Short communication: Initial evidence supporting existence of potential rumen epidermal stem and progenitor cells

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ABSTRACT

The bovine rumen epidermis is a keratinized multi-layered tissue that experiences persistent cell turnover. Because of this constant cell turnover, epidermal stem cells and their slightly more differentiated daughter cells, epidermal progenitor cells, must exist in the stratum basale of rumen epidermis. To date, these 2 epidermal cell populations and any unique cellular markers they may possess remain completely uncharacterized in the bovine rumen. An important first step in this new research area is the demonstration of the relative abundance and existence of markers for these cells in rumen tissue. A related second step is to document rumen epidermal proliferative responses to an extrinsic signal such as nutrient concentration within the rumen. The objectives of this experiment were to evaluate the extrinsic effect of diet on (1) gene expression of 6 potential rumen epidermal stem or progenitor cell markers and (2) rumen epidermal cell proliferation within the stratum basale. Twelve preweaned Holstein heifers were fed either a restricted diet (R) or an enhanced diet (EH). Animals on R received a milk replacer (MR) diet fed at 0.44 kg of powder dry matter (DM)/d (20.9% crude protein, 29.8% fat, DM basis) and EH received MR at 1.08 kg of powder dry matter/d (28.9% crude protein, 26.2% fat, DM basis). All calves had access to a 20% crude protein starter and were weaned during wk 7 of the experiment. Lifetime DM intake was 0.73 kg of DM/calf per day for R (5.88 Mcal of net energy/calf per day) and 1.26 kg of DM/calf per day for EH (10.68 Mcal of net energy/calf per day). Twenty-four hours before slaughter heifers received an intravenous dose of 5-bromo-2'-deoxyuridine to label proliferating cells. Heifers were slaughtered at 8 wk of age, and rumen samples from the ventral sac region were obtained and stored in RNA preservative and processed for routine histology. Quantitative real-time reverse transcriptase PCR was used to analyze relative abundance of genes.

Candidate genes for markers of epidermal stem and progenitor cells were β 1-integrin (*ITGB1*), tumor protein p63 (*TP63*), keratin-14 (*KRT14*), Notch-1 (*NOTCH1*), Leu-rich repeat-containing G protein-coupled receptor 5-expressing (*LGR5*), and musashi-1 (*MSI1*). All genes were detected in the rumen tissue; *ITGB1* was increased in EH compared with R. 5-Bromo-2'-deoxyuridine immunohistochemistry revealed that both R and EH rumen tissue had proliferating cells within the stratum basale of the rumen epidermis at the time of analysis. The EH diet resulted in an additive effect on cell proliferation. The percentage of cells in the stratum basale synthesizing DNA in preparation for mitosis nearly doubled ($23.8 \pm 2.4\%$ for EH vs. $14.7 \pm 2.0\%$ for R) compared with calves fed R. This work represents the first attempt at characterizing rumen epidermal stem and progenitor cells. We demonstrated the relative abundance and existence of potential markers in rumen tissue and showed a rumen epidermal proliferative response to the extrinsic stimulus of nutrient concentration in the form of diet.

Key words: dairy calf, cell proliferation, mitosis, epidermis, epithelium

Short Communication

The epidermis of bovine rumen is a multilayered keratinized, stratified squamous epithelium. It is situated on top of the dermis, hence its name. The rumen epidermis is the first line of defense against microbial infiltration and also the first site for nutrient absorption; it therefore has barrier and absorptive functions. Rumen epithelium is composed of 4 strata, plus an outer layer of keratin and is collectively called the epidermis. Keratin is the most superficial layer, followed by the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. A basement membrane is situated below the stratum basale, followed by the dermis, which contains blood vessels (see Figure 2). The rumen epidermis is dynamic and must be maintained throughout life. Epidermal homeostasis requires cellular proliferation and differentiation events that originate in the stratum basale (Figure 2). From studies with other epidermal tissues, it is known that

