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Apical Na⁺-dependent neutral amino acid exchanger ASCT2 (ATB⁰) and mTOR-signaling components are expressed along the entire jejunal crypt-villus axis in young pigs fed a liquid milk replacer

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Running title: *EXPRESSION OF THE ASCT2 EXCHANGER IN THE PORCINE GUT*

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Abbreviations: AA, amino acid(s); AM, apical membrane; ASCT2, system ASC sodium-dependent neutral AA exchanger 2; ATB⁰, Na⁺-dependent neutral amino acid exchanger; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; Gln, glutamine; Leu, leucine; mTOR, mechanistic & mammalian target of rapamycin; PMSF, phenylmethanesulfonyl fluoride; S6K1, ribosomal S6 kinase 1; SLC1A5, solute carrier family 1 member 5; TLCK, Na-*p*-tosyl-L-Lysine chloromethyl ketone

ABSTRACT

Yang, C., Yang, X. and Fan, M. Z. 2016. **Apical Na⁺-dependent neutral amino acid exchanger ASCT2 (ATB⁰) and mTOR-signaling components are expressed along the entire jejunal crypt-villus axis in young pigs fed a liquid milk replacer.** Can. J. Anim. Sci. xxxxx. The Na⁺-dependent neutral amino acid (AA) exchanger ASCT2 (ATB⁰) plays an important role for the apical exchange of large neutral AA, and is essential to maintain gut mucosal growth. Objectives of this study were to investigate expression of ASCT2/ATB⁰ (SLC1A5) and key mTOR-signaling components in epithelia along the jejunal crypt-villus axis in young pigs fed a liquid milk replacer. Real time RT-PCR analyses showed that the ASCT2/ATB⁰ (SLC1A5) mRNA abundance was higher ($P < 0.05$) in the crypt than in the upper villus and the middle villus cells. There were no differences ($P > 0.05$) in the ASCT2/ATB⁰ protein abundances in the cell homogenate, the intracellular fraction and the apical membrane among the upper villus, middle villus and crypt cells. Abundance of phosphorylated mTOR protein (p-mTOR) was higher ($P < 0.05$) in the middle villus than in the upper villus cells. Phosphorylated p70 S6 kinase protein (p-S6K1) abundance was higher ($P < 0.05$) in the crypt than in the upper villus cells. Thus, the ASCT2/ATB⁰ (SLC1A5) gene mRNA and protein ATB⁰ and the key mTOR-signaling components p-mTOR and p-S6K1 were expressed in the epithelia along the entire jejunal crypt-villus axis in the young pigs fed a liquid milk replacer.

Key words: system ASC sodium-dependent neutral AA exchanger 2, mechanistic & mammalian target of rapamycin signaling, small intestinal crypt-villus axis, young pigs

INTRODUCTION

Mammalian intestinal growth is rapid during suckling (Schwarz and Heird 1994). Small intestinal mucosal growth is a dynamic process dominated by the proliferation, differentiation and eventual apoptosis of enterocytes along the crypt-villus axis (Henning et al. 1994). This process is regulated by luminal amino acid (AA) availability (Ruemmele et al. 2002). Luminal AA not only serve as metabolic fuels and biosynthetic precursors (Burrin et al. 2000) but also act as important signaling molecules in modulating cellular metabolism and growth (Kilberg et al. 2005). There are two known pathways employed by mammalian cells to sense AA availability including the AA response (AAR) pathway triggered by AA deprivation (Kilberg et al. 2005), and the mechanistic & mammalian target of rapamycin (mTOR) pathway (Yang et al. 2008; Abraham 2015).

As gate keepers, membrane-bound AA transporters such as the B⁰-system sodium-dependent neutral AA co-transporter (B⁰AT1) and exchangers such as the system ASC sodium-dependent neutral AA exchanger 2 (ASCT2/ATB⁰) have been shown to serve as the putative cellular surface AA transceptor for AA signaling in cells including the gut epithelia via the mTOR-signaling pathway (Fuchs et al. 2007; Yang et al. 2008; Hundal and Taylor 2009; Yang et al. 2016). The system ASC was shown to be responsible for Na⁺-dependent obligatory exchange activities of only certain specific small and large neutral AA (e.g., Gln) in the small intestine (Del Castillo et al. 2002; Kanai and Hediger, 2004; Yang, 2011). The intestinal AA exchanger ASCT2/ATB⁰ was identified on the apical membrane (Torres-Zamorano et al. 1998; Avissar et al. 2001). The ASCT2/ATB⁰ encoding gene

SLC1A5 was cloned in several animal species (Kekuda et al. 1996; Utsunomiya-Tate et al. 1996; Kekuda et al. 1997; Bröer et al. 1999; Pollard et al. 2002). The ASCT2/ATB⁰ (SLC1A5) gene was originally cloned by Kekuda et al. (1996) and had been mistakenly regarded as the B⁰-system sodium-dependent neutral AA co-transporter gene for several years (Kekuda et al. 1996; Avissar et al. 2001) until that the real B⁰-system transporter B⁰AT1 (SLC6A19) gene was later cloned and the confusion was clarified (Bröer et al. 2004; 2006; Yang, 2011). Expression of the B⁰AT1 (SLC6A19) gene has been recently reported (Yang et al. 2016), however, little is known about expression of the ASCT2/ATB⁰ exchanger encoding gene SLC1A5 and its protein along the small intestinal crypt-villus axis in fast growing young animals.

