EFFECTS OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I ON MILK PROTEIN GENE EXPRESSION AND NUTRIENT UPTAKE AND CELL PROLIFERATION IN CLONAL BOVINE MAMMARY EPITHELIAL CELLS

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ABSTRACT

The overall objective of this research was to further understand the mechanism by which growth hormone (GH) stimulates milk production in cattle. Three studies were conducted toward this objective. In the first study, the effects of GH and insulin-like growth factor-I (IGF-I), a major mediator of GH action in vivo, on cell proliferation, nutrient transport, and milk protein gene expression in bovine mammary epithelial cell line MAC-T cells were determined. GH increased ($P < 0.01$) expression of four major milk protein genes in MAC-T cells transfected with GHR expression plasmid. Cotransfection analyses indicated that GH also stimulated ($P < 0.01$) luciferase reporter gene expression from the promoters of the four milk protein genes in MAC-T cells. These findings together with the fact that GHR mRNA and protein are expressed in the epithelial cells of the bovine mammary gland suggest that GH may directly stimulate milk protein gene expression in the mammary gland. This study also showed that IGF-I increased the proliferation ($P < 0.01$) and amino acid transport ($P < 0.05$) in MAC-T cells. Because GH is known to stimulate IGF-I production in animals, IGF-I-mediated mammary epithelial cell proliferation and amino acid uptake may be additional mechanisms by which GH increases milk

production in cattle. In the second study, the role of connective tissue growth factor (CTGF) on IGF-I-stimulated proliferation of MAC-T cells was investigated. A microarray analysis revealed that IGF-I decreased CTGF mRNA expression in MAC-T cells ($P < 0.01$). This effect of IGF-I was further found to be mediated through the PI-3 kinase/Akt signaling pathway from the IGF-I receptor (IGF-IR). CTGF alone stimulated MAC-T cell proliferation ($P < 0.01$). However, together with IGF-I, CTGF attenuated the proliferating effect of IGF-I on MAC-T cells, and this attenuation was reversed by additional IGF-I. Therefore, IGF-I inhibition of CTGF expression may benefit IGF-I stimulation of MAC-T cell proliferation. CTGF had no effect on IGF-I-induced phosphorylation of IGF-IR or total IGF-IR expression in MAC-T cells, suggesting that CTGF may attenuate IGF-I stimulation of MAC-T cell proliferation through a postreceptor inhibition of the IGF-IR signaling pathway. In the third study, whether a milk yield-associated T/A polymorphism in exon 8 of the bovine GHR gene affected GHR signaling was determined. It was found that the two corresponding GHR variants did not differ in mediating GH induction of gene expression, suggesting that the two GHR variants are not functionally different and hence are unlikely to mediate different effects of GH on milk production. In summary, the results of this dissertation research suggest that GH may directly stimulate milk protein gene expression and indirectly stimulate mammary epithelial cell proliferation and amino acid uptake through IGF-I, thereby stimulating milk production in cattle. The results also suggest that IGF-I stimulation of mammary epithelia cell proliferation may involve an inhibition of CTGF expression in the cells.

Key Words: Growth hormone, Insulin-like growth factor I, Mammary epithelial cells, Milk production, Proliferation

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Introduction

Growth hormone (GH or somatotropin) plays a key role in postnatal development and growth in animals. GH exerts its actions by direct effects on target organs and by stimulating the production of insulin-like growth factor I (IGF-I). Many of the effects of GH are believed to be mediated by IGF-1. One of the important effects of GH in cattle is on milk production. Administration of GH to lactating cows increases milk yield without changing milk composition. Theoretically, GH may stimulate lactation by either direct action or by stimulating IGF-I production. The direct effect of GH on mammary gland can be mediated by two possible mechanisms: GH directly acts on the milk-producing epithelial cells, or GH acts on the stromal tissue from which factors including IGF-I cause the epithelial cells to secrete more milk. The studies reported in this dissertation were conducted to further understand the contributions of direct actions of GH and IGF-I-mediated actions of GH on bovine mammary epithelial cells to increase milk production. The research was started with determining the effects of GH and IGF-I on cell proliferation, nutrient uptake, and milk protein gene expression in clonal bovine mammary epithelial cells. The second part of this research was conducted to determine the mechanism by which IGF-I stimulates bovine mammary epithelial cell proliferation, in particular, the role of connective tissue growth fator (CTGF) in this process. The last portion of this research was conducted to understand whether a milk yield-associated single nucleotide polymorphism in the GHR gene mediates different effects of GH on milk production.

Chapter I Review of Literature

Introduction

Growth hormone (GH), also called somatotropin, is a polypeptide hormone synthesized and secreted by the anterior pituitary. It plays a key role in the control of postnatal development and growth in mammals (Butler and Le Roith, 2001). One well-established biological action of GH is stimulation of insulin-like growth factor I (IGF-I) production (Argetsinger and Carter-Su, 1996). It is believed that many of the effects of GH are mediated by IGF-I.

One of the important effects of GH and IGF-I in ruminants is on mammary gland development and lactation. During mammary gland development, GH plays important roles in ductal elongation and differentiation of ductal epithelia into terminal end buds (Coleman et al., 1988). Administration of GH increases milk production in cows by about 15% (Knight, 1992), but it does not alter the gross composition of milk (i.e., milk fat, protein, and lactose concentrations; Bauman, 1992). In this review, I will attempt to summarize current understanding of the mechanism by which GH and IGF-I regulate mammary gland development and lactation. The potential of the mammary gland to synthesize milk is determined by many hormones in addition to GH and IGF-I. Therefore, this review will also touch on the roles of other hormones in regulation of milk production.

The GH-IGF axis

The GH-IGF axis is intimately involved in the integration of a lot of signals that regulate systemic growth and metabolism throughout fetal and postnatal development. The GH-IGF axis includes GH, the GH receptor (GHR), the GH binding proteins (GHBP), IGF-I, IGF-II, IGF receptors and the six IGF binding proteins (IGFBP). Although GH is produced mainly by the

anterior pituitary, its receptor is expressed in many tissues (Mertani and Morel, 1995), indicating this hormone has widespread effects. IGF are produced by many tissues during both fetal and postnatal life, and IGF-I receptor (IGF-IR) is also expressed in most tissues during at least one period of development (Schuller et al., 1993). So both GH and IGF-I have important effects on the development and physiology of the organism. The actions of GH are initiated by binding to GHR on the cell membrane of various target tissues. Many of these receptors activate tyrosine kinases of the Janus Kinase (JAK) family, in particular JAK2. Upon GH binding to its receptor, JAK2 associates with the receptor, leading to autophosphorylation of the kinase and phosphorylation of the intracellular domain of the receptor. Then members of the signal transducer and activator of transcription (STAT) family, in particular STAT5, become phosphorylated. The phosphorylated STAT5 in turn activates gene expression, including production of IGF-I (Argetsinger et al., 1993). Besides STAT, activated JAK2 can also interact with other signaling molecules, such as the insulin receptor substrate (IRS)-1 and IRS-2, which initiate the PI-3 kinase pathway, and the Ras/Raf/MAPK pathway (Carter-Su and Smit, 1998). The GH-GHR signaling pathway is shown in Figure 1.1.

GH and GHR

GH is a polypeptide hormone synthesized and secreted by acidophilic or somatotropic cells of the anterior pituitary gland. It was first discovered by Evans and Long in 1921 in the extracts of the anterior pituitary which can promote growth and maturation (Evans and Long, 1921). The bovine GH gene contains approximately 1,793 nucleotides and consists of 5 exons (Gordon et al., 1983). The bovine GH protein has a molecular mass of about 22,000 Daltons. Bovine GH has four variants, generated by differential cleavage of the signal peptide. These

variants have either a leucine or valine substitution at position 127 and an alanine (191-amino acid sequence) or a phenylalanine (190-amino acid sequence) at the NH_2 terminus (Wood et al., 1989). GH binds two GHR molecules and induces signal transduction through receptor dimerization (Sundstrom et al., 1996).

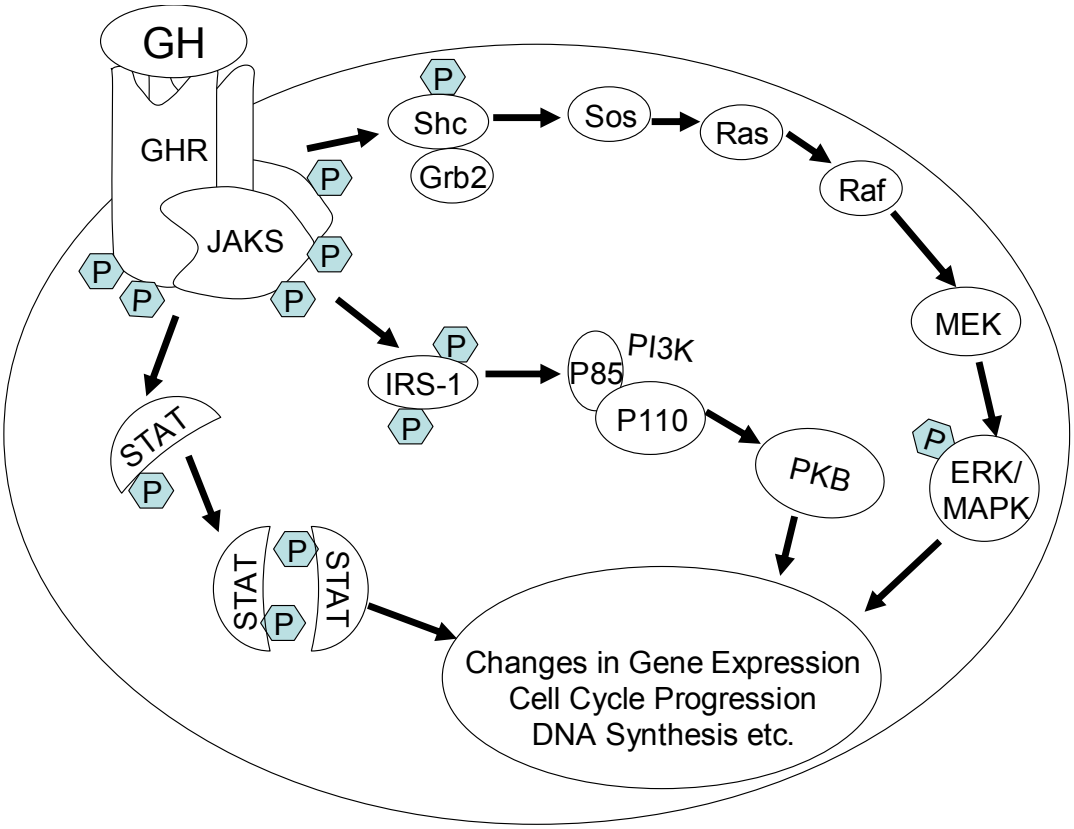


Figure 1.1. Intracellular signaling cascade for the GHR. Dimerization of GHR following GH binding initiates phosphorylation and activation of Janus Kinase (JAKs). Activated JAKs catalyze the following three primary tyrosine phosphorylation events: (1) Signal transducer and activator of transcription (STAT) pathway lead to a direct stimulation of target gene transcription; (2) Activation of insulin receptor substrate PI-3K, including P85 and P110, by tyrosine phosphorylation of insulin receptor substrate (IRS)-1, and the following activation of PI-3K-protein kinase B (PKB) may be important for the metabolic, differentiative, and proliferative effect of GH; (3) Activation of the SHC-Grb2-Ras-Raf-MEK-ERK pathway resultes in the activation of mitogen-activated protein kinase (MAPK), which contributes to cell proliferation. (Adopted from Carter-Su and Smit, 1998).

GH releasing hormone (GHRH) from the arcuate nucleus of the hypothalamus and ghrelin from stomach can promote the release of GH, and somatostatin from the periventricular nucleus of the hypothalamus can inhibit it (Pombo et al., 2001). GH plays a key role in the control of postnatal development and growth in mammals, either directly or indirectly via IGF-I. The hypothalamus-pituitary GH-IGF-I axis is shown in Figure 1.2.

The GHR gene has 9 exons that encode the receptor and several additional exons that form the 5' untranslated region (Godowski et al., 1989). The GHR protein consists of an extracellular domain, a single transmembrane domain, and a cytoplasmic domain (Godowski et al., 1989).

JAK2

The Janus tyrosine kinases (JAK) are the predominant non-receptor tyrosine kinases required for the initiation of GH signal transduction (Herrington and Carter-Su, 2001; Schwartz et al., 2002) Among the four known JAK family tyrosine kinases, JAK2 is the most predominant tyrosine kinase that is activated and phosphorylated in GH stimulated cells (Herrington and Carter-Su, 2001; Zhu et al., 2001). Human JAK2 gene encodes a 1,132-amino acid protein that shares 95% sequence similarity to rat and pig JAK2 (Saltzman et al., 1998). Highest expression of JAK2 was found in spleen, peripheral blood leukocytes, and testis (Saltzman et al., 1998). JAK2 is suggested to mediate signals through a variety of single-chain receptors for ligands such as prolactin (PRL), GH, erythropoietin (EPO), and thrombopoietin as well as to the multichain interleukin 3 receptor family (e.g., interleukin 3 receptor and granulocyte-macrophage colony-stimulating factor receptor) and members of the gp130 receptor family (Kisseleva et al., 2002).

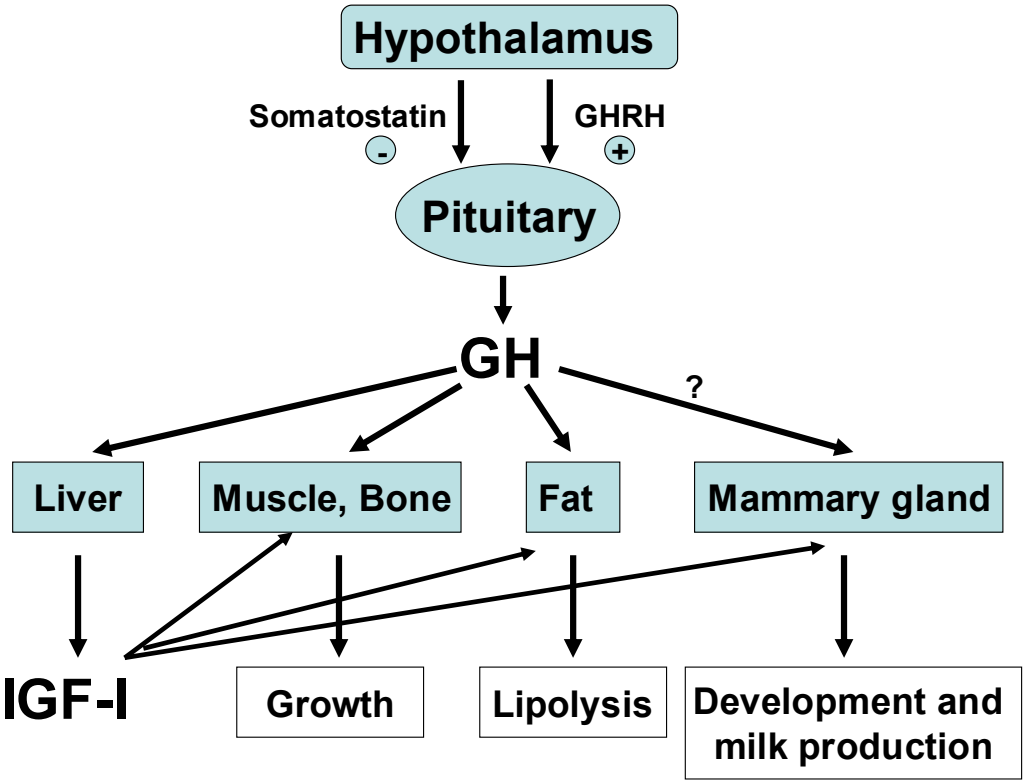


Figure 1.2. Hypothalamic-pituitary GH-IGF-I axis. Pituitary GH production and secretion is regulated by the hypothalamic hormones somatostatin and GH releasing hormone (GHRH). GH has direct effect on muscle, bone, fat, and mammary gland for growth, lipolysis, mammary gland development and milk production. GH also has indirect effect on those organs by stimulating liver to secrete IGF-I.

JAK2 interacts with the membrane proximal proline-rich Box 1 region of GHR (Herrington and Carter-Su, 2001). JAK2 phosphorylates STAT1, STAT2, STAT3, STAT4, and STAT5, but not STAT6 (Saltzman et al., 1998).

JAK2-deficient mammary epithelial cells showed a reduced proliferation index in animals treated with estrogen and progesterone, and the lack of JAK2 impaired the specification of secretory epithelial cells (Shillingford et al., 2002). JAK2-deficient females were unable to

lactate as a result of impaired alveolar formation, and JAK2 deficiency also impaired pregnancy-induced branching morphogenesis (Wagner et al., 2004). Using a conditional knockout approach, signaling through JAK2 was suggested to control not only the expression of the Cyclin D1 mRNA but also the accumulation of Cyclin D1 protein in the nucleus by inhibiting signal transducers that mediate the phosphorylation and nuclear export of Cyclin D1 in proliferating mammary epithelial cells (Sakamoto et al., 2007).

STAT5

STAT5 was firstly identified as mammary gland factor (MGF) in nuclear extracts from lactating mice (Wakao et al., 1994; Wakao et al., 1992). It is essential for mammary gland development and milk protein gene expression (Miyoshi et al., 2001; Shillingford et al., 2002). Expression, activation by phosphorylation, and nuclear translocation of STAT5 are tightly linked to mammary differentiation (Kazansky et al., 1995; Liu et al., 1996). STAT5 is expressed at all stages of mammary development, with a minor induction during the final stages of gestation and the onset of lactation (Kazansky et al., 1995; Liu et al., 1995). During pregnancy, a marked induction in STAT5 phosphorylation occurs, peaking in late pregnancy and early lactation and declining through involution (Liu et al., 1995).

STAT5 has two variants, STAT5a and STAT5b, which have 96% similarity in peptide sequence. The difference between STAT5a and STAT5b is within their carboxyl termini (Liu et al., 1995). Both of them are required for normal milk gene expression and lactation at parturition, (Liu et al., 1996; Teglund et al., 1998), but targeted gene disruption in mice yields distinctive phenotypes. STAT5a is essential for prolactin-induced terminal differentiation of the mammary gland during pregnancy and lactation (Kazansky et al., 1995; Liu et al., 1997). STAT5a-deficient mice exhibited decreased lobuloalveolar development but developed normally and were unable

to lactate after their first gestation period (Liu et al., 1997). STAT5b is an important molecule involved in GH-signaling and disruption of STAT5b results in severe growth retardation of male mice (Udy et al., 1997; Horvath, 2000). STAT5b null mice also demonstrated decreased lobuloalveolar development, which was less severe than that of the STAT5a null mice (Teglund et al., 1998). Overexpressing STAT5a in the mammary glands of transgenic mice stimulated lobuloalveolar development and delayed apoptosis (Liu et al., 1995; Iavnilovitch et al., 2002).

STAT5 can be activated by PRL, GH, and EGF in mammary gland. PRL activates STAT5 only in the epithelium; GH and EGF activate STAT5 preferentially in the stroma (Gallego et al., 2001). STAT5 activity is increased during pregnancy and lactation but rapidly decreased after initiation of involution (Groner and Hennighausen, 2000).

IGF-I

IGF-I is an endocrine factor, which belongs to a growth factor family involved in the regulation of normal and malignant cell growth, differentiation and development (Martin and Stoica, 2002). IGF-I is a polypeptide hormone with similar molecule structure to insulin. The molecular weight of human IGF-I is 7649 Daltons and consists of 70 amino acids in a single chain with three disulfide bridges (Rinderknecht and Humbel, 1978). IGF-I is produced primarily by the liver as an endocrine hormone (Combes et al., 1997). IGF-I is also produced by many other tissues and functions in a paracrine/autocrine fashion to stimulate cell growth (Yee, 1994). The production of IGF-I is stimulated by GH and can be retarded by undernutrition (Gautsch et al., 1998). Approximately 98% of IGF-I is bound to one of the six IGFBPs (Yakar et al., 1999). IGF-I plays an important role in prepubertal growth and continues to have anabolic effects in adults by binding to specific receptors expressed on many cell types in many tissues.

Most of the biological activities of IGF-I are mediated through IGF-IR which can bind both IGF-I and IGF-II with high affinity (Zhu and Kahn, 1997). IGF-IR is a transmembrane tyrosine kinase with similar structure to the insulin receptor (Ullrich et al., 1986). IGF-IR is composed of two extracellular α subunits and two intracellular β subunits. The α subunits bind IGF-IR ligands, while the β subunits transmit ligand-induced signals (Mauro et al., 2003).

The binding of IGF-I to the receptor induces its intrinsic tyrosine kinase activity, resulting in the recruitment and phosphorylation of multiple substrates, such as insulin receptor substrate 1 (IRS-1) and src- and collagen-homology (SHC) protein (Chow et al., 1998). The two main transduction pathways are the phosphatidylinositol 3-kinase (PI-3K) pathway and the mitogen-activated protein kinase (MAPK) pathway (Cheatham et al., 1994). IRS-1 stimulates PI-3K, which then transmits signal to the serine/threonine kinase Akt. Activated Akt phosphorylates and blocks a variety of pro-apoptotic proteins, including Bcl-2-associated death promoter (BAD), Caspase 9, forkhead transcription factors, and the glycogen synthase kinase-3 β (GSK-3β) kinase. Furthermore, Akt induces the expression of antiapoptotic proteins, such as Bcl-2 (Brazil et al., 2002). Atk also activates mTOR and p70S6 kinase, which controls cell cycle and protein synthesis (Harada et al., 2001). Other mitogenic/survival IGF-IR pathways involve STATs that are phosphorylated and activated by IGF-I through JAK1/2 and PI-3K/Akt pathways (Zong et al., 1998; Zong et al., 2000). IGF-IR can also induce proliferation or prevent cell death via the SHC/Ras/Raf/ERK/MAPK pathway (Prisco et al., 1999). The MAPK pathway is involved in proliferation and differentiation, while the PI3K pathway plays a major role in metabolic functions, mainly via the activation of the Akt cascade (O'Connor, 2003). Activation of Akt stimulates glycogen synthesis, protein synthesis, cell survival, inhibition of lipolysis, and glucose

uptake. This pathway is also considered to be important for adipogenesis (Damm and Turner, 1958). IGF-I activated signaling pathways via IGF-IR are shown in Figure 1.3.

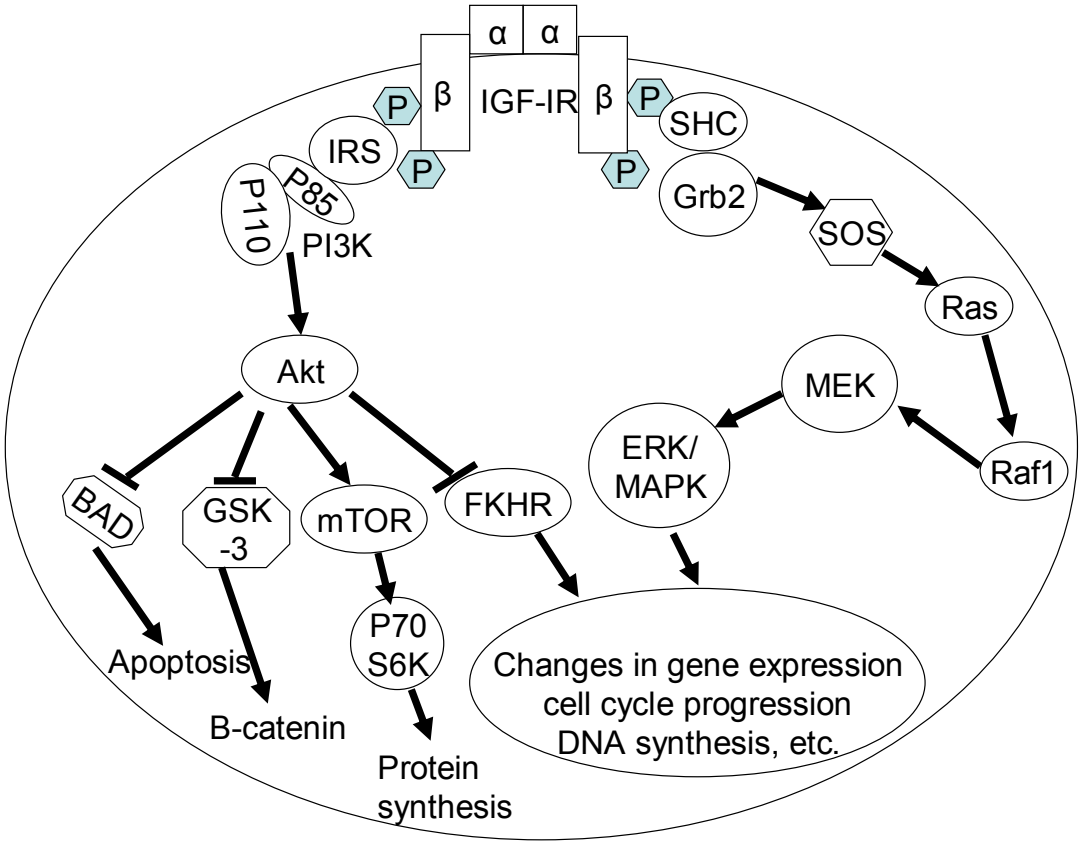


Figure 1.3. Signaling pathways activated by the IGF-IR. Binding of IGF-I to IGF-IR results in recruitment of Insulin Receptor Substrate (IRS) or src- and collagen-homology (SHC) and activate phosphatidylinositol 3-kinase (PI-3K) or mitogen-activated protein kinase (MAPK) pathway. The phosphorylation of IRS/P85 activates Akt, which can block a variety of pro-apoptotic proteins including Bcl-2-associated death promoter (BAD), forkhead transcription factors (FKHR), and the glycogen synthase kinase-3 β (GSK-3β) kinase. Atk also activates mammalian target of rapamycin (mTOR) and p70S6 kinase, which controls protein synthesis. IGF-IR can also induce proliferation or prevent cell death via the SHC-SOS-Ras-Raf-MEK-ERK/MAPK pathway. (Redrawn from O'Connor, 2003.)

Roles of GH-IGF axis

GH-IGF axis and cell proliferation and apoptosis

GHR mRNA and protein were detected in bovine mammary gland tissue (Glimm et al., 1990; Sinowatz et al., 2000; Plath-Gabler et al., 2001). In lactating cows, bovine GH induced proliferation of mammary parenchymal and growth of epithelial cells (Knight et al., 1990), and also increased the cell renewal in the mammary gland (Capuco et al., 2001). In vitro study showed GH had no effect on proliferation of bovine mammary epithelial cells or bovine mammary explants (Akers et al., 2005). Both IGF-I and IGF-IR mRNA expression were detected in mammary tissue of heifers during pregnancy, lactogenesis and galactopoiesis (Plath-Gabler et al., 2001). IGF-I can stimulate proliferation of both the bovine mammary epithelial cell line MAC-T and primary mammary epithelial cells (pMEC) (Silva et al., 2002; Thorn et al., 2006). The DNA synthesis of MAC-T cells increased linearly with increasing concentrations of IGF-I in the media (Silva et al., 2002).

IGF-I can stimulate cell proliferation by regulation of cell cycle progression (Evan and Vousden, 2001). Studies in fibroblasts demonstrated that entry from G_0 into G_1 and overcoming the G_1-S checkpoint are required for the recruitment of quiescent cells into the cell cycle (Stiles et al., 1979). The cyclin-dependent protein kinases regulate cell cycle progression through both the G_1-S and G_2-M cell cycle checkpoints (Kamalati et al., 1998). Regulation of cyclin expression plays an important role in developing mammary tissue. Genetic deletion of cyclin D1 selectively affects mammary gland development (Sicinski et al., 1995). Overexpression of cyclin D1 in mammary epithelial cells in vitro accelerates cell cycle progression by decreasing the length of G_1 and reducing the requirement for growth factors (Kamalati et al., 1998). In addition, overexpression of either cyclin D1 or cyclin E in mammary glands of transgenic mice results in

epithelial hyperplasia and carcinoma (Wang et al., 1994; Bortner and Rosenberg, 1997). These results demonstrate that regulation of cyclin levels is a critical point in regulating cell cycle progression and proliferation of mammary epithelial cells in both normal and abnormal growth of this tissue.

IGF-I can regulate cell cycle progression by inducing cyclin expression (Kenney and Rowitch, 2000). IGF-I induces cyclin D1 expression in many tumor cell lines including breast cancer cells (Dupont et al., 2000). Expression of antisense cyclin D1 abolished the ability of IGF-I to stimulate proliferation of human pancreatic cells (Kornmann et al., 1998). IGF-I induction of cyclin D1 expression can occur through transcriptional regulation (Furlanetto et al., 1994; Altucci et al., 1996) and through stabilization of cyclin D1 mRNA (Dufourny et al., 2000). IGF-I also induces cyclin E in human breast cancer cells, and estrogen can enhance both cyclin D1 and E expression by IGF-I induction in these cells (Dupont et al., 2000; Dupont and Le Roith, 2001). IGF-I is required to induce cyclins essential for late G_1, S and G_2 progression and IGF-I is also essential for EGF-mediated progression of mammary epithelial cells into S phase in the intact mammary gland (Stull et al., 2002). In ventricular myocytes, IGF-I induces cyclins D1 and E in addition to cyclins A2 and B1, cyclins essential for progression through S and G_2 phases of the cell cycle, respectively (Reiss et al., 1997). IGF-I can also induce cyclin A2 expression in skeletal muscle satellite cells (Chakravarthy et al., 2000).

IGF-I is a potent stimulator of cell survival and an inhibitor to apoptosis in many cell types (Barres et al., 1993). Study of transgenic mice that overexpress des(1-3)hIGF-I (truncated analogue of IGF-I and lack a few of the teminal amino acdis, low affinity to IGFBP) within the mammary gland during the pregnancy and lactation suggested that IGF-I inhibited apoptosis and expression of apoptosis-associated genes, which were known to be activated by the transcription

factor AP-1 (Hadsell et al., 2000). Besides AP-1, a number of potential regulator proteins were found to be involved with mammary involution, including c-myc (Strange et al., 1992), NF-1 (Furlong et al., 1996), Bcl-2 (Heermeier et al., 1996) and STAT (Li et al., 1997) families. Inhibition of apoptosis in HC11 mammary cells by insulin caused increased abundance of Bcl-2 (Merlo et al., 1995). The mechanisms of IGF-I inhibition of apoptosis were studied in other cell models. IGF-I inhibition of apoptosis in PCL2 cells is associated with increased Bcl-x mRNA and increased bcl-x_L protein (Parrizas and LeRoith, 1997). Activation of PI-3K and a serine/threonine kinase termed Akt/PKB was involved in IGF-I-dependent protection of fibroblasts from apoptosis (Kulik et al., 1997). Activation of Akt also inhibited caspase activation (Ahmed et al., 1997) and caused activation of the Bcl-2 family member, BAD (del Peso et al., 1997). Inactivation of BAD by this phosphorylation prevented it from heterodimerizing with Bcl-2 (Yang et al., 1995), thereby providing a potential mechanism for the inhibition of apoptosis.

