

The Effects of Tamoxifen on Mammary Development in Prepubertal Heifers

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Abstract

Our purpose was to determine the effects on mammary gland development in prepubertal heifers given the anti-estrogen tamoxifen. Sixteen Holstein calves were randomly assigned to one of two treatment groups: tamoxifen-injected (TAM) or control (CON). Calves were subcutaneously injected daily from 28 to 120 days of age with 0.3 mg/kg tamoxifen or carrier. At 120 days calves were euthanized and udders removed. Weight of trimmed parenchymal tissue (left rear quarter) was dramatically lower in TAM calves than in CON calves ($p < 0.0003$; 16.1 vs. 34.8 g). Parenchymal samples from three regions of the left rear quarter (lower, middle and outer regions) were processed for immunohistochemical staining for Estrogen Receptor α and Progesterone Receptor, myoepithelial cells, and label retaining cells. Overall, the proportion of neither ER nor PR labeled cells was impacted by TAM treatment. However, imaging analysis indicated a markedly higher intensity of ER expression in CON calves. TAM caused an increase in myoepithelial cell differentiation similar to what is seen in ovariectomy. We were able to effectively use a new technique of multispectral imaging to identify label retaining cells, which led to the discovery of an increase in the percentage of label retaining cells in TAM compared to CON. While treatment with the anti-estrogen tamoxifen reduced mammary parenchymal mass similarly to OVX, the mechanism(s) involved appear to differ. This suggests that the impacts of ovariectomy are only partially explained by the absence of estrogen.

Dedication

The first people I have to thank are my parents. My childhood was full of all different kinds of animals that helped develop my love of all species. There were no looks of disgust when I ran into the house covered in mud from head to toe carrying my new “friend”. You both were super supportive of helping me learn about anything I dragged into the house.

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List of Abbreviations

Abbreviation	Definition
BrdU	5-bromo-2'-deoxyuridine
BW	body weight
CON	control group
Ct	cycle threshold
EGF	epidermal growth factor
ER α	estrogen receptor alpha
ER β	estrogen receptor beta
HH	hip height
kg	kilogram
LRC	label retaining cell
MEC	mammary epithelial cell
MFP	mammary fat pad
mg	milligrams
ml	milliliter
nm	nanometers
PR	progesterone receptor
SEM	standard error of the mean
TAM	tamoxifen injected group
TDU	terminal ductal unit
wk	week
wt	weight

Literature Review

Overview of Bovine Mammary Gland Development

Mammary tissue is unique because most of its development is postnatal. Dairy cattle are a practical model for mammary gland research because of the daily manipulation of their glands and their importance to the dairy industry, milk. However, the mechanisms for the development of this tissue in the bovine are only partially understood. This is especially true of mammary development in the neonate and prepubertal animal. These periods are important because development in these physiological stages form the foundation for future growth and functional differentiation. To be able to increase production researchers need to be able to understand these mechanisms to increase the amount of parenchyma because milk production is directly proportional to the amount of parenchyma and degree of differentiation of this tissue (Akers, 2000).

At birth, the parenchyma is rudimentary consisting of only a few ducts branching from the gland cistern. However, the small mammary fat pad (MFP) and circulatory system is almost fully developed (Sejrsen, 1994). The prepubertal period is when heifers first experience allometric growth of the parenchyma tissue in the mammary gland (Sinha et al., 1969). In bovine, development within the parenchyma is dissimilar when compared with mice. For example, there is concurrent branching and elongation of ducts versus elongation and filling of the MFP prior to puberty in mice compared with the bovine (Figure 1.1). However, in the bovine mammary gland developing ducts are surrounded by loose connective tissue to form intralobular stroma at the terminal ductal units (TDU; Figure 1.2) (Capuco et al., 2002). While development of the gland is occurring the parenchyma is invading the MFP. Histologically, prepubertal heifers

exhibit parenchymal tissue with closely packed ducts that branch out from the gland cistern in broccoli-like fashion as these structures integrate into the MFP (Akers et al., 2000). Prior to parenchyma integration the MFP is clear of any mammary epithelial cells (MEC) (Akers, 1990). The MFP remains a distinctively different tissue type until late into pregnancy when the MFP is completely integrated by MEC.

In mice, end buds integrate into the MFP until the limit of the MFP is reached. The end buds also avoid contact with each other and prefer to grow in regions of the MFP absent of any other parenchyma (Williams et al., 1983). This can be visualized as a MFP filled with elongated mammary ducts with very few side branches. In the prepubertal bovine, the MFP typically accounts for one-third of the area of developing udder (Sejrsen et al., 1992). Any ductal growth that occurs requires the MFP, which is also developing at a slower rate than the parenchyma at this time (Faulkin et al., 1960).

For development to occur there has to be proliferating cells as well as changes in ductal morphology. Peripheral MEC, close to the surrounding MFP, are more sensitive to growth factors, like IGF-1, and are more active in the invasion of the MFP compared to cells positioned medially in the gland (Ellis et al., 2000). IGF-1 is known to have receptors in the prepubertal mammary gland. There is also increased sensitivity to IGF-1 in prepubertal heifers compared to lactating cows as indicated by the increased binding capacity in prepubertal tissue compared to lactating tissue (Purup et al., 1995). With a greater ability to be affected by growth factors like IGF-1 this supports the idea that the prepubertal period is a critical window to manipulate the development of the mammary gland.

However, development of the mammary gland depends on multiple hormonal influences. In heifers, the ovaries are a major source of mammogenic hormones even prior to puberty. The ovary is needed for growth in the mammary gland for at least the first 5 months in the bovine (Velayudhan et al., 2012). Heifers that are ovariectomized prior to the allometric growth period have a marked reduction in the amount of parenchyma tissue. However, ovariectomy does not affect prepubertal parenchymal growth in sheep (Ellis et al., 1998). When heifers are ovariectomized it is believed to reduce both estrogen and progesterone, which are required for normal development of the mammary gland (Turner et al., 1956). In mice, estrogen is necessary for induction of progesterone receptor (PR) gene expression (Bocchinfuso et al., 2000). In ovary intact prepubertal calves the concentrations of circulating estrogen are low and difficult to accurately measure. However, small differences between intact and ovariectomized heifers (0.1 pg/ml lower after ovariectomy) have been reported (Sejrsen, 1994). These minute reductions in circulating estradiol seem to suggest that estrogen is likely not the only component responsible for the decrease in parenchyma growth measured when calves are ovariectomized. Moreover, the decrease in parenchyma tissue in ovariectomized calves can be prevented by giving exogenous estrogen, which stimulates proliferation in epithelial cells. Ovary intact calves also positively respond to treatment with exogenous estrogen with increased parenchymal growth (Li et al., 2006). Based on induced lactation studies, it is believed that estrogen and progesterone both need to be present in a finely tuned ratio for optimal development and subsequent milk production (Sud et al., 1968).

Ovariectomy changes the appearance of parenchyma tissue histologically by reducing the number of ducts per unit area as well as the degree ductal branching (Berry et al., 2003b). Ovary intact calves also have more stratified areas of epithelium compared to ovariectomized heifers.

These areas are thought to be regions where side branches are more likely to develop. However, whether or not ovariectomy impacts relative proportion of the epithelium compared to stroma within tissue sections may depend on when ovariectomy occurs and/or length of time after ovariectomy when tissues are sampled (Berry et al., 2003b, Safayi et al., 2012, Velayudhan et al., 2012).

With all these differences occurring in the histology of the parenchyma it is logical to expect changes in the gene expression of the tissue. Interestingly, many of the most dramatic changes in gene expression occur in the MFP rather than the parenchyma. In ovariectomized calves the MFP had more up regulated genes compared to the parenchyma (Li et al., 2008). This is despite the fact that estrogen controls regulation of genes associated with the cell cycle in the parenchyma not the MFP (Li et al., 2006). Regardless, recent genomic evaluations demonstrated that more than 9,000 genes were differently expressed between parenchyma and MFP in prepubertal heifers (Piantoni et al., 2010).

Individual influences of hormones need to be investigated further by blocking individual hormones systemically. The ovary is not the only source of estrogen. It is known that estrogen is also produced locally in the mammary gland (Janowski et al., 2002). This complicates the matter of being able to block estrogen not only from the ovary, but also from the mammary gland itself. Therefore, instead of blocking the source another approach is to block estrogen signaling. Tamoxifen is a selective estrogen receptor antagonist in the mammary tissue (Smith, 2003).

Tamoxifen when bound to ER prevents the binding of helix 12, which activates transcription of AF-2. Depending on the tissue where the ER are housed, if genes are reliant on AF-2 there is no further activation and is part of reason tamoxifen is considered an antagonist

(Shiau et al., 1998). Pure anti-estrogens are able to inhibit the activity of both AF-1 and AF-2 and block the classical pathway of ER α and ER β in all tissue types (Hall et al., 2001). However, in the presence of ER β tamoxifen acts as an agonist in the AP1 pathway (Nilsson et al., 2001).

The result of giving tamoxifen to the mammary gland is multifaceted. Overall, there is a decrease in parenchyma tissue when tamoxifen is given to mice (Kotoula et al., 1993).

Histologically, the mammary glands of mice given tamoxifen show a decrease in the size of the lobular structures as well as atrophic alveoli. The decrease in size is in direct contrast to mice given estrogen, which stimulates lobulo-alveolar and ductal growth (Cheng et al., 2004). Mice given tamoxifen also experience an increase in the number of differentiated myoepithelial cells (Kotoula et al., 1993). There is a dramatic decrease in the amount of Ki67 labeling similar to what is seen in mice given pure anti-estrogen ICI 182, 780. There is also a decrease in expression of IGF-1 receptor but no change in the amount of IGF-1 mRNA (Chan et al., 2001).

Estrogen Receptor α /Progesterone Receptor

Estrogen is known to induce proliferation in MEC; whereas, progesterone increases proliferation only in the presence of estrogen and does not induce proliferation directly. Prepubertal heifers show no proliferative response to treatment with progesterone alone (Woodward et al., 1993). This is not to say progesterone is an unnecessary hormone. Progesterone is known to stimulate lobular-alveolar development during gestation (Lyons, 1958). Estrogen and progesterone are known to act synergistically. In ovariectomized virgin mice exogenous estradiol increased the number of progesterone receptor (PR) expressing cells (Haslam et al., 1979). Expression of several growth factor genes is also stimulated by estradiol and progesterone in mice (Bocchinfuso et al., 1997).

Since hormone action requires expression of receptors in target cells, estrogen receptor (ER) and PR are often evaluated in research on the mammary gland. In ER knockout mice, the mammary gland is underdeveloped (Bocchinfuso et al., 1997). In mice there are two ER expressed, α and β (Kuiper et al., 1996). However, in bovine there are only very low concentrations of ER β transcripts in all stages of mammary development. Furthermore, ER β is undetectable via immunohistochemistry in the bovine mammary gland (Connor et al., 2005). Therefore, in the bovine mammary gland researchers have focused on ER α . For example, there was an increased in the portion of ER α positive cells in ovariectomized heifers compared to control heifers (Berry et al., 2003b). However, it is not the ER α positive cells that are usually proliferative. Instead it seems that estrogen works in a paracrine manner to increase growth in adjacent cells (Capuco et al., 2002).

Simple tabulation of the presence/absence of receptor expressing cells explains only a part of the story behind the effect of estrogen and progesterone. It may be that the location or

position of receptor expressing cells within the developing ducts is also important. In mice, PR positive cells are most often localized in the luminal face of the mammary ducts (Silberstein et al., 1996). During the prepubertal period ER α and PR were usually found in the nuclei of the intermediate layer of epithelium (Connor et al., 2005). ER α positive cells are mostly located in a medial position within ducts in ovariectomized calves (Berry et al., 2003b). Moreover, the greatest expression of ER α and PR transcripts is during the prepubertal period (Connor et al., 2005). Therefore, during the prepubertal period there needs to be focus on ER α and PR presence and position to better able understand the estrogen and progesterone signaling during this developmental phase.

Myoepithelial Cells

Most prior studies of early mammary development have focused on ductal development and specifically on the epithelial cells. However, myoepithelial cells are also present during the prepubertal period. In mice, the cap cell layer which is associated with the end bud, is continuous with a layer of myoepithelial cells arranged in a single row on the subtending ducts (Williams et al., 1983). MEC are not always surrounded by myoepithelial cells. In the developing terminal end buds of mice there is a general lack of myoepithelial cells (Sonnenberg et al., 1986). This may allow for MEC to be more readily exposed to different growth factors.

In the developing bovine mammary gland, myoepithelial cells are typically round in shape and do not form a continuous ring around the circumference of the ducts. However, in calves ovariectomized prior to the allometric growth phase, the morphology of myoepithelial cells is altered. Specifically they appear more elongated in shape, which suggests the cells are becoming prematurely differentiated. The idea that greater elongation of myoepithelial cells equates to greater differentiation is based on observations that in fully developed mammary glands, the myoepithelial cells surrounding the alveoli are distinctly stellate and elongated so that they create an inter-woven layer around the alveoli. In this fully differentiated state it is hypothesized that the myoepithelial cells have the potential to block the MEC from the effects of growth factors (Ballagh et al., 2008). If MEC are impaired from responding to important growth factors because of the myoepithelial cells, the blocking of MEC from growth factors could explain the marked decrease in the growth of mammary parenchyma which occurs when prepubertal calves are ovariectomized. In ovary-intact calves there are also fewer differentiated myoepithelial associated with the developing ducts (Ballagh et al., 2008).

Increased myoepithelial differentiation in ovariectomized calves happens concurrently with the appearance of larger and less arborescent ductal structures in the developing parenchyma. Ovariectomy also alters the morphology of the cellular projections that are found on bovine myoepithelial cells. These cell extensions are believed to be important in the regulation of tissue architecture as the ducts develop (Safayi et al., 2012).

In mice and other mammals it is well recognized that the myoepithelial cells surrounding the mammary alveoli express smooth muscle cell markers. However, it is known now that these markers are expressed by myoepithelial cells present in the developing mammary ducts, as early as 1.5 weeks of age in mice (Deugnier et al., 1995). This supports the idea that at least some aspects of myoepithelial cells differentiation can occur long before formation of alveoli and that these changes may play a role in regulation of normal mammary development. If this differentiation normally occurs early in development there are likely signaling pathways that are activated to initiate these responses. Since ovariectomy acts to promote premature myoepithelial differentiation, it seems likely that factors directly from the ovary or agents induced because of ovarian signals normally act to prevent inappropriate differentiation. Cultured bovine mature myoepithelial cells proliferate in response to the addition of IGF-1 and Epidermal Growth Factor (EGF) (Zavizion et al., 1996). Perhaps ovarian signals depress the capacity of myoepithelial cells to respond by reducing IGF-1 or EGF receptor expression in myoepithelial cells. If this hypothesis is correct, ovariectomy would essentially release the suppression of the myoepithelial cells allowing greater than normal myoepithelial cell proliferation and perhaps increased differentiation as well.

