Unique Aspects of Mammary Growth and Development in

Dairy Heifers and Ewe Lambs

A Thesis

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By

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ABSTRACT

Mammals possess mammary glands which are composed of two tissue types; secretory parenchyma (PAR), and non-secretory stroma. Amount of PAR is positively correlated with milk production. This work contains results from two separate projects. In the first, estrogen signaling in PAR from dairy heifers was more fully characterized using an ovariectomy (OVX) model. In the second, maternal body condition score (BCS) of gestating ewes was imposed as a treatment and PAR characteristics of female progeny were measured.

Estrogen regulates bovine mammary growth and development and acts mainly through estrogen receptors (ER), specifically ERα, to regulate estrogen-responsive genes. One such gene, CREB1, has recently been identified, but has not been characterized in bovine mammary tissue. The CREB1 gene codes for cAMP response element-binding protein (CREB). OVX of prepubertal heifers inhibits mammary development while causing an increase in ERα-positive cells, and increased abundance of myoepithelial cells which are identifiable by staining for α-smooth muscle actin (SMA). Our objective was to study the effects of OVX on tissue localization of CREB. PAR samples were obtained from 16 prepubertal heifers in a 2 × 2 factorial experiment. The first factor was ovarian status (intact or OVX); the second factor was estrogen treatment (control or estradiol). OVX was performed at ~4.5 mo of age and estrogen treatments began ~5.5 mo. After 54 h of estrogen treatment, heifers were slaughtered, udders removed and PAR sampled.

Tissue sections underwent staining for CREB and ERα, or for CREB and SMA. CREB was more abundant in the luminal and embedded layer compared to the basal layer. Neither OVX, estrogen replacement, nor the combination of the two affected the amount of CREB protein. CREB has previously been identified as being estrogen-responsive at the transcript level. Our data indicate that tissue abundance of CREB protein does not show the same pattern. CREB may play a key role in signaling pathways that influence development of the bovine mammary gland because it was constitutively present in all treatment groups examined.

Mammary PAR is formed in utero, so maternal BCS during gestation may affect progeny mammary growth and composition. Pregnant ewes (n = 96; ~80 d of gestation) were grouped based on initial BCS of 2, 3, or 4 (1 to 5 scoring system; 1 = emaciated and 5 = obese). Ewes received gestation diets formulated to maintain initial BCS throughout pregnancy. Post-lambing diet and management were equivalent across treatments. Female progeny (n = 73) were slaughtered at similar BW and age. Udders were removed and mammary tissue subjected to biochemical analyses. Total mammary gland weights did not differ by treatment. However, PAR weight of BCS 2 progeny tended to be greater than that of BCS 3 or BCS 4 progeny. Protein mass within PAR tended to be highest in BCS 2 progeny, as did DNA mass within PAR. Despite detectable differences in PAR due to treatment, no differences in weight or composition of mammary stroma were

found. Our observations suggest that BCS during gestation may have important lactation performance implications for female progeny.

For my family.

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CHAPTER 1

Review of Literature

MAMMOGENESIS IN DAIRY HEIFERS AND THE IMPORTANCE OF OVARIAN HORMONES

Mammogenesis is vital to the productive life and value of a dairy cow. The foundation for functional mammary secretory tissue that is laid down early in life directly relates to future milk production. Hormones are necessary for growth and development of the mammary gland, but much research is still needed in this area. The essentiality of hormones for bovine mammogenesis can be illustrated by ovariectomy (**OVX**) before puberty. This has been shown to have dramatic negative effects on subsequent mammary growth and development (Berry et al., 2003). When the stimulus, estrogen, is removed at the level of the ovary, mammary growth and development cease, but if estrogen is added back into the system, then mammary growth and development are reestablished. It is therefore clear that estrogen-responsive genes and ultimately proteins are involved in this process. The first study presented in this thesis examined molecules involved in estrogen signaling using an OVX model. One such molecule, cyclic AMP response element-binding protein (**CREB**), has been shown capable of phenotypic modulation in blood vessels (Klemm et al., 2001). "Phenotypic modulation" means a switch in cellular

phenotype that occurs under pathological conditions (e.g. hypoxia, mechanical injury, hyperlipidemia, oxidative stress). During vascular stress, smooth muscle cells (**SMC**) in the intimal and medial compartments of the arterial wall have been observed to become proliferative, migratory, and produce excess matrix proteins (Klemm et al., 2001). It was further shown that the nuclear content of CREB influenced the cellular phenotype (Klemm et al., 2001). In blood vessels from neonatal calves exposed to chronic hypoxia, CREB content was depleted and smooth muscle cell proliferation was accelerated. Overexpression of active CREB arrested cell cycle progression, and decreased the expression of genes encoding growth factors, growth factor receptors, and cytokines (Klemm et al., 2001). It is possible that CREB may also play a role in the phenotypic modulation of bovine mammary epithelial cells (**MEC**).

Evidence supporting a role for CREB in modulating mammary tissue can be found in a microarray study of bovine mammary parenchyma (**PAR**) and mammary fat pad (**MFP**) in response to OVX and estrogen replacement (Li and Capuco, 2008). Mammary samples were taken from OVX and intact (**INT**) heifers that were either treated or not treated with exogenous estrogen (17β-estradiol). Parenchyma and MFP gene expression were then evaluated using a high-density oligonucleotide microarray. From these microarray data, a regulatory network was developed to show the relationship among genes influenced by estrogen in bovine mammary gland. Notably, CREB1 (a gene that encodes CREB protein) was an essential part of this network. The biological pathways most significantly influenced by estrogen included cell cycle, cell-to-cell signaling and interaction, and cellular assembly and organization (Li and Capuco, 2008).

Crosstalk between ERα and cAMP signaling pathways has been documented in human breast cancer cells by Lazennec and colleagues (2001). The authors found that CREB is required for the transcriptional synergy between cAMP and estrogen signaling pathways. Their data suggest that CREB is involved in the crosstalk between ERα and protein kinase A (**PKA**) signaling pathways. They further proposed that CREB is linked to DNA by an ERα-coactivator complex and that CREB can in turn increase transcriptional activity in genes. They also noted that their hypothesis may have further implications for the regulation of estrogen-responsive genes by estrogen and antiestrogen in breast cancer and other ERα-containing cells, especially if PKA is upregulated or overexpressed in these cells (Lazennec et al., 2001). This situation could possibly exist in the bovine mammary gland.

Previous research has shown that ERβ protein is not detectable in bovine mammary gland (**MG**) and seems not to play a part in development (Connor et al., 2005). Therefore, it is most likely that the estrogen-responsive genes identified by Li and Capuco (2008) are indeed regulated directly or indirectly, by 17β-estradiol (**E_2**) binding ERα and causing downstream cellular events.

Previous studies have shown that OVX of prepubertal heifers increases the proportion of ERα-positive cells (Berry et al., 2003) and also advances myoepithelial cell differentiation (Ballagh et al., 2008). Therefore, it is of interest to examine the presence of CREB in relation to myoepithelial cells. Myoepithelial cells can be characterized by their presence in the basal layer of PAR epithelium, their distinct morphology, and the presence of α-smooth muscle actin (**SMA**) (Deugnier et al., 1995; Ballagh et al., 2008).

In dairy heifers, ERα-positive cells are present primarily in the luminal and embedded layers of PAR epithelium (Capuco et al., 2002).

Remaining Questions

1. Is the abundance of CREB in bovine mammary PAR influenced by ovarian status (INT or OVX)?
2. Is the abundance of CREB in bovine mammary PAR influenced by exogenous estrogen treatment (control or 17β-estradiol)?
3. Is CREB preferentially localized to either the basal or embedded plus luminal layers of bovine mammary PAR?
4. Is abundance of CREB influenced by a combination of the above three factors?
5. Does CREB colocalize with ERα in bovine mammary PAR?

Hypothesis

We hypothesized that abundance of CREB would vary in relation to either ovarian status (INT or OVX), exogenous estrogen treatment (control or 17β-estradiol), epithelial layer (basal or luminal plus embedded), or some combination of the previous, in PAR of bovine heifers.

We also hypothesized that CREB would colocalize with many, but not all, ERα-positive cells in bovine mammary PAR.

Research Synopsis

This study examined the influence of ovarian hormones on MG development in prepubertal heifers. The objective of this experiment was to more fully characterize the distribution patterns of CREB, ERα, and SMA proteins within bovine MG during "normal" or "altered" (i.e. ovariectomy, with or without exogenous estrogen) situations. Prepubertal bovine MG tissue samples from a previous experiment (Li et al., 2006; Li and Capuco, 2008) were used as a starting point for our experiments.

Rationale and Significance

Any factor that promotes MEC growth can be assumed to have an impact on milk production once the animal reaches lactation (Tucker, 1987). This study investigated known and suspected estrogen-responsive proteins and their impact on developing bovine mammary tissue. Promoting mammary growth early in life is potentially beneficial and may enhance both lactation efficiency and milk production once the animal reaches lactation (Capuco et al., 2001).

MAMMOGENESIS IN EWE LAMBS AND THE IMPORTANCE OF THE FETAL ENVIRONMENT

Nutrition has been shown to influence mammogenesis as early as the pre-weaning period in dairy heifers (Daniels et al., 2009a) and the prepubertal period in ewe lambs (McCann et al., 1989). The effect of maternal nutrition on fetal mammary development in ewe lambs has recently been explored (van der Linden et al., 2009), but further research is needed in this area. Dam body condition score (**BCS**) from mid gestation to parturition may affect progeny postnatal MEC proliferation or mammary composition via in utero metabolic programming. The foundation for functional mammary secretory tissue is established early in life; mammary development is directly related to the milk production potential of a lactating animal.

Isometric mammary growth in dairy heifers occurs during the pre-weaning period of life up until 3 mo of age. Ductal, though not alveolar, growth occurs during this period. Brown et al. (2005) showed that higher energy and protein intake from 2 to 8 wk of age increased PAR mass without increasing deposition of PAR fat. However, in heifers from 8 to 14 wk of age, increased protein and energy intake encouraged deposition of intra- and extraparenchymal fat, and did not increase PAR mass. In a separate study on 65 d old heifers fed milk replacer diets of varying fat and protein content, altered nutrient intake was shown to increase the mass and alter the composition of MFP, but did not have an effect on PAR mass or composition (Daniels et al., 2009a).

According to a classical study by Sinha and Tucker (1969), the period of life from ~3 mo of age until puberty (~10 mo of age) is a time of allometric mammary growth for

dairy heifers. Extensive duct growth occurs during this time. The duct network formed during pre-puberty and puberty helps lay the framework for lobular and alveolar development that take place during gestation. The effect of nutrition on mammogenesis during this time is disputed. Previous research found that a higher level of nutrition had negative effects on development of mammary secretory tissue and subsequent milk production (Swanson, 1960). Recently, it was shown that mammary composition measured at various time points from birth through puberty was not affected by feed intake (Meyer et al., 2006a,b). Body weight and age, rather than rate of gain, were emphasized to have the greatest impact on mammary development (assessed histologically) in heifers (Daniels et al., 2009b). After puberty, mammary growth returns to an isometric rate and nutrition does not positively or negatively affect its composition (Sejrsen et al., 1982). Nutritional effects on mammogenesis in dairy heifers are still being examined.

Even less is known about mammary development in sheep and the influence that nutrition may have on mammary growth and development. As in dairy heifers, accelerated growth during rearing has been shown to impair milk production and number of alveoli present in ewes (Umberger et al., 1985). Similarly, McCann et al. (1989) showed that rapid weight gain to puberty in ewe lambs led to lower milk production and an increase in MFP area. There is a lack of knowledge regarding the gestational environment and subsequent mammary development of the progeny for both sheep and dairy heifers.

The importance of maternal nutrition during gestation has previously been emphasized through the "fetal origins" hypothesis proposed by Barker (1995). This hypothesis suggests that fetal undernourishment results in permanent detrimental changes leading to the development of disease later in life (Barker, 1995). Furthermore, Lucas (1991) has defined "metabolic programming" as the process whereby nutritional manipulation during a critical period of development has lasting or lifelong significance. Such long-term effects have been further studied by Patel and Srinivasan (2002). Using their "pup in a cup" model, neonatal rat pups were raised on a low-fat, high-carbohydrate milk replacer providing 56% of the calories from carbohydrates (compared to rat milk, which typically contains 8% of calories from carbohydrates). These high-carbohydrate fed pups showed hyperinsulinemia, increased body weight, and lower glucose tolerance during the suckling period and through adulthood when compared to pups that were allowed to nurse their mothers (Patel and Srinivasan, 2002). A further consequence was that pups born to these adult rats showed the same characteristics, even though they never received the low-fat, high-carbohydrate milk replacer. Further crossbreeding experiments showed that these attributes were only transmitted to progeny through the mother, suggesting that transmission occurred in utero (Patel and Srinivasan, 2002). Such evidence shows that changes in the gestational environment can influence progeny development. Because the developing fetus relies on the mother for nutrients, it is interesting to speculate that a change in the gestational environment may alter embryonic mammogenesis so much so, that effects persist postnatally.

