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Original Research Article

The developmental changes in intestinal epithelial cell proliferation, differentiation, and shedding in weaning piglets

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ABSTRACT

Intestinal epithelial homeostasis plays an important role in intestinal morphology and function. However, the developmental changes in intestinal epithelial cell turnover in piglets during early weaning are unknown so far. Thus, the aim of this work was to detect changes in piglet gut development from weaning to post-weaning d 14. Accordingly, 40 piglets were used in the present study, and 8 piglets were randomly selected for sampling at d 0, 1, 3, 7 and 14 post-weaning, respectively. The results showed that weaning stress significantly affected small intestinal morphological architecture, and this impact was the worst on d 3, and then returned to normal on d 14. Furthermore, the number of the marker of proliferation Ki-67 (Ki67) positive cells was decreased on d 1 and 3, and then recovered on d 14 ($P < 0.001$). Also, weaning strikingly increased jejunal epithelial cell shedding on d 1 to 7 compared on d 0 ($P < 0.05$). Moreover, weaning remarkably affected the number of small intestinal enterocytes, goblets and endocrine cells ($P < 0.05$), and there were also significant differences in genes expression related to proliferation and differentiation ($P < 0.05$). Additionally, the mechanistic target of rapamycin (mTOR) phosphorylation level was higher on d 3 ($P < 0.05$). However, the Wingless/Int1 (WNT)/β-catenin pathway was not influenced by post-weaning days. Taken together, weaning induced noteworthy changes in intestinal epithelial cell proliferation, differentiation and shedding, and the mTOR signaling pathway was involved in this process. Our findings provide a cellular mechanism for intestinal developmental changes during weaning periods. This may provide nutritionists with better insight into designing efficient in-feed alternatives for preventing the unfavorable gut development in weaning piglets.

1. Introduction

The small intestinal epithelium is organized by millions of crypt-villus units, and one unit consists of a villus which is a finger-like protrusion of the intestinal wall, surrounded by multiple invaginations called crypts (Gehart and Clevers, 2019). The villus mediates the absorption of nutrients and is regarded as a barrier that protects body health. The crypt contains stem cells, which give rise to progenitor cells that rapidly proliferate and eventually migrate up the villus and differentiate into absorptive enterocytes and secretory cells (endocrine cells and goblet cells), or differentiate into Paneth cells that migrate towards the base of the crypt

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(Yang et al., 2016; Verdile et al., 2019). Absorptive enterocytes constitute up to 90% of the epithelial cells in the intestinal epithelium, and are responsible for the absorption of nutrients (Fan et al., 2004). To maintain a constant and effective barrier function in a harsh intestinal environment and ensure the functions of the small intestine, villus epithelial cells undergo rapid renewal via highly coordinated processes of cellular proliferation, lineage-specific differentiation, and apoptosis along the crypt-villus axis (CVA) (Yang et al., 2016; Verdile et al., 2019). Briefly, after exiting the crypt, differentiated epithelial cells migrate until they arrive at the tip of the villus, where they eventually undergo apoptosis and are shed into the intestinal lumen (Gehart and Clevers, 2019; Krndija et al., 2019).

Weaning is one of the most significant events during the life of a pig (Yang et al., 2013a). Numerous studies have reported that weaning stress is associated with intestinal morphological structure, including shorter gut length, villus atrophy and crypt hyperplasia (Hampson, 1986; Beers-Schreurs et al., 1998; Montagne et al., 2007). In addition, it was established that early weaning induced the expression of proinflammatory cytokines, decreased enzymatic activities and resulted in a sustained impairment of intestinal barrier function (Pluske et al., 1997; Montagne et al., 2007). These changes in intestinal structures and function usually result in slow growth and post-weaning diarrhea (Pluske et al., 1997). Besides, it has been reported that compromising changes in intestinal structure are common during the weaning transition in piglets (Spreeuwenberg et al., 2001). Marion et al. (2002) also reported villous atrophy on d 3 and recovery to higher villus on d 14 after weaning. However, the concrete mechanism of weaning stress leading to changes in intestinal morphology and function is not well understood and research on changes in gut development within 14 d after weaning is limited.

Previous studies have shown that the mechanistic target of rapamycin (mTOR) and Wingless/Int1 (WNT)/β-catenin are best known as critical control factors in normal orderly development/differentiation, homeostasis and apoptosis (Korinek et al., 1998; Mariadason, 2001). Thus, we hypothesized that weaning stress could affect intestinal cell turnover through the mTOR or WNT/β-catenin signaling pathway in piglets. Accordingly, the objective of the present study was to determine the changes in intestinal morphology, epithelial cell proliferation, differentiation, and shedding as well as the activity of mTOR and WNT/β-catenin signaling in the small intestine of piglets after weaning.

2. Materials and methods

2.1. Animal ethics

The experimental procedure was reviewed and approved by the Animal Care and Use Committee of Hunan Normal University, Changsha City, Hunan, China.

2.2. Animals and diets

Forty piglets (Landrace × Yorkshire × Duroc; 6.55 ± 0.15 kg) were weaned at 21 d old, and all of them were fed the same corn-soybean meal diets. This experiment lasted 14 days. Diets were formulated in accordance with the nutritional specifications of piglet, and the detailed list of feed ingredients is available from our other published article related to this experiment (Zhou et al., 2019). Furthermore, all piglets had free access to feed and water during the experiment.

