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Butyrate inhibits the bovine rumen epithelial cell proliferation via downregulation of positive regulators at G0/G1 phase checkpoint

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Abstract: Short-chain fatty acids (SCFAs) butyrate promote the postnatal rumen epithelial development and maturation in ruminants. However, molecular mechanisms of effects of butyrate on the bovine rumen epithelial cells (BRECs) proliferation remain elusive. Therefore, purpose of this study was to investigate the effects of butyrate on the expression of genes and proteins at G0/G1 and S phase of BRECs cycle. Our results showed that BRECs treated with butyrate inhibited ($P < 0.05$) the proliferation of BRECs, relatively to control. Flow cytometric assays revealed that butyrate triggers the BRECs cycle arrest at the G0/G1 phase. qRT-PCR analyses of mRNA level of genes involved in the G0/G1 phase of cell cycle showed that butyrate significantly upregulated ($P < 0.001$) the expression of mRNA encoding $p21^{Cip1}$ compared with control group, but it decreased ($P < 0.05$) the mRNA levels of cyclin D1 and CDK4 genes at G0/G1 phase checkpoint compared with control. Moreover, Western blot also revealed that butyrate downregulated the expression of cyclin D3, CDK6, p-Rb, and E2F1 proteins involved in the modulation of G0/G1 phase of cell cycle. In conclusion, our results demonstrated that butyrate inhibits the proliferation of BRECs via downregulation of positive regulators at G0/G1 phase checkpoint.

Introduction

The high diet fiber is fermented into short-chain fatty acids (SCFAs) by rumen microorganisms in ruminants (Aschenbach *et al.*, 2011). The SCFAs can provide the main energy requirement for ruminants. The total SCFAs mainly constitute acetate, propionate, and butyrate (Kristensen *et al.*, 1998). The rumen papilla shows significant changes during calf rumen development before weaning (Lyford, 1988). The rumen shows the short papillae, and it accounts for less than 30% of the total stomach in newborn calves. However, the rumen accounts for 80% of the total stomach and has abundant and long papillae at 4 week-age. The rumen exhibits significant changes from birth to 4 weeks-age, which is probably involved in SCFAs. In particular, butyrate plays a vital role for rumen epithelial growth and development (Lane and Jesse, 1997).

Previous studies have demonstrated that addition of sodium butyrate in dietary ration can promote the ruminal papillae growth and maturation in young ruminants (Cavini *et al.*, 2015; Gorka *et al.*, 2009; Gorka *et al.*, 2018). However, the culture medium containing butyrate disrupted the rumen epithelial cells proliferation *in vitro* (Gálfi *et al.*, 1981) and other types of cells (Comalada *et al.*, 2006; Fu *et al.*, 2004; Liu *et al.*, 2019), which is not consistent with results *in vivo*. The inhibitory effect of butyrate on the rumen epithelial cells proliferation *in vitro* has been speculated that the stimulatory effect of butyrate on the ruminal papillae growth and epithelial development is indirect signal pathway *in vivo* (Comalada *et al.*, 2006; Fu *et al.*, 2004).

The histone deacetylases (HDACs) can regulate the cellular proliferation, differentiation, and apoptosis (Abramova *et al.*, 2006). Previous studies have shown that butyrate, a HDACs inhibitor, are able to inhibit the cell proliferation and stop the progression from G0/G1 and S phase of cell cycle (Khleif *et al.*, 1996; Johnstone, 2002; Piekarz and Bates, 2004). The abnormal proliferation of transformed cells is commonly accompanied by the dysfunctions of negative regulators of the cell cycle, including the $p16^{INK4A}$, $p15^{INK4B}$, $p21^{Cip1}$, $p27^{Kip1}$ cyclin-dependent kinase inhibitors (CDKI), phosphorylation of Rb tumor suppressor and an elevation in the expression of positive regulators of the cell cycle, including the cyclin D/E, cyclin-dependent kinases (CDK), and E2F transcription factor (E2F), leading to bypass of G0/G1 and S phase of cell cycle (Khleif *et al.*, 1996; Kiyono *et al.*, 1998). In particularly, the CDK/E2F signal pathway play a central role in the control of

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cellular proliferation (Chong *et al*., 2009; Wu *et al*., 2001). In this study, we tested the possibility that butyrate affected the proliferation of bovine rumen epithelial cells (BRECs) by changing in key regulator involved in CDK/E2F signal pathway *in vitro*. Therefore, objective of the study was to determine mechanisms underlying the regulatory effects of butyrate on the BRECs proliferation *in vitro*.

