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Expression of proteins in intestinal middle villus epithelial cells of weaning piglets

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1. ABSTRACT

Weaning affects intestinal development in mammals, and pigs are often used as an animal model for analyzing the metabolism and physiology of differentiating middle villus intestinal epithelial cells (DIECs). To assess the impact of weaning on the proteomes of DIECs, we weaned piglets on day 14 and collected their jejunum on days 0, 1, 3, 5, and 7 after weaning. Levels of proteins associated with (a) Golgi vesicle transport and protein glycosylation; (b) monosaccharide, lipid, phospholipid, and nucleotide metabolism; and (c) Krebs cycle and respiratory electron transport chain were decreased in DIECs after weaning. These results indicate that weaning decreases nutrient metabolism in DIECs. Moreover, these results suggest that dietary interventions (e.g., supplementation with functional amino acids) are required to counter these changes.

2. INTRODUCTION

The small intestinal epithelium can be divided into the crypt and villi. The crypt is a flask-shaped region that invaginates into the underlying mesenchyme, and the villi are finger-like regions that project into the lumen of the intestine (1, 2). The intestinal epithelium undergoes continual renewal that involves highly coordinated cell proliferation, differentiation, migration,

and apoptosis along the crypt–villus axis (CVA) (3). Continual renewal of epithelial cells along the CVA is accompanied by changes in the functional specialization of the gut (2). The activities of digestive enzymes were gradually changed along the CVA, with the maximal activities at the top of villi and the lowest activities in the bottom of crypt, whereas the activities of enzymes in the epithelial cells of middle villi were between the upper villi and the crypt (4). Results of experiments involving the measurement of Na^+-dependent L-glutamate transport into apical membrane vesicles showed that maximal transport rate, transporter affinity, and apparent transmembrane diffusion rate were different among epithelial cells of the upper villi, middle villi, and crypt in piglets (5). Similar results were obtained when the rates of Na^+-dependent D-glucose cotransport into apical membrane vesicles were measured (6). Moreover, expression of genes associated with cell cycle, RNA processing, protein translation, cytoskeleton assembly, and lipid biosynthesis and uptake was different among epithelial cells of the upper villi, middle villi, and crypt in mice (7). Because epithelial cells in the upper villi, middle villi, and crypt represent differentiated, differentiating, and proliferating epithelial cells, respectively, these results indicate that intestinal epithelial cells at different levels of maturation have different metabolic patterns and functions (5, 6).

Weaning transition markedly changes the structure and function of the small intestine, thus playing an important role in regulating the development of the intestinal mucosa (2). Major structural changes in the small intestine during weaning transition include increased crypt depth and decreased villus height (5). Moreover, the morphology of the villi changes, with the shape of the villi in the duodenum and jejunum changing from long finger-like projections to a leaf-like structure (8, 9). These structural changes may result from changes in epithelial cell apoptosis and proliferation, which in turn may result from reduced intake of nutrients, particularly amino acids, which are required for maintaining cellular activities (10, 11). Hansson *et al*. (12) reported that expression profiles of proteins associated with glycolysis, carbohydrate uptake, fatty acid metabolism, and lactose metabolism changed in mice after weaning. Similarly, Xiong *et al*. (13) found that levels of proteins associated with energy metabolism were altered in small intestinal epithelial cells of early-weaned pigs compared with those in small intestinal epithelial cells of suckling piglets. Middle villus epithelial cells are differentiating cells that play key roles in the small intestine (2). However, to our knowledge, the effects of weaning on differentiating intestinal epithelial cells (DIECs) have not been determined in any mammal to date. Results of our recent studies involving weanling piglets indicate that weaning affects the metabolism and physiology of DIECs in mammals.

3. CHANGES IN PROTEIN EXPRESSION IN INTESTINAL MIDDLE VILLUS EPITHELIAL CELLS DURING THE POST-WEANING PERIOD

3.1. General study protocols

Dl-β-Hydroxybutyrate sodium salt was purchased from J&K Chemical Ltd. (USA), trypsin was purchased from Promega (Madison, WI, USA), and iTRAQ reagent was purchased from Applied Biosystems (Foster City, CA, USA). Bovine serum albumin (BSA, fraction V), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

In all, 40 piglets (Duroc × [Landrace × Yorkshire]) from 8 litters (5 piglets per litter; birth weight, 1604 ± 138 g) were used in the present study. The piglets were weaned at the age of 14 days and were fed creep diet that met the nutrient specifications of the National Research Council for 5–10 kg BW pigs, as described previously (13, 14). One piglet from each litter was euthanized after intravenously injecting (jugular vein) 4% sodium pentobarbital solution (40 mg/kg body weight) on days 0 (w0d; 4279 ± 138 g), 1 (w1d; 3967 ± 196 g), 3 (w3d; 4399 ± 324 g), 5 (w5d; 4813 ± 263 g), and 7 (w7d; 5355 ± 196 g) after weaning. Intestinal middle villus epithelial cells were isolated using a distended intestinal sac method, as described previously (4, 13) but with slight modifications. Divided mid-jejunum segments were rinsed thoroughly with ice-cold physiological saline solution and were incubated with oxygenated phosphate-buffered saline (PBS) at 37°C for 30 min. After incubation with oxygenated isolation buffer (5 mM Na_2EDTA, 10 mM HEPES [pH 7.4], 0.5 mM DTT, 0.25% BSA, 2.5 mM d-glucose, 2.5 mM L-glutamine, and 0.5 mM Dl-β-hydroxybutyrate sodium salt oxygenated with an O_2/CO_2 mixture [19:1, v/v]) at 37°C for 40 min, the jejunum segments were filled with the same buffer and were incubated at 37°C for 50 min to isolate intestinal middle villus epithelial cells. Isolation buffer containing the cells was collected and was centrifuged at 400 × *g* and 4°C for 4 min. The collected cells were washed twice with an oxygenated cell suspension buffer (1.5 mM $CaCl_2$, 2.0 mM $MgCl_2$, and 10 mM HEPES [pH 7.4]) and were pelleted by centrifugation at 400 × *g* and 4°C for 4 min. The isolated cells were immediately frozen in liquid nitrogen and were stored at -80°C until analysis. Experimental design and procedures used in this study were in accordance with the Chinese Guidelines for Animal Welfare and Experimental Protocols and were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture at the Chinese Academy of Sciences.

The harvested cells were resuspended and disrupted in a lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v 3-([3-cholamidopropyl] dimethylammonio) propanesulfonate, 20 mM tributyl phosphate, 0.2% Bio-Lyte (pH 3–10), and protease inhibitor cocktail

(Roche Diagnostics Ltd, Mannheim, Germany). DNase I (Invitrogen, Carlsbad, CA, USA) and RNase A were added to the lysate at final concentrations of 1 and 0.25 mg/mL, respectively. After cell disruption, proteins were separated from cell debris by centrifugation at 12,000 × *g* and 4°C for 5 min. Crude protein extracts were purified using ReadyPrep 2D Cleanup Kit (Bio-Rad Laboratories, USA) and were subjected to a reductive alkylation reaction. Protein concentration was determined using 2D Quant Kit (GE Healthcare, USA). Trypsin digestion and iTRAQ labeling of the proteins were performed according to the manufacturer's protocol (Applied Biosystems). Briefly, 100 µg total cell protein fraction was reduced, alkylated, and digested overnight at 37°C with trypsin (Promega) and was labeled with iTRAQ reagent as follows: w0d, iTRAQ reagent 115; w1d, iTRAQ reagent 116; w3d, iTRAQ reagent 117; w5d, iTRAQ reagent 118; and w7d, iTRAQ reagent 121.