The IGFBP have been shown to play a major role in regulating the survival effects of IGF-I. Secretion of IGFBP-5 was involved in the initial stages of apoptosis, inhibiting IGF-1-mediated cell survival (Tonner et al., 1997). In normal physiology, upregulation of IGFBP-5 was associated with initiation of apoptosis during involution of the mammary gland (Flint et al., 2000).

GH-IGF axis and nutrient transport and metabolism

Nutrient provision to the lactating mammary gland involves three factors: blood nutrient concentration, blood flow, and cellular uptake. Administration of GH to dairy cows increases milk yield by 10-15%. This increased milk production is associated with increased requirement for milk precursors. Since treatment of GH for 1-3 weeks increases milk yield without increasing

feed intake (Bauman and Currie, 1980), one of the hypothesis of GH action is nutrient repartitioning. After GH treatment, increased milk yield requires the repartition of nutrients to accommodate the increase in rates of milk synthesis. This view is supported by many studies: GH increases glucose uptake in the mammary gland (Davis et al., 1988; Fullerton et al., 1989), while it decreases glucose uptake in skeletal muscle and adipose tissues (Davidson, 1987; McDowell et al., 1987). Because in lactating cows, GH treatment does not alter plasma glucose concentration (Bauman et al., 1988), the increased glucose availability in the mammary gland may be due to the increased blood flow rate to the mammary gland (McDowell et al., 1987) or the decreased glucose usage in other tissues.

Mammary blood flow (MBF) is assumed to be an important factor in regulation of nutrient supply to the mammary gland and thus milk synthesis (Davis et al., 1979), and the close correlation between MBF and milk yield in lactating ruminants indicates that local mechanisms are involved in the regulation of MBF to the mammary gland. It is well-known that GH has a marked stimulating effect on MBF in cows and goats (Mepham et al., 1984; Davis et al., 1988) which may be associated with the role for prostaglandin I_2 (PGI2) and prostaglandin E_2 (PGE2) in local mammary blood flow regulation during lactation. Increased mammary uptake of these two prostanoids may be involved in the mammary blood flow response to GH (Nielsen et al., 2004).

GH-IGF axis in amino acids transport and metabolism — Amino acid (AA) uptake capacity of the alveolar cells and the amount/activity of protein translation components limit the rate of milk protein synthesis in the high-producing dairy cow (Maas et al., 1997; Pohl et al., 2005). GH has acute stimulatory effects on AA transport and protein synthesis in a variety of tissues. Growth hormone is able to stimulate AA transport and utilization by the perfused rat

liver. Intracellular concentrations of seven amino acids, including threonine, serine, proline, glycine, alanine, lysine, and arginine, are increased significantly in livers perfused with medium containing growth hormone (Jefferson et al., 1975). But whether GH also stimulates AA transport in the bovine mammary gland is unknown.

IGF can increase the cellular uptake of amino acids and stimulate protein synthesis (Dimitriadis et al., 1992). The study in MCF7 cells overexpressing IGF indicated that IGF-I upregulated the expression of genes which were involved in amino acid transport and metabolism, protein biosynthesis and stability, including SLC7A11, SLC7A5 and SLC1A4 (Pacher et al., 2007). SLC7A11 and SLC7A5 both bind to the activating peptide SLC3A2, which was also upregulated by IGF-I and IGF-II (Verrey et al., 2004). SLC7A5 is a transporter for large neutral amino acids, particularly branched and aromatic amino acids such as Gln, His, Ile, Leu, Met, Phe, Trp, and Tyr (Wolf et al., 1996; Yanagida et al., 2001). SLC7A5 is expressed in most tumors and tumor cell lines, indicating an important role in cancer. SLC7A11 primarily exchanges extracellular anionic cysteine for intracellular glutamate (Verrey, 2003; Verrey et al., 2004). The SLC1A4 transporter exchanges Na^+ for small neutral amino acids such as Ala, Ser, Cys and Thr (Kanai and Hediger, 2004). In addition to these transmembrane proteins required for amino acid import, key factors involved in amino acid biosynthesis and metabolism are upregulated by IGF: methionyl-tRNA synthetase (MARS), the only mammalian methionyl-tRNA synthetase and ASNS (asparagine synthetase) and ASS (argininosuccinate synthetase), the essential precursor for the synthesis of pyrimidine bases (Berg et al., 2002). Overexpression of IGF-I may also increase amino acid transport in porcine lactating mammary tissue (Gronlund et al., 2003).

GH-IGF axis in glucose transport and metabolism—In milk production, glucose is the primary substrate for synthesis of lactose which controls milk volume by maintenance of milk osmolarity. Lactose synthesis and hence overall milk yield has been found to correlate positively with glucose uptake in the mammary gland in ruminants (Nielsen and Jakobsen, 1993).

Glucose transport across the plasma membrane of mammary epithelial cells is mediated by a family of tissue-specific facilitative glucose transporters (GLUT) (Kahn and Pedersen, 1992). Currently, thirteen members of GLUT have been identified; only GLUT1, GLUT3, GLUT4 and GLUT8 have been cloned in bovine tissues. Before conception, the two isoforms GLUT1 and GLUT4 are present in rat mammary tissue (Burnol et al., 1990). However, during pregnancy, expression of the insulin-dependent GLUT4 decreases (Bell et al., 1990) and virtually disappears during lactation, whereas the expression of GLUT1 localized in the basolateral membrane of mammary epithelial cells increases (Burnol et al., 1990; Camps et al., 1994). This agrees with the reported dominance of GLUT1 mRNA expression in lactating bovine mammary tissue (Zhao et al., 1993). Treatment of GH did not affect the overall mammary glucose uptake despite the fact that GH can increase mammary blood flow and hence glucose supply, indicating that glucose supply is not a major determinant of mammary uptake (Nielsen et al., 2001). GH has no effect on glucose transporter GLUT1 mRNA and protein expression (Zhao et al., 1996), which is the major glucose transporter expressed in the mammary gland (Madon et al., 1990). Mammary glucose uptake was also found to be unrelated to long term, as well as acute, changes in glucose supply (Zhao et al., 1996). So it is very possible that GH did not alter mammary glucose transporter capability, but changed the intracellular glucose metabolism favoring lactose synthesis (Nielsen et al., 2001).

GH-IGF axis in fatty acid transport and metabolism—The effects of GH on lipid metabolism are well-documented in pigs and especially in adipose cells. Exogenous GH treatment consistently decreased lipid deposition in pigs regardless of sex, genotype or age (Louveau and Gondret, 2004). The most striking effect of GH administration in humans is a significant increase in free fatty acids after 1-2 h (Moller et al., 1990), reflecting stimulation of lipolysis and ketogenesis. In vitro studies indicated that the lipolytic actions of GH may involve stimulation of gene expression after binding of the GH receptor and activation of intracellular signaling (Argetsinger et al., 1993), including activation of adenylyl cyclase and stimulation of cAMP production, triggering the hormone-sensitive lipase (Yip and Goodman, 1999). Co-infusion of GH with nicotinic acid (antilipolytic agent) abolished the effects of GH on glucose tolerance (Davidson, 1987). Nicotinic acid derivative, which can inhibit lipolysis, can also reduce the ability of GH to decrease insulin sensitivity (Nielsen et al., 2001). The other opinion considered the reduction in lipid deposition results primarily from a decrease in lipogenesis rather than from an increase in lipolysis, and involves a decrease in adipocyte insulin sensitivity. This decreased insulin sensitivity leads to a marked decrease in insulin-regulated events such as glucose transport and lipogenic enzyme activities as assessed in vivo and in vitro (Dunshea et al., 1992; Harris et al., 1993; Liu et al., 1994; Wang et al., 1999). The finding that GH decreases the activity of lipoprotein lipase (LPL) in culture of adipose tissue from neonatal and growing pigs indicates that GH also regulates lipid uptake (Wang et al., 1999). The effect of GH on the regulation of fatty acid synthase (FAS) is especially well-documented. Both activity and mRNA levels of FAS (Harris et al., 1993; Liu et al., 1994) are decreased in adipose tissue of GH-treated pigs. In primary culture of mature porcine adipocytes, GH antagonizes the stimulatory effect of insulin on FAS expression (Louveau and Gondret, 2004). These latter data associated with data

obtained in various mammalian species have led to the conclusion that FAS is controlled primarily at the level of gene transcription (Semenkovich, 1997; Yin et al., 1998). Inhibition of GH and PRL action decreased the mRNA expressions of acetyl CoA carboxylase, fatty acid synthase, and lipoprotein lipase in the mammary gland of rat, which could also be prevented by treatment with GH, but not IGF-I or IGF-II (Barber et al., 1992).

Much less is known about the effect of IGF-I on free fatty acid (FFA) metabolism. Increased lipolysis in adipose tissue from GH-deficient humans (Hussain et al., 1994) and decreased LPL activity in adipose tissue of hypophysectomized rats (Oscarsson et al., 1999) have been reported after IGF-I treatment. In hypophysectomized rats, IGF-I treatment reduced basal and insulin-stimulated glucose incorporation into lipids; however, the effect of IGF-I seems to involve decreased serum insulin levels rather than direct effects (Frick et al., 2000). In vitro, the effect of IGF-I on adipocytes is observed only at high hormone concentrations and is believed to be mediated through a cross-reaction with the insulin receptor, because IGF-I receptors are not present on fat cells (Le Roith, 1997).

GH-IGF axis and mammary development and lactation

Process of mammary development

To synthesize milk, the mammary gland undergoes dramatic changes in morphology and function during development, puberty and adult life. Although the rudimentary ductal structures appear in utero, the development of the mammary gland largely occurs postnatally (Coleman et al., 1988). During puberty, elongation and bifurcation of these primary ducts occur (Borst and Mahoney, 1982). Mammary epithelial cells proliferate, invade the surrounding stromal extracellular matrix (ECM) during the first part of pregnancy, and form lobulo-alveolar structures that prepare the gland for lactation (Traurig, 1967; Borst and Mahoney, 1982;

Zwierzchowski et al., 1984). During late pregnancy, prior to parturition, breast epithelial cells stop proliferation and invasion, and functionally differentiate into cells that express and secrete milk proteins. Throughout lactation, epithelial cells continue to express milk proteins (Traurig, 1967). After weaning, the mammary gland undergoes involution, characterized by degradation of ECM and epithelial cell death by apoptosis (Strange et al., 1992; Lund et al., 1996).

Fetal development—The development of mammary gland in the embryo is comparable in all species (Damm and Turner, 1958). The slight thickening of the ventrolateral ectoderm in the embryo is the first indication for the presence of bovine mammary development. This thickening, also known as mammary band, first appears when the limb buds begin to lengthen (Deome et al., 1959). The mammary band then narrows and forms a mammary line at about 30 days in the bovine embryo (Akers, 2002). Mammary lines give rise to mammary buds which consist of congregation of ectodermal cells to form epithelial nodules, which occur at day 49 of gestation. In the bovine, there are four mammary buds in the inguinal region, two on each side of the former mammary band. Mammary buds then 'sink' into mesenchyme (embryonic connective tissue) to form the secretory and duct systems of the gland which will represent the future glandular portion (parenchyma) of the udder (Faulkin and Deome, 1960). Development patterns of male and female have no difference before this period (Akers, 2002). Injection of GH into the mouse fetus increased the size of the developing mammary gland in both male and female fetuses, suggesting that this hormone is also involved in development of the mammary gland during the fetal stage (Topper and Freeman, 1980)

Birth to conception—The mammary tissue contains a mammary cord that is embedded in mesenchyme and indicates some branching at the distal end. Prepubertal mammary

development of bovine consists of branching and elongation of the mammary ducts into the surrounding mammary fat pad (MFP). Prior to puberty, mammary growth occurs at an allometric rate and returns to an isometric rate after puberty (Sinha and Tucker, 1969). Increased nutrient intake during this allometric growth phase results in reduced parenchymal (PAR) mass and DNA (Sejrsen et al., 1982; Capuco et al., 1995), which may be due to the impaired epithelial cell proliferation because of reduced circulating growth hormone (Sejrsen et al., 1999). At puberty, the ductal system begins to develop from the nipple, and is characterized by specialized structures-the terminal end buds (TEB) - capping the end of the ducts. TEB grow out across the fat pad and, by bifurcation, lay down the minimally branched structure that covers the fat pad. There is little or no lobulo-alveolar development that occurs before conception. The mammary growth and development are stimulated by ovarian activity at puberty. Ovariectomy in many prepubertal species inhibits mammary growth (Lyons et al., 1958; Nandi, 1958). Additionally, it has consistently been demonstrated that parenchymal (PAR) mass, DNA content, or both are reduced in heifers reared on an elevated level of nutrient intake during this period of prepubertal allometric mammary growth (Sejrsen et al., 1982; Petitclerc et al., 1984). After puberty, under the influence of the hormones with each oestrous cycle, more extensive branching, and elongation of the duct system is stimulated, until the final buds develop into secretory alveolar cells. It is widely believed that estrogen is primarily responsible for duct growth, and progesterone for alveolar formation (Chatterton, 1978). During these processes, GH has been shown to play important roles in ductal elongation and the differentiation of ductal epithelia into terminal end buds (Coleman et al., 1988).

Conception to parturition—Pregnancy is the greatest promoter of natural mammary growth, which is associated with hormonal and physiological changes. For the hamster, 94% of

mammary development takes place during gestation. For other species, estimates range from 78% for the mouse and sheep to 66% in rabbits and 60% for rats (Anderson, 1976). In the early stage of pregnancy, the ductal growth occurs extensively, producing a further branching of the ducts and formation of the end buds (Coleman et al., 1988). Changes in weight, total DNA, or percentage epithelium illustrate a marked increase in parenchymal tissue between day 80 and 115 of gestation (Smith et al., 1987). The masses of interstitial adipose tissues disappear for the most part and make room for proliferation of epithelial cells, with appearance of a rudimentary lobulo-alveolar system by about 5 mo of gestation (Chatterton, 1978). There occurs a dramatic outgrowth of secondary branches throughout the estrus cycle. This together with the formation of the lobulo-alveolar structures, results in the milk-producing gland.

Involution—When milking ceases, involution occurs characterized by apoptosis of alveolar epithelial cells and tissue remodelling of the bovine mammary gland (Wilde et al., 1997). By removing suckling young or by suspending milking in dairy animals, mammary involution can be initiated at any stage of lactation (Akers, 2002). Evidence for involution can be detected by the marked changes in gene expression in 24 h - mRNA expression for the caseins is reduced 95 percent in rats, and mRNA expression for acetyl-CoA carboxylase is reduced 98 percent within 24 h of involution (Travers et al., 1996). Ultrastructurally, the involuting epithelium showed progressive loss of tight junction integrity, followed by the alteration in composition of mammary secretions during the dry period (Athie et al., 1996). The organelles intimately involved in milk protein synthesis and secretion (rough endoplasmic reticulum and Golgi apparatus) degenerated during involution (Holst et al., 1987).

Lactating mammary gland extracts glucose, amino acids, fatty acids, and minerals from blood and produces the carbohydrate, protein, and fat in milk. Due to bacterial fermentation of dietary carbohydrates in ruminants, acetate and β-hydroxybutyrate (BHBA) are also substrates. The pathway of milk component synthesis is shown in Figure 1.4.

Milk components and biosynthesis

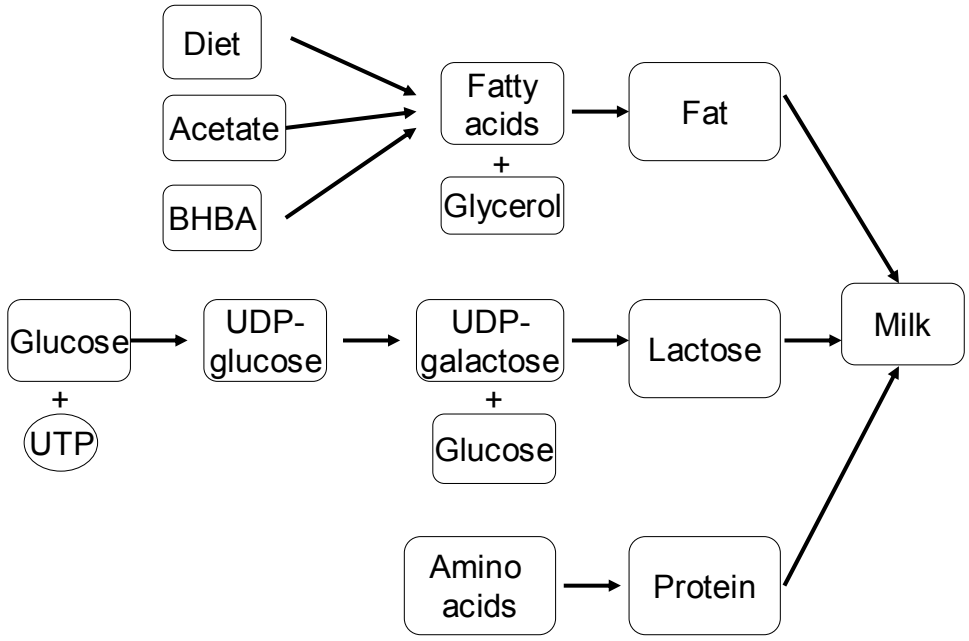


Figure 1.4. Diagram of milk component synthesis. Fat, lactose, and protein are three major components of milk. Most of the milk fat is composed of triglycerides from fatty acids and glycerol. Fatty acids in milk are derived either from diet or from acetate and β-hydroxybutyrate (BHBA). Lactose is the most common carbohydrate in milk. One glucose, together with uridine triphosphate (UTP), is converted to uridine diphosphate (UDP)-glucose, which in turn is converted to one UDP-galactose. Lactose is synthesized from UDP-galactose and another glucose by lactose synthase (a combination of galactosyl transferase and α-lactalbumin) without modification. The milk specific proteins are synthesized from amino acids derived either from the bloodstream (essential amino acids) or from amino acids synthesized by the secretory cells.

Milk protein biosynthesis— The basic process of milk protein synthesis is the same as the processes by all eukaryotic cells. The milk specific proteins include the caseins (α-casein, β-casein, κ-casein, and γ-casein) and the whey proteins (α-lactalbumin, β-lactoglobulin; Miller et al., 1990). These milk specific proteins are synthesized from amino acids derived either from the bloodstream (essential amino acids) or from amino acids synthesized by the secretory cells (Shennan and Beechey, 1995). The α, β, κ, and γ-casein accounts for about 49, 35, 10, and 5 percent respectively of the total milk. The major whey protein in the milk of cows is β-lactoglobulin, which is absent in the milk of humans, rats, and guinea pigs (Akers, 2002).

The proteins that are synthesized on the rough endoplasmic reticulum (RER) include the proteins to be secreted (such as the caseins, α-lactalbumin, and β-lactoglobulin) and membrane bound proteins (such as proteins involved in cell-cell contacts and membrane bound enzymes) (Craig et al., 1978). Milk proteins for secretion pass to the Golgi apparatus, where caseins undergo phosphorylation and associate with calcium to form micelles. Caseins and other proteins undergo post-translational processing in the Golgi. Movement of Golgi vesicles to release their contents from the apical cell membrane by reverse pinocytosis is thought to involve microtubules since milk secretion is inhibited by drugs which disrupt microtubules (Patton, 1974).

Lactose biosynthesis—Lactose, a disaccharide of glucose and galactose, is the major carbohydrate found in milk. Lactose is synthesized from UDP (uridine diphosphate)-galactose and glucose within the Golgi by lactose synthase (Kuhn and White, 1976). The high concentrations of lactose present in the Golgi during lactation lead to osmotic influx of water that contributes to the fluidity of milk. Lactose synthase is a combination of galactosyl transferase and α-lactalbumin that come together in the Golgi apparatus (Kuhn, 1968). β1,4-galactosyltransferase (β4GalT-I) is a constitutively expressed, trans-Golgi resident, type II

membrane-bound glycoprotein that is widely distributed in vertebrates, and its substrate specificity can only be activated by α-lactalbumin. β4GalT-I catalyzes the transfer of galactose to *N*-acetylglucosamine residues, forming the β4-N-acetyllactosamine (Galβ4GlcNAc) (Beyer and Barondes, 1982). In mammals, β4GalT-I has been recruited for a second biosynthetic function, the tissue-specific production of lactose (Galβ4Glc), which takes place exclusively in the lactating mammary gland. The synthesis of lactose is carried out by a protein heterodimer assembled from β4GalT-I and α-lactalbumin, a noncatalytic mammalian protein expressed de novo exclusively in the mammary gland during lactation (Brodbeck et al., 1967; Brew et al., 1968; Hill et al., 1968).

Glucose transport systems were detected in the mammary gland at both the apical and basal plasma membrane, and on Golgi and secretory vesicle membranes. Two distinct glucose transport mechanisms have been identified in the mammary gland: a GLUT1 transporter mechanism (Burnol et al., 1990) and a sodium dependent glucose transporter (Shennan and Beechey, 1995). GLUT1 appears to mediate glucose transport at the basal and Golgi membranes, but not contribute to glucose transport at the apical membrane (Camps et al., 1994).

Milk fat biosynthesis—Milk fat is synthesized in the smooth endoplasmic reticulum in the basal region of the cell from precursor fatty acids and glycerol (Mather and Keenan, 1998). Most of the milk fat is composed of triacylglycerides. The fatty acid composition of milk varies between species. For ruminants, the two major sources of the fatty acids in milk triglycerides include (1) diet via hydrolysis of chylomicra, (2) de novo synthesis within mammary epithelia cells from nonglucose source (Short et al., 1977). In cows half of the milk fatty acids are derived from the diet, including most of the C_{18} (stearic, oleic, or linoleic) fatty acids and about 30

percent of the C_{16} (palmitic) fatty acids. Shorter chain fatty acids are derived from de novo synthesis of acetate and BHBA from the blood (Hansen and Knudsen, 1980).

Process of milk production

Milk production is determined by number, synthetic and secretory activity of mammary alveolar cells and low amounts of residual milk after milking (Blum, 1992). Optimal mammary development and sufficient availability of substrates for milk synthesis are two important factors to achieve high milk yield. The major components of milk are water, lactose, fat, protein, vitamins and minerals, which differ dramatically across species. Milk from Holstein cows has about 3.2% protein, 3.4% fat, and 4.6% lactose.

Milk is synthesized and secreted from the extensive lobulo-alveolar clusters of the lactating mammary gland (Neville and Watters, 1983). A single layer of polarized secretory epithelial cells surrounds each alveolus within these clusters. In turn the alveoli are surrounded by myoepithelial cells that function in milk ejection, and a vascularized connective-tissue stroma that contains lipid-depleted adipocytes and fibroblasts. Ejection of milk from alveoli and ducts requires contraction of myoepithelial cells, stimulated by oxytocin released from the posterior pituitary as part of a suckling induced neuroendocrine reflex.

During pregnancy and the first few days postpartum, milk supply is controlled by the endocrine system. At the latter part of pregnancy, the secretory activity of the alveolar cells becomes apparent and the breasts are making colostrum, but high levels of progesterone inhibit milk secretion and keep the volume “turned down” (Palade, 1975). At birth, the delivery of the placenta results in a sudden drop in progesterone/estrogen levels, which leads to the copious milk production in the presence of high prolactin levels (Neville et al., 2001). It has been shown that during pregnancy, there is a progressive increase in progesterone followed by a sharp decrease

preceding delivery. This decrease is accompanied by a rapid increase in circulating estrogens, which is essential for parturition and lactation (Vermouth and Deis, 1972). Progesterone also prevents the release of prolactin from the anterior pituitary (Vermouth and Deis, 1974). Other hormones (insulin, thyroxine, and cortisol) are also involved, but their roles are not yet well understood. In the maintenance stage of milk production, milk removal is the primary control mechanism for supply.

Role of GH-IGF in mammary development

Both GHR mRNA and protein are expressed in the epidermis of bovine mammary gland during prenatal development (Knabel et al., 1998), suggesting that GH is involved in development of the fetal bovine mammary gland. Administration of GH to prepubertal heifers stimulates mammary growth (Akers et al., 2005). And there is much evidence indicating that GH mediates postnatal mammary development and differentiation (Divisova et al., 2006). In rats, GH stimulates the differentiation of ductal epithelia into TEB, and induces the differentiation of the alveolar structures. GH can also promote the morphogenesis of TEB in the mammary fat pad in rats (Walden et al., 1998). In GH-treated goats, the mammary glands are heavier; a potential cell hypertrophy and a tendency to increase or maintain mammary cell numbers are induced (Boutinaud et al., 2003). An antagonist of GHR dalayed pubertal ductal development resulting in a block of ductal elongation and branching in the mammary gland of mice (Divisova et al., 2006). This antagonist reduced the liver and serum IGF-I levels and also blocked both GH and IGF-I signaling within the mammary gland (Divisova et al., 2006).

The role of IGF-I in mammary ductal development can be inferred from several pieces of evidence. First, IGF-I can stimulate the proliferation of mammary epithelial cells in organ culture at low concentrations (Richert and Wood, 1999). Overexpression of IGF-1 in the mammary

gland led to premature parenchymal development (Weber et al., 1998; Su and Cheng, 2004) and delayed involution (Hadsell et al., 1996). Second, IGF-I mRNA was detected in mammary tissue from pregnant and lactating cows (Hauser et al., 1990). Mammary tissue from pregnant heifers was separated into fractions of epithelium, stroma, and blood components and IGF-I mRNA was found to localize in the stromal component of the mammary gland (Cohick, 1998). IGF-I and IGF-IR are also expressed within both the epithelial and stromal compartments of the virgin mammary gland (Hovey et al., 1998; Richert and Wood, 1999; Berry et al., 2001). The expression of IGF-I within the stromal compartment, coupled with the observation that both IGF-I and the IGF-IR are expressed within TEB supports the idea that IGF-I acts as a paracrine signal originating from the stroma and from select populations of cells within the TEB (Richert and Wood, 1999). IGF-I can also induce some degree of mammary development in the form of TEB development, indicating other mechanisms of IGF-I action are likely in addition to inhibition of apoptosis (Kleinberg, 1998). Third, expression of IGF-I in mammary tissue can be elevated by exogenous GH and E2 (Kleinberg et al., 1990). Last, localized administration of IGF-I to the mammary gland of E2-treated hypophysectomized-ovariectomized rats can stimulate ductal development (Ruan et al., 1992).

Direct demonstration of the role of IGF-I in this process was obtained through the analysis of mice with targeted mutations in the genes for IGF-I and IGF-IR (Ruan and Kleinberg, 1999; Hadsell and Bonnette, 2000). Mice with a targeted mutation in the IGF-I gene have several phenotypic abnormalities, including reduced viability, dramatically reduced postnatal growth, and reduced fertility (Liu et al., 1993; Baker et al., 1996). In addition, the development of the mammary ductal system in the IGF-I knockout mice is tremendously impaired (Ruan and Kleinberg, 1999). The impaired ductal development was associated with diminished TEB

development and could be restored only by the administration of exogenous E2 and IGF-I (Ruan and Kleinberg, 1999).

The need for IGF-IR in mediating the actions of IGF-I on mammary gland development was demonstrated by targeted mutation of IGF-IR gene (Igf1r-/-) in mice (Hadsell and Bonnette, 2000). Mutation of the IGF-IR gene in mice impairs fetal growth and is perinatally lethal. This lethality is caused by respiratory failure as a result of the impaired development of the intercostal muscles. The transplantation of fetal mammary tissue of the Igf1r-/- mice demonstrated that virgin mammary ductal development was dramatically impaired by loss of the IGF-IR (Hadsell and Bonnette, 2000). Mammary gland grafts from Igflr-/- mice also displayed dramatic reductions in both TEB number and size in comparison with Igflr+/+mice (Bonnette and Hadsell, 2001).

In ruminants, rapid mammary development is correlated with increased IGF-1 expression (Hovey et al., 1998) and anti-IGF-1 antibodies are able to block the mitogenic effects of bovine mammary tissue extracts (Weber et al., 1999). Compared with liver or other tissues, mammary gland expresses less IGF-I, but this mammary derived IGF-I is potentially more meaningful than circulating IGF-I in mammary development (Weber et al., 1998). IGF-I from mammary extract induced much more mitogenic activity compared with the same amount of IGF-I from serum (Weber et al., 1999).

IGFBPs are synthesized and secreted by mammary epithelial cells (Werner and LeRoith, 1996). Primary cultures of bovine mammary epithelial cells secrete IGFBP-2, -3, -4, and -5, which by molecular weight analysis are identical to those present in bovine milk (Gibson et al., 1999). IGFBP-3 is the predominant mammary IGFBP and its concentration also declines in blood and milk during lactation compared to prepartum and involution periods.

Role of GH-IGF in milk production

Administration of bGH to high yielding Holstein cows resulted in increased milk yield (Peel et al., 1981). GH has been used in the dairy industry to increase milk yield for more than 10 years. Treatment of rat with anti-GH for 24 h and 48 h reduced milk yield by 20% and 24%, respectively, without any change in concentrations of lactose, fat, protein, (Flint and Gardner, 1994). Lactation inhibition caused by GH deficiency was reinitiated by GH administration either systemically or into the mammary gland (Flint and Gardner, 1994), suggesting the direct effect of GH was to stimulate milk synthesis. Currently, an indirect action of GH on lactating mammary cells is widely believed for several reasons. Firstly, there is no evidence for the presence of high-affinity GHR on mammary epithelial tissue (Akers, 1985; Hauser et al., 1990). Secondly, GH has no stimulatory effects on milk synthesis in most in vitro studies of mammary tissue (Bauman and Vernon, 1993; Etherton and Bauman, 1998), except for a report that it acts synergistically with prolactin to increase synthesis of casein by goat mammary tissue in culture (Skarda et al., 1982). Thirdly, GH stimulated neither proliferation of mammary epithelia nor milk protein and fatty acid synthesis in mammary gland explants (Bolander and Topper, 1980). Finally, unilateral close arterial infusion of GH into one-half of the mammary gland of sheep did not increase milk yield of the infused udder-half (Gluckman et al., 1987).

The effects of GH on milk production is also considered to be mediated by stimulating local production of IGF-I (Cohick, 1998). The effects and evidence of IGF-I on milk synthesis remain controversial. IGF-1 has been shown to stimulate mammary cell (epithelial and stromal) proliferation, casein gene expression and synthesis, and glucose transport (Baumrucker and Stemberger, 1989), and IGF-I could inhibit apoptosis of the mammary gland (Forsyth, 1996). Overexpressed des(1–3)IGF1 enhanced milk synthesis and mammary development during

prolonged lactation (Hadsell et al., 2005). In normal lactating goats, close arterial infusion of IGF-I into the mammary gland increased milk production, which was associated with increased blood flow (Prosser et al., 1994), suggesting a direct effect of IGF-I in mammary gland on milk production. Bovine α-lactalbumin gene promoter can be used to increase local IGF-I expression in transgenic mice. Milk yield in α-lactalbumin-IGF-I transgenic mice was significantly greater than non-transgenic littermates (Su and Cheng, 2004), indicating that local overexpression of IGF-I in the transgenic mice can stimulate milk yield during the first lactation. This may be due to the mitogenic effect of IGF-I on mammary gland development. But Monaco et al. (2005) failed to detect the effects of IGF-I on milk production and composition in mammary specific transgenic swine, which over-expressed IGF-I. They found IGF-I over-expression did not positively or negatively impact milk production of swine (Monaco et al., 2005). This is consistent with other studies in which no statistically significant difference is found in milk yield in IGF transgenic rabbits comparing to control lactating rabbits (Wolf et al., 1997). Pups of IGF-I transgenic mice had about the same growth rates compared as the offspring of control mice (Hadsell et al., 1996). Systemic administration of IGF-I had no effect on milk production in rats (Nielsen et al., 1990; Flint et al., 1992; Flint and Vernon, 1998).

