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Characterization of Differentiation of Bovine Mammary Epithelial Cells

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Abstract

In American society, dairy products are vital for nutrition and the economy. Optimizing the process of producing milk can benefit the consumers, producers, and the animals involved. Understanding the mechanisms of the development of the mammary gland can increase the efficiency of milk production, as well as improve animal health. Mammary epithelial cells (MEC) are the functional unit of the mammary gland. Although, there is a well-established MEC cell line, known as MAC-T, the use of a primary cell line is preferred because it more closely mimics an *in vivo* model. To better understand how mammary cell differentiation is regulated, it is vital to understand key mechanisms involved. One of the key genes involved in differentiation of MEC is the casein gene, which is expressed during mammary development and can be used to indicate differentiation of MEC. The main goal of this research is to establish the optimal methods for differentiation of primary bovine MEC in culture. Previous studies suggest that growth hormone (GH), also known as bovine somatotropin, is a promoter of MEC differentiation. Therefore, we hypothesize that GH will promote differentiation in MEC in culture. To test our hypothesis, we isolated and cultured bovine MEC from lactating dairy cows postmortem. Cells were cultured in standard media (DMEM + 10% FBS) and bovine insulin, prolactin, and dexamethasone were added to induce differentiation. In addition, cells were cultured in the absence (DMEM + 0.2% BSA) or presence of GH (DMEM + 0.2% BSA + 10ng/mL GH) for 8 days. To evaluate differentiation of the cells, we determined the expression of the α-casein gene by real-time reverse transcriptase-polymerase chain reaction (PCR) analysis at 0, 2, 4, 6, and 8 days of culture. The expression in primary MEC cultured was low or not detectable, indicating that the cells have not differentiated into mature MEC. In addition, cells treated with GH did not have greater expression of α-casein at day 8 compared to the controls,

suggesting that GH did not further differentiate MEC. Further studies are needed to identify optimal conditions to differentiate primary bovine MEC in culture.

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Review of Literature

Introduction

In American society, dairy products are vital for nutrition and the economy. According to USDA National Agricultural Statistics Service, New England's profits from milk sales in 2009 totaled $554 million. In addition, milk, the only nutrition source of early postnatal development in mammals, is an excellent source of vitamins and minerals for the human diet (Huynh et al., 1991). Optimizing the process of milk production will benefit consumers, producers, and the animals involved by increasing production efficiency and improving animal health. In order to improve efficiency of milk production, we must gain a better understanding of the mechanisms involved in regulating the development of the mammary gland. To do this, we first need to establish a model to evaluate the key mechanisms involved in regulation of the mammary gland development. We have chosen to use a primary bovine mammary epithelial cells (MEC) culture system.

Development of the Mammary Gland

General Overview of the Anatomy of the Mammary Gland

The mammary gland is the feature that distinguishes the Mammalian class from the other taxonomic classes in the ranking system (Akers, 2002). It is the structure responsible for milk synthesis and secretion. For cows, the mammary glands are contained in the udder. It consists of teats, ducts, alveoli that contain the secretory cells, and supporting tissue (Akers, 2002). The udder, which is split into two halves, contains four mammary glands, each containing a teat. The halves are separated by the median suspensory ligament that is responsible for the attachment of the udder to the body wall. The purpose of the teats is to allow milk to exit the cow. The gland cistern located at the base of the teat, becomes the teat cistern, which leads to the inside opening

of the teat, known as a streak canal. The milk drains from the secretory tissue into the gland cistern from the primary mammary ducts (Akers, 2002).

There are three basic supporting tissues in the mammary gland, which are known as the mammary fat pad, stroma, and parenchyma. The mammary fat pad is adipose tissue that the duct systems grows within (Neville et al., 1998). The stroma is the connective tissue that surrounds the alveoli and provides structural support and anchorage to the mammary gland (Akers, 2002). The parenchyma is the secretory tissue, which contains a duct system and lobes. The lobes are made up of several lobules, which include groups of alveoli and their surrounding ducts. There are billions of alveoli in the udder (Tyler & Ensminger, 2006). The alveoli are lined with two layers of epithelial secretory cells, the functional unit of the mammary gland that synthesize and secrete milk (Akers, 2002). These cells will be the focus of our research.

Fetal Development

The development of the mammary gland is a continuous process throughout life that begins when the animal is a fetus and continues through adulthood (Robinson, 2007). The four main stages of development of the mammary gland include fetal, pre- and post- pubertal, gestational, and lactational development (Akers, 2002). The first sign of the mammary gland forming is the presence of the mammary band, the thickening of the ectoderm, which occurs around day 30 for a bovine embryo (Akers, 2002). The mammary band then develops into the mammary streak, which eventually becomes the mammary line by the fifth week of embryonic development. As proliferation of the ectoderm cells and mesenchymal cells continues, the mammary line forms the mammary crest. This structure begins to round and matures into the mammary bud at day 43 of gestation. Bovine have four mammary buds, each corresponding with the four parts of the udder (Akers, 2002).

The formation of the mammary buds marks the time in which different patterns of development between the species and the sexes occur. After this time point in the development of the bovine fetus, the females have buds that are smaller, more oval and closer to the surface, as well as more pointed teats (Akers, 2002). In male bovine, mammary development slows at this point. The development of the teat at day 65 of gestation starts with the mammary bud cells being forced to the surface by the proliferation of the mesenchyme (Akers, 2002). This develops into the primary sprout as blood vessels begin to form and proliferation of epithelial cells continues and elongation occurs (Robinson, 2007). The primary sprout, which eventually forms the teat and gland cistern, produces the secondary sprouts that become the major ducts. The solid sprouts need to be canalized for it to develop into a teat that can expel milk, but this mechanism is not fully understood. However, it is believed that it may involve cell death or migration. Finally, the streak canal is formed by an invagination of the tip of the teat (Akers, 2002).