Previous studies have shown the effects of varied gestational environments on several aspects of fetal growth and development. In sheep, it has been shown that both nutrient restriction of the adult dam and overnourishment of the adolescent dam during pregnancy suppress placental cell proliferation and vascularity. Undernourished dams have also been shown to have low birth weight offspring (Redmer et al., 2004). Swanson and colleagues (2008) showed that improper nutrition from mid to late pregnancy in ewes altered colostrum quality and quantity and reduced offspring birth weight, which may have negative implications for lamb health and survival during the early postnatal period.

Furthermore, effects of feeding varied amounts of the same maternal diet on embryonic development have been studied by Quigley et al. (2005) in an ovine model. Donor ewes were fed different amounts of the same diet at either 1.5 × maintenance or 0.5 × maintenance during the time surrounding ovulation. Embryos were collected and transferred to recipient ewes. Organ and muscle weights from the resulting pregnancies (examined at d 75 of gestation) showed that restricting feed intake over the peri-conception period reduced or delayed myogenesis in sheep (Quigley et al., 2005). Armed with this information, it would be interesting to determine how alterations in the gestational environment may affect another essential developmental process, namely mammogenesis. These effects have not been reported previously.

More recently, promising data from Wallace et al. (2010) showed that maternal body mass index at conception and gestational nutrient intake in ewes had a profound influence on pregnancy outcome in offspring. Interestingly, initial colostrum yield, total IgG, and nutrient supply were reduced in overnourished dam groups (fed to promote

adiposity) but their low birth weight lambs exhibited rapid catch-up growth to weaning (Wallace et al., 2010). A similar trial using beef heifers also examined compensatory fetal growth in progeny from dams placed on a lower plane of nutrition during gestation (Micke et al., 2010). Researchers reported that fetal development of cattle may be affected by maternal nutrition as early as day 39 of gestation. This may be followed by compensatory fetal growth that is dependent upon maternal nutrition (Micke et al., 2010).

The effects of various prepartum feeding systems on both ewe and lamb performance have recently been explored (Radunz et al., 2011a,b). Ewes that received a prepartum forage diet had lower BCS and that progeny from these ewes tended to have lower birth weights when compared to progeny from ewes receiving limit-fed corn or limit-fed dried distillers grains (limit-fed to achieve isocaloric intake as those fed forage) during the prepartum period (Radunz et al., 2011b). When progeny were examined after weaning, prepartum diet during mid to late gestation was shown to alter weaning weight, dressing percent, and muscle deposition (Radunz et al., 2011a). The examination of putative alterations during the prepartum period are therefore of interest. Progeny MG may undergo changes in composition or patterns of growth when evaluated postnatally.

To date, only one paper on this topic currently exists. Van der Linden and colleagues (2009) showed that mammary duct area of ewe lambs from dams maintained on a higher plane of nutrition during pregnancy was less than mammary duct area of ewe lambs from dams given a maintenance diet during pregnancy. The fetal MG measurements were taken at d 100 of gestation, just before the third trimester, and total duct area and duct number were the only measurements taken. More quantitative methods

and data from ewe lambs at postnatal time points are needed to determine gestational effects on mammary composition in ewe lambs. Our research methods provided us with a more comprehensive picture of gestational influences on progeny mammary development.

Remaining Questions

1. Is progeny mammary composition influenced by maternal over- or undernourishment during mid to late gestation in sheep?
2. Is progeny mammary epithelial cell proliferation influenced by maternal over- or undernourishment during mid to late gestation in sheep?

Hypothesis

We hypothesized that variations in dam BCS during mid to late gestation would affect, either positively or negatively, progeny postnatal mammary composition and mammary epithelial cell proliferation.

Research Synopsis

The objective of this study was to explore the effects of varying maternal BCS during mid to late gestation on MG composition in ewe lamb offspring. This experiment was conducted as part of a larger trial done by A.E. Radunz et al. (S.C. Loerch, OARDC, The Ohio State University, Wooster, OH, personal communication). The ewe lambs used for the current trial were obtained from dams maintained at a set BCS from mid gestation

to parturition. Composition of both PAR and MFP of ewe lambs were evaluated. Composition was measured through analysis of mass, and lipid, protein, and DNA content. Proliferative status of mammary epithelial cells was determined through immunohistochemical assessment of Ki67 antigen abundance, a nuclear proliferation antigen.

Rationale and Significance

Very little is known about maternal nutrition during gestation and resultant effects on progeny mammary composition in domestic animals. Lambs used in this trial represent a valuable resource and offer a unique opportunity to study gestational effects on mammary composition and cell proliferation. Information gained from this exploratory study will be used to further our knowledge in this area. Through better understanding of the role of dam BCS during pregnancy on female progeny mammary composition, we hope to one day contribute to the refinement of gestational nutrition programs for sheep and dairy cattle. These programs will have both the dam and the progeny in mind and will not hinder the performance (i.e. growth, lactation) of either.

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CHAPTER 2:

Effects of Ovarian Status and Estrogen Treatment on the Distribution of cAMP Response Element-Binding Protein in Developing Bovine Mammary Gland

In preparation for submission to Journal of Dairy Science as a short communication

ABSTRACT

Estrogen has been shown to regulate bovine mammary growth and development, though many of the exact mechanisms of estrogen action are still unknown. In cattle, estrogen acts mainly through estrogen receptors (ER), specifically ERα, to regulate estrogen-responsive genes. One such estrogen responsive gene, cAMP response element-binding 1 (CREB1), has recently been identified, but it has not been characterized in bovine mammary tissue. CREB1 is a gene that codes for CREB protein. Ovariectomy of prepubertal heifers has been shown to inhibit mammary development while causing an increase in ERα-positive cells, as well as increase the abundance of myoepithelial cells as identified by staining for α-smooth muscle actin (SMA). Effects of ovariectomy on mammary localization of CREB remain unknown. Our objective was to study the tissue localization of CREB using immunohistochemistry. Mammary parenchyma (PAR) samples were obtained from 16 prepubertal heifers in a 2 × 2 factorial experiment, with

ovarian status (intact or ovariectomized) as the first factor and estrogen treatment as the second (control or estradiol). Heifers were ovariectomized at ~4.5 mo of age and estrogen treatments began 1 mo later (daily subcutaneous injection of 17β-estradiol dissolved in corn oil, 0.1 mg/kg body wt, for 3 consecutive days). After estrogen treatment, heifers were slaughtered and udders removed. Mid-PAR was subsampled for immunohistochemistry. Five-μm thick tissue sections underwent immunofluorescent staining for CREB and ERα, or for CREB and SMA. As expected, ERα was present almost exclusively in the luminal and embedded cell layer in animals not receiving estrogen treatment and not present in animals that received exogenous estrogen. Also to be expected, SMA was present almost entirely in the basal layer. CREB was more abundant in the luminal and embedded layer as opposed to the basal layer. Neither ovariectomy, estrogen replacement, nor the combination of the two affected the amount of CREB protein detected. CREB has previously been identified as being estrogen-responsive at the transcript level. Our data indicate that the tissue abundance of CREB protein does not show the same pattern. Although not elucidated here, CREB may still play a key role in signaling pathways that influence development of the bovine mammary gland because it was constitutively present in all treatment groups examined.
Key words: estrogen receptor, CREB, mammary

INTRODUCTION

Mammogenesis is vital to the productive life and value of a dairy cow. The foundation for functional mammary secretory tissue that is laid down early in life directly

relates to future milk production. Hormones are necessary for growth and development of the mammary gland, but there is still much research to be done in this area. The essentiality of hormones for bovine mammogenesis can be illustrated by ovariectomy (**OVX**) before puberty. This has been shown to have dramatic negative effects on subsequent mammary growth and development (Berry et al., 2003). When the stimulus, estrogen, is removed at the level of the ovary, mammary growth and development cease, but if estrogen is added back into the system, mammary growth and development are reestablished. Therefore, it is clear that estrogen-responsive genes and ultimately proteins are involved in this process. Through this study, we examined molecules involved in estrogen signaling using an OVX model. One such molecule, cyclic AMP response element-binding protein (**CREB**), has been shown capable of phenotypic modulation in blood vessels (Klemm et al., 2001). "Phenotypic modulation" means a switch in cellular phenotype that occurs under pathological conditions (e.g. hypoxia, mechanical injury, hyperlipidemia, oxidative stress). During vascular stress, smooth muscle cells (**SMC**) in the intimal and medial compartments of the arterial wall have been observed to become proliferative, migratory, and produce excess matrix proteins (Klemm et al., 2001). This group showed that nuclear content of CREB influenced cellular phenotype (Klemm et al., 2001). In blood vessels from neonatal calves exposed to chronic hypoxia, CREB content was depleted and smooth muscle cell proliferation was accelerated. Overexpression of active CREB arrested cell cycle progression, and decreased expression of genes encoding growth factors, growth factor receptors, and cytokines (Klemm et al., 2001). CREB may

also play a role in phenotypic modulation of bovine mammary epithelial cells (**MEC**) and mammary stromal cells.

Evidence supporting a role for CREB in modulating mammary tissue can be found in a microarray study of bovine mammary parenchyma (**PAR**) and mammary fat pad (**MFP**) in response to OVX and estrogen replacement (Li and Capuco, 2008). Mammary samples were taken from OVX and intact (**INT**) heifers that were either treated or not treated with exogenous estrogen (17β-estradiol; $\mathbf{E_2}$). Parenchyma and MFP gene expression were then evaluated using a high-density oligonucleotide microarray. From microarray data, a regulatory network was developed to show the relationship among genes influenced by estrogen in bovine mammary gland (Li and Capuco, 2008). Notably, CREB1 (a gene that encodes CREB protein) was an essential part of this network. The biological pathways most significantly influenced by estrogen included cell cycle, cell-to-cell signaling and interaction, and cellular assembly and organization (Li and Capuco, 2008).

Crosstalk between ERα and cAMP signaling pathways has been documented in human breast cancer cells by Lazennec and colleagues (2001). The authors found that CREB is required for transcriptional synergy between cAMP and estrogen signaling pathways. Their data suggest that CREB is involved in the crosstalk between ERα and protein kinase A (**PKA**) signaling pathways. They further proposed that CREB is linked to DNA by an ERα-coactivator complex and that CREB can in turn increase transcriptional activity in genes. They also noted that their hypothesis may have further implications for the regulation of estrogen-responsive genes by estrogen and antiestrogen

in breast cancer and other ERα-containing cells, especially if PKA is upregulated or overexpressed in these cells (Lazennec et al., 2001). This situation could possibly exist in bovine mammary gland.

ERβ protein is not detectable in bovine mammary gland (**MG**) and seems not to play a part in development (Connor et al., 2005). Therefore, it is most likely that the estrogen-responsive genes identified by Li and Capuco (2008) are regulated directly or indirectly, by E_2 binding ERα and causing downstream cellular events.

Previous studies have shown that OVX of prepubertal heifers increases the proportion of ERα-positive cells (Berry et al., 2003) and also advances myoepithelial cell differentiation (Ballagh et al., 2008). For this reason, abundance of CREB in relation to myoepithelial cells was examined to look for phenotypic changes associated with OVX. Myoepithelial cells can be characterized by their presence in the basal layer of PAR by their basket-like morphology, and the presence of α-smooth muscle actin (**SMA**) (Deugnier et al., 1995; Ballagh et al., 2008). In dairy heifers, ERα-positive cells are expressed only in the luminal and embedded layer of PAR (Capuco et al., 2002). Accordingly, our study evaluated the PAR epithelial layers separately. CREB protein abundance in relation to ERα was examined in the luminal and embedded epithelial layer, while CREB protein abundance in relation to SMA was examined in the basal epithelial layer.

We hypothesized that abundance of CREB would vary in relation to either ovarian status (INT or OVX), exogenous estrogen treatment (control or 17β-estradiol), epithelial layer (basal or luminal plus embedded), or some combination of the previous,

in PAR of bovine heifers. We also hypothesized that CREB would colocalize with many, but not all, ERα-positive cells in bovine mammary PAR. Colocalization of CREB and SMA would prove difficult to determine, as CREB is a nuclear stain while SMA is cytoplasmic.