Isotopically labeled samples were pooled and fractionated into 12 fractions by using Ultremex SCX column containing 5-µm particles (Phenomenex, USA). Eluted fractions were desalted using Strata X C18 column (Phenomenex) and were dried under vacuum. Final average peptide concentration in each fraction was found to be approximately 0.2.5 µg/µL. Dried peptides were stored at -80°C before performing MS analysis. Nanospray ion source system (Waters, USA) 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 used for online trapping and desalting. Nanofluidic columns packed with BEH130 C18 (1.7 µm, 100 µm × 100 mm) were used for analytical separation. Solvents were prepared using water, acetonitrile, and formic acid (solvent A: 98%/2%/0.1%; solvent B: 2%/98%/0.1%). Next, 2.25 µg (9 µL) sample was loaded, trapped, and desalted. Analytical separation was established at a flow rate of 300 nL/min by maintaining 5% solvent B for 1 min. In the following 64 min, a linear gradient to 35% solvent B occurred in 40 min. After peptide elution, the gradient was increased to 80% solvent B within 5 min and was maintained for 5 min. Initial chromatographic conditions were restored within 2 min.

Data were acquired using Triple TOF 5600 System (AB SCIEX, USA) equipped with Nanospray III source (AB SCIEX) and a pulled quartz tip as an emitter (New Objectives, USA). Data were acquired using an ion spray voltage of 2.5. kV, curtain gas of 30 Psi, nebulizer gas of 15 Psi, and interface heater temperature of 150°C. The MS was operated with an RP of ≥30,000 FWHM for TOF MS scans. For information-dependant acquisition, survey scans were acquired in 250 ms. As many as 30 product-ion scans were collected if they exceeded a threshold of 120 counts/s, with a 2^+ to 5^+ charge state. Total cycle time was fixed at 3.3 s, and Q2 transmission window was 100 Da for 100%. Four time bins were summed for each scan at a pulsar frequency value of 11 kHz by monitoring 40 GHz multichannel TDC detector with four-anode/channel detection. Sweeping collision energy 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 at half of the peak width (18 s), and the precursor was refreshed of the exclusion list.

Mascot software (version 2.3.0.2.; Matrix Science) was used to simultaneously identify and quantify the proteins. Searches were made against a non-redundant NCBI database of mammalian proteins (31786 sequences). Spectra from the 12 fractions were combined into one Mascot generic format (MGF) file after loading raw data, and the MGF file was searched against the database. Search parameters were as follows: (i) trypsin was chosen as the enzyme and was allowed one missed cleavage, (ii) fixed modifications of carbamidomethylation were set as Cys, (iii) peptide tolerance was set as 0.05 Da and MS/MS tolerance was set as 0.1 Da. Automatic decoy database search strategy was employed to estimate false discovery rate (FDR). FDR was calculated as false-positive matches divided by total matches. In the final search results, the FDR was <1.5%. The search results were passed through additional filters before exporting the data. Filters for protein identification were set as follows: significance threshold of $P < 0.05$ (with 95% confidence) and ion score or expected cutoff of <0.05 (with 95% confidence). Filters for protein quantification were set as follows: "median" was used for protein ratio type (16), minimum precursor charge was set as 2^+, and minimum peptides were set as 2. Only 2 and >2 unique peptides were used to quantify the isolated proteins. Median intensities were set as normalization, and outliers were removed automatically. Peptide threshold was set as above for identity.

Differentially expressed proteins were functionally annotated using Blast2GO program against a non-redundant database of *Sus scrofa* proteins (15). KEGG database (16) and WEGO program (17) were used to classify and group the differentially expressed proteins by using Cluster 3.0. and k-means clustering (18). Upregulated and downregulated proteins were selected, and their cellular component, molecular function, and biological process ontologies were identified using the WEGO program. GO terms with a *P* value of ≤0.05 (Pearson chi-square test by using the numbers of upregulated and downregulated proteins) were selected (17).

3.2. Weaning inhibits various cellular processes in metabolism in intestinal middle villus epithelial cells

Cell differentiation involves the expression cell-specific genes and functional specialization of cells undergoing differentiation (19). Middle villus epithelial

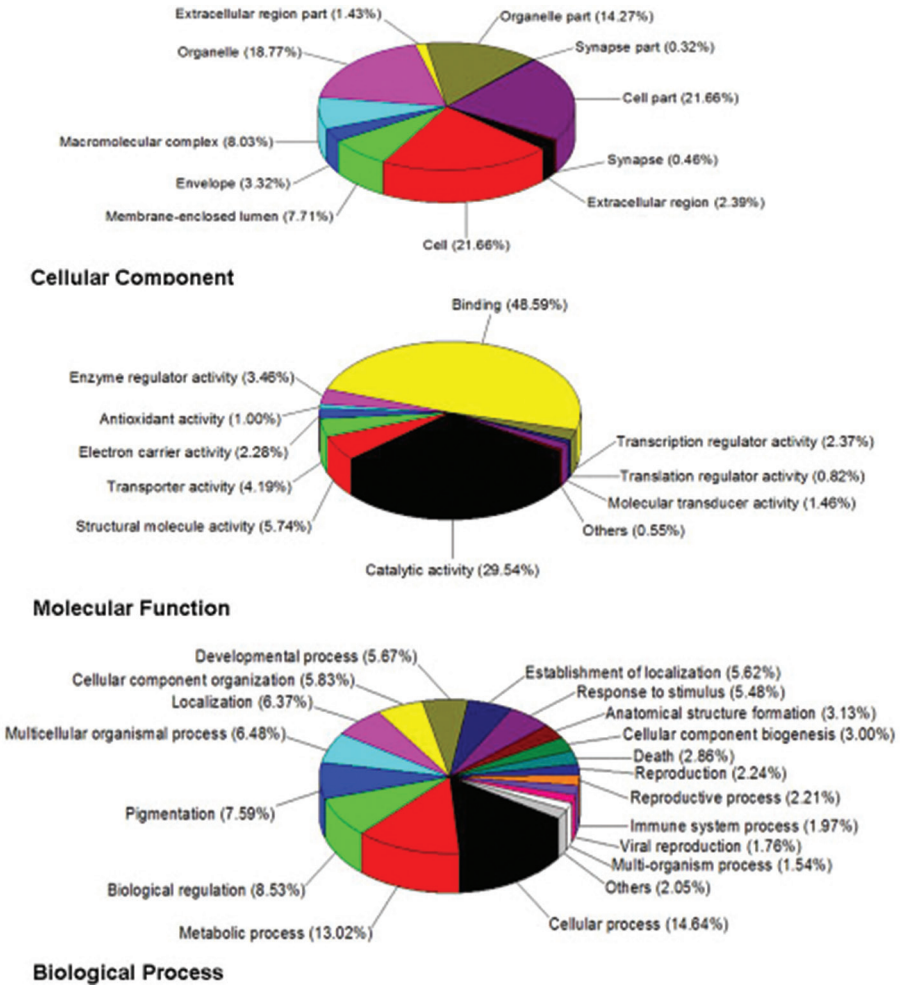

Figure 1. Cellular distribution and functions of differentially expressed proteins in the jejunal middle villus epithelial cells of piglets during the post-weaning period. A protein exhibiting ≥1.2- or ≤0.8-fold difference in expression in the jejunal middle villus epithelial cells between w1d, w3d, w5d, or w7d and w0d, with a *P* value of ≤0.05, was regarded as being differentially expressed. Differentially expressed proteins were classified using the WEGO program.

