Ovarian and Growth Hormone Regulation of Mammary Growth and Transcript Abundance in Prepubertal Dairy Heifers

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ABSTRACT

Ovarian secretions and growth hormone (**GH**) are major endocrine regulators of mammary growth and development in bovine mammary gland; but information on endocrine regulation during early prepubertal period is limited. Our overall objective was to study the regulation of mammary growth and development as well as transcript abundance in early prepubertal bovine mammary gland by ovarian secretions and exogenous bovine somatotropin (**bST**). In the first study, we determined the effect of staged ovariectomy on mammary growth and development in two to four month old Holstein heifers. In the second study, effects of bST on mammary growth and development, and also on putative stem cell population were evaluated by beginning bST treatment in one month old Holstein heifers. Mammary growth and development was determined by mass of mammary tissue, biochemical analyses, histological examination, transcript abundance and protein expression in mammary parenchyma (**PAR**) and fat pad (**MFP**). Ovariectomy reduced mass and lipid content of PAR without affecting the histological characteristics or rate of epithelial cell proliferation. There was a marked reduction in progesterone receptor expression both at the mRNA and protein level. Ovariectomy also reduced transcript abundance in GH receptor (**R**), insulin-like growth factor (**IGF**)-1, IGF binding protein -6, estrogen responsive and proliferation marker genes, but increased the mRNA abundance of IGF-1R in PAR. On the other hand, administration of bST did not have an impact on PAR growth, epithelial proliferation, putative stem cell population or transcript abundance of IGF-axis genes. However, bST reduced the relative abundance of GHR, signal transducers and activators of transcription-5b and suppressors of cytokine signaling-2 in mammary PAR. Transcript abundance of IGF-axis molecules, estrogen responsive genes and proliferation markers in MFP was not affected by ovariectomy or bST. Overall, our data suggest that ovary is a predominant regulator of mammary growth and development in prepubertal heifers and that exogenous bST is not effective as a mammary specific mitogen in very young prepubertal heifers.

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CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

Mammary gland is unique to the mammalian species and it is critical in nourishing the neonates. Mammary gland is a complex exocrine gland. Though the mammary gland structures are formed during the fetal life, the epithelial tissue is rudimentary at birth. There are four major phases in the post natal development in mammary epithelial cells such as proliferation, differentiation, secretion and involution. Growth and development of mammary gland is regulated by the interaction of a wide variety of mammogenic and lactogenic hormones as well as growth factors. Historical evidence proves that pituitary as well as ovarian secretions are equally important for normal growth and functional development of mammary gland. However, the mechanisms involved in hormonal regulation of mammary growth and development are different from species to species as well as during different physiological stages within an animal. Enhancement of lobulo alveolar growth during later stages of pregnancy is significant for ensuring maximum milk yield in the following lactation. Mammary growth during the prepubertal period is also critical because initial allometric growth and expansion of ductular tree occurs at this time which is vital for alveolar development during gestation. During allometric growth period, rapid growth occurs both in the fat pad and ducts. However, in some species the size of the fat pad is detrimental to ductular growth. Poor nutrition in heifers during prepubertal period caused reduced ductular growth in mammary gland and resulted in reduced milk yield in the subsequent lactation (Sejrsen, 1994). Therefore, it is desirable to build a better foundation of alveolar bed during prepubertal period to ensure maximum milk yield during lactation. Many experimental approaches involving extraneous administration of hormonal preparations have proven to enhance mammary growth. It is known that growth hormone (**GH**) and estrogen enhances mammary epithelial proliferation, thereby augmenting mammary development in heifers (Tucker, 1981; Sejrsen et al., 1986; Woodward et al., 1993; Berry et al., 2001). However, the underlying mechanism of hormonal regulation of epithelial cell proliferation is not clearly understood. Insulin-like growth factor-1 (**IGF-1**) and its binding proteins (**IGFBP**) are likely to be involved in mediating these effects. Information on the regulation of local expression of IGF-1 and related proteins during prepubertal mammary development is also scarce in cattle. Further

investigation into the regulatory mechanisms and delineating the underlying processes in the signaling pathways in prepubertal bovine mammary gland are desirable.

1.2. Role of GH-IGF axis in mammary development

The components of the GH-IGF-1 axis such as GH, GH receptor (**R**), IGF-1, IGF-1R, and IGFBP are important in the regulation of proliferation, differentiation and fate of mammary epithelial cells. Insulin-like growth factor-1 and IGFBP are involved in cell proliferation during mammary growth and development as well as apoptosis during mammary involution. Though the synthesis and secretion of IGF-1 is greatly associated with GH and nutritional status, IGFBP modulates the availability and actions of IGF-1. Regulation of local expression of IGFBP in the bovine mammary gland is ambiguous. Feeding level and exogenous GH are known to be involved in the regulation of IGFBP in bovine mammary gland (Weber et al., 2000). However, a previous study in our laboratory showed that protein and fat content in the diet as well as dry matter intake did not have an effect on the mRNA expression of IGF-1 and IGFBP in the mammary gland of prepubertal heifers (Daniels, 2008). The importance of GH (Sejrsen et al., 1986; Purup et al., 1995) and IGF-I (Akers et al., 2000; Berry et al., 2001) in development of mammary gland in heifers has been established. Berry et al. (2001) reported that exogenous GH injections can stimulate up to a 6-fold increase in cell proliferation in older heifers. However, the mechanisms involved in GH-induced enhanced proliferation in bovine mammary gland are not well understood. Though GHR are present in mammary epithelial cells (Hauser et al., 1990), several lines of evidence suggest that the effect of GH on mammary gland is mediated through IGF-1. Ligand binding assays failed to demonstrate direct binding of GH to its receptors in bovine mammary cells, suggesting an indirect effect of GH on mammary epithelial cell proliferation, possibly through serum IGF-1 (Akers, 1985). It is also reported that mammary ductal development was considerably reduced in hypophysectomized rats which was later restored by IGF-1 mammary implants (Ruan et al., 1992; Walden et al., 1998), and administration of GH in IGF-1-null mice did not promote mammary development (Ruan and Kleinberg, 1999). Furthermore, predominant presence of GHR and IGF-1 in the stromal cells and the expression of IGF-1R in the mammary epithelial cells (Forsyth et al., 1999; Gallego et al.,

2001) strongly suggest the importance of stromal-epithelial association for mammary growth and development.

It has been reported that all six IGFBPs (1 - 6) were found in the bovine mammary gland with IGFBP-3 and -5 being the predominant ones (Plath-Gabler et al., 2001). There are conflicting reports about the effect of exogenous bovine somatotropin (**rbST**) on the mammary expression of IGFBP-3. There was no difference in the abundance of IGFBP-3 in the mammary gland between placebo and rbST treated heifers (Weber et al., 2000), whereas IGFBP-3 expression was reduced in mammary gland of heifers in response to rbST resulting in increased free IGF-I (Berry et al., 2001). Insulin-like growth factor binding protein -5 is involved in apoptosis during mammary involution (Tonner et al., 1997) and also in ductal and alveolar differentiation during pregnancy in murine mammary gland (Allar and Wood, 2004). A recent report showed that both basal and IGF-1-induced expression of IGFBP-5 in bovine mammary cells was suppressed by the mitogen-activated protein kinase pathway (Fleming et al., 2007), suggesting a role for IGFBP-5 in apoptosis.

1.3. Ovarian influence on mammary development

Ovarian regulation of mammary growth and development in cattle has been studied historically. Removal of ovary abolished the allometric growth phase of normal mammary growth in calves (Wallace, 1953). However, ovariectomy did not affect allometric mammary growth in sheep (Ellis et al., 1998) suggesting the differential regulatory mechanisms among different ruminant species. Effect of ovaries on mammary development is believed to be mediated through estrogen. The fact that estrogen is a potent mitogen for proliferation of MEC is well documented in rodents and heifers (Berry et al., 2001; Marshman and Streuli, 2002). Administration of estrogen increased IGF-1 mRNA expression in mammary fat pad in heifers with intact mammary gland, whereas there was no increase in IGF-1 mRNA when the parenchyma was removed surgically (Berry et al., 2001). It has been shown in humans (Clarke et al., 1997b) and in prepubertal heifers (Capuco et al., 2002) that estrogen receptors are expressed on the mammary epithelial cells which are not involved in DNA synthesis. Therefore, estrogen-induced proliferation of epithelial cells is likely mediated through local IGF-1 in a paracrine manner. Taken together, the so called “cross-talk” between the epithelium and stromal tissue to

regulate the estrogen-induced IGF-1 production is believed to exist also in bovine mammary gland (Fig 1.1). Moreover, estrogen and progesterone receptors are co-localized in a subset of bovine mammary epithelial cells, but not in stromal cells (Capuco et al., 2002). These findings together indicate an important role for ovarian secretions in the modulation of cell proliferation and local IGF-1 production in bovine mammary gland.

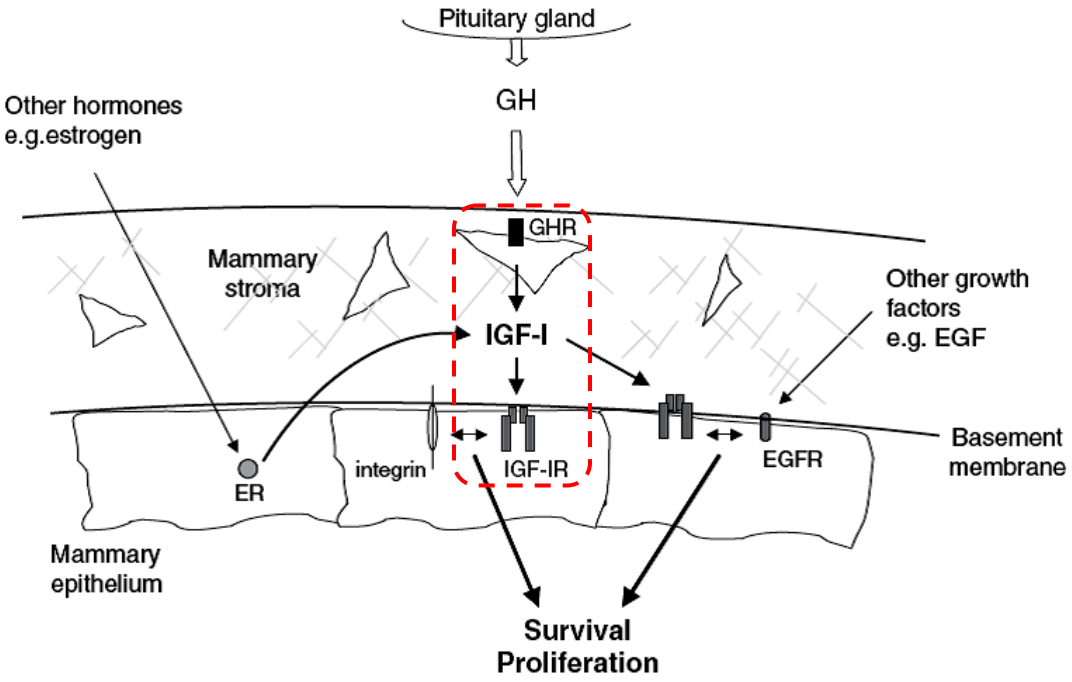


Figure 1. 1. Insulin-like growth factor-1 (IGF-1) signaling networks in the mammary gland

It is proposed that GH acts on the growth hormone receptor (GHR) in stromal cells to induce IGF-I release, which subsequently acts at the IGF-1R on epithelial cells to mediate survival and proliferation. This pathway indicated in red dotted box is not yet proved; only speculated. Estrogen can also induce IGF-1 expression, which may then act on adjacent mammary epithelial cells. The basement membrane provides an interface between stroma and epithelial cells, and it can contribute to the signals required for mammary development *via* integrin receptors. Epidermal growth factor (EGF) can synergize with IGF-1 and IGF-1 can trans-activate the EGF receptor (EGFR). ER, estrogen receptor (adapted from Marshman and Streuli, 2002).

Mammary growth retardation as a result of the absence of ovarian secretions in cattle is effective at a very early age. There was a reduction in mammary parenchymal tissue when heifers were ovariectomized at 2.5 months of age (Purup et al., 1993), and treatment with GH did not restore parenchymal development in ovariectomized heifers. This implies that secretions from the ovary, presumably estrogen, are necessary for growth promoting effects of GH. It was also reported that there was a small but significant reduction in serum estrogen concentration (~0.1 ng/L) in ovariectomized heifers. It is not clear if this reduction in the circulating estrogen concentration was enough to produce a change in mammary gland development. The effects of ovariectomy on mammary growth may be either due to a reduced activity of estrogen or due to changes in the local pathways in mammary gland. We already know from the literature that GH-induced proliferation of mammary epithelial cells is mediated through IGF-1 and that ovariectomy reduced the binding affinity of IGF-1 in mammary parenchymal tissue (Berry et al., 2003c). On the contrary, there was no difference in binding affinity or number of IGF-1 receptors in mammary tissue from ovariectomized heifers (Purup et al., 1995). These observations collectively indicate a probable modification in the locally acting proliferative mechanism involving estrogen and IGF-1 in mammary tissue. The reduction in mammary parenchymal growth in ovariectomized heifers could be due to a decreased local response of estrogen in mammary gland.

1.4. Signaling molecules involved in regulation of cell proliferation response

Pituitary hormones like GH and prolactin manifest their proliferative response through interaction with transmembrane cytokine receptors. Binding of these hormones to their receptors causes phosphorylation of tyrosine kinases of Janus kinase **(JAK)** family and subsequent activation of the latent transcription factor signal transducers and activators of transcription (**Stat**). Phosphorylated Stat forms dimmers and get translocated to the nucleus to induce cell-specific responses. Specific inhibitors of JAK2 suppressed GH-induced IGF-1 gene expression, while mitogen-activated protein kinase or phosphatidyl inositol 3 kinase inhibitors did not block IGF-1 expression (Yoshizato, 2004). This indicates that GH-induced IGF-1 gene expression is mediated through the JAK signaling pathway. However, the transcription factor Stat5 is also activated by prolactin, GH and epidermal growth factor receptors in mouse mammary epithelium

(Gallego et al., 2001), suggesting a common signaling pathway downstream of the receptors. The multiple signaling pathways initiated by GH when it binds with its receptor is shown in Fig. 1.2 (Smit and Cartersu, 1996). Intracellular signaling molecules known as the suppressors of cytokine signaling (**Socs**)-2 are recognized to terminate GH signaling by reducing the ability of GH to activate downstream molecules like JAK and Stat (Greenhalgh et al., 2002; Leung et al., 2003). This negative feedback loop can be further explained as follows; GH induces

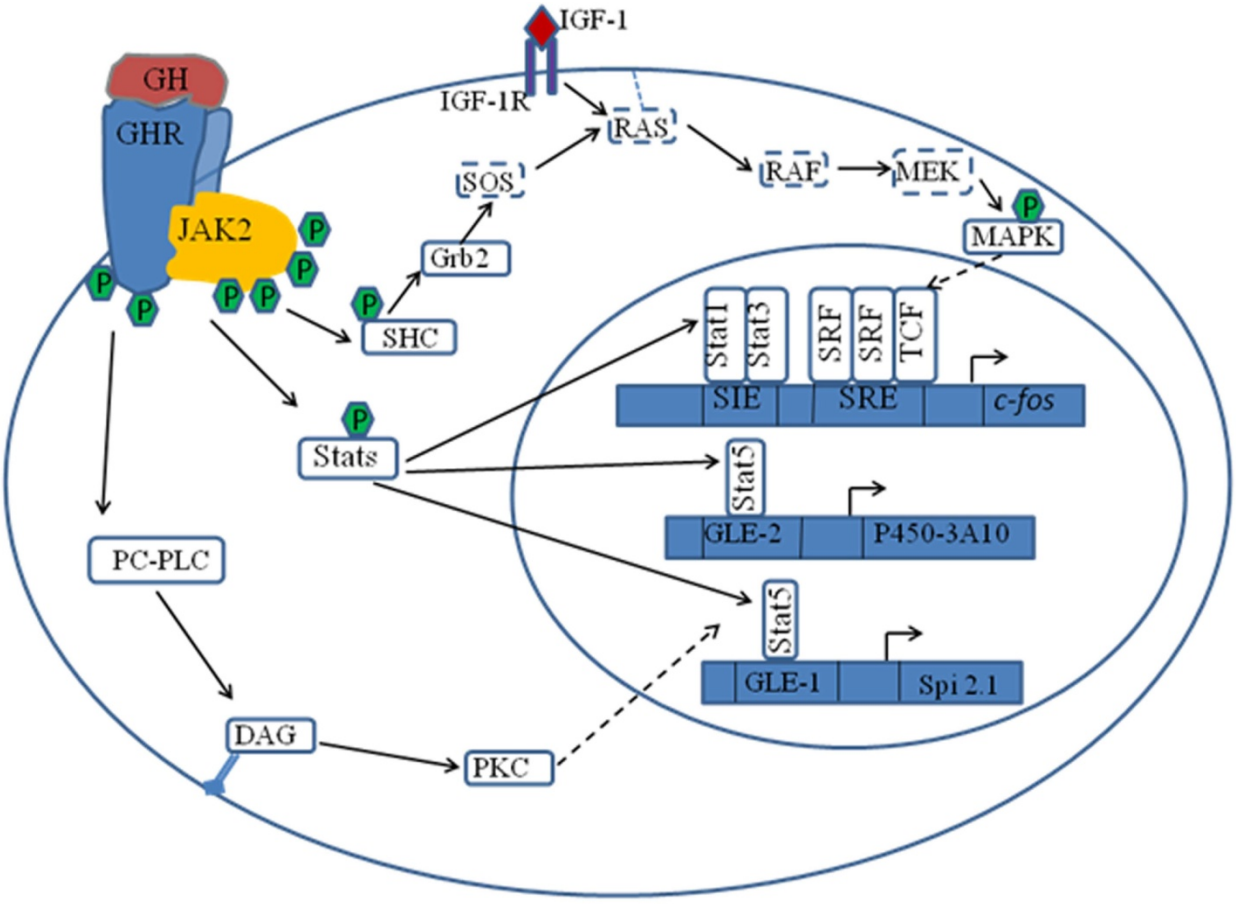


Figure 1. 2. GHR and IGF-1R signaling pathways

Possible signaling pathways initiated by binding of GH and IGF-1 to their receptors are shown in Fig1.1. Solid boxes and arrows indicate molecules and pathways regulated by GH. Dotted line boxes and arrows indicate molecules and pathways utilized by IGF-1 and other growth factors (adapted from Smit and Cartersu, 1996).

production of Socs-1, -2 and -3 and these molecules in turn will inhibit the activity of Stat transcriptional factors and thereby inhibit GH action (Ram and Waxman, 1999). The

mechanisms involved in IGF-1production in mammary gland are still vague. However, the role of Stat5 in GH-induced IGF-1 production in the liver is well understood (Davey et al., 2001). In the intestinal cells of rats, Socs-2 is induced by GH and changes in Socs-2 modify cell proliferation mediated through the GH-IGF-1 axis (Fruchtman et al., 2005). The importance of Stat and Socs is evident in growth performance in mice. For example, disruption of the Stat5b gene reduced body weight gain in male mice (Udy et al., 1997). In another study, Socs-2 inhibited the GH-stimulated JAK-Stat signaling and Socs-2-deficient mice grew larger than their wild-type counterparts (Metclf et al., 2000). Also, skeletal muscle cells responded rapidly to GH by increasing the phosphorylation of JAK2 and Stat5 thereby increasing IGF-1 mRNA expression (Sadowski et al., 2001). These results suggest that Stat and Socs-2 have opposing effects in skeletal muscle, where Stat promote proliferation but Socs-2 is inhibitory.

There is evidence of interaction between steroid hormones and cytokine receptors. Ligand-bound glucocorticoid receptor is directly associated with Stat5 and thereby enhances prolactin-activated JAK/Stat signaling (Wyszomierski et al., 1999). Estrogen is shown to be inhibitory on GH signaling in different cell lines. Estrogen reduced GH-induced phosphorylation of Stat in breast cancer cells but neither GH nor estrogen changed the abundance of Stat protein (Leung et al., 2003). In addition, estrogen increased the Socs-2 mRNA abundance, whereas abundance of Socs-1 and-3 mRNA were unaffected by estrogen or GH. These observations indicate that estrogen inhibits GH signaling by suppressing activation of Stat5 and simultaneously stimulating the expression of the inhibitory protein Socs-2. Administration of estrogen down-regulated GH signaling in hepatocytes (McCarty, 2003).

Information available in other species and in different tissues contradicts the proliferation promoting effects of estrogen in the prepubertal bovine mammary gland. There is only a little information available on the underlying mechanisms to explain how ovarian secretions regulate bovine mammary epithelial proliferation. But it is clear that exogenous estrogen induces proliferation of mammary epithelial cells in prepubertal heifers. If the effect of estrogen is indirect, this suggests that there are alternative mechanisms to explain how estrogen induces mammary cell proliferation. Hence, the effect of estrogen on GH signaling and in turn in the proliferative response in cells could be tissue- as well as species-specific.

1.5. Mammary stem cells

The ability of stem cells for self-renewal is perpetuated by symmetric and asymmetric cell divisions. Symmetric division gives rise to daughter cells that maintain the pool of stem cells while asymmetric division produces one daughter stem cell and a progenitor cell that can proliferate and differentiate into different cell lineages (Chepko and Smith, 1999). A large amount of evidence shows that mammary tumors and hyperplasias are the clonal progeny of a single mutated cell (Young et al., 1971; Cardiff, 1984), implying the presence of mammary stem cells. It is believed that any part of the mammary epithelium is capable of generating a fully functional epithelium in murine mammary gland. For example, random fragments from lactating and involuting mammary glands reproduced lactating outgrowths in mice (Smith and Medina, 1988; Kordon and Smith, 1998). The cyclic pattern of mammary ductular growth, alveolar development, secretory cell differentiation and involution in mammary gland across multiple lactations indicate the existence of stem cells in bovine mammary gland. Limited information is available on bovine mammary stem cell characteristics and the methods to identify them. Ellis and Capuco (2002) reported a method of identifying bovine mammary stem cells by histological features and staining characteristics. Similar to Chepko and Smith (1997), Ellis and Capuco (2002) identified putative mammary stem cells in prepubertal mammary parenchyma by their less differentiated and light staining phenotype. Later Capuco (2007) reported a new method of identifying the putative stem cell population in bovine mammary gland by DNA label retention. Stem cells retain labeled DNA for prolonged period because of the selective segregation of DNA template during cell division. A dual labeling technique involving DNA labeling along with staining for a general proliferating nuclear antigen helps to indentify putative stem cells from the regularly proliferating epithelial cells (Capuco, 2007).

Endocrine regulation of bovine mammary stem cells is not well understood. Estrogen induces epithelial proliferation, but almost all of the proliferating epithelial cells in bovine mammary gland are negative for estrogen receptor-α (**ERα**) (Capuco et al., 2002). Therefore, it is suggested that different population of cells are involved in estrogen-induced epithelial proliferation. The cells that detect estrogen stimulus send paracrine signal to cells that proliferate (Capuco and Ellis, 2005). Furthermore, a large number of putative stem cells in human breast are

ERα-positive, and in murine mammary gland ERα-positive epithelial cells give rise to ERα-negative cells (reviewed in Capuco and Ellis, 2005). There are a few reports on IGF-1 regulation of progenitor cells. Treatment with GH and IGF-1 in aged mice increased the number and function of endothelial progenitor cells (Thum et al., 2007). Overexpression of IGF-1 increased the telomerase activity and functional capacity of aging cardiac stem cells (Torella et al., 2004). Moreover, expression of IGF-1R has been reported in osteogenic and neural progenitor cells (Jia and Heersche, 2000; Zaka et al., 2005). Understanding the mechanisms by which lineage decisions are made during mammary growth and development would be helpful in designing strategies to enhance milk production.

1.6. Rationale and significance

Many studies have been done in the past that evaluated regulation of mammary growth and development in dairy cattle in older heifers and cows, but information specific to early prepubertal heifers is limited. Nonetheless, growth and development of mammary tissue during the prepubertal life is critical for the functional development of mammary gland during gestation and lactation. The allometric mammary growth period is a potential phase during which therapeutic interventions might be applied to enhance mammary growth, thus improving milk production later during lactation. The mechanisms involved in the regulation of hormonal control of mammary growth at the tissue level are still not explained well. Different studies described in this dissertation are aimed at providing a better understanding of the underlying mechanisms involved in the ovarian and GH regulation of mammary growth and development in prepubertal dairy heifers. These experiments are designed such that specific emphasis is given to the regulatory mechanisms occurring locally in the mammary gland through histological methods, as well as by gene expression approaches utilizing relatively modern techniques like quantitative real-time polymerase chain reaction method.

CHAPTER 2: GENERAL LABORATORY METHODS

2.1. Determination of relative gene expression by quantitative real-time polymerase chain reaction

2.1.1. Tissue collection and total RNA isolation

The skin covering the mammary gland was clipped and sanitized by wiping with 70 % ethanol prior to euthanasia of heifers. The udder was removed from the rest of the carcass immediately after exsanguination using sterile razor blades. Whole mammary gland was weighed and then divided into half by cutting through the medial suspensory ligament. Both mammary parenchyma (**PAR**) and fatpad (**MFP**) were dissected from the left hind quarter, cut into small pieces, snap frozen in liquid nitrogen and stored at -80^{o}C in cryoprotected sterile plastic vials.

Total RNA from PAR and MFP were isolated using TRIZOL reagent (Life Technologies; Grand Island, NY) according to the manufacturer's instructions. Briefly, 100-200 mg tissue was homogenized in 1.5 ml Tri reagent in a tissue homogenizer (PRO Scientific Inc., Oxford, CT) at maximum speed for 30 s. Samples were kept on ice at all times. After incubating the homogenate for 5 min at room temperature, 300 µL chloroform was added, incubated again for 15 min and then centrifuged at 12,000xg for 15 min at 4^{o}C. The aqueous phase was separated and then 750 µL of isopropanol was added. Tubes were undisturbed for 5-10 min followed by centrifugation at 12,000xg for 8 min. The supernatant was decanted and the pellet was washed with 2 ml of 75 % ethanol in RNase-free water and mixed well followed by centrifugation at 12,000xg for 5 min. Ethanol was decanted and the pellet was air dried before dissolving in 100 µL RNase-free water. All the subsequent incubations and centrifugations were performed at room temperature unless specified otherwise. Isolated total RNA was then purified by treating with DNase I and RNeasy kit (Qiagen Inc., Valencia, CA) following the product protocol. Quantity of RNA was determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). Samples with a ratio of optical measurements at 260 and 280 nm (OD 260nm/OD 280nm) greater than 1.8 were accepted. To evaluate the integrity of 18S and 28S ribosomal RNA, 5 µg RNA from each sample was electrophoresed on 1 % agarose gel and visualized by ethidium bromide staining under UV light.

2.1.2. Reverse transcription and real-time PCR

Single stranded cDNA were synthesized by reverse transcribing the total RNA by using the High Capacity cDNA Archive kit from Applied Biosystems (Foster City, CA) according to the manufacturer's instructions. Briefly, a total of 4 μg RNA was reverse transcribed to single stranded cDNA in a final reaction volume of 40 μL using random primers. A control sample containing no reverse transcriptase enzyme was used for each sample and this was later used in real-time PCR. The cDNA produced was then diluted 10-fold with sterile nuclease-free water. For quantitative real-time PCR, a total of 20 ng cDNA was used in each reaction, along with 12.5 μL of SYBR Green dye (Applied Biosystems; Foster City, CA), 9.5 μL of sterile distilled water, 0.5 μL of 10 μM forward primer, and 0.5 μL of 10 μM reverse primer. The PCR conditions were: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min and repeated for 40 cycles. Real-time PCR reactions were performed in a 7300 Series Real-Time System and data were collected and analyzed using SDS software (Applied Biosystems, Foster City, CA).

Each sample was assayed in triplicates and replicate samples having a standard error greater than 0.05 were not accepted. Each PCR plate also contained a no-RT template control for each sample analyzed and a no-template control (nuclease-free water instead of cDNA template) per primer pair. No-RT template control was to make sure there was no DNA contamination in the original RNA sample and the no-template control was to test for contamination of reagents used. Average cycle threshold (**Ct**) values of replicate samples collected using the SDS software for each target gene and endogenous reference genes were exported to Microsoft Office Excel. All the primers used were either published in literature or designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Three endogenous reference genes; 1) protein phosphatase 1 regulatory (inhibitor) subunit 11 (**PPP1R11**; forward primer 5'- CCA TCA AAC TTC GGA AAC GG -3' and reverse primer 5'- ACA GCA GCA TTT TGA TGA GCG -3'), 2) ribosomal protein S15A (**RPS15A**; forward primer 5'- GAA TGG TGC GCA TGA ATG TC -3' and reverse primer 5'- GAC TTT GGA GCA CGG CCT AA -3') and 3) mitochondrial GTPase 1 homolog (**MTG1**; forward primer 5'- CTT GGA ATC CGA GGA GCC A -3' and reverse primer 5'- CCT GGG ATC ACC AGA GCT GT -3') (Piantoni et al., 2008) were used for normalization of data. Primer pairs were tested for amplification efficiency. All the primer pairs

used were having amplification efficiency between 90 – 110 %. Additionally, the use of endogenous control genes were validated for each target gene by confirming that the slope of ΔCt vs. log cDNA input is < 0.1. A dissociation curve was performed after each real-time PCR run to rule out formation of primer dimers, genomic DNA contamination, and other impurities in the PCR amplicons. In addition, the PCR products for each primer pair were electrophoresed on 2 % agarose gel containing ethidium bromide and visualized under UV light to confirm purity and size of the amplicon. Relative gene expression was determined by comparative Ct method, also known as the ΔΔCt method (Livak and Schmittgen, 2001). Normalization of Ct value for each target gene from each animal was done by subtracting the geometric mean of Ct for the three endogenous reference genes from the Ct value of each target gene (Piantoni et al., 2008). This normalized value is called the ΔCt for each target gene. Geometric mean of endogenous reference genes were calculated in Excel and then data were imported to SAS using the data import wizard. Calculation of ΔCt for each target gene was done in SAS and statistical analyses were performed on the ΔCt data set. Least squares of means of ΔCt were then used to calculate the fold change in mRNA expression for each target gene for the treatment group relative to control ($2^{-\Delta\Delta Ct}$).

Steps involved in calculating the fold change in gene expression:

ΔCt for each gene = Ct for target gene – Geometric mean of Ct for three reference genes

$2^{-\Delta\Delta Ct}$ = Least square means of treatment ΔCt – Least square means of control ΔCt

2.2. Histological techniques

2.2.1. Tissue collection and processing

Mammary gland was carefully removed from the carcass by incising the skin surrounding the gland immediately after exsanguination. After weighing the whole udder, it was divided into two halves by incising through the medial suspensory ligament. Both PAR and MFP were dissected out in small pieces consistently from the left front quarter from each animal and fixed in 10 % normal formalin. Tissues were transferred to 70 % ethanol after 24 h or later assuring successful fixation. When ready to be embedded, tissues were dehydrated in ascending strengths of ethanol and then cleared in two baths of xylene for 1 hr each. Paraffin infiltration of tissues was done for an hour and then tissues were embedded in melted paraffin using plastic

molds. Tissue sections of 5 µm thickness were made using a rotary microtome (Model HM340E, Microm, International GmbH, Germany). Four to five serial sections were mounted onto charged 25 x 75 x 1 glass slides (VWR International, West Chester, PA).

2.2.2. Histomorphometry

To perform histomorphometry, tissue sections were cleared off paraffin by two washes in xylene for 2 min each. Sections were then rehydrated in descending strengths of ethanol, 100 %, 95 %, and 70 % ethanol, 2 min each and then followed by a final wash in distilled water for 5 min to ensure hydration of the tissue. Hematoxylin and eosin stain (Sigma-Aldrich, St. Louis, MO) was applied for 10 min followed by washing in distilled water for 5 min. Tissue sections were then dehydrated by treating with ascending grades of ethanol, from 70 % to 100 %, and then two washes of xylene for 5 min each. Stained sections were mounted using Permount tissue mounting solution (Fischer Scientific, Pittsburgh, PA) for permanent storage. Photomicrographs were taken using Olympus BH2 light microscope connected to a QColor3 digital camera (Olympus America Inc, Center Valley, PA) at 4x magnification using the Q-Capture suite software program (QImaging; Surrey, BC, Canada). Images were saved as .JPEG files and histological measurements were made using image analyzer software program (Image-Pro Plus Version 6.2; Media Cybernetics; Silver Springs, MD). Using the Image-Pro, epithelial area measurements were made by outlining the epithelial structures using the polygon drawing tool (Fig 2.1). Ten microscopic fields were analyzed per sample. Area measurements were then exported to Microsoft Office Excel and necessary calculations were made in Microsoft Excel.

2.2.3. Immunohistochemistry

Both bright-field and fluorescent immunohistochemistry to identify and locate as well as to quantify some specific proteins in the bovine mammary gland were performed using formalin-fixed paraffin-embedded tissue sections (Capuco et al., 2002). After deparaffinization and hydration of the tissue sections, a quenching reaction was done by treating with 3 % hydrogen peroxide followed by three PBS washes to remove the endogenous peroxidase activity in the tissue. Heat-induced antigen epitope retrieval was performed to recover or enhance antigenicity of proteins which was lost during formalin fixation and paraffin embedding. Antigen

recovery was done by heating the tissue sections in 10 mM citrate buffer for 15 min on a hot plate and then cooling it down for 30 min on bench top. After that the tissues were treated with a blocking solution (5 % goat serum or CAS block) to inhibit non-specific binding of the primary antibody. Individual tissue sections on a slide were isolated using a liquid blocking PAP pen (cat # NC9720458; Fisher Scientific). Sections were incubated overnight at 4^{o}C with primary

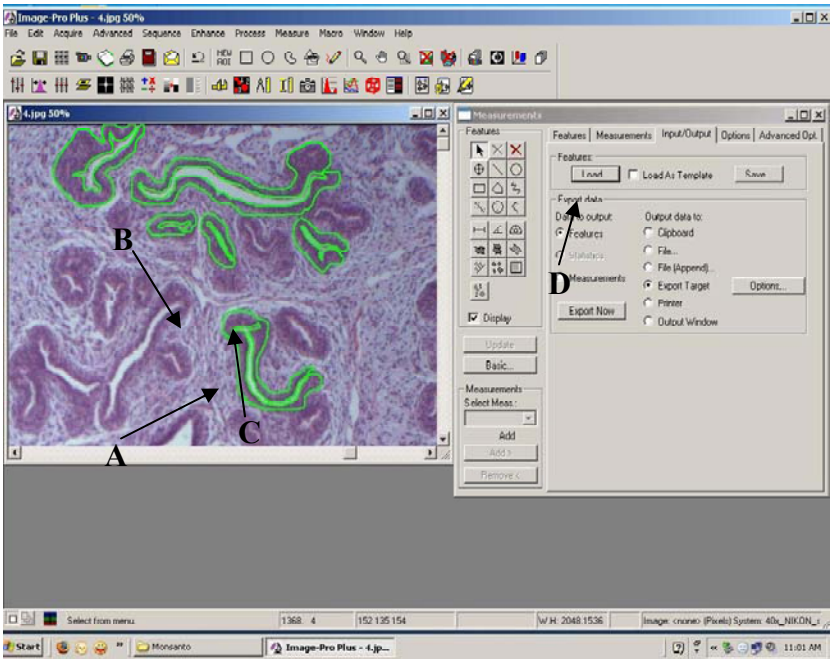


Figure 2. 1. Illustration of making histological measurements.

Screen-capture view of photomicrograph showing histological measurements using ImagePro software. Epithelial areas are outlined in green using the polygonal drawing tool. Black arrows indicate different types of ducts classified based on lumen size such as (A) large, (B) medium, (C) small, and (D) buds.

antibody which was diluted to appropriate concentrations. Each slide had a negative internal control which did not get treated with primary antibody, but was incubated with the blocking reagent. After the overnight incubation, excess antibodies were removed by three washes with PBS for 5 min each. Then, depending on the detection system used, tissue sections were incubated for 1 hr at room temperature with secondary antibodies conjugated either with

fluorescent tags or biotinylated antibody. Any residue left behind from PAP pen was removed by wiping with xylene swabs before applying cover slip.

For the bright field detection system, streptavidin-peroxidase along with diaminobenzidine system was used. After secondary antibody incubation, sections were washed with PBS, treated with streptavidin-peroxidase (Reagent 2 of Histostain Kit; Zymed Laboratories Inc., Invitrogen, Carlsbad, CA) and incubated for 10 min. Sections were then washed thrice with PBS for 5 min each. Two to three drops of diaminobenzidine solution (Liquid DAB substrate kit; Zymed Laboratories Inc., Invitrogen, Carlsbad, CA) was added to each section on the slide and incubated for 5 min. Sections were counterstained with 2 - 3 drops of hematoxylin (Gill's formulation #2 Hematoxylin; Santa-Cruz Biotechnology Inc, Santa Cruz, CA) for 30 sec and washed with water. Tissues sections were then mounted using Permount after dehydration and xylene washes. Positive staining for the specific antigen was indicated by brown staining cells.

If fluorescent detection system was used, CAS block (Invitrogen (cat # 00-8120), Carlsbad, CA) was used for blocking and as diluent for primary and secondary antibodies. Secondary antibodies were tagged with fluorophores. Depending on the species to which primary antibody had been raised, 50 μL of either Alexa 488 goat anti-rabbit or Alexa 594 goat anti-mouse IgG (Molecular probes, Invitrogen, Carlsbad, CA) was used per tissue section. The mixture was spun at 10,000 x g for 10 min before adding on to the tissue to remove aggregates. Slides were incubated with secondary antibody for 60 min in the dark at room temperature. This was followed by three washes in PBS, 5 min each and a brief rinsing in distilled water. Counter staining for DNA was done by addition of 4'-6-diamidino-2-phenylindole (DAPI) which was a part of the Prolong Gold anti-fade mounting solution (Invitrogen, Carlsbad, CA). Slides were stored flat in dark for 24 hr before further processing.

Photomicrographs were taken using Olympus BH2 light microscope connected to a QColor3 digital camera (Olympus America Inc, Center Valley, PA) at 20x and 40x magnifications using the Q-Capture suite software program (QImaging; Surrey, BC, Canada). Images were saved as .JPEG files, and positively stained cells were counted using the Image-Pro software (Media Cybernetics). Using Image-Pro, first of all an index of epithelial cells per unit area was determined. The cell number index was calculated by counting the number of cells

(total of ~2500-3000 cells) in multiple epithelial areas including different locations and types of ducts (for example, epithelial area with single or multiple layers of cells, large and small ducts and also epithelial buds). While counting the labeled cells, epithelial area was first measured and then cell number index was applied to determine the number of cells in a measured area (Fig 2.2). Proportion of labeled cells was then expressed as a percentage of total mammary epithelial cells.

