Prewaning nutrient supply alters mammary gland transcriptome expression relating to morphology, lipid accumulation, DNA synthesis, and RNA expression in Holstein heifer calves

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ABSTRACT

The objective of this study was to analyze the mammary gland transcriptome to determine how preweaning nutrient supply alters the molecular mechanisms that regulate preweaning mammary development. Holstein heifers were fed via milk replacer (MR) either an elevated level of nutrient intake (ELE; on average, 5.9 ± 0.2 Mcal of ME in 8.4 L of MR/d, $n = 6$) or a restricted amount of nutrients (RES; 2.8 ± 0.2 Mcal of ME in 4 L of MR/d, $n = 5$) for 54 d after birth, at which point they were slaughtered and samples of mammary parenchyma tissue were obtained. Parenchymal mRNA was analyzed, and the fold change (FC) of 18,111 genes (ELE relative to RES) was uploaded to Ingenuity Pathway Analysis (IPA) software (Qiagen Bioinformatics, Redwood City, CA) for transcriptomic analysis. Using a threshold of $P < 0.05$, IPA identified that the FC of 1,931 of 18,811 differentially expressed genes (DEG) could be used for the analysis. A total of 18 molecular and cellular functions were relevant to DEG arising from the treatments; the 5 functions most associated with DEG were cell death and survival, cellular movement, cellular development, cellular growth and proliferation, and lipid metabolism. Based on the directional FC of DEG, the mammary gland of ELE heifers was predicted to have increased epithelial-mesenchymal transition ($Z = 2.685$) and accumulation of lipid ($Z = 2.322$), whereas the synthesis of DNA ($Z = -2.137$), transactivation of RNA ($Z = -2.254$),

expression of RNA ($Z = -2.405$), transcription ($Z = -2.482$), and transactivation ($Z = -2.611$) were all predicted to be decreased. Additionally, IPA predicted the activation status of 13 upstream regulators with direct influence on DEG as affected by ELE feeding that were ligand-dependent nuclear receptors ($n = 2$), enzymes ($n = 1$), or transcription regulators ($n = 10$). Of these, 6 were activated ($Z > 2$) and 7 were inhibited ($Z < -2$). In summary, feeding ELE preweaning altered the mammary transcriptome of Holstein heifers, affecting cell functions involved in the morphological and physiological development of the mammary gland.

Key words: transcriptome, mammary parenchyma, metabolizable energy, Holstein, preweaning

INTRODUCTION

Restricted milk or milk replacer (MR) allowance preweaning has been a common strategy within the dairy industry as a method to reduce feeding frequency and costs and encourage greater preweaning starter intake (Kertz et al., 1979; Otterby and Linn, 1981; Anderson et al., 1987). During the postweaning period, additional benefit from limiting energy intake is believed to be derived from improved mammary function and milk production (Lammers and Kensinger, 1999; Radcliff et al., 2000) as a result of lesser fat deposition relative to parenchymal mass accumulation during the allometric growth phase between 7 to 11 and 15 mo of age (Sejrsen et al., 1982, 2000; Sejrsen and Purup, 1997). Decreases in mammary parenchymal DNA and mass have been previously associated with greater prepubertal energy intake (Sejrsen et al., 1982; Petitclerc et al., 1984; Capuco et al., 1995), suggesting that increasing energy intake reduces parenchyma proliferation. Therefore,

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producers have restricted preweaning energy supply in part to limit rearing costs but also in an aim to increase future milk yield and productive capacity of heifer calves.

However, Van Amburgh et al. (1998) found that first-lactation milk yield was not compromised by higher rates of BW gain (i.e., 1.1 as opposed to 0.6 to 0.8 kg/d) during the prepubertal period. Similarly, Waldo et al. (1998) found no difference in milk yield from changing ADG, even though pair-fed animals fed higher energy diets postweaning had significantly less parenchymal DNA postpuberty (Capuco et al., 1995). Moreover, recent work has demonstrated that increased nutrient supply during the preweaning phase can increase yield at first lactation (Moallem et al., 2010; Soberon et al., 2012; Soberon and Van Amburgh, 2013). Contradicting previous research, total mammary gland mass has been observed to be greater for calves consuming elevated quantities of nutrients compared with calves consuming restricted quantities (Meyer et al., 2006b; Soberon and Van Amburgh, 2017). Similarly, calves fed more energy also had greater parenchymal mass, when expressed as a proportion of BW, relative to their restricted counterparts (Brown et al., 2005; Soberon and Van Amburgh, 2017). The resultant increase in mass is due to greater parenchyma proliferation (Brown et al., 2005; Meyer et al., 2006c; Geiger et al., 2016). As past research applied dietary treatments postweaning to assess mammary gland samples collected at 11 and 15 mo of age (Serjensen et al., 1982; Petitclerc et al., 1984; Capuco et al., 1995) and recent work has evaluated preweaning mammary physiology, it is possible that the timing of nutrient provision and specific cell type might be an important factor in determining prepubertal mammary development and future lactational capacity.