Artificial rearing suckling neonatal pigs and feeding early-weaned pigs on milk replacers are practiced to support rapid gut and whole body growth in the young pigs, and to increase production efficiency in commercial swine production (Zijlstra et al. 1996; Veum and Odle 2001). Effect of dietary supplementation of trophic AA on gut mucosal growth and positive association between gut mucosal growth and the small intestinal apical AA transporter expression have been well established (Yang et al., 2012; 2013; Zhang et al., 2014; Yang et al. 2016). Understanding of the expression of ASCT2/ATB⁰ and key mTOR-signaling components in the gut mucosa particularly in the crypt will lead to the development of nutritional strategies to enhance gut mucosa growth and health in young pigs.

We hypothesized that the ASCT2/ ATB⁰ exchanger encoding gene SLC1A5, the ASCT2/ATB⁰ protein, and the mTOR-signaling components would be expressed along the entire small intestinal crypt-villus axis particularly in the crypt region in support of rapid

luminal AA-dependent mucosal growth in young pigs. Objectives of this study were to investigate expression of the ASCT2/ATB⁰ encoding gene SLC1A5, the ASCT2/ATB⁰ protein, and key mTOR-signaling components in epithelia along the jejunal crypt-villus axis in young pigs fed a commercial liquid milk replacer.

MATERIALS AND METHODS

Materials

Dye reagent for protein analysis was purchased from BIO-RAD Laboratories (Hercules, CA). Bovine serum albumin (fraction V), D-mannitol, Tris-HCl, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), diethylpyocarbonate (DEPC), Na-*p*-tosyl-L-Lysine chloromethyl ketone (TLCK-HCl), aprotinin, leupeptin, pepstatin A, and other chemicals and reagents, unless specified, were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Sources of antibodies for the Western blotting analyses and kits for the real time RT-PCR work were specified.

Animals and Tissue Preparation

Six Yorkshire neonate piglets at the age of d 7 were acquired from three different Yorkshire sows from the Arkell Swine Research Station at the University of Guelph and were individually housed in metabolic crates equipped with a liquid milk replacer drinking feeder secured within each cage in an environmentally controlled room (23°C). Two extra infra-red heating lamps were provided to the headspace in each of our metabolic cages to maintain the environment within each of the cages at about 28-29°C for the duration of the study. On the

day of removal from their sows, the piglets in their crates were immediately fed three times daily (8 am, 12 noon and 4 pm) a liquid milk replacer that was freshly mixed by using the Soweena®-Litter Life™ formula powder (Merricks Inc., Middleton, WI, USA). The piglets were able to reach their *ad libitum* intake of the milk replacer in about 2 days. The dairy milk based commercial milk replacer powder contained (% on air-dry basis) 52.7 lactose, 25 protein, 10 fat and 12.7 other supplements including minerals, vitamins, and antibiotics (Fan et al. 2001). The milk replacer was daily made by mixing 1 part of the formula powder with 5 parts of portable water (wt/wt). The use of a commercial milk replacer for feeding our experimental piglets was for the following reasons. Artificial rearing suckling pigs with a commercial dairy milk-based milk replacer is practiced in swine industry (Veum and Odle 2001). Feeding all of our test piglets with the same milk replacer to replace natural suckling could minimize potential individual variability in the gut function among the testing piglets, which would be potentially contributed from suckling from different sows because of variable milk nutrient composition among the sows.

At the age of 16 d, the piglets were given their milk replacer at 8 am, completed their meal by 9 am, and were anesthetized by inhalation of 5% isoflurane (Aerrane, Anaquest, WI) via a facial mask on the sampling day. The sampling process took about 25-30 min for one piglet, and all the six piglets were sampled before the 12 noon on the sampling day. Thus, all piglets were sampled for their small intestinal tissues under the fed condition. The abdomen was immediately opened and a 480-cm jejunal segment, starting at 30 cm posterior to the pyloric sphincter and at 60 cm anterior to the ileo-cecal ligament of Treitz, was removed and flushed twice with 60 ml of ice-cold phosphate-buffered saline (0.2 mM PMSF and 0.5 mM

DTT, at pH 7.4) (Fan et al. 2001). The isolated jejunal segment was divided for cell isolation as further described below. The piglets were sacrificed with an overdose ($50 \text{ mg} \cdot \text{kg}^{-1}$ body weight) of sodium pentobarbital (Sigma-Aldrich). The experimental protocol was approved by the Animal Care and Use Committee at the University of Guelph.

Sequential Isolation of Jejunal Epithelial Cells Along the Crypt-villus Axis

We have adopted the Weiser distended intestinal sac technique for sequentially isolating jejunal epithelial cells along the crypt-villus axis from the piglets by adapting a procedure that was developed from our previous studies (Fan et al. 2001). Sequentially isolated jejunal epithelial cells along the crypt-villus axis from the piglets were shown to be highly viable and were effectively used to characterize intestinal apical membrane nutrient transporter gene expression in our previous studies (Fan et al. 2004; Yang et al. 2011).