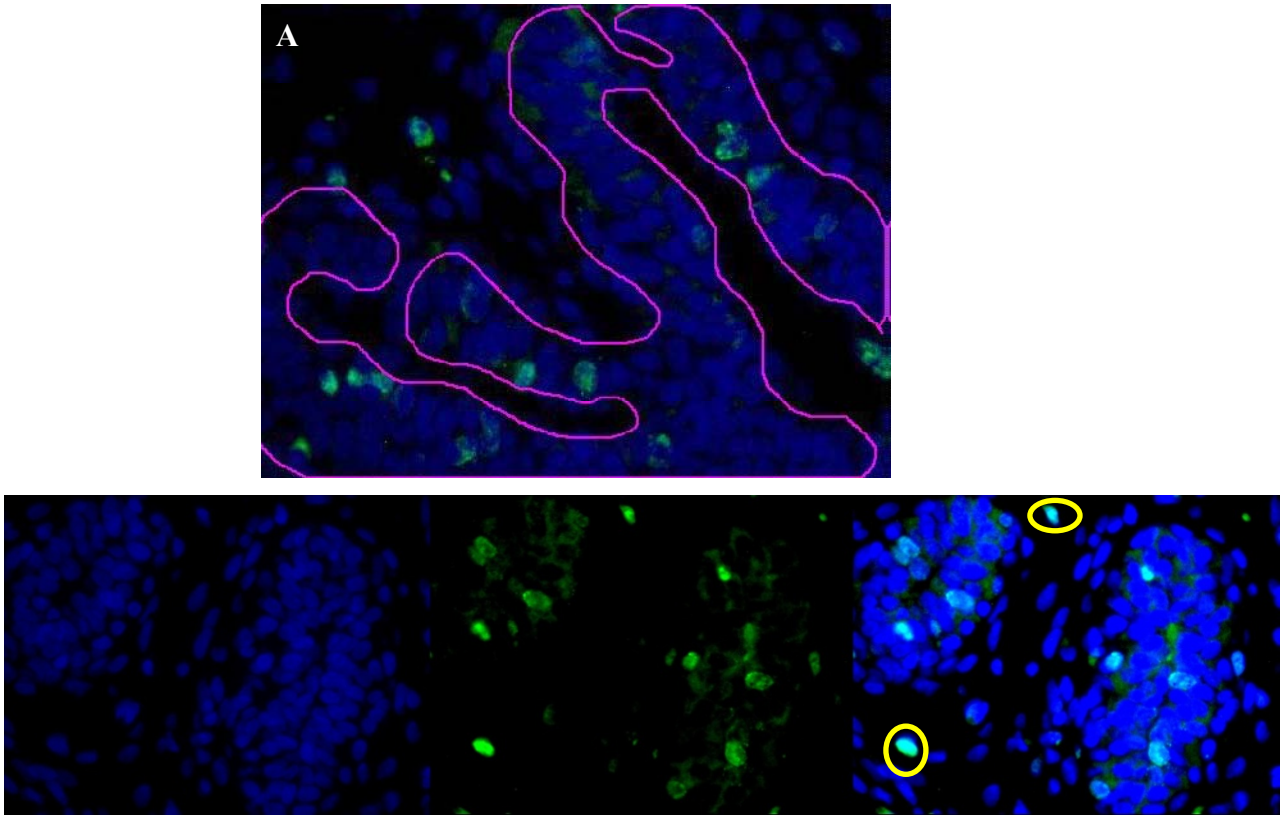


Figure 2. 2. Quantitative immunofluorescence using ImagePro software.

Epithelial area is outlined using the drawing tool in ImagePro to count mammary epithelial cells (A). Total number of cells in the outlined area is determined using the cell number-area index number. (B) DAPI channel showing all nucleated cells in blue by staining the DNA with DAPI. (c) FITC channel showing Ki67 positive cells in green. (d) Computer generated composite image showing both DAPI and Ki67 positive cells. The cells encircled are not included in the count because they are in the stroma.

2.3. Proximate analysis of mammary parenchyma and fat pad

2.3.1. Tissue collection and preparation

The udder was removed from the rest of the carcass immediately after exsanguination using sterile razor blades. The udder was weighed and then divided into half by cutting through the medial suspensory ligament. The right hemi-udder was snap frozen in liquid nitrogen after wrapping in sterile aluminum foil packs. Snap frozen tissues were then stored at -80^{o}C until further processing. Frozen tissues from -80^{o}C were dissected without thawing. Both PAR and MFP were separated out by dissection and weighed separately. Tissue samples were then pulverized in a freezer mill (SPEX SamplePrep LLC, Metuchen, NJ) and again stored at -80^{o}C until assayed. For determination of total protein and DNA in the sample, 250 mg of powdered sample was homogenized for 30 s in ice-cold 1.5 ml high-salt homogenization buffer (see Appendix C.2) using a tissue homogenizer (PRO Scientific Inc., Oxford, CT). The homogenate was centrifuged at 1000xg for 5 min at 4^{o}C and the supernatant was decanted and saved at -80^{o}C for protein and DNA estimation.

2.3.2. Estimation of DNA

The concentrations of DNA in mammary tissue homogenates were determined using Hoefer DQ 300 Fluorometer (Hoefer Inc., San Francisco, CA). The fluorometer was calibrated using the high range assay buffer and DNA standard solutions for PAR samples while low range assay buffer and DNA standards were used for MFP samples (See Appendix C.4). Two µL of the soluble fraction of the tissue homogenate was added to 2 ml of DNA assay buffer in a disposable cuvette and the readings were recorded. Concentrations of DNA were calculated by using the optical density reading of both samples and DNA standard and the concentration of the standard used. Each sample was done in triplicates and average of triplicate samples were used for statistical analysis.

2.3.3. Estimation of total proteins

Total proteins in PAR and MFP were determined using BCA^{TM} protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Different standard concentrations were prepared using bovine serum albumin which had a working range of 20 – 2000 µg/ml.

Soluble fractions from tissue homogenate was diluted before protein estimation and 25 µL of duplicate diluted samples were added to 200 µL of the working reagent and incubated at 37^{o}C for 30 min. Absorbance was measured at 562 nm wavelength on a plate reader (µQuant, Biotek instruments Inc., Winooski, VT). Protein concentrations in the samples were calculated using the optical density readings and corresponding standard curve.

2.3.4. Estimation of total lipids

Pulverized tissue samples were weighed and transferred to acid washed and dried glass tubes. Hexane-isopropanol extraction mixture (HIP; see Appendix C.5) in the ratio of sample to HIP volume (1 gm to 18 ml) was added and mixed well by vortexing. Sodium sulfate at a ratio of 1 gm to 9 ml was then added and mixed well. After 5 min to allow for phase separation, samples were again vortexed. After centrifugation at 1000 x g for 5 min at room temperature, the upper solvent phase was transferred to pre-weighed tubes with a Pasteur pipette. The lower aqueous layer was re-extracted in a similar manor as described above and the solvent layer was transferred and combined with that from the previous extraction. The combined solvent fractions were then evaporated in N-Evap system (Organomation Associates Inc., Berlin, MA) using nitrogen vapor. The tubes with lipid residue were weighed and the weight of lipid residue was calculated by subtracting the initial empty tube weight.

CHAPTER 3: EFFECT OF STAGED OVARIECTOMY ON MEASURES OF MAMMARY GROWTH AND DEVELOPMENT IN PREPUBERTAL DAIRY HEIFERS

3.1. Abstract

Previous studies in prepubertal heifers suggest that the magnitude of reduction in mammary parenchymal growth in response to ovariectomy varies depending on the age at which surgery is performed. We hypothesized that ovarian secretions are essential for initiating mammary development but not required to maintain allometric mammary growth in prepubertal dairy heifers. The objectives of this study were to determine the effect of staged ovariectomy during prepuberty on mammary growth and composition as well as on gene expression in bovine mammary gland. Prepubertal Holstein heifers either at two, three or four months of age were randomly assigned to one of two treatments, ovariectomized (**OVX**; n = 8) or sham operated (**INT**; n = 12). Mammary parenchyma (**PAR**) and fat pad (**MFP**) were harvested 30 d after surgery. Proximate composition of PAR and MFP (DNA, protein and lipid) as well as expression of estrogen receptor-α (**ERα**), the estrogen responsive genes *stc1* and *tfpi*, progesterone receptor (**PR**) and proliferating cell nuclear antigen (**PCNA**) were determined in PAR and MFP by quantitative real time polymerase chain reaction. The relative amount of epithelium as well as localization of PR and Ki67 positive cells in PAR were determined by histological and immunohistochemical analyses, respectively. Mammary PAR growth was reduced in OVX mainly due to reduction in lipid accretion and not due to changes in protein or DNA. In MFP, protein was reduced in OVX while DNA and lipid remained unchanged. Expression of PR was reduced in OVX at both protein and message level, but there was no difference in the proliferation of epithelial cells. Expression of *stc1*, *tfpi*, and PCNA mRNA were reduced in OVX while ERα mRNA was not different between OVX and INT. These data did not support our hypothesis. We did not find an increased response to ovariectomy when surgery was performed at two months rather than at three or four months of age. Our data suggest that a 30 d period after ovariectomy in early prepubertal life in dairy heifers may not be long enough to produce marked reduction in proliferation rates in mammary epithelial cells despite the changes in gene expression.

3.2. Introduction

The significance of the ovary in mammary growth and development has been studied since early 1900s. Even though classic experiments were conducted in rats and mice, a number of studies with ruminants have also been reported. An early study performed in calves and lambs showed that the two species respond differently to ablation and replacement treatments. There was no apparent reduction in mammary parenchyma in sheep when ovaries were removed at birth while in cattle mammary growth was almost blocked (Wallace, 1953). Later studies also supported this difference in ovarian regulation of mammary growth and development between ruminant species (Purup et al., 1995; Ellis et al., 1998; Berry et al., 2003c). Regulation of mammary growth by ovarian secretions is believed to be mainly because of estrogen but a local rather than a systemic pathway has also been proposed. It was reported that there was only a 0.1 pg/ml reduction in circulating concentration of estrogen between intact and ovariectomized heifers (Purup et al., 1993), which seems to be inadequate to elicit the observed reduction in parenchymal growth. Moreover, literature shows that a daily dose of 0.1 mg/ kg BW estradiol is required to elicit positive responses in mammary epithelial cell (**MEC**) proliferation (Woodward et al., 1993; Capuco et al., 2002; Meyer et al., 2006a). On the other hand, remarkable increase in the expression of estrogen receptor-α (**ERα**) along with a significant reduction in MEC proliferation in ovariectomy (Berry et al., 2003b) suggests there are locally active mechanism(s) involving estrogen and cell proliferation pathways.

The age at which ovariectomy is performed is important in eliciting changes in mammary growth. Ovariectomy performed at birth almost completely blocked mammary development in calves (Wallace, 1953) while there was up to a 90 % reduction in mammary parenchymal growth when heifers were ovariectomized at 2.5 mo of age (Purup et al., 1995). When ovaries were removed from heifers between 2.5 to 3.0 mo of age, there was a reduction in mammary development but not complete cessation (Berry et al., 2003b). Therefore, we hypothesized that ovarian secretions are required to initiate mammary development but not required to maintain allometric mammary growth in prepubertal dairy heifers. The main objective was to determine whether there is a difference in the magnitude of response to ovariectomy performed at different stages of early prepubertal period on mammary growth and development. The specific objectives were to determine the effect of staged ovariectomy during early prepuberty on mammary growth

and composition as well as its impact on MEC proliferation and associated biochemical, histological and gene expression responses of mammary tissue.

3.3. Materials and methods

3.3.1. Animals and treatments

Twenty four Holstein heifers were purchased from regional dairies and housed in the Simpson Experiment Station at Clemson University. All animal care and use protocols used in the study were approved by the Clemson University Institutional Animal Care and Use Committee. Heifer health was monitored continuously by checking the body temperature, assessing feed and water intake and observing behavioral changes. Body weights were recorded at birth and weekly thereafter. All animals were fed with commercial milk replacers and calf starter diets according to the manufacturer's instructions prior to weaning and with grains and hay thereafter. After a week's adaptation period at the facility, heifers were randomly assigned to either ovariectomy (**OVX**; n = 12) or sham operation (**INT**; n = 12) when they were 60 ± 1, 93 ± 3 or 122 ± 2 d of age. Animals of different age groups were acquired in batches and hence surgery was performed at different time points. Surgeries were performed by laparoscopic vertical incision of about 10 cm on the left flank after desensitizing the area with local anesthetic (Lidocane HCl, 2% injectable solution). In OVX heifers, ovaries were isolated and removed with a Meagher Ovary Flute (www.spaytool.com). In INT heifers, ovaries were palpated during a sham surgery, but left intact. One of the heifers (animal# 4882) from the OVX group was euthanized at surgery because of the accidental puncturing of the intestine which contaminated the peritoneal cavity. Body weights were recorded a week before the surgery (wk 0) and then once weekly after surgery (wk 1 to 4). Both INT and OVX heifers were healthy and there was no difference in BW gain between treatment groups (Fig 3.1). Thirty days after surgery, animals were humanely sacrificed by captive bolt pistol stunning followed by exsanguination and tissues were harvested immediately.

All ovariectomies were confirmed at slaughter by careful inspection and histological analysis of any suspect tissues. Two heifers in OVX (animal# 1616 and 4876) were found to have pieces of ovary-like tissue attached to the reproductive tract. Histological evaluation of

these suspect tissue pieces revealed characteristic features of ovarian tissue, including obvious follicle structures. Therefore, those two heifers were also excluded from final data analyses.

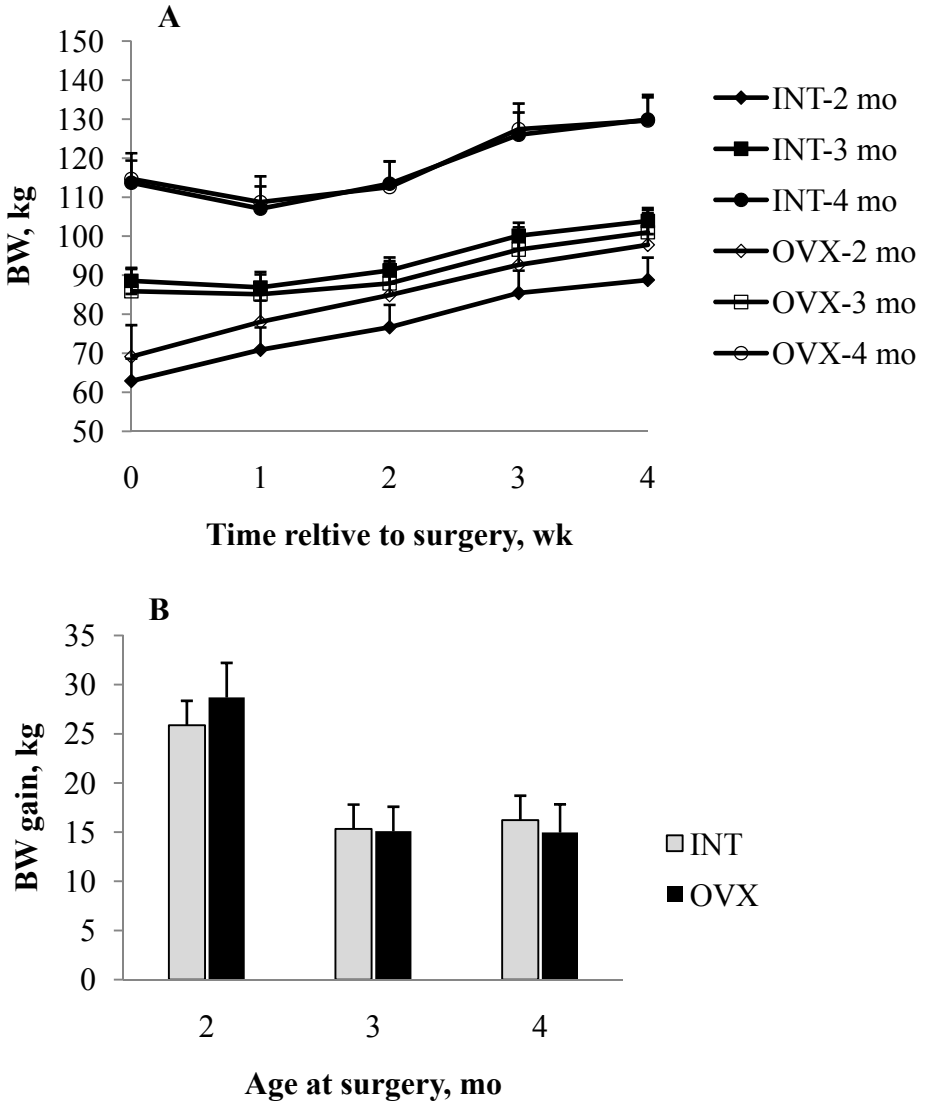


Figure 3. 1. Effect of ovariectomy on weekly BW and BW gain over the treatment period

(A) Weekly BW and (B) BW gain in INT (n = 12) and OVX (n = 8) dairy heifers operated at 2, 3, or 4 mo of age and sacrificed 30 d after surgery. Data presented as LSM and the bars represent SEM.

Mammary PAR was barely detectable (only a narrow streak) in one of the heifers in OVX (animal# 3110) and therefore this heifer could not be included in data analyses due to inadequate

tissue. Thus the total number of OVX animals used for statistical analyses of data was 8; n = 2, 3 and 3 for age at surgery 2, 3, and 4 mo, respectively.

3.3.2. Sample collection and analyses

Mammary gland was separated from the carcass immediately after exsanguination and weights of the whole gland, hemigland, dissected mammary parenchyma (**PAR**) and fat pad (**MFP**) were recorded. Detailed description of sample collection methods is given in Chapter 2. Mammary PAR and MFP were harvested for biochemical, histological and gene expression analyses. Biochemical analyses included spectrometric determination of DNA and protein as well as hexane-isobutanol extraction of lipid in mammary PAR and MFP (Chapter 2).

For histological evaluation, formalin fixed and paraffin embedded mammary PAR tissue sections were used for measuring the percent area of epithelium and also to determine the number of different sized ducts using image analyzer software (Image-Pro Plus Version 6.2 Media Cybernetics, Inc. Silver Spring, MD) (Chapter 2). Epithelial cell proliferation was assessed by determining the labeling index for the proliferating cell antigen Ki67 in MEC (Capuco et al., 2002). Briefly, formalin fixed paraffin embedded tissue sections of 5 μm thickness were deparaffinized in xylene and then rehydrated in descending strengths of ethanol. Antigen retrieval was performed by boiling in 10 mM citrate buffer followed by washing with PBS and blocking in CAS block (Invitrogen; cat # 00-8120; Carlsbad, CA). Primary antibody was prepared by 1:200 dilution of anti-Ki67 monoclonal antibody raised in rabbit (Clone SP6, Fisher, Cat# RM-9106-S0) in CAS block solution and sections were incubated for overnight at 4^{o}C. The secondary antibody was a fluorophore tagged goat anti-rabbit IgG (Alexa 488; Invitrogen) diluted 1:200 in CAS block and sections were incubated in room temperature for 1 h. Counter staining and mounting was done using ProLong Gold antifade reagent with DAPI (Invitrogen).

Immunohistochemistry of progesterone receptors (**PR**) was performed on five μm thick paraffin embedded tissue sections. Briefly, tissue sections were deparaffinized in xylene before rehydration through graded ethanol to deionized water and phosphate buffered saline (**PBS**). Sections were washed with 0.3 % Triton X in PBS for 15 min and then blocked for endogenous peroxidase activity using 0.9 % hydrogen peroxide for 10 min. Antigen retrieval was performed

by boiling in a 10 % citrate buffer (Diagnostic Biosystems, Pleasanton, CA) for 35 min with subsequent cooling for 10 min. Sections were then rinsed in PBS followed by PBST prior to blocking of endogenous biotin activity with an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA). Non-specific binding was prevented by blocking the sections with 10 % horse serum in PBS for 60 min at room temperature. Tissue sections were incubated with anti-PR antibody (Santa Cruz Biotechnology # SC-7208, Santa Cruz, CA; 1:50 in 10 % horse serum/ PBS) overnight at 4˚C. Negative controls were incubated with 10 % horse serum only. Sections were washed three times in PBST prior to incubation with a biotinylated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA #711-065-152; 1:1000 in 10 % horse serum/PBS) for 45 min. After rinsing twice in PBS and once in PBST, sections were incubated with a streptavidin-peroxidase conjugate (Zymed Laboratories, South San Francisco, CA) for 30 min at room temperature. This was followed by washing in PBS and PBST prior to the detection of immunoreactivity using NovaRED or DAB plus nickel chromogens (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin and protected by coverslip using Permount. Slides were stained in groups of 6-12, where each group contained representative samples from either treatment. An average of 2000-2500 mammary epithelial cells were counted per sample for determining the percentage of Ki67 and PR positive cells. Basal layer of myoepithelial cells were excluded from counting.

Relative gene expression for the estrogen responsive genes stanniocalcin1 (*stc1*) and a gene similar to tissue factor pathway inhibitor precursor (*tfpi*) (Li et al., 2006), two steroid receptor genes such as ERα (Connor et al., 2005) and PR as well as the proliferation marker gene proliferating cell nuclear antigen (PCNA) were determined using quantitative real-time PCR by comparative Ct method (Chapter 2). Target gene Ct value in triplicates was normalized using geometric mean of Ct values for three housekeeping genes; PPP1R11, RPS15A and MTG1 (Piantoni et al., 2008). The sequences of forward and reverse primers are given in Table 3.1.

3.3.3. Statistical analysis

Data were analyzed using Mixed procedure of SAS (SAS 9.2; Cary, NC). Effects of treatment, age at surgery and interaction between treatment and age were tested with heifer within treatment and age as the random effect. Proximate composition data for PAR and MFP

were analyzed separately. Positive interaction effects are further analyzed using Slice to determine the treatment differences between age groups. Data distribution was analyzed by using "Proc Mixed Boxplot" statement (Appendix A.6). Significance was declared at $P \leq 0.05$ for all analyses.

The model used in all analyses was

$Y_{ijk} = \mu + T_i + A_j + (TA)_{ij} + e_{(ij)k}$ where

Y_{ijk} = variable being tested

μ = overall mean

T_i = fixed effect of treatment (INT or OVX) (i = 1, 2)

A_j = fixed effect of age at surgery (2, 3 or 4 mo) (j = 1, 2, 3)

$(TA)_{ij}$ = fixed interaction of treatment and age

$e_{(ij)k}$ = residual error

Gene expression data for PAR and MFP were analyzed combined as well as separately. When analyzed combined, main effects of tissue, interactions between tissue and treatment as well as tissue and age were also included in the model (Appendix A.5). The ΔCt data were used for statistical analyses and significance was determined based on the *P* values for ΔCt data (Appendix A.1 and A.2). However, data was presented as fold change in gene expression for OVX animals relative to INT control animals using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Chapter 2). As mentioned in section 3.3.1., the number of animals in each group used for statistical analyses is for INT, n = 4 for all age groups and for OVX n = 2, 3 and 3 for age at surgery 2, 3, and 4 mo, respectively.

3.4. Results

3.4.1. Mammary growth and composition

Weight of tissues per 100 kg BW for whole udder, mammary PAR and MFP are given in Fig 3.2. When tissue weights were adjusted to BW, there was no difference in udder weight

between treatments or between age groups, but PAR mass was reduced in OVX compared with INT ($P < 0.05$). Body weight adjusted mass of PAR was also increased over time ($P < 0.01$; Fig 3.2). Mass of PAR per 100 kg BW was reduced 56, 12 and 59 % in OVX for 2, 3, and 4 mo age surgery groups, respectively. In the case of MFP, there was no difference in tissue weight between treatments and there was no interaction between treatment and age. However, there were differences in MFP weights between age groups (Fig 3.2). Amount of MFP was greater in 2 mo heifers compared with 3 and 4 mo heifers ($P < 0.01$) but there was no difference in MFP weights between 3 and 4 mo heifers ($P = 0.561$).

Table 3. 1. Primer pair sequences of target and endogenous reference genes

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
stc1	CTACTGGACTGTGACGAAGAC	GCTCGCTGCTGTGTATGG
tfpi	TGCTATGAATAATGCCTCCTTGAC	GTAGTAGAATCTGGTCACATTGGC
ERα	TTGCTGGCTACTTCGTCT C	GGTGGATGTGGTCCTTCTC
PR	CAGTGGTCAAGTGGTCTAAATC	TCTCCATCCTAGTCCAAATACC
PCNA	TCGTCTCAGGCGTTCATAGTC	AACATGGTGGCGGAGTCG
PPP1R11	CCATCAAACTTCGGAAACGG	ACAGCAGCATTTTGATGAGCG
RPS15A	GAATGGTGCGCATGAATGTC	GACTTTGGAGCACGGCCTAA
MTG1	CTTGGAATCCGAGGAGCC A	CCTGGGATCACCAGAGCTGT

Proximate composition of DNA, protein and lipid in mammary PAR in terms of tissue concentration (wt/wt), tissue concentration adjusted to BW as well as total amount present in PAR are given in Table 3.1. In mammary PAR, there was no treatment effect for DNA and protein. The amount of lipid in PAR per 100 kg BW was reduced in OVX compared with INT ($P = 0.049$). There was an overall increase in protein and lipid contents in PAR with increase in age

($P < 0.01$). The amounts of DNA and protein were reduced in PAR of OVX compared with INT when surgery was performed at 4 m of age, but not in earlier surgery groups (treatment and age interaction effect, $P < 0.01$). In MFP, content of DNA and lipid did not differ between treatments, but protein was reduced in OVX compared with INT (Table 3.2). All the variables measured for the proximate composition of MFP were affected by age ($P < 0.05$). Overall, there was an increase in the amount of DNA with age while protein and lipid reduced with age (Table 3.2).

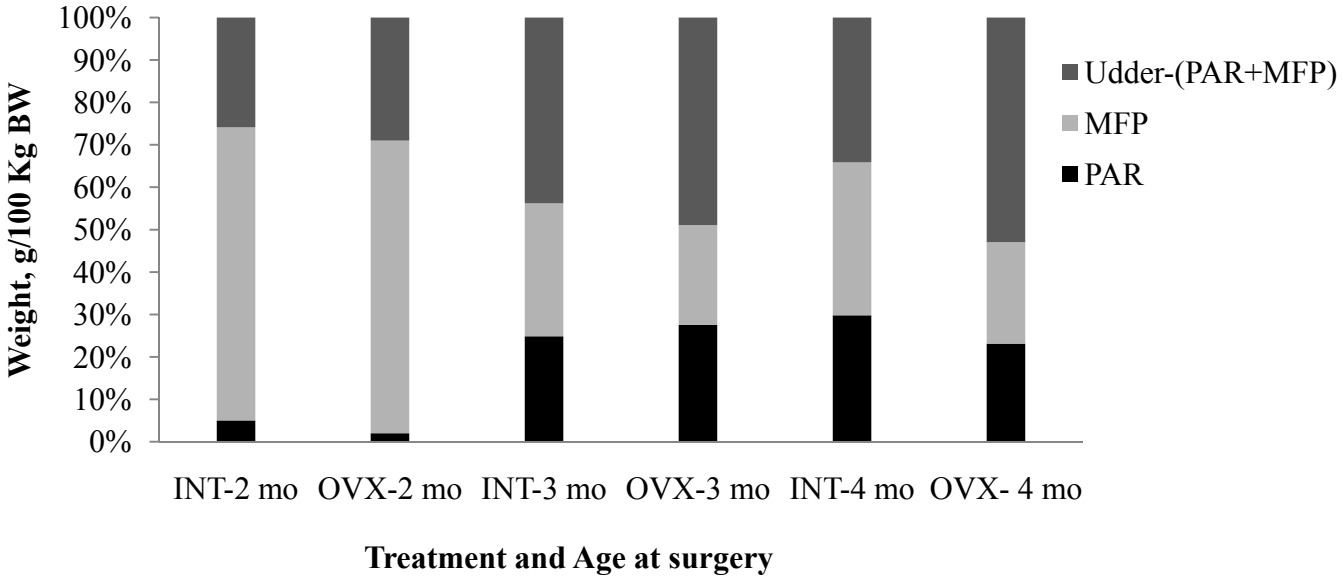


Figure 3. 2. Effect of ovariectomy on udder, mammary parenchyma and fat pad mass

Weight of tissues adjusted to BW for mammary parenchyma (PAR), fat pad (MFP) and the rest of the udder from heifers ovariectomized (OVX; n = 8) or sham operated (INT; n = 12) either at 2, 3 or 4 mo of age and tissue harvested 30 d after surgery

3.4.2. Histomorphometry

Epithelial proportion of mammary PAR in INT and OVX heifers are shown in Fig 3.3. The percent area of epithelium present in PAR was not different between INT and OVX (13.5 ±

1 vs. 13.9 ± 1 % per unit area of PAR; $P = 0.845$). Additionally, numbers of different types of epithelial structures were also not different between treatments (Table 3.3). We did not find any

Table 3. 2. Effect of ovariectomy on proximate composition of mammary parenchyma

Amount of DNA, protein and lipid in the mammary parenchyma from heifers ovariectomized (OVX; n = 8) or sham operated (INT; n = 12) either at 2, 3 or 4 mo of age and tissue harvested 30 d after surgery[†]

	Age at surgery, mo						*P* values[1]		
Item	2		3		4				
	INT	OVX	INT	OVX	INT	OVX	Trt[2]	Age[3]	Trt*Age[4]
DNA									
μg/mg tissue	2.6 ± 0.6	3.1 ± 0.9	2.1 ± 0.6	3.9 ± 0.7	3.7 ± 0.6	3.2 ± 0.7	0.36	0.66	0.28
mg/100 kg BW	40.3 ± 35	20.0 ± 50	119.5 ± 35	166.3 ± 41	308.4 ± 35	115.5 ± 41	0.11	<.01	0.02
Total, mg	35.4 ± 42	20.7 ± 60	124.1 ± 42	187.8 ± 48	418.1 ± 42	155.4 ± 49	0.09	<.01	<.01
Protein									
μg/mg tissue	40.3 ± 3.2	38.9 ± 4.5	34.3 ± 3.2	45.0 ± 3.7	41.0 ± 3.2	38.7 ± 3.7	0.44	0.99	0.15
g/100 kg BW	0.6 ± 0.4	0.2 ± 0.6	1.2 ± 0.4	2.3 ± 0.5	3.4 ± 0.4	1.3 ± 0.5	0.09	<.01	0.06
Total, g	0.5 ± 0.5	0.2 ± 0.7	2.1 ± 0.5	2.5 ± 0.5	4.6 ± 0.5	1.8 ± 0.5	0.06	<.01	0.02
Lipid									
mg/g tissue	131.6 ± 19	192.4 ± 26	133.3 ± 19	122.7 ± 21	142.2 ± 19	195.3 ± 21	0.07	0.14	0.21
g/100 kg BW	2.0 ± 1	1.1 ± 2	7.8 ± 1	5.3 ± 2	11.8 ± 1	7.0 ± 2	0.05	<.01	0.50
Total, g	1.8 ± 2	1.1 ± 2	8.5 ± 2	5.9 ± 2	15.9 ± 2	9.4 ± 2	0.06	<.01	0.33

[†]Data presented as LSM ± SEM. [1]Significance declared at $P \leq 0.05$. [2]*P* value for treatment effect. [3]*P* value for age effect. [4]*P* value for interaction effect between treatment and age.

Table 3. 3. Effect of ovariectomy on proximate composition of mammary fat pad

Amount of DNA, protein and lipid in the mammary fat pad from heifers ovariectomized (OVX; n = 8) or sham operated (INT; n = 12) either at 2, 3 or 4 mo of age and tissue harvested 30 d after surgery[†]

	Age at surgery, mo						*P* values[1]		
Item	2		3		4				
	INT	OVX	INT	OVX	INT	OVX	Trt[2]	Age[3]	Trt*Age[4]
DNA									
μg/mg tissue	0.9 ± 0.1	0.6 ± 0.2	1.2 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.55	0.03	0.48
mg/100 kg BW	114.2 ± 23	66.7 ± 33	151.0 ± 23	121.0 ± 33	86.4 ± 23	77.7 ± 27	0.22	0.15	0.77
Total, mg	103.2 ± 17	66.4 ± 24	139.0 ± 17	148.0 ± 24	116.3 ± 17	104.2 ± 19	0.42	0.04	0.55
Protein									
μg/mg tissue	24.7 ± 2	18.8 ± 3	33.2 ± 2	32.5 ± 3	30.2 ± 2	27.2 ± 2	0.13	<.01	0.59
g/100 kg BW	4.6 ± 0.6	3.9 ± 0.8	2.5 ± 0.6	1.3 ± 0.8	2.9 ± 0.6	0.9 ± 0.7	0.04	<.01	0.62
Total, g	4.1 ± 0.6	4.0 ± 0.8	2.5 ± 0.6	1.6 ± 0.8	3.9 ± 0.6	1.3 ± 0.7	0.05	0.04	0.20
Lipid									
mg/g tissue	606.9 ± 39	652.6 ± 55	258.8 ± 39	276.7 ± 55	425.7 ± 39	429.0 ± 45	0.56	<.01	0.90
g/100 kg BW	118.3 ± 17	137.8 ± 24	21.5 ± 17	11.2 ± 24	43.7 ± 17	15.0 ± 20	0.70	<.01	0.50
Total, g	103.1 ± 15	139.6 ± 22	23.0 ± 15	14.4 ± 22	58.4 ± 15	20.2 ± 18	0.82	<.01	0.15

[†]Data presented as LSM ± SEM. [1]Significance declared at $P \leq 0.05$. [2]*P* value for treatment effect. [3]*P* value for age effect. [4]*P* value for interaction effect between treatment and age.

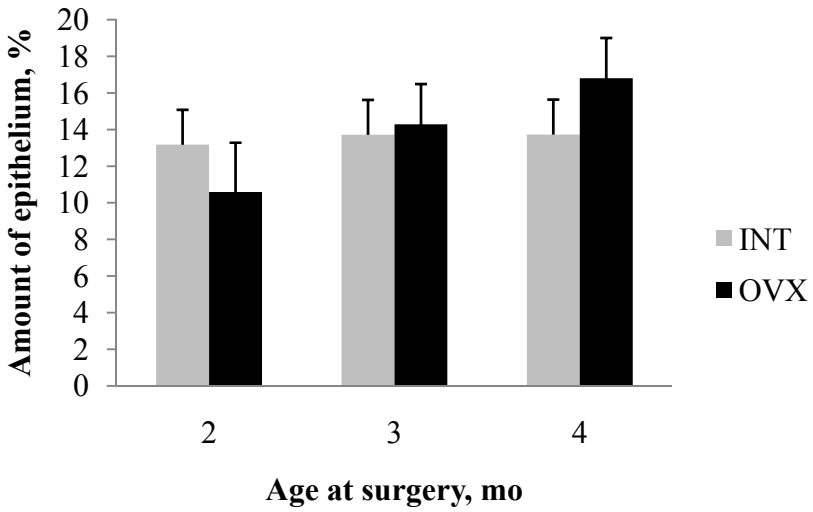


Figure 3. 3. Effect of ovariectomy on the percent area of epithelium present in mammary parenchyma

Proportion of epithelium present in unit area of mammary parenchyma from Holstein heifers ovariectomized (OVX; n = 8) or sham operated (INT; n = 12) either at 2, 3 or 4 mo of age and tissue harvested 30 d after surgery. Data presented as LSM and the bars represent SEM. There was no difference in the amount of epithelium present between INT and OVX heifers (P = 0.845) or between age at surgery (P = 0.334) and there was no interaction effect between treatment and age (P = 0.459).

difference in the proportion of epithelium present in unit area of PAR between different age groups (P = 0.334) and there was no interaction between treatment and age (P = 0.459; Fig 3.3). However, as the heifers got older there was an increase in the number of medium and small sized ducts as well as buds resulting in an overall increase in the total number of epithelial structures (Table 3.3). The number of large sized ducts remained the same among different age groups (P = 0.083).

3.4.3. Immunohistochemistry for Ki67 and PR

We did not find any difference in the number of proliferating MEC between INT and OVX heifers based on Ki67 immunohistochemistry (P = 0.652; Fig 3.4). Overall, out of the total epithelial cells counted, ~2 % cells were immunoreactive to Ki67 antibody in both groups. There was no difference in the number of Ki67 positive cells between different age groups (P = 0.167)

and there was no interaction between treatment and age ($P = 0.099$). However, the number of PR positive cells was significantly reduced in OVX than in INT heifers (4 % and 30 %; $P < 0.05$; Fig 3.5)

3.4.4. Gene expression for Stanniocalcin, TFP1, PR, ER and PCNA

Relative mRNA expression of estrogen-responsive genes *stc1* and *tfpi*, PR as well as the proliferating cell marker PCNA were down regulated in PAR from OVX compared with INT ($P < 0.05$; Fig 3.6A). However, there was no difference in the mRNA expression of ERα between

Table 3. 4. Effect of ovariectomy on the number of different sized ducts in mammary parenchyma

Number of different sized ducts in mammary parenchyma in heifers ovariectomized (OVX; n = 8) or sham operated (INT; n = 12) either at 2, 3 or 4 mo of age and tissue harvested 30 d after surgery†

	Age at surgery, mo								
	2		3		4		*P* values[1]		
Item	INT	OVX	INT	OVX	INT	OVX	Trt[2]	Age[3]	Trt*Age[4]
Large	0.7 ± 0.4	0.7 ± 0.6	1.8 ± 0.4	1.7 ± 0.5	1.8 ± 0.4	1.7 ± 0.5	0.90	0.08	0.98
Medium	4.5 ± 2	1.7 ± 3	10.4 ± 7	7.4 ± 2	8.7 ± 2	11.3 ± 2	0.59	0.03	0.40
Small	5.1 ± 5	16.5 ± 7	27.8 ± 5	23.5 ± 5	48.8 ± 5	52.5 ± 5	0.42	<.01	0.38
Buds	3.9 ± 2	9.3 ± 2	12.9 ± 2	8.9 ± 2	12. 2 ± 2	14.0 ± 2	0.53	0.03	0.11
Total	14.2 ± 7	28.3 ± 10	52.9 ± 7	41.5 ± 8	71.5 ± 7	79.4 ± 8	0.62	<.01	0.31

†Data presented as LSM ± SEM. [1]Significance declared at $P \leq 0.05$. [2]*P* value for treatment effect. [3]*P* value for age effect. [4]*P* value for interaction effect between treatment and age.

OVX and INT ($P = 0.196$). Relative expression of *stc1* was increased linearly with age ($P = 0.053$) but no other genes evaluated was changed by age. There was no interaction between treatment and age for mRNA expression for the genes measured in PAR. Relative mRNA

expressions of *stc1*, *tfpi*, ERα, PR and PCNA in MFP were not affected by treatment or age (Fig 3.6B) and there was no interaction between treatment and age.

3.5. Discussion

We observed a significant reduction in PAR growth per unit BW in ovariectomized heifers at different stages in the early prepubertal life which falls in line with previous findings (Purup et al., 1993; Berry et al., 2003c). Ovariectomies were performed at 2, 3 or 4 mo of age and mammary tissues were harvested 30 d after surgery. Even though these heifers gained BW equally, since the age at slaughter was different for each group, it is more appropriate to express tissue weights and composition on a BW basis. In contrast to Purup et al. (1993) there was no difference in MFP weights between OVX and INT which suggests that the reduction in udder weight was solely due to reduced parenchymal growth. Amounts of DNA, protein and lipid in PAR and MFP were comparable with previous reports taking into account the age and BW of the animals at slaughter (Purup et al., 1993; Meyer et al., 2006b; Daniels, 2008). In response to ovariectomy, protein and DNA content of PAR were reduced only in the 4 mo age group. We did not see the same response when surgery was performed earlier. However, there was a significant reduction in the lipid content of PAR in ovariectomized heifers. Therefore, the overall reduction in PAR mass is mainly attributed to reduced lipid accumulation. In the case of MFP, only the amount of protein was reduced in response to ovariectomy with no change in DNA or lipid. There was a significant reduction of protein and DNA in parenchymal and extra-parenchymal tissues in heifers ovariectomized at 2.5 mo of age and mammary tissues were collected 6 mo after surgery (Purup et al., 1993). Another report on reduction in PAR and MFP was also based on observations 6 mo after ovariectomy (Wallace, 1953). In our study the time period between ovariectomy and sample analyses was only 30 d which may not be sufficient to produce observable changes in composition. Another possible reason for the absence of treatment effect in composition could be the high animal to animal variation in biochemical parameters within treatment groups (Table 3.2 and 3.4). Small sample size for 2 mo surgery group because of limited tissue resources is also a major limitation in declaring significance. Histological appearance of mammary PAR resembles that seen in previous reports (Capuco et al., 2002). Similar to Capuco et al. (2002), we observed extensive branching and ductular elongation in

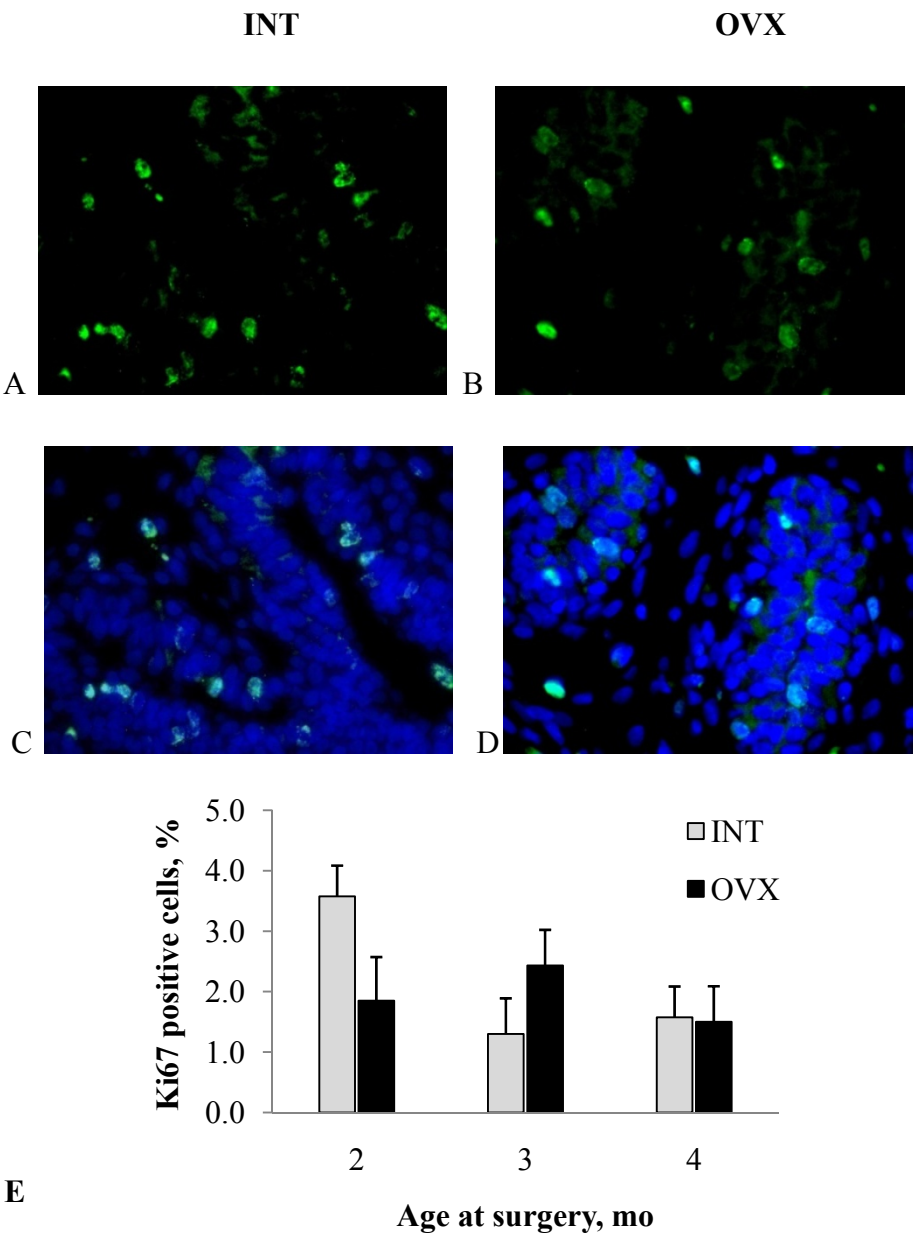


Figure 3. 4. Effect of ovariectomy on mammary epithelial proliferation

Immunofluorescence staining for Ki67 in the mammary epithelial cells from INT (n = 12) and OVX (n = 8) heifers. A and B show Ki67 positive cells and C and D are computer generated composite images showing both DAPI and Ki67 positive cells. (E) Number of Ki67 positive cells was not different between treatments ($P = 0.652$) or age groups ($P = 0.167$) and there was no interaction between treatment and age ($P = 0.099$). Data presented as LSM and the bars represent SEM.

