Differential expression of proteins involved in energy production along the crypt-villus axis in early-weaning pig small intestine

Xia Xiong,1* Huansheng Yang,1,7* Bie Tan,1 Chengbo Yang,1 Miaomiao Wu,1 Gang Liu,1 Sung Woo Kim,2 Tiejun Li,1 Lili Li,1 Junjun Wang,5 Guoyao Wu,3 and Yulong Yin1,4,6

1Chinese Academy of Science, Institute of Subtropical Agriculture, Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory for Agroecological Processes in Subtropical Region, Scientific Observation and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Hunan, China; 2Department of Animal Science, North Carolina State University, Raleigh, North Carolina; 3Department of Animal Science, Texas A&M University, College Station, Texas; 4Southwest Collaborative Innovation Center of Swine for Quality and Safety, Chengdu, China; 5State Key Laboratory of Animal Nutrition, China Agricultural University, Beijing, China; 6School of Life Sciences, Hunan Normal University, Changsha, China; and 7Fujian Aonong Biotechnology Corporation, Xiamen, Fujian, China

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Xiong X, Yang H, Tan B, Yang C, Wu M, Liu G, Kim SW, Li T, Li L, Wang J, Wu G, Yin Y. Differential expression of proteins involved in energy production along the crypt-villus axis in early-weaning pig small intestine. Am J Physiol Gastrointest Liver Physiol 309: G229–G237, 2015. First published June 4, 2015; doi:10.1152/ajpgi.00095.2015.—Weaning of piglets reflects intestinal dysfunction and atrophy and affected the physiological state of enterocytes. However, few studies have defined physiological state of enterocytes along the crypt-villus axis in early-weaning piglets. A total of 16 piglets from 8 litters were used in the experiment. One group of piglets was nursed by sows until age 21 days, and another group was weaned at age 14 days and then fed creep feed instead of breast milk for 7 days. Piglets were killed at 21 days, and the jejunum segments were dissected. After sequential isolation of jejunum epithelial cells along the crypt-villus axis, their proteins were analyzed through the isobaric tags for relative and absolute quantification, and proteins involved in the mammalian target of rapamycin signaling pathway and proliferating cell nuclear antigen abundances in jejunal epithelial cells of weaning or suckling group were determined by Western blotting. The differential proteins in three cell fractions were identified and analyzed. The results showed that proteins involved in the tricarboxylic acid cycle, β-oxidation, and the glycolysis pathway were significantly downregulated in the upper and middle villus of the early-weaned group. However, proteins involved in glycolysis were significantly upregulated in crypt cells. In addition, Western blot analysis showed that the expression of mammalian target of rapamycin pathway-related proteins was decreased (P < 0.05) in the early-weaned group. The present results showed that early-weaning differentially affect the expression of proteins involved in energy production of enterocytes along the jejunal crypt-villus axis.

function, and an imbalance of intestinal microbiota (13, 30). Inadequate food intake immediately after weaning and insufficient energy intake may contribute to the changes in intestinal morphology (25, 35).

The intestinal epithelium is characterized by rapidly proliferating cells organized into structures called crypts, which invaginate into the underlying mesenchyme, and villi, which project into the lumen (6). Furthermore, epithelial cells undergo functional and morphological differentiation during migration along the crypt-villus axis from the crypt to the tip of the villus (22, 43). Intestinal epithelial cell proliferation, differentiation, and apoptosis play important roles in intestinal development, maintenance, and recovery from tissue damage (44). In addition, epithelial cells in the pig intestine have notably high energy demands due to the rapid renewal of the epithelium (within few days) (2, 36). Previous studies showed the activities of key metabolic enzymes in enterocytes changed during the suckling-weaning transition in pigs (20, 21). However, the effect of early weaning on the metabolism of enterocytes along the crypt-villus axis has not yet been elucidated.

In view of the foregoing, we hypothesize that early weaning could differentially affect metabolism along the crypt-villus axis of intestinal cells. Therefore, the objective of the present study was to investigate the metabolism of enterocytes along the crypt-villus axis in jejunum of early-weaned and suckled pigs by isobaric relative and absolute quantification (iTRAQ) liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) proteomics.

