**Growth hormone alters components related to differentiation, metabolism and milk
synthesis and secretion in MAC-T cells**

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TITLE: GROWTH HORMONE ALTERS COMPONENTS RELATED TO
DIFFERENTIATION, METABOLISM AND MILK SYNTHESIS AND SECRETION
IN MAC-T CELLS

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ABSTRACT

**GROWTH HORMONE ALTERS COMPONENTS RELATED TO
DIFFERENTIATION, METABOLISM AND MILK SYNTHESIS AND
SECRETION IN MAC-T CELLS**

Tasha L. Johnson

The mammary alveolar cell-T (MAC-T) cell line is able to uniformly differentiate and secrete casein proteins in response to dexamethasone, insulin and prolactin and is extensively used to study bovine mammary epithelial cell function. Growth hormone (GH) has been shown to increase milk protein synthesis both *in vivo* and in mammary cell models, and induce cytoskeletal rearrangement in 3T3 fibroblast cell line and a Chinese hamster ovary (CHO) cell line. Few studies have focused on identifying the mechanisms involved in differentiated MAC-T cells' response to GH. We tested the hypothesis that MAC-T cells would respond directly to GH and that the response would include alterations in milk protein gene expression, leading to a more appropriate model for mammary cell function than treatment with dexamethasone, insulin and prolactin alone. To identify mechanisms that are involved in MAC-T cells' response to GH, global protein was assessed through two-dimensional gel electrophoresis and differentially expressed proteins were identified through mass spectrometry. Differentiated cells expressed GH receptor mRNA, and addition of GH to the differentiation medium increased production of α_{s1} casein and α -lactalbumin mRNA. Proteins that were differentially expressed are related to metabolism, the cytoskeleton, protein folding, RNA and DNA processing, detoxifying and calcium metabolism. These results indicate that GH is an important factor in inducing a lactogenic phenotype in the MAC-T cell line, and supports GHs involvement in differentiation, while altering cell metabolism in preparation for synthesis and secretion of milk components.

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DEDICATION

I would like to dedicate this thesis to Dr. Dan Peterson who has been an amazing mentor and friend.

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CHAPTER 1

Literature Review

1.1 Introduction

Milk components are synthesized and secreted from mammary epithelial cells (MEC) providing essential nutrients for fetal life and are a good source of nutrients for the adult population. The older population primarily consumes ruminant milk, usually produced by dairy cows. Milk consists of lipids, proteins, carbohydrates, vitamins and minerals, although the composition varies between and within species. The composition of bovine milk varies due to genetics, breed, age, diet, season, environment and stage of lactation. However, ratios of milk components stay relatively constant, consisting of approximately 87.8 % water, 3.1% protein, 3.5 % milk fat, 4.9 % lactose and 0.7 % ash (Schmidt, 1971).

Ruminants provide the primary source of milk consumed by the older human population, which on their own don't have the ability to breakdown plant sources. The breakdown of plant material occurs in the rumen, one of the compartments in the stomach, housing anaerobic microorganisms that have the ability to break down cellulosic fiber, cereal grains, forage and silage crops, pasture and range forage and crop residues for use by the ruminant. In turn the ruminant uses these products to produce meat and milk products (Baldwin, 1984).

During lactation, milk production increases until peak lactation, when production begins to decline. The initial increase is attributed to continued differentiation of alveolar epithelial cells, opposed to increased proliferation of MEC, while the decline is attributed to apoptosis of MEC, rather than decreased secretory activity of the alveolar epithelial cells (Capuco et al., 2001). A complex interaction of hormonal stimuli, transcription

factors, cell-to-cell interactions and basement membrane proteins control proliferation and differentiation of MECs. Many *in vitro* culture systems, such as primary cells, explant culture and continuous cell lines have been employed to study mammary epithelial cell proliferation, differentiation, apoptosis and milk synthesis and secretion.

Growth hormone (GH), also termed somatotropin, increases milk yield without altering the composition of milk (Bauman, 1999). The use of synthetic bovine growth hormone (bGH) appears to increase milk yield through increasing cellular proliferation and therefore reducing the rate of mammary regression (Capuco et al., 2001) through insulin-like growth factor I (IGF-I) and increasing available nutrients for milk synthesis (Bauman, 1999). The identification of growth hormone receptor (GHR) mRNA in mammary epithelium and mammary alveolar cell T (MAC-T) cells (Plath-Gabler et al., 2001, Zhou et al., 2008a) suggests that GH also has a direct effect on MEC development, although the mechanisms involved are not fully characterized. The following thesis will assess a direct effect of GH has on protein expression and lipid synthesis.

1.2 Lipid production

Lipid composition in milk varies greatly between and within species and provides approximately 50% of the calories for the bovine neonate (Fox and McSweeney, 1998, Van Soest, 1994). Lipids are also an important source of essential fatty acids, which cannot be synthesized *in vivo*, such as linoleic acid, and fat-soluble vitamins A, D, E, and K (Fox and McSweeney, 1998). The proportion of milk lipid classes stays relatively constant across species and is comprised primarily of triacylglycerols (TAG), with minor lipid classes consisting of diacylglycerols, monoglycerols, nonesterified fatty acids, phospholipids and sterols (Fox and McSweeney, 2006). Despite the similarities in lipid class distribution, the fatty acid composition of total lipids varies greatly between species (Dils, 1986).

Milk lipids are predominantly TAG in which the fatty acids vary in molecular weight and degree of unsaturation (Fox and McSweeney, 2006). In the ruminant mammary gland, production of TAG entails of esterification of fatty acids obtained from the diet, adipose tissue or *de novo* synthesis to a glycerol backbone. In ruminants, long chain fatty acids are obtained from dietary or adipose source, while medium to short chain fatty acids are synthesized *de novo* in the mammary gland (Anderson et al., 1985). The synthesis of short and medium chain fatty acids, and the desaturation of saturated fatty acids is unique to the ruminant. Short and medium chain fatty acids are hydrolyzed by lipase more rapidly in the neonate and the desaturation by stearoyl CoA desaturase (SCD) keeps milk fat liquid at bovine body temperature when there are low levels of short chain fatty acids (Dils, 1986, German et al., 1997, Van Soest, 1994).

De novo fatty acid synthesis, in ruminants uses acetate and occurs in the cytosol of MECs. The first step is to activate acetate to acetyl-CoA in the cytosol of the mammary epithelial cell. Acetyl-CoA is then converted to malonyl-CoA with the enzyme

acetyl-CoA carboxylase. Malonyl-CoA is complexed with acyl carrier protein (ACP) and binds to fatty acid synthetase (FAS) to assemble fatty acids by two carbon increments by repeating a four-step sequence usually producing the 16 carbon fatty acid palmitate. The first step condenses malonyl-ACP and acetyl-ACP to form acetoacetyl-ACP and CO₂. Acetoacetyl-ACP is reduced to β-hydroxybutyryl-ACP with the enzyme β-ketoacyl-ACP reductase and NADPH. β-hydroxybutyryl-ACP is dehydrated to produce crotonyl-ACP and H₂O with β-hydroxyacyl-ACP dehydrogenase. Crotonyl-ACP is reduced with enoyl-ACP reductase and NADPH, adding a two carbon increment (Nelson and Cox, 2008) (Figure 1).

Termination of fatty acid synthesis is catalyzed by the enzyme thioesterase I predominantly producing palmitate (C_{16:0}). Interestingly, in the mammary gland of non-ruminants, FAS terminates synthesis after the 8 and 10 carbon with an additional enzyme thioesterase II (Anderson et al., 1985, Dils, 1986, Neville and Neifert, 1983). Ruminant mammary FAS may contain medium-chain acetyl transferase, which can produce medium chain fatty acids released as acetyl-CoA esters that can be immediately esterified into milk TAG. Ruminants also have the ability to esterify the short chain fatty acids butyryl-CoA (C_{4:0}-CoA) and hexanoyl-CoA (C_{6:0}-CoA) directly to TAG. Butyryl-CoA can be converted from 3-hydroxybutyrate and hexanoyl-CoA can be synthesized *de novo* in the mammary gland. The alterations in ruminant mammary gland FAS make their milk fat unique, the short and medium chain fatty acids lower the melting point and are

Figure 1: Steps involved in fatty acid synthesis. Acetate is activated to acetyl-CoA, which can be converted to acetyl—S—ACP or malonyl—CoA for the first step in fatty acid synthesis (A). The second part of the pathway is mediated by fatty acid synthetase, beginning with condensation of acetyl-ACP and malonyl-ACP, which is reduced to add two carbons during each cycle. * Butyryl-ACP is the first fatty acid formed by addition of two carbon molecules (B).
hydrolyzed more rapidly by lipase, when compared to nonruminants (Dils, 1986).

Fatty acid synthesis is relatively similar across species although the source of acetyl-CoA and the reducing equivalent NADPH differ between ruminants and nonruminants. In non-ruminants, glucose is the main source of carbon for acetyl-CoA. Glucose is converted to pyruvate and transported into the mitochondria where it is

converted to acetyl-CoA and enters the TCA cycle. Since acetyl-CoA cannot be transported out of the mitochondria it binds to oxaloacetate forming citrate and CoA, which is transported across the mitochondrial membrane. Citrate is cleaved by the enzyme ATP citrate lyase forming acetyl-CoA for fatty acid synthesis and oxaloacetate for NADPH production in the malate transhydrogenation cycle. Glucose can also be converted to glucose-3-phosphate and enter the pentose phosphate pathway to produce NADPH, while small amounts can be produced by the isocitrate cycle. The majority of NADPH is produced through the malate transhydrogenation cycle and the pentose phosphate pathway in nonruminants (Anderson et al., 1985, Neville and Neifert, 1983).

Acetate and not glucose serves as the primary carbon source for acetyl-CoA in ruminants. Acetate is a volatile fatty acid produced by microbial fermentation in the rumen and is activated to acetyl-CoA in the cytosol for fatty acid synthesis. The lack of glucose utilization in ruminants is due to microbial fermentation of feed and low absorption of glucose from the gastrointestinal tract. The majority of glucose is supplied through gluconeogenesis by the liver and kidney (Larson and Smith, 1974b). Glucose is spared in the ruminant mammary gland for lactate synthesis by using acetate to generate energy, acetyl-CoA, and NADPH for fatty acid synthesis.

