Effects of milk replacer formulation on measures of mammary growth and composition in Holstein heifers

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ABSTRACT

Overfeeding prepubertal heifers may impair mammary parenchymal growth and reduce milk production, but evidence suggests that increased intake of a high-protein milk replacer before weaning may be beneficial. This study was designed to evaluate effects of milk replacer (MR) composition on mass and composition of mammary parenchyma and fat pad, growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis gene expression, and putative mammary epithelial stem cells. Specifically, we hypothesized that positive effects of faster rates of gain during the preweaning period alter the development, persistence, or activity of populations of putative mammary epithelial stem cells, possibly through involvement of GH/IGF-I axis molecules. Twenty-four newborn heifers were fed 1 of 4 MR diets ($n = 6/\text{diet}$): control [20% crude protein (CP), 21% fat MR fed at 441 g of dry matter (DM)/d], high protein, low fat (28% CP, 20% fat MR fed at 951 g of DM/d), high protein, high fat (27% CP, 28% fat MR fed at 951 g of DM/d), and high protein, high fat+ (27% CP, 28% fat MR fed at 1,431 g of DM/d). Water and starter (20% CP, 1.43% fat) were offered ad libitum. Animals were killed on d 65 and mammary tissue was subjected to biochemical, molecular, and histological examination. No differences in mammary parenchymal mass or composition, with or without adjusting for empty body weight, were detected. Mass was increased and composition of the mammary fat pad was altered by nutrient intake. No diet differences in putative mammary epithelial stem cell abundance or abundance of transcripts for genes of the GH/IGF-I axis were detected. In this study, growth of the mammary epithelium, size of the mammary epithelial stem cell population, and components of the GH/IGF-I axis did not depend on diet. However, an underlying positive correlation between telomerase, a marker of mammary

stem cells, and growth of the mammary parenchyma was detected. Implications of diet-induced effects on mammary fat pad and possible effects on subsequent development and function remain to be determined.

Key words: stem cell, mammary gland, milk replacer, prepubertal heifer

INTRODUCTION

The effect of nutrition on prepubertal development of the bovine mammary gland has yet to be fully elucidated. Accelerated rearing of heifers reduces the age and mass of mammary parenchyma (**PAR**) at puberty (Sejrsen et al., 1982; Capuco et al., 1995). This apparent reduction in PAR mass was universally interpreted to represent an inhibition of mammary epithelial proliferation induced by overnutrition during this allometric phase of mammary growth, which in turn supported the concept of a critical period during which the mammary gland was sensitive to nutrient intake. Recent data indicated that the rate of mammary growth through the majority of the prepubertal period was not affected by feed intake (Meyer et al., 2006a,b); thus, an effect of diet may be caused by truncation of the allometric phase of mammary growth or an artifact of comparing heifers at different ages or developmental stages. The authors pointed out that when mammary development of heifers reared at different rates of gain is assessed at the same or similar BW, dietary effects are confounded by heifer age. Nonetheless, recent studies focusing on the period from birth to weaning suggest a beneficial role of elevated nutrient intake on PAR growth (Brown et al., 2005; Meyer et al., 2006a,b).

Growth hormone (**GH**), IGF-I, their receptors, and IGF binding proteins (**IGFBP**) are components of the GH/IGF-I axis. Some components of this axis are influenced by nutrition and have been implicated as mediators of inhibited mammary growth in heifers fed elevated levels of nutrients (Sejrsen et al., 1983; Berry et al., 2003b; Weber et al., 1999, 2000). In other cases, specific components of the GH/IGF-I axis were not affected by level of nutrient intake (Meyer et al., 2007). These conflicting results demonstrate the need for fur-

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ther evaluation of the role of the GH/IGF-I axis in development of the bovine mammary gland (**MG**), with respect to both PAR and mammary fat pad (**MFP**) development.

Mammary epithelial stem cells provide for growth and development of the mammary epithelium (Capuco and Ellis, 2005). The response of these adult stem cells to nutrition may be important in determining the effect of nutrition on mammary growth during the prepubertal period (Meyer et al., 2006a). When mammary epithelial cell proliferation was assessed at a common BW of 100 kg, Meyer et al. (2006a) found that it was 44% higher in heifers fed at elevated levels of nutrient intake, compared with heifers fed for restricted intake; this effect was lost at 150 kg of BW. Meyer et al. (2006a) postulated that elevated nutrient intake increased epithelial cell proliferation, which may have resulted from increased proliferation of mammary stem cells or their daughters, or both. Although no mention of mammary stem cells appeared in the paper of Brown et al. (2005), evidence presented demonstrated that increased energy and protein intake associated with accelerated calf growth programs increased PAR growth in heifers from 2 to 8 wk of age. This growth may also have been influenced by stem cell activity. Further support for the idea that stem cells can be modulated by nutrition comes from Drummond-Barbosa and Spradling (2001), who noted that in some model organisms, stem cells and their more differentiated daughter cells change proliferation patterns according to nutritional status. When the data of Meyer et al. (2006a) and Brown et al. (2005) are considered together, the possibility of nutritional regulation of stem cell proliferation is plausible.

This study addresses the hypothesis that increased nutrient intake by preweaned heifers increases PAR mass by promoting proliferation of mammary epithelial stem cells, which is promoted by local changes in the GH/IGF-I axis. There were 3 objectives: The first was to confirm a positive effect of nutrient intake on growth of the mammary parenchyma in preweaned heifers and to expand upon previous studies by evaluating the effect of composition of milk replacer (**MR**) on growth of the PAR and MFP. The second objective was to characterize components of the GH/IGF-I axis in PAR and MFP of young heifers fed different diets. The third objective was to determine whether diet altered the number of putative mammary epithelial stem cells. To address these objectives, preweaned Holstein heifers of similar ages were fed different diets to achieve various rates of BW gain. Four MR regimens were used to make 3 diet comparisons at the end of the experiment. Heifers were harvested at a common age and mammary tissue was subjected to biochemical, molecular, and histological examination to assess effects of diet.

Results of diet effects on blood metabolite and hormone concentrations, body growth and carcass composition, and gastrointestinal tract development and selected gene expression are reported elsewhere (Daniels et al., 2008; Hill et al., 2008; Velayudhan et al., 2008; respectively).

