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Gene expression in bovine rumen epithelium during weaning identifies molecular regulators of rumen development and growth

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Abstract During weaning, epithelial cell function in the rumen transitions in response to conversion from a pre-ruminant to a true ruminant environment to ensure efficient nutrient absorption and metabolism. To identify gene networks affected by weaning in bovine rumen, Holstein bull calves were fed commercial milk replacer only (MRO) until 42 days of age, then were provided diets of either milk + orchardgrass hay (MH) or milk + grain-based calf starter (MG). Rumen epithelial RNA was extracted from calves sacrificed at four time points: day 14 ($n=3$) and day 42 ($n=3$) of age while fed the MRO diet and day 56 ($n=3$/diet) and day 70 ($n=3$/diet) while fed the MH and MG diets for transcript profiling by microarray hybridization. Five two-group comparisons were made using Permutation Analysis of Differential Expression® to identify differentially expressed genes over time and developmental stage between days 14 and 42 within the MRO diet, between day 42 on the MRO diet and day 56 on the MG or MH diets, and between the MG and MH diets at days 56 and 70. Ingenuity Pathway Analysis (IPA) of differentially expressed genes during weaning indicated the top 5 gene networks involving molecules participating in lipid metabolism, cell morphology and death, cellular growth and proliferation, molecular transport, and the cell cycle. Putative genes functioning in the establishment of the rumen microbial population and associated rumen epithelial inflammation during weaning were identified. Activation of transcription factor PPAR-α was identified by IPA software as an important regulator of molecular changes in rumen epithelium that function in papillary development and fatty acid oxidation during the transition from pre-rumination to rumination. Thus, molecular markers of rumen development and gene networks regulating differentiation and growth of rumen epithelium were identified for selecting targets and methods for improving and assessing rumen development and function, particularly in the growing calf.

Keywords Cattle · Growth · Microarray · Rumen · Weaning

Introduction

The rumen is a critical organ mediating nutrient uptake and use in cattle. In calves, efficient nutrient delivery, absorption, and metabolism depend upon the transition of the rumen during weaning from a pre-ruminant to a ruminant state. In the mature animal, the rumen epithelium is also responsible for several physiologically important functions, including absorption, transport, volatile fatty acid (VFA) metabolism, and protection. Unlike many organs, which comprise a smaller proportion of empty body weight (EBW) as the animal matures, the rumen increases from 30 to 70 % of the capacity of the gut during weaning (Warner et al. 1956) and increases as a percentage of EBW throughout lactation (Baldwin et al. 2004). Moreover, given the high metabolic activity of intestinal tissues in general, the rumen epithelium is central to net efficiency of feed conversion in cattle during multiple stages of production.

In order to create nutritional management strategies that improve nutrient use efficiency in cattle, knowledge and understanding of regulatory control points governing differentiation,

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development, and growth of the rumen are needed. Previous studies in growing calves have demonstrated differential morphological development of the rumen mucosa in response to variation in VFA produced during ruminal fermentation, especially butyrate, and to a lesser extent propionate and acetate (Flatt et al. 1958; Sander et al. 1959; Sakata and Tamate 1978, 1979; Mentschel et al. 2001). High-concentrate diets induce significant increases in the density and length of rumen papillae (Stobo et al. 1966), increasing the absorptive surface area of the rumen epithelium (Žitnan et al. 1998) relative to roughage-based diets, which primarily increase rumen size (volume displacement) (Warner et al. 1956; Stobo et al. 1966). Similarly, increased concentrate supplied to neonatal calves increases surface area of rumen papillae (Žitnan et al. 2006), whereas a diet of only milk results in little to no papillary development (Warner et al. 1956; Wilson 1962). Despite knowledge of differential VFA effects on the development of the rumen mucosa, little is known about the molecular mechanisms regulating these morphological changes. The goals of this study were to identify genes differentially expressed between the pre-ruminant and ruminant stages of differentiation induced by diet and to assess diets differing in their potential to produce VFA during establishment of a viable ruminal fermentation (i.e., diets known to enhance rumen growth [hay-based] and varied in their ablity to promote rumen papillary development [grain-based]). Identification of gene networks contributing to rumen growth and development targeted the mechanisms regulating these processes to improve weaning strategies for young calves, as well as manage dietary changes on rumen growth and papillary development in the adult cow.

Materials and methods

All procedures involving animals were approved by the Beltsville Area Animal Care and Use Committee Protocol Number 07-025.

Animals and tissue collection

Eighteen Holstein bull calves (89 ± 14 [SD] kg BW) were used for the study. All calves were purchased from a local dairy farm in New Windsor, MD, USA and transported to the Beltsville Agricultural Research Center (BARC), Beltsville, MD, USA by 3 days of age (1.3 ± 0.7 [SD] days of age at arrival). All calves received colostrum at the first feeding. All calves tested negative for bovine viral diarrhea and received standard vaccinations. Health of the source herd was assessed by a negative PCR test for mycoplasma using a milk sample collected from the source herd bulk tank.