MATERIALS AND METHODS

Mammary Tissue

Animal procedures were previously described (Li et al., 2006; Li and Capuco, 2008) and were approved by the USDA's Animal Care and Use Committee. Briefly, 16 3-mo-old Holstein heifers were blocked by body weight and used in an experiment with a 2 × 2 factorial design with ovarian status, INT or OVX, as the first factor and estrogen treatment (17β-estradiol or control) as the second. Bilateral OVX were performed when heifers reached ~150 kg (4.5 months old) under general anesthesia. Estrogen was supplied as 17β-estradiol dissolved in corn oil (Sigma, St. Louis, MO). Treatment and control injections (corn oil) were initiated 30 d after OVX, using daily subcutaneous injections of 17β-estradiol (0.1 mg/kg body weight) for three consecutive days. Similarly, control animals received corn oil at 0.1 mg/kg body weight. Injections were administered at 24 h intervals, and heifers were euthanized approximately 6 h after the final injection or 54 h after initiation of treatment. PAR samples from the left MG were sampled for histology. Total RNA from samples obtained from mid regions of PAR and MFP of the left mammary glands were interrogated by microarray analysis, as described previously (Li et al., 2006). In addition, quantitative real-time PCR validation experiments were

carried out on 21 genes, including 11 genes relevant to the pathways and networks identified in the analysis of microarray data (Li and Capuco, 2008).

Immunohistochemistry

CREB–ERα Dual Labeling. Paraffin sections of mammary tissue were obtained from the lab of A.V. Capuco (USDA-ARS, Beltsville, Maryland). Half of the tissue sections on one microscope slide (per heifer) were subjected to dual immunofluorescent labeling of CREB and ERα. Slides were processed similar to Daniels et al. (2009a). Briefly, slides were deparaffinized in xylene (3 × 5 min), hydrated through a series of graded ethanol washes (100%, 2 × 3 min; 95% 2 × 3 min; 70% 1 × 3 min), and rinsed with deionized water (2 × 2 min). Slides were then microwaved (2 × 5 min) in 10 m*M* citrate buffer (pH 6.0) for epitope unbinding. Slides were cooled for 30 min and rinsed in PBS (3 × 2 min). Before blocking, individual tissue sections were circled with a PAP barrier pen (cat no. 71312, Electron Microscopy Sciences, Hatfield, PA). Nonspecific binding sites were blocked with 30 min incubation in CAS block (cat no. 008120, Invitrogen, Carlsbad, CA). Slides were then subjected to 60 min incubation with the primary antibody solution. Each tissue section received 100 µL of the primary antibody solution which was a mixture of CREB rabbit monoclonal antibody (1:1,000; cat no. 9197, Cell Signaling Technology, Danvers, MA) and ERα mouse monoclonal antibody (1:100; cat no. sc-787, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in CAS Block. Control tissue sections received 100 µL of CAS Block. After incubation, slides were washed in PBS (3 × 2 min) and 100 µL of the secondary antibody solution was added to each section. The secondary antibody solution consisted of Alexa 488 donkey

anti-rabbit IgG (cat no. A21206, Invitrogen, Carlsbad, CA) and Alexa 594 donkey anti-mouse (cat no. A21203, Invitrogen) diluted in CAS Block (final antibody dilutions: 1:200). Incubation with the secondary antibody solution was performed in the dark and lasted 60 min. The secondary antibody solution was aspirated and slides were incubated for 15 s with DAPI (4'6-diamidino-2-phenylindole, dilactate, cat no. D3571, Invitrogen). Afterwards, slides were washed, in the dark, in PBS (3 × 2 min) and dipped in deionized water. Water was aspirated and 1 to 2 drops of ProLong Gold antifade reagent (cat no. P36939, Invitrogen) were added to each slide. A glass coverslip was placed on top of each slide and slides were allowed to dry 24 to 48 h in the dark before viewing by fluorescence microscopy. Negative control tissue sections (no primary antibody) were included in each staining batch.

CREB–SMA Dual Labeling. Half of the tissue sections on one microscope slide (per heifer) were also subjected to dual immunofluorescent labeling of CREB and SMA. CREB-SMA dual labeling was carried out in the same manner as CREB-ERα dual labeling. The primary antibody solution was a mixture of CREB rabbit monoclonal antibody (1:1,000; cat no. 9197, Cell Signaling Technology, Danvers, MA) and SMA goat polyclonal antibody (1:100, cat no. SAB2500963-100UG, Sigma-Aldrich, St. Louis, MO) diluted in CAS Block. The secondary antibody solution consisted of Alexa 488 donkey anti-rabbit IgG (cat no. A21206, Invitrogen, Carlsbad, CA) and Alexa 594 donkey anti-goat (cat no. A11058, Invitrogen) diluted in CAS Block (final antibody dilutions: 1:200). Negative control tissue sections (no primary antibody) were included in each staining batch.

Image Acquisition and Analysis

Slides were viewed on an Olympus IX81 microscope fitted with an epifluorescence attachment (Olympus Corporation, Shinjuku-ku, Tokyo, Japan) and images were captured with an Olympus DP72 camera. The DAPI, fluorescein isothiocyanate (FITC), and tetramethyl rhodamine isothiocyanate (TRITC) filter blocks were used for visualization of DAPI, CREB, and ERα or SMA, respectively. Digital images were obtained from the mid-parenchymal region of the udder for each animal, using one stained microscope slide (with one half of the sections stained for CREB/ERα/DAPI, and the other half of the sections stained for CREB/SMA/DAPI). These consisted of 10 sets of CREB, ERα, and DAPI images, and 10 sets of CREB, SMA, and DAPI images. Areas that contained primarily epithelium, as opposed to stroma, were selected at random by blurring the objective lens and moving the microscope stage prior to re-focusing. Obtained images were 8-bit monochrome images taken at 32 × magnification (20 × objective lens * 1.6 × manual optical zoom). Exposure lengths were 1.9, 160.2, and 204.8 ms for DAPI, CREB, and ERα or SMA, respectively. Captured images for each animal were further processed using CellSens Standard Software (Olympus Corporation, Center Valley, PA). Images were individually opened in the CellSens program and processed using the "count and measure" feature. Ductal tissue was manually circumscribed with the computer mouse to create two regions of interest (**ROI**) around each duct within every picture. The luminal and embedded epithelial cells were circumscribed together as one ROI, and surrounding basal epithelial cells were circumscribed as a second ROI (Figure 1). This was done so that a separate analyses

could be performed on the basal epithelial cells compared to the luminal and embedded epithelial cells. After all ductal tissue within each picture was outlined as an ROI, a manual threshold (or phase) was created to detect the positively stained cells within the ROI. Selection of the "count and measure on ROI" feature allowed only positive cells within ROI to be quantified. Colocalization and presence of CREB and ERα was evaluated, as well as presence of CREB and SMA. Cells positive for CREB and ERα as well as dual-labeled cells were counted manually. Additionally, the total number of epithelial cells was determined from area values given by the image analysis software and a cell conversion factor (different for luminal and embedded or basal cells). To examine the abundance of each protein, labeling indices were calculated, within each cell layer, to represent the percent of tissue that was positively stained. In the case of ERα and CREB data, this was done by taking the number positive for each particular stain / the total number present within the layer * 100. In the case of SMA and CREB data, this was done by taking the area occupied by each particular stain / the total area present within the layer * 100. These data were measured on an area basis because SMA is a cytoplasmic stain and hard to accurately quantify on a cell-basis

Statistical Analyses

All statistical analyses were performed using the Mixed Procedure of SAS (version 9.2, SAS Institute, Cary, NC) using a model that included the main effects of estrogen treatment (control or E_2 replacement), ovarian status (OVX or INT), and epithelial layer (basal or luminal and embedded), and all two-way interactions between the main effects. The random term was heifer within estrogen status and ovarian status.

The three-way interaction of OVX, estrogen replacement, and epithelial layer was considered, but was not included in the final model due to lack of significance. The CREB-ERα colocalization data were analyzed using essentially the same model. However, the colocalization dataset was a subset of our original data; animals that received E_2 replacement (INT + E and OVX + E) did not express ERα, and were not included in the colocalization dataset. Therefore, E_2 replacement was not included as a main effect because this dataset only included animals not treated with E_2.

RESULTS

CREB–ERα Dual Labeling

CREB-ERα data are presented on a cell basis. The approximate number of cells was determined from the total area outlined within each ROI. As expected, there was an interaction between E_2 replacement and epithelial layer ($P = 0.001$; Table 1). ERα was essentially absent from both basal and luminal and embedded layers in E_2 treated animals (Table 1; Figure 2). When present in non-E_2 treated animals, ERα was more prevalent in the luminal and embedded layer as opposed to the basal layer (Table 1). For CREB, there was no interaction between E_2 replacement and epithelial layer, as observed for ERα ($P = 0.092$), but there was an effect of layer ($P = 0.001$; Table 1). On a percentage basis, CREB was present more in the luminal and embedded layer as compared to the basal layer, regardless of estrogen treatment or ovarian status (Table 1).

Colocalization analysis of CREB and ERα was performed on the subset of animals that did not receive exogenous estrogen treatment (Figure 3). As expected, the

percent of MEC that were only ERα positive was higher in the luminal and embedded layer compared to the basal layer, regardless of ovarian status ($P = 0.002$; Figure 4). Percent of MEC that showed ERα and CREB colocalization was higher in the luminal and embedded layer compared to the basal layer ($P = 0.001$), regardless of ovarian status. Accordingly, the percent of MEC that was only CREB positive was higher in the basal layer compared to the luminal and embedded layer, regardless or ovarian status ($P = 0.001$).

CREB–SMA Dual Labeling

Unlike the CREB-ERα data, the CREB-SMA data are presented on an area basis. Because of their unique morphology, it proved difficult to estimate the approximate size of the myoepithelial cells (stained for SMA). Therefore, the CREB-SMA data are presented on an area basis; the values were not converted or compared on a cell basis. Also to be expected, SMA was present almost entirely in the basal layer and nearly absent from the luminal and embedded layer ($P = 0.001$; Figure 5). CREB was again present in higher amounts in the luminal and embedded layer compared to the basal layer, regardless of estrogen treatment or ovarian status ($P = 0.001$). Data for percent of MEC stained positive for either SMA or CREB are presented in Table 2. Figure 5 contains examples of histology images.

Colocalization of CREB and SMA was not determined; this would be extremely difficult. While CREB is localized in the nucleus, SMA is found in the cytoplasm. Because of the unique morphology of myoepithelial cells within the basal layer, the user could not confidently pair each nucleus with its corresponding cytoplasm (Figure 6). For

that reason, the CREB-SMA dual labeling data are presented as percent positively stained area within each layer (Table 2), while the CREB-ERα dual labeling data are expressed as percent positively stained cells within each layer, because both antigens are nuclear (Table 1).

DISCUSSION

This study investigated estrogen-responsive proteins and their localization in prepubertal bovine mammary tissue. Previous work (Li and Capuco, 2008) pointed to CREB as a possible mediator in estrogen signaling pathways in bovine mammary gland. The sample set used in the current experiment provided us with the opportunity to study the influence of OVX as well as estrogen treatment on the presence of CREB protein in bovine mammary tissue. Ideally, we had hoped to simultaneously stain for CREB, ERα, and SMA on one tissue section. Availability of microscope filter cubes required that red, green, and blue conjugate dyes be used if this were to be achieved. After experiencing poor signal intensity with the blue conjugate dye in our initial antibody staining, we decided to pursue the next option, dual labeling for CREB and ERα, followed by dual labeling for CREB and SMA on serial tissue sections from each animal. This plan negated the use of blue conjugate dyes for labeling antigens of interest and instead used the blue channel in the more traditional role of DAPI counterstain visualization (Figures 2 and 5).

We hypothesized that abundance of CREB would vary in relation to either ovarian status (INT or OVX), exogenous estrogen treatment (control or 17β-estradiol),

epithelial layer (basal or luminal plus embedded), or some combination of the previous, in PAR of bovine heifers. CREB protein localization in relation to ERα was examined in the luminal and embedded epithelial layer, while CREB protein localization in relation to SMA was examined in the basal epithelial layer. The response of ERα after estrogen treatment was, as expected, dramatic, but a similar pattern was not seen in CREB abundance. CREB was present within both the basal and luminal and embedded epithelial layers. The presence of CREB was more abundant in the luminal and embedded layer, regardless of estrogen treatment or ovarian status. Berry and colleagues (2003) had previously shown that OVX of prepubertal heifers increases the proportion of ERα-positive cells. Results from the current student showed only a numerical, not a significant ($P = 0.260$), increase in the proportion of ERα-positive cells. This may possibly be explained by age at OVX as animals in the current study were older at the time of OVX compared to the previous work by Berry et al. (2003).

We also hypothesized that CREB would colocalize with many, but not all, ERα-positive cells in bovine mammary PAR. Our colocalization data support this hypothesis and show that CREB did not require the presence of ERα.

The current study showed only a tendency of increased abundance of SMA in OVX animals in the interaction of OVX * layer ($P = 0.086$). This is of particular interest when compared to the work of Ballagh and colleagues (2008) who found a marked increase of SMA in myoepithelial cells of OVX heifers. This may partially be explained by OVX occurring later in life for the heifers in the current study; around 135 d of age (~4.5 mo) compared to an earlier OVX at 40 d of age in the previous study (Ballagh et

al., 2008). This then implies that age at OVX likely plays a large role in subsequent mammary development. Evidence to that end can be gleaned from Berry and others (2003). They found that OVX performed earlier in life (before 6 wk of age) more severely inhibited mammary development when compared to OVX at a later age (Berry et al., 2003).