Label Retaining Cells

Stem cells have ability to self-renew and generate multiple cell lineages. Adult stem cells have a more limited capacity compared to embryonic stem cells, but are multipotent. One stem cell can regenerate the entire mammary gland parenchyma in a mouse with a previously epithelial cleared MFP (Kordon et al., 1998).

The majority of the research done on mammary stem cells is in humans and mice, but turnover of cells are what allow cows to have multiply lactations. Therefore, there has to be cells that are programmed to provide the progenitor cells that replace lost apoptotic cells. These are the mammary stem cells. Some of the research that has occurred in mice and humans can also translate into the bovine. Bovine, murine, and human stem cells share the same markers beta 1-integrin and alpha 6-integrin (Li et al., 2009). However, bovine stem cell research is still in its infancy in comparison to humans and mice.

There are two types of cell divisions involving stem cells, symmetric and asymmetric. Asymmetric is self-renewing because the end result is the same cell and a progenitor cell. The incorporation of 5-Bromo-2'-deoxyuridine (BrdU) and its retention is used to identify putative stem and progenitor cells because BrdU incorporates into the DNA and remains in the DNA through cell divisions. After a sufficient 'wash out' period, retention of BrdU in a small population of epithelial cells along with the lack of expression of other proliferation markers (Ki67) and ER receptor have been suggested as an index to identify putative bovine mammary stem cell and/or progenitor cells (Capuco et al., 2012). These are described as label retaining cells (LRC).

Ideally, it would be better if researchers were able to confine their search to a specific area in the mammary tissue to look for these stem cells. However, growth within the bovine TDU is not confined to the invading tips of the structure. Proliferating cells are found throughout the TDU (Capuco et al., 2002). In the basal region of the ductal structures there are cells that are aligned with the myoepithelial cells, but they do not express myoepithelial cell markers. It is proposed that these cells may be stem or progenitor cells (Safayi et al., 2012). The incorporation of basement membrane components is shown to increase the ability for mammary organoids in cell cultures to produce putative stem and progenitor cells (Holland et al., 2007). Based on locations where stem and progenitor cells are likely most common a focus on the search for stem cells near the basement membrane/myoepithelial cell layer of the developing mammary gland is logical.

Another way of trying to narrow down the search for the stem cells is to determine what they do not express. For example, actively proliferating epithelial cells rarely express ER α (Capuco et al., 2002). Therefore, when trying to find markers for stem cells the absence of ER α expression seems a likely requirement. Variation in the retention of stains or dyes has also been evaluated as a marker for possible stem cells. For example, lightly stained (pale) cells are found singly and in pairs within the epithelial cell layer. These cells also account for 80-90% of the BrdU label retaining cells. This suggests that stem cells are part of this pale cell population. However, this population is far too large to be stem cells alone. A pale cell found singly has been proposed to be a stem cell that has undergone a division to produce a copy as well as a daughter cell that has subsequently differentiated into one of the cell lineages of a MEC or a myoepithelial cell. A pale cell found next to an intermediate stained cell is thought to have undergone a division to produce a progenitor cell (intermediate staining). These are both examples of

asymmetric division. When there were two pale cells next to each this was proposed to represent cells that underwent symmetric division to produce two stem cells. Interestingly, these pale cells are also located in the basal layer and rarely come in contact with the lumen. This location characteristic matches speculation about where the population of stem cells are likely housed in the developing bovine mammary ducts (Ellis et al., 2002).

To identify the best time to manipulate stem cells it would be important to know when they were available in the greatest quantity. Assuming the population of lightly stained cells includes stem cells, the abundance of pale cells decreased in heifers from 2 and 8 months of age. However, given pale stained cells accounted for about 10 percent of the total population of epithelial cells it seems clear that this population includes cells other than stem cells i.e. progenitor cells (Ellis et al., 2002).

There is potential to manipulate the number of stem cells. Xanthosine, a nucleoside, is able to inhibit the transcription factor p53, which increases the number of asymmetric divisions in the stem populations. Protein p53 does this by down-regulating inosine-5'-monophosphate dehydrogenase, which is the rate limiting enzyme for guanine nucleotide synthesis. Xanthosine treatment increased the number of BrdU retaining cells in the prepubertal bovine mammary gland; however, this is thought to be only a temporary increase (Capuco et al., 2009). If researchers were able to increase the number of stem or progenitor cells then the ease of replacing cells in subsequent lactations could be increased. However, it is also known that stem and progenitor cells develop at slower rates compared to normal cells. For example, when MEC are kept in cell culture with longer times to develop there were more stem and progenitor cells. Faster development times lead to there being more differentiated cells (Holland et al., 2007). If this also occurs *in vivo* then accelerated growth programs may be hampering the ability of the

mammary gland to use the stem cells to their optimal potential of renewing the cell population.

The better the cow's ability to regenerate their mammary gland the greater the number of lactations the cow can experience.

Objectives

The overall objective of this study was to block the action of estrogen and determine the effect on mammary development in prepubertal heifers.

Objective 1: To determine if treatment of heifers with tamoxifen alters the proportion of mammary epithelial cells which expressed ER α /PR as well as the degree of expression and the location of expressing cells within the developing mammary ducts.

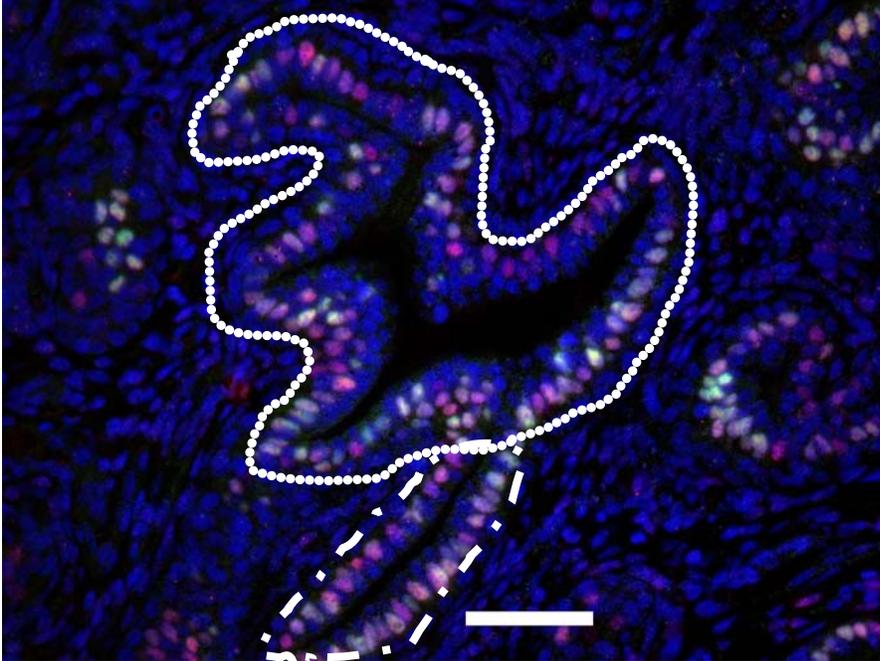
Objective 2: To determine the effect of blocking estrogen action with tamoxifen on the myoepithelial cell population.

Objective 3: To determine the impact of tamoxifen treatment on the number and location of label retaining cells in the developing mammary gland.

Figure 1.1: An example of developing murine parenchyma tissue invading the MFP taken by Akers *et al.*, 2000.



Figure 1.2: An example of developing bovine parenchyma tissue. The scale bar represents 50 μm . The structure surrounded by the dotted line is the TDU. The structure surrounded by the dashed line is a developed duct.



Materials and Methods

Animals

This experiment was conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee, 11-208-DASC. Sixteen female heifer Holsteins at 2 weeks of age were purchased and brought to the Virginia Tech Dairy Farm on February 1, 2012. They were individually housed in calf hutches and fed twice daily with milk replacer, in accordance with standard farm practices. Land O'Lakes milk replacer Cow's Match® Cold Front™ was given at the rate of 6 pints per feeding with a dry matter of 17.6%. Water and pelleted grain was always available. On February 3 heifer 84 (CON) was given an IV of fluid and electrolytes due to dehydration. On February 27 heifer 92 (TAM) was treated for colic by being given 1 ml Banamine (Merck Animal Health; Summit, NJ). The heifers were weaned to receive only pelleted grain and hay at 8 wks of age. Once weaned the heifers were comingled in group housing based on their age. Heifer 88 in the CON group died for unknown reasons on April 26, 2012. The final numbers were $n = 7$ for CON and $n = 8$ for TAM.

Injection Management

The carrier for the tamoxifen injections was composed of 30% ethanol, 30% benzyl benzoate, and 40% corn oil. The CON group animals were injected with the carrier solution. A 10 mg/ml stock solution of tamoxifen citrate (catalog number AAH27260-06; VWR-International Inc.) was prepared by dissolving the tamoxifen citrate in a solution of 50% ethanol, and 50% benzyl benzoate. The stock solution was sterilized by passing through a 0.45 μm filter into sterile bottles. Corn oil was sterilized by autoclave and finished injectable solutions were

created by aseptically combining corn oil and tamoxifen citrate stock solution or ethanol/benzyl benzoate solution in sterile vials with septum closures.

Heifers were randomly assigned to CON or TAM groups (Table 2.1). The first injection day was February 17, 2012. The heifers were enrolled into the experiment when they reached 28 days of age. They were injected subcutaneously daily until 120 days of age with a weight dependent dosage of each treatment. Heifers in the TAM were given of 0.3 mg/kg of tamoxifen. Heifers in the CON were injected with an equivalent volume of the carrier. Injection volumes were adjusted as required biweekly when the heifers were weighed and hip heights recorded.

To minimize possible site of injection reaction four sites were used. The left and right shoulder on each heifer and either the upper or lower portion of the scapula. Each site was rotated throughout the whole trial. If a heifer had evidence of injection site reaction the site was not used until the swelling visibly decreased.

At an average 101.7 days of age (range of 101-103 days) heifers were injected intravenously for five consecutive days with BrdU (5-Bromo-2'-deoxyuridine; Alfa Aesar; Ward Hill, MA; catalog number H27260), at a dosage of 5 mg/kg. The BrdU was prepared in sterile saline with a pH of 8.5.

Slaughter Procedure

The heifers were euthanized May 21-23 at Virginia Tech's Veterinary Facility. The heifer's averaged 120.7 days old (range of 120-122 days). Control animals were euthanized first on each day. Heifers were sacrificed using a commercial phenobarbital solution administered intravenously (Euthasol, 10 mg/kg BW; Butler Schein Animal Health Supply) and then exsanguinated.

Mammary Tissue Sampling

The mammary gland was collected within 20 minutes of slaughter. The mammary gland was bisected medially into left and right hemispheres. The right hemisphere was immediately weighed as a gross measurement of udder size. It was then wrapped in aluminum foil and snap frozen by immersion in liquid nitrogen. The left fore quarter was used to collect samples of parenchyma and MFP tissue that were immediately snap frozen in liquid nitrogen. The left rear quarter was sampled to collect tissues for histological analysis and immunohistochemistry. Prior to these analyses this quarter was trimmed of excess fat pad tissue, butterflied (Figure 2.3), and immediately placed into a container with 10% neutral buffered formalin for 48 hours (Table 2.3). The quarter was then transferred to 70% ethanol for storage. After the quarter was fixed the MFP and any remaining fragments of skin or extraneous tissue were removed from the parenchyma tissue. A small bit of skin on the distal end of the teat remained to allow for appropriate positioning for subsampling. Parenchymal tissue from the left rear quarter was then weighed and recorded. Parenchymal tissue samples were collected from one of the butterflied segments along three zones (1-3). Zone 1 was just above the cisternal portion of the quarter nearest the teat. Zone 2 was medial to zone 1 and 3. Zone 3 was at the parenchyma and MFP interface (Figure 2.4).

Embedding of Tissue and Preparation of Slides

Formalin fixed tissues were prepared for paraffin embedding using an automated tissue processor (Leica TP 1020; Leica Microsystems Inc.; Buffalo Grove, IL). Samples were dehydrated through increasing concentrations of ethyl alcohol (70% x 2, 80% x 2, 95% x 2, 100% x 2), cleared in xylene (x 2) and infiltrated in melted paraffin (x 2). Paraffin infiltrated samples were then embedded in paraffin blocks.

Tissue sections were cut at 5 μm using a microtome (Model HM 340 E, Microm, International GmbH, Germany) and mounted on SuperFrost Plus glass slides (VWR International, Radnor, PA). Slides were dried on a warming plate overnight.

Hydration and Antigen Retrieval of Slides

Equal numbers of CON and TAM slides were processed in each staining run. Slides, less than one month old, were deparaffinized in xylene (3 x 3 min) and hydrated through a descending grade of ethanol washes (100%, 3 min x 2, 95%, 3 min x 2, 70%, 3 min x 1) into distilled water (3 min). Slides were deparaffinized, hydrated, and processed for antigen retrieval in a single day.

Antigens were retrieved by boiling slides in 10 mM citrate buffer, pH 6.0, (Table 2.3) in a microwave for 5 minutes, cooling for 5 minutes, and boiling for another 5 minutes. Slides were then cooled in the buffer for 30 minutes and subsequently washed in PBS, 3 x 2 minutes (Table 2.3). Individual tissue sections were circled with a PAP pen (Ted Pella Inc.; Redding, CA) to facilitate individual treatment of tissue sections on slides. All tissue sections were then blocked with CAS Block (Life Technologies Corporation; Grand Island, NY) for 15 minutes.

Primary Antibody

After aspiration of CAS Block, approximately 50 μL of fresh primary antibody solution was added to each tissue section as appropriate. Dilutions for each primary solution are given in Table 2.4. Slides were incubated with primary antibody overnight at 4 °C in a humidified chamber. One section per a slide served as a negative control and received CAS Block as a substitute for primary antibody solution. The remaining sections on the slide served as replicates for the desired immunofluorescent labeling.

Secondary Antibody

After removal of the primary antibody via a vacuum aspiration the slides were rinsed in PBS three times for five minutes each time. Dilutions for each secondary antibody are given in Table 2.5. All tissue sections received 50 μ L of the secondary antibody mixture and were incubated for 60 minutes at room temperature. Secondary antibody solutions were then aspirated and slides were rinsed in PBS (2 x 5 minutes) PAP pen circles were removed with a cotton swab dipped in xylene. Slides were rinsed again in PBS, aspirated to remove excess liquid, and coverslips were mounted using Slowfade Gold antifade reagent containing DAPI, a counter stain that stains all cell nuclei (Life Technologies Corporations; Grand Island, NY). Completed slides were maintained a minimum of 1 hour in the dark before imaging.

Nuance System

Photomicrographs were taken within 48 hours of finishing the staining procedures. Images were acquired using a Nuance FX multispectral imaging system mounted on a Nikon Eclipse 800 epi-fluorescence microscope that was fitted with Plan Fluor 20x and 40x objectives. Excitation light was generated using a mercury lamp light source and standard filter cubes fitted with long pass emission filters (Table 2.6). The Nuance system was configured to use multiple customized emission filter sets. The combination of standard filter cube and custom Nuance emission settings for each fluorophore is listed in Table 2.7.