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physiological cues induce cells in the stratum basale to detach from the underlying basement membrane, withdraw from the cell cycle, and begin the process of terminal differentiation and migration to the surface (Zouboulis et al., 2008). These committed cells traverse through the epidermal layers before being shed from the epidermal surface as cornified cells (Figure 2; Lavker and Matoltsy, 1970). In rumen epidermis, as with other types of epidermis, cellular death and loss through cornification occurs continuously. Thus, new differentiated cells must be generated throughout life to replenish lost cells. Because of this persistent epidermal cell turnover, it is hypothesized that epidermal stem cells and their slightly more differentiated daughter cells, epidermal progenitor cells (also known as transit-amplifying cells), must exist in the stratum basale of the rumen epidermis. Further, to function as true stem and progenitor cells, these candidate cells must be able to respond to extrinsic stimuli, such as nutrient concentration. Last, these cell populations must have unique cellular markers that distinguish them from other epidermal cells.

To date, these 2 epidermal cell populations and any unique cellular markers they may possess remain poorly understood in bovine rumen. Over the years, rumen epidermal cell proliferation has been quantified in numerous ways. Some methods include quantification of the proportion of stratum basale cells: with mitotic figures (Sakata and Tamate, 1978; Shen et al., 2004; Malhi et al., 2013), labeled with tritiated thymidine (Goodlad, 1981; Ohwada et al., 1984; Neogrady et al., 1989), Ki-67 labeled (Mentschel et al., 2001), or 5-bromo-2'-deoxyuridine (**BrdU**) and Ki-67 labeled cells (Baldwin et al., 2004), but an attempt has never been made to link these proliferation events to the presence of potential stem and progenitor cells. An important first step in this new research area is the demonstration of abundance and existence of these potential markers in rumen tissue. A related second step is to document rumen epidermal proliferative responses to the extrinsic signal of nutrient concentration within the rumen itself.

Regarding the existence of rumen epidermal stem and progenitor cell markers, β 1-integrin (**ITGB1**), tumor protein p63 (**TP63**), keratin-14 (**KRT14**), and Notch-1 (**NOTCH1**) are documented epidermal stem and progenitor cell markers in other species and organs (Zouboulis et al., 2008; Ambler and Maatta, 2009; Eckhart et al., 2013). Also, Leu-rich repeat-containing G protein-coupled receptor 5-expressing (**LGR5**), and musashi-1 (**MSI1**), a RNA-binding protein, are commonly accepted intestinal stem cell markers in other animals (Barker, 2014; Clevers et al., 2014; Tan and Barker, 2014). Because the rumen has properties that

are characteristic of both epidermis (barrier function) and intestine (nutrient absorptive function), we rationalized that presence of these markers may indicate not only the existence of bovine rumen epidermal stem or progenitor cells, but also that these markers are potentially specific for rumen epidermal stem or progenitor cells.

The primary objective of this experiment was to evaluate the extrinsic effect of diet on (1) gene expression of 6 potential rumen epidermal stem or progenitor cell markers and (2) rumen epidermal cell proliferation within the stratum basale.

The Virginia Tech (**VT**) Animal Care and Use Committee approved the live animal experimental portion of this work (protocol #14-045-DASC). Twelve Holstein heifers were used in an experiment that lasted approximately 2 mo; they were part of a larger experiment that dealt with effects of diet on mammary gland growth and full experimental details are reported (Geiger et al., 2016). Briefly, the 12 heifers used here were purchased from a single commercial dairy producer located ~145 km from VT; at VT, heifers were individually housed in outdoor hutches on crushed rock without bedding. Heifers had ad libitum access to water. The experimental unit was heifer. Heifers were approximately 1 wk old (5.3 ± 2.3 d; mean $\pm$ SD) at the time of arrival to VT, had serum total protein ≥ 5.5 g/dL, and weighed 40.4 ± 4.6 kg (mean $\pm$ SD). Heifers used here were in the original treatment groups of restricted (**R**) or enhanced (**EH**), which is in reference to the treatment diet (Geiger et al., 2016). In the original experiment, one heifer on EH died within 48 h of arrival to VT and was not replaced. The final animal numbers were R, n = 6, and EH, n = 5.

