Gene Expression Is Altered in Piglet Small Intestine by Weaning and Dietary Glutamine Supplementation\(^1\)\(^-\)\(^3\)

Junjun Wang,\(^4\)\(^,\)\(^5\) Lixiang Chen,\(^5\)\(^-\)\(^7\) Peng Li,\(^5\) Xilong Li,\(^5\) Huaijun Zhou,\(^5\) Fenglai Wang,\(^4\)\(^\ast\) Defa Li,\(^4\) Yulong Yin,\(^3\)\(^,\)\(^6\)\(^\ast\) and Guoyao Wu\(^4\)\(^-\)\(^6\)\(^\ast\)

\(^{4}\)State Key Laboratory of Animal Nutrition, China Agricultural University, Beijing, China 100094; \(^{5}\)Texas Agricultural Experiment Station, The Texas A&M University System, College Station, TX 77843; \(^{6}\)Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, Hunan, China 410128; and \(^{3}\)College of Animal Science and Technology, Hunan Agricultural University, Changsha, Hunan, China 410128

Abstract

Dietary supplementation of glutamine prevents intestinal dysfunction and atrophy in weaning piglets, but the underlying mechanism(s) are largely unknown. This study was conducted to test the hypothesis that weaning or glutamine may modulate expression of genes that are crucial for intestinal metabolism and function. In Expt. 1, we obtained small intestine from 28-d-old pigs weaned at 21 d of age and from age-matched suckling piglets. In Expt. 2, piglets were weaned at 21 d of age and then had free access to diets supplemented with 1% L-glutamine (wt:wt) or isonitrogenous L-alanine (control). At d 28, we collected small intestine for biochemical and morphological measurements and microarray analysis of gene expression using the Operon Porcine Genome Oligo set. Early weaning resulted in increased (52–346%) expression of genes related to oxidative stress and immune activation but decreased (35–77%) expression of genes related to macronutrient metabolism and cell proliferation in the gut. Dietary glutamine supplementation increased intestinal expression (120–124%) of genes that are necessary for cell growth and removal of oxidants, while reducing (34–77%) expression of genes related to oxidative stress and immune activation. Functionally, the glutamine treatment modulates cell proliferation and mucosal integrity.

Introduction

Recent studies have identified high concentrations of free glutamine (0.5–2 mmol/L) as well as peptide-bound glutamine plus glutamate in the milk of mammals, including pigs (1,2). This conditionally essential amino acid is a major energy substrate for rapidly dividing cells [including enterocytes and lymphocytes (3)]. It is also required for the synthesis of purine and pyrimidine nucleotides that are essential for the proliferation of cells, including intestinal mucosal cells and intraepithelial lymphocytes (4). Additionally, glutamine is a major substrate for the endogenous synthesis of arginine in most mammals (including humans and pigs) via the intestinal-renal axis (5). This synthetic pathway compensates for a marked deficiency of arginine (an essential amino acid for neonates) in milk during the suckling period (6). Furthermore, glutamine is required for the synthesis of N-acetylgalactosamine-6-phosphate, a common substrate for the synthesis of glycoproteins that are particularly rich in intestinal mucosa (7).

Weaning is associated with reduced food consumption by young mammals, including piglets (8), therefore decreasing the intake of glutamine from the diet. Notably, weanling piglets often experience intestinal dysfunction and atrophy (9). Recent work has shown that dietary supplementation with glutamine prevents these gut problems (10). However, the underlying mechanism(s) are largely unknown. We hypothesized that weaning or glutamine may modulate expression of genes that are crucial for intestinal metabolism and function. This hypothesis was tested in the present study using microarray technology.
which provides a powerful discovery tool to simultaneously
analyze expression of thousands of genes in a tissue (11).

Materials and Methods

All animals used in this study were humanely managed according to the
established guidelines of the USDA. The experimental protocol was
approved by the Texas A&M University Institutional Animal Care
and Use Committee.

Animals and tissue collection. Pregnant sows were fed daily a 2-kg
gestation diet during the entire period of pregnancy that met the NRC-
recommended requirements for nutrients (12). After farrowing, sows
had free access to a corn- and soybean meal-based diet that also met
NRC-recommended requirements (13). Each sow freely nursed 9 piglets
before weaning at 21 d of age. All sows had free access to drinking water
during gestation and lactation periods. Neonatal piglets were used in 2
series of experiments.