Though the timing of increased nutrient intake can positively affect mammary development and future milk production (Moallem et al., 2010; Soberon et al., 2012; Soberon and Van Amburgh, 2013), the mechanisms by which this occurs have not been completely elucidated. Furthermore, genetic-hormonal interactions are complex. It can be difficult to determine a cause-and-effect pattern, and very seldom are physiological responses unifactorial. Transcriptome analysis within a tissue can be a useful tool because it can predict the activity status of the physiological pathways to help explain the observed phenotypic responses. Therefore, the objective of this study was to evaluate how nutrient supply (elevated vs. restricted) during the preweaning phase affects the expression of the transcriptome within the mammary gland of female Holstein calves. It was hypothesized that increased preweaning nutrient supply would lead to changes in mRNA expression associated

with growth, differentiation, and nutrient metabolism in the mammary gland.

MATERIALS AND METHODS

Heifer Husbandry, Experimental Design, and Treatments

All heifers were cared for as approved (protocol number 2009-1920) by the Cornell University Institutional Animal Care and Use Committee. Heifer management, feeding, animal care, performance data, and the histological and physical development of the mammary gland have been previously reported in Soberon and Van Amburgh (2017). Briefly, 12 Holstein heifers were randomly assigned at birth to 1 of 2 treatments in which they were offered either an elevated level of nutrient intake (**ELE**; $n = 6$), whereby ME was provided at 0.3 Mcal of ME/kg of BW^{0.75} in MR, or a restricted level of nutrient intake (**RES**; $n = 6$) that was designed to replicate standard industry MR feeding strategies. Heifers consuming RES were fed twice per day [2.8 ± 0.2 (mean $\pm$ SE) Mcal of ME intake in 4 L of MR/d], whereas ELE heifers consumed their ME allotment in 3 feedings per day (4.2–8.4 Mcal of ME intake in 6–12 L of MR/d, 5.9 ± 0.2 Mcal of ME intake in 8.4 L of MR/d; data reported in Soberon and Van Amburgh, 2017). Milk replacer composition was 28% protein and 15% fat (Table 1; Excelsior, Milk Specialties Inc., Eden Prairie, MN). Both ELE and RES heifers were offered starter grain (22% CP, Table 1; Cargill Inc., Minneapolis, MN) ad libitum from d 18 after birth. Treatments were fed for 54 d, adjusting weekly for changes in BW, until calves were slaughtered.

Table 1. Milk replacer and starter grain composition as reported by the manufacturer¹

Analysis, DM basis	Milk replacer ²	Starter grain ³
CP, %	28.5	22
Crude fat, %	15.0	4.3
NDF, %	0.2	33.1
Calcium, %	1.0	1.6
Phosphorus, %	>0.6	1.0
Vitamin A, IU/g	>16.5	43.1
Vitamin D ₃ , IU/g	>5.5	NA ⁴
Vitamin E, IU/g	>110.3	197.0
Gross energy, Mcal/kg	5.1	1.8

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²Excelsior, Milk Specialties Inc., Carpentersville, IL.

³Cargill Inc., Minneapolis, MN.

⁴Not available.

Slaughter and Tissue Collection

At 54 ± 2 d, calves were fed, weighed, and transported to the Cornell University abattoir (Ithaca, NY) for slaughter and tissue collection. Calf slaughter and the removal and dissection of the mammary gland were described by Soberon and Van Amburgh (2017). After the mammary gland was isolated and total weight was recorded, the fat pad was removed from the parenchyma. Tissue samples of the mammary parenchyma were then collected, placed in foil bags, snap-frozen in liquid N₂, and stored at -80°C until RNA isolation. Although 6 ELE and 6 RES heifers were killed and their mammary glands were harvested, the parenchyma of a heifer from the RES group was too small and could not be separated from the fat pad; therefore, it was not available to be sampled due to minimal development of the mammary gland. Thus, data from the RES treatment calves are representative of 5 heifers, as opposed to 6 heifers in the ELE group.

