THE EFFECTS OF GROWTH HORMONE ON PRIMARY BOVINE MAMMARY
CELL MODELS

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ABSTRACT

THE EFFECTS OF GROWTH HORMONE ON PRIMARY BOVINE MAMMARY CELL MODELS

Lisa McDonnell

The ability of exogenous growth hormone (GH) to increase milk yield through insulin-like growth factor-1 (IGF-I) in dairy cows is well characterized. However, recent studies utilizing mammary epithelial cell lines indicate a direct effect of GH on mammary epithelial cells (MEC). To test if these observations are relevant *in vivo* and if this response differs between dairy breeds, three mammary models were utilized. Mammary explants from a lactating Jersey cow were cultured in classical lactogenic media (dexamethasone, insulin, and prolactin) with 0 or 10 ng/mL of recombinant bovine GH for 12h. Primary MEC from lactating Holstein and Jersey cows were cultured in classical lactation media with 0 or 10 ng/mL of GH for 2, 4, and 7 days. And lastly, MEC isolated from pooled Holstein or pooled Jersey milk were cultured in the same conditions as primary MEC. The response to GH was quantified by the relative abundance of mRNA for two milk protein genes (α -lactalbumin and α_{s1} -casein), the GH receptor, IGF-I and insulin-like growth factor binding protein-3 (IGFBP-3) as determined by quantitative RT-PCR. The abundance of α -lactalbumin mRNA in explants was increased in response to GH. After 2 days, Jersey primary MEC showed an increase in GH receptor mRNA, in addition to a noteworthy trend of increasing abundance of IGFBP-3 regardless of GH treatment. After 4 days, Holstein primary cells cultured with GH had decreased IGFBP-3 mRNA. After 7 days, primary cells isolated from Holstein and Jersey mammary tissue showed a slight response to GH. Mammary cells from milk mirrored the responses to GH observed in primary cells: MEC isolated from Holsteins had decreased IGFBP-3 mRNA after 4 days of treatment with GH and MEC isolated from Jerseys showed the same trend of increasing IGFBP-3 abundance between 2 and 4 days, but with no difference between GH treatments. These results indicate that the effect of GH may differ between breeds and indicate GH has a direct effect on mammary epithelial cells, possibly including effects on the abundance of IGFBP-3 mRNA.

Keywords: mammary, primary cells, growth hormone, IGF-I, IGFBP-3

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CHAPTER 1

LITERATURE REVIEW

The Mammary Gland

The mammary gland is a secretory organ unique to the class Mammalia. The mammary gland synthesizes and secretes the components of milk, which provides nutrition to their young. Milk is a complete and easily digestible nutrient source, providing all of the necessary nutrients for growth including proteins, sugars, lipids, vitamins, and minerals. In addition to nutrients, milk also provides immunological components, such as immunoglobulins and white blood cells, which aid in protecting the neonate from disease (Schmidt, 1971; Larson & Smith, 1974). All of these components are secreted into the gland's complex duct system for storage and eventual ejection.

The mammary gland undergoes four distinct stages of development: mammogenesis, lactogenesis, galactopoiesis, and involution. The development of the mammary gland is a continual process because the gland is capable of proliferation, differentiation, and involution many times throughout the adult life of the female animal. Each stage of development is directed by a unique hormonal milieu. Estrogens, growth hormone (GH), progesterone, prolactin, insulin, and glucocorticoids all have a role in the development, function, and regression of the mammary gland which will be described in this section.

Structure

The form of the mammary gland differs by species, with some having breasts, rows of teats, or udders. Each type of mammary gland has unique characteristics. The breast is type of mammary gland found in humans and non-human primates. Breasts, in

particular, are usually two symmetric glands and are thoracically located. The structure of the mammary glands of canines, pigs, and rodents is markedly different from an udder. Mammary glands in these species can be more numerous (1-9 pairs of glands) and are located along the ventral side of the animal and teats are located from the thoracic region through the inguinal region. Additionally, species with breasts or rows of teats have significantly less connective tissue, though the structure of alveoli and ductal systems remain similar (Sheffield, 1988). Ruminants, such as cattle, sheep and goats, exemplify the udder mammary structure.

The udder is comprised of a secretory duct system, circulatory and lymph systems, and a physical support system. The udder is inguinally located below the body and is formed by one or two pairs of mammary glands ending in teats. The gland is supported internally by extensive connective tissue and ligaments, notably the median suspensory ligament, which aids in suspending the udder below the abdomen while keeping it close to the body wall. Each teat is the drainage canal for one cistern. The gland cistern collects secreted milk that drains from many ducts. The ductal system is highly branched and leads from millions of lobuloalveoli. Lobuloalveoli are clusters of alveoli, where each alveolus is made up of functionally differentiated mammary epithelial cells arranged in a single layer surrounding an open lumen into which components of milk are secreted. The single layer of cells around the lumen is surrounded by myoepithelial cells that contract the alveolus to aid in the flow of milk through the ducts and ejection of milk into the cistern and out of the gland (Larson & Smith, 1974). Surrounding the alveoli is the mammary stroma, comprised of connective and adipose

tissue and a well developed vasculature (Larson & Smith, 1974; McManaman & Neville, 2003).

Mammogenesis

The origination and development of the mammary gland begins during embryogenesis, during which time the mammary fat pad also develops (Sheffield, 1988; Daniel & Smith, 1999). Interaction with the mesenchyme appears to be the most influential factor over the development of the mammary rudiment during embryogenesis and this interaction remains critical for proper development and function later in life (Daniel & Smith, 1999; Kleinberg, 1997). Growth of the mammary gland prior to puberty is proportional to body growth (Knight, 2000). The rudimentary ductal system present at birth will elongate, but with limited branching. Development of secretory alveoli does not occur until the onset of pregnancy (Knight, 2000). Other mammary gland tissues, such as connective, circulatory and lymph tissues, are developed during pre-pubertal growth (Takeishi *et al.*, 1980).

At the onset of puberty, the combined effects of pituitary and ovarian hormones stimulate further development of the mammary gland. The synergistic effects of estrogen and GH serve to lengthen and branch the rudimentary ductal system (Kleinberg, 1997). Estrogen receptors begin to appear in high concentrations in the mammary gland during puberty, leading to ductal development within the gland in response to estrogen (Mallepell *et al.*, 2006; Robinson *et al.*, 1995). Prolactin, secreted from the anterior pituitary in response to rising levels of estrogen, acts through its receptor on mammary cells to stimulate some post-pubertal development of alveoli and limited ductal elongation (Ceriani, 1974). It has been suggested that prolactin stimulates paracrine

release of insulin-like growth factor II (IGF-II) within the mammary gland, which stimulates development of alveoli (Briskin *et al.*, 2002). GH also promotes mammary epithelial proliferation, but to a much lesser extent than seen during gestation (Carstens *et al.*, 1997; Sejrsen *et al.*, 1986). Stromal-epithelial interactions during puberty are especially critical, as the effects of GH are exerted through GH receptors (GHR) on stromal cells that produce local IGF-I that acts directly on mammary epithelial cells (MEC). This paracrine action is essential for normal mammary development (Kleinberg & Ruan, 2008; Ruan & Kleinberg, 1999; Akers *et al.*, 2000). The presence of GH is critical for the proper development of the mammary gland. In studies where hypophysectomized, ovariectomized, and adrenalectomized mice were given exogenous injections of hormones, no mammary development occurred at puberty in the absence of GH (Kleinberg, 1997). In addition to GH, estrogens are critical for normal mammary development during puberty, particularly for hormone-dependent ductal elongation and side branching (Briskin & O'Malley, 2010). For example, the mammary gland failed to develop in wild type mice with a cleared fat pad implanted with estrogen receptor negative epithelial cells (Mallepell *et al.*, 2006). Pubertal development of the mammary gland continues with each successive estrous cycle, until ducts reach the edges of the mammary fat pad, which marks the end of major pubertal development (Richert *et al.*, 2000).

The onset of pregnancy elicits the next stage of development in the mammary gland. Pregnancy induces major changes in the mammary gland to support terminal differentiation of alveolar structures in preparation for lactation. The ductal system undergoes significant side-branching, alveolar development occurs, and MEC begin to

differentiate into functional secretory cells (Hovey *et al.*, 2002; McManaman & Neville, 2003). Proliferation of MEC and development of alveoli are critical for milk production (Knight, 2000). Progesterone and prolactin play major roles in the proliferation of MEC and development of alveoli. Under the influence of progesterone, branching of the ductal system becomes extensive and there is considerable development of lobulo-alveolar structures (Larson & Smith, 1974). Sensitized by the effects of progesterone, the mammary gland responds to prolactin through the extensive development of lobuloalveolar structures (Hovey *et al.*, 2003). Additionally, the development of the mammary gland during pregnancy is modulated by the number of previous pregnancies. The mammary gland of multiparous animals responds more rapidly and robustly to progesterone and estrogen through increased proliferation and lobuloalveolar development (Ceriani, 1974).

Lactation

The sum of the changes acquired during pregnancy results in full functional differentiation of the MEC in preparation for synthesis and secretion of milk. The secretory capacity gained by MEC during pregnancy is fully functional at the time of parturition. There is an increase in Golgi, mitochondria, and endoplasmic reticulum in differentiated MEC, in addition to more secretory vesicles (McManaman & Neville, 2003; Larson & Smith, 1974). Increasing levels of prolactin and decreasing levels of progesterone, which allows for secretion and ejection of milk, induce the onset of lactation (Knight, 2000). Synergistic effects of prolactin and GH maintain the production of milk components. Prolactin and GH, through activation of their receptors, activate signal transducer and activator of transcription (STAT) variants, and particularly STAT5.

One of the major roles of STAT5 is increasing the transcription of milk protein genes (Rosen *et al.*, 1999). Prolactin stimulates the production of milk proteins in the MEC. GH indirectly acts on MEC, through the production of IGF-I, to stimulate lipid synthesis by maintaining activation of lipoprotein lipase and acetyl-CoA carboxylase (Knight, 2001; Flint & Knight, 1997; Braun & Severson, 1992). Additionally, cortisol is released during milking and is required to maintain secretory activation of MEC (Gorewit *et al.*, 1992; Sejrsen *et al.*, 2006). Although hormones play an important role in maintaining lactation, one of the most important and fundamental signals is suckling and/or milk removal (Svennersten-Sjaunja & Olsson, 2005). Tactile stimulation of the teat results in release of the hormone oxytocin from the pituitary, which is required for full milk ejection (Svennersten-Sjaunja & Olsson, 2005; Gorewit *et al.*, 1992). The absence of any of these signals leads to the cessation of lactation through involution of the mammary gland.

Involution

Involution of the mammary gland at the end of lactation is the result of a complex signaling process through which the gland sheds MEC and mammary tissue is remodeled to a pre-pregnancy like structure (Baxter *et al.*, 2007). Regression of the mammary gland following the lactation cycle is a result of extensive apoptosis of MEC (Marti *et al.*, 1994). The major signal for mammary involution is the cessation of suckling which in turn leads to other signals resulting in activation of apoptosis. The accumulation of milk in the alveolar lumen may be a signal for involution itself, or may contribute to the accumulation of apoptotic signals in the gland (Li *et al.*, 1997). Additionally, falling levels of hormones required for lactation contribute to regression of the gland. These

signals lead to the activation of apoptotic pathways within MEC and regression of the gland to pre-gestational quiescence (Baxter *et al.*, 2007).

The result of involution is a loss of active secretory cells; however the gland retains a population of MEC that have not terminally differentiated. These cells are retained and protected from the process of involution. This population of stem cells is capable of responding to hormonal stimuli at the onset of the next gestation and proliferate to generate new secretory cells and a “new” functional gland (Malinowski, 2005).

The mammary alveolar epithelial cell

The major developmental stages of the mammary gland revolve around the proliferation and differentiation of mammary epithelial cells (MEC). The functional units of the mammary gland are MEC, which synthesize and secrete the components of milk. This specialized cell absorbs nutrients from the blood stream, synthesizes components of milk, and secretes them into the alveolar lumen. The alveolar lumen is composed of MEC arranged in a single layer surrounding the lumen, into which milk components are secreted. Nutrients are absorbed through the basolateral membrane of MEC and milk components are secreted through the apical membrane of MEC (Wilde *et al.*, 1995). Attaining this polarization is critical to the cell's ability to successfully secrete milk components. Without it, functional lactation fails (Tateyama *et al.*, 1988).

During lactation MEC synthesize and secrete proteins unique to milk— caseins, lactalbumins, and lactoglobulins. Milk protein gene expression is controlled through the synergistic interactions of prolactin, insulin, and dexamethasone (Menzies *et al.*, 2009; Svennersten-Sjaunja & Olsson, 2005). These hormones, through a signal cascade activated by binding their respective receptors, activate transcription factors that promote the transcription of milk protein genes (Rosen *et al.*, 1999). Caseins, which are hydrophobic milk proteins, are secreted in a micelle that also assists in the transport of calcium, lactose, and phosphate into milk (Kumar *et al.*, 1994; Shennan & Peaker, 2000). α -lactalbumin is a protein synthesized in MEC in response to hormonal stimuli during lactation. In addition to being secreted as a milk protein to provide nutrients to the neonate, α -lactalbumin also complexes with galactosyltransferase within the cell to form lactose synthase (Kresge & Simoni, 2006). Lactose synthase catalyzes the synthesis of

lactose from galactose and glucose in the Golgi (Larson, 1969). Without α -lactalbumin, lactose synthase is not functional (Kresge & Simoni, 2006). In this way, the expression of α -lactalbumin, in response to prolactin, is directly linked to successful production of lactose during lactation. The concentration of lactose, in turn, regulates the volume of milk produced by changing osmotic pressure in the alveolar lumen which draws water from the blood into the lumen to balance the pressure (Shennan and Peaker, 2000).

In addition to milk specific proteins, MEC also synthesize and secrete lipids, predominately triacylglycerol (Shennan & Peaker, 2000). The synthesis of fatty acids is markedly different between mammalian species. Non-ruminants are able to utilize glucose for the synthesis of fatty acids through its conversion to citrate, which in turn can be cleaved by citrate lyase resulting in the formation of acetyl CoA, which can be utilized for fatty acid synthesis (Larson & Smith, 1974). In ruminants, the use of glucose is prevented due to the absence of citrate lyase. Fatty acids in ruminant milk are either derived from the blood by MEC or are synthesized by MEC from acetate and β -hydroxybutyrate, both of which are in high concentration in the ruminant as a result of microbial fermentation in the rumen (Larson & Smith, 1974). Lipids are synthesized at the endoplasmic reticulum and are secreted as globules, surrounded by a portion of the apical cell membrane (Ernens *et al.*, 2007). Lipid droplets formed in the cytoplasm of MEC protrude out against the plasma membrane, eventually becoming enveloped, and extruded from the cell resulting in a secreted milk fat globule surrounded by plasma membrane (Keenan, 2001).

Nutrients are absorbed by MEC through the basolateral membrane and used to synthesize the components of milk. Glucose and ions, such as potassium and sodium,

cross the MEC basolateral membrane through facilitated diffusion (Zhao *et al.*, 1999; Shennan & Peaker, 2000). Trace elements, such as zinc and copper, are moved by active transporters (Mather & Keenan, 1998; Truchet & Ollivier-Bousquet, 2009; Lonnerdal, 2007). Nutrients absorbed from the blood will be incorporated into larger molecules in milk, such as proteins or triacylglycerol, and secreted into the alveolar lumen, or be transported directly to the alveolar lumen. Components that characterize milk are caseins, milk fat (predominately triglycerides), and lactose (Akers, 1985). These are synthesized by MEC and secreted into milk along with other nutrients, such as vitamins and minerals.

Growth hormone

Impact of GH

Milk is a complete nutritional source and widespread production and distribution provides a source of complete nutrition to an ever-growing population. Efficiency of milk production will be a key issue in both production of food for humans and efficient use of available resources. Improving the utilization of nutrients by production animals, in addition to genetic improvements, can make the production of milk more efficient (Capper *et al.*, 2008). Early experiments in dairy cattle using crude pituitary extracts showed the presence of a factor which was a potent enhancer of milk yield (Cotes *et al.*, 1949). Further purification identified it as growth hormone (GH). Injections of exogenous GH or recombinant bovine somatotropin (rbST) improve feed conversion efficiency and increase milk yield in dairy cows. Recently, recombinant technologies have allowed the widespread use of exogenous GH in the dairy industry. In addition to providing a valuable food source, utilizing and maximizing exogenous GH technologies allows the current dairy cattle population to produce more milk while utilizing less feed. Efficiently using land and feed resources will continue to be a priority as the population expands and competition for land and resources increases (Capper *et al.*, 2009; Capper *et al.*, 2008).

Despite improvements to production, the widespread use of rbST in dairy cattle has been controversial due to a perceived risk to the consumer as a result of ingestion of milk from cows treated with rbST. There has been some concern that administration of rbST will increase the levels of IGF-I in milk, however any observed changes have been within the range of IGF-I normally present in milk (Dahl *et al.*, 1991; Collier *et al.*, 1999).

Growth hormone structure and secretion

GH, also called somatotropin, is a peptide hormone consisting of 190 or 191 amino acids. Four variants of GH have been described: either a leucine or valine at position 127, and either a 190-amino acid variant with a phenylalanine at the N-terminus, or a 191-amino acid protein with an alanine at the N-terminus (Etherton & Bauman, 1998; Bauman & Vernon, 1993). The potency of variants has been studied and the variant with a valine at position 127 has been reported to be a more potent stimulator of galactopoiesis, while no difference between N-terminal variants was observed with respect to galactopoiesis (Eppard *et al.*, 1992).

Growth hormone releasing hormone, secreted from the hypothalamus, stimulates the secretion of GH from the pituitary gland. Somatostatin, also secreted from the hypothalamus, inhibits the release of GH (Tuggle & Trenkle, 1996). GH is secreted from the anterior pituitary gland in a pulsatile fashion (Gluckman *et al.*, 1987) and acts on target tissues to promote growth. Secretion from the pituitary is the major source of GH, however several other tissues also synthesize GH, including brain, placental, mammary and pineal gland (Butler & Le Roith, 2001).

The GH receptor

The GH receptor (GHR) is a member of the cytokine receptor super-family and transmits signals through its membrane spanning structure via several signal transduction pathways (Shafiei *et al.*, 2006). The GHR shares a similar homology with other receptors, such as the prolactin and interleukin receptors (Butler & Le Roith, 2001). Activation of the GHR results in the phosphorylation and activation of Janus kinase 2 (JAK2) that results in the phosphorylation of STAT proteins (STAT1, 3, and 5), mitogen-

activated protein kinase (MAPK), and phosphatidylinositol-3 kinase (PI3K) (CarterSu *et al.*, 1996; Barclay *et al.*, 2010). The JAK2 pathway is particularly important for expression of IGF-I and IGF-I binding protein transcripts. Activated STAT5 is able to regulate gene expression by binding DNA and activating gene transcription. The critical importance of STAT5 activation, by both GH and prolactin, is highlighted by the discovery that STAT5 deficient mice are unable to lactate (Liu *et al.*, 1997).

The GHR is present on several tissue types including liver, muscle, bone, mammary, adipose, brain, and immune tissue (Kopchick & Andry, 2000). Via these receptors, GH is able to alter metabolic processes which result in an increase body mass and length. There have been conflicting reports on the expression of the GHR on the MEC. Early studies claimed that while the GHR was expressed in the mammary gland, it was most likely on stromal cells and not MEC (Peel & Bauman, 1987; Glimm *et al.*, 1990). More recent studies utilizing more sensitive technologies have reported the expression of GHR mRNA and GHR on the membrane of MEC (Sakamoto *et al.*, 2005; Plath-Gabler *et al.*, 2001). These findings led to investigations into the possibility of GH acting directly on MEC, in addition to its effects through somatomedins.

Effects of GH

GH influences the development, growth, and metabolism of cells and tissues. Both direct and indirect effects of GH have been described: the direct effects of GH include changes in gene expression influencing and metabolism and differentiation of tissues, such as bone and mammary (Bolander, 1989). Indirect effects of GH are mediated by somatomedins (Peel *et al.*, 1981; Bauman & Vernon, 1993). The major somatomedin associated with GH is insulin-like growth factor-1 (IGF-I), which is

produced and secreted by the liver in response to GH. GHR is present in high concentrations on hepatocytes and allows GH to stimulate the production and secretion of IGF-I by the liver (Etherton & Bauman, 1998; Gluckman *et al.*, 1987). IGF-I acts through its receptor, present on most tissue types to induce changes to cell metabolism, division, and survival (Le Roith *et al.*, 2001). High serum concentrations of IGF-I contribute to negative feedback and inhibit the release of GH (Kopchick & Andry, 2000; Berelowitz *et al.*, 1981).