The above divided jejunal segments were quickly filled with a pre-incubation buffer. The pre-incubation buffer contained 27 mM sodium citrate, 0.2 mM PMSF and 0.5 mM DTT at pH 7.4, was oxygenated with O_2/CO_2 mixture (19:1, vol/vol), and was pre-warmed in a water bath (37°C) prior to its use. The pre-incubation buffer-filled jejunal segments were sealed with two pairs of hemostatic forceps, and were immersed in saline (154 mM NaCl) in 2-l glass beakers, and were incubated for 15 min. After the pre-incubation, the jejunal segments were filled with an isolation buffer. The isolation buffer contained 1.5 mM Na_2EDTA , 0.2 mM PMSF, 0.5 mM DTT and 2 mM L-glucose, was oxygenated with the O_2/CO_2 mixture, and was warmed at 37°C . The jejunal segments were repeatedly filled with the isolation buffer for three consecutive incubations for 40, 50, and 60 min for the further

sequential isolation of 3 epithelial cell fractions, representing the upper villus, the middle villus and the crypt epithelial cells, respectively (Fan et al. 2004). Each isolated epithelial cell fraction was washed twice with 150 ml of oxygenated cell suspension buffer (155 mM KCl at pH 7.4) and the cells were retained through centrifugation at 400 x g for 4 min at 4°C (Fan et al. 2001). Washed jejunal epithelial cell fractions were also pooled between the proximal and the distal jejunal segments within each of the piglet.

Freshly isolated and retained small intestinal epithelial cells had a cell viability of 90-95% by trypan blue exclusion prior to their immediately being frozen in liquid nitrogen for storage. The frozen cells were further pulverized to be a homogenous powder under liquid nitrogen by a mortar and pestle based procedure (Yang et al. 2011). Ground samples were stored at -80°C for further analyses.

Preparation of Jejunal Enterocyte Apical Membrane and Intracellular Fraction

Enterocyte apical membrane was prepared by Mg^{2+} -precipitation and differential centrifugation according to our previously established procedures (Fan et al. 2004). Specifically, about 1.3 g of individually pulverized frozen cell epithelial sample was thawed in an ice-cold homogenizing buffer (50 mM D-mannitol and 0.1 mM PMSF at pH 7.4) at a ratio of 20 mL of the homogenizing buffer per gram of frozen cell sample, and was homogenized using a polytron homogenizer. The remaining homogenate was centrifuged at 2,000 x g for 15 min.

The supernatant was mixed with 1 M $MgCl_2$ to contain a final concentration of 10 mM $MgCl_2$, stirred for 15 min, and then centrifuged at 2,400 x g for 15 min. The top foamy layer

was discarded and the resultant supernatant was centrifuged at 19,000 x *g* for 30 min to pellet the crude apical membrane. The further resulting supernatant from the 19,000 x *g* centrifugation was regarded as the intracellular soluble fraction according to Lackeyram et al. (2010), and was sampled for Western blotting analysis of the ASCT2/ATB⁰ AA exchanger protein abundances.

The crude apical membrane pellets were then suspended in a suitable amount of a membrane suspension buffer (150 mM KCl, 180 mM D-mannitol at pH 7.4) and centrifuged at 39,000 x *g* for 30 min to generate the final apical membrane vesicle pellets (Fan et al. 2004). The final pellets were re-suspended with a 25-gauge needle in a suitable volume of the same membrane suspension buffer. The final membrane vesicle suspension was assayed for protein content by using a commercial kit (Bio-Rad) with bovine serum albumin (fraction V) as a standard and diluted with the same buffer to appropriate concentration for subsequent Western blotting analyses. The apical membrane marker, aminopeptidase N specific activity, was typically enriched between 6 and 10 folds in the prepared apical membrane fractions compared with their cell homogenate samples. K⁺-stimulated phosphatase was the basolateral membrane marker, while other intracellular membrane markers include the mitochondrial membrane marker succinate dehydrogenase, the endoplasmic reticulum marker D-glucose-6-phosphatase and the lysosomal membrane marker acid phosphatase (Fan et al. 2004). Specific activities of the basolateral membrane and the other intracellular membrane marker enzymes were regularly assayed and were typically shown to be below one fold in the prepared apical membrane vesicle fraction in comparison with their corresponding cell homogenate samples, and suggested little contamination of the apical membrane preparation

with the basolateral and the other intracellular membrane fractions (Fan et al. 2004).

RNA Extraction and Generation of Partial–Length Porcine SLC1A5 cDNA

Total RNA was isolated from 100 mg of the pulverized isolated jejunal epithelial cell samples using TRIZOL reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Invitrogen) according to the manufacturer's instructions. The RNA quality was checked by 1 % agarose gel electrophoresis with bands stained with 10- μ g/mL ethidium bromide. The RNA had an OD₂₆₀:OD₂₈₀ ratio between 1.8 and 2.0. Synthesis of the first strand cDNA was performed with a total of 1 μ g RNA, random and oligo(dT)₂₀ primers, and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Primers to recognize the partial-length porcine sequence were designed with Primer 3 (http://frodo.wi.mit.edu/primer3/primer3_code.html) based on conserved region of the human SLC1A5 gene sequence. The PCR primers used for the amplification of the partial cDNA sequence of ASCT2 were 5'-CTACAACTCTGTGCACAACA-3' (forward) and 5'-CGCCCTCCATGTTCCCAAAC-3'(reverse). The expected size of the SLC1A5 gene RT-PCR product was 526 bp (corresponding to bp 1394-1920 of the human SLC1A5 gene sequence, GenBank accession no. NM_005628). PCR conditions were as follows: 95 °C denature 2 min, 40 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 10 min. Each of 50- μ L PCR reactions contained 38.1 μ L of 0.1 % diethylpyrocarbonate (DEPC)-treated water, 1 μ L each of forward and reverse primer (10 μ M), 5 μ L of 10 $\times$ PCR Buffer; 1 μ L of dNTP mix (10 mM), 0.4 μ L of TaqDNA polymerase

(5 U/ μ L), 1.5 μ L of $MgCl_2$ (50 mM), 2 μ L cDNA. All PCR products were electrophoresed on a 2 % agarose gel in Tris-borate-EDTA buffer and visualized by staining with 10 μ g/mL ethidium bromide. To confirm the identify of each PCR product, PCR products were purified and sequenced by dideoxy-mediated chain termination sequencing at the University of Guelph Molecular Supercenter. The nucleotide sequence of the SLC1A5 gene reported in this paper resides in the GenBank/EMBL data bank (accession no. DQ231578). Sequence multiple alignments and comparisons to other GenBank entries were performed using CLUSTAL W software (<http://genome.cs.mtu.edu/map.html>).