2.3. Sampling

Eight piglets per time-point were sacrificed at d 0, 1, 3, 7, and 14 after weaning for sampling. The small intestine was removed and its length and weight were measured. The duodenum, jejunum, and ileum were separated. After the contents were flushed thoroughly with a 0.9% NaCl solution, the mucosal layer was scraped away from the jejunum, rapidly frozen in liquid nitrogen, and stored at −80 °C for analysis of gene expression and protein synthesis. In addition, each intestinal tissue segment (approximately 2 cm) was fixed in 4% neutral-buffered formalin and stored at 4 °C before histological and immunohistochemical measurements.

2.4. Immunohistochemistry analysis

The immunohistochemistry procedure was conducted according to a previous study (Zhou et al., 2019). The formalin-fixed tissue samples were embedded in paraffin wax and sectioned onto glass slides (4-μm) by using a microtome (RM2235; Leica; Germany). The deparaffinized and rehydrated slides were incubated with 3% hydrogen peroxide to inhibit endogenous peroxidase. Subsequently, antigen retrieval was performed by using sodium citrate buffer (0.01 mol/L, pH 6.0). The sections were blocked with 5% bovine serum albumin (BSA; Boster Biological Technology Co. Ltd, Wuhan, China) and then incubated with antibodies (maker of proliferation marker Ki-67 [Ki67], Abcam, ab15580; chromogranin A [ChgA], Abcam, ab45179; 1:600 dilutions) overnight. Each step, except blocking, was followed by three washes with PBS for 5 min each. The positive cells were visualized with a diaminobenzidine Kit (ZSGB-BIO, Beijing, China). Images were acquired at 10× magnification with a light microscope (Leica DM3000, Leica Microsystems, Wetzlar, Germany).

The villus height (VH) and crypt depth (CD) were measured blindly using an Image-Pro Plus 6.0 software (Media Cybernetics; San Diego, CA, USA). For proliferation, the results were expressed as the number of Ki67 positive cells in each crypt. The average number of ChgA positive cells, a marker of endocrine cell, in the crypt and villus per piglet was determined from 30 complete villi and crypts. The number of columnar epithelial cells was also counted at 40× magnification on Ki67 immunohistochemical preparations. Epithelial cell shedding was evaluated according to the method of Bullen et al. (2006).

2.5. Alcian blue-periodic acid-shiff (AB-PAS) staining

The goblet cells were visualized by staining with AB-PAS staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. The stained slides were cover-slipped for examination under a light microscope (Leica DM3000; Germany). Goblet cells were counted in 30 villi and 30 crypts. The mean from 30 values of each sample was calculated and reported for each piglet (Zhou et al., 2019).

2.6. RNA extraction and real-time quantitative PCR

Total RNA was extracted from jejunal mucosa tissue samples using RNAiso Plus (TaKaRa, Dalian, China). One microgram of RNA was reverse-transcribed (RT) to cDNA using an RT reagent kit (TaKaRa, Dalian, China). The primers for β-actin, proliferation, and differentiation-related genes were designed with Primer Premier Version 5.0 (Premier Biosoft International, Palo Alto, California,

Table 1
Primers used for real-time PCR analysis.

Genes	Primers	Sequences (5′–3′)	Size	Accession no.
PCNA	Forward	AATGTTGATAAAGAGGAGGAAGCAG	116	NM_001291925.1
	Reverse	ACTGTAGGAGAGAGTGGAGTGGCTT		
Villin	Forward	ACGTGTCTGACTCCGAGGGAAAGGT	201	XM_030973673.1
	Reverse	ACTGCTTCGCTTTGATAAAGTTCAG		
ALP	Forward	GCTCTCCCTTGGCTTCATCC	140	AH012163.2
	Reverse	CATCCCCCAGAAAGAAATGAGGTT		
Hes1	Forward	AAGCTGGAGAAGGCGGACAT	152	NM_001280569.1
	Reverse	AAGCGGGTCACCTCGTTCAT		
TFF3	Forward	ATGTTCTGGCTGCTAGTGGTGCTCC	231	NM_001243483.1
	Reverse	TCAGAAGGTGCATTCTGTTTCCTGC		
MUC2	Forward	ACGCCATCCTGGGTGAGCT	121	XM_021082584.1
	Reverse	ACGCTGCCGTCCGACTTGA		
LYZ	Forward	AATAGCCGCTACTGGTGTAATGATG	148	NM_214392.2
	Reverse	ATGCTTTAACGCCTAGTGGATCTCT		
CHGA	Forward	CCAGCACCCACCCCTTAGCC	192	NM_001164005.2
	Reverse	CTTCTTCCTCCGGGACCGCC		
Atoh1	Forward	GGTGGTAGACGAGCTGGTTTG	170	XM_003129319.4
	Reverse	CGTTGTTGAAGGACGGGATAA		
NGN3	Forward	ACCAGACCGAGCAGCCTTTC	246	XM_021072424.1
	Reverse	GCATTCGATTGCGCTCACG		
SOX9	Forward	GCCTCTACTCCACCTTCACCTA	185	NM_213843.2
	Reverse	ATCACGGGCCATCATCACT		
β-actin	Forward	AGTTGAAGGTGGTCTCGTGG	215	XM_003357928.4
	Reverse	TGCGGGACATCAAGGAGAAG		

PCNA = proliferating cell nuclear antigen; *ALP* = alkaline phosphatase; *Hes1* = hairy enhancer of split 1; *TFF3* = trefoil factor 3; *MUC2* = mucin 2; *LYZ* = lysozyme; *CHGA* = chromogranin A; *Atoh1* = atonal homolog 1; *NGN3* = neurogenin 3; *SOX9* = sex determining region Y-box 9.