In Expt. 1, 24 21-d-old piglets with similar body weights from 6
litters (4 piglets per litter) were assigned randomly to 1 of the 2 groups on
the basis of their litter origins (n = 12/group). Piglets in group 1
continued to be nursed by sows, whereas piglets in group 2 were
weaned and housed in pens (2 piglets per pen) of the same animal
facilities and had free access to a corn- and soybean meal-based diet and
drinking water, as previously described (10). Milk consumption by
suckling piglets was measured with another similar group of 6 piglets
over an 8-h period at d 21, 24, and 28 of age, using the weight-suckle-
weight technique (14) and the mean value for the 3 measurements was
used to represent nutrient intake by a piglet. An additional group of
piglets was used for the measurement of milk consumption, because this
technique was associated with stress on piglets. Feed intake by weaning
piglets was determined during the 7-d period after weaning. On d 28, at
1 h after sucking or feeding, blood samples (3 mL) were obtained from
the jugular vein and piglets were then humanely killed after anesthesia,
as we previously described (3). Plasma was obtained after centrifugation
at 12,000 × g; 1 min and stored at −80°C. The whole small intestine was
weighed and its length was measured after careful removal of luminal
contents. The luminal contents were centrifuged at 12,000 × g; 1 min. The
supernatant fluid was placed in liquid nitrogen and stored at −80°C
until analysis for glutamine. In neonatal pigs, the small intestine was
defined as the portion of the digestive tract between the pylorus and the
ileocecal valve, with the first 10-cm segment being duodenum (13). The
jejenum and ileum constituted ~40 and 60%, respectively, of the small
intestine below the duodenum (13). A portion of mid-jejenum (~3 cm
each in length) was placed in 4% paraformaldehyde for subsequent
analysis of morphology (14) and another set of samples (~10 cm long)
were obtained for glutathione analysis (15). Jejunal samples (~5 g) were
placed in RNA later solution (Ambion) and stored at −80°C before use
for the isolation of total RNA.

Expt. 2 was conducted as for Expt. 1, except that 24 piglets were
weaned and then assigned randomly to 1 of the 2 treatment groups (n =
12/group), representing supplementation with 1% L-glutamine (wt:wt)
and 1.22% l-alanine (wt:wt; isonitrogenous control) to the corn- and
soybean meal-based diet (10). The basal diet, which contained 21.0%
protein (as an as-fed basis; 89.6% dry matter), was analyzed for
crude protein (on an as-fed basis; 89.6% dry matter), was analyzed for
amino acids, except for leucine and lysine, are potential substrates for hepatic
catabolism of increased amounts of cysteine leads to the production of H2SO4, which
disrupts acid-base balance in animals (17). Finally, all amino acids,
except for leucine and lysine, are potential substrates for hepatic
acetyl-CoA synthesis (10) and glutathione (16). Third, both glutamate and proline are major precursors for endogenous
synthesis of arginine (5) and, thus, their addition to the diet may augment arginine provision in vivo (18). Fourth, in animals, serine and
asparagine are readily converted to glycine and aspartate, respectively
(4). Fifth, tyrosine metabolism via tyrosine hydroxylase requires
tetrahydrobiopterin (17); therefore, its supplementation may reduce the availability of this essential cofactor for the generation of nitric oxide
[a major vasodilator and a key regulator of smooth muscle relaxation
(19)]. Sixth, cysteine is a sulfur-containing amino acid and a substrate for
the synthesis of glutathione (an antioxidant) (16). The catabolism of
phosphoethanolamine provides a powerful discovery tool to simultaneously
analyze expression of thousands of genes in a tissue (11).

Histological and biochemical analyses of the small intestine. Villus
height, crypt depth, and lamina propria depth in jejunum were
determined with the aid of a microscope (10× magnification), as we previously described (10,13). Free glutamine in the jejunal luminal
content was analyzed using the HPLC method involving precolumn derivatization with o-phthalaldehyde (1). Jejunal reduced glutathione (GSH)8
and oxidized glutathione (GSSG) were measured using an HPLC
method of Jones et al. (15), except that: 1) fluorescence detection (Waters 2475 Multi λ Fluorescence Detector) was set at 590-nm excitation and
610-nm emission (0.0–7.5 min) to eliminate the appearance of amino
acid peaks and at 335-nm excitation and 610-nm emission (7.5–38 min)
for GSH and GSSG detection; and 2) gain of the detection was set at 100
(0–32.2 min) for GSH detection and at 1000 (32.2–38 min) for GSSG
detection. GSH and GSSG were quantified on the basis of authentic
standards (Sigma Chemicals) using the Millennium-32 software and
workstation.

RNA extraction. Frozen tissue (0.5 g) was homogenized in 5 mL TRIzol
reagent (Invitrogen) and total RNA was isolated according to the
manufacturer’s recommendations. The RNA extracted was treated with
Algen 2100 Bioanalyzer and RNA 6000 NanoLabChip kit (Agilent
catalog no. 5065–4474). The 28S ribosome:18S ribosome peak areas ratio was ≥1.80 for all samples, indicating little degradation of RNA.

Generation of labeled antisense RNA for microarray. One micro-
gram of total RNA was used as the starting material for amplification using Amino Allyl MessageAmp II aRNA Amplification kit (Ambion
catalog no. 1753). The procedure consisted of reverse transcription with an oligo(dT) primer bearing a T7 promoter and arrayscript, a reverse
transcriptase enzyme that catalyzes the synthesis of a full-length cDNA.
The cDNA underwent second-strand synthesis and then used as template
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catalog no. 1753). The procedure consisted of reverse transcription with an oligo(dT) primer bearing a T7 promoter and arrayscript, a reverse
transcriptase enzyme that catalyzes the synthesis of a full-length cDNA.

8 Abbreviations used: aRNA, antisense RNA; GSH, reduced glutathione; GSSG, oxidized glutathione; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; KLF-10, transforming growth factor β-inducible early growth response protein 1; MAPK, mitogen-activated protein kinase; iRNA, ribosomal RNA.

