Comparative transcriptome analysis of rumen papillae in suckling and weaned Japanese Black calves using RNA sequencing

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ABSTRACT: The length and density of rumen papillae starts to increase during weaning and growth of ruminants. This significant development increases the intraruminal surface area and the efficiency of VFA (acetate, propionate, butyrate, etc.) uptake. Thus, it is important to investigate the factors controlling the growth and development of rumen papillae during weaning. This study aimed to compare the transcriptomes of rumen papillae in suckling and weaned calves. Total RNA was extracted from the rumen papillae of 10 male Japanese Black calves (5 suckling calves, 5 wk old; 5 weaned calves, 15 wk old) and used in RNA-sequencing. Transcript abundance was estimated and differentially expressed genes were identified and these data were then used in Ingenuity Pathway Analysis (**IPA**) to predict the major canonical pathways and upstream regulators. Among the 871 differentially expressed genes screened by IPA, 466 genes were upregulated and 405 were downregulated in the weaned group. Canonical pathway analysis showed that "atherosclerosis" was the most significant pathway, and "tretinoin," a derivative of vitamin A, was predicted as the most active upstream regulator during weaning. Analyses also predicted IgG, lipopolysaccharides, and tumor-necrosis factor-α as regulators of the microbe-epithelium interaction that activates rumen-related immune responses. The functional category and the up-regulators found in this study provide a valuable resource for studying new candidate genes related to the proliferation and development of rumen papillae from suckling to weaning Japanese Black calves.

Key words: atherosclerosis pathway, Japanese Black calves, RNA sequencing, rumen papillae, transcriptome, tretinoin

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INTRODUCTION

The rumen has physiologically essential functions such as nutrient uptake, transportation, and metabolism (Roh et al., 2007; Roh et al., 2016). Therefore, this organ is crucial for ruminant life, body maintenance, and growth (Warner et al., 1956; Tamate et al., 1962). A dramatic morphological development of rumen papillae occurs when feed is converted to roughage or concentrated from milk. Highly concentrated diets induce significant increases in rumen papillae density and length, as VFA (acetate, propionate, butyrate, among others) and lactate derive from intraruminal fermentation (Stobo et al., 1966). In neonatal Holstein calves, diets based on grain and on orchardgrass hay significantly increased the length and density of rumen papillae, whereas a milk-based diet had little effect on the development of these structures (Connor et al., 2013). Feeding VFA, particularly butyrate, significantly induced morphological development and promoted an increase in the length and density of the rumen epithelium (Sakata and Tamate, 1978; Shen et al., 2004; Gorka et al., 2011; Kato et al., 2011). Although many studies have been conducted on rumen development, only a few reported gene expression variations, regulatory factors, and gene networks in the rumen epithelium of calves fed different diets. Transcription factors, such as **CREBBP** (cAMP response element binding protein-binding protein) and **TTF2** (transcription termination factor 2), were found to control several regulatory networks in the rumen epithelium by butyrate infusion using RNA-sequencing (RNA-seq) and Gene Ontology (**GO**) (Baldwin et al., 2012). Connor et al. (2013) reported that lipid metabolism, cell morphology and death, cellular growth and proliferation, molecular transport, and the cell cycle were predicted by GO analysis using Ingenuity Pathway Analysis (**IPA**) on microarray data obtained from the rumen epithelium of Holstein calves fed on a milk replacer and solid feed. Using digital differential display (**DDD**), Kato et al. (2015) identified candidate genes related to rumen development in suckling and weaned Japanese Black cattle (Kato et al., 2015). The present study reports the first application of RNA-seq to comprehensively investigate the transcriptomes of rumen papillae in suckling and weaned Japanese Black calves, aiming to identify differentially expressed genes (**DEG**) related to the development of rumen papillae.

MATERIALS AND METHODS

This study was conducted in accordance with the "Guidelines for the National Agriculture and Food Research Organization (NARO) Institute of Livestock and Grassland Science" and was approved by the Animal Care Committee of the NARO Institute of Livestock and Grassland Science.

Animals

Ten Japanese Black male calves were used in the experiment. Information on sires, dams, natural dams, and calves used in this study is presented in Supplementary Tables 1 and 2. All calves were bred at the Grassland Management Research Division, NARO Institute of Livestock and Grassland Science. Feed and management practices were as described previously (Kato et al., 2015; Suzuki et al., 2016). Japanese Black male calves born from March 2013 to May 2014 were assigned sequentially to the suckling and weaned groups. Finally, 5 calves in each group were used for this experiment. All calves were raised by natural suckling, and although none were creep fed, all had free access to dams' feed. Calves in the suckling group were slaughtered at 5 wk of age, and their average body weight at slaughter was 51 ± 2 kg. Calves in the weaned group were separated from their dams at 12 wk of age and then raised by group feeding. All weaned calves were slaughtered at 15 to 16 wk of age, and their average body weight was 126 ± 8 kg. From 12 to 15–16 wk, weaned calves were only fed grower feed and timothy hay at 09:00 and 16:00 h to reach an average daily gain of about 0.6 kg, following the Japanese Feeding Standard for Beef Cattle (Agriculture, Forestry and Fisheries Research Council Secretariat, 2000). Grower feed components (CP 19.1% dry matter basis) were 39% grain (corn, milo, and wheat), 36% chaff and bran (bran, corn gluten meal, corn distiller's dried grains with solubles, and draff), 11% vegetable oil cake (soybean and rapeseed), and 14% others (alfalfa, alfalfa meal, molasses, and minerals) in raw matter basis. Calves were allowed ad libitum access to water and mineral blocks. The blood hormones and metabolites of suckling and weaned calves were reported in our previous study (Suzuki et al., 2016).