PAR sections. Terminal ductular units were present both in INT and OVX heifers. Purup et al. (1993) reported histological changes including less epithelium and lumen and more stroma along with more number of large multilayered ducts in ovariectomized heifers. In contrast, we did not find any difference in the percent epithelial area or the presence of different sized ducts between the PAR of ovariectomized and intact heifers. We observed large multilayered ducts along with branching and budding of small ducts in both OVX and INT. Berry et al. (2003b) reported that in heifers ovariectomized before 6 wk of age epithelial development was virtually terminated while in heifers ovariectomized between 8 and 12 wk of age epithelial development was inhibited but not blocked.

Heifers used in this study were ovariectomized at 8, 12 and 16 wk of age, but age at ovariectomy did not have an effect on histological measurements evaluated. However there were morphological changes in the PAR as the heifers aged. There was a greater number of small and medium sized ducts in older heifers indicating more branching in 5 mo old heifers compared to 3 mo. Coinciding with the histomorphometry, ovariectomy did not affect the proliferation of MEC as evidenced by Ki67 immunohistochemistry. Immunostaining with Ki67 antibody is used to identify proliferating MEC population in bovine mammary gland (Capuco et al., 2001; Capuco et al., 2002). Our failure to note a reduction in epithelial proliferation coincides with the lack of changes in protein and DNA content of PAR. There was a tenfold decrease in epithelial proliferation along with an increase in the number of ERα-positive MEC in ovariectomized heifers when ovaries were removed between 1 - 3 mo of age and samples collected at 6 mo of age (Berry et al., 2003b). There was 3 – 6 mo time interval between ovariectomy and sample collection in their study. Even though the age at which surgeries were performed overlap between the two studies, we failed to demonstrate a reduction in MEC proliferation in our study. Again this may well reflect the relative short interval (30 d) to note an effect. However, we could not analyze the samples from two of the ovariectomized animals because there was not enough parenchyma for histological sampling. Therefore, we suggest that our results might be skewed because we could not incorporate the extreme observations of effect of ovariectomy due to the

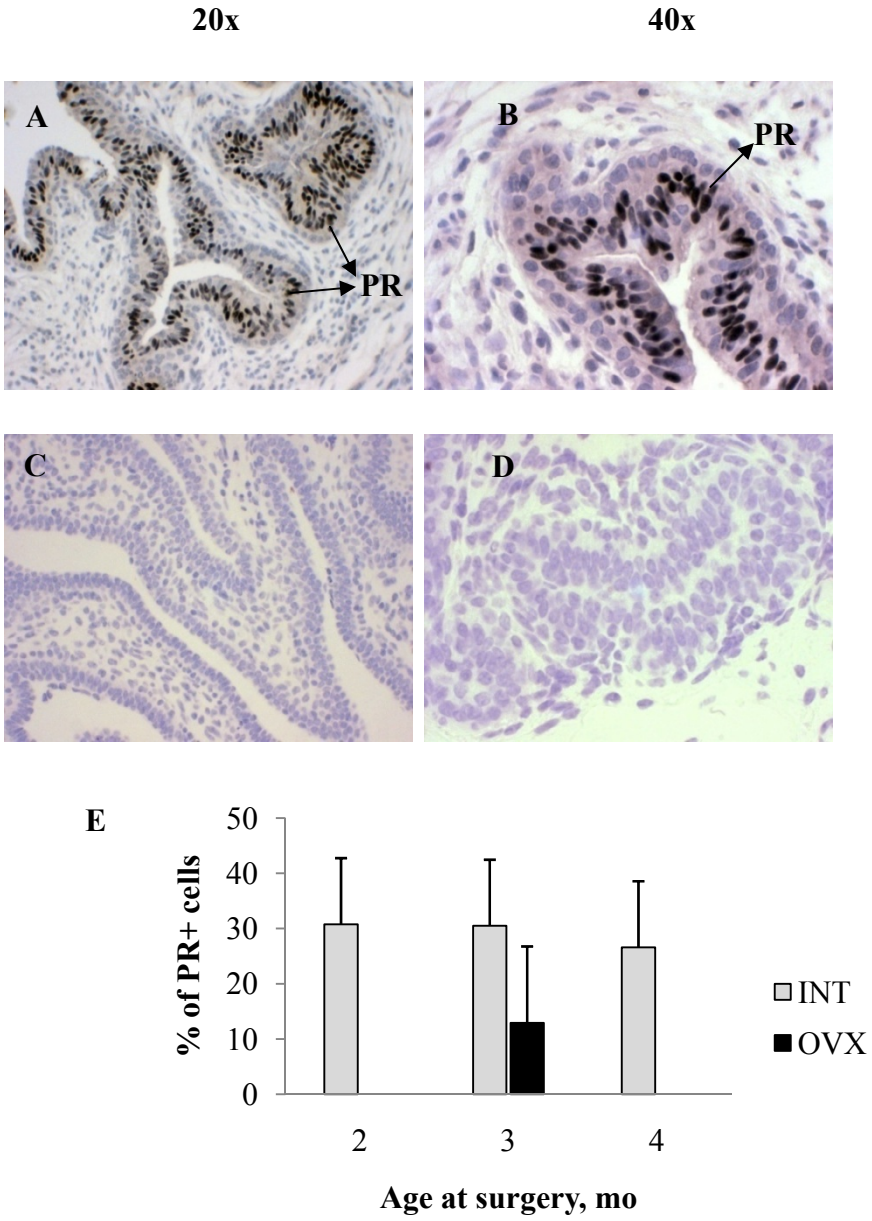


Figure 3. 5. Effect of ovariectomy on progesterone receptor expression in the mammary epithelial cells

Localization of progesterone receptor (PR) in the mammary epithelial cells from INT (n = 12) and OVX (n = 8) heifers. Dark stained cells represent PR positive mammary epithelial cells in INT (A and B) while there was no positively stained cells in OVX (C and D). (E) Quantitative data for PR immunohistochemistry in mammary epithelial cells shows that PR were absent in OVX ($P < 0.05$) except for one heifer in the 3 mo age group. Data presented as LSM and bars represent SEM.

technical constraints in the methods used for analysis. On the other hand, in support to our data, Meyer et al. (2006a) did not find an effect of ovariectomy on MEC proliferation when the heifers were ovariectomized at 4.6 mo and samples harvested after 33 d. In a recent study it was observed that when heifers were ovariectomized at 40 d of age and samples collected at every 15 d interval, reduction in MEC proliferation in OVX was more pronounced between 70 d to 160 d of age, that is 30 to 120 d after surgery (Korn et al., 2008). Another possible reason for failure to demonstrate reduction in proliferation may be due to the method of sampling of PAR. Capuco et al. (2002) reported greater proliferation rate at the periphery or outer region of PAR where it is closer to MFP. In our study, PAR samples were collected randomly from the inner part of PAR. Additionally, there is a possibility that regional differences may be present between parenchymal samples collected from intact and ovariectomized animals. Even though we collected samples from the inner middle of the PAR from all the animals, because of the reduced size of PAR in ovariectomized animals the samples may be more peripheral compared with the samples collected from the larger sized PAR from intact heifers. Therefore, the response to ovariectomy could be masked by the difference between the more peripheral OVX samples compared with the inner INT samples. Thus sampling may have impacted variability and therefore the capacity to detect differences. Although progesterone (**P4**) is known to be functionally important in mammary growth and development during pregnancy and lactation, protein expression of PR was demonstrated in prepubertal heifer mammary gland by immunohistochemistry (Connor et al., 2005). In agreement with Connor et al. (2005), we observed PR localization in the nuclei of the intermediate layer of epithelial cells in mammary ducts. Interestingly, we found a drastic reduction in PR-positive MEC (4 % and 30 %) as well as reduced mRNA expression of PR in the PAR of ovariectomized heifers. The action of P4 in prepubertal mammary gland differs considerably with species. While P4 increases ductal branching and terminal end bud formation thereby enhancing ductal morphogenesis in prepubertal mice (Hovey et al., 2002), ductal branching occurs considerably in prepubertal heifers even in the absence of P4 (Capuco et al., 2002). Moreover, PR knock-out mice develop normal mammary gland (Lydon et al., 2000)

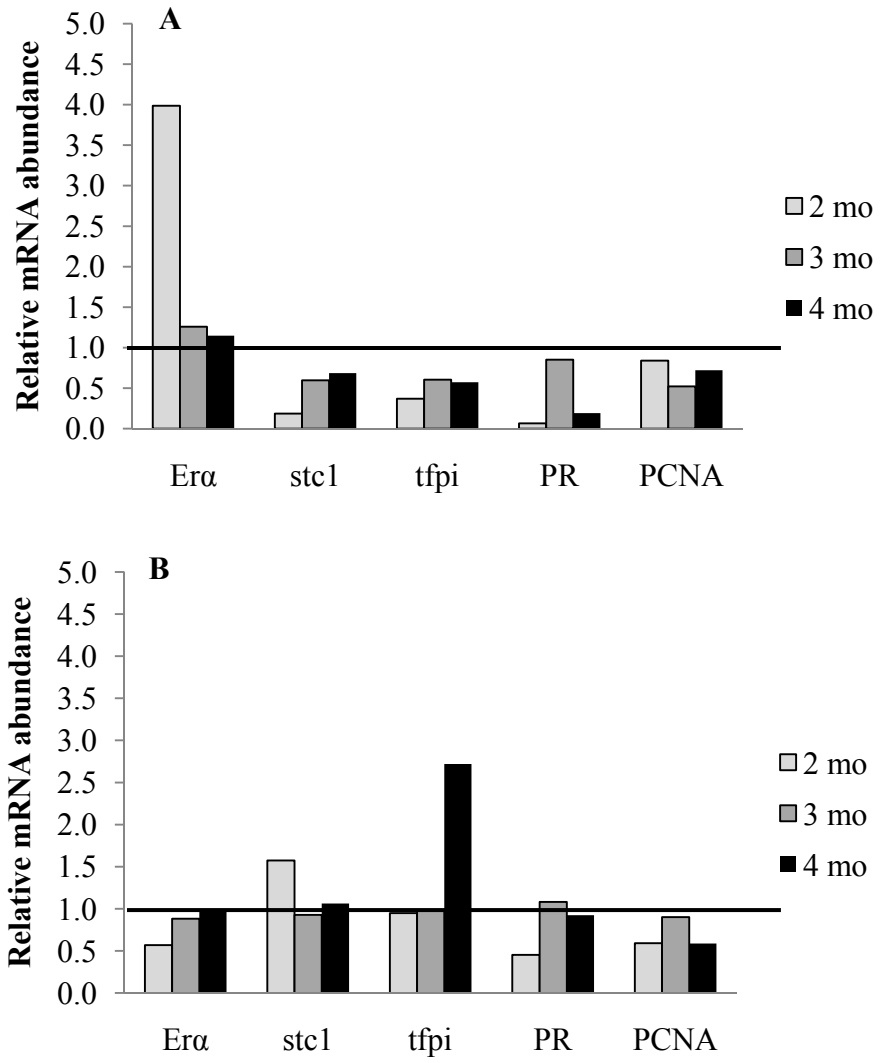


Figure 3. 6. Effect of ovariectomy on relative mRNA expression in mammary parenchyma (PAR) and fat pad (MFP)

Relative mRNA expression ($2^{-\Delta\Delta Ct}$) of ERα, *stc1*, *tfpi*, PR and PCNA in mammary PAR and MFP from OVX heifers (n = 8) relative to INT (n = 12) heifers either at 2, 3 or 4 mo of age and tissue harvested 30 d after surgery. The horizontal line at 1 fold level indicates the mRNA expression in INT heifers. (A) Relative expressions of *stc1*, *tfpi*, PR and PCNA in PAR were down regulated in OVX relative to INT ($P < 0.05$), whereas ER was not different between OVX and INT ($P = 0.196$). There was an age related increase in the expression of *stc1* ($P = 0.053$). (B) Relative gene expressions in MFP were not different between treatments or different age groups and there was no interaction between treatment and age.

implying that P4 is not an absolute necessity for prepubertal mammary growth even in mice. Our findings indicate that PR expression is highly regulated by ovarian secretions in prepubertal heifers and that the presence of a functional ovary is inevitable for expressing PR in the mammary epithelial cells. Complete absence of PR immunoreactivity in ovariectomized animals suggests the possibility of using PR immunoreactivity as a biological marker analysis for studying the effects of ovariectomy in young heifers. From the results of ablation and replacement studies performed earlier, ovarian regulation of mammary growth can be mainly attributed to the lack of estrogen from ovaries. Local implants of anti-estrogen compounds in the mammary gland inhibit epithelial proliferation in mice (Silberstein et al., 1994). Although estrogen mediated MEC proliferation is well documented in rodents, only a few reports are available on the estrogen induced proliferative responses in prepubertal bovine mammary gland. Capuco et al. (2002) reported that proliferation of MEC increased within 72 h of estradiol administration in prepubertal heifers. Woodward et al. (1993) showed that exogenous estradiol but not progesterone dramatically increased mammary epithelial cell proliferation in prepubertal heifers. Effects of estrogen in mammary gland are believed to be predominantly mediated through the nuclear receptor ERα (Connor et al., 2005). But, ironically, most of the proliferating MEC are ERα negative (Saji et al., 2000; Capuco et al., 2002). This suggests a paracrine action of estrogen in eliciting its mitogenic effects. Co-localization of PR and ER is observed in MEC in heifers (Capuco et al., 2002) and PR expression can be induced by low concentrations of estrogen in plasma lower than the concentration required for cell proliferation (Clarke et al., 1997b). This explains the difference in the response of ERα and PR expression in ovariectomy. Preliminary findings from our laboratory show that aromatase is present in bovine prepubertal mammary gland, especially in the intra lobular stroma. Moreover, ovariectomy did not affect the expression of aromatase mRNA or protein (Huderson, unpublished data). This recent finding gives insight to the possibilities of local production of estrogen in the prepubertal bovine mammary gland which is not under ovarian influence. This local estrogen might act to dampen the magnitude of response to ovariectomy, at least for a short period of time. Furthermore, there was no difference in the mitogenic activity of mammary extracts from OVX and control heifers (Berry et al., 2003a) suggesting a locally active mitogenic system independent of ovarian status. Therefore, we hypothesize that the short interval (30 d) between ovariectomy and sample

collection was not sufficient to elicit changes in the local environment of mammary gland to induce observable changes in MEC proliferation.

Like the number of proliferating MEC, ERα mRNA expression in PAR was not affected by ovariectomy. Meyer et al. (2006a) also noted that exogenous estrogen injections reduced (50 %) the mRNA abundance of ERα but ovariectomy did not have an effect on ERα mRNA expression. These two observations strongly suggest that the responses in mammary PAR to exogenous estrogen are not exactly reversed in ovariectomy. Obviously the response to exogenous estrogen treatments depends greatly on the dose and duration of estrogen administered. Likewise, apart from the age at surgery the responses to ovariectomy may also be dependent on the duration of time interval between surgery and sample collection. Taken together, our data suggest that it requires a certain period of time to manifest the inhibitory effects of ovariectomy on proliferation in prepubertal mammary gland. However, unlike Ki67-positive cells we observed significant reduction in the mRNA expression of a proliferation marker gene, PCNA in the PAR as a result of ovariectomy. Even though we chose two different proliferation markers (Ki67 and PCNA) for evaluation at protein and message level, they both are not limited to actively dividing phase but are expressed during synthetic phase of the cell cycle (Bravo and Macdonald-Bravo, 1987; Capuco et al., 2001). Likewise, contrasting ERα mRNA expression, estrogen responsive genes were reduced in ovariectomy. Stanniocalcin is involved primarily in calcium and phosphorus homeostasis (Lu et al., 1994) and both circulating levels as well as local expression of stanniocalcin-1 in mammary gland increase in response to exogenous estrogen (Li et al., 2006; Tremblay et al., 2009). Tissue factor pathway inhibitor precursor is a serine protease inhibitor and gene expression of *tfpi* in mammary gland increases in response to estrogen (Li et al., 2006). Both *stc1*and *tfpi* were reported not to be affected by ovariectomy in a recent study using microarray analysis (Li et al., 2006). They reasoned that the failure to find a difference in gene expression in their study may partially be due to older age (~18 wk) of heifers at ovariectomy. Li et al. (2006) also suggest that impact of ovariectomy on mammary gland may be more pronounced at protein level rather than gene expression. However, our observations contradict their suggestion since we found more changes at the message level than at protein level in response to ovariectomy.

In mice and heifer mammary glands the non-epithelial cells in MFP are required for normal as well as estrogen induced proliferation of MEC (Cunha et al., 1997; Akers et al., 2005). Further, proliferation in epithelial cells as a result of exogenous estrogen administration was greatest in PAR regions adjacent to MFP (Capuco et al., 2002). Even so, very little work has been done in evaluating the ovarian regulation of gene expression in prepubertal MFP. We demonstrated considerable abundance of mRNA expression of ERα, PR, *stc1*, *tfpi* and PCNA in MFP. Nevertheless, gene expression in MFP was not affected by ovariectomy. Relative mRNA expression of ERα or estrogen responsive genes in MFP was similarly expressed in both OVX and INT heifers in a study by Meyer et al. (2006a). Based on our data and recent evaluation of aromatase expression we propose that the absence of ovarian secretions for a month period during early prepubertal life induce changes in PAR at mRNA level, but this time interval is not long enough to induce changes in proteins important in locally active proliferation pathways which may be independent of circulating estrogen. Unlike the effect of estrogen, there is only limited information on the impact of ovariectomy on gene expression in prepubertal bovine mammary gland. Additional studies will be necessary to make conclusive statements on ovarian regulation at message and protein levels in mammary gland.

CHAPTER 4: EFFECT OF EXOGENOUS BOVINE SOMATOTROPIN TREATMENT ON MAMMARY GROWTH AND DEVELOPMENT AND PUTATIVE MAMMARY STEM CELL POPULATION IN PREPUBERTAL HOLSTEIN HEIFERS

4.1. Abstract

Growth hormone is known to increase milk production in lactating cows and improve mammary growth in heifers. The early prepubertal period proposes an ideal therapeutic intervention point to enhance mammary parenchymal growth and development in dairy heifers. We hypothesized that treatment with exogenous bovine somatotropin (**bST**) before the onset of allometric mammary growth phase can enhance mammary epithelial proliferation by modifying the putative stem cell population in bovine mammary gland. Thus our objectives were to determine the effect of bST treatment on mammary growth and putative stem cell proliferation. Eighteen Holstein heifers were randomly assigned to one of two treatments; bST (500 mg; n = 9) or Sal (0.9 % saline; n = 9) beginning on day 29 ± 4 with treatments administered every 21 d. All animals received bromodeoxyuridine (**BrdU**, 5 mg/kg BW) for five consecutive days when they were 50 ± 4 d old. Mammary tissues were harvested after either the sixth or 12th wk of treatment start date and used for biochemical, histological and gene expression analyses. Dual labeling technique using fluorophore tagged antibodies against BrdU and Ki67 were used for determining the percentage of putative stem cells. Transcript abundance of proliferation marker genes was analyzed by quantitative real time PCR. Main effects of treatment and time of harvest as well as their interaction were tested using the Mixed model procedure of SAS and statistical significance was declared at $P \leq 0.05$. We did not find differences in parenchymal mass or composition between treatments, but total udder mass was reduced as a result of reduction in fat pad. There was an increase in the number of epithelial structures and a decrease in BrdU label retaining cells in older compared with younger heifers. However, bST did not impact epithelial cell proliferation, mRNA expression or the number of putative stem cells. Overall, our data suggest that exogenous bST is not effective as a mammary specific mitogen in very young prepubertal heifers.

4.2. Introduction

Growth hormone (**GH**), also known as somatotropin (**ST**), is important for mammary gland growth and development in humans and animals. It has been established that an increase in concentration of endogenous GH and administration of exogenous GH enhance mammary parenchymal growth in dairy heifers (Sejrsen, 1983; Sejrsen et al., 1986; Purup et al., 1995). Exogenous bovine ST (**bST**) can stimulate cell proliferation up to six-fold in mammary glands of six month old heifers (Berry et al., 2003c). However, those studies were focused on the effect of GH on mammary growth in peripubertal heifers. A rapid allometric pattern of growth occurs in the mammary gland beginning at about two to three months of age and continues till puberty (Sinha and Tucker, 1969). Therefore, the beginning of the allometric growth phase could be a potential time point for therapeutic intervention for enhancing parenchymal growth rate. We hypothesize that giving bST at an earlier age would enhance mammary growth and development in dairy heifers.

The existence of mammary stem cells is suggested by the cyclic processes of development and involution during lactation and pregnancy in mammals. Presence of morphologically distinct and pale staining cells which contribute to epithelial cell population was described in murine mammary gland (Chepko and Smith, 1997; 1999). But very little is known about stem cells in bovine mammary gland owing to a major limitation of proper identification methods. In an earlier report, the presence of cells with similar morphological and staining properties as of murine mammary stem cells was described in bovine mammary gland (Ellis and Capuco, 2002). Later, DNA label retention property of stem cells was employed as a method to identify the population of putative mammary stem cells in bovine mammary gland (Capuco, 2007). Similar population of cells were identified in mammary epithelium of prepubertal dairy heifers as early as 60 d of age by BrdU label-retention method (Daniels, 2008). An ATP-binding cassette transporter abc3 protein is a marker for mammary epithelial proliferation which may also serve as a potential stem cell marker (Li et al., 2006). We suggest that induction of this protein might provide a possible mechanism of stem cell mediated regulation of mammary epithelial proliferation in early prepubertal dairy heifers in response to bST within the mammary PAR. The overall objective of this study was to determine the effect of bST treatment on

mammary growth and development in early prepubertal dairy heifers. The specific objectives were:

1. To determine the effect of bST treatment on mammary growth and composition in early prepubertal dairy heifers
2. To determine the effect of bST on mammary epithelial proliferation by histological and gene expression measurements
3. To determine the effect of bST treatment on the population of putative stem cells in bovine mammary gland.

4.3. Materials and Methods

4.3.1. Animals and Treatments

Twenty Holstein heifers in their first week of life (average age at arrival, 7 ± 1 d) were purchased from a private farm in Florida and transported to Virginia Tech Dairy Center. All protocols used in the study were in agreement with the animal use protocols approved by the Virginia Tech Institutional Animal Care Use Committee. Upon arrival one heifer died due to transportation stress. The remaining 19 heifers were used for the study. Heifers were housed in individual open hutches and were fed with a commercial milk replacer using nipple buckets twice a day (0700 and 1900 h). All heifers also received calf starter (20 % CP and 1.4 % fat) and fresh water free of choice. In addition, the morning feeding of milk replacer was supplemented with 30 g of Gammulin powder (APC, Inc.; Ames, IA) which was given to all heifers during the first week.

After the heifers arrived at the Virginia Tech Dairy Center, a three week adaptation period was given before the treatments began. Heifers were blocked by age and BW and at an average age of 29 ± 4 d, they were randomly assigned to one of the treatment groups, bST (n = 10) or Sal group (n = 9). Heifers in the bST group received a sustained release formulation of 500 mg recombinant somatotropin (Posilac; Monsanto Co., St. Louis, MO) administered subcutaneously in the neck region while Sal heifers were injected with 0.9 % saline solution. Treatments were repeated every three week. At an average age of 50 ± 4 d (three weeks after the initial treatment) all heifers were intravenously injected with 5-Bromo-2'-deoxyuridine (**BrdU**;

Sigma-Aldrich, Inc.; St. Louis, MO) solution prepared in saline (pH 8.5) at a dose rate of 5 mg/kg BW for five consecutive days. Body weight was recorded before the treatments started and then once every two weeks.

4.3.2. Sample Collection and Analyses

Animal sacrifice and tissue harvest: Heifers were sacrificed and mammary tissues were harvested in two stages. The first set of heifers were sampled three weeks after the second injection (average age of 72 ± 1 d; treatment period of six week) and the second set of heifers were sampled three weeks after the fourth injection (average age of 116 ± 1 d; treatment period of 12 wk). Heifers were humanely sacrificed using Phenobarbital injection i.v. (Euthasol, 10 mg/kg BW). Mammary parenchyma (**PAR**) and fat pad (**MFP**) were collected to determine proximate composition, histological appearance as well as selected gene expression analysis (see Chapter 2 for detailed methods of sample collection and analysis).

Biochemical, histological and gene expression analyses: Proximate composition of PAR and MFP were determined by measuring the DNA, protein and lipid present in each tissue fraction (Chapter 2). For histological evaluation, percent area of epithelia present in PAR was determined by area measurements using image analyzing software (Image-Pro Plus Version 6.2; Media Cybernetics; Silver Springs, MD). Ten microscopic fields at 4x magnification were used per sample (Chapter 2). In addition to the area measurements, epithelial structures were classified into large, medium and small according to the lumen size and structures without a lumen were classified as buds. The number of different sized ducts and buds as well as total number of epithelial structures were determined.

An estimate of the putative stem cell population was determined using the BrdU label-retention method (Capuco, 2007). This technique is described in Chapter 2 in detail. Briefly, five micrometer thick PAR tissue sections were subjected to immunofluorescence staining for identifying the presence of BrdU and the nuclear proliferation antigen Ki67 in epithelial cell nuclei. Tissue sections were incubated in CAS block (Invitrogen, Carlsbad, CA) to inhibit non-specific binding of primary antibodies. The primary antibody solution used was a mixture of polyclonal anti-Ki67 antibody raised in rabbit (clone SP6, Fisher Scientific, 1:200 dilution in

CAS block) and mouse monoclonal anti-BrdU antibody (clone BMC-9318, Fisher Scientific, 1:66.7 dilution in CAS block). Tissue sections were incubated overnight at 4^{o}C with 50 μL of the antibody mixture per section. The secondary antibody solution was a combination of Alexa 488 goat anti-rabbit IgG (Invitrogen) and Alexa 594 goat-anti mouse IgG (Invitrogen) in CAS Block at a final dilution of 1:200 each. Slides were stained in two batches. In each batch, a PAR tissue section from a BrdU pulse-labeled heifer was used as a positive control. One microscope slide containing multiple tissue sections from each animal was used. In each slide, one section was used for dual labeling and one section served as negative control where only CAS block was added instead of the primary antibody mixture. Tissue sections were circled by PAP pen in order to prevent mixing of reagents between sections. Stained sections were protected by coverslip using Prolong Gold antifade mounting solution which contains DAPI as a general counter stain (Invitrogen). Slides were allowed a curing period of 24 h in the dark. Photomicrographs were taken using Nikon Eclipse E600 microscope (Nikon Instruments, Inc.; Melville, NY) fitted with an epifluorescence attachment connected to a QColor3 digital camera (Olympus America Inc, Center Valley, PA) at 20x and 40x magnifications using the Q-Capture suite software program (QImaging; Surrey, BC, Canada). The UV-2E/C DAPI, FITC, and G-2A filter blocks were used for visualization of DAPI, Ki67 and BrdU, respectively. Ten microscopic fields per each sample were photographed as monochrome images under DAPI, Ki67 and BrdU channels (total 30 images per sample). Monochrome images were then processed in ImagePro Plus (Media Cybernetics) for counting the BrdU labeled and Ki67 positive cells as well as dual-labeled cells (Appendix D.3). Immunohistochemistry of progesterone receptors (**PR**) was performed on five micrometer thick paraffin embedded tissue sections as described in Chapetr 3. An average of 2000-2500 mammary epithelial cells was counted per sample for determining the percent of BrdU labeled as well as Ki67 and PR positive cells. Basal layer of myoepithelial cells were excluded from counting.

Quantitative real-time PCR (Chapter 2) was used for relative quantification of the proliferation marker genes, proliferating cell nuclear antigen (**PCNA**; forward primer 5'-TCGTCTCAGGCGTTCATAGTC -3' and reverse primer 5'- AACATGGTGGCGGAGTCG -3') and ATP-binding cassette transporter (**abc3**; forward primer 5'-

GCCACCTTCCTCGTTGTC -3' and reverse primer 5'- AAGTTGCTCACTGCCATCC-3'). Target gene Ct value in triplicates was normalized using geometric mean of Ct values for three housekeeping genes; PPP1R11 (forward primer 5'- CCATCAAACTTCGGAAACGG -3' and reverse primer 5'- ACAGCAGCATTTTGATGAGCG -3'), RPS15A (forward primer 5'- GAATGGTGCGCATGAATGTC -3' and reverse primer 5'- GACTTTGGAGCACGGCCTAA -3') and MTG1(forward primer 5'- CTTGGAATCCGAGGAGCC A -3' and reverse primer 5'- CCTGGGATCACCAGAGCTGT -3') (Piantoni et al., 2008).

4.3.3. Statistical Analyses

Statistical analyses of all data were performed using Mixed analysis procedure of SAS (SAS 9.2; Cary, NC) testing for main effects of treatment and time of harvest as well as their interaction. For weekly BW analysis, week was used as repeated measure with heifer within treatment as the subject. First-order autoregressive (AR (1)) as well as heterogeneous first-order autoregressive (ARH (1)) covariance structures were tested and ARH (1) was determined to be most appropriate. Data for early harvest and late harvest groups were combined until the first harvest time point and analyzed as a single data set to test main effects until that point of time. Weekly BW for both harvest groups were also analyzed separately because of the difference in the end points between groups. Proximate composition data for PAR and MFP were analyzed separately.

Histological measurements and data for BrdU labeled, Ki67 and PR positive cells were analyzed similarly for main effects of treatment, time of harvest and their interaction. Different chromogens used in the PR immunohistochemistry method was tested in the model and found to be non-significant; hence deleted from the final analysis model.

Gene expression data for PAR and MFP were analyzed combined as well as individually. When analyzed combined, main effects of tissue, interactions between tissue and treatment as well as tissue and time were also included in the model (Table A.6). The ΔCt data were used for statistical analyses and significance was determined based on the *P* values for ΔCt data (Table A.3 and A.4). However, data was presented as fold change in gene expression for bST treated animals relative to control animals using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (see Chapter 2).

The model used was

$Y_{ijk} = \mu + T_i + A_j + (TA)_{ij} + e_{(ij)k}$ where

Y_{ijk} = variable being tested

μ = overall mean

T_i = fixed effect of treatment (bST or Sal) (i = 1, 2)

A_j = fixed effect of harvest time (early or late) (j = 1, 2)

$(TA)_{ij}$ = fixed interaction of treatment and time

$e_{(ij)k}$ = residual error

Data distribution was analyzed using the "Proc Mixed Boxplot" statement (Appendix A.6). Significance was declared at $P \leq 0.05$ for all analyses.

4.4. Results

4.4.1. Body growth, mammary gland mass and composition

Initial BW was the same for bST and Sal heifers and there was no difference in weekly BW between treatments either for the early ($P = 0.526$) or the late harvest group ($P = 0.186$; Fig 4.1A). Overall, there was an increase in BW over time ($P < 0.0001$) for both treatment groups. However, BW gain during the treatment period was greater in the bST compared with the Sal ($P < 0.05$; Fig 4.1B), and the magnitude of BW gain was greater in the late harvest than the early harvest heifers ($P < 0.001$). Gross tissue weights for total mammary gland, PAR and MFP are shown in Fig 4.2A. Overall, total mammary gland was not different between the Sal and bST (228.5 ± 16 g and 202.6 ± 15 g, respectively; $P = 0.262$), but as expected, there was an increase ($P < 0.05$) in mammary weight in the late harvest group (259.6 ± 14 g) compared with the early harvest group (171.5 ± 17 g). Overall, dissected PAR weights were similar between bST and Sal (30.1 ± 6 g and 39.3 ± 6 g, respectively; $P = 0.301$) while PAR mass increased in late harvest group (58.3 ± 6 g) compared with early harvest (11.1 ± 6 g; $P < 0.01$). Treatment effect was not observed in MFP mass (87.0 ± 9 g for Sal and 65.6 ± 9 g for bST; $P = 0.117$). However, MFP weight tended to be greater ($P = 0.056$) in late harvest (89.7 ± 8 g) compared with the early

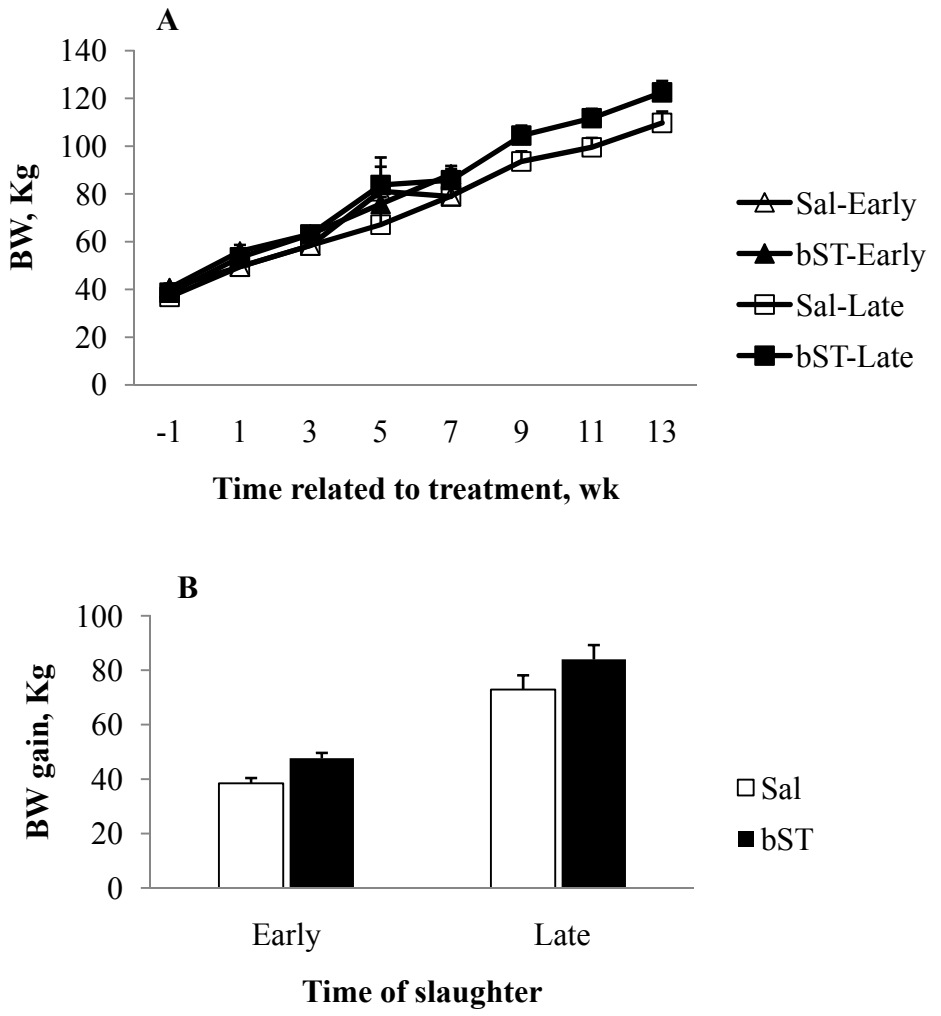


Figure 4. 1. Effect of bST treatmnt on body weight (kg) and overall BW gain

Body weight (kg) and overall BW gain for Holstein heifers that received either Sal and harvested early (closed triangle; n = 4) or late (open triangle; n = 5) or received bST and harvested early (closed square; n = 4) or late (open square; n = 5). (A) There was no difference between bST and Sal groups in the early ($P = 0.526$) or in the late ($P = 0.186$) harvest groups. However, there was an overall increase in BW over time ($P = <0.0001$). (B) There was an increase in the BW gain ($P < 0.05$) in bST-treated heifers compared with Sal and BW gain increased in late harvest compared with early harvest heifers ($P < 0.001$). Data are presented as LSM and bars represent SEM.

harvest group (62.9 ± 9 g). When tissue weights were adjusted to BW, total mammary gland weight was reduced in bST in both harvest groups while MFP weight was reduced in bST only in the early, but not in the late harvest group ($P < 0.05$, Fig 4.2B). Mammary PAR weight per 100

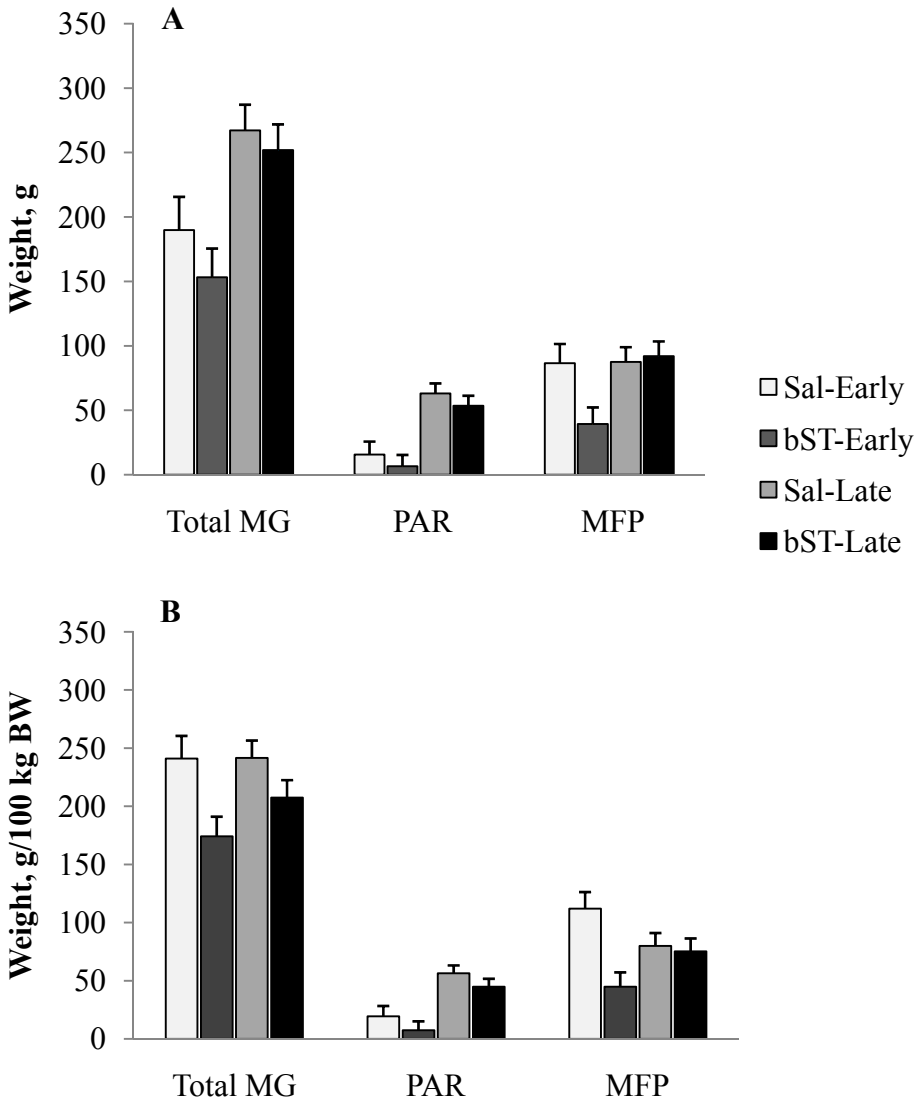


Figure 4. 2. Effect of bST treatment on coarse weight and weight adjusted to BW of total mammary gland (MG), PAR and MFP

Gross weight and weight adjusted to BW of total mammary gland (MG), PAR and MFP for Holstein heifers that received either Sal and harvested early (n = 4) or late (n = 5) or received bST and harvested early (n = 4) or late (n = 5). (A) There was no difference in coarse tissue weights between Sal and bST for heifers in either early or late harvest groups. (B) Total udder weight per 100 kg BW was decreased in bST compared with Sal ($P = 0.01$) and BW adjusted MFP weight was reduced in bST in the early harvest, but not in the late group (treatment and time interaction $P < 0.05$). However, there was no change in PAR weight per 100 kg BW between treatments. Data are presented as LSM and bars represent SEM.

kg BW was not different between treatments, but weight increased in late harvest compared with the early group ($P < 0.05$).