Heifers on R received fewer nutrients through milk replacer (**MR**) than heifers on EH. The MR fed to R heifers was 20.9% CP and 19.8% fat (DM basis), offered at 0.43 kg of MR powder/heifer per d (DM basis). The MR fed to EH heifers was 28.9% CP, 26.2% fat (DM basis), offered at 1.08 kg of MR powder/heifer per d (DM basis). Both MR were reconstituted to 13% solids and fed twice daily in equal portions at 0600 and 1700 h. During the eighth week of the trial, heifers were weaned from MR by removing one daily feeding. The weaning process lasted 7 d. Throughout the experiment, heifers had access to a 25.6% CP, 4.0% fat, and 19.8% NDF (DM basis) texturized calf starter. The amount of calf starter offered per calf was adjusted daily so that starter intake of R approximated that of EH (described in Geiger et al., 2016). Refusals of MR and starter, if any, were recorded daily and are reported elsewhere (Geiger et al., 2016). Likewise, heifer BW were obtained weekly and are summarized in Geiger et al. (2016).

Approximately 24 h before slaughter, which occurred at 62 ± 2 d of age (mean $\pm$ SD), each heifer was administered a single intravenous injection of BrdU at 5 mg/kg of BW; sterile BrdU solution was prepared according to Daniels et al. (2009). Bromodeoxyuridine is a thymidine analog and incorporates into newly synthesized DNA strands, thus marking actively proliferating cells.

Feed was withheld in the 12 h before slaughter. Heifers were euthanized via intravenous administration of a commercial phenobarbital solution (Fatal-Plus, 10 mg/kg of BW, Vortech Pharmaceuticals, Dearborn, MI), followed by immediate exsanguination. The entire gastro-intestinal tract was then ligated, removed, and weighed full; forestomach organ measurements are summarized elsewhere (Geiger et al., 2016). Rumen samples intended for gene expression and immunohistochemistry analyses were obtained exclusively from the ventral sac of each emptied and washed rumen.

Samples for rumen gene expression were processed as follows. First, a punch biopsy tool with an internal diameter of 2.54 cm was used to obtain full-thickness rumen biopsy samples (epidermis + dermis + muscle layers) from the ventral sac within each washed rumen. Forceps were then used to peel the muscle layers away from the epidermis + dermis; the muscle-containing portion was discarded and the epidermis + dermis portion of each sample was placed in tubes containing an RNA preservative (700 g of ammonium sulfate, 40 mL of 0.5 M EDTA at pH 8, and 25 mL of 1 M sodium citrate in 935 mL of purified water and brought to pH 5.2 using ~ 1.3 mL of sulfuric acid and ~ 330 μ L of 10 M sodium hydroxide) and stored on wet ice until permanent storage in a -70°C freezer later that same day. This sample collection technique ensured that only rumen papillae (dermis + epidermis) were submitted for later analyses.

Total RNA was extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Approximately 30 mg of rumen epidermal tissue was taken out of the RNA preservative and placed into 600 μ L of lysis buffer (10 μ L of β -mercaptoethanol in every 1 mL of buffer RLT Plus) in a 14-mL Falcon tube (Fisher Scientific, Waltham, MA). Each sample was homogenized using a Bio-Gen PRO200 homogenizer (Pro Scientific, Oxford, CT) at its top speed for 30 s and placed on ice. After centrifugation at $20,817 \times g$ for 3 min at 23°C , supernatant was then placed in a genomic DNA eliminator column and centrifuged for $10,621 \times g$ for 30 s at 23°C . The flow-through was then processed using an RNeasy Plus mini kit. The RNA purity and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). Furthermore, RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa

Clara, CA) with an average RNA integrity number of 6.87 ± 0.13 (SEM).