Tissue Collection

After slaughter, rumen papillae tissues (approximately 1 × 1 cm) were collected from the ventral cranial sac. Excised rumen papillae tissues of suckling and weaned calves are shown in Figure 1. The papillae layer was manually separated from the muscular layer using surgical scissors and rinsed with PBS to

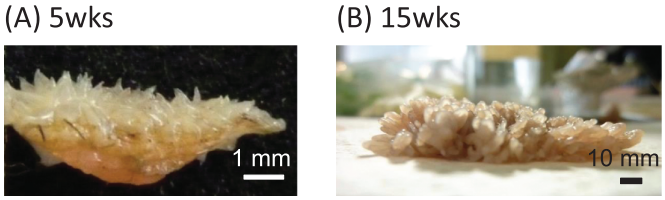

Figure 1. Rumen papillae in suckling (A) and weaned (B) calves of Japanese Black cattle. Calves in the suckling group were slaughtered at 5 wk of age. Weaned calves were separated from their dams at 12 wk of age, and, after weaning, they were raised by group feeding. All weaned calves were slaughtered at 15 to 16 wk of age. The bar represents the scale (mm).

remove residual feed particles. All tissue samples were frozen immediately in liquid nitrogen and stored at −80 °C until total RNA extraction was performed.

Total RNA Purification

Tissues were soaked in 200 µL RNAiso Plus (TAKARA Bio Inc., Shiga, Japan) in liquid nitrogen and homogenized using a Multibeads shocker (YASUIKIKAI Inc., Osaka, Japan), as indicated by the manufacturer. Homogenization was carried out twice at 2,000 rpm for 10 to 15 s, and 300 µL RNAiso was then added at 25 °C. The homogenate was collected in 1.5-mL tubes, 100-µL chloroform was added to the homogenate, and the solution was thoroughly mixed. After settling for 3 min at room temperature, the solution was centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant was collected. The supernatant was then mixed with 700 µL 70% ethanol. Total RNA was further purified and gDNA was removed using NucleoSpin RNA columns (MACHEREY-NAGEL, Düren, Germany), following the manufacturer's instructions. Purified total RNA was quantified in a NanoDrop ND-1000 Spectrophotometer V3.7.1 (THERMO FISHER SCIENTIFIC, Waltham, MA, USA) at wavelengths of 230, 260, and 280 nm. The purity of total RNA was determined as the A_{260}/A_{280} ratio, with expected values over 1.8, and was verified by running samples on 1.0% agarose gels. All samples were stored at −80 °C.

RNA-sequencing

The RNA Integrity Number (RIN) was confirmed in a Bioanalyzer 2100 using RNA nano kit (AGILENT, Palo Alto, CA, USA) to check if the purified total RNA could be used in RNA-seq. The average RIN in suckling and weaned groups was 7.1 (5.9 to 7.9) and 8.0 (7.5 to 8.4), respectively. Quality check of total RNA, library preparation, and RNA-seq were conducted at the NODAI Genome Research Center, Tokyo University of Agriculture. Single 50-bp reads were performed on a HiSeq 2500 platform (ILLUMINA, San Diego, CA, USA) using the TruSeq RNA Sample Preparation Kit v2 (ILLUMINA). Analysis of high-quality sequences was performed as in a previous report (Endo et al., 2013). Sequences were retained as high quality when passing the Illumina quality-filtering pipeline GERALD under default settings with the following conditions: only contain reads that passed filtering based on cluster intensities and noise estimates; demultiplexing; adapter masking; and ignore the first and last bases. These sequences were then aligned to the bovine reference genome (bosTau6) and to the exon–exon splice junction database downloaded from the University of California Santa Cruz (UCSC) sequence and annotation database (http://hgdownload.cse.ucsc.edu/downloads.html#cow). Alignment was performed in CLC Genomics Workbench (Qiagen, Valencia, CA, USA) using the defaults parameters. The EM estimation algorithm was used to iteratively estimate the abundance of transcripts and to assign reads to transcripts according to their abundances (Cappe and Moulines, 2009; Li and Dewey, 2011). Raw and normalized (i.e., Reads Per Kilobase of transcript per Million mapped reads [RPKM]) read counts (Mortazavi et al., 2008) were also calculated in CLC Genomics Workbench. After data normalization and fold change computation based on the estimated relative abundances of reads, the statistical significance of each pairwise comparison (suckling vs. weaned groups) was determined using the digital gene expression (**DGE**) empirical analysis tool incorporated in the EdgeR Bioconductor package (Robinson et al., 2010) and in the CLC Genomics Workbench. The DGE tool implements an "exact test" for two group comparisons, which is similar to Fisher's exact test. The test is applicable to count data only and is designed for the analyses of differentially expressed RNA-seq data. In addition to *P*-values, FDR-corrected *P*-values were also calculated (Benjamini et al., 2001). Differentially expressed genes (fold change ≥ 2 and FDR-corrected *P*-value < 0.05) were analyzed regarding their biological processes and functions, and assigned to canonical pathways using IPA with the default parameters in General Settings, Networks,

Data sources, Confidence, Species, Tissues & Cell Lines, and Mutation (Qiagen, www.qiagen.com/ingenuity). Fisher's exact test was used in the analysis of gene set enrichment in the functional categories.

