Ontogenic and nutritional regulation of steroid receptor and IGF-I transcript abundance in the prepubertal heifer mammary gland

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Abstract

In prepubertal cattle, mammary development is characterized by the growth of an epithelial-rich parenchyma (PAR) into the mammary fat pad (MFP). This proliferation and accumulation of mammary epithelial cells require estrogen. Paradoxically, both epithelial cell proliferation and PAR accumulation rate decline with rising plasma estrogen as puberty approaches. The possibility that variation in abundance of estrogen receptors (ERs) in PAR or MFP accounts for a portion of these effects has not been examined in cattle. Additionally, we recently demonstrated that MFP is highly responsive to exogenous estrogen, suggesting that this tissue may play a role in coordinating estrogen's effects on PAR; however, the developing bovine MFP has yet to be studied in detail. To address these hypotheses, Holstein heifers were assigned to planes of nutrition supporting body growth rates of 950 (E) or 650 (R) g/day and harvested every 50 kg from 100 to 350 kg body weight (BW). Post-harvest, their mammary glands were dissected into PAR and MFP

compartments. Transcript abundance of genes encoding members of the ER family (ER α , ER β , and estrogen-related receptor α -1 (ERR α)) and estrogen-responsive genes (IGF-I and progesterone receptor (PR)) were measured in both mammary compartments by quantitative real-time RT-PCR. Significant expression was detected for all genes in both compartments, with the exception of the ER β gene. Transcript abundance of both ER α and IGF-I decreased linearly with increasing BW within both compartments. ERR α and PR expressions decreased with increasing BW in PAR but not in MFP. Nutrition stimulated ER α and ERR α expression in the PAR but had no effect on IGF-I or PR in either PAR or MFP. Overall, ER α and IGF-I transcript abundance are consistent with the drop in mammary epithelial cell proliferation and PAR accretion observed over development, but do not support a negative effect of nutrition on PAR growth.

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Introduction

Growth of the mammary parenchymal compartment does not occur when prepubertal cattle are ovariectomized (Wallace 1953, Purup *et al.* 1993). The apparent requirement of ovarian stimulation presumably reflects an obligatory role for the low plasma concentration of estrogen present in prepubertal cattle (Evans *et al.* 1994). Estrogen's influence is mediated by the estrogen receptor (ER) family, which include ER α , ER β , and three estrogen-related receptors (ERRs). At this time, the functional significance of these receptors for mammary development of prepubertal cattle can only be inferred from rodents. In the mouse, ER α is the most important receptor as shown by the absence of mammary ductal elongation in the ER α knockout mouse but normal development in the ER β knockout mouse (Couse & Korach 1999). In rodents, ER α is expressed in mammary epithelial

and stromal cells, and paracrine signaling appears to mediate mammogenic effects of estrogen. Although initial studies indicated that estrogen-mediated developmental signals require ER α expression in stromal cells (Cunha *et al.* 1997, Mueller *et al.* 2002), recent studies show that ER α expression in mammary epithelium is most important (Mallepell *et al.* 2006). However, stromal ER α expression allows estradiol-dependent synthesis of growth factors, such as insulin-like growth factor-I (IGF-I), which then stimulate epithelial cell proliferation (Imagawa *et al.* 1986, Shamay *et al.* 1988). We recently demonstrated a similar effect of estrogen on IGF-I transcript abundance in the bovine mammary fat pad (MFP; Li *et al.* 2006, Meyer *et al.* 2006a). The essential role of IGF-I in the developing murine mammary gland is illustrated by the absence of ductal elongation and branching in the IGF-I knockout mouse (Ruan & Kleinberg 1999). In contrast, far less is known about the role of the three ERRs in mammary

gland development. One of them, the $ERR\alpha$ shares considerable homology with $ER\alpha$ (Giguere 2002). Although unable to bind estrogen, $ERR\alpha$ can modulate estrogen-dependent responses (Kraus *et al.* 2002).