In comparison with earlier CREB studies done by Klemm and others (2001), we did not observe anything suggestive of phenotypic regulation because the percentage of CREB positive cells did not differ across treatments. However, the current study examined only mammary PAR; CREB may be present in, and could possibly mediate signaling pathways within MFP, or at the interface of PAR and MFP.

CREB has previously been identified as being estrogen-responsive at the transcript level. Our data indicate that the tissue abundance of CREB protein does not show the same pattern. Additional post-transcriptional, translational, or post-translational modifications may account for this discrepancy. Estrogen-responsive genes and their proteins do not always follow the same pattern. For instance, Vandenberg et al. (2006) showed a constant response in gene expression (homeobox gene Msx-2, Wnt-4, and progesterone receptor) to varying doses of estrogen, while patterns for the encoded proteins varied in response to lower or higher doses of estrogen.

CONCLUSIONS

The presence of CREB was more abundant in the nuclei of cells in the luminal and embedded layer, regardless of ovarian status or estrogen treatment. Our

colocalization data show that CREB did not require the presence of ERα. Staining of both ERα and SMA was as expected. In contrast to previous work (Ballagh et al., 2008), the current study observed only a tendency for OVX to increase the abundance of SMA, which may be due to variations in the time of OVX. Our findings do not exclude CREB from playing a role in estrogen signaling in the bovine mammary gland. Although not elucidated here, CREB may still play a key role in signaling pathways that influence development of the bovine mammary gland because it was constitutively present in all treatment groups examined.

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Table 1. Percent mammary epithelial cells stained positive for either estrogen receptor alpha (ERα) or cAMP response element-binding protein (CREB) within mammary parenchyma from heifers in a 2 × 2 factorial design with ovarian status (intact or ovariectomized) as the first factor and estrogen treatment (control or 17β-estradiol) as the second. This table does not show colocalization data for ERα and CREB

						Test of fixed effects, *P*-value					
Item	OVX[1]	E rep[2]	Layer[3]	Estimate	SEM[4]	OVX	E rep	Layer	OVX × E rep	OVX × Layer	E rep × Layer
% ERα positive cells[5]	N	N	B	1.40	2.56	0.260	0.001	0.001	0.260	0.276	0.001
	N	N	LE	31.67							
	N	Y	B	0†							
	N	Y	LE	0†							
	Y	N	B	2.87							
	Y	N	LE	40.02							
	Y	Y	B	0†							
	Y	Y	LE	0†							
% CREB positive cells[6]	N	N	B	42.83	4.60	0.766	0.420	0.001	0.931	0.841	0.092
	N	N	LE	61.87							
	N	Y	B	41.84							
	N	Y	LE	54.97							
	Y	N	B	40.63							
	Y	N	LE	60.71							
	Y	Y	B	40.74							
	Y	Y	LE	54.23							

[1]OVX = ovariectomy at ~4.5 mo of age; no (N) or yes (Y).

[2]E rep = estrogen replacement, 17β-estradiol, at ~5.5 mo of age for 54 h; no (N) or yes (Y).

[3]Layer = epithelial layer; basal (B) or luminal and embedded (LE).

[4]SEM = standard error of the mean for OVX × E rep × Layer.

[5]% ERα positive cells = number of ERα positive cells / total number of epithelial cells * 100. Denominator in equation is the same as in the % CREB positive cells calculation.

[6]% CREB positive cells = number of CREB positive cells / total number of epithelial cells * 100. Denominator in equation is the same as in the % ERα positive cells calculation.

†Approximated from least squares means.

Table 2. Percent mammary epithelial cells stained positive for either α-smooth muscle actin (SMA) or cAMP response element-binding protein (CREB) within mammary parenchyma from heifers in a 2 × 2 factorial design with ovarian status (intact or ovariectomized) as the first factor and estrogen treatment (control or 17β-estradiol) as the second. This table does not show colocalization data for SMA and CREB

						Test of fixed effects, *P*-value					
Item	OVX[1]	E rep[2]	Layer[3]	Estimate	SEM[4]	OVX	E rep	Layer	OVX × E rep	OVX × Layer	E rep × Layer
% SMA positive area[5]	N	N	B	17.57	3.79	0.107	0.520	0.001	0.591	0.086	0.588
	N	N	LE	0.83							
	N	Y	B	24.07							
	N	Y	LE	1.01							
	Y	N	B	11.11							
	Y	N	LE	0.50							
	Y	Y	B	23.97							
	Y	Y	LE	0.49							
% CREB positive area[6]	N	N	B	31.51	3.54	0.445	0.688	0.001	0.678	0.617	0.517
	N	N	LE	47.89							
	N	Y	B	33.87							
	N	Y	LE	52.56							
	Y	N	B	32.82							
	Y	N	LE	46.67							
	Y	Y	B	32.92							
	Y	Y	LE	48.67							

[1]OVX = ovariectomy at ~4.5 mo of age; no (N) or yes (Y).

[2]E rep = estrogen replacement, 17β-estradiol, at ~5.5 mo of age for 54 h; no (N) or yes (Y).

[3]Layer = epithelial layer; basal (B) or luminal and embedded (LE).

[4]SEM = standard error of the mean for OVX × E rep × Layer.

[5]% SMA positive area = SMA positive area / total area * 100. Denominator in equation is the same as in the % CREB positive area calculation. Percent area data presented, not percent cells; see text.

[6]% CREB positive area = CREB positive area / total area * 100. Denominator in equation is the same as in the % SMA positive area calculation. Percent area data presented, not percent cells; see text.

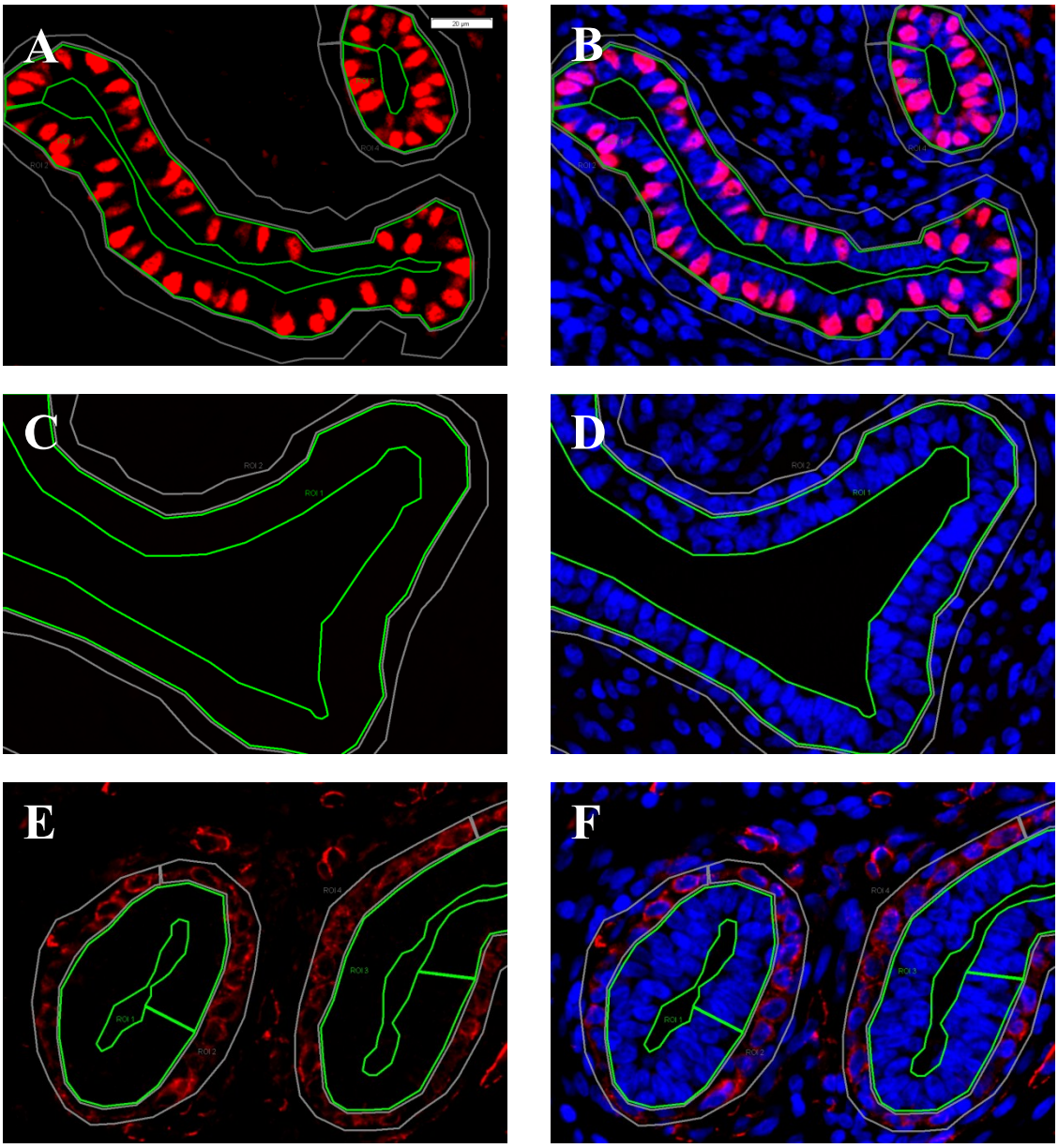

Figure 1. Mammary epithelium was characterized as either basal or luminal plus embedded, and was then outlined by the user as a region of interest (ROI). Basal cells were identified based on location, morphology, and staining for α-smooth muscle actin (SMA), when present. Luminal and embedded cells were identified based on location, morphology, and staining for estrogen receptor alpha (ERα), when present. Examples of basal (gray outline) and the luminal plus embedded (green outline) cell layers are shown above. A) ERα in an OVX animal; B) ERα + 4'6-diamidino-2-phenylindole (DAPI) in an OVX animal; C) ERα in an OVX + estrogen replacement animal; D) ERα + DAPI in an OVX + estrogen replacement animal; E) SMA in an OVX animal; and F) SMA + DAPI in an OVX animal. Scale bar = 20 μm.

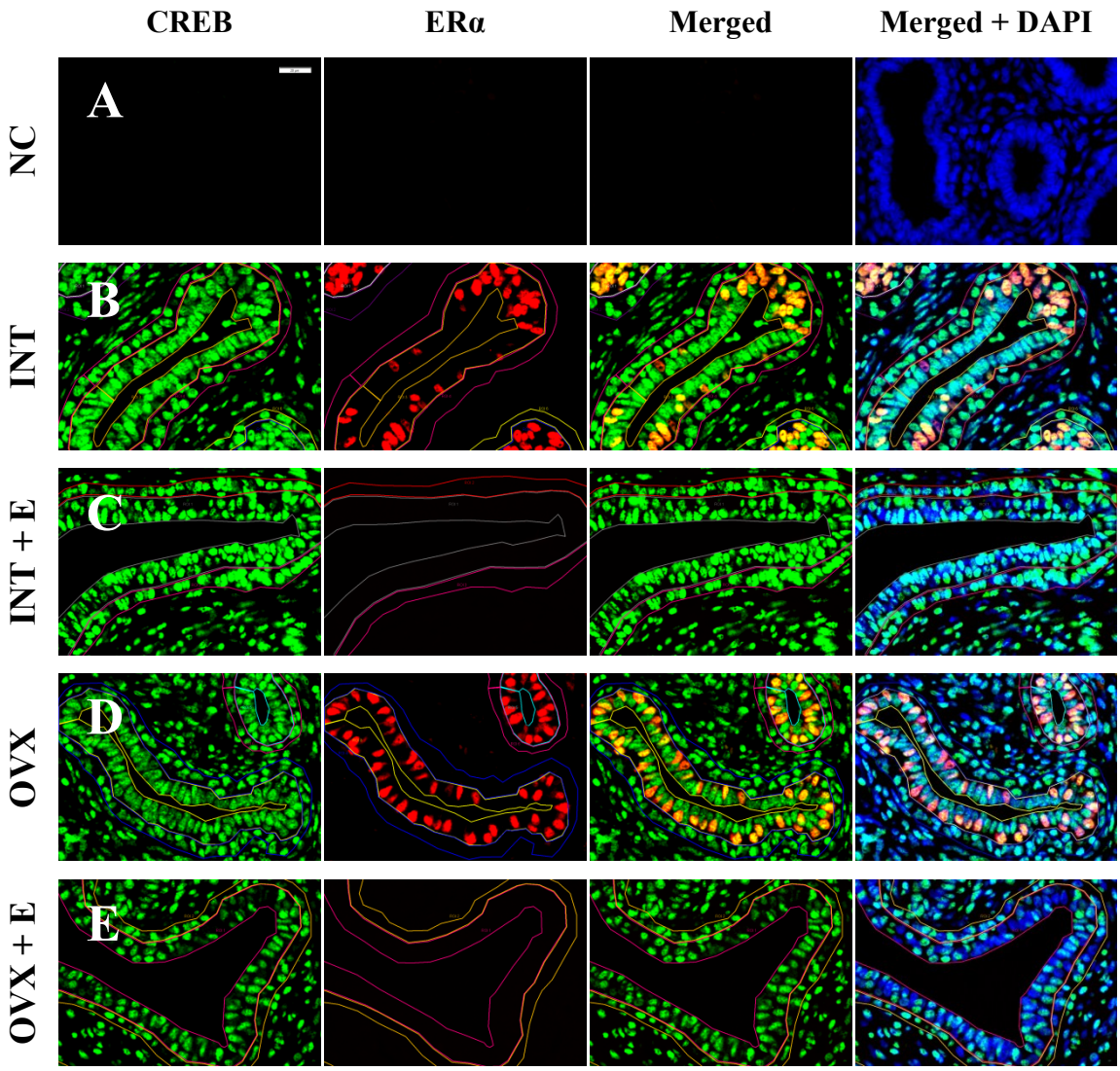

Figure 2. Localization of cAMP response element-binding protein (CREB) and estrogen receptor alpha (ERα) within mammary parenchyma from heifers in a 2 × 2 factorial design with ovarian status (intact, INT, or ovariectomized, OVX, ~4.5 mo of age) as the first factor and estrogen treatment (control or 17β-estradiol (E) ~5.5 mo of age) as the second. Mammary epithelium was outlined as regions of interest. A) Negative control (NC); primary antibodies were substituted with control sera; B) representative staining of an INT animal; C) representative staining of an INT + estrogen replacement animal; D) representative staining of an OVX animal; and E) representative staining of an OVX + estrogen replacement animal. DAPI = 4'6-diamidino-2-phenylindole. Scale bar = 20 µm.