Acetate is the main carbon source for the TCA cycle generating citrate, which cannot be converted to acetyl-CoA in the cytosol due to the lack of ATP citrate lyase enzyme. Therefore, when citrate is transported to the cytosol it enters the isocitrate cycle where it is converted to isocitrate, which is further converted to α -ketoglutarate by the enzyme isocitrate dehydrogenase producing the reducing agent NADPH for lipid production. Citrate can also re-enter the mitochondria or diffuse to the golgi and be secreted in milk. The majority of NADPH produced in the cytosol of the ruminant

mammary gland is through the isocitrate cycle providing the reducing equivalents for fatty acid synthesis while small amounts of NADPH are generated from the pentose phosphate pathway (Anderson et al., 1985, Neville and Neifert, 1983, Van Soest, 1994).

Once lipids are synthesized they are transported out of the MEC through a unique lipid secretion pathway. Newly synthesized TAG form small milk protein coated storage structures termed lipid globules that increase in size as they move from the ER through the cytoplasm toward the apical membrane. Once lipid globules reach the apical membrane they are secreted through a unique budding process that encases them in an envelope of apical plasma membrane. This process may allow for the transfer of a small amount of cytoplasmic material into the lumen of alveolar cells and subsequently into milk (Anderson et al., 1985, McManaman and Neville, 2003).

1.3 Protein production

Milk proteins provide a good source of most amino acids, minerals and antibodies to the neonate. The majority of milk proteins are synthesized in the mammary gland from free amino acids derived from the blood stream. Four casein proteins, α_{s1} , α_{s2} , β and κ , and the two major whey proteins, α -lactalbumin and β -lactoglobulin, make up 95% of total milk proteins and are specific to milk. Minor constituents consist of immunoglobulins, proteose-peptones, serum albumin, lactoferrin and transferrin (Mephram, 1983). Casein proteins are the primary source of amino acids from cow's milk

and are loosely folded with few α -helix and β -sheet structures making casein susceptible to proteolysis prior to denaturation and more readily digestible for the neonate (Fox and McSweeney, 1998).

To initiate transcription, DNA structure is selectively loosened and RNA polymerase II binds to synthesize mRNA. mRNA is responsible for encoding the sequence information of amino acids in proteins. After mRNA transcription the primary transcript immediately undergoes a modification at its 5' end, called capping. The cap protects the end of the mRNA from exonuclease attack and it is involved in initiation of translation. The 3' end undergoes polyadenylation, the addition of a polyA tail by RNA polymerase II. The polyA tail aids mRNA in the termination of transcription, export from the nucleus, translation, and protects it from 3' to 5' degradation by exonucleases through the binding of polyA-binding proteins (Elliott and Elliott, 2005).

Proteins are made from amino acids corresponding to three nucleotides in mRNA that make up a codon. Each codon is translated into one specific amino acid, which becomes activated by ATP and attachment to transfer RNA (tRNA) forming aminoacyl-tRNA complex. Protein synthesis occurs in the ribosome and newly synthesized proteins emerge from the ribosome in a denatured state and may be chaperoned by heat shock proteins (HSP) that attach to the hydrophobic regions preventing incorrect hydrophobic association (Berg et al., 2007, Liang and MacRae, 1997). When HSP release, proteins have been folded into three-dimensional structures that determine function and properties. Once a protein has folded into its three-dimensional structure, post-translational modifications may occur, giving the protein greater specificity (Anderson et al., 1985).

Casein, the major milk protein, is easily digestible and provides a good source of amino acids, inorganic calcium and phosphate to neonates as well as non-neonatal consumers. The relatively loose and open three-dimensional structure increases digestibility by proteolysis prior to denaturation (Anderson et al., 1985, Fox and McSweeney, 1998). Casein is phosphorylated by casein kinase which adds phosphate groups to threonine and serine residues. Phosphorylation, coupled with the high concentration of glutamic acid gives a strong negative charge to the amino terminus, which may be responsible for the binding of calcium in casein micelles (Neville and Neifert, 1983). Micelles function to fluidize casein molecules and transport inorganic calcium and phosphate. Micelles are formed through aggregation of α_{s1} , α_{s2} , β -casein, and the binding of calcium phosphate, which are stably maintained in milk through their interaction with κ -casein. (Fox and McSweeney, 2003, Threadgill and Womack, 1990).

Whey proteins are soluble proteins that do not precipitate with casein (Anderson et al., 1985). β -lactoglobulin is the primary whey protein found in ruminant milk but is not present in all species. The biological role is not well defined although it has been shown to bind retinol, non-esterified fatty acids, low molecular weight phosphate molecules, vitamin D2, cholesterol and mercury ions (Farrell and Thompson, 1971, Kontopidis et al., 2004, Perez and Calvo, 1995). Further research is needed to define a specific function for β -lactoglobulin.

Unlike β -lactoglobulin, enzymatic activity of α -lactalbumin is well defined as part of the enzyme lactose synthetase and is coupled with the enzyme galactosyltransferase (Blackburn, 1993) in the biosynthesis of lactose. α -lactalbumin also possesses a single calcium binding site, has several partially folded intermediate states

and some forms can induce apoptosis in tumor cells suggesting that α -lactalbumin has many biological roles (Permyakov and Berliner, 2000).

Milk proteins are primarily transported by exocytosis along with water, lactose, oligosaccharides, phosphate, calcium and citrate. These substances are sequestered by the golgi, sorted and packaged into secretory vesicles which move directly toward the apical plasma membrane. The vesicles fuse with the membrane and release their substances via exocytosis into the alveolar lumen (McManaman and Neville, 2003).

1.4 Lactose synthesis

Lactose is the main carbohydrate found in milk, it functions as a source of energy for the neonate and is responsible for approximately 50% of the osmotic pressure in milk (Fox and McSweeney, 2009). Neonatal ruminants are able to digest lactose and therefore require it as a source of digestible carbohydrate. They lack sucrase and have a limited amount of amylase making them unable to digest other sugars such as sucrose and starch. The nutritional physiology of neonatal ruminants and nonruminants is similar, in that they rely heavily on the dam's milk until their rumen is fully developed. Lactose can be easily broken down by lactase to form glucose and galactose providing energy to the

infant. As nonruminants mature they produce less lactase and therefore their ability to breakdown lactose decreases. As ruminants mature, the development of rumen fermentation allows digestion of carbohydrates other than lactose (Van Soest, 1994).

Glucose is the major substrate for lactose synthesis and may also play a minor role in the pentose phosphate pathway by producing NADPH in the ruminant mammary gland. Synthesis of lactose occurs in the golgi with the enzyme complex lactose synthetase consisting of two components, galactosyl transferase and α -lactalbumin. The membrane bound galactosyl transferase is found in the golgi of many tissues and regulates the transport of galactosyl groups from uridine diphosphate (UDP)-galactose to carbohydrate groups of glycoprotein's. Glucose is a very poor acceptor of galactosyl residues but is altered in the presence of α -lactalbumin by increasing the binding affinity of galactosyl groups from UDP-galactosyl to glucose, ultimately forming lactose.

Lactose is synthesized in the golgi and cannot diffuse across the membrane, which causes an influx of water across the golgi, secretory vesicle and apical membrane due to the osmotic pressure, which is mainly responsible for the volume of milk produced. The osmotic properties are illustrated by the correlation of copious amounts of milk secretion with increased lactose production. Other carbohydrates in milk consist of monosaccharides, sugar phosphates, nucleotide sugars, free neutral and acid oligosaccharides, and glycosyl groups of peptides and proteins (Anderson et al., 1985, Neville and Neifert, 1983). Once synthesized, lactose is secreted from the cell through exocytosis (Larson and Smith, 1974a, Neville and Neifert, 1983)

1.5 Glucose utilization in the mammary gland.

Glucose is required for lactose synthesis in the mammary gland of all animals and is the major energy source in mammary cells of nonruminants while acetate is responsible for mammary cell metabolism in ruminants. Due to the unique digestive system of ruminants, the manner in which glucose is produced and utilized is different from that of nonruminants. The ruminant's stomach consists of four compartments, the reticulum, rumen, omasum and abomasum. The rumen and reticulum, often referred to as the reticulorumen, house anaerobic microbes responsible for the breakdown of carbohydrates such as cellulose, hemicellulose, pectin, fructans and starch to volatile fatty acids (VFA) which are the main source of metabolizable energy (Van Soest, 1994). The principle VFAs produced by the rumen fermentation are acetate, propionate and butyrate.

Small amounts of isobutyrate, valerate and isovalerate are also produced. The majority of glucose obtained from the diet is utilized by the microbial population within the rumen, with minimal absorption of glucose in the small intestine. To support lactose production during lactation, the majority of glucose is produced through hepatic gluconeogenesis with propionate serving as the primary precursor (Van Soest, 1994).

At the onset of lactation there is a two- to three-fold increase in hepatic glucose production and a dramatic increase in glucose uptake and utilization by the mammary gland in high producing dairy cows (Garnsworthy, 1998). The increase in glucose uptake is correlated with the increase in milk secretion because glucose is required for lactose synthesis and lactose is an important osmotic solute in milk (Bell and Bauman, 1997). In goats, the mammary gland uses 60-85% of glucose produced and absorbed and the majority of glucose taken up by the mammary gland is immediately used for lactose synthesis in the golgi. The remaining portion of glucose is phosphorylated to glucose-6-phosphate by hexokinase and can be utilized for energy through glycolysis, produce NADPH through the pentose phosphate pathway, converted to galactose for lactose synthesis, or used as a glycerol backbone for TAG synthesis (Anderson et al., 1985, Larson and Smith, 1974a). Since the majority of glucose is used for lactose synthesis, the majority of metabolizable energy is obtained from VFA's; for example acetate not only supplies carbon for fatty acid synthesis but also generates usable energy in the form of ATP through oxidation in the TCA cycle (Larson and Smith, 1974a).

During lactation, mammary cells utilize glucose and acetate as their main source of energy. In nonruminants, glucose is the primary precursor for lactose synthesis, is also utilized for fatty acid synthesis by production of acetyl-CoA and is also the primary substrate for ATP generation in the TCA cycle. In ruminants glucose is spared for lactose

production and acetate enters the mitochondria and is oxidized by the TCA cycle to produce energy. Acetate also acts as the primary precursor for fatty acid synthesis through conversion to acetyl-CoA for fatty acid synthesis. The lack of the ATP citrate lyase enzyme in ruminants prevents the conversion of glucose to acetyl-CoA and inhibits the production of NADPH through the malate transhydrogenation cycle. Instead acetate is used for fatty acid synthesis by conversion to acetyl-CoA and used for fatty acid synthesis in adipose tissue and the mammary gland (Larson and Smith, 1974a, Van Soest, 1994). In ruminants, NADPH is primarily produced by the isocitrate cycle, the conversion of isocitrate to α -ketoglutarate with the enzyme isocitrate dehydrogenase, due to the lack of ATP citrate lyase and inhibition of the malate transhydrogenation cycle. Small amounts of NADPH are also generated from glucose through the pentose phosphate pathway although the majority of glucose is used for lactose production (Anderson et al., 1985).