Single-stranded cDNA was synthesized from each RNA sample according to the method of Swank et al. (2013). Each real-time quantitative (q)PCR assay was performed on all samples in duplicate, with each reaction mixture (20 μ L) containing 0.5 μ L each forward and reverse primer, 10 μ L of SYBR Green Supermix (Bio-Rad, Hercules, CA), 8 μ L of RNase/DNase free water, and 1 μ L of cDNA (1:1 stock). The qPCR assays were performed using an iQ5 Multicolor Real Time PCR Detection System (Bio-Rad) with the following cycling conditions: 95°C for 3 min; 45 repeating cycles of 94°C for 15 s (denaturation), respective temperature (Table 1) for a set of primers for 30 s (annealing), and 72°C for 30 s (extension); 95.0°C for 2 min, 55.0°C for 30 s, and then a temperature increase at 1.0°C increments to 95.0°C (melting curve). Each assay included a no-template control and a no-reverse-transcriptase control, with the no-template control receiving 1 μ L of RNase/DNase free water instead of cDNA, whereas the no-reverse-transcriptase control received a 1- μ L sample of the reverse transcription product to which no reverse transcriptase was added. The qPCR was repeated when the resulting intra-assay coefficient of variation was greater than 5%.

Primers were designed with Primer3 (v. 0.4.0; <http://bioinfo.ut.ee/primer3-0.4.0/>; Rozen and Skaletsky, 2000) for all genes used. Validation of primers consisted of 2 steps. Melting curve analyses were performed after each qPCR assay to determine whether primer dimers or genomic DNA contamination were present during the assay. Also, product size variation using gel electrophoresis was performed after each qPCR assay. For product size variation, representative samples of qPCR products for each primer pair were electrophoresed on a 3.5% agarose gel to confirm the expected size of each amplicon. All qPCR products were visualized as single bands that corresponded with predicted product size (data not shown).

Target genes of interest were normalized to the geometric mean of ubiquitously expressed transcript (*UXT*), ribosomal protein S9 (*RPS9*), ribosomal protein S15 (*RPS15*), and ribosomal protein S26 (*RPS26*; target gene Ct – reference genes Ct = ΔCt ; Vandesompele et al., 2002). The Ct values for *UXT*, *RPS9*, *RPS15*, and *RPS26* were evaluated using algorithms used in the GeNorm software for analysis of qPCR data to create M stability values for each reference gene (Hellemans et al., 2007). The M stability values calculated were 0.095 (*UXT*), 0.101 (*RPS9*), 0.086 (*RPS15*), and 0.135 (*RPS26*). A lower M stability value indicates higher stability; Pérez et al. (2008) deemed M stability values below 1.5 as acceptable, so for this study *UXT*, *RPS9*,

RPS15, and *RPS26* were deemed acceptable reference genes. Delta Ct data were transformed to $2^{(-\Delta Ct)}$ to represent fold differences relative to the reference genes (Velayudhan et al., 2008). Raw data were not normally distributed (Shapiro Wilk's test) so all genes were then transformed by the natural log of the ΔCt value before statistical analysis. Data were omitted for one R calf due to poor sample quality, which makes final numbers for qPCR analysis: R, n = 5, and EH, n = 5.

Full-length rumen tissue (epidermis + dermis + muscle layers) from each heifer was fixed overnight in 10% neutral buffered formalin. Samples were then routinely processed, embedded in paraffin, cut to 5- μ m thickness on a microtome, and mounted onto positively charged glass microscope slides (Yohe et al., 2015).

The BrdU immunohistochemistry was performed as previously reported (Daniels et al., 2009), with the following noted modifications. The primary antibody was mouse monoclonal anti-bromodeoxyuridine, clone AH4H7-1 (Millipore, catalog # MAB3424; used at 1:100 dilution). The secondary antibody was Alexa Fluor 488 goat anti-mouse IgG (H⁺L, catalog #A11029 used at 1:200 dilution; Invitrogen, Carlsbad, CA). Coverslips were mounted with SlowFade Gold with DAPI (Invitrogen). Cells positive for BrdU in the stratum basale layer of the rumen epidermis were quantified in the following manner. Digital images were acquired at 40 $\times$ magnification using a Nikon Eclipse E600 epi-