During teat development, mammary epithelium proliferates into undifferentiated embryonic mesenchyme. The mesenchyme contains two different precursors. Located very close to the mammary bud are precursors for stroma cells, such as fibroblast, but closer to the posterior end of the mammary bud precursors for the mammary fat pad are developed at day 80 of gestation (Sheffield, 1988). Little development occurs after month three of gestation. The mammary bud changes only slightly into the mammary pit, more secondary sprouts develop, and canalization continues. Also the lumen of teat starts to form due to canalization, the gland cistern, teat cistern, and streak canal develop. The gestation period of a cow on average is 280 days, which marks the end of fetal development (Akers, 2002).

Prepubertal and Postpubertal Development

The development of the mammary gland that occurs between a cow's birth and the conception of its offspring is associated with the duct system, adipose tissue, and connective tissue increasing in development (Akers, 2002). This period of development can be split into two groups, development pre- and post- pubertal. Before puberty, growth of the mammary gland occurs isometrically compared to the rest of the body. The amount of growth of the mammary gland during this time is only a very small amount in relation to the amount that occurs later during gestation (Akers, 2002). In contrast, this period of time sets the initial groundwork necessary for development of the mammary gland. At this point, improper development can greatly affect later mammary function and milk production (Berry et al., 2003). A short time before puberty, at about 3 months age of cattle, the growth of the mammary gland becomes allometric. Mammary gland growth is stimulated by activity of the ovary occurring before and during puberty (Hovey et al., 2002).

During the estrous cycle in cows, opposing changes in estrogen and progesterone are observed (Hovey et al., 2002). After the first few estrous cycles, mammary gland growth occurs isometrically again (Akers, 2002). After the onset of puberty, estrogen, secreted by the ovary during each estrous cycle, stimulates the development of the mammary gland duct system causing it to become more intricate with each subsequent estrous cycle (Tyler & Ensminger, 2006). Also, growth hormone (GH) stimulates mammary development but only if estrogen is present as well (Hovey et al., 2002). During this developmental time, even though the duct system becomes more complex, the growth and development of the lobulo-alveolar system is minimal (Hovey et al., 2002).

Development During Pregnancy

Pregnancy is a natural promoter of mammary growth and the majority of the development of the mammary gland occurs during pregnancy (Akers, 2002). At this time, the duct system continues to develop extensively during the first three to four months of gestation (Tyler & Ensminger, 2006). The lobulo-alveolar system starts to develop after five months. Alveoli eventually occupy the entire area by individually increasing in size and number (Akers, 2002). High concentrations of mammogenic hormones, such as GH, estrogen, and progesterone, during this time are responsible for the mammary growth. Together they stimulate MEC proliferation and form mature alveoli (Topper & Freeman, 1980). Estrogen is present in even greater concentrations during gestation than during the estrous cycles and increases throughout gestation (Akers, 2002). Progesterone, known as the hormone of pregnancy, is maintained at very high concentrations throughout pregnancy (Akers, 2002). Estrogen and progesterone are essential for final duct growth. Also, the reproductive hormones estrogen, progesterone, and prolactin are vital for lobulo-alveolar development (Brisken, 2002). The mammary gland develops enough to be capable of producing milk by the seventh month (Tyler & Ensminger, 2006).

Development During Lactation

Lactogenesis, the production of milk by the mammary gland, occurs in two stages (Neville et al., 2002). The first stage starts during pregnancy and is characterized by the expression of necessary genes for milk synthesis, such as casein and lactalbumin. Hormonal regulation is not well understood for this stage. The second stage begins at parturition and involves the development of the mammary gland that prepares it for the secretion of colostrum and then milk. The expression of milk protein genes increases, the tight junctions between the

alveolar close, and lipid droplets and casein micelles move to the lumen of the alveolar (Neville et al., 2002). A few days after parturition, an increase in growth of the mammary gland occurs if suckling or removing milk take place and continue to be a regular activity. These activities indicate that stimulation of the teat sends signals that are vital for mammary growth during lactation (Akers, 2002). There is little mammary growth during lactation; mammary cell proliferation becomes slower compared to the rates during other stages of development. Alveoli are dependent on the removal of milk and will degrade and even undergo apoptosis if milk removal ceases (Akers, 2002). Lactation is maintained by prolactin and oxytocin. Prolactin maintains milk secretion by acting on MEC and oxytocin is involved in the ejection of milk by acting on the myoepithelial cells (Neville et al., 2002). Also GH plays an important role in this stage of development by helping maintain lactation and increasing milk yield (Barber et al., 1992).

Mammary Gland Involution

Mammary gland involution occurs when the young is removed from suckling or milk removal ceases. Involution is characterized by apoptosis or loss of the alveolar epithelial cells and proteolytic degradation (Accorsi et al., 2002). In some animals, such as mice, more apoptosis occurs than in others, such as cattle (Akers, 2002). Apoptosis, usually caused by DNA fragmentation, is seen to be correlated with the decrease in prolactin, GH, and insulin growth factor I (IGF-I; Accorsi et al., 2002). In cattle, there are isolated areas of tissue degradation containing undifferentiated cells, but also areas that have the structure fully intact. Since cells are still alive in cattle, if milking is resumed after 12 days of nonmilking, milk production will come close to reaching production levels before milking ceased (Akers, 2002). However, after 28 days of nonmilking, milk production can resume, but levels will only reach about 50% of before

(Akers, 2002). Tissue proteinases restructure the mammary gland to prepare for a new reproductive cycle. However, the tissue does not regress the same throughout the mammary gland. The tissue closer to the teat gets degenerated more than the tissue further away (Akers, 2002). The alveolar are eventually regenerated to prepare for the next gestation and lactation (Tyler & Ensminger, 2006).