1.6 Mammary gland development

The endocrine system plays a key role in mammary development and lactation. Hormones involved in lactation can be divided into two groups, reproductive hormones (estrogen (E), progesterone (P), prolactin (PRL) and oxytocin) and metabolic hormones (growth hormone (GH), glucorticoids, thyroid hormones and insulin). Reproductive hormones change with the reproductive state of the animal and all have direct action on the mammary gland. Metabolic hormones are responsible for coordinating the body's response to the stresses of pregnancy and major nutrient demands of lactation. Metabolic hormones have many functions in the body and many also have direct effects on the mammary gland (Neville et al., 2002).

Mammary gland development can be divided into four distinct stages: mammogenesis, lactogenesis, galactopoesis and involution. Mammogenesis is characterized by proliferation of epithelial cells and development of the lobule-alveolar system in response to E and P. Lactogenesis is characterized by the production of milk

components and the onset of lactation while galactopoesis is the maintenance of lactation, mainly controlled by the peptide hormones PRL and GH. Involution is the gradual regression of the mammary gland in preparation for subsequent lactations (Svennersten-Sjaunja and Olsson, 2005).

Mammogenesis occurs during gestation, creating a system of ducts and alveoli for proper milk synthesis and lactation. The mammary fat pad (MFP) is slowly replaced with ducts, lobule alveoli, blood vessels, lymph vessels, and connective tissue which is generally synchronized with an increase in E, P, PRL and GH (Anderson et al., 1985). E and GH are required for ductal growth while P and PRL are responsible for lobulo-alveolar growth (Lamote et al., 2004). The lobulo-alveolar system is made up of many epithelial cells that make up alveoli, small sac like structures surrounded by blood vessels, myoepithelial cells and a connective tissue basement membrane (Anderson et al., 1985, Svennersten-Sjaunja and Olsson, 2005). The dense connection of lobes and lobules are separated by thin layers of connective tissue and drain milk through a ductal network to the teat (Lamote et al., 2004).

There are two distinct stages of lactogenesis. Stage one begins mid to late pregnancy after sufficient lobulo-alveolar development. Alveolar cells continue to differentiate and begin to synthesize genes involved in the synthesis of different milk components, such as casein and whey proteins. The second stage begins around parturition and is characterized by synthesis of milk components, closure of tight junctions between epithelial cells and the secretion of milk components to the lumen in the mammary gland, and drained through the ductal network (Briskin and Rajaram, 2006). Hormones that are involved in lactogenesis are PRL, placental lactogens (PL), P and GH (Neville et al., 2002). The onset of milk secretion is marked by the substantial

decline of progesterone, a steroid hormone that is released from the corpus luteum that is secreted during pregnancy. Progesterone's main role is to inhibit lactation through partial suppression of the ability of PRL to stimulate synthesis of milk constituents such as lactose, α -lactalbumin and casein (Tucker, 1981).

MECs synthesize and secrete milk components after undergoing differentiation and polarization becoming highly active secretory cells. The cytoplasm is filled with mitochondria, extensive rough endoplasmic reticulum and a well-developed golgi providing the tools to synthesize milk components (Lamote et al., 2004, McManaman and Neville, 2003). During cellular differentiation cells undergo cytoskeletal rearrangement effectively changing their shape, the organization and motion of organelles in the cytoplasm, and degree of attachment. The cytoskeleton is also responsible for polarity, enabling the cells to decipher the basal membrane, where the MEC obtain nutrients to synthesis milk components, from the lumen where milk components are secreted from the cell.

In vitro, MECs are commonly differentiated with lactogenic hormones, dexamethasone, insulin and prolactin (DIP) (Desrivieres et al., 2003, Matitashvili et al., 1997, Topper and Freeman, 1980, Zhou et al., 2008a). Differentiation has also been attributed to GH and the transcription factor STAT5 and is commonly measured by milk protein mRNA expression. Cloned bovine mammary epithelial cells treated with GH exhibit a significant increase in α -casein over cells treated with DIP alone, and an even greater increase was observed when treated with DIP and GH (Sakamoto et al., 2005). Recently, GHR and signal transducer and activator of transcription 5 (STAT5) were ectopically expressed in MAC-T cells and subsequent treatment with GH induced four

major milk protein genes, α_{s1} -casein, α_{s2} -casein, β -casein and α -lactalbumin (Zhou et al., 2008a).

The STAT transcription factors play a central role in cellular proliferation and differentiation that may be involved in maintaining galactopoiesis. Janus kinase 2 (JAK2)-mediated phosphorylation of STAT5 is activated via the cytokine receptors growth hormone receptor (GHR) and prolactin receptor (PRLR). Phosphorylation initiates the homo or heterodimerization of STAT5a and STAT5b proteins mediating their translocation to the nucleus and activating transcription of target genes such as milk proteins (Piwien-Pilipuk et al., 2002, Wang and Jiang, 2005). PRL activation is well established although GH activation of STAT5 has only been observed in mice and the MAC-T cell line when STAT5 was ectopically expressed (Gallego et al., 2001, Zhou et al., 2008a).

PRL is one of the most widely studied endocrine hormones related to lactation due to its role in stimulating the initiation and maintenance of milk secretion. The role of PRL in milk synthesis has been well defined, increasing casein and α -lactalbumin synthesis (Akers, 1985). Studies assessing ^{32}P and ^3H labeled amino acid incorporation in the presence of PRL showed that physiological concentration of PRL in conjunction with insulin or insulin with adrenal corticoids are essential for maintaining casein synthesis in bovine explants from the lactating mammary gland (Gertler et al., 1982). An increase in α -lactalbumin secretion, an enzyme involved in lactose synthesis, was also observed in bovine mammary explants in response to PRL and adrenal corticoids (Goodman et al., 1983). PRL has an important role in milk protein and lactose synthesis and PRL, insulin and adrenal corticoids are required for maintaining lactation in culture.

Hormonal influences on lactation vary greatly between species. In some species such as the lactating rabbit, lactation is completely depressed when PRL is suppressed with bromocriptine (Taylor and Peaker, 1975) which only results in partial suppression of lactation in lactating rats and mice (Flint and Knight, 1997). Interestingly suppression of PRL does not affect milk yield in lactating ruminants and the administration of exogenous PRL fails to increase milk yield or composition. In contrast to PRL, exogenous GH increases milk yield in lactating cows through nutrient partitioning and IGF-I production in the liver and mammary gland, although a direct effect on the mammary gland is not well established (Flint and Knight, 1997). The particular effects of GH will be addressed in the next section.

1.7 Growth Hormone

Growth hormone (GH), also termed somatotropin, is a peptide hormone made up of 190 or 191 amino acids with four naturally occurring variants composed of leucine or valine at position 127 in the protein with alanine or phenylalanine residues at the amino terminal. The difference in length and amino acid residues at the amino terminal result from cleavage of the signal peptide alanine or phenylalanine, making the 190 or 191 amino acid variants, respectively (Bauman and Vernon, 1993, Eppard et al., 1992). It was first discovered as a crude extract from the bovine anterior pituitary gland in the 1920s (Bauman, 1999) and in 1933 the ability of subcutaneous injections of anterior pituitary derived GH to stimulate growth in rats was demonstrated (Lee and Schafer, 1933). Shortly after, the ability to increase milk yield in dairy cows was demonstrated through injection of crude pituitary extract, temporally increasing milk yield in lactating cows. Repeated injections yielded the same results (Asimov and Krouze, 1937). The isolation method for GH from crude pituitary extracts was published in 1945 (Evans et al., 1945). Short-term studies confirmed the ability of pituitary bovine growth hormone (bGH) to increase milk yield and multiple injections for ten weeks further improved the effect (Machlin, 1973).

The increase in milk yield observed from pituitary bGH was promising for commercial application to the dairy industry but insufficient quantities isolated from the pituitary gland made commercial use impractical (Bauman, 1999, Peel and Bauman, 1987, Wood et al., 1989). The development of synthetic bGH using recombinant DNA technology alleviated the previous shortage and possible contamination of pituitary hormones for future research. In 1993 the U.S. food and drug administration (FDA) approved the use of POSILAC, recombinant bovine growth hormone for milk production (Monsanto, St. Louis, MO). Recombinant bovine growth hormone approved for use is the 190-amino acid variant with leucine at position 127, which is the most commonly occurring pituitary derived variant with an extra methionine at the amino terminus (Etherton and Bauman, 1998). The addition of methionine showed no effect on milk yield and the leucine variant was used even though the valine variant induced a greater increase in milk production (Eppard et al., 1992).

The first study using recombinantly derived GH was completed in 1982. Since then it has been widely demonstrated that the use of recombinant bGH does not alter gross composition of milk although the concentrations of milk fat and protein may vary due to other factors such as genetics, breed, stage of lactation, season, diet and nutritional status (Bauman, 1992, Etherton and Bauman, 1998). Both synthetic and pituitary derived bGH does not bind to GHR in human tissue and the FDA concluded that products from dairy cattle treated with recombinant bGH are not harmful for human consumption (Juskevich and Guyer, 1990). Exogenous GH increases milk yield by increasing the availability of nutrients and indirectly acting on the mammary gland through increasing synthesis of IGF-I by the liver (Bauman, 1999, Bauman and Vernon, 1993). Mechanisms

involving a direct effect on the mammary gland have been hypothesized but not fully characterized.

The partitioning of nutrients during lactation in ruminants treated with exogenous GH occurs through many diverse physiological processes, resulting in improved production efficiency through the redirection of nutrients used for body fat (NRC, 1994). Treatment with GH produces effects similar to what is observed at the onset of lactation: the increase in milk yield is immediate, although voluntary feed intake does not meet production needs until the fifth or seventh week of treatment (Prosser and Mepham, 1990). During treatment the most pronounced metabolic change occurs in adipose tissue. The initial negative energy balance in response to GH treatment increases the rate of lipid mobilization (lipolysis), indicated by the increased plasma concentration of free fatty acids, mobilizing nutrients for increased milk synthesis. As feed intake increases and the dairy cow re-enters a positive energy balance lipolysis decreases and lipid synthesis remains low making more nutrients available for milk synthesis. In both negative and positive energy balance, GH acts as an antagonist to insulin, specifically in adipose tissue, decreasing the uptake of glucose and reducing lipogenesis (Bauman, 1999, Bauman and Vernon, 1993, Vernon, 1990). The changes in glucose metabolism in response to GH is important for increased milk synthesis, with the majority of glucose being derived from hepatic gluconeogenesis. When treatment with bGH is initiated glucose turnover increases, oxidation of glucose decreases and there is a reduction in hepatic response to insulin, allowing the liver to maintain high levels of gluconeogenesis supporting increased lactose synthesis (Bauman and Vernon, 1993, NRC, 1994).