Real-time RT-PCR Analysis

Primers for the SLC1A5 and β -actin genes were designed with Primer 3 (http://frodo.wi.mit.edu/primer3/primer3_code.html) based on their respective porcine sequences to produce an amplification product. The SLC1A5 and β -actin gene PCR primers used were 5'-GCCAGCAAGATTGTGGAGAT -3' (forward) and 5'-GAGCTGGATGAGGTTCCAAA -3'(reverse), and 5'-GGATGCAGAAGGAGATCACG-3' and 5'-ATCTGCTGGAAGGTGGACAG-3', respectively. The expected size of the ATB⁰ (SLC1A5) gene RT-PCR product was 206 bp (corresponding to bp 52 to 257 of the partial porcine SLC1A5 gene sequence that was obtained by us, GenBank accession no. DQ231578). The expected size of the β -actin RT-PCR product was 130 bp (corresponding to bp 569 to 698 of the previously reported porcine β -actin sequence, GenBank accession no. U07786). Real-time PCR was performed using one-step SYBR Green RT-PCR Mix (Qiagen Inc.,

Valencia, CA), containing $MgCl_2$, dNTP, reverse transcriptase and Hotstar Taq polymerase. Equal amounts of DNase-I-treated RNA (100 ng) from all samples were added to a total volume of 25 μ L containing 12.5 μ L SYBR Green mix, 0.25 μ L RT mix and 1 μ M each of forward and reverse primers. We used the following protocol: (i) reverse transcription program (30 min at 50 °C); (ii) denaturation program (15 min at 95 °C); (iii) amplification and quantification program, repeated 45 cycles (15 s at 95 °C, 15 s at 54 °C, 15 s at 72 °C); and (iv) melting curve program (60-99 °C with heating rate of 0.1 °C/s and fluorescence measurement). We used β -actin as the internal control to normalize the amount of starting RNA used in the real-time RT-PCR for all the samples. Amplification and melt curve analysis were performed in a Smart Cyclex (Cepheid, Sunnyvale, CA). Melt curve analyses were conducted to confirm the specificity of each product, and the size of products was verified on ethidium bromide-stained 2 % agarose gels in Tris acetate–EDTA buffer. The identity of each product was confirmed by dideoxy-mediated chain termination sequencing at the University of Guelph Molecular Supercenter. Real-time RT-PCR analyses were all performed in duplicate for each sample.

Western Blotting Analyses

We previously demonstrated that the commonly used housekeeping gene β -actin protein abundance was fairly constant on the apical membrane along the young porcine small intestinal crypt-villus axis (Yang et al. 2011; 2016). Thus, for the Western blotting analyses of the ASCT2/ATB⁰ protein, the phosphorylated mTOR (p-mTOR), and the phosphorylated

S6K1 (p-S6K1), a linear range of sample protein loading to densitometry reading responses, from 5 to 15 μ g protein, was established before samples were analyzed.

Epithelial cell homogenate samples were prepared as described below for the Western blotting analyses of the targeted proteins. Specifically, 0.5 g of individually pulverized frozen cell sample was weighed out with a 1.5-mL Eppendorf tube and homogenized with 1 mL of lysis buffer on ice for 2 min. The lysis buffer contained 20 mM Tris-HCl, 2 mM EGTA, 0.2 mM Na₂EDTA, 50 mM NaF, 50 mM β -glycerophosphate, 1 mM bezamidine, 1 mM DTT, 0.5 mM Na₃VO₄, 0.1 mM PMSF, and 10 μ g/mL each of aprotinin, leupeptin, pepstatin A, and TLCK at pH 7.4 (Yang et al., 2011).

For the Western blotting analyses of the targeted ASCT2/ATB⁰ abundances, samples prepared from the prepared whole cell homogenate, the apical membrane and the intracellular soluble fraction of the upper villus, the middle villus and the crypt epithelial cells were boiled in 2 $\times$ SDS loading buffer (250 mM tris-HCL, 500 mM β -mercaptoethanol, 2 % SDS, 0.1 % phenol blue, 10 % glycerol at pH 6.8) for 5 min. The same aliquots (10 μ g protein) of samples were subjected to 10 % SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were blocked at room temperature for 1 h with 6 % non-fat dry milk powder dissolved in 1 $\times$ TBS (25 mM tris-HCl, 0.150 M NaCl at pH 7.4) and then were incubated at 4 °C with goat anti-rabbit ASCT2/ATB⁰ antibody (kindly provided by Professor Nelly E. Avissar at the University of Rochester, New York) (Avissar et al. 2001). Following the overnight incubation, membranes were washed (6 $\times$ 10 min) in TBS with 0.1 % Tween-20 and incubated at room temperature for 1 h with secondary anti-rabbit HRP-conjugated IgG (Promega Corporation, Madison, WI) or anti-mouse HRP-conjugated

IgG (Promega). Blots were visualized using the enhanced chemiluminescence detection system (Sigma-Aldrich) (Yang et al., 2016).