RNA Extraction and Quality

The frozen parenchymal samples were homogenized using an Ultra-Turrax homogenizer (IKA Laboratory Technology, Staufen, Germany) in TRIzol reagent (Thermo Fisher, Waltham, MA), and purification was done using Qiagen RNeasy columns (Qiagen, Hilden, Germany). A total of 100 ng of RNA was used for whole-transcript cDNA synthesis and terminal labeling using the Ambion WT expression kit (Life Technologies, Blijswijk, the Netherlands) following confirmation of RNA quality (RNA integrity number >7.0) using chip analysis (Agilent 1000 Bioanalyzer, Agilent Technologies, Amsterdam, the Netherlands) according to the manufacturer's instructions. The RNA samples were considered appropriate for hybridization if intact bands corresponded to the 18S and 28S ribosomal RNA subunits and did not display chromosomal peaks or RNA degradation products.

Affymetrix GeneChip Oligoarray Hybridization and Quality Control

Samples were analyzed on Affymetrix Bovine Gene 1.1 ST arrays (Affymetrix, Santa Clara, CA). Hybridization, washing, staining, and scanning of the arrays were performed automatically on an Affymetrix GeneTitan instrument. Packages from the Bioconductor project (Gentleman et al., 2004), integrated in an on-line pipeline (Lin et al., 2011), were used to analyze the array data. To control for the quality of the array across probes, the average background signal, percent present calls, and scales factors were determined

and used to assess array quality (Heber and Sick, 2006; Brettschneider et al., 2008). The library "AffyPLM" was used to fit the linear model to the probes in order to provide individual parameter estimates. Pseudoimages were created for each array, as ascertained by the weights and residuals computed by fitPLM, and inspected for any stains, scratches, or other artifacts. Artifacts covered less than 5% of the area of the array. Probes were also assessed in their numerical quality based on two distributions calculated from the probeset level: the normalized unscaled standard error and relative log expression. Arrays were used for transcriptome analysis based on clearance of the following quality control parameters: arrays were reasonably centered around the median normalized unscaled standard error = 1, and boxplots for relative log expression had a small spread and were centered at relative log expression = 0 (Heber and Sick, 2006; Brettschneider et al., 2008).

The Bovine Gene ST 1.1 array probes were redefined using current genome definitions available from the NCBI database (Bos Taurus NCBI Build 6.1, https://www.ncbi.nlm.nih.gov/assembly/GCF_000003205.5/; custom CDF version 18, <http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/18.0.0/ense.asp>) to represent 18,811 unique genes (Gene Expression Omnibus accession number GSE121715). Normalized gene expression estimates were determined using the raw intensity values and applying the robust multiarray analysis preprocessing algorithm (Irizarry et al., 2003).

Data Analysis

Statistical Analysis. In brief, a linear model was applied to identify differentially expressed genes (**DEG**) after using a moderated *t*-test that incorporated a Bayesian model (Smyth, 2004; Sartor et al., 2006) to moderate standard error across genes. Gene fold change (**FC**) was considered different when $P < 0.05$.

Analysis of Functional Implications. The mammary gland data set (18,811 gene IDs and their respective FC and *P*-values) was uploaded into the Ingenuity Pathway Analysis (**IPA**) software (Qiagen Bioinformatics, Redwood City, CA), and a core analysis was performed using default settings. The amount of DEG available for analysis with IPA was tested using different levels of significance ($P < 0.10$, 3,043 DEG; $P < 0.05$, 1,931 DEG; $P < 0.01$, 587 DEG; $P < 0.001$, 99 DEG), and the threshold $P < 0.05$ was determined to be appropriate for the analysis. The transcriptomic analysis, as indicated by IPA program function (i.e., downstream effects analysis and upstream regulator analysis), was filtered by the relevance of the predictions to the mammary tissue. For downstream effects

analysis, any predictions regarding physiology relating to other organ systems were excluded, as were those relating to metastasis and neoplasia within the mammary gland. Only direct relationships were considered when evaluating potential upstream regulators. Molecular and cellular function activity and upstream regulator activation state were determined by IPA, accounting for the sum (Σ_i) of the weighted contribution (w_i) of known up- or downregulated genes (x_i) to calculate the Z-score (z) statistic (formula shown below) for each specific function or upstream regulator (x) by its associated variance (σ_x):

$$z = \frac{x}{\sigma_x} = \frac{\Sigma_i w_i x_i}{\sqrt{\Sigma_i w_i^2}}.$$

Predictions were considered significantly increased or decreased (cellular and molecular functions) and activated or inhibited (upstream regulators) when $Z \geq 2$ or ≤ -2 , respectively.

The regulator effects analysis was used to explore how the predicted activated and inhibited upstream regulators affected downstream DEG and subfunctions. The upstream regulators and subfunctions and their predicted states were selected to create a customized network to directly link upstream regulator states with affected functions in the cellular context of the mammary gland. Data visualization was created using the Pathway Designer tool in the IPA program.