RESULTS

The Sequencing Results and the Dataset of Analyzed Genes

Sequencing of total RNA from rumen papillae in two lanes yielded, on average, 1067.4 and 1011.8 Mb and 21,353,595 and 20,238,186 sequence reads for suckling and weaned groups, respectively. The numbers of raw reads and mapped reads per sample are shown in Supplementary Table 3. Sequence data from RNA-seq were deposited in Sequence Read Archive (**DRA**) of DNA Data Bank of Japan (**DDBJ**). The accession number is DRA005801. After sequence alignment, 82% to 86% of the reads within each sample were mapped to the reference genome (Supplementary Table 3). Gene expression levels were then compared between groups based on these data, using the CLC Genomic Workbench. Overall, 17,001 of the 24,616 genes in the UCSC annotation were expressed, and 871 of these genes were differentially expressed, with fold change ≥ 2 and FDR < 0.05. Compared with the suckling group, weaning calves presented 466 upregulated and 405 downregulated genes (Supplementary Table 4). However, it should be noted that 43 of the 871 DEGs showed low expression level (RPKM < 1), which might have affected the large fold change detected.

Canonical Pathway Analysis

The seven major canonical pathways ($P < 0.05$) of DEGs found through IPA are displayed in Figure 2. Seventeen DEGs were found in the top canonical pathway "Atherosclerosis Signaling" (Figure 3, Table 1).

Upstream Regulators

Based on the differentially increased or decreased expression of genes in the dataset, IPA revealed the upstream regulators that were activated or inhibited within each group. Tables 2 and 3 show the top 5 upstream regulators with $P < 0.05$ and z-scores, respectively. Tretinoin was predicted as the most significant regulator (Table 2); the P-value of 112 target molecules downstream of tretinoin also changed (Table 4). Seventy target molecules were upregulated during weaning, whereas 42 were downregulated (Table 4). Seven molecules were predicted by IPA as the most activated (z-score > 2.000), whereas "estrogen receptor" was inhibited (z-score < −2.000) (Table 3).

DISCUSSION

This study comprises the first application of RNA-seq to investigate the transcriptional changes and to predict the genes underlying the physiological and morphological modifications accompanying the transition from suckling to weaning in the rumen papillae of Japanese Black calves. In our previous study, *HMGCS2*, *AKR1C1*, and *FABP3* were identified as candidate up-regulated genes in

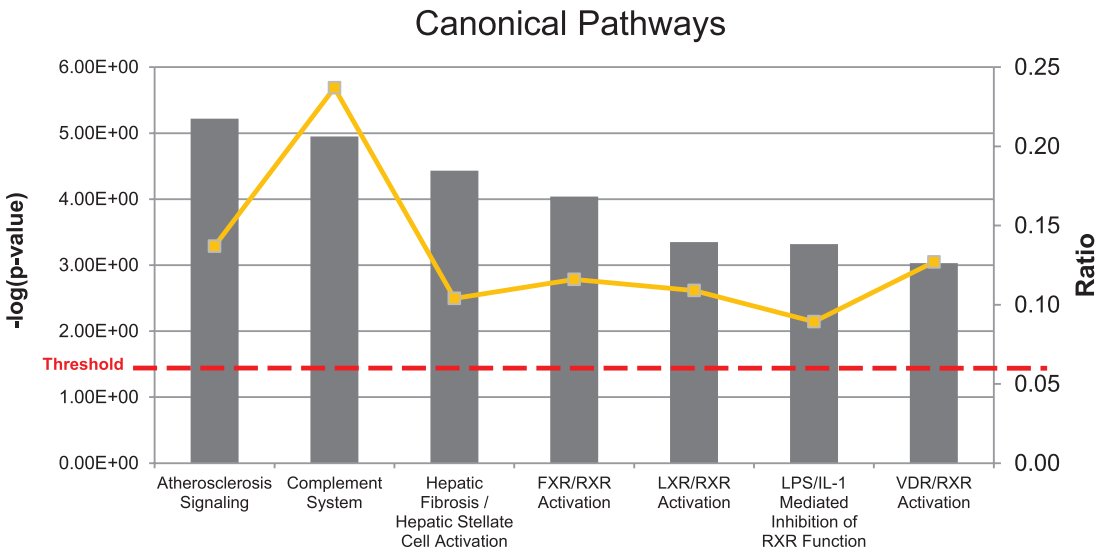

Figure 2. Canonical pathway analysis generated by Ingenuity Pathway Analysis (IPA). The P-values and ratios of the 7 major pathways in suckling and weaned groups are displayed. Pathways were considered as most important at $P < 0.05$.