fluorescence microscope fitted with a Nuance FX Multispectral Imaging System (Perkin-Elmer, Waltham, MA). Captured image files were unmixed to generate monochromatic images from the blue DAPI and green FITC channels, to detect any cell with a nucleus and BrdU-positive cells, respectively. Unmixed images were opened with and analyzed in Image-Pro Plus version 7.0 (Media Cybernetics, Rockville, MD). Pseudo-colored composite images of DAPI labeled (blue) and Alexa Fluor 488 labeled nuclei (green) were generated. These pseudo-colored composite images were individually opened; line segments were created using a freehand drawing tool that followed the contour of the stratum basale. The DAPI-labeled nuclei in the stratum basale were manually marked and counted. In addition, nuclei dual labeled with DAPI and Alexa Fluor 488 in the stratum basale were also counted. Ten images were obtained per animal with an average of 900 basale cells counted per animal. The BrdU labeling index within the stratum basale is defined as the number of dual-labeled cells divided by the number of all counted cells, multiplied by 100. An example of BrdU staining is in Figure 2. Data were omitted for one EH calf due to poor sample quality, which makes the final numbers for immunohistochemistry analysis: R, n = 6, and EH, n = 4.

Variance analyses for gene expression and BrdU data were performed using SAS (version 9.2, SAS Institute

Table 1. PCR primers used to evaluate gene expression in rumen epidermal tissue

Gene target ¹ (GenBank accession number)	Primer name	Primer sequence (5' to 3')	Annealing temperature, °C	Amplicon length, bp
Intestinal stem cell markers				
<i>LGR5</i> (NM_001192520.1)	F.813 R.943	GATACCGGAGAAGGCCCTTTGTA CGCCATTCAAAGTCAGTGTCTT	62.3	131
<i>MSH1</i> (NM_001081527.1)	F.552 R.680	CCAGCCAAAGGAGGTGATGT TAGGTCGTGGCTTGGAAC	62.3	129
Epidermal stem cell markers				
<i>TP63</i> (NM_001191337.1)	F.1122 R.1261	GAAGCGCCCTTTCCGTCA GGGATTCTTTGATCTTCAGCAGC	62.3	140
<i>KRT14</i> (NM_001166575.1)	F.662 R.806	ATCAAGGACTACAGCCCCCTACTT CCGTCTCGTACTTGGTGCG	64.1	145
<i>ITGB1</i> (NM_174368.3)	F.1057 R.1204	TTCAGCCTGTTTACAAGGAAGTGA GGCAATTTGCTGTTTCCAAAATGA	60.5	148
<i>NOTCH1</i> (XM_010819254.1)	F.6183 R.6308	GACCTGGGCAAGTCCGC CAGGAACAAGGGCGTCTCC	64.1	126
Endogenous control genes				
<i>UXT</i> (NM_001037471.2)	F.68 R.196	TCATCTCTGACGTGCTGCAG CCGAGTGGTTAGCTTCCTGG	62.4	129
<i>RPS9</i> (BC148016.1)	F.122 R.229	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTCGAACAGA	62.4	108
<i>RPS15</i> (NM_001024541.2)	F.183 R.329	CAAGGCCAAGAAAGATGCGC TCAGGCTTGATTTCCACCTGG	62.4	147
<i>RPS26</i> (NM_001015561.2)	F.219 R.347	CTGCGTGAGTTGTGCCATTC TACATGGGCTTTGGTGGAGG	62.4	129

¹*LGR5* = leucine-rich repeat containing G protein-coupled receptor 5; *MSH1* = Musashi RNA-binding protein 1; *TP63* = tumor protein p63; *KRT14* = keratin 14; *ITGB1* = integrin, β 1; *NOTCH1* = Notch 1; *UXT* = ubiquitously expressed transcript; *RPS9* = ribosomal protein S9; *RPS15* = ribosomal protein S15; *RPS26* = ribosomal protein S26.

Inc., Cary, NC) with a mixed model that included the fixed effect of diet and the random effect of heifer within diet. Rumen epidermal gene data were transformed to natural log values before statistical analysis to satisfy ANOVA requirements for normality. Data were back-transformed to generate Figure 1. Significance was declared when $P \leq 0.05$. Least squares means $\pm$ standard errors of the mean are reported for these data.