The indirect action of GH on the mammary gland is mediated by IGF-I, mainly produced by the liver with small amounts produced locally by stromal cells in the

mammary gland. IGFs prevents apoptosis as well as mediates growth, development and differentiation in mammary cells (Baumrucker and Stemberger, 1989, Plath-Gabler et al., 2001). Treatment with growth hormone *in vivo* increases circulating plasma levels of IGF-I and high affinity insulin like growth factor binding proteins (IGFBP), which modulate the activity of IGF-I. The binding of IGFBPs to IGFs have been shown to both enhance and inhibit IGFs activity *in vitro*. IGFBPs enhance IGFs by prolonging the half-life and transporting IGFs from vasculature to specific cell types and inhibit IGF action by competing with the insulin-like growth factor receptor (IGFR) (Cohick, 1998, Etherton and Bauman, 1998, Forsyth, 1996).

Discovery of growth hormone receptor (GHR) protein expression and GHR mRNA in mammary alveolar epithelial cells and in MAC-T cells indicates a possible direct effect of GH on the mammary gland (Glimm et al., 1990, Sakamoto et al., 2005, Sinowatz et al., 2000, Zhou et al., 2008a). Cloned MECs have been shown to express and synthesize α -casein in the presence of lactogenic hormones DIP which further increases with the addition of GH (Sakamoto et al., 2005). Increased expression of β -casein mRNA was observed in mammary tissue in cows treated with GH compared to a control cow. Explant cultures also showed an increase in β -casein mRNA when treated with GH or PRL in comparison to tissue cultured in basal medium (Yang et al., 2005). Although it has been confirmed that GHR is expressed in the mammary alveolar epithelial in dairy cattle, whether GH affects milk synthesis is still unclear.

1.8 Mammary gland models

To examine the cellular mechanisms involved in mammary epithelial cell development and lactation, *in vitro* cell culture models have been employed, creating isolated environments that are reproducible at a low cost. Explant culture, primary mammary epithelial cells and continuous mammary epithelial cell lines have been used to examine the effects of hormonal stimuli, cell-cell interactions and an extracellular matrix or basement membrane on mammary epithelial cell function.

Explants, primary cells and continuous cell lines are the most extensively used models to study the mechanisms involved in lactation and mammary gland growth. Explants can be obtained from the mammary gland through tissue samples from a biopsy or at the time of slaughter and can be thought of as cells left in their surrounding extracellular matrix to more accurately mimic the mammary gland *in vivo*. Multiple cell types, such as myoepithelial cells, stromal cells and epithelial cells are present in mammary explants. This provides a partially isolated, 3-dimensional model maintaining similar characteristics of the mammary gland to assess hormonal action (Trott et al., 2008). Although this model offers the most similar environment to the mammary gland, there are many disadvantages making it difficult to interpret results. There is the possibility of latent hormones, growth factors or inhibitors in the tissue that could be activated during incubation. The heterogeneous cell population makes it difficult to determine the target of exogenously added factors. It also is difficult to estimate cell

number in explant culture versus a monolayer of single cells. Additionally, tissue can only be cultured for a finite period of time (Matitashvili et al., 1997).

Alternatively, primary cells are isolated directly from the mammary gland so they have similar metabolic and physiological characteristics as epithelial cells *in vivo*. Primary cells can be isolated from milk or by removal of the mammary gland by biopsy or at slaughter, although transfer of cells from the body to a culture dish may impact the viability, responsiveness to hormonal stimulation, and their ability to synthesize milk components in culture (Matitashvili and Bauman, 1999). Primary cells can be difficult to maintain in culture, although they have the ability to synthesize milk components when stimulated with DIP. Though primary cells share similarities with mammary epithelial cells *in vivo* they also have limitations in culture which include the small number of passages they can undergo before the loss of their original characteristics such as the loss of polarization of organelles and the alteration in milk synthesis and secretion of most milk proteins (Matitashvili and Bauman, 2001, Matitashvili et al., 1997).

Continuous cell lines are derived from primary cells to circumvent senescence and crisis through the expression of certain viral or oncogenes (Huynh et al., 1991). The use of continuous cell lines is an excellent alternative to primary cells eliminating biological variability between cells and making observations between research groups more reliable (Matitashvili et al., 1997). Unfortunately most continuous cell lines do not maintain all of the characteristics of MEC, such as the ability to assume the typical mammary cell morphology, the production of milk proteins, or lipid droplets (Huynh et al., 1991). Rodent, human and ruminant cell lines have been established but are not necessarily reflective of one another due to species differences as well as the establishment of both normal and tumor cell lines for rodents and humans. A variety of

non-carcinogenic cell lines have been established and include the murine COMMA-D cell line (Danielson et al., 1984) and the HC11 cell line (Han et al., 1996). There are also a variety of human and rat carcinogenic cell lines that cause tumor formation in nude mice and show little or no anchorage-independent growth (Stampfer and Bartley, 1985). There are relatively few ruminant mammary cell models and the most widely used *in vitro* bovine cell line is the MAC-T cell line (Matitashvili and Bauman, 2001).

MAC-T cells were derived by transfection of Holstein primary mammary epithelial cells with the simian virus 40 (SV40) large T-antigen (Huynh et al., 1991). The SV40 large-T antigen binds p53 inactivating the ability of p53 to inhibit cellular proliferation therefore allowing MAC-T cells to undergo unlimited cellular proliferation (Ali and DeCaprio, 2001). This cell line has been shown to undergo more than 350 serial passages while nontransfected mammary epithelial cells typically enter crisis after 16-25 passages (Haug et al., 2007). MAC-T cells have shown the ability to differentiate and secrete milk specific proteins when grown with lactogenic hormones making it an extensively used *in vitro* cellular model for ruminant epithelial cell proliferation, differentiation and early lactation (Huynh et al., 1991, Zavizion et al., 1995).

MAC-T cells have been used to assess many areas of research such as *de novo* synthesis of fatty acids (McFadden and Corl, 2009), cellular proliferation (Woodward et al., 1996, Zavizion et al., 1993, Zhou et al., 2008b) and effects of GH on mammary epithelial cells (Zhao et al., 1992, Zhou et al., 2008a). MAC-T cells possess IGF-I receptors and low levels of GHR mRNA. Treatment with IGF-I stimulates proliferation (Zhao et al., 1992) although the mechanisms involved are not fully understood. MAC-T cells have the ability to secrete IGFBP -2, -3, -4, and -6 and when treated with IGF-I there is an increase in IGFBP-3 mRNA (Cohick, 1998), which supports proliferation and

increases the availability of IGF-I to mammary epithelial cells (Leibowitz and Cohick, 2009). Recently, GHR and STAT5 were ectopically expressed in MAC-T cells, and treatment with GH induced an increase in α s1-, α s2- and β -casein, as well as α -lactalbumin mRNA (Zhou et al., 2008a).

1.9 Summary

There has been extensive research on the secretory nature of the mammary gland. Early research focused on milk composition, synthesis and secretion and hormonal regulation of mammary development (Fox and McSweeney, 1998, Tucker, 1979). The isolation of primary MECs and the development of continuous cell lines provided a controlled environment to study hormonal regulation of the mechanisms and transcription factors involved in MEC proliferation, differentiation, apoptosis and milk synthesis (Matitashvili et al., 1997).

MAC-T cells are a continuous cell line stably transfected with the SV40 large-T antigen and are the most commonly used secretory model for bovine MECs. MAC-T cells synthesize and secrete casein in the presence of DIP and appear to have the same morphological characteristics of primary MECs (Huynh et al., 1991).

The effect of exogenous GH on milk yield has been well documented. Treatment with GH increases milk yield by 10-40% without altering the gross composition and it is widely accepted that GH acts through nutrient partitioning and on a cellular level through IGF-I produced by the liver (Bauman, 1999). The identification of GHR mRNA and through *in situ* hybridization in mammary gland (Plath-Gabler et al., 2001, Sinowatz et al., 2000) and mRNA abundance in MAC-T cells (Zhou et al., 2008a) suggests that GH may have a direct galactopoietic effect on MECs (Sakamoto et al., 2005, Zhou et al., 2008a).

Understanding the mechanisms involved in differentiation, proliferation, involution and milk synthesis and secretion will increase the ability to manipulate lactation, better understand the irregular growth that results in mammary tumors and increase the body of knowledge pertaining to lactation biology. Using an *in vitro* cell

model to begin explaining the mechanisms involved in differentiation and lactation due to GH, direct effects of GH were determined and assessed in MAC-T cells by qPCR, measuring the relative mRNA abundance of GHR, α_{s1} -casein and α -lactalbumin. To assess some of the mechanisms involved in response to GH, changes to global protein expression in differentiated MAC-T cells in response to GH were assessed through 2D gel electrophoresis. Gels were analyzed with bioinformatics software Delta 2D (DECODON, Germany) and differentially expressed proteins were identified with MALDI-TOF/TOF mass spectrometry (Bruker Daltonics, Billerica, MA).

CHAPTER 2¹

¹ Previously Published: Johnson, T. J., Fujimoto, B. A., Jimenez-Flores, R., & Peterson, D. G. (2010). Growth hormone alters lipid composition and increases the abundance of

Growth Hormone Alters Lipid Composition² and Increases the Abundance of Casein and Lactalbumin mRNA in the MAC-T Cell Line

2.1 Introduction

The mammary epithelial cell is responsible for the synthesis and secretion of milk components and, therefore, is a primary focus for the study of mammary function. The Mammary Alveolar Cell-T (MAC-T) is a continuous cell line derived from bovine mammary tissue by stable transfection with the SV-40 large T-antigen (Huynh et al., 1991) and has been used extensively for the study of mammary epithelial cell function. Induction and maintenance of a lactogenic phenotype in mammary cell and explant models, including the MAC-T cell line, has commonly been achieved using a combination of dexamethasone, insulin and prolactin (PRL; e.g. (Huynh et al., 1991, Matitashvili et al., 1997)).