Mammary Epithelial Cells

Development of Epithelial Cells

As previously stated, MEC are found lining the alveolar cells and are responsible for synthesizing and secreting milk (Akers, 2002). The primary function of MEC is to remove nutrients from the blood, transform these nutrients to milk, and release the milk into the lumen of the alveolus. The nutrients are extracted from the capillaries around the alveoli (Tyler & Ensminger, 2006). During gestation, these secretory cells start to develop as the alveoli are formed. Mammary epithelial cells contain cytoplasm, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, and lysosome (Akers, 2002). The endoplasmic reticulum is responsible for the synthesis of milk protein. The ribosomes attached to the outside of the rough endoplasmic reticulum are the site of protein synthesis (Tyler & Ensminger, 2006). The Golgi apparatus releases casein and lactose containing secretory vesicles. There are numerous mitochondria present, the organelles that house the energy releasing reactions, and their number increases as energy demand during lactation increases. The purpose of the lysosome is to destroy the MEC after it becomes old and nonfunctional in order to make room for new cells (Tyler & Ensminger, 2006). Tight junctions create a milk/blood barrier limiting transportation between the cells to keep the components of each separate from each other.

Estrogen and progesterone stimulate MEC proliferation. Not all researchers agree but some believe that GH may also stimulate MEC proliferation (Topper & Freeman, 1980; Capuco et al., 2001). MEC differentiation depends on the hormones prolactin and glucocorticoids. Prolactin is involved with the development of the Golgi apparatus and secretory vesicles. Glucocorticoids, the primary one cortisol, develop the rough endoplasmic reticulum. Insulin may also be necessary but there is some controversy whether it is insulin or IGF-I that are responsible (Akers, 2002).

Bovine Epithelial Cell Line, MAC-T

Huynh et al. (1991) established an *in vitro* bovine MEC line, known as mammary alveolar cell- T (MAC-T). MAC-T cells are MEC transfected with simian virus-40 (SV-40) large T-antigen, which gives the cells immortality and the capability to not deteriorate for over 350 serial passages in culture. The result for the tumorigenesis test of injecting the cells subcutaneously into mice exhibited that MAC-T cells were not transformed because no tumor formed (Huynh et al., 1991). The cells were also tested at different temperatures using the SV-40 temperature-sensitive A gene but the results concluded that MAC-T cells were not dependent on temperature and can grow the same amount at each temperature tested. According to Zavizion et al. (1995) the MAC-T cell line is not homogenous, but instead is heterogeneous population of cells, containing at least two different subtypes of MEC, one cuboidal typically epithelial-like cells and the other are large, multinucleated cells, the type of cell is unknown. This proposition was based on differences in cell morphology, cell size, growth, and cytogenetic characteristics (Zavizion et al., 1995).

The results obtained by Huynh et al. (1991) illustrate that MAC-T cells need to be anchored onto either plastic cell culture plates or on attached collagen gels and do not grow on

soft agar. Also, they are dependent on serum and do not proliferate without it. This cell model demonstrates the typical "cobblestone" morphology when grown on plastic (Huynh et al., 1991). However, MAC-T cell were most similar to the differentiated bovine mammary alveoli when they were grown on collagen gels. Dome structures were apparent and even duct-like structures connecting the domes appeared later in culture (Huynh et al., 1991). MAC-T cells are different from other established MEC lines because they can differentiate and secrete milk specific products (Huynh et al., 1991).

In vivo experiments are the best model of MEC but experiments using dairy cows are very costly and generally difficult to perform because they require a large commitment of animal resources and technical labor (Zavizion et al., 1995). The next best model to *in vivo* is primary MEC *in vitro.* Although MEC do not respond well to over 16 passages in culture, transfected MAC-T cells have many more drawbacks that make MEC a better cell model (Huynh et al., 1991). Essentially, primary MEC more precisely represent the cells in lactating bovine compared to cell lines, such as MAC-T cells (Zhou et al., 2008).

Caseins

Caseins are a major group of milk-specific proteins. They are hydrophobic and have the capacity to form casein micelles. Micelles consist of mostly casein but also transport calcium, inorganic phosphate, small amounts of citrate and magnesium, as well as provide a nutritious source of amino acids such as proline and glutamic acid (Akers, 2002). Casein gene expression synthesizes caseins in the ribosomes of the mammary gland from free amino acids predominantly during lactation (Choi et al., 1988). The Golgi apparatus is involved in the maturation of casein micelles and also packages the protein into vesicles for secretion. There are

three subtypes including α-, β-, and κ- casein (Akers, 2002). The focus of this research is on α-casein.