We recently showed that proliferation and accumulation of mammary epithelial cells decrease as heifers progress from birth through puberty (Ellis & Capuco 2002, Meyer *et al.* 2006c). In addition, nutrient intake sustaining average daily gains in excess of 850 g/day has been associated with decreased growth of the mammary epithelial compartment (Sejrsen *et al.* 1982, 2000, Capuco *et al.* 1995). The possibility that changes in the expression of members of the ER family ($ER\alpha$, $ER\beta$, and $ERR\alpha$) and their response genes ($IGF-I$ and progesterone receptor (PR)) could explain variation in parenchyma (PAR) growth has not been studied during prepubertal development. This hypothesis is supported by variation in PAR $ER\alpha$ expression from mammogenesis through lactogenesis and involution in cattle (Schams *et al.* 2003, Connor *et al.* 2005). This information is also lacking for the MFP, which is known to modulate epithelial growth in an estrogen-dependent manner in mice (Cunha *et al.* 1997, Mueller *et al.* 2002) and is hypothesized to do so in the bovine (Meyer *et al.* 2006a). Significantly, we were the first to demonstrate that $ER\alpha$ is expressed and functionally active in the bovine MFP (Meyer *et al.* 2006a). The current experiment characterizes developmental and nutritional regulation of $ER\alpha$ within the bovine MFP in detail not previously described.

To determine whether changes in expression of genes of the ER family and select target genes within the PAR or MFP can explain variation in PAR growth, heifers were fed to gain at a restricted rate of 650 g/day or at an elevated rate of 950 g/day between birth and puberty, and PAR and MFP were obtained at 50 kg body weight (BW) intervals from 100 to 350 kg BW. We then measured the expression of genes encoding members of the ER family ($ER\alpha$, $ER\beta$, and $ERR\alpha$) and estrogen-responsive genes ($IGF-I$ and PR) in both mammary compartments.

Materials and Methods

Animals and tissue collection

The Cornell University Animal Care and Use Committee approved all procedures used in this study. Seventy-two Holstein heifers were purchased from commercial dairy farms and assigned at day 10 of life to a 2×6 factorial design. The first factor consisted of the plane of nutrition, with heifers fed to gain at the restricted rate of 650 g/day (R) or at the elevated rate of 950 g/day (E). The second factor was BW at harvest (100, 150, 200, 250, 300, or 350 kg). Once heifers reached 225 kg BW, blood was collected twice weekly via jugular venipuncture and assayed for progesterone (Coat-A-Count progesterone RIA, Diagnostic Products Corporation, Los Angeles, CA, USA). Heifers were declared pubertal if plasma progesterone exceeded 1 ng/ml. If pubertal at the

assigned harvest BW, heifers were harvested in the luteal phase of their reproductive cycle. Humane slaughter was conducted by stunning with a captive bolt and exsanguination at the Cornell University abattoir.

Mammary gland dissection and immunohistochemistry

The mammary gland was removed and weighed at harvest. The left half was immediately dissected and tissue from the mid-PAR and MFP were snap frozen in liquid nitrogen for RNA extraction. Additional tissue samples from these same regions were fixed overnight in 10% neutral buffered formalin at 4 °C and then stored in 70% ethanol until paraffin embedding and sectioning.

Tissue sections were processed for immunohistochemical detection of ER and PR using the Histostain SP kit (Zymed Laboratories, San Francisco, CA, USA) as described previously (Capuco *et al.* 2002). Primary antibody for ER localization was mouse monoclonal antibody C-311 produced against amino acid sequence 405–595 of the bovine $ER\alpha$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Primary antibody for PR localization was mouse monoclonal antibody, Clone PR-AT4.14 (Research Diagnostics Products Inc., Flanders, NJ, USA) that recognizes A and B forms of PR. To quantify the number of labeled cells, photographs of stained sections were captured as digital images. For each animal, ten random areas from a single-stained slide were photographed with a Spot digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) on a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY, USA) at 400 $\times$ magnification. Labeled epithelial cells and total epithelial cells per micrograph were counted manually. At least 4000 mammary epithelial cells were scored per animal.

RNA isolation and quantitative real-time RT-PCR

Of the 12 heifers slaughtered at each BW, five E heifers and five R heifers were randomly selected (for a total of 60 heifers) for isolation of total RNA and quantitative real-time RT-PCR analysis. Total RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987) and quantified by absorbance at 260 nm. Fifteen micrograms of total RNA were treated with DNA-free (Ambion, Austin, TX, USA) DNase to remove any DNA contamination. Quality and quantity of the DNase-treated RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). DNase-treated RNA (990 ng) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) in a 20 μ l reaction mix. The reverse transcription conditions were as follows: 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C.

