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The Expression of T-Box (Tbx3) in the Bovine Mammary Gland

Maria L. Procopio

B.S., University of Rhode Island, 2009

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APPROVAL PAGE

Master of Science Thesis

The Expression of T-Box (Tbx3) in the Bovine Mammary Gland

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2011

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ABBREVIATIONS

ABI	Applied Biosystems
ANOVA	Analysis of Variance
BMP	Bone Morphogenic Protein
bST	Bovine Somatotropin
DMEM	Dulbecco's Minimum Essential Media
DNA	Deoxyribose Nucleic Acid
ELF5	Ets Transcription Factor-5
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GATA	GATA Binding Protein
GH	Growth Hormone
GRF	Growth Hormone Releasing Factor
HBSS	Hank's Balanced Salt Solution
IGFBP3	Insulin-Like Growth Factor Binding Protein-3
IGF-I	Insulin-Like Growth Factor-I
Lef	Lymphoid Enhancer Factor
LS Means	Least Square Means
MAC-T	Bovine Mammary Alveolar Cell Line
MEC	Mammary Epithelial Cell
mRNA	messenger Ribonucleic Acid
P19-ARF	Promotor 19-Alternate Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PRF DMEM	Phenol Red Free Dulbecco's Minimum Essential Media
RPS15	Ribosomal Protein Subunit 15
RT	Reverse Transcription
SF	Serum Free
Shh	Sonic Hedgehog
STAT5	Signal Transducer and Activator of Transcription-5
Tbx2	T-Box 2
Tbx3	T-Box 3
TGF	Transforming Growth Factor
UMS	Ulnar Mammary Syndrome

Development of the bovine mammary gland is a complex process that is regulated by several hormones, growth factors and transcription factors including but not limited to growth hormone (GH), insulin-like growth factor (IGF)-I and T-box (Tbx)2 and 3. Tbx2 and Tbx3 are transcription factors required for mammary gland development in humans and known to regulate cell cycle. In addition, there is recent evidence that GH increases expression of Tbx3 in osteoblasts independent of IGF-I. Based on these findings, we hypothesized GH and IGF-I will increase Tbx2 and Tbx3 expression in bovine mammary epithelial cells (MEC), the cell responsible for milk production. To test our hypothesis, MAC-T cells (MEC line) were treated with GH at 100 (GH100) or 500 (GH500) ng/mL or IGF-I at 100 (IGF100) or 200 (IGF200) ng/mL for 24 and 48 hours. As determined by real-time RT-PCR, we did not observe a change in Tbx3 expression in cells treated with GH ($P \geq 0.74$). However, both IGF-I treatments increased Tbx3 expression ($P \leq 0.03$). Surprisingly, expression of Tbx2 was not detectable in MEC cells; however it was expressed in mammary fibroblast cells. In fibroblast cells treated with GH500 and IGF200, we did observe a change in Tbx2 or Tbx3 expression ($P \geq 0.76$). In conclusion, IGF-I regulates Tbx3 expression in bovine MEC and Tbx2 and Tbx3 expression are cell type specific.

REVIEW OF LITERATURE

Introduction

Within the United States dairy industry, maintaining udder health and maximizing efficiency of production is essential for a dairy operation to be profitable. In 2009, the United States dairy industry generated $24.3 billion dollars in revenue from dairy sales (USDA: NASS, 2009). However, with rising costs of feed, it is becoming increasingly more expensive to produce milk. For example, between 2005 and 2007 there was a 110% increase in corn prices and a 59% increase in alfalfa prices (USDA: NASS, 2010). When feed prices increase significantly, it not only increases the cost of producing milk, but also reduces the profit made by the producer. As a result, farmers need to alter their management practices to maximize efficiency in an effort to maintain a profitable dairy operation. Efficiency of production can be impacted in several ways, one of which is animal health.

In 2006, 23% of all dairy cattle culled from herds suffered from either mastitis or other mammary gland abnormalities. An additional 16.1% of animals culled were due to poor production (USDA: APHIS, 2007). This demonstrates that poor udder health and function is accounting for 40% of the animal losses within the dairy industry and therefore is an issue within dairy production. In an effort to improve efficiency of production and udder health, research has focused on key elements within the mammary gland in order to better understand mammary gland function. The data obtained from these studies can be used to improve udder health and milk production which in turn will benefit the dairy industry. Specifically, our research focuses on understanding the mechanisms involved in regulating mammary gland development and its response to key

hormones and growth factors. This literature review will focus on general mammary gland anatomy, development and function, as well as, discuss how the function of the mammary gland is regulated in response to specific hormones, growth factors, and transcription factors.

Bovine Mammary Gland Anatomy

Understanding the structure of the gland is necessary not only in production agriculture but in mammary research. The bovine mammary gland can be divided into three segments: 1) The mammary fat pad, 2) The stroma, and 3) The parenchyma (Tucker, 1987). Each segment of the mammary gland contributes to the overall function of the gland which is to produce milk and its components (protein, fat and carbohydrates).

The mammary fat pad is a large deposition of fat on the dorsal aspect of the bovine mammary gland and infiltrates into the mammary stroma. The mammary fat pad plays a role in the ductile branching that occurs in mammary development, especially during embryonic stages (Watson and Khaled, 2008). The stroma of the mammary gland contains the support elements and connective tissue that surrounds the parenchyma. The stroma is comprised of several cell types (fibroblasts, adipocytes, and myoepithelial cells), collagen, lymphatic vessels and a vascular system (Akers, 2002; Connor et al., 2007). In addition to providing support for the parenchyma, stromal tissue facilitates the paracrine actions of growth factors that are required for proper mammary development (Akers, 2006; Flint and Knight, 1997). The functions and roles of these growth factors will be discussed in more detail later in the review. While the mammary fat pad and

stroma play both supportive and developmental roles within the mammary gland they are not responsible for the production of milk.

The parenchyma is the portion of the mammary gland that is considered to be the functional aspect of the gland as this region contains the mammary epithelial cells (MEC). Mammary epithelial cells are responsible for producing milk which includes milk proteins, carbohydrates and fats (Akers, 2002). As a result, MEC are the focus of the majority of mammary gland research in cattle (De Vries et al., 2010). In the mammary gland, there are two main types of epithelial cells; luminal and basal epithelial cells (Watson and Khaled, 2008). The basal epithelium is comprised of the myoepithelial cells which aid in milk ejection from the gland. The luminal epithelial cells are comprised of two cell types: 1) ductile epithelial cells and 2) secretory epithelial cells (Watson and Khaled, 2008). The secratory epithelial cells line the walls of the alveolus and are responsible for milk production (Watson and Khaled, 2008). Each individual alveolus is grouped into lobules which are comprised of many alveoli. These cells are the focus of our research and the literature review will focus on growth and development of these cells from this point forward.

Mammary Gland Growth and Development

Mammary development occurs during four stages in mammals: 1) embryonic, 2) pubertal (pre and post), 3) pregnancy, and 4) lactation (Akers, 2002; Tucker, 1987; Platonova et al., 2007). Within these stages, physiologic and anatomical changes will occur that are specific to that phase of mammary development.

Embryonic Mammary Development

Mammary development in the bovine commences with the formation of the mammary band which occurs at day 30 of embryonic development (Akers, 2002). The formation of the mammary band originates from the ectoderm germ layer of the embryo. This occurs with the aid of several transcription factors and cell signals that are present at different points throughout embryonic mammary development (See Tbx2 and Tbx3 in Mammary Gland Development). In the absence of these factors, mammary gland formation will arrest or fail to occur. From the structure of the mammary band, the mammary bud will form by day 40 of embryonic development in cattle (Akers, 2002). Embryonic mammary development will continue and eventually cease once the rudimentary gland has been established (Hens and Wysolmerski, 2005; Watson and Khaled, 2008).

Pubertal Mammary Development

Mammary development that occurs during puberty is synonymous with ductile development and branching as opposed to lobulo-alveolar development which does not occur until pregnancy (Tucker, 1987). In regards to mammary tissue growth, the stromal elements of the mammary gland grow prior to puberty. In addition to stromal tissue growth, allometric growth of the duct system of the mammary gland occurs just prior to the onset of puberty (Tucker, 1987). Mammary development during this stage is facilitated through the production of ovarian steroid (i.e., estrogen) resulting from the onset of ovarian development (See Hormones and Growth Factors section). Allometric

growth of the gland will continue through the initial estrus cycles after which the mammary gland will continue isometric growth until the animal becomes pregnant (Tucker, 1987).

Gestational and Lactational Mammary Development

Mammary development during pregnancy is synonymous with the establishment of the lobulo-alveolar system. In addition to the development of these mammary structures, several other changes occur in the anatomy of the mammary gland at this stage. Prior to pregnancy the stroma is the more predominate tissues in the mammary gland. During pregnancy, mammary ducts will elongate and there will be an increase in mammary cell proliferation and alveoli formation. As result, these alveoli will replace the mammary fat pad and "rearrange" the stromal elements of the mammary gland resulting in the parenchyma becoming the dominate tissue type (Tucker, 1987). This change in tissue predominance is necessary for the mammary gland to prepare for lactation. As observed during puberty, allometric growth of the mammary gland will occur. This trend in growth will continue, resulting in a 25% increase in the mass of the parenchyma with every month of gestation (Swanson and Poffenbarger, 1979; Tucker, 1987).

Within the mammary gland, proliferation of MEC is needed in order to maximize milk production. Cellular proliferation can be increased during pregnancy and lactation through the action of hormones and growth factors (i.e., IGF-I; see Hormones and Growth Factors section). Mammary epithelial cell proliferation will eventually slow which correlates with the stage of lactation when milk yield begins to decrease (Tucker, 1987).

Mammary Epithelial Cell Development

On the cellular level, MEC must also change cytologically in order to be capable of milk production. Organelles within the cell, specifically the Golgi apparatus and the endoplasmic reticulum, are responsible for the production of milk components. Therefore these organelles must undergo significant changes during pregnancy and lactation. In non-pregnant cattle, the Golgi apparatus and endoplasmic reticulum (ER) are underdeveloped. This is in contrast to late gestation and lactation in which the ER will produce milk proteins and fatty acids while the Golgi apparatus will synthesize lactose, process casein proteins and package cellular products (Akers, 2002; Oka and Yoshimura, 1986; Mills and Topper, 1970). In order for these cytological changes to occur there must be differentiation of MEC. Differentiation of the MEC requires the presence of several hormones, two of which are glucocorticoids and prolactin. Glucocorticoids are needed to develop the rough ER and prolactin is needed for the maturation of the Golgi apparatus as well as the secretory vesicles (Akers, 2002; Mills and Topper, 1970). Once MEC have proliferated and differentiated, with the aid of hormones and growth factors, the mammary gland is then capable of lactation.