However, recent evidence suggests that GH may regulate milk production by direct action on the mammary gland. This new evidence includes: (1) GHR mRNA and protein were detected in the bovine mammary epithelia in most stages of mammary gland development except involution (Hauser et al., 1990; Ilkbahar et al., 1999); (2) GHR expression in mammary gland varies with stages of mammary gland development (lactation and galactopoiesis). In dry cows, the expression of GHR protein is much lower than in lactating cows (Sinowatz et al., 2000). (3) By using slow-release plastic implants, GH has been shown to have an effect on the formation of

terminal end buds, reinitiation of epithelial DNA synthesis, and increase in ductal diameter of the mammary gland in mice (Coleman et al., 1988); (4) GH stimulates DNA binding activity of STAT5, a key component of GHR signaling, in the mammary gland of cattle in vitro and in vivo (Yang et al., 2000). GH treatment can also significantly increase the expression of STAT5 protein and mRNA in the mammary gland of lactating goat (Boutinaud et al., 2004).

GH-IGF and milk protein gene expression

Milk proteins, including caseins and whey proteins, are specific molecular markers for functional differentiation in the mammary gland (Topper and Freeman, 1980). The genes for these proteins display both tissue-specific and developmental patterns of expression (Hobbs et al., 1982). The principal whey proteins in rodents, ruminants, and man are whey acidic protein (WAP), β-lactoglobulin, and α-lactalbumin, respectively. Each is encoded by a relatively small, single-copy gene. The casein proteins are encoded by a cluster of single-copy genes. In bovine, the αS1-, β-, αS2-, and κ-casein genes are clustered in this order in a 250-kb region on chromosome 6 (Rijnkels et al., 1997). In ruminant, the αS1- and β- casein genes are closely linked and arranged in 5'-3'and 3'-5' transcriptional orientations, respectively (Rosen et al., 1999). The three genes encoding the calcium-sensitive caseins (αS1-, β-, and αS2-) have evolved from a common ancestral gene (Stewart et al., 1987; Groenen et al., 1993) and share common regulatory motifs in the proximal and distal 50 bp flanking regions (Groenen et al., 1992; Winklehner-Jennewein et al., 1998). The κ-casein gene is, however, not evolutionarily related to these genes, although its expression pattern is similar and its protein product is essential for micelle formation and stability (Alexander et al., 1988).

The genes encoding these milk proteins are regulated by the complex interplay of peptide and steroid hormones. Milk protein gene expression is controlled by hormones at the

transcriptional level (Teyssot and Houdebine, 1980), at the level of mRNA stability (Hobbs et al., 1982), as well as the translational level (Rosen et al., 1999). The relative specific activity of cytoplasmic mRNA for αS1-, αS2-, β-, and κ-casein were about 3.2, 4.6, 3.3, and 4.5- fold higher in mammary tissues of lactating cows than in those of 8 month pregnant cows (Choi et al., 1988). The combination of insulin, hydrocortisone, and prolactin can induce the accumulation of β-casein mRNA and the secretion of the milk protein by epithelial cells from bovine mammary tissue (Choi et al., 1988). GH increased ß-casein mRNA expression in mammary explants from lactating cows (Yang et al., 2005). GH increased αS1-casein mRNA expression in a bovine mammary epithelial cell (BMEC) clonal line (Sakamoto et al., 2005; Yonekura et al., 2006). The expression of κ-casein RNA in mammary tissue of goat was not affected by GH (Boutinaud and Jammes, 2004). GH treatment can also significantly increase the expression of STAT5 at both transcriptional and protein levels in lactating goat mammary gland (Boutinaud and Jammes, 2004).

Roles of other hormones in mammary gland development and milk production

Growth and differentiation of the mammary gland during development and lactation are controlled by complex hormonal mechanisms. There are some other hormones and growth factors that can control mammary gland development and lactation in addition to GH and IGF-I.

Prolactin

Prolactin (Prl), a peptide hormone synthesized and secreted by the anterior pituitary, can also stimulate mammary growth and milk production. The requirement for Prl maintenance of lactation varies with species. For rodents, Prl can stimulate both mammary gland development

and functional differentiation of the alveolar cells during pregnancy. Treatment of cows with exogenous Prl has little effect on milk production or composition (Plaut et al., 1987), suggesting the circulating concentration of Prl is not rate limiting to continuing milk secretion in cows. Prl was demonstrated to play a key role in lactogenesis in ruminants but certainly a lesser role in galactopoiesis, while exogenous Prl is very galactopoietic in rodents. Prl receptors which are members of the cytokine receptor superfamily are expressed in mammary epithelial cells of many species. Concentrations of Prl receptors in rodent mammary tissue correlate with milk yields (Ormandy et al., 1997). The number of receptors increases to the time of peak milk production and then declines. The metabolic clearance and secretion rate of Prl is also positively correlated with milk production across stages of lactation of cows. Prl can also help to maintain the population of mammary secreting cells and thus promote lactational persistency (Flint and Gardner, 1994). Experiments that disrupt the normal Prl-signaling cascade so that one STAT5 is inactivated result in lactation failure (Hynes et al., 1997).

Insulin

Insulin plays an important role in the regulation of nutrient utilization during lactation. In ruminants, insulin has no effect on the mammary uptake of glucose, acetate, β-hydroxybutyrate, or amino acids, but exogenous insulin inhibits milk production through the metabolic effects on other tissue (Tucker, 2000). In adipose tissue, insulin promotes the uptake of glucose and acetate and stimulates lipogenesis while inhibiting lipolysis. In liver, insulin inhibits gluconeogenesis. It is unlikely that insulin plays a role in regulating the number of mammary epithelial cells. The mitogenic activity of insulin is attributed to the ability of insulin to cross-react with IGF receptors and to elicit IGF-related responses.

Transforming Growth Factor (TGF)

TGF-β plays an important role in embryonic development, cell differentiation and tissue regeneration. It can be upregulated in some human cancers. TGF-β1 usually exerts a growth inhibitory role on epithelial cells. TGF-β1 is the best described related to mammary function (Li et al., 2003) among all TGF-β family members which include TGF-β1, TGF-β2 and TGF-β3. In heifers, the ductal epithelial cells of the mammary gland show extensive presence of type I and type II receptors (Purup et al., 2000). Most of the mammary-associated effects of TGF-β are inhibitory. TGF-β can inhibit IGF-I or serum-stimulated growth of primary bovine mammary epithelial cells in culture (Weber et al., 2000). TGF-β1 reversibly inhibits the growth of mammary ducts. In the mouse, overexpression of TGF-β at puberty reduces the rate of duct development and the degree of ductal tree expansion (Akers, 2006). Expression of TGF-β during pregnancy impairs lobulo-alveolar development and lactation (Serra and Crowley, 2003). TGF-β1 also affects morphology of bovine mammary cells in culture (Plaut et al., 2003).

Expression of TGF-β has been detected in human (Barrett-Lee et al., 1990) and in bovine mammary tissue (Maier et al., 1991), in addition to being secreted in cow's milk (Cox and Burk, 1991), where TGF-β2 was found to be the predominant molecule (Jin et al., 1991). TGF-α stimulated proliferation of bovine mammary epithelial cells in cultures (Collier et al., 1993; Zurfluh et al., 1990). These data support a role for TGF-α as a growth promoting factor during mammary development.

Fibroblast Growth Factors (FGF)

FGF include a family of small peptide growth factors most of which can stimulate DNA synthesis in cultured fibroblasts (Dvorak et al., 2006). Several members of the FGF family have emerged as stroma-derived mitogens. They act in a paracrine manner to locally influence

epithelial cell proliferation and glandular morphogenesis by controlling appearance of side branching of mammary ductal growth (Powers et al., 2000). Three of the FGF variants (FGF-1, FGF-2 and FGF-3) are involved in ruminant mammary development (Deugnier et al., 2002). These FGF and their receptors are expressed throughout the lactation cycle with highest level during virgin stage and involution. Interaction between epithelium and the surrounding stroma influence paracrine FGF-2 expression. The expression is greater in the stroma adjacent to the developing parenchymal tissue compared with more distant mammary stromal tissue (Deugnier et al., 2002).

Leptin

Leptin is a 16-KDa protein primarily produced in adipose cells (Havel, 2000). Leptin is also expressed in mammary gland and secreted into milk. The long form of the leptin receptor was found to be expressed in mammary epithelial cells (Yonekura et al., 2006). Leptin can inhibit IGF-I or serum induced proliferation of bovine mammary epithelial cells. Increased local secretion of leptin in the developing udder may explain the inhibitory effects of high-energy diets on mammary development in heifers (Smith and Sheffield, 2002). Leptin is increased in the serum of animals fed high energy diets, which may be related to the decreased mammary development in these animals. Leptin appears in milk and is present in cultured bovine mammary epithelial cells (Silva et al., 2002). Bovine mammary cells express mRNA for leptin and the expression is increased by insulin and IGF-I, both of which are known mediators of mammary function (Silva et al., 2002). This suggests that leptin may be an autocrine or paracrine signaling molecule in the mammary gland.

Chapter II Effects of Growth Hormone and Insulin-Like Growth Factor-I on Milk Protein Gene Expression, Nutrient Uptake, and Proliferation in Bovine Mammary Epithelial Cell Line MAC-T

Abstract

Administration of growth hormone (GH) stimulates milk production in cattle and this effect is widely believed to be mediated by GH-stimulated insulin-like growth factor-I (IGF-I). However, recent findings that both GH receptor (GHR) mRNA and protein are expressed in the epithelial cells of the bovine mammary gland suggest that GH may stimulate milk production by directly affecting the milk-producing epithelial cells. The objectives of this research were to determine whether GH and IGF-I can directly stimulate proliferation, glucose, amino acid, and fatty acid uptake, and milk protein gene expression in MAC-T cells, a cell line that was derived from bovine mammary epithelia. The research showed that IGF-I increased ($P < 0.05$) cell proliferation and uptake of amino acids, decreased ($P < 0.05$) oleic acid uptake and had no effect on uptake of 2-deoxyglucose, a nonmetabolizable analog of glucose, in MAC-T cells. IGF-I had no effect on mRNA abundance of αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin, the six major milk proteins. The MAC-T cells express barely detectable GHR mRNA. To help detect any effect of GH in these cells, MAC-T cells were transfected with expression plasmids for GHR and signal transducer and activator of transcription 5 (STAT5), a major transcription factor mediating GH induction of gene expressio. GH treatment of the transfected MAC-T cells markedly stimulated ($P < 0.05$) the expression of αS1-casein, αS2-casein, β-casein and α-lactalbumin mRNA, whereas it had no effect ($P > 0.1$) on the expression

of κ-casein, β-lactoglobulin, IGF-I, IGF-II or IGF binding protein 1 (IGFBP-1) to IGFBP-6 mRNAs. GH also did not affect 2-deoxyglucose, amino acid or oleic acid transport, or cell proliferation in the transfected MAC-T cells. A sequence analysis revealed that the promoters of the bovine αS1-casein, αS2-casein, β-casein and α-lactalbumin genes contain putative binding sites for STAT5. Cotransfection analyses confirmed that GH could stimulate ($P < 0.01$) luciferase reporter gene expression from each of these promoters in MAC-T cells, further suggesting that GH can directly stimulate milk protein gene expression. Furthermore, GH administration stimulated ($P < 0.05$) αS1-casein mRNA expression and tended to increase several other milk protein mRNA in the mammary gland of lactating cows. These observations together with the fact that GHR mRNA and protein are expressed in the epithelial cells of the bovine mammary gland suggest that GH may directly stimulate milk protein gene expression in the mammary gland as one of the mechanisms by which GH increases milk protein production in cattle. Additional mechanisms may include IGF-I-mediated mammary epithelial cell proliferation and amino acids uptake.

Key Words: Cattle, Growth hormone, Insulin-like growth factor-I, Mammary epithelial cells

Introduction

Growth hormone (GH), also known as somatotropin, is a polypeptide hormone synthesized and secreted by the anterior pituitary, and plays a key role in the control of postnatal growth and metabolism. One of the important effects of GH in ruminants is stimulation of lactation. Administration of GH to lactating cows increases milk production (Knight, 1992), but does not alter the gross composition of milk (i.e., fat, protein, and lactose concentrations) (Barbano et al., 1992; Bauman, 1992). In response to GH injection, milk secretion of dairy cows increases within a day and is maximized within a week. The increase in milk production is maintained as long as GH treatment is continued (Bauman, 1992). The positive effect of GH on milk production is also implied by the fact that plasma GH is significantly higher in high-yielding than in low-yielding cows (Nebel and McGilliard, 1993).

The effect of GH on lactation is believed to be mediated by indirect action of GH on the mammary gland because using GH binding assay, there was no evidence for the presence of functional GH receptor in the mammary gland (Akers, 1985). Earlier studies that GH stimulated neither proliferation of mammary epithelia nor milk protein (casein) and fatty acid synthesis in mammary gland explants (Bolander and Topper, 1980) also supported an indirect mechanism for GH stimulation of milk production.

Administration of GH to dairy cows increases milk yield by 10-15%. This increase is associated with increased requirement for milk precursors. Since treatment of GH for 1-3 weeks increases milk yield without increasing feed intake (Veerkamp, 1998), it is hypothesized that GH causes a repartitioning of nutrients to accommodate the increased requirement for milk precursors. This view is supported by many studies. For example, GH increases glucose uptake

in the mammary gland (Davis et al., 1988; Fullerton et al., 1989), while it decreases glucose uptake in skeletal muscle and adipose tissues (Davidson, 1987; McDowell et al., 1987).

It is further believed that the indirect effect of GH on the mammary gland is at least in part mediated by endocrine IGF-I. The evidence supporting this action includes that most of the blood IGF-I comes from the liver under GH stimulation; IGF-1 has been shown to stimulate mammary cell (epithelial and stromal) proliferation, casein gene expression and synthesis, and glucose transport (Baumrucker and Stemberger, 1989); and IGF-I could inhibit apoptosis of the mammary gland (Forsyth, 1996).

However, recent evidence suggests that GH may still regulate milk production by direct action on the mammary gland. This new evidence includes: (1) GH receptor (GHR) mRNA and protein were detected in the bovine mammary epithelia in most stages of mammary gland development except involution (Hauser et al., 1990; Ilkbahar et al., 1999); (2) GHR expression in mammary gland varies with stages of mammary gland development (lactation and galactopoiesis). In dry cows, the expression of GHR protein is much lower than in lactating cows (Sinowatz et al., 2000). (3) Slow-release plastic implants of GH stimulate the formation of terminal end buds, reinitiate epithelial DNA synthesis, and increase ductal diameter of the mammary gland in mice (Coleman et al., 1988); (4) GH stimulates DNA binding activity of STAT5, a key component of GHR signaling, in the mammary gland of cattle in vitro and in vivo (Yang et al., 2000). GH treatment can also significantly increase the expression of STAT5 protein and mRNA in the lactating goat mammary gland (Boutinaud et al., 2004).

The overall goal of this study was to understand the mechanism by which GH stimulates milk production in cattle. This goal was achieved by determining the effects of IGF-I on cell proliferation, nutrient uptake, and milk protein gene expression in MAC-T cells, and determining

the effects of GH on these processes in MAC-T cells overexpressing GHR. MAC-T cells are clonal cells derived from the primary bovine mammary alveolar cells by stable transfection with SV-40 large T-antigen (Huynh et al., 1991). MAC-T cells retain their ability to differentiate and secrete milk-specific products, such as α-casein and β-casein proteins. These cells are also responsive to extracellular matrix and to the lactogenic hormones (Huynh et al., 1991) and are considered as a representive in vitro model for bovine lactation.

Materials and Methods

Cell culture

MAC-T cells (provided by Dr. R. M. Akers from Virginia Polytechnic Institute and State University, Blacksburg, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mM of L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (FBS). The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. All reagents used in cell culture were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Genomic DNA and total RNA extraction from cells or tissues

Genomic DNA from the bovine liver was isolated by standard proteinase K digestion followed by phenol-chloroform extraction.

Total RNA from MAC-T cells or bovine mammary tissues was extracted using TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. DNA or RNA concentration was calculated based on the absorbance at 260 nm. DNA or RNA quality was confirmed by gel electrophoresis.

cDNA and DNA constructs

A standard reverse transcription-polymerase chain reaction (RT-PCR) was used to clone the cDNA fragments for six bovine milk protein genes, including αS1-casein (CSNAS1), αS2-casein (CSNAS2), β-casein (CSNB), κ-casein (CSNK), α-lactalbumin (LALBA), and β-lactoglobulin (LGB). Briefly, 0.2 μg of bovine mammary gland RNA was reverse transcribed using 2 pmol of dNTPs, 50 nmol of DTT, 40 ng of random hexamers, 2 U of RNasin ribonuclease inhibitor, and 10 U of reverse transcriptase (Promega, Madison, WI) in 10 μL of 1 x reverse transcription buffer at 42 °C for 2 h. For PCR amplification of each milk protein cDNA, 2 μL of the reverse transcription products were mixed with 12.5 μL of 2 x PCR Master Mix (Promega) and 10 pmol of milk protein mRNA-specific forward and reverse primers (bCAS1F1 and bCAS1R1 for bCSNAS1; bCSN1S2F1 and bCSN1S2R1 for bCSNAS2; bCSNBF1 and bCSNBR1 for bCSNB; bCSNKF1 and bCSNKR1 for bCSNK; bLALBAF1 and bLALBAR1 for bLALBA; bLGBF1 and bLGBR1 for bLGB) (Table 2.1) in a total volume of 25 μL. The PCR amplification was initiated by heating at 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. The PCR products were resolved on 1.0 % agarose gels containing ethidium bromide.

The DNA bands with the expected sizes were extracted from the gel using gel extraction kits (Qiagen, Fremont, CA), according to the manufacturer's instructions. The gel-extracted milk protein gene cDNAs were ligated into pGEM-T Easy vector (Promega), essentially according to the manufacturer's instructions. The ligation was transformed into competent DH10B *Escherichia (E) coli* (Invitrogen, Carlsbad, CA) by electroporation and the positive clones were selected on Luria-Bertani (LB)/Ampicillin/5-bromo-4-choloro-3-indolyl-ß-D -galactoside (X-Gal)/isopropyl thiogalactoside (IPTG) plates. The plasmid DNA from the

selected *E. coli* cells was extracted using the Qiagen miniprep kit (Qiagen) according to the manufacturer's instructions and analyzed for inclusion of inserts by digestion with restriction enzyme EcoR I.

A 2,013 bp promoter of bovine αS1-casein gene (CSNAS1) was amplified by standard PCR from bovine genomic DNA using primers bCSNAS1PF1 and bCSNAS1PR1 (Table 2.1). The PCR product was digested with restriction enzymes Sma I and Xho I, and cloned into pGL2-basic (pGL2b) vector (Promega) digested with the same restriction enzymes to generate plasmid bCSNAS1P/pGL2b. A 2,195 bp promoter of bovine αS2-casein gene (CSNAS2) and a 1,648 bp promoter of bovine β-casein (CSNB) promoter were amplified by PCR with primers bCSNAS2PF1 and bCSNAS2PR1, and bCSNBPF1 and bCSNBPR1, respectively (Table 2.1). These two PCR products were digested with Xba I and Xho I, and cloned into pGL2b vector between Nhe I and Xho I sites to generate plasmid bCSNAS2P/pGL2b and bCSNBP/pGL2b, respectively. A 1,988 bp promoter of bovine α-lactalbumin gene was amplified by PCR using primers bLALBAPF1 and bLALBAPR1 (Table 2.1), cloned into the pGL2b vector between the Kpn I and Xho I sites, resulting in bLALBAP/pGL2b. The conditions of these PCR were 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C. The PCR products were analyzed by standard gel-electrophoresis.

All newly cloned plasmids were sequenced to verify the inserts. The sequencing reaction was set up with 400 ng of plasmid and 5 pmol of vector specific primer in a volume of 10 μL using the ABI Prism Big Dye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA), run at 96 °C for 1 min, followed by 49 cycles of 96 °C for 10 s, 50 °C for 10 s, and 60 °C for 4 min. The sequencing reactions were analyzed on ABI 377 Automated DNA Sequencers (Applied Biosystems). These steps of sequencing were performed by the Virginia

Bioinformatics Institute (Blacksburg, VA). The nucleotide sequences of the cloned milk protein genes or mRNAs were compared with the sequences in GenBank using the BLAST program at http://www.ncbi.nlm.nih.gov.

The expression plasmid encoding wild-type mouse STAT5b was provided by Dr. Kouichi Ariyoshi (The University of Tokyo, Tokyo, Japan). The bovine GHR expression construct was made previously by our laboratory (Wang and Jiang, 2005). The pSPI-LUC plasmid was provided by Tim Wood (Karolinska Institute).

Transient transfection and luciferase assay

For the transfection to test milk protein gene promoter response to GH, MAC-T cells were plated onto 24-well plates at a density of 3×10^4 / well. Cells in each well were transfected with 0.5 μg of the respective milk protein gene promoter reporter construct, 0.5 μg of GHR expression plasmid, 0.5 μg of wild-type STAT5b expression plasmid, and 2 ng of pRL-CMV as transfection efficiency control, using FuGene 6 as transfection reagent (Roche, Indianapolis, IN). 24 h after transfection, the medium was replaced with serum-free DMEM, and the culture was continued for 8 h. The transfected cells were subsequently treated with 500 ng/mL of recombinant bovine GH (provided by Dr. A. F. Parlow, National Hormone and Peptide Program, Torrance, CA) or PBS, the vehicle for GH, for 16 h before being lysed for dual-luciferase assay. The cell lysates were prepared and assayed for luciferase activity using the Dual-LuciferaseTM Reporter Assay System (Promega) according to the manufacturer's instructions. This transfection analysis was repeated four times. The luciferase activity expressed from the promoter construct was divided by that from pRL-CMV in the same well to normalize well-to-well variation in transfection efficiency.

Cell proliferation assay

MAC-T cells were cultured to 70% confluency. The cells were transfected with GHR and STAT5 expression plasmids as described above. 24 h after transfection, the cells were detached with Trypsin-EDTA (Sigma) and transferred to 96-well plate at 1 x 10^4 cells/well. The cells were cultured in DMEM containing 10% FBS for about 4 h and were then cultured in serum-free DMEM for 8 h. The cells were then treated with 0.5 ng/mL, 5 ng/mL, 50 ng/mL, 100 ng/mL, or 500 ng/mL of bGH or PBS (control) for 16 h in serum-free DMEM. The nonradioactive CellTiter 96 assay (Promega) was used to measure number of viable cells and this assay was performed following the manufacture's instruction. Briefly, the cells in each well were treated with 15 μL of dye solution from the kit for 4 h. Then 100 μL of solubilization/stop solution was added to each well and the plate was incubated overnight before the absorbance at 570 nm was recorded using a 96-well plate reader. The absorbance reading was linearly proportional to the number of viable cells per well.

The effects of IGF-I on cell proliferation of MAC-T cells were performed on untransfected MAC-T cells. Briefly, MAC-T cells were seeded in 96 well plates in DMEM containing 10% FBS for about 4 h and were then cultured in serum-free DMEM for 8 h. The cells were then treated with 200 ng/ml of recombinant human IGF-I (Sigma) or PBS (control) in serum-free DMEM for 16 h. 10% FBS was used as a positive control. The nonradioactive CellTiter 96 assay was used to measure the number of viable cells as described above.

[^{3}H]-2-deoxyglucose uptake assay

MAC-T Cells were seeded in 100 mm diameter culture dishes. When cells reached 60 % confluency, they were co-transfected with 10 μg of GHR and 10 μg of STAT5b expression plasmids using FuGene 6 as described above. 24 h after transfection, the cells were detached

with Trypsin-EDTA and seeded in 24 well plates at 3 x 10^4 / well. The cells were allowed to grow in DMEM and 10% FBS for ~4 h and then starved with serum free DMEM for 8 h. The cells were treated with PBS, bGH (0.5 ng/mL, 5 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, or 500 ng/mL) for 16 h. Each treatment was done in triplicate. The plates were set at room temperature for 20 min to make sure the temperature of the medium reached room temperature. The cells were rinsed three times in Krebs-Ringer phosphate buffer (KRB) containing 128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5.0 mM Na_2HPO_4, pH 7.4, and were then incubated in KRB containing 1 μL of [^{3}H]-2-deoxyglucose (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) at room temperature. For time-course studies, the incubation lasted from 5 to 60 min. For the uptake assays, a 20-min incubation was used. The uptake was terminated by washing the cells 3 times with ice-cold KRB solution. Nonspecific [^{3}H]-2-deoxyglucose uptake was measured in the presence of 500 mM of glucose. The cells were lysed in 0.5 mL of 0.1 N NaOH for 30 min and the lysates were neutralized by addition of 0.5 mL of 0.1 N HCl. 0.5 mL of the lysates was taken for counting [^{3}H] activity and another 0.5 mL was taken for measuring protein concentration. The counts (cpm/mL) were normalized with the protein concentrations (μg/mL) of the same wells to make sure the difference in 2-deoxyglucose uptake was not due to difference in the number of cells. The experiment was repeated three times.

The effect of IGF-I on [^{3}H]-2-deoxyglucose uptake was determined in untransfected MAC-T cells essentially as described for the GH effect except that the cells were treated with 200 ng/mL of IGF-I or PBS (control) for 16 h.

[^{3}H]-amino acid mixture uptake assay

To determine if GH or IGF-I stimulated MAC-T cells to take up amino acids, the MAC-T-GHR cells or MAC-T cells were treated with bGH or IGF-I, respectively as described in the

[^{3} H]-2-deoxyglucose uptake assay. For time-course studies, amino acids uptake was terminated after incubation with [^{3}H]-amino acid mixture (Amersham Biosciences, Amersham, UK) at 1.0 μCi/well for 5 to 60 min at room temperature. For GH or IGF-I effect on amino acid uptake, the cells were incubated with [^{3}H]-amino acid mixture for 20 min. The cells were then washed with KRB and the accumulation of [^{3}H]-amino acid within the cells was analyzed as described for [^{3}H]-2-deoxyglucose. Nonspecific [^{3}H]-amino acid mixture uptake was determined in the presence of 1000 ng/mL of unlabeled amino acids mixture (Sigma).

[^{3}H]-oleic acid uptake assay

[^{3}H]-oleic acid uptake assay was performed essentially following the method of Gao and Serrero (Gao and Serrero, 1999). [^{3}H]-oleic acid (PerkinElmer Life and Analytical Sciences, Boston, MA) was dissolved in 10 mL of KRB at 37 °C to give a concentration of ~ 1.60 μM, or 2.5 μCi/mL. When the solution was completely clear after ~20 min, fatty acid-free BSA (Sigma) from a concentrated stock solution (12.54 μg/μL) was added with gentle mixing to obtain an oleic acid/BSA molar ratio of 1.0. The MAC-T-GHR or MAC-T cells were treated with GH or IGF-I, respectively, as described before. Before initiation of the oleic acid uptake, the cells were incubated at room temperature for 20 min. The time course study was performed by incubating the cells with the [^{3}H]-oleic acid/BSA solution from 2 seconds to 1 h. For the oleic uptake assay to measure GH or IGF-I effect, the cells were incubated with [^{3}H]-oleic acid/BSA mixture at 2.5 μCi/well for 20 min at room temperature. The uptake was stopped by removal of the solution followed by addition of 1 mL of an ice-cold stop solution containing 200 μM phloretin. Phloretin can adsorb to lipid surfaces and change the membrane permeability, thereby stopping fatty acid uptake (Cseh and Benz, 1999). The cells were then washed with ice-cold KRB and the accumulation of [^{3}H]-oleic acid within the cells was analyzed as described for [^{3}H]-2-

deoxyglucose. Background radioactivity representing [^{3}H]-oleic acid trapped extracellularly and bound nonspecifically by the cells was measured from 5-second incubation of the cells in the [^{3}H]-oleic acid/BSA solution.

Real-time PCR

Two μg of total RNA extracted from MAC-T cells were used for the synthesis of cDNA. Reverse transcription was performed in a reaction mixture containing 0.5 mM dNTPs, 2.5 μM random hexamers, 0.4 U/μL RNase inhibitor, 1.25 U/μL reverse transcriptase, 5.5 mM $MgCl_2$ and 1 x RT buffer in a final volume of 10 μL. Reverse transcription was run for 10 min at 25 °C, followed by 30 min at 48 °C, and 5 min at 95 °C. PCR was performed in a total volume of 25 μl, containing 12.5 μL of SybrGreen PCR Master Mix (ABI), 2 μL (or 0.2 μg) of the cDNA, 0.2 μM of forward and reverse primers, under the following thermal cycling conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control since the Ct (cycle threshold) value for GAPDH was not different between control and treatment samples. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative abundance of mRNAs (Writzl et al., 2006).

Animal tissue

Mammary parenchymal tissues from 12 lactating Holstein cows were provided by Dr. Anthony Capuco (USDA, ARS, Beltsville, MD). Those samples were taken during a previous study (Capuco et al., 1989). Briefly, 6 cows were injected intramusculary with 40 mg recombinant bGH/day for 5 consecutive days, and 6 cows were injected with an equal volume (4 ml) of excipient. The mammary gland tissue was collected at slaughter, 20-23 h after the last injection.

Ribonuclease protection assay (RPA)

The RPA was used to determine the relative abundance of αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin mRNA in bovine mammary tissues. To synthesize antisense RNA probes, 150 ng of linearized milk protein cDNA plasmid were mixed with 10 nmol of ATP, UTP, GTP, and 100 pmol of CTP (Promega), 50 μCi of ^{32}P-CTP (3,000 Ci/mmol, 10 mCi/mL) (PerkinElmer Life and Analytical Sciences), 250 nmol of DTT, 20 U of RNase inhibitor (Promega) and 15 U of T7 or SP6 RNA polymerase (Promega) in 40 μL of 1 x transcription buffer (Promega). The mixture was incubated at 37 °C for 1 h, and then treated with 2 U of DNase I (Promega) at 37 °C for 20 min. The ^{32}P-labeled probes were purified with phenol-chloroform extraction followed by filtration (1,100 x g at 4 °C for 4 min) through Quick Spin Sephadex G-50 columns (Roche Applied Science). The activity of the probes was estimated by liquid scintillation counting. Similarly, the bovine 18s rRNA antisense probe was generated from a bovine 18s rRNA plasmid. The RPA was performed using the RPA II kit (Ambion, Austin, TX). Briefly, 0.2 μg of total RNA was mixed with 5 x 10^5 dpm of αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin or β-lactoglobulin antisense riboprobe, and 2 x 10^4 dpm of 18S rRNA probe in a total volume of 20 μL hybridization buffer. The mixture was incubated at 42 ºC for 16 h and then digested with 200 μL of 1:100 diluted ribonucleases A and T1 at 37 °C for 45 min. The ribonuclease-protected RNA fragments were then precipitated and resolved on 6% polyacrylamide gels containing 7 M urea. After gel electrophoresis, gels were dried and exposed to phosphor screens. Exposed phosphor screens were scanned on a Molecular Imager FX System (Bio-Rad Laboratories, Hercules, CA). The measured abundance of milk protein mRNA in a sample was divided by that of the 18S rRNA in the same sample to normalize potential variation in loading amount of RNA and in performing RPA.