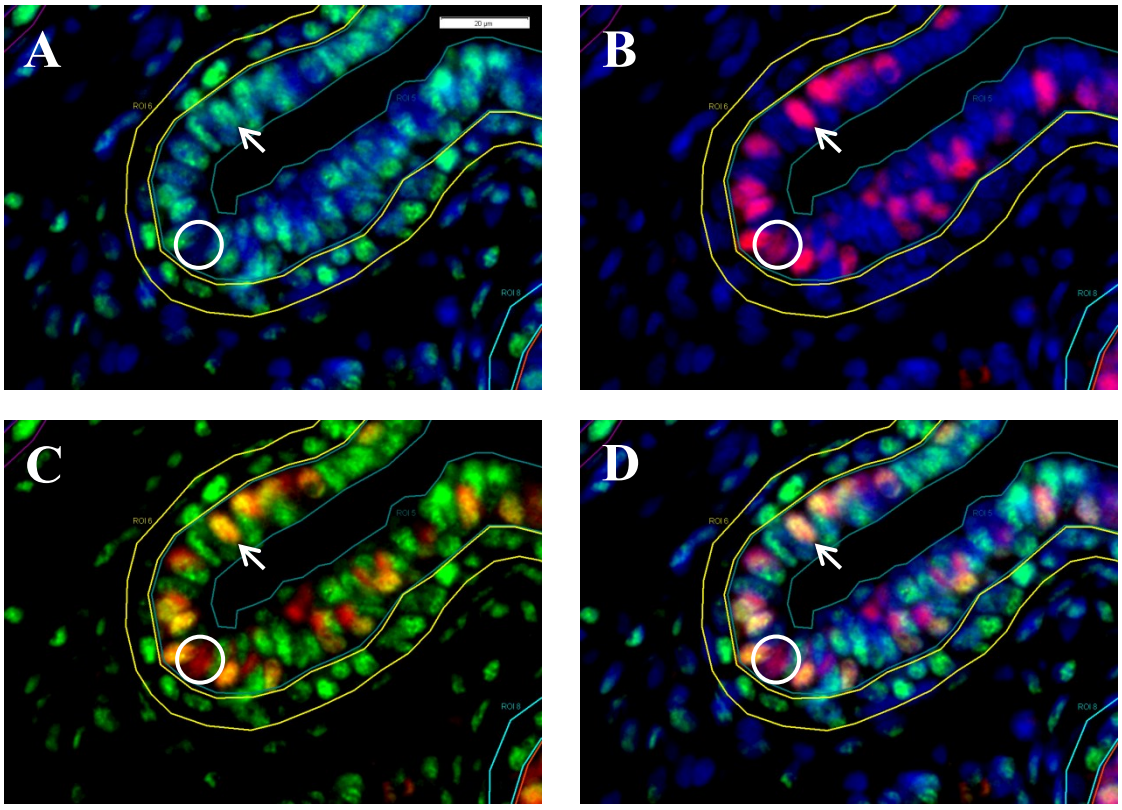

Figure 3. Example of a cAMP response element-binding protein (CREB)/estrogen receptor alpha (ERα) dual-labeled mammary epithelial cell (white arrow), and an ERα-labeled, CREB negative mammary epithelial cell (white circle) in an intact animal that did not receive estrogen treatment. Mammary epithelium was outlined as a region of interest. A) CREB + 4'6-diamidino-2-phenylindole (DAPI); B) ERα + DAPI; C) CREB + ERα; and D) CREB + ERα + DAPI. Scale bar = 20 μm.

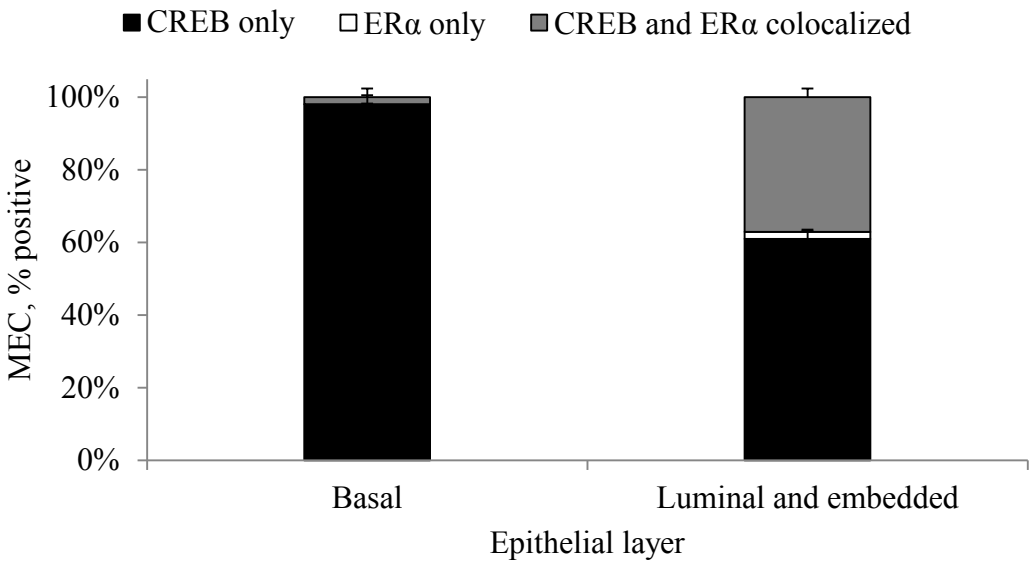

Figure 4. Percent of mammary epithelial cells (MEC) positive for cAMP response element-binding protein (CREB) only, estrogen receptor alpha (ERα) only, or both CREB and ERα (colocalized) differed by epithelial layer ($P = 0.001$) in animals that did not receive exogenous estrogen (INT (n = 4) and OVX (n = 4)). The interaction of ovarian status and epithelial cell layer was not significant ($P = 0.190$) nor was the main effect of ovarian status ($P = 0.216$). Heifers were operated on at ~4.5 mo of age and evaluated at ~5.5 mo of age.

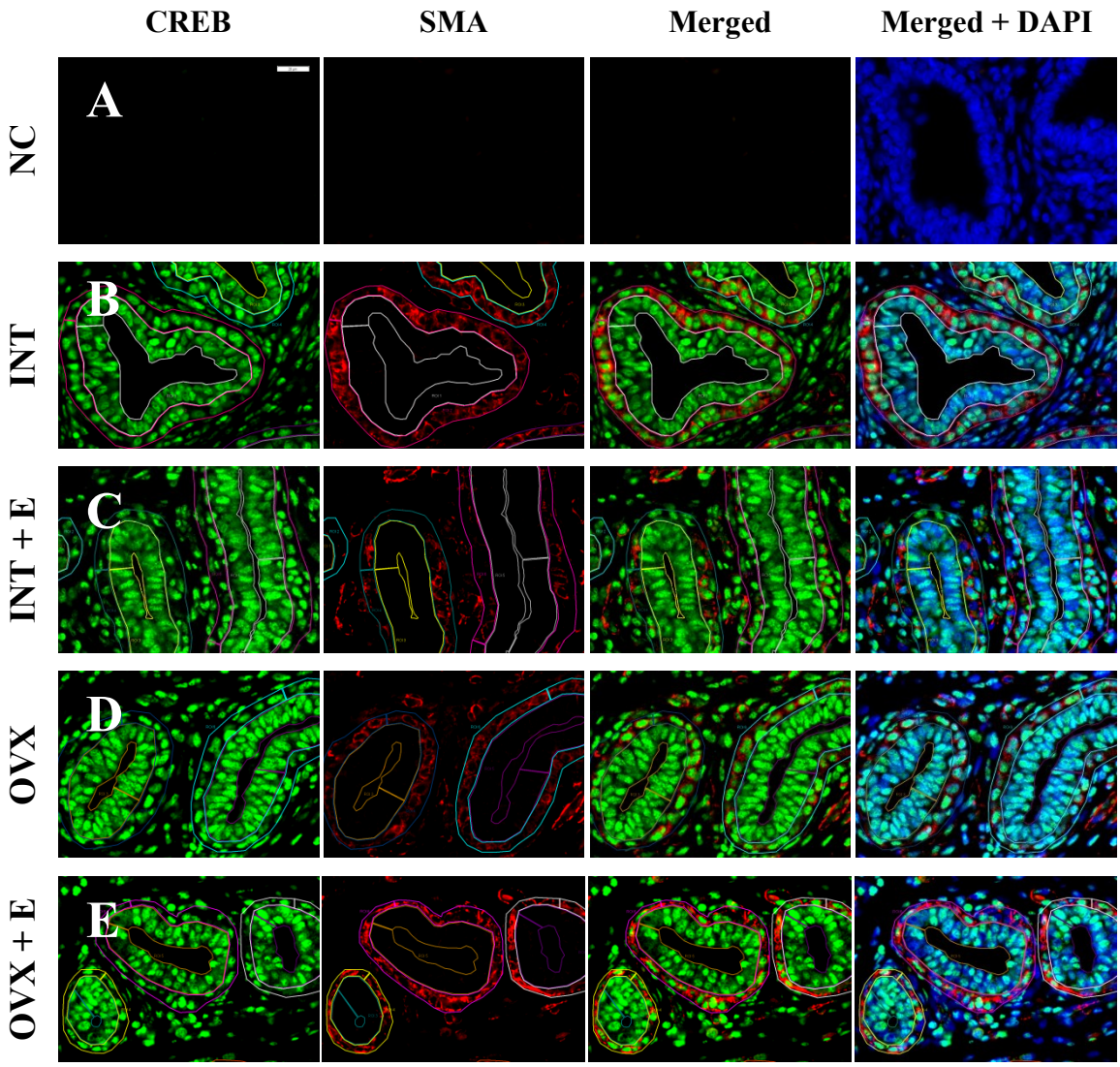

Figure 5. Localization of cAMP response element-binding protein (CREB) and α-smooth muscle actin (SMA) within mammary parenchyma from heifers in a 2 × 2 factorial design with ovarian status (intact, INT, or ovariectomized, OVX, ~4.5 mo of age) as the first factor and estrogen treatment (control or 17β-estradiol ~5.5 mo of age) as the second. Mammary epithelium was outlined as regions of interest. A) Negative control (NC); primary antibodies were substituted with control sera; B) representative staining of an INT animal; C) representative staining of an INT + estrogen replacement animal; D) representative staining of an OVX animal; and E) representative staining of an OVX + estrogen replacement animal. DAPI = 4'6-diamidino-2-phenylindole. Scale bar = 20 μm