Regulation of Mammary Development

The development of the mammary gland is a complex process that consists of many stages. Therefore, it is not surprising that there is a considerable amount of regulation that occurs throughout mammary gland development. Key regulatory factors of mammary development include 1) hormones, 2) growth factors, and 3) transcription

factors. Understanding the roles of these regulatory mechanisms in MEC function allows for a better understanding of the mammary gland. These regulatory factors will be the focus of the remainder of this literature review.

Hormones and Growth Factors

Estrogen

The ovarian steroid, estrogen, aids in the growth of the mammary gland by facilitating ductile system development and assisting in the establishment of the lobulo-alveolar system during pregnancy (Connor et al., 2007). Like all hormones and growth factors, receptors must be present on a specific tissue type in order for the action of the hormone to be carried out. Within mammary tissue, estrogen receptors are present on the ductile MEC, which coincides with the role of estrogen in ductile development (Capuco et al., 2002). One of the ways that estrogen facilities ductile development within the mammary gland is by increasing proliferation of MEC. This was observed in studies where animals were treated with exogenous estrogen and an increase in ductile MEC was observed (Woodward et al., 1993; Connor et al., 2007).

In addition, growth of the mammary gland in the absence of estrogen has been evaluated through the use of overiectomized animals. In 2007, Connor et al, evaluated mammary development in overiectomized heifers compared to a group of control prepubertal heifers (Connor et al., 2007). It was observed that in overioectomized heifers there is a decreased amount of epithelial cell proliferation in the mammary gland. However within this study, mammary development was restored in ovariectomized animals when exogenous estrogen was administered (Connor et al., 2007), thus

demonstrating that estrogen is needed during prepubertal and pubertal mammary gland development.

Prolactin

The hormone prolactin also plays a critical role during mammary development. Secreted by the anterior lobe of the pituitary gland, prolactin is responsible for inducing MEC differentiation (Riley et al., 2009). Prolactin regulates several transcription factors (i.e., STAT-5 and ELF-5) during differentiation and facilitates the production of casein, a major milk protein, in differentiated cells. Prolactin facilitates these changes by interacting with specific cell signaling pathways (i.e., Jak2-Stat5: Riley et al., 2009) which will be discussed in the transcription factor portion of this literature review.

Growth Hormone

Growth hormone has been demonstrated to have a positive effect on lactation in cattle and is commonly used in commercial dairy operations to improve production. Growth hormone, also known as bovine somatotropin (bST), increases milk yield in the bovine by partitioning nutrients to the mammary gland for use in milk production (Bauman, 1999). In regards to mammary development, studies in rodents have determined that GH is needed for proper growth of mammary ducts during puberty and the development of the lobulo-alveolar system during pregnancy (Kleinberg and Ruan, 2008).

Growth hormone can act on target tissues through two mechanisms: 1) indirect action and 2) direct action. Within both mechanisms, GH is released from the anterior

lobe of the pituitary gland in response to growth hormone releasing factor (GRF). Alternately, GH release is inhibited through the actions of somatostatin (Roelfsema and Clark, 2001; Renaville et al., 2002). When GH acts indirectly, GH will bind receptors present on tissue (bone, adipose, and muscle, liver) and cause the production and release of the somatomedian, IGF-I. Insulin-like growth factor-I will in turn carry out the actions of GH on specific tissues. It is through GH binding to the liver and causing production of IGF-I that GH acts indirectly on mammary tissue (Kleinberg and Ruan, 2008; Cohick, 1998). Growth hormone can also act directly on target tissues such as the bone, muscle, and adipose (Le Roith et al., 2001; Govoni et al., 2006). In order for there to be direct action of GH on a tissue, functional GH receptors (GHR) must be present (Le Roith et al., 2001). Unlike the aforementioned tissues, it is questionable as to whether a functional GHR is present within the mammary gland (Akers, 2002; Cohick, 1998).

Studies have demonstrated that GHR mRNA and receptor protein are present in the bovine mammary gland (Plath and Gabler, 2001). In contrast, early experiments which conducted ligand binding assays were not able to detect binding of GH to the GHR of mammary tissue (Akers, 2002). It is hypothesized that GH is able to act directly on the stromal elements of the mammary gland (Flint and Knight, 1997). Specifically, the fibroblasts within the stroma are responsive to GH treatment (Flint and Knight, 1997). In response to GH, fibroblast cells will produce IGF-I. This locally produced IGF-I will act on the surrounding tissue in a paracrine action and it is believed that this IGF-I plays a critical role in the mammary gland development (Baumrucker and Erondu, 2000; Flint and Knight, 1997). This also suggests that the stroma of the mammary gland plays a role beyond a structural support element.

Insulin-Like Growth Factor-I

Insulin-like growth factor-I has been found to regulate cell cycle, increase proliferation, and prevent apoptosis of MEC (Cohick, 1998). The mechanisms through which IGF-I regulates cell cycle was evaluated in mice where treatment with IGF-I increased several cyclin molecules, in particular cyclin D1 and cyclin E (Stull et al., 2004). These molecules are needed within the G1 phase of the cell cycle. Within this phase, the cell increases in size and prepares for the S phase, where DNA replication will occur (Stull et al., 2004). A relationship has been found between the concentrations of IGF-I in the blood of the animal and stage of mammary development. Specifically, IGF-I concentrations increase during pregnancy and then decreases immediately following parturition (Baumrucker and Erondu, 2000). However, as the lactation period advances IGF-I concentrations in the blood will continue to increase (Baumrucker and Erondu, 2000). The increase in IGF-I concentrations during lactation could be a way through which apoptosis of existing MEC is prevented, thus sustaining lactation for a prolonged period of time.

The need for IGF-I for mammary development has been demonstrated in several experiments. Studies using IGF-I knockout mice have demonstrated that animals deficient in IGF-I lack proper mammary development which is not restored when treated with GH (Kleinberg and Ruan, 2008). These results suggest that IGF-I may be mediating the actions of GH on the mammary gland during development as well (Kleinberg and Ruan, 2008).

In summary, hormones and growth factors are key regulators of the different stages of mammary development. In particular, they facilitate growth of the different

structural elements of the gland (ductile and lobuloaleveolar system) as well as the development and differentiation of MEC. However there is also another subset of regulatory elements that are needed throughout mammary gland development and are directly involved in gene expression. These elements are transcription factors.

Transcription Factors

One of the ways in which hormones and growth factors can facilitate mammary growth and development is through transcription factors. Three transcription factors are needed during mammary development are GATA binding protein-3 (GATA-3), signal transducer and activator of transcription-5 (STAT-5), and ets transcription factor-5 (ELF-5) (Oakes et al., 2006; Siegal and Muller, 2010).

GATA-3

GATA-3 is a transcription factor that has been found to be involved in luminal mammary cell type specification and maintains differentiated mammary epithelial cells (Siegel and Muller, 2010). In mice, the use of a cre/lox system to conditionally disrupt GATA-3 resulted in a knockdown of GATA-3 function in the mammary gland. In these mice, the MEC lacked the ability to differentiate. As a result, these animals demonstrated improper lobulo-alveolar development and did not lactate or produce milk proteins like the wild type animals (Siegal and Muller, 2010). This demonstrates that GATA-3 is necessary for differentiation of MEC and proper mammary development for lactation to occur.

Signal Transducer and Activator of Transcription-5 (STAT-5)

Another transcription factor that regulates mammary development is STAT-5. Signal transducer and activator of transcription-5 expression is induced by prolactin and is responsible for aiding in alveolar development, establishing cellular polarity, milk protein production and cell-cell interactions (Oakes et al., 2006). By conditionally knocking-down STAT5A and STAT5B gene expression, researchers observed a decrease in MEC differentiation in pregnant animals and a failure of these animals to lactate (Siegel and Muller, 2010). In STAT5 knockout animals there was also a decrease in the number of luminal alveolar progenitor cells (Siegal and Muller, 2010). However, if expression of this gene was restored, the mice resumed the normal mammary phenotype (Siegal and Muller, 2010). This demonstrates the role of STAT-5 in establishing differentiated mammary epithelial cells.

Ets Transcription Factor-5 (ELF-5)

Ets transcription factor-5, like GATA-3 and STAT5, is responsible for controlling alveolar development. This transcription factor, like GATA-3, plays a role in switching the mammary gland from a state of cellular proliferation to cellular differentiation during pregnancy. Ets transcription factor-5 expression is induced by prolactin. This was determined through the use of prolactin-receptor deficient mice (Oakes et al., 2006). Similar to the other transcription factors, a knockdown of ELF-5 gene expression results in failure of luminal mammary epithelial cells to differentiate (Siegal and Muller, 2010). These aforementioned transcription factors are an example of some of the downstream targets of hormones and growth factors that regulate mammary development. By altering

the expression of these transcription factors regulation of mammary development is able to occur.

T-Box Genes and the Mammary Gland

Transcription factors are not just present during lactation and pregnancy. The establishment of the rudimentary mammary gland during embryonic mammary development also relies on transcription factors. Two of these transcription factors are T-box (Tbx) 2 and Tbx3. These transcription factors are examples of Tbx genes, which are a family of transcription factors that were first discovered in 1927. Today, 18 Tbx genes have been identified and can be categorized into five subfamilies (T, Tbx 1, 2, 6 and Tbr1; Naiche et al., 2005). These genes are expressed in several different tissue types and are critical during embryonic development when these transcription factors aid in the establishment of germ layers and organs (Abrahmas et al., 2010).

All Tbx transcription factors contain a "T-box" DNA binding domain also known as the Tbx binding element. The Tbx binding element is 180 amino acids in size and is highly conserved between all members of the family (Naiche et al., 2005; Coll et al., 2002). Both Tbx2 and Tbx3 contain this highly conserved domain however there are differences within their structure. In contrast to other Tbx family members Tbx2 and Tbx3 regulate development by binding DNA and repressing transcription (Rowley et al., 2004). In order to do so, Tbx2 and Tbx3 contain specific structural domains, called repressor domains. Tbx2 contains two repressor domains located near the N-terminal domain (amino acids 1-53) and carboxy terminal domain (amino acids 529 -573) of the protein (Abrahams et al., 2010). In contrast to Tbx2, Tbx3 contains only one repression domain between amino acids 567 to 623 located near the carboxy terminal domain

(Weiwei Fan et al., 2004). Despite variation in protein structure, the DNA sequence encoding for the Tbx2 and Tbx3 transcription factors are 95% homologous to one another (Coll et al., 2002). Consequently, these transcription factors are considered to be very similar to one another in both form and function.