For the Western blotting analyses of the phosphorylated p-mTOR and the mTOR-signaling pathway down-stream component phosphorylated p-S6K1 protein abundances, the epithelial cell homogenate samples were incubated on ice for 30 min with gentle inversion of the tube every 5 min and then centrifuged at 12,000 $\times$ g for 20 min at 4°C. A suitable amount of the prepared supernatants was diluted with Milli-Q H₂O and boiled in 2 $\times$ SDS loading buffer (250 mM Tris-HCl, 500 mM β -mercaptoethanol, 2 % SDS, 0.1 % phenol blue, 10 % glycerol at pH 6.8) for 5 min. The same aliquots (10 μ g protein) of samples were subjected to 10 % SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore) via semi-dry transfer apparatus (Bio-Rad). The membranes were blocked at room temperature for 1 h with 6 % non-fat dry milk powder dissolved in 1 $\times$ TBS (25 mM Tris-HCl, 0.15 M NaCl at pH 7.4) and then were incubated at 4 °C with a primary antibody overnight. A primary rabbit polyclonal antibody against the human mTOR phosphorylated at Ser2481 (1:1,000 dilution in 6 % skim milk powder, 2974S, Cell Signaling Technology, Inc., Danvers, MA) was used for the detection of the porcine mTOR. A primary goat polyclonal antibody against the human p-S6K1 phosphorylated at Thr389 (Sc-11759, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for detection of the phosphorylated porcine p-S6K1 protein. Following the overnight incubation, membranes were washed (6 $\times$ 10 min) in 1 $\times$ TBS with 0.1 % Tween-20 and incubated at room temperature for 1 h with a secondary donkey anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (1:10000 dilution in 6% skim milk powder, Promega) (Yang et al.,

2016).

Aliquots of extracted sample protein were loaded at the same amount of protein across all the cell fractions for the Western blotting analyses. Blots were visualized using the enhanced chemiluminescence detection system (Sigma-Aldrich). Photographs of the film were scanned and densitometry was performed with Scion Imaging software (Scion Corporation). Western blotting analyses were all performed in duplicate for each sample.

Calculations for Real-time PCR and Statistical Analyses

The relative SLC1A5 gene mRNA abundance ratio (R) in the upper villus, the middle villus, and the crypt epithelial cells was calculated by using the value obtained from the crypt epithelial cell as the control according to the Delta and delta method (Livak and Schmittgen 2001).

$$R = 2^{-\Delta\Delta Ct(\text{sample-control})}$$

where $-\Delta\Delta Ct(\text{sample-control}) = (Ct \text{ of SLC1A5} - Ct \text{ of } \beta \text{ actin})_{\text{sample}} - (Ct \text{ of SLC1A5} - Ct \text{ of } \beta \text{ actin})_{\text{control}}$. The Ct value is the cycle number at which the both the target and the housekeeping genes were amplified beyond the threshold of 30 fluorescence units. Control was defined as the crypt epithelial cell and samples were defined as the middle or upper villus epithelial cells. Data were expressed in being relative to the crypt cell value (value = 1) and were presented as means $\pm$ SE ($n = 6$) in arbitrary units. Real-time PCR efficiencies were acquired by amplification of dilution series of RNA according to the equation $10^{(-1/\text{slope})}$ and were consistent between the SLC1A5 and the β -actin genes. Negative controls were performed in

which water was substituted for RNA.

There were no significant sow effects and the sow interactions with the small intestinal epithelial cell fraction effects in the biochemical and molecular endpoints in the young piglets in our previous studies (Fan et al. 2004; Yang et al. 2011). Thus, these sources of variability, i.e., sow effects and the sow interactions with the small intestinal epithelial cell fraction effects, were included in the random experimental error for the following the analysis of variance without further removal. Data for the epithelial cell fractions obtained from the Western blotting analyses and real time RT-PCR analyses were subjected to the analysis of variance using the General Linear Model procedure of SAS (the SAS Institute, Cary, NC). Comparisons of differences for the molecular endpoints among the epithelial cell fractions were further conducted by using the Tukey's multiple comparisons of SAS. Comparison of all the endpoint differences with a level of $P < 0.05$ was considered significant, $n = 6$.

RESULTS

The 526-bp porcine partial cDNA sequence of the SLC1A5 gene was obtained by RT-PCR according to the conserved region in the human gene sequence. The partial porcine SLC1A5 cDNA sequence had 90, 84 and 89 % homology with the known human, mouse and rabbit sequences, respectively (Fig. 1). The relative abundances of the SLC1A5 mRNA were analyzed by real-time RT-PCR using β -actin as the housekeeping control (Fig. 2). The Ct values for the amplification of β -actin cDNA were similar in all samples (data not shown). The SLC1A5 mRNA abundance was higher ($P < 0.05$) in the crypt epithelial cells than in the

upper villus and the middle villus epithelial cells by 54 and 32 %, respectively. Moreover, The SLC1A5 mRNA abundance was higher ($P < 0.05$) in the middle villus epithelial cells than in the upper villus epithelial cells by 31%.