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transcription generated hundreds to thousands of aRNA copies of each mRNA in the sample. The quality and quantity of the amplified mRNA were analyzed via capillary electrophoresis using RNA nano-assay technique (Agilent 2100 Bioanalyzer). No ribosomal RNA (rRNA) contamination was detected for all samples, indicating the high quality of the aRNA obtained.

Five micrograms of aRNA was used for the coupling reaction using the Mono-Reactive Cy3 and Cy5 dyes (Amersham Biosciences). In Expt. 1, jejunal RNA samples from each of 4 sow-reared piglets were used as controls and labeled with Cy3 (green), whereas RNA samples from each of 4 weaned piglets were labeled with Cy5 (red). In Expt. 2, jejunal RNA samples from alanine (control)- and glutamine-supplemented weaned piglets (n = 4/group) were labeled with Cy3 and Cy5, respectively. A dye swap was adopted for each experiment.

Microarray hybridization and analysis. One microgram of each labeled aRNA was used for hybridization to the porcine oligomicroarray. The paired aRNA samples were combined and fragmented using fragmentation reagents (catalog no. 8740, Ambion) and hybridized overnight with hybridization buffer (SlideHyb catalog no. 8861, Ambion) at 48°C to glass arrays with the Operon Pig Genome Oligo Set containing 11,000 oligonucleotides (genes spotted by the Microarray Core Facility, Department of Systems Biology and Translational Medicine, Texas A&M Health Science Center, Temple, TX). Following the hybridization, the arrays were washed and scanned using a GenePix 4000A scanner (Axon Instruments).

Real-time RT-PCR confirmation of gene expression. Real-time RT-PCR technology was employed to verify changes in the mRNA levels of select genes (Supplemental Table 1) obtained from the microarray analysis. First-strand cDNAs were synthesized from 1 μg of total RNA (10 ng for internal standard: 18S RNA) using oligo (deoxythymidine) primers, random hexamer primers, and SuperScript II Reverse Transcriptase as described (20). RT-PCR analysis was performed using the SYBR Green method and the ABI 7900 Sequence Detection System (Applied Biosystems). The thermal cycling parameters were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers were designed using Primer Express Software version 1.5 (Applied Biosystems) (Supplemental Table 1). The values of cycle threshold, the value of cycle at which the fluorescence reaches a predetermined threshold, were determined using the Applied Biosystems Software. The cycle threshold values were analyzed using the generalized estimating equations model and the PROC GENMOD procedure of the Statistical Analysis System, as we described (21). All of the data were normalized with the 18S rRNA gene in the same samples and are expressed as the relative values to those of piglets fed the control diet.

Statistical analysis. Results are expressed as means ± SE. Data on tissue metabolite concentrations and intestinal morphology were statistically analyzed using an unpaired t test. In microarray analysis, the acquired data were transformed (to accommodate the dye swap), normalized, and filtered using the GeneSpring v7.2 software package (Silicon Genetics, Agilent Technologies). Gene expression significance was assessed using multiple t tests and the Benjamini Hochberg false discovery rate multiple testing correction (20,21). P-values ≤ 0.05 were considered significant. The GeneSpring v7.2 software was used to categorize the genes that were up- and downregulated.

Results

Food intake, body weights, and small intestine weight. Food intake and daily body weight gain between 21 and 28 d of age were reduced (P < 0.01) by 36 and 47%, respectively, in weaned pigs compared with age-matched suckling piglets (Expt. 1; Table 1). At d 28, the small intestine weight was 26% lower (P < 0.01) in weaned than in sow-reared piglets (Table 1). Supplementing 1.0% l-glutamine to the diet for weaned piglets did not affect feed intake but increased (P < 0.05) daily body weight gain between 21 and 28 d of age by 19% compared with the control group (Expt. 2; Table 1).

Glutamine concentrations in plasma and jejunum. Glutamine concentrations in plasma and jejunum of 28-d-old pigs were affected (P < 0.01) by weaning and dietary glutamine supplementation (Table 2). Glutamine concentrations in jejunal lumen fluid, jejunal tissue, and plasma were 70, 38, and 30% lower (P < 0.01), respectively, in weaned than in sow-reared piglets (Expt. 1). Dietary glutamine supplementation increased (P < 0.01) concentrations of glutamine in jejunal lumen fluid, jejunal tissue, and plasma by 638, 107, and 46% (P < 0.01), respectively, compared with alanine-supplemented (control) piglets (Expt. 2).

Glutathione concentrations in jejunum. Concentrations of GSH in jejunal tissue of 28-d-old pigs were 25% lower (P < 0.01) in weaned than in sow-reared piglets (Expt. 1, Table 1). In contrast, weaning increased (P < 0.01) jejunal concentrations of GSSG. As a result, the GSSG:GSH ratio (an indicator of oxidative stress) was 59% greater (P < 0.01) in weaned than in sow-reared piglets. Dietary glutamine supplementation enhanced (P < 0.01) jejunal concentrations of GSH by 29% while reducing (P < 0.01) jejunal concentrations of GSSG by 18% and the GSSG:GSH ratio by 38% (Table 2).