Tbx2 and Tbx3 are responsible for regulating the expression of many different genes during embryonic and postnatal development. One of the genes that these transcription factors regulate is the p19-Alternate Reading Frame (P19ARF), promoter also known as p14-ARF in humans (Carlson et al., 2002; Platonova et al., 2007; Rowley et al., 2004). This alternate reading frame is involved in the P19ARF-Mdm2-P53 pathway which is responsible for controlling cell cycle (Carlson et al., 2002; Platonova et al., 2007; Rowley et al., 2004). When Tbx2 and Tbx3 represses transcription of the p19ARF, there is an increase in cellular proliferation, inhibition of cellular senescence, and a decrease in cellular apoptosis (Platonova et al., 2007).

By inhibiting cellular senescence, which is a state in which cellular growth is arrested (Collado et al., 2007); Tbx2 and Tbx3 alter cellular growth. This is believed to be one of the mechanisms through which Tbx2 and Tbx3 are involved in tumerogenisis within the mammary gland (Rowley et al., 2004). The implications of what is known about Tbx2 and Tbx3 in inhibiting senescence and how it translates to our research will be discussed later in the rationale and hypothesis section.

Role of Tbx3 and Tbx2 in the Mammary Gland

Embryonic Mammary Development

Tbx3 has been found to play a key role in the formation of the mammary placodes from the mammary line. These placodes will eventually become the parenchyma of the mammary gland. In mice, Tbx3 is expressed at day 10.5 gestation, which is typically the time in which the mammary placodes are established. It is also thought that Tbx3 plays an active role in the formation of the mammary gland from the cells of the ectoderm (Hens and Wysolmeske, 2005). In humans, a haploinsufficency of Tbx3 is not lethal but results in Ulnar Mammary Syndrome (UMS). This disorder is characterized by mammary gland hypoplasia which further demonstrates the critical role of Tbx3 in mammary gland development (Carlson et al., 2002; Platonova et al., 2007).

As stated previously, Tbx2 is a repressor much like Tbx3; however Tbx2 is not expressed within the epithelial buds of the mammary gland. Instead, Tbx2 is expressed in the mesenchyme of the developing embryonic mammary gland (Chapman et al., 1996). Tbx2 and Tbx3 function independently of each other and Tbx2 has been found to play a role in the regulation of adhesion molecules such as cadherins and integrans during embryonic development (Abrahams et al., 2010; Chen et al., 2001). As a result, it has been hypothesized that Tbx2 could play a role in the migration as well as invagination of the cells when the mammary placodes are being formed (Hens and Wysolmeske, 2005). Within our research we want to determine if Tbx2 is expressed within the stromal tissue of the mammary gland of adult cattle and if its function is similar to Tbx3 within this tissue type.

Role of GH and IGF-I on Tbx gene expression

Within the mammary gland GH, IGF-I, and the transcription factors Tbx2 and Tbx3 are important regulators of mammary gland development and function. However, there is limited information on the interactions of these factors and their role in postnatal mammary gland development and function. Recent research has demonstrated that GH regulates Tbx3 gene expression in bone (Govoni et al., 2006). Within this study, GH deficient *lit/lit* mice were treated with exogenous GH and gene expression of Tbx3 was quantified (Govoni et al., 2006). In treated animals, Tbx3 expression significantly increased within bone tissue. Similar results were observed in a MC3T3-E1 osteoblast cell line when cells were treated with 100 ng/mL of GH. To determine if the increase in Tbx3 gene expression was mediated directly by GH, cells were pre-treated with IGFBP-4, a known inhibitor of IGF-I, and a similar increase in Tbx3 was observed. These findings suggest that the increase in Tbx3 gene expression is mediated directly by GH, independent of IGF-I (Govoni et al., 2006). Knowing that GH and IGF-I are needed during mammary development and that GH alters Tbx3 expression in bone, one of the objective of our research was to evaluate the effect of these growth factors on Tbx3 gene expression within the mammary gland.

RATIONALE AND HYPOTHESES

It is well established that hormones, growth factors, and transcription factors regulate mammary gland development. Two of these transcription factors are Tbx2 and Tbx3. Tbx2 and Tbx3 are expressed in the early stages of embryonic development and are responsible for establishing the early structures of the mammary gland. Little is known about the postnatal role, gene expression and regulation of Tbx2 and Tbx3 in the mammary gland. The majority of what is known about these genes in postnatal mammary development is often pertaining to cancer rather than their function in healthy mammary tissue (Rowley et al., 2004). In regards to the bovine mammary gland, to our knowledge, the role of these genes in bovine mammary development is not known and has yet to be evaluated.

It is also well known that mammary development is regulated by two growth factors, GH and IGF-I as previously discussed in the literature review. Recent research also suggests that the somatotropic axis may regulate Tbx3 expression in other tissues. Knowing that Tbx2 and Tbx3 are essential for mammary development and that GH and IGF-I are involved in mammary development we developed the following hypotheses:

1) GH and IGF-I will increase Tbx3 expression in MEC.
2) GH and IGF-I will increase Tbx2 expression in MEC.

MATERIALS AND METHODS

Mammary Tissue

Mammary tissue for RNA extraction was obtained from mammary parenchymal tissue from lactating cattle at slaughter. Tissues were excised and snap frozen in liquid nitrogen and stored at -80 ºC.

Cell Culture

MAC-T cells used in experiments were generously provided by W. Cohick (Rutgers University) and M. Akers (Virginia Polytechnic Institute and State University). Primary MEC were established using a protocol from Wellnitz and Kerr, 2004. Mammary cells were obtained from lactating dairy cows at slaughter. Immediately following slaughter, the mammary gland was removed from the animal and tissue was aseptically removed from the mammary gland and transported back to lab on ice in Hank's Balanced Salt Solution (HBSS; Invitrogen, Carlsbad,CA), 5 µg/mL Fungizone (Sigma-Aldrich, St. Louis, MO), 20 µg/mL Penicillin (Thermo Fischer Scientific, Waltham, MA), 20 µg/mL Streptomycin (Thermo Fischer Scientific, Waltham, MA) , and 25 µg/mL Gentamycin (Sigma-Aldrich, St. Louis, MO). When culturing primary MEC, culture contamination with fibroblasts was common. To remove this cell type selective trypsinization was performed and the fibroblasts were cultured separately.

When subculturing cells, MEC were grown to 80% confluence in DMEM (Invitrogen, Carlsbad,CA) supplemented with 10% FBS (Thermo Fischer Scientific, Waltham, MA), 10 µg/ml Penicillin/Streptomycin, 0.1 µg/mL Fungizone, 25 µg/mL

Gentamycin and 5 µg/mL Bovine Insulin (Sigma-Aldrich, St. Louis, MO). Cells were incubated at 37º C/ 5% CO_2. MACT cells and fibroblasts were cultured in the same conditions as MEC.

For experiments, MAC-T and MEC were plated at a density of 100,000 cells/well in Phenol Red Free (PRF) DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS. When plating fibroblasts, 150,000 cells/well were plated. At 70% to 80% confluence, all cells were serum deprived for 24 hours in PRF/Serum-Free DMEM supplemented with 0.2% BSA (Sigma Aldrich, St. Louis, MO) and 30nM sodium selenite (Sigma-Aldrich, St. Louis, MO). After serum depravation, cells were rinsed with PBS and treated with either control media containing 0.02% BSA and 30 nM sodium selenite (Sigma-Aldrich, St. Louis, MO) or control media plus 100 ng/mL of bovine GH, 500 ng/mL bovine GH, 100 ng/mL human IGF-I or 200 ng/mL human IGF-I (National Hormone and Peptide Program, Torrance, CA) for 24h or 48h

RNA extraction

For cell cultures, media was removed from cells and cells were rinsed with PBS prior to addition of 1mL TriReagent (Sigma-Aldrich, St. Louis, MO). For mammary tissue, tissue was ground in a mortar cooled with liquid nitrogen prior to the addition of TriReagent. RNA was extracted using a Qiagen Mini Kit according to the manufacturer`s protocol (Qiagen, Valencia, CA). Genomic DNA was removed from samples through the use of a DNA Free Kit (Ambion, Foster City, CA). Samples were diluted to a concentration of 30 ng/µL using a Nanodrop spectrophotometer. Quality of RNA was determined by using Experion analysis (BioRad, Hercules,CA).

Reverse Transcription (RT) - PCR

RT-PCR was performed using 300 ng total RNA with OligoDT primer (Ambion) and master mix containing 5.5 µL of 5x Buffer (Invitrogen, Carlsbad, CA), 1 µL dNTP (Promega, Madison, WI), 2 µL DTT and 0.5 µL Superscript II (Invitrogen, Carlsbad, CA) for a total reaction volume of 20 µL. The samples and master mix underwent a standard reverse transcription protocol starting at 70 ºC for 10 min, 4ºC for 20 min, 37ºC for 3 min, 42ºC for one hour, 4ºC for 3 min, 90ºC for 2.5 min. Resulting cDNA was then diluted using 80 µL of Nuclease Free Water (Thermofisher Scientific, Waltham, MA) and stored at -20ºC.

Real-time RT-PCR

Primers were designed using Primer3 and NCBI BLAST. DNA oligos were synthesized by Integrated DNA Technologies (Coralville, IA; Table 1). The endogenous control gene selected was ribosomal protein subunit (RPS) 15 (Bionaz and Loor, 2007).
Real-time RT-PCR was performed using Power SybrGreen Master Mix (Invitrogen, Carlsbad, CA) and the ABI 7900 Fast Real-time PCR machine (Applied Biosystems, Foster City, CA). The total volume of the reaction mixture was 25 µL containing 5 µL of cDNA, 3 uL of nuclease free water, 1 µl each of 10 nM forward and reverse primer and 10µL of Sybergreen. For the Tbx3 master mix only 0.5µL of each forward and reverse primer was used along with 4 µL of nuclease free water. PCR cycling conditions used were a standard cycle with a starting stage of 50 ºC for 2 min and 95 ºC for 10 min. The PCR entered the second stage at 95 ºC for 15 sec and 60 ºC for 1 min for 40 cycles. The third and final stage consisted of 95 ºC for 15 sec and 60ºC for 15 sec with a 2% ramp to

95 ºC for 5 min. $^{\Delta}$Ct values were obtained and these values were used to calculate the $^{\Delta\Delta}$Ct values to determine changes in gene expression (Livak and Schittgen, 2001). Changes in gene expression are expressed as fold changes from the control treatments.

PCR

PCR was used as a method to optimize primers and visualize gene expression. Primers used were the same as those that were described in the real time RT-PCR portion of the methods section. The total volume of the reaction mixture was 25 µL containing 5 µL of cDNA, 5.5µL of nuclease free water, 12.5 µL of Go Taq Green Master Mix (Promega, Madison, WI) and 1.0 µL of forward and reverse primers. PCR cycling conditions with a starting stage at 95ºC for 3 min. The thermocycling parameters for the second stage were 94ºC for 30 sec, 60ºC for 1 min, 72ºC for 1 min repeating for 40 cycles concluding at 72ºC for 5 min. PCR products were then run in a 2% agarose gel and visualized using UV light.

