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


Department of Animal Science, Cornell University, 272 Morrison Hall, Ithaca, New York 14850, USA
1Bovine Functional Genomics Lab, USDA-ARS, Beltsville, Maryland 20705, USA
(Correspondence should be addressed to M E Van Amburgh; Email: mev1@cornell.edu)

M J Meyer is now at Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, National Institutes of Health, 37 Convent Drive, Building 37, Room 1108, Bethesda, Maryland 20892, USA
R P Rhoads is now at Department of Animal Science, University of Arizona, Tucson, Arizona 85721, USA

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 (ERα, ERβ, and estrogen-related receptor α-1 (ERRα)) 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 ERβ gene. Transcript abundance of both ERα and IGF-I decreased linearly with increasing BW within both compartments. ERRα and PR expressions decreased with increasing BW in PAR but not in MFP. Nutrition stimulated ERα and ERRα expression in the PAR but had no effect on IGF-I or PR in either PAR or MFP. Overall, ERα 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.


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 ERα, ERβ, 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, ERα is the most important receptor as shown by the absence of mammary ductal elongation in the ERα knockout mouse but normal development in the ERβ knockout mouse (Couse & Korach 1999). In rodents, ERα 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 ERα expression in stromal cells (Cunha et al. 1997, Mueller et al. 2002), recent studies show that ERα expression in mammary epithelium is most important (Mallepell et al. 2006). However, stromal ERα 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 ERRα shares considerable homology with ERα (Giguere 2002). Although unable to bind estrogen, ERRα 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 (ERα, ERβ, and ERRα) 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. ERα expression from mammmogenesis 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 ERα is expressed and functionally active in the bovine MFP (Meyer et al. 2006a). The current experiment characterizes developmental and nutritional regulation of ERα 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 (ERα, ERβ, and ERRα) 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 at gain at a 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-31 produced against amino acid sequence 405–595 of the bovine ERα (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× 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 DNase (Ambion, Austin, TX, USA) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) in a 20 μl 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 20 μl RT reaction mix was used in each PCR. Primers and PCR conditions for the ERα, ERβ,
ERRα1, 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′-CATCCTCCCTG-CCATCTCTTC-3′ and 5′-GCACACGAACTGGAGAGC-3′ respectively (Tm = 57·0 °C; 180 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 ERα gene transcript was not affected by the plane of nutrition (data not shown). However, there was a negative linear relationship between PAR ERα mRNA abundance and BW, with ERα transcript abundance decreasing by a factor of five between 100 and 350 kg BW (P<0.01; Fig. 1). As shown previously, ERα 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).

ERα mRNA abundance in MFP was comparable with that in PAR (Figs 1 and 2A and B) and ERα transcript abundance in R heifers’ MFP was ~60% of that in E heifers (P=0·03; Fig. 2A). A cubic relationship between ERα transcript abundance and BW at slaughter was observed, wherein expression reached a peak at 100 and 250 kg and a nadir at 150 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 ERα 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. ERα 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 ERα protein.

ERRα mRNA was more abundant in both PAR and MFP of E than R heifers (P≤0·05, Fig. 4A and B). A negative linear relationship was observed between ERR1α 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 ERβ 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. 2003b). PR RT-PCR assays were described recently (Connor et al. 2005). The plane of nutrition did not alter PAR or MFP PR abundance (P>0·05). As with ERα, PR expression was negatively related to BW in PAR (P=0·04; Fig. 5A) but not in MFP (data not shown). These findings indicate that oestrogen sensitivity of mammary parenchyma increases with increasing BW. Presumably, this is due to an increase in the fraction of PAR cells expressing ERα, the predominant isoform of the receptor in this compartment (Connor et al. 2005). However, for the PR gene, the effect of BW was not as pronounced as for ERα in PAR.
cell proliferation occurs in the region of PAR adjacent to
studies in cattle have examined only PAR (Schams et al. 2006)
expression to a greater extent in MFP than in PAR (Meyer for the MFP. Specifically, estrogen stimulated
IGF-I MFP compartments (Cunha 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 ERα differed between PAR and MFP. We observed that ERα 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 ERα 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 ERα expression in PAR, but not in MFP (Meyer et al. 2006a). A second difference between PAR and MFP may be the
relation between ERα expression and the fraction of ER-positive cells. We showed that these two variables are
well correlated in the PAR (Meyer et al. 2006a), but this was not seen in the MFP in the present study, with the fraction of adipocytes or fibroblasts expressing ERα remaining constant even when the transcript copy number reached its nadir at 350 kg BW. This implies decreased immunoreactive ERα 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, ERβ 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 ERβ mRNA in the bovine MFP, and in agreement with previous work of ours

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 estrogens in both species (Wallace 1953, Purup et al. 1993). Proliferating epithelial cells, however, rarely
express ERα, suggesting that estrogen induces the synthesis of
locally acting growth factors such as IGF-I in ERα-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

Figure 2 Effect of nutrition and body weight (BW) on expression of the gene encoding estrogen receptor α (ERα) 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 ERα 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 ± S.E.M. (B) Main effect of BW on transcript abundance (n= 7, 10, 10, 8, and 9 for 100, 150, 200, 300, and 350 kg respectively). Bars represent means ± S.E.M. Variation in BW was partitioned into linear, quadratic, and cubic contrasts, and any significant contrast was reported.
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 ERRα which shares considerable homology with ERα (Giguere 2002) and is known to influence transcription of estrogen-responsive genes (Zhang & Teng 2001). In the current study, ERRα1 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 ERα and epithelial cell...
proliferation in these same heifers (Meyer et al. 2006). 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 ERα 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 (Brisken 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 4 Effect of nutrition and body weight (BW) on expression of the gene encoding estrogen-related receptor α-1 (ERRα) 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 ERRα 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 ERRα transcript abundance in PAR (E (solid bar), n = 27 versus R (open bar), n = 28). Bars represent means ± S.E.M. (B) Main effect of plane of nutrition on ERRα transcript abundance in MFP (E (solid bar), n = 28 versus R (open bar), n = 26). Bars represent means ± 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 ± 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 ERRα expression in MFP was not detected (data not shown).
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 ERα 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 ERα 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 ERα 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 ERα 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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