 250 ng ($P$=0.04) and 500 ng ($P$=0.06).

IGFBP-3 (300 ng/ml), added to equimolar concentrations of IGF-I (50 ng/ml), reduced DNA synthesis of primary bovine mammary epithelial cells ($P$=0.03, Figure 17).

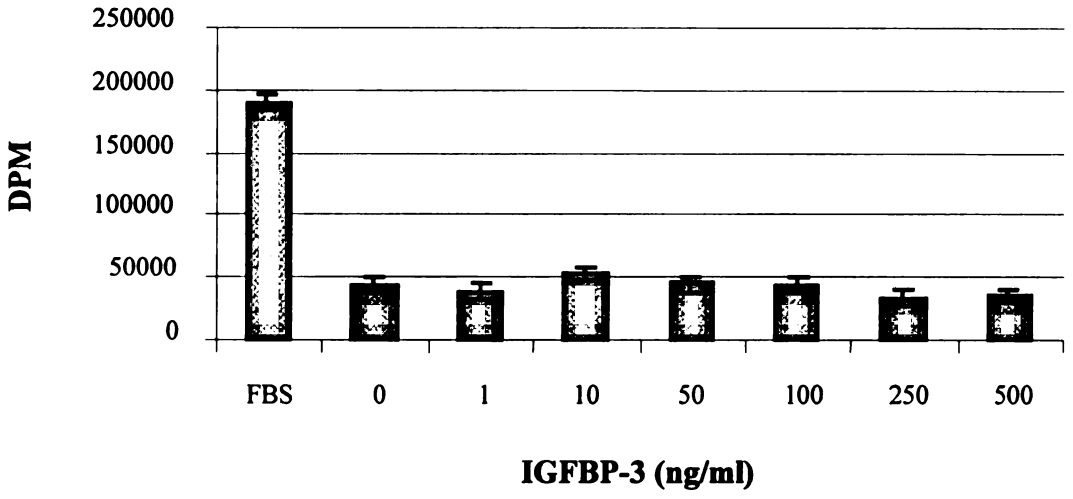


**Figure 17.** Effect of increasing doses of IGFBP-3 (1, 10, 50, 100, 250 and 500 ng/ml of media) on [$^3$H] thymidine incorporation into DNA of primary bovine mammary epithelial cells. Results are reported as DPM (disintegrations per minute). Data are presented as LS means ± SEM. Basal medium (0) and 10% fetal bovine serum (FBS) were included as controls.

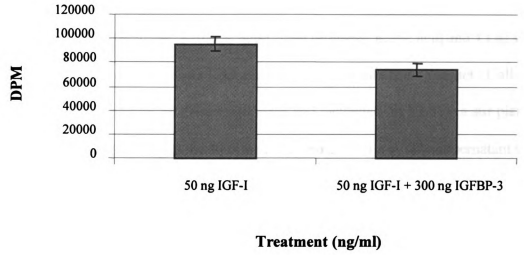


**Figure 18.** DNA synthesis of primary bovine mammary epithelial cells ([$^3$H] thymidine incorporation into DNA) in medium containing IGF-I alone (50 ng/ml) or with IGFBP-3 (300 ng/ml). IGFBP-3, added to equimolar concentrations of IGF-I, reduced DNA synthesis of primary bovine mammary epithelial cells ($P$=0.03). Results are reported as DPM (disintegrations per minute). Data are presented as LS means ± SEM.

Preparation of Rat Tail Collagen:

Rat tails, which had previously been frozen at -20°C, were placed in 70% ethanol for 30 min. Tails were dissected to collect collagen threads and threads were transferred to a petri dish containing 70% ethanol. Five grams of collagen threads were weighed out in a clean tared petri dish to make 1 liter of collagen. Collagen threads were incubated 30 min in 70% ethanol. In a sterile culture hood, 1 ml of glacial acetic acid and 1 l of sterile deionized $H_2O$ were placed into an Erlenmeyer flask that contained a magnet. Collagen threads were transferred to the Erlenmeyer flask and incubated for 72 h on a stir plate at 4°C. Collagen was centrifuged for 60 min at 4°C and 13,000 x *g*. The supernatant was poured into a sterile bottle and centrifuged for 45 min at 4°C and 35,000 x *g*. The supernatant from the second spin was filtered through 75-μm Nitex (Sefar America, Kansas City, MO) and placed in a sterile bottle.