Materials and Methods

Bovine rumen epithelial cells culture

All procedures involving dairy cows were complied with the guidelines of the Institutional Animal Care and Use Committee of Yang Zhou University (SYXK (Su) IACUC 2012-0029). Bovine rumen epithelial tissues from 6- to 7-month-old Holstein calves were obtained from the Experimental Farm of Yang Zhou University. Primary bovine rumen epithelial cells (BRECs) were immortalized by lentiviruses expressing SV40 large T antigen and validated by cytokeratin 18 (Zhan *et al*., 2019), and the immortal BRECs were collected at the Institute of Animal Culture Collection and Application (IACCA), Yangzhou University. These immortal BRECs used in this study were provided by IACCA, Yangzhou University (China). Cells were incubated in the DMEM/F12 medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% non-essential amino-acids, 4 mm/L glutamine, 1× Insulin-Transferrin-Selenium (1× ITS; Invitrogen, Shanghai, China), 15 ng/mL EGF (Peprotech, Shanghai, China).

Cell proliferation test

The BRECs were seeded at a density of 1×10^3 cells/well in 96-well plates. After overnight, the BRECs were treated in the absence (control group) or presence of 10, 20, 40 mM butyrate. Cells were then incubated for 1, 2, 3, 4, 5, or 6 days, respectively. Next, cell viability was detected using Cell Counting Kit-8 (CCK-8) Assay Kit (Dojindo, Shanghai, China) according to the manufacturer's protocol. Briefly, 10 μL of CCK-8 solution was added to 200 μL of DMEM/F12 medium. After 2 h of incubation at 37°C, absorbance was measured using Multiskan Go microplate reader (Thermo Scientific, Shanghai, China) at 450 nm.

Flow cytometric assay

The BRECs were seeded at a density of 2×10^5 cells/well in 25-cm^2 tissue culture plates and incubated in culture medium at 37°C under 5% CO_2. Cells were isolated by trypsinization and washed by adding 40 mL PBS, and then centrifuged at 300 × *g* for 10 min to obtain the cell pellet. Cells were mixed with 5 mL cold 75% ethanol and stored at −20°C for 2 h. Thereafter, cells were washed in PBS and then incubated with stain buffer containing 5% horse serum. The supernatants were carefully discarded, and cells were treated by 100 μL PI/RNase Staining Buffer (BD Biosciences, Shanghai, China) for 15 min. DNA content were measured by flow cytometry (BD Biosciences, Shanghai, China), and characteristics of cell cycle phase were analyzed using ModFit LT software (Verity Software House, Topsham, USA).

RNA extraction and real-time quantitative PCR

Total RNA was extracted from BRECs using a TRIzol kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The 1 μg of total RNA reversely transcribed into cDNA using a 1× PrimeScript RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) reactions were performed using SYBR® Premix Kit (Takara), and the qRT-PCR reaction was carried out on LightCycler96 System in a final volume of 20 μL using 96-well microwell plates. The qRT-PCR reactions were initially denatured at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The primers were designed by Primer 6 software. The GAPDH gene was used as references. These primers used are listed in Table 1 and were synthesized by Suzhou Genewiz Biological Co. (Suzhou, China). The qRT-PCR analysis was calculated by the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Cells were extracted total protein using a radioimmunoprecipitation (RIPA) lysis buffer containing protease inhibitor buffer, and the cell lysates were incubated on ice for 30 min. Following centrifugation, cells were centrifuged at 20,000 × *g* for 10 min. Protein concentrations in the lysates were measured using a BCA protein assay kit (Thermo Scientific, Shanghai, China). Equal amounts of protein lysates were subjected to SDS-PAGE on a 10% polyacrylamide gel, following which polyacrylamide gels were transferred to nitrocellulose membranes (Pall, Shanghai, China). The membranes were blocked for 2 h with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST) or 5% horse serum (for phosphorylation primary antibody). After blocking, membranes were incubated with the primary antibody at 4°C overnight by gentle shaking. The primary antibodies were obtained from Cell Signaling Technology (CST, Shanghai, China): GAPDH (1:1000; #2118), CDK2 (1:1000; #2546), CDK6 (1:1000; #3136), and cyclin D3 (1:1000; #2936). The primary antibodies were obtained from Abcam: phosphorylated (p)-Rb (1:1000; ab184796) and E2F1 (1:1000; ab179445). The following primary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Shanghai, China): E2F3 (1:200; SC-878). The membranes were incubated by a second incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000; CST, Shanghai, China). The chemiluminescence detection of HRP-conjugated secondary antibodies were performed using the Western Blotting Detection Reagent kit Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Statistical analysis