Upon arrival at BARC, calves were weighed and subsequently weighed every 7 days thereafter until sacrifice. Calves were housed individually in concrete pens (7.3 m^2)

within a single environmentally controlled building. Each pen had a rubber mat as a bedding surface but no bedding material was used, which could confound dietary treatments if consumed by the calves. Calves were assigned randomly to one of three dietary treatments (six calves/treatment): milk replacer only (MRO), MRO+grain-based commercial calf starter (MG; Bel Air Calf Starter 22 %, Bel Air, MD, USA; 22.0 % crude protein, 4.0 % crude fat; 5.5 % crude fiber), or MRO+orchardgrass hay (MH; fed free choice). All calves were provided continuous access to water and fed twice daily (1.9 L per feeding for a total of 3.8 L/day as recommended by the manufacturer) with only commercial milk replacer (Cornerstone 22:20, Purina Mills, St. Louis, MO, USA; 22.0 % crude protein, 20.0 % crude fat, 0.15 % crude fiber, 0.75 to 1.25 % Ca, 0.70 % P, 66,000 IU/kg vitamin A, 11,000 IU/kg vitamin D$_3$, and 220 IU/kg vitamin E) until 42 days of age (bottle fed and transitioned to bucket feeding by 6 days of age) at which time they were transitioned to either MH or MG diets. Milk replacer (1.9 L per feeding) was still provided twice daily in addition to hay or calf starter. Consumption of hay was not quantified but was monitored by animal caretakers to ensure calves were consuming dry feed and that ad libitum access was maintained. Consumption of calf starter was monitored on an as-fed basis and recorded daily. Calves were initially provided 0.23 to 0.45 kg of calf starter daily, depending on their body weight. Once calves consumed all grain provided for three consecutive days, the amount given was increased by 0.23 kg, according to standard weaning practices at our facility. Calves within each dietary treatment group were sacrificed by captive bolt followed by exsanguination at two time points: day 14 ($n=3$) and day 42 ($n=3$) within the MRO group to represent two stages of pre-ruminant development and day 56 and day 70 within the MH and MG groups to represent development at two stages of weaning on either a grain- (concentrate) or hay-based (forage) diet. Previous research from our laboratory demonstrated that a sample size of three is sufficient to detect differences in gene expression related to organ development in dairy cattle (Connor et al. 2008). At sacrifice, rumen epithilial tissue was photographed and collected from the anterior portion of the ventral sac of the rumen beneath the reticulum and below the rumen fluid layer. Subsamples (approximately 2 cm×2 cm) were fixed in neutral buffered formalin for gross evaluation of mucosal development. In addition, the epithelial layer of the rumen tissue was manually separated from the muscular layer and rinsed in tap water to remove residual feed particles. Samples were further rinsed in ice-cold saline and subsamples of epithelial tissues (approximately 600 mg) were fixed in RNAlater (Life Technologies, Grand Island, NY, USA) RNA stabilization solution according to the manufacturer's instructions and stored at −80 °C until RNA extraction and hybridization to a custom bovine whole-genome array (USDA Bovine 60mer 344-k Array; Li et al. 2006).

RNA extraction and transcript profiling

Frozen tissues were homogenized in Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted using the RNeasy Midi Kit (Qiagen, Valencia, CA, USA) with on-column DNase digestion. Quality of RNA was assessed using a 2100 Bioanalyzer and RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA, USA). Mean (± SD) RIN value was 8.4±0.7. Concentration of RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Probe labeling, hybridizations of probes to the high-density oligonucleotide (60-mer) microarray, and array scanning were performed according to standard procedures (http://www.nimblegen.com) by the Roche NimbleGen Systems, Inc. Microarray Core Facility in Reykjavik, Iceland. All samples met the NimbleGen Core Facility's quality control requirements for cDNA synthesis yield and quality.

Raw estimated fold changes for each gene transcript based on microarray hybridization were calculated as the simple ratio of the geometric means of the signal values for each dietary group being compared. Five separate two-group comparisons of differential gene expression were made including day 14 versus day 42 within the MRO group, MRO at day 42 versus MH at day 56 or MG at day 56, and MH group versus MG group at day 56 and day 70. Differential expression was evaluated by Expression Analysis, Inc. (Durham, NC, USA) using a robust implementation of permutation testing called Permutation Analysis of Differential Expression® or PADE, as described in greater detail on the Expression Analysis website (http://www.expressionanalysis.com/images/uploads/tech_notes/Tech_note_Illumina_Two-Group_Comparison_v5.pdf). Briefly, a modified t-statistic (Di) was calculated for each transcript when comparing groups, and a difference (Δ) was computed between Di and the average or expected t-statistic ordered values from a reference distribution (D [i]) calculated by computing all possible random permutations of our samples. Lists of differentially expressed transcripts for each two-group comparison were then created by first selecting any transcript with a suitable difference associated with a small false discovery rate (<0.05) and further narrowed by selecting for transcripts with an estimated absolute raw fold change ≥1.5. (Connor et al. 2008). Identity of target transcripts was determined by aligning the oligonucleotide sequences used on the array to the bovine genome (UMD 3.1; ENSEMBL Genebuild v65.0) using TopHat (Trapnell et al. 2009) with a cutoff of two mismatches tolerated per 60 bp of input sequence. For the purpose of this report, detailed gene pathway analysis and discussion of findings is limited to genes commonly affected by a transition from a milk-based diet to a diet supplemented with grain or hay (i.e., overlapping genes differentially expressed in the same

direction in both the MG and MH groups at day 56 relative to the MRO group at day 42). Grain-specific and hay-specific changes in gene expression during weaning will be reported separately.