ImagePro-Cell Counting Estrogen Receptor α /Progesterone Receptor

All of the TIFF pictures taken by the Nuance System were converted into JPEGs, for use by the ImagePro software. This allowed for reduction of background as necessary to readily identify stained cells. The individual files for each image were pseudo colored; DAPI blue, PR

green, and ER red. The individual channel files were used to create composite images for display or evaluation.

Individual areas within each composite image were then outlined for evaluation. Areas were defined by having at least 20 cells that were stained with DAPI that were within the parenchyma area. There were 3-4 areas selected per image. Two types of areas were selected for detailed analysis: (1) Class 1 or (2) Class 2. The Class 2 areas were deemed less uniform than the Class 1 areas. The Class 1 areas had 3-4 well developed layers of cells. Class 2 areas generally had cells that were grouped together and frequently “bulged” into the stroma or lumen (Figure 2.5).

Pilot counting was done to determine the optimal number of areas or structures that needed to be counted to minimize within animal variation balanced by overall workload. To accomplish the count two randomly selected animals one CON and one TAM had all of their areas counted across all the zones. I determined that counting 5-6 Class 1 areas per zone for each animal and 3-4 Class 2 areas per zone was satisfactory. Specifically, counting additional regions had minimal impact on average values or variation.

Areas selected for evaluation were used to determine eight different response variables. First the total number of epithelial cells was counted. These were cells whose nuclei were stained with DAPI. Cells that expressed PR and ER α were also counted. In addition the location of each labeled cell was also classified. That is cells were recorded as having a lumen, medial, or basal position. Luminal cells were defined as being in contact with the lumen of the ductal structure. Basal cells were in contact with the basement membrane. Medial cells were in contact with neither the lumen nor basement membrane (Figure 2.5). This gave 6 of the categories used to

classify steroid receptor expressing cells: PR luminal, PR medial, PR basal, ER α luminal, ER α medial, and ER α basal. The last category counted were cells that were labeled for both ER α and PR. The position of these cells was not noted. Based on these classifications the number of unlabeled cells was also determined. The following formula was used total number of cells/area minus (ER α luminal+ ER α medial+ ER α basal-dual labeled cells) minus (PR luminal+PR medial+PR basal-dual labeled cells) minus dual labeled cells. All the counts were recorded in an Excel spreadsheet.

ImagePro-Estrogen Receptor α /Progesterone Receptor Intensity

Photomicrographs were evaluated by identifying the ten cells in each image that expressed the most intense signal of antibody. Tissues from zones 1 and 3 were used to evaluate ER α and PR expression intensity. ImagePro software was then used to measure the mean intensity of each selected cell. The same photomicrographs used to count of the ER α /PR expressing cells were used for this analysis.

Cellprofiler

Cellprofiler (Carpenter et al., 2006) is a software program that uses the black white pictures collected in correspondence with output from selected wavelengths (particular filter cube combinations) and overlays these pictures to identify target areas where individual cells are analyzed. The computer system utilizes complex algorithms to evaluate populations of cells with common morphological characteristics. Briefly, each channel (DAPI, FITC, and Texas Red) is captured into the program. The programmer is then able to lower the “noise” in each channel by amplifying the signal to identify target areas. The specific channel used in this case was p63/Texas Red because this stain identifies the nuclei of myoepithelial cells. This channel is then overlaid on the other channels, so that the background noise is erased from the raw

photomicrograph. The software algorithms then use the unprocessed pictures corresponding to the selected target areas. Data from every pixel in each of the target areas provides the output data used for the analysis. The different algorithms allow multiple characterizations of the target area, in this case characteristics of myoepithelial cell nuclei. For example, area is the number of pixels in the target area i.e. the cell nucleus. Perimeter is the number of pixels on the outer edge of the nucleus. Eccentricity is a measure of how circular or elongated the nucleus is based on a scale of 0-1. Zero is a symmetric circle and 1 is a line segment. Neighbor Contact Percentage is how much of the nucleus is touching another nearby nucleus. The Zernike numbers represent localization of patterns within the shape of the nuclei with no influence from intensity or texture of the pixels (Boland et al., 1998). Difficult to easily visualize, the Zernike number classification nonetheless provides an ability to further characterize cell morphology based on staining features, in this case, staining of p63 expression in the nucleus. Other stains for example could be used to characterize the cell nucleus, the cytoplasm, or both depending on the specificity of the staining and the capacity and ability to define the desired populations of cells.

ImagePro-Cell Counting Label Retaining Cell

There were 10 photomicrographs taken for each animal in each zone. All images were counted for an average of 900 cells evaluated for each animal. LRC were defined as cells that did not express ER α or Ki67, but heavily expressed BrdU. These LRC served as a proxy for putative mammary stem cells. Definition of heavy expression was determined by the focus, range in the histogram, consistency of coverage, and percent of stain coverage on the cell. The cell had to be in focus at the time that the image was taken. The cell had to express BrdU at a minimum of 5,000 mean density units. The density of the expression of BrdU had to be consistent over the nuclear area and the stain had to cover at least 75 percent of the nuclear area.

Estradiol Assay

Plasma concentrations of estradiol were determined using a commercially available RIA kit (Ultra-sensitive estradiol assay; DSL-4800 (Diagnostic Systems Laboratory, Webster, TX) which is currently sold by Beckman Coulter (Brea, CA 92822). Procedures were essentially as described by Gibbons et al. (1999) for the bovine. To prepare for assay, 5 ml samples of plasma **Error! Hyperlink reference not valid.** assay were placed in glass 25 ml acid washed screw cap tubes with Teflon liners and 10 ml of diethyl ether (E138-4, Fisher Scientific, Pittsburg, PA 15275) added. The tubes were vortexed for two minutes (VWR Model DVX-2500 Multi-tube vortexer, VWR Scientific, Radnor, PA 19087) operating at 2500 rpm. The tubes were then placed in a dry ice ethanol bath and the lower aqueous layer was frozen and the upper organic phase was decanted into an acid washed glass 15 screw top tubes. The decanted tubes were placed into an N-EVAP 111 nitrogen evaporator (Organomation Associates, Inc. Berlin, MA 01503) and the organic phase allowed to evaporation under a stream of nitrogen and gentle heating (38-40°C). The extraction process was repeated three times and the dried tubes capped until further processing. Once all the samples were extracted, the dried tubes were rinsed with 1 ml of diethyl ether and contents transferred to a 12 x 75 mm assay tube. The tubes were allowed to evaporate then the dried contents reconstituted in RIA assay buffer (0.01 M phosphate buffered saline, pH 7.2 with 1g/L) gelatin. For the assay we followed the manufacturer's instructions with one exception, we utilized a stock estradiol solution to create a standard curve which ranged from 0.156 pg/tube to 40 pg/tube. All samples were run in a single assay. Extraction efficiency was estimated from the recovery of radiolabeled estradiol diluted in plasma and averaged 86.2 % \pm 1.7. Presented estradiol values are not corrected for extraction efficiency.

DNA Content

The DNA content of parenchymal tissue was determined essentially as previously described Daniels et al. (2009). Briefly, 450 mg of tissue was weighed and homogenized in 1.5 ml of high salt buffer (0.05 M Na₂HPO₄ + 2 M NaCl + 0.002 M Na₂EDTA pH 7.4). Homogenates were centrifuged 1,000x g for 10 minutes at 4 °C to produce a supernatant for DNA assay. The supernatant was transferred to a microfuge tube for subsequent analysis. Samples of the supernatant (2 µl) were transferred to a tube containing 2 ml of assay solution. The assay solution was composed of 100 µl of 1mg/ml Hoechst H 33258 + 10 ml of 10 mM Tris-Cl + 90 ml of H₂O. Triplicate samples were measured using a Hoefer DQ 300 fluorometer (Hoefer Inc., San Francisco, CA). Intraassay CV's averaged 6.2%.

Real-time qPCR

Parenchyma was taken from the left fore quarter of the udder at time of slaughter. Samples were then stored -80 °C until RNA was isolated. Protocols for isolation were used following purification of total RNA from animal tissues from the RNase Mini Kit (Qiagen; Velencia, CA; catalog number 74104) and DNase 1 digestion (Qiagen Inc.; Velencia, CA; catalog number 79254). Integrity of RNA was evaluated with a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc.; Wilmington, DE). Only samples with a ratio of optical measurements at 260 and 280 nm (OD 260nm/OD 280nm) greater than 1.8 were accepted. The integrity of 18S and 28S ribosomal RNA was evaluated with gel electrophoresis with 1% agarose gel and visualized by ethidium bromide staining under UV light.

Reverse transcription involved synthesizing single stranded cDNA via High Capacity cDNA Archive Kit (Life Technologies Corporations; Grand Island, NY). Briefly, 4 µg of RNA was reverse transcribed to single stranded cDNA in a final reaction volume of 40 µl using

random primers. The cDNA and no reverse transcriptase controls products were diluted 1:100 in sterile nuclease-free water. A total of 2 μ l of cDNA was used in the quantitative real-time PCR in conjunction with 12.5 μ l of SYBR Green dye (Applied Biosystems; Foster City, CA), 9.5 μ l of sterile distilled water, 0.5 μ l of 10 μ M forward primer, and 0.5 μ l of 10 μ M reverse primer. The PCR conditions were: 95 $^{\circ}$ C for 10 min., 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 1 min. this repeated for 40 cycles. Reactions were performed in a 7300 Series Real-Time System and data were collected and analyzed using SDS software (Applied Biosystems; Foster City, CA).

Triplicates of each sample were assayed and average standard error was 0.046. Each PCR plate contained a no reverse transcriptase control for each sample and a no template control (nuclease-free water instead of cDNA template). Average cycle threshold (Ct) values of replicate samples were collected using the SDS software for each target gene and endogenous reference genes and were exported to Microsoft Office Excel. All primers used were published in literature. Three endogenous reference genes were used to normalize the data: protein phosphatase 1 regulatory (inhibitor) subunit 11 (PPP1R11), ribosomal protein S15A (RPS15A), and mitochondrial GTPase 1 homolog (MTG1) (Piantoni et al., 2008).

Statistical Analysis

Data were analyzed using the Glimmix procedure of SAS (SAS Institute, Inc., Cary, NC). The main effects for the ER α /PR data were treatment, zone of parenchymal tissue, type of area count, and the interactions between these three effects. The main effects of the myoepithelial data were treatment, zone of the parenchymal tissue, eccentricity label, and interactions. The main effects for the label retaining cell data were treatment, and the interaction of treatment and zone. Pair wise comparisons were done with the Tukey-Kramer procedure. Least squares means \pm standard error of the means were reported, significance was declared at $P < 0.05$.

Table 2.1: Calf data that includes the ID number, treatment, beginning weight (Wt) in kg, beginning hip height (HH) in centimeters, first day of injection, and day the calves would 120 days old.

Calf	Treatment	Beginning Wt	Beginning HH	1 st Day Injection	End Day
80	TAM	46.4	77.5	2/19/2012	5/21/2012
81	TAM	53.6	82.6	2/21/2012	5/23/2012
82	CON	54.5	83.8	2/18/2012	5/20/2012
83	CON	47.7	76.2	2/21/2012	5/23/2012
84	CON	50.9	80	2/18/2012	5/20/2012
85	TAM	58.3	81.3	2/19/2012	5/21/2012
86	TAM	49.5	77.5	2/17/2012	5/19/2012
87	CON	40.5	76.2	2/20/2012	5/22/2012
88	CON	41	73.7	2/22/2012	5/24/2012
89	CON	47.3	78.4	2/17/2012	5/19/2012
90	TAM	47.7	78.4	2/20/2012	5/22/2012
91	CON	43.7	78.4	2/21/2012	5/23/2012
92	TAM	54	81.3	2/21/2012	5/23/2012
93	TAM	50.4	80	2/17/2012	5/19/2012
94	TAM	62.1	83.8	2/21/2012	5/23/2012
95	CON	50.4	83.8	2/19/2012	5/21/2012

Table 2.2: The dosage of injections given on a ml basis. The dosages changed biweekly.

Calf ID	Treatment	wk0	wk2	wk4	wk6	wk8	wk10	wk12
80	TAM	2.3	2.5	3.2	3.5	3.6	4.1	4.5
81	TAM	2.7	3.2	3.6	4.3	4.8	5.5	6.1
82	CON	2.7	3.2	3.8	4.6	5.2	5.9	6.1
83	CON	2.3	2.7	3.2	4.1	4.5	5.2	5.7
84	CON	2.5	3.2	3.8	4.5	5.2	5.7	6.1
85	TAM	2.4	2.5	3.2	4.0	4.7	5.0	5.9
86	TAM	2.5	2.7	3.6	4.3	5.0	5.5	5.9
87	CON	2.0	2.7	3.6	4.3	4.5	5.2	5.7
88	CON	2.0	2.5	2.7	3.0	3.2	-	-
89	CON	2.3	3.0	3.2	3.8	4.4	5.0	5.5
90	TAM	2.6	2.7	3.2	3.6	4.1	4.8	5.2
91	CON	2.2	2.5	3.2	4.1	4.5	5.2	5.7
92	TAM	2.7	3.2	4.1	4.8	5.5	6.4	7.0
93	TAM	2.5	3.0	3.6	4.5	5.2	5.5	5.9
94	TAM	2.6	3.0	3.6	4.3	4.8	5.5	6.1
95	CON	2.5	3.0	3.6	4.3	5.0	5.2	5.9

Figure 2.3: Parenchyma tissue “butterflied” for histological use.



Figure 2.4: The positioning of the different zones taken from the trimmed left rear quarter.

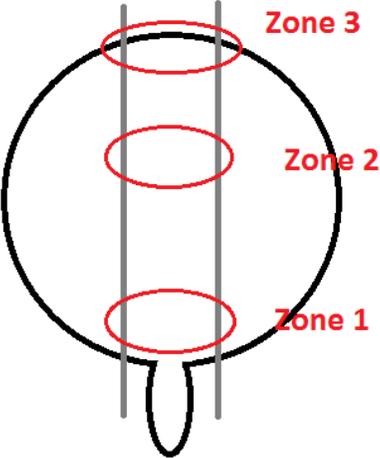


Table 2.3: Reagents used in the immunohistochemistry protocol.

Reagent	Composition
10 x Citrate Buffer	2.9 g sodium citrate (adjust volume to 1 L with distilled H ₂ O and adjust pH to 6.0)
1 x Citrate Buffer	100 ml 10 x Citrate Buffer + 900 ml distilled H ₂ O
10 % Neutral Buffered Formalin	4.0 g Sodium phosphate (monobasic) + 6.5 g Sodium phosphate (dibasic) + 100 ml 37 % Formaldehyde + 900 ml distilled H ₂ O
10 x PBS	21.4 g Na ₂ HPO ₄ + 5.76 g NaH ₂ PO ₄ + 87.8 g NaCl (adjust volume to 1 L with distilled H ₂ O and adjust pH to 7.4)
1 x PBS	100 ml 10 x PBS + 900 ml distilled H ₂ O

Table 2.4: Listing of the primary antibodies used for immunohistochemistry.