MATERIALS AND METHODS

Animals and Treatments

The Virginia Tech Institutional Animal Care and Use Committee approved all animal procedures. This study was designed as a randomized complete block experiment. Heifers were managed and fed according to Daniels et al. (2008). Briefly, 24 Holstein heifers were acquired from a single commercial dairy farm within 3 d of birth (40.4 ± 2.2 kg of BW) and blocked into groups of 8 in the order acquired. Heifers were housed individually and fed 1 of 4 MR diets ($n = 6/\text{diet}$): control (**CON**; 20% CP, 21% fat MR fed at 441 g of DM/d), high protein, low fat (**HPLF**; 28% CP, 20% fat MR fed at 951 g of DM/d), high protein, high fat (**HPHF**; 27% CP, 28% fat MR fed at 951 g of DM/d), and high protein, high fat plus (**HPHF+**; 27% CP, 28% fat MR fed at 1,431 g of DM/d). Heifers were fed twice daily; water and starter (20% CP, 1.43% fat) were offered ad libitum. Diets are further characterized in Table 1. At 1 mo of age ($32 \text{ d} \pm 0.4$; $\text{LSM} \pm \text{SEM}$) heifers were injected intravenously with 5 mg of 5-bromo-2'-deoxyuridine (**BrdU**) per kg of BW daily for 4 d. The BrdU (Sigma Chemical Co., St. Louis, MO, cat no. B5002-5G) solution was made in sterile 0.9% NaCl and contained 20 mg of BrdU/mL (pH 8.5). One heifer (group 1; HPLF) died unexpectedly at 6 wk of age from acute peritonitis and endotoxemia and was not replaced; all data from that animal were excluded.

Mammary Tissue Sampling

Thirty days after the last BrdU injection (65 d of age ± 0.4 ; $\text{LSM} \pm \text{SEM}$) and an overnight fast, heifers were harvested to evaluate body composition (reported in Hill et al., 2008) and mammary development. Heifers were killed by phenobarbital injection (Euthasol, 10 mg/kg BW; Virbac AH Inc., Fort Worth, TX) and immediately exsanguinated. Udders were removed. The whole udder was weighed and bisected along the median suspensory ligament. The right hemi-udder was reweighed, wrapped in foil, submerged in liquid nitrogen, and stored at -80°C until later compositional analysis. The left rear quarter was sampled for later RNA analysis and the left front quarter was sampled for histology. At the time of slaughter, it was noted that

Table 1. Milk replacer and starter composition and nutrient intake in Holstein heifer calves¹

Item	Dietary treatment ²				SEM ³	<i>P</i> <			
	CON (n = 6)	HPLF (n = 5)	HPHF (n = 6)	HPHF+ (n = 6)		Diet	CON vs. all	HPLF vs. HPHF	HPHF vs. HPHF+
Milk replacer intake ⁴									
CP, % of DM	19.8	28.3	27.1	27.1					
Fat, % of DM	21.1	19.8	27.6	27.6					
Gross energy, Mcal/kg	4.69	4.90	5.24	5.24					
DM, g/d	441	951	951	1,431					
N, g/d	13.7	42.2	40.4	60.8					
Fat, g/d	91.3	191	242	365					
Gross energy, Mcal/d	2.07	4.66	4.98	7.50					
Starter intake ⁵									
CP, % of DM	20.3	20.3	20.3	20.3					
Fat, % of DM	1.43	1.43	1.43	1.43					
Gross energy, Mcal/kg	3.52	3.52	3.52	3.52					
DM, g/d	560	449	401	221	52.4	0.01	0.01	0.51	0.02
N, g/d	18.4	14.5	13.0	7.2	1.74	0.01	0.01	0.53	0.03
Fat, g/d	8.01	6.78	5.72	3.15	1.31	0.06	0.06	0.56	0.15
Gross energy, Mcal/d	1.97	1.67	1.41	0.78	0.32	0.06	0.06	0.56	0.15
Total intake ⁶									
DM, g/d	1,001	1,400	1,352	1,652	52.4	0.01	0.01	0.51	0.01
N, g/d	32.1	56.7	53.4	68.0	1.74	0.01	0.01	0.19	0.01
Fat, g/d	99	198	248	368	1.31	0.01	0.01	0.01	0.01
Gross energy, Mcal/d	3.61	5.41	5.57	6.94	0.33	0.01	0.01	0.74	0.01

¹From Daniels et al. (2008).²Diets: CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPLF = high-protein, low-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d.³Standard error of the mean for diet; highest listed.⁴No statistical analysis was completed because within treatment, equal amounts were fed daily and probability of difference could not be calculated.⁵Starter contained 44.4% ground corn grain, 44.4% soybean meal, 11.2% cottonseed hulls, and 1.0% dried molasses.⁶Total intake = MR plus starter.

one heifer (group 3; CON) had an irregularly shaped reproductive tract and underdeveloped mammary tissue. This heifer was suspected to be a freemartin; her mammary data are not reported.

Right Hemi-Udder Analysis

Mammary Tissue Dissection. Right hemi-udders were removed from the freezer, reweighed, allowed to partially thaw, and dissected into 1 of 4 fractions. These fractions were hide/teats, lymph node, PAR, and MFP; the weight of each fraction was recorded. The hide/teats and lymph node fractions were discarded. At this time, PAR and MFP subsamples were obtained, placed in cryovials, and submerged in liquid nitrogen. Cryovials were shipped to the University of Illinois for microarray analysis of gene expression (reported in Piantoni et al., 2007, 2008). Remaining tissue portions were refrozen for later analysis.

Biochemical Analyses of PAR and MFP. Dissected PAR and MFP fractions were removed from the freezer, reweighed, and pulverized to a powder in a freezer mill (6850 Freezer Mill, Spex Sample Prep,

Metuchen, NJ). The remaining powder was subsampled and used in separate assays for determination of lipid, protein, and DNA content.

Lipid content was determined gravimetrically essentially according to the method of Hara and Radin (1978). Briefly, 9 mL of hexane:isopropanol (3:2; vol/vol) was added to 500 mg of tissue powder. Butylated hydroxytoluene (Sigma Chemical Co.) was added to the hexane:isopropanol mixture at 0.005% and served as an antioxidant. Tubes were vortexed and 6 mL of aqueous sodium sulfate (1 g of anhydrous salt per 15 mL of H₂O) was then added. The suspension was vortexed, phases were allowed to separate, and tubes were vortexed again. Tubes were then centrifuged at 1,000 × *g* for 5 min. The upper lipid-rich solvent layer was transferred to a clean, preweighed tube. Residual lipids were resuspended by adding 5 mL of the hexane:isopropanol mixture to the original tubes. Tubes were vortexed and then centrifuged at 1,000 × *g* for 5 min; the upper solvent layer was removed and decanted into a preweighed tube. Solvent was evaporated under N gas at 40°C on an analytical evaporator (N-EVAP model no.112, Organomation Associates Inc., South Berlin, MA). Tubes

were reweighed after evaporation of solvent and solidification of lipid residue. Lipid mass was calculated as the final tube weight minus the initial tube weight. Samples were measured in duplicate; the intraassay coefficient of variation (CV) averaged 3.95%.