Early experiments with pituitary extracts showed that they could promote growth and increase milk yield (Cotes *et al.*, 1949; Lee & Schaffer, 1934). Exogenous injections of GH have been shown to increase milk yield and improve lactation persistency in dairy cattle without altering the composition of milk (Hutton, 1957; Akers, 2006). Advances in recombinant technologies made the commercial application of GH feasible to the dairy industry. Recombinant bovine GH was approved in 1993 by the FDA and marketed as Posilac® by Monsanto (Capper *et al.*, 2008). The increase in milk yield by GH is accomplished through several mechanisms. Firstly, nutrient partitioning shunts nutrients in favor of the mammary gland through alterations in nutrient usage by other tissues, by increasing lipolysis in adipose tissue and decreasing glucose oxidation in muscle (Bauman & Currie, 1980; Pocius & Herbein, 1986). These actions result in more nutrients being available to the mammary gland for synthesis of milk components (McDowell *et al.*, 1987). Secondly, GH contributes to cell survival by inhibiting apoptotic pathways and activating pathways involved with cell survival (Flint & Knight, 1997). This effect is primarily directed by IGF-I produced by the liver.

The metabolic effects of GH are a result of a direct action on target tissues, notably adipose and liver, altering their utilization of nutrients. GH increases lipolysis in adipose tissue to release nutrients for use by other tissues, such as bone, muscle and mammary tissue (Butler & Le Roith, 2001). GH increases lipid synthesis in animals with a positive energy balance, particularly growing animals, and increases lipolysis in animals with a negative energy balance, for example a lactating dairy cow. This allows animals to grow and animals with high energy demands to better utilize available nutrients—such as in growing pigs and lactating dairy cows (Bauman & Currie, 1980). GH also increases protein accumulation, but this effect is largely mediated by increased concentrations of IGF-I (Etherton & Bauman, 1998). Additionally, GH can stimulate glucose uptake by tissues in an insulin-dependent manner and this may be mediated by recruitment of the insulin receptor substrate by the GHR (Butler & Le Roith, 2001).

Insulin-like growth factors

There is clear evidence of a close relationship between GH and insulin-like growth factors (IGF). The IGF family includes two growth factors, IGF-I and IGF-II, which share high amino acid homology. Insulin-like growth factors (IGF) also share high homology with insulin (~50%); however IGF retain the C peptide that is cleaved from pro-insulin to make the functional form of insulin. IGF-I and IGF-II both stimulate growth, however IGF-II is more involved in fetal growth, while IGF-I is involved in post-natal growth (Bolander, 1989; Le Roith *et al.*, 2001). IGF-I and -II also promote mammary development, facilitating ductal elongation and branching, and alveolar development (Rowzee *et al.*, 2009). In particular, IGF-I increases protein synthesis, cellular proliferation, and cell survival (Berry *et al.*, 2001).

IGF are capable of binding three receptors with varying affinities: the IGF-I receptor (IGFR-1), the IGF-II receptor (IGFR-2), and the insulin receptor (IR). IGF receptors are present on a wide variety of tissues as is evident by the wide spread effects of IGF-I on multiple body systems. IGFR-1 is the primary receptor for signaling the actions of IGF-I, but some functions may be mediated through IGFR-2. Additionally, the IR binds IGF with 100-fold lower affinity than insulin (Stewart & Rotwein, 1996; Leroith *et al.*, 1995). The IR and the IGFR-1 share 70% homology (Baserga *et al.*, 2003). Both of these receptors are members of the tyrosine kinase receptor family and recruit the IR substrate and activate downstream signaling pathways such as PI3K and MAPK (Bolander, 1989). At high concentrations, IGF-I can bind the IR and can induce the uptake of glucose by tissues (McGuire *et al.*, 1992).

Synergistic hormonal interactions are critical to the development and function of the mammary gland (Topper & Freeman, 1980). The secretion of IGF-I, the major somatomedin associated with many of the effects of GH, is induced by GH (Berry *et al.*, 2001). IGF-I is primarily secreted by the liver. Metabolic effects of IGF-I include increased protein synthesis and decreased proteolysis, in addition to increased proliferation through stimulation of cell cycle progression (Sejrsen *et al.*, 2006; Le Roith *et al.*, 2001). Additionally, IGF-I promotes cell survival through activation of Akt (Kulik *et al.*, 1997).

Although the liver is the primary source of IGF-I production, other tissues such as mammary stroma can secrete IGF-I, which can act in a paracrine manner on MEC. MEC have not been shown to secrete IGF-I (Baumrucker & Erondur, 2000). When considered with MEC expression of the GHR, this further supports investigation into a separate, direct role of GH on MEC.

IGF binding proteins

The majority of IGF-I and IGF-II are bound to binding proteins in serum (Cohick & Clemmons, 1993). The involvement of IGF binding proteins (IGFBP) in the GH/IGF axis affects the ability of IGF to interact with their receptors. There are at least six different IGF binding proteins whose secretion pattern and function differ (Knight, 2000). IGFBP aid in transport of IGF, prolong the half-life of IGF in circulation, and localize IGF to particular tissues through interactions with cell surface proteins and extracellular matrix proteins (Cohick, 1998; Cohick & Clemmons, 1993; Yamada & Lee, 2009). The binding affinity of IGF for each binding protein differs between isoforms, tissues, and their interaction with other molecules (Rowzee *et al.*, 2009).

IGFBP-3 and IGFBP-5 both have major functions with respect to the mammary gland. In dairy cattle, the predominant IGFBP in serum is IGFBP-3 which binds the majority of IGF-I in circulation (Baumrucker & Erond, 2000; Plath-Gabler *et al.*, 2001). IGFBP-3 is synthesized and secreted by mammary epithelial cells both *in vivo* and *in vitro* (Berry *et al.*, 2001; Gibson *et al.*, 1999; Cohick & Turner, 1998). IGFBP-3 concentrations have been shown to be higher during mammogenesis and involution than during lactation (Plath-Gabler *et al.*, 2001; Baumrucker & Erond, 2000). It has been shown that IGFBP-5 is in high concentrations in the mammary gland during involution and appears to be involved in mediating the process of involution (Flint *et al.*, 2003; Sakamoto *et al.*, 2007).

Mammary functional models

The mammary gland provides an excellent model in which to study development, differentiation, and lactation; however, due to the numerous interconnected systems in a whole animal, a more independent model may be useful in deducing the role and functions of specific stimuli. Apart from the whole-animal, there are several models that are useful in understanding the function of the mammary gland, each of which seek to best mimic the *in vivo* conditions of the mammary gland while eliminating confounding systemic factors. Many studies report mammary tissue and cells *in vitro* are capable of responding to structural, nutritional, and hormonal stimuli (Keys *et al.*, 1997; Campbell *et al.*, 1991; Mills & Topper, 1970; Riley *et al.*, 2008; Mallepell *et al.*, 2006; Gibson *et al.*, 1999). However, each of these models has limitations and varying culture requirements to achieve function *in vitro*.

One of the most fundamental requirements for normal function of MEC is polarization, which in some models must be achieved by a three-dimensional culture system. Failure to determine cellular orientation, and consequently polarization, results in reduced expression of indicators of differentiation and lactation in MEC *in vitro* (Tateyama *et al.*, 1988; Kawano *et al.*, 1988). For some *in vitro* MEC models, a collagen gel matrix is needed to provide support for proper polarization and expression of genes associated with the differentiated phenotype (Riley *et al.*, 2010; Richards *et al.*, 1983). Matrices can also be made from various materials designed to mimic the extra cellular matrix and thereby assist the orientation and polarization of cells *in vivo* (Grant *et al.*, 1985). These matrices help to replicate the 3D structure and orientation of mammary tissue.

Explants

Explants are small pieces of excised mammary tissue and are composed of the many cells types present in the mammary gland including connective, adipose, vascular, and MEC. Structural orientation is a key component of MEC differentiation and lactogenesis. By culturing excised tissue, the physical orientation of the cells is maintained. (Ebner *et al.*, 1961). Explants, however, can only be cultured for a relatively short time (up to 5 days) *in vitro* which makes long term studies difficult (Ip & Darcy, 1996). Additionally, it can be a costly and invasive procedure to continually excise mammary tissue; new tissue samples need to be obtained for every experiment because explants do not undergo proliferation *in vitro* and, unlike isolated primary cells, will not survive cryopreservation. Due to the invasiveness of excising mammary tissue, tissue is often collected after slaughter; however this does not allow repeated sampling from the same animals. Despite these drawbacks, explants provide an excellent model of the mammary gland *in vitro* and have been used by many groups to study functional differentiation, lactation, and involution. Explants respond to hormonal stimuli in a similar fashion as the mammary gland; they are capable of differentiating, synthesizing and secreting milk proteins, IGFBP and, when co-cultured with liver and adipose tissue, lipids (Campbell *et al.*, 1991; Keys *et al.*, 1997; Keys *et al.*, 1992). However, they are not a homogeneous population of cells and investigating the response of a single cell type within the tissue can be confounded by stromal interactions between various cell types present.

Primary cells

Primary cells are isolated from mammary tissue and, depending on the method of isolation, can be a relatively homogenous population of MEC, free of other cell types found in the mammary gland. The isolation of this singular cell type allows for investigation into the direct effects of signaling molecules, such as GH, upon the MEC without confounding influences from the surrounding tissue, which is especially important given the prevalence of paracrine signaling in the mammary gland. However, isolated MEC have been dissociated from their connective tissue, and therefore the use of extra-cellular matrix scaffolds is common in single cell culture systems. Despite these extra-cellular changes, primary cells provide an excellent model to study the proliferation and differentiation of MEC. Using primary cell models, the importance of insulin, progesterone and epidermal growth factor in MEC proliferation has been shown (Ip & Darcy, 1996). Additionally, the importance of prolactin, hydrocortisone, and insulin to the expression of milk proteins, especially casein, has been shown (Suard *et al.*, 1983; Wheeler *et al.*, 1995). Studies using primary cells show their capability to synthesize and secrete lactose and lipids (Rohlfes *et al.*, 1993; Taketani & Oka, 1981).

Mammary tissue is not the only source of primary MEC; primary MEC can also be isolated from milk. Several groups have isolated MEC from raw milk from humans, cows, and goats (Gaffney, 1982; Boutinaud *et al.*, 2002; Boutinaud *et al.*, 2008; Buehring, 1990; Ceriani *et al.*, 1979). MEC are sloughed from the alveolus into milk and can be collected from samples of raw milk (Boutinaud & Jammes, 2002). Several factors affect the concentration and viability of MEC in milk, such as stage of lactation, parity, infection, and milking frequency (Boutinaud & Jammes, 2002). The suitability of milk-

derived MEC to represent the responses and functions of MEC has been evaluated by comparing the gene expression of mammary tissue to the gene expression of MEC derived from milk through RT-PCR (Boutinaud *et al.*, 2008; Boutinaud *et al.*, 2002). MEC derived from milk and mammary tissue have a significant correlation between the quantity and types of genes expressed (Boutinaud *et al.*, 2002).

Continuous cell lines

Cell lines are immortalized cells capable of continuous division *in vitro*. They originate from cells isolated from tissue and through transfection or spontaneous mutation acquired the ability for indefinite proliferation. Cell lines provide a virtually infinite source of cells, however their infinite proliferative ability calls into question their accurate representation of normal biological systems. There are many mammary epithelial cell lines in use for multiple species. In particular, the MAC-T cell line was derived from normal Holstein bovine mammary tissue by Huynh *et al.* (1991) and transfected with the simian virus 40 large-T antigen. This transfection inhibits p53, a protein that regulates the cell cycle, allowing the cells to remain proliferative rather than reach senescence. MAC-T cells are capable of synthesizing milk proteins and lipids. In addition to the bovine derived MAC-T cell line, cell lines exist in other species including mice (COMMA-D, HC11) and humans (MCF-10A) (Ball *et al.*, 1988; Soule *et al.*, 1973; Danielson *et al.*, 1984). Cell lines have also been developed from tumor cells in several species, such as mice (4T1), human (MCF-7), and rats (Rama 25) (Dulbecco *et al.*, 1979; Aslakson & Miller, 1992). There are limitations to using transformed cell lines, however. These limitations stem from the inherent dysregulation of the cell cycle required to

achieve infinite proliferation and the possibilities of spontaneous mutations that may alter the ability of the cell line to respond in a similar fashion to its tissue of origin.

Project Rationale

Utilizing two mammary cell models, this project aims to evaluate the response of primary bovine mammary cells to GH with an underlying comparison of the response between two dairy breeds. This investigation is based on the clear influence of GH on milk yield in several dairy breeds and previous observations in mammary cell lines that support a direct action of GH on MEC. The use of primary cells is critical for investigating the direct role of GH independent of IGF-I in normal cells. Isolated primary cells provide a model where the response of MEC can be evaluated, independent of other stromal interactions. While investigations utilizing cell lines are certainly valuable, validating their responses in models that more closely resemble normal cells is an important step in elucidating the active mechanisms at work.

CHAPTER 2

THE EFFECTS OF GROWTH HORMONE ON PRIMARY BOVINE

MAMMARY CELL MODELS

Introduction

Early experiments in dairy cattle using crude pituitary extracts showed the presence of a factor which was a potent enhancer of milk yield (Cotes *et al.*, 1949). Further purification identified it as growth hormone (GH) and its administration could increase milk yield (Hutton, 1957). With the utilization of recombinant technologies, commercial administration of exogenous GH became feasible. Lactating animals respond to administration of exogenous GH by increasing milk production without altering composition (Hutton, 1957). Previous studies have reported that high-producing animals differ from low-producing animals in their response to exogenous GH and suggest that this difference is due to the animal reaching maximum potential of milk production (Nytes *et al.*, 1990; Gibson *et al.*, 1992) or differences in the potency of GH variants (Etherton & Bauman, 1998).

GH stimulates growth throughout the body through activation of the GH receptor (GHR). The GHR activates several signal transduction pathways, such as Janus kinase 2 (JAK2) and signal transducers and activators of transcription (STAT5). The activation of these pathways promotes changes in cellular metabolism, differentiation, and transcription of GH-activated genes (Piwien-Pilipuk *et al.*, 2002). In particular, GH stimulates the production and secretion of insulin-like growth factor-1 (IGF-I) by the liver (Wilde *et al.*, 1995). The direct action of IGF-I on target tissues affects cell metabolism, proliferation, and survival (Etherton & Bauman, 1998; Campbell *et al.*,

1991). In the mammary gland, IGF-I improves cell survival and increases protein synthesis in the mammary epithelial cell (MEC) (McGuire *et al.*, 1992). However, the actions of IGF-I are mediated by insulin-like growth factor binding proteins (IGFBP). There are several isoforms of IGFBP that have been described (IGFBP-1 to -6) (Knight, 2000). IGFBP serve many functions and expression levels differ between isoforms and stage of the gland: development, lactation, or involution (Baumrucker & Erondy, 2000; Marshman & Streuli, 2002). The major function of IGFBP is to bind IGF. Through interactions with extracellular matrix proteins, cellular membrane proteins, and proteases, IGFBP regulate the localization and availability of IGF to its receptor (Marshman & Streuli, 2002; Le Roith *et al.*, 2001).

It is well established that GH increases the production of IGF-I by the liver and that IGF-I can act on many cell types, including MEC, resulting in increased milk production. But, despite the well defined indirect action of GH on the mammary gland, the discovery of the GHR on the mammary epithelium led to the investigation of a direct action of GH on MEC (Plath-Gabler *et al.*, 2001; Sakamoto *et al.*, 2005; Hauser *et al.*, 1990). A bovine derived mammary epithelial cell line, MAC-T, was shown to express the GHR (Zhou *et al.*, 2008). When the GHR and STAT5 were over-expressed, MAC-T cells responded to treatment with GH by increasing mRNA levels of major milk proteins (Zhou *et al.*, 2008). Additionally, it was observed that native MAC-T responded to treatment with GH resulting in an increase in mRNA levels of major milk proteins and altered lipid distribution (Johnson *et al.*, 2010). Previous research has also shown the proteome of native MAC-T cells is altered in response to treatment with GH. GH induced changes in metabolic, cytoskeletal, and other proteins in differentiated MAC-T

cells (Johnson, 2010). These observations indicate the possibility of a direct action of GH on MEC.

Although MAC-T cells were originally derived from normal bovine mammary tissue, they were immortalized with the simian virus 40 large T antigen (SV40), which inhibits the ability of p53 and retinoblastoma protein to regulate cell division (Huynh *et al.*, 1991). Due to these known changes in cell cycle regulation, and the potential for changes to other regulatory mechanisms to have also been affected by SV40, the use of models which may more closely resemble conditions *in vivo* may better represent the response of MEC to GH.

Mammary explants from several species have been widely used to study the mammary gland (Keys *et al.*, 1992; Sheehy *et al.*, 2004; Brennan *et al.*, 2008). Explants retain the structure and orientation of the tissue in culture and preserve interactions between the stroma and epithelium (Sheehy *et al.*, 2004; Keys *et al.*, 1997). However, mammary explants retain the surrounding stromal tissue in the gland and are not a homogenous cell population. Due to the multiple cell types present, explants may not accurately represent the response of the MEC, but rather the complex intercellular interactions of the gland as a whole.

To further segregate the direct effect of GH on MEC, primary MEC can be isolated and used to evaluate their response. Primary cells have previously been used to study the metabolic functions and gene expression of the mammary gland (Suard *et al.*, 1983; Matitashvili & Bauman, 1999; Wheeler *et al.*, 1995). However, primary MEC are difficult and costly to obtain due to the invasive nature of sampling tissue from a lactating gland. In addition, proliferation rates are limited and cultures often require complex

substrata (Ip & Darcy, 1996). Limited proliferation of MEC impedes achieving adequate cell numbers for analysis and substrata can interfere with downstream protein analyses. Primary MEC are also shed into milk and can be isolated from a raw milk sample, and it has previously been shown that MEC in milk are viable (Taylor-Papadimitriou *et al.*, 1977; Stoker & Perryman, 1984; Ceriani *et al.*, 1979; Buehring, 1990). Transcript levels from mammary tissue and MEC from milk have previously been compared and found to be highly correlated (Boutinaud *et al.*, 2002; Murrieta *et al.*, 2006). They may provide a MEC model that is non-invasive and easy to collect.

Here, we compare the response of several MEC models to GH: explants, primary cells from tissue, and primary cells from milk. To assess the potential of *in vitro* models to reflect differences between breed, models originating from two major dairy breeds, Holstein and Jersey, were studied.

Methods

All procedures involving animals were reviewed and approved by California Polytechnic State University, San Luis Obispo's Institutional Animal Care and Use Committee. Neomycin, enzymes in the collagenase cocktail, and metal mesh filters were obtained from Fisher Scientific (Pittsburg, PA). Fetal bovine serum (FBS) was obtained from Innovative Research (Novi, MI). All other materials were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Primary Cell Isolation

Primary mammary cell isolation procedures were modified from earlier published reports (Ebner *et al.*, 1961; Matitashvili & Bauman, 1999; Richards *et al.*, 1983). Mammary tissue (200-300 g) was collected from multiparous (2nd lactation), lactating dairy cows (Jersey: 190 d in milk (DIM); Holstein: 125 DIM) immediately post mortem and placed in sterile saline with 1% penicillin-streptomycin-neomycin and 1% amphotericin B solution. Excised tissue was washed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DME:F12) with 1.25% penicillin-streptomycin-neomycin and 1.25% amphotericin B and minced into 3-5 mm³ pieces. Tissue was digested with a collagenase cocktail (DME:F12, 1% penicillin-streptomycin-neomycin, 1% amphotericin B, 1 mg/mL collagenase, 0.5 mg/mL hyaluronidase, 1 U/mL elastase, 20 µg/mL DNase) for 2.5 h at 30°C. The cell suspension was filtered through three sterile metal mesh filters of decreasing size (380 µm, 230 µm, 100 µm); and tissue remaining on the filters was digested for an additional 2.5 h in fresh collagenase cocktail and filtered again. Following filtration, the cell suspension was washed by centrifugation (5 min, 1000g, 15°C) in Wash 1 (DME:F12 with 1% penicillin-streptomycin-neomycin,

1% amphotericin B, 5% FBS and 40 $\mu\text{g/mL}$ DNase) until the supernatant was clear. The pellet was resuspended in Wash 1 and loaded atop a 3-step Ficoll-BSA gradient (5%, 10%, 15% BSA in Ficoll) and centrifuged (1 h, 1420g, 15°C) with no brake. The epithelial cell band (Figure 1) was removed and washed 3 times by centrifugation (5 min, 1000g, 15°C) in Wash 2 (DME:F12 with 1% penicillin-streptomycin-neomycin, 1% amphotericin B, 5% FBS). Each wash step consisted of resuspending the pellet in 50 mL of Wash 2, centrifuging, and discarding the supernatant. Purified epithelial cells were cryopreserved in 1mL aliquots ($\sim 6 \times 10^6$ cells/vial) of freezing media with DMSO. Cells were initially stored for 24 h at -80°C and then transferred to liquid nitrogen for long term storage.