Growth hormone (GH), also termed somatotropin, is known to have stimulatory effects on milk production *in vivo* (reviewed by (Bauman, 1992, 1999, Bauman and Vernon, 1993), and while it is well accepted that GH acts through somatomedins such as the insulin-like growth factors produced locally and by the liver, it remains somewhat in question whether the effects of GH include direct influence on the mammary epithelium (for example, see (Akers, 2006, Bauman and Vernon, 1993, Svennersten-Sjaunja and Olsson, 2005). GH receptor (GHR) has been detected in mammary tissue (eg. (Hauser et al., 1990, Plath-Gabler et al., 2001) and investigation into the effects of GH administration on the mammary epithelium in particular have subsequently been pursued in cell models of lactation. Sakamoto and co-workers (2005) observed GH receptor

casein and lactalbumin mRNA in the MAC-T cell line. *Journal of Dairy Research*(77), 1-6.

² The research pertaining to lipid composition was completed and presented by Brent Fujimoto in his master's thesis.

(GHR) expression on the surface of a novel bovine mammary cell line cloned from a Holstein heifer, and that GH administration to these cells enhanced their secretion of α -casein both independently and in synergy with the classical combination of dexamethasone, insulin and PRL (DIP). Additionally, these investigators noted that treatment with DIP but without GH enhanced the expression of GHR in the mammary cells. More recently, both the GHR and the signal transducer and activator of transcription 5 (STAT5) were ectopically expressed in MAC-T cells, and subsequent treatment with GH led to significant increases in the abundance of α_{s1} -, α_{s2} - and β -casein, as well as α -lactalbumin mRNA (Zhou et al., 2008a). While these investigators were able to detect GHR mRNA in MAC-T cells both by PCR and ribonuclease protection assay, the overexpression of GHR and STAT5 was intended to exaggerate any response to GH that may have been observed; there were no data reported on the effects of GH in the untransfected cells.

The influence of lactogenic hormones on milk protein production is an important means of assessing the quality of a mammary cell model, though the lipogenic profile of mammary cells could also be used to determine the pertinence of such a model. Milk lipids consist mainly of triacylglycerol (TAG), and while freshly dispersed mammary cells of ruminant origin synthesize predominantly TAG (Hansen and Knudsen, 1987a, b), the MAC-T cell line produces predominantly polar lipids under the traditional lactogenic hormone milieu (present work and D. Peterson, unpublished observations). Treatments that increase the proportion of TAG synthesized by cultured mammary cells could be useful, particularly when studying mammary lipogenesis.

The recent findings with respect to GH treatment of mammary cell lines led us to the hypothesis that GH may act directly to alter milk protein synthesis and the lipid class

distribution of MAC-T cells to better mimic the mammary epithelium *in vivo*. Our objectives were to determine whether the cells expressed GHR mRNA, and whether the addition of GH to the differentiation medium would affect the abundance of α -lactalbumin and α -S1-casein mRNA, as well as the lipid class distribution as determined by thin-layer chromatography (TLC).

2.2 Materials and Methods

All reagents were acquired from Sigma Aldrich (St Louis, MO) unless otherwise noted.

2.2.1 Cell culture and treatments

MAC-T cells were grown to confluence in 100mm diameter CellBIND treated plastic cell culture dishes (Corning Inc., Corning, NY) using a proliferation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS; Innovative Research, Novi, MI), 1% penicillin/streptomycin, 5 µg/ml insulin, 1

µg/ml progesterone. To ensure that any effect of serum was consistent between treatments, all FBS used was from a single lot. Once confluent, MAC-T cells were treated for 7 days with differentiation medium consisting of DMEM, 10% FBS, 1% penicillin/streptomycin, 10 µg/ml dexamethasone, 5 µg/ml insulin, 5 µg/ml prolactin and either 0 or 10 ng/ml bovine GH (generously provided by G. Bogosian [gregg.bogosian@monsanto.com] at Monsanto, St. Louis, MO). Each experiment was conducted in triplicate.

2.2.2 mRNA abundance

For quantification of mRNA abundance, cells were harvested from one confluent 100mm plate per replicate (n = 3 replicates) by scraping, total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and 500ng was reverse transcribed using oligo dT primed iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA).

Abundance of GHR, α -lactalbumin (LALBA) and α S1-casein (CSN1S1) mRNA was

determined by quantitative PCR using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and the primers specified in Table 1. All primers were obtained from Integrated DNA Technologies (Coralville, IA). Reactions were carried out using a 7500Fast PCR system (Applied Biosystems, Foster City, CA) with an initial denaturing step of 20 s at 95°C followed by 55 cycles of 3 s at 95°C and 30 s at 60°C. Initial target mRNA abundance was calculated using the $2^{-\Delta\Delta C_t}$ method with abundance of β -actin mRNA serving as an internal control to correct for the efficiency of reverse transcription and data for each treatment were expressed relative to the undifferentiated cells (Livak and Schmittgen, 2001). PCR product quality and specificity were verified by melt curve analysis and subsequent agarose gel electrophoresis.

Target	Sense Primer (5'-3')	Antisense Primer (5'-3')	Source ¹
GHR	CTAACTAGCAATGGCGGT	GGGTGGATCTGGTTGTACTA	NM_176608
LALBA	AAAGACGACCAGAACCCTCA	GCTTTATGGGCCAACCAGTA	Zhou et al., 2008
CSN1S1	AATCCATGCCCAACAGAAAG	TCAGAGCCAATGGGATTAGG	Zhou et al., 2008
ACTB	GCGTGGCTACAGCTTCACC	TTGATGTCACGGACGATTTC	Bionaz and Loor, 2007

Table 1. Primer sequences used for quantitative PCR analysis in this study.

¹GHR primers were designed from GenBank accession number NM_176608 using PrimerQuest software available at www.idtdna.com and primer quality was further assessed using Beacon Designer (Premier Biosoft International).

2.2.3 Lipid composition

For lipid separation, cells were harvested by scraping from two confluent 100mm plates of cells per replicate (n = 3 replicates) and subsequently pelleted by centrifugation (500 x g, 10 min, 4°C). Total lipid was extracted from the cell pellet using 2:1 chloroform-methanol (Folch et al., 1957) and separated on the basis of polarity by thin layer chromatography (TLC) using a 65:25:4 chloroform-methanol-water mobile phase

(Christie, 1982). TLC plates were placed in a sealed chamber with iodine crystals for 24 hours followed immediately by scanning and quantification by densitometry using Molecular Analyst (Bio Rad, Hercules, CA). Relative abundance was calculated as the density of each band normalized to the total density of all bands for each lane on each TLC plate. Bands were identified by comparison to standards representing TAG, cholesterol, phosphatidylethanolamine (PE), phosphatidyl choline (PC), phosphatidylinositol (PI), and sphingomyelin (SM) that were included with each plate; two bands did not correspond to any of the chosen standards and are designated Unknown.

2.2.4 Cell viability

Cell viability was assessed in parallel cultures grown as described above. Viability was assessed in cells grown to confluence in proliferation medium, as well as in cells differentiated with DIP alone and DIP + 10 ng/ml GH using flow cytometry with the ViaCount cell viability assay (Guava Technologies, Hayward, CA) according to the manufacturer's recommendation in a Guava EasyCyte Plus flow cytometer (Guava Technologies, Hayward, CA). Prior to incubation with the ViaCount reagent, cells were

detached from the culture substrate by incubation with trypsin-EDTA solution and washed in complete medium before pelleting and resuspension in PBS.

2.2.5 Statistical analysis

Lipid composition data were analyzed using the general linear model of SAS (SAS Institute, Cary, NC) for differences between treatments. Data were transformed by arcsine-square root for analysis due to a tendency for non-normality at the extremes of values represented as a proportion (between 0 and 1; (Freeman and Tukey, 1957).

Pairwise comparisons between means were made using the Tukey test. For statistical analysis of mRNA abundance and cell viability data, normalized abundance for each treatment was analyzed using the general linear model of SAS for differences between

treatments. Tukey's simultaneous test was used to compare all means to each other. In all cases, differences were considered significant at $P < 0.05$.

2.3 Results

To determine whether any effect of GH could be due to direct, receptor-mediated effects, we used the classical DIP differentiation protocol with either 0 or 10 ng/ml GH and quantified the abundance of GHR mRNA, as well as that of two milk protein genes, LALBA and CSN1S1. Differentiation with DIP in the absence of GH led to a 14-fold increase in GHR mRNA, while DIP with 10 ng/ml GH led to a 35-fold increase in GHR mRNA compared to the undifferentiated cells (Figure 2A). Differentiation with DIP led to small numerical increases in LALBA and CSN1S1 mRNA that were not significant, while GH addition to the differentiation media led to significant 28- and 32-fold increases

in LALBA and CSN1S1 mRNA, respectively, as compared to the undifferentiated controls (Figures 2B, C).

Observations of our TLC plates indicate that PC and PI migrated as one band with the current protocol, and SM eluted as two separate bands as has been described previously (Ramstedt et al., 1999). Semi-quantitative analysis of lipid class distribution was achieved by densitometry and the results are shown in Table 2. Differentiation with 10 ng/ml GH led to an 80% increase in the proportion of TAG (Figure 3), and a more than three-fold decrease in the proportion of the SM2 fraction ($P < 0.05$). It is

noteworthy that between 10 and 14% of the total lipid did not correspond to any of our chosen standards (designated Unknown) and while these fractions were not significantly affected by GH, it would be of interest to identify these lipid classes. Based on their migration in relation to the known lipid classes, these are polar lipids with Unknown 1 being more polar than SM, while Unknown 2 had a polarity in between SM and PC + PI fraction.

Figure 2. Abundance of growth hormone receptor (GHR; panel A), α -lactalbumin (LALBA; panel B) and α_{s1} -casein (CSN1S1; panel C) mRNA in undifferentiated MAC-T cells as well as those differentiated with either 0 or 10 ng/ml GH. Undifferentiated cells were grown to confluence in DMEM supplemented with 10% FBS, 5 μ g/ml insulin and 1 μ g/ml progesterone; differentiated cells were grown to confluence as the undifferentiated cells then cultured for 7d in DMEM supplemented with 10% FBS, 5 μ g/ml insulin, 10 μ g/ml dexamethasone, 5 μ g/ml PRL, and the GH concentrations noted. Relative mRNA abundance was assessed by quantitative PCR and normalized to the expression of β -Actin. Values represented are means (n=3); error bars represent the standard error of the mean; values with different letters differ ($P<0.05$).