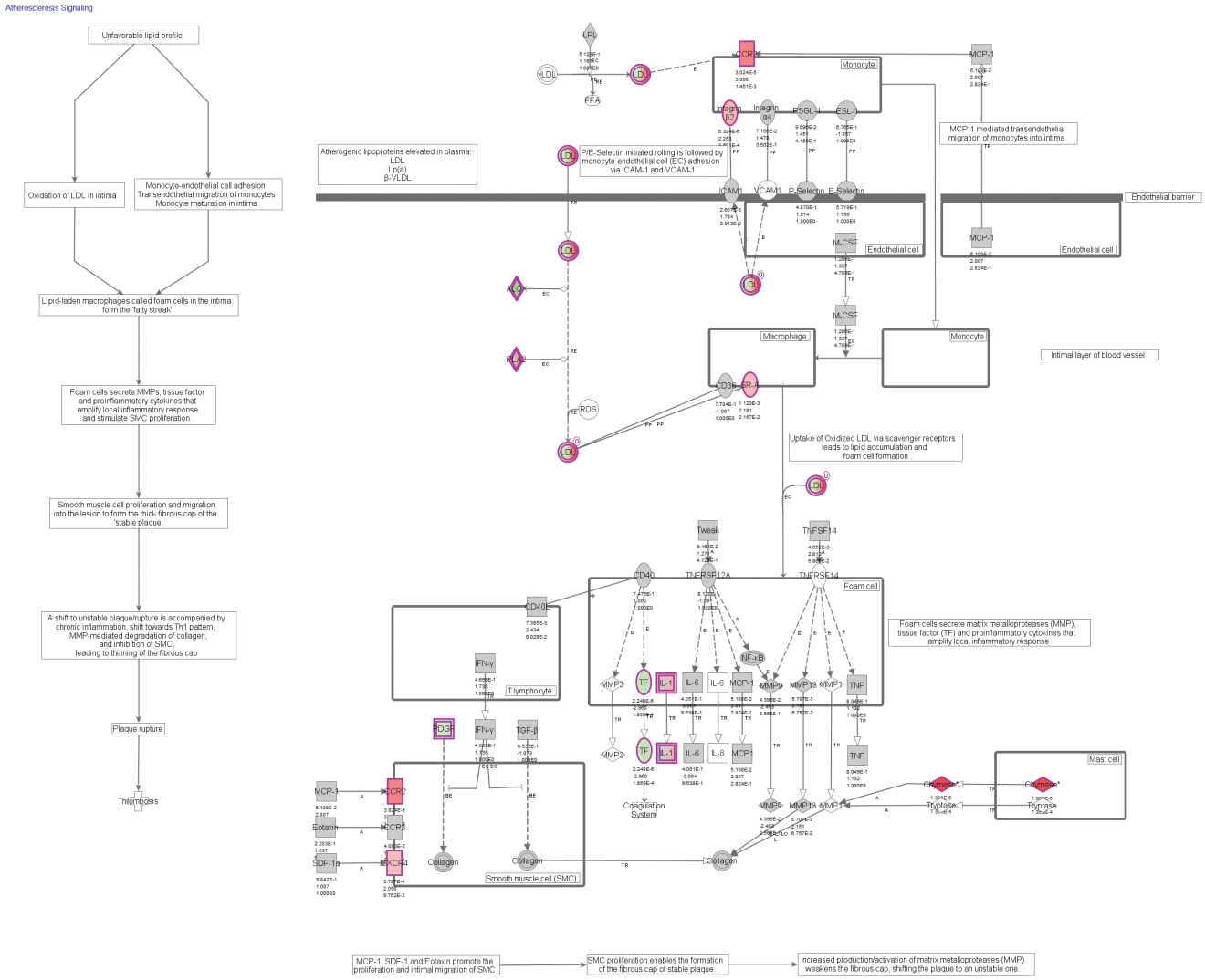

Figure 3. The atherosclerosis-signaling pathway in rumen papillae. The Ingenuity Pathway Analysis (IPA) indicated that 17 genes were related to this pathway, and 12 of them functioned in the rumen papillae as demonstrated in this figure.

the rumen epithelium after weaning and growth (Kato et al., 2015). However, based on RNA-seq analysis, only the expression of *HMGCS2* increased in weaned calves compared with suckling calves. These results reflected the limited dataset in the EST library in silico. However, the present results from RNA-seq analysis revealed several possible pathways and upregulators by analyzing global expression profiles during the weaning, growth, and development of rumen papillae.

The IPA data used in the present study were based on human, rat, and mouse and therefore had limitations for bovine pathway analysis. However, analysis revealed that 7 pathways significantly changed (FDR < 0.05) from suckling to weaned calves, especially "Atherosclerosis signaling." Atherosclerosis is a subsequent, nonadaptive response of blood vessels to damage, induced by chronic inflammation. So far, there are no direct evidences of a relationship between rumen epithelial cells and atherosclerosis-related genes. The main factors regulating atherosclerosis are thought to be high-cholesterol levels (in particular low-density lipoprotein [**LDL**] cholesterol), hypertension, and blood fat (Levine et al., 1995; Glasser et al., 1996). During the atherogenesis, circulating monocytes are recruited and differentiated, and tissue macrophages capture cholesterol and oxidize LDL in order to form lipid-foam cells (Moreno and Mitjavila, 2003). Intraruminal VFA are thought to be the substrate of cholesterol, and the in vivo synthesis of cholesterol may become active by increasing VFA levels through feeding concentrate or high-grain diets (Sakata and Tamate, 1978). Holstein cattle fed grain-based feed presented changes in the expression level of genes related to cholesterol synthesis and homeostasis, and the synthesis of cholesterol was regulated via intracellular sterol regulatory element-binding protein (**SREBP**) (Steele et al., 2011). In the present study, 12 of the 17 DEGs in the atherosclerosis

Table 1. Genes integrating the atherosclerosis pathway

Symbol	Entrez gene name	Fold change[1]	FDR[2]	Location	Type(s)
RBP4	Retinol binding protein 4, plasma	6.461	1.13E-09	Extracellular space	Transporter
PLA2G4D	Phospholipase A2, group IVD (cytosolic)	6.146	2.98E-03	Cytoplasm	Enzyme
CMA1	Chymase 1, mast cell	5.912	7.36E-04	Extracellular space	Peptidase
CCR2	Chemokine (C-C motif) receptor 2	3.998	1.45E-03	Plasma membrane	G-protein coupled receptor
IL33	Interleukin 33	2.655	2.40E-05	Extracellular space	Cytokine
PLA2G4F	Phospholipase A2, group IVF	2.533	3.50E-02	Cytoplasm	Enzyme
APOA2	Apolipoprotein A-II	2.389	7.94E-06	Extracellular space	Transporter
ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	2.253	3.60E-04	Plasma membrane	Transmembrane receptor
PON1	Paraoxonase 1	2.232	1.48E-02	Extracellular space	Phosphatase
PLA2G16	Phospholipase A2, group XVI	2.21	9.76E-04	Nucleus	Enzyme
MSR1	Macrophage scavenger receptor 1	2.181	2.17E-02	Plasma membrane	Transmembrane receptor
CXCR4	Chemokine (C-X-C motif) receptor 4	2.09	9.76E-03	Plasma membrane	G-protein coupled receptor
PDGFA	Platelet-derived growth factor alpha polypeptide	−2.225	3.31E-04	Extracellular space	Growth factor
PLA2G3	Phospholipase A2, group III	−2.448	3.23E-03	Extracellular space	Enzyme
APOA1	Apolipoprotein A-I	−2.681	1.01E-02	Extracellular space	Transporter
F3	Coagulation factor III (thromboplastin, tissue factor)	−2.96	1.87E-04	Plasma membrane	Transmembrane receptor
ALOX12B	Arachidonate 12-lipoxygenase, 12R type	−5.499	1.67E-02	Cytoplasm	Enzyme