MEC Isolation from Milk

To isolate mammary epithelial cells from milk, raw milk (800mL) was collected from four lactating dairy cows (125 ± 15 DIM) 2 h after parlor milking and placed on ice. 0.5mM EDTA was added and raw milk was centrifuged at 500g for 20 minutes. The cream layer and supernatant were discarded. The remaining pellets were pooled by resuspension in 1X PBS with 1% penicillin-streptomycin and 0.5mM EDTA and then passed through a 100 μm cell strainer. The pooled cell suspension was washed 3 times in 1X PBS-penicillin-streptomycin by centrifugation (10 min, 500g, 4°C); after each wash, the supernatant was discarded and the pellet resuspended. Finally, the pellet was resuspended in 10 mL DMEM with 1% penicillin-streptomycin and layered on top of a 3-step Ficoll-BSA gradient (5%, 10%, 15% BSA in Ficoll) and centrifuged (1 h, 1420g, 15°C) with no brake. The epithelial cell band (Figure 1) was removed and washed 3 times in DMEM with 1% penicillin-streptomycin. Each wash step consisted of

resuspending the pellet in 50mL of DMEM with 1% penicillin-streptomycin, centrifuging (5 min, 1000g, 15°C), and discarding the supernatant. Purified epithelial cells were cultured immediately.

Explant Culture

Explants were obtained at the same time as tissue for primary cell isolation. Tissue was rinsed in a solution containing 49% DME:F12, 49% Hanks' buffered saline solution, 1% penicillin-streptomycin-neomycin and 1% amphotericin B and cut into 1 mm³ pieces. 10-15 pieces of tissue were transferred to Falcon tubes containing DMEM with 10% FBS, 1% penicillin-streptomycin, 5 µg/mL porcine insulin, 5 µg/mL ovine prolactin, 1 µg/mL dexamethasone with 0 ng/mL or 10 ng/mL GH and cultured for 12 h at 37°C with 5% CO₂.

Primary Cell Culture

Prior to seeding, plates were coated with 10 µg of bovine collagen per cm² of growth area (Santa Cruz Biotechnology, Paso Robles, CA) diluted in 0.01M HCl. The collagen was allowed to incubate at room temperature for 1-2 h. The plates were rinsed three times with 1X PBS prior to seeding. Primary cells were then cultured on collagen-coated 6-well plates in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 µg/mL porcine insulin, 1 µg/mL progesterone, 1 µg/mL dexamethasone, 5 µg/mL bovine transferrin, 5 µM ascorbic acid, 5 mM sodium acetate (**INS/PROG**) at 37°C with 5% CO₂. At 75-80% confluence, media was changed to M199:NCTC 135 with 12% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% amphotericin B, 5 µg/mL porcine insulin, 5 µg/mL ovine prolactin, 1 µg/mL dexamethasone, 5 µg/mL bovine transferrin, 5 µM ascorbic acid, 5 mM sodium acetate

with 0 ng/mL (DIP) or 10 ng/mL GH (**DIP+GH**). Primary cells were cultured for the duration indicated in Figures 5, 6, 8 and 9.

Culture of MEC from Milk

Milk epithelial cells were cultured on collagen-coated wells (10ug/cm²), prepared as described above, in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 µg/mL porcine insulin, 1 µg/mL progesterone, 1 µg/mL dexamethasone, 5 µg/mL bovine transferrin, 5 µM ascorbic acid, 5 mM sodium acetate (**INS/PROG**) at 37°C with 5% CO₂. At 75-80% confluence, media was changed to M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 µg/mL porcine insulin, 5 µg/mL ovine prolactin, 1 µg/mL dexamethasone, 5 µg/mL bovine transferrin, 5 µM ascorbic acid, 5 mM sodium acetate with 0 ng/mL (**DIP**) or 10 ng/mL GH (**DIP+GH**). Cells were cultured for the durations listed in Figures 7, 10, and 11.

Immunocytochemistry

Isolated primary cells were stained with an anti-type II cytokeratin antibody to confirm epithelial origin. Type II cytokeratins are intermediate filaments of the intracytoplasmic skeleton (Alberts *et al.*, 2008). All steps were performed at room temperature. At 60% confluence, cells were fixed for 30 minutes with 4% formalin in PBS with 0.1% Triton X100. Cells were blocked for 20 minutes with 10% FBS in PBS and then incubated with a mouse anti-cytokeratin (type II) antibody (sc-57004, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour on a plate rotator. A fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody was used for visualization (sc-2010, Santa Cruz Biotechnology). Nuclei were stained with propidium

iodide (PI) (Santa Cruz Biotechnology). Cells were visualized using an upright microscope with appropriate filters. FITC and PI images were then overlaid using Adobe Photoshop CS3.

Flow Cytometry

Cryopreserved primary cells were thawed and immediately fixed for 15 minutes at room temperature using 0.01% formaldehyde in PBS. Permeabilizing solution (PBS with 0.5% Tween 20) was added and cells were incubated at room temperature for 15 minutes. An additional volume of permeabilizing solution was added and cells were pelleted by centrifugation at 280g for 5 minutes. Cells were resuspended in dilution buffer (PBS with 10% FBS and 1% sodium azide) and diluted to 500,000 cells/mL. Cells were incubated overnight at 4°C with 3% BSA and primary antibody, mouse anti-cytokeratin (type II) antibody (sc-57004, Santa Cruz Biotechnology). The cells were then pelleted and resuspended in dilution buffer, followed by incubation with 3% BSA and the secondary FITC-conjugated goat anti-mouse IgG secondary antibody (sc-2010, Santa Cruz Biotechnology) overnight at 4°C. Cells were then pelleted and washed twice with ice cold dilution buffer. Fluorescently labeled cells were quantified using a Guava EasyCyte flow cytometer (Millipore, Billerica, MA).

RNA Extraction and cDNA synthesis

Total RNA was extracted from all tissue and cell cultures using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Up to 1µg of total RNA was reverse transcribed using the IScript Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Quantitative PCR

The relative abundance of α_{S1} -casein, α -lactalbumin, GH receptor, IGF-I, and IGFBP-3 mRNA transcripts was determined with quantitative PCR using Fast SYBR Green Master mix (Applied Biosystems, Foster City, CA). For primer sequence and design, see Table A. Quantitative PCR was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001) with mRNA transcripts of 18S RNA (18S), β -actin (ACTB), TATA-binding protein (TBP), or RNA polymerase I, polypeptide D (POLRD1) serving as internal controls.

Statistical Analysis

For explants and primary cells, tissue was collected from one Holstein cow (125 DIM) and one Jersey cow (190 DIM). For milk derived MEC, milk from four cows was pooled (125 $\pm$ 15 DIM). Each experiment was carried out in triplicate. Normalized data were analyzed using the general linear model in SAS 9.0 (SAS Institute, Inc., Cary, NC). Differences were considered significant at $P < 0.05$. Data are represented as the mean $\pm$ SEM.

Figure 1: MEC band at the interface of media and 5%BSA-Ficoll layers.

MEC were isolated from either digested mammary tissue or raw milk using a 3-step BSA-Ficoll density gradient (5%, 10%, 15% layers). Cell suspensions were layered on the top of the 5% BSA-Ficoll gradient layer and centrifuged. Cell types separated according to density and a heavy band of epithelial cells is visible at the interface of the media layer and 5%BSA-Ficoll layer, as indicated by the green arrow.

Target	Sense Primer (5' – 3')	Antisense Primer (5'-3')	Source*
LALBA	AAAGACGACCAGAACCCTCA	GCTTTATGGGCCAACCAGTA	Zhou et al., 2008
CSN1S1	AATCCATGCCCAACAGAAAG	TCAGAGCCAATGGGATTAGG	Zhou et al., 2008
GHR	CTAACTAGCAATGGCGGT	GGGTGGATCTGGTTGTACTA	Johnson et al., 2010
IGF-I	TCACATCCTCCTCGCATCTCTTCT	AACTGGAGAGCATCCACCAACTCA	BC126802.1
IGFBP3	AAAGAGATGTTTGAAATGCCTAGTTTT	TCAAACCTCGGTTTCACTGACTACTG	Voge et al, 2004
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	Zhou et al., 2008
ACTB	GCGTGGCTACAGCTTCACC	TTGATGTCACGGACGATTTC	Bionaz and Loor, 2007
TBP	TGAAACGACTTAGCAGCAGCAGCA	TGCTCGGCTCTGTGGGTTATCTTT	BC113308.1
POLRD1	GCCCGCACGCTTCGAGGCAT	GTCCCAGCCGCCTGGACCAT	NM_001076144.1

Table A: Primers based on bovine mRNA sequences used for quantitative PCR in this study.

Primers used for quantitative PCR analysis of gene expression in mammary epithelial cells cultured with 0 or 10 ng/mL GH. * Primers were obtained based on sequences reported in the reference listed, or were designed and analyzed using Primerquest Software (Integrated DNA Technologies, Coralville, IA) and NetPrimer (Premier Biosoft International, Palo Alto, CA) based on the GenBank accession numbers listed.

Results

Isolated cells were confirmed to be epithelial using immunocytochemistry (Figure 2) and flow cytometry. Cultured cells were stained using a monoclonal cytokeratin type II antibody. Cell populations in culture predominately stained positive for cytokeratin type II indicating their epithelial origin. Additionally, primary cell isolates were quantified as 53% and 58% positive for cytokeratin (Jersey and Holstein respectively) by flow cytometry (Figure 3, Table B)

Explants

Mammary explants from a lactating Jersey cow showed an increase in α -lactalbumin mRNA abundance in response to GH ($P < 0.05$), however, no other targets measured were affected at 12 h by treatment with GH (Figure 4).

Primary cells

Primary cells from both breeds responded to classical lactogenic media through continued expression of milk protein genes. Primary cells isolated from Jersey mammary tissue showed few changes in response to GH. GHR mRNA abundance was increased in response to GH when measured at 2 and 7 d ($P < 0.05$), but no difference was observed at 4 d (Figure 5 and Figure 9). The abundance of milk protein mRNA, α -lactalbumin and α_{s1} -casein, did not change in response to GH. However, a noteworthy trend occurred in IGFBP-3 mRNA expression. The mRNA abundance of IGFBP-3 tended to increase between d 2 and d 4 of treatment, however, this difference was not significant.

After 7 d, primary cells isolated from Holstein mammary tissue did not show a significant difference in the mRNA abundance of any target measured (Figure 6). However, a shorter treatment duration, 4 d, showed a decrease in IGFBP-3 mRNA

abundance in response to GH, as seen in Figure 8, but no other targets were affected. After 4 d of treatment with GH, IGFBP-3 mRNA abundance was significantly decreased in comparison to cells not treated with GH ($P < 0.05$) (Figure 8). Although not significant, a similar trend was also observed at 2 d of treatment ($P < 0.1$).

MEC from Milk

MEC isolated from milk showed a limited response to GH. In cells from Holstein milk, an increase in α -lactalbumin mRNA abundance was observed in response to GH ($P < 0.05$). The increase in α_{s1} -casein mRNA, although not statistically significant, exhibited changes similar to what was previously observed in MAC-T cells. Johnson *et al.* observed an increase in α_{s1} -casein mRNA in differentiated MAC-T cells, compared to undifferentiated, and between MAC-T differentiated with GH, compared to those differentiated without. GHR mRNA abundance was significantly increased at 7 d in Holstein milk cells cultured in lactogenic media when compared to cells cultured in growth media ($P < 0.05$) but was not affected by treatment with GH (Figure 7). Additionally, a decrease in IGFBP-3 mRNA abundance in response to GH was observed at day 4 (Figure 10). This pattern was also observed in Holstein primary cells (Figure 8). MEC from Jersey milk showed no changes in mRNA abundance of any target measured in response to GH. However, a similar trend in IGFBP-3 mRNA abundance was observed between Jersey primary cells and MEC from Jersey milk—IGFBP-3 mRNA abundance was increased at day 4 in response to GH (Figure 9 and 11).

Figure 2: Isolated cells are of epithelial origin.

Immunocytochemistry showed cultured cells to be predominately positive for cytokeratin expression, indicating their epitheliality. Images are representative of immunocytochemistry performed on isolated MEC. Panel A shows nuclei stained with propidium iodide; panel B shows cells positive for cytokeratin type II stained using FITC; panel C shows the two images over-layed using Adobe Photoshop.

A

B

Figure 3: Isolated cells are positive for epithelial marker proteins.

Primary MEC were stained with a primary antibody against cytokeratin type II and a FITC-conjugated secondary antibody, then quantified using flow cytometry (A: Jersey, B: Holstein). Gates were set relative to control samples (cells only incubated with secondary antibody). Unstained cells are represented by black dots (control); cells stained with fluorescent antibody against cytokeratins (type II) are represented by red dots (stained). Quantified results are displayed in Table B.

Source	Sample	Cell count		Total	% positive
		Lower left quadrant	Upper left quadrant		
Jersey	control	4000	0	4000	0
	stained	1782	2218	4000	55.45
Holstein	control	3986	14	4000	0.35
	stained	1661	2339	4000	58.48

Table B: Primary MEC flow cytometry values.

Primary cells were stained with an antibody against cytokeratin type II and a FITC conjugated antibody. Cells were quantified using flow cytometry. Four individual samples were tested. Counts were compiled into the represented values.

Figure 4: α -lactalbumin mRNA abundance in Jersey mammary explants is increased after 12 h in response to GH.

Mammary explants from a lactating Jersey cow show a limited response to GH, with only the mRNA abundance of α -lactalbumin being affected by GH. Explants taken from lactating mammary tissue from a Jersey cow were cultured for 12 h in DMEM with 10% FBS, 1% penicillin-streptomycin, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone with 0 ng/mL GH (**DIP**) or 10 ng/mL GH (**DIP+GH**) and cultured for 12 hrs at 37°C with 5% CO₂. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM. An asterisk (*) denotes a significant difference ($P < 0.05$).

Figure 5: In primary MEC from a Jersey cow, GHR mRNA abundance is increased in response to GH after 7 d.

Primary MEC from a Jersey cow show an increase in GHR mRNA abundance after 7d in response to treatment with GH. Confluent primary MEC isolated from lactating Jersey mammary tissue were cultured for 7 d in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate with 0 ng/mL (**DIP**) or 10 ng/mL GH (**DIP+GH**) at 37°C with 5% CO₂. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM. An asterisk (*) denotes a significant difference ($P < 0.05$).

Figure 6: Primary MEC from a Holstein do not respond to treatment with GH after 7 d.

No change in the mRNA abundance of targets in primary MEC from Holstein mammary tissue was observed after 7 d in response to GH. Confluent primary MEC isolated from lactating Holstein mammary tissue were cultured for 7 d in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate with 0 ng/mL (**DIP**) or 10 ng/mL GH (**DIP+GH**) at 37°C with 5% CO₂. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM.

Figure 7: In MEC from Holstein milk, α -lactalbumin mRNA abundance is increased in response to treatment with GH after 7 d.

Abundance of α -lactalbumin mRNA in MEC from Holstein milk cultured with GH was increased after 7 d in culture. Additionally, α_{s1} -casein mRNA abundance was greater in MEC differentiated with GH, than undifferentiated MEC. GHR mRNA abundance was greater in differentiated MEC. MEC isolated from milk were cultured in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 1 μ g/mL progesterone, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate (**INS/PROG**) until confluent. At 75-80% confluence, media was changed to M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate with 0 ng/mL (**DIP**) or 10 ng/mL GH (**DIP+GH**) and cultured for 7 d. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM. A superscript denotes a significant difference ($P < 0.05$).

Figure 8: Primary Holstein MEC have decreased IGFBP-3 mRNA abundance after 4 d of treatment with GH.

Primary MEC from a Holstein cow responded to GH with a decrease in mRNA abundance of IGFBP-3 after 4 d; a similar pattern is also seen after 2 d, although not statistically significant. MEC isolated from Holstein mammary tissue were cultured in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 1 μ g/mL progesterone, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate (**INS/PROG**) until confluent. At 75-80% confluence, media was changed to M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate with 0 ng/mL (**DIP**) or 10 ng/mL GH (**DIP+GH**) and cultured for 2 and 4 d. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM. A superscript denotes a significant difference ($P < 0.05$)

Figure 9: Primary Jersey MEC have increased GHR mRNA abundance after 2 d in response to treatment with GH.

GHR mRNA abundance was significantly greater in MEC after 2 d in response to treatment with GH. No other targets were affected by GH. MEC isolated from mammary tissue from a Jersey cow were cultured in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 1 μ g/mL progesterone, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate (**INS/PROG**). At 75-80% confluence, media was changed M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate with 0 ng/mL (**DIP**) or 10 ng/mL GH (**DIP+GH**) and cultured for 2 and 4 d. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM. A superscript denotes a significant difference ($P < 0.05$).

Figure 10: Holstein MEC from milk have decreased mRNA abundance of IGFBP-3 after 4 d in response to treatment with GH.

IGFBP-3 mRNA abundance is decreased in MEC from Holstein milk after 4 d in response to treatment with GH. No other targets were affected by GH. MEC isolated from Holstein milk were cultured in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 1 μ g/mL progesterone, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5mM sodium acetate (INS/PROG). At 75-80% confluence, media was changed M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5mM sodium acetate with 0ng/mL (DIP) or 10ng/mL GH (DIP+GH) and cultured for 2 and 4 d. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM. A superscript denotes a significant difference ($P < 0.05$)

Figure 11: In MEC from Jersey milk, the mRNA abundance for any transcript evaluated was not affected by GH.

Although mRNA transcript abundance was not affected by GH, α_{s1} -casein mRNA abundance was greater in MEC differentiated with GH than in undifferentiated MEC after 2 days. MEC isolated from Jersey milk were cultured in M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 1 μ g/mL progesterone, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate (INS/PROG). At 75-80% confluence, media was changed M199:NCTC 135 with 12% FBS, 1% penicillin-streptomycin, 1% amphotericin B, 5 μ g/mL porcine insulin, 5 μ g/mL ovine prolactin, 1 μ g/mL dexamethasone, 5 μ g/mL bovine transferrin, 5 μ M ascorbic acid, 5 mM sodium acetate with 0 ng/mL (DIP) or 10 ng/mL GH (DIP+GH) and cultured for 2 and 4 d. Total RNA was extracted and reverse transcribed. Targets were analyzed using quantitative PCR. Bars represent the mean $\pm$ SEM. A superscript denotes a significant difference ($P < 0.05$).

Discussion

Recently, two studies utilizing MAC-T cells have reported a direct response to GH (Johnson *et al.*, 2010; Zhou *et al.*, 2008). One study utilizing transformed MAC-T cells which over-express the GHR and STAT5 reported a significant increase in the mRNA expression of α -lactalbumin and α_{s1} -casein, and other milk proteins in response to GH (Zhou *et al.*, 2008). GHR mRNA was over-expressed in MAC-T cells because it appeared to be in very low abundance when quantified by a ribonuclease protection assay. Additionally, Johnson *et al.* reported that native MAC-T cells cultured for 7 d in lactation media with 0 or 10ng/mL GH showed increased α -lactalbumin and α_{s1} -casein mRNA in response to GH, in addition to increased abundance of the GHR mRNA (Johnson *et al.*, 2010). Prior proteomic work in the MAC-T cell line has shown a direct effect of GH which induced changes in abundance of proteins involved in cytoskeletal arrangement, metabolism and lactogenesis, indicating that GH plays a role in stabilizing the cell and preparing it for lactation (Johnson, 2010). Although MAC-T cells provide a useful model of MEC, undergoing immortalization may have altered their ability to model normal MEC function. Here, the direct effect of GH on MEC was investigated using three primary *in vitro* MEC models: explants, primary MEC isolated from whole mammary tissue, and primary MEC derived from milk. Mammary explants and primary cells did not express the same response to GH as previously observed in MAC-T cells. While some primary mammary models showed an increased abundance of milk proteins in response to GH, no model reproduced the robust response to GH observed in MAC-T cells.