Values were expressed as mean ± standard error of the results. All results were evaluated by one-way analysis of variance (ANOVA) for post-hoc multiple comparisons using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered significant differences and $P < 0.01$ was considered extremely significant differences.

Results

Butyrate inhibited bovine rumen epithelial cells proliferation

The immortalized BRECs have the ability to proliferate rapidly (Fig. 1). Compared with control, addition of 10, 20, and 40 mM butyrate to culture medium significantly inhibited the proliferation of BRECs ($P < 0.05$; Fig. 1). At 3

TABLE 1

Primers for real-time PCR analyses

Gene	Primer sequence, 5' to 3'	Accession number	Product size (bp)	Source
GAPDH	F: GGGTCATCATCTCTGCACCT	NM_001034034	176	Zhou *et al.* (2008)
	R: GGTCATAAGTCCCTCCACGA			
$p15^{INK4B}$	F: ACCCGGAAGTCACCTCAATT	NM_001075894	226	Present study
	R: GGGGCTCTCTGAATCCTACC			
$p16^{INK4A}$	F: CCTCTGAAGTCAAAAGGCGG	XM_010807758	121	Present study
	R: AAATCCTGACTCGTGGTGGG			
$p21^{Cip1}$	F: GCAGACCAGCATGACAGATT	NM_001098958	205	Wang and Jiang (2010)
	R: GTATGTACAAGAGGAGGCGT			
$p27^{Kip1}$	F: GACCTGCCGCAGATGATTCC	NM_001100346	249	Wang and Jiang (2010)
	R: CCATTCTTGGAGTCAGCGAT			
Cyclin E1	F: TTGACAGGACTGTGAGAAGC	XM_612960	229	Wang and Jiang (2010)
	R: TTCAGTACAGGCAGTGGCGA			
Cyclin E2	F: CTGCATTCTGAGTTGGAACC	NM_001015665	210	Wang and Jiang (2010)
	R: CTTGGAGCTTAGGAGCGTAG			
CDK4	F: ACTCTGGTATCGTGCTCCAGAAG	NM_001037594	114	Totty *et al.* (2017)
	R: CAGAAGAGAGGCTTTCGACGAA			
Cyclin D1	F: GCACTTCCTCTCCAAGATGC	NM_001046273	204	Wang and Jiang (2010)
	R: GTCAGGCGGTGATAGGAGAG			
Cyclin D2	F: CCAGACCTTCATCGCTCTGT	XM_024992177	163	Wang and Jiang (2010)
	R: GATCTTTGCCAGGAGATCCA			
HDAC1	F: CTCCATCCGCCCAGATAACA	NM_001037444	124	Wang *et al.* (2011)
	R: CACAGAGCCACCAGTAGACAG			
HDAC7	F: GCTTCTCAATAAGGACAAGA	NM_001193141	121	Present study
	R: ATTAGGATGAACCGTTCTCT			
HDAC8	F: GCGAAGATGGAGATGATGAT	NM_001076231	168	Present study
	R: CAGACCAGTTGATTGCTACT			

Note: F, forward; R, reverse.

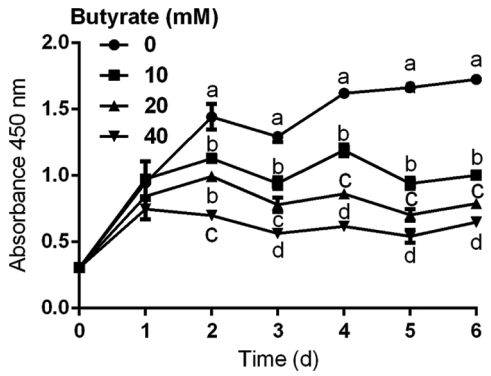

FIGURE 1. Effects of 10, 20, and 40 mM butyrate on the proliferation of BRECs. The BRECs were cultured in the absence (control group) or presence of 10, 20, and 40 mM butyrate. These cells were cultured for 1, 2, 3, 4, 5, or 6 days (N = 3 per group). Absorbance at 450 nm was measured in each well using an auto-microplate reader. Data are presented as means ± SEM (N = 3). Means with different letters (a–d) differed significantly according to time point.

and 6 days of culture, there were significant differences in the proliferation of BRECs between control and butyrate treatment groups ($P < 0.05$; Fig. 1).