Primary Antibody	Manufacture	Catalog Number	Working Dilution
ER α (CON)	Santa Cruz Biotechnology	SC 787	1:400
ER α (TAM)	Santa Cruz Biotechnology	SC 787	1:200
PR (CON)	Santa Cruz Biotechnology	SC 708	1:50
PR (TAM)	Santa Cruz Biotechnology	SC 708	1:100
p63	Invitrogen	37-9500	1:500
CD10	Vector Laboratory Inc.	6009311	1:40
ER α (LRC)	Santa Cruz Biotechnology	SC 787	1:100
Ki67	Thermo Scientific	RM 9106 S0	1:150
BrdU	Millipore	MAB3424	1:100

Table 2.5: Listing of the secondary antibodies used for immunohistochemistry.

Secondary Antibody	Manufacture	Catalog Number	Working Dilution
Alexa 594 Goat Anti-mouse IgG _{2A} (ER α)	Invitrogen	A21135	1:200
Alexa 488 Goat Anti-rabbit IgG (PR)	Invitrogen	A11008	1:200
Alexa 594 Goat Anti-mouse IgG _{2B} (p63)	Invitrogen	A21145	1:400
Alexa 488 Goat Anti-mouse IgG ₁ (CD10)	Invitrogen	A21121	1:400
Alexa 532 Goat Anti-rabbit IgG (Ki67)	Invitrogen	A11009	1:200
Alexa 488 Goat Anti-mouse IgG ₁ (BrdU)	Invitrogen	A21121	1:200

Table 2.6: Excitation and emission wavelengths used for the Nuance System.

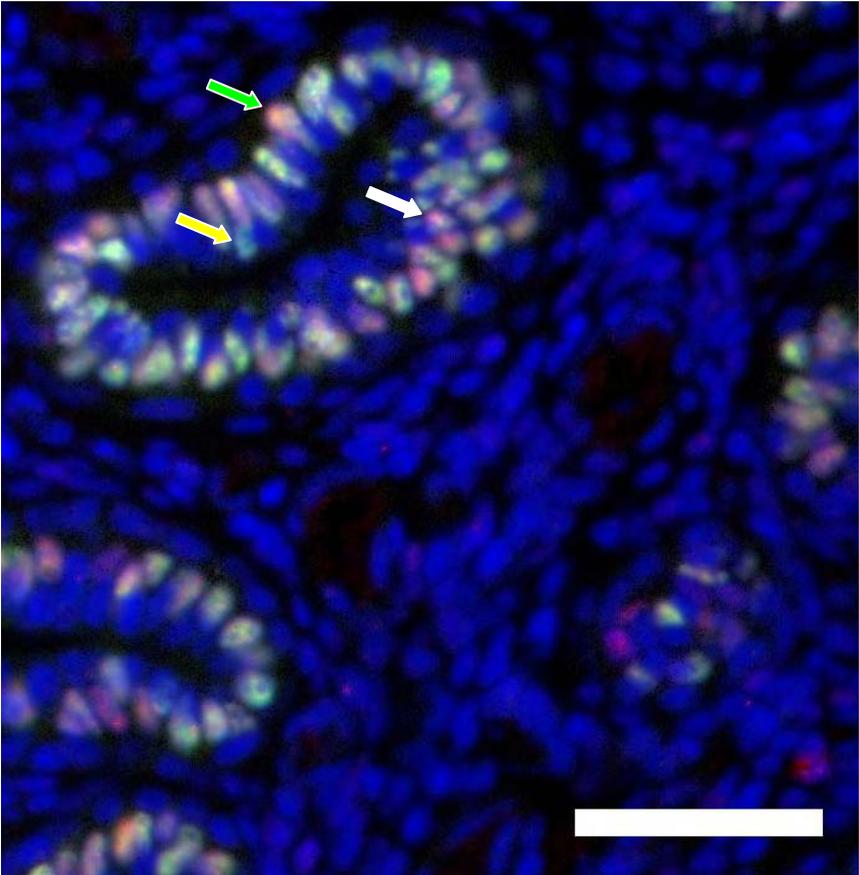
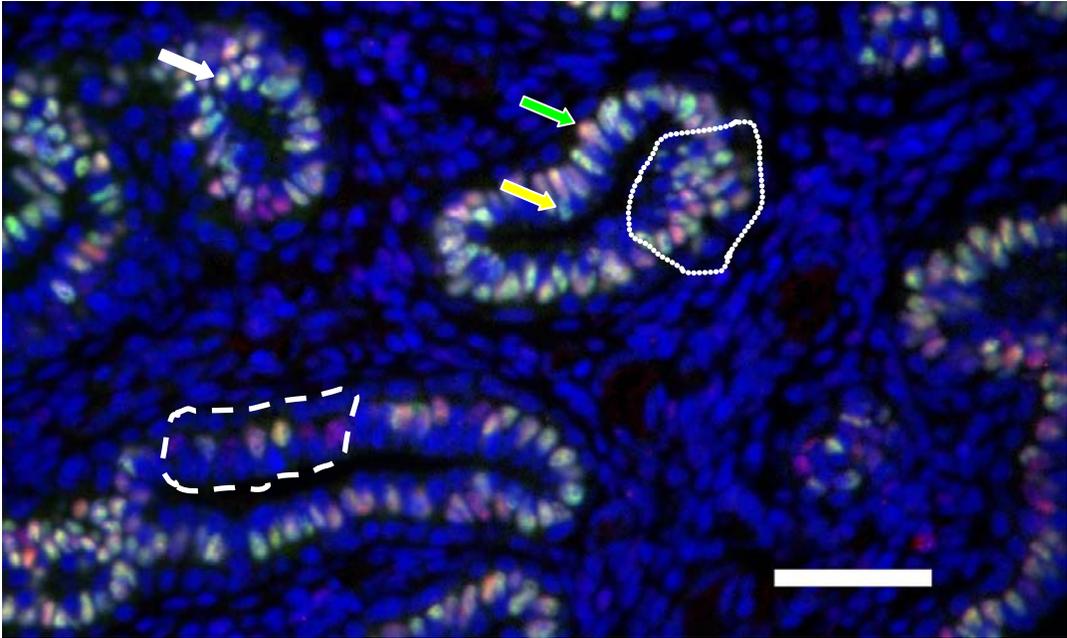
Cube	Excitation Filter (nm)	Emission Filter (nm)
UV-2E/C DAPI	325-375	420LP ¹
FITC	460-500	515LP
Texas Red	532-587	590LP

*¹LP Long pass filter

Table 2.7: The cube and emission setting used in the Nuance System for each secondary used with immunohistochemistry.

Fluorophore	Nikon Cube	Nuance Emission Setting (nm)
DAPI	UV-2E/C DAPI	490-560
Alexa 488	FITC HYQ	520-550
Alexa 532	FITC HYQ	540-570
Alexa 594	Texas Red HYQ	610-640

Figure 2.5: An example of parenchyma tissue with a scale bar that equals 50 μm . The dotted line represents a Class 2 area, the dashed line represents a Class 1 area, and the arrows indicate examples of cell positions luminal (yellow), medial (white), and basal (green).



Results

Biometrics

There was no significant difference between the body weights (BW) at the beginning or end of the trial between the treatments (Table 3.8). Neither was there a difference in estradiol concentrations in serum at the time of slaughter, CON 2.1 ± 1.0 pg/ml versus TAM 1.7 ± 0.6 $P = 0.37$ (Figure 3.6). There was a significant difference between CON (116 ± 9) versus TAM (55 ± 8) for udder parenchyma weight $P = 0.0002$ (Figure 3.7). Due to this difference in weight there is a difference in total amount of DNA content (Figure 3.8). However, the concentration of parenchymal DNA was nearly identical for the two treatments i.e. CON (1.7 ± 0.13) vs. TAM (1.8 ± 0.12).

Estrogen Receptor α /Progesterone Receptor

The position of ER α positive cells was not influenced by treatment. Luminal cells averaged (12.1% \pm 1.4 in CON and 10.7% \pm 1.3 in TAM). Medial ER α positive cells were present in the greatest abundance overall but proportions were not impacted by treatment (CON 28.7 % \pm 1.3 versus TAM 28.5 % \pm 1.2). Basal ER α positive cells were very rare averaging only 0.24% of total epithelial cells with similar proportions for each treatment (CON 0.22 % \pm 0.11 versus TAM 0.26 % \pm 0.27; Figure 3.9). ER α positive cells adjacent to the ductal lumen were more frequently ($P < 0.0001$) in the Class 1 areas (14.2 % \pm 1.1) than the Class 2 areas (8.6 % \pm 1.1). ER α cells found medially occurred more frequently ($P = < 0.0001$) in the Class 2 areas (35.1 % \pm 1.0) compared Class 1 areas (22.1 % \pm 1.1) of the epithelium. Similarly ER α positive cells in the basal layer were also more abundant ($P < 0.0001$) in the Class 2 areas of the epithelium (0.29 % \pm 0.09) versus (0.20 \pm 0.09).

PR positive cells followed a similar pattern, to ER α positive epithelial cells. Namely, the position (luminal, medial, or basal) was not significantly influenced by treatment. Proportions of expressing cells within position were also similar. Averages were luminal CON (11.6 % \pm 1.3) versus TAM (10.1 % \pm 1.3), medial (CON 27.5 % \pm 1.3 versus TAM 25.5 % \pm 1.2), and basal CON (0.22 % \pm 0.09) versus TAM (0.12 % \pm 0.08). By far the majority of the cells that expressed ER α or PR were medially oriented. Proportions of all PR expressing cells were significantly affected by area of epithelial structure evaluated. PR stained lumen cells were more common ($P < 0.0001$) in Class 1 areas (13.7 % \pm 1.0) versus Class 2 areas (8.0 % \pm 1.0), medial cells expressing PR were more common ($P < 0.0001$) Class 2 areas (32.6 % \pm 1.0) versus (20.4 % \pm 1.0). Like ER α positive cells, the proportion of PR expressing cells in the basal region were

much lower but there was a higher percentage ($P < 0.0001$) in the Class 2 ($0.21 \% \pm 0.07$) areas compared to the Class 1 areas ($0.14 \% \pm 0.07$).

While the proportions of ER α expressing cells were not impacted by treatment, the intensity of ER α expression was 6.3 times greater ($P = 0.00005$) in the CON treatment versus TAM (Table 3.9). This difference in level of expression was consistent for ER α positive cells in all positions. The intensity of PR intensity expression showed the opposite pattern, i.e. greater intensity in TAM calves ($P = 0.03$) but the difference in expression was only 42 % vs. 6.3 fold (Table 3.9). There were no apparent differences in treatment related PR expression intensity dependent on cell position. ER α intensity expression was corroborated with mRNA relative expression levels. CON had a 2-fold increase of mRNA levels over TAM ($P < 0.0001$; Table 3.10). PR mRNA relative expression levels were not significant; however, expression in TAM numerically was 60% greater compared to CON (Table 3.10). Both ER α and PR followed the trend that was seen with the receptor intensities.

In summary, the ER α /PR staining patterns were not impacted by treatment. For both ER α /PR the majority of the cells that were stained were in the medial layer of the epithelium (Figure 3.9; Figure 3.10).

Myoepithelial Cells

There was not a significant difference between treatments for overall myoepithelial cell eccentricity values, CON 0.64 ± 0.004 versus TAM 0.63 ± 0.004 (Table 3.11). But overall eccentricity value differed depending on the region in which the tissue was collected. Values for cells in zone 3 (peripheral area of the parenchyma) were lower ($P = 0.005$) than for zone 1 and 2 (0.63 ± 0.003 vs. 0.64 ± 0.003). More importantly, eccentricity values were used to classify myoepithelial cells based on the shape of the nucleus. Specifically, eccentricity values of 0.6 or less signify cells with a rounded nucleus, values greater than 0.8 indicate elongated nuclei and values of 0.6-0.8 indicate cells with an intermediate shape.

There was a significant effect of treatment ($P = 0.05$) on myoepithelial cell nuclear area, CON 1134 ± 36 versus TAM 1030 ± 33 (Table 3.11). Irrespective of treatment, there were also significant differences ($P < 0.0001$; Table 3.13) in pixel area per nucleus depending on shape classification. Elongated nuclei were largest (1211 ± 25) followed by intermediate (1055 ± 25) and round (977 ± 25) classified nuclei.

As with area values, nuclear perimeter values were also significantly different between treatments ($P = 0.02$; Table 3.11), CON 145 ± 3 versus TAM 133 ± 3 . As would be expected from area values, perimeter values were also significantly different ($P < 0.0001$; Table 3.13) depending on nuclear shape classification. Elongated averaged 155 ± 2 intermediate 136 ± 2 and round 127 ± 2 . Treatment differences were also detected within each of the shape classifications as follows: elongated 161 ± 3 CON vs. 150 ± 3 TAM ($P = 0.04$); intermediate 142 ± 3 CON vs. 130 ± 3 TAM ($P = 0.006$); round 134 ± 3 CON vs. 121 ± 3 TAM ($P = 0.002$).

Treatment altered the proportion ($P = 0.01$; Table 3.11) of myoepithelial cells that were immediately adjacent (touching) a neighboring myoepithelial cell; CON 5.6 ± 0.2 vs. TAM $4.8 \pm$

0.2. Myoepithelial cells with rounded nuclei were also more likely to be adjacent to other myoepithelial cells than those that were elongated ($P = 0.0003$; Table 3.13) or intermediate in shape ($P = 0.002$).

Histologically, the only noticeable difference between treatments was from the spacing of myoepithelial cells. TAM myoepithelial cells had wider spacing between them compared to CON (Figure 3.14). This difference is in agreement with the measurements from Cellprofiler.

Label Retaining Cells

There was a significant difference (2.9-fold; $P = 0.01$) between treatments for the percentage of label retaining cells measured, CON $0.08 \% \pm 0.03$ vs. TAM $0.23 \% \pm 0.04$ (Table 3.14). Overall, there were no significant differences between the zones for percentage of label retaining cells. However, there was a significant ($P = 0.02$) interaction between treatment and zone. The difference between treatments was greatest for tissue collected from zone 3. Specifically, in CON zone 3 label retaining cells averaged $0.05 \% \pm 0.03$ compared with $0.32 \% \pm 0.09$ in zone 3 tissue from tamoxifen treated heifers i.e. a 6.4-fold difference (Figure 3.15).

The label retaining cells routinely noted in one of two positions 1) the cell that was touching the lumen of the duct and appeared to be “entering” the lumen i.e. the majority of the cell was protruding into the lumen of the duct or 2) the cell was positioned in the basal layer of the duct touching the basement membrane. It seems likely that many of these cells were also apparent in the myoepithelial cell stained images. Specifically, these were cells that were in the basal layer of the duct that did not stain for p63 (Figure 3.16). Other cells that were positive for BrdU and were not in these two positions were not as heavily or consistently stained across the entire cell nucleus and were not included in the label retaining cell population.