RPA was also used to confirm the increased expression of GHR mRNA in the transfected MAC-T cells. GAPDH mRNA expression was used as endogenous control. The bovine GHR and GAPDH antisense probes were generated from a bovine GHR and GAPDH cDNA plasmid (Kobayashi et al., 1999) as described before. This RPA was performed as described above.

Statistical analysis

All data were analyzed by one-factor analysis of variance (ANOVA). Multiple comparisons of means were done using the Tukey test. These analyses were performed using General Linear Model (GLM) of SAS (SAS Institute). All of the data were expressed as the means ± SEM. Differences at $P < 0.05$ were considered significant.

Results

Establishment of GH responsive MAC-T cells through transient transfection

There was no detectable GHR mRNA in untransfected MAC-T cells (Figure 2.1A). Following transfection with GHR and STAT5 expression plasmids, the expression of GHR mRNA was readily detectable (Figure 2.1A). GH treatment of the transfected MAC-T cells caused a nearly 80-fold induction ($P < 0.001$) in reporter gene activity expressed from a construct containing STAT5-binding sites (Figure 2.1B), demonstrating that these transfected cells became GH responsive. The untransfectd MAC-T cells did not respond to GH. The following experiments to detect the effects of GH on MAC-T cells were performed on the transfected MAC-T cells designated MAC-T-GHR cells.

Effects of GH and IGF-I on MAC-T-GHR and MAC-T cell proliferation, respectively

As shown in Figure 2.2A, GH at various concentrations had no effect on the proliferation of MAC-T-GHR cells ($P = 0.21$), whereas 10% FBS increased cell proliferation

by 30% ($P < 0.01$). As shown by Figure 2.2B, 200 ng/mL of IGF-I stimulated MAC-T cell proliferation by 50% ($P < 0.01$), compared with PBS.

Effects of GH and IGF-I on nutrient uptake in MAC-T-GHR and MAC-T cell, respectively

For each nutrient uptake assay, time course experiment was done first to determine the time period within which accumulation of the radio-labeled substance increased linearly. As shown in Figure 2.3, [^{3}H]-2-deoxyglucose (Panel A), [^{3}H]-amino acid mixture (Panel B), and [^{3}H]-oleic acid (Panel C) increased linealy within 60 min of incubation with the cells. Therefore, 20 min was used in the uptake assays to test the effects of GH or IGF-I. As shown in Figure 2.4A and 2.5A, both GH and IGF-I had no effects on 2-deoxyglucose uptake in MAC-T-GHR and MAC-T cells ($P = 0.56$, 0.24, respectively). IGF-I increased the uptake of [^{3}H]-amino acids in MAC-T cells ($P < 0.05$) (Figure 2.5B), while GH had no effect ($P = 0.65$) (Figure 2.4B). IGF-I decreased [^{3}H]-oleic acid uptake by MAC-T cells ($P < 0.05$) compared with PBS (Figure 2.5C). GH had no effect on [^{3}H]-oleic acid uptake by MAC-T-GHR cells ($P = 0.87$) (Figure 2.4C).

Effects of GH and IGF-I on expression of milk protein, IGF, and IGFBP mRNA in MAC-T-GHR and MAC-T cell, respectively

Real-time PCR was performed to detect mRNA expression of six milk protein genes, IGF-I, IGF-II, and six IGFBP genes. In MAC-T-GHR cells, GH increased αS1-casein (CSNAS1), αS2-casein (CSNAS2), β-casein (CSNB), and α-lactalbumin (LALBA) mRNA expression significantly ($P < 0.05$) (Figure 2.6A). The αS1-casein, αS2-casein, and β-casein mRNAs were almost undetectable in MAC-T-GHR cells before GH treatment (Figure 2.6C). IGF-II and IGFBP-1, 2, 3, 4, 5, and 6 mRNAs were readily detectable in those cells, but their expressions were not affected by GH treatment ($P = 0.91$, 0.45. 0.70, 0.10, 0.99, 0.14, 0.37, respectively) (Figure 2.6B). IGF-I, k-casein (CSNK), and β-lactoglobulin (LGB) mRNAs were not detectable

in GH treated or untreated MAC-T-GHR cells. IGF-I had no effect on αS1-casein, αS2-casein, β-casein, α-lactalbumin, k-casein, and β-lactoglobulin gene expression ($P = 0.83$, 0.27, 0.78, 0.33, 0.91, 0.93, respectively) (Figure 2.7A). IGF-I increased IGFBP-1 ($P < 0.05$) and IGFBP-3 ($P < 0.01$) mRNA expression in MAC-T cells.

Effects of GH on reporter gene expression from milk protein gene promoters

The promoters of αS1-casein, αS2-casein, β-casein, and α-lactalbumin were cloned into pGL2 basic vector, generating bCSNAS1P/pGL2b, bCSNAS2P/pGL2b, bCSNBP/pGL2b, and bLALBAP/pGL2b, respectively. Transient transfection analysis was performed to determine whether GH could enhance reporter gene expression from these milk protein gene promoters. As shown in Figure 2.8A, GH stimulated ($P < 0.01$) luciferase reporter gene expression from each of these promoters in MAC-T-GHR cells, with the greatest increase from the bCSNAS2P/pGL2b construct.

Sequencing analysis (Figure 2.8B) revealed that each of these four promoters contained at least one consensus sequence for STAT5 binding site (TTCNNNGAA, where N is A, C, G, or T) indicating that the increased reporter gene expression from these promoters may be mediated by GH induced STAT5 binding to the promoters.

Effect of GH administration on milk protein gene expression in mammary gland

As determined by RPA (Figure 2.9A), αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin mRNA were abundantly expressed in the mammary gland of lactating cows, with αS1-casein mRNA being most abundant. GH treatment stimulated αS1-casein mRNA expression ($P < 0.05$) by 18%. The abundance of the other five milk protein mRNAs also tended to be greater in GH-treated cows than in control cows, although these

differences were not statistically significant (P = 0.28, 0.40, 0.21, 0.19, 0.56, respectively) (Figure 2.9B).

Discussion

Continuous GH administration increases milk yield in lactating cows (Etherton and Bauman, 1998; Bauman and Vernon, 1993). Many studies on the mechanisms of GH action on milk production were conducted using mammary explants (Forsyth and Turvey, 1984; H. Yang et al., 2000; J. Yang et al., 2000a; Yang et al., 2005). Although explants can be easily and rapidly prepared, they are a mixed cell population, and hence are not appropriate for addressing questions like "Does GH or IGF-I act directly on the mammary epithelial cells to affect milk production?" In this study, we used a clonal cell line MAC-T cell which was derived from primary bovine mammary alveolar cells, and was considered an appropriate in vitro model for bovine mammary epithelial cells (Huynh et al., 1991). The MAC-T cells have significant expression of IGF-I receptor and are sensitive to IGF-I action (Cohick and Turner, 1998). We therefore used them directly in testing the effects of IGF-I on proliferation, nutrient transport and gene expression in them. The MAC-T cells, however, do not express much GHR mRNA. We made these cells maximally GH responsive by transfecting them with GHR and STAT5 expression plasmids and these transfected MAC-T cells allowed us to determine whether GH has any effects on proliferation, nutrient transport and milk protein gene expression in mammary epithelial cells. However, a potential drawback of these transfected MAC-T cells is that they are not exactly the same as MAC-T cells and hence they may be less representative of mammary epithelial cells than MAC-T cells.

The ability to produce milk is determined by both activity and the number of epithelial cells in the mammary gland. Lactation is typically characterized by continued proliferation of secretory tissue during early lactation, followed by a gradual loss of secretory cells and milk yield declines after peak lactation. This study showed that IGF-I could significantly stimulate MAC-T cell proliferation, while GH did not have this effect, suggesting that following GH administration, GH stimulated IGF-I, but not direct action of GH, may stimulate the proliferation of mammary epithelial cells, thereby contributing to increased milk production. Our observation of IGF-I stimulation on MAC-T cell proliferation is also consistent with the reports that IGF-I had a stimulatory effect on mammary cell (epithelial and stromal cell) proliferation (Baumrucker and Stemberger, 1989), and that IGF-I was a potent mitogen for the developing mammary gland (Sell et al., 1995).

Administration of GH to lactating cows increases milk production by about 15% (Knight, 1992), while it does not alter milk concentrations of fat, protein, and lactose (Bauman, 1992). This means that GH must stimulate lactose, protein and fat synthesis by about 15% in the mammary epithelial cells. So the increased lactose, protein and fat synthesis might result partially from increased mammary uptake of the substrates for these milk components, i. e. glucose, amino acids, and fatty acids. In this study, we found neither GH nor IGF-I had effects on glucose uptake of MAC-T-GHR or MAC-T cells, suggesting that GH administration-increased lactose synthesis is not mediated by direct stimulation of GH or IGF-I on glucose uptake by the mammary epithelial cells. Our observation is consistent with earlier reports that bGH did not change glucose uptake by the mammary gland of lactating dairy cows (Davis et al., 1988; McDowell et al., 1987; Miller et al., 1991). It was also found GH had no effect on glucose transporter expression in the mammary cells (Zhao et al., 1996), and that glucose uptake of

mammary gland was independent of IGF-I concentration during lactation in goat (M. O. Nielsen et al., 2001).

We found IGF-I significantly stimulated amino acid uptake of MAC-T cell, while GH had no such effect, indicating that GH-stimulated IGF-I, but not GH itself, may increase amino acid transport into the epithelial cells of mammary gland, thereby increasing milk protein synthesis. This effect of IGF-I on amino acid transport is consistent with the findings that overexpression of IGF-I in porcine lactating mammary tissue increases amino acid transport (Gronlund et al., 2003). A previous study found GH had no effect on amino acid uptake of mammary gland (Miller et al., 1991) in lactating cows, which is consistent with our data. IGF-I was reported to increase amino acids uptake in both MAC-T cell line and MAC-T-based cell line transfected to secrete IGF-I under the constitutive SV40 promoter (Robinson et al., 2001). Recently, IGF-I was found to upregulate expression of some genes that were involved in transport and biosynthesis of amino acids, including several amino acid transport proteins, argininosuccinate and asparagine synthetases (Pacher et al., 2007) in MCF7 breast cancer cells. It remains to be determined whether IGF-I upregulates amino acids transporters in MAC-T cells, thereby increasing amino acids uptake.

In the mammary gland, the long-chain fatty acids incorporated into milk triglycerides are derived from mobilized fat stores and from dietary sources, whereas short- and medium chain fatty acids are synthesized within mammary tissue (Bauman and Griinari, 2003). The mechanism of fatty acid uptake by mammary epithelial cells has not been identified. There have been few reports on the effect of GH or IGF-I on fatty acid uptake in the mammary gland and the reports were conflicting. Mammary uptake of nonesterified free fatty acids (NEFA) increased in mid-lactating cows by GH administration (McDowell et al., 1987), while, early data also showed that

GH administration did not change free fatty acid uptake in mammary tissue of cows (Davis et al., 1988). Oleic acid (C18:1) is the most abundant fatty acid in milk (27.1%, according to the total milk fatty acids) (Jenkins, 2000). Our data showed that IGF-I significantly decreased oleic acid uptake by MAC-T cells and GH had no effects on the uptake. These data suggest that increased milk fat synthesis in the mammary gland following GH administration is due to increased de novo fatty acid synthesis rather than increased long chain fatty acid uptake.

Major milk proteins are coded by a small group of genes expressed exclusively in the epithelium of the mammary gland during pregnancy and lactation. In vitro studies showed that the expression of β-casein gene was induced by the synergistic action of insulin, glucocorticoid and prolactin (Rosen et al., 1980; Topper and Freeman, 1980) and was inhibited by progesterone (Terada et al., 1988). Several studies have reported the effects of GH on milk protein levels. Administration of GH to pregnant rabbits induced expression of casein and whey proteins in the mammary gland (Malewski et al., 2002). Bovine GH was also shown to induce β-casein gene expression in cultured whole mouse mammary gland (Plaut et al., 1993). Infusion of GH for 63 d to the mammary gland of lactating dairy cows significantly increased β-casein mRNA levels (Yang et al., 2005). Further investigation with cultured mammary explants showed that increased β-casein mRNA resulted from a direct effect of bST on the mammary gland (Yang et al., 2005). GH increased αS1-casein mRNA expression in a bovine mammary epithelial cell (BMEC) clonal line (Sakamoto et al., 2005; Yonekura et al., 2006). Consistent with these reports, our results demonstrated that GH can stimulate milk protein gene αS_1-casein, αS_2-casein, β-casein, and α-lactalbumin RNA expression in MAC-T cells over-expressing bovine GHR. Co-transfection analysis further demonstrated that GH can activate αS1-casein, αS2-casein, β-casein, and α-lactalbumin promoter activity. Sequencing these four milk protein gene promoters showed that

all contained at least one potential STAT5 binding site. GH stimulates the DNA binding activity of STAT5 in cattle in vitro (Yang et al., 2000a). Together these observations suggest that GH stimulation may increase milk protein gene expression through STAT5 signaling.

Our in vivo study showed that GH treatment increased αS1-casein mRNA expression in the mammary gland of lactating cows by 18%. GH administration tended to increase αS2-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin mRNA expression, although these effects were not statistically significant, perhaps because these changes were too slight to be reliably detected by the RPA.

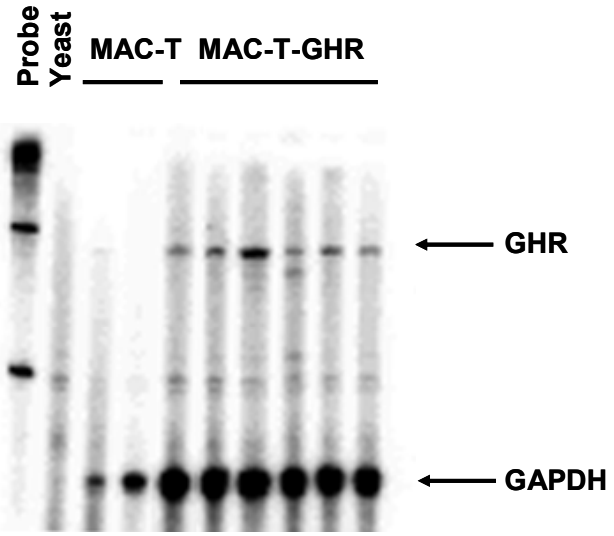
In conclusion, we demonstrated some specific effects of GH or IGF-I on the bovine mammary epithelial cell line MAC-T. GH could directly induce expression of αS1-casein, αS2-casein, β-casein, and α-lactalbumin mRNA in these cells. We also showed that GH administration significantly increased αS1-casein mRNA expression in the lactating mammary gland and tended to increase the expression of other major milk protein genes. These observations together with the fact that GH receptor mRNA and protein are expressed in the epithelial cells of the bovine mammary gland suggest that GH may have direct effects on milk protein gene expression in these cells in cattle, thereby increasing milk protein production. GH probably has no direct effect on glucose, amino acids, or fatty acid transport in mammary epithelial cells in cattle. We also demonstrated that IGF-I could stimulate proliferation of MAC-T cells, and increase amino acids uptake of MAC-T cells, indicating that IGF-I may have similar effects in cattle, thereby partially mediating the GH effect on milk production.

Table 2.1. Primers used in this study.

Name	Sequence[1]	Template and Genbank Accession #	Product size (bp)
bCASA1F1	AATCCATGCCCAACAGAAAG	αS1-casein mRNA,	189
bCAAS1R1	TCAGAGCCAATGGGATTAGG	BC109618	
bCSNAS2F1	AGCTCTCCACCAGTGAGGAA	αS1-casein mRNA,	150
bCSNAS2R1	GCAAGGCGAATTTCTGGTAA	NM_174528.2	
bCSNBF1	GTGAGGAACAGCAGCAAACA	β-casein mRNA,	115
bCSNBR1	TTTTGTGGGAGGCTGTTAGG	NM_181008	
bCSNKF1	CCAGGAGCAAAACCAAGAAC	κ-casein mRNA,	148
bCSNKR1	TGCAACTGGTTTCTGTTGGT	NM_174294	
bLALBAF1	AAAGACGACCAGAACCCTCA	α-lactalbumin mRNA,	143
bLALBAR1	GCTTTATGGGCCAACCAGTA	NM_174378	
bLGBF1	CTTGTGCTGGACACCGACTA	β -lactoblobulin mRNA,	146
bLGBR1	TTGAGGGCTTTGTCGAATTT	NM_173929	
bGAPDHRNAF1	GGGTCATCATCTCTGCACCT	GAPDH mRNA,	177
bGAPDHRNAR1	GGTCATAAGTCCCTCCACGA	XM_001252479	
bCSNAS1PF1	AGAGCTAGCTGACCCAGATAGCCACAGTT	αS1-casein DNA,	2013
bCSNAS1PR1	AGACTCGAGGATGGCAGACTTTTGCTTCC	X59856	
bCSNAS2PF1	AGAGAGCTCCAAACCAGATCCTCCCTCAA	αS1-casein DNA,	2195
bCSNAS2PR1	AGACTCGAGATCTCTGAGGGATTGGCACA	M94327.1	
bCSNBPF1	AGAGAGCTCTCTTCCTGAGAAAAGGGAAATG	β-casein DNA,	1648
bCSNBPR1	AGACTCGAGCCGAAGGAGGAGCTGAATGGAT	X14711.1	
bLALBAPF1	AGAGAGCTCGGACTGATGCTGCAGTTGAA	α-lactalbumin DNA,	1988
bLALBAPR1	AGACTCGAGATGCCTACCAGGAGCAGAGA	U63109.1	
bIGF1F1	GTTGGTGGATGCTCTCCAGT	IGF-I mRNA	148
bIGF1R1	CTCCAGCCTCCTCAGATCAC	XM001251168.1	
bIGF2F1	GCTCAACCAGGGAACTACA	IGF-II mRNA	236
bIGF2R1	CAGAGGCATACAGCACTCCA	XM606794.3	
bIGFBP-1F1	ACCAGCCCAGAGAATGTGTC	IGFBP-1 mRNA	243
bIGFBP-1R1	GTTTGTCTCCTGCCTTCTGC	NM174554.2	
bIGFBP-2F1	CAAGGGTGGCAAACATCAC	IGFBP-2 mRNA	198
bIGFBP-2R1	GAGGTTGTACAGGCCATGCT	NM174555.1	
bIGFBP-3F1	CAGAGCACAGACACCCAGAA	IGFBP-3 mRNA	230
bIGFBP-3R1	TGCCCCGTACTTATCCACACA	AF305199.1	
bIGFBP-4F1	GCCGCACACACGTCTATCTA	IGFBP-4 mRNA	219
bIGFBP-4R1	CGCTTGCATGATTTACACGA	XM581740.3	
bIGFBP-5F1	GTGCGGCGTCTACACTGAG	IGFBP-5 mRNA	155
bIGFBP-5R1	TCACGGGAGTCTCTTTTCGAT	S52657.1	
bIGFBP-6F1	TCTCTAGAAAGGAGAGTAAGCCCCAAGC	IGFBP-6 mRNA	161
bIGFBP-6R1	GAGAATTCAGCACGGAGTCCAGATGTTT	NM001040495.1	
b18sRNAF1	AAACGGCTACCACATCCAAG	18s rRNA	155
b18sRNAR1	CCTCCAATGGATCCTCGTTA	AF176811	

[1]All sequences are written from 5' to 3'. The top sequence of a pair of primers is the forward primer and the bottom sequence the reverse primer. Underlined are restriction enzyme recognition sites added for cloning.

A.



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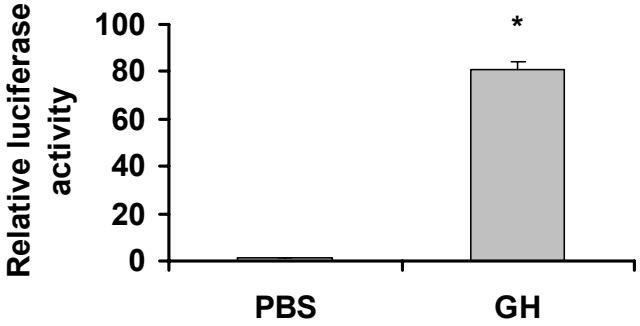
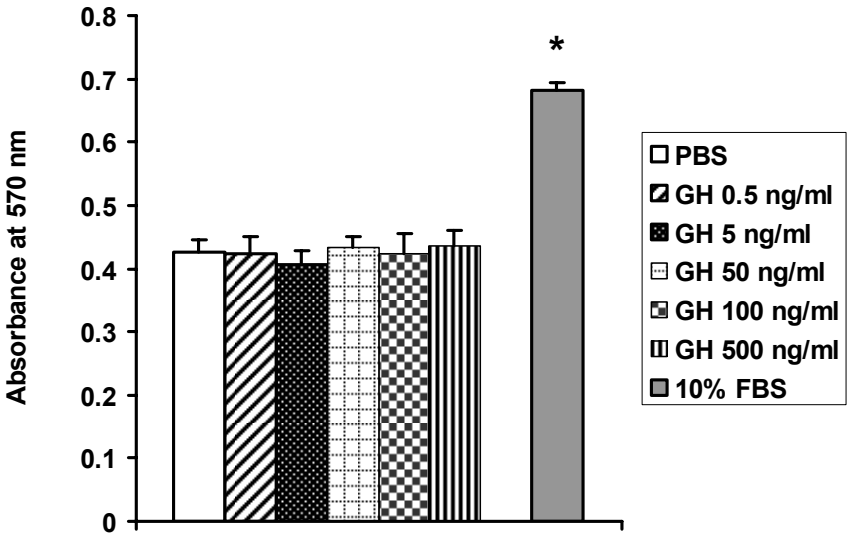


Figure 2.1. MAC-T cells made GH-responsive by transient transfection of GHR and STAT5 expression plasmids. **(A)** Expression of GHR mRNA was confirmed in the transfected MAC-T cells, or MAC-T-GHR cells by RPA. Yeast RNA served as a negative control. **(B)** The MAC-T-GHR cells were co-transfected with a GH-responsive element-containing reporter construct pSPI-LUC. Luciferase reporter assay showed GH stimulated a ~ 80-fold increase in reporter gene activity from pSPI-LUC. Values are expressed as means ± SEM (n = 4). "*" indicates $P < 0.01$ vs. PBS.

A.



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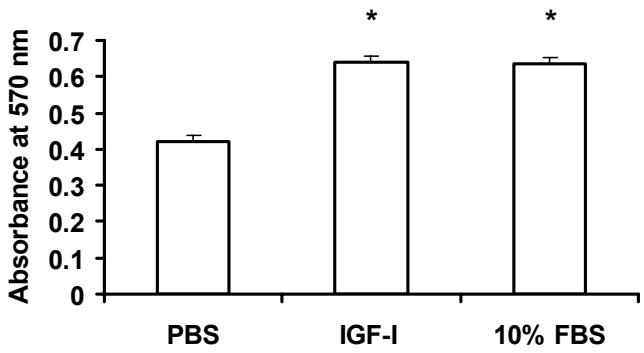
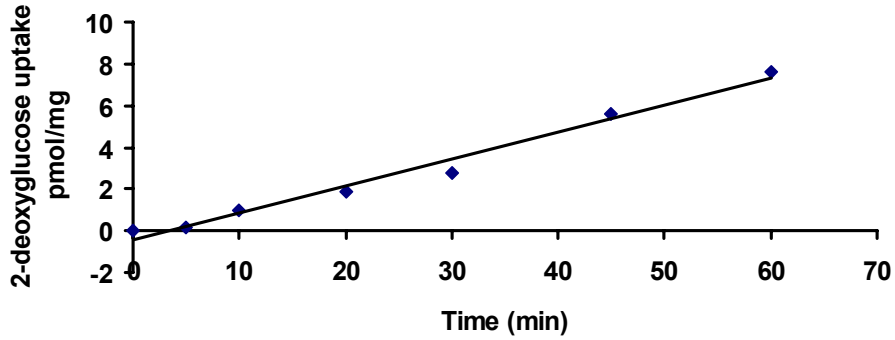


Figure 2.2. Effects of GH and IGF-I on proliferation of MAC-T-GHR and MAC-T cells, respectively. **(A)** Effects of GH on proliferatrion of MAC-T-GHR cells. MAC-T cells were transfected with GHR and STAT5 expression plasmids, and were treated with indicated concentrations of GH for 16 h. Nonradioactive CellTiter 96 assay showed GH had no effect on proliferation of these cells ($P = 0.21$). 10% FBS was used as a positive control. **(B)** Effects of IGF-I on cell proliferation of MAC-T cells. MAC-T cells were treated with PBS, 200 ng/mL of IGF-I, or 10% FBS for 16 h. IGF-I increased MAC-T cell proliferation by 50%. "*" indicates $P < 0.01$, compared to PBS. Values are expressed as means ± SEM (n = 4).

A.



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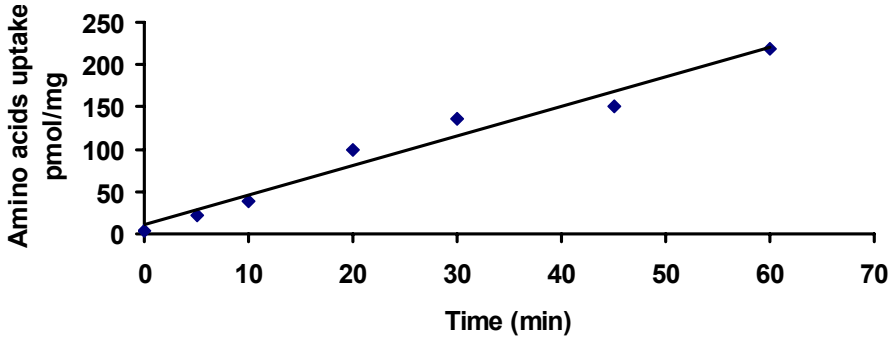

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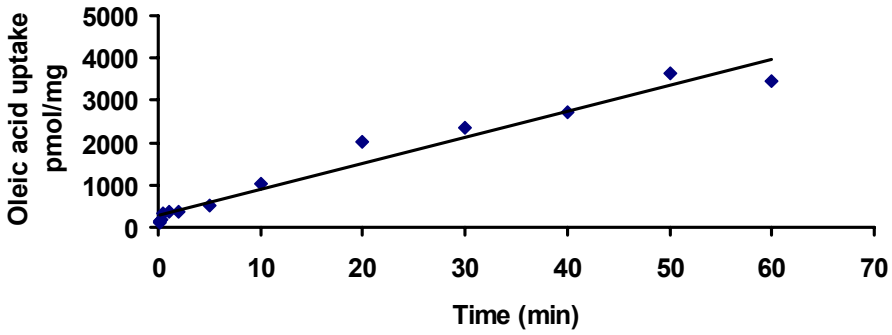
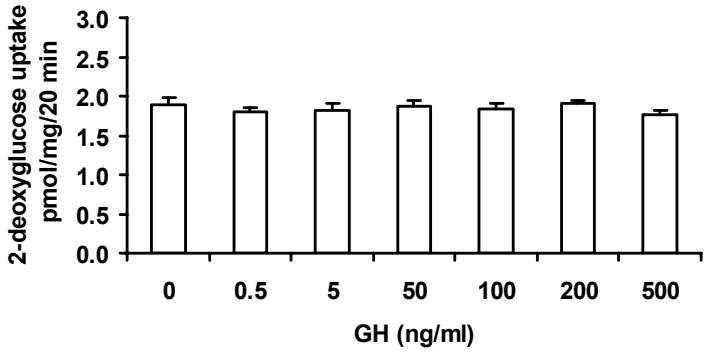


Figure 2.3. Time-course study of [^{3}H]-2-deoxyglucose **(A)**, [^{3}H]-amino acids **(B)**, and [^{3}H]-oleic acid uptake **(C)** by MAC-T cells. The MAC-T cells were incubated with [H^3]-labeled 2-deoxy-glucose, mixture of amino acids, or oleic acid for different times. The cells were lysed and the radioactivity of the cell lysates was measured. The accumulated radioactivity in the cells was normalized to protein concentration of the cell lysates. The accumulation of [^{3}H]-2-deoxyglucose, amino acids, and oleic acid increased linearly with time over 60 min.

A.



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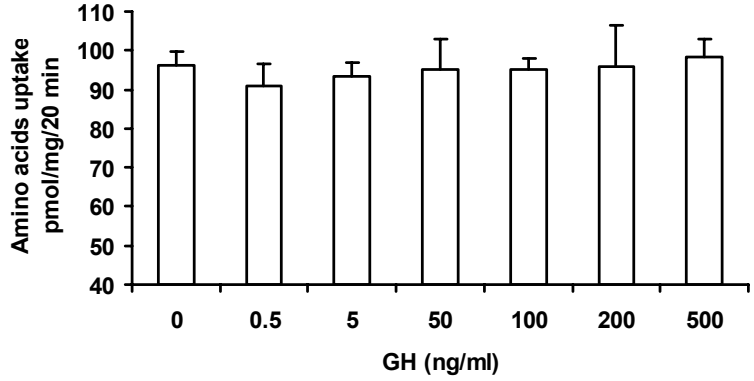

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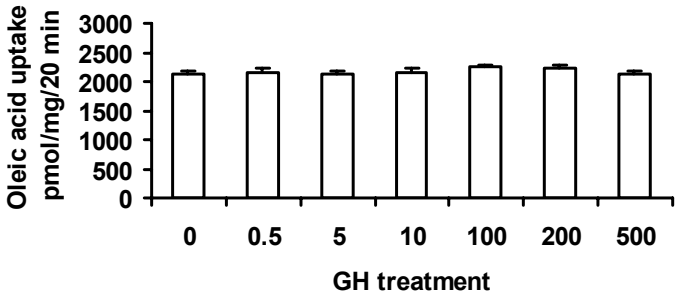
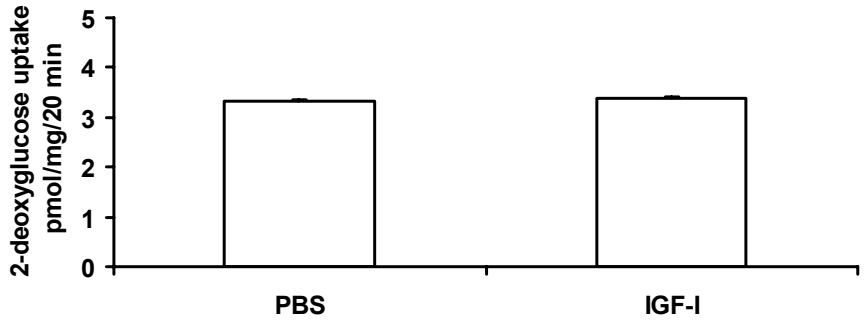


Figure 2.4. Effects of GH on nutrient uptake in MAC-T-GHR cells. Effects of GH on [^{3}H]-2-deoxyglucose **(A)**, [^{3}H]-amino acids **(B)**, and [^{3}H]-oleic acid uptake **(C)** of MAC-T-GHR cells. The MAC-T-GHR cells were treated with 0, 0.5, 5, 10, 100, 200, or 500 ng/mL of GH for 16 h. Then they were incubated with [^{3}H]-2-deoxyglucose, or [^{3}H]-amino acid mixture, or [^{3}H]-oleic acid at room temperature for 20 min. Radioactivity of cells was normalized to protein concentration. Values are expressed as means ± SEM (n = 3). GH had no significant effect on 2-deoxyglucose uptake ($P = 0.56$), amino acids uptake ($P = 0.65$), or oleic acid uptake ($P = 0.58$).