Collagen (25 ml) was added to 4 ml neutralizing solution (10 ml 10X M199, 5 ml 0.33 *M* NaOH). The collagen solution had a cloudy appearance before it was plated, possibly due to protein degradation because of a pH change. The first collagen gel was firm and attached to plates. However, the second collagen gel containing primary cells did not attach to the first gel, but floated on top of medium. Collagen was diluted (800 ml collagen, 200 ml sterile deionized $H_2O$, 800 μl 25% acetic acid) and 25 ml of diluted collagen was added to 4 ml of neutralizing solution. Again, the first gel was firm but medium addition dissociated the second gel from the first gel. After these complications, it was decided that rat tail collagen purchased from Becton Dickinson (Type I, Bedford, MA) would be used for future experiments.

MAC-T Cell Culture Experiments:

MAC-T cells were grown on a collagen monolayer to achieve the following: 1) observe the proliferation and morphology of MAC-T cells in culture, 2) test the MAC-T cells' response to increasing doses of IGF-I, 3) determine if IGFBP-3 regulates the proliferation of epithelial cells dependently and independently of IGF-I, and 4) evaluate the regulation of IGFBP-3 synthesis by TGF-β in MAC-T cells.

MAC-T cells of a single passage were grown up for use in cell culture experiments. Basal medium (DMEM/F12, Invitrogen) containing HEPES (Invitrogen, Carlsbad, CA, 7.4 g) and $NaH_2CO_3$ (cell culture grade, Sigma, St. Louis, MO, 2.478 g), was titrated to a pH of 7.3 and sterile filtered. Basal medium also contained soybean trypsin inhibitor (Type I-S, Sigma, 1 mg/L), transferrin (apo-bovine, Sigma, 5 mg/L), glutathione (Sigma, mg/L), insulin (bovine, 0.01 mg/L), sodium selenite (Sigma, 5 mg/L), antibiotic/ antimycotic (Invitrogen, 10 ml/L), gentamicin (Invitrogen, 2.5 ml/L), and bovine serum albumin (BSA) (Invitrogen, 2.625 g/L). Cells were plated at 1.5 X $10^4$ cells per well in a 24-well plate. Cells were plated on top of a collagen monolayer (Type I, Becton Dickinson, Bedford, MA) in a mixture of BM supplemented with 10% fetal bovine serum (FBS, Invitrogen). After 24 hours in culture, medium was aspirated, cells were washed, and 1 ml of BM was applied to each well. For the IGFBP-3 experiments, all cells were pre-incubated for 24 h with 80 ng IGFBP-3/ml on day 3. After 72 h in culture, treatments were applied to MAC-T cells.

MAC-T cells were treated with increasing doses of IGFBP-3 (human recombinant, Upstate Biotechnology, Lake Placid, NY) at 0.5, 1, 10, 20, 40 and 80 ng/ml of medium. Equimolar concentrations of IGFBP-3 (40 ng/ml) and IGF-I (8 ng/ml) were