Western blotting analyses showed the presence of a 57-kDa immune-reactive ASCT2/ATB⁰ band in the isolated epithelial cell homogenate, the intracellular soluble pool and the apical membrane preparation (Fig. 3). There were no differences ($P > 0.05$) in the ASCT2/ATB⁰ abundances in the cell homogenate, the intracellular soluble pool and the apical membrane among the upper villus, the middle villus and the crypt epithelial cells (Fig. 3).

Phosphorylated mTOR protein abundance was higher ($P < 0.05$) in the middle villus epithelial cells than in the upper villus epithelial cells by 20 %. However, there were no differences ($P > 0.05$) in the p-mTOR protein abundances between the middle villus and the crypt epithelial cells. Although the p-mTOR protein abundance was relatively higher in the crypt epithelial cells, there were no differences ($P > 0.05$) in the p-mTOR protein abundances between the upper villus and the crypt epithelial cells (Fig.4). The p-S6K1 protein abundance was higher ($P < 0.05$) in the crypt epithelial cells than in the upper villus epithelial cells by 24 %. However, there were no significant differences in the p-S6K1 abundances ($P > 0.05$) between the upper villus and the middle villus epithelial cells as well as between the middle villus and the crypt epithelial cells (Fig. 5).

DISCUSSION

Our major objective of this study was to test if the ASCT2/ATB⁰ AA exchanger (SLC1A5) gene, and the ASCT2/ATB⁰ AA exchanger protein were expressed in epithelia cells along the

entire jejunal crypt-villus axis in the young pigs fed a milk replacer. Results from this study suggested that both the ASCT2/ATB⁰ AA exchanger (SLC1A5) mRNA, and the ASCT2/ATB⁰ exchanger protein were expressed in the epithelial cells along the entire jejunal crypt-villus axis in the fed young pigs. Significantly higher ASCT2/ATB⁰ AA exchanger (SLC1A5) mRNA abundance was observed in the crypt epithelial cells, whereas there were no significant differences in the ASCT2/ATB⁰ protein abundances among the upper villus, the middle villus and the crypt epithelial cells. There is a scarcity of literature reports regarding expression of the ASCT2/ATB⁰ AA exchanger (SLC1A5) gene, and the ASCT2/ATB⁰ protein in epithelia along the small intestinal crypt-villus axis in fast growing animals. These results from this study are not consistent with the pattern of the ASCT2/ATB⁰ (SLC1A5) gene expression previously reported in adult animals in showing the expression of the ASCT2/ATB⁰ (SLC1A5) gene was enterocyte differentiation dependent and the ASCT2/ATB⁰ AA exchanger protein abundances were associated with a decreased density in the crypt epithelial cells (Avissar et al. 2001). However, our results were consistent with a previously reported neonatal pig functional study in showing that Na⁺-dependent neutral AA transport activities were expressed in the small intestinal crypt cells (Smith, 1981). Therefore, our results suggest that expression of the ASCT2/ATB⁰ AA exchanger (SLC1A5) gene, and the ATB⁰ protein in the epithelia along the entire jejunal crypt-villus axis was unique in fast growing young animals such as the milk replacer fed young pigs. The high level expression of the ASCT2/ATB⁰ AA exchanger (SLC1A5) gene, and the ASCT2/ATB⁰ protein in the epithelia along the entire jejunal crypt-villus axis would support large neutral AA-dependent trophic gut mucosal growth in young pigs fed a milk replacer.

The partial porcine ASCT2/ATB⁰ AA exchanger (SLC1A5) gene cDNA sequence obtained in this study showed high homology (more than 80%) to human, mouse and rabbit, which indicated that the partial sequence belongs to the porcine ASCT2/ATB⁰ AA exchanger (SLC1A5) gene. Moreover, the 57-kDa bands were determined with the goat anti-rabbit ATB⁰ in the cell homogenate, intracellular soluble, and the apical membrane along the three cell fractions. The porcine ASCT2/ATB⁰ AA exchanger protein size is similar to the calculated molecular weight of 57-63 kDa of the rabbit ATB⁰ based on its amino acid sequence but is much smaller than the 85- to 88-kDa band ATB⁰ observed in adult rabbit intestine (Avissar et al. 2001). The deglycosylation study demonstrated that the ASCT2/ATB⁰ AA exchanger protein in the adult rabbit intestine was highly glycosylated and glycosylation was responsible for the changes of the AA exchanger protein ATB⁰ sizes between 78 and 88-kDa (Avissar et al. 2001). Moreover, except two *N*-glycosylation sites, the ASCT2/ATB⁰ AA exchanger protein also had two protein kinase C phosphorylation sites (Utsunomiya-Tate et al. 1996; Kekuda et al. 1997; Bröer et al. 1999). Thus, the degree of phosphorylation might have been another contributing factor. These results suggest differences in the level of glycosylation and phosphorylation were likely responsible for the variability in the ASCT2/ATB⁰ AA exchanger protein sizes between the aforementioned studies.