USA). The sequences of the PCR primers are listed in Table 1. The cDNA sample was diluted (1:5) with sterile, double-distilled water (ddH_2O) prior to use in PCR reactions. Each PCR reaction was performed in triplicate as described by a previous study (Yang et al., 2013b), and had a 10-μL reaction volume containing 5 μL of SYBR Green quantitative PCR mix (TaKaRa, Dalian, China), 1 μL of cDNA, 0.3 μL each of forward and reverse primers and 3.4 μL of ddH_2O. The β-actin served as an internal control to calculate the relative expression levels of target genes using the $2^{-\Delta\Delta Ct}$ method (Yang et al., 2013b).

2.7. Western blotting

Western blot was performed as described earlier (Yan et al., 2018). Frozen jejunal mucosa samples were powdered under liquid nitrogen and lysed in RIPA buffer with protease inhibitor PMSF (Beyotime Biotechnology, Shanghai, China), followed by centrifugation at 12,000 × *g* for 10 min at 4 °C to obtain the supernatants. Briefly, the denatured proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes at 200 mA for 45 min. The membranes were blocked with 5% nonfat milk at room temperature for 2 h and then incubated with primary antibody (β-actin [Santa, SC-47778; 1:3,000 dilution], mTOR [Cell Signaling Technology, 7C10; 1:1,000], phosphorylated mTOR [p-mTOR; Cell Signaling Technology, D9C2; 1:1,000], non-phospho-β-catenin [non-p-β-catenin; Cell Signaling Technology, D2U8Y; 1:1,000 dilution], β-catenin [Cell Signaling Technology, D10A8; 1:1,000 dilution]) at 4 °C overnight. After being washed three times with TBST, the membranes were incubated with a secondary antibody (1:3,000 dilution) at room temperature for 2 h. Finally, the membranes were washed with TBST, and chemiluminescence was used to visualize the protein bands. Densitometric analysis of band intensities was performed with the Gel-pro Analyzer software (Media Cybernetics, Inc., USA). The density of each band, representing the abundance of the protein expression, was expressed relative to the density of the corresponding β-actin band.

2.8. Statistical analysis

All data analysis was performed by SPSS software (version 22.0; IBM Corp., Chicago, IL, USA). The differences among means were examined by a one-way ANOVA followed by Duncan's multiple comparisons. The normality of the data distribution was detected by using the histograms and Shapiro–Wilk test, and any value was excluded if it departed more than three standard deviations from the standardized mean. The *P*-values < 0.05 were considered as statistically significant, whereas $P < 0.10$ was used to indicate a tendency towards significance. Data are expressed as the mean and the standard error of the mean (SEM) in the Tables and figures ($n = 8$).

3. Results

3.1. Effect of days after weaning on intestinal morphology

Dramatic changes in small intestinal morphological structure were observed following weaning (Table 2). In the duodenum, VH decreased by approximately 20% on d 3, and then gradually recovered on d 7 and 14 compared to d 0 ($P < 0.01$). The duodenal CD was greatest on d 14 and decreased 16% on d 3 compared to d 0 ($P < 0.001$). The villus width was decreased on d 3 and 7 compared to d 0 and 1 ($P < 0.001$). The ratio of VH to CD (VH:CD) decreased by approximately 21% on d 14 compared with d 0 ($P < 0.05$). Besides, in the jejunum, the VH was reduced by 24% on d 1 and 3 compared to d 0 ($P < 0.05$). The CD increased by 26% on d 7 and 14 compared to d 1 and 3 post-weaning ($P < 0.001$). The VH:CD tended to decrease on d 7 post-weaning ($0.05 < P < 0.1$). For the ileum, the VH was lowest on d 3 and greatest on d 0 and 14 compared to another groups ($P < 0.001$), and the ileal CD and villus width were higher on d 14 than on other days post-weaning

Table 2
Small intestinal morphology in weaning piglets.[1]

Item	Day post-weaning, d					SEM	*P*-value
	0	1	3	7	14		
Duodenum							
Villus height, μm	353.51[a]	284.36[c]	279.36[c]	300.70[bc]	347.06[ab]	8.87	0.008
Crypt depth, μm	239.11[bc]	211.93[c]	206.85[c]	256.84[b]	311.69[a]	7.99	<0.001
Villus width, μm	99.44[a]	99.37[a]	83.85[c]	79.12[c]	91.37[b]	1.61	<0.001
VH:CD, μm:μm	1.51[a]	1.36[ab]	1.36[ab]	1.19[b]	1.14[b]	0.04	0.042
Jejunum							
Villus height, μm	355.74[a]	284.17[c]	271.59[c]	305.91[bc]	343.73[ab]	8.44	0.010
Crypt depth, μm	174.55[bc]	159.16[c]	152.22[c]	205.21[a]	195.47[ab]	4.88	<0.001
Villus width, μm	84.03	82.11	79.21	80.60	79.82	0.97	0.537
VH:CD, μm:μm	2.06	1.83	1.80	1.52	1.78	0.06	0.057
Ileum							
Villus height, μm	332.71[a]	282.39[b]	218.24[c]	278.34[b]	328.83[a]	8.48	<0.001
Crypt depth, μm	181.57[ab]	134.76[c]	147.84[bc]	210.79[a]	224.60[a]	8.39	<0.001
Villus width, μm	87.48[b]	84.75[b]	80.08[b]	83.50[b]	100.14[a]	1.64	<0.001
VH:CD, μm:μm	1.85[ab]	2.13[a]	1.48[b]	1.51[b]	1.49[b]	0.07	0.004

VH:CD = the ratio of villus height to crypt depth.
[a to c] Within a row, means without a common superscript differ significantly at $P < 0.05$.
[1] Values are presented as means with SEM ($n = 8$).