Validation of differential gene expression by quantitative polymerase chain reaction

Twenty-four genes (Table 1) were selected for validation by quantitative polymerase chain reaction (qPCR) using the iCycler iQ and SYBR green detection (Bio-Rad Laboratories, Hercules, CA, USA). Differentially expressed genes (both upregulated and downregulated) identified from each two-group comparison representing a range of fold changes were selected for validation. In addition, genes of interest identified from the IPA output (i.e., *ACAT1* and *HMGCS2* identified as downstream targets of PPAR-α) were selected for validation by qPCR. For each primer pair, amplification products were purified by agarose gel electrophoresis and extraction using the QIAquick Gel Extraction Kit (Qiagen). Identity of intended amplification products was verified by direct DNA sequencing using a CEQ 8000 Genetic Analysis System and GenomeLab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA). First-strand cDNA synthesis was performed using 500 ng of total RNA per 20-μL sample reaction and the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Reaction conditions were 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C. Negative control reactions in the absence of reverse transcriptase were performed on each sample to verify the absence of genomic DNA contamination of the RNA samples. Subsequent qPCR was performed at a minimum in duplicate using 2 μL of cDNA, 7.5 pmol of each primer, and 5 μL (or 12.5 μL) of iQ SYBR Green Supermix in a 10-μL (or 25-μL) reaction volume. Cycling conditions consisted of 95 °C for 3 min, 45 cycles of 94 °C for 15 s, annealing temperature (see Table 1) for 30 s, and 72 °C for 30 s, followed by a melting curve analysis. A standard curve (10^2 to 10^6 molecules) using gel-purified PCR amplicons of each gene target as template for qPCR was generated for each gene assay and used to interpolate absolute transcript copy numbers for each sample. A reaction blank using molecular grade water as template was included with each standard curve to verify absence of target contamination in PCR reagents. Efficiency of amplification for each assay and interpolation of sample concentrations from the standard curve were determined automatically by the iCycler software. Amplification efficiency of all assays ranged from 85.0 to 107.2 %, and linearity of standard curves was acceptable ($R^2 \geq 0.996$). Fold changes in gene expression between developmental stages were calculated as the ratio of absolute copy numbers between the two groups.

Table 1 Primer sequences and amplification conditions used for quantitative real-time polymerase chain reaction

Gene symbol	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (bp)	Annealing temperature (°C)
ACAT1	GCAGATGCAGCTAAGAGGCT	ATATGCAGGCGCAAGTGGAA	102	60.2
B4GALNT2	CTGTGGCCAAGCCACTAAAT	ATCCACTGACTCCCAAGGTG	113	63.0
BNIP3	CTGCGAGTCGTTTCTTCTGG	TCTGAAGAAGGTTACTACCGAATTA	149	63.0
BPIFA1	GCTTGGACGTCACTCTGGTA	AGCCTCAGGAAACGGTTCAG	126	62.6
C29H11orf67	GAAAAGGTGAGAGGTAAAGCAC	GAAGCAATTTCAGGGGATGA	101	63.0
CBLN2	GGGGAAGGGGACATTGTATC	AGGGGAAGAAAAACCCACAC	119	63.0
DMBT1	GCTCGGACTCAAGCTACCAG	GGTGTCGTTATCCACCTGCT	107	63.0
ERRFI1	GACACGGACTTCCTTCTGGA	GGTTCCTGGCTGAGATCCAC	146	63.0
FAIM	ACTCTGGATGTGTGGTGCAA	TAATCCCTTCTTTCCGCTTG	142	63.0
FUT1	ACAGCACACCAGTGGCATAA	TCACTTGTGCAAAGCCAGAC	124	63.0
HEY1	CGCCCTTGCTATGGACTATC	GCTGGGAGGCATAGTTGTTC	146	63.0
KCNQ1	TCGAGCAGTATGTCGCCCTGG	CACCACCACGATGAGGTCGATGATG	188	56.7
HIF3A	GGCCGGGGTCCCCAATCTGA	CCGGGGACCAGAGGTGTCAAGT	186	62.6
HMGCS2	ACTCCTCCGAAAATGCAGCG	ACCTCCATGGCAAGAATACCC	194	62.6
KRT4	AAGGTGCGGTTCTTAGAGCA	CTTCCTCAGGGCATTGATGT	132	63.0
KRT19	GCCCGCGACTACAGCCACTA	CCGTCTCAAACTTGGTGCGGA	145	56.7
LBH	GTGTTTCTGTGTCCCCGTCT	AAACCACCCCCATAAACACA	127	63.0
MIF	GTTCCCGGGCTGCCTTAGCG	CGCGATGTACTGTGCCGGCTT	186	62.6
O3FAR1	CCCGGCGCTGACCAGGAAAT	CCGAGTAGGCCAGGCTCATCGT	193	62.6
PTHLH	GCCGCCGACTCAAAAGGGCT	CGGCTGTGTGGATTTCCGCGA	120	62.6
SLC46A2	CGAAAGAACTGCCAACTGTG	CTTCAAATGCCTGAGCCCTA	139	63.0
SPOCK1	GTCTCCCTTGCCAGAATGAA	GGCACTGTGTGGCTTTGTAA	117	63.0
TMEM40	TCAGGAAAAGCCATGGAGAC	TCCTTGAGAAACGAGTCTGG	132	63.0
TRIM40	CCACGGGAGAAACTGAGAATG	AGACACAACTCAGCCCTCCAA	99	62.6

To validate microarray results, transcript abundances as measured by real-time qPCR of 24 genes selected from among the five-group comparisons described above were compared using *t* test. *P* values were determined based on a one-tailed test, as a specific direction in fold change was expected. Differential gene expression as detected by microarray hybridization was considered validated if the direction of the fold change was consistent between microarray and qPCR approaches, and differences in transcript abundance between the dietary groups as detected by qPCR had a *P* value<0.05.