cells undergo differentiation and play important roles in digestion and absorption of luminal nutrients (4-6). Digestive enzyme activities and nutrient uptake capacities in middle villus epithelial cells were mainly between the upper villus and crypt epithelial cells (4-6). Weaning is usually associated with intestinal dysfunction (20, 21). In the present study, 651 proteins were found to be differentially expressed in the middle villus epithelial cells of w0d, w1d, w3d, w5d, and w7d piglets. Cellular component GO enrichment analysis indicated that the differentially expressed proteins were associated with metabolism, organelle function, macromolecular complex formation, membrane-enclosed lumen structure formation, and extracellular matrix formation (Figure 1). Molecular function GO enrichment analysis indicated that the differentially expressed proteins were mainly involved in binding and enzyme catalysis and functioned as structural molecules, transporters, enzyme regulators, transcription regulators, electron carriers, molecular transducers, and antioxidants (Figure 1). Biological process GO enrichment analysis indicated that the differentially expressed proteins were mainly associated with metabolism, biological regulation, pigmentation, multicellular organization, localization, development, and signal transduction (Figure 1). KEGG pathway enrichment analysis indicated that the differentially expressed proteins were mainly associated with carbon metabolism, ribosomes, oxidative phosphorylation, protein processing in the endoplasmic reticulum, spliceosomes, glycolysis/gluconeogenesis, amino acid biosynthesis, fatty acid degradation, fatty acid metabolism, branched-chain amino acid degradation, proteasomes, and PPAR signaling pathway (Figure 2).

The differentially expressed proteins were clustered into 9 distinct groups (bins 0–8) by using k-means clustering and Cluster 3.0. We observed that the expression of proteins in bins 1 and 4 was upregulated after weaning and that of proteins in bins 2, 3, 6, and 7 was downregulated after weaning. The WEGO program

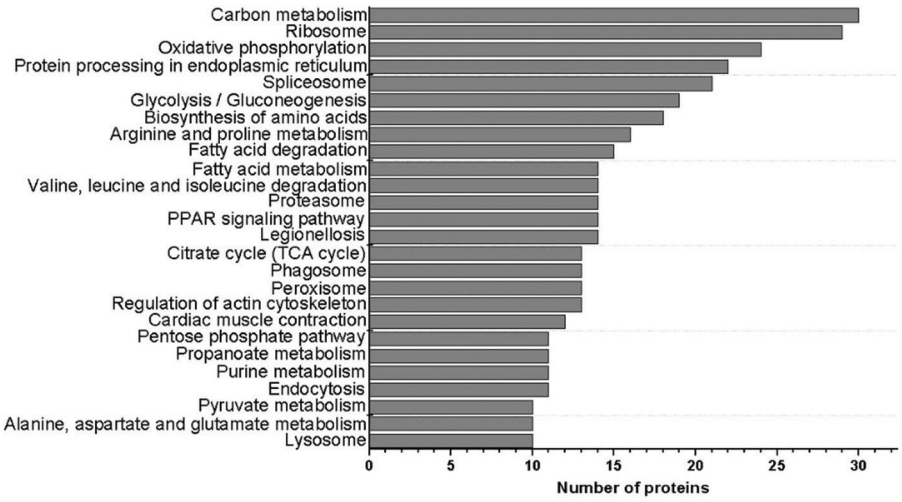

Figure 2. KEGG pathways involving differentially expressed proteins in the jejunal middle villus epithelial cells of weaning piglets. A protein exhibiting ≥1.2- or ≤0.8-fold difference in expression between w1d, w3d, w5d, or w7d and w0d, with a *P* value of ≤0.05, was regarded as being differentially expressed.

was used to analyze the GO enrichment of upregulated and downregulated proteins, and GO terms differing ($P < 0.05$) among these proteins were selected. On the ontology type of cellular components, low percentage of proteins associated with the endoplasmic reticulum, Golgi membrane, mitochondrial membrane, mitochondrial matrix, vesicle, and ribosomes was upregulated (Figure 3). On the ontology type of molecular function, high percentage of proteins associated with peptidase activity was upregulated ($P < 0.05$; Figure 3). In contrast, low percentage of proteins associated with NAD or NADH, ribonucleotides, and nucleotides as well as proteins that functioned as substrate-specific transporters, structural constituents of ribosomes, transferases, and oxidoreductases was upregulated ($P < 0.05$; Figure 3). On the ontology type of biological processes, high percentage of proteins associated with protein catabolism was upregulated ($P < 0.05$; Figure 3). In contrast, low percentage of proteins associated with energy derivation; intracellular protein transport; intracellular localization of macromolecules; Golgi vesicle transport; respiratory electron transport chain; oxidation–reduction reactions; and carbohydrate, organic acid, glycoprotein, lipid (including phospholipids), nucleotide, fatty acid, and alcohol metabolism was upregulated (Figure 3).

In addition to the DIECs, the differentially expressed proteins changes in the proteomes of villus epithelial cells of weaning piglets were similar to those in the DIEC (13, 22). These results suggest that all jejunal epithelial cells show similar changes in cellular processes during the post-weaning period. Moreover, KEGG pathway enrichment analysis showed that enriched pathways of differentially expressed proteins in differentiating cells were similar to those in differentiated cells. However, some differences existed between differentiating and differentiated cells (our unpublished data). These differences may have resulted from the different physiology and functions or different location of the differentiating and differentiated cells in the small intestinal epithelium (23). High percentage of enriched proteins in many subcellular locations such as the mitochondria, ribosomes, Golgi, and endoplasmic reticulum was downregulated, suggesting that the functions of these organelles in the jejunal DIECs of piglets were compromised after weaning. Moreover, high percentage of proteins with oxidoreductase and transferase activities, associated with NAD or NADH and nucleotide binding, and functioning as structural constituents of ribosomes and substrate-specific transporters was downregulated.

4. CHANGES IN VESICLE-MEDIATED TRANSPORT, GOLGI VESICLE TRANSPORT, AND PROTEIN GLYCOSYLATION IN INTESTINAL MIDDLE VILLUS EPITHELIAL CELLS DURING THE POST-WEANING PERIOD

4.1. General study protocols

Differentially expressed proteins associated with vesicle-mediated transport, Golgi vesicle transport, and protein glycosylation were grouped using the WEGO program (17). The grouped proteins were clustered using Cluster 3.0. and k-means clustering (18).

4.2. Weaning inhibits vesicle-mediated transport, Golgi vesicle transport, and protein glycosylation in intestinal middle villus epithelial cells

In all, 41 proteins involved in vesicle-mediated transport were found to be differentially expressed, with