($P < 0.001$). In addition, ileal VH:CD decreased by approximately 20% on d 3, 7 and 14 compared with d 0 and 1 ($P < 0.01$).

3.2. Effect of days after weaning on proliferation and shedding of intestinal epithelial cell

As shown in Table 3 and Fig. 1, the results of Ki67 immunohistochemistry showed that the number of Ki67 positive cells in the duodenum decreased by 22% on d 1 and 3 compared to d 0, 7 and 14 ($P < 0.001$). In the jejunum and ileum, the Ki67 positive cell numbers decreased by 44% and 36% on d 1 and 3, respectively, and gradually recovered from d 7 to d 14 ($P < 0.001$). However, cell shedding increased by 7% to 8% on d 7 compared with the rates on d 0 and 14 in the jejunum ($P < 0.05$). In the duodenum and ileum, alterations in cell shedding were not influenced by weaning time.

3.3. Effect of days after weaning on intestinal epithelial cell differentiation

Compared to the pre-weaning stage (d 0), the enterocyte numbers in the duodenum were greatest on d 14, but lowest on d 1 after weaning (Table 3; $P < 0.001$). This number in the jejunum decreased by 17% on d 1 (Table 3; $P = 0.025$). However, in the ileum, the number of epithelial cells was the lowest on d 7 ($P = 0.002$) among all days post-weaning (Table 3). The number of goblet cells (Table 4; Fig. 2) in both the duodenal and jejunal villi (46% and 59%) and jejunal crypts (34%) increased on d 14 ($P < 0.001$). In the ileal villus, the goblet cell number transiently increased on d 1 post-weaning compared with other days, and then increased on d 7 and 14 ($P < 0.001$). Furthermore, the goblet cell numbers in the duodenum and jejunum crypt were greater on d 14 than on other days ($P < 0.001$), but the goblet cell number in the ileal crypt significantly increased ($P = 0.040$) on d 1, 7, and 14 compared with d 0 and 3 (Table 4; Fig. 2). In addition, there was a significant decrease in the number of ChgA positive cells in the duodenal ($P = 0.003$) and jejunal ($P = 0.014$) villi on d 7 post-weaning, and the number of ChgA positive cells in the ileum villi also tended to decrease on d 7 and 14 ($P = 0.095$). In the jejunal crypt, the ChgA positive cell numbers were higher on d 1 and 3 than on d 0, 7, and 14 ($P = 0.006$). However, the number of ChgA positive cells was not significantly different when comparing between the ileal and duodenal crypts (Table 4; Fig. 3).

3.4. Effect of days after weaning on the mRNA expression of proliferation and differentiation-related genes

As shown in Table 5, in the jejunum, the mRNA abundance of *Villin* was lowest on d 1 ($P = 0.003$). The hairy enhancer of split 1

Table 3
Number of epithelial cells, proliferation and shedding cells in weaning piglets.[1]

Item	Day post-weaning, d					SEM	*P*-value
	0	1	3	7	14		
Proliferating cell number							
Duodenum	23.89[a]	18.56[b]	16.61[b]	25.37[a]	24.23[a]	0.73	<0.001
Jejunum	28.59[a]	15.88[d]	14.99[d]	19.87[c]	24.94[b]	0.98	<0.001
Ileum	23.09[a]	13.85[c]	14.78[c]	19.53[b]	24.93[a]	0.81	<0.001
Shedding cell number							
Duodenum	0.21	0.24	0.23	0.22	0.22	0.01	0.660
Jejunum	0.21[b]	0.28[a]	0.28[a]	0.29[a]	0.24[ab]	0.01	0.021
Ileum	0.19	0.19	0.19	0.18	0.20	0.01	0.950
Epithelial cell number							
Duodenum	169.64[bc]	128.85[d]	195.39[b]	160.81[c]	250.77[a]	7.86	<0.001
Jejunum	172.76[abc]	143.60[c]	159.42[bc]	183.58[ab]	200.05[a]	6.07	0.025
Ileum	181.14[a]	184.32[a]	169.75[a]	134.94[b]	179.50[a]	4.57	0.002

[a to d] Within a row, means without a common superscript differ significantly at $P < 0.05$.
[1] Values are presented as means with SEM ($n = 8$).