Gene pathway and network analysis

Gene pathway and network analysis was performed using Ingenuity Pathway Anaysis (IPA) software (www.ingenuity. com; Ingenuity Systems, Inc., Redwood City, CA, USA). Differentially expressed genes identified by PADE that were commonly affected by a transition from a milk-based diet to a diet supplemented with grain or hay as described above and their corresponding raw estimated fold changes (cutoff of 1.5) were uploaded in the software application, and each gene was mapped to its corresponding object in the Ingenuity

Knowledge Base. These molecules were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base to form algorithmically generated networks based on their connectivity. The gene list was evaluated using the Ingenuity Knowledge Base (Genes Only) reference set and included direct and indirect relationships, as well as endogenous chemicals. A filter summary was used that considered only molecules or relationships where the species equaled mouse, rat, or human. To determine biological functions of networks, network molecules associated with biological functions in the Ingenuity Knowledge Base were analyzed using a right–tailed Fisher's exact test to calculate a *P* value for the probability that each biological function assigned to that network was due to chance alone. A score was assigned where the score equals the negative log of the *P* value. Identification of upstream transcription factors was assessed using IPA wherein the activation or inhibition of a transcription factor was determined from expression patterns of the transcription factor and its downstream genes present within the differentially expressed list using an algorithm. The absolute value of the *z*-score≥2.0 was considered statistically significant wherein a positive value indicates activation and a negative value indicates inhibition of the transcription factor.

## Results

Dietary effects on gross rumen papillary development

Calves in the MRO, MH, and MG groups gained an average (± SE) of 0.2 (±0.1), 0.4 (±0.0), and 0.4 (±0.1) kg/day of body weight, respectively. Figure 1 illustrates differences observed in gross rumen papillary development induced by the MRO diet at day 14 and day 42 of age and the MH and MG diets at day 56 and day 70 of age. The luminal surface of the rumen epithelium of calves fed the MRO exhibited a smooth appearance with no apparent papillary development at day 14 or day 42 (Fig. 1a and b, respectively), as expected. Under magnification, small protrusions of the rumen epithelium existed that were ≤0.5 mm in height. Feeding of the MG diet induced pigmentation and visible papillary development on the luminal surface of the rumen epithelium by day 56 (Fig. 1c). Under magnification, the papillae appeared to be in the range of 1.5 to 2.0 mm in height. Papillary development appeared more prominent (greater length and width of papillae) at day 70 of age (Fig. 1d) relative to day 56, with papillae of approximately 2.5 mm in height. Rumen epithelium of calves fed the MH diet exhibited very little papillary development at day 56 or day 70

of age (Fig. 1e and f, respectively) and no pigmentation. Estimates of papillae height were approximately 0.5 and 1.5 mm in the day 56 and day 70 samples, respectively. Overall, desired visible differences in rumen mucosal development and morphology were accomplished by feeding the MRO, MG, and MH diets for subsequent transcript profiling of rumen epithelium.

Differential gene expression by microarray analysis

The complete microarray dataset is available as accession number GSE18382 in the Gene Expression Omnibus repository at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). There were no differentially expressed genes detected in rumen epithelium between samples collected at day 14 versus day 42 within the MRO group due to high variability among samples. Expression of one gene, tripartite motif-containing protein 40 (TRIM40) was found to be 20.7-fold greater in the MH versus the MG groups at day 56. At day 70, four transcripts were expressed more highly in the MG group relative to the MH group. Only one of the four transcripts was annotated as BPI fold containing family A, member 1 (BPIFA1). Its