METHODS

Sequential Isolation of Epithelial Cells Along the Crypt-Villus Axis

The experimental design and procedures used in this study were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. A total of 16 piglets [Duroc × (Landrace × Yorkshire)] from 8 litters were used in the experiment. One group of piglets (suckled group) were kept in pens and nursed by sows until age 21 days, and another group (early-weaned group) were weaned at age 14 days and then fed creep feed (Table 1) instead of breast milk for 7 day (45). The two groups had eight replicates with one pig separately. There were no differences in initial body weight (4.27 ± 0.13 vs. 4.27 ± 0.17 kg, P = 0.978) and final body weight (5.79 ± 0.47 vs. 5.38 ± 0.47 kg, P = 0.101) between suckled and early-weaned group. Piglets in both groups were...
Table 1. Ingredient composition of diet (as fed basis)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content, g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extruded maize meal</td>
<td>505</td>
</tr>
<tr>
<td>Fermented soybean meal (54% crude protein)</td>
<td>150</td>
</tr>
<tr>
<td>Plasma protein powder</td>
<td>30</td>
</tr>
<tr>
<td>Fish meal</td>
<td>40</td>
</tr>
<tr>
<td>Whey powder</td>
<td>150</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.7</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>30</td>
</tr>
<tr>
<td>Fat powder</td>
<td>25</td>
</tr>
<tr>
<td>Calcium hydrogen phosphate</td>
<td>8</td>
</tr>
<tr>
<td>Calcium formate</td>
<td>6</td>
</tr>
<tr>
<td>L-Lysine HCl (78.8% lysine)</td>
<td>1.0</td>
</tr>
<tr>
<td>t-t-Methionine (99% methionine)</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Threonine (98% threonine)</td>
<td>2</td>
</tr>
<tr>
<td>L-Tryptophan (98% tryptophan)</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin and mineral premix*</td>
<td>10</td>
</tr>
</tbody>
</table>

Nutrient composition

| Digestible energy ‡, kcal/kg        | 3,510         |
| Crude protein‡                      | 201           |
| Calcium‡                            | 9.2           |
| Total phosphorus‡                   | 7.1           |
| Total lysine †                      | 16.2          |

*Provided the following amount (in mg/kg diet): 0.76 retinol acetate; 0.055 cholecalciferol; 16 t-t-a-tocopherol acetate; 3 menadione; 0.02 vitamin B12; 4 riboflavin; 30 niacin; 12 pantothenic acid; 600 choline chloride; 0.3 folic acid; 1.5 thiamin; 3 pyridoxine; 0.1 biotin; 80 Zn (as ZnSO4·7H2O); 20 Mn (as MnSO4·H2O); 83 Cu (as CuSO4·5H2O); 0.48 I (as KI); 0.36 Se (as Na2SeO4·5H2O). †Calculated. ‡Analyzed.

weighed and killed by CO2 asphyxiation at age 21 days, and the small intestine was removed and rinsed thoroughly with ice-cold physiological saline solution (10, 47). Mid-jejunal segments were dissected, and sequential isolation of pig small intestinal epithelial cells along the crypt-villus axis was performed according to the methods of Fan et al. (10). The jejunal segment was flushed once with phosphate-buffered saline and then incubated at 37°C for 30 min in 15 ml of oxygenated phosphate-buffered saline. The fluid contents of the intestinal segments were drained and discarded (17, 46). After the preincubation, the jejunal segments were then filled with 15–30 ml of isolation buffer [5 mM Na2EDTA, 10 mM HEPES, 0.5 mM DTT, 0.25% BSA, 2.5 mM D-glucose, 2.5 mM L-glutamine, 0.5 mM DL-β-hydroxybutyrate sodium salt, oxygenated with an O2/CO2 mixture (19:1 vol/vol)] for sequential isolation of three epithelial cell fractions (upper villus: F1; middle villus: F2; crypt: F3) from the villus tip to the crypt bottom. Isolated cell fractions were also pooled into fractions (upper villus: F1; middle villus: F2; crypt: F3) from the jejunal segments and sequentially isolated by using a reductive alkylation reaction, and protein concentration was determined. Trypsin digestion and iTRAQ labeling were performed according to the manufacturer’s protocol (Applied Biosystems). The labeled samples were pooled and resolved into 12 fractions using an Ultremex SCX column containing 5-μm particles (Phenomenex). The eluted fractions were then desalted using a Strata X C18 column (Phenomenex) and dried under vacuum. The final average peptide concentration in each fraction was ~0.25 μg/μl. Dried peptides were stored at ~80°C before MS analysis.