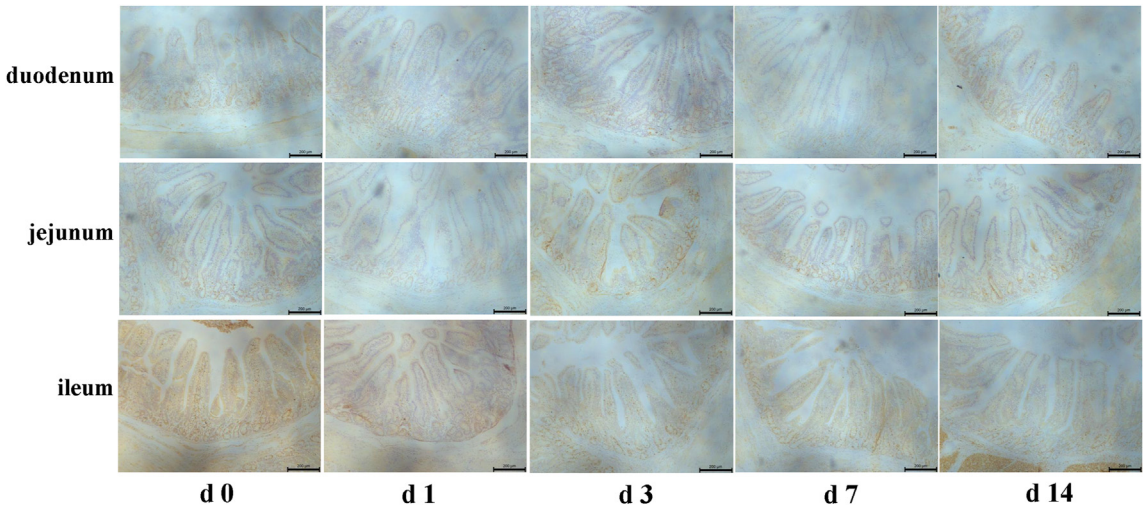

Fig. 1. Representative immunohistochemistry images of a piglet small intestine show proliferating cells, in brown, using the maker of proliferation Ki-67 (Ki67) antibody. Images were taken at 10× magnification. Bar = 200 μm.

Table 4
Number of goblet cells and endocrine cells in weaning piglets.[1]

Item	Day post-weaning, d					SEM	*P*-value
	0	1	3	7	14		
Goblet cells in villus							
Duodenum	12.85^{b}	13.02^{b}	11.52^{b}	8.83^{b}	18.83^{a}	0.78	<0.001
Jejunum	9.05^{c}	11.51^{b}	9.04^{c}	8.66^{c}	14.38^{a}	0.49	<0.001
Ileum	19.10^{b}	23.99^{a}	12.24^{c}	18.92^{b}	19.94^{b}	0.81	<0.001
Goblet cells in crypt							
Duodenum	6.04^{b}	5.80^{b}	6.78^{ab}	5.92^{b}	7.10^{a}	0.16	<0.001
Jejunum	9.91^{b}	9.92^{b}	10.10^{b}	11.25^{b}	13.27^{a}	0.28	<0.001
Ileum	6.63^{b}	8.27^{a}	6.38^{b}	7.68^{a}	8.30^{a}	0.18	0.040
Endocrine cells in villus							
Duodenum	4.08^{a}	2.90^{ab}	2.92^{ab}	1.41^{b}	2.22^{bc}	0.23	0.003
Jejunum	1.82^{abc}	2.25^{a}	2.19^{ab}	1.38^{c}	1.65^{bc}	0.07	0.014
Ileum	2.10	2.28	1.92	1.59	1.61	0.09	0.095
Endocrine cells in crypt							
Duodenum	1.91	1.59	1.80	1.21	1.93	0.09	0.123
Jejunum	1.50^{b}	1.89^{a}	1.93^{a}	1.42^{b}	1.39^{b}	0.06	0.006
Ileum	1.43	1.65	1.54	1.47	1.38	0.06	0.144

[a] to [c] Within a row, means without a common superscript differ significantly at $P < 0.05$.
[1] Values are presented as means with SEM ($n = 8$).

(*Hes1*) mRNA level tended to increase on d 14 compared with d 0 and 1 ($P = 0.082$). The mRNA expression of trefoil factor 3 (*TFF3*) was significantly upregulated 2.2-fold on d 14 compared to d 0 ($P < 0.001$). Moreover, the mRNA expression of *MUC2* (mucin 2) was upregulated 3 to 5-fold on d 7 and 14 ($P < 0.001$) compared to d 0. The gene expression of lysozyme (*LYZ*) tended to be elevated on d 7 and 14 compared to d 1 and 3 ($P = 0.077$). Furthermore, the atonal homolog 1 (*Atoh1*) increased approximately 1.3-fold on d 7 and 14 compared to d 0 to 3 ($P < 0.001$). However, the neurogenin 3 (*NGN3*) mRNA level was higher on d 1, 3, and 14 than on d 0 and 7 ($P = 0.035$).

3.5. Effect of days after weaning on WNT/β-catenin and mTOR signaling pathway

The effects of weaning on the mTOR and WNT/β-catenin signaling pathway were studied in piglet jejunal mucosa (Fig. 4). The ratio of p-mTOR/mTOR was higher on d 3 than on those on all other days post-weaning ($P < 0.05$). However, the ratio of non-p-β-catenin/β-catenin did not significantly change on different days

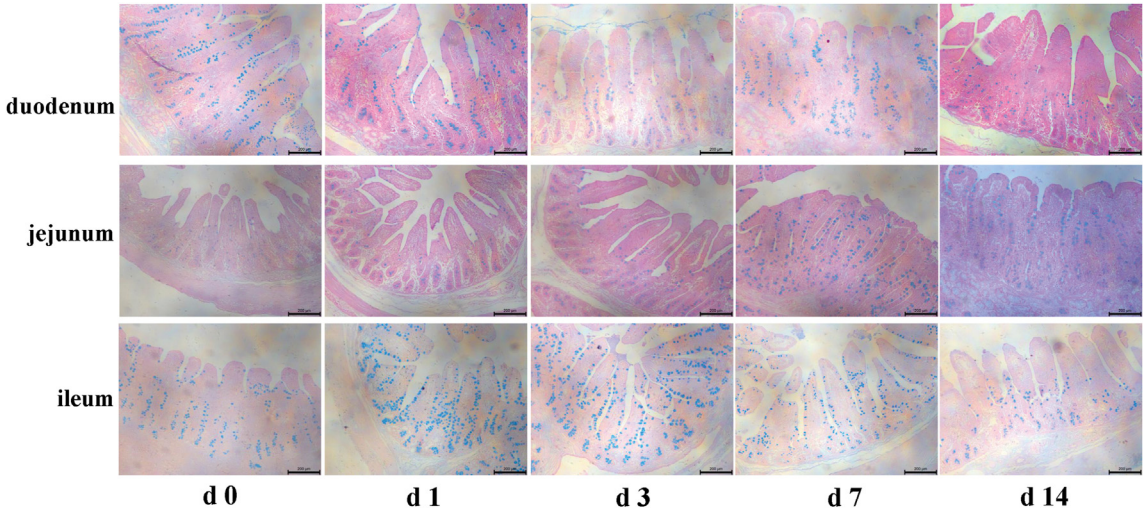

Fig. 2. Representative images of Alcian blue-periodic acid-Schiff (AB-PAS) staining of piglet small intestine. Images were taken at 10× magnification. Bar = 200 μm.