Table 3.8: The mean and SEM for the beginning and end BW for each treatment group.

Treatment	Beginning BW	<i>P</i> -value	End BW	<i>P</i> -value
CON	47.6 ± 1.6	0.2	115.2 ± 5	0.95
TAM	50.4 ± 1.4		114.8 ± 4	

Figure 3.6: Serum estradiol concentration (pg/ml) immediately prior to slaughter in pg/ml from CON and TAM calves.

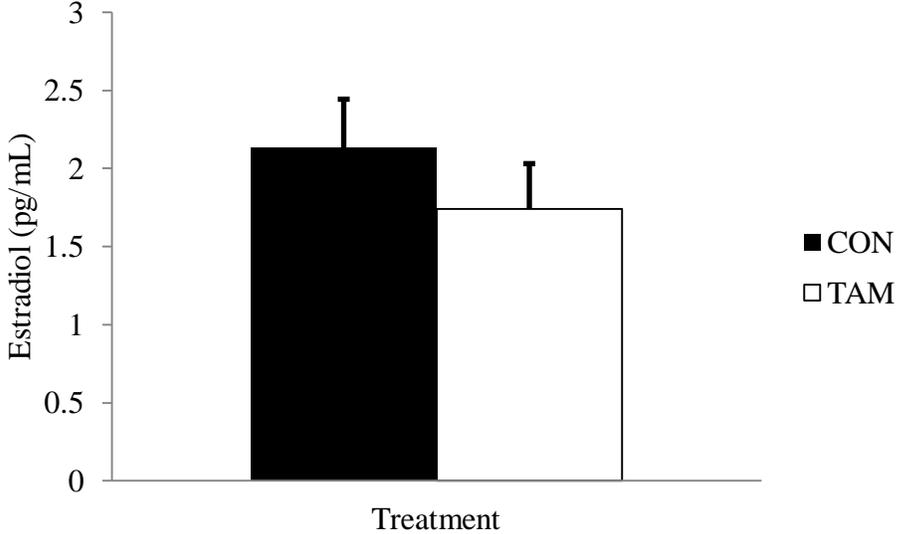
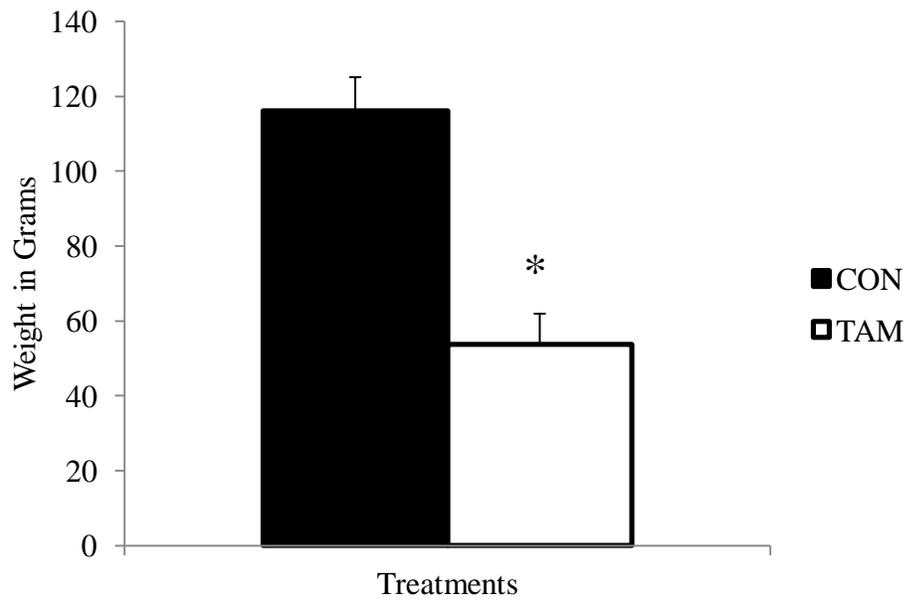
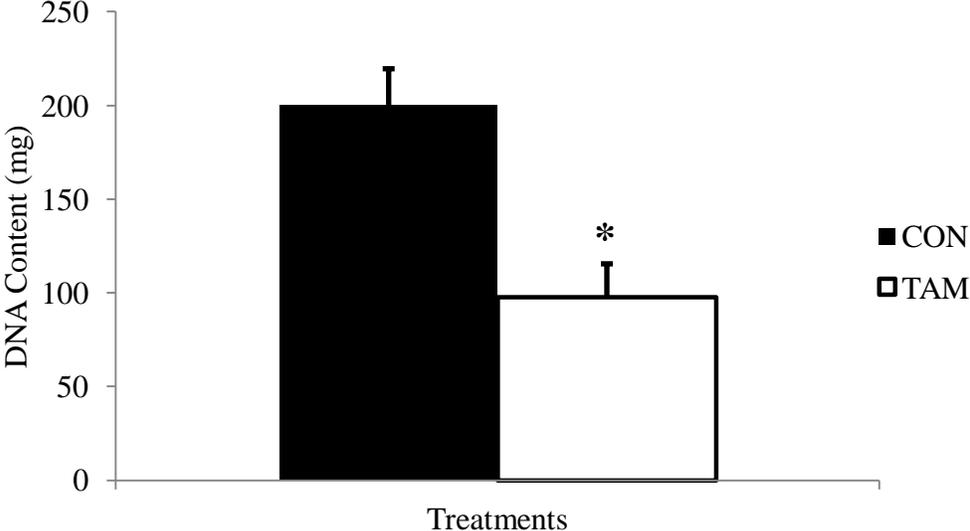


Figure 3.7: Parenchyma weights for the udder.



• $P = 0.0002$

Figure 3.8: Udder parenchymal DNA content in tamoxifen treated heifers.



- *($P = 0.002$)

Figure 3.9: ER α position in percentages by treatment.

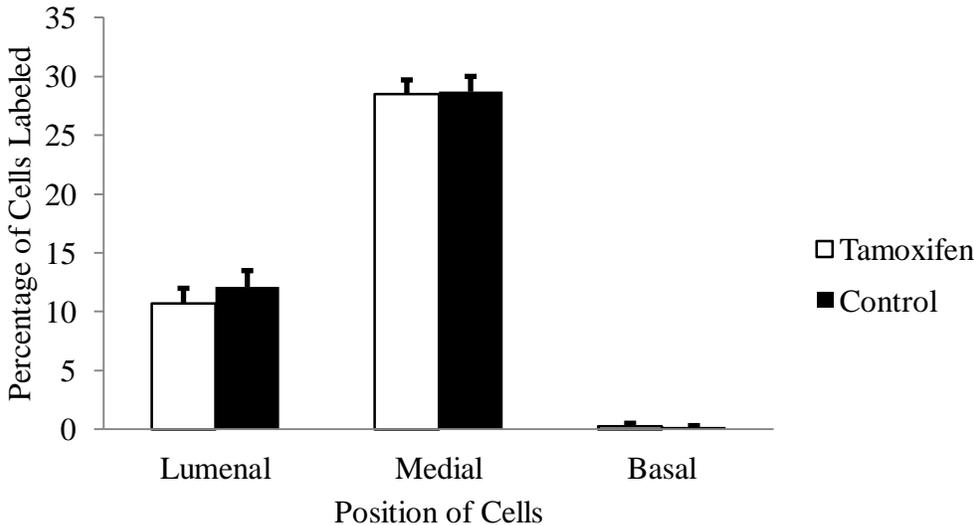


Figure 3.10: PR position in percentages by treatment.

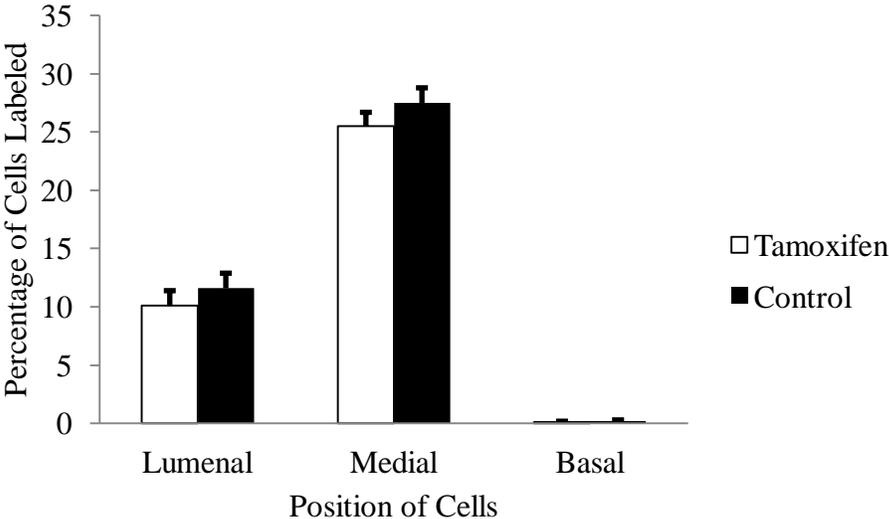


Table 3.9: ER α and PR intensity levels LSmean and SEM for each treatment group.

Treatment	ER Intensity	<i>P</i> -value	PR Intensity	<i>P</i> -value
CON	1259 \pm 146	0.00005	4950 \pm 712	0.028
TAM	202 \pm 42		7044 \pm 496	

Figure 3.11: ER α staining intensity in mammary tissue from CON (panel A) and TAM (panel B). The scale bar represents 50 μ m.

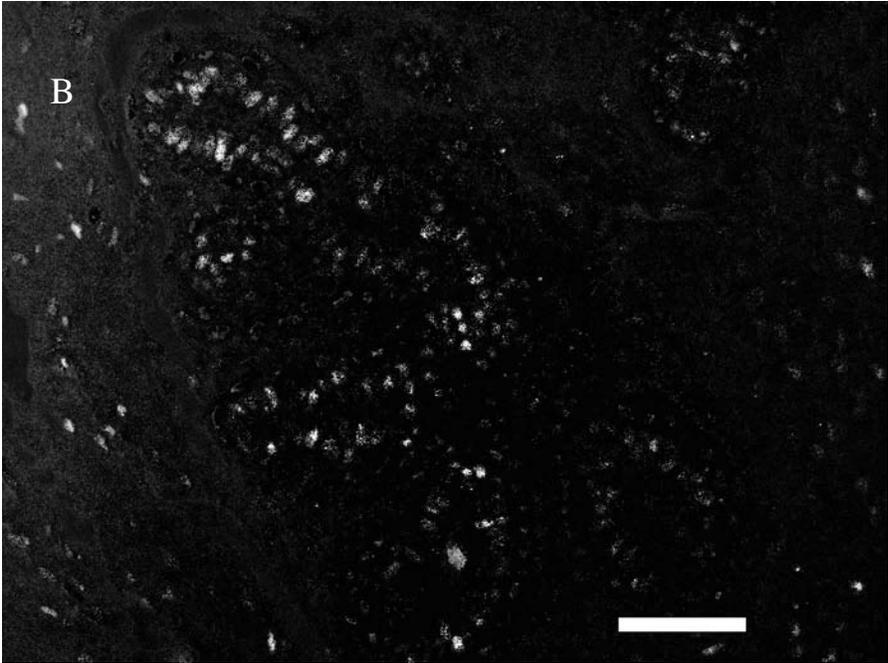
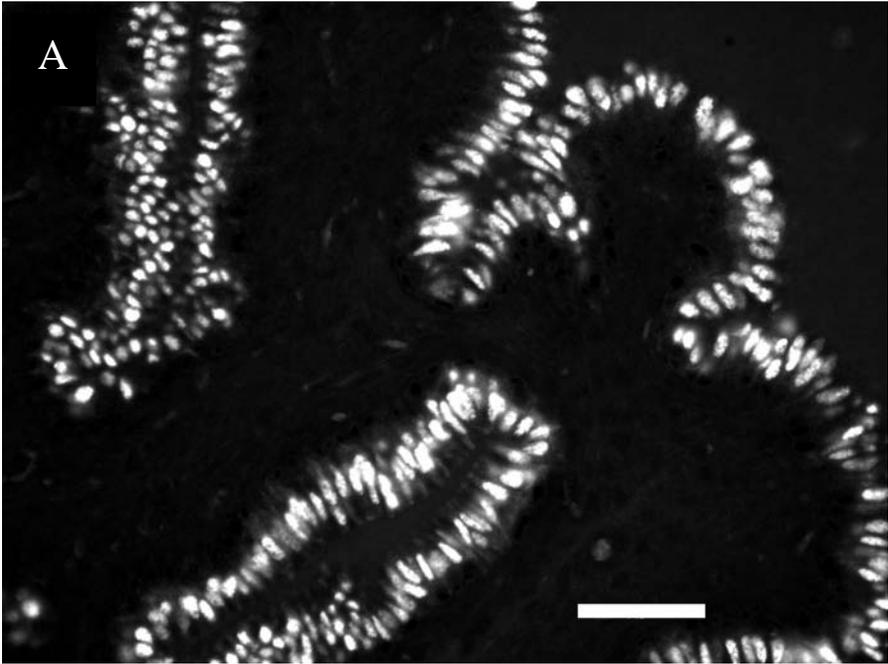
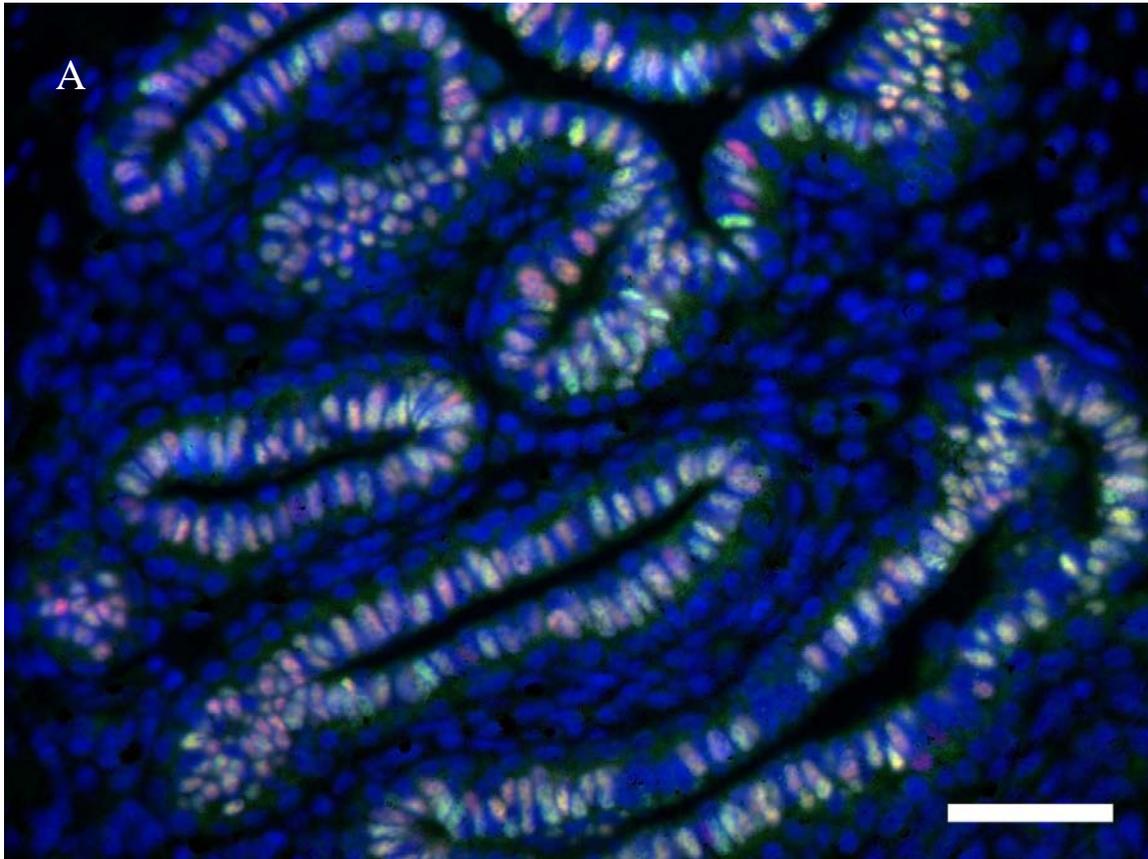


Table 3.10: Delta Ct lsmeans and SEM for ER α and PR by treatment group.