Protein and DNA contents of PAR and MFP were determined after homogenization of each fraction in a high-salt buffer. Briefly, 250 mg of tissue was weighed and homogenized (PRO200 Homogenizer, PRO Scientific, Oxford, CT) in 1.5 mL of buffer (0.05 M Na₂HPO₄ + 2 M NaCl + 0.002 M Na₂EDTA). Homogenates were centrifuged briefly at 1,000 × *g* at 4°C to clear samples of lipid and residual connective tissue; the soluble fraction was removed with a Pasteur pipette and transferred to a clean microfuge tube. For the DNA assay, 2 µL of each homogenate fraction was added to 2 mL of assay solution. Assay solution contained 100 µL of 1 mg/mL Hoechst H33258, 10 mL of 2 M NaCl + 100 mM Tris + 10 mM Na₂EDTA, pH 7.4 buffer, and 90 mL of H₂O. DNA was quantified against calf thymus DNA (Sigma Chemical Co.); samples were measured in triplicate on a Hoefer DQ 300 fluorometer (Hoefer Inc., San Francisco, CA) set on the UV fluorescence channel. Intraassay CV averaged 4.90%. Protein in PAR and MFP homogenates was determined via the bicinchoninic acid assay (Pierce, Rockford, IL); BSA was used as the standard. Intraassay CV averaged 2.63% for the protein assay.

For all composition assays, obtained values were multiplied by 2 to determine total lipid, DNA, and protein content of the udder. Parenchyma and MFP weights are expressed on a raw- and empty BW (EBW)-adjusted basis. All calculations were made before statistical analysis.

Left Rear Quarter Analysis

RNA Isolation and Quantitative Reverse Transcription-PCR. At slaughter, samples of PAR and MFP from left rear quarters were excised, snap frozen in liquid nitrogen, and stored at −80°C until RNA was isolated. Isolation of RNA and quantitative reverse transcription-PCR (qRT-PCR) were carried out according to Velayudhan et al. (2008) except a different data normalization strategy was utilized (discussion follows). The RNA yield data were similar to those reported by Piantoni et al. (2008) for PAR and MFP for the same heifers (data not shown).

Primers and Data Normalization. In a recent study that utilized mammary tissue from these heifers (Piantoni et al., 2008), it was concluded that the geometric average of 3 housekeeping genes (HKG) is ideal for normalization of qRT-PCR data obtained from prepubertal bovine mammary tissue, as opposed to using a single HKG such as β-actin. Specifically,

PPP1R11 (plays a role in protein phosphatase-1 inhibition), *RPS15A* (a component of the 40S ribosomal subunit), and *MTG1* (a GTPase) were deemed the most suitable HKG for normalization (Piantoni et al., 2008). Consequently, for relative quantification of target gene mRNA, the geometric mean of HKG cycles to threshold (Ct) values was used to normalize gene expression data. Specifically, relative mRNA abundance of IGF-I, IGF-I receptor, IGFBP 1–6, and growth hormone receptor for each heifer was determined by subtracting the Ct value for the geometric mean of the 3 HKG genes from the target gene Ct (Ct_{target} − Ct_{geometric mean HKG}). Fold difference in target gene expression was calculated as 2^(−ΔCt).

All primers were from Integrated DNA Technologies (Coralville, IA) and were diluted to 10 µM with RNase- and DNase-free water before use in qRT-PCR experiments. Primer sequences used for GH/IGF-I axis genes are reported in Velayudhan et al. (2008), and sequences for HKG are reported in Piantoni et al. (2008). Primer efficiencies for HKG and each target gene were determined using 5 dilutions of PAR and MFP cDNA in triplicate according to the equation: % efficiency = (10^{−1/slope} − 1) × 100. Primer efficiencies were approximately equal for target genes and HKG and averaged 98%.

Telomerase Assay. Snap-frozen PAR was also used to determine telomerase activity. Telomerase is an enzyme that synthesizes telomeric repeats on chromosome ends to prevent continued truncation of DNA strands during DNA replication (Greider and Blackburn, 1985; Gillis et al., 2008). Telomerase activity is specifically associated with immortal cells, including stem and cancer cells. For the assay, a quantitative PCR-based Telomerase Assay Kit (US Biomax, Rockville, MD) was used as described previously (Capuco et al., 2009) and in accordance with the manufacturer's instructions. Briefly, for each heifer, 40 to 100 mg of PAR was homogenized for 20 s in the provided 1× lysis buffer. Samples were incubated on ice for 30 min and then centrifuged for 30 min at 12,000 × *g* at 4°C; the supernatant was removed and aliquoted. One aliquot was used for protein determination by the bicinchoninic acid assay (described above) and one aliquot was diluted 1:200 with 1× lysis buffer and kept at 4°C until further processing. A portion of each diluted sample was removed and placed in a new tube, which underwent heat inactivation (85°C, 10 min). Heat-inactivated samples served as negative controls in the assay. Snap frozen bull testes served as an additional positive control in the assay. A standard curve was prepared with eight 1:5 serial dilutions of the provided control template oligonucleotide (0.5 amol/µL); a no-template control was also used. Master mix was prepared by mixing 12.5 µL of the provided 2×

Quantitative Telomerase Detection Premix with 11.5 μL of PCR qualified H_2O . For the assay, 1 μL of tissue extract (or standard) was added to 24 μL of master mix. Samples were measured in duplicate in a 96-well plate. The PCR conditions were 25°C for 25 min, 95°C for 10 min, followed by 3-step cycling (40 cycles) of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. A melting curve analysis was included at the end of the assay and yielded a single PCR product that dissociated at approximately 76°C (data not shown). Telomerase activity was quantified against a standard curve that had an R^2 of 0.97. For each sample, activity of the heat-inactivated aliquot was subtracted before statistical analysis and data are presented as attomoles of telomerase per milligram of PAR protein.

Left Front Quarter Analysis

Microscope Slide Preparation. At slaughter, left front quarters of each udder were bisected to expose the tissue regions and PAR ($\sim 4 \text{ mm}^2$) from 4 regions within the quarter (2 cisternal and 2 peripheral) were excised and placed into vials of 10% formalin. Tissues were allowed to fix overnight; formalin solution was then removed and replaced with 70% ethanol. Tissues remained in 70% ethanol until embedding in paraffin. At least 2 tissue blocks were prepared for each region; in most cases only one replicate block was processed further. Microscope slides were prepared by slicing 5- μm -thick sections from the paraffin-embedded tissue blocks with a microtome (Reichert-Jung Model 2040 Autocut, Leica Microsystems, Wetzlar, Germany). Four or 5 serial tissue sections from each sample were mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Approximately 5 microscope slides were prepared from each tissue block.