[1]Fold change: Values indicate the relative gene expression. Positive values indicate higher expression in weaned than in suckling calves, and negative values indicate the opposite.

[2]FDR = false discovery rate.

Table 2. The 5 major upstream regulators generated by ingenuity pathway analysis (IPA)

Upstream regulator	Fold change[1]	Predicted activation state	Activation *z*-score[2]	*P*-value of overlap[3]	Mechanistic network[4]
Tretinoin			1.918	2.19E-16	301 (20)
TGFβ1[5]	−1.073		1.089	1.15E-14	285 (17)
Phorbol myristate acetate			0.371	1.24E-14	253 (17)
PPARA[6]	1.364	Activated	2.155	3.17E-14	207 (16)
Lipopolysaccharide		Activated	2.516	2.35E-13	283 (16)

[1]Fold change: Values indicate the relative gene expression. Positive values indicate higher expression in weaned than in suckling calves, and negative values indicate the opposite.

[2]*z*-Score: Values indicate a statistically significant pattern match between up- and down-regulation patterns.

[3]*P*-value of overlap: Values indicate a statistically significant enrichment of the network-regulated genes in the database.

[4]Mechanistic network: The number of molecules that are downstream of the regulators and the number of regulators involved in that particular network in parentheses.

[5]TGFβ1: tumor growth factor beta-1.

[6]PPARA: Peroxisome proliferator-activated receptor alpha.

pathway were upregulated during weaning, whereas 5 genes were downregulated (Table 1). In monogastric animals, all these genes, except APOA1, may act as regulators or initiators of atherosclerosis (Miller-Kasprzak et al., 2004; Takahashi et al., 2005; Sato et al., 2008; Meisel et al., 2011). Thus, cholesterol synthesis might be promoted by grain-based feeds during weaning, and atherosclerosis might advance. Decreasing the expression of the genes related to cholesterol synthesis may help relieve the inflammation caused by atherosclerosis, at least partly. This suggests that cholesterol homeostasis and inflammation might have been activated in the rumen epithelium during weaning, by feeding starter and roughage.

Among the upstream regulators indicated by IPA, tretinoin was predicted as the most active molecule in the rumen papillae of Japanese Black cattle during weaning (P = 2.19E-16; Table 2). However, tretinoin was not yet reported as an upregulating factor in the rumen epithelium. Other important factors controlling the differentiation of rumen epithelium at weaned are TGFB1 and PPARA (Connor et al., 2014). Tretinoin is also known as "All-trans retinoic acid (ATRA)," which is a derivative of vitamin A. Alitretionin, a form of vitamin A, is predicted to play a role on rumen development (Table 3). There are some reports that vitamin A or its derivatives are related to epithelium functioning. For example, vitamin A was related

Table 3. Upstream regulators activated (*z*-score > 2.00) and inhibited (*z*-score < -2.00) in the rumen of Japanese Black cattle during weaning

Upstream regulator	Molecule type	*P*-value of overlap[1]	Predicted activation state	Activation *z*-score[2]
IgG	Complex	1.38E-09	Activated	3.039
Lipopolysaccharide	Chemical drug	2.35E-13	Activated	2.516
Alitretinoin	Chemical drug	7.57E-06	Activated	2.241
PPARδ[3]	Ligand-dependent nuclear receptor	3.40E-07	Activated	2.232
PPARα[4]	Ligand-dependent nuclear receptor	3.17E-14	Activated	2.155
Pirinixic acid	Chemical toxicant	2.34E-08	Activated	2.075
TNF[5]	Cytokine	6.08E-12	Activated	2.007
Estrogen receptor	Group	1.97E-07	Inhibited	−2.232

[1]P-value of overlap: Values indicate a statistically significant encrichment of the network-regulated genes in the database.

[2]*z*-Score: Values indicate a statistically significant match between up- and down-regulation patterns.

[3]PPARδ = peroxisome proliferator-activated receptor delta.

[4]PPARα = peroxisome proliferator-activated receptor alpha.

[5]TNF = tumor necrosis factor.

to the maintenance of lung epithelial cells in rat (Takahashi et al., 1993). In addition, vitamin A and its derivatives, including retinol, retinal, and retinoic acid, function as effective inhibitors of mammary epithelial cell proliferation in cattle, and it is possible that retinoid, an analogue of vitamin A, can regulate mammary growth and development (Purup et al., 2001). The expression of SLC26A3, downregulated in adenoma (Makela et al., 2002), is increased by ATRA in intestinal epithelial cells (Priyamvada et al., 2015). Only a few studies have reported the effects of vitamin A in rumen papillae. Although the effects of vitamin A differ according to gastrointestinal track sites, villus height in the ileum and jejunum was enhanced by the supplementation of vitamin A in newborn calves (Schottstedt et al., 2005). The present study suggests that tretinoin (probably also retinol or retinoic acid) might be an important factor mediating the development of the rumen epithelium during the transition period from suckling to weaned.