Casein gene expression is increased during mammary differentiation by hormonal stimulation of transcription (Teyssot & Houdebine, 1980). Choi et al. (1988) concluded that mammary cells in culture were capable of hormone-induced milk protein gene expression. The combination of hydrocortisone, prolactin, and insulin increased the amount of milk proteins secreted. Also, the study indicated that the addition of prolactin could induce the secretion of milk proteins because of the increase in β-casein mRNA concentration observed (Hobbs et al., 1982). This supports the idea that prolactin plays a vital role in transcription or turnover rate of casein mRNA (Hobbs et al., 1982). Insulin has the lowest effect on casein expression and therefore may not be essential for casein expression in MEC (Ono et al., 1981).

Casein can be used as an indicator of MEC differentiation. According to Talhouk et al. (1990), casein synthesis and secretion indicates complete mammary differentiation of lactation since casein is a vital protein in mature milk. Huynh et al. (1991) used α-casein and β-casein proteins to indicate differentiation of the MAC-T cells. The existence of these proteins could only be due to the secretory abilities of MAC-T cells when differentiated. An increase of β-casein mRNA was observed when plated on collagen gels. The maximum amount detected occurred with the addition of prolactin. Also, MAC-T cells produced and secreted both α-casein and β-casein when plated on floating collagen gels; β-casein appearing first and α-casein needing more time (Zavizion et al., 1995). However, much less β-casein was produced when the cells were grown on attached collagen (Zavizion et al., 1995). Finally, the presence of prolactin increased the expression of casein (Huynh et al., 1991). Therefore, the presence of the casein gene can be used as an indicator of MEC differentiation.

Growth Hormone (GH)

Growth hormone and prolactin are the two main hormones secreted by the anterior pituitary that effect mammary development. Growth hormone uses a receptor tyrosine kinase signal transduction pathway by binding to GH receptors activating Janus kinase 2 (JAK2), which phosphorylates STAT5 and other substrates (Zhu et al., 2001). STAT5 is a signal transducer and transcription factor. This pathway causes a change in gene expression, such as IGF-I (Zhu et al., 2001).

Growth hormone regulates animal growth and metabolism (Etherton & Bauman, 1998). More specifically in relationship with the mammary gland, it is vital for duct and lobulo-alveolar development and is known to stimulate milk production without altering milk composition (Sejrsen et al., 1999). In contrast, experiments conducted administering GH in the time period before puberty showed a nonsignificant increase in milk yield or no increase (Buskirk et al., 1997). This indicates that increased pubertal growth due to GH does not affect milk yield. One key function of GH is its effect during lactation (Sejrsen et al., 1999). An increase in milk yield is exhibited, which may be a result of GH coordinating changes in metabolism of tissues that encourages an increase of nutrients and energy to the mammary gland (Akers, 2002). For example, in adipose tissue it either inhibits lipogenesis or promotes lipolysis and in the liver it promotes gluconeogenesis according to the amount of energy that is critical for milk production (Akers, 2002). At first, the lack of GH binding found in the mammary gland and GH effects *in vitro* indicated that it works indirectly to affect the mammary gland (Sejrsen et al., 1999). On the contrary, more recent research supports the idea that it also works by direct contact because GH receptor mRNA and protein were expressed in the bovine mammary gland (Plath-Gabler et al.,

2001). Since the exact mechanisms of GH action are still relatively unknown, there is much speculation (Akers, 2002).

Zhou et al. (2008) determined that GH significantly affects α-casein and β-casein by increasing mRNA expression in MAC-T cells. This is believed to be a consequence of GH directly affecting the MEC. Therefore milk protein concentrations remain at a constant percentage of milk when treated with GH (Zhou et al., 2008). Johnson et al. (2010) found MAC-T cells that were differentiated with dexamethasone, insulin, and prolactin had a significant increase in GH receptor mRNA, which increased even more with the presence of GH. Sakamoto et al. (2005) also reported that GH was shown to have a positive effect on milk protein production, specifically the secretion of α-casein on cloned bovine MEC. In this research, the cells were treated in the absence and the presence of dexamethasone, insulin, and prolactin, which were also found to enhance the expression of GH receptors without GH present in MEC. Alpha-casein expression and synthesis was stimulated in both circumstances. Therefore, there is evidence to suggest that GH may enhance differentiation of MEC.

Rationale

It is important to understand key mechanisms involved in regulating mammary gland development. Specifically, we will try to better comprehend the function of bovine MEC by understanding the mechanisms in which they differentiate. Other researchers have used *in vitro* primary bovine MEC as a model but it is new to our research laboratory, therefore we must first establish and optimize the primary bovine MEC culture system before being able to perform experiments to evaluate the mechanisms of differentiation. Since we wanted to evaluate the key mechanisms involved in regulating the MEC, we first cultured these cells in conditions known to induce differentiation. In addition, based on the role of GH in mammary gland development and

MEC differentiation, we hypothesized that the addition of GH would promote differentiation of primary bovine MEC into a more mature cell, by determining expression patterns of the casein gene, as an indicator of differentiation.

Materials and Methods

Excising Tissue From Bovine Mammary Gland

Tissue samples of the mammary parenchyma were obtained from four lactating dairy cows postmortem from Rhode Island Beef and Veal, ranging from young first calf heifers to older cows that have gone through multiple lactations. The work area was covered with white bench top paper after being washed with 10% bleach solution and then 70% ethanol. The udder was observed for signs of abnormalities or disease. The mammary fat pad on the udder was washed with betadine or chlorhexidine and then 70% ethanol. After the adipose tissue was removed the parenchyma tissue was excised carefully with minimal undesirable tissues, such as adipose tissue. The samples were put in 50 mL falcon tubes containing Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+} (HBSS; Sigma-Aldrich, MO), penicillin G (100 μg/mL; Fisher, UT), streptomycin (100 μg/mL; Fisher, UT), gentamicin (100 μg/mL; Sigma-Aldrich, MO), and Fungizone (5 μg/mL; Sigma-Aldrich, MO) then stored and transported to the laboratory on ice.