BrdU–Ki-67 Dual Labeling. One microscope slide per region per heifer was subjected to dual immunofluorescent labeling of BrdU and Ki-67. The labeling method was carried out essentially according to Capucio (2007) and was designed to identify mammary epithelial stem cells by their retention of labeled DNA strands. Microscope slides were deparaffinized in xylene ($3 \times 5 \text{ min}$) and hydrated by passage through a series of graded ethanol washes. Ethanol washes were 100% ($2 \times 3 \text{ min}$), 95% ($2 \times 3 \text{ min}$), and 70% ($1 \times 3 \text{ min}$). The final step in hydration consisted of soaking slides in distilled water ($2 \times 2 \text{ min}$). Antigen sites were retrieved by boiling slides in 500 mL of 10 mM citrate buffer (pH 6.0) continuously for 15 min. Slides were allowed to cool completely ($\sim 30 \text{ min}$), and were then washed in PBS (pH 7.4; $3 \times 2 \text{ min}$). Residual PBS was aspirated with a vacuum and slides were blocked with CAS Block (1–2 drops CAS Block/section, cat no. 00-8120, Invitrogen,

Carlsbad, CA). In the case of positive/reagent control slides (mammary tissue from a BrdU pulse-labeled heifer), individual tissue sections were circled with a PAP barrier pen (cat no. NC9720458, Fisher Scientific) before blocking to prevent commingling of antibodies in subsequent steps. All slides were incubated for 30 min. The CAS Block was aspirated and 50 μL of combined primary antibody solution was added per section. The primary antibody solution consisted of a mixture of Ki-67 rabbit monoclonal antibody (1:200; clone SP6, cat no. RM-9106-SO, Fisher Scientific) and BrdU mouse monoclonal antibody (1:66.7; clone BMC-9318, cat no. MAB3424, Fisher Scientific), diluted in a total of 3 mL of CAS Block. Reagent controls received 50 μL of CAS Block instead of primary antibody mixture. Slides were incubated overnight at 4°C. The next morning, slides were aspirated and washed in PBS ($3 \times 5 \text{ min}$). Residual PBS was aspirated, and 50 μL of combined secondary antibody solution was added per section. The combined secondary antibody solution consisting of 15 μL of Alexa 488 goat anti-rabbit IgG (cat no. A11008, Invitrogen), and 15 μL of Alexa 594 goat-anti mouse IgG (cat no. A11005, Invitrogen) was added to 3 mL of CAS Block (final dilution of antibodies, 1:200). Before use, the secondary antibody mixture was centrifuged at $10,000 \times g$ for 10 min to remove aggregates. Slides were incubated with secondary antibody for 60 min in the dark. Afterward, PAP Pen residue was removed from positive/reagent control slides with a xylene-soaked cotton swab. All slides were then washed in PBS ($3 \times 2 \text{ min}$, in the dark, on a rocker platform). Next, individual slides were rinsed by dipping in distilled water; residual water was aspirated, and 1 to 3 drops of Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, cat no. P36935, Invitrogen) was added to each slide. A glass coverslip was then added and slides were allowed to cure 24 h in the dark before viewing by fluorescence microscopy.

Microscopy and Imaging. Slides were visualized using a Nikon Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY) fitted with an epifluorescence attachment and photographed with an Olympus QColor 3 digital camera (Olympus America Inc., Center Valley, PA). The UV-2E/C DAPI, fluorescein isothiocyanate (FITC), and G-2A filter blocks were used for visualization of DAPI, Ki-67, and BrdU, respectively. Eighteen to 30 digital images were obtained per slide (depending on epithelium content); these consisted of sets of BrdU, Ki-67, and DAPI images. Each set represented an independent area composed primarily of mammary epithelium. At least 1,000 epithelial cells were scored per slide ($1,687 \pm 100$; LSM $\pm$ SEM) and this number did not differ by treatment (data not shown). All images obtained were 12-bit monochrome images taken at

40× magnification. Exposure lengths were 5 to 30, 100, and 100 ms for DAPI, BrdU, and Ki-67, respectively.

BrdU and Ki-67 Quantification. Images were analyzed using Image Pro Plus software (version 6.2, Media Cybernetics, Silver Spring, MD). Cells positive for BrdU and Ki-67 as well as dual-labeled cells were counted manually by a single observer blinded to dietary treatment, and the total number of epithelial cells per image was determined using image analysis software. Labeling indexes for BrdU and Ki-67 were calculated by dividing the number of positive cells counted per image by the total number of mammary epithelial cells in that image. The BrdU-positive cells were further categorized as being heavily labeled or lightly labeled according to degree of nuclear stain present. To classify as heavily labeled, 100% of the apparent nuclear area must have been stained, whereas a classification of lightly labeled was reserved for nuclei with less than 100% of apparent nuclear area stained. The heavily labeled BrdU cells were further characterized by presence or absence of Ki-67 stain.

Statistical Analysis

All data were analyzed using the Mixed Procedure of SAS (version 9.1.3, SAS Institute, Cary, NC). Mammary composition and telomerase assay data were analyzed with a model that included the main effects of diet, group, and their interaction. The residual for testing fixed effects was heifer within diet and group. Heat inactivation values were used as a covariate for the telomerase data.

The qRT-PCR data submitted to SAS consisted of $2^{(-\Delta Ct)}$ values. Normality of data distribution for each gene, diet, and tissue fraction combination was determined using the Shapiro-Wilk test in the univariate procedure of SAS. For this test, the null hypothesis that the data were from a random sample from a normal distribution was rejected when $P < 0.05$. Because some gene, diet, and tissue fraction combinations appeared to be non-normally distributed and data were not transformed, a more conservative α was selected for ANOVA; differences were declared significant at $P < 0.01$. Additionally, before ANOVA, heterogeneity of variance for each gene was evaluated with a Hartley F -max test; variances were deemed homogeneous so no transformations were made.

Stem cell and gene expression analyses included location as the repeated measure and the subject used in tests was heifer within diet and group. An autoregressive covariance structure [AR(1)] was used; no denominator degrees of freedom approximation method was specified. Model terms included main effects of diet, group, and location, as well as all 2-way interactions.

All data are reported as least squares means $\pm$ standard error of the means; differences were declared significant at $P \leq 0.05$ unless otherwise noted. Nonorthogonal preplanned contrast statements were used to examine the main effect of diet, if significant. These were CON versus all other treatments, HPLF versus HPHF, and HPHF versus HPHF+.