Proximate composition in PAR and MFP were evaluated by determining protein, DNA and lipid content of each tissue fraction. Protein, DNA and lipid contents were calculated as concentration in the tissue (wt/wt of tissue), total amount present in the entire gland as well as the amount adjusted to BW (wt/100 kg BW). In PAR, there was no effect due to treatment or interaction between treatment and time for any of the proximate components except for total DNA amount when it was expressed per 100 kg BW of the animal (Table 4.1). There was an unexpected decrease in the amount of parenchymal DNA per 100 kg BW in bST heifers compared with Sal (0.41 ± 0.1 vs. 0.85 ± 0.1 mg DNA/100 kg BW; $P = 0.037$). Concentrations as well as total amounts of DNA and protein in PAR were greater ($P < 0.05$) in late harvest heifers while neither lipid concentrations nor total lipid were different between Sal and bST or between harvest groups (Table 4.1). Proximate composition of MFP is given in Table 4.2. Total protein and the amount of protein per 100 kg BW were reduced in bST treated heifers in the early harvest group (interaction effect between treatment and time; $P = 0.016$ and 0.009, respectively; Table 4.2). Other measurements for DNA, protein and lipid were not affected by treatment, time or treatment and time interaction.

4.4.2. Histomorphometry

Percent area of epithelium present in PAR was not different between heifers treated with bST or Sal ($P = 0.524$) or between times of harvest ($P = 0.083$; Fig 4.4) and there was no interaction ($P = 0.958$). In early harvest group Sal and bST heifers had 9.9 ± 2 and 8.7 ± 2 % epithelia in PAR, respectively while in late harvest heifers the amount of epithelium present in the PAR for Sal and bST was 13.5 ± 2 and 12.2 ± 2 %, respectively (Fig 4.4). Classification of different sized ducts and epithelial structures and their counts for bST and Sal heifers from early and late harvest groups are given in Table 4.3. The number of large and medium sized ducts was not different either between treatments or between harvest groups. However, there was an increase in the number of small sized ducts, buds and total number of epithelial structures in the late harvest heifers compared with early harvest ($P < 0.05$).

Table 4. 1. Effect of bST treatment on proximate composition of mammary parenchyma

Proximate composition of mammary parenchyma of bST (Posilac 500mg; n = 9) and Sal (0.9% NaCl; n = 9) heifers and samples harvested at two different time points; early or late[1]

		Time of tissue harvest						
		Early		Late				
		Treatment				*P* values		
Item		Sal	bST	Sal	bST	Trt[2]	Time[3]	Int[4]
DNA:	µg/mg tissue	1.57 ± 0.5	1.51 ±0.5	2.92 ± 0.4	1.85 ± 0.4	0.23	0.08	0.28
	mg/100 kg BW	0.15 ± 0.2	0.08 ± 0.2	1.56 ± 0.2	0.75 ± 0.2	0.04	<.01	0.07
	Total in PAR, mg	11.69 ± 28	7.73 ± 28	176.5 ± 22	97.8 ± 22	0.13	<.01	0.16
Protein:	µg/mg tissue	5.92 ± 15	36.8 ± 15	61.5 ± 12	52.7 ± 12	0.44	0.02	0.17
	mg/100 kg BW	0.7 ± 1.3	1.5 ± 1.3	38.1 ± 10	22.2 ± 10	0.53	0.03	0.49
	Total in PAR, g	0.06 ± 1.7	0.13 ± 1.7	4.5 ± 1.3	2.8 ± 1.3	0.59	0.04	0.56
Lipid:	mg/g tissue	394.9 ±125	125.3 ± 125	184.3 ± 97	84.9 ± 97	0.13	0.28	0.46
	mg/100 kg BW	37.9 ± 44	5.7 ± 44	94.3 ± 34	37.9 ± 34	0.28	0.28	0.76
	Total in PAR, g	3.1 ± 5	0.5 ± 5	10.5 ± 4	4.6 ± 4	0.33	0.20	0.69

[1]Data presented as LSM ± SEM. [2]*P* value for treatment effect; α =0.05. [3]*P* value for time of harvest effect; α =0.05. [4]*P* value for interaction effect between treatment and time; α =0.05.

4.4.3. Gene expression of proliferation markers

Abundance of mRNA expression for proliferation marker genes PCNA and abc3 in PAR and MFP of bST treated heifers relative to Sal controls from early and late harvest groups are shown in Fig 4.5. There was no difference in relative mRNA expression of PCNA in the PAR of bST treated heifers compared with Sal in early or late harvest groups (*P* = 0.075; Fig 4.5 A). Expression of abc3 mRNA tended to be down regulated in bST heifers compared with Sal controls in early and late harvest (0.7 and 0.3 fold reduction, respectively; *P* = 0.054). Neither PCNA nor abc3 gene expression in PAR was different between early and late harvest groups (*P* = 0.067 and 0.315, respectively; Fig 4.5 A). Gene expression for PCNA and abc3 in MFP was

Table 4. 2. Effect of bST treatment on proximate composition of mammary fat pad

Proximate composition of mammary fat pad of bST (Posilac 500mg; n = 9) and Sal (0.9% NaCl; n = 9) heifers and samples harvested at two different time points; early or late[1]

		Time of tissue harvest						
		Early		Late				
		Treatment				*P* values		
Item		Sal	bST	Sal	bST	Trt[2]	Time[3]	Int[4]
DNA:	µg/mg tissue	1.9 ± 0.7	2.7 ± 0.7	1.9 ± 0.6	1.7 ± 0.6	0.65	0.42	0.44
	mg/100 kg BW	2.2 ± 0.7	1.1 ± 0.7	1.6 ± 0.6	1.2 ± 0.6	0.32	0.67	0.58
	Total in MFP, mg	166.7 ± 64	100.1 ± 64	175.9 ± 50	139.8 ± 50	0.39	0.68	0.80
Protein:	µg/mg tissue	28.2 ± 3	22.9 ± 3	26.6 ± 2	32.8 ± 2	0.88	0.18	0.07
	mg/100 kg BW	29.5 ± 4	8.2 ± 4	20.7 ± 3	23.8 ± 3	0.04	0.40	0.01
	Total in MFP, g	2.3 ± 0.4	0.74 ± 0.4	2.3 ± 0.3	2.9 ± 0.3	0.26	0.02	0.02
Lipid:	mg/g tissue	329.2 ± 115	316.5 ± 115	286.6 ± 89	347.2 ± 89	0.82	0.95	0.73
	mg/100 kg BW	399.7 ± 107	161.2 ± 107	217.9 ± 83	245.3 ± 83	0.29	0.62	0.19
	Total in MFP, g	31.4 ± 10	14.3 ± 10	23.4 ± 8	31.4 ± 8	0.62	0.62	0.18

[1]Data presented as LSM ± SEM. [2]*P* value for treatment effect; α =0.05. [3]*P* value for time of harvest effect; α =0.05. [4]*P* value for interaction effect between treatment and time; α =0.05.

not affected by either treatment (*P* = 0.204 and 0.918) or time of harvest (*P* = 0.056 and 0.535) and there was no interaction between treatment and time (*P* = 0.218 and 0.728) (Fig 4.5 B).

4.4.4. Labeling index for BrdU and Ki67

The percentage of mammary epithelial cells that are positive for BrdU and Ki67 antigens in the PAR of bST and Sal animals from early and late harvest heifers are shown in Fig 4.6. the population of BrdU label-retaining epithelial cells was not affected by bST treatment (P = 0.374). In the early harvest group, 0.8 ± 0.4 and 1.9 ± 0.4 % cells in Sal and bST, respectively, retained

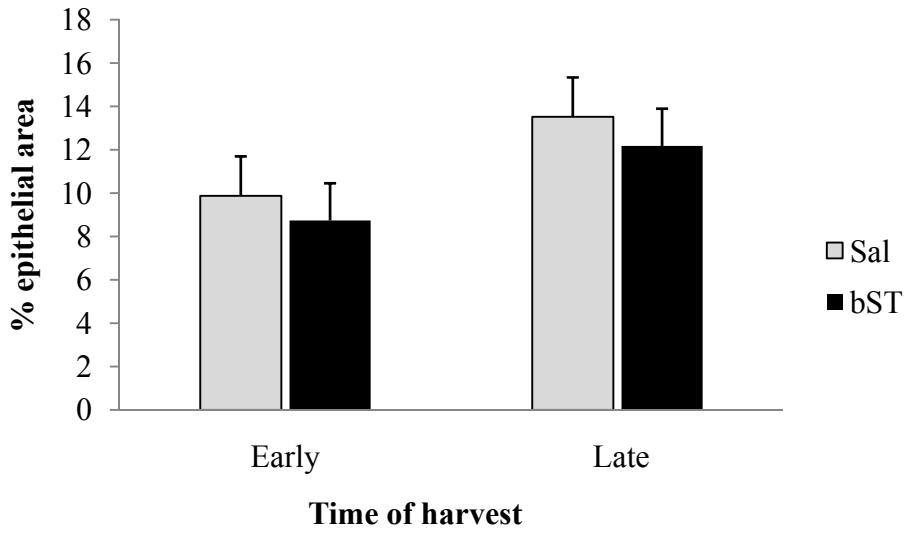


Figure 4. 3. Effect of bST treatment on the percent area of mammary epithelium present in parenchyma

Proportion of mammary epithelium present in unit area of PAR tissue section from bST (Posilac 500mg; n = 9) and Sal (0.9% NaCl; n = 9) heifers and samples harvested at two different time points; early or late. There was no difference in the amount of epithelium present in PAR from bST and Sal heifers ($P = 0.524$) nor was there an effect due to harvest time ($P = 0.083$). Data are presented as LSM and bars represent SEM.

BrdU labeling, but they were negative for Ki67. In the late harvest group, 0.5 ± 0.3 and 0.1 ± 0.3 % cells were positive only for BrdU in Sal and bST, respectively (Fig 4.6 A). However, there was an overall decrease in the percentage of BrdU label-retaining cells in late harvest heifers compared with early heifers (P = 0.016). The percentage of Ki67 positive cells in the mammary epithelium was not affected by bST treatment (P = 0.421) or time of harvest (P = 0.271) and there was no treatment by time interaction (P = 0.907). Heifers treated with bST in the early harvest group had 2.9 ± 1.0 % Ki67 positive cells in the mammary epithelium compared with 2.2 ± 1.0 % in Sal (Fig 4.6 B). In the case of late harvest heifers the percentages of Ki67 positive

Table 4. 3. Effect of bST treatment on number of epithelial structures in the mammary parenchyma

Number of epithelial structures in the mammary parenchyma from early and late harvest Holstein heifers treated with bST or Sal[†].

	Treatment						
	Sal		bST		*P* values[1]		
Item	Early	Late	Early	Late	Trt[2]	Time[3]	Int[4]
Large ducts	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.9 ± 0.2	0.64	0.61	0.64
Medium ducts	4.0 ± 1.4	6.6 ±1.3	2.9 ± 1.4	4.9 ± 1.2	0.32	0.10	0.88
Small ducts	4.1 ± 3.4	15.8 ± 3.0	3.1 ± 3.4	8.4 ± 3.0	0.21	0.02	0.33
Buds	4.0 ± 1.5	7.8 ± 1.3	7.3 ± 1.2	6.6 ± 1.3	0.49	0.02	0.87
Total	12.7 ± 5.8	30.9 ± 5.2	9.7 ± 5.8	20.8 ± 5.7	0.25	0.02	0.52

[†]Data presented as LSM ± SE. [1]Significance declared at $P \leq 0.05$. [2]*P* value for treatment effect. [3]*P* value for time effect. [4]*P* value for interaction effect between treatment and time.

cells in the mammary epithelium were 1.0 ± 0.9 and 1.9 ± 0.8 % in Sal and bST, respectively. Of the total cells counted in the mammary epithelium of the early harvest heifers, 0.3 ± 0.3 % in Sal and 0.8 ± 0.3 % in bST were dual labeled (positive for both BrdU and Ki67 antigens (Fig 4.6 C)), while in the late harvest heifers 0.4 ± 0.3 and 0.5 ± 0.3 % cells were dual labeled, respectively. The population of dual labeling cells in the mammary epithelium was not different between treatments ($P = 0.366$) or time of harvest ($P = 0.930$) and there was no interaction between treatment and time ($P = 0.581$). Overall, 0.8 % cells were retaining only BrdU labeling, 2.1 % cells were positive only for Ki67 and 0.5 % were retaining both BrdU and Ki67 labeling. Out of the total number of BrdU label-retaining cells, 53 % in older and 33 % in younger animals were dual labeled for BrdU and Ki67.

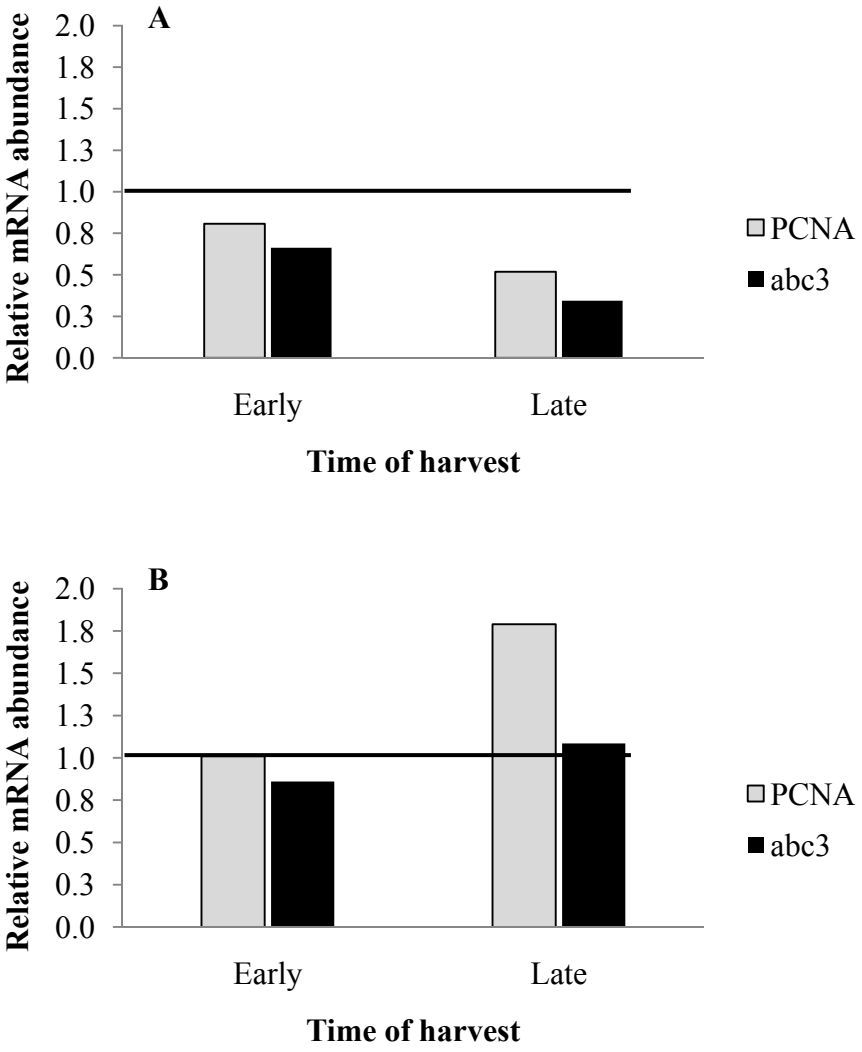


Figure 4. 4. Effect of bST treatment on relative mRNA expression of proliferative marker genes in mammary parenchyma

Relative mRNA expression ($2^{-\Delta\Delta Ct}$) of proliferative marker genes in mammary parenchyma (PAR) and fat pad (MFP) from bST treated Holstein heifers (Posilac 500mg; n = 9) in the early and late harvest groups relative to Sal controls (0.9% NaCl; n = 9) in the respective harvest groups. Horizontal line at 1.0 on Y-axis represents the mRNA abundance in Sal. (A) Relative mRNA abundance in the PAR of bST treated heifers in early and late harvest groups were not different from Sal for PCNA ($P = 0.075$), but abc3 was decreased in bST relative to Sal ($P = 0.054$). There was no difference in gene expression for both PCNA and abc3 between harvest times and there was no interaction between treatment and time of harvest. (B) Relative mRNA abundance of PCNA and abc3 in MFP from bST treated heifers in the early and late harvest groups were not different from Sal ($P = 0.204$ and 0.918, respectively). There was no effect due to harvest time or treatment and time interaction for gene expression in MFP.

4.4.5. Immunohistochemistry for PR

The percentage of PR positive cells in Sal and bST were 29.1 ± 3 and 33.7 ± 3 %, respectively. We observed the majority of PR positive cells in the middle layer of the multilayered epithelium. Treatment with bST did not affect the number of PR positive cells in mammary epithelium ($P = 0.365$; Fig 4.7 B). Furthermore, percentage of PR positive epithelial cells were not affected by time of harvest ($P = 0.941$) and there was no interaction between treatment and time of harvest ($P = 0.244$).

4.5. Discussion

Total udder weight was reduced as a result of decrease in extra-parenchymal tissue when prepubertal dairy heifers were treated with exogenous bST (Sejrsen et al., 1986). Increased BW gain and reduced MFP mass suggests that there was a favorable response to bST in terms of overall body growth and less fat accumulation in mammary gland. Despite this change in whole udder mass as a result of bST treatment, we did not find a difference in PAR mass between treatments. Therefore, like in Sejrsen et al. (1986), the reduced udder weight was primarily due to reduction in fat tissue suggesting that bST treatment is indirectly creating a complimentary environment for PAR growth by eliminating the inhibitory effect of adipose tissue on mammary epithelial proliferation. In a 2 x 2 factorial study examining the effect of ovariectomy and bST treatment, only intact animals showed bST induced increase in PAR protein content (Purup et al., 1993) suggesting that GH depends on ovarian secretions to exert its effects on mammary gland. Sejrsen et al. (1986) also reported a 27 % increase in PAR protein in bST treated heifers. Contrasting these reports we did not find a significant increase in PAR protein in bST treated heifers but there was a reduction in PAR DNA per unit BW. However, decrease in PAR DNA was not enough to make a difference in overall tissue mass.

A comparison of the results from previous studies and our current study is given in Table 4.4 (Sejrsen et al., 1999). We administered 500 mg bST every three weeks which is assumed to be 23.8 mg/d, if the sustained release of Posilac is linear. Hence the bST dose used in the current study falls in the range of previous reports. However, it is speculated that if the established Posilac injection dose of once in two weeks for increasing milk production is based on the fact that the sustained release of hormone lasts only for 14 d, then the timing of possible mitogenic

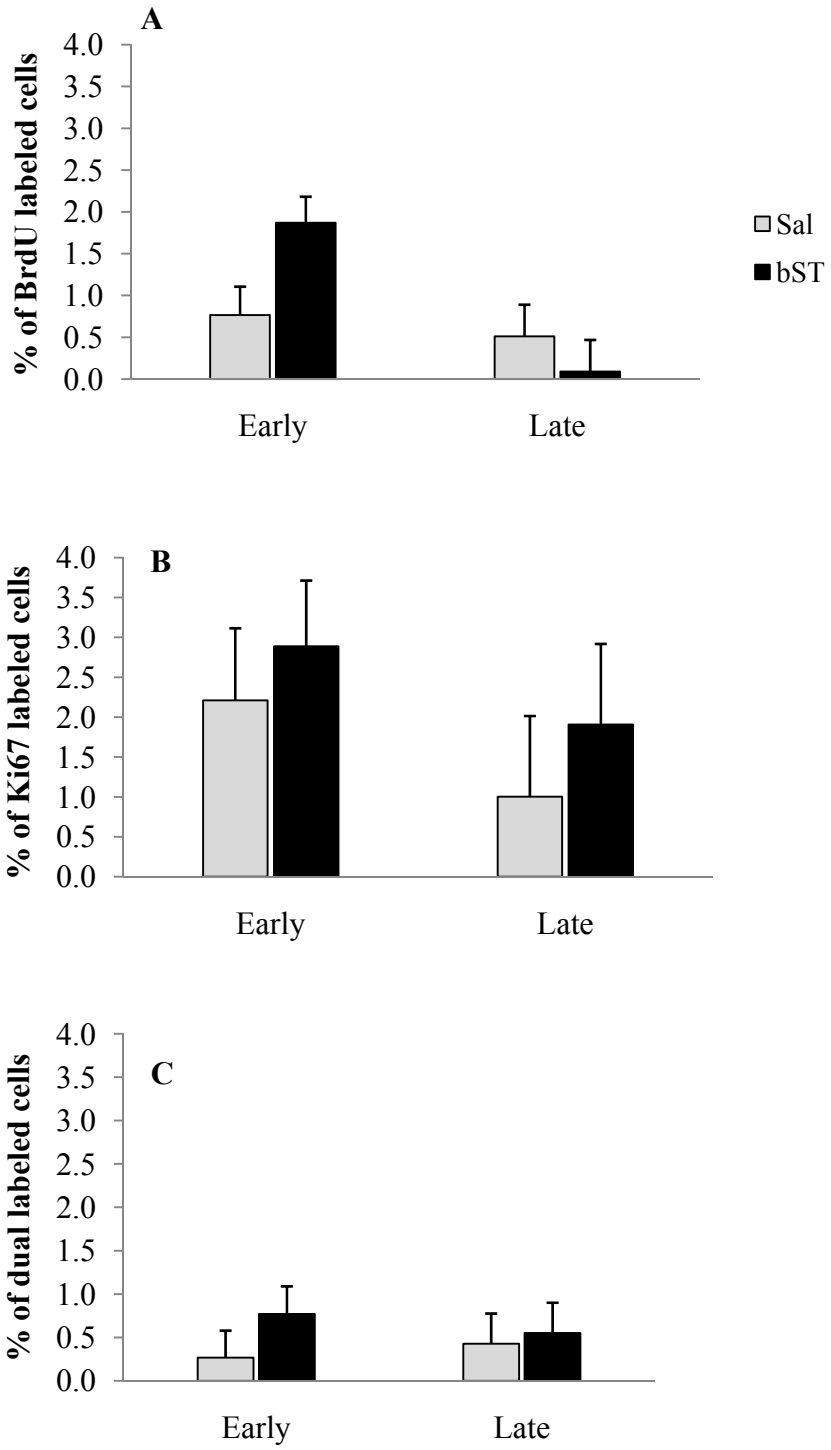


Figure 4. 5. Effect of bST treatment on cell proliferation and putative stem cell population in the mammary epithelial cells

Labeling index for proliferation and putative stem cell markers in the mammary epithelial cells of Holstein heifers that received either bST (Posilac 500mg; n = 9) or Sal (0.9% NaCl; n = 9) and samples harvested at two different time points; early or late. (A) Labeling index for BrdU positive cells was not different between Sal and bST ($P = 0.374$) and there was no interaction effect between treatment and time (P = 0.059). But, early harvest heifers had a greater percentage of BrdU label-retaining cells compared with heifers harvested late ($P = 0.016$). (B) Labeling index for Ki67 positive cells was not affected by treatment ($P = 0.421$), time of harvest ($P = 0.271$) or interaction between treatment and time ($P = 0.907$). (C) Labeling index for cells which are positive for both BrdU and Ki67 was neither different between bST and Sal ($P = 0.366$) and nor between early and late harvest time ($P = 0.930$) and there was no interaction between treatment and time of harvest ($P = 0.581$). Data are presented as LSM and bars represent SEM.

effects of bST and our tissue sampling may have been mistimed. Serum IGF-1 concentrations in these heifers support this hypothesis. Concentrations of serum IGF-1 in bST treated and control animals were not different in the samples collected on the day before each injection (three wk after the preceding injection) while there was a significant difference immediately after the injections (data not shown). Concentration of serum IGF-1 is significant since bST acts on mammary gland via circulating IGF-1 (Sejrsen and Purup, 1997). In addition, despite the fact that the heifers were treated with prolonged release bST, by three weeks after treatment concentrations of bST are markedly reduced in heifers (Jobst et al., 2000). Age of the animals at the beginning of bST treatment in previous studies ranged from 3.5 mo to 8.0 mo and the BW at slaughter was between 171 to 396 kg. However, in our study, the average age of animals at the beginning of bST treatment was 30 d and the average BW at slaughter for the second harvest group was only 123 kg. Therefore, major differences between the previous studies and our study are the earlier start time and shorter duration of bST treatment. Studies performed in beef animals for evaluating the growth performance in response to bST demonstrated that the response to bST is more pronounced in older and heavier compared with younger and lighter animals (Rausch et al., 2002; Velayudhan et al., 2007). Age related changes in the magnitude of response were due to difference in the regulation of somatotropic axis components in serum by exogenous bST. As in the case of body growth responses, the age factor may also be true for a mammary gland response to bST because the effects of bST on mammary growth is believed to be mediated through circulating IGF-1 (Sejrsen and Purup, 1997). Since earlier evidence show

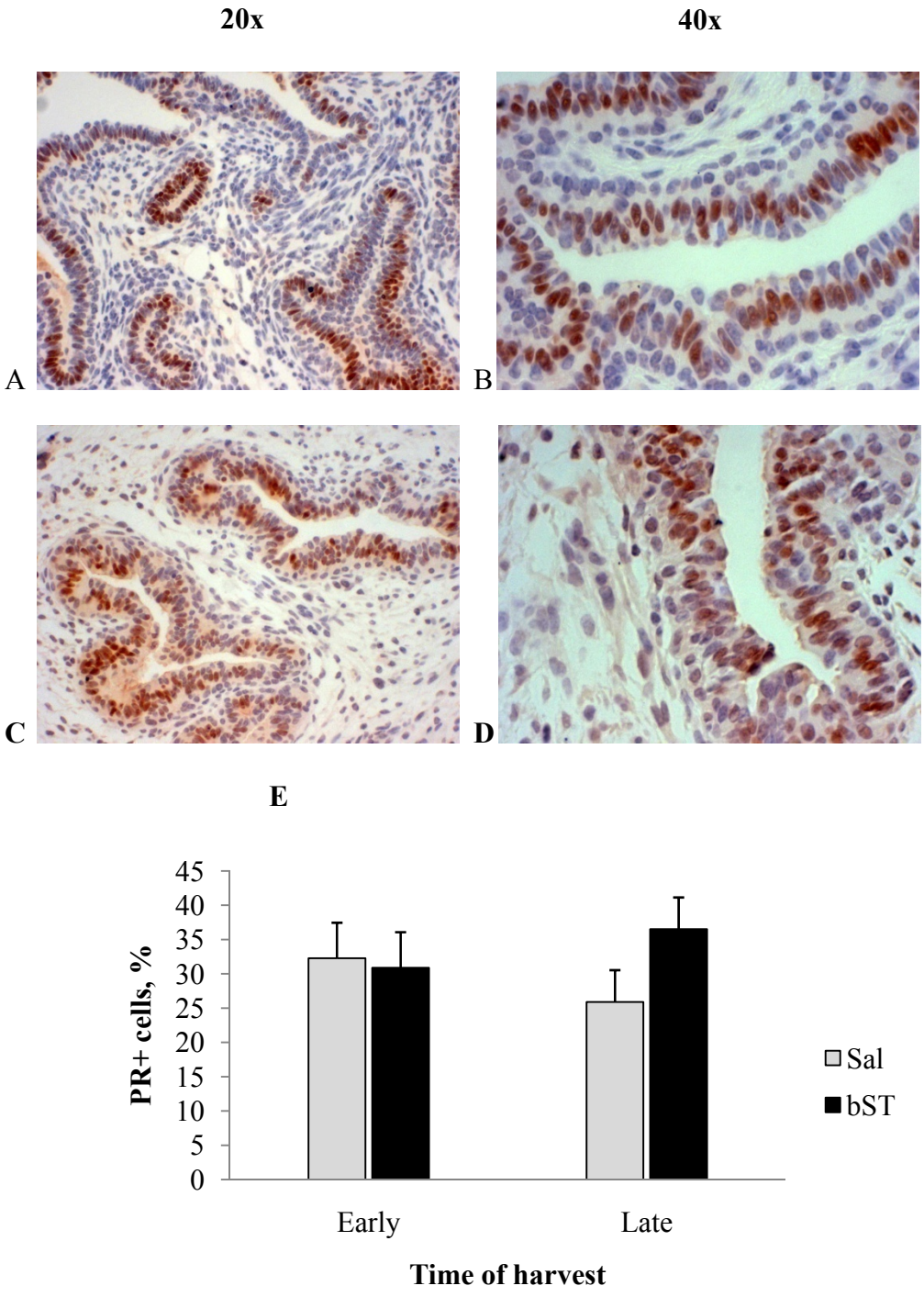


Figure 4. 6. Effect of bST treatment on progesterone receptor expression

Representative samples for localization of PR positive cells in mammary epithelium from heifers treated with Sal (A & B) and bST (C & D) by immunostaining with anti-PR antibody and detected by NovoRed chromogen. (E) Percentage of PR positive cells was not affected by treatment with bST or time of harvest. Data are presented as LSM and bars represent SEM.

that exogenous bST increased mammary gland growth and epithelial proliferation in older heifers, our data suggest that bST treatment is not effective in enhancing mammary growth rate in animals at one month of age. In agreement with our observations, total PAR mass or composition was not affected in prepubertal dairy heifers when a daily dose of 0.1 mg of bST/ kg BW was given (Capuco et al., 2004). In Capuco et al. (2004), treatment with bST began at 3.0 mo of age (Table 4.4) which was still younger than the other studies. Similar observations were made in beef heifers when treated with bST (Buskirk et al., 1996).

Supporting previous reports (Schams et al., 2003; Connor et al., 2005), we demonstrated the presence of PR in the mammary epithelium of prepubertal heifers, but the number of receptors were unaffected by bST treatment. In agreement with previous reports, mammary histology was not affected by treatment with bST (Sejrsen et al., 1986; Purup et al., 1993). We found similar histological architecture of large, medium and small sized ducts, subtending ducts and terminal ductal units extending into the surrounding loose connective tissue as described earlier (Capuco and Ellis, 2005) in both bST and Sal heifers. Proliferation in mammary epithelial cells assessed by Ki67 immunostaining was also not affected by bST, which also supports previous findings (Capuco et al., 2004). Coinciding with Ki67 positive cells, mRNA expression of PCNA was also not different between treatments further supporting our conclusion that mammary epithelial proliferation is not affected by exogenous bST in prepubertal heifers. Contrasting our findings, short-term treatment with exogenous bST resulted in increased incorporation of ^{3}H-thymidine in mammary epithelial cells (Berry et al., 2001; Berry et al., 2003b). We suggest the possibility that mitogenic effects of exogenous bST on epithelial cells could be transient; i.e. bST may stimulate epithelial proliferation soon after the injections and may not sustain for longer periods of time.

To our knowledge the current study is the first to evaluate the effect of exogenous bST treatment on putative stem cell population in prepubertal bovine mammary gland. Capuco et al. (2007) described a label-retention method using BrdU labeling of epithelial cells to identify putative stem cells in bovine mammary gland. Our working hypothesis was that exogenous bST would increase the putative stem cell population in prepubertal mammary gland. By the dual labeling technique we could demonstrate epithelial cells retaining BrdU labeling at the same time

Table 4. 4. Comparison of previous reports (modified from Sejrsen et al., 1999) and our study on the effect of bST on mammary growth in prepubertal heifers

Study	Breed used	bST dose	Age or BW at start	Treatment period	BW (kg) at slaughter	% change in PAR weight compared with control
(Sejrsen et al., 1986)	Danish Friesian x Danish Red	20 U/d	8.0 m	16 wk	273	+18
(Sandles et al., 1987)	Jersey x British Friesian	0.6 mg/ $kg^{0.75}$/d	3.5 m	21 wk	383	+20
(Singh et al., 1991)	Holstein Friesian	8.2 mg/d	6.9 m	17 wk		-9
(Purup et al., 1993)	Danish Friesian	15 mg/d	5.8 m	15 wk	214	+13
(Carstens et al., 1997)	Holstein Friesian x Angus	500 mg/ 2 wk	6.0 m	to 390 kg	385	+48
(Radcliff et al., 1997)	Holstein Friesian	25 μg/kg/d	126 kg	to 5th estrus	366	+46
(Capuco et al., 2004)	Holstein Friesian	500 mg/ 2 wk	3.0 m	2 m 7 m	300	No effect
Our study	Holstein Friesian	500 mg/ 3 wk	1.0 m	6 wk 12 wk	88 123	No effect

negative for Ki67 staining suggesting that these cells are not actively proliferating. Not supporting our hypothesis, we failed to demonstrate an increase in BrdU-label retaining cells in prepubertal mammary gland in response to bST treatment. However, there was a decrease in the number of BrdU label retaining cells in the late harvest compared with early harvest heifers. This could be because of the difference in the interval between BrdU injections and slaughter in early and late harvest groups. Identification of BrdU label retaining cells is based on the principle of

dilution effect. At the time of injection, BrdU, being a thymine analogue, will be incorporated into newly synthesized DNA and subsequent cell divisions in an actively proliferating epithelial cell causes dilution of BrdU in daughter cells by symmetric cell division. As the time interval between injection and slaughter increases there will be more dilution and hence fewer detectable label retaining cells. Information is scarce on hormonal regulation of bovine mammary stem cells albeit some speculations on ovarian regulation of stem cell population based on data available in the murine species have been proposed. It is suggested that a similar mechanism of estrogen-mediated cell proliferation, as in the case of epithelial cells, also exists for stem cells because of the similar pattern of expression of estrogen receptors in proliferating epithelial cells and putative stem cells (Capuco and Ellis, 2005). Supporting their hypothesis, a recent study reported that ovariectomy in prepubertal heifers reduced the number of BrdU-label retaining cells in mammary epithelium (Korn et al., 2008). Mammary stem cells are capable of symmetric cell division producing two identical stem cells and at the same time they can undergo asymmetric division to give rise to one stem cell and a progenitor cell of committed lineage (Chepko and Smith, 1999). It is proposed that putative mammary stem cells may be sensitive to mitogenic stimuli but not responsive to lactogenic stimuli (Capuco and Ellis, 2005). Exogenous bST increased the rate of cell renewal in lactating mammary gland and thus resulted in high rate of cell turn over (Capuco et al., 2001). Taken together, this observation and several other historic reports on increased milk production in bST treated heifers (Asimov and Krouze, 1937; Bauman et al., 1999) along with our current findings of failure of increased epithelial proliferation in bST treated heifers (also supported by Capuco et al., 2004) seem to establish GH as a lactogenic rather mitogenic hormone for bovine mammary gland.

CHAPTER 5: TRANSCRIPT ABUNDANCE OF INSULIN-LIKE GROWTH FACTOR-1 SYSTEM IN PREPUBERTAL BOVINE MAMMARY GLAND IN RESPONSE TO OVARIECTOMY AND TREATMENT WITH EXOGENOUS BOVINE SOMATOTROPIN

5.1. Abstract

Mammary epithelial cell proliferation and differentiation are believed to be mediated through local synthesis of somatotropic axis components. We hypothesized that at an early age, ovariectomy would down regulate, while treatment with exogenous bovine somatotropin (**bST**) would up regulate transcript abundance of insulin-like growth factor (**IGF**)-1 and its binding proteins (**IGFBP**) in bovine mammary gland. Our specific objectives were to determine the effects of ovariectomy and treatment with bST on mRNA expression of IGF-1, IGF-1 receptor (**R**) and IGFBP-1 to -6 by quantitative real time polymerase chain reaction (**qRT-PCR**). In the first study, prepubertal dairy heifers either at 2, 3 or 4 mo of age were randomly assigned to one of two treatments, ovariectomized (OVX; n = 8) or sham operated (INT; n = 12) Mammary parenchyma (**PAR**) and fat pad (**MFP**) were harvested 30 d after surgery. In the second study, 18 heifers (7 ± 4 d old) were randomly assigned to one of two treatments; bST (500 mg Posilac; n = 9) or Sal (0.9% saline; n = 9) beginning on day 29 ± 4 and received treatments every 21 d. Mammary tissues were harvested either after six week or 12 wk of treatment. Relative abundance of mRNA was determined by qRT-PCR by comparative Ct method. Main effects of treatment and age as well as their interaction were tested using the Mixed model procedure of SAS and statistical significance was declared at $P \leq 0.05$. Overall, IGF-1and IGFBP-6 were decreased while IGF-1R was increased in PAR of OVX, but there was no difference in mRNA expression of IGFBP-1 to -5 between OVX and INT. However, bST did not have any effect on mRNA abundance of IGF-1 and IGFBP. Transcript abundance in MFP was mostly regulated by age of animals. IGFBP-1 and IGFBP-5 were up regulated in older animals whereas IGFBP-3 and IGFBP-4 were down regulated over time. In conclusion, ovarian secretions are major regulators of gene expression in PAR while ontogeny plays a major role in regulation of gene expression in MFP in prepubertal dairy heifers. Expression of IGF-I axis molecules in mammary glands of very young prepubertal heifers appear largely refractory to treatment with exogenous bST.