included to evaluate the effects of IGFBP-3 and IGF-I addition concurrently. Also, the effects of increasing doses of TGF-$\beta_1$ (human recombinant, Sigma) at 5, 10, 50, 100, 500 and 1000 pg/ml were tested on MAC-T cells. All experiments included BM, FBS at 10% concentration of medium and an IGF-I dose-response curve of 0.5, 1, 2, 4, 8 and 16 ng/ml of medium. After 24 h in treatment media, 1 μCi (2.2 x $10^6$ DPM) of [$^3$H] thymidine was added to each well and incubated for 2 h. Media was aspirated and a series of washes was conducted: Hanks' Buffered Salts (HBSS, Sigma, 1 ml, 2X), 90% ethanol (250 μl, 2X), Trichloroacetic acid (TCA, Sigma, 250 μl, 2X), and 90% ethanol (500 μl, 2X). Cells were then air-dried for 10 min and 250 μl of triton (0.1% NaOH) was added per well. Plates of cells were gently rocked for 30 min and a 100 μl sample was taken from each well and placed into scintillation vials. Scintillation fluid (5-ml) was added to each vial and [$^3$H] was quantified on a beta counter (TM Analytical Mark V 32 ARI). Calculated counts per minute (CPM) were adjusted to disintegrations per minute (DPM) assuming 40% efficiency of the counter.

Results of the IGFBP-3 experiment showed that IGFBP-3 did not inhibit DNA synthesis independently of IGF-I (Figure 18). An equimolar concentration of IGFBP-3 did inhibit IGF-I-stimulated DNA synthesis by 51% in MAC-T cells (Figure 19). TGF-$\beta_1$ experiments showed that at levels ≥ 10 pg/ml, TGF-$B_1$ reduced [$^3$H] thymidine incorporation in MAC-T cells as compared to treatment with BM (Figure 20).

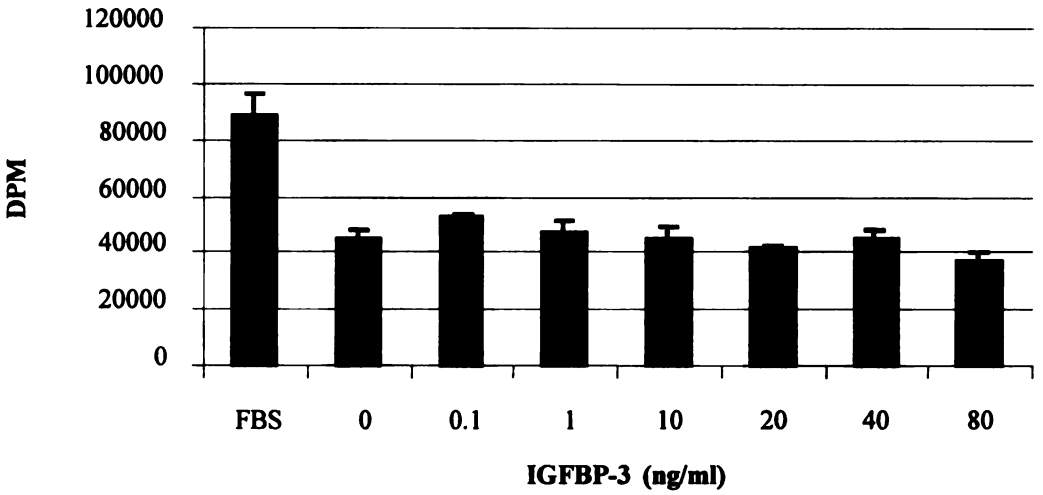


**Figure 19.** Effect of increasing doses of IGFBP-3 (0.1, 1, 10, 20, 40 and 80 ng/ml of media) on [$^3$H] thymidine incorporation into DNA of MAC-T cells. Results are reported as DPM (disintegrations per minute). Data are presented as means ± standard error of the mean. Basal medium (0) and 10% fetal bovine serum (FBS) were included as controls.