Intestinal morphology and glutathione concentrations. Villus height, crypt depth, and lamina-propria depth in the jejunum of 21-d-old sow-reared piglets were 345 ± 14, 203 ± 12, and 205 ± 13 μm, respectively. Villus height in the jejunum was reduced (P < 0.01) by 43% in 28-d-old weaned piglets than in age-matched suckling pigs (Expt. 1, Table 3). Crypt depth or lamina propria depth did not differ between

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Food intakes and body weights of piglets1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
</tr>
<tr>
<td></td>
<td>Dry matter</td>
</tr>
<tr>
<td>Expt. 1</td>
<td>Suckling</td>
</tr>
<tr>
<td></td>
<td>Weaned</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>W+Ala</td>
</tr>
<tr>
<td></td>
<td>W+Gln</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 6 (feed intake) and 12 (other parameters). One gram dry matter contained 234 mg crude protein. *P < 0.01 vs. age-matched suckling piglets; †P < 0.05 vs. the W+Ala group. W+Ala, weaned pigs receiving dietary supplementation with isonitrogenous l-alanine (1.22%, wt:wt; control); W+Gln, weaned pigs receiving dietary supplementation with l-glutamine (1%, wt:wt).
weaned and sow-reared piglets. Dietary glutamine supplementation increased ($P < 0.01$) jejunal villus height by 38% but had no effect on crypt depth or lamina propria depth (Expt. 2, Table 3).

**Effect of weaning on gene expression in the piglet small intestine.** Compared with age-matched suckling piglets, mRNA levels for 18 genes were reduced ($P < 0.05$) in the jejum of 28-d-old weaned piglets (Table 4). These downregulated genes were acyl-CoA dehydrogenase, N-acyl-d-glucosamine 2-epimerase, adenylate cyclase, ADP-ribosylation factor GTPase activating protein I, aminopeptidase A, apolipoprotein A-IV precursor, carnitine transporter 2, cathepsin F, DNA-binding protein inhibitor ID-2, fatty acid binding protein, insulin-like growth factor II precursor, leukocyte antigen-related protein, oxysterol binding protein-related protein 10, preprogalanin, sodium- and chloride-dependent creatine transporter 1, somatostatin precursor, ubiquitin carboxyl-terminal hydrolase, and vanin-1.

There were 52–346% increases ($P < 0.05$; Table 5) in expression of 21 genes in the jejum of weanling pigs compared with suckling piglets. The upregulated genes were aquaporin 8, core 2-β-16-N-acetylgalactosaminyltransferase, cytochrome P450, galactoside 2-α-1-fucosyltransferase, glutathione transferase mitochondrial precursor, diphosphoesterosterol decarboxylase, hydroxymethylglutaryl-CoA synthase, 3-hydroxy-3 methylglutaryl-CoA reductase, 3-hydroxysteroid-δ-5, 7, 20-desaturase, lysozyme, C-4 methyl sterol oxidase, nudix hydrolase-5, polymeric immunoglobulin receptor precursor, septin 5, and squalene epoxidase.

**Effect of dietary glutamine supplementation on gene expression in the small intestine of weaned piglets.** Intestinal expression of 8 genes decreased ($P < 0.05$) by 34–75% in weaned piglets supplemented with glutamine compared with alanine-supplemented (isonitrogenous control) weaned pigs (Table 6). These downregulated genes were casein kinase Iε, intercellular adhesion molecule-1 (ICAM-1) precursor, la protein homolog, mitogen-activated protein kinase 6 (MAPK-6), peptidyl-prolyl isomerase, Rho-related GTP-binding protein RhoE, pre-mRNA cleavage complex II protein, and transforming growth factor β-inducible early growth response factor (TGF-β-1).

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Villus height</th>
<th>Crypt depth</th>
<th>Lamina propria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling</td>
<td>468 ± 8.5</td>
<td>204 ± 8.1</td>
<td>205 ± 8.2</td>
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<tr>
<td>Weaned</td>
<td>265 ± 7.0*</td>
<td>223 ± 8.7</td>
<td>224 ± 8.8</td>
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<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>W+Ala</td>
<td>269 ± 7.5</td>
<td>228 ± 7.9</td>
<td>228 ± 8.0</td>
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<tr>
<td>W+Gln</td>
<td>371 ± 9.2*</td>
<td>246 ± 7.7</td>
<td>248 ± 7.8</td>
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### Table 4