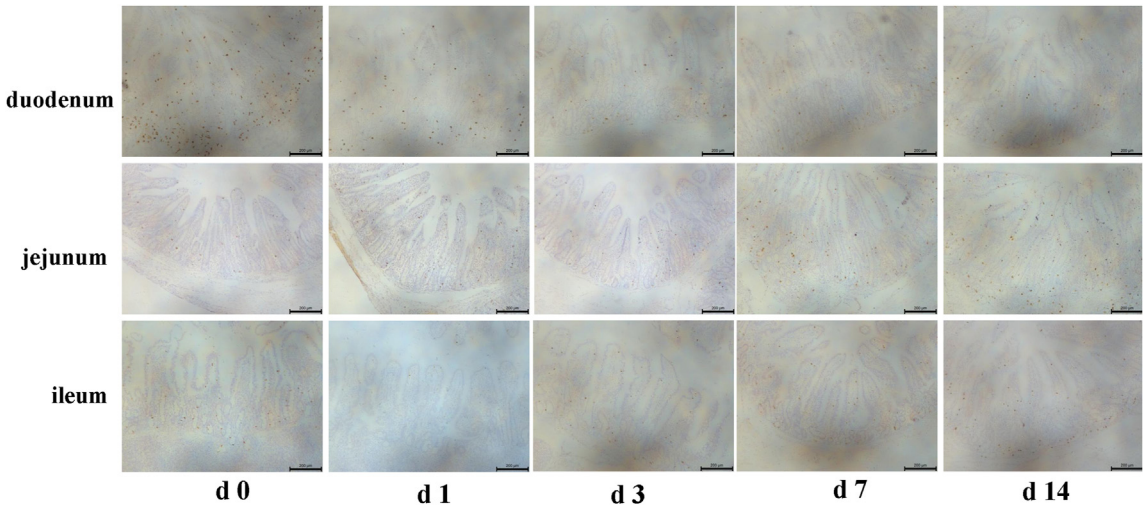

Fig. 3. Representative immunohistochemical images of a piglet small intestine show enteroendocrine cells, in brown, using chromogranin A antibody. Images were taken at 10× magnification. Bar = 200 μm.

Table 5
Expression of proliferation and differentiation-related genes in weaning piglets.[1]

Item[2]	Day post-weaning, d					SEM	*P*-value
	0	1	3	7	14		
PCNA	1.05^{a}	0.78^{b}	0.47^{b}	0.75^{b}	0.71^{b}	0.06	0.103
Villin	1.10^{bc}	0.69^{c}	1.63^{ab}	1.98^{a}	1.98^{a}	0.16	0.003
ALP	1.10	0.65	1.27	1.03	1.22	0.10	0.315
Hes1	1.07	0.98	1.17	1.34	1.63	0.08	0.082
TFF3	1.04^{c}	2.05^{ab}	1.78^{bc}	2.17^{ab}	3.29^{a}	0.19	<0.001
MUC2	1.04^{b}	1.59^{b}	1.74^{b}	4.63^{a}	6.23^{a}	0.47	<0.001
LYZ	1.45	1.12	0.69	1.93	1.41	0.14	0.077
CHGA	1.04	0.95	1.25	1.18	1.16	0.07	0.668
Atoh1	1.07^{b}	1.25^{b}	1.26^{b}	2.84^{a}	2.41^{a}	0.15	<0.001
NGN3	1.05^{b}	1.89^{a}	2.35^{a}	0.68^{b}	1.53^{a}	0.22	0.035
SOX9	1.16	1.51	1.02	1.86	1.93	0.16	0.129

[a to c] Within a row, means without a common superscript differ significantly at $P < 0.05$.

[1] Values are presented as means with SEM ($n = 8$).

[2] *PCNA* = proliferating cell nuclear antigen; *ALP* = alkaline phosphatase; *Hes1* = hairy enhancer of split 1; *TFF3* = trefoil factor 3; *MUC2* = mucin 2; *LYZ* = lysozyme; *CHGA* = chromogranin A; *Atoh1* = atonal homolog 1; *NGN3* = neurogenin 3; *SOX9* = sex determining region Y-box 9.

post-weaning, although this ratio was higher on d 0, 3 and 14 compared to d 1 and 7.

4. Discussion

Previous studies reported that weaning induced dramatic changes in the intestinal morphology of weaning piglets (Cera et al., 1988; Tsukahara et al., 2016), which was similar to our previous data (Zhou et al., 2019). Besides, we found that the duodenal, jejunal and ileal VH significantly decreased on d 1 and 3 after weaning compared with d 0, and the values recovered to normal levels on d 14. It has been reported that a marked change in piglets after weaning involves the gastrointestinal tract structure and function, which may be due to decreased feed intake, particularly in the small intestinal mucosa (Pluske et al., 1997). Our previous study discovered that the average daily feed intake was lowest on d 1, and individually recovered from d 3 to 14. Compared with other days after weaning, the average daily gain significantly decreased on d 1 and 3 (Zhou et al., 2019), which was consistent with the change in intestinal morphology. Following weaning, the most obvious changes in the small intestine are reduced VH and increased CD. Such changes in small intestinal morphology resulted in greatly reduced VH:CD in weaning piglets in accordance with earlier observations (Hu et al., 2013).