Isolation of Primary MEC

Once in the laboratory, all of the procedures were performed in a sterile environment. According to Wellnitz and Kerr (2004), the tissue samples were minced to approximately 1-5 mm^3 in a 50mL falcon tube using surgical scissors and rinsed multiple times with HBSS in order to remove blood and milk. Then, the samples were placed in a 1L Erlenmeyer flask for 3 hours on a magnetic stirrer, which contained the digestive mixture [HBSS with collagenase IV (0.5 mg/mL; Gibco, NY), DNAse I (0.4 mg/mL; Ambion, TX), hyaluronidase (0.5 mg/mL; Sigma-Aldrich, MO), gentamicin (50 μg/mL), Fungizone (2.5 μg/mL)]. Next, the cells were filtered through a metal strainer with a pore size of 1 mm and centrifuged for 5 minutes at 40 x g. The cell pellet was resuspended in HBSS, filtered through a metal strainer with a pore size of 0.5 mm

and centrifuged for 5 minutes at 40 x g. Again, the cells were resuspended in HBSS and then filtered through a cell strainer (100 μm) and centrifuged for 5 minutes at 40 x g. The final cell pellet was resuspended in standard media [Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY) with fetal bovine serum (FBS; 10%) containing bovine insulin (5 μg/mL; Sigma, MO), gentamicin (50 μg/mL), penicillin (20 U/mL), streptomycin (20 μg/mL), and Fungizone (2 μg/mL)].

Cell Culture

After isolation, the cells contained a mix population including MEC and fibroblast cells. The cells were plated on 100 mm plastic cell culture dishes (USA Scientific, FL) and incubated for 30 minutes at 37°C, 5% CO_2 to allow some of the fibroblast cells to attach. The cells were then decanted off and counted using a hemocytometer. They were plated in the same standard media described previously on 100 mm plastic cell culture plate (1 million cells/plate) and incubated at 37°C, 5% CO_2. The media was changed every 48 hours. Cells were passed at 75 to 80% confluency by washing with phosphate buffered saline (PBS), adding a solution containing 0.025% trypsin (MP Biomedicals, OH), 0.25mM EDTA, and 50% 1X PBS, incubating for 15 minutes, and then gently scraping the cells to lift them off of the plate. MEC were cryopreserved in liquid nitrogen in 1 mL aliquots, each containing 2 million cells and 10% dimethyl sulfoxide (Sigma-Aldrich, MO), for future use. MAC-T cells were also cultured in the same standard media, without Fungizone, on 100 mm plastic cell culture dishes. The same steps were performed for cell culture, except the trypsin solution was added and only incubated for 7-10 minutes at 37°C, 5% CO_2.

Removal of Fibroblast Cells

At first, when the cells were cultured, contamination of fibroblasts was observed. Two techniques were used to remove the fibroblast cells from the MEC. The first method used HBSS to lift the fibroblast cells (Pal & Grover, 1983). Media on cells were removed and cells were washed with HBSS solution previously described (Wellnitz & Kerr, 2004). HBSS solution was added to the cells and incubated for 2-3 hours. The fibroblasts lifted off the bottom of the plate, while the MEC stayed adhered to the plate. After the plate was incubated, the HBSS containing fibroblast were vacuumed off. The cells were washed with media. Then the standard media was added to the cells and further incubated. A second method, which included partial trypsinization, was used to remove fibroblast cells. This was usually performed during the cell passage. First, the media was vacuumed off. Then the cells were washed with PBS, the trypsin solution was added, and the cells were incubated for 5 minutes at 37°C, 5% CO_2. This caused the fibroblasts to change shape and lift off the plate. The trypsin and fibroblasts were vacuumed off and the cells were washed with PBS once more. Both procedures were done at least four times until the cells were devoid of fibroblasts, leaving only primary bovine MEC in culture.

Differentiation of MEC

Cells were cultured in media that Johnson et al. (2002) found to differentiate MEC [DMEM + 0.2% BSA (Sigma-Aldrich, MO) + bovine insulin (5 μg/mL) + prolactin (3 μg/mL; A.F. Parlow NHPP, CA) + dexamethasone (10 μg/mL; Sigma-Aldrich, MO) + gentamicin (50 μg/mL) + 1% penicillin/streptomycin + sodium selenite (30 μM; Sigma-Aldrich, MO)] in the absence or presence of GH (10 ng/mL; A.F. Parlow NHPP, CA) for 8 days. RNA was extracted at days 0, 2, 4, 6, 8 of culture using Tri Reagent (trizol; Sigma-Aldrich, MO) and RNeasy mini kit (Qiagen, MD) according to the manufacturer's protocol. The RNA sample was rid of DNA by

using the TURBO DNA-free kit (Ambion, TX). The quantity and quality of RNA were determined using a NanoDrop ND-1000 Spectrophotometer and Bio-Rad Experion (CA), respectively. All RNA samples were diluted to 30 ng/μL. The RNA was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen, CA). Lastly, the cDNA was used for real-time RT-PCR (Applied Biosystems, UK) to quantify gene expression of α-casein gene and RPS15 (endogenous control). The α-casein primer (Johnson et al., 2010) and RPS15 primer (Bionaz & Loor, 2007) were designed for real-time RT-PCR by searching NCBI to identify the coding sequence of the gene of interest to ensure the correct gene would be amplified. Primer3 was used to identify primers appropriate for real-time RT-PCR, and these primers were BLAST to guarantee they targeted the gene of interest. Primer sequences are listed in Table 1. The PCR conditions were optimized by running a thermogradient with temperatures between 53˚C and 63˚C. Real-time PCR conditions were as follows: 50˚C for 2 minutes, 95˚C for 10 minutes then 55 cycles at 95˚C for 15 seconds and 60˚C for 1 minute, followed by 95˚C for 15 seconds, 60˚C for 15 seconds and 95˚C for 5 seconds.