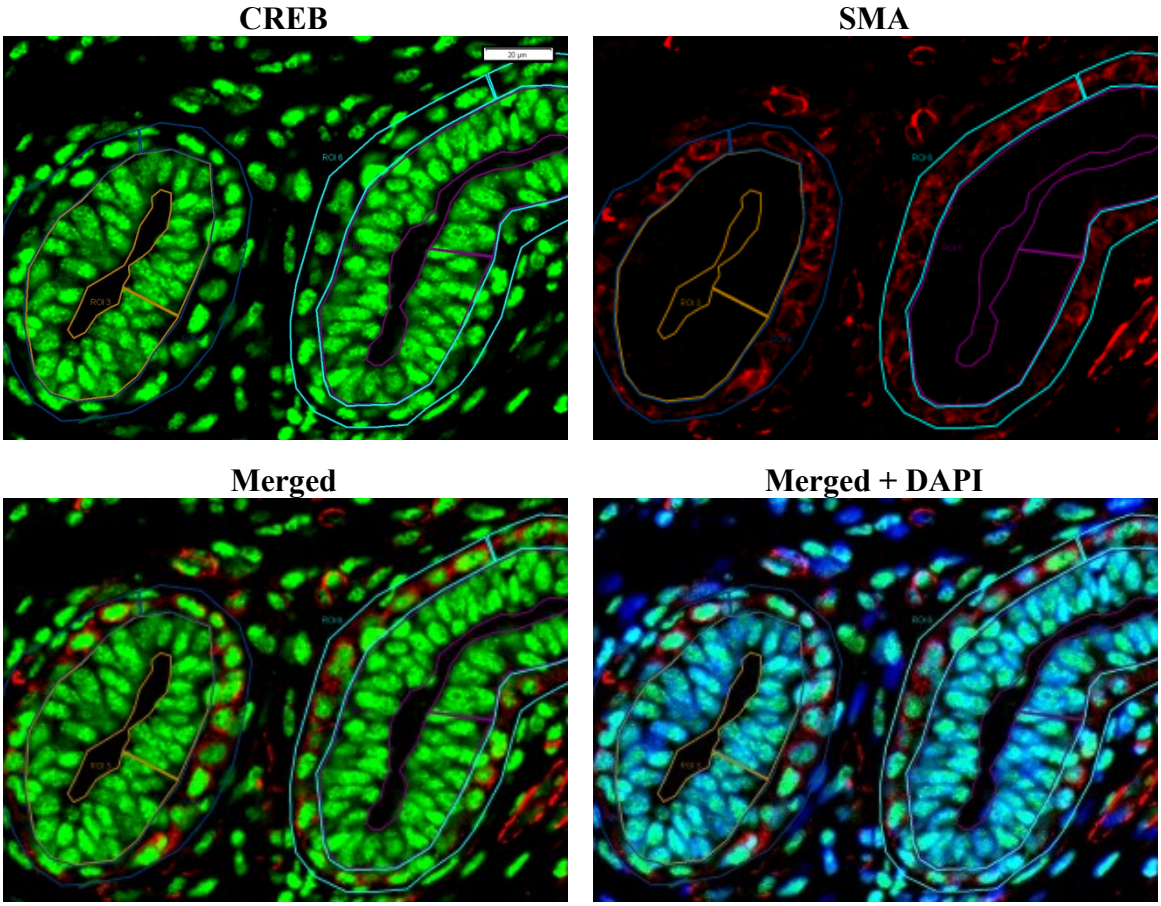

Figure 6. Colocalization of CREB and SMA was not determined; this would be extremely difficult. While CREB is localized in the nucleus, SMA is found in the cytoplasm. Because of the unique morphology of myoepithelial cells within the basal layer, the user could not confidently pair each nucleus with its corresponding cytoplasm. Example images are from an OVX animal. Mammary epithelium was outlined as a region of interest. DAPI = 4'6-diamidino-2-phenylindole. Scale bar = 20 μm.

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CHAPTER 3:

Effect of Ewe Body Condition Score During Mid to Late Gestation on Mammary Composition and Epithelial Cell Proliferation of Female Progeny

In preparation for submission to Journal of Dairy Science

ABSTRACT

The foundation for functional mammary secretory tissue, parenchyma (PAR), is established early in life; amount of PAR directly relates to future milk production. Dam body condition score (BCS) during mid to late gestation may affect progeny postnatal mammary growth and composition via in utero metabolic programming events. Pregnant ewes (n = 96; ≈80 d of gestation) were allotted to treatment groups based on initial BCS of 2, 3, or 4 (on a 1 to 5 scoring system with 1 being emaciated and 5 being obese). Ewes were housed in 18 pens (6 pens per treatment) and fed a prescribed diet of corn silage (1.1 kg DMI/d), to which whole shelled corn was supplemented at 0.12, 0.26, or 0.47 kg DMI/d for BCS groups 2, 3, and 4, respectively. Amount of corn was adjusted every 2 wk to maintain desired BCS throughout pregnancy. Prior to weaning, lambs nursed their mothers and were fed a common starter using creep feeders. Lambs were weaned (≈56 d

of age; 23.68 kg) and placed on a common finishing diet that met NRC requirements. Female progeny from the three BCS groups (n = 73) were slaughtered at similar BW (46.9 ± 0.5 kg), and age (126.3 ± 2.8 d). Udders were removed and mammary tissue subjected to biochemical analysis. Total mammary gland weights (179.2, 167.4, and 175.6 ± 8.8 g for BCS 2, 3, 4, respectively) did not differ by treatment. However, PAR weight of progeny from BCS 2 ewes (25.3 g) tended to be greater than that of BCS 3 (18.5 g) or BCS 4 (18.8 g) progeny. Protein mass within PAR (BCS 2 = 1.43, 3 = 1.02, and 4 = 1.07 ± 0.13 g) varied by treatment, as did DNA mass within PAR (BCS 2 = 134.8, 3 = 93.1, and 4 = 103.0 ± 13.4 mg). Lipid mass within PAR did not differ by treatment and averaged 7.10 ± 1.17 g. Despite detectable differences in PAR due to treatment, no differences in weight or composition of the mammary fat pad were found. Factors that promote mammary PAR growth may have a positive impact on future milk production. Our observations suggest that BCS during gestation may have important lactation performance implications for female progeny.

Key words: body condition score, sheep, mammary, gestation

INTRODUCTION

The foundation for functional mammary secretory tissue is established early in life; mammary development is directly related to the milk production potential of a lactating animal (Tucker, 1987). Nutrition has been shown to influence mammogenesis as early as the pre-weaning period in dairy heifers (Brown et al., 2005; Daniels et al., 2009a) and the prepubertal period in ewe lambs (Umberger et al., 1985; McCann et al., 1989).

Increased nutrition prior to 2 mo of age in dairy heifers, mainly a time of isometric mammary growth (Sinha and Tucker, 1969), has yielded a positive effect on development of the mammary gland (**MG**). Brown et al. (2005) showed that higher energy and protein intake from 2 to 8 wk of age increased PAR mass without increasing deposition of PAR fat. In a separate study on 65 d old heifers fed milk replacer diets of varying fat and protein content, altered nutrient intake was shown to increase the mass and alter the composition of MFP and did not have an effect on PAR mass or composition (Daniels et al., 2009a).

From 3 mo of age through the first few estrous cycles, mammary growth is allometric for dairy heifers (Sinha and Tucker, 1969). Extensive duct lengthening and branching occurs during this time, and increased nutrient intake during this time has been shown to negatively influence MG growth (Sejrsen et al., 1982; Capuco et al., 1995). Brown and colleagues (2005) also showed that in heifers from 8 to 14 wk of age, increased protein and energy intake increased deposition of PAR fat, but did not increase PAR mass. Recently, it was shown that composition of the MG from birth through puberty was not affected by feed intake (Meyer et al., 2006a,b). Daniels et al. (2009b) emphasized that BW and age, not rate of gain, have the greatest impact on mammary development (viewed histologically) in dairy heifers.

Nutritional influences early in life have also been shown to affect subsequent milk production. Radcliff and others (2000) showed that heifers reared on a higher plane of nutrition during the prepubertal and pubertal periods had decreased first lactation milk yields when compared to heifers reared on a standard diet meeting NRC requirements.

While nutritional effects on mammogenesis in dairy heifers are still debated, even less is known about mammary development in ewe lambs and the influence that nutrition may have. Previous studies in ewes have shown results similar to those in dairy heifers, namely that increased nutrition during the prepubertal period can hinder development of the MG. Umberger and others (1985) found that accelerated growth during rearing impaired milk yield, as well as the number of alveoli present in ewes. Similarly, McCann et al. (1989) showed that rapid weight gain to puberty in ewe lambs led to lower milk production and an increase in MFP area.

One novel area of interest is the influence of gestational environment and subsequent mammary growth of the progeny. This is an essentially unexplored research area in both dairy cattle and sheep. The importance of maternal nutrition during gestation has previously been emphasized through the "fetal origins" hypothesis proposed by Barker (1995) suggesting that fetal undernourishment results in permanent detrimental changes leading to the development of disease later in life (Barker, 1995). Furthermore, Lucas (1991) has defined "metabolic programming" as the process whereby nutritional manipulation during a critical period of development has lasting or lifelong significance. Because the developing fetus relies on the mother for nutrients, it is interesting to speculate that a change in the gestational environment may possibly alter embryonic mammogenesis so much so, that effects persist postnatally.

Previous studies have shown the effects of varied gestational environments on several aspects of fetal growth and development, other than mammary development. Most recently, promising data from Wallace et al. (2010) showed that overnourished ewe

dams had reduced initial colostrum yield and total IgG yield, but their low birth weight lambs showed rapid compensatory growth to weaning (Wallace et al., 2010). A similar trial using beef heifers also examined compensatory fetal growth in progeny from dams placed on a lower plane of nutrition during gestation (Micke et al., 2010). Therefore, it is of interest to examine if the MG also undergoes compensatory growth during the pre-weaning period of normally isometric growth.

To date, no studies have been published on gestational effects on progeny mammary development in dairy heifers, and only one paper on this topic currently exists in the ewe lamb literature. Van der Linden and colleagues (2009) showed that mammary duct area of ewe lambs from dams maintained on a higher plane of nutrition during pregnancy was less than mammary duct area of ewe lambs from dams given a maintenance diet during pregnancy. However, the fetal MG measurements were taken only at d 100 of gestation, just before the third trimester, and total duct area and duct number were the only measurements taken. More quantitative methods are needed to determine differences in progeny mammary growth and composition in ewe lambs. Our proposed research methods will supply a more comprehensive picture of gestational influences on progeny mammary growth and development.

The objective of this study was to explore the effects of varying body condition score (**BCS**) during gestation on MG composition in ewe lamb offspring. This experiment was conducted as part of a larger trial done by A.E. Radunz et al. (S.C. Loerch, OARDC, The Ohio State University, Wooster, OH, personal communication).

We hypothesized that variations in dam BCS during mid to late gestation would affect progeny postnatal mammary composition and mammary epithelial cell proliferation.

MATERIALS AND METHODS

Animals and Treatments

Animal procedures were approved by Ohio State University's Institutional Animal Care and Use Committee. Lambs used were part of a larger trial studying the effects of ewe BCS during mid to late gestation on ewe performance and progeny postnatal growth (S.C. Loerch, OARDC, The Ohio State University, Wooster, OH, personal communication). Pregnant ewes (n = 96; ≈80 d of gestation) were allotted to treatment groups based on initial BCS of 2, 3, or 4 (on a 1 to 5 scoring system with 1 being emaciated and 5 being obese; a single individual made the BCS measurements throughout the trial). Ewes were bred by natural service to one of 8 rams; these included 4 Dorset, 2 Hampshire, and 2 Suffolk rams. Sires were randomized across all BCS ewes. At the initiation of the trial, ewes were housed in 18 pens (6 pens per treatment) and fed a prescribed amount of corn silage (1.1 kg DMI/d), to which whole shelled corn was supplemented at 0.12, 0.26, and 0.47 kg DMI/d for BCS groups 2, 3, and 4, respectively. Body weight and BCS were measured for two consecutive days at the start of the trial and every 2 wk during the trial. The amount of corn offered was adjusted as needed to maintain targeted BCS within treatments. Feed samples were taken every 2 wk, composited, and analyzed for DM, N, ether extract, NDF, ADF, Ca, and P (data not

shown; A.E. Radunz et al., unpublished data). Lamb weight and vigor score were recorded at parturition. Ewe weight and BCS were also recorded at that time.

Lactating ewes were housed in 18 pens (6 pens per treatment) and fed a common lactation diet. Lambs were weighed at 28 d postpartum (near peak lactation); milk production and composition were measured using a weigh-suckle-weigh approach at this time (Radunz et al., 2011b). Briefly, ewes were separated from their lambs 28 ± 1 d after lambing, given an injection of oxytocin (1 mL; 10 IU) into a jugular vein, and milked out by hand. Ewes were kept separate from their lambs for a 3-h period after which ewes were given a second injection of oxytocin (1 mL; 10 IU), milked out by hand, and 3-h milk weights were determined. A subsample of milk was collected and treated with bronopol and natamycin and held at 4°C until analyzed by a commercial DHI laboratory.

Lambs nursed their own mother prior to weaning, which took place when offspring averaged 23.68 ± 0.76 kg BW ($P = 0.766$) and 56.97 ± 0.99 d ($P = 0.054$). Lambs then entered a feedlot phase, grouped by original dam pen, and were fed an identical finishing diet that met requirements for feedlot lambs (NRC, 1985).