Statistical Analysis

Data were analyzed by ANOVA using SAS program (Version 2.9, SAS Institute Inc, Cary, NC, USA). Data were evaluated by comparing control treated cells verses hormone treated cells. The differences between the least square means of control vs. hormone treated cells were used to determine P values. Statistical significance was considered at $P \leq 0.05$

RESULTS

IGF-I stimulates Tbx3 expression in bovine MEC

At both 24h and 48h time points we did not observe an effect of GH treatment on Tbx3 gene expression (Figure 1; $P \geq 0.74$). However, IGF-I at both doses increased Tbx3 expression significantly from controls (Figure 2; $P \leq 0.03$) at 24h but not at 48h (Figure 2; $P \geq 0.17$). To determine if IGF-I treatments were effective at both time points, IGFBP3 mRNA expression, which is known to increase in response to IGF-I, was determined in treated and control cells. A significant increase in IGFBP3 mRNA was observed at both time points for both doses (Figure 3; $P \leq 0.001$), demonstrating that IGF-I treatment was effective.

Tbx3, but not Tbx2, is expressed similarly in the mammary gland, fibroblasts and MEC.

To evaluate our second hypothesis we determined Tbx2 gene expression in MAC-T cells that were treated with either GH or IGF-I. We were unable to detect expression of Tbx2 in the MAC-T cell line. To determine if this lack of expression was specific to the MAC-T cell line, we isolated primary MEC and evaluated Tbx2 expression. Similarly, Tbx2 expression was not detectable (Figure 4 and Figure 5). Based on our preliminary findings that Tbx2 is expressed in mammary gland tissue (Figure 4 and Figure 5) and previous findings that Tbx2 is highly expressed in lung fibroblasts (Teng et al., 2007), we hypothesized that Tbx2 expression may be in mammary fibroblasts. Fibroblasts were then isolated from the stroma of the mammary gland to detect Tbx2 expression. We observed that Tbx2 is highly expressed in fibroblasts when compared to the mammary gland (Figure 4 and 6 ; $P = 0.01$). To determine if Tbx3 was also differentially expressed we

evaluated Tbx3 gene expression within the mammary gland and all cell types. We determined that all cell types and mammary tissue expressed Tbx3 mRNA and gene expression did not vary significantly from the expression in the mammary gland (Figure 6; $P \geq 0.14$).

GH and IGF-I treatment did not alter Tbx2 and Tbx3 gene expression in fibroblasts

Based on our findings that Tbx2 expression in the bovine mammary gland was primarily in fibroblasts, these cells were used as the model to determine the effect of GH and IGF-I on Tbx2 gene expression. In addition, we wanted to determine if treatment with GH or IGF-I would have an effect on Tbx3 expression in these cells. We did not observe an effect of GH or IGF-I treatment on either Tbx2 or Tbx3 gene expression (Figure 7; $P \geq 0.76$). To determine if the cells were responsive to treatment, IGFBP3 gene expression was evaluated. As expected, when cells were treated with IGF-I, IGFBP3 expression increased 63-fold from control fibroblasts cells (Figure 7; P value < 0.0001).

FIGURE LEGENDS

Figure 1: GH treatment did not alter Tbx3 gene expression. All MAC-T cells were cultured in control media (0.02% BSA) and treated with 100 ng/mL GH (GH100), 500 ng/mL GH (GH500) or without GH (control) for 24h or 48h. Data are presented as fold change from the control and expressed as mean ± SE. No significant differences were observed at $P \geq 0.74$. GH = growth hormone, IGF-I = insulin-like growth factor-I, h= hours, SE= standard error, BSA = bovine serum albumin.

Figure 2: IGF-I treatment increased Tbx3 expression. All MAC-T cells were cultured in control media (0.02% BSA) and treated with 100 ng/mL IGF-I (IGF100), 200 ng/mL IGF-I (IGF200) or without IGF-I (control) for 24h and 48h. Data are presented as fold change from the control and expressed as a mean ± SE. * indicates $P \leq 0.03$. IGF-I = insulin-like growth factor-I, h= hours, SE= standard error, BSA = bovine serum albumin.

Figure 3: Treatment with IGF-I significantly increased IGFBP-3 expression in MAC-T cells. IGFBP-3 expression was quantified for cells treated with 100 ng/mL IGF-I (IGF100), 200 ng/mL IGF-I (IGF200) or without IGF-I (control) for 24h and 48h. Data are presented as fold change from the control and expressed as a mean ± SE. * indicates $P \leq 0.001$. IGF-I = insulin-like growth factor-I, h= hours, SE= standard error, BSA = bovine serum albumin, IGFBP3 = insulin-like growth factor binding protein-3.

Figure 4: Tbx2 expression is present in the mammary gland and fibroblasts. PCR products from mammary gland tissue (MG), MAC-T cells, MEC cells, and fibroblasts were run in an 2% agarose gel at 130 V. Bands were visualized using UV light. RSP15 = Ribosomal protein subunit 15, MG = mammary gland, MEC= mammary epithelial cells, UV = ultraviolet, PCR = polymerase chain reaction.

Figure 5: Tbx2 mRNA is expressed in fibroblasts in the bovine mammary gland. Data are presented as fold change from the control and expressed as a mean ± SE. * indicates $P \leq 0.02$. ND = Not detectable, MEC = mammary epithelial cells, MG = mammary gland.

Figure 6: Tbx3 is expressed similarly in all cell types in the bovine mammary gland. Data are presented as fold change from the MG and expressed as a mean ± SE. A significant different was not observed at $P \geq 0.13$. MEC = mammary epithelial cells, MG = mammary gland.

Figure 7: GH and IGF-I treatment did not alter Tbx2 or Tbx3 expression in fibroblasts. Fibroblast cells were cultured in control media (0.02% BSA) and treated with 500 ng/ml GH (GH500), 200 ng/mL IGF-I (IGF200) or without growth factors (control) for 24h. Data are presented as fold change from the control and expressed as a mean ± SE. * indicates $P \leq 0.0001$. GH = growth hormone, IGF-I = insulin-like growth factor-I, SE = standard error, IGFBP-3 = insulin-like growth factor binding protein-3.

TABLES

Table 1: Bovine Specific Primers

Gene	Primer	Amplicon length (bp)	Sequence (5' to 3')
RPS15	Forward	151	GCA GCT TAT GAG CAA GGT CGT
RPS15	Reverse	151	GCT CAT CAG CAG ATA GCG CTT
Tbx3	Forward	101	ATC GCT GTG ACT GCA TAC CA
Tbx3	Reverse	101	TCT CTC CTG CCA TTT CCA GT
Tbx2	Forward	124	CTT GCA GTG CTC CTC CTA
Tbx2	Reverse	124	CAC GCA GCT TAA GAT CGA CA
IGFBP-3	Forward	231	CAG AGC ACA GAC ACC CAG AA
IGFBP-3	Reverse	231	TGC CCC CTA CTT ATC CAC ACA

Primers were designed using Primer3 and NCBI Blast

RPS15 = ribosomal protein subunit-15, Tbx2 = T-box 2, Tbx3 =T-box 3,

IGFBP-3 = Insulin-like binding protein-3

Figure 1:

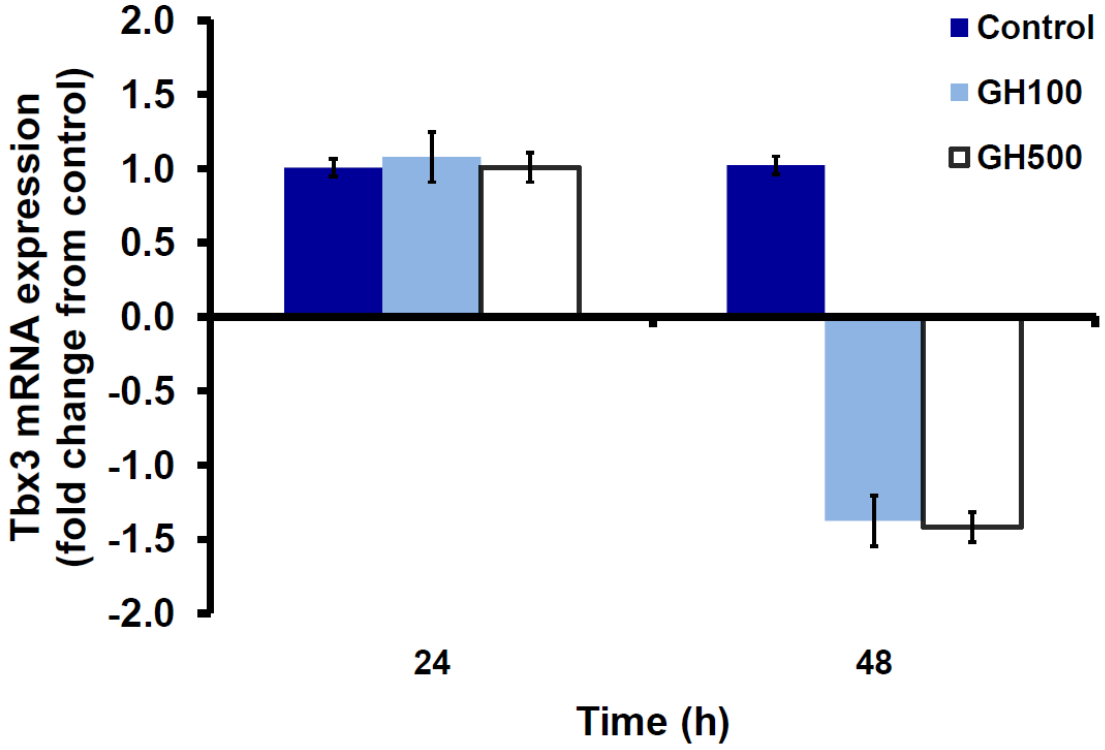

Control
GH100
GH500
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
Tbx3 mRNA expression
(fold change from control)
24
48
Time (h)

Figure 2:

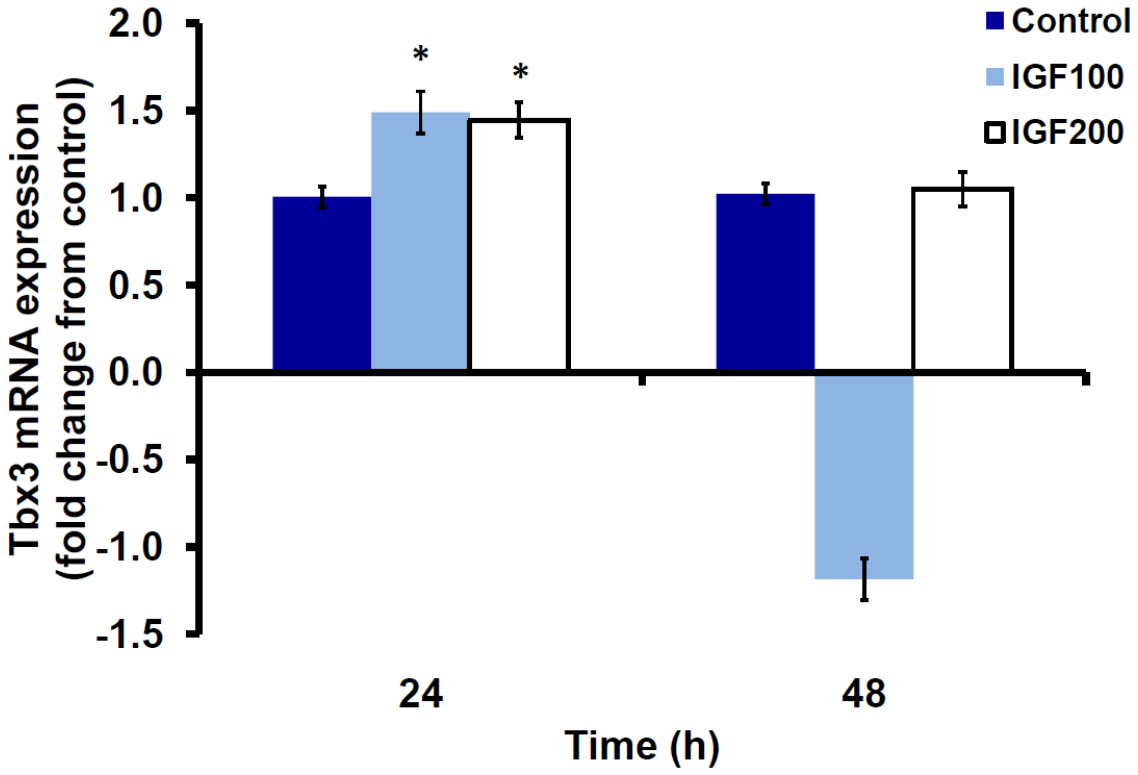

Control
IGF100
IGF200
Tbx3 mRNA expression
(fold change from control)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
*
*
24
48
Time (h)

Figure 3:

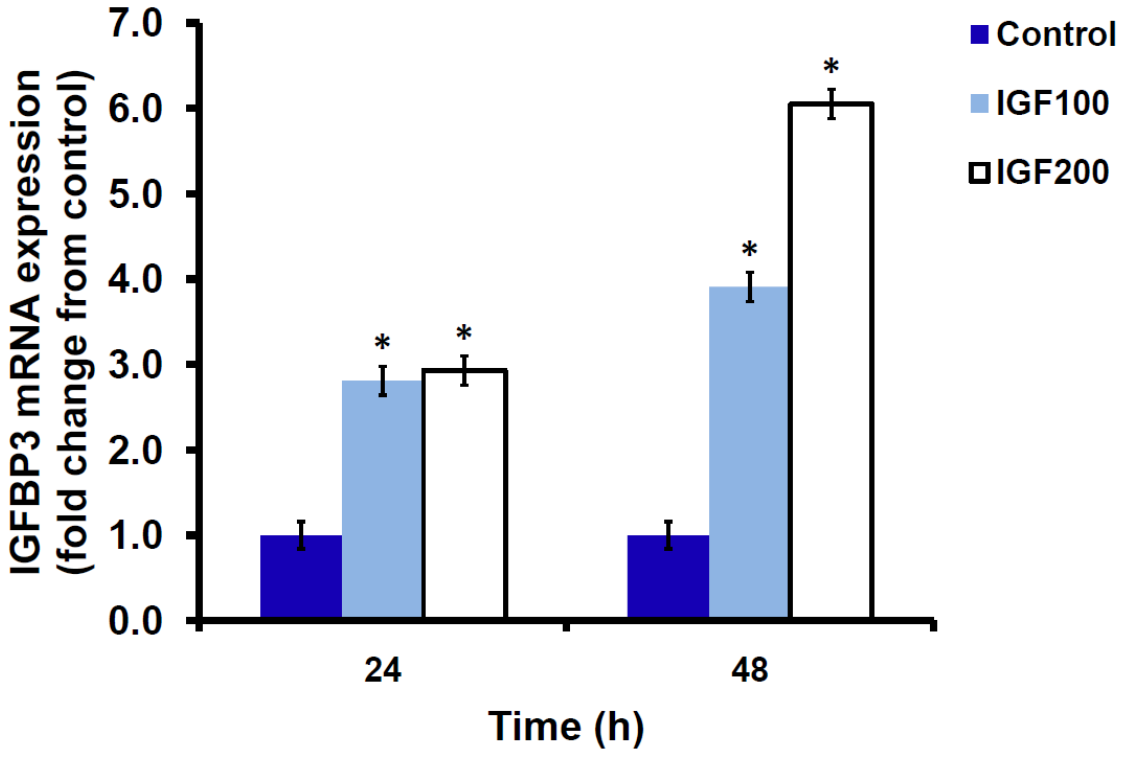

IGFBP3 mRNA expression
(fold change from control)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
24
48
Time (h)
Control
IGF100
IGF200
*
*
*
*

Figure 4:

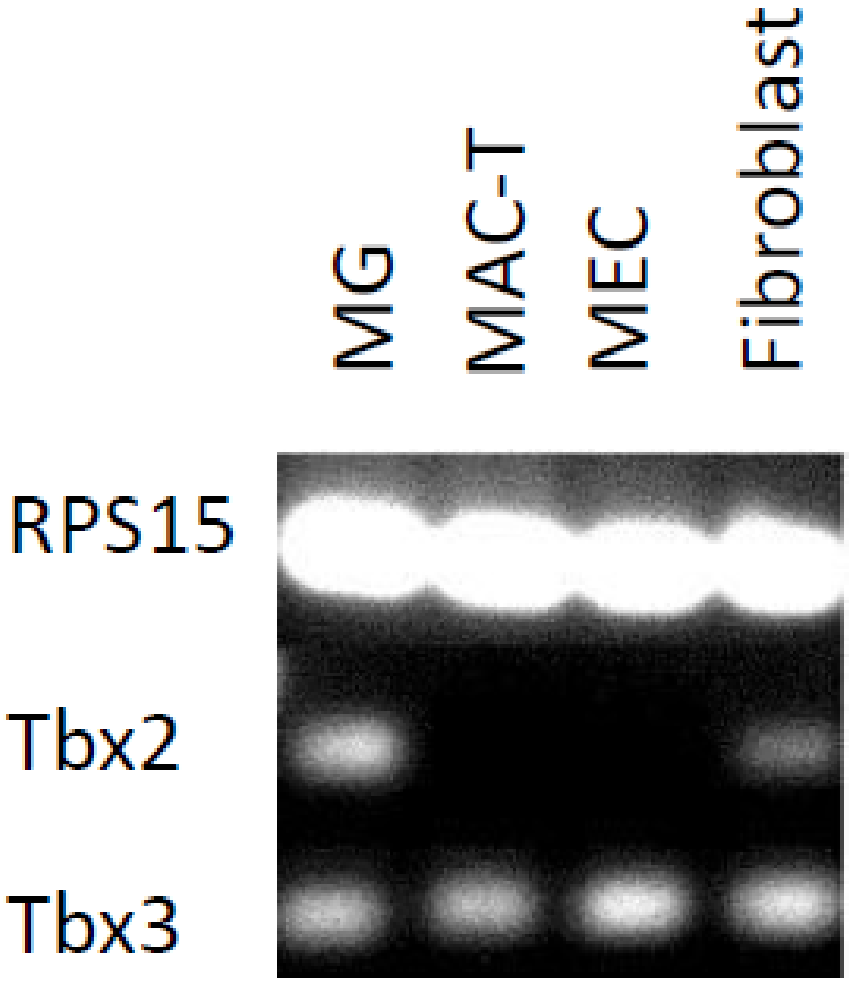


Figure 5:

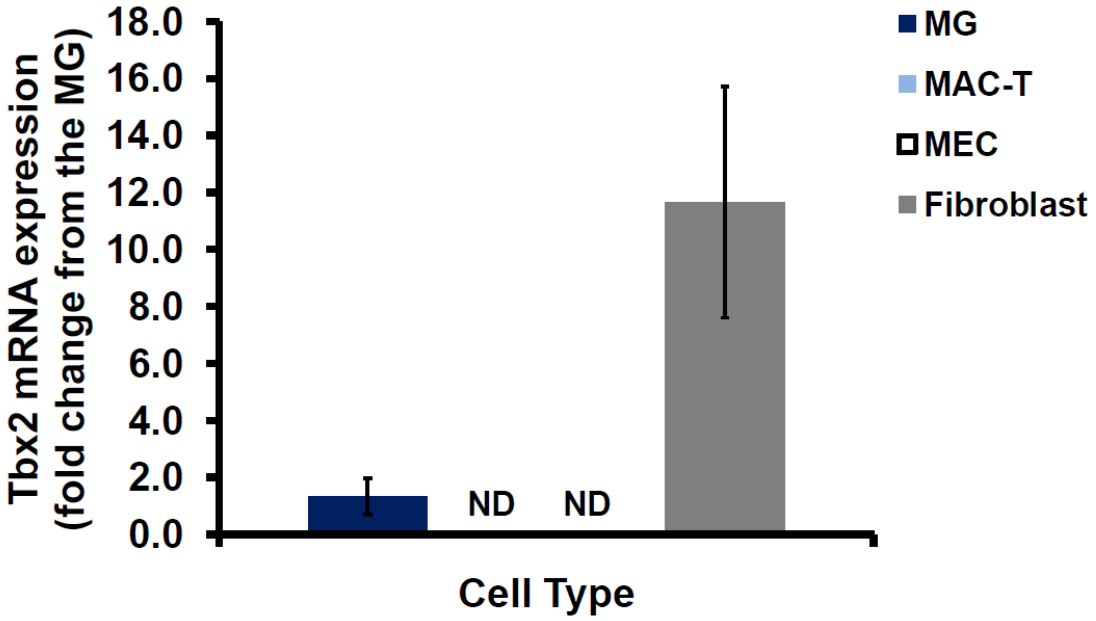

Tbx2 mRNA expression
(fold change from the MG)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
ND
ND
Cell Type
MG
MAC-T
MEC
Fibroblast

Figure 6:

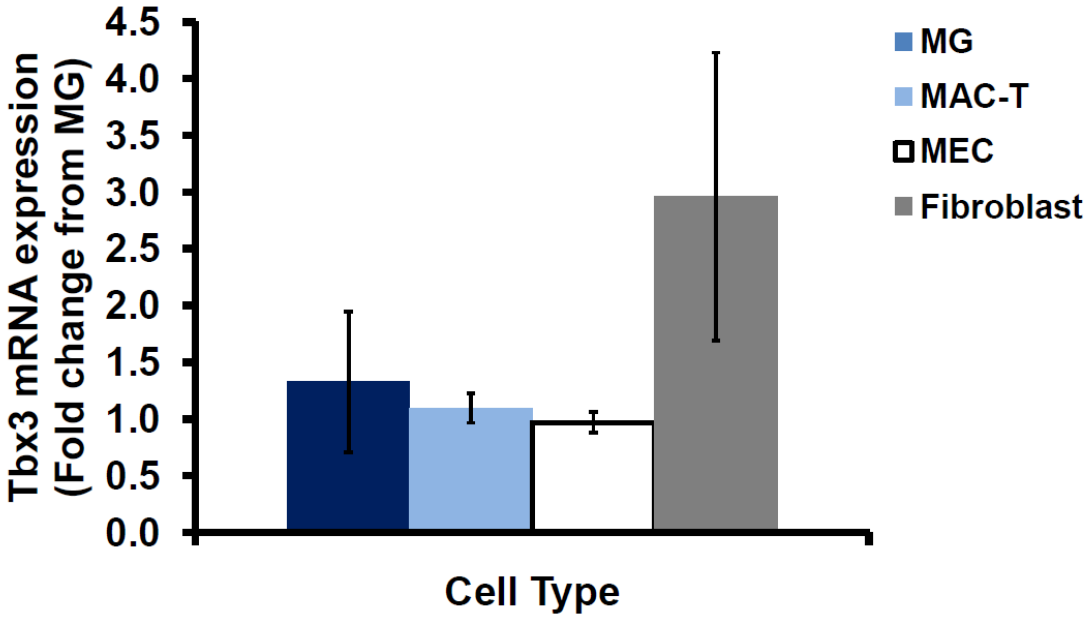
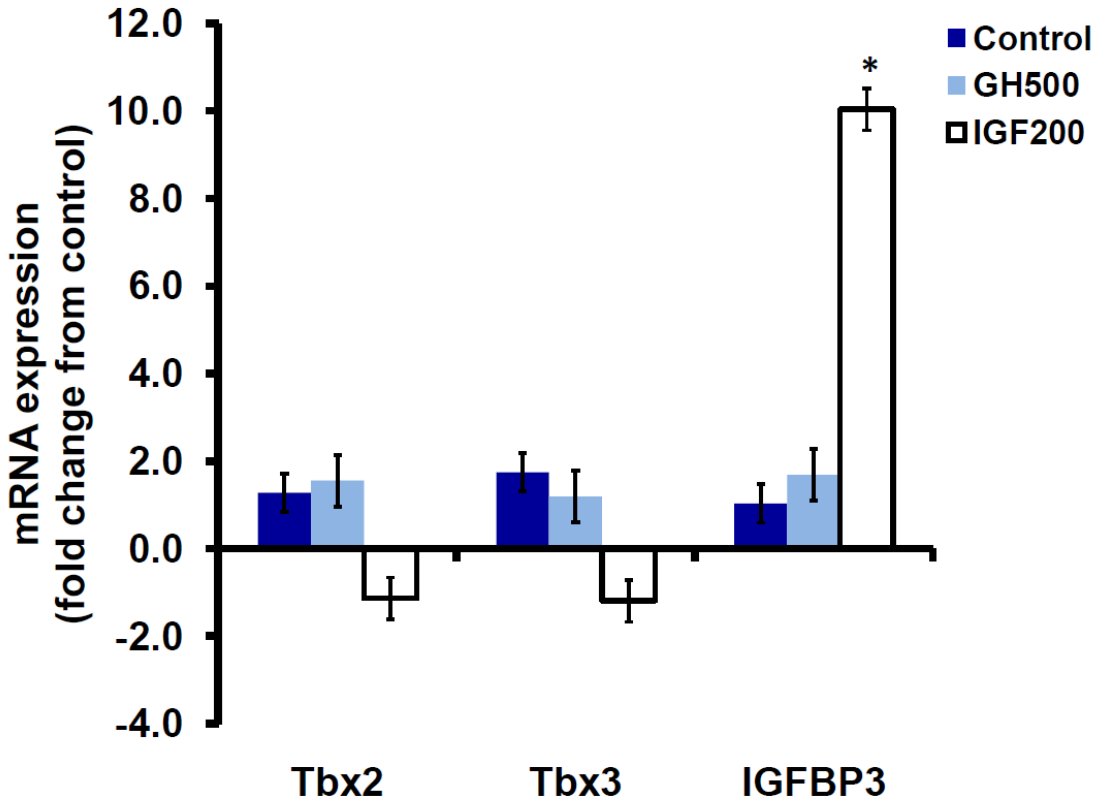

Tbx3 mRNA expression
(Fold change from MG)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cell Type
MG
MAC-T
MEC
Fibroblast

Figure 7:


12.0
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
mRNA expression
(fold change from control)
Control
GH500
IGF200
*
Tbx2
Tbx3
IGFBP3

DISCUSSION

Tbx2 and Tbx3 expression in the bovine mammary gland

There is a limited amount of information about Tbx2 and Tbx3 gene expression in adult mammary tissue. Consequently, the regulatory mechanisms, expression patterns, and roles of these genes in postnatal mammary development are not known. Within our research, we observed that there is differential expression patterns and regulation of Tbx2 and Tbx3 within the bovine mammary gland. We determined that Tbx2 expression is localized within mammary fibroblasts whereas we were unable to detected expression in MEC. Our observations of Tbx2 expression in fibroblasts are in agreement with data from a study conducted by Teng et al in 2007. Within this study researchers observed endogenous Tbx2 expression in the WI-38 fetal lung fibroblast cell line (Teng et al., 2007). Other studies have evaluated Tbx2 expression in fibroblasts. However these fibroblasts did not express Tbx2 endogenously and were transfected with a plasmid containing a Tbx2 gene construct (Teng et al., 2007; Chen et al., 2001). In adult human tissues it is known that heart, kidney, lung, mammary and ovarian tissue express Tbx2 (Law et al., 1995). However differential gene expression of Tbx2 and Tbx3 has yet to be evaluated within these tissue types. Further research is needed to evaluate the expression patterns within these tissues.

The expression pattern of Tbx2 in the fibroblasts in adult mammary tissue is similar to what is observed in the embryonic mammary gland. Tbx2, but not Tbx3, is expressed within the mammary mesenchyme which will later become the stroma (Chapman et al., 1996). This conserved expression pattern suggests that it may be necessary for postnatal mammary gland development and therefore must be maintained.

In embryonic development, Tbx3 but not Tbx2 is expressed within the mammary placodes which will later become the parenchyma (Chapman et al., 1996). Interestingly, within our experiments we did observe that all cell types evaluated express Tbx3 similar to one another. This suggests that Tbx3 expression within the fibroblasts may be necessary postnatally however expression is prevented during the embryonic stages. Overall, a potential reason for this differential gene expression between mammary fibroblasts and MEC could be the need for tissue predominance to change throughout mammary gland development.

As the mammary gland matures and transitions through puberty, pregnancy and lactation, the predominate tissue type will change. There are several examples of regulatory factors within the bovine mammary gland that will increase or decrease based on the stage of mammary gland development and effect mammary tissue predominance. An example of one of these factors is the transforming growth factor-β1 (TGFβ1: Akers, 2002; Plaut et al., 2003; Plath et al., 1997). This growth factor has been found to alter the predominance of the mammary stroma and parenchyma especially as the gland prepares for lactation to cease during involution. Transforming growth factor-β1 does this by facilitating an increase in stromal cell proliferation, ECM break down, and MEC apoptosis (Akers, 2002; Plaut et al, 2003; Plath et al., 1997). Transforming growth factor-β1 concentrations within the mammary gland will remain low throughout pregnancy and lactation. However, as the gland prepares for involution TGFβ1 concentration will increase significantly (Akers, 2002; Plath et al., 1997).

The temporal variation in TGFβ1 concentration is in direct correlation with the stages at which a greater number of MEC are needed. This regulatory mechanism not

only demonstrates that tissue remodeling occurs frequently within the mammary gland but that Tbx2 and Tbx3 could be facilitating a similar role with the gland. Studies have evaluated the effects of over expressing Tbx2 in the embryonic fibroblast cell line, NIH3T3 (Chen et al., 2001). The results from this study suggest that Tbx2 reduces collagen gene expression as well as cell adhesion molecules such as cadherins (Chen et al., 2001). Within the mammary gland, collagen comprises the ECM of the stroma and is produced by the fibroblasts. Without the ECM, MEC cannot differentiate properly and therefore are not able to produce milk proteins. It is possible that Tbx2 may play a role in regulating collagen production of mammary fibroblasts and therefore may affect MEC as the gland transitions from one state to another. It is known that Tbx3 increases cellular proliferation however it has also been determined that Tbx3 gene expression prevents differentiation. Therefore further research needs to be conducted in MEC and fibroblasts to determine how Tbx2 and Tbx3 expression changes during differentiation.

Within our research, we also observed differential regulation of the Tbx genes within the mammary gland. In MAC-T cells, IGF-I increased Tbx3 gene expression. However, in mammary fibroblasts, both Tbx2 and Tbx3 expression remained unaltered in the presence of GH and IGF-I treatment. This suggests that IGF-I affects Tbx3 in MEC and potentially does not affect Tbx2 or Tbx3 expression in fibroblasts of the stroma. Therefore, this could be a mechanism through which proliferation of the MEC increases differently from the stroma of the gland. It is believed that Tbx2 and Tbx3 are expressed and function similar to one another (Coll et al., 2002). However, our findings suggest that there is differential expression as well as differential regulation of expression of Tbx2 and Tbx3 in the bovine mammary gland. Further studies are need to evaluate Tbx2 and

Tbx3 expression during the different stages of development in order to determine how Tbx2 and Tbx3 expression changes within the parenchyma as well as the stroma over time

Regulation of Tbx2 and Tbx3 by the Somatotropic Axis

In order for Tbx2 and Tbx3 to facilitate cellular proliferation and inhibit senescence, the genes for these transcription factors must be transcribed. However, in an effort to prevent uncontrolled cellular proliferation to allow for cellular senescence, the expression of these genes is tightly regulated. Within embryonic development it has been determined that Tbx2 and Tbx3 expression is regulated by a several signaling molecules in many different tissue types (i.e., BMP, Shh: Trumpel et al., 2002). However, the mechanisms for regulating Tbx2 and Tbx3 expression in the adult bovine mammary gland have yet to be determined. Within our research, we observed that IGF-I increased Tbx3 gene expression within MAC-T cells. This suggests Tbx3 may be a key mediator of IGF-I action within the mammary gland. Further research is needed to determine if IGF-I is directly mediating the increase of Tbx3 expression within the gland or if there are other regulatory mechanisms involved in this process.