RESULTS

Molecular and Cellular Functions Relevant to DEG

Eighteen cellular and molecular functions met the threshold [$P < 0.05$ or $-\log(P\text{-value}) > 1.3$] to be considered relevant to the 1,931 DEG within the mammary gland by feeding ELE versus RES during the preweaning period. Of these 18 functions, cell death and survival, cellular movement, cellular development, cellular growth and proliferation, and lipid metabolism were the most enhanced ($P \leq 3.46\text{E-}16$; Figure 1) and free radical scavenging, cellular compromise, cell cycle, DNA replication, recombination and repair, and protein synthesis were the least enhanced ($0.05 > P \geq 3.21\text{E-}10$; Figure 1).

Predicted Activity of Cellular Functions Within the Mammary Gland

Downstream effects analysis was used to predict which biological processes were increased or decreased based on the directional expression of the involved

DEG. The analysis revealed that heifers consuming ELE rations would have an increased rate of epithelial-mesenchymal transition occurring in the mammary gland ($Z = 2.685$; Table 2) based on the expression of 61 genes. Thirty-two genes (*AHNAK*, *AKT2*, *CDH11*, *CLIC4*, *EFEMP1*, *EGFR*, *EIF3E*, *EIF4E*, *ESR2*, *FFAR4*, *FOXA1*, *FOXO3*, *GLIS2*, *GRHL2*, *HNRNPAB*, *LEP*, *MALAT1*, *NDRG1*, *NRP1*, *PCBP1*, *PLAU*, *POSTN*, *PRKAA2*, *PTPN6*, *SIM2*, *SNAI2*, *STMN1*, *TGFBR1*, *TNF*, *WISP1*, *WWC1*, and *ZEB1*) had a measurement direction consistent with an increase in this function, 15 genes (*AGT*, *CDH1*, *CDKN1B*, *CTNNB1*, *CUL7*, *CXCL9*, *DACT3*, *GH1*, *MAP3K3*, *NAMPT*, *S100A4*, *SKIL*, *SP1*, *SPARCL1*, and *STAT5A*) had a measurement direction consistent with a decrease, and 14 genes (*EFNA1*, *EPB41L5*, *Epb41l5*, *ERBB3*, *GATA3*, *GSK3B*, *HIF1A*, *IDH1*, *IGF1R*, *IKBKB*, *MMP2*, *MSX2*, *TP53*, and *TRIP10*) were known to affect the function, but IPA findings are not consistent to predict whether they increase or decrease epithelial-mesenchymal transition (Figure 2). Accumulation of lipid in the mammary gland was also predicted to be increased ($Z = 2.322$) in ELE calves as a result of measurement direction of 42 genes (*ABCD1*, *ACACA*, *ACAT1*, *ACAT2*, *AGPAT2*, *ASAH1*, *ATP8B1*, *BCL2L1*, *CCL21*, *EDN1*, *ENPP2*, *ERN1*, *FASN*, *GGT5*, *GPAM*, *GSK3B*, *HRASLS*, *IDH1*, *IL6*, *LPL*, *MGEA5*, *MID1IP1*, *MYOD1*, *NCOA2*, *NOS2_NPC2*, *PDK4*, *PIP5K1A*, *PLA2G4A*, *PLAU*, *PLIN2*, *PLTP*, *PPARD*, *PARKAA2*, *PRKAR2B*, *PSAP*, *SCD*, *SCP2*, *SOAT1*, *TNF*, *TP53*, and *YY1*) supporting increased lipid accumulation, whereas 23 genes (*AGT*, *APOE*, *BCL2*, *CEBPD*, *CSF2RB*, *ERCC1*, *GBA*, *HMOX1*, *IL2*, *KDM1A*, *LEP*, *MARCKS*, *MGLL*, *PEMT*, *PEX2*, *PRKD1*, *SMPD2*, *SREBF2*, *STAT5A*, *UGCG*, *VLDLR*, *WNT10B*, and *ZBTB7A*) had a measurement direction compatible with decreased function and 4 genes (*AHR*, *IGHG1*, *LMNA*, and *NR2C2*) were inconsistent by their directional FC with the IPA prediction (Figure 3). The synthesis of DNA, transactivation of RNA, expression of RNA, transcription, and transactivation were all predicted to have decreased activity ($Z < -2$) within the mammary gland of ELE heifers. For full details of the molecules included by IPA to predict the activity of these molecular and cellular subfunctions, refer to Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15699>).