Peptide Fractionation and LC-MS/MS Acquisition

A nanospray ion source (Waters) system coupled with Triple TOF was used for analytical separation. Microfluidic traps and nanofluidic columns packed with Symmetry C18 (5 μm, 180 μm × 20 mm) were utilized for online trapping and desalting, and nanofluidic columns packed with BEH130 C18 (1.7 μm, 100 μm × 100 mm) were employed in analytic separation. Solvents were composed of water-acetonitrile-formic acid (solvent A: 98:2:0.1%; solvent B: 2:98:0.1%). A portion of a 2.25 μg (9 μl) sample was loaded, trapping, and desalted. At a flow rate of 300 nl/min, analytic separation was established by maintaining 5% solvent B for 1 min. In the following 64 min, a linear gradient to 35% solvent B occurred in 40 min. Following the peptide elution window, the gradient was increased to 80% solvent B in 5 min and maintained for 5 min. Initial chromatographic conditions were restored in 2 min.

Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX) fitted with a Nanospray III source (AB SCIEX) and a pulled quartz tip as the emitter (New Objectives). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150°C. The MS was operated with a resolving power ≥30,000 full-width half-maximum for the TOF MS scans. For information dependent acquisition, survey scans were acquired in 250 ms, and as many as 30 product ion scans were collected if they exceeded a threshold of 120 counts per second (counts/s) with a 2+ to 5+ charge state. The total cycle time was fixed to 3.3 s, and the Q2 transmission window was 100 Da for 100%. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40-GHz multichannel thermal conductivity detector with four-anode/channel detection. A sweeping collision energy setting of 35 ± 5 eV, coupled with iTRAQ adjust rolling collision energy, was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for one-half of the peak width (18 s), and the precursor was then refreshed off the exclusion list.

Database Analysis and Quantification

Mascot software (version 2.3.02, Matrix Science) was used to simultaneously identify and quantify proteins. Searches were made against the National Center for Biotechnology Information nonredundant database consisting of mammalian proteins (31,786 sequences). Spectra from the 12 fractions were combined into one Mascot generic format file after the raw data were loaded, and the Mascot generic format file was searched. The search parameters were as follows: 1) trypsin was chosen as the enzyme with one missed cleavage allowed; 2) the fixed modifications of carbamidomethylation were set as Cys; 3) peptide tolerance was set as 0.05 Da, and MS/MS tolerance was set as 0.1 Da. An automatic decoy database search strategy was employed to estimate the false discovery rate (FDR). The FDR was calculated as the false positive matches divided by the total matches. In the final search results, the FDR was < 1.5%. The search results were passed through additional filters before data exportation. For protein identification, the filters were set as follows: significance threshold P < 0.05 (with 95% confidence) and ion score or expected cutoff < 0.05 (with 95% confidence). For protein quantitation, the filters were set as follows: “median” was chosen for the protein ratio type (24); the minimum precursor charge was set to 2+ and minimum peptides were mined. 

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set to 2; only 2 and >2 unique peptides were used to quantify proteins. The median intensities were set as normalization, and outliers were removed automatically. The peptide threshold was set as above for identity.

Bioinformatics Analysis

Functional annotations of the proteins were conducted using Blast2GO program against nonredundant database consisting of mammalian proteins. The KEGG database (14) and the COG database (27) were used to classify and group these identified proteins.