A.



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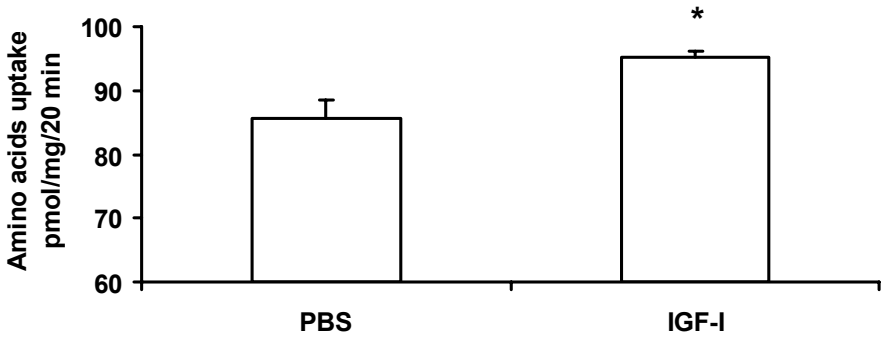

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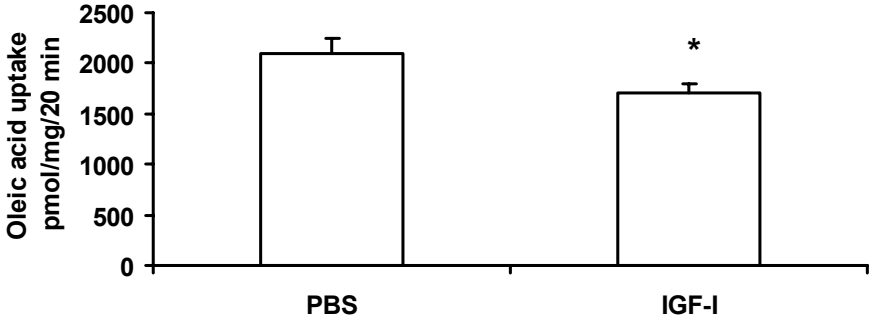
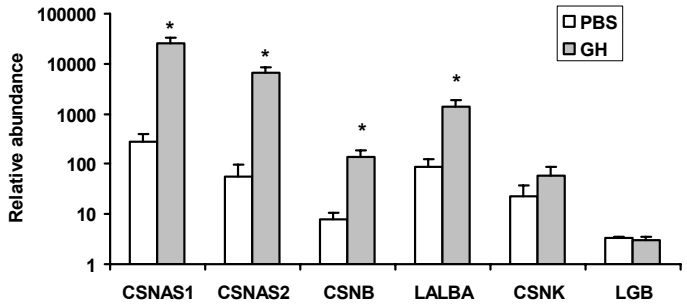


Figure 2.5. Effects of IGF-I on nutrient uptake in MAC-T cells. Effects of IGF-I on [^{3}H]-2-deoxyglucose **(A)**, [^{3}H]-amino acids **(B)**, and [^{3}H]-oleic acid uptake **(C)** by MAC-T cells. The MAC-T cells were treated with 200 ng/mL of IGF-I or PBS for 16 h. Then they were incubated with [^{3}H]-2-deoxyglucose, or [^{3}H]-amino acid mixture, or [^{3}H]-oleic acid at room temperature for 20 min. Radioactivity of cells was normalized to protein concentration. IGF-I had no effect ($P = 0.24$) on 2-deoxy glucose uptake of MAC-T cells **(A)**, stimulated amino acids uptake ($P < 0.05$) **(B)**, and decreased oleic acid uptake ($P < 0.05$) **(C)**. Values are expressed as means ± SEM of independent cultures (n = 3). "*" indicates $P < 0.05$ vs control.

A.



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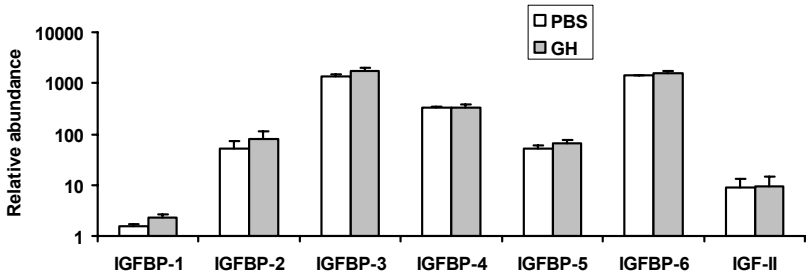

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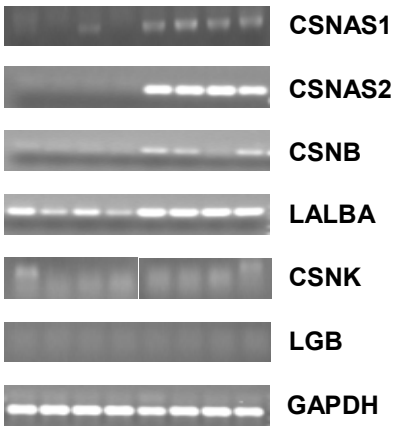
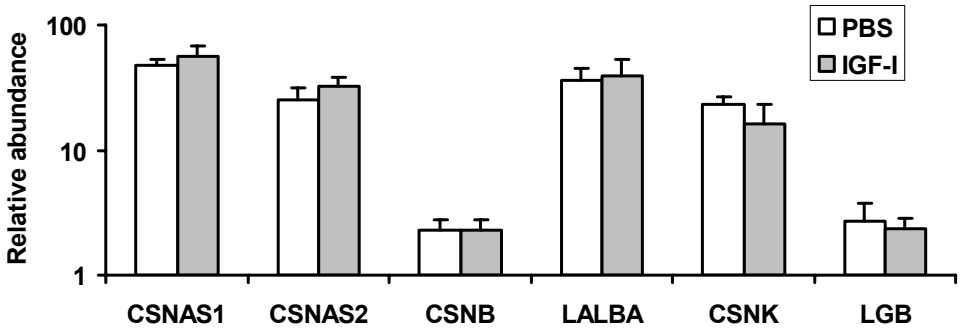


Figure 2.6. Effects of GH on milk protein, IGF and IGFBP mRNA expression in MAC-T-GHR cells. The MAC-T-GHR cells were treated with GH or PBS for 16 h before total RNA was isolated. Real-time PCR was performed to quantify mRNA expression. Gene expression values were normalized to GAPDH mRNA and are expressed as relative values. Values are expressed as means ± SEM (n = 4). "*" indicates a significant difference from the respective control ($p < 0.01$). **(A)** GH increased CSNAS1, CSNAS2, CSNB and LALBA mRNA expression in MAC-T-GHR cells ($P < 0.01$). **(B)** GH had no effects on IGFBP-1 to IGFBP-6 or IGF-II mRNA expression (P = 0.45, 0.70, 0.10, 0.99, 0.14, 0.37, 0.91, respectively). IGF-I mRNA was not detetable in MAC-T-GHR cells (data not shown). **(C)** Agarose gel images of products of RT-PCR of CSNAS1, CSNAS2, CSNB, LALBA, CSNK, and LGB, and GAPDH mRNA.

A.



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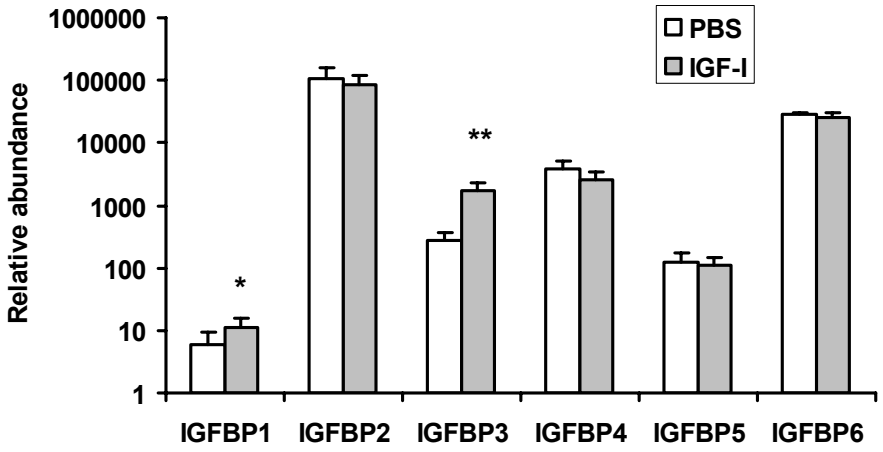
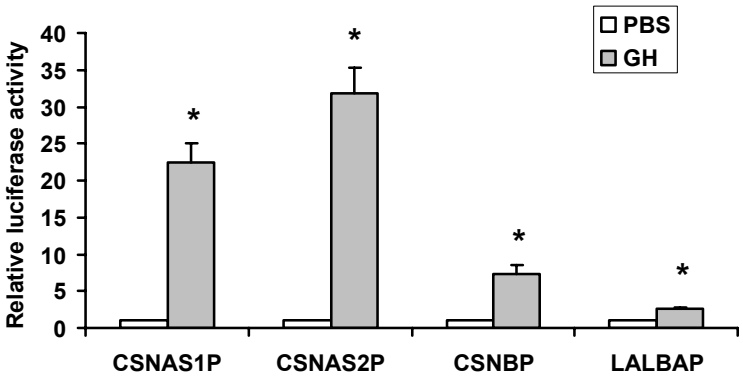


Figure 2.7. Effect of IGF-I on milk protein and IGFBP gene expression in MAC-T cells. The MAC-T cells were treated with 200 ng/mL of IGF-I or PBS for 16 h before total RNA was isolated. Real-time PCR was performed to quantify mRNA expression. **(A)** IGF-I had no significant effect on milk protein gene expression. For CSNAS1, CSNAS2, CSNB, LALBA, CSNK, and LGB, $P = 0.83, 0.27, 0.78, 0.33, 0.91, 0.93$, respectively. **(B)** IGF-I increased IGFBP-1 ($P < 0.05$) and IGFBP-3 ($P < 0.01$) mRNA expression in MAC-T cells, but had no significant effect on expression of the other IGFBP mRNA. For IGFBP-2, 4, 5, 6, $P = 0.61, 0.15, 0.52, 0.68$, respectively. Values are expressed as means ± SEM (n = 4). "*" indicates $P < 0.05$ and "**" indicates $P < 0.01$ vs PBS.

A.



B.

CSNAS1P

```
...TCACTTC TTTGTTGTAA ACTCTCCTTA GAATTTCTTG GGAGAGGAAC TGAACAGAAC    -120
ATTGATTTCC TATGTGAGAG AATTCTTAGA ATTTAAATAA ACCTGTTGGT TAAACTGAAA    -60
CCACAAAATT AGCATTTTAC TAATCAGTAG GTTTAAATAG CTTGGAAGCA AAAGTCTGCC    +1
```

CSNAS2P

```
...CTATCGA ATTTTTGTAC CCCATATTCC TCAGAATGCT TCTACTCAGT TTGATTTAGA    -120
TGGTTTGAAA TACCTGAACA GAATTTGAAT TCTAAGAAGT CATGACGATT AGAATTGCTG    -60
GATTCAGGTA TTTCAAACCA CAGAATTACC ATATTATGGA GGAACAAGGT ATAAATAGTG    +1
```

CSNBP

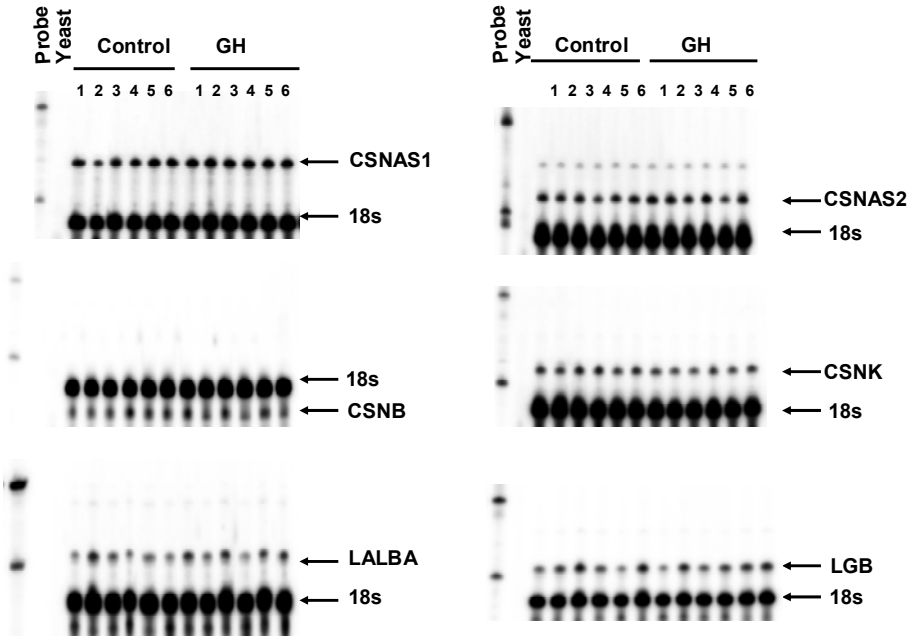
```
...GAATTAT TCCCTTTAAA ATGCTCCCCA GAATTTTTGG GGACAGAAAA ATAGGAAGAA    -120
TTCATTTTCT AATCATGCAG ATTTCTAGGA ATTCAAATCC ACTATTGGTT TTATTTCAAA    -60
AGGCTCCACT ATTTCCAGTT AAGAATACTG GAGTGGATTG TTGTGCCAAT CCCTCAGAGA    +1
```

LALBAP

```
...TCTGTAT GTCTCTAAGA GGAAGGAGGA GTTGGCCGTG GACCCTTTGT GCATTTTCTG    -120
ATTGCTTCAC TTGTATTACC CCTGAGGCCC CCTTTGTTCC TGAAATAGGT TGGGCACATC    -60
TTGCTTCCTA GAACCAACAC TACCAGAAAC AACATAAATA AAGCCAAATG GGAAACAGGA    +1
```

Figure 2.8. Transfection analysis of bovine milk protein gene promoters in MAC-T cells **(A)**. Four milk protein gene promoters (CSNAS1P, CSNAS2P, CSNBP, and LALBAP) were cloned into pGL2b. The MAC-T cells were co-transfected with a milk promoter-reporter gene plasmid, and STAT5 and GHR expression plasmids, and pRL-CMV. Twenty-four h after transfection, the cells were serum-starved for 8 h, followed by 500 ng/mL of GH or PBS treatment for 16 h before dual-luciferase assay. The transfection was repeated four times. The firefly luciferase activity from a milk protein promoter-reporter construct was divided by the *renilla* luciferase activity from pRL-CMV in the same well to normalize variation in transfection efficiency. "*" indicates $P < 0.01$ between PBS and GH-treated cells. Each of these promoters contains at least one potential STAT5 binding site (TTCNNNGAA), which is underlined **(B)**.

A.



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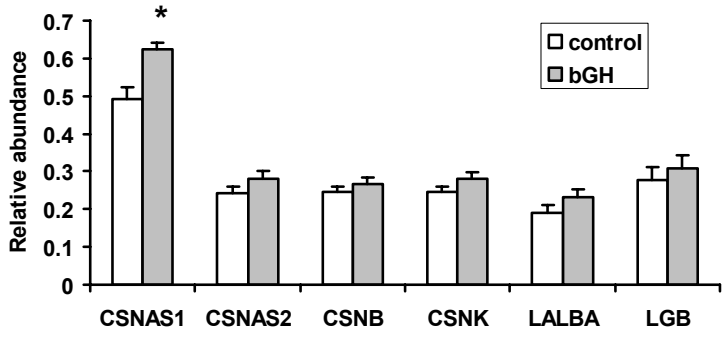


Figure 2.9. Effect of GH administration on milk protein gene expression in bovine mammary gland. The mammary gland RNA was isolated from six non-pregnant lactating cows administerd with bGH and six cows injected with excipient. (A) Ribonuclease protection assays of milk protein αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin mRNA. A milk protein mRNA and 18s rRNA (loading control) was quantified simultaneously with Yeast RNA serving as a negative control. Arrows point to ribonuclease-protected bands. (B) Density analysis of the abundance of the six milk protein mRNA relative to that of 18s rRNA. "*" indicates $P < 0.05$ compared to control.

Chapter III Role of Connective Tissue Growth Factor (CTGF) in IGF-I Induced Proliferation of Bovine Mammary Epithelial Cells

Abstract

The bioactivity of IGF-I in the cellular microenvironment is modulated by both inhibitory and stimulatory IGF binding proteins (IGFBP), whose production is partially under the control of IGF-I. Connective tissue growth factor (CTGF), a 38-kDa cysteine-rich peptide, is a multifunctional growth factor for fibroblasts, chondrocytes and vascular endothelial cells, and is also considered one of the low affinity IGFBP. This study determined the role of CTGF in IGF-I induced proliferation of MAC-T, a bovine mammary epithelial cell line. Both microarray and real-time PCR analyses demonstrated that IGF-I decreased CTGF mRNA expression in MAC-T cells. GH had no effect on CTGF mRNA expression in MAC-T cells, but GH administration to cows decreased CTGF mRNA expression in the mammary epithelia. Because GH is known to increase IGF-I production, these in vitro and in vivo data suggest that IGF-I may also decrease mammary CTGF mRNA expression in cows. IGF-I and CTGF each stimulated MAC-T cell proliferation ($P < 0.05$). However, added together, CTGF attenuated the proliferating effect of IGF-I on MAC-T cells, and this attenuation was reversed by additional IGF-I. The p42/44 MAPK pathway inhibitor PD098059 and mTOR inhibitor rapamycin had no effect on IGF-I suppression of CTGF gene expression in MAC-T cells. In contrast, blocking the PI-3 kinase signaling pathway by LY294002 reversed IGF-I-decreased CTGF mRNA expression, indicating that IGF-I suppresses CTGF mRNA expression in MAC-T cells through the PI-3K/Atk pathway. CTGF had no effect on IGF-I induced phosphorylation of IGF-I receptor (IGF-IR) or total IGF-IR expression in MAC-T cells, indicating that CTGF attenuation of IGF-I stimulation of cell

proliferation is not mediated by decreased availability or activity of IGF-I or IGF-IR expression, and rather that CTGF may attenuate IGF-I stimulation of mammary epithelial cell proliferation through a postreceptor interaction with the IGF-IR signaling pathways.

Key Words: CTGF, IGF-I, cell proliferation

Introduction

Insulin-like growth factor-I (IGF-I) is essential for normal growth and development (Cohick and Clemmons, 1993). It is one of the most potent stimulators of cell growth and proliferation and a potent inhibitor of programmed cell death. The proliferation of various cells can be stimulated by IGF-I, including fibroblasts, vascular smooth muscle cells, epithelial cells, and endothelials (Nicosia et al., 1994).

Biological actions of IGF-I are mediated by the IGF-I receptor (IGF-IR) (Mauro et al., 2003). Binding of IGF-I to IGF-IR activates the receptor kinase, leading to receptor autophosphorylation and tyrosine phosphorylation of multiple substrates, including insulin receptor substrate (IRS) and Src Homologous and Collagen (SHC) proteins (Grimberg, 2003). Through these initial tyrosine phosphorylation reactions, IGF-I signals are transduced to a complex network of intracellular lipid and serine/ threonine kinases that are ultimately responsible for cell proliferation, modulation of tissue differentiation, and protection from apoptosis (Hadsell et al., 2002).

IGF-I is present in plasma and other biological fluids predominantly in a complex with IGF binding proteins (IGFBP). IGFBP comprise a family of six homologous proteins that bind IGF-I with a high affinity, equal or higher than the affinity of IGF-I for IGF-IR (Baxter et al., 1998). IGFBP have both IGF-dependent and IGF-independent effects (Firth and Baxter, 2002). IGFBPs regulate growth and development by regulating IGF-I transport to tissues and IGF-I bioavailability to IGF-I receptors at the cell membrane level. Excess IGFBP lead to inhibition of IGF-I action and growth retardation with impaired organogenesis (Hwa et al., 1999).

Connective tissue growth factor (CTGF, also known as hypertrophic chondrocyte – specific gene product 24 or Hcs24), is a member of the CCN family (including Cry61, CTGF, and Nov) of immediate early proteins (Takigawa, 2003) that are multifunctional and involved in cell proliferation, differentiation, migration, angiogenesis, and tumorigenesis (Lau and Lam, 1999). CTGF is a secretory protein and contains a motif that is homologous to IGFBP, and it can specifically bind IGF-I with relatively low affinity (Kim et al., 1997). CTGF can also be a negative regulator of cell growth and it does this through interactions with growth modifiers inside the cell (Moritani et al., 2003).

In the previous chapter, IGF-I was found to stimulate MAC-T cell proliferation. The objective of this study was to determine the underlying mechanism. Microarray analysis was performed to detect the genes that were regulated by IGF-I in MAC-T cells. CTGF was among the genes that were down-regulated by IGF-I. Further experiments were conducted to determine whether CTGF was involved in IGF-I induced proliferation of MAC-T cells as well as mammary epithelial cells in vivo. We also determined the signaling pathway that mediates IGF-I downregulation of CTGF expression in MAC-T cells.

Materials and Methods

Cell culture and treatment

MAC-T cells were cultured as described in Chapter 2. For the cell proliferation assay, the cells were treated with 1) Phosphate buffered saline (PBS), 2) 200 ng/mL IGF-I (Sigma, St. Louis, MO), 3) 200 ng/mL CTGF (Fitzgerald, Concord, MA), 4) 200 ng/mL IGF-I + 200 ng/ml CTGF, 5) 300 ng/mL IGF-I + 200 ng/mL CTGF, 6) 400 ng/mL IGF-I + 200 ng/mL CTGF, 7) 500 ng/mL IGF-I + 200 ng/mL for 16 h followed by cell proliferation assay or Western blotting analysis. The MAC-T cells were also treated with: 1) PBS + DMSO (control); 2) 200 ng/mL

IGF-I, 3) 20 μM Rapamycin (Sigma), an inhibitor of p70 S6 kinase; 4) 50 μM LY294002 (Calbiochem, San Diego, CA), a PI-3 kinase/Akt inhibitor; 5) 50 μM PD098059 (Calbiochem), a MEK1/2 inhibitor; 6) 200 ng/mL IGF-I and 20 μM Rapamycin; 7) 200 ng/mL IGF-I and 50 μM LY294002; 8) 200 ng/mL IGF-I and 50 μM PD098059 for 16 h followed by total RNA isolation and mRNA expression analysis.

Total RNA extraction

Total RNA from MAC-T cells or bovine mammary tissues was extracted as described in Chapter 2. The bovine mammary tissue used in this study was the same as described in Chapter 2.

Microarray analysis

MAC-T cells were grown until 60% confluency. The cells were serum-starved for 8 h and were then treated with 200 ng/mL of IGF-I for 16 h or PBS (control). Total RNA was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) and further purified using RNeasy MiniElute Cleanup kit (Qiagen). RNA quality was confirmed using the Agilent Bioanalyzer 2100. Three IGF-I and three PBS- treated RNA samples were used in microarray analysis. Microarray analysis (RNA labeling, hybridization, scanning, preliminary data analysis) was conducted by the Virgina Tech Core laboratory Facilities using the Affymetrix Genechip Bovine Genome Arrays that contained ~24,000 bovine transcripts (Affymetrix, West Sacramento, CA). Microarray data were analyzed using Genesifter software (Cox et al., 2006). mRNA expression differences > 1.5 fold and with $P < 0.05$ (by t-Test, Benjamini and Hochberg adjustment) were considered significant.

Real-time PCR

Real-time PCR, performed as described in Chapter 2, was used to quantify mRNA in MAC-T cells and mammary gland tissue. GAPDH mRNA was used as an endogenous control in

real time PCR of MAC-T mRNA and β-actin mRNA was used as an endogenous control in real time PCR of mammary tissue mRNA. The primers used in this real time PCR were also shown in Table 2.1. The relative expression level of a target mRNA was calculated as $2^{-\Delta Ct}$, where ΔCt was obtained directly by subtracting the Ct for this mRNA from the Ct for the endogenous control mRNA.

Cell proliferation assay

This assay was performed using the Nonradioactive CellTiter 96 assay (Promega), as described in Chapter 2.

Western blot analysis

The effect of CTGF on IGF-I-induced proliferation of MAC-T cells was determined by treating cells with IGF-I, CTGF, or IGF-I together with CTGF. To detect the effects of CTGF on phospho-IGF-I receptor expression, the MAC-T cells were treated as described before. The cellular protein lysates were prepared by lysing the cells in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40) containing Complete Mini protease inhibitor (Roche, Indianapolis, IN). Total protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. For Western blot analysis, 40 μg of MAC-T cell protein were separated by electrophoresis in a 12% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% nonfat dried milk in TBST (20 mM Tris-HCl pH 7.5, 500 mM NaCl and 0.05% Tween 20) for 3 h, the membrane was incubated with 1:1000 dilutions of rabbit anti-Phospho-IGF-IR antibody (Cell Signaling, Boston, MA, catalog # 3021s) at 4 ºC overnight. After being washed 3 times in TBST, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology) at 1:2,000 dilutions for 2 h at room temperature. The

membrane was subsequently incubated in SuperSignal West Pico chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL) for 5 min, and the chemiluminescent signals were detected by exposure to X-ray films. The membrane was then stripped by immediately incubating the membrane in Restore™ Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL) for 30 minutes at room temperature. After blocking and washing, the membrane was incubated with 1:1000 dilutions of rabbit anti- total IGF-I R (Cell Signaling, catalog # 3027) at 4 ºC overnight. The same membrane was stripped again and reprobed with a beta-actin antibody (Cell Signaling, catalog # 4967). The intensities of phospho-IGF-IR, total IGF-IR, and beta-actin bands were measured using ImageJ and the measured band intensity of phospho-IGF-IR protein or total IGF-IR protein in a sample was divided by that of beta-actin in the same sample to normalize variation in protein loading.

Statistic Analysis

The pairwise comparisons were made using t-Test. Multiple comparisons were done using ANOVA followed by the Tukey test. All these procedures were performed using the GLM of SAS. All data were expressed as mean ± SEM.

Results

Effects of IGF-I on mRNA expression in MAC-T cells

On average, the mRNA for ~30% of the 24,000 bovine genes in the MAC-T cells were detectable in the microarray analysis. 781 transcripts were at least 1.5 fold different ($P < 0.05$) in abundance between PBS- and IGF-I-treated MAC-T cells. Among them, 498 transcripts were up-regulated and 283 transcripts down-regulated by IGF-I. Table 3.2 lists the top 10 IGF-I upregulated genes and Table 3.3 lists the top 10 downregulated genes in MAC-T cells, including GABA(A)RAPL1, whose involvement in IGF-I action on the mammary gland is not obvious.

Table 3.4 listed 10 genes that were involved in the regulation of cell proliferation, apoptosis or nutrient metabolism. The microarray based expressions of most of these 10 genes were confirmed by real-time PCR (Table 3.4). Of these 10 genes, CTGF was particulary interesting, because it is a binding protein of IGF-I (Kim et al., 1997), and the relation of CTGF to IGF-I was therefore further investigated in this study.

Effect of CTGF on IGF-I induced proliferation of MAC-T cells

Treatment with 200 ng/mL of IGF-I, or 200 ng/mL of CTGF stimulated MAC-T cell proliferation as compared with PBS control ($P < 0.05$) (Figure 3.1A). Treatment with 200 ng/mL of CTGF together with 200 ng/mL of IGF-I decreased the proliferation of MAC-T cells compared with 200 ng/mL of IGF-I alone ($P < 0.05$) (Figure 3.1A), but increased the proliferation compared with PBS ($P < 0.05$). Treatment of these cells with 200 ng/mL of CTGF and 300 ng/mL of IGF-I stimulated a greater increase in MAC-T cell proliferation compared to 200 ng/mL of CTGF and 200 ng/mL of IGF-I, and this increase was even greater when IGF-I concentration was increased to 400 ng/mL or 500 ng/mL. ($P < 0.05$) (Figure 3.1B).

Effect of CTGF on IGF-I activation of IGF-I receptor

To determine if CTGF inhibits IGF-I activation of MAC-T cells proliferation by an IGF-I dependent mechanism, phospho-IGF-IR expression was compared in the MAC-T cells treated with 1) PBS; 2) 200 ng/mL IGF-I; 3) 200 ng/mL IGF-I + 200 ng/mL CTGF; 4) 200 ng/mL CTGF by western blotting analysis. It was found that there was no phospho-IGF-IR expression in PBS or CTGF treatment groups (Figure 3.2A). Both IGF-I and "IGF-I + CTGF" groups induced phospho-IGF-IR expression, but there was no difference in phospho-IGF-IR expression between them ($P = 0.78$) (Figure 3.2B). Neither IGF-I nor CTGF affected total IGF-IR expression in MAC-T cells (Figure 3.2B).

Signaling pathway mediating IGF-I inhibition of CTGF expression

To determine the signaling pathway by which IGF-I inhibits CTGF mRNA expression, MAC-T cells were treated with several inhibitors of proteins involved in IGF-I signaling pathways (Figure 3.3A). Real-time PCR data showed that LY294002 increased CTGF mRNA expression compared with PBS (Figure 3.3B). Neither Rapamycin nor PD98059 alone had effects on CTGF mRNA expression in MAC-T cells (P = 1.0, 0.49, respectively). LY294002 and IGF-I together increased CTGF mRNA expression, compared with IGF-I alone ($P < 0.05$), but it had no effect on CTGF expression compared with PBS (P = 1.00). Combination of IGF-I and Rapamycin or of IGF-I and PD98059 had no effects on CTGF mRNA expression (P = 1.00, 0.97 respectively) compared with IGF-I alone (
Figure 3.3).