Predicted Activity of Upstream Regulators Within the Mammary Gland

A total of 371 upstream regulators were predicted ($P\text{-value of overlap} < 0.05$) to directly regulate the expression of DEG. Of these, 275 were expressed in mam-

Table 2. Molecular and cellular functions that were predicted to be increased¹ or decreased² at 54 d after birth by the differentially expressed genes of heifers that were fed elevated quantities of nutrients (5.9 ± 0.2 Mcal of ME/d) compared with those consuming restricted amounts of nutrients (2.8 ± 0.2 Mcal of ME/d)

Category	Functional annotation	P-value	Activity Z-score	Involved genes ³
Cellular development	Epithelial-mesenchymal transition	1.96E-10	2.685	26, 35
Lipid metabolism	Accumulation of lipid	3.00E-08	2.322	40, 29
DNA replication, recombination, and repair	Synthesis of DNA	2.30E-08	−2.137	47, 38
Gene expression	Transactivation of RNA	3.36E-09	−2.254	50, 60
Gene expression	Expression of RNA	3.43E-11	−2.405	197, 209
Gene expression	Transcription	1.54E-12	−2.482	185, 208
Gene expression	Transactivation	9.94E-11	−2.611	54, 67

¹Predicted increases in biological function activity were based on a Z-score >2.

²Predicted decreases in biological function activity were based on a Z-score <−2.

³Values are upregulated genes, downregulated genes.

Figure 1. Molecular and cellular functions relevant to differentially expressed genes [threshold: $-\log(P\text{-value}) = 1.3$ or $P < 0.05$] for female calves that were fed elevated quantities of nutrients (5.9 ± 0.2 Mcal of ME/d) relative to those that consumed restricted amounts of nutrients (2.8 ± 0.2 Mcal of ME/d).

mary gland parenchyma. However, only 13 (Table 3) of these were predicted to be activated ($Z > 2$, $n = 6$) or inhibited ($Z < -2$, $n = 7$) based on the downstream directional change of their known target genes. Those predicted to be activated were peroxisome proliferator-activated receptor gamma (*PPARG*; $Z = 4.011$), sterol regulatory element-binding protein 1 (*SREBF1*; $Z =$

4.007), estrogen receptor 1 (*ESR1*; $Z = 3.072$), sterol regulatory element-binding protein 2 (*SREBF2*; $Z = 2.429$), Krüppel-like factor 15 (*KLF15*; $Z = 2.214$), and POU 5 class homeobox 1 (*POU5F1*; $Z = 2.181$), whereas those predicted to be inhibited were Parkinson protein 7 (*PARK7*; $Z = -2.176$), zinc finger and BTB domain containing 7B (*ZBTB7B*; $Z = -2.207$), Msh homeobox

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Figure 2. The differential expression and respective fold change of the genes involved in the epithelial-mesenchymal transition within the mammary gland of heifers provided elevated quantities of nutrients (5.9 ± 0.2 Mcal of ME/d) as opposed to restricted quantities of nutrients (2.8 ± 0.2 Mcal of ME/d). The difference in color (i.e., yellow, orange, or gray) corresponds to the known effect of the specific gene and its directional fold change on the process of the epithelial-mesenchymal transition. Yellow lines indicate a decrease in function, orange lines indicate an increase in function, and gray lines indicate direction fold changes that are inconsistent with Ingenuity Pathway Analysis software (Qiagen Bioinformatics, Redwood City, CA) predictions. The termination of the line with an arrow indicates a stimulatory effect, whereas the termination of the line with a cap indicates an inhibitory effect of the gene on the epithelial-mesenchymal transition.

2 (*MSX2*; $Z = -2.213$), mediator complex subunit 13 (*MED13*; $Z = -2.236$), activating transcription factor 3 (*ATF3*; $Z = -2.360$), myotrophin (*MTPN*; $Z = -2.366$), and JunD proto-oncogene (*JUND*; $Z = -2.415$). For full details of the affected downstream molecules and their directional change, refer to Supplemental Tables S2 and S3 (<https://doi.org/10.3168/jds.2018-15699>). Six up-

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Table 3. The effect of feeding elevated quantities of nutrients (5.9 ± 0.2 Mcal of ME/d) relative to restricted amounts of nutrients (2.8 ± 0.2 Mcal of ME/d) to female calves on the predicted activation status of upstream regulators by the differential expression of their target molecules at 54 d after birth

Upstream regulator	FC ¹	Class ²	State	Z-score	P-value ³	Target molecules ⁴
<i>PPARG</i>	1.450	LDNR	Activated	4.011	2.91E-09	52, 26
<i>SREBF1</i>	1.012	TR	Activated	4.007	5.67E-07	29, 18
<i>ESR1</i>	-1.326	LDNR	Activated	3.072	4.02E-12	92, 69
<i>SREBF2</i>	-1.364	TR	Activated	2.429	1.81E-04	13, 4
<i>KLF15</i>	1.058	TR	Activated	2.214	1.92E-03	8, 2
<i>POU5F1</i>	1.094	TR	Activated	2.181	4.87E-04	26, 21
<i>PARK7</i>	1.067	Enzyme	Inhibited	-2.176	5.25E-03	1, 4
<i>ZBTB7B</i>	-1.353	TR	Inhibited	-2.207	1.27E-03	3, 5
<i>MSX2</i>	-2.134	TR	Inhibited	-2.213	1.73E-01	1, 4
<i>MED13</i>	1.14	TR	Inhibited	-2.236	1.55E-02	7, 1
<i>ATF3</i>	-1.297	TR	Inhibited	-2.36	3.53E-01	5, 4
<i>MTPN</i>	1.054	TR	Inhibited	-2.366	3.74E-06	4, 14
<i>JUND</i>	-1.276	TR	Inhibited	-2.415	9.86E-06	6, 12