As previously indicated, *in vitro* primary cell models provide an isolated cell model of MEC. However, because there is some dispute as to whether MEC themselves express and secrete IGF-I, or if it is only produced by near-by stromal cells, and because the *in vivo* role of GH is coupled with IGF-I, the mRNA abundance of IGF-I was investigated to evaluate the influence, if any, IGF-I may play in *in vitro* mammary models. IGF-I mRNA was found in all models studied. This may be expected in explants because it is well documented that the mammary stroma synthesizes and secretes IGF-I (Kleinberg, 1997; Fleming *et al.*, 2005). However, in isolated populations of MEC, IGF-I mRNA was also detected. It has been previously reported that the mammary epithelium does not express IGF-I mRNA (Campbell, 1988). Zhou *et al.* reported the expression of IGF-I mRNA in transfected MAC-T; however, its abundance was not affected by treatment with GH (Zhou *et al.*, 2008). Here, IGF-I mRNA was detected in both mammary explants and primary cells, however IGF-I mRNA abundance was not affected by treatment with GH. This indicates the observed changes in IGFBP-3 mRNA abundance are likely a result of a direct action of GH, and not due to the autocrine or paracrine effects of increased IGF-I being secreted in response to GH.

Expression of GHR mRNA was detected in mammary explants and primary cells from tissue and milk. The presence of GHR mRNA supports the possibility of a direct role of GH on MEC; however, that role does not appear to be directly mediating an increase in milk components to the same magnitude observed in MAC-T. Only explants and MEC from Holstein milk showed a significant increase in α -lactalbumin in response to GH. Based on these results, and proteomic changes previously observed in the MAC-T cell line, the direct action of GH on the MEC may have influence over differentiation

and regulate binding proteins to alter the availability of IGF-I, rather than exerting a direct effect on milk synthesis and secretion.

A previous study evaluating the response of Holstein and Jersey cows to GH reported differences in their capacity to respond to GH (West *et al.*, 1990). West *et al.* reported that in Holsteins as the dose of GH increased, milk yield increased in a linear fashion in Holsteins; however, in Jerseys, as the dose of GH increased over the same range, milk yield only increased until the dosage of GH reached 15mg and then declined at higher doses. A similar difference in breed response is observed in the current results. IGFBP-3 mRNA abundance is altered by GH and the effect differs between breeds. Primary and milk cells from Holsteins showed a decrease in IGFBP-3 mRNA abundance in response to GH after 4 d. In both primary and milk MEC from Jersey cows, no change was observed in response to GH, however, IGFBP-3 abundance tended to increase from day 2 and day 4 regardless of GH treatment. Changes in the abundance of IGFBP-3 may affect the actions of the GH-IGF axis, because IGFBP-3 is the major binding protein for IGF-I (Baumrucker and Erondur, 2000). For example, mammary epithelial cells isolated from Holsteins may be able to better respond to GH because GH inhibits the production of IGFBP-3, allowing more IGF-I to remain unbound and able to interact with its receptor. This proposed model is diagramed in Figure 12.

The roles of IGFBP are not fully understood, however, it is well established that they bind IGF-I, can extend its half-life in serum, limit the availability of IGF-I, can aid in the localization of IGF-I to a particular tissue through interactions with extracellular matrix proteins and membrane proteins. IGFBP also have important IGF-independent actions by binding of extracellular membrane proteins and can induce changes in gene

expression, metabolism and apoptosis (Marshman & Streuli, 2002; Gluckman *et al.*, 1987; Baumrucker, 2005; (Yamada and Lee, 2009). As a result, an increase in IGFBP-3 by GH may influence the gene expression of MEC, independently of IGF-I.

The direct effects of GH and MEC are not fully defined; however, these data indicate GH may play a role in regulating the expression of IGFBP-3. Whether the observed changes in IGFBP-3 mRNA abundance are translated into changes in protein has yet to be tested. However, despite the complex relationship of IGFBP-3 with IGF-I and MEC, there is an influence of GH over IGFBP-3 mRNA abundance which substantiates further investigation into the roles and relevance of the relationships between IGFBP-3 and GH and their effects on MEC *in vivo*.

Figure 12: Proposed model of direct effect of GH on primary bovine mammary epithelial cells.

GH stimulates the production of IGF-I by the liver. IGF-I is a potent growth factor which stimulates gene transcription and protein synthesis through its receptor. The direct action of GH on MEC alters the mRNA abundance of IGFBP-3. IGFBP-3 binds IGF-I in circulation, it can sequester it from interacting with its receptor, in addition to aiding in localization to the receptor through interaction with nearby membrane proteins. By regulating IGFBP-3 mRNA abundance, GH in turn effects the systemic availability of IGF-I. GH: growth hormone, GHR: growth hormone receptor, MEC: mammary epithelial cell, IGF-I: insulin-like growth factor-1, IGFR: insulin-like growth factor-1 receptor, MP: membrane proteins.

Chapter 3

LIMITATIONS AND CONCLUSIONS

Limitations and Implications

Due to culture conditions, the nature of the models utilized, and the limited number of animals available, there are several important factors that limit the depth of inference of these results.

Attempts to culture primary MEC and milk derived MEC without the use of FBS were unsuccessful. Particularly in milk derived MEC, the low number of cells obtained by each isolation made an initial dense seeding impossible. Additionally, these cells did not proliferate at all without the presence of serum and with serum, adequate proliferation took 10-12 days. It has previously been suggested that due to their lack of *in vitro* proliferation, milk cells must represent a fully differentiated cell type (Boutinaud & Jammes, 2002). As a result of observing poor growth in milk derived MEC and the wide spread use of serum reported in the literature, primary MEC were always cultured with serum. Even with the addition of serum, primary cells exhibited better proliferation than milk derived cells. Investigation into the requirements of serum for both primary and milk derived MEC may prove useful in designing future experiments.

In addition to limited proliferation without serum, unknown quantities of growth factors and other proteins present in serum represent a confounding variable. Additionally, because GH is present in serum, it is difficult to determine the actual dose of GH during experimentation. However, despite unknown concentrations of GH, serum lots were the same for each experiment. During the course of these experiments, charcoal-stripped serum was not used. In light of these limitations, it may be useful in

future to evaluate primary cell proliferation with charcoal-filtered serum and use it during future experiments. If primary cells fail to proliferate using charcoal-stripped serum, it may be appropriate to use standard serum during proliferation and charcoal-stripped during experimentation.

Given the concern of the addition of an unquantified amount GH through the use of serum in addition to the treatment concentration of 10ng/mL, it may also be useful to evaluate a dose-response curve for GH in primary cells. GH concentrations in serum can vary between 4.1-167ng/mL (Honn et al., 1975). The treatment value (10ng/mL GH) of GH in this study was selected based on previous dose-response curves in MAC-T and values reported in the literature (Johnson et al., 2010).

Considering the integrated role GH and IGF-I play *in vivo*, evaluating their synergistic effects on IGFBP-3 in primary MEC would lend clarity to the proposed model and the observed differences between dairy breeds.

MEC populations for these experiments were all derived from the campus dairy herd. For milk-derived cells, milk was pooled from four dairy cows and a total of 800mL of milk could be collected at one time. These limitations were a result of the equipment available and feasibility of time. The centrifuge used was capable of holding 800mL of contents and the time required to obtain the milk samples became a limiting factor in order to quickly return to the laboratory and begin isolating MEC. As a result, only a small number of MEC were able to be collected at one time. By collecting a larger volume of raw milk, a larger initial population of MEC could be collected with which to begin experiments. Additionally, because milk was collected from a small number of animals, it is difficult to draw inferences that apply to breeds as a whole.

For explants and primary MEC, only two animals were used for this study (one each of Holstein and Jersey). Because all primary cells from each breed were originally derived from the same animal, it is difficult to extrapolate results obtained from these two individual animals to their respective breed populations as a whole. However, although only one animal was used for the initial isolation, the experiment was replicated three times. Additionally, most cells lines, MAC-T in particular, also originate from cells isolated from one individual. Despite this, MAC-T have been widely used to model bovine MEC *in vitro*. However, it is common to validate results obtained from cells lines by evaluating primary models. Therefore, in this study using primary cells that have not been widely studied, it would have been valuable to have more individuals from which to obtain samples to aid in reducing the impact that individual variations in genotype, health status, and stage of lactation may play in this model. It is important to note, though, that the pattern of IGFBP-3 abundance in response to GH was similar between MEC from the same breed, regardless of the isolation technique used. Although the sample size in this study was low, different MEC models obtained from different animals showed the same pattern of response to GH across breeds. This lends support to the claim that GH has differing effects between Jerseys and Holsteins.

In spite of these limitations, these results remain valuable initial data that can serve to guide future investigations. Future studies utilizing these primary models would benefit from the availability of more sample animals, particularly animals at the same stage of lactation, and perhaps a broader collection of samples beyond the campus herd. Increased sampling would more accurately represent the response of breeds as a whole, and not only the isolated population in the campus herd.

Conclusion

In consideration of this study's limitations, further investigation is required in order to convincingly describe the proposed model of the direct effect of GH and its potential regulation of IGFBP-3. Differences observed between breeds were conserved across all models evaluated, although not at the same magnitude. Additionally, these conclusions have been drawn from mRNA abundance data and it is critical to verify if these differences in mRNA abundance are still present in protein abundance. It is also important to note that there are other regulators of IGFBP-3 *in vivo* that were not measured here. Investigation into any effect GH may play on other regulators of IGFBP-3 may yield a more complete, and accurate, model of the direct role of GH.

The reported results taken with consideration of their limitations provide a useful potential model of the direct role of GH on bovine MEC. Although extrapolation of these results to whole populations is difficult, it provides a plausible explanation of one of the differences between Jersey and Holstein cattle's response to GH.

Literature Cited

- Akers RM** 1985 Lactogenic hormones - binding-sites, mammary growth, secretory-cell differentiation, and milk biosynthesis in ruminants. *Journal of Dairy Science* **68**(2) 501-519
- Akers RM** 2006 Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. *Journal of Dairy Science* **89**(4) 1222-1234
- Akers RM, McFadden TB, Purup S, Vestergaard M, Sejrsen K & Capuco AV** 2000 Local IGF-I axis in peripubertal ruminant mammary development. *Journal of Mammary Gland Biology and Neoplasia* **5**(1) 43-51
- Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P** 2008 *Molecular Biology of the Cell. 5th Edition.*: Garland Science, Taylor & Francis Group, LLC.
- Aslakson CJ & Miller FR** 1992 Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Research* **52**(6) 1399-1405
- Ball RK, Friis RR, Schoenenberger CA, Doppler W & Groner B** 1988 Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. *EMBO J* **7**(7) 2089-2095
- Barclay JL, Kerr LM, Arthur L, Rowland JE, Nelson CN, Ishikawa M, d'Aniello EM, White M, Noakes PG & Waters MJ** 2010 In Vivo Targeting of the Growth Hormone Receptor (GHR) Box1 Sequence Demonstrates that the GHR Does Not Signal Exclusively through JAK2. *Molecular Endocrinology* **24**(1) 204-217
- Baserga R, Peruzzi F & Reiss K** 2003 The IGF-1 receptor in cancer biology. **107** 873-877
- Bauman DE & Currie WB** 1980 Partitioning of nutrients during pregnancy and lactation - a review of mechanisms involving homeostasis and homeorhesis. *Journal of Dairy Science* **63**(9) 1514-1529
- Bauman DE & Vernon RG** 1993 Effects of exogenous bovine somatotropin on lactation. *Annual Review of Nutrition* **13** 437-461
- Baumrucker CR** 2005 Intracrine signaling in the mammary gland. *Livestock Production Science* **98**(1-2) 47-56
- Baumrucker CR & Erondur NE** 2000 Insulin-like growth factor (IGF) system in the bovine mammary gland and milk. *Journal of Mammary Gland Biology and Neoplasia* **5**(1) 53-64
- Baxter F, Neoh K & Tevendale M** 2007 The Beginning of the End: Death Signaling in Early Involution. *Journal of Mammary Gland Biology and Neoplasia* **12**(1) 3-13
- Berelowitz M, Szabo M, Frohman LA, Firestone S, Chu L & Hintz RL** 1981 Somatomedin-C Mediates Growth Hormone Negative Feedback by Effects on Both the Hypothalamus and the Pituitary. *Science* **212**(4500) 1279-1281

- Berry SD, McFadden TB, Pearson RE & Akers RM** 2001 A local increase in the mammary IGF-1: IGFBP-3 ratio mediates the mammary effects of estrogen and growth hormone. *Domestic Animal Endocrinology* **21**(1) 39-53
- Bolander FF** 1989 *Molecular endocrinology*. Academic Press
- Boutinaud M, Ben Chedly MH, Delamaire E & Guinard-Flament J** 2008 Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. *Journal of Dairy Science* **91**(3) 988-998
- Boutinaud M & Jammes H** 2002 Potential uses of milk epithelial cells: a review. *Reproduction Nutrition Development* **42**(2) 133-147
- Boutinaud M, Rulquin H, Keisler DH, Djiane J & Jammes H** 2002 Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. *Journal of Animal Science* **80**(5) 1258-1269
- Braun JEA & Severson DL** 1992 Regulation of the synthesis, processing and translocation of lipoprotein-lipase. *Biochemical Journal* **287** 337-347
- Brennan AJ, Sharp JA, Lefevre CM & Nicholas KR** 2008 Uncoupling the mechanisms that facilitate cell survival in hormone-deprived bovine mammary explants. *J Mol Endocrinol* **41**(3) 103-116
- Briskin C, Ayyannan A, Nguyen C, Heineman A, Reinhardt F, Jan T, Dey SK, Dotto GP & Weinberg RA** 2002 IGF-2 Is a Mediator of Prolactin-Induced Morphogenesis in the Breast. *Developmental cell* **3**(6) 877-887
- Briskin C & O'Malley B** 2010 Hormone action in the Mammary Gland. Cold Spring Harbor Perspectives in Biology **2**
- Buehring GC** 1990 Culture of mammary epithelial-cells from bovine-milk. *Journal of Dairy Science* **73**(4) 956-963
- Butler AA & Le Roith D** 2001 Control of growth by the somatotropic axis: Growth hormone and the insulin-like growth factors have related and independent roles. *Annual Review of Physiology* **63** 141-164
- Campbell PG** 1988 Insulin-like growth factor-I and insulin-like growth factor binding proteins in the bovine mammary gland: Receptors, endogenous secretion, and appearance in milk.
- Campbell PG, Skaar TC, Vega JR & Baumrucker CR** 1991 Secretion of insulin-like growth factor-1 (IGF-1) and IGF-binding proteins from bovine mammary tissue *in vitro*. *Journal of Endocrinology* **128**(2) 219-228
- Capper JL, Cady RA & Bauman DE** 2009 Increased production reduces the dairy industry's environmental impact. Proceedings of the 18th Annual Tri-State Dairy Nutrition Conference, Fort Wayne, Indiana, USA, 21-22 April, 2009 71-82
- Capper JL, Castaneda-Gutierrez E, Cady RA & Bauman DE** 2008 The environmental impact of recombinant bovine somatotropin (rbST) use in dairy production. Proceedings of the National Academy of Sciences of the United States of America **105**(28) 9668-9673

- Carstens GE, Glaser DE, Byers FM, Greene LW & Lunt DK** 1997 Effects of bovine somatotropin treatment and intermittent growth pattern on mammary gland development in heifers. *Journal of Animal Science* **75**(9) 2378-2388
- CarterSu C, Schwartz J & Smit LS** 1996 Molecular mechanism of growth hormone action. *Annual Review of Physiology* **58** 187-207
- Ceriani R, Taylor-Papadimitriou J, Peterson J & Brown P** 1979 Characterization of cells cultured from early lactation milks. *In Vitro Cellular & Developmental Biology - Plant* **15**(5) 356-362
- Ceriani RL** 1974 Hormones and other factors controlling growth in mammary-gland - review. *Journal of Investigative Dermatology* **63**(1) 93-108
- Cohick WS** 1998 Role of the insulin-like growth factors and their binding proteins in lactation. *Journal of Dairy Science* **81**(6) 1769-1777
- Cohick WS & Clemmons DR** 1993 The insulin-like growth-factors. *Annual Review of Physiology* **55** 131-153
- Cohick WS & Turner JD** 1998 Regulation of IGF binding protein synthesis by a bovine mammary epithelial cell line. *Journal of Endocrinology* **157**(2) 327-336
- Collier R, McGrath MF & Vincini JL** 1999 Somatotropin and the U.S. dairy industry. *Medecin Veterinaire du Quebec* **29**(2) 89-92
- Cotes P, Crichton J, Folley S & Young F** 1949 Galactopoietic activity of purified anterior pituitary growth hormone. *Nature* **164**(4180) 992-993
- Dahl GE, Chapin LT, Allen MS, Moseley WM & Tucker HA** 1991 Comparison of somatotropin and growth hormone-releasing factor on milk-yield, serum hormones, and energy status. *Journal of Dairy Science* **74**(10) 3421-3428
- Daniel CW & Smith GH** 1999 The mammary gland: A model for development. *Journal of Mammary Gland Biology and Neoplasia* **4**(1) 3-8
- Danielson KG, Oborn CJ, Durban EM, Butel JS & Medina D** 1984 Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. *Proceedings of the National Academy of Sciences* **81**(12) 3756-3760
- Dulbecco R, Bologna M & Unger M** 1979 Differentiation of a rat mammary cell-line *in vitro*. *Proceedings of the National Academy of Sciences of the United States of America* **76**(3) 1256-1260
- Ebner KE, Hoover CR, Hageman EC & Larson BL** 1961 Cultivation and properties of bovine mammary cell cultures. *Experimental Cell Research* **23**(2) 373-385
- Eppard PJ, Bentle LA, Violand BN, Ganguli S, Hintz RL, Kung L, Krivi GG & Lanza GM** 1992 Comparison of the galactopoietic response to pituitary-derived and recombinant-derived variants of bovine growth hormone. *Journal of Endocrinology* **132**(1) 47-56

- Ernens I, Clegg R, Schneider YJ & Larondelle Y** 2007 Short communication: Ability of cultured mammary epithelial cells in a bicameral system to secrete milk fat. *Journal of Dairy Science* **90**(2) 677-681
- Etherton TD & Bauman DE** 1998 Biology of somatotropin in growth and lactation of domestic animals. *Physiological Reviews* **78**(3) 745-761
- Fleming JM, Leibowitz BJ, Kerr DE & Cohick WS** 2005 IGF-I differentially regulates IGF-binding protein expression in primary mammary fibroblasts and epithelial cells. *Journal of Endocrinology* **186**(1) 165-178
- Flint DJ, Beattie J & Allan GJ** 2003 Modulation of the actions of IGFs by IGFBP-5 in the mammary gland. *Hormone and Metabolic Research* **35**(11-12) 809-815
- Flint DJ & Knight CH** 1997 Interactions of prolactin and growth hormone (GH) in the regulation of mammary gland function and epithelial cell survival. *Journal of Mammary Gland Biology and Neoplasia* **2**(1) 41-48
- Gaffney EV** 1982 A cell-line (HBL-100) established from human-breast milk. *Cell and Tissue Research* **227**(3) 563-568
- Gibson CA, Staley MD & Baumrucker CR** 1999 Identification of IGF binding proteins in bovine milk and the demonstration of IGFBP-3 synthesis and release by bovine mammary epithelial cells. *Journal of Animal Science* **77**(6) 1547-1557
- Gibson JP, van der Meulen M, McBride BW & Burton JH** 1992 The Effects of Genetic and Phenotypic Production Potential on Response to Recombinant Bovine Somatotropin. *Journal of Dairy Science* **75**(3) 878-884
- Glimm DR, Baracos VE & Kennelly JJ** 1990 Molecular evidence for the presence of growth-hormone receptors in the bovine mammary-gland. *Journal of Endocrinology* **126**(3) R5-R8
- Gluckman PD, Breier BH & Davis SR** 1987 Physiology of the Somatotrophic Axis with Particular Reference to the Ruminant. *Journal of Dairy Science* **70**(2) 442-466
- Gorewit RC, Svennersten K, Butler WR & Uvnasmoberg K** 1992 Endocrine responses in cows milked by hand and machine. *Journal of Dairy Science* **75**(2) 443-448
- Grant DS, Kleinman HK, Leblond CP, Inoue S, Chung AE & Martin GR** 1985 The basement-membrane-like matrix of the mouse ehs tumor .2. Immunohistochemical quantitation of 6 of its components. *American Journal of Anatomy* **174**(4) 387-398
- Hauser SD, McGrath MF, Collier RJ & Krivi GG** 1990 Cloning and *in vivo* expression of bovine growth-hormone receptor messenger-RNA. *Molecular and Cellular Endocrinology* **72**(3) 187-200
- Honn, K., Singley J, & Chavin W** 1975 Fetal Bovine Serum: A Multivariate Standard. *Experimental Biology and Medicine* **149**: 344-347.
- Hovey RC, Harris J, Hadsell DL, Lee AV, Ormandy CJ & Vonderhaar BK** 2003 Local insulin-like growth factor-II mediates prolactin-induced mammary gland development. *Molecular Endocrinology* **17**(3) 460-471