RESULTS

Body Growth and Intake

Growth data are summarized in Table 2. Age and BW at initiation of dietary treatments were not different between diets and averaged ($\pm$ SEM) 3.5 ± 0.39 d and 40.2 ± 1.93 kg, respectively. As intended, diet had no effect on age at harvest; average age at harvest was 64.6 ± 0.38 d. In contrast, BW at harvest was affected by diet ($P = 0.001$). Heifers fed CON weighed less than the average of the other 3 diets. Heifers fed HPLF weighed more than those fed HPHF at the end of the experiment, despite ADG being statistically equivalent. Heifers fed HPHF+ gained 30% more weight than did heifers fed HPHF and as a result weighed more than HPHF at the end of the experiment (91.6 ± 2.7 kg vs. 75.9 ± 2.7 kg for HPHF+ and HPHF, respectively).

Lifetime ADG for heifers on each diet were 0.47, 0.70, 0.66, and 0.85 kg/d for CON, HPLF, HPHF, and HPHF+, respectively ($P = 0.002$; Table 2). During the final 2 wk of the experiment there was a tendency for ADG to converge ($P = 0.078$; Table 2). Likewise, lifetime differences in DMI due to diet were detected ($P = 0.006$; Table 2), but there was a tendency for DMI convergence in the final 2 wk of the experiment ($P = 0.053$; Table 2). No differences in gain:feed were detected due to diet, and lifetime averages were 0.43, 0.47, 0.51, and 0.49 for CON, HPLF, HPHF, and HPHF+, respectively (Table 2).

Proportion of MG Occupied by PAR and MFP

No differences in PAR weight due to diet were detected (Table 3). In contrast, diet-induced differences in total MG and MFP weights were noted (Table 3). Total MG (skinned right half of the udder including both PAR and MFP, $\times 2$) and MFP weights were lowest in CON compared with the average of the other 3 diets. The addition of fat to our isonitrogenous diet had no effect on unadjusted total MG and MFP weights. However, when adjusted for EBW, HPHF had heavier total MG and MFP than did HPLF. Feeding an increased volume of a high-fat MR increased unadjusted total MG and MFP weights. However, when adjusted for EBW, total MG and MFP weights only tended

Table 2. Initial and final heifer BW, age, average daily gains (ADG), DMI, and gain:feed on treatment diets at various times during the experiment

Item	Diet ¹				SEM ²	Diet, <i>P</i>	Diet contrasts		
	CON (n = 5)	HPLF (n = 5)	HPHF (n = 6)	HPHF+ (n = 6)			CON vs. all	HPLF vs. HPHF	HPHF vs. HPHF+
Initial BW, kg	40.1	44.1	36.3	40.1	1.93	0.075	—	—	—
Initial age, d	3.5	3.3	3.3	3.7	0.39	0.809	—	—	—
Final BW, kg	68.4	86.7	75.9	91.6	2.66	0.001	0.001	0.012	0.001
Final age, d	64.8	64.6	64.5	64.6	0.38	0.921	—	—	—
ADG, kg/d									
Lifetime	0.47	0.70	0.66	0.85	0.05	0.002	0.001	0.532	0.012
wk 5 to wk 9	0.57	0.78	0.66	0.86	0.05	0.003	0.004	0.068	0.005
wk 7 to wk 9	0.58	0.83	0.71	0.94	0.09	0.078	—	—	—
DMI, kg/d									
Lifetime	1.04	1.40	1.35	1.65	0.10	0.006	0.005	0.585	0.014
wk 5 to wk 9	1.29	1.59	1.39	1.70	0.08	0.011	0.013	0.097	0.010
wk 7 to wk 9	1.38	1.70	1.40	1.74	0.10	0.053	0.078	0.071	0.031
Gain:feed									
Lifetime	0.43	0.47	0.51	0.49	0.05	0.534	—	—	—
wk 5 to wk 9	0.47	0.49	0.48	0.51	0.03	0.847	—	—	—
wk 7 to wk 9	0.46	0.49	0.52	0.55	0.07	0.821	—	—	—

¹Diets: CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPLF = high-protein, low-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d.

²Standard error of the mean for diet (n = 5).

to differ ($P = 0.067$ and $P = 0.071$, respectively) for HPHF versus HPHF+.

PAR and MFP Composition

No differences in lipid, protein, or DNA composition of PAR due to diet were detected (Table 3). In contrast, MFP composition was markedly influenced by diet (Table 3). Lipid concentration and total lipids in MFP were lowest in CON compared with the average of the other 3 diets. Heifers fed HPHF had greater lipid concentration and total lipids in MFP than did HPLF. Feeding an increased volume of the high fat MR did not affect MFP lipid concentration; however, it increased total MFP lipids relative to HPHF because the MFP of HPHF+ heifers were larger and heavier than those of HPHF heifers.

In MFP, concentrations (mg/g of MFP) of protein and DNA were higher and the concentration of lipid lower when CON was compared with the average of the other 3 diets (Table 3). The same was true when HPLF was compared with HPHF. Protein and DNA concentrations in MFP were not affected by feeding an increased volume of a high fat MR.

Deserving mention here is a methodological note regarding our mammary protein determination assay. Compared with other recent reports (Brown et al., 2005; Meyer et al., 2006b) the protein amounts contained herein are low. This is because our homogenate preparation protocol (from which protein content was

determined) included a brief centrifugation step followed by a decanting step that effectively excluded insoluble proteins from further study. This was an unintended oversight; the reader is asked to keep that in mind when considering Table 3.

GH/IGF-I Axis Genes in PAR and MFP

Messenger RNA for IGFBP-1 was not readily detectable in PAR or MFP, whereas inclusion of hepatic cDNA as a positive control for the assay verified the ability of the primer to amplify a single product of expected size. Most PAR and MFP samples failed to reach threshold amplification after 40 cycles of PCR. This indicated that IGFBP-1 mRNA is absent in heifers of this age. The IGFBP-1 data were not analyzed, are labeled non-detectable, and are not discussed further.

No diet differences in expression of transcripts for components of the GH/IGF-I axis within PAR or MFP were detected (Table 4). A few genes were differentially expressed according to tissue fraction (PAR or MFP): IGFBP-2, IGFBP-5, and GH-R mRNA were more abundant in MFP than in PAR, whereas IGF-IR mRNA was more abundant in PAR (Table 4).