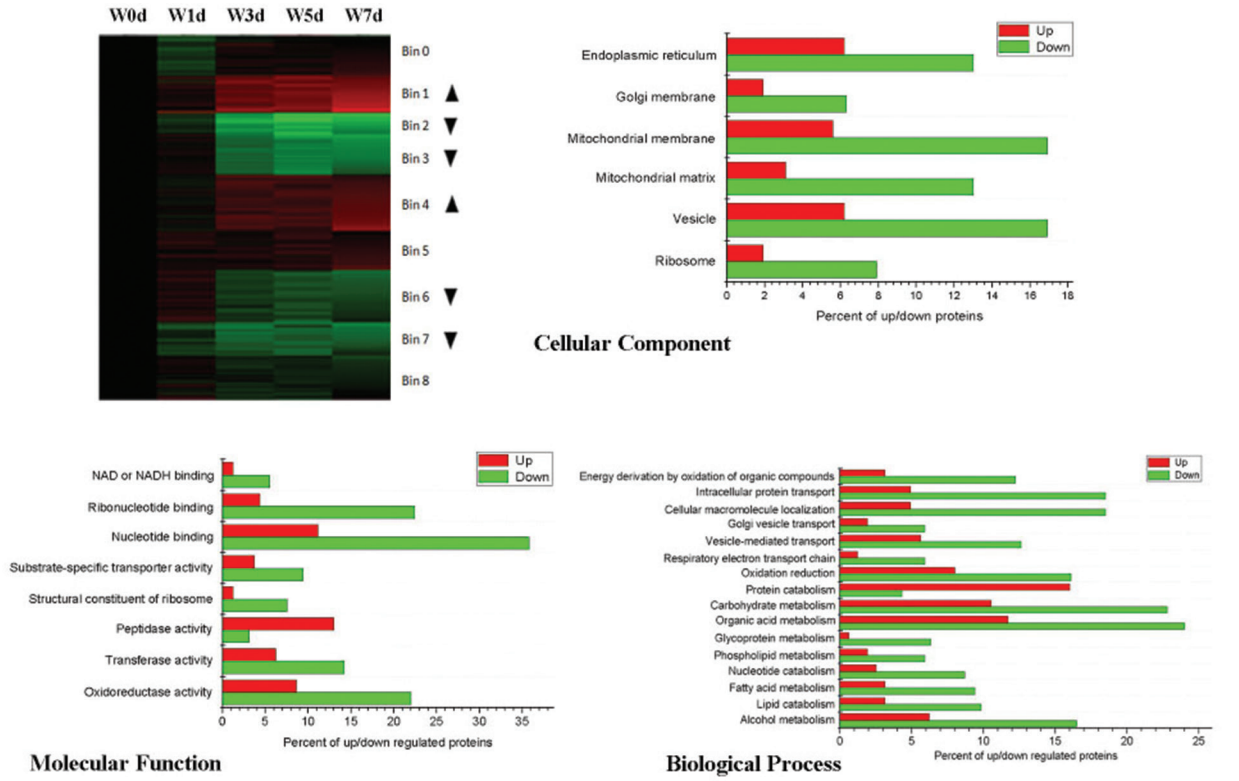

Figure 3. Functional categorization of upregulated and downregulated proteins in the jejunal middle villus epithelial cells of weaning piglets. Differentially expressed proteins were clustered using Cluster 3.0. and k-means clustering. Upregulated and downregulated proteins are indicated by arrowheads. Intracellular distribution and function of the upregulated (arrowheads pointing up) and downregulated proteins (arrowheads pointing down) were identified using the WEGO program. GO terms with a *P* value of ≤0.05 (Pearson chi-square test between the numbers of upregulated and downregulated proteins) were selected.

32 proteins being downregulated after weaning. The downregulated proteins were mainly involved in Golgi vesicle transport, platelet degranulation, and endocytosis (Figure 4). Eighteen proteins involved in Golgi vesicle transport were found to be differentially expressed, of which 15 proteins were downregulated after weaning (Figure 5). The downregulated proteins were involved in retrograde vesicle-mediated transport (Golgi to ER), intra-Golgi vesicle-mediated transport, COPII coating of Golgi vesicles, COPI coating of Golgi vesicles, and post-Golgi vesicle-mediated transport (Figure 5). In all, 13 proteins involved in protein glycosylation were found to be differentially expressed. All the identified proteins were downregulated after weaning and were involved in *N*-linked glycosylation (Figure 6).

Proteins involved in *N*-linked glycosylation were downregulated in jejunal DIECs. *N*-Linked glycosylation of proteins occurs in the endoplasmic reticulum. These *N*-linked glycosylated proteins are then transported to the Golgi complex to undergo *O*-linked glycosylation (24). Thus, our data suggest that weaning affects the functions of the endoplasmic reticulum (including protein glycosylation). High percentage of proteins associated with the endoplasmic reticulum was downregulated. However, proteins involved in Golgi vesicle transport and vesicle-mediated transport were also downregulated in the DIECs of post-weaning piglets. Golgi vesicle-mediated transport (such as endocytosis) plays a key role in transporting glycosylated proteins to their appropriate locations (25), indicating that weaning regulates the functions of glycosylated proteins by affecting the Golgi complex. Glycosylated proteins such as mucins play important protective role against pathogens in the gastrointestinal tract (26). Weaning decreases the levels of mucins in the intestine of piglets (27, 28), thereby impairing the function of DIECs.

5. CHANGES IN TRANSLATION INITIATION, CELLULAR MACROMOLECULE LOCALIZATION, AND PHOSPHOLIPID METABOLISM IN INTESTINAL MIDDLE VILLUS EPITHELIAL CELLS DURING THE POST-WEANING PERIOD

5.1. General study protocols

Differentially expressed proteins involved in vesicle-mediated transport, Golgi vesicle transport, and protein glycosylation were grouped using the WEGO

Figure 4. Expression of proteins involved in vesicle-mediated transport in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be associated with vesicle-mediated transport based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

program (17). The grouped proteins were then clustered using Cluster 3.0. and k-means clustering (18).

5.2. Weaning inhibits translation initiation, cellular macromolecule localization, and phospholipid metabolism in intestinal middle villus epithelial cells

Twenty proteins associated with translation initiation were found to be differentially expressed, with 19 proteins being downregulated after weaning (Figure 7). In all, 55 proteins associated with cellular macromolecule localization were found to be differentially expressed, with 47 proteins being downregulated after weaning. These downregulated proteins were involved in intracellular protein transport, SRP-dependent protein targeting to membrane, and protein localization in organelles (Figure 8). In all, 18 proteins involved in phospholipid metabolism were differentially expressed, with majority of these proteins being downregulated after weaning. These downregulated proteins were involved in phospholipid biosynthesis (Figure 9).

Differentiation of intestinal epithelial cells involves cell polarization (29). During this process,

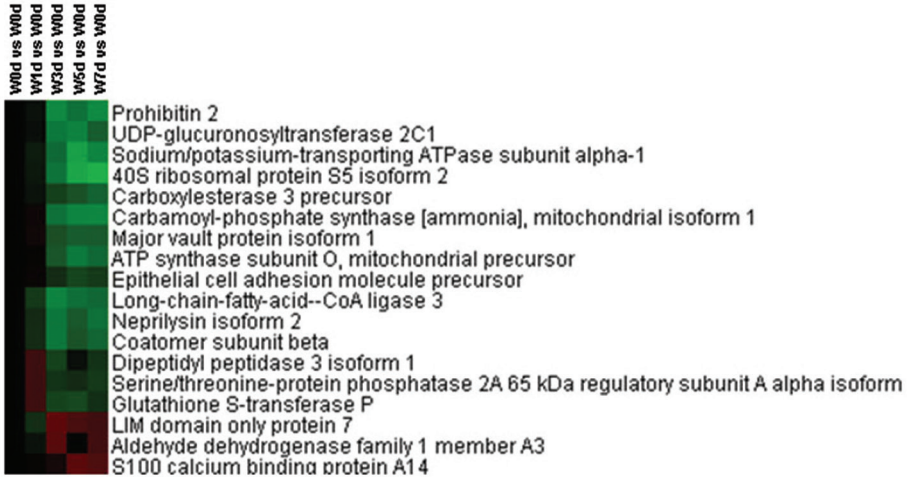

Figure 5. Expression of proteins involved in Golgi vesicle transport in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be associated with Golgi vesicle transport based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