Our observations are in contrast to what was found in a study by Govoni et al., in 2006 where GH treatment increased Tbx3 gene expression independent of IGF-I (Govoni et al., 2006). Within our cells, GH did not have an effect on Tbx3 gene expression. The difference in findings between our work and the previous study could be due, in part, to variation in the receptors present on these two tissue types and as a result the response of the tissues to GH and IGF-I. It is well established that GHR are present on the germinal

portion of bone (Le Roith et al., 2001; Hadley and Levine, 2007). However the presence of a functional GHR in the MEC is debatable (see Hormones section of literature review). Therefore, it is not clear if GH does not alter Tbx3 expression in MEC or if GH cannot have an effect on these cells due to a lack of a functional GHR.

When fibroblast cells were treated with either GH or IGF-I we did not observed a change in Tbx2 and Tbx3. It is known that GHR are present on fibroblasts and that these cells also produce paracrine IGF-I which in turn can act on MEC. However, our results suggest that within the mammary fibroblast cells of lactating animals there is no effect of the somatotropic axis on Tbx3 and Tbx2 expression. Further studies are needed to determine if this remains the same throughout the different stages of mammary development. While IGF-I effects Tbx3 gene expression in MEC there may be other growth factors or hormones that are needed in order to facilitate an increase in Tbx2 or Tbx3 gene expression within mammary fibroblasts

Role of Tbx3 in mediating the Somatotropic Axis

Within the mammary gland one of the mechanisms through which mammary growth occurs is through cellular proliferation induced by IGF-I (Akers., 2002; Cohick., 1998). IGF-I increases cellular proliferation by activating several cell signaling cascades that result in alterations of the cell cycle. Some of these pathways that are activated are the ras/raf/mapk pathway and β-catenin which in turn will activate cyclin D1 and c-myc (Akers, 2002; Hadsell et al., 2002). In a study conducted by Carlson et al., mammary fibroblast cells were transfected with plasmids that would cause over expression of Tbx3, H-Ras, and Myc (Carlson et al., 2002). When Tbx3 was over expressed in combination

with H-Ras and/or Myc there was an increase in the immortalization of the cells (Carlson et al., 2002). These results suggests that Tbx3 was working in cooperation with Myc and H-Ras by down regulating the p19ARF resulting in cellular immortalization of MEF (Carlson et al., 2002). As stated previously, IGF-I activates several cell signaling cascades of which myc and ras belong to. Therefore, it is possible that by repressing P19ARF that Tbx3 facilitates the actions of IGF-I resulting in an increase in cellular proliferation of MEC. Further research is needed within this area to identify the downstream targets of Tbx3 when MEC are treated with IGF-I.

In summary, we know that Tbx2 and Tbx3 are needed during embryonic mammary development. Within our data, we observed that these transcription factors are expressed in the adult mammary gland and the differential expression pattern observed during embryogenesis is maintained into adulthood. We also observed in MEC that IGF-I increases Tbx3 gene expression within these cells. However we did not observed an effect of GH treatment on Tbx3 expression in MEC. This suggests that Tbx3 may mediate the actions of IGF-I on the mammary gland and that the effects of GH treatment on Tbx3 gene expression may be tissue type specific. Alternately, we did not observe an effect of GH or IGF-I treatment on Tbx2 or Tbx3 expression in mammary fibroblasts. Further research is needed to evaluate growth factors and hormones that may be involved in modulating this genes expression in this cell type. In future research, Tbx2 and Tbx3 expression needs to be evaluated at the different stages of mammary development especially during differentiation. We observed that Tbx3 may be mediating the actions of IGF-I on MEC, but further research is needed to identify the downstream targets of Tbx3 within the bovine mammary gland.

APPENDICES

Appendix 1: Protocol for excising tissue from the bovine mammary gland

1. Set up the area that you will be using as a work surface. This entails washing down the counters with a 1:10 bleach solution following up by wiping the surface down with 70% Ethanol. Place white lab bench top covering over the work surface.

2. Set up you tools that will be utilized to excise the tissue. Keep autoclaved scissors and tweezers in tin foil until they are ready to be used. Affix scalpel blade to the scalpel holder. Leave the blade covered by the protective foil wrapper so the sterility is maintained.

3. Once the udder has been presented to you, prepare the udder for tissue excision. Observe the udder for any signs of abnormalities (i.e.: slack quarter ect). Avoid any regions that appear to be damaged due to mastitis. If there are any gross abnormalities with the gland obtain samples from another gland.

4. Wash a portion of the gland with 70% Ethanol, taking care to clean the udder to remove any debris. Make and incisions with the scalpel. Remove adipose tissue and connective tissue using forceps

5. Utilize alcohol to clean instruments, changing for new sterile ones if necessary. Take care not to damage the tissue with the Ethanol. Excise the parenchymal tissue as carefully and cleanly as possible. Large vessels, mammary fat pad, and other undesired tissue should be avoided however this can always be removed prior to mincing once back at the lab

6. Once the tissue has been excised, carefully place the tissue in a falcon tube containing 35 mL of HBSS containing Penn/Step, Fungizone, Glucose and Gentamycin that has been kept on ice. Do not leave at room temperature as this can decrease the efficacy of the antibiotics.

7. Transport the tissue in media on ice back to the lab where it will be prepped for isolation

- Take tissue samples from the different quarters of the mammary gland ensuring that you clean each surface well before making an incision. By doing this you are not only getting tissue from different portions of the mammary gland but checking the udder for any signs of mastitis or other abnormalities that could impact the success of the isolation.

- If collecting from more than one animal ensure that all tubes are labeled properly

Appendix 2: Protocol for the isolation of bovine mammary epithelial cells

1. Clean the outside of the falcon tubes clean them with 70% Ethanol so as not to introduce any contaminates to the hood.

2. Take fresh flacon tubes and add 35 mL HBSS . Aspirate off the HBSS that you transported the tissue in and remove the tissue from the transport tube and place it in the new tube. Swirl tissue around and then remove this HBSS. This is done to rinse the tissue prior to mincing in an attempt to remove any foreign matter and detritus

3. Mince tissue in flacon tube using a pair of scissors. Change scissors in between tubes so as to prevent contamination. Rinse tissue frequently with HBSS to remove milk as well as blood. Use transfer pipettes as it is easier than using the vacuum. Mince until the tissue is approximately 1-5 mm3 in size.

4. Once tissue has been minced and cleaned it will be added to the digesting mixture. The digesting mixture should contain the following: 250 mL of HBSS, .5 mg/mL collagenase IV, .4 mg/mL Dnase I, .5 mg/ml hyaluronidase, 50 ug/mL Gentamycin, and 2.5 ug/mL fungi zone. The digesting mixture should be STERILE FILTERED prior to use. In the hood add the digesting mixture to an AUTOCLAVED Erlenmeyer flask with a spinner bar. Allow the tissue to remain in this mixture for three hours

5. Upon completing of the incubation utilize a large metal strainer to remove large pieces of undigested tissue as well as fat. Place the resulting liquid into falcon tubes and centrifuge at 40 G (rcf) for 5 min.

6. Resuspend in fresh HBSS and filter again through a smaller metal filter. Place the resulting liquid into falcon tubes and centrifuge at 40 G (rcf) for 5 min. Remove HBSS and resuspend in fresh HBSS and filter through a cell strainer (100 um). Centrifuge at 40 G (rcf) for 5 min.

7. Resuspend the cell pellet in culture media. If there appears to be still some debris use the cell strainer again but do not re-centrifuge. Plate out the cells and allow them to incubate for 30 min at 37 degrees Celsius/5% CO

8. After 30 minutes gently decant off the media. The cells left behind should be fibroblasts

9. Watch cells very carefully for ANY signs of contamination. Also watch for fibroblast growth and use the “Removing Fibroblast Protocol” to prevent overgrowth of the culture with fibroblasts.

Appendix 3: Standard and PRF Dulbecco's Modified Eagle Medium Preparation

To Prepare 1X Liquid Medium

1. Measure out 5% less distilled water than desired total volume of medium, using a mixing container that is as close to the final volume as possible

2. Add powdered medium to 15 to 30°C (room temperature) water with gentle stirring (DO NOT heat water)

3. Rinse out inside of package to remove all traces of powder

4. Add 3.7 g of Sodium Bicarbonate per liter of medium

5. Dilute to a desired volume with water. Stir until dissolved taking care not to over-mix

6. Adjust pH of medium to 0.2-0.3 below desired final working pH. Use of 1N Sodium Hydroxide or 1N Hydrochloric Acid is recommended. After pH has been adjusted, keep container closed until media is filtered

7. Sterilize immediately by membrane filtration

Appendix 4: MAC-T/MEC/Fibroblast cell subculture

1. Bottles of media, PBS, and 50% Trypsin-EDTA/50% PBS should be warmed prior to usage. Care should be taken so that the media does not stay in the water bath for prolonged periods of time. Trypsin should never be placed directly in the water bath.

2. Clean work area prior to usage with 70% Ethanol. Clean bottles from the water bath with 70% Ethanol prior to inserting them into the hood.

3. Remove media from the plates using vacuum aspiration. Take care so as not to damage or disturbed the cells

4. Add 2-3mL of warmed PBS to each plate. Do not add directly to the cells as this could dislodge them. Gently swirl PBS on the plate so as to remove any remaining media. Aspirate off PBS prior to addition of Trypsin-EDTA/PBS solution.

5. Add 2-3 mL of Trypsin-EDTA/PBS to each plate. Place plates in incubator.
    a. For MAC-T: Allow cells to incubate in the presence of Trypsin-EDTA/PBS for 7-10 min
    b. For MEC: Allow cells to incubate in the presence of Trypsin-EDTA/PBS for 25 min
    c. Fibroblasts: Allow cells to incubate in the presence of Trypsin-EDTA/PBS for 6 min

6. Check plates at the end of trypsinzation to ensure that all cells have lifted then add media to stop the action of the enzyme. Using a cell scraper is sometimes needed for these cells. Be gentle when using the cell scraper so as to prevent cellular damage and cell clumping.