Unquestionably, the maintenance of intestinal morphology of the small intestine relies on a permanent turnover of the intestinal epithelium (Yang et al., 2013a, 2016). The intestinal epithelium is composed of a monolayer of epithelial cells, which plays a major role in the digestion and absorption of nutrients, and forms a gut barrier against luminal antigens, pathogens, and toxins (Gehart and Clevers, 2019). Intestinal epithelial cells undergo continual renewal that involves highly coordinated processes of cellular proliferation, lineage-specific differentiation, migration and apoptosis along the CVA (Pácha, 2000; Yang et al., 2016). The continual and normal renewal of epithelial cells along the CVA is accompanied by functional specialization, thereby maintaining the integrity of intestinal morphology and function (Yang et al., 2013a, 2016).

A previous study showed that the decrease in VH is either the result of increased cell loss or a brief reduction in the crypt cell population (Pluske et al., 1997). In order to explore the mechanism of weaning on the decrease of VH and increase of CD, we further investigated the effect of weaning on cell shedding and proliferation of intestinal epithelial cells. The findings showed that cell shedding in the jejunum was higher from 1 to 7 d post-weaning, whereas the number of Ki67 positive cells, a proliferation marker, was lower during the same period. Therefore, the decrease in VH may be due to increased cell shedding and decreased cell proliferation. However, the increased intestinal epithelial cell shedding at the early stage after weaning has generally been associated with a reduced digestive and absorptive capability (Pluske et al., 1996), which may be an important factor that causes the poor growth performance. The majority of gut mucosal cells are absorptive enterocytes (Traber et al., 1991). Along with the reduction in cell production and the increase in cell shedding, the number of villus enterocytes also decreased. Fortunately, these changes recovered on d 14 post-weaning, and this was confirmed by the expression of *villin*, which is expressed in all intestinal lineages and in their multipotential precursors.

In addition to the enterocytes mentioned above, crypt-generated cells also differentiate into other cell types by expressing specific genes, mainly including enteroendocrine, goblet, and Paneth cells (Gehart and Clevers, 2019). The results showed that

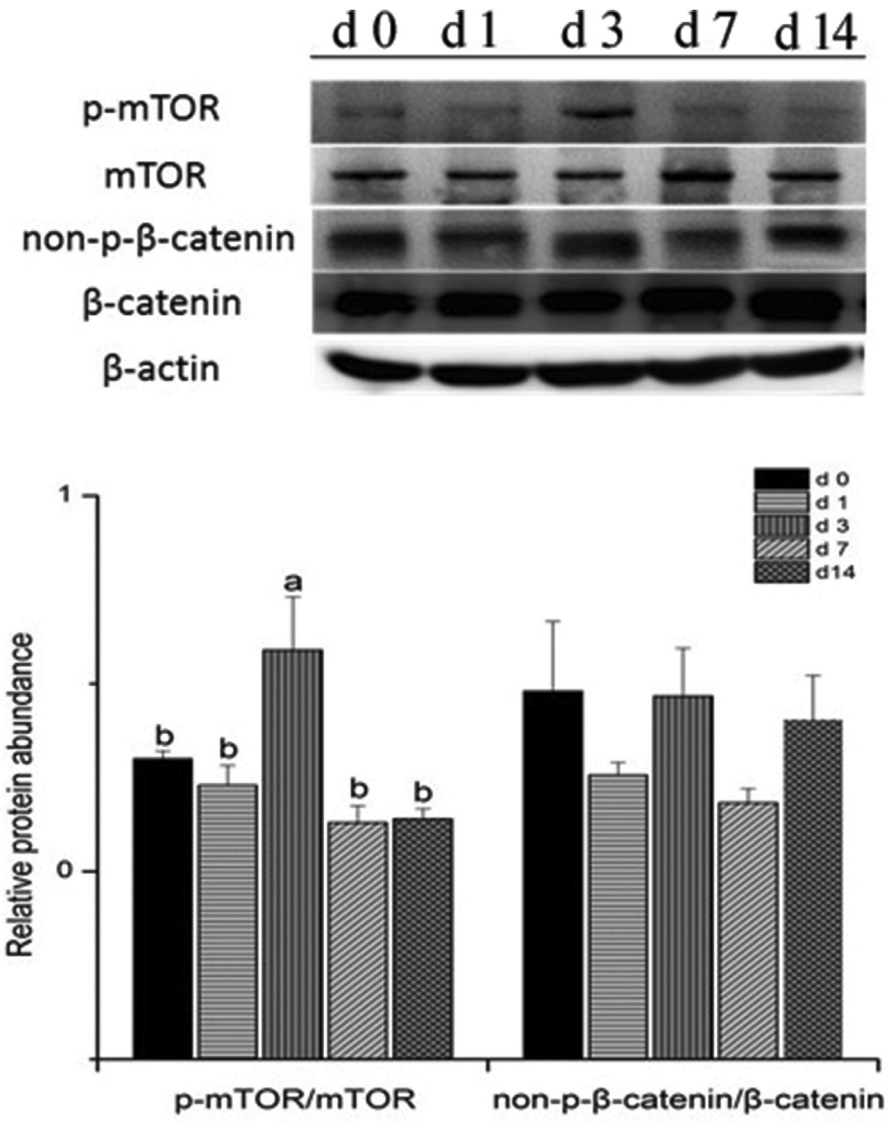

Fig. 4. Effects of weaning on mTOR and Wingless/Int1 (WNT)/β-catenin signaling pathway in piglet jejunal mucosa tissues. The expression of proteins was measured using Western blotting and β-actin was used as an internal control to normalize abundance. [a,b] Within a row, means without a common superscript differ significantly at $P < 0.05$. mTOR = mechanistic target of rapamycin.