Transcript abundance was determined by SYBR green quantitative real-time RT-PCR (iQ SYBR Green Supermix, Bio-Rad Laboratories) using the iCycler iQ (Bio-Rad Laboratories). A total of 2.0 μ l RT reaction mix was used in each PCR. Primers and PCR conditions for the $ER\alpha$, $ER\beta$,

ERR α 1, and PR RT-PCR assays were described recently (Connor *et al.* 2005). Forward and reverse primers for evaluation of IGF-I gene expression were 5'-CATCCTCCTCG-CATCTCTTC-3' and 5'-GCACACGAAGTGGAGAGC-3' respectively (T_m =57.0 °C; 118 bp product). Amplicons were verified for each assay by sizing on an Agilent 2100 Bioanalyzer and sequencing. Copy numbers for each transcript were calculated from standard curves based on purified amplicons. Melting curve analysis confirmed the presence of a single product for each real-time RT-PCR assay.

Statistical analysis

Data were analyzed by a general linear model accounting for plane of nutrition, BW at slaughter and their interaction, with the level of statistical significance set at $P < 0.05$. When BW at slaughter was significant, variation was partitioned into linear, quadratic, and cubic contrasts. This analysis excluded outliers, defined as data with residuals falling two S.D.s beyond the residual mean.

Results

Mammary development

R and E heifers grew at the average rate of 0.66 and 0.93 kg/day respectively. Their mammary development has previously been described (Meyer *et al.* 2006b,c). Briefly, at a common BW, the mass of PAR DNA was less in E than R heifers. This effect of nutrition was not explained by a slower PAR DNA accretion rate or mammary epithelial cell proliferation in E heifers, but rather by a shorter lifespan between birth and slaughter. Finally, irrespective of level of nutrient intake, mammary epithelial cell proliferation was greatest at 100 kg BW, after which it decreased with increasing BW. Additionally, PAR DNA accretion dropped markedly as heifers approached puberty.

Expression of ER family members

Within the PAR, abundance of the ER α gene transcript was not affected by the plane of nutrition (data not shown). However, there was a negative linear relationship between PAR ER α mRNA abundance and BW, with ER α transcript abundance decreasing by a factor of five between 100 and 350 kg BW ($P < 0.01$; Fig. 1). As shown previously, ER α protein was localized to epithelial cells and to a rare fraction of fibroblasts (Capuco *et al.* 2002, Schams *et al.* 2003), which appear primarily in the interlobular connective tissue (Capuco, unpublished data).

ER α mRNA abundance in MFP was comparable with that in PAR (Figs 1 and 2A and B) and ER α transcript abundance in R heifers' MFP was ~60% of that in E heifers ($P = 0.03$; Fig. 2A). A cubic relationship between ER α transcript abundance and BW at slaughter was observed, wherein expression reached a peak at 100 and 250 kg and a nadir at 150

Figure 1 Effect of body weight (BW) on expression of the gene encoding estrogen receptor α (ER α) within the mammary parenchyma (PAR). Dairy heifers were offered a restricted or an elevated plane of nutrition starting at day 10 of age and harvested at fixed BW (100–350 kg). Total RNA was isolated from PAR and assayed by quantitative RT-PCR for ER α transcript copy number. In all cases, transcript copy number was normalized to the amount of total RNA used in the reverse transcription reaction. The plane of nutrition did not influence transcript abundance so only main effect of BW is presented ($n = 10, 7, 9, 9, 10$, and 9 for 100, 150, 200, 250, 300, and 350 kg respectively). Bars represent means $\pm$ S.E.M. Variation in BW was partitioned into linear, quadratic, and cubic contrasts, and any significant contrast was reported.

and 350 kg ($P = 0.03$; Fig. 2B). Given the dearth of information on the bovine MFP, relative to PAR, expression and localization of ER protein across prepubertal development were also assessed. Consistent with first published reports of ER α localization in the bovine MFP (Meyer *et al.* 2006a), we observed expression of the receptor in both fibroblasts (Fig. 3A) and adipocytes (Fig. 3A) in the current study. ER α immunoreactivity was detected in ~30% of MFP fibroblasts and adipocytes (Fig. 3C and D). Neither increasing BW nor level of nutrient intake affected the fraction of either cell type expressing the ER α protein.