7. Add contents of the plate to a 50 mL falcon tube and centrifuge to form a cell pellet
    a. For MAC-T and MEC: 600 rpm for 6.5 min
    b. For Fibroblasts: 1400rpm for 4.5 min

8. After centrifugation, aspirate off media and resuspend cells in fresh media. The amount of media added to the pellet will depend on the purpose for these cells. If subculturing pass add 10 mL of media for every plate passed.

9. Plate cells and return them to the incubator. Cells will have to be subcultured again once they reach 70%-80% confluence

Appendix 5: Removal of Fibroblasts from Primary Bovine Mammary Epithelial Cell Culture

HBSS Removal Technique

1. Remove media from the primary BMEC and rinse gently with warm PBS several times.

2. Add HBSS solution containing .5 ug/mL fungizone,100,000 U/mL Penn/Strep, and Gentamycin.

3. Allow cells to incubate in the presence of HBSS for 3hrs at 37 degrees Celsius/5% CO2.

4. Remove HBSS and wash again several times with PBS. Wash slightly vigorously so as to remove fibroblasts that may have changed morphology but not lifted.

5. Add fresh media to the plate and return to the incubator

Selective Trypsinization

1. Add 2 mL of 25% Trypsin-EDTA/75% PBS

2. Allow cells to incubate in the presence of trypsin for 3 min

3. Remove cells from the incubator and examine the plate. Look to ensure that the fibroblasts have lifted and NOT the mammary cells

4. Remove the trypsin and fibroblasts and rinse with PBS. Use some force when rising but do not be too vigorous as this will cause the mammary cells to detach.

5. Add 2 mL of 25% Trypsin-EDTA/75% PBS to the plate

6. Allow cells to incubate in the presence of trypsin for 10-12 min. Monitor the cells periodically throughout this incubation. Once the cells have changed morphology add media to deactivate the trypsin.

7. Remove cells from plate and place media and cells in centrifuge. Spin cells at 600 rpm for 6 min

8. Resuspend cells and plate out onto new plates containing on 5 mL of media per a plate. Allow the plates to incubate for half an hour. This is to remove any residual fibroblasts that may have remained after the first trypsinization.

9. Decant off the media ensuring not to disturbed the fibroblasts that have adhered to the plate. Plate out decanted cells (performing a cells count at this point if need be) in culture media and allow to grow until confluence.

Appendix 6: RNA extraction

1. Rinse experimental plates with PBS and remove. Then add 1 ml TRI Reagent (Sigma Aldrich, at no. T9424) to each well and scrape well with pipette tip so as to remove the cells. Transfer the contents of the well to a 1.5 mL eppendoff tube.

2. Vortex the TRI Reagent mixture to homogenize. Allow the tubes to incubate for 5 min if extracting RNA immediately. If not, tubes should be stored in the freezer at -80 °C.

3. Add 200 uL chloroform to <u>each</u> tube. Mix by inverting 5 -7 times and allow for it to incubate at room temperature for 2-3 min. *Centrifuge these tubes at 12000 x g for 15 min

4. Transfer the aqueous phase into a new eppendorf tube taking care not to disturb the other layers. The total volume transferred will be close to 500 µL. Next Add one volume (500 uL) of 70% EtOH to <u>each</u> tube and vortex

5. Transfer 700 uL of the sample into the RNA east Spin Column and centrifuge 8,000 X , 30s. Discard the resulting flow through and add 700 µL RWI wash buffer. Take care to avoid adding any liquid directly onto the filter as this could cause a decrease in RNA yield. Centrifuge spin column again at 8,000 X g 30s

6. Discard the flow through and collection tube put the filer on the new collection tube. Add 500 uL of Buffer RPE to spin column ensuring that 100% Ethanol has been added to the buffer prior to use. Centrifuge 8,000 x g, 30s and discard the flow through.

7. Add 500 uL of Buffer RPE and centrifuge at 8,000 X g for 2 min. Discard the flow through and centrifuge at 8,000 X g 1 min. Discard the flow through and collection tube, put the filter on the new collection capped tube

8. Add 50uL of Rnase free water to the spin column and centrifuge at 8,000 X g for 1 min. Take 35-40 uL from the collection tube and put back on the filter centrifuge at 8000 X g, 1 min

9. Collect the eluted RNA, in the capped collection tube, keep on ice, and spec it in the Nanodrop. Aliquot it and store in the -80 freezer

- When extracting RNA from fibroblasts centrifuge for 15 min at 4°C

Appendix 7: Turbo DNA-Free Protocol

1. Thaw the reagents (10x Turbo DNase Buffer, Turbo DNase, DNase inactivation reagent) on ice.

2. Add 0.1 volume of 10x turbo Dnase Buffer (i.e.: 5 uL of 50 uL RNA sample) and 1 uL Turbo Dnase to the RNA sample and mix gently. This is for routine DNase treatment with RNA concentration of 10ug/50uL of sample. If it is more than this con. Dilute your sample to have 10 ug/50 uL using C1V1=C2V2

3. Incubate at 37 °C for 20-30 min

4. Vortex the Dnase Inactivation reagent well as this mixture is very turbid. If performing DNA-Free treatment on a significant number of samples be sure to vortex frequently to prevent settling. Add 0.1 volume of resuspended DNase Inactivation reagent.

5. Incubate for 5 min at room temp while mixing occasionally by flicking or tapping

6. Centrifuge at 10000 x g for 1.5 min

7. Transfer the supernatant to a fresh tube Note: Avoid disturbing the pellet

8. The final DNA-free RNA con. Is determined by Nano drop 1000.

Appendix 8: Reverse Transcriptase PCR

1. Thaw the reagents on ice(Ambion OligoDT, Invitrogen Superscript kit II containing DTT, 5X buffer and reverse transcriptase)

2. Add 1uL of OligoDT to each 10uL of normalized RNA sample (300ng/10ul) in 8 tube strips. This makes it a total of 11uL.NOTE: Always include a positive and negative control

3. Place these tubes into the thermocycler and state the desired RT PCR program (see below) and start step 1, 70˚C for 10min

   70˚C for 10min
   4˚C for 20min
   37˚C for 3min
   42˚C for 1hr
   4 ˚C for 3min
   90˚C for 2.30 min
   10˚C forever

4. During the time of step 1, make the master mix. Do not add superscript until you are ready to add the master mix to the samples.

   Master Mix

5X Buffer-	5.5uL
dNTP-	1uL
DTT-	2uL
SuperscriptII (Reverse transcriptase)	0.5 uL

   NOTE: The above calculations are per sample, multiply it by the no. of samples+2 for pipetting errors.

5. Pause the thermocycler when the temperature is dropping to 4˚C for the next step (step 2). Take the samples with RNA and Oligo DT out of the machine and keep it on ice.

6. Add SSII to the master mix, mix gently. Add 9uL of master mix to each sample , making it a total of 20uL ensuring that samples are kept on ice while adding the master mix. Mix contents of tube with master mix by pippetting up and down several times.

7. Place the samples back into the machine hit proceed and hit proceed again to move to step# 3. When RT is complete, add 80uL of Nuclease free water to samples and store in -20˚C freezer.

Appendix 9: Designing Primers for PCR

1. Go to www.pubmed.com. Ensure that you search "nucleotide" and then type in your gene of interest. Include the species

2. Find your gene of interest and click on FASTA. This will present you with your gene of interest's sequence. Copy only **CDS or CODING REGION** of the sequence. If you do not use this your primers will not bind the correct sequence.

3. Blast this sequence using the link entitled " RUN BLAST" along the upper right of the webpage. This will compare your gene sequence to other sequences to ensure that you will be amplifying the right gene.

4. Once the blast has been run, check the results to ensure that there are no matches for other genes in the species that you will be working with.

5. Go to: http://frodo.wi.mit.edu/primer3/. Paste your sequence of interest in the large box. This program will construct your primers for you .Change the product size range. Usually you want your product to be between 80-300bp in size. Click "Pick Primers"

6. Once you have the primers return to BLAST on the pubmed website and run a BLAST on these primers to ensure that they will amplify only the gene you want to look at in your species.

7. Go to: http://genome.ucsc.edu/. Click on the tab entitled PCR located at the top of the webpage. Change the Genome tab to match the species you are working in. Enter your Forward and Reverse primer as determined by Primer3 (you can copy and paste it)

8. Observe the temperatures at which these primers anneal. If they are close together for both the forward and reverse primer then you are all set

9. Order your primers through Integrated DNA Technologies (IDT) at the following website: http://www.idtdna.com/Home/Home.aspx

   *Dilute and optimize your primers once you receive them by running both PCR and RT-PCR with an efficiency curve.

Appendix 10: Polymerase Chain Reaction

1. Thaw reagents on ice (Promega Green Master mix, Tbx2 114 forward and reverse primer working aliquots, cDNA sample)

2. Add 5uL of the cDNA to 8 tube strips once the master mix is added there will be a total volume of 25µL

3. Make Master mix of 20uL for one reaction

Nuclease free water	5.5µL
Green master mix	12.5µL
Forward Primer	1.0µL
Reverse Primer	1.0µL

   NOTE: This is for one sample, multiply it by the number of samples plus two for pipetting errors. Be sure to include a negative and positive control. Transfer 20uL of the master mix to 8 tube strips

4. Place the tubes into PCR machine and run the following program:

   94˚C for 3min

   94˚C for 30s

   60˚C for 1min

   72˚C for1min

   X 40cycles

   72˚C for 5min

   10˚C forever

5. Once the PCR is complete (about 21/2 hr), it is stored at 4˚C.

Appendix 11: Real time PCR

1. Thaw the Reagents on ice.(Syber green wrapped in foil, forward and reverse primers, cDNA)

2. Add 5uL of cDNA (diluted 1:5, 20uL of cDNA + 80uL of Nfree water) to the tubes. Make sure to pipette to the bottom of the tube.

3. Make Master Mix. Amount will vary depending on the number of samples and number of genes. Turn off lights when using the syber green as it is light sensitive. Always add plus two for pippeting error and include negative and positive controls.

   Master Mix

2X Syber Green	10uL
Forward Primer	1uL
Reverse primer	1uL
NFree water	3uL

4. Add 15uL of master mix to each well in 96 PCR optical plate. Gently pipette contents of well with master mix to facilitate mixing. Avoid forming bubbles as this can result in incorrect values.

5. Seal it with a PCR adhesive film tightly to prevent evaporation and centrifuge the plate at 1000RPM for 1-2minutes

6. Run plate in the ABI 7900 HT Fast real time machine using the following protocol:

   Stage 1: 50 °C for 2 min
   95°C for 10 min

   Stage 2: 95°C for 15 sec
   60°C for 1 min
   *Repeat for 40 cycles

   Stage 3: 95°C for 15 s
   60 °C for 15 s
   95 °C with a 2% ramp for 2 min

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