Telomerase, BrdU, and Ki-67 in PAR

No differences in telomerase activity in PAR were detected (for diet, $P = 0.442$; Figure 1). No diet differences for percentage of BrdU- or Ki-67-labeled epithelial cells

Table 3. Mammary parenchyma (PAR) and fat pad (MFP) mass and composition for heifers fed 1 of 4 milk replacer (MR) diets

Item	Diet ¹				SEM ²	<i>P</i> > <i>F</i> ³	Diet contrasts (<i>P</i> > <i>F</i>)		
	CON (n = 5)	HPLF (n = 5)	HPHF (n = 6)	HPHF+ (n = 6)			CON vs. all	HPLF vs. HPHF	HPHF vs. HPHF+
PAR									
Weight, g	5.94	8.78	10.97	13.66	3.66	0.466	—	—	—
Weight, g/100 kg of empty BW	9.41	11.1	15.53	15.12	4.48	0.683	—	—	—
Lipids, mg/g of PAR	113 ⁴	132	165	161	21.6	0.241	—	—	—
Total lipids, g	0.9 ⁴	1.1	1.8	2.2	0.54	0.255	—	—	—
Protein, mg/g of PAR ⁵	46	38	41	38	2.4	0.085	—	—	—
Total protein, g	0.3	0.4	0.45	0.5	0.16	0.646	—	—	—
DNA, mg/g of PAR	2.12	1.79	1.97	2.13	0.233	0.680	—	—	—
Total DNA, mg	11.5	16.9	22.5	32.1	10.41	0.942	—	—	—
MFP									
Weight, g	84.08	131.33	192.21	324.83	35.07	0.002	0.008	0.219	0.012
Weight, g/100 kg of empty BW	139.23	168.29	272.51	364.08	36.96	0.003	0.012	0.059	0.071
Lipids, mg/g of MFP	455	475	687	706	46.4	0.003	0.010	0.006	0.742
Total lipids, g	39.7	64.6	132.4	233.7	28.25	0.002	0.009	0.100	0.015
Protein, mg/g of MFP ⁵	20	19	9	6	3.4	0.022	0.053	0.042	0.486
Total protein, g	1.6	2.4	1.6	1.6	0.41	0.477	—	—	—
DNA, mg/g of MFP	0.74	0.65	0.23	0.13	0.149	0.026	0.038	0.056	0.601
Total DNA, mg	59.0	79.0	42.5	37.1	14.07	0.177	—	—	—
Total udder									
Weight, g	90.00	140.11	203.16	338.50	34.86	0.002	0.006	0.202	0.010
Weight, g/100 kg of empty BW	148.68	179.40	288.04	379.23	36.18	0.002	0.009	0.047	0.067

¹Diets: CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPLF = high-protein, low-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d.

²Standard error of the mean for diet (*n* = 5).

³*F*-test *P*-value for diet.

⁴*n* = 4.

⁵See text, protein values represent total soluble tissue protein.

in PAR were detected (Table 5), but labeling varied by sampling region (Table 5). Percentage of heavily and total BrdU-labeled epithelial cells was largest in cisternal regions of the gland (0.41% heavy; 2.49% total) and decreased toward the periphery (0.13% heavy; 0.79% total; Table 5). The opposite was true for Ki-67-labeled epithelial cells (9.06% cisternal vs. 10.94% peripheral; Table 5). Only 4.9% of heavily BrdU-labeled cells were also positive for Ki-67 (data not shown), which suggests that label-retaining epithelial cells proliferate infrequently. Figure 2 and Figure 3 contain examples of histology images.

DISCUSSION

Meyer et al. (2006b) observed that although increased energy intake directly influenced MFP growth, its influence on PAR was indirect and time dependent. In that study, heifers were evaluated at the same BW (50-kg increments from 100 to 350 kg), so heifers with elevated levels of nutrient intake were, by default, younger compared with their cohorts with a restricted energy intake. This age effect, not level of nutrient intake, was hypothesized to be the single greatest determinant of

total PAR DNA content. Of particular note, within that data set, there was a positive effect of nutrient intake on PAR DNA in heifers at 100 kg of BW; however, it was confounded by age (Meyer et al., 2006b). Brown et al. (2005) demonstrated that increased intake of a high protein milk replacer stimulated growth of PAR when the study was controlled for heifer age. The purpose of the current experiment was to confirm the positive effects of nutrient intake on mammary growth in preweaned calves, while also evaluating the influence of protein and fat consumption on development of PAR and MFP, and to assess potential mechanisms whereby growth is promoted. There were 3 primary objectives pertaining to the effect of nutrition on mammary growth and development.

Our first objective was to determine if MR composition and intake influenced mammary gland growth and tissue composition. Data of Meyer et al. (2006b) suggested that growth of PAR was refractory to the level of nutrient intake, whereas growth of MFP was not. Brown et al. (2005) showed that PAR mass was enhanced by increased consumption of protein and energy during the preweaning interval. In the current study, total MG weight was affected by diet and this

Figure 1. Telomerase abundance in mammary parenchyma was not affected by diet ($P = 0.442$). Diets: CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPLF = high-protein, low-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d.

is assumed to be due primarily to accumulation of MFP because no diet differences in PAR weight were detected (discussed below), whereas differences in MFP weight were. This direct effect of diet on MFP is simi-

lar to previous studies (Sejrsen et al., 1982; Capuco et al., 1995; Brown et al., 2005; Meyer et al., 2006b). As expected, total MG and MFP weights were lowest in CON and increased with feeding more nutrient-dense diets or with increasing nutrient intake. Adding fat to an isonitrogenous diet resulted in heifers that weighed less than cohorts fed HPLF. However, when scaled to BW, those fed HPHF had heavier MFP than did those fed HPLF. Additionally, heifers fed HPHF had more lipids, less protein, and less DNA per gram of MFP than did their cohorts fed HPLF, suggesting adipocyte hypertrophy in HPHF.

In contrast to Brown et al. (2005), we observed no effect of diet on PAR weight or composition, despite marked changes in MFP. Importantly though, just as these data do not confirm a positive effect of diet, neither do they support the notion that diet has no effect on PAR. In the least, they are supportive of the concept that an increased level of nutrition during the preweaning period is not detrimental to growth of PAR. Furthermore, our data lend support to a recent study and conclusions by Thorn et al. (2008). They concluded that although MFP expansion causes increased production of certain inflammation-related peptides that may affect PAR growth, it is unlikely to contribute to the

Table 4. Relative mRNA abundance (expressed as $2^{-\Delta Ct}$ values; higher values equate to more mRNA) of selected genes in parenchyma (PAR) and mammary fat pad (MFP) in preweaned heifers fed 1 of 4 milk replacer (MR) diets