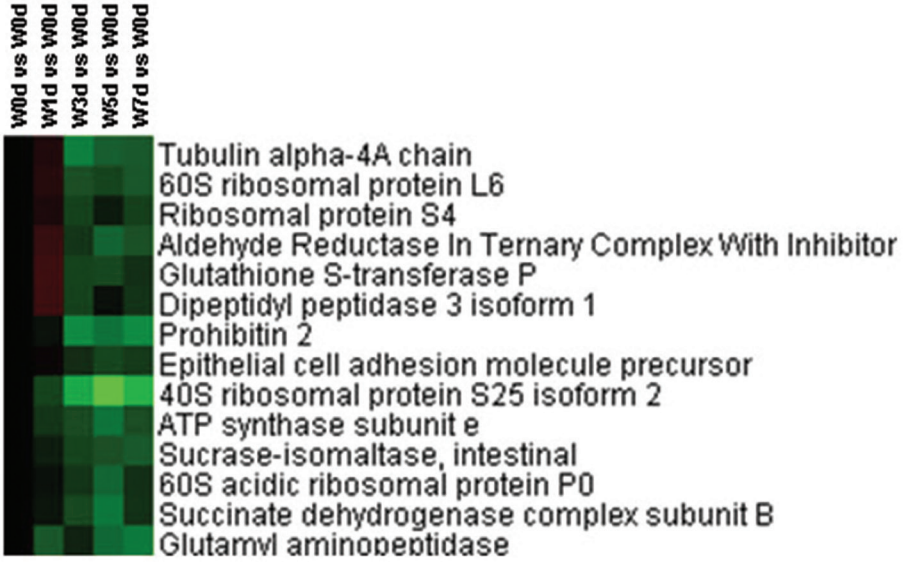

Figure 6. Expression of proteins involved in protein glycosylation in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be associated with protein glycosylation based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

cytoplasmic membranes of intestinal epithelial cells form two major domains, namely, apical and basolateral membranes (30, 31). Basolateral membranes adhere to the basement membrane of the extracellular matrix while apical membranes are exposed to the luminal content (30, 32). Microvilli appear on the apical side during differentiation, and many proteins such as transporters, receptors, and membrane-anchored hydrolase are localized on the microvilli surface (32). Phospholipids are the basic structural components of cell membranes and regulate the functions of mammalian cells (33). Differentiation of intestinal epithelial cells is accompanied with membrane restructuring and functional specialization, phospholipid metabolism, and protein expression and localization to the cell membrane (2, 34). Results of our studies indicate that expression of proteins associated with phospholipid metabolism, translation initiation, and cellular macromolecule localization is downregulated in the middle villus epithelial cells of post-weaning piglets. These results suggest that membrane restructuring and functional specialization may be abnormal processes in the DIECs of weanling piglets, which decrease the activities of digestive enzymes and compromise the integrity of intestinal mucosal barrier (10, 28).

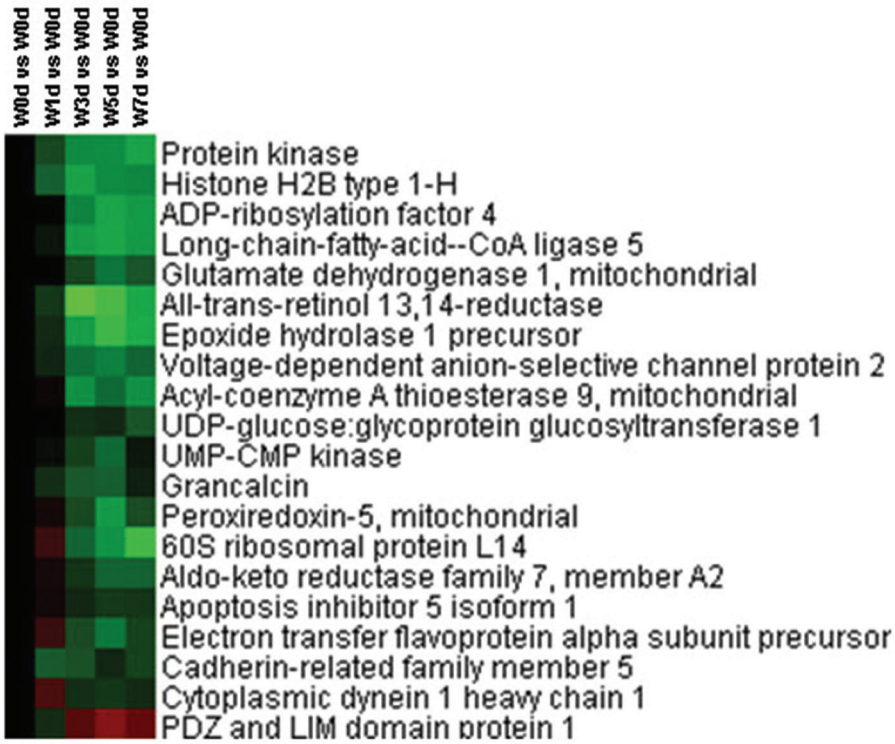

Figure 7. Expression of proteins involved in translation initiation in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in translation initiation based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

6. CHANGES IN ENERGY METABOLISM IN INTESTINAL MIDDLE VILLUS EPITHELIAL CELLS DURING THE POST-WEANING PERIOD

6.1. General study protocols

Differentially expressed proteins involved in vesicle-mediated transport, Golgi vesicle transport, and protein glycosylation were grouped according to the KEGG database (16) or WEGO program (17). The grouped proteins were then clustered using Cluster 3.0. and k-means clustering (18).

To measure the mRNA levels of target genes, approximately 100 mg DIECs from each piglet were pulverized in liquid nitrogen. Total RNA was isolated from the cells by using TRIZOL reagent (Invitrogen) and was treated with DNase I, according to the manufacturer's instructions. RNA quality was determined by performing agarose gel electrophoresis on a 1.2% gel and by staining the gel with ethidium bromide. The isolated RNA was reverse transcribed to cDNA, and the resulting cDNA was amplified by performing RT-PCR. Primers for RT-PCR were designed using Oligo 6.0. software (Molecular Biology Insights, CO, USA; Table 1). PCR was performed using the following program: denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C~60°C for 1 min, extension at 72°C for 1 min; and final extension at 72°C for 10 min. Amplification products were electrophoresed on a 1.5% agarose gel and were visualized by staining the gel with ethidium bromide (35).

6.2. Weaning inhibits energy metabolism in intestinal middle villus epithelial cells

Thirty proteins associated with lipid catabolism were found to be differentially expressed, of which 25 proteins were downregulated after weaning. These downregulated proteins were involved in fatty acid β-oxidation, membrane lipid turnover, and glycerolipid metabolism (Figure 10). In all, 36 proteins involved in monosaccharide metabolism were found to be differentially expressed, of which 30 proteins were downregulated after weaning. These downregulated proteins were involved in glycolysis, gluconeogenesis, and fructose metabolism (Figure 11). Proteins involved in glycolysis were identified using the KEGG database, which showed that expression of these proteins decreased after weaning (Figure 12). Proteins involved in the Krebs cycle were identified using the KEGG database and were found to be downregulated after weaning (Figure 13). The mRNA levels for genes associated with glycolysis, fatty acid catabolism, and Krebs cycle were quantified by performing RT-PCR. Results of RT-PCR showed that the mRNA levels for genes encoding PFK

Figure 8. Expression of proteins involved in cellular macromolecule localization in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in cellular macromolecule localization based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

and PYK (glycolysis); CPT1, CPT2, M-ACD, L-ACD, and ACO (fatty acids catabolism); and CISN, ICDH, and OxoGDH (Krebs cycle) were elevated in the DIECs of pigs after weaning (Figure 14).

Thirty-five proteins associated with energy metabolism were found to be differentially expressed in the DIECs of piglets. Of these, 31 proteins associated with the respiratory electron transport chain, Krebs cycle, and glycogen metabolism were downregulated after weaning (Figure 15). In all, 18 proteins associated with the respiratory electron transport chain were found to be differentially expressed. Of these, 16 proteins were downregulated after weaning (Figure 16). In all, 26 proteins associated with nucleotide metabolism were found to be differentially expressed. Of these, 22 proteins were downregulated after weaning. Twelve of these downregulated proteins were associated with GTP metabolism and 10 proteins were associated with ATP metabolism (Figure 17).