ERR α mRNA was more abundant in both PAR and MFP of E than R heifers ($P \leq 0.05$, Fig. 4A and B). A negative linear relationship was observed between ERR1 α mRNA abundance within PAR and BW at slaughter (Fig. 4C; $P = 0.04$); no such effect was detected in MFP (data not shown). Finally, copy number of ER β transcripts was below the lowest standard (equivalent to one transcript per ng total RNA) in both mammary compartments, irrespective of BW or nutrition (data not shown).

Expression of E-dependent genes

Plane of nutrition did not alter abundance of IGF-I transcripts in PAR or MFP (data not shown). In both mammary compartments, IGF-I mRNA abundance decreased linearly with increasing BW ($P < 0.05$; Fig. 5A and B). In the MFP, the effect of BW was dominated by a drop in transcript abundance between 100 and 150 kg BW with little additional variation at higher BW.

We also assessed mammary expression of the PR gene, which is stimulated by estrogen in the PAR (Meyer *et al.*

Figure 2 Effect of nutrition and body weight (BW) on expression of the gene encoding estrogen receptor α (ER α) within the mammary fat pad (MFP). Dairy heifers were offered a restricted (R) or an elevated (E) plane of nutrition starting at day 10 of age and harvested at fixed BW (100–350 kg). (A and B) Total RNA was isolated from the MFP and assayed by quantitative RT-PCR for ER α transcript copy number. In all cases, transcript copy number was normalized to the amount of total RNA used in the reverse transcription reaction. (A) Main effect of plane of nutrition (E (solid bar), $n=28$ versus R (open bar), $n=26$). Bars represent means $\pm$ S.E.M. (B) Main effect of BW on transcript abundance ($n=7, 10, 10, 10, 8$, and 9 for 100, 150, 200, 250, 300, and 350 kg respectively). Bars represent means $\pm$ S.E.M. Variation in BW was partitioned into linear, quadratic, and cubic contrasts, and any significant contrast was reported.

2006a). PR gene expression was approximately tenfold higher in the PAR than in the MFP (data not shown). Plane of nutrition had no influence on PR mRNA abundance in PAR or MFP (data not shown). PR mRNA abundance decreased linearly with BW in PAR ($P<0.01$; Fig. 5C), but remained invariant in MFP (data not shown). Consistent with the low abundance of PR mRNA in MFP, few if any cells in this mammary compartment stained positive for PR by immunohistochemistry (data not shown).

Discussion

In both mice and cattle, mammary development between birth and puberty is characterized by the accumulation of epithelial ductal structures (Silberstein 2001, Capuco *et al.* 2002). The ducts elongate until they reach the edge of the fat pad in the mature nulliparous mouse, whereas they grow as a dense mass within the fat pad in cattle and do not appear to reach the margins of the fat pad until gestation in cattle. Despite these morphological differences, epithelial growth is dependent on estrogen in both species (Wallace 1953, Purup *et al.* 1993). Proliferating epithelial cells, however, rarely express ER α , suggesting that estrogen induces the synthesis of locally acting growth factors such as IGF-I in ER-positive, non-proliferating cells. Experiments involving genetically altered mice show that these cells reside in both PAR and MFP compartments (Cunha *et al.* 1997). Although most studies in cattle have examined only PAR (Schams *et al.* 2003, Connor *et al.* 2005), recent data from our group support a role for the MFP. Specifically, estrogen stimulated IGF-I gene expression to a greater extent in MFP than in PAR (Meyer *et al.* 2006a), and the greatest degree of mammary epithelial cell proliferation occurs in the region of PAR adjacent to

MFP (Capuco *et al.* 2002). For these reasons, we studied the expression of ER family members and E-dependent genes in both mammary compartments. Further, ER protein expression was evaluated in the MFP due to the lack of data on this mammary compartment in the bovine.

The regulation of ER α differed between PAR and MFP. We observed that ER α transcript in PAR gradually declines from a peak at 100 kg BW to a nadir at 350 kg BW, when puberty was reached. This indicates that ER α transcript abundance decreases prior to puberty and is consistent with data from gestating heifers (Connor *et al.* 2005). A similar ontogenic effect was not obvious in MFP between 100 and 250 kg BW, although a nadir was clearly seen at 350 kg BW. This difference may relate to the ability of rising E to repress ER α expression in PAR but not in MFP (Meyer *et al.* 2006a). A second difference between PAR and MFP may be the relation between ER α expression and the fraction of ER-positive cells. We showed that these two variables are well correlated in the PAR (Meyer *et al.* 2006c), but this was not seen in the MFP in the present study, with the fraction of adipocytes or fibroblasts expressing ER α remaining constant even when the transcript copy number reached its nadir at 350 kg BW. This implies decreased immunoreactive ER α per cell and/or significant post-transcriptional regulation of this receptor in the MFP.