Table 1. Primers for Real-Time RT-PCR

α-Casein	Forward	5'- AATCCATGCCCAACAGAAAG -3'
	Reverse	5'- TCAGAGCCAATGGGATTAGG -3'
RPS15	Forward	5'- GCAGCTTATGAGCAAGGTCGT -3'
	Reverse	5'- GCTCATCAGCAGATAGCGCTT -3'

The primers for RPS15 (endogenous control) and α-casein were obtained from integrated DNA Technologies (IDT) but first Primer3 was used to identify primers appropriate for RT-PCR, and these primers were run on NCBI BLAST to guarantee they targeted the gene of interest.

Results

We successfully isolated MEC from the parenchyma tissue in the udder of lactating bovine after slaughter and were able to propagate the cells and cryopreserve them in liquid nitrogen for future experiments. Previous research has shown that MEC have a cobblestone appearance that is characteristic of this cell type's morphology (Wellnitz & Kerr, 2004). The MEC grew in a monolayer adhered to the plastic dish and exhibited the cobblestone organization that is distinctive of the MEC *in vivo* (Figure 1A). To compare morphology of the primary MEC, we also looked at the transfected MEC line, MAC-T. MAC-T cells were successfully cultured and the cell morphology was similar to primary MEC (Figure 1B). The MAC-T cells were cuboidal and displayed the same cobblestone shape. Also, both cell lines formed a monolayer and clusters on the plastic cell culture plate.

We were able to detect mRNA expression of α-casein at each time point in the primary bovine MEC (Table 2). However, the overall expression was low and α-casein expression was not detectable in some samples at each time point. Even though there was some α-casein expression at each time point, due to the lack of expression in some samples, a limited number of samples were available for analysis. To quantify the values, we used the ΔCT method. For the not detectable samples, we gave a value of 55 cycles, the maximum cycles of amplification. The mean values were then calculated at each time point for the mRNA expression of α-casein. A low ΔCT value means greater α-casein expression. We did not detect a change in α-casein expression between day 0 and day 8 (Table 2). The endogenous control, RPS15, was expressed similarly for all of the time points ($P > 0.05$; data not shown) signifying that the low expression of α-casein was not due to the RNA quality or different amounts of RNA between samples. We

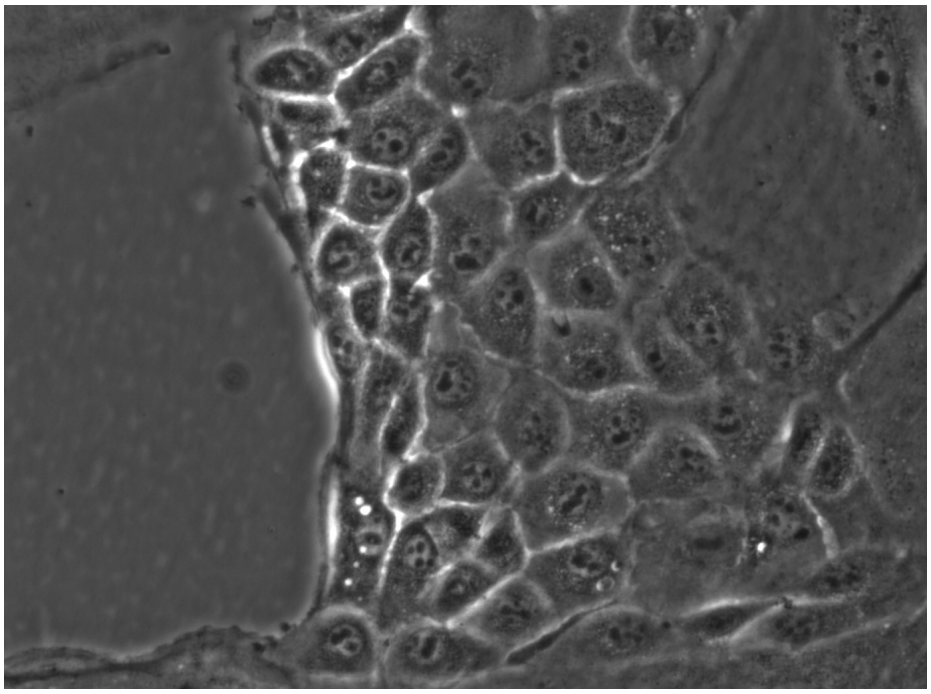
did not observe an effect of GH on the mRNA expression of α-casein between day 0 and day 8 of culture (Table 2).

Figure Legends

Figure 1. Primary bovine mammary epithelial cells (A) and MAC-T cells (B) in culture. Primary bovine MEC and MAC-T cells were cultured on a plastic cell culture dish in standard DMEM media. Images were taken at 20x using a phase contrast microscope. The MEC and MAC-T cells both display the cobblestone shape that is characteristic of this cell type.

Figure 1.

A.



B.