weaning conspicuously lowered the duodenal and jejunal goblet cell numbers in villi and crypts on d 0 to 7 compared with d 14. This occurred in parallel with the change in the mRNA expression of *Muc2*, which is a marker of goblet cells, and *NGN3*. But the increased expression of the latter may also imply an increase in enterocytes (Li et al., 2012), which may be a crucial factor that leads to the reduction of goblet cells in the jejunum on d 1 and 3. Interestingly, the number of goblet cells in the ileum was highest on d 1, which may be a protective mechanism for microbial disorders resulting from acute weaning, through the secretion of mucus (Smith et al., 2009; Birchenough et al., 2015). In addition, the altered expression of *TFF3* produced by goblet cells may be related to the healing of epithelial injury (Jagla et al., 1999; Matsuoka et al., 1999). Moreover, we speculated in the early pre-weaning period (d 1 to 3), poor feed intake resulted in an insufficient energy supply (Zhou et al., 2019), thereby causing the number of goblet cells to be reduced in the small intestine, and gradually recovering on d 14 when the feed intake increased in piglets. The *Hes1* and *Atoh1* are differentiation-related drivers of absorptive (enterocytes) and secretory (enteroendocrine, goblet, and Paneth cells) lineages, respectively (Jensen et al., 2000; Yang et al., 2002). It has been reported that *Hes1* negatively regulates cell differentiation by suppressing *Atoh1* (also known as *Math1*) (Yang et al., 2002; Bjerknes et al., 2012). We found that the *Atoh1* was significantly elevated on d 7 and 14. This means that intestinal epithelial cells increase in differentiation in the later stage post-weaning, suggesting that the stem/progenitor cell fate is pushed towards a secretory lineage (Formeister et al., 2009; Bjerknes et al., 2012; Lo et al., 2017). Although endocrine cells change after weaning, there is no difference in the mRNA expression of *ChgA* and sex determining region Y-box 9 (*SOX9*), which may indicate the presence of mostly immature endocrine cells (Formeister et al., 2009). In addition, a trend of increased *LYZ* expression was observed during the late period after weaning, suggesting that the differentiation of Paneth cells was elevated (Mori-Akiyama et al., 2007). However, Crissey et al. (2011) concluded that the number of goblet and Paneth cells may increase at the expense of other cell types. Thus, fewer enteroendocrine cells may also be due to a

simultaneous increase in the number of goblet and Paneth cells. However, this must be further confirmed in the future.

The expression of enterocytic alkaline phosphatase (*ALP*) is cell differentiation dependent along the CVA, and early weaning reduces the expression of jejunal *ALP* (Lackeyram and Yang, 2010). These findings are inconsistent with our research, which may be attributed to the different research paradigms. Weaned piglets and suckling piglets were used in the former study, but we used weaned piglets herein. In our study, although jejunal CD was significantly different, proliferating cell nuclear antigen (*PCNA*) expression was unchanged. It may be implied that there is no correlation between CD and *PCNA* expression, which was also observed in the study of Xie et al. (2019). Taken together, weaning stress affects cell proliferation and differentiation, and cell shedding in the intestines of piglets.

The canonical WNT/β-catenin signaling pathway is likely closely associated with stem cell maintenance and differentiation in the intestinal epithelium, and the key switch in this pathway is the cytoplasmic protein β-catenin (Nusse and Clevers, 2017; Gehart and Clevers, 2019). Our data demonstrated that there was no difference in the β-catenin protein level; thus, weaning days did not affect WNT-dependent crypt stem cells. In addition, mTOR also plays a central role in regulating most major cellular functions, including proliferation, differentiation, apoptosis, and cell growth, because of its ability to integrate signals from nutrients and growth factors (Fingar and Blenis, 2004; Yang et al., 2016). Our previous study showed that the mTOR signaling pathway is involved in regulating intestinal epithelial cell renewal along the CVA in piglets (Yang et al., 2016). In this study, weaning stress affected both cell renewal and the mTOR signaling pathway. In particular, mTOR protein expression significantly increased on d 3, which may be related to an increase in sudden feed intake (2.38 g on d 1, 61.02 g on d 3) (Zhou et al., 2019). We speculated that weaning stress resulted in sharp decline in the proliferation of jejunal stem cells in piglets on d 1 and 3, which was compensated by increased protein synthesis induced by mTOR that may be connected with increased nutrient ingestion on d 3 (Fingar and Blenis, 2004; Zhou et al., 2019).

5. Conclusions

In summary, this study revealed developmental changes in piglet intestinal epithelial cell proliferation, differentiation, and shedding during the 14 d after weaning, and mTOR signaling is likely involved in this process. Our findings provide a cellular mechanism for the intestinal morphology changes that occur after early weaning.

Author contributions

Min Wang: Investigation, Writing – original draft. **Lixia Wang:** Data curation, Visualization, Investigation. **Xian Tan:** Investigation. Lei Wang: Investigation. **Xia Xiong:** Resources. **Yancan Wang:** Investigation. **Qiye Wang:** Visualization. **Huansheng Yang:** Conceptualization, Methodology, Software, Writing – review & editing. **Yulong Yin:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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