5.2. Introduction

Like in many other tissues insulin-like growth factor (**IGF**) -1 and IGF binding proteins (**IGFBP**) are also identified as critical mediators of proliferation and survival of mammary epithelial cells. These growth factors and related proteins act on the mammary gland in an endocrine as well as paracrine and/or autocrine manner. Actions of growth hormone (**GH**) in mammary gland is believed to be mediated through both circulating as well as locally produced IGF-1 resulting in proliferation of mammary epithelial cells (Forsyth, 1996). Growth hormone receptors (**GHR**) and IGF-1 are predominantly expressed in stromal cells (Gallego et al., 2001) while IGF-1 receptor (**IGF-1R**) is mainly expressed in the epithelial cells (Forsyth et al., 1999), supporting the concept of stroma-epithelial interactions. Local mRNA expression of IGF-1 and IGFBP -1 to -6 through various physiological stages were previously reported for bovine (Plath-Gabler et al., 2001) and murine (Boutinaud et al., 2004) mammary glands. However data for young prepubertal ruminants is largely absent. Among the IGFBP detected, IGFBP-3 and -5 are typically the most abundant proteins present in mammary tissue. However, very little is known about the functional role played by individual IGFBP in mammary tissue. Biological activity and availability of IGF-1 is generally modulated by IGFBP. The mechanism of either inhibitory or stimulatory regulation of IGF-1 by IGFBP occurs primarily as a consequence of competition between IGF-1 and IGFBP which limits IGF-1 binding to the IGF-1R (Cohick, 1998). The relative affinity of IGFBP for IGF-1 depends on the status of proteolysis, phosphorylation and adherence to either cell-surface proteins or extra cellular matrix of the IGFBP (Clemmons, 1997). In addition, each of the IGFBP is independently regulated so their biological actions vary between tissues and also across species. For example, IGFBP-3 is growth promoting but IGFBP-2 is a negative indicator of growth in humans (Underwood et al., 1994) and bovine (Govoni et al., 2004). On the contrary, IGFBP-1, -2, -3 and -5 are identified as suppressers of IGF-1-mediated survival of murine, bovine and human mammary epithelial cells (Purup et al., 2000; Strange et al., 2002; Marshman et al., 2003). The hormones, estrogen and GH regulate mammary growth at least in part by modulating the synthesis and functionality of IGF-1 and IGFBP. Estrogen increases the mitogenic effects of IGF-1 by inducing expression of IGF-1 and IGF-1R in human breast tissue (Clarke et al., 1997a). Estrogen significantly increased transcription of IGF-1 gene in various tissues across many species (Umayahara et al., 1994). Ovarian secretions

clearly are involved in regulating the local IGF-1 axis in bovine mammary gland of older pre- or peri-pubertal heifers (Berry et al., 2003c). In addition increased DNA synthesis in mammary parenchyma occurred in correspondence with increased local expression of IGF-1 in the mammary gland of heifers that received exogenous GH (Berry et al., 2001).

Previous studies evaluating the hormonal regulation of local expression of IGF-1 and related proteins were done in older heifers and results were based on the observations using methods less sensitive than those currently available. Quantitative real time reverse transcription polymerase chain reaction (**qRT-PCR**) has recently been identified as a powerful tool to evaluate gene expression profiles in tissues. The extreme sensitivity of the real time PCR method ensures the detection of transcripts in very low abundance in contrast to the comparatively lower sensitivity shown by earlier detection methods such as Northern blots or quantification of PCR amplification products on agarose gels. Therefore, the objective of our study was to evaluate the gene expression profile of IGF-1 and IGFBP in prepubertal bovine mammary glands from ovariectomized and bST-treated heifers using qRT-PCR and to particularly evaluate the responsiveness of mammary tissue in very young prepubertal heifers. Our working hypothesis was that ovariectomy would decrease relative abundance of IGF-1 and IGFBPs while treatment with bST would produce the opposite responses.

5.3. Materials and Methods

5.3.1. Animals and Treatments

This study consisted of two experiments. In the first experiment, 20 prepubertal Holstein heifers were used to study the ovarian regulation of local expression of IGF-1 and related proteins. All animal care and use protocols used in this study were approved by the Clemson University Institutional Animal Care and Use Committee. Heifers were fed with commercial milk replacers and calf starter diets according to the manufacturer's instructions prior to weaning and fed with grains and hay thereafter. After a week's adaptation period at the facility, heifers were randomly assigned to either ovariectomy (OVX; n = 8) or sham operation (INT; n = 12) when they were either 60 ± 1, 93 ± 3 or 122 ± 2 d of age. Surgeries were performed by laparoscopic vertical incision of about 10 cm on the left flank after desensitizing the area with

local anesthetic (Lidocane HCl, 2% injectable solution). Thirty days after surgery animals were humanely sacrificed by captive bolt pistol stunning followed by exsanguination and mammary tissues were harvested immediately. See Chapter 3 for more information on experimental design.

The second experiment was conducted to study the effect of bST on the local expression of IGF-1 and related proteins in the bovine mammary gland. All protocols used in the study were in agreement with the animal use protocols approved by the Virginia Tech Institutional Animal Care Use Committee. Eighteen Holstein heifers were blocked by age and BW and at an average age of 29 ± 1 d heifers were randomly assigned to one of the treatment groups, either bST or control. Heifers in the bST group (n = 9) received a sustained release formulation of 500 mg recombinant somatotropin (Posilac; Monsanto Co., St. Louis, MO) subcutaneously in the neck region. Control heifers (Sal; n = 9) were injected with 0.9 % saline solution subcutaneously. The treatments were repeated at every 21 d interval. Body weight was recorded before the treatments started and then once in two week. All heifers also received calf starter (20 % CP and 1.4 % fat) and fresh water free of choice. (See Chapter 4 for further details on experimental design). Heifers were humanely sacrificed in two stages using Phenobarbital injection i.v. (Euthasol, 10 mg/kg BW) and mammary tissue was harvested. The first set of heifers were sampled three week after the second injection (average age of 72 ± 1 d; treatment period of six weeks) and the second set of heifers were sampled three week after the fourth injection (average age of 116 ± 1 d; treatment period of 12 wk).

5.3.2. Sample Collection and Analyses

In both experiments, mammary parenchyma (**PAR**) and fat pad (**MFP**) were collected consistently from the left hind quarter for gene expression analyses. Sample collection procedures have been described previously in Chapter 2. For gene expression analyses parenchyma was bisected and PAR samples were collected randomly from the inner region of the tissue approximately mid-gland. Samples of MFP were collected from regions distinctly away from the parenchyma.

Total RNA isolation and quantitative real time RT-PCR method as described in Chapter 2 were used to determine relative mRNA abundance. Yield of total RNA was not different between treatments in either of the experiment (Table 5.1 and Table 5.2). Relative gene

expression of IGF-1, IGF-1R, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 were determined. For normalization of the cycle threshold (**Ct**) of target genes, geometric mean of the Ct values of three endogenous control genes were used (Piantoni et al., 2008). Relative mRNA expression was determined as fold change ($2^{-\Delta\Delta Ct}$) in gene expression in treatment group relative to control by comparative Ct method (Chapter 2). Primer pair sequences are given in Table 5.3.

5.3.3. Statistical Analysis

Gene expression data from both ovariectomy and bST experiments were analyzed similarly. Data for PAR and MFP were analyzed combined as well as separately. When analyzed combined, main effects of tissue, interactions between tissue and treatment as well as tissue and time were also included in the model (Table A.5 and A.6). When there was significant age effect, it was further tested by contrast statements. The ΔCt data were used for statistical analyses and significance was determined based on the *P* values for ΔCt data (Table A.3 and A.4). However, data was presented as fold change in gene expression for bST treated animals relative to control animals using the $2^{-\Delta\Delta Ct}$ method of calculation (Chapter 2).

Table 5. 1. Yield of total RNA (µg/ mg tissue) for mammary PAR and MFP from ovariectomy experiment

Yield of total RNA (µg/ mg tissue) for mammary PAR and MFP from Holstein heifers ovariectomized (OVX; n = 8) or sham operated (INT; n = 12) either at 2, 3 or 4 mo of age and tissues were harvested 30 d after surgery[†].

	Age at surgery, m									
	2		3		4			*P* values[1]		
	INT	OVX	INT	OVX	INT	OVX	SEM[2]	Trt[3]	Age[4]	Trt*Age[5]
PAR	0.66	0.82	0.99	1.19	1.02	0.68	0.26	0.98	0.25	0.34
MFP	0.40	0.29	0.20	0.29	0.20	0.17	0.10	0.78	0.05	0.28

[†]Data presented as LSM. [1]Significance is declared at $P \leq 0.05$. [2]Highest standard error of the means. [3]Treatment effect. [4]Effect of age at surgery. [5]Interaction between treatment and age

Table 5. 2. Yield of total RNA (µg/ mg tissue) for mammary PAR and MFP from bST experiment

Yield of total RNA (µg/ mg tissue) for mammary PAR and MFP from Holstein heifers treated with bST (500 mg Posilac; n = 9) or Sal (0.9% Saline; n = 9) and tissues were harvested either at an early or later period of time[†].

	Treatments							
	Sal		bST			*P* values[1]		
	Early	Late	Early	Late	SEM[2]	Trt[3]	Time[4]	Trt*Time[5]
PAR	0.38	0.40	0.33	0.34	0.12	0.68	0.90	0.98
MFP	0.18	0.15	0.20	0.23	0.04	0.24	0.94	0.45

[†]Data presented as LSM. [1]Significance is declared at $P \leq 0.05$. [2]Highest standard error of the means. [3]Treatment effect. [4]Effect of age at surgery. [5]Interaction between treatment and age

Table 5. 3. Primer pair sequences of target and endogenous reference genes

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
IGF-1	GTTGGTGGATGCTCTCCAGT	CTCCAGCCTCCTCAGATCAC
IGF-1R	TCAAGGACGGAGTCTTCACC	GCTCAAACAGCATGTCAGGA
IGFBP-1	ACCAGCCCAGAGAATGTGTC	GTTTGTCTCCTGCCTTCTGC
IGFBP-2	CAAGGGTGGCAAACATCAC	GAGGTTGTACAGGCCATGCT
IGFBP-3	CAGAGCACAGACACCCAGAA	TGCCCCGTACTTATCCACACA
IGFBP-4	GCCGCACACACGTCTATCTA	CGCTTGCATGATTTACACGA
IGFBP-5	GTGCGGCGTCTACACTGAG	TCACGGGAGTCTCTTTTCGAT
IGFBP-6	CGCAGAGACCAACAGAGGAACT	GGGACCCATCTCAGTGTCTTG
PPP1R11	CCATCAAACTTCGGAAACGG	ACAGCAGCATTTTGATGAGCG
RPS15A	GAATGGTGCGCATGAATGTC	GACTTTGGAGCACGGCCTAA
MTG1	CTTGGAATCCGAGGAGCC A	CCTGGGATCACCAGAGCTGT

The model used was

$Y_{ijk} = \mu + T_i + A_j + (TA)_{ij} + e_{(ij)k}$ where

Y_{ijk} = variable being tested

μ = overall mean

T_i = fixed effect of treatment (bST/OVX or control) (i = 1, 2 for both experiments)

A_i = fixed effect of time (early or late harvest time for bST and age at surgery (2, 3 or 4 mo) for ovariectomy experiment) (j = 1, 2 for bST and j = 1, 2, 3 for ovariectomy)

$(TA)_{ij}$ = fixed interaction of treatment and time

$e_{(ij)k}$ = residual error

Data distribution was analyzed using the "Proc Mixed Boxplot" statement (Appendix A.6). Significance was declared at $P \leq 0.05$ for all analyses.

5.4. Results

5.4.1. Effect of ovariectomy on gene expression

Relative abundance of IGF-1 and IGFBP-6 mRNA in PAR were decreased in OVX relative to INT controls ($P < 0.05$) whereas there was an increase in the expression of IGF-1R mRNA in PAR of OVX ($P < 0.05$; Fig 5.1). Ovariectomy did not affect the abundance of mRNA of IGFBP-1, -3, -4 and -5 in PAR but IGFBP-2 mRNA expression tended to decrease ($P = 0.057$) in OVX. We did not find a change in gene expression in PAR due to age of heifers and there was no interaction between treatment and age at surgery. In MFP, transcript abundance of IGF-1 and related proteins were not affected by ovariectomy and there was no interaction between treatment and age at surgery (Fig 5.2). However, we observed age related changes in mRNA of IGFBP in MFP (Fig 5.3). There was a linear decrease in the mRNA expression of IGFBP-3 and IGFBP-4 in MFP with increased age ($P < 0.01$), while IGFBP-5 expression was increased in older animals compared with younger animals ($P < 0.05$). In older animals there was a numerical increase in the IGFBP-1 transcript abundance but this was not significantly different than younger animals ($P = 0.086$).

5.4.2. Effect of bST on gene expression

Transcript abundance of IGF-1 and related proteins were not affected by treatment with bST in PAR or MFP (Table 5.3 and 5.4, respectively). However, relative abundance of IGF-1R in MFP tended to increase in bST ($P = 0.066$). In both PAR and MFP, there was change in the expression of IGFBP-1 with age of animals. Expression of IGFBP-1 was increased in PAR when animals got older ($P < 0.01$), while in MFP, there was a decrease in IGFBP-1 expression in older compared with younger heifers ($P < 0.05$). Except for IGFBP-6 expression in MFP, there was no interaction between treatment and time of harvest for mRNA abundance of IGF-1 and related proteins. In MFP, treatment with bST increased the expression of IGFBP-6 in early harvest while there was a reduction in IGFBP-6 in bST heifers from the late harvest (treatment and time interaction; $P < 0.05$).

5.5. Discussion

Our study provides strong evidence of mRNA synthesis of IGF axis molecules in both PAR and MFP components of mammary gland in prepubertal heifers, supporting a previous report (Plath-Gabler et al., 2001). Regulation of local expression of IGF-1 and IGF-1R mRNA were primarily by ovarian secretions, while bST did not affect gene expression which is in agreement with previous report (Berry et al., 2001). Ligand-binding assays demonstrated that bST does not bind with GHR in bovine mammary gland, suggesting an indirect effect of bST on mammary gland, possibly through serum IGF-1 (Akers, 1985). Serum concentrations of IGF-1 were not different between bST and Sal animals except for the samples collected immediately after injections (data not shown). Absence of bST-induced proliferation of mammary cells in these heifers could be due to a failure to maintain greater serum IGF-1 concentration. IGF-1 is more important in mammary development than GH or estrogen because mammary development was impaired in IGF-1-knockout mice despite normal production of GH and estrogen (Ruan and Kleinberg, 1999). However, in rodents, injections of human GH increased IGF-1 mRNA within a12 h period (Kleinberg and Ruan, 2008), suggesting species difference in the response to exogenous GH. Hormonal regulation of gene expression of IGF-1, IGF-1R and IGFBPs were more evident in PAR than MFP. Estrogen induced synthesis of growth factors in MFP supports the estrogen dependency as well as the concept of stromal-epithelial interaction in mammary

growth and development. In prepubertal heifers estrogen increased IGF-1 mRNA expression in PAR and MFP (Meyer et al., 2006a) and the response was greater in MFP. As we hypothesized, IGF-1 mRNA was down regulated by ovariectomy in agreement with previous reports (Berry et al., 2003c). However, we did not find a similar response in MFP. This could be due to a difference in the region of sample collection. We collected MFP samples distinctly distant from

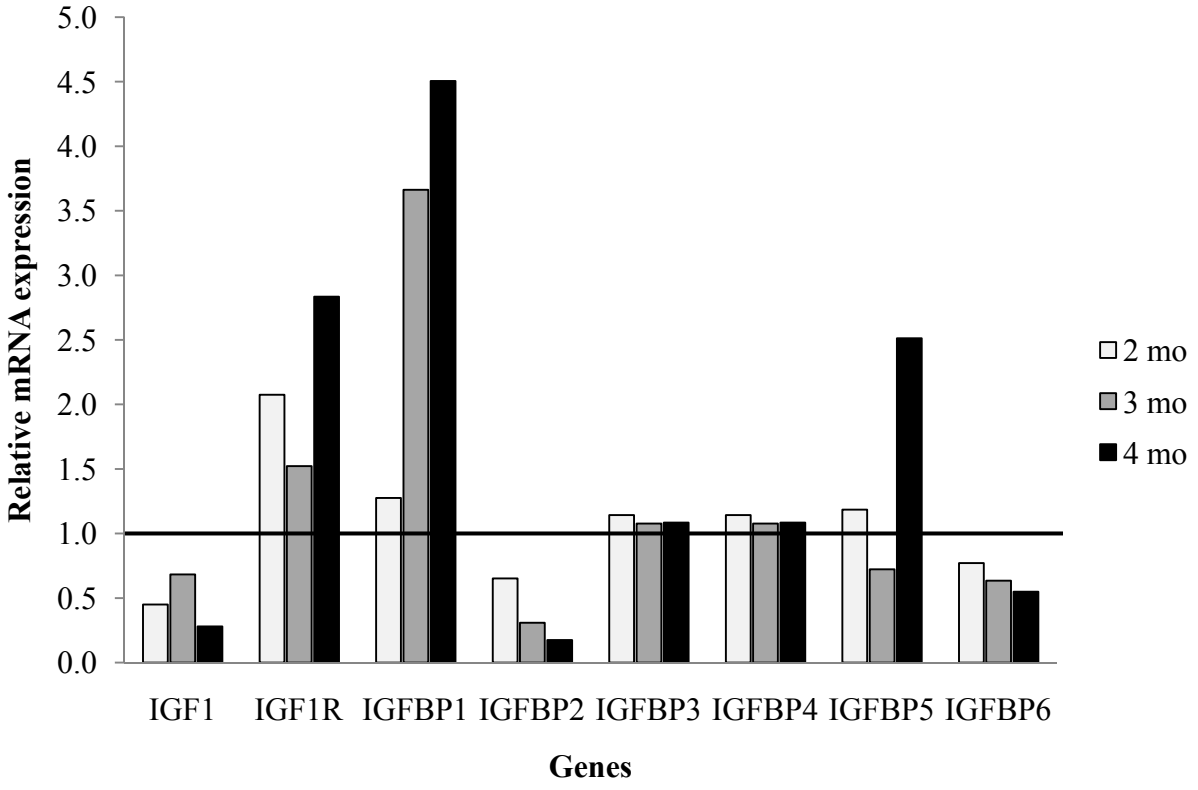


Figure 5. 1. Relative mRNA abundance of IGF-1 and related proteins in the PAR from ovariectomy experiment

Relative mRNA abundance ($2^{-\Delta\Delta Ct}$) of IGF-1 and related proteins in the PAR of OVX (n = 8) ovariectomized either at 2, 3 or 4 mo of age relative to their INT controls (n = 12). The horizontal line at 1.0 in the Y axis represents the mRNA expression in INT. Local expression of IGF-1 and IGFBP-6 mRNA in PAR were down regulated in OVX ($P < 0.05$) while IGF-1R mRNA was up regulated in OVX compared with INT ($P < 0.05$). Local expressions of IGFBP-2 to -5 were not different between OVX and INT. Gene expression of IGF-1 and related proteins in PAR was not affected by age at surgery and there was no interaction between treatment and age at surgery.

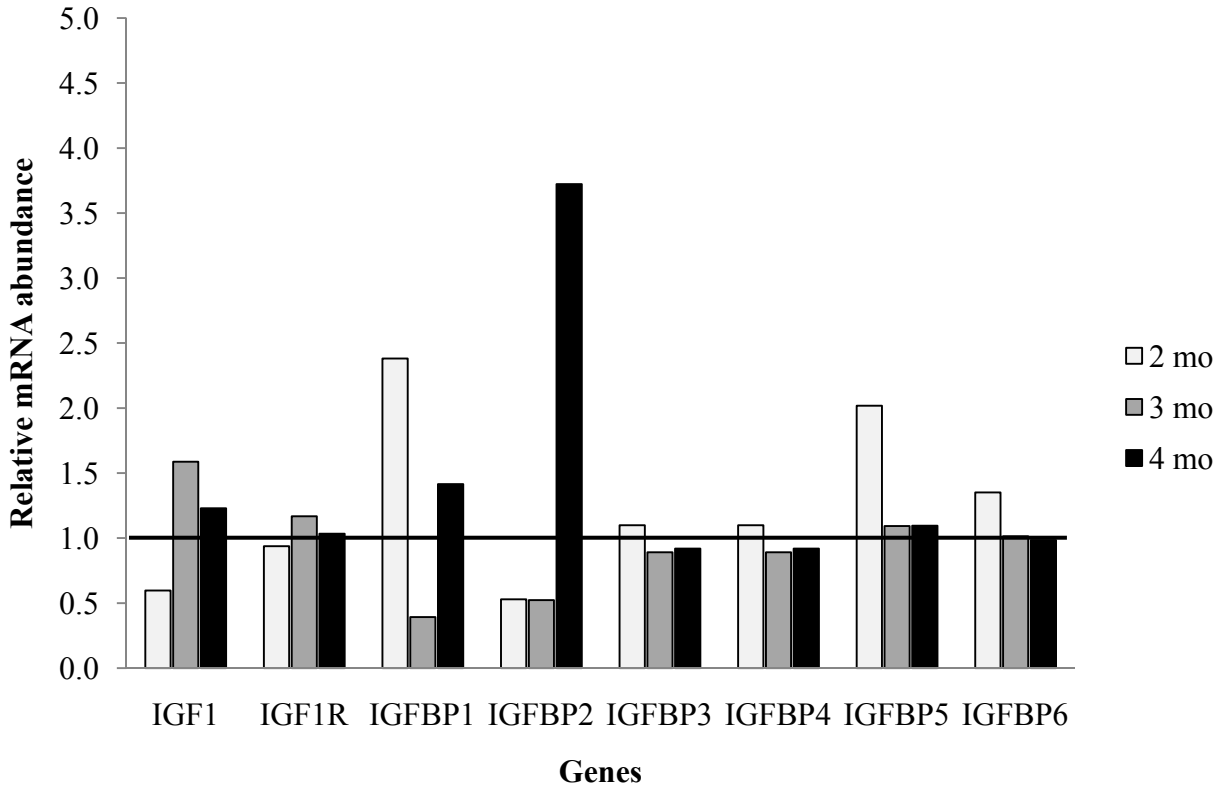


Figure 5. 2. Relative mRNA expression of IGF-1 and related proteins in MFP from ovariectomy experiment

Relative mRNA abundance ($2^{-\Delta\Delta Ct}$) of IGF-1 and related proteins in MFP of OVX (n = 8) ovariectomized either at 2, 3 or 4 mo of age relative to their INT controls (n = 12). The horizontal line at 1.0 in the Y axis represents the mRNA expression in INT. Gene expression of IGF-1 and related proteins in MFP were not affected by treatment and there was no interaction between treatment and age at surgery. However, local expressions of IGFBP-3, -4 and IGFBP-5 mRNA in MFP were different in different age groups ($P < 0.05$).

PAR and therefore these tissues may not reflect the response of MFP cells adjacent to epithelial cells as described by Meyers et al. (2006a). Estrogen induced proliferation of epithelial cells by modulating IGF-1 expression is demonstrated by in vivo and in vitro studies in many cell types

such as bone, endometrium, hypothalamus and mammary gland. Mechanism responsible for estrogen-induced epithelial proliferation in bovine mammary gland is not clear yet, but is under active research. In bovine mammary gland IGF-1 expression is almost entirely to stromal cells while ERα immunoreactivity is nearly exclusive to the epithelial cells (Capuco et al., 2002; Berry et al., 2003b) suggesting that estrogen induced IGF-1 mRNA expression is not directly through ERα signaling. However, cell culture experiments showed that ERα is required for rapid activation of IGF-1R signaling cascade (Kahlert et al., 2000). Furthermore, decreased sensitivity for IGF-1 binding was exhibited by parenchymal cells collected from ovariectomized heifers (Berry et al., 2003c). These observations suggest the interdependency between ERα signaling

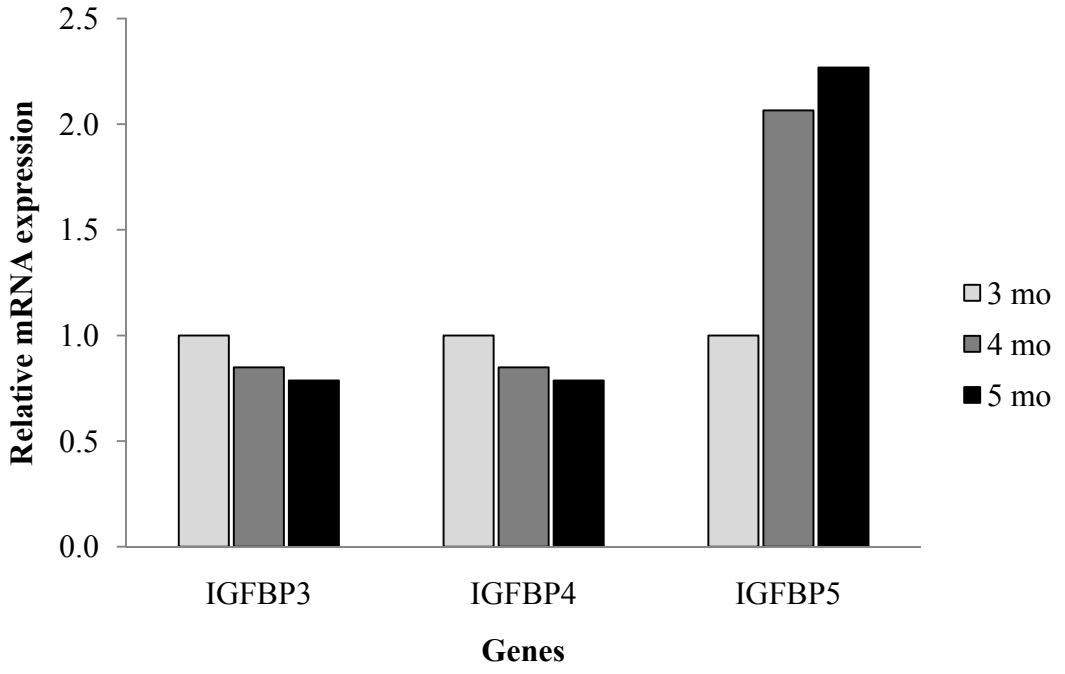


Figure 5. 3. Ontogenic regulation of gene expression in mammary fat pad

Relative mRNA abundance in MFP at 3 (n = 6), 4 (n = 7) and 5 (n = 7) m of age relative to 3 mo of age. Since there was no treatment effect on gene expression in MFP, INT and OVX data were pooled within age group. Local gene expressions of IGFBP-3 and -4 in MFP were down regulated with age ($P < 0.01$) while IGFBP-5 mRNA was up regulated with age ($P < 0.05$).

and IGF-1 local synthesis. The increase in IGF-1R mRNA in response to ovariectomy could be explained as a compensatory up-regulation of receptor expression because of reduced availability of ligands. Reciprocal expression pattern was observed for IGF-1 and IGF-1R mRNA during different stages of mouse mammary development wherein IGF-1R mRNA decreased as IGF-1 mRNA increased (Boutinaud et al., 2004), supports our suggestion.

Table 5. 4. Relative mRNA abundance of IGF-1 and related proteins in PAR from bST experiment

Relative mRNA abundance of IGF-1 and related proteins in PAR of Holstein heifers treated with bST (n = 9) and tissues harvested either at an early or late harvest time relative to Sal controls (n = 9) †

	Time of harvest		*P* values[1]		
	Late	Early	Trt[2]	Time[3]	Trt*Time[4]
IGF-1	1.20	2.32	0.33	0.31	0.52
IGF-1R	0.45	0.77	0.21	0.46	0.52
IGFBP-1	0.44	0.20	0.08	0.01	0.54
IGFBP-2	0.45	0.74	0.22	0.15	0.57
IGFBP-3	0.63	0.46	0.15	0.95	0.69
IGFBP-4	0.73	0.82	0.21	0.48	0.77
IGFBP-5	0.53	1.09	0.58	0.14	0.48
IGFBP-6	0.61	1.02	0.45	0.15	0.41

†Data presented as relative mRNA expression ($2^{-\Delta\Delta Ct}$) in bST relative to Sal. [1]Significance was declared at $P \leq 0.05$. [2]Effect due to treatment with bST. [3]Effect due to early or late harvest time. [4]Interaction between treatment and time

Many studies have been conducted in murine and human breast cell lines to delineate the association between estrogen and growth factors in enhancing epithelial proliferation. Proliferation induced by IGF-1 is a result of signaling via phosphorylation of IGF-1R which in

turn activates a cascade of phosphorylation of adapter proteins such as insulin-receptor substrates (**IRS**) and mitogen-activated protein kinases (**MAPK**) (Sachdev and Yee., 2001). In a recent study conducted with breast cancer cells estrogen phosphorylated IGF-1R and epidermal growth factor receptor but specific inhibitors of IGF-1R inhibited estrogen-induced MAPK activation thereby inhibiting cell growth (Santen et al., 2008). Interdependency of estrogen and GH in promoting epithelial cell proliferation was demonstrated in mice mammary gland (Walden et al., 1998) and the proliferation was mediated by increased expression of IGF-1 mRNA. In another

Table 5. 5. Relative mRNA abundance of IGF-1 and related proteins in MFP from bST experiment

Relative mRNA abundance of IGF-1 and related proteins in MFP of Holstein heifers treated with bST (n = 9) and tissues harvested either at an early or late harvest time relative to Sal controls (n = 9) [†]

	Time of harvest		*P* values[1]		
	Late	Early	Trt[2]	Time[3]	Trt*Time[4]
IGF-1	0.19	0.94	0.24	0.45	0.28
IGF-1R	2.13	1.21	0.07	0.21	0.25
IGFBP-1	2.67	0.56	0.60	0.01	0.06
IGFBP-2	0.89	1.29	0.83	0.20	0.54
IGFBP-3	3.28	0.45	0.75	0.43	0.11
IGFBP-4	1.35	0.48	0.43	0.99	0.07
IGFBP-5	3.05	0.27	0.88	0.54	0.10
IGFBP-6	2.59	0.47	0.81	0.67	0.04

[†]Data presented as mRNA expression in bST relative to Sal ($2^{-\Delta\Delta Ct}$). [1]Significance was declared at $P \leq 0.05$. [2]Effect due to treatment with bST. [3]Effect due to early or late harvest time. [4]Interaction between treatment and time

study involving human mammary epithelial cells, estrogen stimulated phosphorylation of IRS-1, which was inhibited by anti-estrogens (Lee et al., 1999). Nevertheless, another study conducted in bovine satellite cells reported induction of IGF-1 mRNA by estrogen through the mediation of a G-protein-coupled membrane receptor 30 (Kamanga-Sollo et al., 2008). Taken together these findings suggest that there are multiple mechanisms to explain the action of estrogen in mediating cell proliferation. These include inducing IGF-1transcription as well as cross-talk involving IGF-1signaling through membrane bound receptors other than the conventional nuclear ERα signaling. Further investigations are warranted to verify whether similar mechanisms exist in bovine mammary gland.

Insulin-like growth factor binding proteins are present in both PAR and MFP components of prepubertal heifer mammary gland and they are regulated by either ovarian status or age of animals, suggesting functional significance of these proteins in mammary growth and development. Abundance of different IGFBP mRNA were not affected by exogenous bST, in agreement with previous report (Weber et al., 2000). Transcript abundance of IGFBPs in MFP was mostly under ontogenic regulation. Expression of IGFBP-1 mRNA was not detected in 2 mo old heifers (Daniels, 2008), whereas we found IGFBP-1 mRNA in the mammary gland of heifers aged 2.5 mo and older. We also found an increase in IGFBP-1 mRNA with increase in age, strongly suggesting existence of an ontogenic regulation of local expression of IGFBP-1 in mammary gland. IGFBP-1 and IGFBP-2 are important in transporting IGF-1 in circulation, but the functional significance for their local synthesis in mammary gland is not well understood.

Transcript abundance of IGFBP-3 and IGFBP-4 in mammary gland decreased while IGFBP-5 increased with age of animals. The age-related changes in IGFBP could be associated with age-related changes in epithelial proliferation. Relative rate of proliferation and accumulation of mammary epithelial cells decreased from birth to puberty (Ellis and Capuco, 2002; Meyer et al., 2006c). It is also reported that IGFBP-5 serves as an inhibitor of cell proliferation during mammary involution by preventing the interaction between IGF-1 and its receptor (Flint et al., 2000). Although we did not see an age-related decrease in IGF-1 mRNA, the changes in IGFBP as age increases could be associated with their IGF-1-dependent roles in

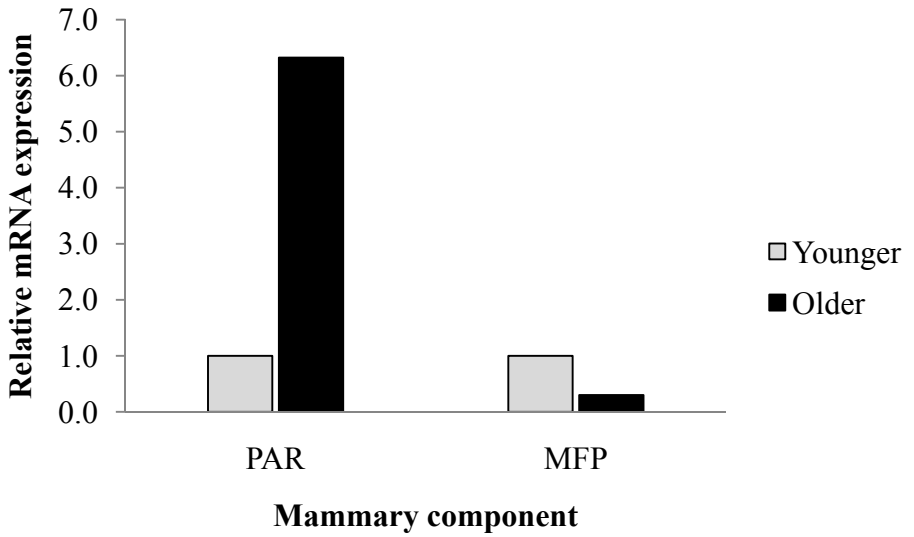


Figure 5. 4. Ontogenic and tissue specific regulation of IGFBP-1 gene expression in mammary PAR and MFP

Relative IGFBP-1 mRNA expressions in mammary PAR and MFP in Holstein heifers at early (younger heifers) and late harvest time (older) relative to age. Data from bST and Sal are pooled because of absence of treatment effect. In MFP expression of IGFBP-1 was reduced in older animals whereas in PAR mRNA expression of IGFBP-1 was increased with age ($P < 0.05$).

cell proliferation. This is supported by the observation that addition of IGF-1 to cultures of bovine mammary cells increased mRNA level of both IGFBP-3 and IGFBP-4 (Fleming et al., 2005). In our study, abundance of IGFBP-3 and IGFBP-4 mRNA were not affected by ovariectomy or bST, in agreement with previous report (Berry et al., 2001). IGFBP-3 mRNA is the most abundant IGFBP in bovine mammary gland (Plath-Gabler et al., 2001) and the most widely studied. The effect of IGFBP-3 on IGF-1 mediated cell proliferation is either stimulatory or inhibitory depending on the species and physiological status (Hunynh et al., 1996; Cohick, 1998; Weber et al., 1999). In situ hybridization in murine mammary gland showed that IGFBP-3 and IGFBP-5 transcripts are localized to epithelial cells (Wood et al., 2000). However, qRT-PCR data from our studies demonstrate that IGFPB transcripts are produced in stromal cells as well. Expression of IGFBP-4 and IGFBP-5 may be regulated by estrogen. For example, greater

abundance of IGFBP-4 and IGFBP-5 were reported in ER-positive breast cancer cells compared with ER-negative cells (McGuire et al., 1994). Local expression of IGFBP-6 was regulated by

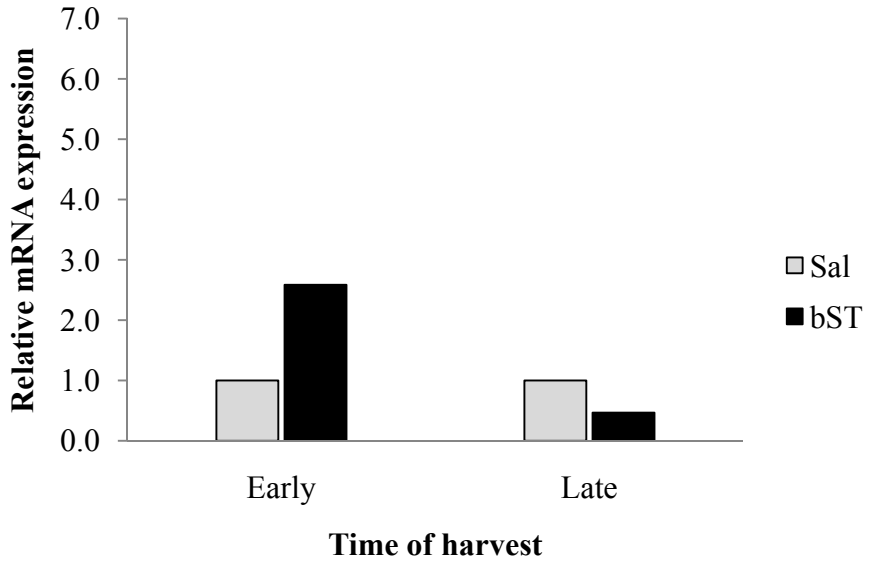


Figure 5. 5. Expression of IGFBP-6 mRNA in MFP

Relative expression ($2^{-\Delta\Delta Ct}$) of IGFBP-6 mRNA in MFP of bST treated from early and late harvest heifers relative to Sal controls. In MFP, bST increased the expression of IGFBP-6 in early harvest while there was a reduction in IGFBP-6 in bST heifers from the late harvest (treatment and time interaction; $P < 0.05$).

both ovarian secretions and bST. While ovariectomy reduced IGFBP-6 mRNA, bST had an age-dependent response. In younger animals bST increased IGFBP-6, but in older animals the response was opposite. Based on the measure of weak expression of IGFBP-2 and IGFBP-6, Plath-Gabler et al. (2001) concluded that these proteins do not have significant physiological roles and therefore they are not regulated in bovine mammary gland. On the contrary, we found synthesis of IGFBP-2 and -6 mRNA in PAR and MFP components of prepubertal mammary gland were regulated by hormones, in particular by ovarian secretions. Moreover, there is an estrogen responsive element in the IGFBP-6 gene which binds with the ER (Zhu et al., 1993).

Therefore, since ovariectomy decreased IGFBP-2 and IGFBP-6 mRNA, this suggests that IGFBP-2 and IGFBP-6 may be mediators of estrogen-induced mammary growth.

Quantitative RT-PCR being very sensitive allowed the detection of transcripts of all the components of IGF axis in prepubertal mammary gland, both in PAR and MFP. Endocrine regulation of gene expression in PAR is primarily under ovarian regulation but in MFP the predominant method of regulation is ontogeny rather than hormonal. Our data suggests that all the six IGFBP may be important in mammary growth and development because they are highly regulated in prepubertal mammary gland. However, further investigation is warranted to elucidate functional significance of each binding proteins.

CHAPTER 6: REGULATION OF TRANSCRIPT ABUNDANCE OF GROWTH HORMONE SIGNALING MOLECULES BY OVARIAN SECRETIONS AND EXOGENOUS BOVINE SOMATOTROPIN IN PREPUBERTAL BOVINE MAMMARY GLAND

6.1. Abstract

Growth hormone regulates insulin-like growth factor (**IGF**)-1 gene transcription through the mediation of intracellular signaling molecules called signal transducers and activators of transcription (**Stat**) 5. Another family of negative regulators called suppressors of cytokine signaling (**Socs**) are induced by GH and feed back to inhibit Stat-mediated GH-signaling. Our specific objectives were to determine the effects of ovariectomy and treatment with exogenous bovine somatotropin (**bST**) on the mRNA expression of GH receptor (**GHR**), Stat5a, Stat5b, Socs-2 and Socs-3 in bovine mammary gland. In the first study, prepubertal dairy heifers either at two, three or four months of age were randomly assigned to one of two treatments, ovariectomized (n = 8) or sham operated (n = 12), and mammary parenchyma (**PAR**) and fat pad (**MFP**) were harvested 30 d after surgery. In the second study, 18 heifers were randomly assigned to one of two treatments; bST (500 mg Posilac; n = 9) or Sal (0.9 % saline; n = 9) beginning on day 29 ± 4 and received treatments every 21 d. Mammary tissues were harvested either after the sixth or 12th wk of treatment period. Relative abundance of mRNA was determined by quantitative real-time PCR. Protein expression of Stat5 was determined in PAR and MFP by Western blot analysis. Main effects of treatment and age as well as their interaction were tested and statistical significance was declared at $P \leq 0.05$. Significant mRNA expression for GHR, Stat5a, Stat5b, Socs-2 and Socs-3 were detected in PAR and MFP of prepubertal mammary gland. Ovariectomy decreased GHR mRNA in PAR and increased Socs2 mRNA in MFP. Treatment with bST decreased mRNA expression of GHR, Stat5b and Socs2 in PAR but decreased Socs2 mRNA in MFP. Western blot analysis demonstrated expression of Stat5a/b which was not affected by bST treatment. However, we did not detect phosphorylated form of Stat5a/b in PAR or MFP. Our data suggest that GH signaling is regulated by ovarian secretions mediated through Socs2 and we hypothesize that an alternate pathway may exist in prepubertal mammary gland for regulation of the IGF-1 gene which is independent of GH action.