- Hovey RC, Trott JF & Vonderhaar BK** 2002 Establishing a Framework for the Functional Mammary Gland: From Endocrinology to Morphology. *Journal of Mammary Gland Biology and Neoplasia* **7**(1) 17-38
- Hutton JB** 1957 The effect of growth hormone on the yield and composition of cows milk. *Journal of Endocrinology* **16**(2) 115-125
- Huynh HT, Robitaille G & Turner JD** 1991 Establishment of bovine mammary epithelial-cells (MAC-T) - an *in vitro* model for bovine lactation. *Experimental Cell Research* **197**(2) 191-199
- Ip MM & Darcy KM** 1996 Three-dimensional mammary primary culture model systems. *Journal of Mammary Gland Biology and Neoplasia* **1**(1) 91-110
- Johnson T** 2010 Growth hormone alters components related to differentiation, metabolism and milk synthesis and secretion in MAC-T cells. Vol. MS. California Polytechnic State University
- Johnson TL, Fujimoto BAS, Jimenez-Flores R & Peterson DG** 2010 Growth hormone alters lipid composition and increases the abundance of casein and lactalbumin mRNA in the MAC-T cell line. *Journal of Dairy Research* **77**(2) 199-204
- Kawano A, Tateyama S, Yamaguchi R, Nosaka D & Kondo F** 1988 Morphology of goat milk-derived mammary epithelial cells cultured in collagen gel. *Nippon Juigaku Zasshi* **50**(6) 1252-1258
- Keenan TW** 2001 Assembly and secretion of the lipid globules of milk. *Bioactive Components of Human Milk* **501** 125-136
- Keys J, Cifrian E, Guidry A & Farrell H** 1997 Bovine mammary explant versus primary cell cultures: Effect of bovine somatotropin and insulinlike growth factor-I on DNA content and protein synthesis. *In Vitro Cellular & Developmental Biology - Animal* **33**(3) 206-211
- Keys JE, Fekry AE, Wood DL & Capuco AV** 1992 The ability of bovine mammary tissue to synthesize lipids for 96-h when co-cultured with liver and adipose-tissue. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire* **70**(5) 343-346
- Kleinberg D & Ruan WF** 2008 IGF-I, GH, and Sex Steroid Effects in Normal Mammary Gland Development. *Journal of Mammary Gland Biology and Neoplasia* **13**(4) 353-360
- Kleinberg DL** 1997 Early mammary development: growth hormone and IGF-1. *J Mammary Gland Biol Neoplasia* **2**(1) 49-57
- Knight CH** 2000 Mammary gland development and function. *The health of dairy cattle.* 203-212
- Knight CH** 2001 Overview of prolactin's role in farm animal lactation. *Livestock Production Science* **70**(1-2) 87-93
- Kopchick JJ & Andry JM** 2000 Growth hormone (GH), GH receptor, and signal transduction. *Molecular Genetics and Metabolism* **71**(1-2) 293-314

- Kresge N & Simoni RD** 2006 Lactose Synthesis in the Mammary Gland: Lactose Synthase and the Work of Robert L. Hill. *Journal of Biological Chemistry* **281**(6) e6
- Kulik G, Klippel A & Weber MJ** 1997 Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Molecular and Cellular Biology* **17**(3) 1595-1606
- Kumar S, Clarke AR, Hooper ML, Horne DS, Law AJR, Leaver J, Springbett A, Stevenson E & Simons JP** 1994 Milk-composition and lactation of beta-casein-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **91**(13) 6138-6142
- Larson BL** 1969 Biosynthesis of Milk. *Journal of Dairy Science* **52**(5) 737-747
- Larson BL & Smith VR** 1974 Lactation: a comprehensive treatise, edited by Bruce L. Larson and Vearl R. Smith. New York: Academic Press
- Le Roith D, Bondy C, Yakar S, Liu J-L & Butler A** 2001 The Somatomedin Hypothesis: 2001. *Endocrine Reviews* **22**(1) 53-74
- Lee MO & Schaffer NK** 1934 Anterior Pituitary Growth Hormone and the Composition of Growth. *The Journal of Nutrition* **7**(3) 337-363
- Leroith D, Werner H, Beitnerjohnson D & Roberts CT** 1995 Molecular and cellular aspects of the insulin-like growth-factor-i receptor. *Endocrine Reviews* **16**(2) 143-163
- Li ML, Liu XW, Robinson G, BarPeled U, Wagner KU, Young WS, Hennighausen L & Furth PA** 1997 Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proceedings of the National Academy of Sciences of the United States of America* **94**(7) 3425-3430
- Liu XW, Robinson GW, Wagner KU, Garrett L, WynshawBoris A & Hennighausen L** 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes & Development* **11**(2) 179-186
- Livak KJ & Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25**(4) 402-408
- Lonnerdal B** 2007 Trace element transport in the mammary gland. *Annual Review of Nutrition* **27** 165-177
- Malinowski E** 2005 Mammary gland involution in cows. *Medycyna Weterynaryjna* **61**(9) 968-971
- Mallepell S, Krust Ae, Chambon P & Briskin C** 2006 Paracrine signaling through the epithelial estrogen receptor $\text{ER}\alpha$ is required for proliferation and morphogenesis in the mammary gland. *Proceedings of the National Academy of Sciences of the United States of America* **103**(7) 2196-2201
- Marshman E & Streuli CH** 2002 Insulin-like growth factors and insulin-like growth factor binding proteins in mammary gland function. *Breast Cancer Research* **4**(6) 231-239

- Marti A, Jehn B, Costello E, Keon N, Ke G, Martin F & Jaggi R** 1994 Protein kinase- α and ap-1 (c-fos/jund) are induced during apoptosis of mouse mammary epithelial-cells. *Oncogene* **9**(4) 1213-1223
- Mather IH & Keenan TW** 1998 Introduction - The cell biology of milk secretion: Historical notes. *Journal of Mammary Gland Biology and Neoplasia* **3**(3) 227-232
- Matitashvili E & Bauman DE** 1999 Culture of primary bovine mammary epithelial cells. *In Vitro Cellular & Developmental Biology-Animal* **35**(8) 431-434
- McDowell GH, Gooden JM, Leenanuruxsa D, Jois M & English AW** 1987 Effects of exogenous growth-hormone on milk-production and nutrient-uptake by muscle and mammary tissues of dairy-cows in midlactation. *Australian Journal of Biological Sciences* **40**(3) 295-306
- McGuire MA, Vicini JL, Bauman DE & Veenhuizen JJ** 1992 Insulin-like growth-factors and binding-proteins in ruminants and their nutritional regulation. *Journal of Animal Science* **70**(9) 2901-2910
- McManaman JL & Neville MC** 2003 Mammary physiology and milk secretion. *Advanced Drug Delivery Reviews* **55**(5) 629-641
- Menzies KK, Lefevre C, Macmillan KL & Nicholas KR** 2009 Insulin regulates milk protein synthesis at multiple levels in the bovine mammary gland. *Functional & Integrative Genomics* **9**(2) 197-217
- Mills ES & Topper YJ** 1970 Some ultrastructural effects of insulin, hydrocortisone, and prolactin on mammary gland explants. *Journal of Cell Biology* **44**(2) 310-&
- Murrieta CM, Hess BW, Scholljegerdes EJ, Engle TE, Hossner KL, Moss GE & Rule DC** 2006 Evaluation of milk somatic cells as a source of mRNA for study of lipogenesis in the mammary gland of lactating beef cows supplemented with dietary high-linoleate safflower seeds. *Journal of Animal Science* **84**(9) 2399-2405
- Nytes AJ, Combs DK, Shook GE, Shaver RD & Cleale RM** 1990 Response to Recombinant Bovine Somatotropin in Dairy Cows with Different Genetic Merit for Milk Production. *Journal of Dairy Science* **73**(3) 784-791
- Peel CJ & Bauman DE** 1987 Somatotropin and lactation. *Journal of Dairy Science* **70**(2) 474-486
- Peel CJ, Bauman DE, Gorewit RC & Sniffen CJ** 1981 Effect of exogenous growth-hormone on lactational performance in high yielding dairy-cows. *Journal of Nutrition* **111**(9) 1662-1671
- Piwien-Pilipuk G, Huo JS & Schwartz J** 2002 Growth hormone signal transduction. *Journal of Pediatric Endocrinology & Metabolism* **15**(6) 771-786
- Plath-Gabler A, Gabler C, Sinowatz F, Berisha B & Schams D** 2001 The expression of the IGF family and GH receptor in the bovine mammary gland. *Journal of Endocrinology* **168**(1) 39-48

- Pocius PA & Herbein JH** 1986 Effects of *in vivo* administration of growth-hormone on milk-production and *in vitro* hepatic-metabolism in dairy-cattle. *Journal of Dairy Science* **69**(3) 713-720
- Richards J, Larson L, Yang J, Guzman R, Tomooka Y, Osborn R, Imagawa W & Nandi S** 1983 Method for culturing mammary epithelial cells in a rat tail collagen gel matrix. *Methods in Cell Science* **8**(1) 31-36
- Richert MM, Schwertfeger KL, Ryder JW & Anderson SM** 2000 An atlas of mouse mammary gland development. *Journal of Mammary Gland Biology and Neoplasia* **5**(2) 227-241
- Riley LG, Gardiner-Garden M, Thomson PC, Wynn PC, Williamson P, Raadsma HW & Sheehy PA** 2010 The influence of extracellular matrix and prolactin on global gene expression profiles of primary bovine mammary epithelial cells in vitro. *Animal Genetics* **41**(1) 55-63
- Riley LG, Williamson P, Wynn PC & Sheehy PA** 2008 Lactoferrin decreases primary bovine mammary epithelial cell viability and casein expression. *Journal of Dairy Research* **75**(2) 135-141
- Robinson GW, McKnight RA, Smith GH & Hennighausen L** 1995 Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development* **121**(7) 2079-2090
- Rohlfes EM, Louie DS & Zeisel SH** 1993 Lipid-synthesis and secretion by primary cultures of rat mammary epithelial-cells. *Journal of Cellular Physiology* **157**(3) 469-480
- Rosen JM, Wyszomierski SL & Hadsell D** 1999 Regulation of milk protein gene expression. *Annual Review of Nutrition* **19** 407-436
- Rowzee AM, Ludwig DL & Wood TL** 2009 Insulin-Like Growth Factor Type 1 Receptor and Insulin Receptor Isoform Expression and Signaling in Mammary Epithelial Cells. *Endocrinology* **150**(8) 3611-3619
- Ruan WF & Kleinberg DL** 1999 Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. *Endocrinology* **140**(11) 5075-5081
- Sakamoto K, Komatsu T, Kobayashi T, Rose MT, Aso H, Hagino A & Obara Y** 2005 Growth hormone acts on the synthesis and secretion of alpha-casein in bovine mammary epithelial cells. *Journal of Dairy Research* **72**(3) 264-270
- Sakamoto K, Yano T, Kobayashi T, Hagino A, Aso H & Obara Y** 2007 Growth hormone suppresses the expression of IGFBP-5, and promotes the IGF-I-induced phosphorylation of Akt in bovine mammary epithelial cells. *Domestic Animal Endocrinology* **32**(4) 260-272
- Schmidt GH** 1971 *Biology of Lactation*. Books on Demand

- Sejrsen K, Foldager J, Sorensen MT, Akers RM & Bauman DE** 1986 Effect of exogenous bovine somatotropin on pubertal mammary development in heifers. *Journal of Dairy Science* **69**(6) 1528-1535
- Sejrsen K, Hvelplund T & Nielsen MO** 2006 Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Xth International Symposium on Ruminant Physiology, Copenhagen, Denmark, September 2004. Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Xth International Symposium on Ruminant Physiology, Copenhagen, Denmark, September 2004. 600 pp.
- Shafiei F, Herington AC & Lobie PE** 2006 Mechanisms of signal transduction utilized by growth hormone. (Elsevier)
- Sheehy PA, Della-Vedova JJ, Nicholas KR & Wynn PC** 2004 Hormone-dependent milk protein gene expression in bovine mammary explants from biopsies at different stages of pregnancy. *Journal of Dairy Research* **71**(2) 135-140
- Sheffield LG** 1988 Organization and growth of mammary epithelia in the mammary-gland fat pad. *Journal of Dairy Science* **71**(10) 2855-2874
- Shennan DB & Peaker M** 2000 Transport of milk constituents by the mammary gland. *Physiological Reviews* **80**(3) 925-951
- Soule HD, Vazquez J, Long A, Albert S & Brennan M** 1973 A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute* **51**(5) 1409-1416
- Stewart CEH & Rotwein P** 1996 Growth, differentiation, and survival: Multiple physiological functions for insulin-like growth factors. *Physiological Reviews* **76**(4) 1005-1026
- Stoker M & Perryman M** 1984 Cultures of exfoliated mammary epithelial cells: Variation between donors. *Breast Cancer Research and Treatment* **4**(1) 11-18
- Suard Y, Haeuptle M, Farinon E & Kraehenbuhl J** 1983 Cell proliferation and milk protein gene expression in rabbit mammary cell cultures. *The Journal of Cell Biology* **96**(5) 1435-1442
- Svennersten-Sjaunja K & Olsson K** 2005 Endocrinology of milk production. *Domestic Animal Endocrinology* **29**(2) 241-258
- Takeishi M, Nakamura S, Tsumagari S, Shibata M, Okuda M, Ishii T, Tsunekane T & Yosai A** 1980 Studies on the development of mammary gland in bovine fetuses. *Japanese Journal of Animal Reproduction* **26**(3) 134-137
- Taketani Y & Oka T** 1981 Hormonal induction of milk protein-synthesis in primary mammary epithelial-cell culture. *Journal of Cell Biology* **91**(2) A217-A217
- Tateyama S, Kawano A, Yamaguchi R, Nosaka D & Kondo F** 1988 Culture conditions and cell morphology of goat milk-derived mammary epithelial cells in plate culture. *Nippon Juigaku Zasshi* **50**(6) 1192-1199

- Taylor-Papadimitriou J, Shearer M & Stoker MGP** 1977 Growth requirements of human mammary epithelial cells in culture. In Book Growth requirements of human mammary epithelial cells in culture, Vol. 20, pp. 903-908 (Ed.^Eds. Editor).Series Growth requirements of human mammary epithelial cells in culture. Wiley Subscription Services, Inc., A Wiley Company
- Topper YJ & Freeman CS** 1980 Multiple hormone interactions in the developmental biology of the mammary gland. *Physiological Reviews* **60**(4) 1049-1106
- Truchet S & Ollivier-Bousquet M** 2009 Mammary gland secretion: hormonal coordination of endocytosis and exocytosis. *animal* **3**(12) 1733-1742
- Tuggle CK & Trenkle A** 1996 Control of growth hormone synthesis. *Domestic Animal Endocrinology* **13**(1) 1-33
- West JW, Bondari K & Johnson JC** 1990 Effects of bovine somatotropin on milk-yield and composition, body-weight, and condition score of Holstein and Jersey cows. *Journal of Dairy Science* **73**(4) 1062-1068
- Wheeler TT, Callaghan MR, Davis SR, Prosser CG & Wilkins RJ** 1995 Milk Protein Synthesis, Gene Expression, and Hormonal Responsiveness in Primary Cultures of Mammary Cells from Lactating Sheep. *Experimental Cell Research* **217**(2) 346-354
- Wilde CJ, Peaker M & Knight CH** 1995 Intercellular signalling in the mammary gland. Plenum Press
- Yamada PM & Lee K-W** 2009 Perspectives in mammalian IGFBP-3 biology: local vs. systemic action. *American Journal of Physiology - Cell Physiology* **296**(5) C954-C976
- Zhao FQ, Okine EK & Kennelly JJ** 1999 Glucose transporter gene expression in bovine mammary gland. *Journal of Animal Science* **77**(9) 2517-2522
- Zhou Y, Akers RM & Jiang H** 2008 Growth hormone can induce expression of four major milk protein genes in transfected MAC-T cells. *Journal of Dairy Science* **91**(1) 100-108

Appendix

QUANTITATIVE PCR DATA

Raw data from quantitative PCR is presented in the following appendix. Briefly, raw C_T values were normalized to the indicated house-keeping gene (HKG). The raw HKG C_T value was subtracted from the gene target's raw C_T value as an absolute value, yielding the $\Delta\Delta C_T$. The $2^{-\Delta\Delta C_T}$ method was used to calculate abundance. The $2^{-\Delta\Delta C_T}$ value was averaged within the treatment group and a standard error of the mean (SEM) was calculated.

Mammary explants from a Jersey cow (Tables 1-5): raw qPCR values corresponding to results presented in Figure 4.

	Sample ID	C_T	HKG ID	C_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	ALAC 1	23.3877	TBP 1	30.7681	7.38040	0.006002	0.001955	0.000936
	ALAC 2	19.7817	TBP 2	31.6413	11.85960	0.000269		
	ALAC 3	20.0330	TBP 3	30.0357	10.00270	0.000975		
	ALAC 6	18.9668	TBP 6	30.3789	11.41210	0.000367		
	ALAC 7	22.0721	TBP 7	30.2745	8.20240	0.003395		
	ALAC 8	20.3920	TBP 8	30.8261	10.43410	0.000723		
DIP+GH	ALAC 10	22.2297	TBP 10	31.0763	8.84660	0.002172	0.067483	0.027305
	ALAC 13	26.8911	TBP 13	29.9393	3.04820	0.120893		
	ALAC 15	27.3917	TBP 15	31.2600	3.86830	0.068474		
	ALAC 16	23.4633	TBP 16	30.0017	6.53840	0.010758		
	ALAC 18	26.9977	TBP 18	29.8854	2.88770	0.135119		

Appendix Table 1: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in mammary explants from a Jersey cow cultured for 12 h.