	GH (ng/ml)		<i>P</i>
	0	10	
TAG	10.3 $\pm$ 1.0	18.6 $\pm$ 0.2	< 0.01
Cholesterol	18.8 $\pm$ 1.7	24.5 $\pm$ 5.9	0.16
PE²	7.0 $\pm$ 0.6	6.7 $\pm$ 0.7	0.80
PC + PI³	7.3 $\pm$ 0.3	6.0 $\pm$ 0.4	0.07
SM1⁴	24.3 $\pm$ 2.2	24.6 $\pm$ 1.7	0.92
SM2⁴	13.4 $\pm$ 0.3	3.7 $\pm$ 0.4	< 0.01
Unknown 1⁵	4.2 $\pm$ 0.4	2.8 $\pm$ 0.6	0.13
Unknown 2⁵	9.6 $\pm$ 1.7	7.2 $\pm$ 1.8	0.39
Origin	5.3 $\pm$ 0.9	6.0 $\pm$ 1.0	0.66

Table 2. Lipid class distribution of MAC-T cells grown to confluence in DMEM supplemented with 10% FBS, 5µg/ml insulin and 1µg/ml progesterone, and then differentiated for 7d in DMEM supplemented with 10% FBS, 5µg/ml insulin, 10µg/ml dexamethasone and either 0 or 10 ng/ml GH¹

¹Values represent means (n=3); effect of GH was deemed significant at $P < 0.05$; *ns*, not significant; SEM represents the pooled standard error of the mean for each dependent variable.

²PE, phosphatidylethanolamine.

³PC, phosphatidylcholine, and PI, phosphatidylinositol, coeluted and are expressed as the combined value.

⁴SM eluted as two separate bands as has been previously observed (Ramstedt et al., 1999) and each band is represented individually.

⁵Two bands did not correspond to any of the chosen standards, and are represented as Unknown.

Figure 3. Effect of growth hormone (GH) on triacylglycerol (TAG) content in MAC-T cells. Cells were grown to confluence in DMEM supplemented with 10% FBS, 5 μ g/ml insulin and 1 μ g/ml progesterone, and then differentiated for 7d in DMEM supplemented with 10% FBS, 5 μ g/ml insulin, 10 μ g/ml dexamethasone and either 0 or 10 ng/ml GH. Total lipids were extracted and lipid classes separated by TLC. Each band resulting from the TLC separation was quantified by densitometry and TAG content was calculated as a percent of the sum of all band densities for that lane. Values represented are means (n=3); error bars represent the standard error of the mean; values with different letters differ ($P < 0.01$).

To ensure that any differences observed were not due to differences in cell number due to GH addition to the differentiation media, we analyzed cell viability and found no significant effect of GH on viable cell number or percent viable cells ($P > 0.20$; data not shown).

2.4 Discussion

There has been much speculation about the exact nature of the effects of GH on lactation *in vivo*, and it remains unclear whether GH has any direct effect on the mammary epithelium. The identification of GHR expression both in the bovine mammary gland (Plath-Gabler et al., 2001) and in bovine mammary cell lines including the MAC-T (Sakamoto et al., 2005, Zhou et al., 2008a) indicate the possible direct influence of GH on the mammary epithelial cell. Accordingly, Sakamoto et al. (2005) showed a positive influence of GH on milk protein production in a mammary cell line. More recently, Zhou et al. (2008) demonstrated that GH administered to MAC-T cells

that were overexpressing GHR and STAT5 also led to increased production of milk proteins.

The demonstration of very low GHR abundance in undifferentiated native MAC-T cells by Zhou et al. (2008) led us to determine the abundance of GHR mRNA in undifferentiated cells as well as those differentiated with either DIP or DIP and GH. We found that differentiation led to a significant increase in the abundance of GHR mRNA, and that this was even more dramatic in the GH treated cells. A similar pattern was observed for two major milk protein genes, CSN1S1 and LALBA, though it was surprising to find a much greater difference between the DIP differentiated cells with and without GH than between undifferentiated cells and those differentiated with DIP alone.

The lipid component of milk represents the major energy cost for milk production, the primary energy source for the consumer of whole milk, and a natural source of bioactive compounds such as conjugated linoleic acids. As such, milk fat synthesis is of interest to many research groups, and a convenient cell model for the study of milk fat synthesis would be of great value. While Hansen and Knudsen (1987a; 1987b) have reported that freshly dispersed mammary cells of bovine and caprine origin predominantly produce TAG, the major lipid class of secreted milk fat, there is no report of the actual secretion of a milk fat globule by a mammary cell model. Fekry et al. (1989) found that milk lipid synthesis was enhanced in bovine mammary explants treated with GH, though only when co-cultured with adipose and liver explants, leading to the conclusion that the effects of GH on mammary lipid synthesis were indirect, possibly through IGF production by the liver explants.

Here, we have demonstrated that the addition of GH to differentiation medium shifts the lipid class distribution of MAC-T cells toward greater TAG content, a

phenotype that more closely resembles lactation *in vivo*, and that this effect was observed without overexpression of GHR or STAT5. It is unclear from this experiment whether the change in TAG content as a proportion of total lipid represented an increase in actual amount of lipid synthesized. Our data indicate that changes in cell number due to proliferative or toxic effects of GH are not responsible for the alterations in lipid composition. Observations *in vivo* of increased output of milk with normal composition without any change in mammary DNA content indicate that GH acts to increase the overall synthetic capacity of the mammary gland (reviewed in (Bauman, 1999)). This overall change, whether direct, indirect, or both, would require a coordinated response within the cell that involved many different pathways, and our results demonstrate that GH affects not only lipid metabolism, but also the mRNA abundance of two milk proteins in MAC-T cells. It is important to note that based on our analysis, the changes in mRNA abundance cannot be attributed specifically to increases in synthetic rate, but may instead represent changes in the stability of mRNA without any change in transcription rates.

These data indicate that GH can directly affect the MAC-T cell line, and based on the presence and inducibility of GHR mRNA, that the effects of GH are likely mediated by GHR. Further, it appears that the effects of GH involve responses in milk protein mRNA abundance as well as lipogenic pathways, consistent with findings of GH treatment of lactating cows (reviewed by Bauman, 1999). It is possible that GH is important in attaining a more differentiated state in the MAC-T cells as opposed to enhancing the production of already differentiated cells. Either way, in developing cell culture models of lactation, it may be important to consider GH as an integral part of the hormone milieu that is required for maximal galactopoietic potential. Finally, these data

support the developing hypothesis that GH may have a direct effect on mammary metabolism *in vivo*.

CHAPTER 3

A proteomic analysis of the effect of growth hormone on differentiated MAC-T cells.

3.1 Introduction

The mammary alveolar cell-T (MAC-T) cell line is a widely used model of the ruminant mammary gland due to their ability to differentiate and secrete milk specific proteins when grown with lactogenic hormones (Huynh et al., 1991, Zavizion et al., 1995). The induction and maintenance of functional differentiation and a lactogenic phenotype by treatment with dexamethasone, insulin and prolactin (DIP) is commonly achieved in mammary explants, primary cell cultures and continuous cell lines including MAC-T cells (Desrivieres et al., 2003, Huynh et al., 1991, Matitashvili et al., 1997).

Growth hormone (GH), also termed somatotropin, is known to increase milk yield without altering milk composition *in vivo*. It is widely accepted that GH acts through somatomedins such as insulin-like growth factor I (IGF-I) and as a nutrient partitioning agent (Bauman, 1999, Bauman and Vernon, 1993). The identification of growth hormone receptor (GHR) in mammary tissue (Plath-Gabler et al., 2001), cloned bovine mammary epithelial cells (Sakamoto et al., 2005, Zhou et al., 2008a) and in MAC-T cells (Johnson et al., 2010, Zhou et al., 2008a) indicates a direct effect of GH on mammary epithelium, however, the mechanisms through which GH may act on this system have not been fully characterized. Cloned bovine mammary epithelial cells treated with GH exhibited a significant increase in α -casein over cells treated with DIP alone, and an even greater increase was observed when treated with DIP and GH (Sakamoto et al., 2005). Recently, GHR and signal transducer and activator of transcription 5 (STAT5) were ectopically expressed in MAC-T cells and subsequent treatment with GH increased four major milk protein genes, α ₁-casein, α ₂-casein, β -casein and α -lactalbumin (Zhou et al., 2008a). An increase in GHR and two major milk proteins, α ₁-casein and α -lactalbumin mRNA was induced in native MAC-T cells in response to DIP differentiation with GH (Johnson et al., 2010). Recent research in our laboratory observed a significant increase in triacylglycerol (TAG) in MAC-T cells grown in DIP with 10 ng/ml GH above cells grown in DIP alone, more closely resembling lactation *in vivo* (Johnson et al., 2010).

To develop the ability to synthesize and secrete milk products mammary epithelial cells (MECs) must undergo differentiation. There has been little research pertaining to hormonal effects on cellular structure, specifically changes in the cytoskeleton on MECs. Using 2D gel electrophoresis, analysis of global protein expression in the mouse mammary HC11 cell line differentiated with DIP compared to

undifferentiated cells was assessed (Desrivieres et al., 2003). Reorganization of the actin cytoskeleton was observed using immunofluorescence microscopy in the Chinese hamster ovary (CHO) cell line in response to GH (Goh et al., 1997a). These studies suggest a hormonal effect on cellular structure and differentiation of MECs.

To assess the mechanisms involved in differentiation in preparation for milk secretion, global protein expression patterns of MAC-T cells differentiated in DIP with or without GH were assessed by 2D gel electrophoresis and proteins that were differentially expressed in response to GH were identified using MALDI-TOF/TOF mass spectrometry.

3.2 Materials and Methods

All reagents were acquired from Sigma Aldrich (St Louis, MO, USA) unless otherwise stated.

3.2.1 Cell culture and treatments

MAC-T cells were grown to confluence in 100mm diameter CellBIND treated plastic cell culture dishes (Corning Inc., Corning, NY) using a proliferation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS; Innovative Research, Novi, MI), 1% penicillin/streptomycin, 5 µg/ml insulin, 1 µg/ml progesterone. To ensure that any effect of serum was consistent between treatments, all FBS used was from a single lot. Once confluent, MAC-T cells were treated for 7 days with differentiation medium consisting of DMEM, 10% FBS, 1% penicillin/streptomycin, 10 µg/ml dexamethasone, 5 µg/ml insulin, 5 µg/ml prolactin and either 0 or 10 ng/ml bovine GH (generously provided by G. Bogosian [gregg.bogosian@monsanto.com] at Monsanto, St. Louis, MO). Each experiment was conducted in triplicate.