Treatment	ER α Δ CT	<i>P</i> -value	PR Δ CT	<i>P</i> -value
CON	-1.5 \pm 0.22	< 0.0001	2.6 \pm 0.24	NS
TAM	0.5 \pm 0.24		2.0 \pm 0.26	

Figure 3.12: ER α /PR staining in mammary tissue from CON (panel A) and TAM (panel B) blue=DAPI, red=ER α , green=PR, and yellow=overlay of red and green. The scale bar represents 50 μ m.



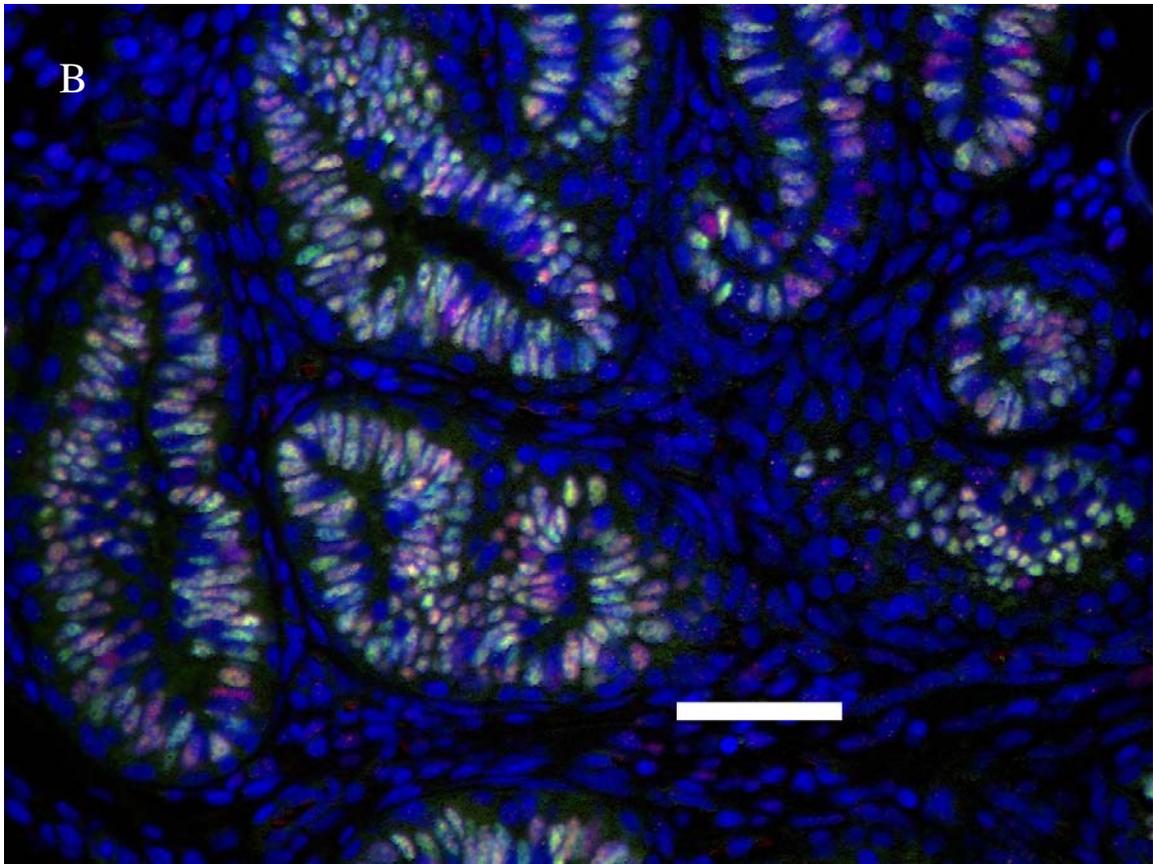


Table 3.11: Treatment lsmeans \pm SEM for characteristics of myoepithelial cell nuclei from Cellprofiler.

Characteristic*	CON	TAM	<i>P</i> -value
Area	1134 \pm 36	1030 \pm 33	0.057
Eccentricity	0.64 \pm 0.004	0.63 \pm 0.004	0.07
Neighbor Contact %	5.6 \pm 0.17	4.8 \pm 0.16	0.01
Perimeter	145 \pm 3	133 \pm 3	0.02
Zernike 1_1	0.056 \pm 0.001	0.050 \pm 0.001	0.002
Zernike 3_1	0.022 \pm 0.0006	0.019 \pm 0.0006	0.004
Zernike 4_2	0.023 \pm 0.0003	0.022 \pm 0.0003	NS
Zernike 4_4	0.023 \pm 0.0002	0.024 \pm 0.0002	NS

*Characteristics for myoepithelial cells are defined in Cellprofiler materials and methods.

Table 3.12: Zone lsmeans \pm SEM of characteristics of myoepithelial cell nuclei from Cellprofiler.

Characteristic	Zone 1	Zone 2	Zone 3	<i>P</i> -value
Area	1027 \pm 42	1096 \pm 42	1123 \pm 42	NS
Eccentricity	0.64 \pm 0.003 ^a	0.64 \pm 0.003 ^a	0.63 \pm 0.003 ^b	<0.0001
Neighbor Contact %	5.0 \pm 0.2	5.5 \pm 0.2	5.2 \pm 0.2	NS
Perimeter	134 \pm 3.5	141 \pm 3.5	143 \pm 3.5	NS
Zernike 1_1	0.050 \pm 0.001 ^a	0.054 \pm 0.001 ^{ab}	0.055 \pm 0.001 ^b	0.02
Zernike 3_1	0.019 \pm 0.0006 ^a	0.021 \pm 0.0006 ^b	0.021 \pm 0.0006 ^b	0.01
Zernike 4_2	0.022 \pm 0.0003	0.022 \pm 0.0003	0.022 \pm 0.0003	NS
Zernike 4_4	0.024 \pm 0.0002	0.023 \pm 0.0002	0.023 \pm 0.0002	NS

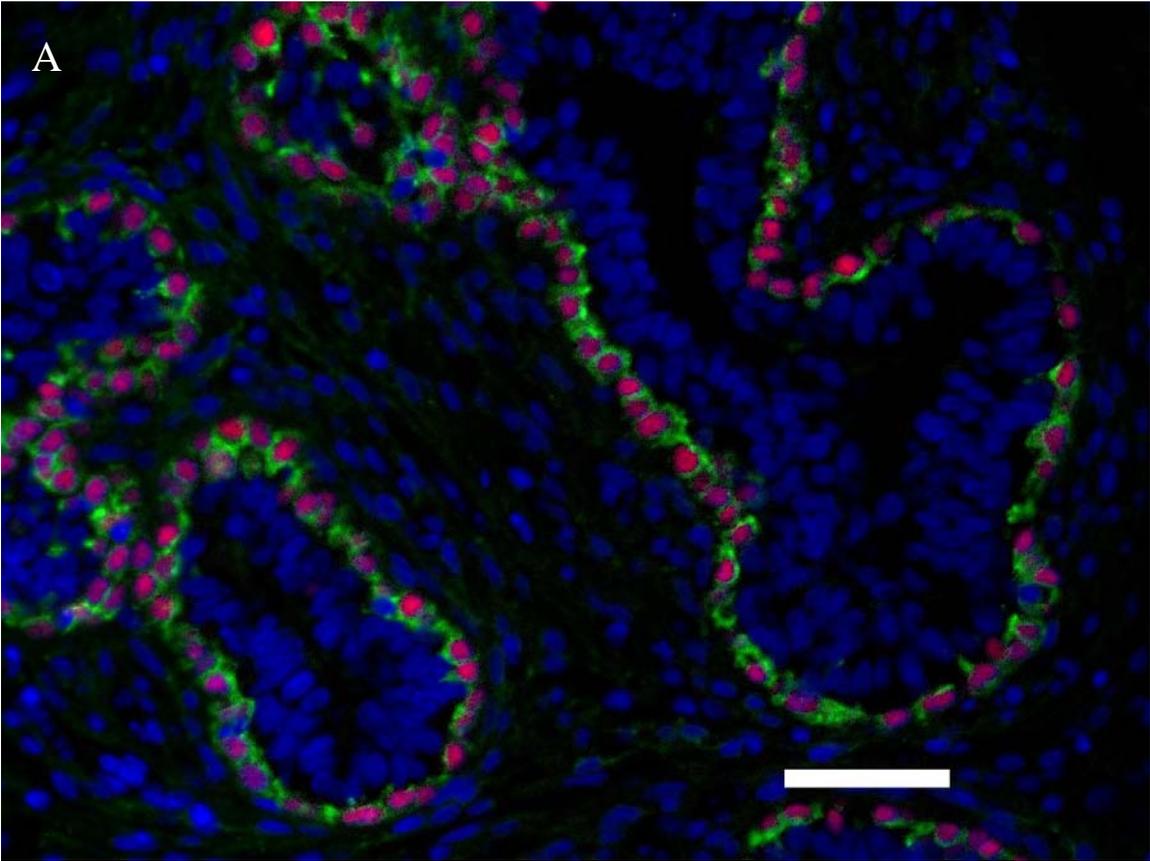
^{abc} Denotes which of the lsmeans are significantly difference from each other within a characteristic based on the Tukey-Kramer pair wise comparison.

Table 3.13: Eccentricity label lsmeans \pm SEM of characteristics of myoepithelial cell nuclei from Cellprofiler.

Characteristic	Elongated	Intermediate	Round	<i>P</i> -value
Area	1211 \pm 25 ^a	1055 \pm 25 ^b	977 \pm 25 ^c	<0.0001
Neighbor Contact %	5.2 \pm 0.1 ^a	5.2 \pm 0.1 ^a	5.3 \pm 0.1 ^b	<0.0001
Perimeter	155 \pm 2.2 ^a	136 \pm 2.1 ^b	127 \pm 2.1 ^c	<0.0001
Zernike 1_1	0.049 \pm 0.001 ^a	0.058 \pm 0.0008 ^b	0.052 \pm 0.0009 ^c	<0.0001
Zernike 3_1	0.029 \pm 0.0004 ^a	0.018 \pm 0.0004 ^b	0.014 \pm 0.0004 ^c	<0.0001
Zernike 4_2	0.036 \pm 0.0002 ^a	0.017 \pm 0.0002 ^b	0.015 \pm 0.0002 ^c	<0.0001
Zernike 4_4	0.029 \pm 0.0002 ^a	0.022 \pm 0.0001 ^b	0.020 \pm 0.0001 ^c	<0.0001

^{abc} Denotes which of the lsmeans are significantly difference from each other within a characteristic based on the Tukey-Kramer pair wise comparison.

Figure 3.13: Myoepithelial cell staining of mammary tissue from CON (A) and TAM (B). Blue is DAPI, red is p63, and green is CD10. The scale bar represents 50 μm .