The IPA analysis also indicated that IgG, lipopolysaccharides (**LPS**), and tumor-necrosis factor-α (**TNF**α) are activated in the rumen papillae of weaned calves. These upstream regulators are involved in immune responses and inflammation. Because IgG is vital to provide adequate immunological protection and resistance to disease (Conneely et al., 2014), in rumen papillae it is needed to induce adequate immunity and protection against pathogenic organisms. Changes in LPS, which are produced from dead bacteria, are caused by the alteration of bacterial diversity during weaning. Grain-induced subacute ruminal acidosis and the increment of a grain-based diet in lactating dairy cows increased the free ruminal LPS (Gozho et al., 2007). The concentration of ruminal LPS increased in steers fed grain-based diets and caused a variety of metabolic and immunologic alterations in the host (Andersen et al., 1994; Gozho et al., 2005). Moreover, the mucosal epithelium becomes more permeable and susceptible to apoptosis by the presence and increment of endotoxins (Chin et al., 2006). In addition, TNFα may be produced in the rumen epithelium by increasing the LPS released from rumen microbes in weaned calves (Pfeffer et al., 1993; Pasparakis et al., 1996; Raabe et al., 1998). Because TNFα is paracrine and endocrine, it may only act on the inflammation of rumen epithelium after weaning. The presence of IgG, LPS, and TNFα in rumen papillae contributes to microbe-epithelium interactions that activate rumen-related immune responses.

The IPA analysis also revealed that PPARα, PPARδ, and pirinixic acid might activate the growth and proliferation of rumen papillae (Table 3). Both PPARα and PPARδ are nuclear-receptor stimulating genes involved in fatty acid intake and metabolism, ketogenesis, and epidermal proliferation (Schoonjans et al., 1996; Burdick et al., 2006; Takahashi et al., 2006; Badman et al., 2007; Laarman et al., 2012; Naeem et al., 2012; Connor et al., 2013; Benesch et al., 2014). These PPARs might be increased to adapt to fatty acid metabolism, ketogenesis, and development of rumen papillae in weaned calves. Both PPARα and PPARδ heterodimerize with retinoid X receptor (RXR) (Klemm et al., 2001). The FXR/RXR-, LXR/RXR-, and LPS/IL-1-mediated inhibition of the RXR function, and the VDR/RXR activation obtained in the canonical pathway analysis suggest the importance of RXR (Figure 1). Tretinoin was identified as the most important upregulator (Table 2), and alitretinoin (9-cis retinoic acid) has been identified

Table 4. Genes regulated by tretinoin in rumen papillae tissue

Symbol	Entrez gene name	Fold change[1]	FDR[2]	Location	Type(s)
SFTPC	Surfactant protein C	15.731	6.95E-05	Extracellular space	Other
CDKN2A	Cyclin-dependent kinase inhibitor 2A	14.833	3.44E-04	Nucleus	Transcription regulator
MSLN	Mesothelin	14.337	5.37E-03	Extracellular space	Other
CYP4F2	Cytochrome P450, family 4, subfamily F, polypeptide 2	14.255	5.63E-17	Cytoplasm	Enzyme
NEU4	Sialidase 4	7.214	1.56E-03	Cytoplasm	Enzyme
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	7.161	2.84E-15	Cytoplasm	Transporter
SIX1	SIX homeobox 1	6.675	1.69E-04	Nucleus	Transcription regulator
TNC	Tenascin C	6.034	8.28E-04	Extracellular space	Other
CES1	Carboxylesterase 1	5.812	3.44E-04	Cytoplasm	Enzyme
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	5.111	4.60E-03	Cytoplasm	Enzyme
SLA	Src-like-adaptor	3.589	9.81E-05	Plasma membrane	Other
AREG	Amphiregulin	3.566	1.95E-03	Extracellular space	Growth factor
A2M	α-2-macroglobulin	3.543	5.08E-03	Extracellular space	Transporter
DUSP4	Dual specificity phosphatase 4	3.494	4.82E-03	Nucleus	Phosphatase
EBI3	Epstein-Barr virus induced 3	3.48	6.85E-04	Extracellular space	Cytokine
BMP6	Bone morphogenetic protein 6	3.453	6.37E-05	Extracellular space	Growth factor
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2	3.445	7.74E-03	Cytoplasm	Enzyme
CCR1	Chemokine (C-C motif) receptor 1	3.425	1.12E-03	Plasma membrane	G-protein coupled receptor
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	3.274	2.77E-03	Cytoplasm	Enzyme
PLEK	Pleckstrin	3.273	1.37E-02	Cytoplasm	Other
ALPL	Alkaline phosphatase, liver/bone/kidney	3.185	8.35E-05	Plasma membrane	Phosphatase
CCR5	Chemokine (C-C motif) receptor 5 (gene/pseudogene)	3.121	7.27E-03	Plasma membrane	G-protein coupled receptor
CTGF	Connective tissue growth factor	3.116	1.03E-02	Extracellular space	Growth factor
TLR5	Toll-like receptor 5	3.1	2.96E-03	Plasma membrane	Transmembrane receptor
PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	2.883	1.53E-02	Cytoplasm	Phosphatase
THBS1	Thrombospondin 1	2.851	1.03E-02	Extracellular space	Other
NOV	Nephroblastoma overexpressed	2.818	4.67E-02	Extracellular space	Growth factor
ANPEP	Alanyl (membrane) aminopeptidase	2.817	2.10E-02	Plasma membrane	Peptidase
SCML1	Sex comb on midleg-like 1 (Drosophila)	2.814	5.60E-03	Nucleus	Transcription regulator
SOAT1	Sterol O-acyltransferase 1	2.774	1.47E-07	Cytoplasm	Enzyme
MMP28	Matrix metallopeptidase 28	2.76	1.13E-09	Extracellular space	Peptidase
CTSL	Cathepsin L	2.678	2.61E-02	Cytoplasm	Peptidase
C1QA	Complement component 1, q subcomponent, A chain	2.625	7.58E-03	Extracellular space	Other
VSIG4	V-set and immunoglobulin domain containing 4	2.623	2.56E-02	Plasma membrane	Other
AMICA1	Adhesion molecule, interacts with CXADR antigen 1	2.523	7.94E-06	Plasma membrane	Other
CD52	CD52 molecule	2.522	5.02E-03	Plasma membrane	Other
CD101	CD101 molecule	2.504	4.32E-03	Plasma membrane	Other
FABP4	Fatty acid binding protein 4, adipocyte	2.496	3.53E-03	Cytoplasm	Transporter
HMGCS2	3-Hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	2.491	4.20E-05	Cytoplasm	Enzyme
IGFBP5	Insulin-like growth factor binding protein 5	2.452	9.78E-04	Extracellular space	Other
CD86	CD86 molecule	2.404	5.74E-03	Plasma membrane	Transmembrane receptor
RBP2	Retinol binding protein 2, cellular	2.401	2.18E-03	Cytoplasm	Transporter
APOA2	Apolipoprotein A-II	2.389	7.94E-06	Extracellular space	Transporter
SEMA7A	Semaphorin 7A, GPI membrane anchor (John Milton Hagen blood group)	2.374	1.44E-02	Plasma membrane	Transmembrane receptor
BAMBI	BMP and activin membrane-bound inhibitor	2.374	2.89E-03	Plasma membrane	Other
GGT1	γ-Glutamyltransferase 1	2.365	2.67E-02	Plasma membrane	Enzyme
POMC	Proopiomelanocortin	2.343	1.19E-04	Extracellular space	Other
CD68	CD68 molecule	2.337	4.21E-03	Plasma membrane	Other
CD38	CD38 molecule	2.332	3.81E-02	Plasma membrane	Enzyme