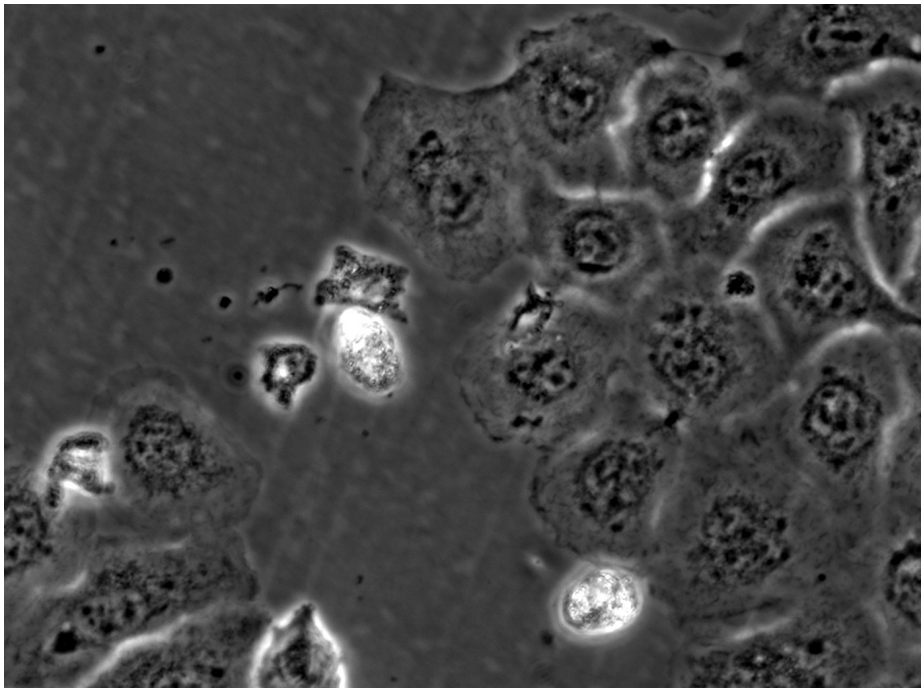


Table 2. Expression of α-casein mRNA in MEC during differentiation.

Growth hormone	Day 0	Day 2	Day 4	Day 6	Day 8
-	26.31 ± 7.55	26.92 ± 5.83	27.07 ± 5.62	25.19 ± 6.44	21.87 ± 5.84
+	24.86 ± 5.75	28.36 ± 3.76	21.58 ± 4.70	24.59 ± 2.58	25.46 ± 4.92

Real-time RT-PCR was performed using RNA from bovine primary MEC. Data are expressed as ΔCT values and presented as mean ± SD. ND = not detectable. All means are representative of 2 to 4 replicates. The lower the ΔCT value, the greater α-casein expression.

Discussion

We were able to successfully proliferate MEC in culture using conditions previously described (Wellnitz & Kerr, 2004; Pal & Grover, 1983). Specifically, previous laboratories have used different digestive mixtures including an enzyme mixture of collagenase, hyaluronidase, and DNase; a trypsin-collagenase digestion; or a series of 30 minutes digestions using collagenase, and pronase (Shamay & Gertler, 1986; German & Barash, 2002; Ahn et al., 1995). To prevent fungal and bacterial contamination we added Fungizone, and gentamicin to the enzyme digestion mixture that contained collagenase, hyaluronidase, and DNase. We were successful in isolating the MEC from the parenchyma of a lactating bovine using this digestive mixture and a similar tissue preparation procedure used by others (Wellnitz & Kerr, 2004; Shamay & Gertler, 1986). There are two basic methods that may be used to culture MEC. Cells may be cultured on extracellular matrices (Emerman and Pitelka, 1977; Talhouk et al., 1993) or directly on the plastic cell culture plates. We chose to use the direct plating on the plastic dish and our method was successful similar to other reports (Wellnitz and Kerr, 2004).

One very common media used for bovine MEC in culture is DMEM with fetal bovine serum (German & Barash, 2002; Ahn et al., 1995). In our experiments using this common media, we observed a similar effect on cell morphology as seen by Wellnitz and Kerr (2004). The cobblestone appearance of the monolayer of MEC on plastic was apparent. This is a characteristic feature of bovine MEC, also visible in MAC-T cells, that supports the fact that we successfully isolated and cultured primary bovine MEC. Once we established the primary cell line, we were able to begin optimization of culture conditions to differentiate these cells into a more mature cell.

There are several different factors that can be utilized to differentiate MEC. We used the

lactogenic hormones dexamethasone, bovine insulin, and prolactin in the culture media. Prolactin was used because it has been known to stimulate MEC proliferation and differentiation during pregnancy, and is essential for the secretion of milk by inducing transcription of milk proteins (Ormandy et al., 2003). The use of these lactogenic hormones to differentiate MEC has previously been performed, resulting in an increase of α-casein expression (Johnson et al., 2010) and an increase in the expression of GH receptors (Sakamoto et al., 2005). Surprisingly, we did not observe a similar increase in α-casein expression using these lactogenic hormones. Based on these findings, we conclude that we did not successfully differentiate the primary bovine MEC into a more mature cell using the differentiation media. This could be due to several factors such as the lack of a collagen matrix, which has been previously demonstrated to improve MEC differentiation or the addition of GH, which is discussed later (Katz & Streuli, 2007; Sakamoto et al., 2005; Talhouk et al., 1993).