6.2. Introduction

Growth hormone (**GH**) brings about its effects on somatic growth, tissue proliferation and regeneration at least in part through the mediation of insulin-like growth factor (**IGF**)-1. When GH binds to the transmembrane GH receptor (**GHR**), an intracellular tyrosine protein kinase called Janus kinase (**JAK**)-2 gets activated. This leads to activation of a multitude of transcription factors including a group of proteins called signal transducers and activators of transcription (**Stat**) 1, 3, 5a and 5b (Rotwein et al., 1997). Involvement of Stat proteins in the regulation of GH-induced IGF-1 gene expression in liver was demonstrated earlier in mice knockout models (Udy et al., 1997; Davey et al., 2001). The subset of Stat protein which gets activated by GH stimulation varies based on species and tissue under investigation. GH stimulates phosphorylation of Stat1 in preadipocytes (Meyer et al., 1994), while Stat5b is a key component in acute GH-stimulated activation of IGF-1 gene in rat liver (Woelfle et al., 2003). Presence of Stat5a mRNA in the mammary gland of virgin sheep (Kazansky et al., 1995) and ablation of Stat5a expression in ovariectomized rats (Santos et al., 2008) were reported previously. Several negative regulatory pathways also act to terminate GH signaling. One of these involve signal inhibitory molecules called suppressors of cytokine signaling (**Socs**), which are immediate-early gene products that can be induced by various cytokines via Stat5-inducible transcriptional mechanism (Yoshimura, 1998). GH-induced Stat5-dependent transcriptional activity was inhibited by both Socs1 and Socs3 in transfected cells (Adams et al., 1998; Favre et al., 1999) while partial inhibition or no inhibition was seen by Socs2 and Socs6, respectively (Ram and Waxman, 1999). Furthermore, estrogen is found to be a negative regulator of GH signaling in human. Oral estrogen administration reduced serum IGF-1 even with elevated GH concentration (Weissberger et al., 1991) in women.

Activation of Stat5 in mammary gland is through prolactin and GH receptors (Gallego et al., 2001) and the expression of GHR in bovine mammary gland was reported early (Hauser et al., 1990; Jiang et al., 1999). Additionally, locally produced IGF-1 in bovine mammary gland during prepubertal period is known to be important in mammary development (Berry et al., 2003a; Berry et al., 2003c). However, to our knowledge there is no data on expression of Stat5 and Socs in prepubertal bovine mammary gland or information regarding regulation. We were

interested in finding whether these signaling molecules are expressed during prepubertal period and whether they are under hormonal regulation in bovine mammary gland. Therefore, the objectives of this study were to determine whether prepubertal bovine mammary gland produces Stat5a, Stat5b, Socs2 and Socs3 and also to determine whether they are regulated by ovarian secretions and exogenous bovine somatotropin (**bST**).

6.3. Materials and Methods

6.3.1. Animals and Treatments

In the first experiment to study the ovarian regulation of expression of molecules in the GH-signaling pathway, 20 prepubertal Holstein heifers were used. All animal care and use protocols used in the study were approved by the Clemson University Institutional Animal Care and Use Committee. Heifers were fed with commercial milk replacers and calf starter diets according to the manufacturer's instructions prior to weaning and fed with grains and hay thereafter. After a week's adaptation period at the facility, heifers were randomly assigned to either ovariectomy (**OVX**; n = 8) or sham operation (**INT**; n = 12) when they were two, three or four months of age. Surgeries were performed by laparoscopic vertical incision of about 10 cm on the left flank after desensitizing the area with local anesthetic (Lidocane HCl, 2% injectable solution). Thirty days after surgery animals were humanely sacrificed by captive bolt pistol stunning followed by exsanguination and mammary tissues were harvested immediately. See Chapter 3 for more information on experimental design.

The second experiment was conducted to study the effect of bST on the local expression of GH-signaling molecules in the bovine mammary gland. All protocols used in the study were in agreement with the animal use protocols approved by the Virginia Tech Institutional Animal Care Use Committee. Preweaned Holstein heifers were blocked by age and BW and at an average age of 29 ± 1 d heifers were randomly assigned to one of the treatment groups, bST or control group (Sal; n = 9). Heifers in the bST group (n = 9) received a sustained release formulation of 500 mg recombinant somatotropin (Posilac; Monsanto Co., St. Louis, MO) subcutaneously in the neck region every three week. Control heifers (n = 9) received 0.9 % saline solution. All the heifers also received calf starter (20 % CP and 1.4 % fat) and fresh water

free of choice. See Chapter 4 for further details on experimental design. Heifers were sacrificed and mammary tissue was harvested at two stages. The first set of heifers were sampled three week after the second injection (average age of 72 ± 1 d) and the second set of heifers were sampled three week after the fourth injection (average age of 116 ± 1 d). Heifers were humanely sacrificed using Phenobarbital injection i.v. (Euthasol, 10 mg/kg BW).

6.3.2. Sample Collection and Analyses

In both experiments, mammary parenchyma (**PAR**) and fat pad (**MFP**) were collected consistently from left hind quarter. Sample collection procedures are described previously in Chapter 2. Mammary parenchyma was bisected and PAR samples were collected randomly from the inner region. Samples of MFP were collected from regions distinctly away from parenchyma.

Gene expression by real-time PCR: Total RNA isolation and quantitative real time PCR methods as described in Chapter 2 were used to determine relative mRNA abundance. Relative gene expression of GHR, Stat5a, Stat5b, Socs2 and Socs3 were determined. For normalization of the cycle threshold (**Ct**) of target genes, the geometric mean of the Ct values of three endogenous control genes were used (Piantoni et al., 2008). Relative mRNA expression was determined as fold change ($2^{-\Delta\Delta Ct}$) in gene expression in treatment group relative to control group using the comparative Ct method (Chapter 2). The sequences of primer pairs for the target and endogenous reference genes are given in Table 6.1.

Stat5 protein expression by Western blot: Expression of both total and phosphorylated (p) Stat5a/b proteins were determined in PAR and MFP of Sal and bST treated heifers by Western blot analysis with modification of the protocol described earlier (Yang et al., 2000). Briefly, mammary PAR and MFP from Sal and bST heifers were collected from left hind quarter and 100 mg tissue was homogenized immediately in 700 µL lysis buffer (Appendix C.2) containing β-glycerophosphate and a protease inhibitor. Homogenization mixture was then centrifuged at 1000 x g for 10 min at 4^{o}C. The supernatant was then decanted and mixed with 2x Laemmli sample buffer (Bio-Rad Life Science Research, Hercules, CA) containing β - mercaptoethanol (Bio-Rad) in 1:1 proportion and mixed well in a vortex. Proteins were then denatured at 95^{o}C for 10 min and stored at -80^{o}C until further processing. Samples from -80^{o}C were thawed and total

amount of protein in each sample was determined by BCA method (Chapter 2). A total of 30 μg protein was aliquoted from each sample and then dried down to an equal volume of ~20 uL in a vacuum operated Speed Vac (RVT4104 Refrigerated Vapor Trap, Thermo Electron Corporation, Milford, MA), and separated on 7.5 % polyacrylamide gel (Appendix C.3) at 70 V overnight. Samples were run in duplicates. After the proteins were separated based on molecular weight, they were electro transferred on to polyvinylidene difluoride (PVDF) membrane (Immnobilon-P PVDF membrane, Millipore Corporation, Bedford, MA) which was soaked in transfer buffer for 30 min (Appendix C.3). The membrane was then incubated in 10 % non-fat dried milk (NFDM) for 50 min in room temperature for inhibiting non-specific antibody binding. The blots were first probed for phosphorylated form of Stat5 by overnight incubation at 4^{o}C with anti-phospho-Stat5a/b antibody (Tyr694/699, rabbit immunoaffinity purified IgG, Upstate Cell Signaling Solutions, Lake Placid, NY; 1: 100 dilution in 1 % NFDM). After multiple rinses and washes in TBST, blots were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Upstate Cell Signaling Solutions; 1:500 dilution in 1 % NFDM) for 1 h at room temperature. Bands were detected using chemiluminescence detection kit (ECL plus Western blotting detection kit, GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions followed by scanning and analyzing in a Chemidoc gel imaging system (Bio-Rad). Antibodies on the membrane were then stripped off using a harsh stripping protocol (Appendix B.4), incubated in blocking buffer (10 % NFDM) and re-probed for total Stat5a/b using 1 μg/ mL Stat5 (N-20) rabbit polyclonal antibody (Santa Cruz Biotechnology, CA) for 1 h at room temperature. Secondary antibody incubation and detection of the bands were performed in the same manner as for pStat5a/b detection. Along with test samples, equal amounts of a standard sample and a molecular weight marker (Precision plus protein standard, Bio-Rad, Catalogue # 161-0375) were also loaded in each gel. Whole cell lysate (20 μL) from CHO cells transfected with a Stat5 expression construct and a GHR expression construct (Wang and Jiang, 2005) and treated with GH for 30 min was used as a positive control for pStat5 analysis while non-transfected, untreated CHO cells (20 μL) served as negative control. Total and pStat5a/b were identified based on molecular weight and by comparing with positive control and were quantified by densitometry. To minimize the gel to gel variation, protein quantity was expressed in arbitrary units as a percentage of the standard used in each gel.

Table 6. 1. Sequences of primer pairs used for target and endogenous reference genes

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
GHR	CGTCTCTGCTGGTGAAAACA	AACGGGTGGATCTGGTTGTA
Stat5a	ACTGCTGTGTGATGGAGTAT	CATGAACGATGACAACCACG
Stat5b	TTTACCCGGACGGAATTACA	TAACTCAGGTCTCCCAAGCG
Socs2	TCGCATCGAATACCAAGATG	GTCCGCTTATCCTTGCACAT
Socs3	TCTCTAGAGTGGCCACTCTCCAACATCT	GAGAATTCGGCTGGATTCTTGTGCTTGT
PPP1R11	CCATCAAACTTCGGAAACGG	ACAGCAGCATTTTGATGAGCG
RPS15A	GAATGGTGCGCATGAATGTC	GACTTTGGAGCACGGCCTAA
MTG1	CTTGGAATCCGAGGAGCC A	CCTGGGATCACCAGAGCTGT

6.3.3. Statistical Analysis

Data were analyzed using Mixed procedure of SAS (SAS 9.2; Cary, NC). For gene expression data from ovariectomy experiment, main effects in the model were treatment, age at surgery and interaction between treatment and age. Similarly, for bST experiment, treatment, time of tissue harvest and interaction between treatment and time were tested as main effects. The random effect tested was heifer within treatment and age/time. Data distribution was analyzed using the "Proc Mixed Boxplot" statement (Appendix A.6). Significance was declared at $P \leq 0.05$ for all analyses.

The model used in all analyses was

$Y_{ijk} = \mu + T_i + A_j + (TA)_{ij} + e_{(ij)k}$ where

Y_{ijk} = dependent variable

μ = overall mean

T_i = fixed effect of treatment (INT or OVX/Sal or bST; i = 1, 2)

A_i = fixed effect of age at surgery/time of harvest (2 mo, 3 mo or 4 mo; j = 1,2,3/ early or late; j = 1,2)

$(TA)_{ij}$ = fixed interaction of treatment and age/time

$e_{(ij)k}$ = residual error

Gene expression data for PAR and MFP were analyzed separately for each experiment. The ΔCt data were used for statistical analyses and significance was determined based on the *P* values for ΔCt data. However, data was presented as fold change ($2^{-\Delta\Delta Ct}$) in gene expression relative to control animals using the comparative Ct method (Chapter 2).

Protein data from PAR and MFP were tested for main effects of tissue (PAR or MFP), treatment, time of harvest, interactions between main effects and three way interactions between tissue, treatment and time. None of the interactions were significant and therefore not included in the final model. Duplicate samples were run on different gels. Therefore, to minimize the gel to gel variation, an equal amount of a standard sample was also loaded in all the gels and arbitrary unit of protein expression in each sample was calculated as the percentage of the standard loaded in each gel.

6.4. Results

6.4.1. Effect of ovariectomy on transcript abundance

In PAR, mRNA expression of GHR was reduced in OVX relative to INT ($P = 0.054$), whereas there was no difference in the expression of Stat5a, Stat5b, Socs-2 and Socs-3 between OVX and INT (Fig 6.1). Parenchymal gene expression was not different between different age groups and there was no interaction between treatment and age at surgery. In MFP, only Socs-2 mRNA expression was affected by ovariectomy while all other genes remained unaffected (Fig 6.2). Expression of Socs-2 was increased in OVX relative to INT ($P < 0.05$). Unlike PAR, mRNA expression of GHR and Socs-2 genes were under ontogenic regulation in MFP. Both GHR and Socs-2 mRNA in MFP were down regulated as the calves aged ($P < 0.05$; Fig 6.2).

6.4.2. Effect of bST treatment on transcript abundance

Relative abundance of GHR, Stat5b and Socs-2 mRNA in PAR were reduced in bST relative to Sal ($P \leq 0.05$) while Stat5a and Socs-3 in PAR were not affected by bST treatment (Fig 6.3). Expression of Socs-2 mRNA in MFP was increased in bST ($P < 0.01$) whereas GHR, Stat5a, Stat5b and Socs-3 were not affected by bST treatment (Fig 6.4). In addition, GHR mRNA in PAR was down regulated in older ($P \leq 0.05$) compared with younger heifers. Transcript abundance of other signaling molecules was not affected by age of the animals and there was no interaction between treatment and time of harvest in PAR and MFP.

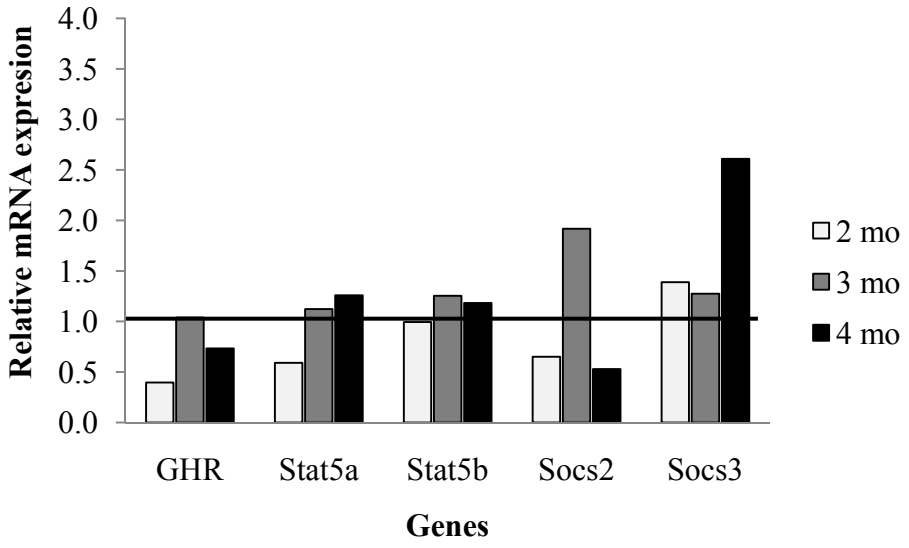


Figure 6. 1. Effect of ovariectomy on transcript abundance of signaling molecules in parenchyma

Relative mRNA abundance ($2^{-\Delta\Delta Ct}$) of signaling molecules in PAR of Holstein heifers ovariectomized (OVX; n = 8) either at 2, 3 or 4 mo of age relative to sham operated (INT; n = 12) controls. The horizontal line at 1.0 on Y-axis represents gene expression in INT. Local expression of GHR mRNA in PAR was reduced in OVX relative to INT ($P = 0.054$) heifers. However, there was no difference in the expression of Stat5a, Stat5b, Socs2 and Socs3 in PAR between OVX and INT.

6.4.3. Effect of bST on expression of Stat5 protein

Western blot analysis detected Stat5a/b proteins of ~92 kDa size in both PAR and MFP tissue fractions, but the phosphorylated form of Stat5a/b was not detectable in either PAR or MFP of Sal and bST heifers (Fig 6.5A). There was an increase in the expression of Stat5a/b in PAR compared with MFP ($P < 0.01$; Fig 6.5B). However, expression of Stat5a/b was not affected by treatment with bST or time of harvest and there was no interaction effect between treatment, time and/or tissue.

6.5. Discussion

Substantial levels of mRNA of GHR, Stat5a, Stat5b, Socs-2 and Socs-3 were measured in PAR and MFP from prepubertal mammary gland. Growth hormone receptor is expressed in both stromal and epithelial cells in bovine mammary gland during prenatal and postnatal life (Hauser et al., 1990; Knabel et al., 1998; Jiang et al., 1999; Plath-Gabler et al., 2001). The interaction between ovary and local IGF-1 axis in mammary gland typically synergized to produce optimization of IGF-1 effects in stimulating mammary gland development in heifers (Berry et al., 2003c). We observed a reduction in GHR mRNA expression in PAR as a result of ovariectomy. Supporting our data, estrogen increased GHR mRNA expression in rat liver (Schwartzbauer and Menon., 1998). Later it was reviewed that estrogen up regulated GHR transcription in rat liver and that the effect was GH-dependent (Leung et al., 2004). However, general regulation of GHR by GH is highly variable because the effect depends on the duration of treatment, the species, and the cell type evaluated. For example, there was an increase in hepatic GHR mRNA and protein in cattle when bST was administered for one week period (Jiang et al., 2007). However, chronic GH deficiency reduced GHR in sheep and rabbit liver (Posner et al., 1980) while GH treatment down regulated GHR expression in fibroblasts (Murphy and Lazarus, 1984). In our study treatment with bST in prepubertal heifers for six and 12 wk reduced GHR mRNA expression in mammary PAR, suggesting that the reduction in GHR mRNA in the mammary PAR in response to bST was because of the intralobular stromal cells rather than the mammary epithelial cells. Moreover, treatment with exogenous GH in swine resulted in an increase in GHR mRNA in liver while GHR expression was not affected in

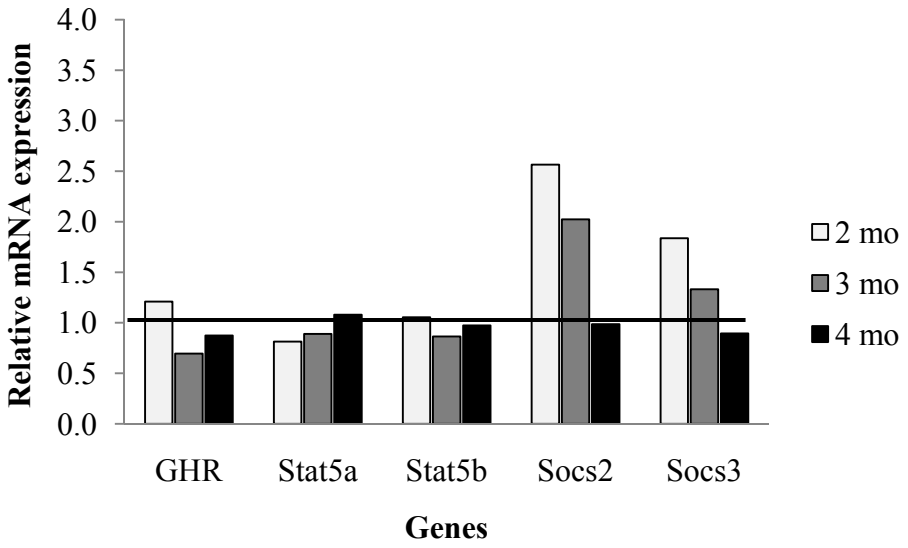


Figure 6. 2. Effect of ovariectomy on transcript abundance of signaling molecules in mammary fat pad

Relative mRNA abundance ($2^{-\Delta\Delta Ct}$) of signaling molecules in MFP of Holstein heifers ovariectomized (OVX; n = 8) either at 2, 3 or 4 mo of age relative to sham operated (INT; n = 12) controls. The horizontal line at 1.0 on Y-axis represents gene expression in INT. Local expression of Socs2 mRNA in MFP was up regulated in OVX relative to INT ($P < 0.05$) while none of the other signaling molecules were affected by ovariectomy. Relative mRNA expressions of GHR and Socs2 were reduced with age ($P < 0.05$).

longissimus dorsi muscle (Xu et al., 2003), demonstrating differential regulation of GHR by GH in different tissues within the same animal.

Very little is known about the regulation of intracellular mediators of GH signaling in prepubertal bovine mammary gland. Among the different Stat proteins, Stat5 which was formerly known as mammary gland factor, is most studied in bovine mammary gland and found particularly important during lactation. Transcript abundance of Stat5a in the mammary gland of prepubertal heifers was affected neither by ovariectomy nor bST. Expression of Stat5a mRNA was detected in mammary gland from virgin and pregnant sheep, but transcript abundance was

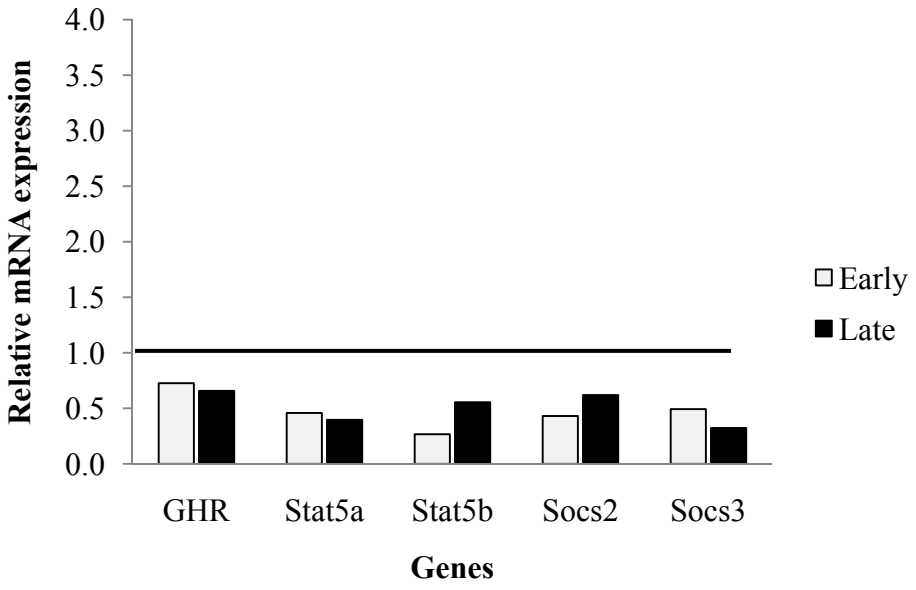


Figure 6. 3. Effect of bST on transcript abundance of signaling molecules in parenchyma

Relative mRNA abundance ($2^{-\Delta\Delta Ct}$) of signaling molecules in PAR of Holstein heifers treated with bST (n = 9) and tissues harvested either early or late time points relative to their Sal controls (n = 9). The horizontal line at 1.0 on Y-axis represents gene expression in Sal. Local expressions of GHR, Stat5b and Socs2 in PAR were reduced in bST treated heifers relative to Sal ($P < 0.05$). Additionally, there was a decrease in GHR mRNA in late harvest compared with early harvest time ($P < 0.05$).

increased during pregnancy and was regulated by prolactin (Kazansky et al., 1995). Additionally, Stat5 DNA-binding activity and phosphorylation status was greatest during late pregnancy in both murine and bovine mammary gland (Wheeler et al., 2001) suggesting a role of Stat5 in terminal differentiation of mammary epithelial cells. Whole mount analysis of mammary gland showed that ductal development in puberty was not affected in Stat5-null mice whereas alveolar development during pregnancy was severely impaired (Miyoshi et al., 2001). However, contrasting our observations in prepubertal heifers, ovariectomy ablated Stat5a expression in 18 wk old virgin mice and treatment with estrogen and progesterone restored Stat5a expression (Santos et al., 2008), suggesting that ovarian regulation of Stat5a is prominent in murine mammary development. Collectively, these observations imply that Stat5a is constitutively expressed but not functionally regulated in prepubertal bovine mammary gland.

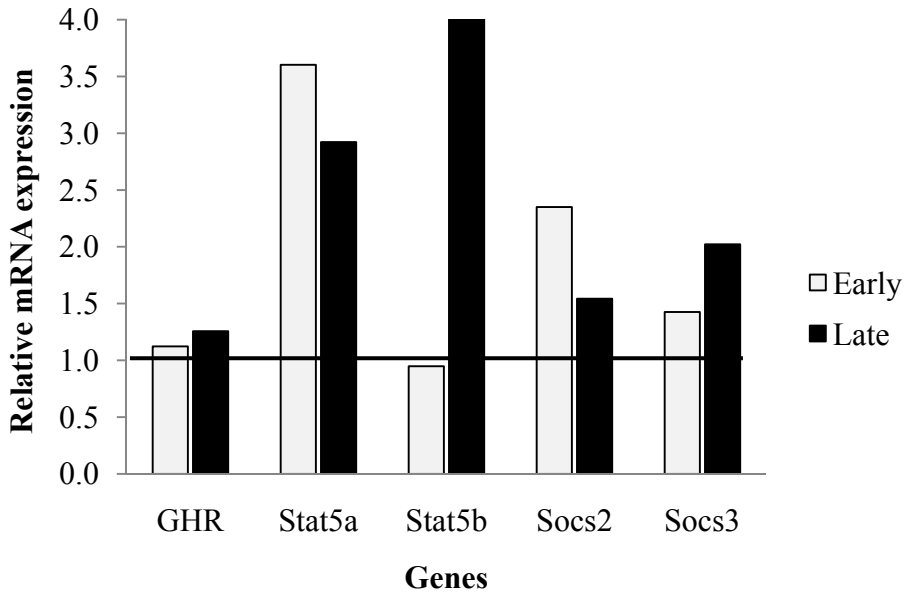


Figure 6. 4. Effect of bST on transcript abundance of signaling molecules in mammary fat pad

Relative mRNA abundance ($2^{-\Delta\Delta Ct}$) of signaling molecules in MFP of Holstein heifers treated with bST (n = 9) and tissues harvested either early or late time points relative to their Sal controls (n = 9). The horizontal line at 1.0 on the Y-axis represents gene expression in Sal. Local expression of Socs2 mRNA in MFP was increased by bST treatment ($P < 0.01$) whereas none of the other genes were affected by treatment with bST.

Administration of GH in Stat5b-null mice failed to increase the circulating concentration of IGF-1 as well as liver synthesis of IGF-1 mRNA (Davey et al., 2001). Therefore, Stat5b is an inevitable factor in GH-induced IGF-1 mRNA gene transcription. In vitro studies using MAC-T cells showed that GH can induce the expression of milk protein genes through stimulation of GHR leading to activation of Stat5b, but GH did not alter cell proliferation (Zhou et al., 2008). Data from Zhou et al. (2008) emphasize that GH-signaling pathway in mammary cell is dissociated from cell proliferation pathway. Therefore, there is a possibility that proliferation in mammary epithelial cells could be mediated through GH-independent IGF-1 gene transcription. Unlike the in vitro stimulation of Stat5b by GH in Zhou et al. (2008), we found lower mRNA expression of Stat5b in PAR of bST treated heifers. The reason for this discrepancy is not clear

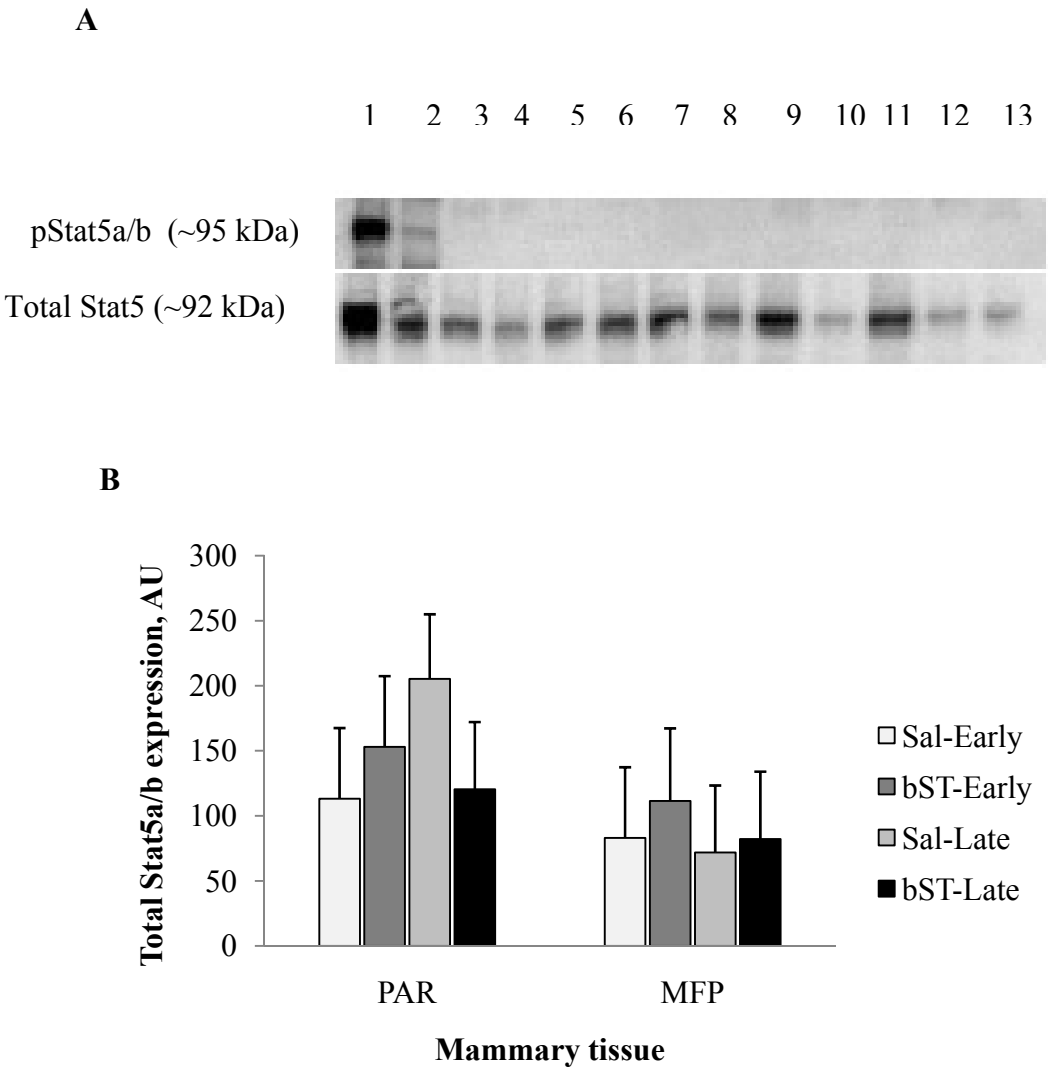


Figure 6. 5. Effect of bST on Stat5a/b protein in mammary parenchyma and fat pad

Expression of Stat5 proteins in PAR and MFP of heifers treated with bST (n = 9) and tissues harvested either early or late time points relative to their Sal controls (n = 9). (A) Western blots showing expression of total and phosphorylated (p) forms of Stat5 in positive control for p-Stat5A/B (lane 1), negative control for p-Stat5A/B (lane 2), PAR (lanes 3, 9 and 11) and MFP (lanes 4, 10 and 12) from Sal, PAR (lanes 5 and 7) and MFP (lanes 6 and 8) from bST treated heifers, and equal amount of standard used in all gels (lane 13). (B) Total Stat5 expression was greater in PAR compared with MFP, but there was not effect due to bST treatment on the expression of total Stat5 in PAR and MFP. Data are presented as LSM and bars represent SEM.

but could relate to differences between in vitro and in vivo responses. Western blot analysis demonstrated expression of total Stat5a/b, but we did not see bST induced phosphorylation of Stat5. Like in Yang et al. (2000), there was no doublet formation for total Stat5a/b in all samples. Densitometry measurements for total Stat5a/b were not affected by bST treatment which is in agreement with Yang et al. (2000), suggesting that Stat5a/b protein synthesis in bovine mammary gland is not affected by GH. However, treatment with GH increased the expression of both protein and mRNA of Stat5 in lactating goat mammary gland (Boutinaud and Jammes, 2004). Mammary explant cultures showed that Stat5 activity was sensitive to high level of GH (50 ng/mL) but low level (5 ng/mL) of GH did not induce Stat5 activity in bovine mammary gland (Yang et al., 2000). In our study, tissues were harvested three weeks after the administration of the sustained release formulation of bST. Therefore, the amount of GH available to the mammary gland at the local level at the time of sampling may not be sufficient to induce phosphorylation of Stat5a/b. Although we did not find pStat5a/b protein expression by Western blot analysis, an unknown high molecular weight (~200 - 250 kDa) protein was detected (data not shown) which suggest possible association of Stat5 with other signaling molecules. The phosphorylated form of GHR provides docking sites for Stat5 and once Stat5 bind to GHR it becomes the substrate for tyrosine kinases (Desrivieres et al., 2006).

Different members of Stat and Socs protein families get activated by GH stimulation depending on species and tissue under investigation. Different members of the Socs family differ in their ability to inhibit GH-signaling pathway. In vitro and in vivo studies in rodent liver showed that Socs1 and Socs3 are potent inhibitors of GH-signaling whereas Socs2 is a weaker inhibitor when expressed at the same rate (Ram and Waxman, 1999). Nevertheless, our data indicate that Socs2 is a major component in the negative regulation of GH-signaling compared with Socs3 in bovine mammary gland because Socs2 was altered by ovarian secretions and bST but Socs3 was not. In vitro studies in human hepatic carcinoma cell lines also showed that Socs2 is a major negative regulator of GH signaling (Niwa et al., 2005). Ovariectomy and treatment with bST down regulated the expression of GHR in PAR while both treatments increased the transcript abundance of Socs2 in MFP. This surprising similarity in the expression pattern of GHR and Socs2 in the absence of ovarian secretions as well as abundance of bST is intriguing,

but poorly understood. One possibility is for a differential mechanism of regulation of GH-signaling in PAR versus MFP because the mechanism of regulation of GH-signaling is known to vary depending on the type of cells, functional status of the cell as well as the species involved. Orally administered estrogen in women inhibited GH signaling resulting in reduced serum concentration of IGF-1 (Weissberger et al., 1991). This inhibitory effect of estrogen on GH action is believed to be mediated through an increased expression of Socs2 as indicated in human cell lines expressing GHR (Leung et al., 2003). Contrary to their observation, absence of ovarian secretions in heifers increased Socs2 in MFP and this corresponded with reduced IGF-1 mRNA in mammary parenchyma (Chapter 5). Therefore, it is possible that mechanisms of estrogen regulation of GH-signaling differ greatly between tissues and across different species. Furthermore, while ovariectomy reduced transcript abundance of IGF-1, bST did not have an effect on IGF-1 mRNA (Chapter 5). Induction of IGF-1 mRNA in liver was blocked in rats overexpressing a negative dominant mutant of Stat5b, whereas IGF-1 mRNA expression was increased in animals overexpressing a constitutively active Stat5b mutant, even in the absence of GH (Woelfle et al., 2003). Taken together our data suggest that ovarian secretions regulate GH signaling in bovine mammary gland through Socs2 and that multiple pathways involving Stats and Socs likely exist to impact GH signal through mechanisms other than the IGF-1 gene transcription. Our data also suggest a plausible alternate pathway for IGF-1 gene transcription in bovine mammary gland which is independent of GH action.

CHAPTER 7: GENE EXPRESSION PROFILE IN PREPUBERTAL BOVINE MAMMARY PARENCHYMA IN RESPONSE TO OVARIECTOMY: A COMPARISON BETWEEN PARENCHYMAL TISSUE HOMOGENATE AND MAMMARY EPITHELIAL CELLS ISOLATED BY LASER MICRODISSECTION

7.1. Abstract

Mammary parenchyma (**PAR**) is a complex tissue consisting of mammary epithelial cells (**MEC**) surrounded by intra lobular stroma composed of fibroblasts, adipocytes and blood vessels. Laser assisted microdissection and capturing have made isolation of even single cells from complex tissues possible. We have seen previously that ovariectomy impacted transcript abundance in PAR. We hypothesized that the effect of ovariectomy on transcript abundance in PAR is mainly due to the stromal cells present in the PAR rather than a direct effect on the MEC. Therefore, the objective of this study was to compare the expression profile of selected genes associated with cell signaling and proliferation as well as steroid receptors in PAR homogenates compared with MEC in response to ovariectomy. Prepubertal dairy heifers were randomly assigned to one of two treatments, ovariectomized (n = 7) or sham operated (n = 12) and tissues were harvested 30 d after surgery. Parenchymal samples were snap frozen in liquid nitrogen and total RNA was isolated from tissue homogenates. Samples of MEC were prepared from cryosections of parenchymal tissues using laser assisted microdissection and capture followed by total RNA isolation. Data were analyzed using Mixed procedure of SAS and significance was declared at $P \leq 0.05$. Coupling two precise technologies like laser capture microdissection and quantitative real-time PCR enabled measurement of transcript abundance in MEC even at a very low level. Ovariectomy reduced the mRNA abundance of insulin-like growth factor-1, progesterone receptor and proliferating cell nuclear antigen, but the abundance of insulin-like growth factor-1 receptor mRNA was increased. However, gene expression was not affected by ovariectomy in MEC except for a reduction in estrogen receptor-α mRNA. Our data strongly indicate a disparity in the response to ovariectomy between MEC and PAR tissue and suggest that ovariectomy very strongly impacted the non-epithelial cells in the PAR rather than the MEC themselves.

7.2. Introduction

Much of our knowledge of cell characteristics and cell behavior under specific conditions is based on cell culture studies. However, it is true for almost all cell types that in vitro observations are not always directly applicable to what is happening in vivo. There are two main reasons for this discrepancy. First of all, the complex interactions between extracellular components and the cells under study are usually poorly replicated in cell culture. Secondly, that most of the cell cultures involves a single cell type and not the array of cells present in tissues. Advent of laser assisted microdissection (**LMD**) of histological preparations in 1990s has enabled precise separation of even single cells from tissues consisting of complex cell types. This powerful tool provides a multitude of research possibilities, specifically in the areas of genetics and proteomics studies. The use of LMD is particularly useful in understanding the molecular basis of tumors because most of the tumors are a heterogeneous mixture of neoplastic and non-neoplastic cells (Becker et al., 1997). A combination of LMD along with quantitative real time polymerase chain reaction (**qRT-PCR**) is widely used to determine transcript abundance in single cell types. A major drawback in using these methods is the poor quality of isolated RNA. Methods of tissue preservation and storage, duration of exposure of tissue to room temperature while processing, quality of solutions and reagents used as well as the method of RNA isolation are very critical in maintaining the integrity of RNA (Saal et al., 2003).

Prepubertal mammary parenchyma is a heterogeneous tissue consisting of ductular epithelium surrounded by intra lobular stroma composed of fibroblasts, adipocytes and blood vessels. It is well known that ovary is an integral regulator of prepubertal mammary development and that the effects of estrogen are mediated through stromal cells (Haslam, 1988; Hovey et al., 1998). This is supported by the finding that isolated mammary epithelial cells in culture did not respond to estrogen (Woodward et al., 1994). Therefore, the interaction between stromal and epithelial cells is important in endocrine control of mammary epithelial cell proliferation. We have seen in previous chapters that ovariectomy impacted transcript abundance of proliferation markers, insulin-like growth factor (**IGF**)-axis and growth hormone (**GH**)-signaling molecules in mammary parenchyma (Chapters 3, 5 and 6). However, the magnitude of gene expression responses to estrogen and ovarian status were recently shown to be greater in mammary fat pad

compared with parenchyma (Meyer et al., 2006a). Furthermore, histology data from our previous experiment showed that the percent area of epithelium present in mammary parenchyma was not affected by ovariectomy (Chapter 3). Nevertheless, the area occupied by epithelium per unit area of parenchyma was only 13-15 %, indicating that tissue homogenates prepared from parenchymal samples were largely composed of cells other than mammary epithelial cells. Therefore, we hypothesized that the response to ovariectomy in the transcript abundance in mammary parenchyma (**PAR**) samples is mainly from the intra lobular stromal cells, rather than the mammary epithelial cells (**MEC**). The objective of the study was to compare the gene expression profile between the PAR homogenate and MEC in response to ovariectomy employing the techniques of LMD and qRT-PCR.

7.3. Materials and Methods

7.3.1. Animals and Treatments

All animal care and use protocols used in the study were approved by the Clemson University Institutional Animal Care and Use Committee. Mammary PAR samples used in this study were collected from the previous ovariectomy experiment using prepubertal Holstein heifers as described in Chapter 3. Briefly, 24 Holstein heifers housed in the Simpson Experiment Station at Clemson University were fed with commercial milk replacers and calf starter diets according to the manufacturer's instructions prior to weaning and fed grains and hay thereafter. After a week's adaptation period at the facility, heifers were randomly assigned to either ovariectomy (**OVX**; n = 12) or sham operation (**INT**; n = 12) either at 2, 3 or 4 mo of age. Animals of different age groups were acquired in batches and hence surgery was performed at different time points. In OVX heifers ovaries were removed surgically while in INT heifers surgery was performed, but ovaries were kept intact. Mammary tissues were harvested 30 d after surgery by humanely sacrificing the heifers using captive bolt pistol stunning and exsanguination. We used only 19 animals (INT, n = 12 and OVX, n = 7) for comparative gene expression analysis because of inadequate amount of tissue available for some animals.