		Diet ²				Test of fixed effects, ⁴ <i>P</i> -value						
Gene ¹	Fraction	CON	HPLF	HPHF	HPHF+	SEM ³	Diet	Group	Fraction ⁵	Diet × group	Diet × fraction	Group × fraction
– Fold differences in mRNA (2 ^{−ΔCt} values) –												
IGFBP-1	PAR	ND ⁶	ND	ND	ND							
	MFP	ND	ND	ND	ND							
IGFBP-2	PAR	0.32	0.24	0.26	1.89	1.20	0.409	0.513	0.001	0.920	0.870	0.831
	MFP	5.51	3.92	5.42	6.58							
IGFBP-3	PAR	13.78	15.13	14.60	19.90	4.99	0.716	0.090	0.030	0.345	0.984	0.142
	MFP	7.82	6.48	7.35	10.53							
IGFBP-4	PAR	0.57	0.51	0.47	0.50	0.09	0.288	0.017	0.594	0.420	0.561	0.011
	MFP	0.70	0.39	0.49	0.59							
IGFBP-5	PAR	1.90	0.96	0.82	1.68	0.96	0.234	0.566	0.010	0.452	0.227	0.514
	MFP	4.80	1.17	3.91	2.33							
IGFBP-6	PAR	1.33	1.10	1.55	2.04	0.83	0.501	0.813	0.071	0.793	0.168	0.435
	MFP	2.54	0.94	3.51	1.96							
GH-R	PAR	0.63	1.06	1.15	1.26	0.40	0.452	0.219	0.002	0.190	0.636	0.350
	MFP	1.85	1.67	2.54	1.89							
IGF-IR	PAR	0.92	0.72	1.07	0.86	0.19	0.548	0.577	0.001	0.815	0.500	0.364
	MFP	0.32	0.16	0.26	0.55							
IGF-I	PAR	1.24	1.05	1.47	1.63	0.34	0.512	0.183	0.104	0.091	0.967	0.017
	MFP	1.78	1.45	1.79	1.87							

¹IGFBP = IGF binding protein; GH-R = growth hormone receptor; IGF-IR = IGF-I receptor.

²Diets: CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPLF = high-protein, low-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d.

³SEM = standard error of the mean for diet $\times$ fraction; ($n = 5$).

⁴Significance declared when $P < 0.01$.

⁵Fraction was either PAR or MFP.

⁶ND = nondetectable.

Table 5. Effects of diet¹ and mammary sampling region on Ki-67 and bromodeoxyuridine (BrdU) labeling indexes in Holstein heifers

Item	Region		SEM ²	Test of fixed effects, ³ <i>P</i> -value		
	Cisternal	Peripheral		Diet	Region	Diet × region
Heavy BrdU labeling index, %						
CON	0.38	0.18	0.209	0.532	0.004	0.478
HPLF	0.25	0.07	0.187			
HPHF	0.32	0.08	0.163			
HPHF+	0.72	0.22	0.163			
Total BrdU labeling index, %						
CON	2.15	1.17	0.843	0.766	0.002	0.413
HPLF	2.68	1.05	0.753			
HPHF	1.82	0.54	0.662			
HPHF+	3.43	0.42	0.662			
Ki-67 labeling index, %						
CON	9.81	10.71	1.218	0.403	0.007	0.881
HPLF	8.69	10.83	1.089			
HPHF	10.23	11.93	0.953			
HPHF+	7.99	10.00	0.953			

¹Diets: CON = control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g of DM/d; HPLF = high-protein, low-fat MR with 28% CP and 20% fat, fed at 951 g of DM/d; HPHF = high-protein, high-fat MR with 27% CP and 28% fat, fed at 951 g of DM/d; HPHF+ = HPHF fed at 1,431 g of DM/d.

²SEM = standard error of the mean for diet × region.

³Other fixed effects tested but not shown include group, diet × group, group × region; all had *P* > 0.05.

reduced PAR growth and development frequently observed with increased nutrition. In the current study, had PAR growth been hindered by inflammatory cues from MFP that affect IGF-I actions, we should have detected diet-induced differences in expression of genes for components of the GH/IGF-I axis in either MFP or PAR. We observed no such differences.

Our second objective was to determine if expression of GH/IGF-I axis genes in PAR and MFP differed among heifers fed different diets. We detected no dietary influence on transcript abundance for components of the GH/IGF-I axis. This suggests, but does not prove, that nutrition does not influence mammary growth in the preweaned calf by modulating expression of genes that are components of the local GH/IGF-I axis. However, because transcript level was assessed in tissues harvested at the conclusion of the study, we cannot rule out changes in gene expression that may have occurred earlier during the preweaning period. Pertinent to this issue is the observation that ADG and DMI of the various dietary groups tended to converge during the final 2 wk of the study (Table 2). Nevertheless, because diet-induced differences in lifetime ADG and DMI were detectable (Table 2) and circulating concentrations of IGF-I remained different throughout the study (see Daniels et al., 2008), had diet induced a change in gene expression, it seems likely that some alterations should have been evident at the time of tissue harvest.

Although perhaps not regulated by diet, several genes of the GH/IGF-I axis appear to be spatially regulated in bovine MG. For instance, IGF-IR mRNA and GH-R

mRNA were inversely related in PAR and MFP, with PAR having relatively more IGF-IR and less GH-R expression than MFP. This is consistent with past findings and supports an indirect mechanism for GH to affect PAR. Additionally, we observed relatively more IGFBP-5 in MFP compared with PAR, which is also consistent with past findings in prepubertal heifers (Berry et al., 2003a).

Overall, the dominant IGFBP detected was IGFBP-3, which was expressed at high levels in both MFP and PAR, followed by IGFBP-2 and IGFBP-5, which were preferentially expressed in MFP compared with PAR. Plath-Gabler et al. (2001) noted IGFBP-3 and IGFBP-5 as the dominant IGFBP present in bovine PAR evaluated at various stages from puberty through involution. They later concluded that because IGFBP-2 expression in PAR was very weak in their study, it is not regulated and does not have a significant physiological role in the bovine MG (Plath-Gabler et al., 2001). We challenge their conclusion and suggest that IGFBP-2 is at least spatially regulated in MG of 2-mo-old heifers.