	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	CAS 1	18.4718	TBP 1	30.7681	12.29630	0.000199	0.001879	0.001761
	CAS 2	16.9761	TBP 2	31.6413	14.66520	0.000038		
	CAS 5	22.8326	TBP 5	30.3654	7.53280	0.005400		
DIP+GH	CAS 11	18.6314	TBP 11	29.9068	11.27540	0.000403	0.000938	0.000575
	CAS 12	16.4670	TBP 12	30.3710	13.90400	0.000065		
	CAS 13	20.0431	TBP 13	29.9393	9.89620	0.001049		
	CAS 14	15.8211	TBP 14	30.0381	14.21700	0.000053		
	CAS 15	22.9361	TBP 15	31.2600	8.32390	0.003121		

Appendix Table 2: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in mammary explants from a Jersey cow cultured for 12 h.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	GHR 1	26.6222	TBP 1	30.7681	4.14590	0.056488	0.134622	0.068005
	GHR 2	26.8406	TBP 2	31.6413	4.80070	0.035879		
	GHR 3	27.613	TBP 3	30.0357	2.42270	0.186507		
	GHR 4	25.96	TBP 4	30.7241	4.76410	0.036801		
	GHR 5	29.6131	TBP 5	30.3654	0.75230	0.593656		
	GHR 6	26.3811	TBP 6	30.3789	3.99780	0.062595		
	GHR 7	26.6273	TBP 7	30.2745	3.64720	0.079815		
	GHR 8	25.5178	TBP 8	30.8261	5.30830	0.025237		
DIP+GH	GHR 10	28.9397	TBP 10	31.0763	2.13660	0.227415	0.143545	0.046488
	GHR 11	26.0177	TBP 11	29.9068	3.88910	0.067494		
	GHR 12	25.128	TBP 12	30.371	5.24300	0.026406		
	GHR 13	26.3044	TBP 13	29.9393	3.63490	0.080498		
	GHR 14	25.9939	TBP 14	30.0381	4.04420	0.060614		
	GHR 15	30.1589	TBP 15	31.26	1.10110	0.466161		
	GHR 16	26.9681	TBP 16	30.0017	3.03360	0.122122		
	GHR 17	28.3196	TBP 17	31.1174	2.79780	0.143806		
	GHR 18	26.5253	TBP 18	29.8854	3.36010	0.097389		

Appendix Table 3: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in mammary explants from a Jersey cow cultured for 12 h.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	IGF-1 1	28.1265	TBP 1	30.7681	2.64160	0.160250	0.143852	0.016590
	IGF-1 2	28.7227	TBP 2	31.6413	2.91860	0.132256		
	IGF-1 6	28.0533	TBP 6	30.3789	2.32560	0.199492		
	IGF-1 7	27.2523	TBP 7	30.2745	3.02220	0.123091		
	IGF-1 8	27.5631	TBP 8	30.8261	3.26300	0.104169		
DIP+GH	IGF-1 11	26.1650	TBP 11	29.9068	3.74180	0.074749	0.155514	0.033081
	IGF-1 12	26.5807	TBP 12	30.3710	3.79030	0.072278		
	IGF-1 13	26.8985	TBP 13	29.9393	3.04080	0.121514		
	IGF-1 15	29.4842	TBP 15	31.2600	1.77580	0.292032		
	IGF-1 16	27.7479	TBP 16	30.0017	2.25380	0.209671		
	IGF-1 17	28.9910	TBP 17	31.1174	2.12640	0.229029		
	IGF-1 18	26.4006	TBP 18	29.8854	3.48480	0.089325		

Appendix Table 4: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in mammary explants from a Jersey cow cultured for 12 h.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	IGFBP3 1	24.028	TBP 1	30.7681	6.74010	0.009355	0.009059	0.001630
	IGFBP3 2	24.1119	TBP 2	31.6413	7.52940	0.005413		
	IGFBP3 3	23.7328	TBP 3	30.0357	6.30290	0.012666		
	IGFBP3 4	23.158	TBP 4	30.7241	7.56610	0.005277		
	IGFBP3 6	24.0665	TBP 6	30.3789	6.31240	0.012583		
DIP+GH	IGFBP3 12	23.0041	TBP 12	30.371	7.36690	0.006058	0.017787	0.004616
	IGFBP3 13	24.0115	TBP 13	29.9393	5.92780	0.016427		
	IGFBP3 14	23.288	TBP 14	30.0381	6.75010	0.009290		
	IGFBP3 16	25.2369	TBP 16	30.0017	4.76480	0.036783		
	IGFBP3 17	25.7785	TBP 17	31.1174	5.33890	0.024708		
	IGFBP3 18	23.67	TBP 18	29.8854	6.21540	0.013458		

Appendix Table 5: Q-PCR raw C_T values and calculations for IGFBP3 mRNA abundance in mammary explants from a Jersey cow cultured for 12 h.

Primary MEC from a Jersey cow (Tables 6-10): raw qPCR values corresponding to results presented in Figure 5.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	ALAC 1	33.8750	18S 1	12.61380	21.26120	3.978699E-07	1.091965E-06	4.989461E-07
	ALAC 2	32.4164	18S 2	13.34990	19.06650	1.821426E-06		
	ALAC 3	33.2099	18S 3	14.32620	18.88370	2.067473E-06		
	ALAC 4	36.0860	18S 4	12.53010	23.55590	8.109004E-08		
DIP+GH	ALAC 5	33.6129	18S 5	12.05600	21.55690	3.241354E-07	8.227119E-07	2.606856E-07
	ALAC 6	33.2850	18S 6	12.95840	20.32660	7.604728E-07		
	ALAC 7	33.1465	18S 7	13.85050	19.29600	1.553550E-06		
	ALAC 8	33.2366	18S 8	12.68950	20.54710	6.526894E-07		

Appendix Table 6: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in primary MEC from a Jersey cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	CAS 1	30.8435	18S 1	12.6138	18.2297	3.253220E-06	7.224671E-06	2.912437E-06
	CAS 2	30.9295	18S 2	13.3499	17.5796	5.105204E-06		
	CAS 3	30.2685	18S 3	14.3262	15.9423	1.588143E-05		
	CAS 4	30.2417	18S 4	12.5301	17.7116	4.658833E-06		
DIP+GH	CAS 5	29.2374	18S 5	12.056	17.1814	6.727957E-06	1.400971E-05	4.422066E-06
	CAS 6	29.6532	18S 6	12.9584	16.6948	9.426803E-06		
	CAS 7	29.0455	18S 7	13.8505	15.195	2.665933E-05		
	CAS 8	28.8959	18S 8	12.6895	16.2064	1.322475E-05		

Appendix Table 7: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in primary MEC from a Jersey cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	GHR 1	26.4361	18S 1	12.6138	13.8223	6.903560E-05	8.035560E-05	5.855804E-06
	GHR 2	27.0933	18S 2	13.3499	13.7434	7.291626E-05		
	GHR 3	27.8546	18S 3	14.3262	13.5284	8.463418E-05		
	GHR 4	25.8943	18S 4	12.5301	13.3642	9.483637E-05		
DIP+GH	GHR 5	25.2033	18S 5	12.056	13.1473	1.102220E-04	1.852639E-04	4.524816E-05
	GHR 6	25.4063	18S 6	12.9584	12.4479	1.789817E-04		
	GHR 8	24.5626	18S 8	12.6895	11.8731	2.665881E-04		

Appendix Table 8: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in primary MEC from a Jersey cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	IGF-1 1	31.885	18S 1	12.6138	19.2712	1.580486E-06	7.413002E-06	3.202423E-06
	IGF-1 2	29.8172	18S 2	13.3499	16.4673	1.103694E-05		
	IGF-1 3	30.392	18S 3	14.3262	16.0658	1.457848E-05		
	IGF-1 4	31.1653	18S 4	12.5301	18.6352	2.456099E-06		
DIP+GH	IGF-1 5	30.7174	18S 5	12.056	18.6614	2.411897E-06	6.658784E-06	2.202387E-06
	IGF-1 6	31.1657	18S 6	12.9584	18.2073	3.304126E-06		
	IGF-1 7	30.4107	18S 7	13.8505	16.5602	1.034863E-05		
	IGF-1 8	29.2191	18S 8	12.6895	16.5296	1.057048E-05		

Appendix Table 9: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in primary MEC from a Jersey cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	IGFBP3 1	19.6852	18S 1	12.6138	7.0714	0.007435	0.003667	0.001535
	IGFBP3 2	24.8863	18S 2	13.3499	11.5364	0.000337		
	IGFBP3 3	22.0761	18S 3	14.3262	7.7499	0.004646		
	IGFBP3 4	21.3261	18S 4	12.5301	8.796	0.002250		
DIP+GH	IGFBP3 5	22.4727	18S 5	12.056	10.4167	0.000732	0.001288	0.000681
	IGFBP3 6	21.1982	18S 6	12.9584	8.2398	0.003308		
	IGFBP3 7	24.1743	18S 7	13.8505	10.3238	0.000780		
	IGFBP3 8	24.2517	18S 8	12.6895	11.5622	0.000331		

Appendix Table 10: Q-PCR raw C_T values and calculations for IGFBP-3 mRNA abundance in primary MEC from a Jersey cow cultured for 7 d.

Primary MEC from a Holstein cow (Tables 11-15): raw qPCR values corresponding to results presented in Figure 6.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	ALAC 1	35.0438	18S 1	14.0686	20.9752	4.851049E-07	4.346979E-07	4.434087E-08
	ALAC 2	35.2205	18S 2	13.7097	21.5108	3.346601E-07		
	ALAC 3	34.9493	18S 3	14.1006	20.8487	5.295609E-07		
	ALAC 4	35.0254	18S 4	13.7334	21.292	3.894658E-07		
DIP+GH	ALAC 5	35.4095	18S 5	13.8183	21.5912	3.165200E-07	4.349154E-07	6.046438E-08
	ALAC 6	34.4291	18S 6	13.5414	20.8877	5.154372E-07		
	ALAC 7	34.3402	18S 7	13.3279	21.0123	4.727891E-07		

Appendix Table 11: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in primary MEC from a Holstein cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	CAS 1	37.6757	18S 1	14.0686	23.6071	7.826269E-08	3.191571E-08	1.660944E-08
	CAS 2	42.9531	18S 2	13.7097	29.2434	1.573473E-09		
	CAS 3	39.9647	18S 3	14.1006	25.8641	1.637307E-08		
	CAS 4	38.6556	18S 4	13.7334	24.9222	3.145359E-08		
DIP+GH	CAS 5	41.7294	18S 5	13.8183	27.9111	3.962066E-09	9.471850E-08	8.463085E-08
	CAS 6	38.8596	18S 6	13.5414	25.3182	2.390355E-08		
	CAS 7	41.7331	18S 7	13.3279	28.4052	2.813084E-09		
	CAS 8	36.3013	18S 8	14.8477	21.4536	3.481953E-07		

Appendix Table 12: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in primary MEC from a Holstein cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	GHR 1	25.2728	18S 1	14.0686	11.2042	0.000423838	0.00030858	0.00004790
	GHR 2	25.6855	18S 2	13.7097	11.9758	0.00024827		
	GHR 3	25.5869	18S 3	14.1006	11.4863	0.000348561		
	GHR 4	25.9259	18S 4	13.7334	12.1925	0.000213645		
DIP+GH	GHR 5	25.7405	18S 5	13.8183	11.9222	0.000257668	0.00035632	0.00011547
	GHR 6	25.5746	18S 6	13.5414	12.0332	0.000238586		
	GHR 7	25.434	18S 7	13.3279	12.1061	0.00022683		
	GHR 8	25.3235	18S 8	14.8477	10.4758	0.000702215		

Appendix Table 13: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in primary MEC from a Holstein cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	IGF-1 1	28.6594	18S 1	14.0686	14.5908	4.052579E-05	5.733957E-05	8.389575E-06
	IGF-1 2	27.4961	18S 2	13.7097	13.7864	7.077503E-05		
	IGF-1 3	27.8476	18S 3	14.1006	13.747	7.273453E-05		
	IGF-1 4	28.1628	18S 4	13.7334	14.4294	4.532291E-05		
DIP+GH	IGF-1 5	28.5876	18S 5	13.8183	14.7693	3.580945E-05	1.042875E-04	6.051191E-05
	IGF-1 6	28.145	18S 6	13.5414	14.6036	4.016783E-05		
	IGF-1 7	27.4571	18S 7	13.3279	14.1292	5.580678E-05		
	IGF-1 8	26.6226	18S 8	14.8477	11.7749	2.853658E-04		

Appendix Table 14: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in primary MEC from a Holstein cow cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
DIP	IGFBP3 1	18.9506	18S 1	14.0686	4.882	0.033913	0.014598	0.006467
	IGFBP3 2	20.942	18S 2	13.7097	7.2323	0.006651		
	IGFBP3 3	21.0318	18S 3	14.1006	6.9312	0.008194		
	IGFBP3 4	20.4311	18S 4	13.7334	6.6977	0.009634		
DIP+GH	IGFBP3 5	20.4531	18S 5	13.8183	6.6348	0.010063	0.010478	0.001857
	IGFBP3 6	20.3526	18S 6	13.5414	6.8112	0.008905		
	IGFBP3 7	20.4487	18S 7	13.3279	7.1208	0.007185		
	IGFBP3 8	20.8353	18S 8	14.8477	5.9876	0.015760		

Appendix Table 15: Q-PCR raw C_T values and calculations for IGFBP-3 mRNA abundance in primary MEC from a Holstein cow cultured for 7 d.

MEC from Holstein milk (Tables 16-20): raw qPCR values corresponding to results presented in Figure 7.

TX	Sample ID	C_T	HKG ID	C_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	ALAC 2	39.0751	TBP 2	22.46722422	16.60787578	1.00122E-05	1.58326E-05	3.22302E-06
	ALAC 3	37.2106	TBP 3	21.57255561	15.63804439	1.96101E-05		
	ALAC 4	38.0784	TBP 4	21.57362486	16.50477514	1.07539E-05		
	ALAC 5	37.166	TBP 5	21.75510852	15.41089148	2.29541E-05		
DIP	ALAC 6	36.6503	TBP 6	22.13608466	14.51421534	4.27352E-05	2.07183E-05	7.90292E-06
	ALAC 7	37.2433	TBP 7	22.45249931	14.79080069	3.52797E-05		
	ALAC 8	37.8777	TBP 8	21.6431689	16.2345311	1.29694E-05		
	ALAC 9	44.9402	TBP 9	21.81917515	23.12102485	1.09617E-07		
	ALAC 10	37.8668	TBP 10	21.57881884	16.28798116	1.24977E-05		
DIP+GH	ALAC 11	35.3894	TBP 11	22.06155041	13.32784959	9.72562E-05	5.65669E-05	1.56171E-05
	ALAC 12	36.2938	TBP 12	22.17592414	14.11787586	5.62466E-05		
	ALAC 13	36.3021	TBP 13	22.45877618	13.84332382	6.80369E-05		
	ALAC 14	41.8127	TBP 14	22.0093804	19.8033196	1.09297E-06		
	ALAC 15	36.1221	TBP 15	22.10227343	14.01982657	6.02021E-05		

Appendix Table 16: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in MEC from Holstein milk cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	CAS 1	39.1774	TBP 1	22.47289944	16.70450056	9.36363E-06	1.15504E-05	2.14207E-06
	CAS 2	39.5444	TBP 2	22.46722422	17.07717578	7.23199E-06		
	CAS 3	37.4151	TBP 3	21.57255561	15.84254439	1.70184E-05		
	CAS 4	38.5574	TBP 4	21.57362486	16.98377514	7.71568E-06		
	CAS 5	37.6491	TBP 5	21.75510852	15.89399148	1.64222E-05		
DIP	CAS 6	34.2626	TBP 6	22.13608466	12.12651534	0.000223643	0.00026227	2.54201E-05
	CAS 7	34.1528	TBP 7	22.45249931	11.70030069	0.00030051		
	CAS 8	33.9849	TBP 8	21.6431689	12.3417311	0.00019265		
	CAS 9	33.3678	TBP 9	21.81917515	11.54862485	0.000333824		
	CAS 10	33.484	TBP 10	21.57881884	11.90518116	0.000260725		
DIP+GH	CAS 11	35.5343	TBP 11	22.06155041	13.47274959	8.79626E-05	0.000585178	0.000189884
	CAS 12	34.6928	TBP 12	22.17592414	12.51687586	0.000170626		
	CAS 13	32.4097	TBP 13	22.45877618	9.950923817	0.001010354		
	CAS 14	32.182	TBP 14	22.0093804	10.1726196	0.000866436		
	CAS 15	32.4072	TBP 15	22.10227343	10.30492657	0.000790511		

Appendix Table 17: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in MEC from Holstein milk cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	GHR 1	34.1009	TBP 1	22.47289944	11.62800056	0.000315953	0.000398088	4.70582E-05
	GHR 2	34.0955	TBP 2	22.46722422	11.62827578	0.000315893		
	GHR 3	33.0429	TBP 3	21.57255561	11.47034439	0.000352438		
	GHR 4	32.7063	TBP 4	21.57362486	11.13267514	0.00044538		
	GHR 5	32.5554	TBP 5	21.75510852	10.80029148	0.000560775		
DIP	GHR 6	31.8345	TBP 6	22.13608466	9.698415344	0.001203611	0.001191864	0.000119915
	GHR 7	31.7484	TBP 7	22.45249931	9.295900687	0.001590945		
	GHR 8	31.413	TBP 8	21.6431689	9.769831095	0.001145481		
	GHR 9	32.0417	TBP 9	21.81917515	10.22252485	0.000836977		
	GHR 10	31.303	TBP 10	21.57881884	9.724181157	0.001182306		
DIP+GH	GHR 11	32.0181	TBP 11	22.06155041	9.956549592	0.001006422	0.001168319	0.00019359
	GHR 12	32.7346	TBP 12	22.17592414	10.55867586	0.000663013		
	GHR 13	31.5495	TBP 13	22.45877618	9.090723817	0.001834085		
	GHR 14	31.6187	TBP 14	22.0093804	9.609319598	0.001280285		
	GHR 15	31.987	TBP 15	22.10227343	9.884726569	0.001057793		

Appendix Table 18: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in MEC from Holstein milk cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	IGF-1 1	33.3659	TBP 1	22.47289944	10.89300056	0.000525872	0.000524181	0.000203493
	IGF-1 2	32.3084	TBP 2	22.46722422	9.841175779	0.001090212		
	IGF-1 3	31.7706	TBP 3	21.57255561	10.19804439	0.0008513		
	IGF-1 4	35.544	TBP 4	21.57362486	13.97037514	6.23014E-05		
	IGF-1 5	35.1754	TBP 5	21.75510852	13.42029148	9.12199E-05		
DIP	IGF-1 6	33.5583	TBP 6	22.13608466	11.42221534	0.000364393	0.000365843	0.000143484
	IGF-1 7	32.6163	TBP 7	22.45249931	10.16380069	0.000871748		
	IGF-1 8	32.8773	TBP 8	21.6431689	11.2341311	0.000415135		
	IGF-1 9	35.1887	TBP 9	21.81917515	13.36952485	9.4487E-05		
	IGF-1 10	35.1275	TBP 10	21.57881884	13.54868116	8.34527E-05		
DIP+GH	IGF-1 11	32.9106	TBP 11	22.06155041	10.84904959	0.000542139	0.001840695	0.001422288
	IGF-1 12	32.9823	TBP 12	22.17592414	10.80637586	0.000558415		
	IGF-1 13	29.5131	TBP 13	22.45877618	7.054323817	0.007523795		
	IGF-1 14	34.3348	TBP 14	22.0093804	12.3254196	0.00019484		
	IGF-1 15	33.4478	TBP 15	22.10227343	11.34552657	0.000384287		

Appendix Table 19: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in MEC from Holstein milk cultured for 7 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	IGFBP3 1	22.1111	TBP 1	22.47289944	0.361799438	0.778193353	0.504184184	0.083298338
	IGFBP3 2	21.69	TBP 2	22.46722422	0.777224221	0.583488359		
	IGFBP3 3	22.918	TBP 3	21.57255561	1.345444386	0.393532752		
	IGFBP3 4	22.6583	TBP 4	21.57362486	1.084675143	0.471498425		
	IGFBP3 5	23.5202	TBP 5	21.75510852	1.765091477	0.294208029		
DIP	IGFBP3 6	21.8865	TBP 6	22.13608466	0.249584656	0.84113854	0.574123684	0.077782049
	IGFBP3 7	21.7177	TBP 7	22.45249931	0.734799313	0.600901608		
	IGFBP3 8	22.4257	TBP 8	21.6431689	0.782531095	0.581345972		
	IGFBP3 9	22.9201	TBP 9	21.81917515	1.100924846	0.466217529		
	IGFBP3 10	22.9709	TBP 10	21.57881884	1.392081157	0.381014773		
DIP+GH	IGFBP3 11	20.9935	TBP 11	22.06155041	1.068050408	0.47696311	0.57993335	0.075945437
	IGFBP3 12	21.4078	TBP 12	22.17592414	0.768124137	0.587180459		
	IGFBP3 13	22.7068	TBP 13	22.45877618	0.248023817	0.842049053		
	IGFBP3 14	22.7416	TBP 14	22.0093804	0.732219598	0.601977055		
	IGFBP3 15	23.4552	TBP 15	22.10227343	1.352926569	0.391497074		

Appendix Table 20: Q-PCR raw C_T values and calculations for IGFBP-3 mRNA abundance in MEC from Holstein milk cultured for 7 d.