Effects of butyrate on the bovine rumen epithelial cells cycle

As shown in Fig. 2, flow cytometric analysis also indicated that BRECs treated with 10, 20, and 40 mM butyrate included a higher proportion of cells in G0/G1 phase relatively to control group ($P < 0.05$; Figs. 2A and 2B). Consistent with this observation, the amount of BRECs treated by 10, 20, and 40 mM butyrate accounted for less cells at the S phase of cell cycle, relatively to control group ($P < 0.05$; Figs. 2A and 2B). These data suggest that butyrate inhibited the BRECs proliferation by disruption of progression from G0/G1 to S phase of the cell cycle.

Effect of butyrate on the mRNA expression of genes involved in the cellular proliferation regulator

As shown in Fig. 3, butyrate treatment increased the $p21^{Cip1}$ expression compared with control ($P < 0.001$; Table 2), while no significant difference was shown in the mRNA level of $p16^{INK4A}$ and $p27^{Kip1}$ between control and butyrate treatment ($P > 0.05$; Table 2). Surprisingly, the expression of $p15^{INK4B}$ was attenuated ($P < 0.001$) in BRECs treated by butyrate compares with control (Table 2). In addition, BRECs treated by butyrate significantly decreased ($P < 0.05$)

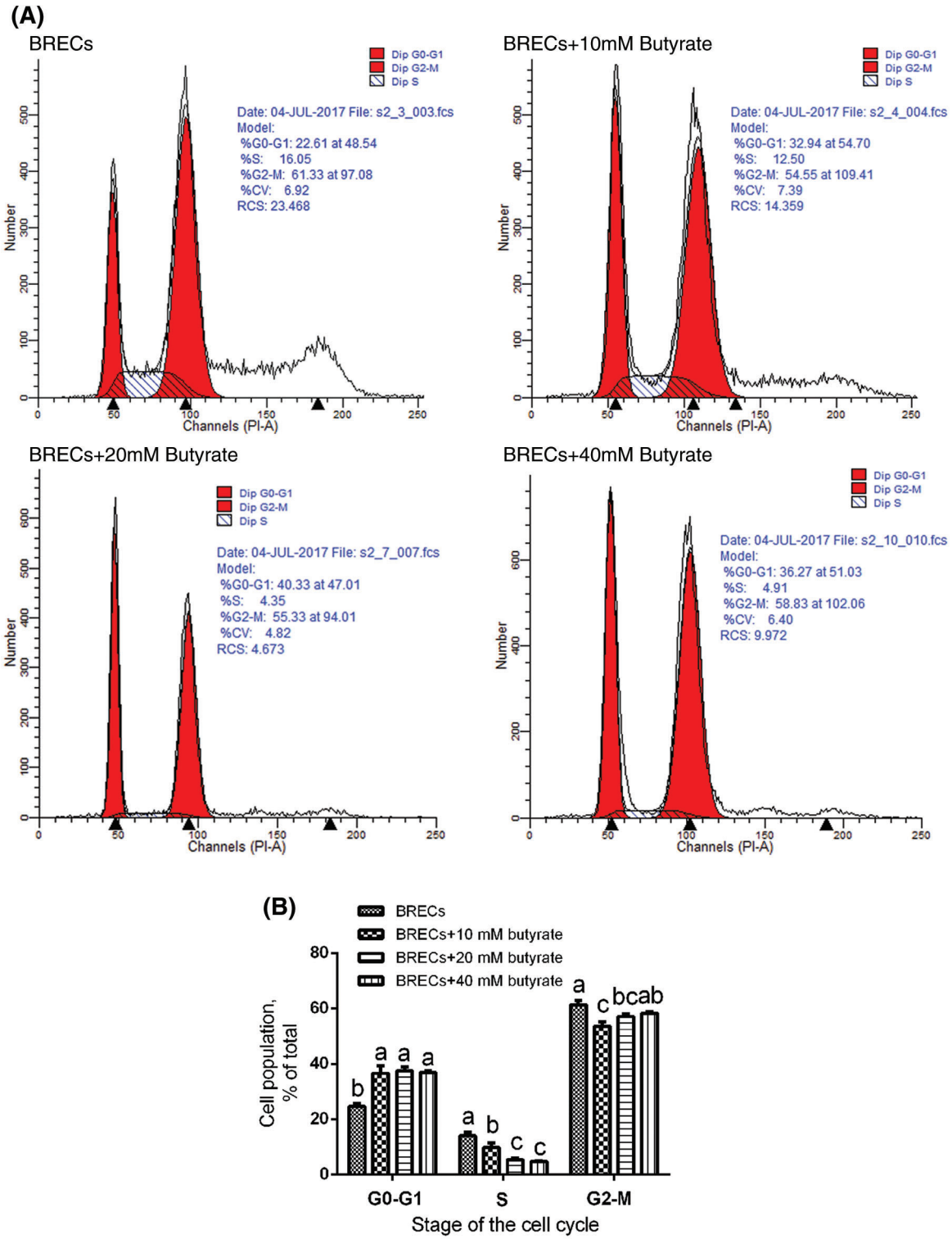

FIGURE 2. Effects of butyrate on cell cycle progression. The BRECs were cultured in the absence (control group) or presence of 10, 20, or 40 mM butyrate for 24 h. After incubation, cells were analyzed by flow cytometry. (A) A representative histogram of flow cytometric analyses. (B) Percentages of cells at different phases of the cell cycle. Data are presented as the means ± SEM (N = 3). Means with different letters (a–c) differed significantly according to treatment group.

the mRNA levels of CDK4 and cyclin D1 genes related to the modulation of G0/G1 phase of cell cycle compared with control, whereas there was no significant difference in the mRNA level of cyclin D2, cyclin E1, and cyclin E2 between control group and butyrate treatment ($P > 0.05$; Table 2). The levels of mRNA encoding HDAC1, HDAC7, and HDAC8 were attenuated ($P < 0.05$) in BRECs treated by butyrate in comparison with the control group (Table 2). The relationships between the mRNA expression of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and CDK4, cyclin D1, HDACs mRNA expression was shown (Fig. 3). There is a negative correlation between $p21^{Cip1}$ mRNA expression and CDK4, cyclin D1, HDAC1, HDAC7, and HDAC8 mRNA expression (Fig. 3; $P < 0.001$). These results