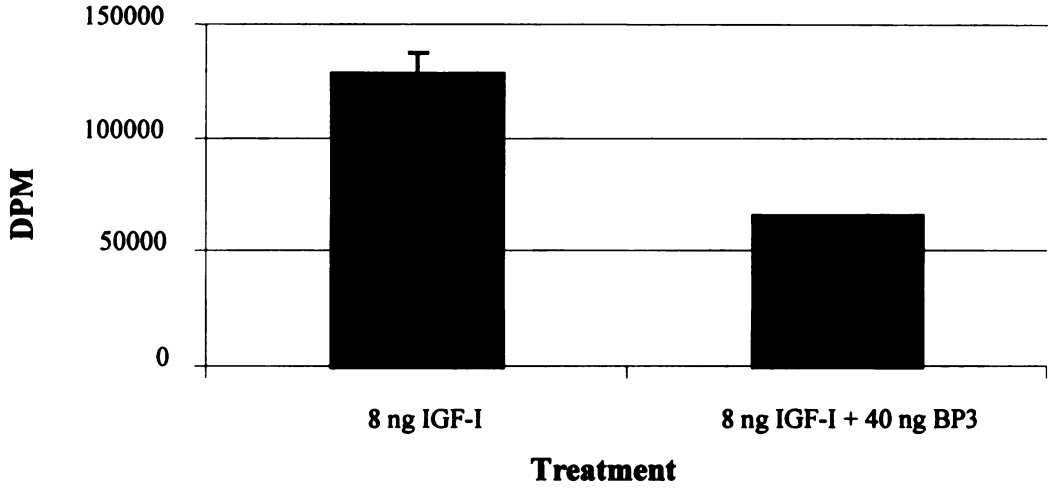


**Figure 20.** Inhibition of DNA synthesis of MAC-T cells in medium containing IGF-I alone (8 ng/ml) or with IGFBP-3 (40 ng/ml). IGFBP-3, added to equimolar concentrations of IGF-I, reduced DNA synthesis of MAC-T cells by 51%. Results are reported as DPM (disintegrations per minute). Data are presented as means ± standard error of the mean.

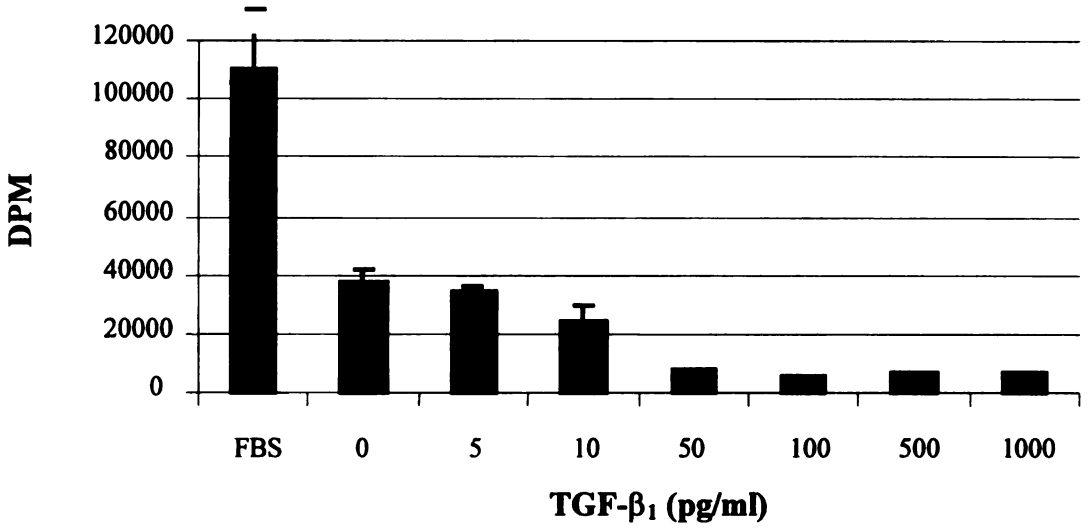


**Figure 21.** Effect of increasing doses of TGF-$\beta_1$ (5, 10, 50, 100, 500 and 1000 pg/ml of media) on [$^3$H] thymidine incorporation into DNA of MAC-T cells. Results are reported as DPM (disintegrations per minute). Data are presented as means ± standard error of the mean. Basal medium (0) and 10% fetal bovine serum (FBS) were included as controls.