Calf growth data are reported elsewhere (Geiger et al., 2016). Calves assigned to EH grew at a faster rate than heifers assigned to R; this was an intended effect of the treatments as total DMI was different by design. Lifetime DMI was 0.73 kg of DM/calf per d for R ($n = 6$) and 1.26 kg of DM/calf per d for EH ($n = 5$). Lifetime net energy intake was 5.88 Mcal/d and 10.68 Mcal/d for R and EH calves, respectively. Calf BW were not different at the beginning of the trial, but beginning in the second week of the trial, EH weighed more than R. At the end of the trial, EH weighed 73.0 ± 0.8 kg (mean $\pm$ SD), whereas R weighed 62 ± 0.8 kg (mean $\pm$ SD).

The research problem, as we defined it, was that rumen epidermal stem and progenitor cell populations and any unique cellular markers they may possess are uncharacterized in bovine rumen. An important first step in this new research area is demonstration of abundance/existence of these potential markers in rumen tissue. We met this goal by doing qPCR experiments and showed that all 6 genes (*LGR5*, *MSI1*, *TP63*, *KRT14*, *ITGB1*, and *NOTCH1*) are expressed in ruminal papillae (full-thickness rumen epidermis + dermis; Figure 1). Further, in decreasing order and regardless of diet, the overall abundance of these genes was *KRT14* > *ITGB1* > *TP63* > *NOTCH1* > *LGR5* > *MSI1* (Figure 1). This pattern is perhaps reflective of the rumen epidermis being more similar in structure to the skin epidermis as opposed to the intestine, as was discussed in the Introduction. Also, we found that feeding a high ADG diet (EH) from wk 1 to 9 of age increased expression of *ITGB1* measured at wk 9 after a 12 h fast (Figure 1). This potentially demonstrates a response to the extrinsic stimulus of nutrient concentration and is something that stem and progenitor cells must be able to do. We caution that when interpreting these data, it must be remembered that the absence of a dietary effect at the gene level does not preclude effects measured at the protein level. Likewise, gene difference in *ITGB1* abundance may not equate to differences at the protein level. Also, it is worth reiterating that these heifers underwent a 12 h fast before slaughter; this may have affected gene expression. However, all animals were subjected to the same conditions so comparison should still be valid. Our documentation of the existence of all 6 of these gene transcripts in bovine epidermis is

Figure 1. Real-time quantitative (q)PCR was used to show that known intestinal stem cell genes musashi-1 (*MSI1*), and leucine-rich repeat containing G protein-coupled receptor 5 (*LGR5*), along with known epidermal stem and progenitor cell genes *p63*, *NOTCH-1*, keratin-14 (*KRT14*), and B-1 integrin (*ITGB1*) are all expressed in bovine rumen epidermis + dermis of 62-d-old dairy calves. Calves were fed either a restricted diet (R; $n = 5$) or an enhanced diet (EH; $n = 5$). Calves on R received a milk replacer (MR) diet fed at 0.44 kg of powder DM/d (20.9% CP, 29.8% fat, DM basis) and EH received MR at 1.08 kg of powder DM/d (28.9% CP, 26.2% fat, DM basis). All calves had access to a 20% CP starter. Lifetime DMI was 0.73 kg of DM/calf per d for R (5.88 Mcal of net energy/calf per d) and 1.26 kg of DM/calf per d for EH (10.68 Mcal of net energy/calf per d). * $P < 0.05$. Data are reported as LSM $\pm$ SEM.

exciting. However, we are not yet sure of the protein localization of these 6 gene transcripts within the full-thickness rumen epidermis + dermis. Further, we acknowledge that some of the signal obtained through our qPCR analyses may have originated from the dermis or supra-basale cell layers of the rumen epidermis. We can definitely say that none of the detected signal arose from smooth muscle because that portion of rumen tissue was dissected and discarded at sample collection. Going forward, these 6 markers will only remain in the stem and progenitor cell marker candidate pool if we show them to be present exclusively in the stratum basale of the rumen epidermis.