7.3.2. Sample Collection and Analyses

Mammary parenchymal samples were collected consistently from the inner parenchymal region of the left hind quarter for PAR homogenate preparation and from the left front quarter for MEC samples. Sample collection method for gene expression analysis in general is explained previously in Chapter 2. For preparing tissue homogenate, PAR samples were immediately frozen by dipping tissue pieces into liquid nitrogen, and observing precautions to minimize contamination with RNase. For preparing MEC samples, parenchymal tissue pieces were first placed in plastic molds containing a tissue embedding compound that ensure optimal cutting temperature (**OCT**) (Sakura Finetek U.S.A. Inc.; Torrence, CA) and then the tissues were also frozen in liquid nitrogen. Both samples were stored at -80^{o}C until further processing. Isolation of total RNA from PAR tissue homogenate was performed as described in Chapter 2.

Cryo-preserved samples in OCT blocks were prepared for LMD following protocols described earlier with modifications (Becker et al., 1997; Saal et al., 2003). Briefly, seven μm thick cryosections were made from OCT blocks and placed on polyethylene naphthalate membrane coated glass slides (Molecular Devices, Sunnyvale, CA). Slides were previously irradiated in a UV cross linker (FB UVXL-1000, Fisher Scientific, Pittsburg, PA) for 30 min at maximum power to ensure cross linking of RNA onto the membrane. Slides were kept on dry ice while sectioning and stored at -80^{o}C until LMD. Cryosections were thawed for less than 30 s and subjected to a quick staining procedure. Tissue sections were first rehydrated in 70 % ethanol followed by a rinse in RNase-free water for 30 s each. Sections were then treated with 1 % Toluidine blue stain (Fisher Scientific) containing 0.1 U/ μL of RNase-inhibitor (Qiagen Inc., Valencia, CA) for 30 s followed by washing in RNase-free water and dehydration in 80, 90 and100 % ethanol solutions, 30 s each. Tissue sections were made completely moisture free by air drying. Mammary epithelial cells were identified and the selected areas were precisely excised (Fig 7.1) using a UV-laser which was coupled through the illumination path of the microscope (PALM micro beam system, Carl Zeiss Micro Imaging, Inc.; Thornwood, NY). The excised tissue zones were then catapulted against gravity into collecting tube caps containing 40 μL of lysis buffer (RNeasy micro kit; Qiagen Inc.). The membrane coating of slides facilitated the laser pressure catapulting procedure. The cells were then lysed immediately by vortexing in

350 µL lysis buffer. The laser microdissection protocol is described in detail in Appendix B.2. On an average 85 to 90 epithelial zones depending on size were collected per heifer. Once the slides were taken out from -80^{o}C, the procedure to the cell lysis step was completed within 60 – 65 min. All procedures including cryosectioning, LMD as well as RNA isolation and purification were performed under RNase-free environment and all the equipment, materials and solutions were made RNase-free using RNase Zap (Ambion Inc.; Austin, TX), following the manufacturer's instructions. Solutions of ethanol and staining materials were made in RNase-free water.

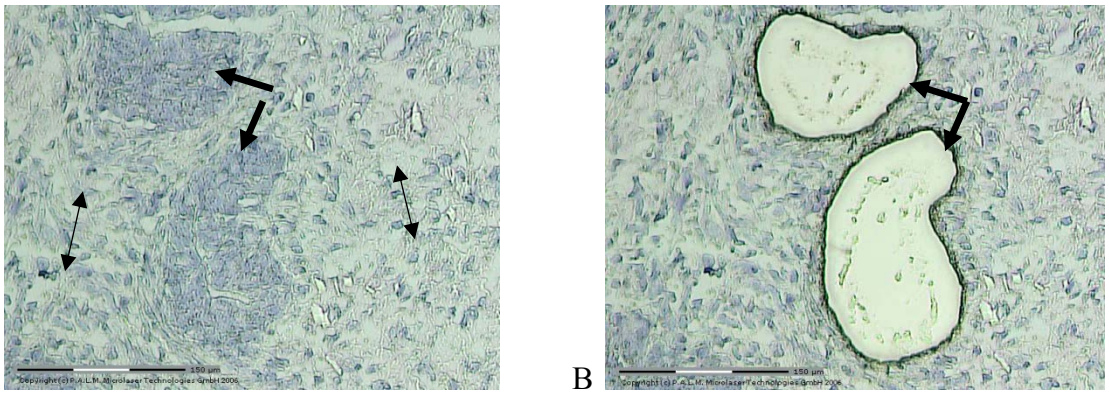


Figure 7. 1. Representative figures of mammary parenchymal tissues before and after microdissection and catapulting.

Mammary PAR tissue sections stained with toluidine blue before (A) and after (B) laser assisted microdissection and capture by catapulting. Thick arrows show the epithelial structures that were dissected and collected for RNA isolation for MEC samples and thin double headed arrows show the intralobular stroma. Tissue homogenates of PAR used for gene expression analysis consisted of a heterogeneous mixture of cell types as seen in panel A whereas in microdissected samples consisted of a homogenous cell lysate from the epithelium.

Total RNA from MEC was isolated and purified using RNeasy Micro Kit (Qiagen Inc.) according to the manufacturer's instructions (Appendix B.3.). Quantity and quality of RNA were determined in a 2100 series Bioanalyzer from Agilent (Quantum Analytics, Inc, Foster City, CA) using pico-chips. The quality of isolated RNA was compared for different processing conditions to ensure that the solutions and procedures used in LMD and RNA isolation processes do not

affect the quality of RNA in the sample. Different conditions tested were 1) sections stained with either 0.1 % or 2) 1.0 % Toluidine blue, 3) sections stained with 1% toluidine blue containing RNase inhibitor (Qiagen Inc.), and 4) sections treated with alcohol solutions but not stained to test the quality of alcohol as well as water used for staining. The quality of RNA isolated from

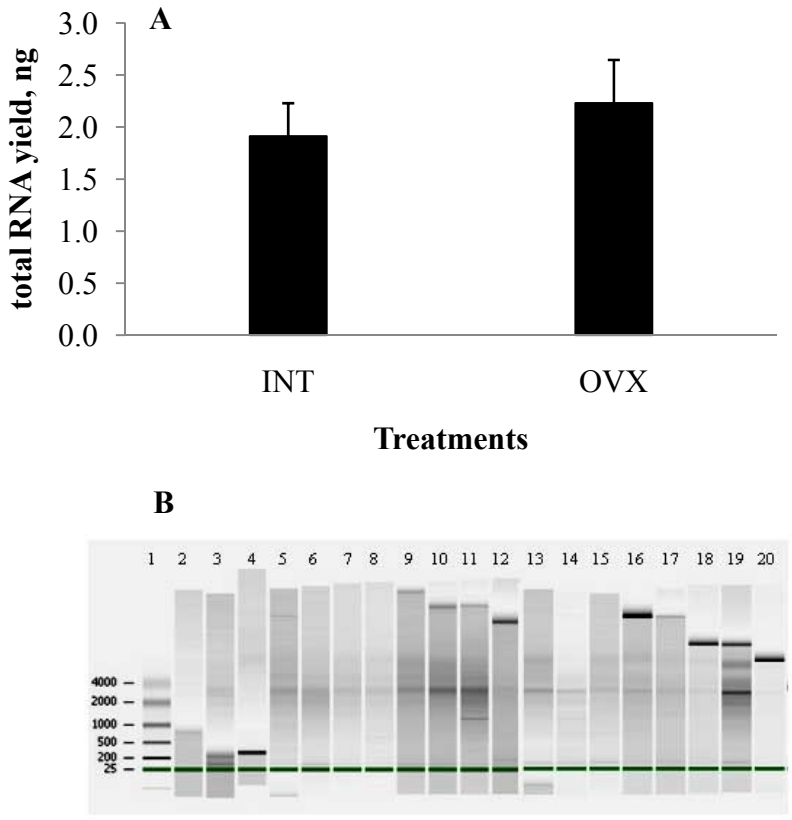


Figure 7. 2. Quantity and quality of total RNA isolated from microdissected mammary epithelial cells.

Total yield (A) and gel picture (B) of RNA isolated from microdissected mammary epithelial cells from sham operated (INT; n = 12) and ovariectomized (OVX; n = 7) Holstein heifers. Total yield of RNA was not different between INT and OVX ($P = 0.549$). Data are presented as LSM and bars represent SEM.

all these test samples were not different from each other suggesting that any of the steps involved in LMD procedure is not significantly affecting the quality of RNA. We preferred 1 % Toluidine blue over 0.1 % because we could obtain better staining in lesser time. Total yield of RNA from

microdissected samples ranged from 0.84 – 4.04 ng and were not different between INT and OVX ($P = 0.549$; Fig 7.2). From each sample a total of 0.4 ng RNA was used to make single stranded cDNA in a 20 µL reaction volume using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. A control sample containing no reverse transcriptase enzyme was used for each sample and this was later used in qRT-PCR as controls. A test run of qRT- PCR for different genes was performed with different concentrations of cDNA (stock and diluted) to determine the lowest amount of cDNA that can be used to produce good amplification. The Ct values of 1:10 diluted cDNA samples were in acceptable range and differed by two cycles from the stock Ct values (Appendix B.4). In addition, the amount of stock cDNA was limited to perform qRT- PCR analysis for several target genes selected. Therefore, 10 fold dilution of cDNA was used for qRT-PCR. A total of 4 pg cDNA was used in each reaction, along with 12.5 µl of SYBR Green dye (Applied Biosystems; Foster City, CA), 9.5 µl of sterile distilled water, 0.5 µl of 10 µM each of forward and reverse primers. The PCR conditions were: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min and ran for 40 cycles in 7300 Series Real-Time System (Applied Biosystems).

Relative mRNA abundances of growth hormone receptor (**GHR**), IGF-1, IGF-IR, IGF binding protein (**BP**)-3, estrogen receptor (**ER**)-α, progesterone receptor (**PR**), proliferating cell nuclear antigen (**PCNA**), signal transducers and activators of transcription (**Stat**)-5b, and suppressors of cytokine signaling (**Socs**)-2 were determined in both PAR and MEC for OVX relative to INT heifers by comparative Ct ($2^{-\Delta\Delta Ct}$) method after normalizing the Ct values to the geometric mean of three endogenous control genes as described in Chapter 2. Primer sequences of target and endogenous reference genes are given in Table 7.1.

7.3.3. Statistical Analysis

Relative abundance of mRNA in PAR homogenates was not different between different age groups and there was no interaction between treatment and age (Chapters 3, 5 and 7). Additionally, the number of samples available in the 2 mo surgery group was very limited for MEC samples due to limited tissue resources. Therefore, for statistical analysis of comparative gene expression between the two types of sample preparations (PAR and MEC) data were pooled within treatments and only treatment effect was tested. All data were analyzed using Mixed

procedure of SAS (SAS 9.2; Cary, NC). Random effect error was heifer within treatment. Data distribution was analyzed using the "Proc Mixed Boxplot" statement (Appendix A.6). Significance was declared at $P \leq 0.05$ for all analyses.

The model used in all analyses was

$Y_{ij} = \mu + T_i + e_{(i)j}$ where

Y_{ij} = variable being tested

μ = overall mean

T_i = fixed effect of treatment (INT or OVX) (i = 1, 2)

$e_{(i)j}$ = residual error

Gene expression data for PAR and MEC were analyzed separately. The ΔCt data were used for statistical analyses and significance was determined based on the *P* values for ΔCt data. However, data was presented as fold change in gene expression for OVX relative to INT control using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Chapter 2).

7.4. Results and Discussion

Relative abundance of individual mRNA species present in MEC samples were very low compared with that of PAR homogenate as indicated by greater Ct values for MEC samples owing to the very low amount of cDNA used in the qRT-PCR reactions. For example, in both sample types ERα mRNA was more abundant and Socs2 mRNA was less abundant. The Ct values of ERα mRNA ranged from 16 – 19 in PAR and 28 – 31 in MEC while Ct values for Socs2 ranged from 19 – 23 and 32 – 34 in PAR and MEC, respectively (Fig 7.3). It should be noted, nonetheless, that there was a moderate degree of disintegration in the RNA isolated from MEC samples for both OVX and INT samples. However, we succeeded in obtaining a sufficient amplification for all the mRNA species evaluated and no-RT template controls had undetectable amounts in all the samples tested. Dissociation curves for each target gene had the same single peak in PAR and MEC samples (Fig 7.4) indicating amplification of the same single product in both sample types. Additionally, there was no difference in the yield of total RNA between treatments. Therefore, we made the assumption that low integrity of RNA did not hamper our

Table 7. 1. Primer pair sequences used in real-time PCR assays

Gene	Forward (5'-3')	Reverse (5'-3')	Target/Endo[1]
GHR	CGTCTCTGCTGGTGAAAACA	AACGGGTGGATCTGGTTGTA	Target
IGF-1	GTTGGTGGATGCTCTCCAGT	CTCCAGCCTCCTCAGATCAC	Target
IGF-1R	TCAAGGACGGAGTCTTCACC	GCTCAAACAGCATGTCAGGA	Target
IGFBP-3	CAGAGCACAGACACCCAGAA	TGCCCCGTACTTATCCACACA	Target
ERα	TTGCTGGCTACTTCGTCTC	GGTGGATGTGGTCCTTCTC	Target
PR	CAGTGGTCAAGTGGTCTAAATC	TCTCCATCCTAGTCCAAATACC	Target
PCNA	TCGTCTCAGGCGTTCATAGTC	AACATGGTGGCGGAGTCG	Target
Stat5b	TTTACCCGGACGGAATTACA	TAACTCAGGTCTCCCAAGCG	Target
Socs2	TCGCATCGAATACCAAGATG	GTCCGCTTATCCTTGCACAT	Target
PPP1R11	CCATCAAACTTCGGAAACGG	ACAGCAGCATTTTGATGAGCG	Endo
RPS15A	GAATGGTGCGCATGAATGTC	GACTTTGGAGCACGGCCTAA	Endo
MTG1	CTTGGAATCCGAGGAGCCA	CCTGGGATCACCAGAGCTGT	Endo

[1]Whether the gene is treated as a target gene or endogenous control gene

ability to evaluate the relative gene expression between treatments using the highly sensitive qRT-PCR methodology. Quantitative determination of transcript abundance even in tissue extracts containing partially fragmented RNA is possible by qRT-PCR because this technique enables amplification of targets of very small size (Gibson et al., 1996; Heid et al., 1996). According to literature, transcript level of mRNA species with half-lives of 2-10 h were detected from RNase-rich liver homogenates using TaqMan qRT-PCR (Specht et al., 2001), indicating that even though RNA undergo degradation, the resulting fragments are still large enough to be detected by qRT-PCR. However, in our study we used custom designed primers in SYBR Green based PCR reactions which are somewhat less specific in its amplification ability compared with TaqMan.

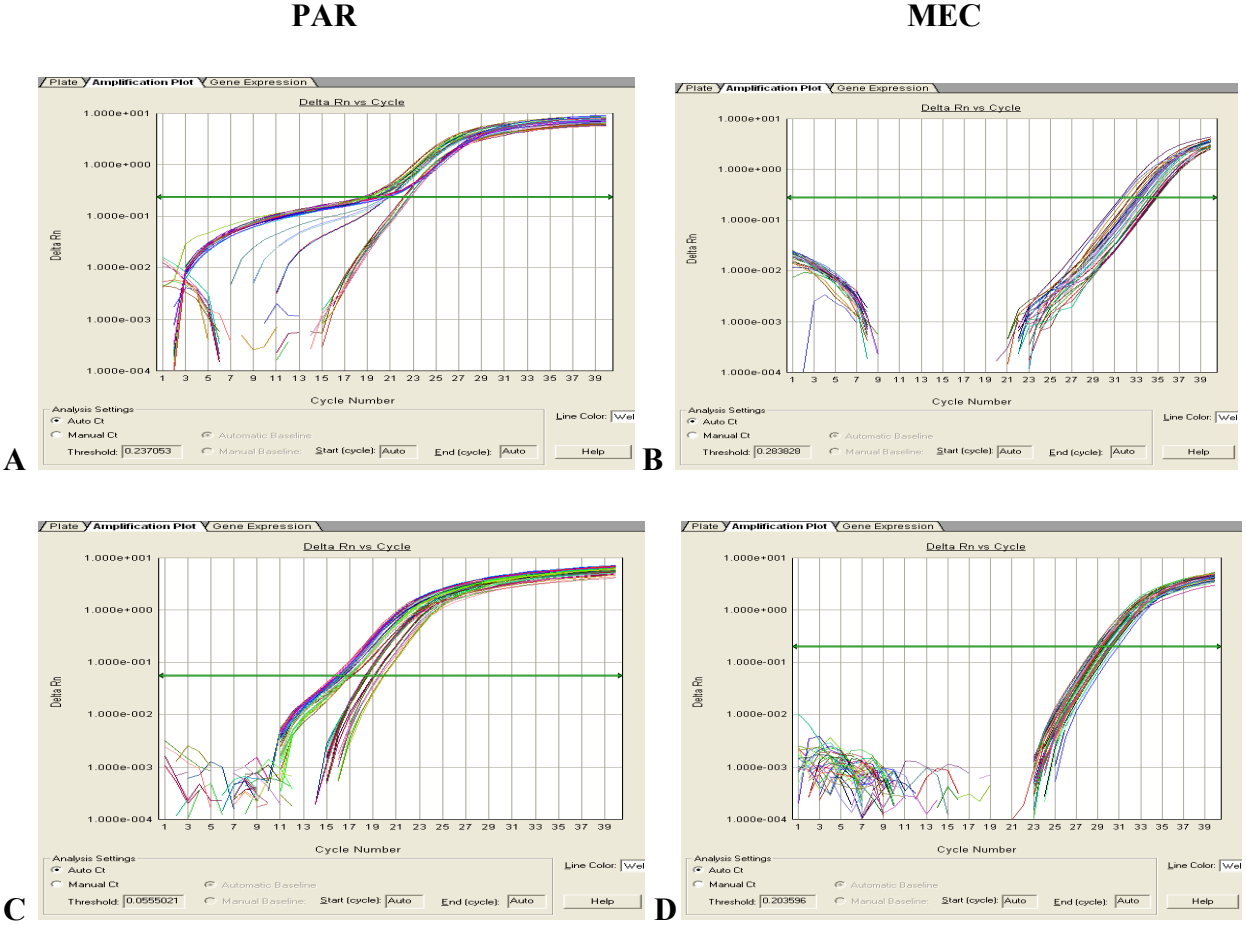
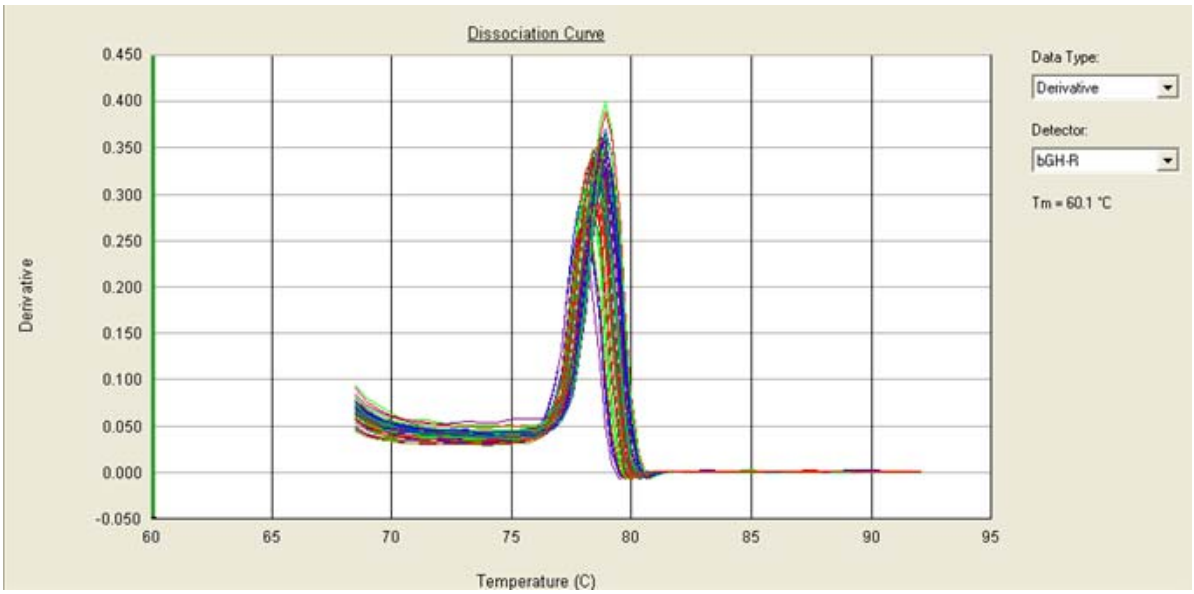


Figure 7. 3. Representative figures of qRT-PCR amplification plots for parenchymal homogenate and mammary epithelial cells.

Relative quantification PCR amplification plots for comparatively less abundant (Socs2; A and B) and more abundant (ERα; C and D) mRNA for parenchymal homogenate (PAR) and mammary epithelial cells (MEC). The shift in Ct values to the right in MEC is due to the huge difference in the total amount of cDNA used in PCR. The amounts of cDNA used in real-time PCR reactions were 20 ng and 4 pg for PAR and MEC samples, respectively.

We measured mRNA expression of GHR, IGF-1, IGF-IR, IGFBP-3, ERα, PR, PCNA, Stat5b and Socs2 in MEC from OVX and INT heifers. Relative mRNA abundance of IGF-1, PR and PCNA was down regulated and IGF-1R mRNA was up regulated in PAR homogenate due to

A



B

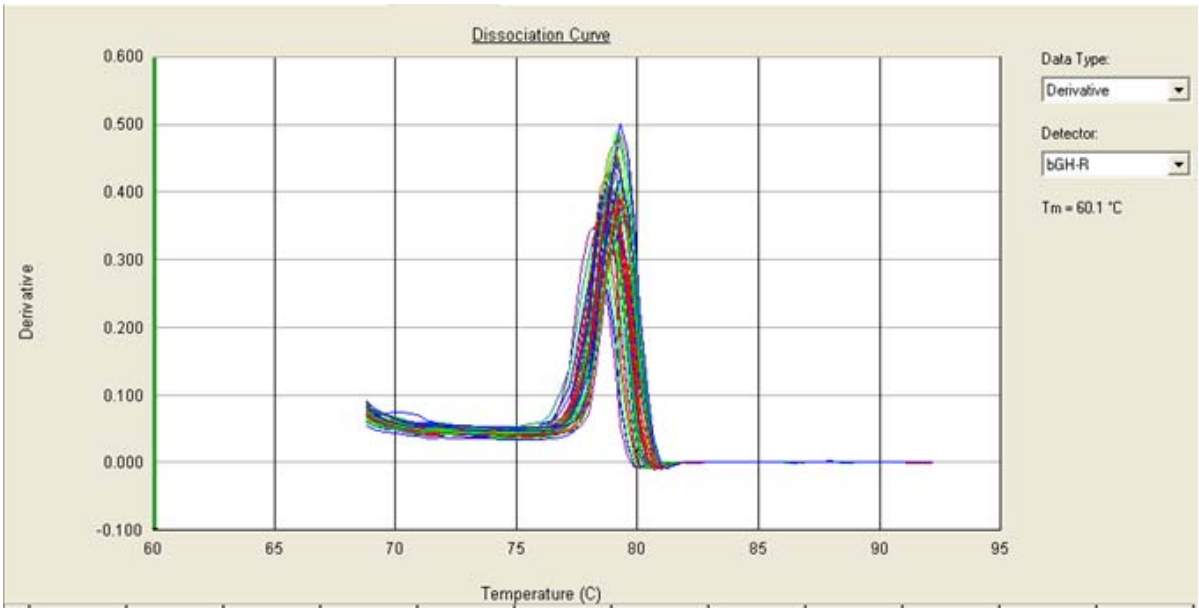


Figure 7. 4. Representative dissociation curves for PAR homogenate and MEC samples

Dissociation curves for GHR performed in PAR homogenate (A) and MEC (B) samples. In both samples the peak is the same indicating amplification of the same single product.

ovariectomy ($P < 0.05$; Fig 7.5). On the other hand, there were no differences in transcript abundance in MEC except for ERα; transcript abundance of ERα was reduced in OVX relative to

INT ($P = 0.056$; Fig 7.6). Even though GHR is expressed in stromal and epithelial cells (Jiang et al., 1999; Plath-Gabler et al., 2001) ligand binding assays failed to demonstrate direct binding of GH in mammary parenchyma (Akers and Keys, 1984). In the current study there was no detectable expression of Stat5b mRNA in MEC. However, GH increased transcription of milk protein genes in MAC-T cells expressing GHR, mediated through Stat5 (Zhou et al., 2008). Therefore, the activity of GHR in MEC may differ in vivo and in vitro conditions as well as under different endocrine states. However, Socs2 mRNA was detected in MEC although transcript abundance was not affected by ovariectomy. We and others observed a decrease of

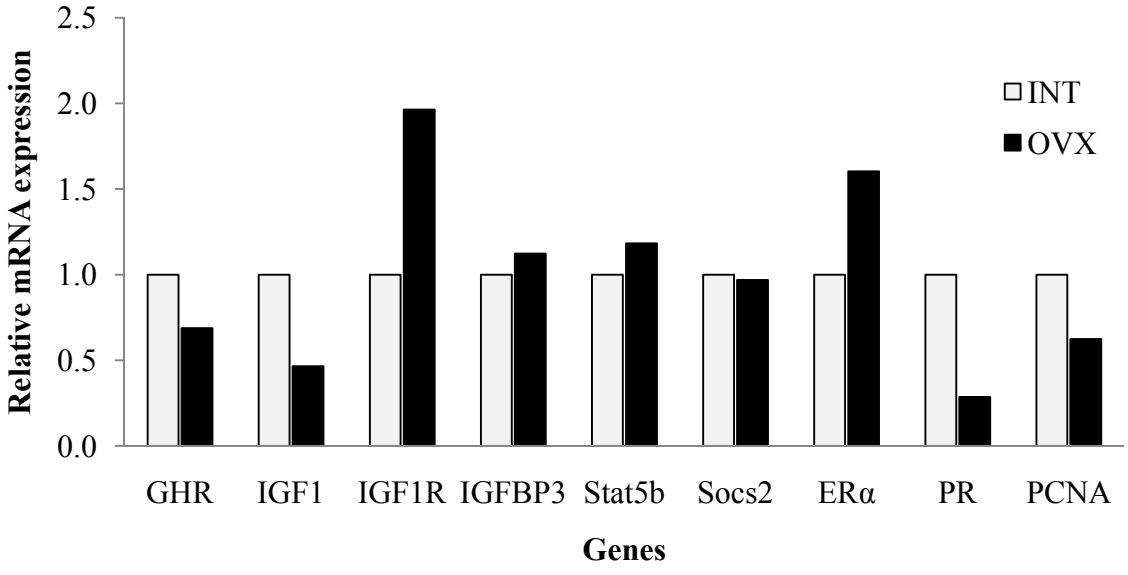


Figure 7. 5. Effect of ovariectomy on transcript abundance in mammary parenchyma

Relative mRNA expression ($2^{-\Delta\Delta Ct}$) in PAR tissue homogenate samples collected from sham operated (INT; n = 12) and ovariectomized (OVX; n = 7) heifers. There was a reduction in transcript abundance for IGF-1, PR and PCNA while IGF-1R mRNA increased in OVX relative to INT.

IGF-1 mRNA in PAR due to ovariectomy (Akers et al., 2000; Berry et al., 2003c). Contrary to previous reports we measured IGF-1 mRNA also in MEC (Berry et al., 2003c). However, IGF-1

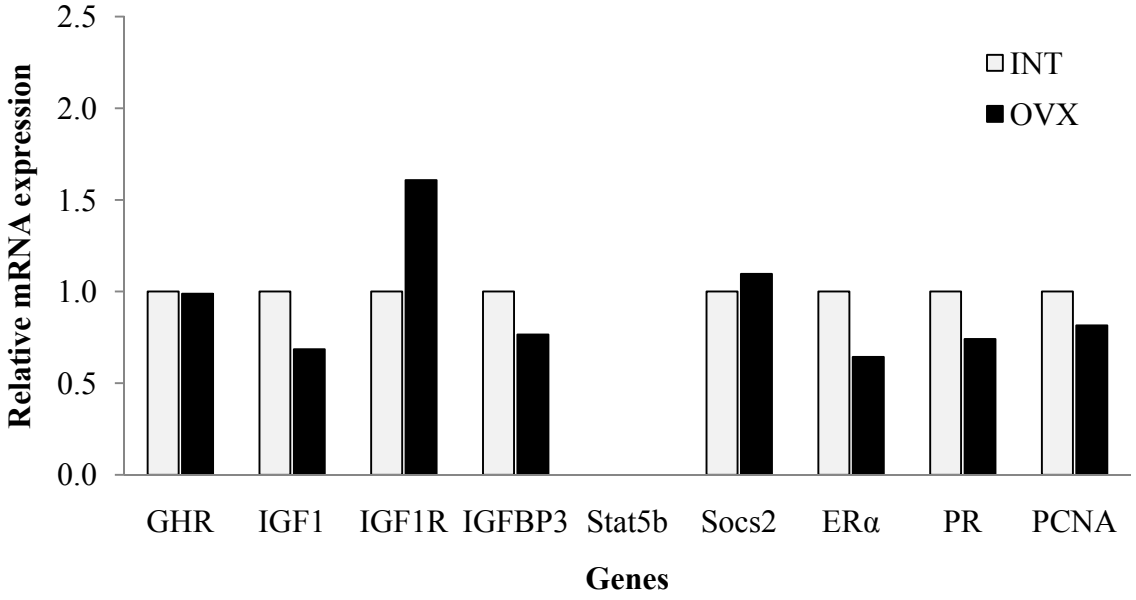


Figure 7. 6. Effect of ovariectomy on transcript abundance in mammary epithelial cells

Relative mRNA expression ($2^{-\Delta\Delta Ct}$) in microdissected MEC collected from sham operated (INT; n = 12) and ovariectomized (OVX; n = 7) heifers. There was no detectable expression of Stat5b in mammary epithelial cells. Transcript abundance in mammary epithelial cells was not affected by ovariectomy except for a reduction in ER mRNA abundance ($P = 0.056$).

mRNA expression in MEC was not affected by ovariectomy. Although there were numerical decreases in IGF-1 (46 %) and PR (35 %) mRNA in MEC by ovariectomy, the decrease was not significant ($P = 0.295$ and 0.540, respectively). It could be due to the high animal to animal variation and comparatively less number of samples used in the assay. Similarly, transcript abundance of IGF-1R in MEC was also increased numerically (38 %), but was not statistically significant ($P = 0.207$). There was a dramatic reduction in the number of PR immunoreactive cells in mammary epithelium in ovariectomized heifers (Chapter 3). The difference in mRNA and protein expression could be attributed to reduced stability of mRNA compared with the protein. Bovine mammary epithelial cells express IGFBP-3 both in vivo and in cell culture systems (Cohick and Turner, 1998; Weber et al., 2000). Like previous reports we did not find

difference in IGFBP-3 mRNA abundance between OVX and INT in PAR or MEC (Berry et al., 2001). Therefore, we conclude that IGFBP-3 in MEC is not regulated by ovarian secretions. Transcript abundance of the proliferation marker gene PCNA was reduced in PAR ($P < 0.05$) but there was no difference in PCNA mRNA in MEC between OVX and INT. In support we did not see a difference in epithelial cell proliferation between intact and ovariectomized heifers (Chapter 3). Therefore, it is evident that the reduction in PCNA mRNA in PAR as a response to ovariectomy was from the non-epithelial cells present in PAR. Estrogen administration caused a 50 % reduction in ERα mRNA in PAR (Meyer et al., 2006a). But similar to our data, Meyer et al. (2006a) did not find a difference in transcript abundance of ERα in PAR due to ovariectomy. Although there was a 37 % increase in ERα mRNA in PAR, it was not statistically significant. However, interestingly, there was a decrease in ERα mRNA in MEC due to ovariectomy. The change in the direction of response in PAR and MEC is surprising. Similar to Meyer et al. (2006a), Shyamala et al. (1992) reported that estrogen reduced ER mRNA expression in mammary gland. Whereas in vitro studies using dissociated cells from mammary gland showed that estrogen reduced ER mRNA but only in non-epithelial cells (Shyamala et al., 1992). Like in Shyamala et al. (1992), it is possible also in bovine mammary gland that PAR and MEC respond differentially because PAR, especially in the young prepubertal heifer has a large complement of non-epithelial cells. Nevertheless, it is reported that within the bovine mammary PAR, immunoreactive ERα are localized mainly in the epithelial cells and that receptor expressing cells are non-proliferating (Capuco et al., 2002). In contrast, Meyer et al. (2006a) reported presence of ER-positive cells also in the intralobular stroma. Additionally, 30 % of the adipocytes and fibroblasts in mammary fat pad contained immunoreactive ERα (Meyer et al., 2006a).

In summary, coupling the technique of laser assisted microdissection and pressure catapulting with the precise and sensitive method of qRT-PCR enabled measurement of transcript abundance in MEC from prepubertal mammary gland in ovariectomized and intact heifers. Our results strongly indicate a disparity in the response to ovariectomy between MEC and PAR tissue and suggest that response to ovariectomy in the gene expression profile in PAR is tremendously impacted by the presence of the stromal cells adjacent to epithelium.

SUMMARY AND CONCLUSION

We used early prepubertal heifers to study the effects of ovarian status and treatment with bST on mammary growth and development employing biochemical, histological and relative gene expression analyses. Ovariectomy reduced PAR mass but there was no significant difference in DNA or protein content of PAR compared with intact heifers. In addition, we did not find a difference in the histological characteristics and percent area occupied by mammary ducts in the PAR between the ovariectomized and intact heifers. These observations were supported by the similar rate of epithelial proliferation in intact and ovariectomized heifers. However, PR immunoreactivity was absent in ovariectomized heifers. This dramatic change in the mammary epithelial cells in the expression of immunoreactive PR in response to ovariectomy proposes the use of PR immunoreactivity as a biomarker for the effect of ovariectomy in young heifers. Transcript abundance of PCNA, IGF-1, IGFBP-6, PR, and estrogen responsive genes were markedly reduced in ovariectomy. However, the changes in the transcript abundance in PAR in response to ovariectomy were absent in mammary epithelial cells. It is to be noted that a major limitation in this study was the inability to incorporate the extreme observations in the analysis because of the very limited amount of parenchyma available in those animals. There was not enough parenchymal tissue in two of the animals from the ovariectomy group and therefore these animals could not be included in data analysis. It is unfortunate that the techniques of biochemical and histological analysis used in this study could not accommodate for these extreme observations. Transcript abundance of IGF-axis genes in MFP was not affected by ovariectomy, but there was a decrease in IGFBP-3 and IGFBP-4 mRNA while IGFBP-5 increased as the calves aged. Furthermore, the magnitude of response to ovariectomy was not different with age at surgery. Based on our data and previous reports on the effects of ovariectomy in prepubertal heifers we propose that absence of functional ovary in prepubertal heifers for a month period elicit changes in PAR at mRNA level, but this time interval may not be long enough to induce changes in proteins important in locally active proliferation pathways which are independent of circulating estrogen concentrations. On the other hand, administration of bST in one month old prepubertal heifers for six and 12 wk period did not affect parenchymal mass or composition, but total udder mass was reduced as a result of reduction in fat pad. Moreover, bST did not impact epithelial cell proliferation or the number of putative stem cells in

PAR. However, there was an increase in the number of epithelial structures and a decrease in cells that retained BrdU label in older compared with younger heifers. We found significant mRNA expression for GH-signaling molecules Stat5a, Stat5b, Socs-2 and Socs-3 was detected in PAR and MFP of prepubertal mammary gland. Ovariectomy and treatment with bST decreased GHR mRNA in PAR and increased Socs2 in MFP. We also provide evidence of local synthesis of GHR and IGF-1 in mammary epithelial cells.

In conclusion our data suggest that ovarian status is a predominant regulator of mammary growth and development in prepubertal heifers than growth hormone and that exogenous bST is not effective as a mammary specific mitogen in very young prepubertal heifers. Our data also suggest that ovariectomy strongly impacted the non-epithelial cells in the PAR rather than the mammary epithelial cells and that an alternate pathway may exist in prepubertal mammary gland for regulation of the IGF-1 gene which is independent of GH action. Further investigation on ovarian regulation of prepubertal mammary growth using more heifers and allowing longer duration between surgery and tissue harvest is needed for understanding the mechanisms involved. Furthermore, utilizing molecular techniques which use minimum sample volumes such as biopsy samples is advocated to account for the extremely low tissue volumes in ovariectomized heifers. We also propose that because the effects of bST in mammary gland is believed to be transient, more frequent sampling during bST administration would provide more confirmative evidence of the effects of bST on gene expression and signaling in the mammary gland.