Primary MEC from a Holstein cow (Table 21-25): raw qPCR values corresponding to results presented in Figure 8.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	ALAC 1	37.8121	POL1 1	22.1496	15.6625	1.92805E-05	0.000102162	2.89675E-05
		ALAC 2	35.0814	POL1 2	21.5752	13.5062	8.59466E-05		
		ALAC 3	35.3206	POL1 3	22.111	13.2096	0.000105564		
		ALAC 4	35.3557	POL1 4	22.0638	13.2919	9.97102E-05		
		ALAC 5	34.3295	POL1 5	22.044	12.2855	0.000200307		
	DIP	ALAC 6	35.0221	POL1 6	22.7134	12.3087	0.000197112	0.000401239	0.00014815
		ALAC 7	35.0082	POL1 7	21.4217	13.5865	8.12935E-05		
		ALAC 8	35.7172	POL1 8	23.8427	11.8745	0.00026633		
		ALAC 9	32.1319	POL1 9	22.0195	10.1124	0.000903367		
		ALAC 10	32.9837	POL1 10	22.1765	10.8072	0.000558096		
4 DAYS	DIP+GH	ALAC 11	33.9591	POL1 11	24.0026	9.9565	0.001006456	0.000282145	0.00018305
		ALAC 12	35.1085	POL1 12	22.4189	12.6896	0.000151373		
		ALAC 13	37.0585	POL1 13	21.7222	15.3363	2.41721E-05		
		ALAC 14	35.8989	POL1 14	21.9122	13.9867	6.16004E-05		
		ALAC 15	35.1477	POL1 15	22.6009	12.5468	0.000167123		
	DIP	ALAC 16	34.6513	POL1 16	22.3455	12.3058	0.000197508	0.00022833	3.22772E-05
		ALAC 17	34.0734	POL1 17	22.1725	11.9009	0.0002615		
		ALAC 18	34.4364	POL1 18	21.9373	12.4991	0.000172741		
		ALAC 19	34.1859	POL1 19	22.6033	11.5826	0.000326054		
		ALAC 20	35.2209	POL1 20	22.8117	12.4092	0.000183848		
	DIP+GH	ALAC 21	35.1489	POL1 21	22.7896	12.3593	0.000190318	0.000280086	0.000146367
		ALAC 22	34.0872	POL1 22	21.7237	12.3635	0.000189765		
		ALAC 23	35.7661	POL1 23	21.9127	13.8534	6.75633E-05		
		ALAC 24	35.3991	POL1 24	22.0464	13.3527	9.55954E-05		
		ALAC 25	34.3812	POL1 25	24.1931	10.1881	0.000857188		

Appendix Table 21: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in primary MEC from a Holstein cow cultured for 2 d and 4 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	CAS 1	38.554	POL1 1	22.1496	16.4044	1.15288E-05	6.67216E-06	1.94398E-06
	CAS 2	40.8805	POL1 2	21.5752	19.3053	1.54357E-06		
	CAS 3	39.1396	POL1 3	22.111	17.0286	7.47964E-06		
	CAS 4	38.6832	POL1 4	22.0638	16.6194	9.93258E-06		
	CAS 5	40.4514	POL1 5	22.044	18.4074	2.87621E-06		
2 DAYS	DIP	CAS 6	POL1 6	22.7134	20.6155	6.22467E-07	7.86088E-06	4.69617E-06
		CAS 7	POL1 7	21.4217	18.5815	2.54924E-06		
		CAS 8	POL1 8	23.8427	17.1491	6.88029E-06		
		CAS 9	POL1 9	22.0195	15.5126	2.13915E-05		
	DIP+GH	CAS 11	POL1 11	24.0026	23.2591	9.96124E-08	6.60311E-06	4.06207E-06
		CAS 12	POL1 12	22.4189	15.5381	2.10168E-05		
		CAS 13	POL1 13	21.7222	19.954	9.84572E-07		
		CAS 14	POL1 14	21.9122	16.5753	1.02409E-05		
		CAS 15	POL1 15	22.6009	20.5014	6.73696E-07		
4 DAYS	DIP	CAS 16	POL1 16	22.3455	14.6241	3.96011E-05	1.17798E-05	8.14008E-06
		CAS 17	POL1 17	22.1725	23.7335	7.16976E-08		
		CAS 18	POL1 18	21.9373	20.2897	7.80174E-07		
		CAS 19	POL1 19	22.6033	17.2347	6.48393E-06		
		CAS 20	POL1 20	22.8117	16.3512	1.19618E-05		
	DIP+GH	CAS 21	POL1 21	22.7896	22.0053	2.37544E-07	1.15479E-05	7.81649E-06
		CAS 22	POL1 22	21.7237	14.8764	3.32474E-05		
		CAS 24	POL1 24	22.0464	26.2494	1.25355E-08		
		CAS 25	POL1 25	24.1931	16.2655	1.26939E-05		

Appendix Table 22: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in primary MEC from a Holstein cow cultured for 2 d and 4 d.

	INS/PROG	GHR 1	25.4279	POL1 1	22.1496	3.2783	0.103070258	0.203376084	0.044766903
		GHR 2	24.8639	POL1 2	21.5752	3.2887	0.102329924		
		GHR 3	23.7643	POL1 3	22.111	1.6533	0.317912137		
		GHR 4	23.8747	POL1 4	22.0638	1.8109	0.285013073		
		GHR 5	24.3055	POL1 5	22.044	2.2615	0.208555028		
2 DAYS	DIP	GHR 6	23.3232	POL1 6	22.7134	0.6098	0.655287538	0.320326449	0.14449317
		GHR 7	24.5285	POL1 7	21.4217	3.1068	0.116080698		
		GHR 8	23.3098	POL1 8	23.8427	0.5329	0.691164009		
		GHR 9	25.6134	POL1 9	22.0195	3.5939	0.082818679		
		GHR 10	26.3277	POL1 10	22.1765	4.1512	0.056281321		
	DIP+GH	GHR 11	23.9434	POL1 11	24.0026	0.0592	0.959796196	0.370030738	0.153459091
		GHR 12	24.5908	POL1 12	22.4189	2.1719	0.221918216		
		GHR 13	24.4729	POL1 13	21.7222	2.7507	0.148578781		
		GHR 14	24.7311	POL1 14	21.9122	2.8189	0.141718499		
		GHR 15	24.0039	POL1 15	22.6009	1.403	0.378141999		
4 DAYS	DIP	GHR 16	24.1323	POL1 16	22.3455	1.7868	0.289814162	0.366387754	0.024716355
		GHR 17	23.5285	POL1 17	22.1725	1.356	0.39066394		
		GHR 18	23.268	POL1 18	21.9373	1.3307	0.39757529		
		GHR 19	24.1414	POL1 19	22.6033	1.5381	0.344338643		
		GHR 20	24.0996	POL1 20	22.8117	1.2879	0.409546736		
	DIP+GH	GHR 21	24.2947	POL1 21	22.7896	1.5051	0.352305768	0.394980124	0.135991159
		GHR 22	24.0402	POL1 22	21.7237	2.3165	0.200753911		
		GHR 23	23.9745	POL1 23	21.9127	2.0618	0.239517006		
		GHR 24	24.0312	POL1 24	22.0464	1.9848	0.252647884		
		GHR 25	24.0879	POL1 25	24.1931	0.1052	0.929676053		

Appendix Table 23: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in primary MEC from a Holstein cow cultured for 2 d and 4 d.

	TX	Sample ID	C_T	HKG ID	C_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
	INS/PROG	IGF1 1	30.859	POL1 1	22.1496	8.7094	0.002388963	0.002448109	0.0003281
		IGF1 2	30.916	POL1 2	21.5752	9.3408	0.001542194		
		IGF1 3	30.5103	POL1 3	22.111	8.3993	0.002961821		
		IGF1 4	30.4938	POL1 4	22.0638	8.43	0.00289946		
2 DAYS	DIP	IGF1 6	31.3717	POL1 6	22.7134	8.6583	0.002475096	0.003799523	0.0012174
		IGF1 7	29.9586	POL1 7	21.4217	8.5369	0.002692384		
		IGF1 8	31.169	POL1 8	23.8427	7.3263	0.006231089		
		IGF1 9	30.0339	POL1 9	22.0195	8.0144	0.003867454		
	DIP+GH	IGF1 11	31.3547	POL1 11	24.0026	7.3521	0.006120648	0.002496836	0.000946923
		IGF1 12	31.9041	POL1 12	22.4189	9.4852	0.001395309		
		IGF1 13	31.3803	POL1 13	21.7222	9.6581	0.001237719		
		IGF1 14	31.7548	POL1 14	21.9122	9.8426	0.001089136		
		IGF1 15	31.1654	POL1 15	22.6009	8.5645	0.002641366		
4 DAYS	DIP	IGF1 16	31.3141	POL1 16	22.3455	8.9686	0.0019961	0.003552774	0.00076245
		IGF1 17	30.7546	POL1 17	22.1725	8.5821	0.002609339		
		IGF1 18	30.3076	POL1 18	21.9373	8.3703	0.003021959		
		IGF1 19	30.0295	POL1 19	22.6033	7.4262	0.005814214		
		IGF1 20	30.6657	POL1 20	22.8117	7.854	0.004322255		
	DIP+GH	IGF1 21	30.6987	POL1 21	22.7896	7.9091	0.004160291	0.003097148	0.000808104
		IGF1 22	29.9946	POL1 22	21.7237	8.2709	0.003237509		
		IGF1 23	31.4874	POL1 23	21.9127	9.5747	0.001311379		
		IGF1 24	31.6112	POL1 24	22.0464	9.5648	0.001320408		
		IGF1 25	31.711	POL1 25	24.1931	7.5179	0.005456154		

Appendix Table 24: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in primary MEC from a Holstein cow cultured for 2 d and 4d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	IGFBP3 1	25.2027	POL1 1	22.1496	3.0531	0.120482874	0.086597734	0.01028331
	IGFBP3 2	25.1948	POL1 2	21.5752	3.6196	0.08135642		
	IGFBP3 3	26.1638	POL1 3	22.111	4.0528	0.060253965		
	IGFBP3 4	25.8117	POL1 4	22.0638	3.7479	0.074433712		
	IGFBP3 5	25.4179	POL1 5	22.044	3.3739	0.096461697		
2 DAYS	DIP	IGFBP3 6	POL1 6	22.7134	3.1301	0.114221014	0.363945544	0.154895353
		IGFBP3 7	POL1 7	21.4217	1.7616	0.294920906		
		IGFBP3 8	POL1 8	23.8427	0.049	0.966606097		
		IGFBP3 9	POL1 9	22.0195	-1.7864	0.289894527		
		IGFBP3 10	POL1 10	22.1765	-2.6982	0.154085178		
	DIP+GH	IGFBP3 11	POL1 11	24.0026	2.7918	0.144405741	0.134156422	0.032723296
		IGFBP3 12	POL1 12	22.4189	2.0381	0.24348419		
		IGFBP3 13	POL1 13	21.7222	4.0099	0.062072583		
		IGFBP3 14	POL1 14	21.9122	3.8068	0.071456051		
		IGFBP3 15	POL1 15	22.6009	2.7431	0.149363547		
4 DAYS	DIP	IGFBP3 16	POL1 16	22.3455	0.4928	0.710644531	0.468753614	0.125223136
		IGFBP3 17	POL1 17	22.1725	2.4661	0.180979727		
		IGFBP3 18	POL1 18	21.9373	1.9872	0.25222794		
		IGFBP3 19	POL1 19	22.6033	-0.4763	0.718818777		
		IGFBP3 20	POL1 20	22.8117	1.0556	0.481097097		
	DIP+GH	IGFBP3 21	POL1 21	22.7896	1.6818	0.311693505	0.209613902	0.030064775
		IGFBP3 22	POL1 22	21.7237	2.5369	0.172312588		
		IGFBP3 23	POL1 23	21.9127	2.2238	0.214076746		
		IGFBP3 24	POL1 24	22.0464	2.931	0.131123666		
		IGFBP3 25	POL1 25	24.1931	2.1919	0.218863002		

Appendix Table 25: Q-PCR raw C_T values and calculations for IGFBP-3 mRNA abundance in primary MEC from a Holstein cow cultured for 2 d and 4 d.

Primary MEC from a Jersey cow (Table 26-30): raw qPCR values corresponding to results presented in Figure 9.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	ALAC 1	POL1 1	21.6008	12.9848	0.000123363	0.000211492	5.24183E-05
		ALAC 2	POL1 2	21.5417	11.3734	0.000376934		
		ALAC 3	POL1 3	22.12	11.7653	0.000287271		
		ALAC 4	POL1 4	21.0434	12.5285	0.000169257		
		ALAC 5	POL1 5	21.5339	13.2786	0.000100634		
	DIP	ALAC 6	POL1 6	21.7818	11.5825	0.000326077	0.000202165	5.18116E-05
		ALAC 7	POL1 7	21.8675	12.0695	0.000232658		
		ALAC 8	POL1 8	22.0168	13.6492	7.78362E-05		
		ALAC 9	POL1 9	21.393	11.7477	0.000290797		
		ALAC 10	POL1 10	21.2424	13.5486	8.34574E-05		
4 DAYS	DIP+GH	ALAC 11	POL1 11	21.1259	12.8693	0.000133646	0.00025843	5.15797E-05
		ALAC 12	POL1 12	21.7264	11.8228	0.000276047		
		ALAC 13	POL1 13	21.7135	11.4406	0.000359779		
		ALAC 14	POL1 14	22.3513	11.3708	0.000377614		
		ALAC 15	POL1 15	21.9469	12.751	0.000145066		
	DIP	ALAC 16	POL1 16	22.3855	11.5307	0.000337997	0.000227131	7.54479E-05
		ALAC 17	POL1 17	21.9234	11.1986	0.000425486		
		ALAC 18	POL1 18	21.2639	13.6767	7.63665E-05		
		ALAC 19	POL1 19	21.1321	13.2548	0.000102308		
		ALAC 20	POL1 20	21.7294	12.3354	0.000193497		
	DIP+GH	ALAC 21	POL1 21	22.1445	10.7842	0.000567064	0.000235285	8.68794E-05
		ALAC 22	POL1 22	20.822	13.2381	0.000103499		
		ALAC 23	POL1 23	21.1386	13.4932	8.67246E-05		
		ALAC 24	POL1 24	22.0556	12.2752	0.000201742		
		ALAC 25	POL1 25	21.848	12.1674	0.000217394		

Appendix Table 26: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in primary MEC from a Jersey cow cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	CAS 1	31.4597	POL1 1	21.6008	9.8589	0.0010769	0.001057859	0.000312418
		CAS 2	32.0113	POL1 2	21.5417	10.4696	0.000705239		
		CAS 3	32.6377	POL1 3	22.12	10.5177	0.000682114		
		CAS 4	31.8314	POL1 4	21.0434	10.788	0.000565573		
		CAS 5	30.3237	POL1 5	21.5339	8.7898	0.00225947		
	DIP	CAS 6	30.8572	POL1 6	21.7818	9.0754	0.00185367	0.002200633	0.000469574
		CAS 7	30.1443	POL1 7	21.8675	8.2768	0.003224296		
		CAS 8	32.8435	POL1 8	22.0168	10.8267	0.000550603		
		CAS 9	29.8539	POL1 9	21.393	8.4609	0.002838019		
		CAS 10	29.8653	POL1 10	21.2424	8.6229	0.002536579		
4 DAYS	DIP+GH	CAS 11	29.9966	POL1 11	21.1259	8.8707	0.002136256	0.002340952	0.000505111
		CAS 12	31.3127	POL1 12	21.7264	9.5863	0.001300877		
		CAS 13	29.7951	POL1 13	21.7135	8.0816	0.003691441		
		CAS 14	30.5838	POL1 14	22.3513	8.2325	0.003324839		
		CAS 15	31.5892	POL1 15	21.9469	9.6423	0.001251349		
	DIP	CAS 16	31.2776	POL1 16	22.3855	8.8921	0.002104802	0.001349554	0.000414668
		CAS 17	31.8075	POL1 17	21.9234	9.8841	0.001058253		
		CAS 18	32.9138	POL1 18	21.2639	11.6499	0.000311194		
		CAS 20	30.7511	POL1 20	21.7294	9.0217	0.001923967		
	DIP+GH	CAS 21	29.1547	POL1 21	22.1445	7.0102	0.00775746	0.002568681	0.001745061
		CAS 22	31.4587	POL1 22	20.822	10.6367	0.000628108		
		CAS 23	32.4016	POL1 23	21.1386	11.263	0.000406911		
		CAS 24	31.4536	POL1 24	22.0556	9.398	0.001482245		

Appendix Table 27: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in primary MEC from a Jersey cow cultured for 2 d and 4 d.

TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
INS/PROG	GHR 1	25.4218	POL1 1	21.6008	3.821	0.070756181	0.051652521	0.005175248
	GHR 2	26.0969	POL1 2	21.5417	4.5552	0.042535169		
	GHR 3	26.4875	POL1 3	22.12	4.3675	0.048445283		
	GHR 4	25.2684	POL1 4	21.0434	4.225	0.053474689		
	GHR 5	26.0717	POL1 5	21.5339	4.5378	0.043051282		
2 DAYS	DIP	GHR 6	POL1 6	21.7818	3.6228	0.081176165	0.072710057	0.010764663
		GHR 7	POL1 7	21.8675	3.2119	0.107924925		
		GHR 8	POL1 8	22.0168	3.8004	0.071773745		
		GHR 9	POL1 9	21.393	4.1054	0.058096699		
		GHR 10	POL1 10	21.2424	4.4875	0.044578751		
	DIP+GH	GHR 11	POL1 11	21.1259	3.6071	0.082064382	0.19177127	0.04091166
		GHR 12	POL1 12	21.7264	2.9467	0.129704461		
		GHR 13	POL1 13	21.7135	2.428	0.185822873		
		GHR 14	POL1 14	22.3513	1.6883	0.310292343		
		GHR 15	POL1 15	21.9469	1.9944	0.250972292		
4 DAYS	DIP	GHR 16	POL1 16	22.3855	2.7258	0.151165414	0.081900973	0.024898724
		GHR 17	POL1 17	21.9234	3.1279	0.114395325		
		GHR 18	POL1 18	21.2639	3.9926	0.062821404		
		GHR 19	POL1 19	21.1321	4.2523	0.052472306		
		GHR 20	POL1 20	21.7294	5.1253	0.028650418		
	DIP+GH	GHR 21	POL1 21	22.1445	2.1814	0.220461708	0.098989872	0.036827619
		GHR 22	POL1 22	20.822	3.9197	0.066077367		
		GHR 23	POL1 23	21.1386	4.2599	0.052196613		
		GHR 24	POL1 24	22.0556	3.8236	0.070628781		
		GHR 25	POL1 25	21.848	3.5465	0.085584895		

Appendix Table 28: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in primary MEC from a Jersey cow cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	IGF1 1	29.6025	POL1 1	21.6008	8.0017	0.00390165	0.003608913	0.000536653
		IGF1 2	29.6337	POL1 2	21.5417	8.092	0.003664927		
		IGF1 3	29.6476	POL1 3	22.12	7.5276	0.005419592		
		IGF1 4	29.7737	POL1 4	21.0434	8.7303	0.002354604		
		IGF1 5	30.0647	POL1 5	21.5339	8.5308	0.002703792		
	DIP	IGF1 6	31.3099	POL1 6	21.7818	9.5281	0.001354429	0.001595369	0.000521966
		IGF1 7	30.1355	POL1 7	21.8675	8.268	0.003244024		
		IGF1 8	33.2513	POL1 8	22.0168	11.2345	0.000415029		
		IGF1 9	30.1739	POL1 9	21.393	8.7809	0.002273452		
		IGF1 10	31.7437	POL1 10	21.2424	10.5013	0.000689912		
4 DAYS	DIP+GH	IGF1 11	29.8128	POL1 11	21.1259	8.6869	0.002426512	0.001716306	0.000446985
		IGF1 12	30.6218	POL1 12	21.7264	8.8954	0.002099993		
		IGF1 13	31.35	POL1 13	21.7135	9.6365	0.00125639		
		IGF1 14	30.933	POL1 14	22.3513	8.5817	0.002610062		
		IGF1 15	34.3195	POL1 15	21.9469	12.3726	0.000188572		
	DIP	IGF1 16	32.9366	POL1 16	22.3855	10.5511	0.000666503	0.000992458	0.000490324
		IGF1 17	30.4395	POL1 17	21.9234	8.5161	0.002731483		
		IGF1 18	32.1584	POL1 18	21.2639	10.8945	0.000525326		
		IGF1 19	32.5702	POL1 19	21.1321	11.4381	0.000360403		
		IGF1 20	32.2546	POL1 20	21.7294	10.5252	0.000678577		
	DIP+GH	IGF1 22	31.9508	POL1 22	20.822	11.1288	0.000446578	0.001900061	0.000735241
		IGF1 23	31.271	POL1 23	21.1386	10.1324	0.00089093		
		IGF1 24	30.573	POL1 24	22.0556	8.5174	0.002729022		
		IGF1 25	29.9926	POL1 25	21.848	8.1446	0.003533712		

Appendix Table 29: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in primary MEC from a Jersey cow cultured for 2 d and 4d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
	INS/PROG	IGFBP3 1	26.3767	POL1 1	21.6008	4.7759	0.036501511	0.039140462	0.001811193
		IGFBP3 2	26.4466	POL1 2	21.5417	4.9049	0.033379358		
		IGFBP3 3	26.7508	POL1 3	22.12	4.6308	0.040363637		
		IGFBP3 4	25.5961	POL1 4	21.0434	4.5527	0.042608941		
		IGFBP3 5	26.2400	POL1 5	21.5339	4.7061	0.038310934		
2 DAYS	DIP	IGFBP3 6	26.3926	POL1 6	21.7818	4.6108	0.040927093	0.041659423	0.013680801
		IGFBP3 7	27.4488	POL1 7	21.8675	5.5813	0.020886289		
		IGFBP3 8	25.6509	POL1 8	22.0168	3.6341	0.080542831		
		IGFBP3 10	26.6064	POL1 10	21.2424	5.364	0.024281477		
	DIP+GH	IGFBP3 11	29.3348	POL1 11	21.1259	8.2089	0.003379674	0.04887478	0.017196604
		IGFBP3 12	26.0112	POL1 12	21.7264	4.2848	0.051303462		
		IGFBP3 13	24.9722	POL1 13	21.7135	3.2587	0.104480094		
		IGFBP3 14	27.7126	POL1 14	22.3513	5.3613	0.024326962		
		IGFBP3 15	25.9847	POL1 15	21.9469	4.0378	0.060883706		
4 DAYS	DIP	IGFBP3 17	26.6946	POL1 17	21.9234	4.7712	0.036620619	0.125632681	0.045810735
		IGFBP3 18	23.8805	POL1 18	21.2639	2.6166	0.163051543		
		IGFBP3 19	23.2112	POL1 19	21.1321	2.0791	0.236662003		
		IGFBP3 20	25.6465	POL1 20	21.7294	3.9171	0.066196557		
	DIP+GH	IGFBP3 21	25.9182	POL1 21	22.1445	3.7737	0.073114431	0.12510767	0.052712988
		IGFBP3 22	23.1279	POL1 22	20.822	2.3059	0.202234354		
		IGFBP3 23	24.4609	POL1 23	21.1386	3.3223	0.099974225		
		IGFBP3 24	24.9623	POL1 24	22.0556	2.9067	0.133350949		

Appendix Table 30: Q-PCR raw C_T values and calculations for IGFBP-3 mRNA abundance in primary MEC from a Jersey cow cultured for 2 d and 4 d.