Effect of GH administration on mammary gland expression of CTGF mRNA in cows

The effect of GH administration on CTGF mRNA expression in the bovine mammary tissue was studied. GH administration to lactating cows decreased mammary expression of CTGF mRNA (P = 0.07) (Figure 3.4).

Effect of GH on CTGF mRNA expression in MAC-T cells

MAC-T cells were transfected with GHR and STAT5 expression plasmid as described in Chapter 2. GH had no effect on CTGF mRNA expression in the transfected MAC-T-GHR cells (P = 0.88) (Figure 3.5).

Discussion

Both microarray and real-time PCR analyses demonstrated that CTGF mRNA expression was decreased by IGF-I in MAC-T cells. This effect of IGF-I may also occur in vivo because GH administration decreased CTGF mRNA in bovine mammary gland and GH is known to increase IGF-I in vivo, and GH had no direct effect on CTGF mRNA expression in MAC-T cells.

Previous studies found that the effect of IGF-I on CTGF expression was cell type-dependent. In renal fibroblasts, CTGF mRNA expression was decreased by IGF-I (Lam et al., 2003). However, IGF-I had no effect on CTGF mRNA expression in retinal vascular endothelial cells (RVEC) (Wunderlich et al., 2000) and IGF-I increased CTGF mRNA expression in Ewing's

sarcoma (ES) cells (Strammiello et al., 2003). To our knowledge, this study is the first to have demonstrated an inhibiting effect of IGF-I on CTGF mRNA expression in mammary epithelial cells.

CTGF is widely considered to be a downstream mediator of some of the fibrogenic actions of TGF-β, particularly in the promotion of fibroblast proliferation and ECM production. TGF-β upregulated CTGF expression in several fibrotic disorders (Paradis et al., 1999; Chen et al., 2000) involving inflammation and connective tissue accumulation (Paradis et al., 1999; Chen et al., 2000). The induction of CTGF by TGF-β is also cell-type specific; for example, CTGF mRNA was increased markedly by TGF-β in human foreskin fibroblasts (Igarashi et al., 1993) but not in epidermal keratinocytes (Grotendorst et al., 1996; Leask et al., 2003). The mechanism by which TGF-β regulates CTGF gene expression is unknown. In this study, IGF-I inhibition of CTGF mRNA expression was reversed when the cells were incubated with the PI-3 kinase/Akt inhibitor LY294002. LY294002 alone also increased basal expression of CTGF mRNA. mTOR inhibitor rapamycin or MEK1/2 inhibitor PD98059 had no effects on CTGF expression. It can therefore be concluded that CTGF mRNA expression is regulated by IGF-I through the PI-3 kinase/Akt pathway, not through mTOR or the MAPK pathway.

Overexpression of CTGF induced lactogenic differentiation of mouse mammary epithelial cell line HC11 with the increased expression of β-casein (Wang et al., 2007). Overexpression of CTGF in MCF-7 cells induced apoptosis (Hishikawa et al., 1999). Low levels of CTGF were associated with increased metastasis and poor prognosis in breast cancer patients (Xie et al., 2001; Jiang et al., 2004). The metastatic breast cancer cell line, MDA-MB-231, expresses lower levels of CTGF than the less invasive MCF-7 cells (Jiang et al., 2004). All these data suggest that CTGF is a differention inducer and a proliferation inhibitor in the mammary

gland. In our study, CTGF stimulated MAC-T cell proliferation. This result suggests a proliferating effect of CTGF on mammary epithelial cells, in contrast to its role in mouse or human mammary epithelial cells (Hishikawa et al., 1999; Xie et al., 2001; Jiang et al., 2004; Wang et al., 2007). The mechanisms underlying these differences remain to be addressed.

Our study showed that CTGF, however, attenuated the proliferating effect of IGF-I on MAC-T cells and that this attenuation could be reversed by excess IGF-I. CTGF has an IGF-binding domain and was considered a low-affinity IGF-I binding protein (Kim et al., 1997). We found CTGF had no effect on IGF-I induced phosphorylation of IGF-IR protein, indicating that CTGF did not attenuate the effect of IGF-I on cell proliferation through inhibition of the availability or activity of IGF-I on cell membrane. Others found that overexpression of CTGF suppressed IGF-I-dependent Akt phosphorylation and ERK 1/2 phosphorylation in NSCLC cell lines (Chien et al., 2006). It is therefore possible that CTGF may inhibit IGF-I stimulation of MAC-T cell proliferation through a similar inhibitory interaction with post-IGF-IR signaling components.

In summary, IGF-I can directly inhibit CTGF mRNA expression in MAC-T cells. IGF-I does this through the PI-3 kinase/Akt signaling pathway. The decreased expression of CTGF may enhance IGF-I induced MAC-T cell proliferation, and this effect of CTGF does not appear to be mediated through binding to IGF-I.

Table 3.1. Primers used in this study.

Name	Sequence[1]	Template and GenBank #	Product size (bp)
bGAPDHRNAF1	GGGTCATCATCTCTGCACCT	GAPDH mRNA, XM001252479	177
bGAPDHRNAR1	GGTCATAAGTCCCTCCACGA		
bCD1qPF1	GCACTTCCTCTCCAAGATGC	CD1 mRNA, NM001046273.1	204
bCD1qPR1	GTCAGGCGGTGATAGGAGAG		
bCD2qPF1	CCAGACCTTCATCGCTCTGT	CD2 mRNA, NM001076372.1	163
bCD2qPR1	GATCTTTGCCAGGAGATCCA		
bSLC1A5qPF1	TCGATTCGTTCCTGGATCTT	SLC1A5 mRNA, BC123803.1	162
bSLC1A5qpR1	CCAGGCCCAGAATGTTCATA		
bRTP801qPF2	ACAGCAGCAACAGTGGCTTT	RTP801 mRNA, NM001075922.1	208
bRTP801qPR2	ACCTGGCTCACCAGCTGAC		
bCTGFF1	AGCTGACCTGGAGGAGAACA	CTGF mRNA, BC113279.1	138
bCTGFR1	GTCTGTGCACACTCCGAAGA		
bBCL2F1	CCTGTGGATGACCGAGTACC	BCL2 mRNA, XM586976.3	133
bBCL2R1	CCTTCAGAGACAGCCAGGAG		
bGDF5F1	AAGCGTATCACTGCGAAGGT	GDF5 mRNA, AB004301.1	120
bGDF5R1	CAAGTTGGAGGCGTTGATTC		
bPETF1	GGAAGATCCAGATGGTACTCCT	PET mRNA, BC123397.1	124
bPETR1	TACACCTGGGAGCTGTGTGT		
bFAB3F1	TGCAGAAGTGGAATGGACAA	Fatty acid binding protein 3	144
bFAB3R1	GCAGTCAGTGGAAGGAGAGG	mRNA, BT021486.1	
bFAB4F1	AATTGGGCCAGGAATTTGAT	Fatty acid binding protein 4	116
bFAB4R1	TGGTGGTTGATTTTCCATCC	mRNA, NM174314.2	
beNOSF1	CACCTACCACCTCCGAGAGA	nitric oxide synthase	141
beNOSR1	ACATCTCCTGTGCTGAGCTG	mRNA, M89952.1	

[1]All sequences are written from 5' to 3'. The top sequence of a pair of primers is the forward primer and the bottom sequence the reverse primer.

Table 3.2. Top 10 IGF-I upregulated genes in MAC-T cells

Gene name*	GenBank #	Ratio (IGF-I to PBS)
RTP801	CK846550	4.64
Hydroxymethylglutaryl-CoA synthase	CK974002	4.58
FABP3	NM174313.2	3.91
phosphoserine aminotransferase	CF763176	3.25
unknown	CK960423	3.18
unknown	CB166901	3.08
unknown	CK949309	2.96
unknown	BF774362	2.93
unknown	CK847195	2.93
unknown	CK972168	2.87

*RTP801, also named HIF1 (hypoxia-inducible factor 1)-responsive gene. RTP801 is strongly upregulated by hypoxia (Shoshani et al., 2002). When induced from a tetracycline-repressible promoter, RTP801 protected human epithelial breast carcinoma cells and rat pheochromocytoma cells from hypoxia in glucose-free medium and from H_2O_2-triggered apoptosis via a dramatic reduction in the generation of reactive oxygen species (ROS) (Brafman et al., 2004).

Hydroxymethylglutaryl-CoA synthase: (HMG-CoA synthase) catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to produce HMG-CoA and CoA].

FABP3 is fatty acid binding protein 3. It is a transport vehicle of fatty acids between the plasma membrane and mitochondria or peroxisomes for beta-oxidation, and between other cellular organelles for lipid synthesis (Peeters et al., 1991).

Table 3.3. Top 10 IGF-I downregulated genes in MAC-T cells

Gene name*	GenBank #	Ratio (IGF-I to PBS)
unknown	CB424375	0.17
connective tissue growth factor (CTGF)	NM174030.2	0.22
unknown	CK953351	0.22
unknown	BP103230	0.27
unknown	BF774834	0.29
unknown	BP106018	0.30
GABA(A) receptor-associated protein like 1	CB440509	0.31
unknown	CK772698	0.31
unknown	CB533350	0.34
GABA(A) receptor-associated protein like 1	CK966100	0.35

*GABA(A) receptor-associated protein like 1: Gamma-Aminobutyric acid type A receptors - associated protein like 1 (GABARAPL1), also called glandular epithelial cell protein 1 (GEC1) (Pellerin et al., 1993). The deduced 117-amino acid protein shares 87% identity with GABARAP (GABA(A)R-associated protein) (Vernier-Magnin et al., 2001). GABA(A)R mediate fast synaptic inhibition in brain and spinal cord. For efficient synaptic transmission, GABA(A)Rs need to be localized to and anchored at postsynaptic sites in precise apposition to presynaptic nerve terminals that release the neurotransmitter GABA (Kneussel, 2002).

Table 3.4. Validation of microarray data by real-time PCR of 10 mRNAs.

Gene*	IGF-I caused changes (fold) in mRNA expression		
	By Microarray	**By real-time PCR**	**Description**
CTGF	0.22 ($P < 0.05$)	0.15 ($P < 0.01$)	connective tissue growth factor
Cyclin D1	0.42 ($P < 0.05$)	0.77 ($P = 0.37$)	cyclin-dependent kinase
Cyclin D2	1.68 ($P < 0.05$)	1.59 ($P < 0.05$)	cyclin-dependent kinase
SLC1A5	1.73 ($P < 0.05$)	1.20 ($P = 0.12$)	neutral amino acid transporter
eNOS	1.76 ($P < 0.05$)	Not detectable	nitric oxide synthase
PET	2.48 ($P < 0.05$)	5.03 ($P < 0.05$)	pro- and anti-apoptotic activities
BCL2	2.57 ($P < 0.05$)	0.99 ($P = 0.99$)	apoptosis inhibitor
FABP4	3.11 ($P < 0.05$)	4.23 ($P < 0.01$)	fatty acid binding protein
FABP3	3.91 ($P < 0.05$)	4.07 ($P < 0.05$)	fatty acid binding protein
RTP801	4.64 ($P < 0.05$)	9.21 ($P = 0.08$)	pro- and anti-apoptotic activities

*CTGF: Connective tissue growth factor, is multifunctional and involved in cell proliferation, differentiation, migration, angiogenesis, and tumorigenesis (Lau and Lam, 1999). It is considered as one of IGF binding proteins (Kim et al., 1997)

Cyclin D: A member of the cyclin family that regulate cyclin dependent kinase type 4 and 6. Cyclin D plays a key regulatory role during the G1 phase of the cell cycle and its gene is amplified and overexpressed in many cancers (Sweeney et al., 1997).

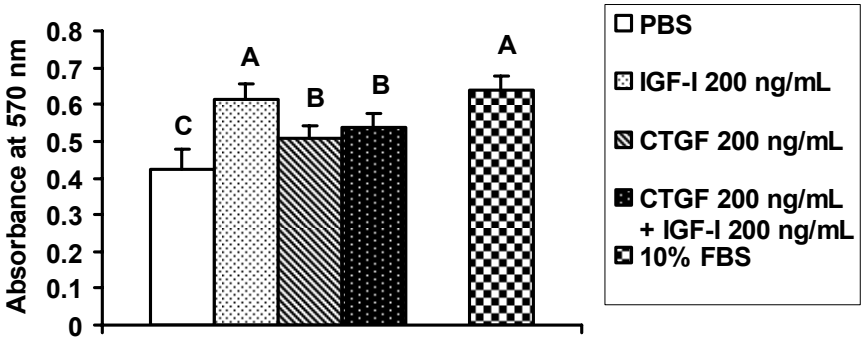
SLC1A5: Solute carrier family 1 (neutral amino acid transporter), member 5. SLC1A5 is a glutamine transporter and involved in glutamine efflux from astrocytes (Brauers et al., 2005).

eNOS: Endothelial nitric oxide synthase. eNOS is important in synthesizing nitric oxide (NO) in endothelial cells from L-arginine. NO accounts for the biologic activity of endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980), which is is important in regulation of vasomotor tone and blood flow by inhibiting smooth muscle contraction and platelet aggregation (Rapoport et al., 1983).

BCL2: A anti-apoptotic protein. Overexpression of Bcl-2 is known to block cytochrome c release, possibly through the inhibition of Bax and Bak (Chao and Korsmeyer, 1998).

FABP4: Fatty acid binding protein 4. It is expressed in human bronchial epithelial cells (HBE) and is strongly upregulated by both IL-4 and IL-13 and downregulated by IFN-gamma (Shum et al., 2006), suggesting a role in allergic airway inflammation. FABP4 is an effective therapeutic agent against severe atherosclerosis and type 2 diabetes in mouse models (Furuhashi et al., 2007).

A.



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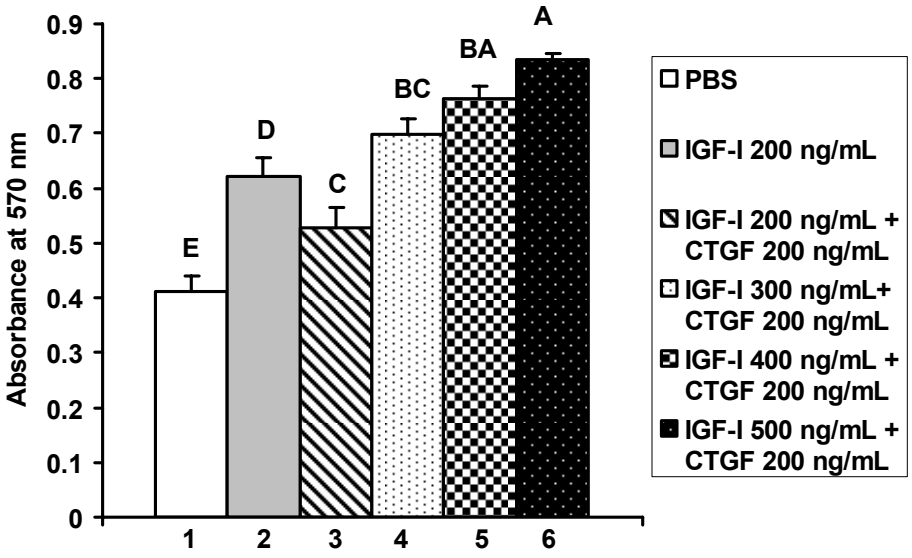
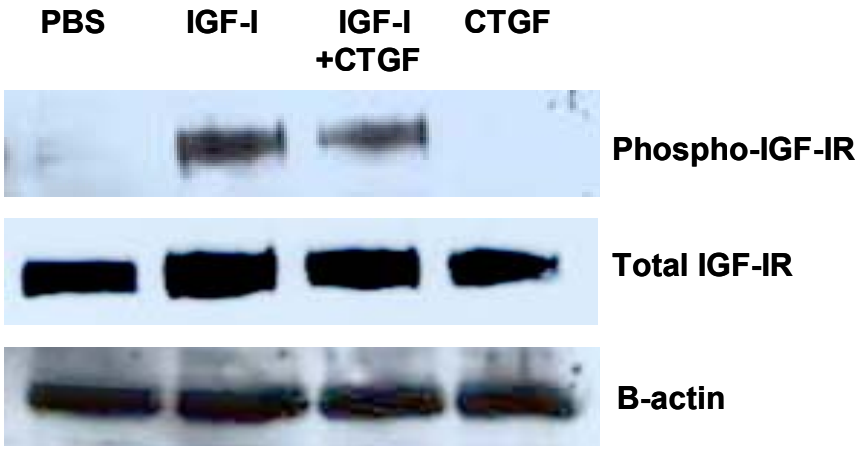


Figure 3.1. Effect of CTGF on IGF-I induced cell proliferation. (A) After 8 h serum starvation, MAC-T cells were incubated with 1) PBS, 2) 200 ng/mL IGF-I, 3) 200 ng/mL CTGF, 4) 200 ng/mL IGF-I + 200 ng/mL CTGF, or 5) 10% FBS for 16 h. Nonradioactive CellTiter 96 assay was performed after these treatments. (B) Incubation of MAC-T cells with PBS, 200 ng/mL IGF-I, or 200 ng/mL CTGF and increasing concentration of IGF-I for 16 h followed by cell proliferation assay. Values are expressed as means ± SEM (n = 4). Means with different letters are significantly different ($P < 0.05$).

A.



B.

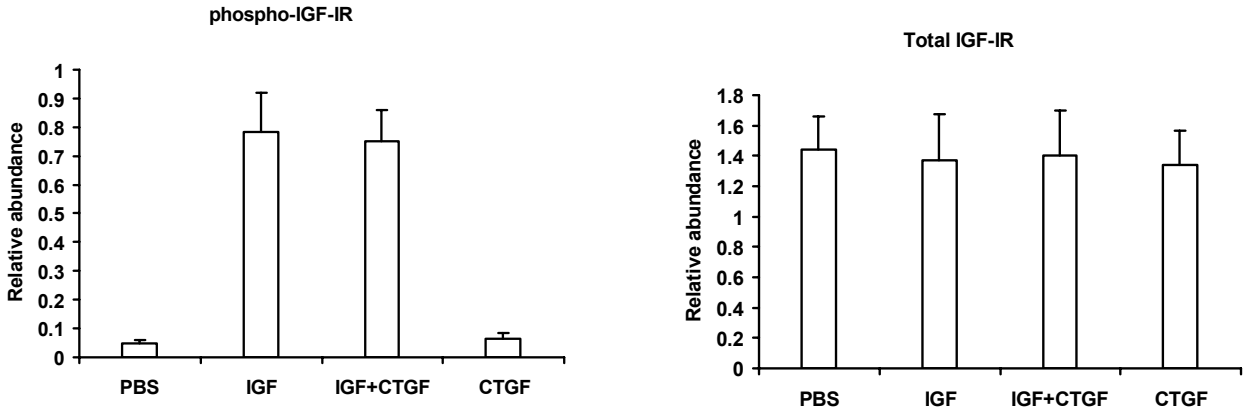
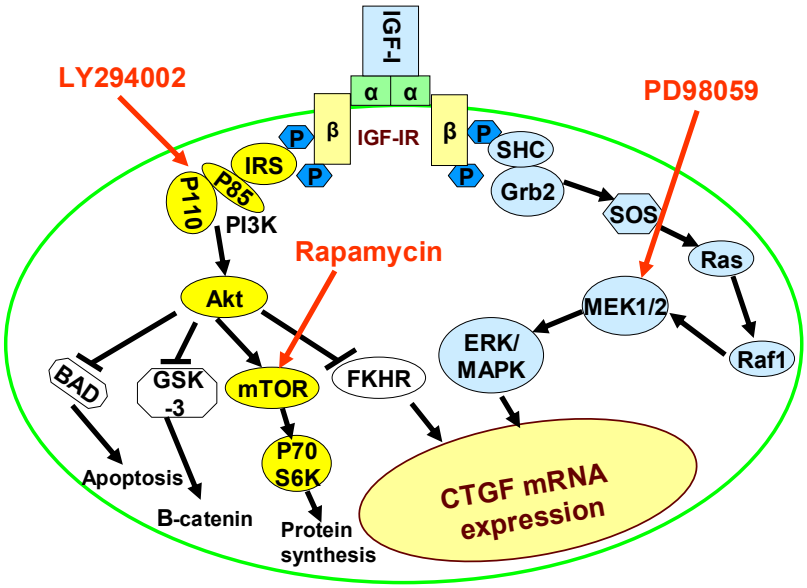


Figure 3.2. Effect of CTGF on IGF-I activation of IGF-I receptor (IGF-IR). MAC-T cells were treated with: 1) PBS; 2) 200 ng/mL of IGF-I; 3) 200 ng/mL of IGF-I + 200 ng/mL of CTGF; 4) 200 ng/mL of CTGF for 1 h. Total cell lysates were extracted from the cells and phospho-IGF-IR and total IGF-IR were detected by Western blotting using specific antibodies. (A) Representative images of Western blotting analysis. (B) Densitometric analysis of the phopho-IGF-IR and total IGF-IR protein bands like those in Panels A from 3 experiments. The density of phospho-IGF-IR and total IGF-IR bands was normalized to that of beta-actin protein in the same sample. There was no difference in phospho-IGF-IR expression between IGF-I and IGF-I plus CTGF samples ($P = 0.88$). Values are expressed as means ± SEM (n = 3).

A



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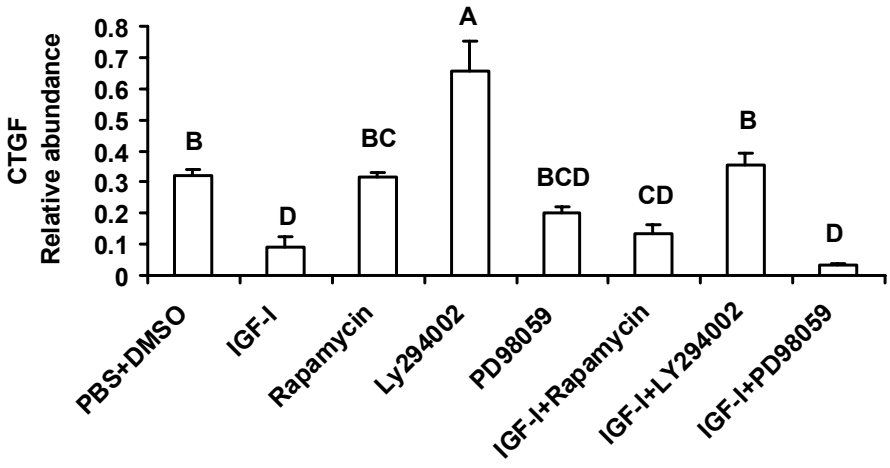


Figure 3.3. Identification of signaling pathways mediating IGF-I inhibition of CTGF expression. **A**, specific inhibitors of the signaling pathways from IGF-IR, including MEK1/2 inhibitor PD98059, PI-3K/Akt inhibitor LY294002 and mTOR inhibitor Rapamycin. **B**, real-time PCR of CTGF mRNA. MAC-T cells were treated with 1) PBS + DMSO (as control), 2) 200 ng/mL IGF-I, 3) 20 µM Rapamycin, 4) 50 µM LY294002, 5) 50 µM PD98059, 6) 200 ng/mL IGF-I + 20 µM Rapamycin, 7) 200 ng/mL IGF-I + 50 µM LY294002, 8) 200 ng/mL IGF-I + 50 µM PD98059 for 16 h. Real-time PCR was performed to quantify CTGF mRNA expression. Values are expressed as means ± SEM (n = 3). Means with different letters were significantly different ($P < 0.05$).

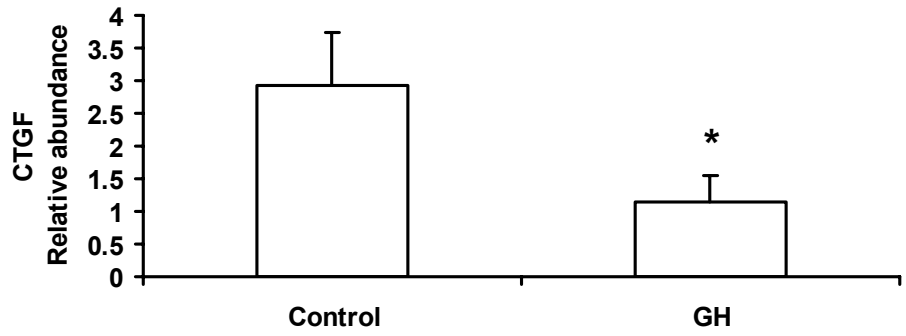


Figure 3.4. Effects of GH administration on mammary gland expression of CTGF mRNA in cows. Total RNA were extracted from mammary gland of lactating cows receiving vehicle (control) or GH. Real-time PCR was performed to measure CTGF mRNA. GH administration tended to decrease mammary gland CTGF mRNA expression. Values are expressed as means ± SEM (n = 6). "*" indicates $P = 0.07$.

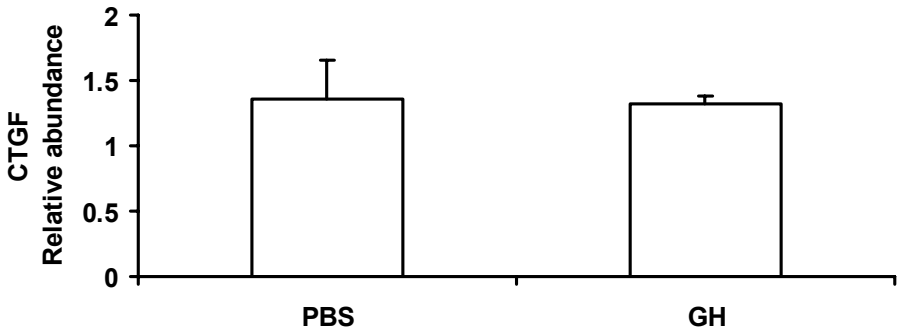


Figure 3.5. Effect of GH on CTGF mRNA expression in MAC-T cells. MAC-T cells were transfected with GHR and STAT5 expression plasmids to be GH responsive. The cells were treated with PBS or GH for 16h. Real-time PCR was performed to measure CTGF mRNA. GH had no effect on CTGF mRNA expression in these cells ($P = 0.88$). Values were expressed as means ± SEM (n = 4).

Chapter IV A Milk Trait-Associated Polymorphism in the Bovine Growth Hormone Receptor Gene Does Not Affect Receptor Signaling

Abstract

Growth hormone (GH), also known as somatotropin, stimulates milk production in cows. At the tissue level, the action of GH is mediated by the GH receptor (GHR) and the receptor-activated intracellular signaling pathway involving Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5 (STAT5). A T to A nucleotide variation in exon 8 of the bovine GHR gene, resulting in a phenylalanine to tyrosine change at a residue of the transmembrane domain of the GHR protein, has been reported to be associated with a major affect on milk yield in cows. The objective of this study was to determine whether the two versions of GHR differ in mediating GH-induced STAT5 activation of gene expression. We created cDNA expression plasmids for the two versions of GHR and cotransfected each of them with a STAT5 expression plasmid and a luciferase reporter gene construct containing STAT5 binding sites into two different cell lines. Treatment of the transfected cells with various concentrations of GH triggered a dose-dependent increase in luciferase activity. However, the GH-induced luciferase activity was not different ($P > 0.05$) between the two GHR expression plasmids, indicating that the two GHR forms do not differ in mediating GH-induced STAT5 activation of gene expression. Thus, if the T/A polymorphism in exon 8 of the GHR gene has a causative effect on milk production, this effect is unlikely mediated by the JAK2-STAT5 pathway, the currently known major signaling pathway from the growth hormone receptor.

Key words: single nucleotide polymorphism, growth hormone receptor, milk production

Introduction

Growth hormone (GH), also known as somatotropin, is a major stimulator of postnatal growth and milk production in cattle (Etherton and Bauman, 1998). At the tissue level, the GH action is mediated by a specific cell membrane receptor, the growth hormone receptor (GHR). Binding of GH to GHR activates Janus kinase 2 (JAK2); activated JAK2 in turn activates signal transducer and activator of transcription 5 (STAT5) through phosphorylation. Phosphorylated STAT5 translocates from the cytoplasm to the nucleus, where it binds to specific DNA regions and thereby activates transcription (Herrington and Carter-Su, 2001). A well-known gene controlled by GH through this JAK2-STAT5 pathway is insulin-like growth factor-I (IGF-I), which is believed to mediate most of the growth- and at least part of the milk production-stimulating effect of GH (Stewart and Rotwein, 1996; Etherton and Bauman, 1998). In addition to STAT5, GH-activated JAK2 also phosphorylates insulin receptor substrate 1, phospholipase C, and SHC protein, leading to changes in gene expression, enzymatic activity or metabolite transport (Herrington and Carter-Su, 2001).

Predicted from its cDNA sequence (Hauser et al., 1990), the bovine GHR protein is a single-chain polypeptide of 634 amino acids, composed of an 18-aa signal peptide (not present in mature GHR protein) encoded by exon 2 of the GHR gene, a 242-aa extracellular domain encoded by exons 3 to 7, a 24-aa single transmembrane domain encoded by exon 8, and a 350-aa intracellular domain encoded by exons 9 and 10. The bovine GHR mRNA is heterogeneous in the 5'-untranslated region, due to initiation of transcription from different leader exons (or alternative exon 1) and alternative splicing (Jiang and Lucy, 2001). Because the action of GH depends on GHR, genetic variations in the GHR gene might change the GHR amino acid

sequence or expression level, thereby affecting GH-controlled traits such as growth and milk production in animals. The 279th residue of GHR, a residue located on the transmembrane domain (Figure 4.1) of the GHR protein, is encoded by the exon 8 of the bovine GHR gene. A T to A single nucleotide variation in exon 8 of the bovine GHR gene (the corresponding two GHR DNA or cDNA forms are designated GHR-E8(T) and GHR-E8(A)), results in a phenylalanine (F) to tyrosine (Y) change at the 279th residue of GHR. Through quantitative trait loci (QTL) mapping, Blott et al. (2003) found this single nucleotide polymorphism of the 279th reside was associated with a major effect on milk yield in Ayreshire, Holstein and Jersey cows. The cows with GHR279Y appear to produce ~200 kg more milk annually than the cows carrying GHR279F (Blott et al., 2003). The same authors also indicated that the single nucleotide polymorphism had a similar effect on live weight in cattle (Blott et al., 2003).

Given the well-known effect of GH on milk production in cows, an obvious hypothesis for why GHR279Y is associated with greater milk yield than GHR279F is that GHR279Y mediates greater GH action than GHR279F. In this study we compared the ability of GHR279Y and GHR279F to mediate GH-induced STAT5 activation of gene expression in both MAC-T and CHO cell cultures.