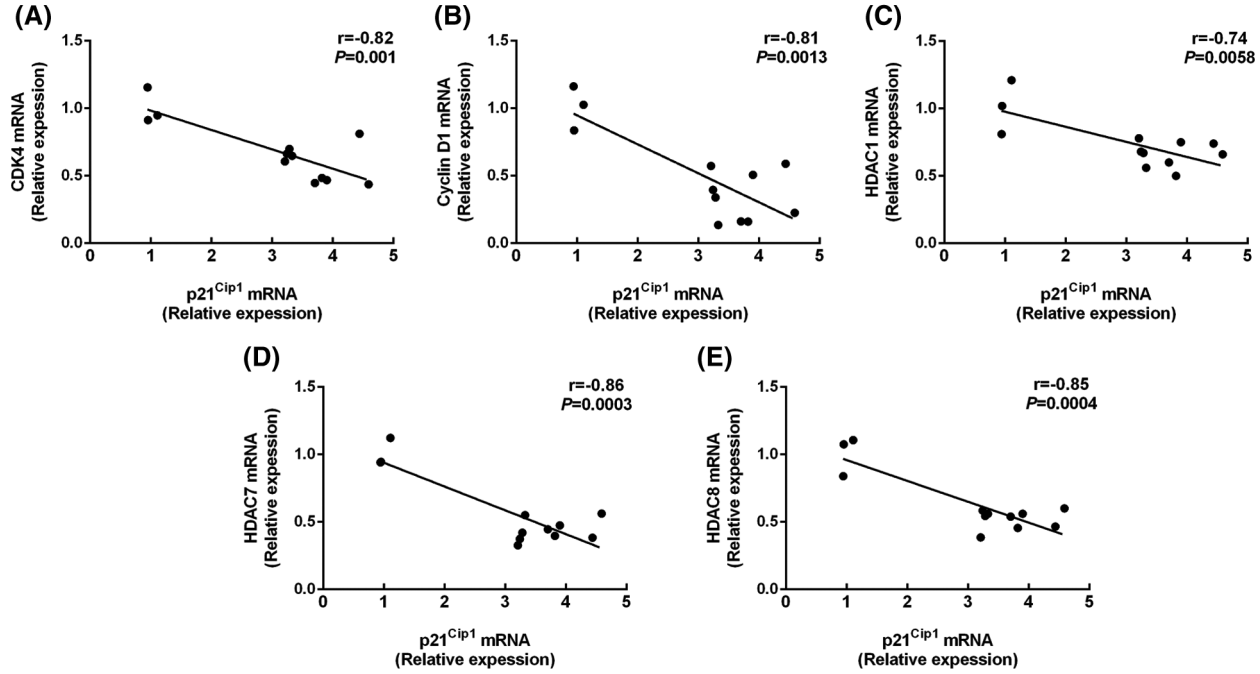

FIGURE 3. Correlations between the mRNA expression of the $p21^{Cip1}$ and CDK4 (A), cyclin D1 (B), HDAC1 (C), HDAC7 (D), and HDAC8 (E) mRNA expression. The figure includes data were obtained from untreated BRECs or butyrate treated BRECs. Data are presented as the means ± SEM (N = 3).

TABLE 2

Expression of genes involved in cell cycle regulators in BRECs incubated with 0 (control) or 10, 20, 40 mM butyrate *in vitro*

	Treatment[1]					
Symbol	**Control**	**10 mM**	**20 mM**	**40 mM**	**SEM**	***P*-value**
$p15^{INK4B}$	1.00^{a}	0.25^{b}	0.16^{bc}	0.13^{c}	0.10	<0.001
$p16^{INK4A}$	1.09	1.17	0.84	0.52	0.11	0.14
$p21^{Cip1}$	1.00^{b}	3.85^{a}	3.70^{a}	3.61^{a}	0.36	<0.001
$p27^{Kip1}$	1.07	0.60	0.95	0.98	0.09	0.62
Cyclin E1	1.01	1.46	1.25	1.20	0.06	0.07
Cyclin E2	1.00	0.84	0.98	0.93	0.03	0.42
CDK4	1.00^{a}	0.62^{b}	0.59^{b}	0.52^{b}	0.06	0.012
Cyclin D1	1.00^{a}	0.55^{b}	0.31^{c}	0.15^{c}	0.09	<0.001
Cyclin D2	1.04	1.17	1.02	0.99	0.05	0.78
HDAC1	1.01^{a}	0.75^{b}	0.67^{bc}	0.55^{c}	0.06	0.011
HDAC7	1.00^{a}	0.39^{b}	0.45^{b}	0.46^{b}	0.07	<0.001
HDAC8	1.00^{a}	0.47^{b}	0.57^{b}	0.51^{b}	0.06	<0.001

Note: ^{a,b,c}Means in the same row with different superscripts differ significantly for treatment effect.
[1]The BRECs were cultured in DMEM/F12 medium in the absence (control group) or presence of 10, 20, and 40 mM butyrate.

indicated that $p21^{Cip1}$ may play a vital role in the inhibitory role of BRECs proliferation.

Effect of butyrate on regulatory proteins at G0/G1 phase checkpoint

Although butyrate inhibited the proliferation of BRECs by upregulation of $p21^{Cip1}$ and downregulation of CDK4 and cyclin D1, key protein expression of CDK/E2F involved in the cyclin-dependent kinase regulator downstream signal pathway should be investigated. To confirm that the effect of butyrate on the CDK/E2F signaling pathway key protein, the protein expression of genes related to the CDK/E2F pathway (i.e., CDK2, CDK6, cyclin D3, p-Rb, E2F1, and E2F3) was determined by Western blot analysis in control and butyrate treatment group (Fig. 4). The level of CDK6 protein involved in the modulation of G0/G1 phase of cell cycle was attenuated by addition of butyrate compared with control, whereas there was no significant difference in the expression of CDK2 protein related to the modulation of S phase of cell cycle between control and butyrate treatment