MEC from Holstein milk (Tables 31-35): raw qPCR values corresponding to results presented in Figure 10.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	ALAC 1	35.4827	18S 1	11.4193	24.0634	5.7042E-08	7.28731E-08	1.0326E-08
		ALAC 2	35.3123	18S 2	11.9428	23.3695	9.2274E-08		
		ALAC 3	35.059	18S 3	11.2765	23.7825	6.93033E-08		
	DIP	ALAC 4	34.6648	18S 4	11.5985	23.0663	1.13855E-07	1.15846E-07	1.9912E-09
		ALAC 5	34.7661	18S 5	11.7494	23.0167	1.17837E-07		
		ALAC 6	34.0765	18S 6	11.054	23.0225	1.17365E-07		
	DIP+GH	ALAC 7	34.8795	18S 7	12.7561	22.1234	2.18873E-07	1.93935E-07	2.49383E-08
		ALAC 8	34.0782	18S 8	11.5817	22.4965	1.68997E-07		
		ALAC 9	33.3754	18S 9	11.1304	22.245	2.01181E-07		
4 DAYS	DIP	ALAC 10	35.7723	18S 10	11.9081	23.8642	6.54877E-08	1.02858E-07	4.92585E-08
		ALAC 11	35.7088	18S 11	11.2243	24.4845	4.26021E-08		
		ALAC 12	34.0201	18S 12	11.7701	22.25	2.00485E-07		
	DIP+GH	ALAC 13	34.7988	18S 13	11.7107	23.0881	1.12147E-07	1.07207E-07	2.80877E-08
		ALAC 14	35.1879	18S 14	11.105	24.0829	5.62762E-08		
		ALAC 15	34.6257	18S 15	11.9876	22.6381	1.53198E-07		

Appendix Table 31: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in MEC from Holstein milk cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	CAS 1	27.2279	18S 1	11.4193	15.8086	1.74236E-05	1.86508E-05	3.68267E-06
		CAS 2	27.1989	18S 2	11.9428	15.2561	2.55538E-05		
		CAS 3	27.5104	18S 3	11.2765	16.2339	1.29751E-05		
	DIP	CAS 4	25.5287	18S 4	11.5985	13.9302	6.40607E-05	3.90745E-05	1.42258E-05
		CAS 5	27.7938	18S 5	11.7494	16.0444	1.47963E-05		
		CAS 6	25.7238	18S 6	11.054	14.6698	3.83663E-05		
	DIP+GH	CAS 7	26.2087	18S 7	12.7561	13.4526	8.91998E-05	6.03257E-05	1.5931E-05
		CAS 8	25.6664	18S 8	11.5817	14.0847	5.7555E-05		
		CAS 9	25.9651	18S 9	11.1304	14.8347	3.42224E-05		
4 DAYS	DIP	CAS 10	26.1668	18S 10	11.9081	14.2587	5.10157E-05	3.7712E-05	9.87572E-06
		CAS 11	26.9529	18S 11	11.2243	15.7286	1.8417E-05		
		CAS 12	26.252	18S 12	11.7701	14.4819	4.37032E-05		
	DIP+GH	CAS 13	26.8458	18S 13	11.7107	15.1351	2.77895E-05	3.08542E-05	1.0219E-05
		CAS 14	27.1406	18S 14	11.105	16.0356	1.48869E-05		
		CAS 15	26.2786	18S 15	11.9876	14.291	4.98862E-05		

Appendix Table 32: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in MEC from Holstein milk cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	GHR 1	29.8442	18S 1	11.4193	18.4249	2.84153E-06	3.43703E-06	2.97809E-07
		GHR 2	29.9773	18S 2	11.9428	18.0345	3.72456E-06		
		GHR 3	29.3031	18S 3	11.2765	18.0266	3.74501E-06		
	DIP	GHR 4	29.6643	18S 4	11.5985	18.0658	3.64462E-06	3.16392E-06	3.89627E-07
		GHR 5	29.8924	18S 5	11.7494	18.143	3.45472E-06		
		GHR 6	29.7271	18S 6	11.054	18.6731	2.39242E-06		
	DIP+GH	GHR 7	30.1897	18S 7	12.7561	17.4336	5.64889E-06	3.67708E-06	1.06131E-06
		GHR 8	29.7598	18S 8	11.5817	18.1781	3.37168E-06		
		GHR 9	30.0543	18S 9	11.1304	18.9239	2.01066E-06		
4 DAYS	DIP	GHR 10	29.1504	18S 10	11.9081	17.2423	6.44986E-06	5.3952E-06	9.49014E-07
		GHR 11	29.348	18S 11	11.2243	18.1237	3.50125E-06		
		GHR 12	29.0614	18S 12	11.7701	17.2913	6.23448E-06		
	DIP+GH	GHR 13	28.8586	18S 13	11.7107	17.1479	6.88601E-06	5.0225E-06	1.29484E-06
		GHR 14	29.6955	18S 14	11.105	18.5905	2.53339E-06		
		GHR 15	29.4214	18S 15	11.9876	17.4338	5.64811E-06		

Appendix Table 33: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in MEC from Holstein milk cultured for 2 d and 4 d.

		TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG		IGF1 1	29.1346	18S 1	11.4193	17.7153	4.6469E-06	3.51106E-06	7.47294E-07
			IGF1 2	29.9543	18S 2	11.9428	18.0115	3.78441E-06		
			IGF1 3	30.1364	18S 3	11.2765	18.8599	2.10186E-06		
	DIP		IGF1 4	29.814	18S 4	11.5985	18.2155	3.2854E-06	1.84255E-06	7.52093E-07
			IGF1 5	32.0904	18S 5	11.7494	20.341	7.5292E-07		
			IGF1 6	30.4109	18S 6	11.054	19.3569	1.48934E-06		
	DIP+GH		IGF1 7	30.8813	18S 7	12.7561	18.1252	3.49761E-06	2.04212E-06	7.33043E-07
			IGF1 8	30.9606	18S 8	11.5817	19.3789	1.4668E-06		
			IGF1 9	30.8454	18S 9	11.1304	19.715	1.16197E-06		
4 DAYS	DIP		IGF1 10	30.1747	18S 10	11.9081	18.2666	3.17107E-06	2.09381E-06	5.53929E-07
			IGF1 11	30.7431	18S 11	11.2243	19.5188	1.33124E-06		
			IGF1 12	30.8705	18S 12	11.7701	19.1004	1.77913E-06		
	DIP+GH		IGF1 13	30.5918	18S 13	11.7107	18.8811	2.0712E-06	1.52172E-06	2.87696E-07
			IGF1 14	30.9002	18S 14	11.105	19.7952	1.09913E-06		
			IGF1 15	31.4391	18S 15	11.9876	19.4515	1.39481E-06		

Appendix Table 34: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in MEC from Holstein milk cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	IGFBP3 1	22.914	18S 1	11.4193	11.4947	0.000346538	0.000497597	0.000130984
		IGFBP3 2	22.3074	18S 2	11.9428	10.3646	0.000758481		
		IGFBP3 3	22.609	18S 3	11.2765	11.3325	0.000387773		
	DIP	IGFBP3 4	21.2056	18S 4	11.5985	9.6071	0.001282256	0.001118501	0.000115328
		IGFBP3 5	21.4797	18S 5	11.7494	9.7303	0.001177302		
		IGFBP3 6	21.1783	18S 6	11.054	10.1243	0.000895946		
	DIP+GH	IGFBP3 7	22.1124	18S 7	12.7561	9.3563	0.001525714	0.000801061	0.000363897
		IGFBP3 8	22.5555	18S 8	11.5817	10.9738	0.00049723		
		IGFBP3 9	22.4912	18S 9	11.1304	11.3608	0.000380241		
4 DAYS	DIP	IGFBP3 10	20.057	18S 10	11.9081	8.1489	0.003523195	0.00319178	0.000226548
		IGFBP3 11	19.7262	18S 11	11.2243	8.5019	0.002758501		
		IGFBP3 12	20.0162	18S 12	11.7701	8.2461	0.003293643		
	DIP+GH	IGFBP3 13	20.3635	18S 13	11.7107	8.6528	0.002484549	0.001986796	0.000497754
		IGFBP3 14	20.4964	18S 14	11.105	9.3914	0.001489042		
		IGFBP3 15	20.9867	18S 15	11.9876	8.9991	0.001954344		

Appendix Table 35: Q-PCR raw C_T values and calculations for IGFBP-3 mRNA abundance in MEC from Holstein milk cultured for 2 d and 4 d.

MEC from Jersey milk (Tables 36-40): raw qPCR values corresponding to results presented in Figure 11.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	ALAC 1	36.9548	ACTB 1	17.3359	19.6189	1.24200E-06	1.64567E-06	5.33074E-07
		ALAC 2	36.2916	ACTB 2	17.7941	18.4975	2.70208E-06		
		ALAC 3	36.4954	ACTB 3	16.5536	19.9418	9.92933E-07		
	DIP	ALAC 4	33.4253	ACTB 4	17.2348	16.1905	1.33713E-05	7.64075E-06	2.88632E-06
		ALAC 5	34.2052	ACTB 5	16.7007	17.5045	5.37800E-06		
		ALAC 6	34.2396	ACTB 6	16.3691	17.8705	4.17295E-06		
	DIP+GH	ALAC 7	35.6313	ACTB 7	16.2588	19.3725	1.47332E-06	5.62285E-06	2.53920E-06
		ALAC 8	34.4467	ACTB 8	17.8703	16.5764	1.02331E-05		
		ALAC 9	34.1958	ACTB 9	16.6322	17.5636	5.16214E-06		
4 DAYS	DIP	ALAC 10	35.7032	ACTB 10	17.827	17.8762	4.15650E-06	5.22433E-06	2.29724E-06
		ALAC 11	33.7961	ACTB 11	17.1318	16.6643	9.62822E-06		
		ALAC 12	35.3421	ACTB 12	16.3276	19.0145	1.88827E-06		
	DIP+GH	ALAC 13	34.0834	ACTB 13	16.5664	17.517	5.33160E-06	5.65707E-06	3.20858E-07
		ALAC 14	35.4741	ACTB 14	17.9596	17.5145	5.34085E-06		
		ALAC 15	34.5708	ACTB 15	17.2943	17.2765	6.29876E-06		

Appendix Table 36: Q-PCR raw C_T values and calculations for α -lactalbumin mRNA abundance in MEC from Jersey milk cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	2 ^{-$\Delta\Delta C_T$}	Average	SEM
	INS/PROG	CAS 1	29.3246	ACTB 1	17.3359	11.9887	0.0002461	0.0002694	0.0000416
		CAS 2	29.2735	ACTB 2	17.7941	11.4794	0.0003502		
		CAS 3	28.758	ACTB 3	16.5536	12.2044	0.0002119		
2 DAYS	DIP	CAS 4	27.5161	ACTB 4	17.2348	10.2813	0.0008036	0.0009078	0.0000617
		CAS 5	26.6418	ACTB 5	16.7007	9.9411	0.0010173		
		CAS 6	26.4829	ACTB 6	16.3691	10.1138	0.0009025		
	DIP+GH	CAS 7	27.4095	ACTB 7	16.2588	11.1507	0.0004399	0.0012001	0.0003878
		CAS 8	27.0591	ACTB 8	17.8703	9.1888	0.0017135		
		CAS 9	26.0651	ACTB 9	16.6322	9.4329	0.0014468		
4 DAYS	DIP	CAS 10	29.2148	ACTB 10	17.827	11.3878	0.0003732	0.0003319	0.0000482
		CAS 11	28.4687	ACTB 11	17.1318	11.3369	0.0003866		
		CAS 12	28.3777	ACTB 12	16.3276	12.0501	0.0002358		
	DIP+GH	CAS 13	28.7063	ACTB 13	16.5664	12.1399	0.0002216	0.0005407	0.0001682
		CAS 14	28.6425	ACTB 14	17.9596	10.6829	0.0006083		
		CAS 15	27.5959	ACTB 15	17.2943	10.3016	0.0007923		

Appendix Table 37: Q-PCR raw C_T values and calculations for α_{s1} -casein mRNA abundance in MEC from Jersey milk cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	2 ^{-$\Delta\Delta C_T$}	Average	SEM
	INS/PROG	GHR 1	28.6422	ACTB 1	17.3359	11.3063	0.00039488	0.00041399	0.00015660
		GHR 2	28.2863	ACTB 2	17.7941	10.4922	0.00069428		
		GHR 3	29.2296	ACTB 3	16.5536	12.676	0.00015281		
2 DAYS	DIP	GHR 4	28.8247	ACTB 4	17.2348	11.5899	0.00032441	0.00017934	0.00007807
		GHR 5	29.3396	ACTB 5	16.7007	12.6389	0.00015679		
		GHR 6	30.4724	ACTB 6	16.3691	14.1033	0.00005682		
	DIP+GH	GHR 7	29.8178	ACTB 7	16.2588	13.559	0.00008286	0.00010245	0.00003682
		GHR 8	30.3612	ACTB 8	17.8703	12.4909	0.00017373		
		GHR 9	30.8976	ACTB 9	16.6322	14.2654	0.00005078		
4 DAYS	DIP	GHR 10	29.6507	ACTB 10	17.827	11.8237	0.00027587	0.00018538	0.00007569
		GHR 11	29.1254	ACTB 11	17.1318	11.9936	0.00024523		
		GHR 12	31.1288	ACTB 12	16.3276	14.8012	0.00003503		
	DIP+GH	GHR 13	29.2169	ACTB 13	16.5664	12.6505	0.00015553	0.00019620	0.00002460
		GHR 14	29.9811	ACTB 14	17.9596	12.0215	0.00024053		
		GHR 15	29.6369	ACTB 15	17.2943	12.3426	0.00019253		

Appendix Table 38: Q-PCR raw C_T values and calculations for GH receptor mRNA abundance in MEC from Jersey milk cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	IGF1 1	30.715	ACTB 1	17.3359	13.3791	9.3862E-05	6.32073E-05	1.94066E-05
		IGF1 2	31.6277	ACTB 2	17.7941	13.8336	6.8497E-05		
		IGF1 3	31.7163	ACTB 3	16.5536	15.1627	2.72629E-05		
	DIP	IGF1 4	31.5126	ACTB 4	17.2348	14.2778	5.03447E-05	3.05633E-05	9.92924E-06
		IGF1 5	32.3723	ACTB 5	16.7007	15.6716	1.91592E-05		
		IGF1 6	31.8291	ACTB 6	16.3691	15.46	2.21859E-05		
	DIP+GH	IGF1 7	32.3105	ACTB 7	16.2588	16.0517	1.47217E-05	2.81245E-05	7.90166E-06
		IGF1 8	33.0166	ACTB 8	17.8703	15.1463	2.75746E-05		
		IGF1 9	31.1688	ACTB 9	16.6322	14.5366	4.20773E-05		
4 DAYS	DIP	IGF1 10	31.871	ACTB 10	17.827	14.044	5.92018E-05	3.92231E-05	1.43526E-05
		IGF1 11	31.5062	ACTB 11	17.1318	14.3744	4.70841E-05		
		IGF1 12	32.7503	ACTB 12	16.3276	16.4227	1.13835E-05		
	DIP+GH	IGF1 13	31.981	ACTB 13	16.5664	15.4146	2.28951E-05	2.42725E-05	1.42617E-05
		IGF1 14	32.2579	ACTB 14	17.9596	14.2983	4.96344E-05		
		IGF1 15	39.0223	ACTB 15	17.2943	21.728	2.87886E-07		

Appendix Table 39: Q-PCR raw C_T values and calculations for IGF-I mRNA abundance in MEC from Jersey milk cultured for 2 d and 4 d.

	TX	Sample ID	C _T	HKG ID	C _T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	Average	SEM
2 DAYS	INS/PROG	IGFBP3 1	20.4037	ACTB 1	17.3359	3.0678	0.119261	0.117997	0.025489
		IGFBP3 2	20.4245	ACTB 2	17.7941	2.6304	0.161499		
		IGFBP3 3	20.325	ACTB 3	16.5536	3.7714	0.073231		
	DIP	IGFBP3 4	19.7434	ACTB 4	17.2348	2.5086	0.175726	0.078838	0.048567
		IGFBP3 5	22.0573	ACTB 5	16.7007	5.3566	0.024406		
		IGFBP3 6	21.1498	ACTB 6	16.3691	4.7807	0.036380		
	DIP+GH	IGFBP3 7	20.0309	ACTB 7	16.2588	3.7721	0.073196	0.179820	0.101473
		IGFBP3 8	19.2561	ACTB 8	17.8703	1.3858	0.382677		
		IGFBP3 9	20.2128	ACTB 9	16.6322	3.5806	0.083586		
4 DAYS	DIP	IGFBP3 10	18.7936	ACTB 10	17.827	0.9666	0.511711	0.559820	0.193940
		IGFBP3 11	17.2565	ACTB 11	17.1318	0.1247	0.917195		
		IGFBP3 12	18.3244	ACTB 12	16.3276	1.9968	0.250555		
	DIP+GH	IGFBP3 13	18.1136	ACTB 13	16.5664	1.5472	0.342174	0.535663	0.200015
		IGFBP3 14	18.0556	ACTB 14	17.9596	0.096	0.935623		
		IGFBP3 15	18.8973	ACTB 15	17.2943	1.603	0.329192		

Appendix Table 40: Q-PCR raw C_T values and calculations for IGFBP-3 mRNA abundance in MEC from Jersey milk cultured for 2 d and 4 d.