The dissociation between the ASCT2/ATB⁰ neutral AA exchanger (SLC1A5) gene mRNA and the AA exchanger protein ATB⁰ abundances along the crypt-villus axis from this study suggested that the ASCT2/ATB⁰ AA exchanger protein expression was likely regulated by post-transcriptional mechanisms in fast growing young animals. Moreover, the lack of differences in the ASCT2/ATB⁰ protein abundances between the cell homogenates, the

intracellular pool and the apical membrane along the intestinal crypt-villus axis from this study would suggest that intracellular trafficking did not seem to play a key role in regulation of the apical ASCT2/ATB⁰ AA exchanger protein abundances in the young pigs. The crypt epithelial cells had 32 – 54 % higher SLC1A5 mRNA abundances in comparison with the villus cells in the young pigs of this study. Previous studies showed that global protein translational efficiency was high in the crypt cells (Mariadason et al. 2005; Gassler et al. 2006; Chang et al. 2008; Yang et al. 2011). It is thus apparent that ASCT2/ATB⁰ neutral AA exchanger (SLC1A5) gene mRNA abundance was not a limiting factor and the high protein translational efficiency did not lead to a much higher neutral AA exchanger ASCT2/ATB⁰ protein abundance in the crypt cells, as shown in Fig. 3. These results of this study would suggest that the ASCT2/ATB⁰ AA exchanger (SLC1A5) gene and the neutral AA exchanger ASCT2/ATB⁰ protein expression in young animals may involve gene-specific transcriptional and translational control mechanisms, such as luminal substrate AA availability, which needs to be further elucidated in future studies.

An intriguing question is how to explain the physiological importance of presence of the high neutral AA exchanger ASCT2/ATB⁰ protein abundance in the crypt epithelial cells because crypt epithelial cells potentially may have limited access to luminal AA supply especially under low-protein feeding conditions in comparison with villus epithelial cells. It is conceivable that high neutral AA exchanger protein ATB⁰ abundance in crypt epithelial cell would contribute to high levels of systemic trophic large neutral AA absorption in young animals. The ASCT2/ATB⁰ neutral AA exchanger protein has been shown to serve as the putative cellular surface AA transceptor for AA sensing and signaling in cells (Peng et al.

2002; Hyde et al. 2003; Hundal and Taylor 2009). Silencing of the A ASCT2/ATB⁰ neutral AA exchanger protein expression in human liver cancer cells suppressed the mTORC1 signaling and protein synthesis rates (Fuchs et al. 2007). Nicklin et al. (2009) showed that loss of the ASCT2/ATB⁰ neutral AA exchanger protein function by adding an inhibitor to the ASCT2 exchanger (L- γ -glutamyl-p-nitroanilide) or knockdown of the SLC1A5 gene inhibited S6 phosphorylation and activated autophagy-dependent uptake of extracellular large neutral AA Gln. Studies also showed that silencing of the SLC1A5 gene expression not only arrested cell growth but also eventually led to apoptosis of cancer cells (Fuchs et al. 2004). These literature results suggest that the ASCT2/ATB⁰ neutral AA exchanger is essential for cell survival and growth by maintaining high activity of the mTOR-signaling pathway. On the other hand, the presence of high ASCT2/ATB⁰ neutral AA exchanger protein abundance in crypt epithelial cells may play a compensatory role in AA absorption under pathophysiological conditions, for example, during infection, as demonstrated by Blikslager et al. (2001) and Talukder et al. (2008). Our results also clearly showed that significantly higher p-mTOR protein and p-S6K1 protein abundances were presented in the crypt epithelial cells compared with the upper villus epithelial cells. These results would suggest that sensitivity and activity of the mTOR signaling pathway were higher in the crypt epithelial cells than in the upper villus epithelial cells. Therefore, expression of the apical ASCT2/ATB⁰ neutral AA exchanger along the small intestinal crypt-villus axis would play an important role to channel the high mTOR-signaling activity, which would be required to promote crypt cell proliferation and differentiation in the gut mucosal growth of the young pigs fed a milk replacer being high in highly digestible protein.

In summary, the apical ASCT2/ATB⁰ neutral AA exchanger and the mTOR-signaling components were highly expressed along the entire crypt-villus in the small intestinal mucosa of young pigs. Thus dietary supplementation of suitable levels of trophic neutral AA could enhance gut mucosal growth in fast growing young pigs.

For Review Only

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Figure Legends:

Figure 1. Partial cDNA sequence of the porcine Na⁺-neutral amino acid (AA) ASCT2 exchanger (SLC1A5). A partial clone of the porcine ortholog of the SLC1A5 gene was obtained by RT-PCR. Numbers at right refer to corresponding nucleotides of previously published full-length human, mouse, and rabbit SLC1A5 gene clones in comparison with the corresponding partial pig cDNA sequence. Underlined stars indicate sequences are conserved among pig, human, mouse, and rabbit. Overall, the partial-length porcine SLC1A5 gene cDNA sequence had 90, 84, and 89% homology to the known human sequence (GenBank accession no. NM_005628), the known mouse sequence (GenBank accession no. NM_009201), and the known rabbit sequence (GenBank accession no. NM_001082378), respectively. This pig partial sequence now resides in GenBank with the accession no. DQ231578.

Figure 2. Real-time RT-PCR analysis of the Na⁺-neutral amino acid (AA) ASCT2/ATB⁰ exchanger SLC1A5 mRNA abundances in the upper villus (U), the middle villus (M) and the crypt (C) epithelial cells isolated along the jejunal crypt-villus axis in young pigs-fed a liquid milk replacer. Results were normalized using β -actin expression as a housekeeping control gene in each real time RT-PCR analysis. Data were expressed in being relative to the crypt cell value (value = 1) and were presented as means $\pm$ SE ($n = 6$) in arbitrary units. Bars with a different letter differ, $P < 0.05$.