Differences in expression of IGF-I, IGFBP-3, IGFBP-4, and IGFBP-6 mRNA due to diet or tissue source were not detected, providing no evidence for or against nutritional or spatial regulation of these components in MG of 2-mo-old heifers. Berry et al. (2003a) also found IGF-I and IGFBP-3 to be equally expressed in PAR and MFP in intact and ovariectomized heifers. In addition, similar to our findings, Meyer et al. (2007) found IGF-I mRNA abundance to be unaltered by level of nutrient intake in PAR and MFP of heifers. Recent

lines of evidence suggest that locally produced IGF-I plays a larger role in mediating mammary development in prepubertal heifers than does IGF-I from circulation (Weber et al., 1999; Berry et al., 2001, 2003a). Although no diet-induced differences in mammary

IGF-I mRNA expression or PAR mass and composition were detected, serum concentrations of IGF-I in these heifers were influenced by diet (Daniels et al., 2008), suggesting that local expression of IGF-I mRNA may indeed be of greater importance to MG development

Figure 2. Localization of bromodeoxyuridine (BrdU)-labeled and Ki-67-labeled epithelial cells in peripheral and cisternal parenchymal regions of the gland. Few BrdU-labeled cells and many Ki-67-labeled cells were evident in peripheral regions of the gland; the opposite was true of cisternal regions. A) Positive control; obtained from a BrdU pulse-labeled heifer; B) negative control; serial section of A, primary antibodies were substituted with control sera; C and D) BrdU and Ki-67 staining in 2 peripheral regions; E and F) BrdU and Ki-67 staining in 2 cisternal regions. DAPI = 4',6-diamidino-2-phenylindole. Scale bar = 50 μ m.

Figure 3. Example of a bromodeoxyuridine (BrdU)/Ki-67 dual-labeled mammary epithelial cell (white arrow). A) BrdU + 4',6-diamidino-2-phenylindole (DAPI); B) Ki-67 + DAPI; C) BrdU + Ki-67; and D) BrdU + Ki-67 + DAPI. Scale bar = 50 μ m.

than systemic IGF-I. In support of this idea, supplementary data analysis of Pearson correlations within diet (using the single time point of slaughter) revealed no definitive relationship between serum IGF-I and PAR weight adjusted for EBW ($r = 0.186$, $P = 0.407$). On the other hand, IGF-I mRNA expression in PAR (but not MFP) tended to correlate, albeit negatively, with EBW-adjusted PAR weight ($r = -0.422$, $P = 0.056$ and $r = -0.212$, $P = 0.356$). When serum IGF-I and expression of IGF-I mRNA in MFP were compared with EBW-adjusted MFP weight, neither was found to be correlated ($r = 0.110$, $P = 0.634$ and $r = 0.072$, $P = 0.757$, respectively). However, IGF-I mRNA in PAR (presumably from stromal cells) tended toward a positive relationship with EBW-adjusted MFP ($r = 0.343$, $P = 0.128$). Interestingly, IGF-IR mRNA in MFP (but not PAR) was also positively associated with EBW-adjusted MFP weight ($P = 0.031$ and $P = 0.364$).

Finally, there was a strong correlation between IGF-I mRNA and IGF-IR mRNA in PAR ($r = 0.796$, $P = 0.001$), but not in MFP ($r = 0.114$, $P = 0.623$). This probably pertains to the epithelial localization of IGF-IR and the production of IGF-I by stromal cells located in both tissue fractions (PAR and MFP) and

lends further support for a paracrine mode of action for IGF-I hypothesized by others (Plath-Gabler et al., 2001; Berry et al., 2003a).

Our final objective was to determine if abundance of mammary epithelial stem cells was modulated by diet in young heifers. We hypothesized that positive effects of faster rates of gain during the preweaning period alter the development, persistence, or activity of populations of mammary epithelial stem cells and thereby affect subsequent mammary development. Putative mammary epithelial stem cells were histologically assessed using the technique described by Capuco (2007). Our results from immunohistochemical analysis and the telomerase assay collectively provide no direct evidence for or against the notion that the number of putative mammary epithelial stem cells is affected by nutrient intake or level of protein and fat in MR fed to preweaned heifers. However, we do not suggest that putative mammary epithelial stem cells of prepubertal heifers are unresponsive to mitotic stimuli. To this point, Capuco et al. (2009) showed that in xanthosine-treated glands of prepubertal heifers, numbers of putative mammary stem cells and telomerase activity were increased compared with contralateral control glands. Brown et

al. (2005) observed an increase in PAR growth with an increased level of nutrition in preweaned calves; they did not evaluate Ki-67 labeling in calves around the time of weaning. They did, however, measure a negative effect on the Ki-67-labeling index of PAR in peripubertal heifers at the conclusion of the study. Finally, it should be pointed out that although telomerase activity and Ki-67 labeling provided indices of stem cell number and cell proliferation at the time of tissue harvest in the current study, BrdU labeling of putative stem cells occurred early during the preweaning period.

Although no diet effects were detected in our histology experiment, we observed regional differences in BrdU and Ki-67 labeling in PAR. This serves as a methodological control of sorts, given that others (Ellis et al., 2000; Capuco et al., 2002) have previously shown that cells from peripheral PAR zones of the developing bovine mammary gland have greater proliferative and morphogenic potential than cells from the medial PAR mass. We speculate that these differences reflect local tissue regulation necessary for sequential ductular and lobuloalveolar development *in vivo*. Our results therefore agree with previous findings and are consistent with decreased proliferation and increased cell cycle arrest in cisternal regions of the gland (Ellis et al., 2000; Capuco et al., 2002; Capuco, 2007).

In the current experiment, although individual measurements pertaining to mammary growth (PAR mass, PAR DNA, Ki-67-labeling index), and stem cell activity (BrdU-label retention, telomerase activity) were not appreciably influenced by preweaning diet, there was a correlation between PAR mass and telomerase activity ($r = 0.586$, $P = 0.011$). In contrast, no correlation between BrdU-label retaining cells and PAR mass or between BrdU-label retaining cells and telomerase activity were detected ($r = -0.116$, $P = 0.646$ and $r = -0.171$, $P = 0.472$, respectively). The suitability of these 2 methods (telomerase activity and BrdU label retention) to identify mammary stem cells remains to be determined. However, the relationship between telomerase and PAR mass is of interest and taken at face value may indicate that there is an underlying effect on stem cells or their progeny (Capuco and Ellis, 2005). Our data clearly support the conclusion that an increased level of nutrition during the preweaning period does not impair PAR growth in heifers, and, together with data of Brown et al. (2005), suggests the possibility it may enhance mammary growth.

CONCLUSIONS

In conclusion, we confirmed that MR composition and intake affect protein and lipid deposition in MFP, as evidenced by detectable differences in mass and

composition of MFP. Our data do not demonstrate an effect, either positive or negative, of diet on measures of PAR: stem cell abundance, GH/IGF-I axis gene expression, or mass and composition. However, we observed a correlation between the stem cell marker telomerase and PAR mass and a numerical increase in PAR mass with increased level of nutrition. Perhaps with more animals and sustained separation in ADG and DMI (as could be achieved by controlling starter intake), or both, more diet effects would have been actualized. Further study in this area is necessary to clarify the underlying nutritional and physiological mechanisms that govern mammary growth and development. These future studies should include careful inspection of both tissue compartments in the udder (PAR and MFP) as each influences the overall architecture and likely, the eventual function of the udder.

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