The small intestine has high demand for energy because of the high rates of renewal and energy metabolism of intestinal epithelial cells (36-38).

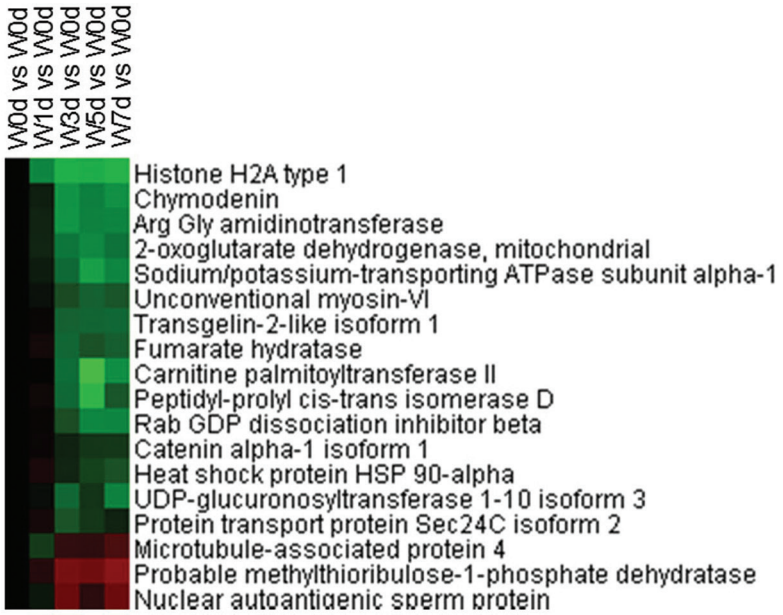

Figure 9. Expression of proteins involved in phospholipid metabolism in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in phospholipid metabolism based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

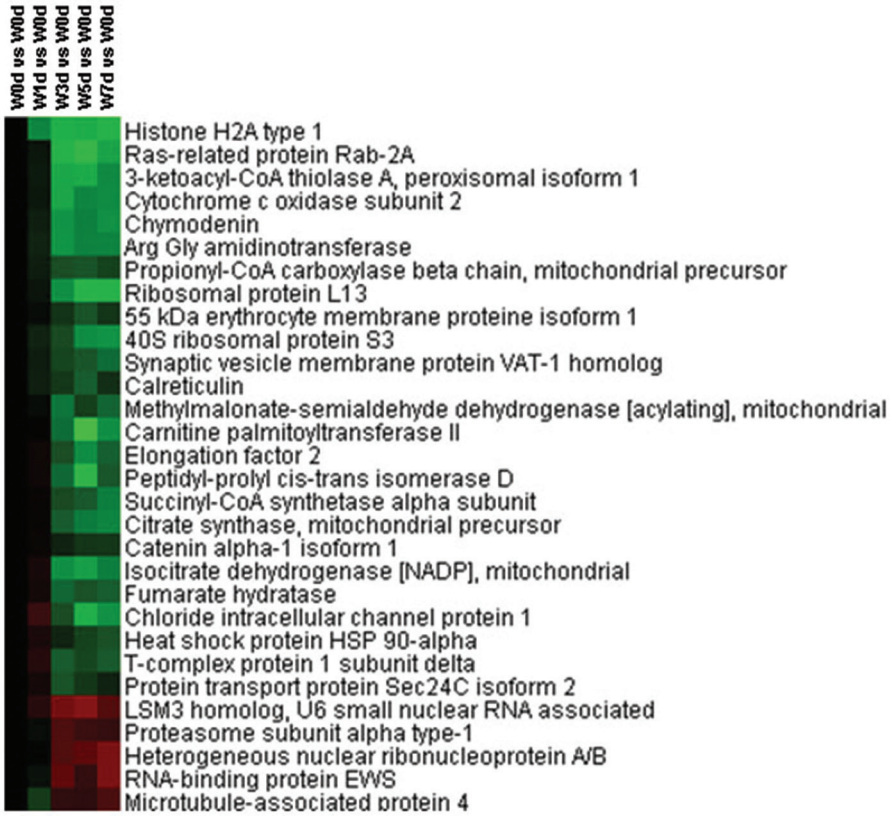

Figure 10. Expression of proteins involved in lipid metabolism in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in phospholipid metabolism based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

Table 1. Primers used for PCR

Genes	Sequences 5'–3'	GenBank accession number	Product size
PFK	TGGATGGCGGAGATAACA	NM_001044550.1	221 bp
	CCACTCAGAGCGGAAGGT		
PYK	TCGCATCTTTCATCCGTAA	XM_005666189.1	124 bp
	TCATCAAATCTCCGAACTCC		
CISN	ATGAAGGTGGCAATGTAAG	NM_214276.1	228 bp
	CCCGTCCTGAGTTGAGTG		
ICDH	ATTCTGAAAGCCTACGACG	NM_001164007.1	143 bp
	GAAGACTTGAGGACCTGAGC		
OxoGDH	CGTGACCGACAGGAACATC	XM_003134891.4	239 bp
	CGTGGACAGTGCCGTGAG		
CPT1	GTGTCGCCAAGCCTATTT	NM_001129805.1	224 bp
	GGAGTGCTCAGCGTTCAT		
CPT2	GCTTTGGCATTGGGTAT	NM_001246243.1	64 bp
	CTTTGGTAGGCGGAGAC		
S-ACD	CCGTGGAAAGAGCGAAAT	D89477.1	143 bp
	CCAAACTACTCCTCCCGAAC		
M-ACD	ACCAGACCTTCGGTAGCA	U40845.1	350 bp
	ATCTTGGCATCCCTCATTA		
L-ACD	GAGTAAGAACAAATGCCAAGA	NM_213897.1	105 bp
	CAGCCACTACAATCACAACA		
ACO	TATGCCTTCCAGTTTGTTG	NM_001101028.1	52 bp
	TAATGCGGTGATAGGTCTCT		
β-actin	TGCGGGACATCAAGGAGAAG	XM_003357928.2	216 bp
	AGTTGAAGGTGGTCTCGTGG		

However, piglets show low feed intake during the first three days after weaning, which results in intestinal atrophy (39). Results of our study showed that proteins associated with the respiratory electron transport chain and GTP and ATP metabolism were downregulated in the DIECs of weaning piglets, indicating impaired energy metabolism in these cells during the post-weaning period. These results support the practice of dietary supplementation with glutamine, glutamate, and aspartate (major metabolic fuels in enterocytes that are now recognized as nutritionally essential amino acids for the small intestine [40-43]) for improving intestinal growth and health of weanling piglets (44-48). In addition, our results showed that proteins involved in lipid and monosaccharide metabolism and Krebs cycle were downregulated in the DIECs of weanling piglets. Therefore, enhancing the supply of metabolic fuels may be an attractive method to improve the differentiation of intestinal epithelial cells of weaning piglets. Interestingly, although the levels of proteins involved in glucose and lipid metabolism and Krebs cycle were decreased, the mRNA levels for genes encoding these proteins were increased in the DIECs of weaning piglets. These findings may be explained by the differences in transcription and translation profiles of pig enterocytes (49-55), as those reported for cell types (56).