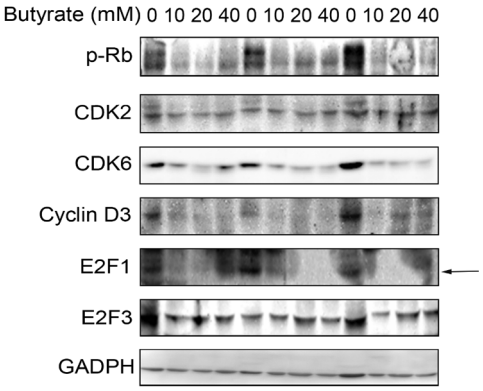

FIGURE 4. Effect of butyrate on regulatory proteins at G0/G1 phase checkpoint. The BRECs were cultured in the absence (control group) or presence of 10, 20, or 40 mM butyrate *in vitro*. After incubation, these cells were collected to extract total protein. Western blot analysis for CDK2, CDK6, cyclin D3, p-Rb, E2F1, and E2F3. Data are based on triplicate experiments.

(Fig. 4). In addition, butyrate treatment downregulated the level of cyclin D3 protein involved in the modulation of G0/G1 phase of cell cycle, relatively to control. Accordingly, the phosphorylation level of Rb was almost not expressed by addition of butyrate, compared to control (Fig. 4). There was no significant difference in the protein level of E2F3 between control and butyrate treatment. However, the protein expression of E2F1 activator was not found in BRECs treated by butyrate compared with control (Fig. 4). These findings suggested that butyrate decreased the expression of proteins (cyclin D3, CDK6, p-Rb, and E2F1) related to G1 progression phase of cell cycle, leading to an inhibition of BRECs proliferation.

Discussion

The short-chain fatty acids (SCFAs), acetate, and propionate, and butyrate, which are produced by fermentation of high diet soluble fiber by the rumen microbiota, play a vital role in the rumen epithelial development and maturation in newborn ruminants. However, the mechanisms underlying the effects of butyrate on the BRECs growth remain largely unknown (Vi *et al.*, 2003). Infusion of sodium butyrate promotes the growth and functional maturation of rumen papillae (Lane and Jesse, 1997; Liu *et al.*, 2019). In contrast, the inhibitory effect of butyrate on the proliferation of colon epithelial cells, neutrophil, and intestinal epithelial cells *in vitro* has been reported (Aoyama *et al.*, 2010; Comalada *et al.*, 2006; Qiu *et al.*, 2017). These reverse effects of butyrate on the cell proliferation between *in vivo* and *in vitro* was believed that rumen butyrate contributes to the development of rumen epithelium by other molecular pathways. Wang and Jiang (2010) reported that the BRECs treated by butyrate did not inhibit the proliferation of cells at the culture of 72 h, whereas the concentration of butyrate is only 1 mM. In fact, the different concentration butyrate should be used to investigate the inhibitory effect of butyrate on the proliferation of BRECs *in vitro*. In addition, the CDK/E2F signal pathway can control the cellular proliferation and the progression of the cell cycle (Chong *et al.*, 2009; Wu *et al.*, 2001). Therefore, we tested the possibility that butyrate affects the proliferation of BRECs by change in key regulator involved in CDK/E2F signal pathway *in vitro*. Our results demonstrated that butyrate significantly inhibited the proliferation of BRECs by downregulating the expression of mRNA and proteins related to G1 progression phase of cell cycle, including the cyclin D1, cyclin D3, CDK4, CDK6, p-Rb, and E2F1.