<table>
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<tr>
<th>No.</th>
<th>Gene name</th>
<th>Common name</th>
<th>$P$-value</th>
<th>Change</th>
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<tbody>
<tr>
<td>W1</td>
<td>NM_001608</td>
<td>Acyl-CoA dehydrogenase (long-chain specific)</td>
<td>0.049</td>
<td>−52</td>
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<tr>
<td>W2</td>
<td>NM_001114</td>
<td>Adenylate cyclase</td>
<td>0.003</td>
<td>−64</td>
</tr>
<tr>
<td>W3</td>
<td>NM_014570</td>
<td>ADP-ribosylation factor</td>
<td>0.036</td>
<td>−65</td>
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<tr>
<td>W4</td>
<td>NM_001977</td>
<td>Aminopeptidase A</td>
<td>0.010</td>
<td>−41</td>
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<tr>
<td>W5</td>
<td>NM_004822</td>
<td>Apolipoprotein A-IV precursor</td>
<td>0.008</td>
<td>−52</td>
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<tr>
<td>W6</td>
<td>TC104654</td>
<td>Cathepsin F</td>
<td>0.015</td>
<td>−35</td>
</tr>
<tr>
<td>W7</td>
<td>NM_002166</td>
<td>RNA-binding protein inhibitor ID-2</td>
<td>0.041</td>
<td>−77</td>
</tr>
<tr>
<td>W8</td>
<td>NM_000134</td>
<td>FZHUI fatty acid-binding protein intestinal</td>
<td>0.012</td>
<td>−50</td>
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<tr>
<td>W9</td>
<td>NM_000612</td>
<td>Insulin-like growth factor II precursor</td>
<td>0.027</td>
<td>−59</td>
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<tr>
<td>W10</td>
<td>NM_130440</td>
<td>Leukocyte antigen related protein precursor</td>
<td>0.047</td>
<td>−36</td>
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<td>W11</td>
<td>NM_002910</td>
<td>N-Acyl-d-glucosamine 2-epimerase</td>
<td>0.046</td>
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<td>W12</td>
<td>NM_003060-2</td>
<td>Carnitine transporter 2</td>
<td>0.040</td>
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<td>W13</td>
<td>NM_017784</td>
<td>Oxysterol binding</td>
<td>0.012</td>
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<td>W14</td>
<td>NM_015973</td>
<td>Protein-related protein 10</td>
<td>0.019</td>
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<tr>
<td>W15</td>
<td>NM_004654</td>
<td>Ubiquitin carboxyl-terminal hydrolase FAF-Y</td>
<td>0.038</td>
<td>−46</td>
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<tr>
<td>W16</td>
<td>NM_005629</td>
<td>Sodium- and chloride-dependent creatine transporter 1</td>
<td>0.002</td>
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<tr>
<td>W17</td>
<td>NM_001048</td>
<td>Somatostatin precursor</td>
<td>0.028</td>
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<tr>
<td>W18</td>
<td>NM_004666</td>
<td>Vanin-1</td>
<td>0.019</td>
<td>−76</td>
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</table>

1. The sign (−) denotes a decrease in mRNA levels in the small intestine of weaned piglets compared with age-matched suckling piglets. FZHUI, fatty acid binding protein human intestine; FAF-Y, Y-linked fat facets protein related; CoA, coenzyme A; ID, inhibitor of DNA-binding protein.

### Table 2

<table>
<thead>
<tr>
<th>Glutamine</th>
<th>In plasma</th>
<th>In jejunal lumen fluid</th>
<th>Jejunal tissue</th>
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<td>Glutamine</td>
<td>μmol/L</td>
<td>mmol/mol</td>
<td>μmol/g tissue</td>
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<tr>
<td>Suckling</td>
<td>507 ± 42</td>
<td>3.76 ± 0.43</td>
<td>1.85 ± 0.11</td>
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<tr>
<td>Weaned</td>
<td>354 ± 30*</td>
<td>1.12 ± 0.16*</td>
<td>1.14 ± 0.06*</td>
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### Table 5

<table>
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<tr>
<th>Treatment</th>
<th>Glutamine</th>
<th>GSH</th>
<th>GSSG</th>
<th>GSSG/GSH</th>
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<tbody>
<tr>
<td>Suckling</td>
<td>507 ± 42</td>
<td>120 ± 6.0</td>
<td>0.049 ± 0.003</td>
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<tr>
<td>Weaned</td>
<td>354 ± 30*</td>
<td>148 ± 7.3</td>
<td>0.078 ± 0.005*</td>
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</tbody>
</table>

1. Values are means ± SEM, n = 12. * $P < 0.01$ vs. age-matched suckling piglets; † $P < 0.01$ vs. the W+Ala group. W+Ala, Weaned pigs receiving dietary supplementation with isonitrogenous L-alanine (1.12%, wt:wt; control); W+Gln, Weaned pigs receiving dietary supplementation with L-glutamine (1%, wt:wt).
Real-time PCR confirmation of gene expression. Changes in mRNA levels for 12 genes were determined using real-time PCR analysis. The 18S rRNA gene was used as an internal standard (house-keeping gene). Results of the RT-PCR analysis indicated that compared with age-matched suckling piglets, mRNA levels for DNA-binding protein inhibitor ID-2 (−81%), insulin-like growth factor II precursor (−77%), ubiquitin carboxyl-terminal hydrolase Y-linked fat facets protein related (−92%), somatostatin precursor (−83%), and vanin (−87%) decreased (P < 0.05), whereas mRNA levels for aquaporin 8 (+315%), glutathione transferase α-1 (+215%), hydroxymethylglutaryl-CoA synthase (cytoplasmic; +63%), and lysozyme (+278%) increased (P < 0.05) in the jejunum of weaned piglets (Expt. 1). Additionally, supplementing glutamine to the diet for weaning piglets decreased (P < 0.05) mRNA levels for casein kinase I epsilon (−34%), MAPK-6 (−67%), and Krueppel-like factor 10 (KLF-10; −51%) compared with alanine-supplemented pigs (Expt. 2). The relative changes in gene expression revealed by RT-PCR analysis were similar to those indicated by the microarray analysis (Tables 4–6).