Western Blot Analysis

Total eukaryotic initiation factor-4E (eIF4E), S6 kinase (S6K), phospho-S6K, mammalian target of rapamycin signaling pathway (mTOR), phospho-mTOR, eukaryotic initiation factor-4E binding protein-1 (4EBP1), phospho-4EBP1, and PCNA protein abundances in epithelial cells along the crypt-villus axis of jejunum were determined by Western blotting, as previously reported (34, 38). Total protein were extracted using ice-cold radioimmunoprecipitation assay lysis buffer (Biyuntian, Shanghai) containing phenylmethylsulfonyl fluoride. The sample was then centrifuged, and the resulting supernatants were sampled for analyses of their protein concentrations before Western blotting of the target proteins.

The following antibodies were used for Western blot analysis. Antibodies for eIF4E and PCNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies for S6K, phospho-S6K (Thr389), mTOR, phospho-mTOR (Ser2448), 4EBP1, phospho-4EBP1 (Thr70), and β-actin were purchased from Cell Signaling Technology (Cedarlane, ON, Canada). β-Actin was used to normalize the abundance of the target proteins. Quantification of the bands was carried out using Quantity-One software (Bio-Rad).

Statistical Analysis

The Western blot data were analyzed using a t-test in SAS (version 9.2; SAS Institute, Cary, NC). The activity of alkaline phosphatase data was analyzed by Wilcoxon tests in SAS. Values are means ± SE, and probability values (P) < 0.05 were used to indicate statistical significance.

RESULTS

Validation of the Fractionation Procedure

We isolated three cell fractions in the present study and validate the fractionation efficiency by alkaline phosphatase (Fig. 1). The specific activity of alkaline phosphatase increased 5.6-fold from 1.11 ± 0.87 (F3) to 6.20 ± 0.06 U/g protein (F1), which is consistent with a previous study (10). These findings demonstrate the efficient fractionation of differentiated villus cells and proliferating crypt cells with this method.

Differentially Expressed Proteins in the Jejunum Along the Crypt-Villus Axis After Weaning

A biological replicate sample was included in the iTRAQ experiment in the early-weaned and suckled group. A total of 1,650 proteins were identified in the present study using Mascot version 2.3.02. In the present study, a protein with ≥1.2-fold or ≤0.8-fold difference and a P value ≤ 0.05 was regarded as being differentially expressed. The differential proteins in the jejunum of suckling and early-weaned piglets were classified into 21 groups based on major intestinal functions, i.e., RNA processing and modification; amino acid transport and metabolism; carbohydrate transport and metabolism; lipid transport and metabolism; secondary metabolite biosynthesis, transport, and catabolism; coenzyme metabolism; inorganic ion transport and metabolism; posttranslational modification, protein turnover, chaperones; energy production and conversion; signal transduction mechanisms; transcription; translation, ribosomal structure, and biogenesis; cell division and chromosome partitioning; cytoskeleton; DNA replication, recombination, and repair; cell motility and secretion; intracellular trafficking and secretion; function unknown; general function prediction only; and no COG information. The COG analysis demonstrated that proteins with a role in RNA processing and modification, transcription, cell cycle/division, inorganic ion transport and metabolism, secondary metabolite biosynthesis, cytoskeleton, and cell motility were mainly upregulated in the villus of early-weaned piglets, and proteins with a role in carbohydrate, amino acid and lipid metabolism-related proteins, energy production and conversion, posttranslational modification, protein turnover, chaperones and DNA replication, recombination, and repair were mainly upregulated in the villus of suckled pigs. However, proteins related to carbohydrate, amino acid, lipid, inorganic ion transport and metabolism, secondary metabolite biosynthesis, coenzyme metabolism and posttranslational modification, protein turnover, and chaperones were mainly upregulated in the crypt of early-weaned piglets (Fig. 2). These results showed that early weaning has differential effects on protein expressions along the crypt-villus axis of epithelial cell.

In the present study, we focused on proteins associated with the energy production, including digestion/absorption of nutrients, energy production and conversion (tricarboxylic acid cycle), lipid metabolism (β-oxidation pathway enzymes), and carbohydrate metabolism (glycolysis pathway) in the three fractions. Biochemical information about these proteins is summarized in the Supplemental Material. (The online version of this article contains supplemental data.)