According to previous research, α-casein signifies that MEC differentiation has occurred because casein is an essential protein in mature milk (Talhouk et al., 1990). Huynh et al. (1991) used α-casein proteins to indicate differentiation of MAC-T cells. Other researchers have observed an increase in α-casein gene expression by using a combination of hormones to induce differentiation (Choi et al., 1988; Riley et al. 2009). In our research, we found that the concentrations of mRNA expression of α-casein were low, suggesting that the primary bovine MEC did not differentiate into a more mature cell. However, the expression of α-casein may have been low due to using primary bovine MEC because the previous research just mentioned was conducted on mammary alveoli and alveoli-like mammospheres in culture instead of primary MEC, which may not be capable of hormone-induced milk protein gene expression on plastic (Choi et al., 1988; Riley et al. 2009). Also, there has been limited work showing α-casein

expression on MEC on plastic cell culture dishes. The majority of the experiments performed were grown on extracellular matrices, such as collagen gel, which will be discussed later (Katz & Streuli, 2007; Sakamoto et al., 2005; Talhouk et al., 1993; Emerman & Pitelka, 1977). Therefore, the low α-casein expression could also be due to the fact that the primary MEC in our research were cultured on plastic cell culture dishes. The mammospheres previously discussed were grown on Matrigel, an extracellular matrix, which supports the idea that MEC may not differentiate well on plastic.

The mRNA expression of α-casein in primary bovine MEC did not increase in the presence of GH. Research has been performed on MAC-T cells on plastic cell culture dishes testing the effects of GH on differentiated cells (Johnson et al., 2010). The cells were differentiated using the combination of the same lactogenic hormones, as used in our research and differentiated MAC-T cells containing GH exhibited a large increase in α-casein mRNA abundance compared to cells lacking GH (Johnson et al., 2010). Also, Zhou et al. (2008) determined that GH significantly effects α-casein and β-casein by increasing mRNA expression in MAC-T cells cultured on plastic. Sakamoto et al. (2005) concluded, GH has a positive effect on α-casein in cloned bovine MEC, and observed that α-casein expression and synthesis was stimulated in the presence of GH. In our research, there was no observed effect of GH on the mRNA expression of α-casein primary MEC cells. This may be due to culturing the MEC on plastic cell culture plates instead of using substratum or using primary MEC instead of MAC-T cells or alveoli. Johnson et al. (2010) was able to see an increase in α-casein expression due to GH but the research was performed on MAC-T cells not primary MEC. The research performed on cloned bovine MEC, cultured the cells on a cell culture insert, type I-C collagen coated flask (Sakamoto et al., 2005).

There is much research on α-casein expression in MAC-T cells, however less is known using the primary bovine MEC model. The research that there is on the primary MEC often also contains surrounding cells that are normally present in the mammary gland, such as myoepithelial cells, when alveoli-like mammospheres or alveoli are cultured as opposed to using only MEC, as does our research (Riley et al., 2009; Choi et al., 1988). Also early studies show that primary mouse MEC from pregnant mice maintained on plastic, lose their differentiation characteristics even in the presence of hormones (Emerman et al., 1977). The amount of α-casein is consistently greater in floating collagen membrane cultures of primary mouse MEC when it is exposed to the three lactogenic hormones, insulin, cortisol, and prolactin, to greater induce differentiation (Emerman et al., 1977). Research has been conducted claiming that prolactin can only help induce differentiation if the cells are grown on the appropriate extracellular matrix with a laminin-rich basement membrane (Katz & Streuli, 2007). In addition, MEC are frequently grown on substratum, a complex extracellular matrix, such as collagen, which can be a regulator of MEC function in culture (Katz & Streuli, 2007; Emerman & Pitelka, 1977). Using a flexible collagen substratum is vital for the development of the cellular morphology and the ability to synthesis and secrete milk proteins. The substratum allows separation into two compartments, which generates a three-dimensional system. This system subsequently simulates MEC *in vivo*, creating polarized cells (Sakamoto et al., 2005). Talhouk et al. (1993) demonstrated that MEC synthesized and secreted α-casein at high levels when differentiated on collagen gel matrix. Also, it was reported that the thickness, as well as detachment of the collagen gel affected the expression of α-casein (Talhouk et al., 1993). The expression increased when the collagen was detached at day 6, after the cells had formed cell sheets, and on thicker collagen gels, as well. These findings suggest that our attempt to differentiation MEC may not have been successful due

to the use of plastic dishes. Additional work is needed using a collagen matrix to determine if our lactogenic hormones and/or GH can induce differentiation of the MEC using a collagen matrix. Future research needs to be performed on primary bovine MEC. The next step to pursue is the use of substratum, specifically collagen gel matrix to culture or mature MEC. Utilizing a collagen gel matrix may be a more ideal condition to differentiate MEC. Thus, causing the mRNA expression of α-casein to increase, indicating differentiation has occurred, by creating a three-dimensional structure that is more similar to an *in vivo* model and creating polarized cells (Talhouk et al., 1993).

Conclusion

Primary bovine MEC were successfully isolated from lactating cows post-slaughter and cultured on plastic cell culture dishes. The monolayer cells displayed the cobblestone organization. Using the α-casein gene as an indicator of differentiation, the expression of α-casein gene in primary MEC cultured on plastic was low or not detectable; suggesting that differentiation into a more mature cell was not successful. The addition of GH did not increase α-casein expression or further differentiate MEC. Further studies are needed to identify optimal conditions to differentiate primary bovine MEC in culture. Based on previous work (Talhouk et al., 1993), use of a collagen matrix may be needed for optimal differentiation of primary MEC in culture.

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