¹FC = fold change in expression.

²LDNR = ligand-dependent nuclear receptor; TR = transcription regulator.

³P-value of overlap.

⁴Values are upregulated genes, downregulated genes.

increases in epithelial-mesenchymal transition and lipid accumulation and decreases in synthesis of DNA, trans-activation of RNA, and expression of RNA (Figure 4).

DISCUSSION

The objective of the current experiment was to analyze the change in mammary gland transcriptome expression profile from preweaned heifers fed an elevated nutrient supply relative to those consuming a restricted nutrient supply and relate these changes to the physiology of the tissue—in particular, functions related to cellular proliferation and differentiation. A total of 1,931 genes out of 18,811, expressed in mammary parenchyma, were differentially expressed ($P < 0.05$). Therefore, the feeding of elevated quantities of ME induced a change in the mammary gland transcriptome of ELE relative to RES heifers to affect genes participating in several metabolic and developmental processes in the mammary gland. Although the majority of relevant functions within the mammary gland were related to cellular development, growth and proliferation, and differentiation, IPA did not directly predict cellular growth and proliferation to be an increased subfunction for ELE heifers. However, it is important to recognize that transcriptomic data only detail predictions to the physiological state of the tissue relative to the time of collection. Soberon and Van Amburgh (2017) reported that ELE heifers had increased mammary growth relative to their counterparts at 54 ± 2 d, as demonstrated by the drastic increase in parenchymal tissue (5.9 times greater mass; 6.48 vs. 1.10 g for ELE compared with RES calves). Soberon and Van Amburgh (2017) were unable to detect changes in putative stem cell proliferation in a localized manner

within the mammary parenchyma when 5-bromo-2'-deoxyuridine (**BrdU**) was administered 30 and 42 d before parenchyma sample collection. Similarly, Daniels et al. (2009) found no differences in BrdU-positive cells between heifers fed greater and lesser quantities of ME from milk preweaning. It is likely that the month between final BrdU administration and sample collection in both studies confounded the results to indicate that there was no difference in proliferation of putative stem cell population between treatments. Indeed, Meyer et al. (2006c) detected increases in cellular proliferation in 100-kg calves fed an elevated plane of energy (81.3 d of age; Meyer et al., 2006b) compared with their counterparts (113.2 d of age; Meyer et al., 2006b) when BrdU was administered at the time of euthanasia. As BW increased in the postweaned phase of growth, differences in cellular proliferation as indicated by BrdU index were no longer apparent (Meyer et al., 2006c). Yet, as Soberon and Van Amburgh (2017) reported at time of slaughter, BW was 61.0 and 83.2 kg for calves consuming 2.8 or 5.9 Mcal of ME/d, respectively; therefore, it is unlikely that calf BW influenced BrdU index within that study.

As with increased parenchymal mass, ELE heifers had 4.5 times greater adipose mass within the mammary gland compared with RES heifers (331.1 vs. 74.4 g, respectively; Soberon and Van Amburgh, 2017). The fat pad was removed from the parenchyma before sampling (Soberon and Van Amburgh, 2017), but there was likely bidirectional paracrine activity, as increased adiposity is known to occur when feeding greater quantities of energy (Meyer et al., 2006a,b,c; Geiger et al., 2016). Additionally, the development of the fat pad is necessary to provide energy and act as a scaffold for the

expansion of the epithelial tissue (Hovey et al., 1999; Meyer et al., 2006a; Thorn et al., 2008). At 15 mo of age the parenchymal cellular composition is reported as 10.1% epithelial cells, 51.1% connective tissue, 36.3% adipocytes, and 2.6% ductular lumen (Sejrsen et al., 1982), and at 20 mo of age the composition is relatively similar (12.3, 51.1, 33.1, and 2.9% epithelia, connective tissue, adipose, and ductular lumen, respectively; Sejrsen et al., 1982). Though a similar characterization of the cellular composition of the preweaning mammary gland is not detailed in the current experiment, one could expect based on IPA's prediction for increased lipid accumulation that the parenchyma of ELE heifers contained more adipocytes than their counterparts. Not unexpectedly, IPA included both leptin (*LEP*) and stearoyl-coenzyme A desaturase (*SCD*) within this function, as both *LEP* and *SCD* are known by their influence in regulating lipid metabolism (Baile et al., 2000; Reidy and Weber, 2000; Flowers and Ntambi, 2008) and appear to function in coordination to promote adiposity (Dobrzyn et al., 2008). Additionally, the ac-