Fig. 1 Representative images of the luminal surface of the calf rumen at different stages of development and in response to different dietary treatments. **a** Day 14 of age fed milk replacer only (MRO); **b** day 42 of age fed MRO; **c** day 56 of age fed milk replacer through 42 days of age then supplemented with commercial calf starter (MG); **d** day 70 of age fed MG; **e** day 56 of age fed milk replacer through 42 days of age then supplemented with orchardgrass hay (MH); and **f** day 70 of age fed MH. Note that magnification of large images is not uniform across panels. On high-magnification panels, *scale bar to right* is in 1-mm increments
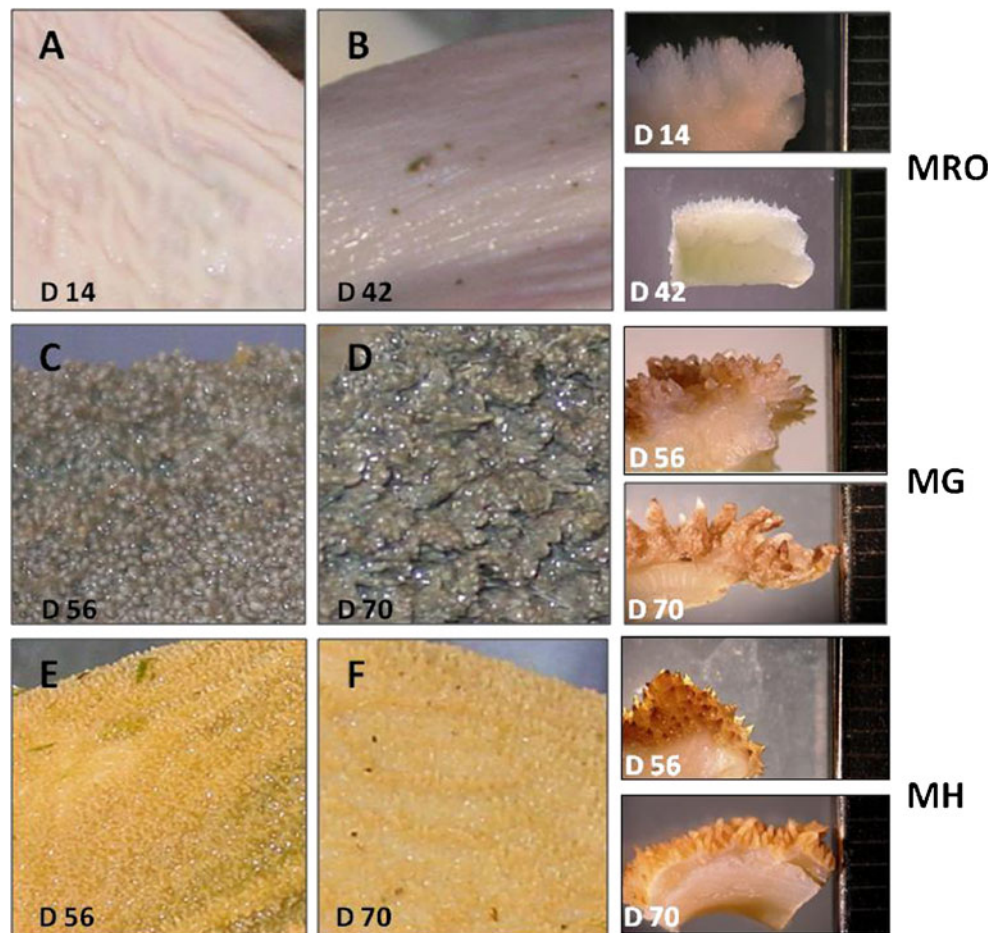
expression level was 39.6-fold greater in the MG group relative to the MH group at day 70.

There were 971 genes in which the transcription level was affected in the same direction by transition from the MRO diet at day 42 to both the MG or MH diets by day 56 of age (Supplementary Table 1). These genes are considered to be those generally influenced by weaning and age and comprised the dataset used for gene network and pathway analysis using IPA software.

Validation of differential gene expression by real-time quantitative polymerase chain reaction

Figure 2 illustrates the fold changes detected by microarray analysis and by absolute real-time qPCR for 24 genes of interest. In all 24 instances, the two methods were in agreement in that the direction of estimated fold changes was consistent between microarray and qPCR approaches. However, differences in gene expression between the dietary treatment groups or developmental stages as detected by real-time qPCR were not statistically significant for two genes (TMEM40 and PTHLH; $0.05 < P \leq 0.10$).

Gene pathway and network analysis

Analysis by the IPA software of the 971 genes differentially expressed between MRO at day 42 and MG or MH at day 56 identified 25 networks, all of which had a score ≥9. The complete list of networks and their component genes with direction of change are summarized in Supplementary Table 2. The top 5 major networks (score ≥30; each containing at least 27 directly interacting genes in the network) involved molecules participating in (1) lipid metabolism, (2) cell morphology and death, (3) cellular growth and proliferation, (4) molecular transport, and (5) the cell cycle. The top molecular and cellular functions associated with these genes were free radical scavenging (12 molecules; $P < 0.02$), molecular transport (96 molecules; $P < 0.04$), and lipid metabolism (86 molecules; $P < 0.04$). Among the 96 molecules participating in molecular transport, 20 were solute carrier family members, which are listed in Table 2 along with their functions. The transcription factor PPAR-α was identified by the IPA software as being activated (regulation z-score= 2.2; $P < 2.5 \times 10^{-6}$; Table 3) based on the expression pattern of these genes. The most significant biological/disease function associated with this group of 971 genes was gastrointestinal tract cancer (206 molecules; $P < 0.04$).

Discussion

In the present study, calves were fed diets to induce differential rumen growth and mucosal development for determination of

gene pathways associated with regulation of these processes with age and in response to weaning. As expected, obvious differences were observed at slaughter among the MRO, MH, and MG groups in the development of rumen papillae (MG>MH >>>MRO at the same ages). No differentially expressed genes between day 14 and day 42 of age within the MRO group were detected as the diet remained the same, viable fermentation presumably had not yet been established, and the only difference between the two groups was the age of calves by 28 days. Although ontogenic changes in gene expression likely occurred over this period, individual calf variation in gene expression in the MRO groups was large (based on principal components analysis; data not shown), prohibiting our ability to detect such differences.