Materials and Methods

Constructs preparation

In a previous study (Wang and Jiang, 2005), a 2026-bp bovine GHR cDNA (GenBank accession number AY748827) containing the entire open reading frame for the 634-aa GHR protein from a Holstein cow was cloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, California) between the EcoR V and Xba I restriction sites. The nucleotide at the

polymorphic position of exon 8 in this GHR cDNA was T (nucleotide 914 in GenBank accession number AY748827) and, for the purpose of this study, this expression plasmid was renamed bGHR-E8 (T)/pcDNA3. We mutated T at the polymorphic position in bGHR-E8 (T)/pcDNA3 to A, generating bGHR-E8 (A)/pcDNA3. The mutagenesis was done by three rounds of PCR, using a mixture (10:1) of *Taq* DNA polymerase and high fidelity DNA polymerase *Tli* (Promega, Madison, WI). The first PCR amplified the GHR cDNA region 1 to 924 (in GenBank accession number AY748827), using forward primer 5'-GCCATAAAGCCTGGAGGAAC-3' and reverse primer 5'-TATGAGTAAA**T**ATAATGTCACTGCTAGC-3', where the nucleotide in boldface was the incorporated mutation; the second PCR amplified the GHR cDNA region 903 to 2026 (in GenBank accession number AY748827), using forward primer 5'-AGTGACATTAT**A**TTTACTCATATTTTC-3' (the nucleotide in boldface was the incorporated mutation) and reverse primer 5'-TCTCTCTAGACCTGTGCCATTCAA TGGGTAG-3', where the sequence underlined is a Xba I restriction site. The products of these two PCR were mixed and from this mixture, the 2026-bp GHR-E8 (A) cDNA was amplified by the third PCR, using the same forward primer as used in the first PCR and the same reverse primer as used in the second PCR. The product of the third PCR was cloned into pcDNA3.1 between the EcoR V and Xba I sites, generating bGHR-E8 (A)/pcDNA3. The GHR cDNA insert in this plasmid was sequenced completely to confirm that it differed from GHR-E8 (T) cDNA only at the polymorphic nucleotide position of exon 8 (Figure 4.1).

Transfection and Luciferase assay

We then cotransfected 0.2 μg of bGHR-E8(T)/pcDNA3 or bGHR-E8(A)/pcDNA3 or pcDNA3.1 (no-GHR control), 0.2 μg of a STAT5b expression plasmid (Ariyoshi et al., 2000), 0.2 μg of pSPI-LUC, a firefly luciferase reporter plasmid that contained a growth hormone

response element consisting of six copies of STAT5 binding site from the Spi 2.1 gene (Wood et al., 1997), and 0.5 ng of pRL-CMV, a Renilla luciferase-encoding plasmid for transfection efficiency control, into Chinese hamster ovary-derived cell line CHO (American Tissue Culture Collection, Manassas, VA) and MAC-T, using FuGENE6 (Roche, Indianapolis, IN) as transfection reagent. Both CHO and MAC-T cells were cultured in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. In both cell lines, endogenous GHR expression was undetectable (Emtner et al., 1990; Jiang et al., unpublished data, respectively). Twenty-four hours after transfection, the medium was replaced with serum-free DMEM, and the culture was continued for 16 hours. The transfected cells were subsequently treated with different concentrations of recombinant bovine GH (provided by Dr. A. F. Parlow, National Hormone & Peptide Program) or phosphate buffered saline (PBS), the vehicle for GH, for 8 hours before being lysed for dual-luciferase assay (Promega). Such transfection analysis was repeated four times in CHO cells and three times in MAC-T cells. The luciferase activity expressed from the pSPI-LUC construct was divided by that from pRL-CMV in the same well to normalize well-to-well variation in transfection efficiency.

Statistical analysis

The adjusted luciferase activity data for the same GHR construct were compared between different concentrations of GH by one-factor ANOVA, followed by the Tukey's procedure. The adjusted luciferase activity data for the two different GHR constructs at each GH concentration were compared by the t-Test. Differences at $P < 0.05$ were considered significant.

Results and Discussion

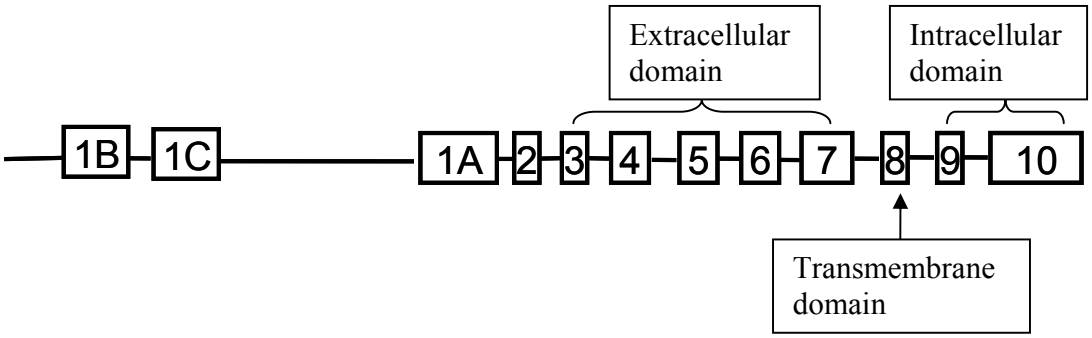
As shown in Fig 4.2, GH caused a dose-dependent increase in luciferase activity, with significant induction ($P < 0.05$) starting to occur at 5 ng/mL, in both CHO and MAC-T cells. This response was dependent on the GHR cDNA plasmid, because GH treatment failed to induce luciferase expression when the GHR expression plasmid was omitted in the transfection (Figure 4.2). The CHO or MAC-T cells transfected with bGHR-E8(T)/pcDNA3 and the same type of cells transfected with bGHR-E8(A)/pcDNA3 expressed similar levels ($P > 0.05$) of luciferase activity in response to various GH concentrations (Figure 4.2), indicating that the two versions of GHR, GHR279Y and GHR279F, expected to be expressed from bGHR-E8(A)/pcDNA3 and bGHR-E8(T)/pcDNA3, respectively, did not differ in mediating GH-induced STAT5 activation of reporter gene expression in CHO or MAC-T cells.

Some genotypes appear to be obvious candidates for selection aiming at improving milk production traits. SSCP analysis revealed in Serra da Estrela sheep, GH2-N and GH2-Z genotypes affect milk yield significantly, and the apparent joint effect of GH2-N and GH2-Z genotype could improve milk yield by 25% as compared with the mean milk production of the analyzed population (Marques et al., 2006). Milk protein such as κ-casein and β-LG genotypes can influence milk traits (Tsiaras et al., 2005). The AB κ-CN genotype had a positive effect on protein yield and content. The B variant of β-LG was associated with higher milk yield, and fat yield and content.

That GHR279Y and GHR279F do not differ in mediating GH activation of JAK2-STAT5 signaling is perhaps not surprising, because the 279th residue of the receptor is located in the transmembrane domain (Figure 4.1), a region that is not involved in association with JAK2 or any other known intracellular signaling components (Argetsinger and Carter-Su, 1996). Hence, if

the T/A nucleotide polymorphism in exon 8 of the GHR gene has a causative effect on milk production in cows, this effect is unlikely mediated by the JAK2-STAT5 signaling pathway, the currently known major signaling pathway from the growth hormone receptor (Herrington and Carter-Su, 2001). Besides a cause-effect relationship, the association between GHR sequence variation and changes in milk yield could be due to linkage disequilibrium of the GHR gene with proximal alleles that control the apparently GH-controlled traits.

A.



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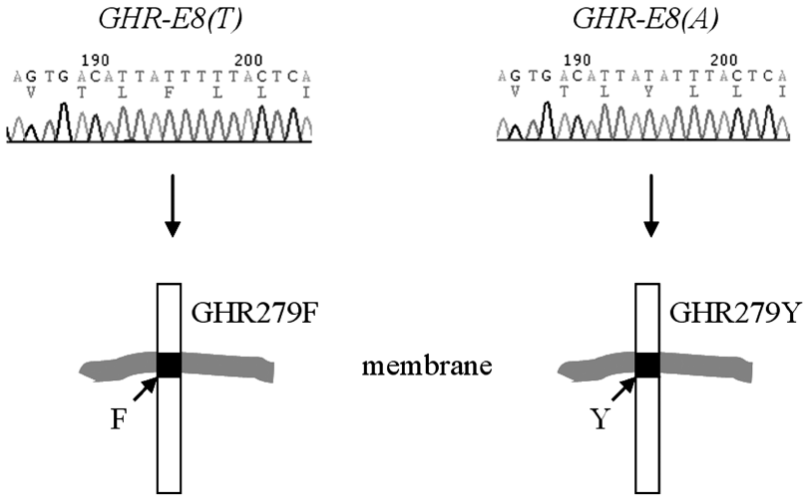

Figure 4.1. Schematic representation of the milk trait-associated single nucleotide in exon 8 (E8) of the bovine GHR gene and the corresponding changes in the GHR protein. **(A)** The GHR gene has 10 exons among which exons 2-10 encode GHR protein with exons 2-7 encoding the extracellular domain, exon 8 encoding the transmembrane domain, and exons 9-10 encoding the intracellular domain. **(B)** The T to A nucleotide variation in exon 8 of the bovine GHR gene (GHR-E8 (T) or GHR-E8 (A)) results in a phenylalanine (F) to tyrosine (Y) change at the 279th residue, a residue that is located within the transmembrane domain, of the receptor.

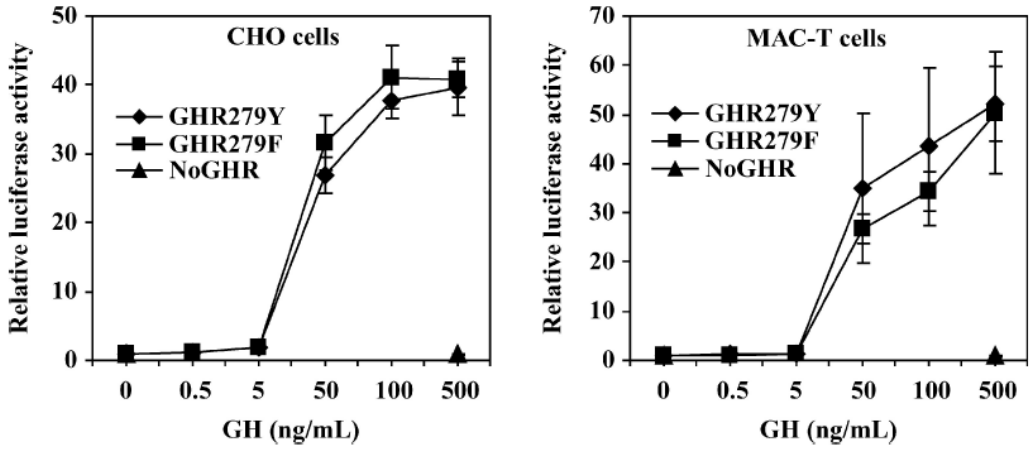

Figure 4.2. Comparison of the abilities of GHR279F and GHR279Y to mediate growth hormone-induced STAT5 activation of reporter gene expression. Chinese hamster ovary cell line CHO and bovine mammary epithelial cell line MAC-T were transfected with a STAT5b expression plasmid, a STAT5 binding site-containing reporter gene plasmid, a transfection efficiency control plasmid, and a GHR279F-encoding plasmid (GHR279F) or a GHR279Y-encoding plasmid (GHR279Y) or the pcDNA3.1 plasmid that did not contain GHR cDNA insert (NO-GHR). The transfected cells were treated with indicated concentrations of recombinant bovine GH for 8 hours (the NO-GHR cells were treated with 500 ng/mL GH only), followed by dual-luciferase assay. Values are expressed as mean ± standard error of the mean. Growth hormone caused a dose-dependent activation of luciferase reporter gene expression in both cell types and this activation depended on inclusion of the GHR expression plasmid in the transfection. Within each cell type, GH-induced luciferase activity was not different ($P > 0.05$) between GHR279F and GHR279Y.

Conclusions

It has been known for decades that GH stimulates milk production in cows, but the underlying mechanism is not clear. Many previous studies in this field suggest that the effect of GH on milk production is mediated by IGF-I. The results of this dissertation research suggest that GH can also directly stimulate milk protein gene expression in the mammary gland, thereby increasing milk production. It has also been well established that IGF-I can stimulate mammary epithelial cell proliferation, thereby mediating GH stimulation of milk production. The results of this dissertation research suggest that IGF-I may stimulate mammary epithelial cell proliferation partly by decreasing CTGF mRNA expression in the mammary epithelial cells because CTGF can attenuate IGF-I stimulation of proliferation of MAC-T cells. The results of this dissertation also indicated that IGF-I inhibits CTGF mRNA expression in MAC-T cells through the PI-3 kinase/Akt signaling pathway from the IGF-IR and that CTGF may attenuate IGF-I stimulation of mammary epithelial cell proliferation through a postreceptor inhibition of the IGF-IR signaling pathway. Finally, the results of this dissertation research suggest that a milk yield-associated polymorphism in the GHR exon 8 does not affects signaling transduction from the GHR and hence unlikely directly affect milk production.

Literature Cited

Ahmed, N. N., H. L. Grimes, A. Bellacosa, T. O. Chan, and P. N. Tsichlis. 1997. Transduction of interleukin-2 antiapoptotic and proliferative signals via akt protein kinase. Proc Natl Acad Sci U S A 94: 3627-3632.

Akers, R. M. 1985. Lactogenic hormones: Binding sites, mammary growth, secretory cell differentiation, and milk biosynthesis in ruminants. J Dairy Sci 68: 501-519.

Akers, R. M. 2002. Lactation and the mammary gland. 22-23.

Akers, R. M. 2006. Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. J Dairy Sci 89: 1222-1234.

Akers, R. M., S. E. Ellis, and S. D. Berry. 2005. Ovarian and igf-i axis control of mammary development in prepubertal heifers. Domest Anim Endocrinol 29: 259-267.

Alexander, L. J., A. F. Stewart, A. G. Mackinlay, T. V. Kapelinskaya, T. M. Tkach, and S. I. Gorodetsky. 1988. Isolation and characterization of the bovine kappa-casein gene. Eur J Biochem 178: 395-401.

Anderson, R. R. 1976. Relation between mammary gland development and body weight. J Dairy Sci 59: 1518-1521.

Argetsinger, L. S., G. S. Campbell, X. Yang, B. A. Witthuhn, O. Silvennoinen, J. N. Ihle, and C. Carter-Su. 1993. Identification of jak2 as a growth hormone receptor-associated tyrosine kinase. Cell 74: 237-244.

Argetsinger, L. S., and C. Carter-Su. 1996. Growth hormone signalling mechanisms: Involvement of the tyrosine kinase jak2. Horm Res 45 Suppl 1: 22-24.

Ariyoshi, K., T. Nosaka, K. Yamada, M. Onishi, Y. Oka, A. Miyajima, and T. Kitamura. 2000. Constitutive activation of stat5 by a point mutation in the sh2 domain. J Biol Chem 275: 24407-24413.

Athie, F., K. C. Bachman, H. H. Head, M. J. Hayen, and C. J. Wilcox. 1996. Estrogen administered at final milk removal accelerates involution of bovine mammary gland. J Dairy Sci 79: 220-226.

Barber, M. C., R. A. Clegg, E. Finley, R. G. Vernon, and D. J. Flint. 1992. The role of growth hormone, prolactin and insulin-like growth factors in the regulation of rat mammary gland and adipose tissue metabolism during lactation. J Endocrinol 135: 195-202.

Barres, B. A., R. Schmid, M. Sendnter, and M. C. Raff. 1993. Multiple extracellular signals are required for long-term oligodendrocyte survival. Development 118: 283-295.

Barrett-Lee, P., M. Travers, Y. Luqmani, and R. C. Coombes. 1990. Transcripts for transforming growth factors in human breast cancer: Clinical correlates. Br J Cancer 61: 612-617.

Bauman, D. E. 1992. Bovine somatotropin: Review of an emerging animal technology. J Dairy Sci 75: 3432-3451.

Bauman, D. E., and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. J Dairy Sci 63: 1514-1529.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu Rev Nutr 23: 203-227.

Baumrucker, C. R., and B. H. Stemberger. 1989. Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue in vitro. J Anim Sci 67: 3503-3514.

Baxter, R. C., M. A. Binoux, D. R. Clemmons, C. A. Conover, S. L. Drop, J. M. Holly, S. Mohan, Y. Oh, and R. G. Rosenfeld. 1998. Recommendations for nomenclature of the insulin-like growth factor binding protein superfamily. Endocrinology 139: 4036.

Bell, G. I., T. Kayano, J. B. Buse, C. F. Burant, J. Takeda, D. Lin, H. Fukumoto, and S. Seino. 1990. Molecular biology of mammalian glucose transporters. Diabetes Care 13: 198-208.

Berg, J. M., J. L. Tymoczko, and L. Stryer. 2002. BIochemistry.

Berry, S. D., T. B. McFadden, R. E. Pearson, and R. M. Akers. 2001. A local increase in the mammary IGF-I: IGFBP-3 ratio mediates the mammogenic effects of estrogen and growth hormone. Domest Anim Endocrinol 21: 39-53.

Beyer, E. C., and S. H. Barondes. 1982. Quantitation of two endogenous lactose-inhibitable lectins in embryonic and adult chicken tissues. J Cell Biol 92: 23-27.

Blum, J. W. 1992. [physiological basis of high milk production in cattle]. Schweiz Arch Tierheilkd 134: 213-229.

Bolander, F. F., Jr., and Y. J. Topper. 1980. Stimulation of lactose synthetase activity and casein synthesis in mouse mammary explants by estradiol. Endocrinology 106: 490-495.

Bonnette, S. G., and D. L. Hadsell. 2001. Targeted disruption of the IGF-I receptor gene decreases cellular proliferation in mammary terminal end buds. Endocrinology 142: 4937-4945.

Borst, D. W., and W. B. Mahoney. 1982. Mouse mammary gland DNA synthesis during pregnancy. The Journal of experimental zoology 221: 245-250.

Boutinaud, M., J. Guinard-Flamenta, and H. Jammes. 2004. The number and activity of mammary epithelial cells, determining factors for milk production. Reprod Nutr Dev 44: 499-508.

Boutinaud, M., C. Rousseau, D. H. Keisler, and H. Jammes. 2003. Growth hormone and milking frequency act differently on goat mammary gland in late lactation. J Dairy Sci 86: 509-520.

Brafman, A., I. Mett, M. Shafir, H. Gottlieb, G. Damari, S. Gozlan-Kelner, V. Vishnevskia-Dai, R. Skaliter, P. Einat, A. Faerman, E. Feinstein, and T. Shoshani. 2004. Inhibition of oxygen-induced retinopathy in rtp801-deficient mice. Investigative ophthalmology & visual science 45: 3796-3805.

Brauers, E., U. Vester, K. Zerres, and T. Eggermann. 2005. Search for mutations in slc1a5 (19q13) in cystinuria patients. Journal of inherited metabolic disease 28: 1169-1171.

Brazil, D. P., J. Park, and B. A. Hemmings. 2002. Pkb binding proteins. Getting in on the akt. Cell 111: 293-303.

Brew, K., T. C. Vanaman, and R. L. Hill. 1968. The role of alpha-lactalbumin and the a protein in lactose synthetase: A unique mechanism for the control of a biological reaction. Proc Natl Acad Sci U S A 59: 491-497.

Brodbeck, U., W. L. Denton, N. Tanahashi, and K. E. Ebner. 1967. The isolation and identification of the b protein of lactose synthetase as alpha-lactalbumin. J Biol Chem 242: 1391-1397.

Burnol, A. F., A. Leturque, M. Loizeau, C. Postic, and J. Girard. 1990. Glucose transporter expression in rat mammary gland. Biochem J 270: 277-279.

Butler, A. A., and D. Le Roith. 2001. Control of growth by the somatropic axis: Growth hormone and the insulin-like growth factors have related and independent roles. Annual review of physiology 63: 141-164.

Camps, M., S. Vilaro, X. Testar, M. Palacin, and A. Zorzano. 1994. High and polarized expression of glut1 glucose transporters in epithelial cells from mammary gland: Acute down-regulation of glut1 carriers by weaning. Endocrinology 134: 924-934.

Capuco, A. V., J. E. Keys, and J. J. Smith. 1989. Somatotrophin increases thyroxine-5'-monodeiodinase activity in lactating mammary tissue of the cow. J Endocrinol 121: 205-211.

Capuco, A. V., D. L. Wood, R. Baldwin, K. McLeod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bst. J Dairy Sci 84: 2177-2187.

Carter-Su, C., and L. S. Smit. 1998. Signaling via jak tyrosine kinases: Growth hormone receptor as a model system. Recent Prog Horm Res 53: 61-82; discussion 82-63.

Chao, D. T., and S. J. Korsmeyer. 1998. Bcl-2 family: Regulators of cell death. Annual review of immunology 16: 395-419.

Chatterton, R. T., Jr. 1978. Mammary gland: Development and secretion. Obstetrics and gynecology annual 7: 303-324.

Chen, M. M., A. Lam, J. A. Abraham, G. F. Schreiner, and A. H. Joly. 2000. Ctgf expression is induced by tgf- beta in cardiac fibroblasts and cardiac myocytes: A potential role in heart fibrosis. Journal of molecular and cellular cardiology 32: 1805-1819.

Chien, W., D. Yin, D. Gui, A. Mori, J. M. Frank, J. Said, D. Kusuanco, A. Marchevsky, R. McKenna, and H. P. Koeffler. 2006. Suppression of cell proliferation and signaling transduction by connective tissue growth factor in non-small cell lung cancer cells. Mol Cancer Res 4: 591-598.

Choi, Y. J., W. L. Keller, I. E. Berg, C. S. Park, and A. G. Mackinlay. 1988. Casein gene expression in bovine mammary gland. J Dairy Sci 71: 2898-2903.

Cohick, W. S. 1998. Role of the insulin-like growth factors and their binding proteins in lactation. J Dairy Sci 81: 1769-1777.

Cohick, W. S., and D. R. Clemmons. 1993. The insulin-like growth factors. Annual review of physiology 55: 131-153.

Coleman, S., G. B. Silberstein, and C. W. Daniel. 1988. Ductal morphogenesis in the mouse mammary gland: Evidence supporting a role for epidermal growth factor. Dev Biol 127: 304-315.

Combes, S., I. Louveau, and M. Bonneau. 1997. Effect of gh administration on gh and IGF-I receptors in porcine skeletal muscle and liver in relation to plasma gh-binding protein. J Endocrinol 155: 19-26.

Cox, D. A., and R. R. Burk. 1991. Isolation and characterisation of milk growth factor, a transforming-growth-factor-beta 2-related polypeptide, from bovine milk. Eur J Biochem 197: 353-358.

Cox, L. A., N. Schlabritz-Loutsevitch, G. B. Hubbard, M. J. Nijland, T. J. McDonald, and P. W. Nathanielsz. 2006. Gene expression profile differences in left and right liver lobes from mid-gestation fetal baboons: A cautionary tale. J Physiol 572: 59-66.

Craig, R. K., A. P. Boulton, and P. N. Campbell. 1978. Intracellular mechanisms involved in the biosynthesis of milk proteins. Biochem Soc Trans 6: 501-505.

Cseh, R., and R. Benz. 1999. Interaction of phloretin with lipid monolayers: Relationship between structural changes and dipole potential change. Biophysical journal 77: 1477-1488.

Damm, H. C., and C. W. Turner. 1958. Effects of hormone dose and length of injection on mouse mammary gland growth. Proc Soc Exp Biol Med 98: 192-195.

Davidson, M. B. 1987. Effect of growth hormone on carbohydrate and lipid metabolism. Endocr Rev 8: 115-131.

Davis, A. J., I. R. Fleet, J. A. Goode, M. H. Hamon, F. M. Walker, and M. Peaker. 1979. Changes in mammary function at the onset of lactation in the goat: Correlation with hormonal changes. J Physiol 288: 33-44.

Davis, S. R., R. J. Collier, J. P. McNamara, H. H. Head, W. J. Croom, and C. J. Wilcox. 1988. Effects of thyroxine and growth hormone treatment of dairy cows on mammary uptake of glucose, oxygen and other milk fat precursors. J Anim Sci 66: 80-89.

Deome, K. B., L. J. Faulkin, Jr., H. A. Bern, and P. B. Blair. 1959. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female c3h mice. Cancer Res 19: 515-520.

Deugnier, M. A., M. M. Faraldo, B. Janji, P. Rousselle, J. P. Thiery, and M. A. Glukhova. 2002. EGF controls the in vivo developmental potential of a mammary epithelial cell line possessing progenitor properties. J Cell Biol 159: 453-463.

Dimitriadis, G., M. Parry-Billings, S. Bevan, D. Dunger, T. Piva, U. Krause, G. Wegener, and E. A. Newsholme. 1992. Effects of insulin-like growth factor i on the rates of glucose transport and utilization in rat skeletal muscle in vitro. Biochem J 285 (Pt 1): 269-274.

Divisova, J., I. Kuiatse, Z. Lazard, H. Weiss, F. Vreeland, D. L. Hadsell, R. Schiff, C. K. Osborne, and A. V. Lee. 2006. The growth hormone receptor antagonist pegvisomant blocks both mammary gland development and mcf-7 breast cancer xenograft growth. Breast Cancer Res Treat.

Dvorak, P., D. Dvorakova, and A. Hampl. 2006. Fibroblast growth factor signaling in embryonic and cancer stem cells. FEBS Lett 580: 2869-2874.

Etherton, T. D., and D. E. Bauman. 1998. Biology of somatotropin in growth and lactation of domestic animals. Physiological reviews 78: 745-761.

Evan, G. I., and K. H. Vousden. 2001. Proliferation, cell cycle and apoptosis in cancer. Nature 411: 342-348.

Evans, H. M., and L. A. Long. 1921. The effect of the anterior lobe of the hypophysis administered intraperitoneally upon growth and maturity and estrous cycles of the rat. Anat Record 21: 62.

Faulkin, L. J., Jr., and K. B. Deome. 1960. Regulation of growth and spacing of gland elements in the mammary fat pad of the c3h mouse. J Natl Cancer Inst 24: 953-969.

Firth, S. M., and R. C. Baxter. 2002. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23: 824-854.

Flint, D. J., and M. Gardner. 1994. Evidence that growth hormone stimulates milk synthesis by direct action on the mammary gland and that prolactin exerts effects on milk secretion by maintenance of mammary deoxyribonucleic acid content and tight junction status. Endocrinology 135: 1119-1124.

Flint, D. J., E. Tonner, and G. J. Allan. 2000. Insulin-like growth factor binding proteins: Igf-dependent and -independent effects in the mammary gland. J Mammary Gland Biol Neoplasia 5: 65-73.

Forsyth, I. A. 1996. The insulin-like growth factor and epidermal growth factor families in mammary cell growth in ruminants: Action and interaction with hormones. J Dairy Sci 79: 1085-1096.

Furchgott, R. F., and J. V. Zawadzki. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373-376.

Furuhashi, M., G. Tuncman, C. Z. Gorgun, L. Makowski, G. Atsumi, E. Vaillancourt, K. Kono, V. R. Babaev, S. Fazio, M. F. Linton, R. Sulsky, J. A. Robl, R. A. Parker, and G. S. Hotamisligil. 2007. Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein ap2. Nature 447: 959-965.

Gao, J., and G. Serrero. 1999. Adipose differentiation related protein (adrp) expressed in transfected cos-7 cells selectively stimulates long chain fatty acid uptake. J Biol Chem 274: 16825-16830.

Gibson, C. A., M. D. Staley, and C. R. Baumrucker. 1999. Identification of igf binding proteins in bovine milk and the demonstration of IGFBP-3 synthesis and release by bovine mammary epithelial cells. J Anim Sci 77: 1547-1557.

Gluckman, P. D., B. H. Breier, and S. R. Davis. 1987. Physiology of the somatotropic axis with particular reference to the ruminant. J Dairy Sci 70: 442-466.

Gordon, D. F., D. P. Quick, C. R. Erwin, J. E. Donelson, and R. A. Maurer. 1983. Nucleotide sequence of the bovine growth hormone chromosomal gene. Mol Cell Endocrinol 33: 81-95.

Groenen, M. A., R. J. Dijkhof, J. J. van der Poel, R. van Diggelen, and E. Verstege. 1992. Multiple octamer binding sites in the promoter region of the bovine alpha s2-casein gene. Nucleic acids research 20: 4311-4318.

Groner, B., and L. Hennighausen. 2000. Linear and cooperative signaling: Roles for stat proteins in the regulation of cell survival and apoptosis in the mammary epithelium. Breast Cancer Res 2: 149-153.

Gronlund, D. E., W. L. Hurley, M. H. Monaco, M. B. Wheeler, and S. M. Donovan. 2003. Over-expression of IGF-I in lactating porcine mammary tissue has a differential effect on amino acid transport systems. . J dairy sci 81: 189.

Hadsell, D. L., and S. G. Bonnette. 2000. Igf and insulin action in the mammary gland: Lessons from transgenic and knockout models. J Mammary Gland Biol Neoplasia 5: 19-30.

Hadsell, D. L., S. G. Bonnette, and A. V. Lee. 2002. Genetic manipulation of the igf-i axis to regulate mammary gland development and function. J Dairy Sci 85: 365-377.

Hadsell, D. L., N. M. Greenberg, J. M. Fligger, C. R. Baumrucker, and J. M. Rosen. 1996. Targeted expression of des(1-3) human insulin-like growth factor i in transgenic mice influences mammary gland development and igf-binding protein expression. Endocrinology 137: 321-330.

Hansen, J. K., and J. Knudsen. 1980. Transacylation as a chain-termination mechanism in fatty acid synthesis by mammalian fatty acid synthetase. Synthesis of butyrate and hexanoate by lactating cow mammary gland fatty acid synthetase. Biochem J 186: 287-294.

Hauser, S. D., M. F. McGrath, R. J. Collier, and G. G. Krivi. 1990. Cloning and in vivo expression of bovine growth hormone receptor mrna. Mol Cell Endocrinol 72: 187-200.

Havel, P. J. 2000. Role of adipose tissue in body-weight regulation: Mechanisms regulating leptin production and energy balance. The Proceedings of the Nutrition Society 59: 359-371.

Herrington, J., and C. Carter-Su. 2001. Signaling pathways activated by the growth hormone receptor. Trends Endocrinol Metab 12: 252-257.

Hill, R. L., K. Brew, T. C. Vanaman, I. P. Trayer, and P. Mattock. 1968. The structure, function, and evolution of alpha-lactalbumin. Brookhaven symposia in biology 21: 139-154.

Hishikawa, K., B. S. Oemar, F. C. Tanner, T. Nakaki, T. F. Luscher, and T. Fujii. 1999. Connective tissue growth factor induces apoptosis in human breast cancer cell line mcf-7. J Biol Chem 274: 37461-37466.

Hobbs, A. A., D. A. Richards, D. J. Kessler, and J. M. Rosen. 1982. Complex hormonal regulation of rat casein gene expression. J Biol Chem 257: 3598-3605.

Holst, B. D., W. L. Hurley, and D. R. Nelson. 1987. Involution of the bovine mammary gland: Histological and ultrastructural changes. J Dairy Sci 70: 935-944.