Mammary Gland Collection

Ewe lambs were harvested at an average body weight of 46.92 ± 0.53 kg ($P = 0.913$) and age 126.31 ± 2.80 d ($P = 0.160$). Lambs were euthanized by captive bolt followed immediately by exsanguination at the Ohio State University Meat Science Lab. The whole udder was removed, weighed, and bisected along the median suspensory ligament. The left MG was sampled for histology. Mid-PAR, MFP, and interface samples to be used for histology were placed into vials of formalin. Formalin was replaced with

70% ethanol 24 h later. Tissues remained in 70% ethanol at 4°C until paraffin embedding. The right MG was weighed, wrapped in foil, flash frozen in liquid nitrogen, transported to the OARDC on dry ice, and stored at -80°C until further composition analysis.

Staining Procedures for Histology

Slides were processed similar to Daniels et al. (2009a) and Brown et al. (2005). Briefly, microscope slides were prepared by slicing 5-µm-thick sections from the paraffin-embedded tissue blocks with a microtome. Two or three serial tissue sections from each sample were mounted on positively charged microscope slide. Slides were deparaffinized in xylene (3 × 5 min), hydrated through a series of ethanol washes, quenched with H_2O_2, and microwaved in a 10m*M* citrate buffer (pH 6.0) for antigen retrieval. Before blocking, individual tissue sections were circled with a PAP barrier pen (cat no. 71312, Electron Microscopy Sciences, Hatfield, PA). Slides were then blocked with CAS block (cat no. 008120, Invitrogen, Carlsbad, CA) for 30 min before incubation with pre-diluted Ki67 rabbit monoclonal antibody (clone SP6, cat no. RM-9106-R7, Thermo Scientific, Waltham, MA) for 60 min. The SuperPicture Polymer Detection kit (Invitrogen, Carlsbad, CA) was then used. A 30 min incubation with the pre-diluted secondary antibody, a broad spectrum poly-HRP conjugate (Invitrogen, Carlsbad, CA), followed. Slides were incubated with DAB chromogen (3,3' diaminobenzidine; Invitrogen, Carlsbad, CA) for 4 min. Samples were washed in deionized water and then counterstained with hematoxylin for 1 min. Slides were then washed in tap water,

dehydrated, and coverslipped with the aid of Permaslip mounting medium (Alban Scientific Inc., St. Louis, MO).

Image Acquisition and Analysis

Slides were viewed on an Olympus IX81 microscope (Olympus Corporation, Shinjuku-ku, Tokyo, Japan). Three digital pictures were obtained from the mid-parenchymal region of the udder for each animal, using one stained microscope slide. Images were selected at random by blurring the objective lens and moving the microscope stage prior to re-focusing and images were captured with an Olympus DP72 camera. The scope was set for brightfield illumination, and 20× magnification. Lamp voltage was set at 9v, with a natural contrast, and 0 exposure compensation. Each picture was white balanced, and the exposure length was locked at 83µs for all images. Images were further processed using CellSens Standard Software (Olympus Corporation, Center Valley, PA). Images were individually opened in the CellSens program and processed using the "count and measure" feature. Ductal tissue was manually circumscribed with the computer mouse to create a region of interest (**ROI**) around the ducts within each picture. After all ductal tissue within the picture was outlined as an ROI, a manual threshold (or phase) was created to detect the Ki67 (brown) cells within the ROI (Figure 7). The phase contained three channels with programmed minimum and maximum wavelengths of: Red, 109 and 530; Green, 37 and 426; and Blue, 30 and 461, respectively. Selecting the "count and measure on ROI" feature allowed only positive cells within ROI to be quantified. To prevent non-nuclear artifacts from being counted, the object filter was then set to remove any objects smaller than $4\mu m^2$ in area. The object

filter was removed and the remaining data were exported to a Microsoft Excel document. Within each image, each ROI that was outlined was assigned a percentage of Ki67 positive cells by the software (Figure 8). These values were then summed and averaged for each image. The image averages for each animal were then likewise averaged, yielding a Ki67 labeling index (number of Ki67 positive cells / total number of epithelial cells × 100) for each animal. Total area occupied by epithelium in each image was also documented and averaged for each ewe lamb prior to statistical analysis.

Mammary Dissection for Composition Analysis

Previously collected and frozen right mammary glands were thawed at 4°C overnight, weighed, and dissected by color into one of four fractions; PAR, MFP, lymph node, or discard (hide, teats, large blood vessels). Weights of each fraction were recorded and the lymph node was discarded along with the discard fraction. Dissected PAR and MFP fractions were wrapped in foil and again stored at -80°C for later biochemical analysis.

Biochemical Analyses

The PAR and MFP portions were later removed from the freezer and ground to a fine powder in the presence of dry ice using a commercial Waring blender. This powder was subsampled for determination of lipid, protein, and DNA content. Water content was not assessed due to the nature of storage and processing methods used. Lipid content was determined gravimetrically with duplicate samples using the method of Hara and Radin (1978), as summarized recently by Daniels et al. (2009a).

Protein and DNA determination methods were adapted from Daniels et al. (2009a). Prior to protein and DNA determination, (~300 mg each) of PAR and MFP fractions were homogenized in 0.9% NaCl. Protein concentrations were determined in triplicate using the bicinchoninic acid assay (Pierce, Rockford, IL) with BSA as the standard. DNA content was determined in triplicate using a DNA quantitation kit and fluorescence assay (Sigma-Aldrich, St. Louis, MO). The bisBenzimide Hoechst 33258 dye reagent was used with calf thymus DNA as the assay standard.

Statistical Analyses

All statistical analyses were performed using the Mixed Procedure of SAS (version 9.2, SAS Institute, Cary, NC) using a model that included the effect of dam BCS. Sire breed, parity, birth type, and rear type were included in the model as covariates if they represented a significant ($P \leq 0.05$) source of variation. No random term was specified, yielding the basic model: $y_{ij} = \mu + T_i + e_{(i)j}$. "Lamb" was used as the experimental unit. When treatment was significant ($P \leq 0.05$), means were separated by the PDIFF procedure of SAS.

RESULTS

Dam Information and Female Progeny Distribution

Ewe and lambing information for the 57 dams yielding female progeny is summarized in Table 3. Seventy-three ewe lambs were obtained from 57 dams. Table 4 lists the twin status and breed of sire for all ewe lambs.

Body Weight, Age, and Average Daily Gain

Body weight, age, and ADG data are summarized in Table 5. Female progeny from BCS 2 dams had lower birth weights (4.69 kg) than progeny from BCS 3 (5.31 kg) or BCS 4 (5.41 ± 0.18 kg) dams ($P = 0.010$). Body weight and age measurements were also taken near the dams' peak milk production (28 ± 1 d into lactation). Progeny from BCS 2 dams still tended to weigh less at this time ($P = 0.082$). These progeny were also younger (27.70 d) on d 28 of lactation compared to BCS 3 (28.17 d) or BCS 4 (27.78 ± 0.13 d) progeny ($P = 0.021$). As designed, weaning weights did not differ across treatments and averaged 23.68 ± 0.76 kg ($P = 0.766$). However, weaning ages tended to be greater in progeny from BCS 2 dams ($P = 0.054$) when compared to either BCS 3 or BCS 4 progeny. As designed, lambs were harvested at a similar BW (46.92 ± 0.53 kg; $P = 0.913$); age did not differ at slaughter (126.31 ± 2.80 d; $P = 0.160$). Average daily gain was calculated for 5 different periods and, though not significantly different, ADG was numerically greater for BCS 3 progeny and lowest for BCS 2 progeny for all intervals measured (Table 5).

Dam Milk Production

Peak milk production and composition of dams are presented in Table 6. Milk yield and composition were similar among treatments.

PAR and MFP Weight

There were no treatment differences in either the total MG weight or MFP weight when compared as actual weights, or when adjusted for equal BW (Table 7). In contrast,

PAR weight tended to be highest from BCS 2 progeny when compared both as actual weights ($P = 0.075$) and when adjusted for equal BW ($P = 0.054$).

PAR and MFP Composition

No differences were detected in the lipid, protein, or DNA composition of MFP due to treatment (Table 7). PAR composition, however, tended to be influenced by dam BCS. The concentration of lipid (mg / g PAR) tended to be higher in BCS 2 progeny ($P = 0.064$). Total amount of lipid (g), though not significantly different, was also numerically highest in progeny from BCS 2 dams. Total amount of protein (g) in PAR tended to be highest from BCS 2 progeny ($P = 0.054$), as did total amount of DNA (mg) in PAR ($P = 0.073$). No differences by treatment were seen in concentrations (mg / g PAR) of either protein or DNA.

Ki67 in PAR

Percentage of Ki67 labeled cells in PAR tended to be lowest in progeny from BCS 4 dams ($P = 0.058$). Epithelial area (outlined by the operator as a region of interest) was also numerically lowest in progeny from BCS 4 dams ($P = 0.108$). Values are presented in Table 8.

DISCUSSION

The majority of ovine mammary growth occurs postnatally. Many factors applied postnatally, such as level of nutrient intake, are known to affect mammary development (Daniels et al., 2009a, Meyer et al., 2006a,b), and therefore future milk production potential of the dam (Capuco et al., 2001). The fetal period of mammary growth

primarily consists of establishment of a primitive branching ductal network that differentiates postnatally into the alveolar system responsible for milk production (Tucker, 1987). Appropriate nutrition is essential for growth and development the mammary gland. Mammogenesis may be influenced by under-or over-nutrition. Our findings point to the importance of the fetal environment for mammary growth of the offspring. The objective of the present study was to determine the influence that maternal BCS during mid to late gestation may have on progeny mammary development.

The effects of different fetal environments, essentially nutrient intake of the mother, on mammary growth and subsequent first lactation performance of the offspring have not been extensively reported; one of the only papers in this area is the work of van der Linden et al. (2009). Their study suggests that ad-libitum feeding of the dam during pregnancy may have negative implications on lactation performance of the offspring (van der Linden et al., 2009).

The current study found that birth weights of female progeny from low BCS dams were significantly lower than birth weights of their cohorts. These progeny then exhibited compensatory growth from birth to harvest as harvest weights were similar when compared across treatments at similar ages. While weaning weights were similar across treatments, weaning ages of BCS 2 progeny were higher than either BCS 3 or BCS 4 progeny, indicating that these progeny did take longer to reach their weaning weight. Accordingly, ADG did not differ across treatments during any of the evaluated times. Compensatory growth of low birth weight progeny was also seen by Wallace et al. (2010). In contrast to our experiment, however, the low birth weight progeny in their

experiment resulted from overnourished ewe dams. The catch-up growth by low birth weight progeny in the current experiment was not caused by differences in dam milk production or composition, as there were no differences among dams across treatments for these measurements. During lactation ewes were no longer being maintained at their initial BCS scores and diets were the same across treatments during lactation.

We hypothesized that variations in dam BCS during mid to late gestation would affect progeny postnatal mammary composition and mammary epithelial cell proliferation. No differences were seen in the weight or composition of the mammary fat pad due to maternal BCS. Dam BCS during gestation did tend to affect PAR weight as well as the total amount of both protein and DNA in PAR. It is difficult to compare these results to previous findings because only total MG weights were reported by van der Linden et al. (2009). The nutritional affects on mammary development reported here do differ from affects seen during previous studies in the prepubertal period (Umberger et al., 1985; McCann et al., 1989). McCann et al. (1989) found differences only in MFP area in prepubertal ewe lambs on varied planes of nutrition.

In the current study, lipid concentration with PAR did tend to be higher in BCS 2 progeny, but this group also tended to have higher PAR weight.

Although the current study focused on a single time point analysis of mammary epithelial cell proliferation in prepubertal ewe lamb offspring, our findings seemingly compliment the findings of van der Linden et al. (2009). Our data show a tendency for lower proliferation in BCS 4 progeny at the time of slaughter, but no differences in total epithelial area were seen. Van der Linden et al. (2009) observed lower epithelial area at

100 d of gestation in progeny from dams on a higher plane of nutrition during gestation (comparable to the BCS 4 dams in the present study). Using van der Linden et al. (2009) as a baseline, it seems that the BCS 4 progeny in the current study may have experienced rapid compensatory mammary duct growth, and that proliferative status only at the time of slaughter had slowed. Taken together, both studies suggest an inverse relationship between dam BCS during mid to late gestation and ewe lamb mammary epithelial cell proliferation and subsequent lactation milk yield. If this relationship does in fact exist, then this demonstrates nutritional imprinting in the mammary gland.

CONCLUSIONS

In conclusion, data presented here show that both progeny mammary composition as well as progeny mammary epithelial cell proliferation are influenced by maternal BCS during gestation. Dam BCS during mid to late gestation affected the birth weight of lambs, with lower BCS ewes having lower birth weight lambs. Mammary fat pad weight or composition of female progeny was not influenced by maternal BCS during gestation. Lower dam BCS during gestation tended to increase the amount of PAR, as well as the protein and DNA content within PAR. Dam BCS tended to affect Ki67 labeling index of progeny. A potential inverse relationship was also seen between dam BCS during mid to late gestation and ewe lamb mammary epithelial cell proliferation.