On the contrary, two genes (TRIM40 and BPIFA1) were differentially expressed in rumen epithelium post weaning during hay versus grain feeding. First, TRIM40 mRNA was substantially upregulated in the MH group relative to the MG group at 56 days of age (14 days after introduction of solid feed). This gene is highly expressed in normal intestinal epithelium at both the mRNA and protein levels, its encoded protein is an inhibitor of NF-κB activity, and its mRNA expression is downregulated in gastrointestinal tumors and in Crohn's disease relative to normal tissues (Noguchi et al. 2011); thus, TRIM40 is thought to function in inhibition of cell growth and inflammation. Morphological differences observed between the MG and MH groups at day 56 indicate that TRIM40 may be an important factor regulating mucosal growth in the bovine rumen. In fact, both the MG and MH groups had increased TRIM40 mRNA expression at day 56 of age compared to the MRO group at day 42. Intestinal inflammation associated with dietary adaptation in ruminants is well documented and is proposed to be due to increased production of VFA and lipopolysaccharide by gram-negative bacteria in the rumen (Penner et al. 2011). We propose that solid feeding, particularly of hay, stimulates TRIM40 mRNA expression, ultimately protecting the epithelial mucosa from inflammation via inhibition of NF-κB activity by TRIM40 protein. Future studies examining the ontogeny of TRIM40 mRNA and protein expression in the developing calf rumen and in mature cattle during dietary transition are needed to assess its role in mediating the inflammatory responses in the bovine gut.

Second, BPIFA1 mRNA expression was greater in the MG group relative to the MH group at day 70 of age. This gene is in the BPI-like family of proteins, members of which appear to participate in innate immunity of the epithelial mucosa. Wheeler et al. (2007) proposed that duplication events in this gene family have played an evolutionary role in the establishment of ruminants. Further, the BPIFA1 protein may be important in regulating specific rumen microbial populations and improving the adhesion of fibrolytic
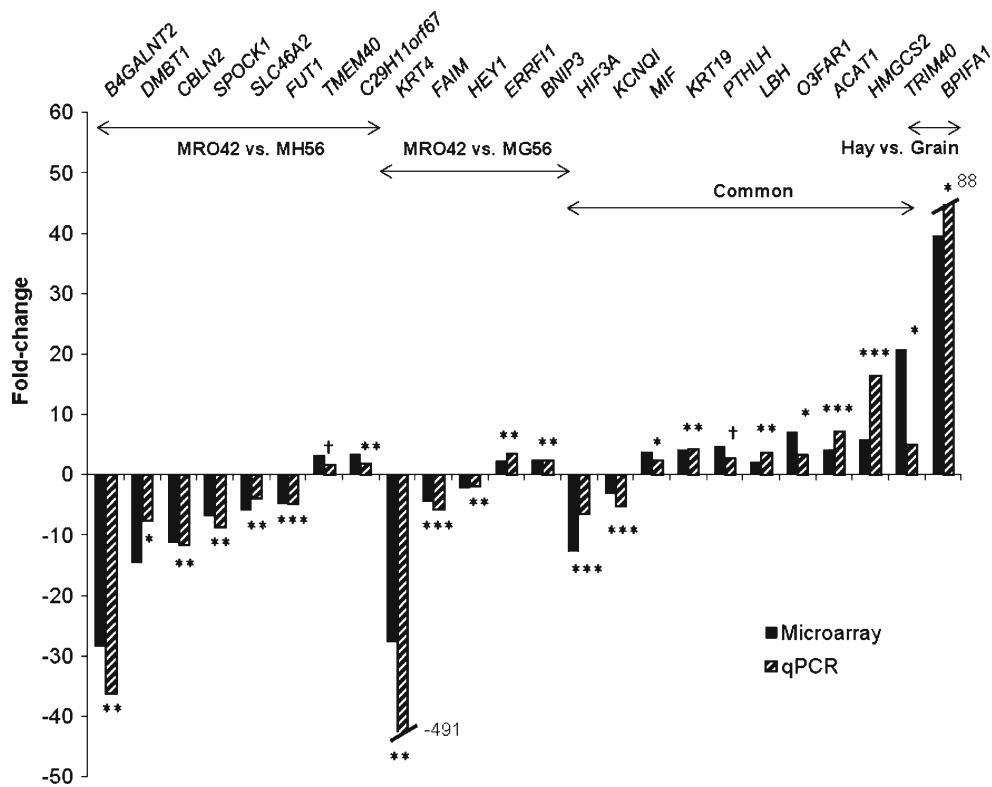
Fig. 2 Validation of differential gene expression in rumen epithelium of dairy calves as detected by microarray hybridization using quantitative real-time polymerase chain reaction (qPCR). Differences between mean transcript abundance between dietary groups detected by qPCR are indicated by $\dagger P \leq 0.10$, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. Differential expression ($P < 0.05$) between dietary groups for 22 out of 24 genes was confirmed by qPCR. Dietary group comparisons are indicated as follows: MRO42 vs. MH56=milk replacer only at day 42 of age versus milk supplemented with orchard grass hay at day 56 of age; MRO42 vs. MG56=MRO42 versus milk supplemented with commercial calf starter (grain) at day 56 of age; Common=genes differentially expressed in the same direction between MRO42 vs. MH56 and MRO42 vs. MG56; Hay vs. Grain=genes differentially expressed at either day 56 (*TRIM40*) or day 70 (*BPIFA1*) between calves fed milk supplemented with orchard grass hay versus commercial calf starter (grain)

bacteria to forages in the rumen (Wheeler et al. 2011). Given that microbial fermentation is being established during this period in MG calves due to the availability of concentrates in the diet, increased expression of *BPIFA1* mRNA relative to the MH group indicates that this gene may be critical to the health and physiology of the developing rumen during establishment of the rumen microbiome.