Table 4. Continued

Symbol	Entrez gene name	Fold change[1]	FDR[2]	Location	Type(s)
SLAMF7	SLAM family member 7	2.316	2.30E-02	Plasma membrane	Other
CYP4F3	Cytochrome P450, family 4, subfamily F, polypeptide 3	2.302	1.12E-03	Cytoplasm	Enzyme
ECI2	Enoyl-CoA delta isomerase 2	2.289	2.31E-05	Cytoplasm	Enzyme
TYROBP	TYRO protein tyrosine kinase binding protein	2.288	2.73E-03	Plasma membrane	Transmembrane receptor
RGS5	Regulator of G-protein signaling 5	2.276	4.61E-02	Plasma membrane	Other
ITGB2	Integrin, β 2 (complement component 3 receptor 3 and 4 subunit)	2.253	3.60E-04	Plasma membrane	Transmembrane receptor
ITGAM	Integrin, α M (complement component 3 receptor 3 subunit)	2.245	2.53E-02	Plasma membrane	Transmembrane receptor
MGST3	Microsomal glutathione S-transferase 3	2.237	2.79E-05	Cytoplasm	Enzyme
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	2.231	2.39E-06	Cytoplasm	Other
HLA-DMB	Major histocompatibility complex, class II, DM beta	2.207	1.58E-04	Plasma membrane	Transmembrane receptor
FOLR2	Folate receptor 2 (fetal)	2.206	1.12E-02	Plasma membrane	Transporter
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	2.177	1.38E-02	Cytoplasm	Enzyme
SPI1	Spi-1 proto-oncogene	2.172	4.23E-03	Nucleus	Transcription regulator
IKZF1	IKAROS family zinc finger 1 (Ikaros)	2.165	8.46E-03	Nucleus	Transcription regulator
CFP	Complement factor properdin	2.165	7.82E-03	Extracellular space	Other
CXCR4	Chemokine (C-X-C motif) receptor 4	2.09	9.76E-03	Plasma membrane	G-protein coupled receptor
CIDEA	Cell death-inducing DFFA-like effector a	2.085	3.59E-02	Cytoplasm	Other
RPL6	Ribosomal protein L6	2.055	1.27E-02	Cytoplasm	Other
COL4A1	Collagen, type IV, alpha 1	2.024	1.40E-03	Extracellular space	Other
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	2.009	3.27E-04	Plasma membrane	Transmembrane receptor
MAOB	Monoamine oxidase B	2.002	7.89E-03	Cytoplasm	Enzyme
KLF9	Kruppel-like factor 9	−2.011	1.35E-04	Nucleus	Transcription regulator
BTC	betacellulin	−2.042	2.68E-02	Extracellular space	Growth factor
MYBL2	v-myb avian myeloblastosis viral oncogene homolog-like 2	−2.109	2.85E-02	Nucleus	Transcription regulator
TSC22D3	TSC22 domain family, member 3	−2.116	1.31E-04	Nucleus	Transcription regulator
SOX6	SRY (sex determining region Y)-box 6	−2.129	2.14E-02	Nucleus	Transcription regulator
DLL1	δ-Like 1 (Drosophila)	−2.154	1.79E-03	Plasma membrane	Enzyme
KRT15	Keratin 15	−2.166	1.11E-03	Cytoplasm	Other
HES1	Hes family bHLH transcription factor 1	−2.169	6.79E-03	Nucleus	Transcription regulator
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	−2.172	1.12E-02	Plasma membrane	Other
KIF23	Kinesin family member 23	−2.181	2.57E-03	Cytoplasm	Other
HOXD4	Homeobox D4	−2.212	1.81E-02	Nucleus	Transcription regulator
HEY1	Hes-related family bHLH transcription factor with YRPW motif 1	−2.22	1.12E-02	Nucleus	Transcription regulator
DUSP1	Dual specificity phosphatase 1	−2.259	3.10E-02	Nucleus	Phosphatase
MYB	v-myb avian myeloblastosis viral oncogene homolog	−2.285	1.84E-02	Nucleus	Transcription regulator
PTCH1	Patched 1	−2.319	3.84E-03	Plasma membrane	Transmembrane receptor
IGFBP3	Insulin-like growth factor binding protein 3	−2.437	7.34E-03	Extracellular space	Other
IGF2	Insulin-like growth factor 2	−2.472	3.57E-05	Extracellular space	Growth factor
FGF2	Fibroblast growth factor 2 (basic)	−2.476	2.82E-02	Extracellular space	Growth factor
COL4A5	Collagen, type IV, alpha 5	−2.481	4.70E-03	Extracellular space	Other
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	−2.507	6.26E-05	Plasma membrane	Transmembrane receptor
GJB5	Gap junction protein, beta 5, 31.1kDa	−2.615	7.49E-03	Plasma membrane	Transporter
IGFBP2	Insulin-like growth factor binding protein 2, 36kDa	−2.711	1.65E-03	Extracellular space	Other
IGFBP6	Insulin-like growth factor binding protein 6	−2.797	1.94E-03	Extracellular space	Other
STC1	Stanniocalcin 1	−2.923	5.90E-03	Extracellular space	Kinase
F3	Coagulation factor III (thromboplastin, tissue factor)	−2.96	1.87E-04	Plasma membrane	Transmembrane receptor
HS3ST1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	−3.044	6.26E-05	Cytoplasm	Enzyme