Mitogenic Activity of Mammary Extracts from Prepubertal and Postpubertal Heifers:

From approximately 3 to 9 months of age, the rate of mammary gland growth is 3.5 times faster than the overall rate of body growth (Sinha and Tucker, 1969). This period of development is considered the allometric phase. Puberty and maturation of the ovaries signal the end of the allometric phase. From approximately the third estrus and until pregnancy, the growth of the mammary gland occurs at the same rate as body growth and is considered an isometric phase of development. Therefore, it would seem logical that the mitogenic activity within the mammary gland would be greater during the prepubertal period than the postpubertal period.

Mammary extracts were prepared as discussed previously (p. 30) from a prepubertal heifer (232 kg body weight at slaughter) and a postpubertal heifer (347 kg body weight at slaughter). Reproductive tracts were dissected to determine whether or not heifers had ovulated. MAC-T cells were plated as described above, except at 1 X $10^4$ cells per well. Mammary extracts were used as treatments at 1, 3 and 5% of BM on MAC-T cells to evaluate the mitogenic activity of the extracts after 72 h in BM. Sixteen hours after treatments were applied, 1 μCi (2.2 x $10^6$ DPM) of [$^3$H] thymidine was added to each well and incubated for 2 h. At 18 h, a [$^3$H] thymidine incorporation assay and a total DNA assay (p. 33) were performed on two separate culture dishes. In addition, the total DNA assay was performed on a third culture dish at 40 h after treatment addition.

Results showed that mammary extracts prepared from parenchymal tissue of a prepubertal heifer increased total DNA at 40 h more than extracts of tissue from a postpubertal heifer. This effect was significant at concentrations of 3% ($P$=0.03) and 5%

of medium ($P$<0.001) and tended to be significant at concentrations of 1% of medium ($P$=0.09).

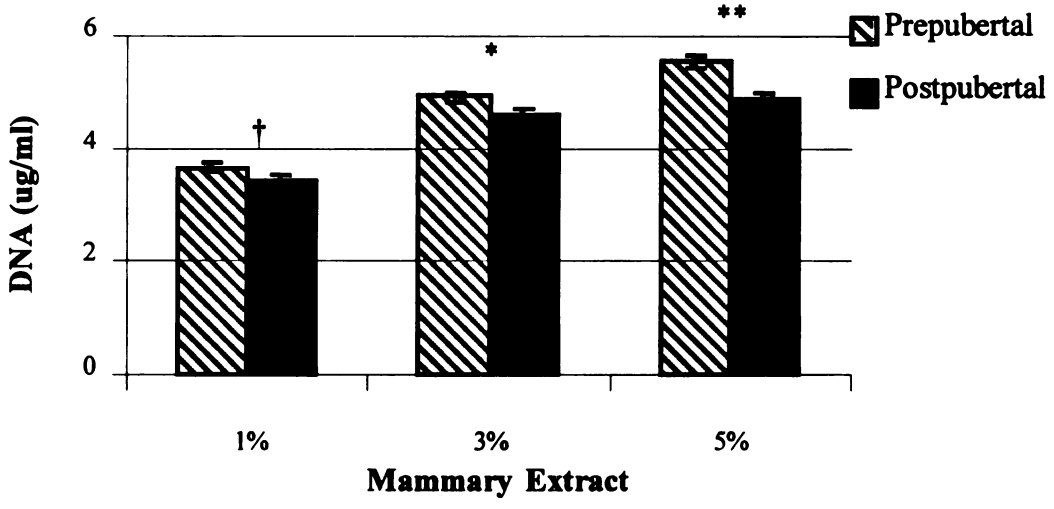


**Figure 22.** Effect of mammary extracts (at 1, 3 and 5% concentrations) from prepubertal and postpubertal heifers (n=1 heifer per stage) on total DNA of MAC-T cells after 40 h. Results are reported as micrograms of DNA per well. Data are presented as LS means ± SEM. Asterisks indicate treatment with extracts from a prepubertal heifer increased DNA more than those from a postpubertal heifer († $P$=0.09; * $P$=0.03; ** $P$<0.001).