Digestion/absorption of nutrients. The downregulated proteins included many transporters of monosaccharides (e.g., sodium/glucose transporter), nucleosides (e.g., ADP/ATP translocase 3), monocarboxylic acid (e.g., solute carrier family
12 member 2; chloride intracellular channel proteins 1 and 5; voltage-dependent anion-selective channel proteins 1 and 2), lipids and sterols (e.g., retinol-binding protein II), and proteins associated with carbohydrate digestion (e.g., sucrose-isomaltase) and protein digestion (e.g., glutamyl aminopeptidase and aminopeptidase N). The upregulated proteins included transporters for fatty acids (e.g., liver-type fatty acid-binding protein), metal ions (e.g., the copper transport protein ATOX1), and nutrients digestion (e.g., aspartyl aminopeptidase; maltase-glucoamylase) (Table 2).

**β-Oxidation.** Eight enzymes for β-oxidation were identified in the three fractions. The expressions of long-chain fatty acid-coenzyme A (CoA) ligase, very-long-chain specific acyl-CoA dehydrogenase, medium-chain acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and acetyl-CoA acyltransferase were downregulated in the three fractions, and those of peroxisomal acyl-CoA oxidase (ACO) and long-chain specific acyl-CoA dehydroge-

lataed in the upper and middle villus in the jejunum of the early-weaned group. Although short-chain specific acyl-CoA dehydrogenase was identified in the three fractions in the early-weaned group, there was no significant difference in its expression (Fig. 3).

**Glycolysis.** Eleven enzymes in the glycolytic pathway were identified in the three fractions in the present study, including aldose 1-epimerase, hexokinase, 6-phosphofructokinase, fructose-bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, pyruvate dehydrogenase, and L-lactate dehydrogenase. The expression of some enzymes was downregulated in F1 and F2 or upregulated in F3 in the early-weaned group (Fig. 4).

**Tricarboxylic acid cycle.** Eight enzymes in the tricarboxylic acid cycle were identified in the three fractions. The expressions of citrate synthase predicted, cytoplasmic aconitate hydratase, isocitrate dehydrogenase, 2-oxoglutarate dehydroge-
nase (OxoGDH), succinyl-CoA ligase, succinyl-CoA synthetase, succinate dehydrogenase, fumarate hydratase, and malate dehydrogenase were simultaneously downregulated in the upper villus of the early-weaned group. In addition, the expressions of citrate synthase, isocitrate dehydrogenase, OxoGDH, succinyl-CoA synthetase, fumarate hydratase, and malate dehydrogenase were downregulated in the middle villus in the early-weaned group. Only malate dehydrogenase and OxoGDH were identified and downregulated in crypt cells (Fig. 5).

**DISCUSSION**

The main function of the intestine concerns the absorption of nutrients arising from intestinal digestion. Early weaning stress results in increased intestinal dysfunction and atrophy; however, these changes are transient, and the level of energy intake is a major factor that accounts for the postweaning villus height (3, 25). The suckling piglets consumed only lactose in milk as carbohydrate, and the activity of lactase enzyme declines after weaning. Peuhkuri et al. (28) showed that the lactase protein expression decreased after weaning and expression and activity were induced by lactose-rich diet. Motohashi et al. (26) showed that lactase was regulated at the transcriptional level and not caused by termination of milk ingestion. In the present study, we identified lactase-like protein in three cell fraction, and its expression did not change between suckled and weaned piglets. In addition, we identified the expression of maltase-glucoamylase is upregulated in jejunal upper villus of early-weaned piglets and reduced ($P < 0.05$) 4EBP1, phospho-4EBP1, mTOR, phospho-mTOR, S6K1, S6K2, and phospho-S6K1 in F1 of early-weaned group (Fig. 6). Compared with suckled piglets, the relative expression abundance of PCNA in F3 was downregulated in the early-weaned group.