tivation of the upstream regulators *PPARG*, *SREBF1*, and *SREBF2* and inhibition of the upstream regulator *MED13* may have mediated some of the increase in adipose content for ELE heifers, as they directly and indirectly relate to lipid metabolism. Peroxisome proliferator-activated receptor gamma is well studied for its relation to adipocyte differentiation (Tontonoz et al., 1994) and adipose accumulation within various tissues (Jain et al., 1998; Medina-Gomez et al., 2007). Specifically, within the mammary gland, *PPARG* has been previously implicated in mediating fat synthesis and secretion (Kadegowda et al., 2009; Mani et al., 2009; Bionaz et al., 2012; Shi et al., 2013) and, interestingly, as being necessary during gestational development of the mammary gland (Jain et al., 1998). However, to our knowledge, our study is the first to report predicted activation of *PPARG* as a regulator within the mammary transcriptome of preweaning heifers in response to nutrient supply. Similarly, *SREBF1* and *SREBF2* are both known by their involvement in lipid metabolism. Sterol regulatory element binding protein-1 has a role

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Figure 4. The relationship of upstream regulators *SREBF1*, *SREBF2*, *KLF15*, *MED13*, *JUND*, and *PARK7* to the increased epithelial-mesenchymal transition and accumulation of lipid functions and decreased synthesis of DNA, transactivation of RNA, and expression of RNA as interpreted by Ingenuity Pathway Analysis software (Qiagen Bioinformatics, Redwood City, CA).

in fatty acid synthesis (Rudolph et al., 2010; Suburu et al., 2014; Xu et al., 2016) and triglyceride accumulation (Xu et al., 2016) within the lactating mammary gland, whereas the function of *SREBF2* is less characterized within the mammary gland but generally understood to have a role in overall cholesterol metabolism (Kim et al., 1998; Mani et al., 2009). Activation of *MED13* promotes systemic lipolysis and protects against diet-induced obesity (Carrer et al., 2012; Yang et al., 2015); therefore, its predicted inhibition within the current study would indicate an antilipolytic state. Although these findings do not derive from mammary tissue of preweaned calves, it is likely that their stimulatory action on lipogenesis and triacylglycerol accumulation within the prepubertal mammary gland is similar to that of the pubertal and lactating gland.

Synthesis of DNA, transactivation of RNA, expression of RNA, transactivation, and transcription were all predicted to be decreased (indicated by IPA to be potentially mediated by the upstream regulators *JUND* and *PARK7*) for the ELE calves. Yet, these predictions refer to rates rather than the total accumulation of DNA and RNA and therefore are not reflective of the total quantity of DNA contained within the tissue. Soberon and Van Amburgh (2017) noted that, given the total parenchymal mass, ELE heifers were expected to have total greater DNA content within the mammary gland compared with RES heifers. In support, results reported by Geiger et al. (2016) agree that greater consumption of energy during the preweaning period yields greater total DNA content within the mammary gland. However, IPA's prediction is relative to the growth rate of the tissue at that time, and the rate of parenchymal area accretion before weaning is demonstrated to accelerate with increasing age (Esselburn et al., 2015; Furini et al., 2018) and respond to nutrient intake (Furini et al., 2018). At present, research has not characterized how the rate of parenchyma growth changes over the entire early life developmental phase. However, if mammary parenchyma growth occurs in a sigmoidal pattern (Berg and Butterfield, 1976; Batt, 1980; Richardson et al., 1991; Bollwein et al., 2017) over the course of development from birth to postpuberty, then it is possible that increased nutrient supply can alter the rate of development and composition of gain. As such, ELE heifers might be expected to reach a greater rate of parenchymal growth earlier than RES heifers (Meyer et al., 2006b,c) but of different cellular composition. Increased energy provision could cause parenchymal adipocyte hypertrophy, to result in greater total mass, but would reduce the density and rate of accumulation of genetic material present in the parenchyma. The rates of growth of mammary tissue between the 2 treatments would differ at 54 d of age, and one could expect

the RES heifer rate of lean tissue accretion, as reflected by rate of DNA synthesis and RNA transactivation and expression, to be greater than that of the ELE calves. This explanation is speculative in nature because we do not have supporting data on parenchymal growth rate or the composition of parenchyma gain during the 2 mo after birth before slaughter. However, it highlights the challenge in assessing and quantifying tissue and organ growth and development from measurements taken at one time period. Thus, it is still unknown how altering nutrient supply (e.g., that in the current study) will influence the rate of parenchyma accumulation and composition of gain at different time points over the course of the preweaning phase.