### Discussion

After mammalian neonates are weaned from their mothers, they undergo tremendous changes in intestinal structure and function (8,9). However, the underlying molecular and cellular mechanisms are largely unknown. Due to the invasive nature of biochemical research on intestinal development, the piglet provides a useful animal model for studying the responses of the neonatal gut to weaning (10). With the recent availability of the microarray technology, which can analyze simultaneously expression of thousands of genes in a tissue (11), we identified significant changes in intestinal expression of key regulatory genes in response to weaning and dietary glutamine supplementation (Tables 4–6). Functionally, the glutamine treatment resulted in increased oxidative-defense capacity (Table 2), prevention of protein I Krueppel-like factor 10 (KLF-10). In contrast, expression of 6 genes increased (P < 0.05) by 120–124% in the jejunum of weaned pigs in response to dietary glutamine supplementation (Table 6). These upregulated genes were AF-9 protein I Krueppel-like factor 10 (KLF-10), the microarray analysis (Tables 4–6).

### Table 5
Enhanced expression of genes in the jejunum of 28-d-old weaned piglets compared with age-matched suckling piglets

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene name</th>
<th>Common name</th>
<th>P-value</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>W19</td>
<td>NM_006579</td>
<td>3-β-Hydroxysteroid-δ-δ&lt;sup&gt;5&lt;/sup&gt; δ-8&lt;sup&gt;-&lt;/sup&gt; δ-8&lt;sup&gt;-&lt;/sup&gt;-isomerase (cholesterol δ-8&lt;sup&gt;-&lt;/sup&gt;-isomerase)</td>
<td>0.031</td>
<td>+55</td>
</tr>
<tr>
<td>W20</td>
<td>NM_00112683</td>
<td>Aquaporin 8</td>
<td>0.002</td>
<td>+220</td>
</tr>
<tr>
<td>W21</td>
<td>NM_006745</td>
<td>C-4 Methyl sterol oxidase</td>
<td>0.019</td>
<td>+76</td>
</tr>
<tr>
<td>W22</td>
<td>NM_006745–2</td>
<td>C-4 Methyl sterol oxidase</td>
<td>0.020</td>
<td>+147</td>
</tr>
<tr>
<td>W23</td>
<td>NM_004751</td>
<td>Core 2 β-18-N-acylglucosaminyltransferase</td>
<td>0.009</td>
<td>+95</td>
</tr>
<tr>
<td>W24</td>
<td>NM_000786</td>
<td>Cytochrome P450 5A1 (sterol 14α-demethylase; lanosterol 14α-demethylase)</td>
<td>0.024</td>
<td>+51</td>
</tr>
<tr>
<td>W25</td>
<td>TC117335</td>
<td>Diposphoehemoglobin</td>
<td>0.010</td>
<td>+54</td>
</tr>
<tr>
<td>W26</td>
<td>TC118958</td>
<td>Farnesyl diposphate synthase</td>
<td>0.042</td>
<td>+102</td>
</tr>
<tr>
<td>W27</td>
<td>NM_000511</td>
<td>Galactoside 2-α-L-fucosyltransferase 2</td>
<td>0.033</td>
<td>+125</td>
</tr>
<tr>
<td>W28</td>
<td>NM_004832</td>
<td>Glutathione transferase α-1</td>
<td>0.037</td>
<td>+206</td>
</tr>
<tr>
<td>W29</td>
<td>NM_002130</td>
<td>Hydroxymethylglutaryl-CoA synthase, cytoplasmic</td>
<td>0.019</td>
<td>+141</td>
</tr>
<tr>
<td>W30</td>
<td>TC104371</td>
<td>Ig α-chain C</td>
<td>0.024</td>
<td>+268</td>
</tr>
<tr>
<td>W31</td>
<td>TC119456</td>
<td>Ig α-chain C region</td>
<td>0.033</td>
<td>+153</td>
</tr>
<tr>
<td>W32</td>
<td>TC103973</td>
<td>Ig λ-chain</td>
<td>0.049</td>
<td>+279</td>
</tr>
<tr>
<td>W33</td>
<td>NM_144646</td>
<td>Ig J-chain</td>
<td>0.021</td>
<td>+192</td>
</tr>
<tr>
<td>W34</td>
<td>NM_214392</td>
<td>Lysosome</td>
<td>0.007</td>
<td>+366</td>
</tr>
<tr>
<td>W35</td>
<td>NM_014142</td>
<td>Nudix hydrolyase-5</td>
<td>0.045</td>
<td>+52</td>
</tr>
<tr>
<td>W36</td>
<td>NM_004585</td>
<td>Plasminogen activator-inducible c54</td>
<td>0.031</td>
<td>+158</td>
</tr>
<tr>
<td>W37</td>
<td>NM_002644</td>
<td>Polymeric-immunoglobulin receptor precursor</td>
<td>0.018</td>
<td>+137</td>
</tr>
<tr>
<td>W38</td>
<td>TC104588</td>
<td>Septin 5 (peanut-like protein-1; cell division control related protein-1)</td>
<td>0.047</td>
<td>+153</td>
</tr>
<tr>
<td>W39</td>
<td>NM_003129</td>
<td>Squalene epoxidase</td>
<td>0.011</td>
<td>+67</td>
</tr>
</tbody>
</table>

1 The signs (−) and (+) denote decreased and increased mRNA levels, respectively, in the small intestine of weaned piglets receiving dietary supplementation with l-glutamine (1%, wt:wt), compared with piglets receiving dietary supplementation with isonitrogenous l-alanine (1.22%, wt:wt; control). La protein, a nuclear phosphoprotein first described as an autoantigen, with the name La deriving from the name of the patient in which the antibody was detected; AF-9, ALL1 fused gene from chromosome 9; Cd, cluster designation; Pcf11, mammalian homolog cleavage and polyadenylation factor II subunit.