**Epithelial cells in the small intestine undergo a spatially and temporally regulated maturation program as they migrate along the crypt-villus axis.** Enterocytes constitute up to 90% of epithelial cells in the crypt and more than 95% of villus cells (10, 32). Pig intestinal epithelial cells have notably high-energy demands due to the rapid renewal of the epithelium (within a few days). Previous studies have shown that the intestinal tissues are capable of oxidizing all three alternative substrates.
(amino acids, glucose, and fatty acid), and amino acids in general (i.e., glutamine, glutamate, and aspartate) are the major contributors to mucosal oxidative energy generation in adequately nourished animals, whereas glucose and fatty acids are of much less importance (39, 40). In addition, the pattern of intestinal substrate oxidation is altered by protein restriction in pigs (37). In the present study, we found that key enzymes in the tricarboxylic acid cycle are mainly downregulated in F1 and F2 of early-weaned piglets compared with suckled piglets, which suggest that the capacity of epithelial cells in the small intestine to produce energy by the tricarboxylic acid cycle pathway is impaired in early-weaned piglets. Glutamine is an abundant amino acid in the sow colostrum and milk (41). The oxidation of glutamine (the other main source for energy production), which can enter the tricarboxylic acid cycle in the form of \( \alpha \)-ketoglutarate, was high during the suckling period and remained unchanged in the postweaning period (16).

Furthermore, alanine aminotransferase is involved in the pathway for glutamine oxidation in the jejunum, and its activity was also higher in suckling rats than in adults (15, 32). In the
In the present study, the expressions of alanine aminotransferase and aspartate aminotransferase were upregulated in all three fractions in early-weaned piglets, which suggest that porcine small intestinal epithelium provides the tricarboxylic acid cycle with oxaloacetate or acetyl-CoA during the suckling-weaning transition. In addition, the expression of glutamate dehydrogenase is downregulated in F1 and F2 of early-weaned piglets compared with that in suckled piglets. Madej et al. (20) also showed that the activity of piglet intestinal glutamate dehydrogenase during the first and second weeks after weaning was lower than suckling. In addition, although most key enzymes of TCA cycle in F3 were identified in suckled and early-weaned group, however, their expression was unchanged. The results showed that weaning decreased energy conversion in upper and middle villus cells, but had little effect on crypt cells after 7 days postweaning. The possible explanation is that villus cells may use luminal as well as systemic nutrients, while crypt cells use arterial nutrition (1).

Fig. 6. Effects of early-weaning on the expression of proteins in mammalian target of rapamycin signaling pathway (mTOR) of F1 or F3 cell fraction. A: the relative abundance of proteins in mTOR signaling pathway in F1. B: the relative abundance of proteins in mTOR signaling pathway in F3. C: the expression of proteins in mTOR signaling pathway was determined by Western blotting. D: the relative abundance of PCNA in F3. β-actin was used as loading control. Values are means ± SE; n = 8. *P < 0.05 compared with suckled group. 4EBP1, eukaryotic initiation factor-4E binding protein-1; p-4EBP1, phospho-4EBP1; p-mTOR, phospho-mTOR; S6K, S6 kinase; p-S6K, phospho-S6K; eIF4E, total eukaryotic initiation factor-4E.
The β-oxidation of short-, medium-, and long-chain fatty acids derived from the diet is catalyzed in mitochondria, and this pathway constitutes the major process by which fatty acids are oxidized to generate energy (34). β-Oxidation occurs in both mitochondria and peroxisomes. Phipps et al. (29) showed that there are more peroxisomes in mature cells than in crypt cells along the crypt-villus axis. The levels of the peroxisomal enzymes catalase and ACO were found to be significantly higher in differentiated and mature cells situated at the villus tip and stem than in the crypt (29). In the present study, enzymes in the β-oxidation pathway, except for ACO and long-chain specific acyl-CoA dehydrogenase, were downregulated in the upper and middle villus in the early-weaned group compared with the suckled group. The ACO gene, which encodes the first enzyme in peroxisomal fatty acid β-oxidation, has been shown to reflect the level of peroxisomal fatty acid catabolism (18). Although ACO was upregulated in F1 of early-weaned piglets, the other enzymes in this pathway were downregulated in the present study. The suckling period is characterized by high lipid availability and oxidation, and more than 95% of the fatty acids in porcine milk are long- and medium-chain fatty acids (11). After weaning, the composition of diet is higher in carbohydrate and lower in fat. Thus we speculated that β-oxidation in early-weaned piglets was limited by the weaned diet. In addition, we identified the downregulated expression of long-chain fatty acid transport protein-4 in the upper villus in the early-weaned group. The results indicated that early-weaning decreased fatty acid transport and metabolism, and these may increase after subsequent adaptation. Little research has been carried out on intestinal peroxisomes, and further studies will be needed to elucidate the effects of weaning on β-oxidation in intestinal epithelial cells.