Given that both amount of PAR and mammary epithelial cell number are positively correlated with milk yield, our observations here require further evaluation as they may have important lactation performance implications for sheep as well as dairy

cattle. Nutrient supply to the fetus of dairy cows may vary greatly as the dam transitions from early to late lactation and into the dry period. Effects of these factors on subsequent mammary development and lactation potential of the progeny are unknown. Additional research is required to determine the optimal gestational environment for fetal mammary development in both the sheep industry and the dairy industry.

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Table 3. Ewe and lambing information for dams maintained at different body condition scores (BCS) during mid to late gestation

Dam BCS[1]	Parity	Birth Type	n
2	1	Single	2
2	1	Twins	11
2	2	Single	1
2	2	Twins	3
3	1	Single	3
3	1	Twins	10
3	2	Single	1
3	2	Twins	3
4	1	Single	4
4	1	Twins	10
4	2	Single	2
4	2	Twins	5
4	3	Twins	1

[1]Dam BCS: Dams were maintained at a BCS of 2, 3, or 4, during mid to late gestation. Scoring system was on a 1 to 5 scale, with 1 being emaciated and 5 being obese.

Table 4. Female progeny distribution for ewe lambs from dams maintained at different body condition scores (BCS) during mid to late gestation

	Dam BCS[1]		
	2	3	4
Sire breed, birth type	(n = 22)	(n = 23)	(n = 28)
Dorset			
Female, female twins	5†	8	6
Female, male twins	4	2	7
Single female	1	2	2
Hampshire			
Female, female twins	6	2	4
Female, male twins	2	3	1
Single female	1	1	1
Suffolk			
Female, female twins	0	2	2
Female, male twins	2	2	2
Single female	1	1	3

[1]Dam BCS: Dams were maintained at a BCS of 2, 3, or 4, during mid to late gestation. Scoring system was on a 1 to 5 scale, with 1 being emaciated and 5 being obese.

†One twin pairing was not collected.

Table 5. Birth, weaning, and harvest BW and age, as well as ADG of lambs from dams maintained at different body condition scores (BCS) during mid to late gestation

	Dam BCS[1]				
Item	2 (n = 22)	3 (n = 23)	4 (n = 28)	SEM[2]	*P*-value
Birth Weight, kg	4.69	5.31	5.41	0.18	0.010
BW near dam's peak milk (28 d target), kg	12.64	13.93	13.60	0.42	0.082
Age near dam's peak milk (28 d target), d	27.70	28.17	27.78	0.13	0.021
Weaning Weight, kg	23.23	23.94	23.86	0.76	0.766
Weaning Age, d	58.77	55.35	56.79	1.00	0.054
Harvest Weight, kg	46.74	47.02	47.01	0.53	0.913
Harvest Age, d	130.64	123.74	124.54	2.80	0.160
ADG, birth to dam's peak milk, kg/d	0.286	0.306	0.295	0.012	0.519
ADG, birth to weaning, kg/d	0.311	0.335	0.328	0.123	0.384
ADG, dam's peak milk to weaning, kg/d	0.339	0.367	0.357	0.014	0.357
ADG, weaning to harvest, kg/d	0.336	0.347	0.341	0.010	0.747
ADG, birth to harvest, kg/d	0.326	0.343	0.337	0.009	0.413

[1]Dam BCS: Dams were maintained at a BCS of 2, 3, or 4, during mid to late gestation. Scoring system was on a 1 to 5 scale, with 1 being emaciated and 5 being obese.

[2]SEM = standard error of the mean for Dam BCS (n = 22).

Table 6. Estimated peak milk production (~ 28 d of lactation) of dams maintained at different body condition scores (BCS) during mid to late gestation

	Dam BCS[1]				
Item	2 (n = 22)	3 (n = 23)	4 (n = 28)	SEM[2]	*P*-value
Milk production[3], kg/d	2.07	2.90	2.57	0.29	0.142
Fat[4], %	8.86	9.29	9.37	0.91	0.665
Protein[4], %	3.97	3.83	3.94	0.13	0.713
Lactose[4], %	5.12	5.29	5.23	0.08	0.291
SCC[4] (×1,000 cells/mL)	842	339	511	197	0.197
Milk Urea Nitrogen[4], mg/dL	10.80	11.83	10.71	0.81	0.534

[1]Dam BCS: Dams were maintained at a BCS of 2, 3, or 4, during mid to late gestation. Scoring system was on a 1 to 5 scale, with 1 being emaciated and 5 being obese.

[2]SEM = standard error of the mean for Dam BCS (n = 22).

[3]24 h milk production was calculated by multiplying 3-h milk weights by 8.

[4]Measurement made in a commercial DHI laboratory with equipment calibrated for bovine milk. No adjustments were made to the values obtained.

Table 7. Mammary fat pad (MFP) and parenchyma (PAR) mass and composition of lambs from dams maintained at different body condition scores (BCS) during mid to late gestation

	Dam BCS[1]				
Item	2 (n = 22)	3 (n = 23)	4 (n = 28)	SEM[2]	*P*-value
Total Mammary Gland					
Weight, g	179.20	167.36	175.61	8.81	0.615
Weight, g / 100 kg BW	383.00	357.52	374.64	19.55	0.637
MFP					
Weight, g	153.18	149.85	156.61	8.89	0.850
Weight, g / 100 kg BW	329.08	317.70	334.48	19.80	0.815
Lipid, mg / g MFP	815.3	817.5	822.0	9.78	0.867
Total lipid, g	125.7	123.3	129.1	7.81	0.856
Protein, mg / g MFP	8.88	8.72	8.58	0.38	0.840
Total protein, g	1.34	1.26	1.33	0.82	0.751
DNA, mg / g MFP	0.26	0.26	0.25	0.02	0.715
Total DNA, mg	39.85	38.87	39.74	3.48	0.975
PAR					
Weight, g	25.27	18.47	18.80	2.38	0.075
Weight, g / 100 kg BW	54.45	39.60	39.92	4.96	0.054
Lipid, mg / g PAR	341.5	312.6	266.7	23.8	0.064
Total lipid, g	8.69	6.54	6.08	1.17	0.228
Protein, mg / g PAR	57.24	55.23	58.14	1.63	0.397
Total protein, g	1.43	1.02	1.07	0.13	0.054
DNA, mg / g PAR	5.38	5.05	5.38	0.18	0.311
Total DNA, mg	134.8	93.1	103.0	13.3	0.073

[1]Dam BCS: Dams were maintained at a BCS of 2, 3, or 4, during mid to late gestation. Scoring system was on a 1 to 5 scale, with 1 being emaciated and 5 being obese.

[2]SEM = standard error of the mean for Dam BCS (n = 22).

Table 8. Ki67 labeling index (a measure of cell proliferation) of lambs from dams maintained at different body condition scores (BCS) during mid to late gestation

	Dam BCS[1]				
	2	3	4		
Item	(n = 22)	(n = 23)	(n = 28)	SEM[2]	*P*-value
Ki67 Labeling Index, %	6.27	6.36	4.08	0.82	0.058
Epithelial Area†, (mm^2)	47.62	47.66	47.26	1.58	0.977
Epithelium, %	32.88	32.91	32.63	1.09	0.977

[1]Dam BCS: Dams were maintained at a BCS of 2, 3, or 4, during mid to late gestation. Scoring system was on a 1 to 5 scale, with 1 being emaciated and 5 being obese.

[2]SEM = standard error of the mean for Dam BCS (n = 22).

†Ductal epithelial tissue was outlined, by the operator, as a region of interest.

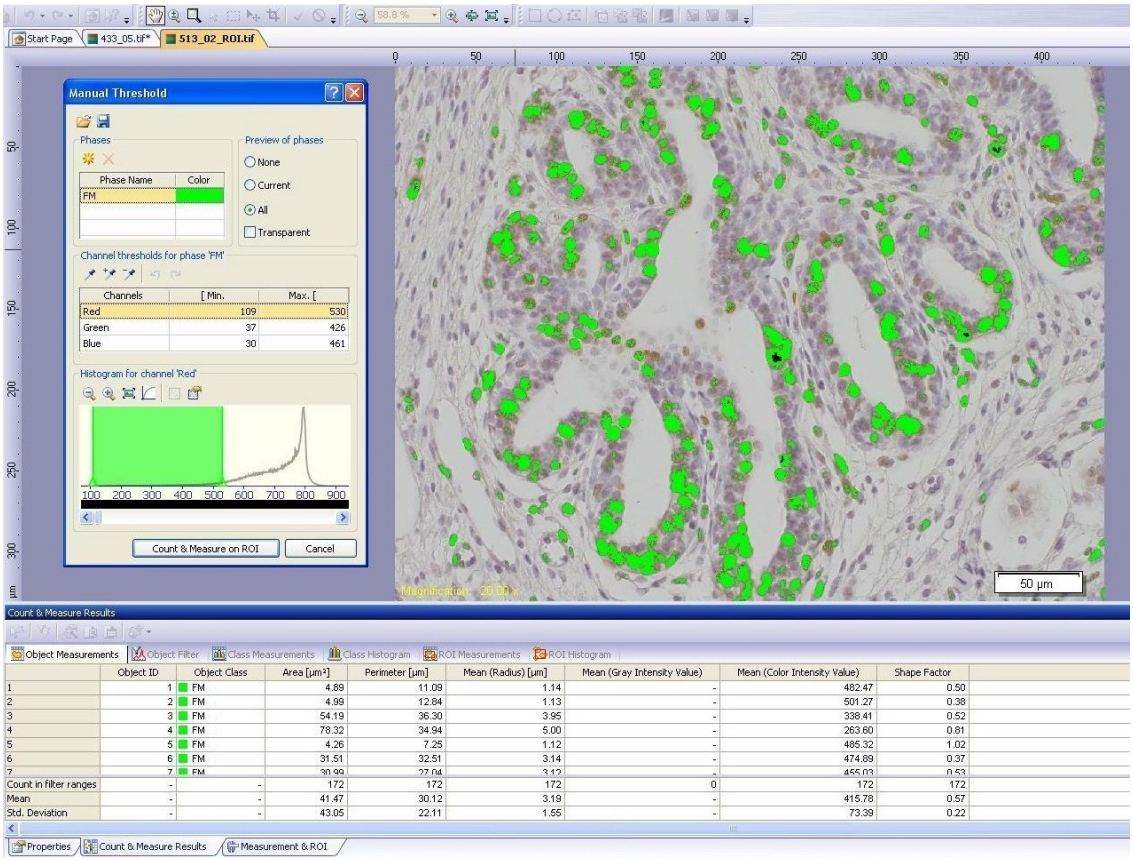

Figure 7. Prior to analyzing Ki67 images, a manual threshold (phase) was created with CellSens Software to automatically detect all Ki67 positive cells (brown stain).

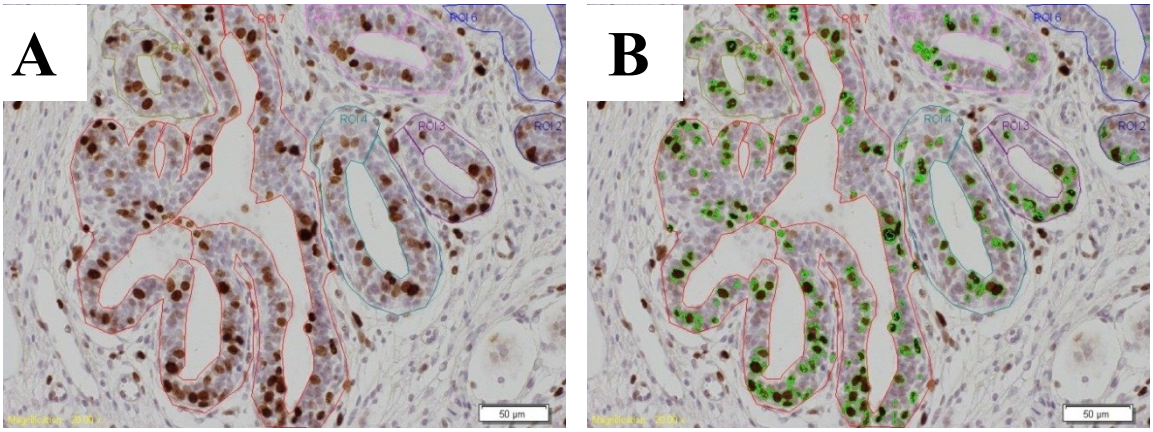

Figure 8. Ki67 image analysis. A) All ductal tissue was outlined by the user and classified by the software as a region of interest (ROI). B) The "count and measure on ROI" option was performed using the phase described in Figure 2. This ensures that only positive cells within ROIs are quantified.

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