7. SUMMARY

In conclusion, results of our and other studies indicate that levels of proteins associated with Golgi vesicle transport; protein glycosylation; Krebs cycle; respiratory electron transport chain; and monosaccharide, lipid, phospholipid, and nucleotide metabolism decreased in the jejunal DIECs of piglets during the post-weaning period. Moreover, various cellular processes were impaired in these cells after weaning. These findings provide new strategies for improving the differentiation and function of intestinal epithelial cells in weanling mammals. In addition, these data may be used as a basis

Figure 11. Expression of proteins involved in monosaccharide metabolism in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in monosaccharide metabolism based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

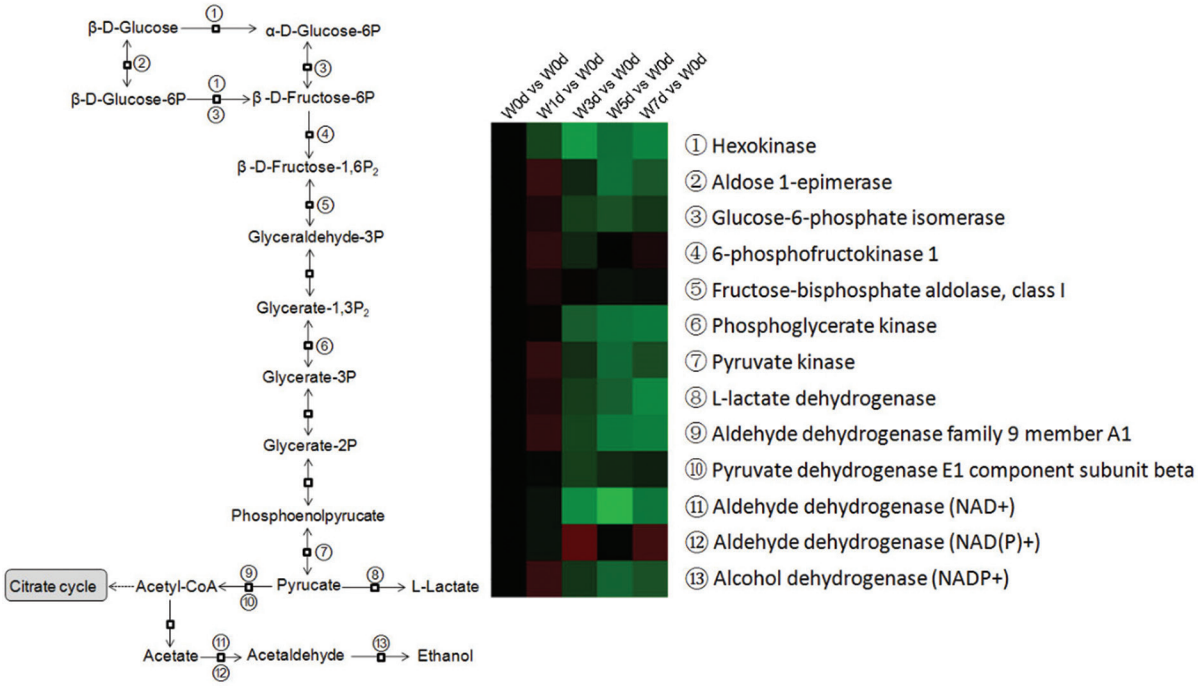

Figure 12. Expression of proteins involved in glycolysis in the jejunal middle villus epithelial cells of weanling piglets. A protein exhibiting ≥1.2- or ≤0.8-fold difference in expression between w1d, w3d, w5d, or w7d and w0d, with a *P* value of ≤0.05, was regarded as being differentially expressed. The glycolysis pathway was analyzed using the KEGG database.

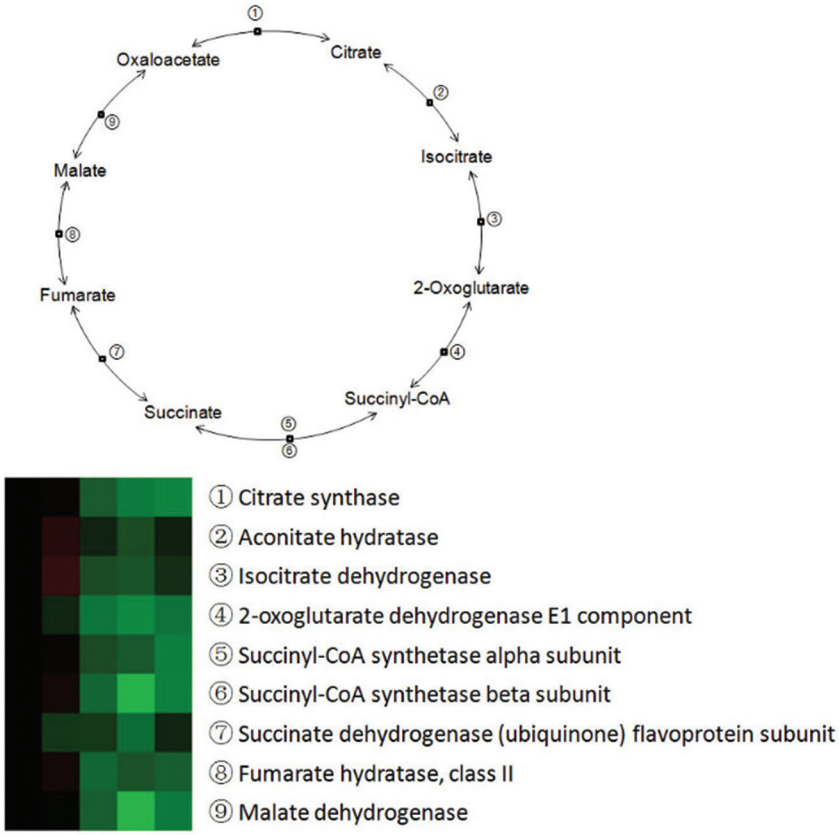

Figure 13. Expression of proteins involved in the Krebs cycle in the jejunal middle villus epithelial cells of weanling piglets. A protein exhibiting ≥1.2- or ≤0.8-fold difference in expression between w1d, w3d, w5d, or w7d and w0d, with a *P* value of ≤0.05, was regarded as being differentially expressed. The Krebs cycle pathway was performed using the KEGG database.

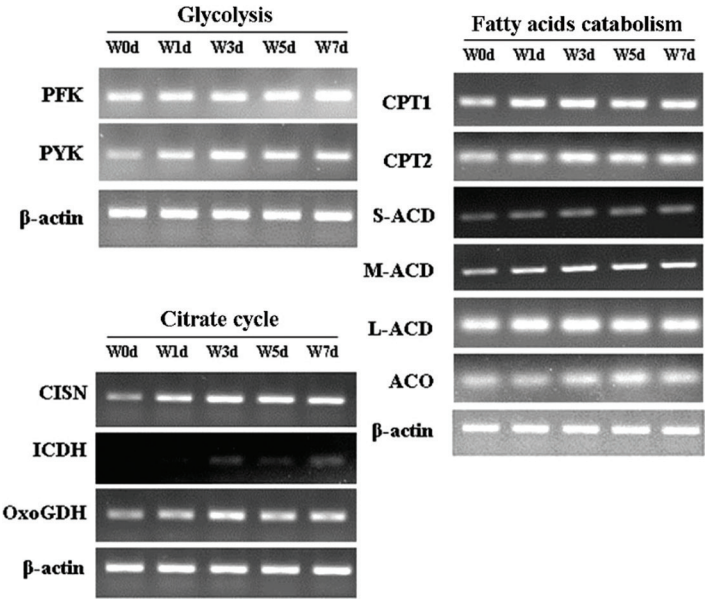

Figure 14. The mRNA levels for genes associated with glycolysis, fatty acid metabolism, and Krebs cycle in the jejunal middle villus epithelial cells of weanling piglets. ACO, acyl-CoA oxidase; CISN, citrate synthase; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase; ICDH, isocitrate dehydrogenase; L-ACD, long-chain acyl-CoA dehydrogenase; M-ACD, medium-chain acyl-CoA dehydrogenase; OxoGDH, oxoglutarate dehydrogenase; PFK, phosphofructokinase; PYK, pyruvate kinase; 2; S-ACD, short-chain acyl-CoA dehydrogenase.