Glycolytic proteins may be linked to the intraluminal absorption of monosaccharides, primarily glucose, fructose, and galactose, which is a key function of the differentiated intestinal epithelium (5). In the present study, the expressions of glycolytic proteins were downregulated in F1 and F2 of early-weaned piglets. Correspondingly, the brush border hydrolyase sucrose-isomaltase, which generates monosaccharides from disaccharides (9), and the glucose transporter Na+/glucose cotransporter-1 were downregulated in F1 and F2 of early-weaned piglets. These results showed that early weaning decreased the absorption of glucose and impaired the glycolytic capacity of enterocytes. Additionally, the glycolytic rates may reflect the rates of protein synthesis and degradation, a process that has been proposed to permit abrupt changes in gut size to match absorptive capacity with nutrient delivery (4). Together, weaning altered the expressions of proteins involved in energy production (amino acids, glucose, and fatty acid metabolism) in pig jejunal epithelial cells, and these changes may result from the decreases in nutrients intake and the changes in energy sources after weaning. These changes in energy metabolism may further affect the functions of epithelial cells, which further reduced the digestibility of energy and energy supply. Further studies should be conducted to improve energy supply to weaning piglets, thus improving intestinal functions.

To our knowledge, mTOR is involved in energy metabolism regulation at the organissmal level (19). Although the proteins involved in the mTOR signaling pathway were not detected by iTRAQ, we examined the relative expression of mTOR-related proteins by Western blotting. The mTOR pathway integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation, and survival. mTOR is activated by amino acids or the phosphati-
dylinositol 3-kinase-akt pathway and phosphorylation of mTOR is a good biomarker of mTOR pathway activation (7). The ribosomal protein S6K and 4EBP1/eIF4E pathways are both critical mediators of mTOR-dependent cell cycle progression (8). In our study, the expression of eIF4E, S6K, phospho-S6K, and phospho-mTOR in F1 was downregulated in the early-weaned group. In addition, the relative expression abundance of phospho-4EBP1, phosphor-mTOR, and phospho-S6K1 in crypt cells was downregulated in F3 of early-weaned piglets. These observations suggest that weaning may decrease protein synthesis in villus and crypt epithelial cells, which corresponds to an adaptation of the gut to the weaning diet.

The present results showed that early weaning differentially affect energy production in villus and crypt. In addition, the present results also showed that weaning has different effects on the villus and crypt cells in suckled and early-weaned piglets. Pigs are a good model for human nutrition and medicine, and the pig gastrointestinal tract is much closer to that in humans than that in rodents (12). Thus the present findings in early-weaning and suckling piglets may be useful for human infants to establish which consequences may derive from a preweaning and possibly to develop future therapeutic interventions.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: X.X., H.Y., C.Y., and Y.Y. conception and design of research; X.X., H.Y., B.T., and M.W. performed experiments; X.X. analyzed data; X.X. interpreted results of experiments; X.X., G.L., and S.W.K. prepared figures; X.X. drafted manuscript; X.X., S.W.K., T.L., L.L., J.W., G.W., and Y.Y. edited and revised manuscript; X.X. and Y.Y. approved final version of manuscript.

REFERENCES


25. McCracken KJ, Kelly D.


23. Madej M, Lundh T, Lindberg JE.