Next, we investigated the possibility that other members of the ER family are involved in bovine mammary development. In the mouse, ER β has been proposed to play a role in coordinating the responsiveness of the mammary gland to estrogen and is actually the primary isoform expressed in MFP (Cheng *et al.* 2004). In contrast, we detected extremely low levels of ER β mRNA in the bovine MFP, and in agreement with previous work of ours

Figure 3 Effect of nutrition and body weight (BW) on expression of the gene encoding estrogen receptor α (ER α) within the mammary fat pad (MFP). Dairy heifers were offered a restricted (R) or an elevated (E) plane of nutrition starting at day 10 of age and harvested at fixed BW (100–350 kg). (A and B) ER α localization within the prepubertal bovine MFP. Twelve prepubertal dairy heifers were raised on an elevated or restricted plane of nutrition from birth and harvested at 150 and 350 kg BW. Tissue collected from the MFP was analyzed for immunoreactive ER α . A representative 150 kg BW heifer is shown. (A) Closed arrow indicates examples of ER-positive fibroblasts and open arrows indicate ER-positive adipocytes. Higher magnification of ER-positive fibroblast (insert A': dashed red box) and ER-positive adipocytes (insert A'': dashed black box) is provided. Magnification bar = 50 μ m. (B) Staining with control serum. Magnification bar = 50 μ m. (C and D) Effect of BW on the percent of adipocytes (C) and fibroblasts (D) within the MFP that stain positive for ER as assessed by immunohistochemistry. Elevated plane of nutrition is represented by the solid bars and restricted plane of nutrition by the open bars. No effect of nutrition or BW was detected. In all cases, bars represent means $\pm$ S.E.M. For (C), 150 kg, $n = 12$ and 350 kg, $n = 11$. For (D), 150 kg, $n = 12$ and 350 kg, $n = 11$.

and others (Schams *et al.* 2003, Connor *et al.* 2005), in PAR. Overall, these data suggest that this gene likely plays no significant role in coordinating mammary development in cattle. We also measured expression of the orphan receptor ERR α which shares considerable homology with ER α (Giguere 2002) and is known to influence transcription of estrogen-responsive genes (Zhang & Teng 2001). In the current study, ERR α 1 transcript abundance within PAR

gradually decreased with increasing BW but no such regulation was observed in MFP.

IGF-I and PR transcript copy numbers are increased by estrogen in PAR (Woodward *et al.* 1993, Meyer *et al.* 2006a) and MFP (Meyer *et al.* 2006a) of prepubertal heifers and consequently could provide an index of ER-dependent effects. IGF-I gene expression in PAR declined with increasing BW, mimicking the age-dependent decline in ER α and epithelial cell

Figure 4 Effect of nutrition and body weight (BW) on expression of the gene encoding estrogen-related receptor α -1 (ERR α) within the mammary parenchyma (PAR) and mammary fat pad (MFP). Dairy heifers were offered a restricted (R) or an elevated (E) plane of nutrition starting at day 10 of age and harvested at fixed BW (100–350 kg). Total RNA was isolated from PAR and MFP and assayed by quantitative RT-PCR for ERR α transcript copy number. In all cases, transcript abundance was normalized to the amount of total RNA used in the reverse transcription reaction. (A) Main effect of plane of nutrition on ERR α transcript abundance in PAR (E (solid bar), $n=27$ versus R (open bar), $n=28$). Bars represent means $\pm$ S.E.M. (B) Main effect of plane of nutrition on ERR α transcript abundance in MFP (E (solid bar), $n=28$ versus R (open bar), $n=26$). Bars represent means $\pm$ S.E.M. (C) Main effect of BW on transcript abundance within PAR ($n=10, 8, 10, 8, 10$, and 9 for 100, 150, 200, 250, 300, and 350 kg respectively). Bars represent means $\pm$ S.E.M. Variation in BW was partitioned into linear, quadratic, and cubic contrasts, and any significant contrast was reported. An effect of BW on ERR α expression in MFP was not detected (data not shown).

proliferation in these same heifers (Meyer *et al.* 2006c). In contrast, in the MFP, there was a marked decline in IGF-I expression only between 100 and 150 kg, perhaps reflecting the absence of E-dependent regulation of ER α in this compartment. The effects of development on PR transcript abundance were more variable but appeared similar to IGF-I mRNA abundance (i.e., reduction with BW in PAR but not in MFP). Our study also shows that PR mRNA is expressed at high levels in the PAR at 100 kg BW, long before progesterone is secreted. The PR is required for ductal branching in the mouse (Briskin *et al.* 1998). In the bovine, however, ductal branching is initiated long before puberty and therefore before progesterone secretion (Capuco *et al.* 2002).