### Table 6
Effects of dietary glutamine supplementation on gene expression in the jejunum of weaned piglets

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene name</th>
<th>Common name</th>
<th>P-value</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>NM_152221</td>
<td>Casein kinase I epsilon</td>
<td>0.048</td>
<td>−34</td>
</tr>
<tr>
<td>G2</td>
<td>TC105498</td>
<td>Intercellular adhesion molecule-1 precursor</td>
<td>0.032</td>
<td>−38</td>
</tr>
<tr>
<td>G3</td>
<td>NM_016648</td>
<td>La protein homolog</td>
<td>0.002</td>
<td>−41</td>
</tr>
<tr>
<td>G4</td>
<td>NM_002748–2</td>
<td>MAPK-6</td>
<td>0.018</td>
<td>−75</td>
</tr>
<tr>
<td>G5</td>
<td>NM_004792</td>
<td>Peptidyl-prolyl isomerase G</td>
<td>0.003</td>
<td>−37</td>
</tr>
<tr>
<td>G6</td>
<td>NM_015885–3</td>
<td>Pre-mRNA cleavage complex II</td>
<td>0.029</td>
<td>−61</td>
</tr>
<tr>
<td>G7</td>
<td>NM_005168</td>
<td>Rho-related GTP-binding protein RhoE (RhoB; Rho3)</td>
<td>0.017</td>
<td>−59</td>
</tr>
<tr>
<td>G8</td>
<td>NM_005855</td>
<td>Transforming growth factor-β-inducible early growth response protein 1 (TGFB-inducible early growth response protein 1); KLF 10)</td>
<td>0.001</td>
<td>−64</td>
</tr>
</tbody>
</table>

1 The signs (−) and (+) denote decreased and increased mRNA levels, respectively, in the small intestine of weaned piglets receiving dietary supplementation with l-glutamine (1%, wt:wt), compared with piglets receiving dietary supplementation with isonitrogenous l-alanine (1.22%, wt:wt; control). La protein, a nuclear phosphoprotein first described as an autoantigen, with the name La deriving from the name of the patient in which the antibody was detected; AF-9, ALL1 fused gene from chromosome 9; Cd, cluster designation; Pcf11, mammalian homolog cleavage and polyadenylation factor II subunit.

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intestinal atrophy (Table 3), and enhanced growth performance (Table 1) of early-weaned piglets.

Weaning was associated with reduced expression of 18 genes out of 11,000 in the piglet small intestine (Table 4). Interestingly, these genes encode for proteins that are related to: 1) the regulation of gene expression [DNA-binding protein inhibitor ID-2 (22)]; 2) protein and peptide degradation [aminopeptidase A, cathepsin F, and ubiquitin carboxyl-terminal hydrolase (23)]; 3) lipid metabolism [acyl-CoA dehydrogenase, carnitine transporter 2, and oysterbinding protein-related protein 10 (24)]; 4) signal transduction [adenylate cyclase and ADP-ribosylation factor GTPase-activating protein I (25)]; 5) immune function [leukocyte antigen-related protein and vanin-1 (26)]; 6) growth regulation (insulin-like growth factor II precursor and somato-growing factor GTPase-activating protein I (25)]; 7) aminosynthysis [N-acetyl-d-glucosamine 2-epinerase (27)]; and 8) intestinal transport [apolipoprotein A-IV precursor, fatty acid-binding protein, sodium- and chloride-dependent creatine transporter I, and preproga1anin (28)].

Downregulation of these genes is expected to reduce oxidative defense capacity, intestinal transport and utilization of dietary nutrients (particularly lipids and proteins), immune response, and synthesis of glycoproteins (major proteins secreted by the intestinal mucosa), as well as proliferation and differentiation of intestinal epithelial cells. This is consistent with the previous report that intestinal atrophy and dysfunction often occur in early-weaned piglets (8,9) in association with an increased GSSG:GSH ratio (Table 3), an indicator of cellular oxidative stress (16).