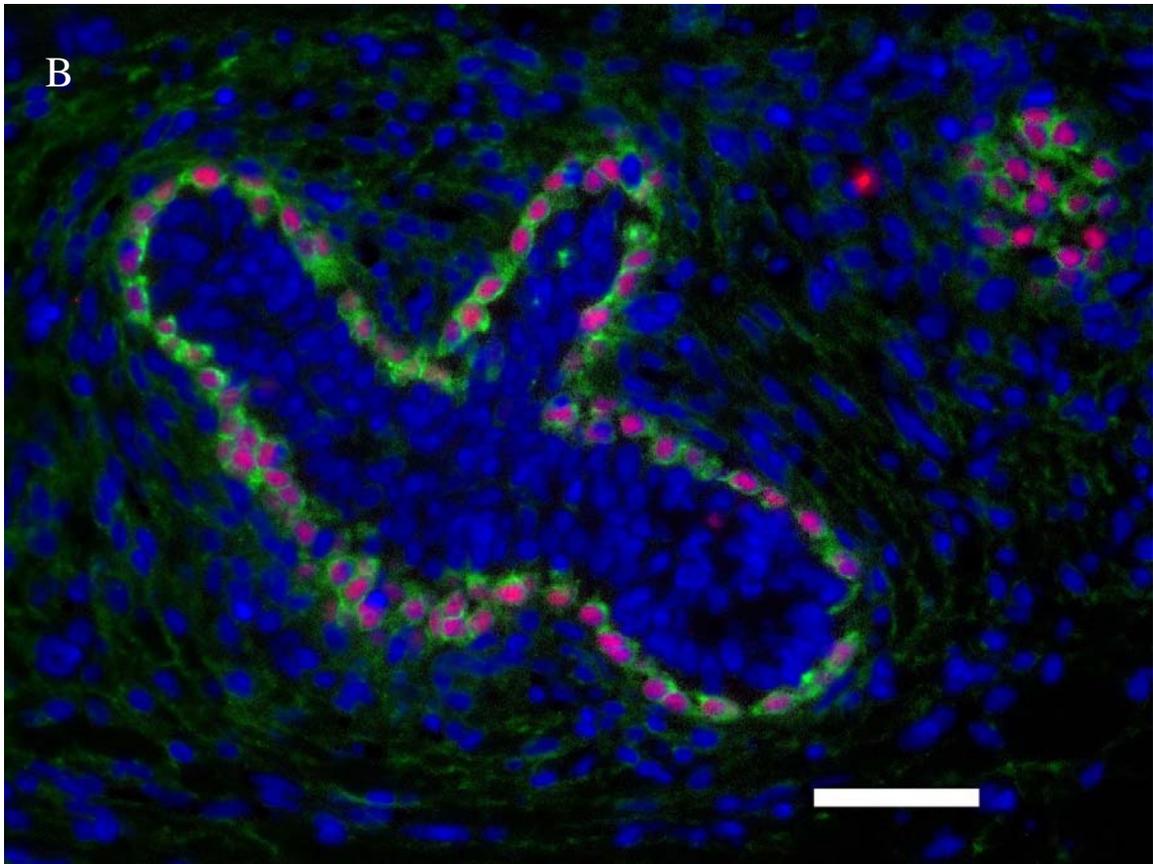
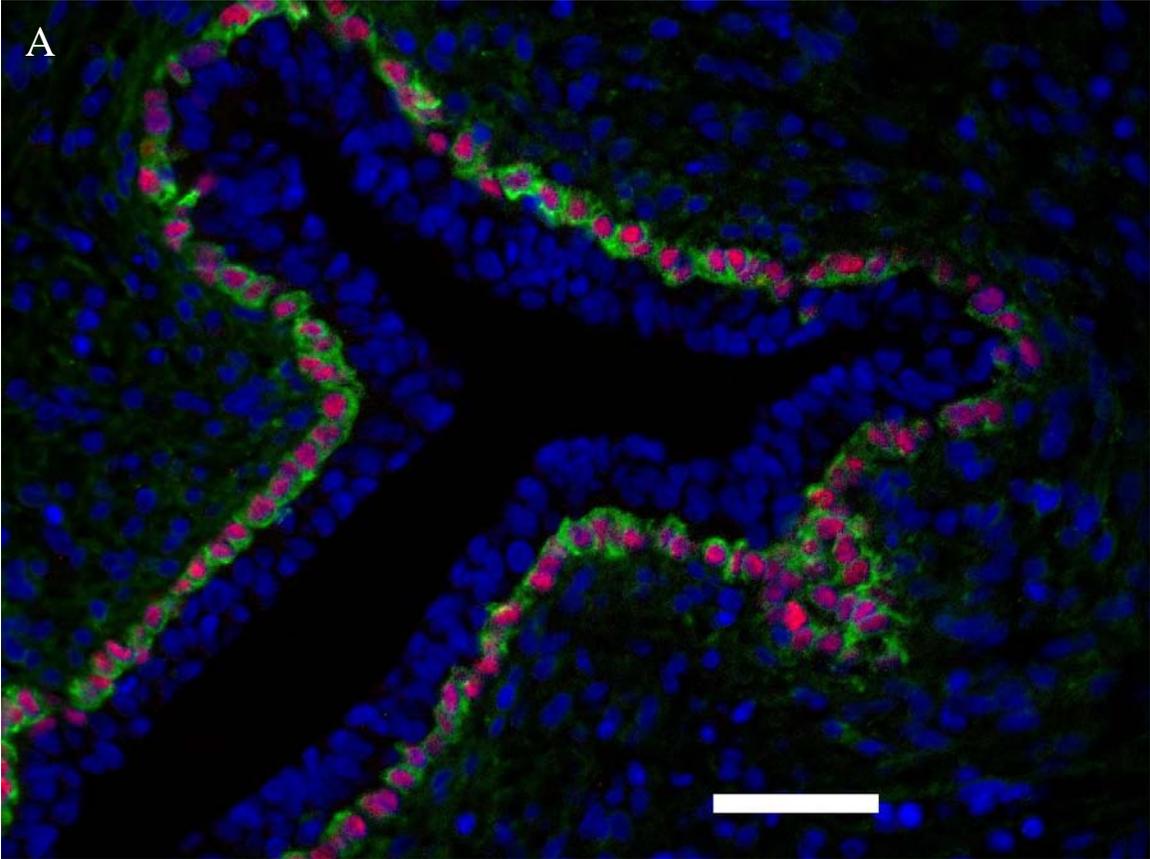


Figure 3.14: Myoepithelial cell staining to illustrate extremes in the spacing of myoepithelial cells in CON (A) and TAM (B). Blue is DAPI, red is p63, and green is CD10. The scale bar represents 50 μm .



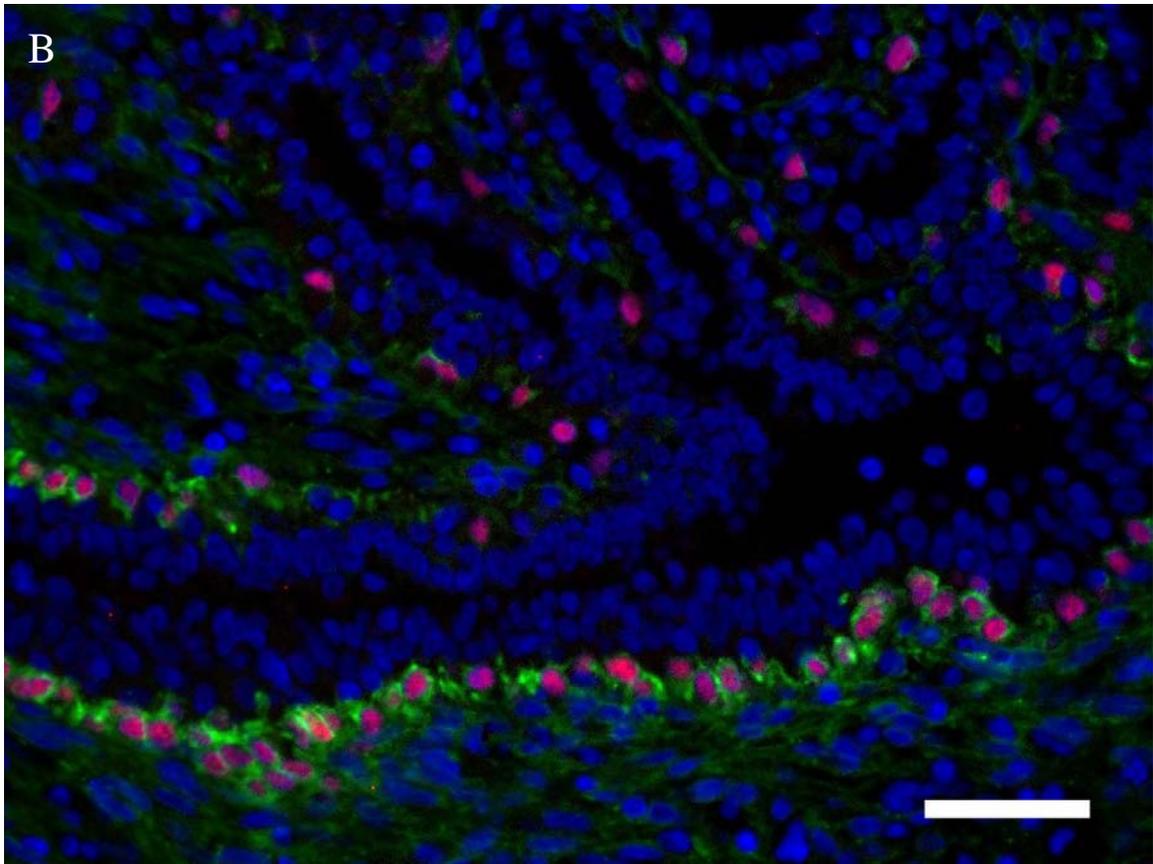
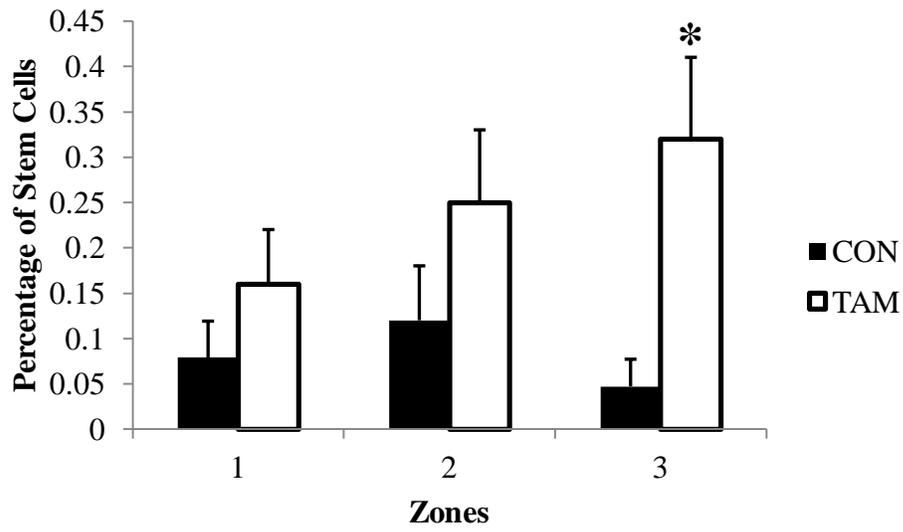


Table 3.14: Label retaining cell percentages lsmeans \pm SEM separated by treatment.

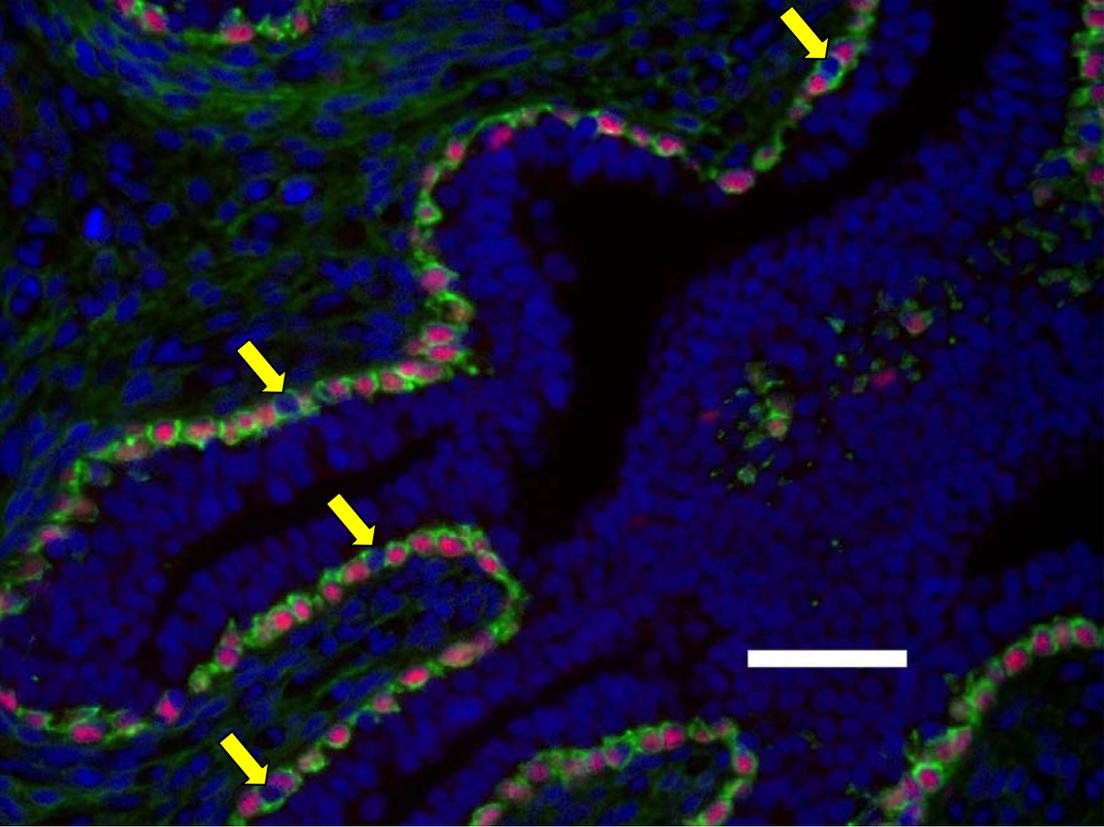
Treatment	Label Retaining Cell %	<i>P</i> -value
CON	0.08 \pm 0.03	0.01
TAM	0.23 \pm 0.04	

Figure 3.15: Label retaining cell percentages separated by treatment and zone.



- * $P = 0.02$ Zone 3 TAM vs. Zone 3 CON

Figure 3.16: Myoepithelial cell staining to illustrate cells found in the basal layer of the duct that did not stain for p63 (yellow arrows). Blue is DAPI, red is p63, and green is CD10. The scale bar represents 50 μm .



Discussion

Overall treatment with the potent anti-estrogen tamoxifen markedly reduced ($P = 0.002$) parenchyma growth (50%) in the developing mammary gland of prepubertal heifer calves based on both parenchymal mass and parenchymal DNA content (Figure 3.7; Figure 3.8). This effect is similar to responses in mammary development noted when prepubertal calves were ovariectomized (Purup et al., 1993, Velayudhan et al., 2012). Thus in general terms, this suggests that the effect of ovariectomy previously reported is at least partially a result of inhibition of production and/or action of estrogen. These data support assumptions from prior ovariectomy studies that a major impact of removal of the ovary on mammary development, even in prepubertal heifers, involves either the loss of circulating estradiol or interference with estrogen mediated actions.

Paradoxically, although Purup et al. (1993) noted a small (0.1 pg/ml) but significant decrease in circulating estradiol concentrations in ovariectomized heifers, Velayuhan et al. (unpublished) observed no difference in control and ovariectomized prepubertal heifers (1.67 ± 0.27 vs. 1.61 ± 0.23 pg/ml). Similarly, in this study there was no difference in circulating estradiol concentrations in CON and TAM heifers at the time of slaughter (Figure 3.6). This suggests that the negative impact of ovariectomy on prepubertal mammary development is not directly driven by changes in circulating concentrations of estradiol. It may be that other factors that are controlled by the ovary or impacted by tamoxifen treatment in the current study ultimately regulate the growth capacity of MEC in the prepubertal heifer.

In this regard, one of our goals in this study was to evaluate in detail the impact of tamoxifen treatment on the expression of ER α and PR in the epithelium of the developing mammary gland. This evaluation included a determination of not just the proportion of epithelial

cells expressing these receptors but also the localization of receptor positive cells within the ductal structure. We also measured the degree of receptor expression at the protein level by quantitative multispectral imaging of immunocytochemical tagged cells and at the mRNA level via quantitative real-time PCR.

In prior ovariectomy studies, the reduction in mammary growth (parenchymal weight and/or DNA content) was proposed to be related to differences in the number of cells that express ER α /PR in control compared with ovariectomized heifers (Berry et al., 2003b). However, in our trial there was no significant difference between CON versus TAM for the number of cells that expressed ER α /PR but the impact on mammary development was similar i.e. a 50 % reduction with TAM treatment. This suggests that the impairment in mammary development after ovariectomy is not simply a reflection of change in the proportion of epithelial cells expressing ER α .

Both treatments did follow the pattern (noted in ovariectomy trials) that the majority of ER α expressing cells were in the medial/embedded layer of the duct. The main difference in ER α /PR was the amount of expression per a cell, represented in intensity levels of expression. To our knowledge, this is the first bovine study to quantitatively evaluate expression of ER α /PR at the protein level via immunocytochemistry and quantitative image analysis. Consequently, it is unknown if there were similar differences in the intensity of ER α or PR expression in previous ovariectomy experiments. Regardless, our data suggests that the difference in parenchymal growth is more closely related to the number of receptors per a cell rather than the proportion of cells that express ER α /PR. Indeed, CON expressed higher amounts of ER α per positive cell and these animals had double the mammary growth (parenchymal weights, DNA content).