In both mice (Shyamala & Ferenczy 1984) and heifers (Capuco *et al.* 2002, Schams *et al.* 2003), PR is localized primarily to mammary epithelial cells. Similarly, abundance of PR mRNA was low in MFP (which is devoid of epithelial cells) and few cells in this mammary compartment stained positive for the PR protein.

In cattle, elevated prepubertal nutrient intake reduces mammary PAR at puberty (Sejrsen *et al.* 1982, 2000, Capuco *et al.* 1995). Nutrition, however, has no effect on epithelial cell proliferation or the rate of DNA accumulation in PAR (Meyer *et al.* 2006c). Rather, we demonstrated that elevated nutrient intake shortens the length of time between birth and puberty, resulting in reduced PAR mass and DNA at puberty,

Figure 5 Effect of body weight (BW) on expression of estrogen-responsive genes *IGF-I* and progesterone receptor (*PR*) within the mammary parenchyma (PAR) and mammary fat pad (MFP). Dairy heifers were offered a restricted or an elevated plane of nutrition starting at day 10 of age and harvested at fixed BW (100–350 kg). Total RNA was isolated from PAR and MFP and assayed by quantitative RT-PCR for *IGF-I* transcript copy number. In all cases, transcript abundance was normalized by the amount of total RNA used in the reverse transcription reaction and variation in BW was partitioned into linear, quadratic, and cubic contrasts, and any significant contrast was reported. Plane of nutrition had no effect on transcript abundance of either gene so only main effect of BW is presented. (A) Main effect of BW on *IGF-I* mRNA abundance in the PAR ($n=9, 8, 9, 8, 10$, and 10 for 100, 150, 200, 250, 300, and 350 kg respectively). Bars represent means $\pm$ S.E.M. (B) Main effect of BW on *IGF-I* mRNA abundance in the MFP ($n=9, 10, 8, 9, 9$, and 10 for 100, 150, 200, 250, 300, and 350 kg respectively). Bars represent means $\pm$ S.E.M. (C) Main effect of BW on *PR* mRNA abundance in the PAR ($n=10, 7, 9, 9, 10$, and 9 for 100, 150, 200, 250, 300, and 350 kg respectively). Bars represent means $\pm$ S.E.M.

when mammary development is commonly assessed (Meyer *et al.* 2006c). Consistent with this conclusion, we demonstrated in the current experiment that elevated plane of nutrition had few effects on transcript abundance in the mammary gland. This was the case even for *IGF-I*, a gene we previously demonstrated to respond dynamically to nutrition in the liver of growing cattle (Smith *et al.* 2002). This lack of effect is consistent with the data of Weber *et al.* (2000) who observed similar *IGF-I* mRNA abundance in the parenchyma of prepubertal heifers growing 0.55 or 1.1 kg/day. We did see a positive effect of nutrition on *ER α* expression in the PAR, but this is the opposite of what would be expected if *ER* were mediating a negative effect of nutrition on PAR growth.

In a previous experiment, we demonstrated that mammary epithelial cell proliferation in the prepubertal bovine decreases from a peak at 100 kg BW through puberty and beyond (Meyer *et al.* 2006c). Likewise, in the current study, we observed a concomitant developmental

decrease in *ER α* and *IGF-I* transcript abundance within both the MFP and PAR. Given the requirement for ovarian secretions in prepubertal mammary development, data presented herein suggest that *ER α* and estrogen-responsive *IGF-I*, in concert, might play a role in developmental regulation of mammary epithelial cell proliferation in the bovine. Strong expression of *ER α* and *IGF-I* genes in MFP provides additional evidence that this compartment possesses the ability to influence development of the adjacent PAR via estrogen-responsive paracrine acting hormones, as has been demonstrated in the mouse.

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