Although few differences were detected in gene expression of MH versus MG calves at days 56 and 70 of age, 971 gene transcripts were commonly up- or downregulated in rumen epithelium of calves 14 days after transition to solid feed. Ingenuity Pathway Analysis assigned these genes to networks functioning primarily in lipid metabolism, cell morphology and death, cellular growth and proliferation, molecular transport, and the cell cycle, as well as the overall disease function of gastrointestinal tract cancer. These top functions are consistent with knowledge that solid feed facilitates fermentaion, and thus, VFA production by rumen microbes and increased absorption of these VFA into circulation, stimulating rapid growth and proliferation of the rumen mucosa and development of the epithelial layer into

papillae of neonatal calves (Flatt et al. 1958; Sander et al. 1959; Sakata and Tamate 1978, 1979). Futhermore, upregulation of multiple pro- and anti-apoptotic genes during rumen mucosal development supports the concept by Mentschel et al. (2001) that butyrate inhibits pro-apoptotic gene expression during rumen development to facilitating papillary development, in addition to a molecular balance between proliferative and apoptotic events that promotes normal tissue and organ development (Elmore 2007).

It is interesting to note that within the network of cell proliferation and growth, the gene ERBB receptor feedback inhibitor 1 (*ERRFI1*) was found to be upregulated during the transition to solid feed. The protein encoded by this gene is one of the four epidermal growth factor receptor (EGFR)-inducible feedback inhibitors recently discovered in mammals that is critical for the regulation of EGFR signaling and other members of the ErbB family to control epithelial cell morphogenesis (Anastasi et al. 2003; Segatto et al. 2011). Epidermal growth factor (EGF) may play a role in rumen epithelial proliferation due to its high mRNA expression in ruminant salivary gland and its high protein concentration in

Table 2 Genes in calf rumen epithelium affected by weaning and age that encode solute carrier proteins and participate in molecular transport. Genes were differentially expressed in calf rumen epithelium during transition from a diet of milk replacer only to a diet of milk replacer supplemented with calf starter (grain) or hay feeding at 42 days of age. Gene expression was assessed at 56 days of age

Gene symbol	Gene name and description	Response
SLC12A2	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2	Down
SLC12A7	Solute carrier family 12 (potassium/chloride transporters), member 7	Up
SLC14A1	Solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	Up
SLC15A1	Solute carrier family 15 (oligopeptide transporter), member 1	Down
SLC16A1	Solute carrier family 16 (monocarboxylic acid transporter 1), member 1	Up
SLC19A3	Solute carrier family 19 (sodium/hydrogen exchanger), member 3	Up
SLC1A3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	Down
SLC25A24	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24	Up
SLC26A3	Solute carrier family 26 (chloride anion exchanger), member 3	Up
SLC26A11	Solute carrier family 26 (sodium-independent sulfate anion transporter), member 11	Up
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	Up
SLC2A8	Solute carrier family 2 (facilitated glucose transporter), member 8	Up
SLC2A11	Solute carrier family 2 (facilitated glucose transporter), member 11	Up
SLC31A1	Solute carrier family 31 (copper transporters), member 1	Up
SLC43A2	Solute carrier family 43 (L-type amino acid transporter), member 2	Down
SLC4A4	Solute carrier family 4 (sodium bicarbonate cotransporter), member 4	Down
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	Up
SLC6A14	Solute carrier family 6 (amino acid transporter), member 14	Down
SLC6A20	Solute carrier family 6 (proline IMINO transporter), member 20	Down
SLCO3A1	Solute carrier organic anion transporter family, member 3A1	Down

ruminant saliva (Onaga et al. 2006), as well as its stimulation of ruminal epithelial cell proliferation in vitro (Baldwin 1999). Observed increases in *ERRFI1* mRNA expression in the developing calf rumen support the hypothesis that ERRFI1 protein plays a role in the regulation of epithelial cell proliferation and morphogenesis by mediating the effects of EGF on its cognate receptor.

Changes in transporter gene expression of the rumen epithelium included 20 different solute carrier (SLC) family members in response to weaning, consistent with previously observed changes in expression of transporters functioning in VFA and ion transport in ruminants fed high-concentrate (Penner et al. 2011) or high-protein (Naeem et al. 2012) diets. Naeem et al. (2012) found that changes in transporter mRNA expression in rumen epithelium of calves fed conventional milk replacer and calf starter versus high-protein milk replacer and calf starter were due primarily to age of the calf rather than diet. Results of the present and earlier studies provide evidence of the critical role of the highly metabolically active rumen mucosa in nutrient absorption and maintenance of ionic balance in the developing calf rumen.

Finally, an interesting discovery was the identification of PPAR-α by IPA as an activated transcription factor regulating expression of genes responsive to weaning in this study. Expression of 32 downstream gene targets of PPAR-α changed during the transition from milk replacer to solid feed in developing calf rumen epithelium. The majority of these genes participate in beta-oxidation of lipids; however, other functions include apoptosis, organ development, nutrient metabolism, and the immune response. Of interest, Bünger et al. (2007) evaluated the functions of PPAR-α in the small intestine of mice and found indentical roles of PPAR-α in this tissue. Their study also showed that mRNA expression of *PPAR-α* itself was greatest in villus cells of the enterocytes relative to the crypt cells. The authors attributed this distinct distribution pattern to its critical role in the absorption of long-chain fatty acids by the intestinal villus and suggested that PPAR-α serves as a mechanism by which fatty acids can influence their own uptake and processing by enterocytes. To date, the distribution of PPAR-α protein or mRNA expression has not been evaluated within the rumen papillae to determine whether localized differences exist throughout the epithelial mucosa, or whether VFA in the rumen can interact with PPAR-α to influence their uptake and metabolism. It does not appear that expression of *PPAR-α* mRNA itself is affected by VFA concentration in bovine rumen (Steele et al. 2011), although it appears that VFA such as butyrate can serve as a PPAR ligand in the colon (Kinoshita et al. 2002). At this time, the specific factors controlling PPAR-α activation during weaning in bovine rumen are not clear, but warrant additional investigation. It should be noted, however, that the expression of two gene targets of PPAR-α, mitochondrial