In recent reports, Ballagh et al. (2008) and Safayi et al. (2012) reported that ovariectomy impacted the development not just of the epithelial portion of the developing mammary gland but also the ontogeny of myoepithelial cells. To follow up on these evaluations, we used multispectral image analysis to evaluate expression of p63 and CD10 as markers to identify myoepithelial cells and specifically p63 staining of myoepithelial cell nuclei to quantitatively evaluate selected morphological characteristics of the nuclei of these cells to further determine effects of tamoxifen treatment on myoepithelial development. Our purpose was to compare and contrast these results with changes reported following ovariectomy.

Myoepithelial cell differentiation is thought to be inhibited by ovarian secretions, which allows for growth factors to more readily impact MEC (Ballagh et al., 2008). If myoepithelial cells can act like a barrier this could explain why ovariectomized calves have a decrease in parenchyma development. Mature myoepithelial cells are described as having dense and irregularly shaped nuclei. They are also typically larger and more stellate than other mammary epithelial cells and have elongated surface projections (Zavizion et al., 1992).

The overall shape-types of myoepithelial cell nuclei in tissues from TAM and CON animals were not different based on overall eccentricity value (Table 3.11). That is nuclei that were classified as elongated, intermediate, or round were present in mammary tissue from animals in both treatments. However, there were marked differences, due to treatment, for myoepithelial cell nuclei in each of the shape classifications. Moreover, there were significant differences between nuclear area and perimeter due to treatment (Table 3.11). Ovariectomized heifer's myoepithelial cells were noted as having smaller nuclei compared to intact calves (Safayi et al., 2012). The TAM treatment significantly decreased myoepithelial cell nuclear area and perimeter. More differentiated myoepithelial cells are characterized as having more compact

nuclei and therefore smaller areas and perimeters. This suggests that treatment with tamoxifen increased the differentiation of myoepithelial cells similar to that of ovariectomized calves.

CON myoepithelial cells also touched more neighboring cells compared to TAM. This could be attributed to myoepithelial cells having smaller spaces in between the cells compared to TAM (Figure 3.14). They are more elongated in shape and have more extreme spacing in between each cell. This agrees with the concept that myoepithelial cells are more differentiated in the TAM treatment compared to the CON. Therefore, the TAM treatment increases the number of differentiated myoepithelial cells and blocks the MEC from the different growth factors. This could be a contributing factor as to why there is a decrease in the parenchyma weight of the TAM group.

Mice that were treated with tamoxifen from 2-12 months of age experienced similar differences in mammary development seen in this experiment. There was an overall decrease in the amount of parenchyma similar to what was seen with the heifers. There was also an increase in differentiation of cells specifically the myoepithelial cells. However, in the mice the MEC differentiated into myoepithelial-like cells (Kotoula et al., 1993). There is no apparent evidence of a difference in the number of myoepithelial cells when comparing CON to TAM in the heifers. There was just an increase in the amount of differentiation undergone in the myoepithelial cell population.

The LRC population identified in this study was very small in comparison to other studies (< 1 % of the total cell population). However, this is the first study to utilize this particular multi-labeling approach for bovine mammary tissue. It is well established that a large population of stem cells is not required for normal development (Kordon et al., 1998). It is

assumed that the population of stem cells is small in comparison to the other cell types in the mammary gland. In this respect, it should not be surprising that only a very small number of MEC are actual stem cells.

Cells that were putatively identified as stem or progenitor cells i.e. the LRC were oriented 1) towards the lumen of the duct or 2) near the basement membrane in close alignment with the myoepithelial cells. It has been previously observed that there is a small population of cells within the myoepithelial cells that do not stain for myoepithelial cell markers and these cells were hypothesized to be possible stem cells (Safayi et al., 2012). Similar cells were identified in this study (Figure 3.16) and are thought to be a part of the stem population located near the basement membrane. LRC that were near the lumen of the duct appeared to be in close contact with another/other cell(s) that were not classified as LRC. The nearby cells could be cells that the LRC is replacing either as fully differentiated cells or progenitor cells. Regardless, our data appear to support the hypothesis that the majority of LRC are positioned within the usual myoepithelial cell compartment within the developing mammary duct.

The purpose of this study was to not only refine the identifiers of LRC in the bovine but to also determine the effect of tamoxifen treatment on the population of LRC. There is evidence of being able to manipulate the number of LRC found in the bovine model; however, these numbers were not thought to be sustained if treatments were continued long term (Capuco et al., 2009). There was an overall significant increase (2.8 fold) in the number of LRC in TAM treated calves. One explanation is that with the decrease in parenchyma growth, the TAM group did not experience enough development to “use” up their LRC. Another explanation is that the TAM group had more progenitor cells that did not have defined cell lineages and were identified as LRC rather than epithelial or myoepithelial cells. This explanation does not seem likely because

LRC that were counted for either treatment were rarely in close proximity to each other and are therefore less likely to be progenitor cells. Finally, the difference in the proportion of LRC was even more pronounced for tissue collected from zone 3 (6.3-fold). Assuming proliferation is more active in this more peripheral region of the developing parenchymal tissue, it may be that LRC were more readily 'used' in CON compared with TAM.

Summary and Conclusions

This study increased our knowledge of the mammary development during the prepubertal period in heifers by complementing ovariectomy research. We now know that the decrease of parenchyma weight can be contributed to blocking the effect of estrogen. The ovariectomy studies attributed this difference to the proportion of ER α /PR positive cells; however, the treatment with tamoxifen did not induce this difference. There was however a marked difference in the intensity of expression of receptor per cell. The difference in the number of receptors per a cell is certainly correlated with the disparity of udder weights between treatments. Further research will be required to see if there is similar difference in the intensity of expression of ER α when heifers are ovariectomized.

We also provide evidence that myoepithelial cells appear more differentiated in the tamoxifen treated heifers as seen in ovariectomized heifers. This result at least partially mirrors responses noted following ovariectomy. However, the degree of difference was not as extreme as with ovariectomy. Myoepithelial cells that are more differentiated are proposed to have the ability to block growth factors from the MEC and hamper the ability for MEC to develop.

In this study we were successful at using multispectral image analysis to identify label retaining cell populations. This led to the discovery of a difference between treatments for the percentage of cells that were identified as LRC. We were also able to corroborate evidence that LRC have a preferred position within the duct. Specifically, they were found touching the lumen or near the basement membrane. The small population of cells that were identified (< 1%) and correspondence with other studies suggests we are now closer to identifying stem cells in the bovine mammary gland. However, this ability is only true for fixed cells. In the future, there

needs to be an ability to identify living stem cells in order to manipulate the number in the bovine gland.

Future Research

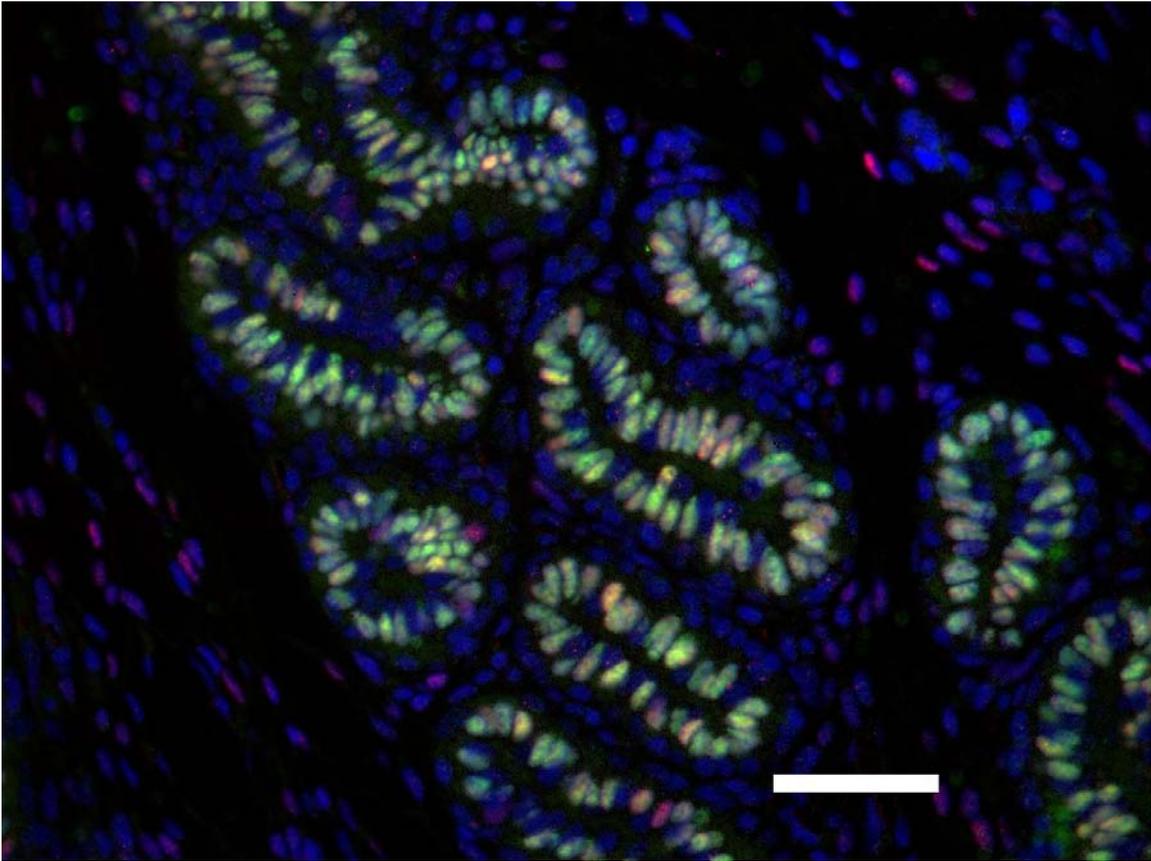
In preliminary data for parenchymal tissue from Zone 3 there was no significant difference for in the area occupied by stromal elements between treatments. However, use of Sirius Red staining (to stain collagen) and associated image quantifications showed that collagen deposition was significantly increased ($P = 0.0002$) in TAM compared to CON. These data did not address possible differences in specific collagen types or the different zones in the parenchyma. However, knowing that stromal composition can impact mammary ductal development, it seems likely that some of the negative impact of tamoxifen treatment on parenchymal development reflects tamoxifen induced differences in stromal tissue. There is some evidence that deposition of collagen and other extra cellular matrix proteins may also be altered by ovariectomy (Berry et al., 2003a) thus providing another parallel between direct inhibition of estrogen receptor (TAM) and ovariectomy.

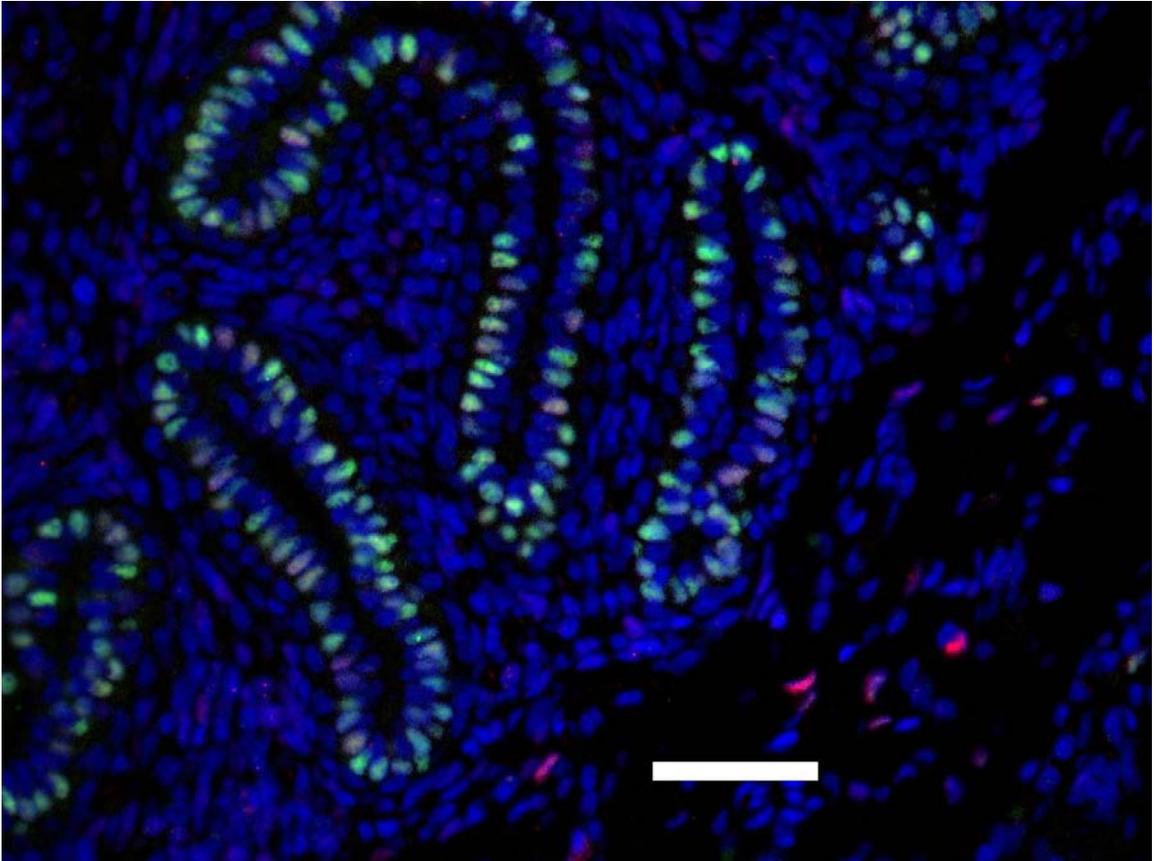
There is potential for a difference in the stroma composition that could affect the parenchyma development. A study that looked at bovine mammary tissue transplanted into a cleared murine MFP noted an increase in the amount of collagen/fibrous tissue found in the immediate area of the epithelial organoids (Sheffield et al., 1986). This observation supports that the composition of supporting tissue is important to the development of parenchyma. In a mouse study there was also an increase in collagen deposition when the mice were injected with tamoxifen (Kotoula et al., 1993). Further research is required that delves into the difference of stroma composition between treatments.

In the stroma ER α positive cells were observed in this study unlike in ovariectomized calf studies where ER α was only found in the epithelium (Berry et al., 2003b). Macrophages in mice

are known to be present in the stroma and express ER α . Macrophage depleted mice also have a decrease in the amount of epithelium and alveolar buds (Chua et al., 2010). This could be a direct affect on the mammary gland that can be seen in the bovine with the decrease of estradiol availability. However, due to the intensity levels of ER α in the CON epithelium we were unable to identify if there was a treatment difference (Figure 4.17). Further research identifying the immune cells in the prepubertal bovine mammary gland is required.

Figure 4.17: Examples of TAM tissue with ER α stain in the stroma. The scale bar represents 50 μm .





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