3.2.2 Two-dimensional gel electrophoresis

To isolate protein, MAC-T cells were washed in PBS, harvested by scraping and placed in a 2 mL centrifuge tube with 150 μ L lysis buffer (7M urea, 2M thiourea, 1% ASB-14, 40mM tris base, 0.1% IPG buffer and 40mM DTT) per plate of cells. Cells were lysed by sonication three times for 10 seconds each with a probe sonicator. Sonicated cells were centrifuged at 10,400 x g for 30 min at 4°C. Impurities were removed from protein sample using a 10% TCA/acetone precipitation and rehydrated with rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 2% NP-40, .002% bromophenol blue, 0.1% IPG buffer and 100mM DTE). Protein concentrations were determined using 2D-Quant kit (GE Healthcare, Uppsala, Sweden). 400 μ g of protein was separated by isoelectric point on 11cm immobilized pH gradient (IPG) gel strips, (GE healthcare, Uppsala, Sweden) pH 3-10 strips where rehydrated using passive rehydration for 16 hours and focused using the preset 11cm rapid program (250V for 15 minutes, 8000V for 2:30 hrs

and 35,000 vhours) with a Protean IEF cell (Bio-Rad Laboratories, Hercules, CA, USA). The protein was then separated by molecular weight on 10% pre-cast tris-HCL gels in a Criterion Dodeca Cell (Bio-Rad Laboratories, Hercules, CA, USA) at 200V for 1hour. Protein spots were stained with colloidal Coomassie Blue G-250. Gels were scanned and analyzed using the 2D gel image analysis software, Delta2D (DECODON, Germany). Using Delta2D, gels were warped to line up corresponding spots to correct for running differences between gels gel images. A t-test, using 200 permutations was used to detect statistically different proteins, significant determined at $P < 0.05$.

3.2.3 Tryptic digestion, MALDI-TOF MS

Protein spots were excised from Coomassie stained 2D gels using a 1.5 mm manual tissue arrayer puncher (Beecher Instruments, Sun Prairie, WI) and placed in 0.5 mL centrifuge tubes. Gel pieces were destained in destaining buffer (25mM NH_4HCO_3 /50% acetonitrile) for two 30 min incubations, then washed with 100% acetonitrile and digested with trypsin solution (1 $\mu\text{g}/\mu\text{l}$ mass spectrometry grade porcine trypsin gold (Promega, Madison, WI) in 40 mM NH_4HCO_3 /10% acetonitrile) overnight at 37°C. To elute peptides out of the gel material, analyte solution (0.1% TFA/ acetonitrile 2:1) was added and incubated and shaken for 30 min at room temperature, repeat to elute remaining peptides out of gel material. Protein samples were dehydrated with a SpeedVac at 45°C and immediately rehydrated with 1 μl analyte solution. Matrix solution (0.2mg/ml α -cyano-4-hydroycinnamic acid (HCCA) in acetonitrile) was added and matrix /analyte sample was plated on an anchorchip target (Bruker Daltonics, Billerica, MA). Each plated spot was washed with 0.1% TFA in 10 mM ammonium

phosphate and recrystallized with recrystallization buffer (EtOH, acetone, 0.1% TFA 6:3:1). The peptides were analyzed using ultraflex II MALDI-TOF/TOF mass spectrometry (Bruker Daltonics, Billerica, MA). Peptide mass fingerprints (PMF) were acquired and externally calibrated using FlexControl software (Bruker Daltonics, Billerica, MA), Spectra were internally calibrated and processed using FlexAnalysis software (Bruker Daltonics, Billerica, MA). PMFs were analyzed using MASCOT server launched from BioTools software (Bruker Daltonics, Billerica, MA) against the NCBI database. When searching MASCOT, carbamidomethylation and oxidation were set as fixed modifications, a maximum of one missed cleavage was allowed and taxonomy category was restricted to mammals. PMF spectra were further analyzed using MS/MS spectra using five to ten of the largest peaks per sample excluding keratin and trypsin during analysis. PMF and MS/MS spectra were combined and queried as described for PMF spectra analysis using the MS/MS spectra.

3.3 Results

Total global protein expression of MAC-T cells differentiated with or without GH identified 265 protein spots (n=3) (Figure 4). Differentially expressed proteins were observed between treatment groups, revealing 40 of 265 protein spots that significantly changed in abundance when stimulated with GH (Figure 5). Of the 40 differentially expressed proteins, 15 proteins were down regulated with a fold change from 1.2 to 5.78 and 25 proteins were up regulated with a fold change of 1.23 to 3.11 in response to 10 ng/ml GH for seven days (Figure 6). Proteins varied in size from 20 kDa to 100 kDa and in pI from 4.0 and 9.0.

Thirty-two of the forty differentially expressed proteins were successfully matched to NCBI database sequences (Table 3). The eight proteins not identified were either too low in abundance to obtain a suitable PMF, or trypsin dominated the spectrum. Proteins that were identified were involved in metabolism, cytoskeleton, protein folding, RNA processing, detoxification, calcium binding, and stress response.

In response to GH, seven differentially expressed proteins were metabolic proteins involved in mammary metabolism and production of NADPH. Four metabolic

proteins, α -enolase, aspartate aminotransferase 2, cytosolic isocitrate dehydrogenase and lactate dehydrogenase were up-regulated. Three metabolic proteins, fructose-bisphosphate aldolase A, chain A mitochondrial F1-ATPase and glucose-6-phosphate dehydrogenase, were down-regulated. Chain A mitochondrial F1-ATPase was found in two spots in the gel and (spot 164 and 165) was inversely expressed, spot 164 increased in abundance with a 2.39 fold change, while spot 165 decreased in abundance with a 3.14 fold change. Eight proteins involved in cytoskeletal rearrangement and support changed

Figure 4. Proteome map of all proteins detected by two-dimensional gel electrophoresis from MAC-T cells treated in DIP with 0 or 10 ng/ml GH. Four hundred micrograms of total protein underwent isoelectric focusing on 11 cm IPG strips, pH 3-10 and electrophoresed on 10% pre-cast Tris-HCl gels.

Figure 5. The numbered spots are differentially expressed proteins identified by Delta 2D with a $P < 0.05$. Two-dimensional gel electrophoresis of total protein from MAC-T cells differentiated in DIP with 0 or 10 ng/ml GH. Four hundred micrograms of total protein underwent isoelectric focusing on 11 cm IPG strips, pH 3-10 and were electrophoresed on 10% pre-cast Tris-HCl gels. Spot number corresponds to Table 3.

Figure 6. GH induces a significant change in the abundance of forty proteins in MAC-T cells. Protein abundance is represented by color, ranging from orange to blue and low to high respectively. The hierarchal tree was constructed using Pearson correlation as the similarity measure between images. Clustering represents proteins with similar behavior that may have similar regulation. Quantitative change in protein abundance is shown in Table 3.

Gel spot	Protein name	gi Number	Theoretical values		Regulation	P	Fold change
Metabolic proteins							
164	Chain A, bovine mitochondrial F1-ATPase complexed with aurovertin B	1827809	7.87	55.2	Down	0.015	2.39
143	Glucose-6-phosphate dehydrogenase	194680257	6.9	62.4	Down	< 0.001	1.84
177	Fructose-bisphosphate aldolase A	156120479	8.45	39.4	Down	0.016	1.34
165	Chain A, bovine mitochondrial F1-Atpase complexed with aurovertin B	1827809	7.87	55.2	Up	< 0.001	3.12
179	Aspartate aminotransferase 2	27807377	9.19	47.5	Up	< 0.001	2.46
53	Lactate dehydrogenase B	154425698	5.72	36.7	Up	0.024	2.30
119	Isocitrate dehydrogenase 1 (NADP+), soluble	75832090	6.13	46.7	Up	< 0.001	1.90
109	α-enolase	4927286	6.44	47.2	Up	0.008	1.88
Cytokeletal proteins							
118	Similar to Actin, cytoplasmic 2 (β-actin-2)	194676388	5.3	41.8	Down	0.047	1.22
29	Lamin A/C	77404182	6.54	65.1	Down	0.009	1.20
34	Fascin homolog 1, actin-bundling protein	78045491	6.55	54.7	Down	0.023	1.20
223	Lamin A/C	77404182	6.54	65.1	Up	< 0.001	3.14
125	Gelsolin-like capping protein	30466254	6.17	38.9	Up	0.015	2.47
234	Zyxin	118151274	6.33	60.1	Up	< 0.001	2.13
94	Emerin	42733600	5.56	29.3	Up	0.039	1.57
108	Keratin 19 isoform 8	194676168	5.08	51.9	Up	0.016	1.45
124	Gelsolin-like capping protein	30466254	6.17	38.9	Up	0.024	1.25
Chaperone proteins							
213	Heat shock cognate 71 kDa protein (Hsc 71)	12653415	5.24	70.7	Down	< 0.001	1.51
240	HSP 65	60903	5.91	60.9	Down	0.007	1.50
214	HSP 70	157832109	6.63	42.4	Down	0.008	1.33
190	Chaperonin containing TCP1, subunit 7 isoform a	114051425	7.55	57.4	Up	0.007	1.33
103	Chaperonin containing TCP1, subunit 2 (beta)	77735435	6.18	57.4	Up	0.032	1.7
Calcium dependent proteins							
52	Annexin I	74	6.44	38.8	Up	0.020	1.70
RNA processing proteins							
205	Similar to Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP1)	73974146	9.51	67.6	Down	0.047	3.93
225	Heterogeneous nuclear ribonucleoprotein K	77736071	5.14	51.0	Down	0.047	1.33
263	Heterogeneous nuclear ribonucleoprotein A2/B1	3329498	8.67	36.0	Up	< 0.001	1.23
Detoxifying proteins							
90	Glutathione transferase	29135329	6.89	23.6	Down	< 0.001	5.77
83	Peroxiredoxin 2	27807469	5.37	22.0	Down	0.015	1.90
219	Stress-induced phosphoprotein 1 (Hsp70/hsp90-organizing protein)	78369310	6.08	62.4	Up	< 0.001	2.27
77	Peroxiredoxin 6	27807167	6.00	25.1	Up	0.039	1.60
57	Thiosulfate sulfurtransferase	29135275	6.72	33.3	Up	0.04	1.40
Unknown proteins							
191	Hypothetical protein LOC525106	62752829	6.77	55.2	Up	0.015	1.41

Table 3: List of all identified differentially expressed proteins from MAC-T cells DIP differentiated with 0 or 10 ng/ml GH. Proteins are separated by functionality, regulation and fold change. Proteins were identified with MALDI-TOF MS and analyzed using Mascot against the complete NCBI database.

in abundance. Five cytoskeletal proteins, gelsolin-like capping protein, emerin, keratin 19, lamin A/C, and zyxin were up regulated. Three cytoskeletal proteins, β -actin, fascin homolog-1 actin bundling protein, and lamin A/C were down regulated. Gelsolin-like capping protein and Lamin A/C were identified in two spots in the gel. Both gelsolin-like capping proteins (spot 124 and 125) increased in abundance with a fold change of 1.25 and 2.47 respectively. Lamin A/C (spot 29 and 223) was inversely expressed, spot 29 decreased in abundance with a 1.20 fold change and spot 223 increased in abundance with a 3.14 fold change. Five chaperone proteins were identified: heat shock protein 65, heat shock protein 70 and heat shock cognate 71 were down regulated and chaperonin containing TCP-1 subunit 2, and subunit 7 were up regulated. The expression of one member from the annexin family, annexin I, involved in calcium dependent cellular proliferation, increased in abundance. Three proteins involved in RNA processing were altered, including two members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family A2/B1 and K, hnRNP A2/B1 was up regulated while hnRNP K and polyadenylate binding proteins were down regulated. Four oxidative stress proteins changed in abundance; glutathione transferase and peroxiredoxin 2 decreased and thiosulfate sulfurtransferase and peroxiredoxin 6 increased in abundance. One stress response protein, stress induced phosphoprotein 1, increased in abundance.