It is likely that the degree of differentiation of the parenchymal tissue was greater for ELE heifers as evident by the predicted increase in epithelial-mesenchymal transition. Epithelial-mesenchymal transition has been extensively investigated in its relationship to metastatic cancer growth (Micalizzi et al., 2010); within the developing mammary gland of pubertal mammals, it is implicated as an essential process for branching and elongation of the parenchymal ductal system (Ewald et al., 2008; Andrew and Ewald, 2010). Clusters of epithelial cells are afforded phenotypic plasticity and can transition to their mesenchymal state (Rørth, 2009), in which they are readily capable of cellular migration to develop the morphogenic features of the ductal network (Ewald et al., 2008; Andrew and Ewald, 2010). Comparatively, the postnatal ruminant mammary gland is more extensively studied than that of the rodent species (Hovey et al., 2002), but immediately after birth little ductal development is noted (Mayer and Klein, 1961; Sheffield, 1988; Meyer et al., 2006b,c). However, during the prepubertal phase, more ductal development as observed by the complexity of branching exists within the ruminant mammary gland relative to the rodent (Hovey et al., 2002). The degree of ductal branching and elongation can be affected by nutrition and endocrine signals, as both Capuco et al. (2002) and Geiger et al. (2016) described that extensive ductal branching and elongation had occurred within the mammary glands of 3- and 2-mo-old heifers in response to increased energy provision or exogenous estrogen, respectively. Thus, similar to pubertal development (Rowson et al., 2012), there exists a preweaning phase when the mammary gland undergoes ductal morphogenic alterations and maturation and the epithelial-mesenchymal transition likely modulates part of the branching and elongation of the ductal system that is likely responsive to energy provision.

Upstream regulator analysis identified 13 upstream regulators as being either activated or inhibited by preweaning ELE feeding. The relevance of some of these

regulators has been previously discussed (i.e., *PPARG*, *SREBF1*, *SREBF2*, and *MED13*), yet most of the remaining regulators can be implicated in the development of the mammary gland as well. The function of certain molecules, such as *ESR1*, is clearly defined in relation to mammary gland development. Estrogen is well known to promote mammary development in prepubescent and pubescent heifers (Woodward et al., 1993; Berry et al., 2003; Geiger et al., 2016) as it is necessary to sustain cellular proliferation during ductal elongation (Paine and Lewis, 2017). Upstream regulator analysis predicted that its function was activated, which may suggest that feeding ELE potentiated its responsiveness to estrogen or its physiological effects. Compared with *ESR1*, *POU5F1* is not as well studied within the mammary gland; however, its known action is to afford epithelial cells pluripotency and, in conjunction with Nanog and Cripto-1, regulate cellular commitment and differentiation of embryonic stem cells (Strizzi et al., 2008). Parkinson disease protein 7, part of the metastatic tumor antigen family of proteins, exhibits a different effect compared with *ESR1* and *POU5F1* when its expression is upregulated, as ductal morphogenesis will be inhibited by Wnt4 signaling (Sen et al., 2014). Therefore, the predicted inhibition of *PARK7* aligns with the functional development of the mammary gland of ELE heifers at 54 d of age. However, the role of the remaining upstream regulators within the mammary gland (i.e., *ZBTB7B*, *MSX2*, *ATF3*, and *MTPN*) is less clear either due to a lack of available literature or because their known function is contradictory to their predicted activity. For example, *MSX2* is expressed in the epithelial cells and mesenchyme of the mammary gland (Hens and Wysolmerski, 2005) and is found to be involved in branching morphogenesis (Sato et al., 2004), yet within the current study *MSX2* is downregulated in ELE and its activity is predicted to be inhibited in the mammary gland. It could be that, within the prepubertal mammary gland, the function of certain molecules, such as *MSX2*, differs relative to their function in the maturing and mature gland. For this reason, transcriptome analysis is a useful tool to identify molecules that may have physiological relevance to the tissue-specific points of development.

CONCLUSIONS

The preweaning mammary gland allometric growth phase has been demonstrated as nutritionally responsive by the FC variation in the transcriptome of ELE and RES heifers. The results suggest that restricted preweaning nutrient supply may hinder the development of the ductal network of the parenchyma by reducing the rate of epithelial-mesenchymal transition. Feeding

ELE may have altered the growth rate and composition of growth of the mammary gland by 2 mo of age and allowed for greater physiological development relative to the RES heifers.

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