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APPENDIX A: STATISTICAL TABLES

Table A.1. Delta Ct LSM and P values for PAR from OVX and INT heifers

	Treatment								
	INT			OVX					
	Age at surgery						*P* values		
Item	2 mo	3 mo	4 mo	2 mo	3 mo	4 mo	trt	age	trt*age
GHR	0.3	1.6	1.4	1.6	1.5	1.8	0.054	0.166	0.161
IGF1	-3.0	-2.8	-2.4	-1.8	-2.2	-0.6	0.023	0.161	0.506
IGF1R	1.4	1.8	1.9	0.3	1.2	0.4	0.022	0.489	0.654
IGFBP1	6.1	5.2	4.1	5.8	3.3	1.9	0.152	0.086	0.736
IGFBP2	5.5	4.5	5.2	6.1	6.2	7.7	0.057	0.485	0.628
IGFBP3	1.4	1.2	1.3	1.2	1.1	1.2	0.358	0.847	0.969
IGFBP4	1.4	1.2	1.3	1.2	1.1	1.2	0.358	0.847	0.969
IGFBP5	4.6	5.4	5.8	4.4	5.8	4.5	0.467	0.230	0.323
IGFBP6	1.6	2.2	2.1	1.9	2.9	2.9	0.047	0.087	0.797
stc1	1.7	1.7	1.2	4.1	2.5	1.8	0.010	0.053	0.186
tfpi	4.6	4.9	5.5	6.0	5.6	6.3	0.021	0.363	0.729
ER	-1.6	-2.2	-2.2	-3.6	-2.6	-2.4	0.196	0.938	0.469
PR	0.1	1.0	0.7	4.1	1.3	3.1	0.017	0.604	0.208
STAT5A	1.3	1.8	2.0	2.1	1.7	1.7	0.730	0.920	0.211
STAT5B	3.1	3.3	3.2	3.1	3.0	3.0	0.410	0.939	0.828
SOCS2	2.8	2.1	1.5	3.4	1.2	2.4	0.762	0.218	0.449
SOCS3	7.8	8.0	8.8	7.3	7.6	7.4	0.112	0.581	0.561
PCNA	1.7	1.8	1.9	2.0	2.8	2.4	0.046	0.394	0.545

Table A.2. Delta Ct LSM and P values for MFP from OVX and INT heifers

	Treatment								
	INT			OVX					
	Age at surgery						*P* values		
Item	2 mo	3 mo	4 mo	2 mo	3 mo	4 mo	trt	age	trt*age
GHR	-2.6	-1.9	-2.2	-2.8	-1.4	-2.0	0.586	0.022	0.502
IGF1	-1.5	-1.1	-0.8	-0.8	-1.7	-1.1	0.854	0.589	0.360
IGF1R	1.1	1.3	1.4	1.2	1.1	1.3	0.732	0.691	0.760
IGFBP1	5.7	4.8	4.9	4.4	6.2	4.4	0.901	0.801	0.600
IGFBP2	7.3	8.0	9.7	8.2	8.9	7.8	0.986	0.599	0.231
IGFBP3	0.1	0.2	0.4	0.0	0.4	0.5	0.403	0.001	0.133
IGFBP4	0.1	0.2	0.4	0.0	0.4	0.5	0.403	0.001	0.133
IGFBP5	2.5	1.0	0.9	1.5	0.9	0.7	0.174	0.014	0.425
IGFBP6	0.2	-0.7	-0.5	-0.3	-0.7	-0.5	0.630	0.233	0.800
stc1	1.7	1.7	1.2	1.0	1.9	1.1	0.720	0.593	0.865
tfpi	2.5	2.1	3.7	2.6	2.1	2.2	0.437	0.464	0.451
ER	-0.8	-0.3	0.2	0.0	-0.1	0.3	0.315	0.283	0.631
PR	2.4	3.4	3.6	3.5	3.3	3.8	0.310	0.292	0.374
STAT5A	1.3	1.5	1.5	1.6	1.7	1.4	0.557	0.828	0.695
STAT5B	2.5	2.7	2.7	2.4	2.9	2.7	0.669	0.197	0.685
SOCS2	1.4	1.9	2.0	0.1	0.9	2.0	0.035	0.027	0.252
SOCS3	6.9	6.2	6.0	6.1	5.8	6.2	0.140	0.292	0.253
PCNA	2.1	2.4	2.8	2.8	2.6	3.6	0.202	0.283	0.780

Table A.3. Delta Ct LSM and P values for PAR from rbST and Sal heifers

	Treatment						
	Sal		rbST				
	Time of harvest				*P* values		
Item	Early	Late	Early	Late	trt	time	trt*time
GHR	0.7	1.2	1.2	1.8	0.052	0.039	0.780
IGF1	-3.0	-2.7	-4.2	-2.9	0.327	0.312	0.518
IGF1R	1.4	1.5	1.8	2.6	0.214	0.463	0.519
IGFBP1	8.9	6.8	11.2	8.0	0.080	0.013	0.545
IGFBP2	1.4	2.0	1.8	3.1	0.220	0.146	0.574
IGFBP3	-2.0	-1.7	-0.8	-1.0	0.148	0.947	0.689
IGFBP4	1.7	1.8	2.0	2.2	0.214	0.481	0.773
IGFBP5	2.3	2.9	2.2	3.8	0.583	0.138	0.479
IGFBP6	1.7	2.0	1.7	2.7	0.452	0.155	0.412
PCNA	2.0	2.3	2.3	3.3	0.075	0.067	0.343
abc3	3.9	4.0	4.5	5.5	0.054	0.315	0.367
Stat5a	3.6	4.1	4.8	5.5	0.073	0.347	0.873
Stat5b	4.8	5.6	6.7	6.4	0.044	0.684	0.411
Socs2	3.4	3.2	4.6	3.8	0.032	0.267	0.521
Socs3	7.5	6.6	8.6	8.2	0.187	0.496	0.753
PR	1.3	1.3	1.4	1.9	0.396	0.580	0.550

Table A.4. Delta Ct LSM and P values for MFP from rbST and Sal heifers

	Treatment						
	Sal		rbST				
	Time of harvest				*P* values		
Item	Early	Late	Early	Late	trt	time	trt*time
GHR	-0.3	-0.1	-0.4	-0.4	0.546	0.855	0.841
IGF1	-0.2	-2.1	-0.1	0.3	0.243	0.455	0.277
IGF1R	1.5	2.4	1.2	1.3	0.066	0.210	0.255
IGFBP1	5.9	8.8	6.7	7.3	0.596	0.007	0.059
IGFBP2	5.7	6.0	5.3	6.2	0.828	0.204	0.540
IGFBP3	-3.8	-1.7	-2.7	-3.4	0.750	0.429	0.111
IGFBP4	2.1	2.9	3.2	2.5	0.431	0.987	0.073
IGFBP5	-2.3	0.1	-0.4	-1.5	0.884	0.540	0.097
IGFBP6	-0.9	0.5	0.1	-0.8	0.811	0.671	0.044
PCNA	2.9	4.0	2.9	3.1	0.204	0.056	0.218
abc3	4.9	5.4	5.2	5.3	0.918	0.535	0.728
Stat5a	4.2	3.9	2.3	2.4	0.066	0.937	0.862
Stat5b	4.4	6.0	4.5	4.0	0.132	0.376	0.107
Socs2	3.2	3.2	2.0	2.5	0.007	0.453	0.315
Socs3	5.3	6.4	4.8	5.4	0.319	0.294	0.738
PR	5.7	5.6	3.9	4.9	0.040	0.438	0.334

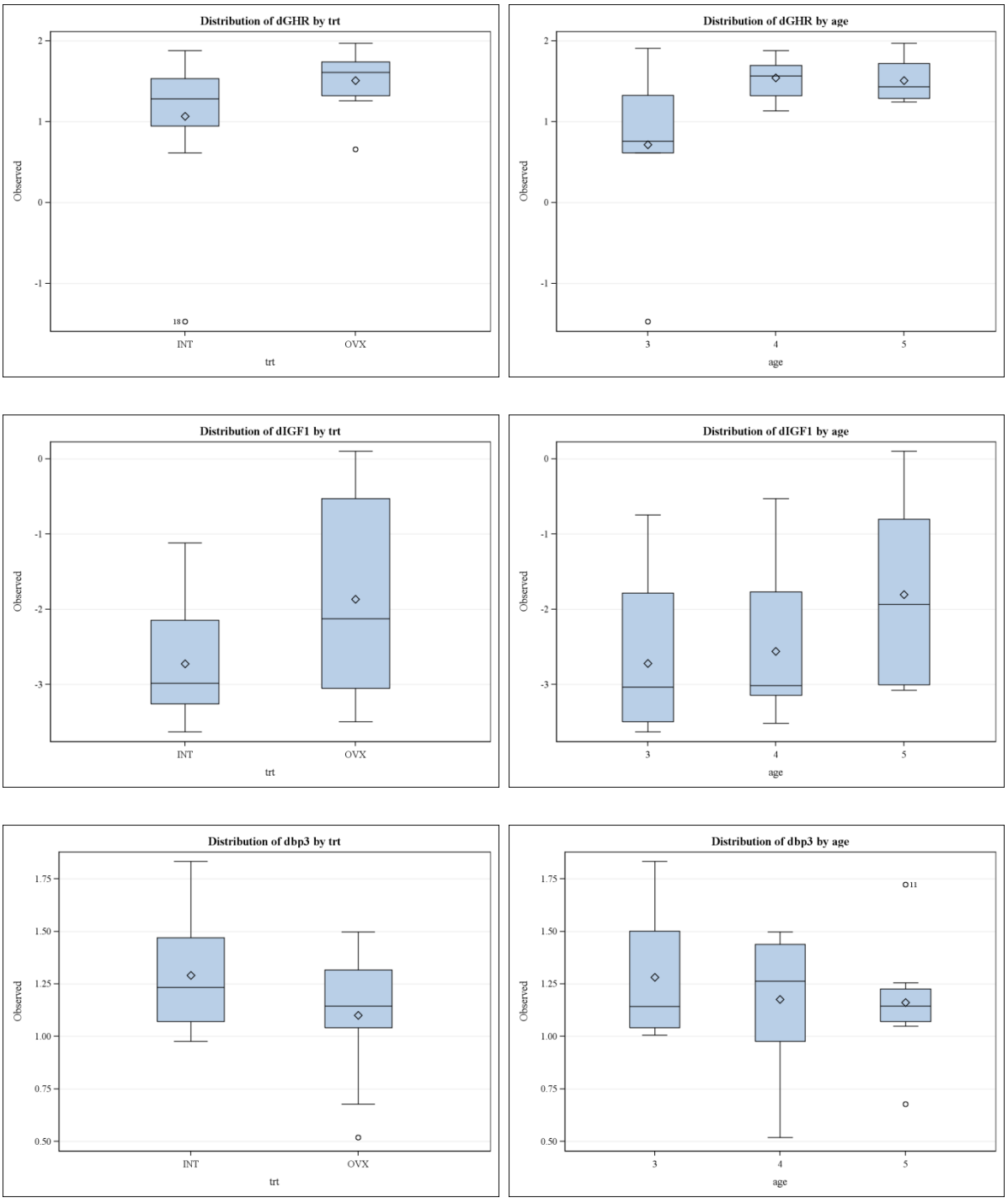
Table A.5. P values for main as well as interaction effects of mammary tissue component (PAR or MFP) for gene expression analysis of OVX and INT heifers

	GHR	IGF1	IGF1R	IGFBP1	IGFBP2	IGFBP3	IGFBP4	IGFBP5	IGFBP6	stc1	tfpi	ER	PR	Stat5a	Stat5b	SOCS2	SOCS3	PCNA
Tissue	<.0001	0.009	0.853	0.227	0.002	<.0001	<.0001	<.0001	<.0001	0.091	<.0001	<.0001	0.004	0.208	0.001	0.071	<.0001	0.022
Trt*tissue	0.519	0.057	0.052	0.233	0.187	0.141	0.141	0.998	0.055	0.096	0.045	0.181	0.096	0.840	0.279	0.282	0.388	0.940
Age*tissue	0.727	0.773	0.622	0.346	0.678	0.075	0.075	0.023	0.014	0.577	0.841	0.731	0.677	0.819	0.732	0.083	0.136	0.344

Table A.6. P values for main as well as interaction effects of mammary tissue component (PAR or MFP) for gene expression analysis of rbST and Sal heifers

	GHR	IGF1	IGF1R	IGFBP1	IGFBP2	IGFBP3	IGFBP4	IGFBP5	IGFBP6	PCNA	abc3	Stat5a	Stat5b	SOCS2	SOCS3	PR
Tissue	<.0001	0.195	0.342	0.007	<.0001	0.007	0.008	<.0001	<.0001	0.006	0.020	0.024	0.020	0.001	0.003	<.0001
Treatment*tissue	0.1052	0.139	0.024	0.043	0.170	0.194	0.777	0.671	0.346	0.021	0.079	0.013	0.013	0.002	0.090	0.009
Age*tissue	0.303	0.478	0.924	<0.001	0.548	0.510	0.679	0.676	0.545	0.967	0.631	0.520	0.745	0.187	0.245	0.241

Appendix A.6. Examples of data distribution plots using “Proc mixed Boxplot” statement.



Note: The bottom of each box is the 25th percentile, the top is the 75th percentile, and the line in the middle is the 50th percentile (indicates the median). The “whiskers” above and below the box shows the spread of the data. The unequal length of whiskers indicates the skewness of data distribution. The diamond within the box represents the mean and the little circles outside the box represent outliers.

APPENDIX B: ADDITIONAL PROTOCOLS

Appendix B.1. Protocol for cryosectioning and laser capture microdissection

Materials required at the Cryostat: Dry ice, slide box, PEN slides, small brush, microtome blades, samples in O.C.T. blocks (carried to be in dry ice), and gloves. All equipments and materials are to be RNase-free.

Irradiate the PEN-slides in the UV-Crosslinker for 30 min at maximum power.

At the cryostat machine: Clean the chuck, apply O.C.T. on it (as a thick smear), place the O.C.T. block on it (remove the plastic mold off the O.C.T. block before placing it on the chuck)

1. Allow the chuck and O.C.T. block to freeze in the cold cutting chamber of the machine (the temp is -20^{o}C). This will take 10-15 min to get the O.C.T. block adhered on to the chuck
2. Place the chuck with O.C.T. block in the machine, tighten the screws and adjust the angles. Adjust the distance between the tissue and blade by either rotating the handle or by moving the blade block.
3. Adjust the blade angle. A little upward slanting from horizontal is good.
4. Trim off the excess O.C.T. by setting at 10 μm.
5. Change the setting to 7 μm once well into the tissue.
    a. Cool the slides in the chamber before collecting the sections
    b. Harvest single section at a time and place it on the slide
    c. Once 2-3 sections are placed on the slide, take it out, allow the tissue sections to melt on to the slide and put it in dry ice immediately and store at -80 C until micro-dissection.

Staining and Laser micro-dissection:

Materials required at the LMD: Tissue sections from -80 C on dry ice, RNase Zap, alcohol solutions and 1% toluidine blue solution made in RNase-free water, RNase-free water, lysis buffer (RNeasy micro kit; Qiagen Inc., Valencia, CA), plastic slide jars (RNase-free), 500uL non-stick RNase-free tubes, 200 and 1000 uL pipettes and tips, gloves, Kim wipes, Sharpie, forceps, Sprout and vortex and timer,

1. Take the tissue sections and place on Kim wipe to thaw, no more than 30sec
2. Wash in 75% ETOH for 30 s
3. Wash in RNase-free H_20 for 30 s
4. Place the slide on Kim wipe and add 100 uL 1% Toluidine blue per tissue section for 30 s
5. Wash in RNase-free H_20 for 30 s
6. Wash in 75% for 30 s
7. Wash in 95% ETOH for 30 s
8. Wash in 100% ETOH for 2min
9. Place slide on Kimwipe and dry in hood for 5 min. Make sure the sections are completely moisture free.

Try to complete LMD within 30 min of staining

(Plastic slide jars can be reused, but must be cleaned between batches of slides. Rinse jars with 100% ETOH, followed by ddH_20, then treat with RNase Zap according to manufacturer's instructions. Rinse in nuclease-free-water and allow drying under the hood.

PALM laser micro-dissection scope directions

Start up:

1. Power on the control unit by turning the key clockwise. After a short period, you should not see any red lights.
2. The laser power supply comes on with the control unit. After you see two green lights, fire the laser with laser on/off button. The right light will turn red, indicating the laser is ready to go.
3. If it has been 1 minute since you powered on the control unit, open the software by clicking on the Palm Robo 3.1 icon.
4. Load your sample on the stage. If needed, use the LOAD setting to load your slide. Push the LOAD button on the top menu of the software menu. This will release the spring that holds the slide holder in place, so the holder will lift out.
5. Alternatively, the condenser turret can be tilted back to load your slide(s).

Calibrate the stage:

1. If either of the two lights on the stage is red instead of green, you must calibrate the stage. Also, if what you mark on the screen and what cuts is not the same, the stage must be calibrated.
2. Select a reference point on your slide. It can be any distinct item.
3. Go to SET UP, CALIBRATION, NEW CALIBRATION, STAGE.
4. Click on your selected reference point and drag it to the center of the stage (marked by red crosshairs).

Focusing and trail dissection

- Use manual focusing
- View under 5x objective first and then move to 20x.
- Select the irregular shape drawing tool from the menu bar at the bottom of the screen. Select “cut” or “close & cut” from the drop down menu.
- Draw an outline of an area other than epithelium.
- Select “element list” from the view menu
- High light the element to be dissected
- Then click on the laser icon. Adjust the values for energy and focus to ensure proper cutting and catapulting. These values will change for different magnifications.
- Make sure the cutting is along the same line of the drawing. If not, re-calibrate the system.

Loading caps:

1. Add 40 uL of lysis buffer in the cap of the 500 uL tube and place the tube and cap in the cap holder.
2. Use the stage controller (Load/Unload) button to move the cap into position.
3. Check the distance of the cap from the slide. Adjust the cap height using the black lever on the cap holder to ≤1 mm above the slide. (The cap should not touch the slide.
4. Center the light in the cap using the joystick on the stage controller.
5. Set the reference setting by pressing and holding the left (Set Ref) red button on the stage controller until the red light flickers once, indicating the position is set.

6. Once the reference is set, pressing the Set Ref button quickly (not holding it down) will reposition the cap holder.
7. Go back to the computer monitor; outline the epithelial structures by using the drawing tool. Highlight the elements to be cut, choose the "cut' option from the drop down menu and click on the laser icon.
8. After cutting, using the dot button mark 2-3 points in the elements especially the areas where cutting was not complete. Then highlight the dots and click the laser icon again to catapult the cut structures. You can draw multiple elements and highlight them all together and click the laser icon.
9. Once the cut and catapulting is completed, remove the cap and add 310 uL lysis buffer with into the tube.
10. Spin the tube to get the dissected tissue pieces to the bottom and then vortex briefly for 30 s to lyse the cells.
11. Transfer the tube immediately to dry-ice and then can be stored at -80 until total RNA is extracted.

Notes: At lower magnification, you have greater depth of cutting and less cutting laser power.

At higher magnification, you have less depth of cutting and more cutting laser power.

The limit for cutting (at least, with a single pass), is about 15 μm.

If you are using a low magnification objective and your sample is >12-15 μm thick, you may need more than one pass to cut. You can define a region using low magnification, and then cut it at a higher magnification.

Appendix B.2. RNA extraction from laser capture microdissected samples

Preparations:

- Add B-mercaptoethanol to buffer RLT (10 uL BME to 1 mL buffer)- good up to 1 month
- Add ethanol to buffer RPE (4 volumes of 96-100% ETOH to one volume of buffer)
- Prepare 80 % and 70 % ETOH by using RNase-free water
- Prepare DNase I stock solution by injecting 550 uL nuclease-free water into the vial. Mix gently. DO NOT VORTEX! Make aliquots and store at -20^{o}C. Thawed aliquots should be stored in refrigerator. DO NOT RE-FREEZE!!
- Prepare DNase incubation mix by adding 10 uL of DNase stock to 70 uL buffer RDD. Mix by gently inverting the tube. DO NOT VORTEX. Prepare fresh each time.
- Microdissected samples would be in the -80^{o}C freezer in lysis buffer. Take the tubes out of the freezer and incubate the lysates at 37^{o}C in a water bath until completely thawed. AVOID PROLONGED INCUBATION

Steps:

- Add 350 uL of 70 % ETOH to the lysate, mix well by pipetting. Do not centrifuge.
- Transfer the sample (including any precipitate if formed) to an RNeasy Mini Elute spin column placed in 2 mL collection tube. Centrifuge @ 8000 x g for 15 s. Discard the flow-through; save the collection tube for next step.
- Add 350 uL RW1 to the column, centrifuge @ 8000 x g for 15 s. Discard the flow-through; save the collection tube.
- Add 80 uL DNase incubation mix **directly to column membrane** and place on the bench-top for 15 min.
- Add 350 uL RW1 to the column, centrifuge @ 8000 x g for 15 s. Discard the flow-through and collection tube.
- Place the column in a new 2 mL collection tube, add 500 uL buffer RPE and centrifuge @ 8000 x g for 15 s. Discard the flow-through; save the collection tube.
- Add 500 uL of 80 % ethanol to RNeasy spin column and centrifuge @ 8000xg for 2 min. ***Remove the spin column carefully without touching the flow-through.*** Discard the flow-through and collection tube.
- Place the column in a new 2 mL collection tube and centrifuge at full speed for 5 min keeping the column lid open to ensure complete removal of ETOH. Discard the flow-through and collection tube.
- Place the column in a new 1.5 mL collection tube. Add 20 uL RNase-free water **directly to the membrane**. Centrifuge at full speed for 1 min to elute the RNA.

Appendix B.3. Harsh stripping protocol for Western blots

(To be used when secondary is the same as the previously used)

1. Put the blots in a container with a tight lid
2. Rinse membrane with methanol
3. Rinse membrane 3 times with TBST
4. Wash membrane 3 times with TBST for 10 min each
5. Heat 100 ml of stripping buffer in microwave until boils

   Recipe for stripping buffer for 1L

1M tris-HCl at pH 6.8	62.5 mL
20% SDS	100 mL
ddH_2O	829.7 mL

   Note: final concentrations of tris-HCl and SDS in 1L of stripping buffer will be 62.5 mM and 2 %, respectively.
6. Immediately add 781 uL of B-mercaptoethanol
7. Add hot stripping buffer to membrane, close the lid and rock for 10 min at RT
8. Rinse membrane in ddH_2O several times until it doesn't smell
9. Rinse membrane 5 times in TBST
10. Wash membrane 3 times in TBST for 10 min each

    Now the membrane is ready for further processing. Start incubation steps, right from blocking step

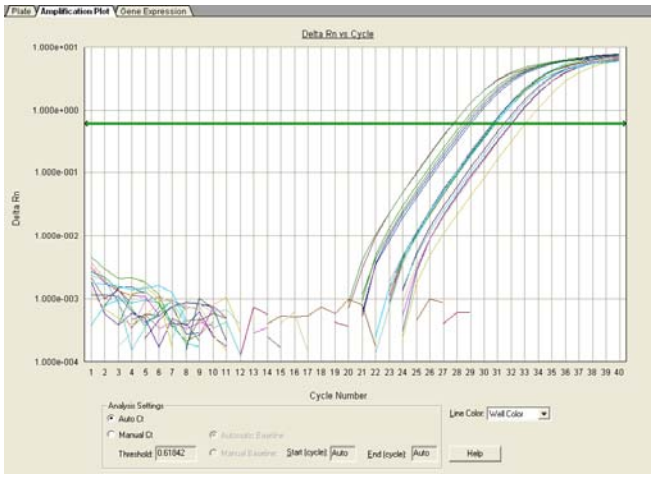
Appendix B.4. Results of test-run of real-time PCR using RNA isolated from LCM samples

A test run of real-time PCR for different genes was performed with different concentrations of cDNA (stock and diluted) to determine the lowest amount of cDNA that can be used to produce good amplification.

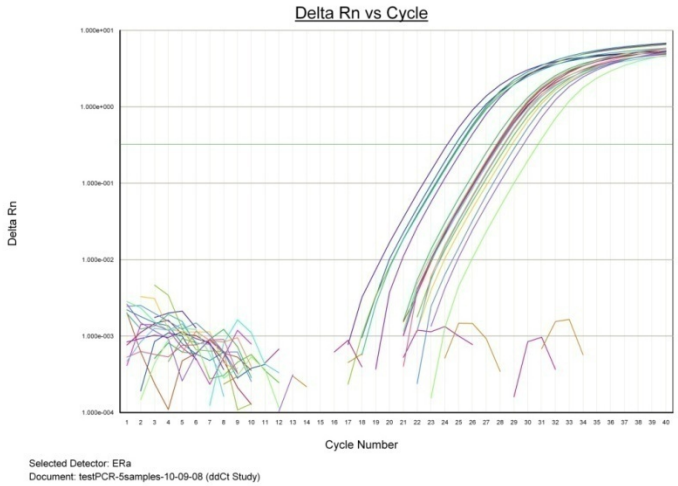
Total RNA and cDNA concentrations used

				Total cDNA in real time PCR, pg	
sample	RNA, pg/μL	volume used, μL	Total RNA in RT reaction, pg	stock	1:10 dilution
1	3	7.5	22.5	2.3	0.23
2	13	7.5	97.5	9.8	0.98
3	20	7.5	150.0	15.0	1.50
4	12	7.5	90.0	9.0	0.90
5	3748	7.5	28110.0	2811.0	281.10

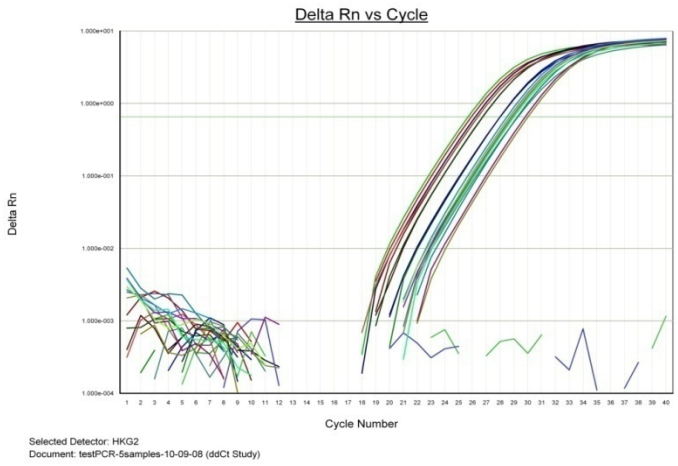
For real-time PCR, both stock and 1:10 diluted cDNA were used. There was an increase in Ct values for the 1:10 dilutions by 2 cycles from the Ct value of the stock solution. I chose one of the housekeeping gene and two target genes which are expressed in low levels (GHR) and high levels (ERα).



Amplification plot for GHR – Average Ct for replicate samples ranged from 27.1 to 32.5.



Amplification plot for ER – Average Ct for replicate samples ranged from 24.4 to 30.1



Amplification plot for RPS15A (HKG) – Average Ct for replicate samples ranged from 25.7 to 29.8

APPENDIX C: REAGENTS AND SOLUTIONS

C.1. Immunohistochemistry

1) 10x PBS (phosphate buffered saline**)**
    (a) 21.4 g Na_2HPO_4
    (b) 5.76 g NaH_2PO_4
    (c) 87.8 g NaCl
    (d) Adjust volume to IL with distilled water and pH to 7.4
2) 1x PBS (referred to as PBS throughout protocol)
    (a) 100 mL 10x PBS
    (b) 900 mL distilled water
3) 10 Mm citrate buffer
    (a) 2.1 g sodium citrate
    (b) Adjust volume to 1L with distilled water and pH to 6.0 with 2 M NaOH
4) 5% Goat serum
    (a) 5 mL goat serum
    (b) 95 mL PBS
5) 1% Goat serum
    (a) 1 mL goat serum
    (b) 99 mL PBS
6) Diaminobenzidine (DAB) Solution (for 10 slides)
    (a) 2 drops Reagent A of DAB kit
    (b) 2 mL of distilled water (mix after adding)
    (c) 2 drops Reagent B of DAB kit
    (d) 2 drops Reagent C of DAB kit (mix after adding)
7) Primary antibody for detecting ERα
    (a) Santa-Cruz ERα (c-311) mouse monoclonal; 200 ug/mL; Cat # Sc 787 lot# D2807
    (b) Dilute 1:100 in 1% goat serum to get 2ug/mL working solution
8) 3% H_2O_2 in PBS (for 10 slides)
    (a) 90 mL PBS
    (b) 10 mL 30% w/w H_2O_2

C.2. Tissue homogenization

1) High-salt homogenization buffer
    (a) 0.05 M NaPO4
    (b) 2 M NaCl
    (c) 0.002 M EDTA

2) Lysis buffer (1L)
    (a) 4.76 g HEPES
    (b) 0.76 g EGTA
    (c) 2.1 g NaF
    (d) 7.44 g KCl
    (e) 0.074 g EDTA
    (f) 15.2 g Beta-glycerophosphate
    (g) Add double distilled water to make up to 1L
    (h) Add protease inhibitor into this buffer in a 1:50 ratio before homogenization

C.3. Electrophoresis

1. 10X MOPS (1L)
    a) 41.8 g of MOPS
    b) 800 mL 100 mM Na acetate (adjust the pH to 7.0)*
    c) Add 20 mL DEPC-treated 0.5 M EDTA (pH 8.0)**

    Make up the volume to 1L. Cover bottle and autoclave

    *100 mM Na acetate prepared by adding 8 g of Na acetate in 1L DEPC water
    ** 0.5 M EDTA pH 8.0 prepared by adding 73.1 g EDTA in RNase-free water. Adjust the pH to 8.0 and make up the volume to 500mL.
2. 1X running buffer (1L) for RNA gel
    a) 100 mL 10X MOPS
    b) 50 mL 37% formaldehyde
    c) 850 mL DEPC water
3. 10X Running buffer for Western blot
    a) 30 g Tris base
    b) 144 g Glycine
    c) 10 g SDS
    d) add DI water to make up to 1L
4. 10X transfer buffer for Western blot
    a) 30 g Tris base
    b) 144 g Glycine
    c) Add DI water to make up to 1L
5. 1X transfer buffer for Western blot (1L)
    a) 100 mL of 10X
    b) 150 mL 100% methanol
    c) 750 mL DI water

6. 7.5 % polyacrylamide gel – volume required to make 2 gels

Reagents	Resolving gel (75 mL)	Stacking gel (20 mL)
30 % Acrylamide + 1 % Bis-	18.75 mL	2.6 mL
1.5 M Tris, pH 8.7	20.25 mL	----------
0.5 M Tris, pH 6.7	----------	2.5 mL
20 % SDS	0.5 mL	130 µL
DI water	35.75 mL	14.45 mL
TEMED	25 µL	20 µL
10 % Ammon. Persulfate	562.5 µL	300 µL

C.4. DNA assay

1) Hoechst dye stock solution (10 mL, 1 mg/mL Hoechst H 33258)
   a) Add 10 mL distilled water to 10 mg H 33258.
   Do not filter. Store at 4C for up to 6 mo in amber bottle

2) 10X TNE buffer (1000 mL, buffer stock solution)
   a) 12.11 g Tris (100 mM)
   b) 3.72 g EDTA Na_2-$2H_2O$ (10 mM)
   c) 116.89 g NaCl(2 M)
   Dissolve in ~800 mL distilled water. Adjust ph to 7.4 with 12 N HCl. Add distilled water to 1000 mL. Filter before use (0.45 um). Can be stored at 4^oC for up to 3 months.

3) High Range Assay Buffer (100 to 5000 ng/mL final DNA concentration) MAKE FRESH DAILY:
   a) 100 uL Hoechst dye stock solution
   b) 10 mL 10X TNE buffer
   c) 90 mL distilled water
4) Low Range Assay Buffer (10 to 500 ng/mL final DNA concentration) MAKE FRESH DAILY:
   a) 10 uL Hoechst dye stock solution
   b) 10 mL 10X TNE buffer
   c) 90 mL distilled water

5) DNA Standard for low range assay (100 ug/mL) = 1:10 dilution of 1 mg/mL DNA stock standard
   a) 100 uL of the1 mg/mL DNA standard stock
   b) 100 uL 10X TNE buffer
   c) 800 uL distilled water

C.5. Lipid extraction

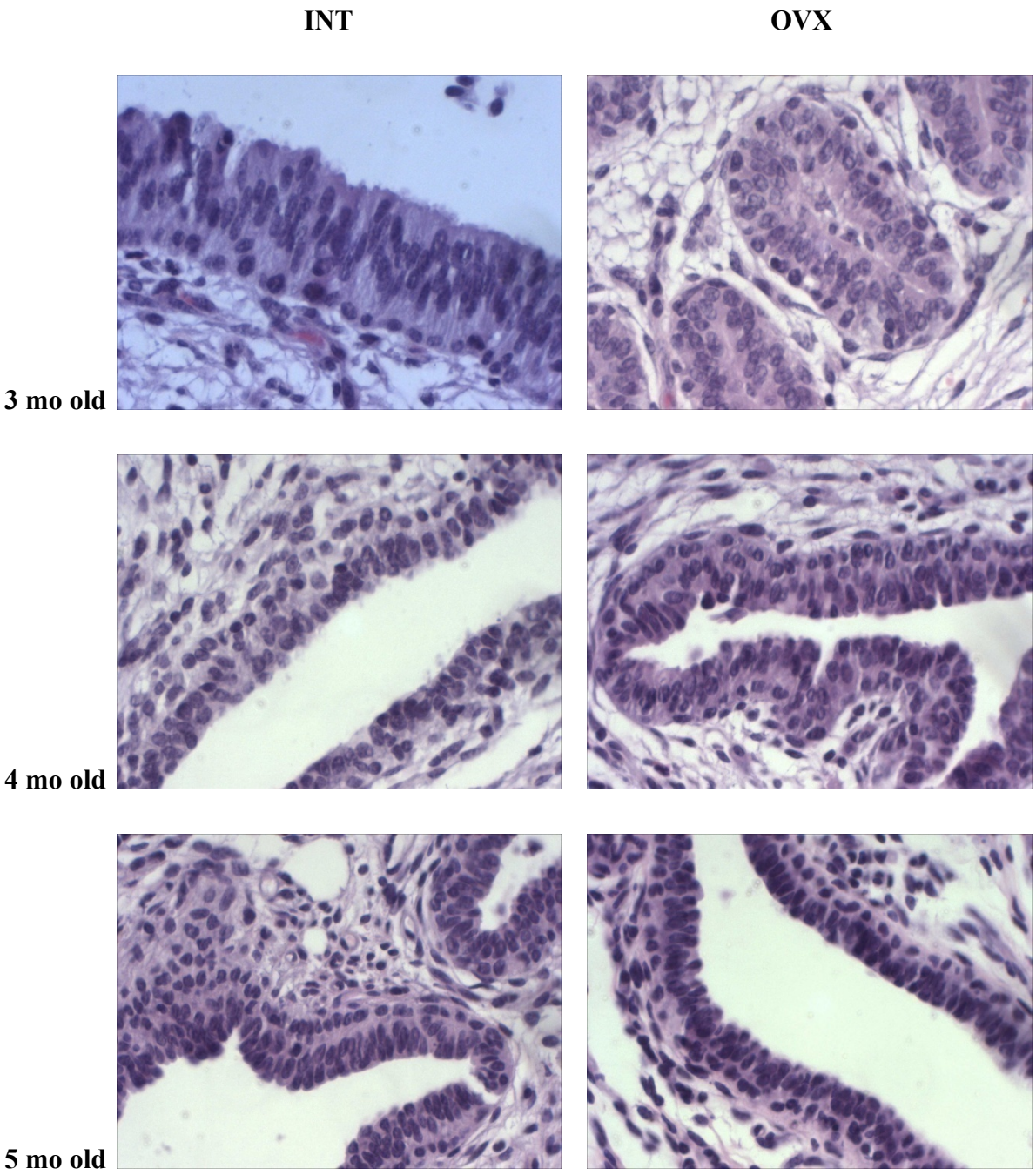
1) Hexane-isopropanol (3:2; HIP)
   (a) 600 mL Hexane
   (b) 400 mL 100% Isopropanol
   (c) 50 mg BHT (omit BHT if no further analysis on lipid is necessary)

2) Sodium sulfate
   a) 6.67 g Na_2SO_4
   b) 100 mL H_2O

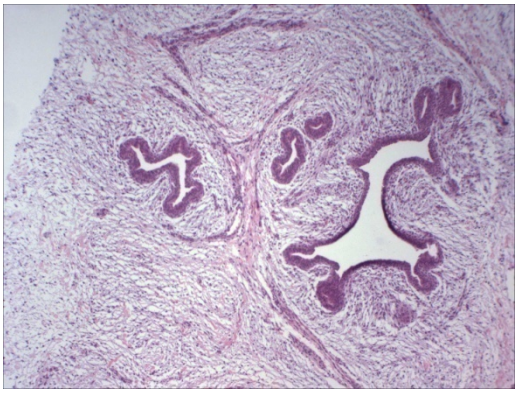
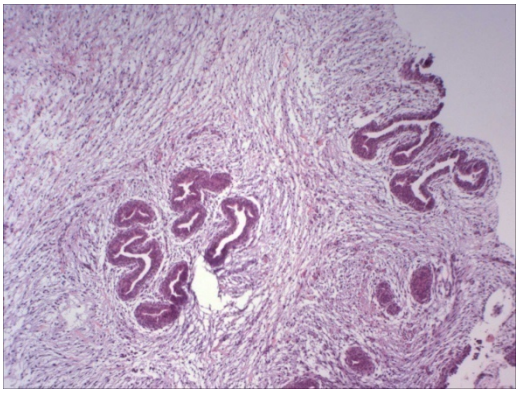
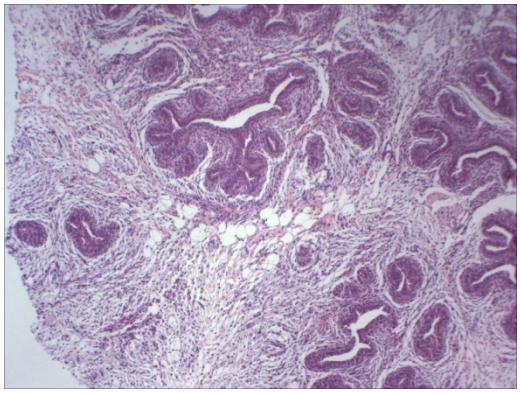
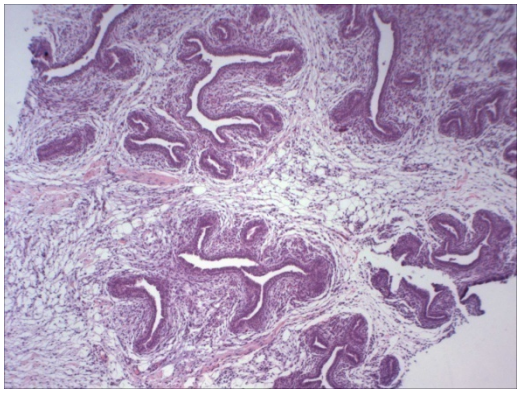
   (these solutions can be stored at room temperature indefinitely)

APPENDIX D: ADDITIONAL HISTOLOGY FIGURES

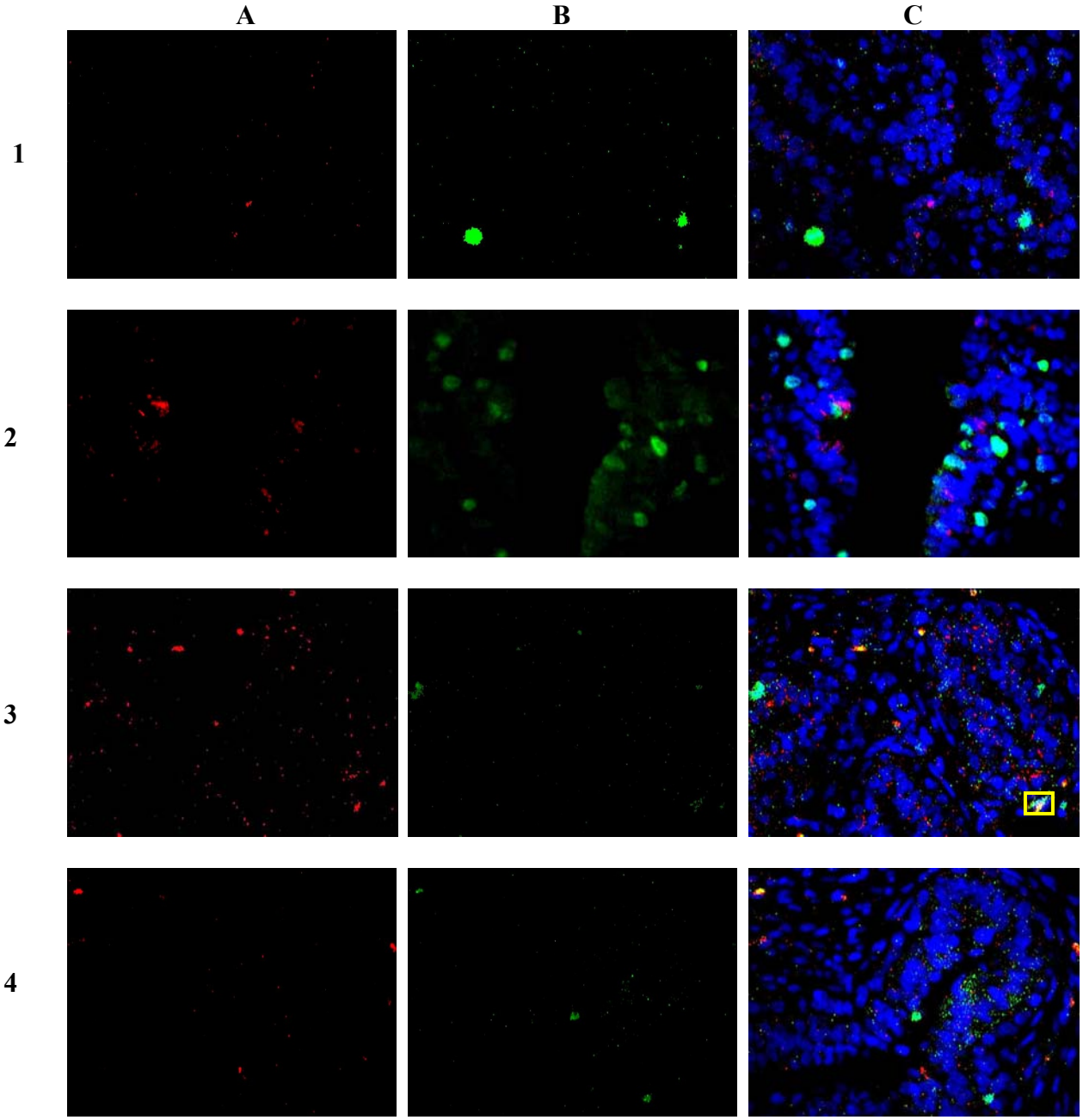
Appendix D.1. Representative photomicrographs of Hematoxylin and Eosin stained PAR sections from INT and OVX heifers (40x magnification).



Appendix D.2. Representative photomicrographs of Hematoxylin and Eosin stained PAR sections from Sal and bST heifers (4x magnification)

	Sal	**bST**
Early Harvest		
Late Harvest		

Appendix D.3. Representative photomicrographs of fluorescent images from dual labeling for BrdU and Ki67 antigens in Sal and bST heifers (40x magnification)



A - BrdU-label retaining cells; B – Ki-67 positive cells; C – Composite image showing DAPI, BrdU and Ki67 staining. 1 – Sal-Early harvest; 2 – bST-Early harvest; 3 – Sal-Late harvest; 4 - bST-Late harvest. Dual-positive cell is shown in yellow rectangle.