Table 3 Genes regulated by transcription factor PPAR-α that were differentially expressed in calf rumen epithelium during transition from a diet of milk replacer only to a diet of milk replacer supplemented with calf starter (grain) or hay feeding at 42 days of age. Gene expression was assessed at 56 days of age. Nineteen of 32 genes have expression direction consistent with activation of PPAR-α (regulation z-score=2.17, $P<0.0001$)

Target molecules	Gene name	Response	GO process
ACAA1[a]	3-Ketoacyl-CoA thiolase, peroxisomal	Up	Fatty acid beta-oxidation
ACAA2[a]	3-Ketoacyl-CoA thiolase, mitochondrial	Up	Fatty acid beta-oxidation; inhibition of apoptosis
ACADS[a]	Short-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	Up	Fatty acid beta-oxidation
ACAT1[a]	Acetyl-CoA acetyltransferase, mitochondrial	Up	Organ/tissue development; nutrient metabolism
ACOX1[a]	Peroxisomal acyl-coenzyme A oxidase 1	Up	Fatty acid beta-oxidation
ACSL1	Long-chain fatty acid—CoA ligase 1	Down	Long-chain fatty acid metabolism
ACSS2[a]	Acetyl-coenzyme A synthetase, cytoplasmic	Up	Lipid biosynthesis
ASS1	Argininosuccinate synthase	Up	Organ development
AURKA	Aurora kinase A	Up	Mitosis
C6	Complement component C6 precursor	Up	Innate immune response; apoptosis
C6orf105[a]	Chromosome 6 open reading frame 105	Up	–
CAT[a]	Catalase	Up	Mitosis; inhibition of apoptosis
CROT[a]	Peroxisomal carnitine O-octanoyltransferase	Up	Fatty acid beta-oxidation
DECR1	2,4-Dienoyl-CoA reductase, mitochondrial	Up	Fatty acid beta-oxidation
ELOVL3	ELOVL fatty acid elongase 3	Up	Lipid biosynthesis
FABP4[a]	Fatty acid-binding protein, adipocyte	Up	Lipid metabolism
GPT	Glutamic-pyruvate transaminase (alanine aminotransferase)	Up	Gluconeogenesis; nitrogen compound metabolism/biosynthesis
H2AFZ	H2A histone family, member Z	Up	Nucleosome assembly
HADH[a]	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	Up	Fatty acid beta-oxidation
HMGCS2[a]	Hydroxymethylglutaryl-CoA synthase, mitochondrial	Up	Organ development
KIF4A	Kinesin family member 4A	Up	Organelle organization
ME2[a]	Malic enzyme 2, NAD(+)-dependent, mitochondrial	Up	Malate metabolism
MGST1[a]	Microsomal glutathione S-transferase 1	Up	Response to oxidation
MGST3[a]	Microsomal glutathione S-transferase 3	Up	Lipid metabolism
MLYCD[a]	Malonyl-CoA decarboxylase, mitochondrial	Up	Fatty acid beta-oxidation
PLIN2[a]	Perilipin-2	Up	Long-chain fatty acid transport
RBP2	Retinol-binding protein 2, cellular	Up	Retinoid metabolism
SCARB1	Scavenger receptor class B member 1	Down	Transport
SLC27A2[a]	Solute carrier family 27 (fatty acid transporter), member 2	Up	Fatty acid alpha-oxidation; fatty acid beta oxidation; long-chain fatty acid transport
TLR5[a]	Toll-like receptor 5	Up	Inflammatory response
TOP2A	Topoisomerase (DNA) II alpha 170 kDa	Up	Mitosis
TSPO	Translocator protein	Up	Induction of apoptosis

[a] Direction of gene expression was consistent with activation of PPAR-α as predicted by Ingenuity Pathway Analysis

acetyl-CoA acetyltransferase (*ACAT1*) and mitochondrial hydroxymethylglutaryl-CoA synthase (*HMGCS2*) that function in organ development, was upregulated during weaning. The products of these genes play a crucial role in ketogenesis by the rumen epithelium of sheep during development (Lane et al. 2002). Furthermore, *ACAT1* expression has been shown previously to be increased in rumen epithelium in heifers fed high- versus low-concentrate diets (Penner et al. 2011). Combined, these observations support the hypothesis that increased production of VFA in response to introducion of solid feed during weaning may promote ketogenesis in rumen epithelial cells via PPAR-α-mediated activation of ACAT1 and HMGCS2 to promote papillary development, as well as activation of genes promoting fatty acid beta-oxidation to support cellular differentiation. Overall, identified gene networks provide insight into the molecular basis of rumen development during transition from a pre-ruminant to a ruminant state and reflect ontogenic changes in rumen epithelium that cannot be separated from dietary effects. Further analysis is needed at the level of protein expression and activity to verify the proposed impacts of gene expression changes observed in the current study.

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