Hovey, R. C., H. W. Davey, D. D. Mackenzie, and T. B. McFadden. 1998. Ontogeny and epithelial-stromal interactions regulate igf expression in the ovine mammary gland. Mol Cell Endocrinol 136: 139-144.

Hussain, M. A., O. Schmitz, A. Mengel, Y. Glatz, J. S. Christiansen, J. Zapf, and E. R. Froesch. 1994. Comparison of the effects of growth hormone and insulin-like growth factor i on substrate oxidation and on insulin sensitivity in growth hormone-deficient humans. J Clin Invest 94: 1126-1133.

Huynh, H. T., G. Robitaille, and J. D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): An in vitro model for bovine lactation. Exp Cell Res 197: 191-199.

Hwa, V., Y. Oh, and R. G. Rosenfeld. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20: 761-787.

Jefferson, L. S., C. M. Schworer, and E. L. Tolman. 1975. Growth hormone stimulation of amino acid transport and utilization by the perfused rat liver. J Biol Chem 250: 197-204.

Jenkins, T. C. 2000. Feeding oleamide to lactating jersey cows 1. Effects on lactation performance and milk fatty acid composition. J Dairy Sci 83: 332-337.

Jiang, H., and M. C. Lucy. 2001. Variants of the 5'-untranslated region of the bovine growth hormone receptor mrna: Isolation, expression and effects on translational efficiency. Gene 265: 45-53.

Jiang, W. G., G. Watkins, O. Fodstad, A. Douglas-Jones, K. Mokbel, and R. E. Mansel. 2004. Differential expression of the ccn family members cyr61, ctgf and nov in human breast cancer. Endocr Relat Cancer 11: 781-791.

Jin, Y., D. A. Cox, R. Knecht, F. Raschdorf, and N. Cerletti. 1991. Separation, purification, and sequence identification of tgf-beta 1 and tgf-beta 2 from bovine milk. Journal of protein chemistry 10: 565-575.

Kahn, B. B., and O. Pedersen. 1992. Tissue-specific regulation of glucose transporters in different forms of obesity. Proc Soc Exp Biol Med 200: 214-217.

Kamalati, T., D. Davies, J. Titley, and M. R. Crompton. 1998. Functional consequences of cyclin d1 overexpression in human mammary luminal epithelial cells. Clinical & experimental metastasis 16: 415-426.

Kanai, Y., and M. A. Hediger. 2004. The glutamate/neutral amino acid transporter family slc1: Molecular, physiological and pharmacological aspects. Pflugers Arch 447: 469-479.

Kenney, A. M., and D. H. Rowitch. 2000. Sonic hedgehog promotes g(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol Cell Biol 20: 9055-9067.

Kim, H. S., S. R. Nagalla, Y. Oh, E. Wilson, C. T. Roberts, Jr., and R. G. Rosenfeld. 1997. Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): Characterization of connective tissue growth factor as a member of the igfbp superfamily. Proc Natl Acad Sci U S A 94: 12981-12986.

Kisseleva, T., S. Bhattacharya, J. Braunstein, and C. W. Schindler. 2002. Signaling through the jak/stat pathway, recent advances and future challenges. Gene 285: 1-24.

Kleinberg, D. L. 1998. Role of IGF-I in normal mammary development. Breast Cancer Res Treat 47: 201-208.

Kleinberg, D. L., W. Ruan, V. Catanese, C. B. Newman, and M. Feldman. 1990. Non-lactogenic effects of growth hormone on growth and insulin-like growth factor-i messenger ribonucleic acid of rat mammary gland. Endocrinology 126: 3274-3276.

Knabel, M., S. Kolle, and F. Sinowatz. 1998. Expression of growth hormone receptor in the bovine mammary gland during prenatal development. Anat Embryol (Berl) 198: 163-169.

Kneussel, M. 2002. Dynamic regulation of gaba(a) receptors at synaptic sites. Brain research 39: 74-83.

Knight, C. H. 1992. Milk yield responses to sequential treatments with recombinant bovine somatotropin and frequent milking in lactating goats. J Dairy Res 59: 115-122.

Knight, C. H., P. A. Fowler, and C. J. Wilde. 1990. Galactopoietic and mammogenic effects of long-term treatment with bovine growth hormone and thrice daily milking in goats. J Endocrinol 127: 129-138.

Kobayashi, Y., C. K. Boyd, C. J. Bracken, W. R. Lamberson, D. H. Keisler, and M. C. Lucy. 1999. Reduced growth hormone receptor (GHR) messenger ribonucleic acid in liver of periparturient cattle is caused by a specific down-regulation of ghr 1a that is associated with decreased insulin-like growth factor i. Endocrinology 140: 3947-3954.

Kornmann, M., N. Arber, and M. Korc. 1998. Inhibition of basal and mitogen-stimulated pancreatic cancer cell growth by cyclin d1 antisense is associated with loss of tumorigenicity and potentiation of cytotoxicity to cisplatinum. J Clin Invest 101: 344-352.

Kuhn, N. J. 1968. Lactogenesis in the rat. Metabolism of uridine diphosphate galactose by mammary gland. Biochem J 106: 743-748.

Kuhn, N. J., and A. White. 1976. Evidence for specific transport of uridine diphosphate galactose across the golgi membrane of rat mammary gland. Biochem J 154: 243-244.

Lam, S., R. N. van der Geest, N. A. Verhagen, F. A. van Nieuwenhoven, I. E. Blom, J. Aten, R. Goldschmeding, M. R. Daha, and C. van Kooten. 2003. Connective tissue growth factor and igf-i are produced by human renal fibroblasts and cooperate in the induction of collagen production by high glucose. Diabetes 52: 2975-2983.

Lau, L. F., and S. C. Lam. 1999. The ccn family of angiogenic regulators: The integrin connection. Exp Cell Res 248: 44-57.

Le Roith, D. 1997. Seminars in medicine of the beth israel deaconess medical center. Insulin-like growth factors. The New England journal of medicine 336: 633-640.

Li, W., W. Qiao, L. Chen, X. Xu, X. Yang, D. Li, C. Li, S. G. Brodie, M. M. Meguid, L. Hennighausen, and C. X. Deng. 2003. Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in smad4/dpc4 conditional knockout mice. Development 130: 6143-6153.

Liu, X., G. W. Robinson, K. U. Wagner, L. Garrett, A. Wynshaw-Boris, and L. Hennighausen. 1997. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev 11: 179-186.

Louveau, I., and F. Gondret. 2004. Gh and insulin affect fatty acid synthase activity in isolated porcine adipocytes in culture without any modifications of sterol regulatory element binding protein-1 expression. J Endocrinol 181: 271-280.

Maas, J. A., J. France, and B. W. McBride. 1997. Model of milk protein synthesis. A mechanistic model of milk protein synthesis in the lactating bovine mammary gland. J Theor Biol 187: 363-378.

Madon, R. J., S. Martin, A. Davies, H. A. Fawcett, D. J. Flint, and S. A. Baldwin. 1990. Identification and characterization of glucose transport proteins in plasma membrane- and golgi vesicle-enriched fractions prepared from lactating rat mammary gland. Biochem J 272: 99-105.

Maier, R., P. Schmid, D. Cox, G. Bilbe, and G. K. McMaster. 1991. Localization of transforming growth factor-beta 1, -beta 2 and -beta 3 gene expression in bovine mammary gland. Mol Cell Endocrinol 82: 191-198.

Malewski, T., M. Gajewska, T. Zebrowska, and L. Zwierzchowski. 2002. Differential induction of transcription factors and expression of milk protein genes by prolactin and growth hormone in the mammary gland of rabbits. Growth Horm IGF Res 12: 41-53.

Marques, M. D., I. C. Santos, N. Carolino, C. C. Belo, R. Renaville, and A. Cravador. 2006. Effects of genetic polymorphisms at the growth hormone gene on milk yield in serra da estrela sheep. J Dairy Res: 1-12.

Martin, M. B., and A. Stoica. 2002. Insulin-like growth factor-I and estrogen interactions in breast cancer. J Nutr 132: 3799S-3801S.

Mather, I. H., and T. W. Keenan. 1998. Origin and secretion of milk lipids. J Mammary Gland Biol Neoplasia 3: 259-273.

Mauro, L., M. Salerno, C. Morelli, T. Boterberg, M. E. Bracke, and E. Surmacz. 2003. Role of theIGF-I receptor in the regulation of cell-cell adhesion: Implications in cancer development and progression. J Cell Physiol 194: 108-116.

McDowell, G. H., J. M. Gooden, D. Leenanuruksa, M. Jois, and A. W. English. 1987. Effects of exogenous growth hormone on milk production and nutrient uptake by muscle and mammary tissues of dairy cows in mid-lactation. Aust J Biol Sci 40: 295-306.

Merlo, G. R., F. Basolo, L. Fiore, L. Duboc, and N. E. Hynes. 1995. P53-dependent and p53-independent activation of apoptosis in mammary epithelial cells reveals a survival function of egf and insulin. J Cell Biol 128: 1185-1196.

Mertani, H. C., and G. Morel. 1995. In situ gene expression of growth hormone (gh) receptor and gh binding protein in adult male rat tissues. Mol Cell Endocrinol 109: 47-61.

Miller, M. J., S. A. Witherly, and D. A. Clark. 1990. Casein: A milk protein with diverse biologic consequences. Proc Soc Exp Biol Med 195: 143-159.

Miller, P. S., B. L. Reis, C. C. Calvert, E. J. DePeters, and R. L. Baldwin. 1991. Relationship of early lactation and bovine somatotropin on nutrient uptake by cow mammary glands. J Dairy Sci 74: 3800-3806.

Moller, N., J. O. Jorgensen, K. G. Alberti, A. Flyvbjerg, and O. Schmitz. 1990. Short-term effects of growth hormone on fuel oxidation and regional substrate metabolism in normal man. J Clin Endocrinol Metab 70: 1179-1186.

Monaco, M. H., D. E. Gronlund, G. T. Bleck, W. L. Hurley, M. B. Wheeler, and S. M. Donovan. 2005. Mammary specific transgenic over-expression of insulin-like growth factor-i (IGF-I) increases pig milk igf-i and igf binding proteins, with no effect on milk composition or yield. Transgenic Res 14: 761-773.

Moritani, N. H., S. Kubota, T. Nishida, H. Kawaki, S. Kondo, T. Sugahara, and M. Takigawa. 2003. Suppressive effect of overexpressed connective tissue growth factor on tumor cell growth in a human oral squamous cell carcinoma-derived cell line. Cancer Lett 192: 205-214.

Nebel, R. L., and M. L. McGilliard. 1993. Interactions of high milk yield and reproductive performance in dairy cows. J Dairy Sci 76: 3257-3268.

Neville, M. C., J. Morton, and S. Umemura. 2001. Lactogenesis. The transition from pregnancy to lactation. Pediatric clinics of North America 48: 35-52.

Neville, M. C., and C. D. Watters. 1983. Secretion of calcium into milk: Review. J Dairy Sci 66: 371-380.

Nicosia, R. F., S. V. Nicosia, and M. Smith. 1994. Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-I promote rat aortic angiogenesis in vitro. Am J Pathol 145: 1023-1029.

Nielsen, M. O., and K. Jakobsen. 1993. Changes in mammary glucose and protein uptake in relation to milk synthesis during lactation in high- and low-yielding goats. Comp Biochem Physiol Comp Physiol 106: 359-365.

Nielsen, M. O., T. G. Madsen, and A. M. Hedeboe. 2001. Regulation of mammary glucose uptake in goats: Role of mammary gland supply, insulin, IGF-I and synthetic capacity. J Dairy Res 68: 337-349.

Nielsen, M. O., S. Nyborg, K. Jakobsen, I. R. Fleet, and J. Norgaard. 2004. Mammary uptake and excretion of prostanoids in relation to mammary blood flow and milk yield during

pregnancy-lactation and somatotropin treatment in dairy goats. Domest Anim Endocrinol 27: 345-362.

Nielsen, S., N. Moller, J. S. Christiansen, and J. O. Jorgensen. 2001. Pharmacological antilipolysis restores insulin sensitivity during growth hormone exposure. Diabetes 50: 2301-2308.

O'Connor, R. 2003. Regulation of IGF-I receptor signaling in tumor cells. Horm Metab Res 35: 771-777.

Ormandy, C. J., N. Binart, and P. A. Kelly. 1997. Mammary gland development in prolactin receptor knockout mice. J Mammary Gland Biol Neoplasia 2: 355-364.

Oscarsson, J., M. Ottosson, and S. Eden. 1999. Effects of growth hormone on lipoprotein lipase and hepatic lipase. J Endocrinol Invest 22: 2-9.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. Science 189: 347-358.

Paradis, V., D. Dargere, M. Vidaud, A. C. De Gouville, S. Huet, V. Martinez, J. M. Gauthier, N. Ba, R. Sobesky, V. Ratziu, and P. Bedossa. 1999. Expression of connective tissue growth factor in experimental rat and human liver fibrosis. Hepatology (Baltimore, Md 30: 968-976.

Parrizas, M., and D. LeRoith. 1997. Insulin-like growth factor-1 inhibition of apoptosis is associated with increased expression of the bcl-xl gene product. Endocrinology 138: 1355-1358.

Patton, S. 1974. Reversible suppression of lactation by colchicine. FEBS Lett 48: 85-87.

Peel, C. J., D. E. Bauman, R. C. Gorewit, and C. J. Sniffen. 1981. Effect of exogenous growth hormone on lactational performance in high yielding dairy cows. J Nutr 111: 1662-1671.

Peeters, R. A., J. H. Veerkamp, A. Geurts van Kessel, T. Kanda, and T. Ono. 1991. Cloning of the cdna encoding human skeletal-muscle fatty-acid-binding protein, its peptide sequence and chromosomal localization. Biochem J 276 (Pt 1): 203-207.

Pellerin, I., C. Vuillermoz, M. Jouvenot, C. Ordener, M. Royez, and G. L. Adessi. 1993. Identification and characterization of an early estrogen-regulated rna in cultured guinea-pig endometrial cells. Mol Cell Endocrinol 90: R17-21.

Petitclerc, D., L. T. Chapin, and H. A. Tucker. 1984. Carcass composition and mammary development responses to photoperiod and plane of nutrition in holstein heifers. J Anim Sci 58: 913-919.

Plaut, K., D. E. Bauman, N. Agergaard, and R. M. Akers. 1987. Effect of exogenous prolactin administration on lactational performance of dairy cows. Domest Anim Endocrinol 4: 279-290.

Plaut, K., M. Ikeda, and B. K. Vonderhaar. 1993. Role of growth hormone and insulin-like growth factor-i in mammary development. Endocrinology 133: 1843-1848.

Plaut, K., R. L. Maple, C. E. Wade, L. A. Baer, and A. E. Ronca. 2003. Effects of hypergravity on mammary metabolic function: Gravity acts as a continuum. J Appl Physiol 95: 2350-2354.

Pohl, J., A. Ring, U. Korkmaz, R. Ehehalt, and W. Stremmel. 2005. Fat/cd36-mediated long-chain fatty acid uptake in adipocytes requires plasma membrane rafts. Mol Biol Cell 16: 24-31.

Pombo, M., C. M. Pombo, A. Garcia, E. Caminos, O. Gualillo, C. V. Alvarez, F. F. Casanueva, and C. Dieguez. 2001. Hormonal control of growth hormone secretion. Horm Res 55 Suppl 1: 11-16.

Powers, C. J., S. W. McLeskey, and A. Wellstein. 2000. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7: 165-197.

Prosser, C. G., S. R. Davis, V. C. Farr, L. G. Moore, and P. D. Gluckman. 1994. Effects of close-arterial (external pudic) infusion of insulin-like growth factor-ii on milk yield and mammary blood flow in lactating goats. J Endocrinol 142: 93-99.

Purup, S., M. Vestergaard, and K. Sejrsen. 2000. Involvement of growth factors in the regulation of pubertal mammary growth in cattle. Adv Exp Med Biol 480: 27-43.

Rapoport, R. M., M. B. Draznin, and F. Murad. 1983. Endothelium-dependent vasodilator-and nitrovasodilator-induced relaxation may be mediated through cyclic gmp formation and cyclic gmp-dependent protein phosphorylation. Transactions of the Association of American Physicians 96: 19-30.

Richert, M. M., and T. L. Wood. 1999. The insulin-like growth factors (IGF) and igf type i receptor during postnatal growth of the murine mammary gland: Sites of messenger ribonucleic acid expression and potential functions. Endocrinology 140: 454-461.

Rijnkels, M., P. M. Kooiman, H. A. de Boer, and F. R. Pieper. 1997. Organization of the bovine casein gene locus. Mamm Genome 8: 148-152.

Rinderknecht, E., and R. E. Humbel. 1978. The amino acid sequence of human insulin-like growth factor i and its structural homology with proinsulin. J Biol Chem 253: 2769-2776.

Robinson, R. M., R. M. Akers, and K. E. Forsten. 2001. IGF-I stimulation of extracellular acidification is not linked to cell proliferation for autocrine cells. Endocrine 15: 205-211.

Rosen, J. M., R. J. Matusik, D. A. Richards, P. Gupta, and J. R. Rodgers. 1980. Multihormonal regulation of casein gene expression at the transcriptional and posttransciptional levels in the mammary gland. Recent Prog Horm Res 36: 157-193.

Rosen, J. M., S. L. Wyszomierski, and D. Hadsell. 1999. Regulation of milk protein gene expression. Annu Rev Nutr 19: 407-436.

Ruan, W., and D. L. Kleinberg. 1999. Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. Endocrinology 140: 5075-5081.

Ruan, W., C. B. Newman, and D. L. Kleinberg. 1992. Intact and amino-terminally shortened forms of insulin-like growth factor i induce mammary gland differentiation and development. Proc Natl Acad Sci U S A 89: 10872-10876.

Sakamoto, K., B. A. Creamer, A. A. Triplett, and K. U. Wagner. 2007. The janus kinase 2 (jak2) is required for expression and nuclear accumulation of cyclin d1 in proliferating mammary epithelial cells. Mol Endocrinol.

Sakamoto, K., T. Komatsu, T. Kobayashi, M. T. Rose, H. Aso, A. Hagino, and Y. Obara. 2005. Growth hormone acts on the synthesis and secretion of alpha-casein in bovine mammary epithelial cells. J Dairy Res 72: 264-270.

Sejrsen, K., J. T. Huber, H. A. Tucker, and R. M. Akers. 1982. Influence of nutrition of mammary development in pre- and postpubertal heifers. J Dairy Sci 65: 793-800.

Sejrsen, K., S. Purup, M. Vestergaard, M. S. Weber, and C. H. Knight. 1999. Growth hormone and mammary development. Domest Anim Endocrinol 17: 117-129.

Sell, C., R. Baserga, and R. Rubin. 1995. Insulin-like growth factor i (IGF-I) and the igf-i receptor prevent etoposide-induced apoptosis. Cancer Res 55: 303-306.

Serra, R., and M. R. Crowley. 2003. Tgf-beta in mammary gland development and breast cancer. Breast Dis 18: 61-73.

Shennan, D. B., and R. B. Beechey. 1995. Mechanisms involved in the uptake of d-glucose into the milk producing cells of rat mammary tissue. Biochem Biophys Res Commun 211: 986-990.

Short, V. J., D. N. Brindley, and R. Dils. 1977. Co-ordinate changes in enzymes of fatty acid synthesis, activation and esterification in rabbit mammary gland druing pregnancy and lactation. Biochem J 162: 445-450.

Shoshani, T., A. Faerman, I. Mett, E. Zelin, T. Tenne, S. Gorodin, Y. Moshel, S. Elbaz, A. Budanov, A. Chajut, H. Kalinski, I. Kamer, A. Rozen, O. Mor, E. Keshet, D. Leshkowitz, P. Einat, R. Skaliter, and E. Feinstein. 2002. Identification of a novel hypoxia-inducible factor 1-responsive gene, rtp801, involved in apoptosis. Mol Cell Biol 22: 2283-2293.

Shum, B. O., C. R. Mackay, C. Z. Gorgun, M. J. Frost, R. K. Kumar, G. S. Hotamisligil, and M. S. Rolph. 2006. The adipocyte fatty acid-binding protein ap2 is required in allergic airway inflammation. J Clin Invest 116: 2183-2192.

Sinha, Y. N., and H. A. Tucker. 1969. Mammary development and pituitary prolactin level of heifers from birth through puberty and during the estrous cycle. J Dairy Sci 52: 507-512.

Sinowatz, F., D. Schams, A. Plath, and S. Kolle. 2000. Expression and localization of growth factors during mammary gland development. Adv Exp Med Biol 480: 19-25.

Skarda, J., E. Urbanova, S. Becka, L. M. Houdebine, C. Delouis, D. Pichova, J. Picha, and J. Bilek. 1982. Effect of bovine growth hormone on development of goat mammary tissue in organ culture. Endocrinol Exp 16: 19-31.

Smith, J. J., A. V. Capuco, and R. M. Akers. 1987. Quantification of progesterone binding in mammary tissue of pregnant ewes. J Dairy Sci 70: 1178-1185.

Smith, J. L., and L. G. Sheffield. 2002. Production and regulation of leptin in bovine mammary epithelial cells. Domest Anim Endocrinol 22: 145-154.

Stewart, C. E., and P. Rotwein. 1996. Growth, differentiation, and survival: Multiple physiological functions for insulin-like growth factors. Physiological reviews 76: 1005-1026.

Stiles, C. D., G. T. Capone, C. D. Scher, H. N. Antoniades, J. J. Van Wyk, and W. J. Pledger. 1979. Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc Natl Acad Sci U S A 76: 1279-1283.

Strange, R., F. Li, S. Saurer, A. Burkhardt, and R. R. Friis. 1992. Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development 115: 49-58.

Su, H. Y., and W. T. Cheng. 2004. Increased milk yield in transgenic mice expressing insulin-like growth factor 1. Anim Biotechnol 15: 9-19.

Sundstrom, M., T. Lundqvist, J. Rodin, L. B. Giebel, D. Milligan, and G. Norstedt. 1996. Crystal structure of an antagonist mutant of human growth hormone, g120r, in complex with its receptor at 2.9 a resolution. J Biol Chem 271: 32197-32203.

Sweeney, K. J., B. Sarcevic, R. L. Sutherland, and E. A. Musgrove. 1997. Cyclin d2 activates cdk2 in preference to cdk4 in human breast epithelial cells. Oncogene 14: 1329-1340.

Takigawa, M. 2003. Ctgf/hcs24 as a multifunctional growth factor for fibroblasts, chondrocytes and vascular endothelial cells. Drug News Perspect 16: 11-21.

Terada, N., H. Wakimoto, and T. Oka. 1988. Regulation of milk protein synthesis by progesterone in cultured mouse mammary gland. Journal of steroid biochemistry 29: 99-104.

Teyssot, B., and L. M. Houdebine. 1980. Role of prolactin in the transcription of beta-casein and 28-s ribosomal genes in the rabbit mammary gland. Eur J Biochem 110: 263-272.

Tonner, E., M. C. Barber, M. T. Travers, A. Logan, and D. J. Flint. 1997. Hormonal control of insulin-like growth factor-binding protein-5 production in the involuting mammary gland of the rat. Endocrinology 138: 5101-5107.

Topper, Y. J., and C. S. Freeman. 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiological reviews 60: 1049-1106.

Traurig, H. H. 1967. A radioautographic study of cell proliferation in the mammary gland of the pregnant mouse. The Anatomical record 159: 239-247.

Tsiaras, A. M., G. G. Bargouli, G. Banos, and C. M. Boscos. 2005. Effect of kappa-casein and beta-lactoglobulin loci on milk production traits and reproductive performance of holstein cows. J Dairy Sci 88: 327-334.

Tucker, H. A. 2000. Hormones, mammary growth, and lactation: A 41-year perspective. J Dairy Sci 83: 874-884.

Ullrich, A. G., A. Tam, A. W. Yang-Feng, T. Tsubokawa, M. Collins, C. Henzel, W., T. Le Bon, S. Kathuria, E. Chen, and et al. 1986. Insulin-like growth factor i receptor primary structure: Comparison with insulin receptor suggests structural determinants that define functional specificity. Embo J 5: 2503-2512.

Veerkamp, R. F. 1998. Selection for economic efficiency of dairy cattle using information on live weight and feed intake: A review. J Dairy Sci 81: 1109-1119.

Vermouth, N. T., and R. P. Deis. 1972. Prolactin release induced by prostaglandin f2 in pregnant rats. Nature: New biology 238: 248-250.

Vermouth, N. T., and R. P. Deis. 1974. Prolactin release and lactogenesis after ovariectomy in pregnant rats: Effect of ovarian hormones. J Endocrinol 63: 13-20.

Vernier-Magnin, S., S. Muller, M. Sallot, J. Radom, J. F. Musard, P. Adami, P. Dulieu, J. P. Remy-Martin, M. Jouvenot, and A. Fraichard. 2001. A novel early estrogen-regulated gene gec1 encodes a protein related to gabarap. Biochem Biophys Res Commun 284: 118-125.

Verrey, F., E. I. Closs, C. A. Wagner, M. Palacin, H. Endou, and Y. Kanai. 2004. Cats and hats: The slc7 family of amino acid transporters. Pflugers Arch 447: 532-542.

Walden, P. D., W. Ruan, M. Feldman, and D. L. Kleinberg. 1998. Evidence that the mammary fat pad mediates the action of growth hormone in mammary gland development. Endocrinology 139: 659-662.

Wang, W., B. Morrison, T. Galbaugh, C. C. Jose, N. Kenney, and M. L. Cutler. 2007. Glucocorticoid induced expression of connective tissue growth factor contributes to lactogenic differentiation of mouse mammary epithelial cells. J Cell Physiol.

Wang, Y., S. K. Fried, R. N. Petersen, and P. A. Schoknecht. 1999. Somatotropin regulates adipose tissue metabolism in neonatal swine. J Nutr 129: 139-145.

Wang, Y., and H. Jiang. 2005. Identification of a distal stat5-binding DNA region that may mediate growth hormone regulation of insulin-like growth factor-i gene expression. J Biol Chem 280: 10955-10963.

Weber, M. S., P. L. Boyle, B. A. Corl, E. A. Wong, F. C. Gwazdauskas, and R. M. Akers. 1998. Expression of ovine insulin-like growth factor-1 (IGF-I) stimulates alveolar bud development in mammary glands of transgenic mice. Endocrine 8: 251-259.

Weber, M. S., S. Purup, M. Vestergaard, R. M. Akers, and K. Sejrsen. 2000. Nutritional and somatotropin regulation of the mitogenic response of mammary cells to mammary tissue extracts. Domest Anim Endocrinol 18: 159-164.

Weber, M. S., S. Purup, M. Vestergaard, S. E. Ellis, J. Scndergard-Andersen, R. M. Akers, and K. Sejrsen. 1999. Contribution of insulin-like growth factor (IGF)-I and IGF-binding protein-3 to mitogenic activity in bovine mammary extracts and serum. J Endocrinol 161: 365-373.

Werner, H., and D. LeRoith. 1996. The role of the insulin-like growth factor system in human cancer. Adv Cancer Res 68: 183-223.

Wilde, C. J., C. V. Addey, P. Li, and D. G. Fernig. 1997. Programmed cell death in bovine mammary tissue during lactation and involution. Experimental physiology 82: 943-953.

Winklehner-Jennewein, P., S. Geymayer, J. Lechner, T. Welte, L. Hansson, S. Geley, and W. Doppler. 1998. A distal enhancer region in the human beta-casein gene mediates the response to prolactin and glucocorticoid hormones. Gene 217: 127-139.

Wolf, E., P. M. Jehle, M. M. Weber, H. Sauerwein, A. Daxenberger, B. H. Breier, U. Besenfelder, L. Frenyo, and G. Brem. 1997. Human insulin-like growth factor i (IGF-I) produced in the mammary glands of transgenic rabbits: Yield, receptor binding, mitogenic activity, and effects on igf-binding proteins. Endocrinology 138: 307-313.

Wood, D. C., W. J. Salsgiver, T. R. Kasser, G. W. Lange, E. Rowold, B. N. Violand, A. Johnson, R. M. Leimgruber, G. R. Parr, N. R. Siegel, and et al. 1989. Purification and characterization of pituitary bovine somatotropin. J Biol Chem 264: 14741-14747.

Writzl, K., B. Zorn, and B. Peterlin. 2006. Preliminary analysis of azfb region duplication by quantitative real-time pcr. Hum Reprod 21: 753-754.

Xie, D., C. W. Miller, J. O'Kelly, K. Nakachi, A. Sakashita, J. W. Said, J. Gornbein, and H. P. Koeffler. 2001. Breast cancer. Cyr61 is overexpressed, estrogen-inducible, and associated with more advanced disease. J Biol Chem 276: 14187-14194.

Yakar, S., J. L. Liu, B. Stannard, A. Butler, D. Accili, B. Sauer, and D. LeRoith. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor i. Proc Natl Acad Sci U S A 96: 7324-7329.

Yang, J., J. J. Kennelly, and V. E. Baracos. 2000. Physiological levels of stat5 DNA binding activity and protein in bovine mammary gland. J Anim Sci 78: 3126-3134.

Yang, J., B. Zhao, V. E. Baracos, and J. J. Kennelly. 2005. Effects of bovine somatotropin on beta-casein mrna levels in mammary tissue of lactating cows. J Dairy Sci 88: 2806-2812.

Yee, D. 1994. The insulin-like growth factor system as a target in breast cancer. Breast Cancer Res Treat 32: 85-95.

Yip, R. G., and H. M. Goodman. 1999. Growth hormone and dexamethasone stimulate lipolysis and activate adenylyl cyclase in rat adipocytes by selectively shifting gi alpha2 to lower density membrane fractions. Endocrinology 140: 1219-1227.

Yonekura, S., K. Sakamoto, T. Komatsu, A. Hagino, K. Katoh, and Y. Obara. 2006. Growth hormone and lactogenic hormones can reduce the leptin mrna expression in bovine mammary epithelial cells. Domest Anim Endocrinol 31: 88-96.

Zamzami, N., C. Brenner, I. Marzo, S. A. Susin, and G. Kroemer. 1998. Subcellular and submitochondrial mode of action of bcl-2-like oncoproteins. Oncogene 16: 2265-2282.

Zavizion, B., R. C. Gorewit, and I. Politis. 1995. Subcloning the mac-t bovine mammary epithelial cell line: Morphology, growth properties, and cytogenetic analysis of clonal cells. J Dairy Sci 78: 515-527.

Zhao, F. Q., D. R. Glimm, and J. J. Kennelly. 1993. Distribution of mammalian facilitative glucose transporter messenger rna in bovine tissues. Int J Biochem 25: 1897-1903.

Zhao, F. Q., W. M. Moseley, H. A. Tucker, and J. J. Kennelly. 1996. Regulation of glucose transporter gene expression in mammary gland, muscle, and fat of lactating cows by administration of bovine growth hormone and bovine growth hormone-releasing factor. J Anim Sci 74: 183-189.

Zhu, J., and C. R. Kahn. 1997. Analysis of a peptide hormone-receptor interaction in the yeast two-hybrid system. Proc Natl Acad Sci U S A 94: 13063-13068.

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