3.4 Discussion

In MAC-T cells, the majority of proteins that are differentially expressed in response to GH are involved in glucose metabolism, NADPH production, the cytoskeleton and RNA processing. The fact that these proteins change, could be indicative of cellular differentiation and preparation for milk synthesis. To prepare for milk synthesis, the MEC must undergo differentiation through cytoskeletal rearrangement and changes in metabolism to synthesize and secrete milk components.

Two metabolic enzymes involved in glucose metabolism changed in response to GH: fructose-bisphosphate aldolase A decreased and α -enolase increased. Fructose bisphosphate aldolase A catalyzes cleavage of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate (G3P), which can be broken down to pyruvate or used as a backbone for TAG synthesis. The decrease in fructose-bisphosphate aldolase A may act as a glucose sparing mechanism due to the mammary gland's ability to phosphorylate glycerol imported from the plasma with glycerol kinase (Rudolph et al., 2007). The increase in α -enolase, which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, indicates that alternate carbon sources, such as glycerol, may be used in the latter half of glycolysis, sparing glucose for lactose synthesis.

GH affected two proteins involved in NADPH generation: cytosolic isocitrate dehydrogenase, involved in the isocitrate cycle increased in abundance while glucose-6-phosphate dehydrogenase, the first step of the pentose-phosphate pathway decreased in abundance. Although both proteins play a role in NADPH production, the majority of NADPH is generated through cleavage of isocitrate to α -ketoglutarate by the enzyme

isocitrate dehydrogenase in ruminants. The increase in isocitrate dehydrogenase indicates that there may be an increase in production of NADPH, supporting a possible increase in lipid synthesis and is consistent with previous observations of an increase in TAG content in the MAC-T cell line in response to DIP with 10 ng/ml GH (Johnson et al., 2010).

Glucose-6-phosphate dehydrogenase produces small amounts of NADPH from glucose for fatty acid synthesis in ruminants. The enzyme glucose-6-phosphate dehydrogenase decreased in abundance, suggesting a decrease in the amount of glucose used for NADPH synthesis, sparing glucose for lactose synthesis.

The main carbon source entering the TCA cycle in ruminants is acetate, although glucose can supply a small amount of pyruvate. Other carbon sources, such as fatty acids, amino acids and monosaccharides enter the TCA cycle, usually as pyruvate or oxaloacetate, to produce energy. Aspartate, obtained from the blood, can be converted to oxaloacetate by aspartate aminotransferase and lactate can be converted to pyruvate by the enzyme lactate dehydrogenase. Both aspartate aminotransferase and lactate dehydrogenase increased in response to GH, indicating that carbon sources other than glucose may be entering the TCA cycle to generate ATP and isocitrate for NADPH production by the isocitrate cycle.

To prepare for lactation, MECs must also undergo polarization through reorganization of the cytoskeleton for milk synthesis and secretion (Desrivieres et al., 2003, Wodarz, 2002). Multiple proteins involved in actin cytoskeletal rearrangement also showed significant changes in response to GH in the present study. Gelsolin-like capping protein (capG) is up regulated in MAC-T cells treated with GH. It is a part of the gelsolin superfamily and is activated by calcium to cap actin filaments without severing them and is involved in cellular locomotion (Silacci et al., 2004, Sun et al., 1995). The increase in

capG may be related to the decrease in cytoplasmic β -actin and a decrease in fascin-homolog-1, through possible stabilization of the actin cytoskeleton. Interestingly, zyxin, a focal adhesion component regulating actin filament assembly, was up regulated in the presence of GH (Han et al., 2009). Zyxin interacts with Ena/VASP to shield the barbed ends of actin from capping, making zyxin's potential role in cytoskeletal rearrangement in MAC-T cells unclear.

Cytoskeletal proteins, emerin and lamin A/C, are located in the nucleolus and are important in maintaining nuclear integrity, although lamin A/C can also be found in the cytosol. Lamin A/C make up nuclear intermediate filaments involved in stabilizing the nucleolus and is inversely regulated in response to GH, possibly due to its ability to translocate from the cytosol. Emerin is up regulated in the presence of GH and is an actin binding protein that may stimulate actin polymerization *in vitro*, binding and stabilizing the pointed end of growing actin filaments in the nucleus (Holaska et al., 2004). The increased stabilization of the nucleolus may be important for increased transcription and translation during differentiation of secretory cells. The changes observed in cytoskeletal proteins in MAC-T cells treated with GH are similar to the proteomic changes in the mammary HC11 cell line when differentiated with DIP (Desrivieres et al., 2003) and rapid reorganization of the actin cytoskeleton in response to GH is also observed in the 3T3 fibroblast cell line and Chinese hamster ovary (CHO) cell line when GHR is ectopically expressed (Goh et al., 1997b).

After translation, most proteins cannot spontaneously fold to attain their functional conformation and are assisted by chaperone proteins (Elliott and Elliott, 2005). Five molecular chaperone proteins were altered in MAC-T cells differentiated in DIP with GH. Two subunits of TCP-1 increased in abundance while HSP60, HSP70 and

Hsc71 decreased. TCP-1 is involved in folding actin, α - and β -tubulin, and the centrosome related protein γ -tubulin (Gao et al., 1993, Liang and MacRae, 1997). HSP70 regulates tubulin polymerization, assists in transporting proteins to the mitochondria and endoplasmic reticulum, as well as protecting proteins under stress and may also play an important role in stabilizing actin filaments through its association with the TCP-1 complex (Liang and MacRae, 1997). HSP60 is similar to the TCP-1 complex and plays an important role in protein folding (Liang and MacRae, 1997). The similarities and association of TCP-1 with HSPs suggests that both proteins play a role as a molecular chaperone in the mammary gland and are important for proper differentiation and protein folding in preparation for lactation. However, the inverse regulation of these proteins warrants further investigation.

Annexins are a family of calcium-dependent phospholipid binding protein that are involved in modulating phospholipase-A₂ activity and inflammation, intracellular calcium mobilization, vesicular transport, exocytosis, immune response, membrane cytoskeletal linkage and intracellular signal transduction (Donnelly and Moss, 1997, Horlick et al., 1991). Annexin I is thought to be involved in signal transduction, cellular proliferation and differentiation and is developmentally regulated in the mammary gland of humans and rats (Ahn et al., 1997, Horlick et al., 1991). The increase in annexin I in response to GH may be associated with further differentiation of the actin cytoskeleton and annexin I and Cap G suggest calcium acts as a cofactor in cellular differentiation of MAC-T cells.

Increased transcription and translation of milk proteins is observed during differentiation and the onset of lactation. The hnRNP family A2/B1 and K are involved in pre-mRNA splicing, remaining associated with pre-mRNA until processing is complete. Polyadenylate binding protein is a member of the ribonucleoproteins (RNP), which binds

the poly-A tail and plays an important role in stabilization and translation of mRNA (Varani and Nagai, 1998). The increase in hnRNP A1/B2 and the decrease in hnRNP K and polyadenylate binding protein indicates there may be possible alterations in production of mRNA in MAC-T cells treated with GH. However, the inverse relationship between these two proteins indicates further research is needed to explain their exact role in MECs during differentiation.

Interestingly, we did not observe changes in milk proteins in response to GH treatment with 2D gel electrophoresis although we previously observed an increase in mRNA for two major milk proteins (Johnson et al., 2010). The lack of detection of differentially expressed milk proteins may be due to a low abundance of translated proteins, post-translational modifications or possible removal from sample during the acetone/TCA precipitation. The smaller proteins α -lactalbumin and β -lactaglobulin are 14 and 18 kDa and were not detected because they were below the molecular weight range analyzed during 2D gel electrophoresis.

The alterations in the proteome indicate GH has a direct effect on MAC-T cells, adding to the existing evidence that GH may act directly on mammary epithelial cells *in vivo*. MAC-T cells differentiated in DIP with GH appear to be preparing for lactation by sparing glucose for lactose synthesis through the regulation of glycolytic enzymes and increasing enzymes involved in NADPH production for fatty acid synthesis. The differentially expressed proteins involved in rearrangement of the actin cytoskeleton suggest that GH influences differentiation and possible stabilization of the actin cytoskeleton of MAC-T cells. These data are supported by an increase in relative mRNA abundance of two major milk proteins, α_{s1} -casein and α -lactalbumin in MAC-T cells differentiated with DIP with 10 ng/ml GH (Johnson et al., 2010). The increase in nuclear

cytoskeletal proteins support increased stabilization of the nucleolus in MAC-T cells while proteins involved in RNA processing and molecular chaperones suggest an increase in transcription and translation of proteins.

It is important to note that only a subset of metabolic, structural and RNA processing proteins were observed through 2D analysis indicating that GH may act to induce structural changes of the actin cytoskeleton and induce changes in metabolic enzymes in MAC-T cells. For greater insight into the mechanisms involved in MEC development in response to GH, further analysis is needed. It may be insightful to look at specific enzymes or proteins that are involved in fatty acid synthesis and lactose synthesis. It would also be interesting to assess changes in the actin cytoskeleton in MAC-T cells in response to DIP differentiation with and without GH through immunofluorescence microscopy.

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