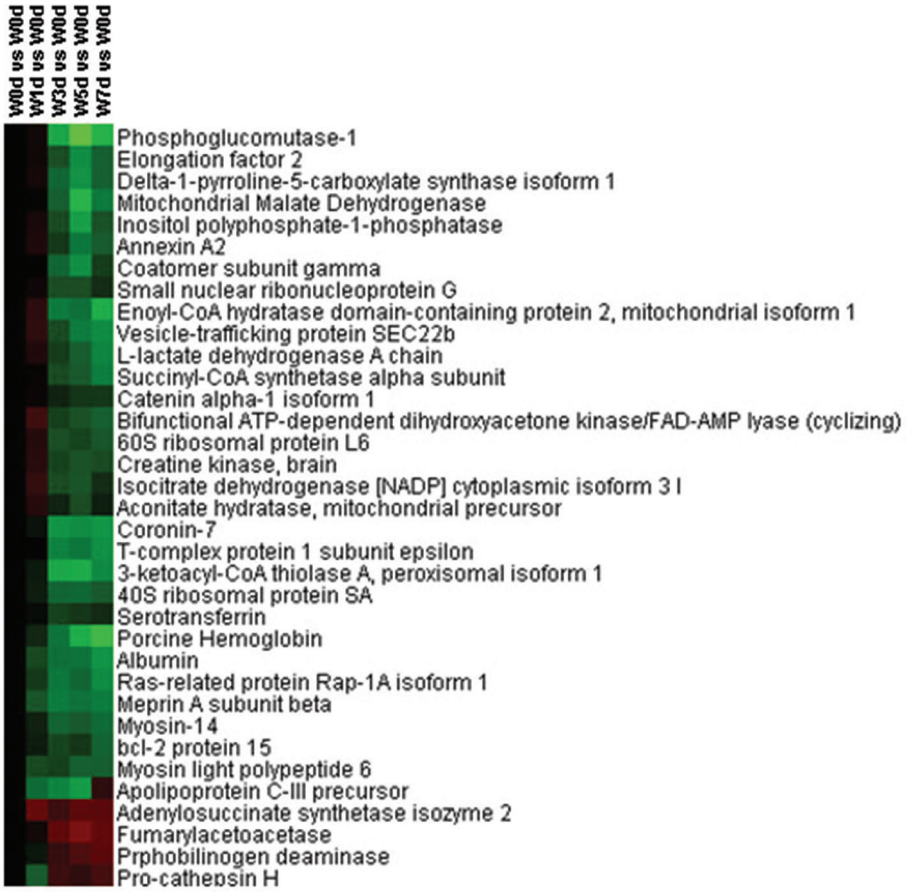

Figure 15. Expression of proteins involved in energy metabolism in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in energy metabolism based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

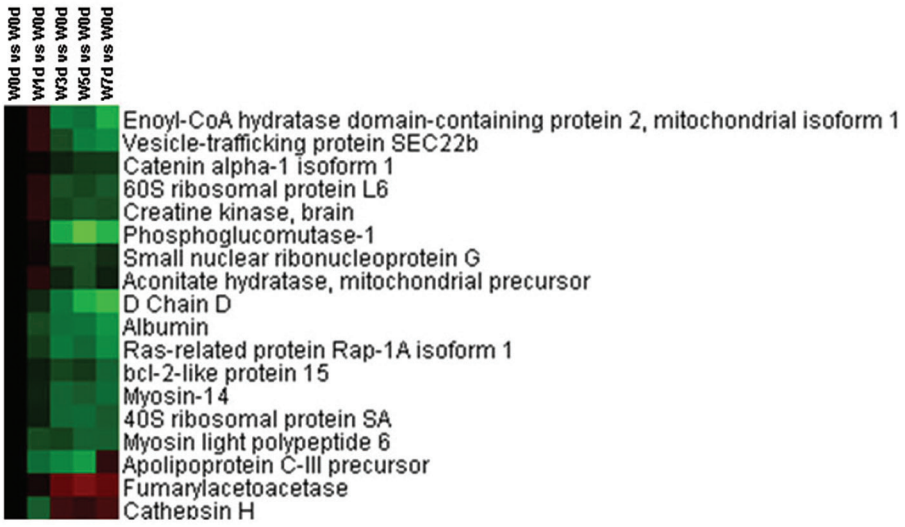

Figure 16. Expression of proteins involved in the respiratory electron transport chain in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in the respiratory electron transport chain based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

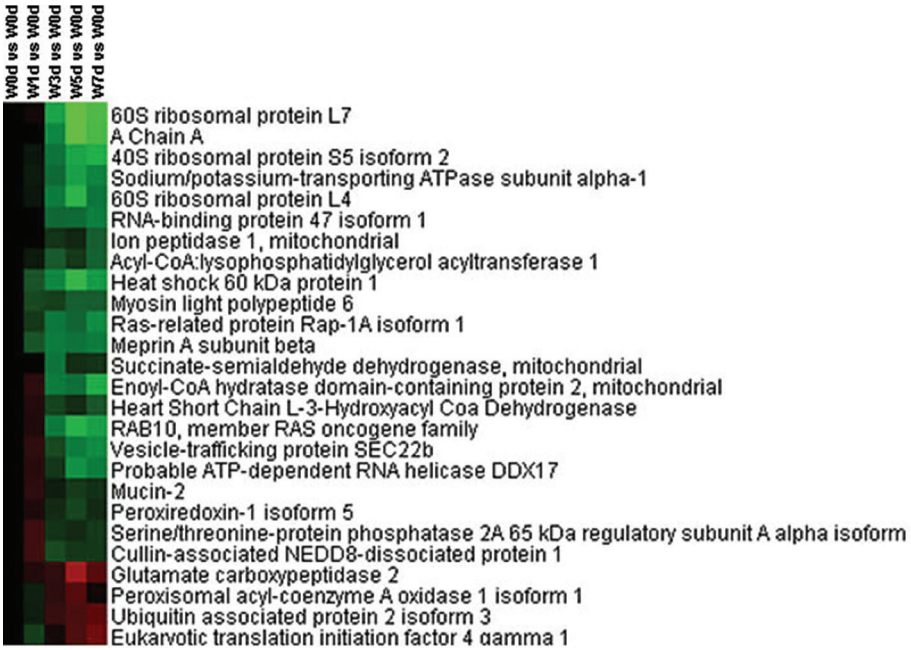

Figure 17. Expression of proteins involved in nucleotide metabolism in the jejunal middle villus epithelial cells of weanling piglets. Differentially expressed proteins (upregulated or downregulated proteins [$P < 0.05$]), which were found to be involved in nucleotide metabolism based on the WEGO program analysis, were selected and clustered using Cluster 3.0.

for providing nutritional support to other young mammals such as calves, lambs, and human infants.

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Abbreviations: ACO, acyl-CoA oxidase; CISN, citrate synthase; CPT1/2, carnitine palmitoyltransferase 1/2; CVA, crypt-villus axis; DIECs, differentiating intestinal epithelial cells; DTT, dithiothreitol; FDR, false discovery rate; ICDH, isocitrate dehydrogenase; L-ACD, long-chain acyl-CoA dehydrogenase; M-ACD, medium-chain acyl-CoA dehydrogenase; OxoGDH, oxoglutarate dehydrogenase; PFK, phosphofructokinase; PMSF, phenylmethylsulfonyl fluoride; PYK, pyruvate kinase; S-ACD, short-chain acyl-CoA dehydrogenase.

Key Words: Weaning, Middle Villi, Epithelial Cells, Piglet, Jejunum, Review

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