Expression of 21 genes was enhanced in the small intestine of piglets in response to early weaning (Table 5). These genes encode for proteins that play crucial roles in regulating: 1) lipid metabolism [3-β-hydroxysteroid-Δ, Δ-isomerase, C-4 methyl sterol oxidase, diphosphomevalonate decarboxylase, hydroxy-methylglutaryl-CoA synthase, and squalene epoxidase (29,30)]; 2) water secretion [aquaporin-8 (31)]; 3) aminosynthysis (core 2-β-16-N-acetyl-glucosaminyltransferase); 4) xenobiotic metabolism and oxidative defense [cytochrome P450, glutathione transferase α-1 (32), and nudix hydrolase-5 (33)]; 5) carbohydrate metabolism [galactoside 2-α-l-fucosyltransferase]; 6) immune function (Ig α chain C, Ig κ chain C, Ig λ chain, Ig J chain, lysozyme, polymeric Ig receptor precursor); and 7) cellular division [septin-5 (34)]. Emerging evidence shows that cAMP mediates expression of aquaporin-8 expression in small intestinal mucosa in response to cholera toxin (35). The enhanced expression of aquaporin-8, which stimulates water permeation across the gut (31), may help explain the increased secretion of water from intestinal mucosal cells and the malabsorption of water from the intestinal lumen and, therefore, diarrhea that frequently occurs in early-weaned piglets (36). Additionally, weaned piglets were fed a typical corn- and soybean meal-based diet that contained high levels of plant-origin antigens (37). Results from the present study indicate that a response to such a diet was increased expression of genes responsible for the synthesis of Ig in the small intestine (Table 4). Also, the increased expression of glutathione S-transferase [which catalyzes the conjugation of GSH to electrophilic substances (32) and nudix hydrolase-5 [which eliminates toxic nucleotide derivatives from cells and regulates the levels of signaling nucleotides (33)] suggests the presence of oxidative stress in the small intestine of weaned piglets. In support of this view, the GSSG:GSH ratio in the jejunum was elevated by 59% in response to early weaning (Table 2).

Previous studies have shown that glutamine regulates MAPK activation and C-Jun signaling in enterocytes (38). In addition, this amino acid enhances intestinal oxidative metabolism, polyamine synthesis, ion transport, and cell proliferation (39,40) as well as cytoprotection via heat shock proteins (41,42). Consistent with these reports, we found that, in response to dietary glutamine supplementation, expression of 8 genes was downregulated in the small intestine (Table 6). These genes are related to cellular signaling transduction [casein kinase 1 epsilon and MAPK-6 (43)], the cell cycle [Rho-related GTP-biding protein (44)], apoptosis [KLF-10 (45)], immune activation (La antoanigen homolog and ICAM-1), protein modification [peptidyl-prolyl isomerase G (46)], and gene expression (pre-mRNA cleavage complex II). Enhanced expression of antigens and activation of leukocytes (e.g. lymphocytes and macrophages) in the intestinal epithelium because of activation of the MAPK-6 signaling may contribute to intestinal dysfunction and diarrhea in weaning pigs (36). In addition, peptidyl-prolyl isomerase G plays a role in posttranslational protein modifications and cell proliferation (46), which is likely unfavorable for intestinal integrity in weaning piglets. Likewise, ICAM-1 associates with receptors of the integrin family proteins, thereby playing an important role in immune activation (47). Also, Rho GTPase inhibits cell cycle progression by decreasing Ras (a signal transduction protein with an abbreviation that originated from rat sarcoma)- and Raf (an oncogene that encodes protein kinase with an abbreviation from rat fibrosarcoma)-induced fibroblast transformation (44). Furthermore, KLF-10 induces and promotes apoptosis through the mitochondrial apoptotic pathway (48). Therefore, downregulation of these genes provides an additional explanation for the effect of dietary glutamine supplementation on reducing intestinal damage and enhancing intestinal cell proliferation in early-weaned piglets (10).

Another beneficial effect of glutamine supplementation is increased expression of 6 genes (Table 6) related to transcription regulation [AF-9 protein (49)], lipid metabolism (endozepine), iron absorption (heme-binding protein), cytoskeletal structure and function (myosin), defense against pathological microorganisms [IL-13R-α1 (50) and endozepine (51)], and regulation of nutrient metabolism (signal recognition particle 72K chain, AF-9 is a transcription factor that regulates gene expression and cell growth (52)]. IL-13 stimulates contractility of intestinal smooth muscle (50) and, thus, the movement of luminal digesta along the small intestine, which facilitates the digestion of macronutrients by various digestive enzymes and the absorption of resultant smaller molecules by enterocytes. Signal recognition particle (a cytoplasmic ribonucleoprotein), which consists of 1 RNA and 6 proteins, regulates gene expression and protein function (53). Additionally, endozepine, which is expressed in intestinal mucosal cells (54), has multiple functions in intestinal metabolism, physiology, and immunology. This polypeptide acts like acyl CoA-binding protein, therefore regulating lipid metabolism, assembly, and trafficking across the small intestine (55). Also, porcine endozepine has a potent antibacterial activity in the porcine gut (56). Because early weaning is often associated with immunological challenges in the intestine (36), elevated expression of endozepine in glutamine-supplemented piglets may protect the gut from infections during weaning. Collectively, these findings provide a mechanism for the effect of dietary glutamine supplementation on preventing intestinal atrophy and improving nutrient digestion and utilization in early-weaned piglets (10).

In conclusion, results of the microarray analysis reveal that early weaning resulted in increased expression of genes that promote oxidative stress and immune activation but decreased expression of genes related to nutrient utilization and cell proliferation in the piglet small intestine. In contrast, dietary supplementation of glutamine to weaning piglets enhanced
expression of genes that prevent oxidative stress, improve antibacterial activity, enhance nutrient absorption, and stimulate cell growth. These novel findings reveal coordinate alterations of gene expression in response to weaning and aid in providing molecular mechanisms for explaining the previous observation that dietary glutamine supplementation prevents intestinal dysfunction and enhances the growth performance of early-weaned piglets. Further work is necessary to determine how these changes in gene expression translate into enhanced gut growth and function.

Acknowledgments
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Literature Cited

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