The second research problem we addressed was to evaluate the extrinsic effect of diet on rumen epidermal cell proliferation within the stratum basale. All animals tested had BrdU-positive cells located within the stratum basale of the rumen epidermis, signifying active epidermal cell proliferation within 24 h of slaughter (includes 12 h of fasting). This was expected because epidermal tissues experience high cell turnover and rely on cell proliferation to help maintain the tissue. Feeding calves a high ADG diet (EH) resulted in an additive effect on cell proliferation. The percentage of cells in

the stratum basale synthesizing DNA in preparation for mitosis almost doubled ($23.8 \pm 2.4\%$ for EH vs. $14.7 \pm 2.0\%$ for R) compared with calves fed a diet that supported a low ADG (Figure 2). These data build on knowledge obtained in previous studies (Sakata and Tamate, 1978; Goodlad, 1981; Sakata and Yajima, 1984) and help provide a link between activity of stem and progenitor cells (i.e., rumen cell proliferation) and diet. The percentages of proliferating rumen epidermal cells in these growing heifers are numerically higher, but in general agreement with, similar measurements in rumens of other growing dairy calves (Mentschel et al., 2001), growing beef steers (Baldwin and McLeod, 2000), and lactating dairy cows (Baldwin et al., 2004).

In closing, we showed that 6 candidate epidermal stem or progenitor cell markers (*LGR5*, *MSI1*, *TP63*, *KRT14*, *ITGB1*, and *NOTCH1*) exist at the gene level

in full-thickness bovine rumen epidermis + dermis. We also showed that the relative gene abundance of one of these (*ITGB1*) was increased in animals fed a high ADG diet. Last, we presented that in 8-wk-old dairy heifers, feeding a high ADG diet compared with a low ADG diet had an additive effect on rumen epidermal cell proliferation and this occurred exclusively in the stratum basale of the epidermis. Rumen epidermal proliferation and differentiation events are likely coordinated with VFA absorption and might therefore affect nutrient utilization and feed efficiency. If true, better understanding of these regulated processes represent a likely point of animal management intervention that may improve whole animal feed efficiency. Continued work in this new area of rumen epidermal stem and progenitor cells, by our research group and others, will add to our knowledge of how the structure of the

Figure 2. The thymidine analog 5-bromo-2'-deoxyuridine (BrdU) can be used to label DNA. Here, BrdU was injected 24 h before slaughter and labeled all cells undergoing DNA synthesis in the S phase of the cell cycle. The top left panel shows that DNA synthesis in the rumen epidermis (bounded by white) occurs exclusively in the stratum basale [BrdU = light gray (green); DAPI nuclear counterstain = dark gray (blue)]. The top right panel is rumen tissue stained with Masson's trichrome provided for histological comparison (trichrome staining methods described in Yohe et al., 2015) with the organization of the proliferative and differentiated cell compartments of rumen epidermis defined. Scale bars are 10 μ m. The bottom panel shows that rumen epidermal DNA synthesis can be affected by diet in 62-d-old calves. Calves were fed either a restricted diet (R; n = 6) or an enhanced diet (EH; n = 4). Calves on R received a milk replacer (MR) diet fed at 0.44 kg of powder DM/d (20.9% CP, 29.8% fat, DM basis) and EH received MR at 1.08 kg of powder DM/d (28.9% CP, 26.2% fat, DM basis). All calves had access to a 20% CP starter. Lifetime DMI was 0.73 kg of DM/calf per d for R (5.88 Mcal of net energy/calf per d) and 1.26 kg of DM/calf per d for EH (10.68 Mcal of net energy/calf per d). * $P = 0.05$. Data are reported as LSM $\pm$ SEM. Color version available online.

rumen epidermis is maintained, how the rumen epidermis functions as a microbial barrier but also as a point of absorption for VFA, and how various nutrition programs affect ruminant nutrient utilization and feed efficiency.

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