Our study has shown that butyrate inhibited the proliferation of BRECs by disruption of progression from G0/G1 to S phase of the cell cycle. The progression of the cell cycle from G0/G1 to S phase is regulated by cyclin, CDK, Rb, and E2F. The cyclin D1, D2, and D3 binds to the CDK4/6 and activates the CDK4/6, leading to the progression of the G0/G1 phase of the cell cycle (Sherr, 1994). However, the cyclin E1 and E2 interact with CDK2, triggering the progression of the S phase of the cell cycle (Lauper *et al.*, 1998). The activity of cyclin/CDK complex are regulated by CDKI, such as $p16^{INK4A}$, $p15^{INK4B}$, $p21^{Cip1}$, and $p27^{Kip1}$ protein (Fridman and Tainsky, 2008). Previous study has shown that rumen fluid enhances the mRNA expression of $p16^{INK4A}$ and $p21^{Cip1}$ to inhibit the progression of BRECs from the G1 phase to the S phase during the cell cycle (Wang and Jiang, 2010). However, the study did not investigate the expression level of CDK. In addition, rumen fluid did not change the cyclin D1, D2, D3, E1, and E2 expression levels (Wang and Jiang, 2010). Butyrate inhibited the progression of porcine intestinal epithelial cells from the G1 phase to the S phase of cell cycle by upregulating the $p21^{Cip1}$ and $p27^{Kip1}$ protein level and downregulating the CDK4 protein level (Qiu *et al.*, 2017). Surprisingly, an increase in butyrate concentration from 0 to 10 mM decreased the CDK6 protein expression. Our study demonstrated that butyrate enhanced the mRNA expression of $p21^{Cip1}$ in BRECs, and decreased the mRNA levels of CDK4, cyclin D1 and the protein expression of CDK6 and cyclin D3 of genes related to the modulation of G0/G1 phase of cell cycle, whereas butyrate did not change the mRNA levels of cyclin E1 and cyclin E2 and the protein expression of CDK2 of genes involved in the modulation of S phase of cell cycle. These results indicated that addition of butyrate inhibits the progression of BRECs at G0/G1 phase checkpoint during the cell cycle by upregulating the expression of $p21^{Cip1}$ and downregulating the expression of cyclin D1, cyclin D3, CDK4, and CDK6.

The molecular mechanism of the inhibitory effect of butyrate on the positive regulators of cell cycle and proliferation of BRECs *in vitro* should be further investigated. The CDK/E2F pathway is believed to play a critical role in control of cellular proliferation (Gupta *et al.*, 2015; Wu *et al.*, 2001). Replicative senescence is commonly accompanied by an elevation in CDKI levels and downregulation of CDK and E2F activators (Khleif *et al.*, 1996). In contrast, the activity of cyclin/CDK complex can trigger the phosphorylation of Rb, and this in turn causes the release of E2F activators that drive cell cycle progression for entering G1 into S-phase (Chen *et al.*, 2009). Previous studies have shown that mice lacking E2F1 and E2F2 are able to survive and develop to adulthood. However, mice deficient for E2F1 and E2F3 or E2F2 and E2F3 activators cannot develop to adulthood during early

embryonic development (Gupta *et al.*, 2015; Wu *et al.*, 2001). In addition, the mouse embryonic fibroblasts deficient for E2F1, E2F2, and E2F3 activators inhibited the expression of CDK and the phosphorylation of Rb, leading to a decrease in E2F target genes and a severe block in the proliferation of cell (Timmers *et al.*, 2007; Wu *et al.*, 2001). The butyrate downregulated the E2F1 expression and the activity of cyclin E/CDK2 complex, and enhanced the expression of $p21^{Cip1}$, leading to the inhibitory effect of primary embryonic fibroblast proliferation (Abramova *et al.*, 2006). Based on above studies, we investigated whether butyrate could mediate its inhibitory effect of cellular proliferation through modulation of the CDK/E2F signal pathway. In present study, our results showed that butyrate treatment caused the loss of p-Rb and E2F1 protein and decreased the cyclin D3 and CDK6 protein involved in the G0/G1 phase checkpoint in BRECs. The phosphorylation of Rb can disrupt the Rb/E2F complex to release of E2F activators during G0/G1 phase, leading to the proliferation of cell (Kiyono *et al.*, 1998; Wu *et al.*, 2001). Conversely, the activity of Rb/E2F complex induced by dephosphorylation of Rb or loss of E2F activators can inhibit the proliferation of cell (Kiyono *et al.*, 1998; Wu *et al.*, 2001). Our results demonstrated that butyrate can trigger the loss of E2F1 activators and a decrease in cyclin D3 and CDK6 protein levels at G0/G1 phase checkpoint, resulting in the block proliferation of BRECs. In conclusion, butyrate inhibits the progression of BRECs by downregulating the expression of genes and proteins involved in the G0/G1 phase checkpoint during the cell cycle.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

Authors' Contribution: Methodology, KZ and MCJ; writing—original draft preparation, KZ and MCJ; visualization, TYY and ZXH; funding acquisition, GQZ and KZ; writing—review and editing, GQZ and KZ; supervision, GQZ. All authors have read and agreed to the published version of the manuscript.

Ethics Approval: The experiments were approved by the Institutional Animal Care and Use Committee of Yang Zhou University (SYXK (Su) IACUC 2012-0029). The date of approval was 06 April 2016.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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