Table 4. Continued

Symbol	Entrez gene name	Fold change[1]	FDR[2]	Location	Type(s)
RNASE1	Ribonuclease, RNase A family, 1 (pancreatic)	−3.056	2.31E-02	Extracellular space	Enzyme
GATA6	GATA binding protein 6	−3.173	1.12E-02	Nucleus	Transcription regulator
TERT	Telomerase reverse transcriptase	−3.239	3.14E-03	Nucleus	Enzyme
GHR	Growth hormone receptor	−3.268	2.20E-05	Plasma membrane	Transmembrane receptor
CHL1	Cell adhesion molecule L1-like	−3.721	1.88E-02	Plasma membrane	Other
SYCP3	Synaptonemal complex protein 3	−4.417	2.38E-05	Nucleus	Other
SLC12A2	Solute carrier family 12 (sodium/potassium/chloride transporter), member 2	−4.847	3.85E-07	Plasma membrane	Transporter
CFTR	Cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	−4.953	1.01E-06	Plasma membrane	Ion channel
OAS2	2′-5′-Oligoadenylate synthetase 2, 69/71kDa	−5.376	7.18E-05	Cytoplasm	Enzyme
BMPR1B	Bone morphogenetic protein receptor, type IB	−6.669	2.95E-03	Plasma membrane	Kinase
PITX2	Paired-like homeodomain 2	−7.073	3.43E-02	Nucleus	Transcription regulator
RXRG	Retinoid X receptor, gamma	−7.723	2.15E-02	Nucleus	Ligand-dependent nuclear receptor
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	−8.077	1.09E-09	Plasma membrane	Kinase
HOXB9	Homeobox B9	−8.378	4.76E-02	Nucleus	Transcription regulator
DSG1	Desmoglein 1	−9.067	3.88E-09	Plasma membrane	Other
EYA2	EYA transcriptional coactivator and phosphatase 2	−19.365	2.07E-05	Nucleus	Phosphatase

[1]Fold change: Values indicate the relative gene expression. Positive values indicate higher expression in weaned than in suckling calves, and negative values indicate the opposite.

[2]FDR = false discovery rate.

as a ligand of RXR (Heyman et al., 1992), whereas pirinixic acid (Wy-14,643) is a ligand of PPARα (Schaefer et al., 2008). Because Wy-14,643 increased the gene expression of monocarboxylate transporters (MCT)1, but not the MCT4 of VFA, in cultured ovine rumen epithelial cells (Benesch et al., 2014), pirinixic acid and PPARα/δ activation of RXR by alitretinoin might be important to adapt to VFA absorption and, therefore, are related to the growth and development of the rumen epithelium during the weaning period. However, estrogen receptors were inhibited in rumen papilla of weaned calves. There are 2 types of estrogen receptors: estrogen receptor 1 (ESR1) and estrogen receptor 2 (ESR2). In the present study, the expression of ESR2, not ESR1, was lower in the rumen papillae of weaned calves, and this molecule is known to have an antiproliferative role in the epithelium of immature uterus (Weihua et al., 2000). Thus, ESR2 might be downregulated in the rumen papillae after weaning to block antiproliferative effects. The upstream regulators predicted in this study need to be further validated.

In conclusion, the RNA-seq analysis of the rumen papillae of suckling and weaned calves provided a comprehensive view of the relative abundance and differential expression of several protein-coding genes. This information provides a valuable resource for studying atherosclerosis signaling, as candidate networks were found, including the activation of PPAR/RXR, which act as upregulators of the proliferation and development of rumen papillae in the transition from suckling to weaning in Japanese Black calves.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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