Figure 3. Western blotting analyses of the Na⁺-neutral amino acid (AA) ASCT2/ATB⁰ exchanger protein abundances in cell homogenate (H), soluble (S), and apical membrane (AM) prepared from the upper villus (U), the middle villus (M), and the crypt (C) epithelial cells along the jejunal crypt-villus axis in young pigs fed a liquid milk replacer. Data were in arbitrary units and were presented as means $\pm$ SE ($n = 6$).

Figure 4. Western blotting analyses of the phosphorylated mechanistic and mammalian target of rapamycin (p-mTOR) protein abundances in the upper villus (U), middle villus (M), and crypt (C) epithelial cells isolated the jejunal crypt-villus axis in young pigs fed a liquid milk replacer. Data were presented at the means $\pm$ SE ($n = 6$) in arbitrary units. Bars with a different letter differ ($P < 0.05$).

Figure 5. Western blotting analyses of the phosphorylated p70 S6 kinase 1 (p-S6K1) protein abundances in the upper villus (U), middle villus (M), and crypt (C) epithelial cells isolated the jejunal crypt-villus axis in young pigs fed a liquid milk replacer. Data were presented at the means $\pm$ SE ($n = 6$) in arbitrary units. Bars with a different letter differ ($P < 0.05$).

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Pig      DQ231578      -----CTGGTCTCCTGG 12
Human    NM_005628      TCCGCTTCTTCAACTCCTCAATGAGGCCACCATGGTTCTGGTCTCCTGG 1436
Mouse    NM_009201      TTCGTTTCTTCAACTCCTCAATGATGCCACCATGGTCTGGTCTCCTGG 1413
Rabbit   NM_001082378    TCCGCTTCTTCAACTCCTCAATGAGGCCACCATGGTCTGGTCTCCTGG 1367
                                     *****

Pig      DQ231578      ATCATGTGGTATGCCCTGTGGGCATCATGTTCTCTGGTGGCCAGCAAGAT 62
Human    NM_005628      ATCATGTGGTACGCCCCGTGTGGGCATCATGTTCTCTGGTGGCCAGCAAGAT 1486
Mouse    NM_009201      ATTATGTGGTACGCACCCGTGGAATCCTGTTCTCTGGTGGCCAGCAAGAT 1463
Rabbit   NM_001082378    ATCATGTGGTACGCCCCGTGTGGGCATCTTGTCTCTGGTGGCCAGCAAGAT 1417
                                     ** ***** ** ** ** ** ***** *****

Pig      DQ231578      TGTGGAGATGGAGGATGTGGGGATGCTCTTCGCCAGTCTGGGCAAGTACA 112
Human    NM_005628      CGTGGAGATGGAGGATGTGGGTTTACTCTTTGCCCGCCTTGGCAAGTACA 1536
Mouse    NM_009201      TGTGGAGATGAAAGACGTCCGCCAGCTCTTCATCAGCCTCGGCAAAATACA 1513
Rabbit   NM_001082378    CGTGGAGATGGACGACGTGGGCGTGCTGTTCGCCAGCCTGGGCAAAATACA 1467
                                     ***** * ** * * ** * * * * *

Pig      DQ231578      TCCTGTGCTGCCTGCTCGGCCACACCATCCATGGGCTCCTGGTGTGCTG 162
Human    NM_005628      TTCTGTGCTGCCTGCTGGGTACGCCATCCATGGGCTCCTGGTACTGCCC 1586
Mouse    NM_009201      TTCTGTGCTGCCTGCTGGGCCACGCCATCCACGGGCTCCTGGTCTGCTG 1563
Rabbit   NM_001082378    TCCTGTGCTGCCTGCTGGGCCACGCCATCCACGGGCTCCTGGTGTGCTG 1517
                                     * ***** ** * * * ***** *****

Pig      DQ231578      CTCATCTACTTCCTCTTCACTCGCAAAAACCCCTACCGCTTCCTGTGGG 212
Human    NM_005628      CTCATCTACTTCCTCTTCACTCGCAAAAACCCCTACCGCTTCCTGTGGG 1636
Mouse    NM_009201      CTCATCTACTTCCTCTTCACTCGCAAAAATCCCTATCGATTCCTGTGGG 1613
Rabbit   NM_001082378    CTCATCTACTTCCTCTTCACTCGCAAGAACCCCTACCGCTTCCTGTGGG 1567
                                     ***** ***** ** * * * * *

Pig      DQ231578      CATCATGACACCACTGGCCACTGCCTTTGGAACCTCATCCAGCTCCGCCA 262
Human    NM_005628      CATCGTGACGCGCTGGCCACTGCCTTTGGGACCTCTTCCAGTTCCGCCA 1686
Mouse    NM_009201      CATCATGACACCCCTGGCCACTGCCTTCGGGACCTCTTCTAGCTCTGCCA 1663
Rabbit   NM_001082378    CATCCTGACGCGCTGGCCATGGCCTTTGGGACCTCTTCCAGCTCGGCCA 1617
                                     **** * * * * * ** * * * * *

Pig      DQ231578      CGCTGCCGCTGATGATGAAGTGCGTGGAGGAGAAGAACGGCGTGGCCAAG 312
Human    NM_005628      CGCTGCCGCTGATGATGAAGTGCGTGGAGGAGAATAATGGCGTGGCCAAG 1736
Mouse    NM_009201      CCTTGCCCTCTGATGATGAAGTGCTAGAGGAGAAGAATGGTGTGGCCAAA 1713
Rabbit   NM_001082378    CGCTGCCGCTGATGATGAAGTGCGTAGAGGAGAGGAACGGCGTGGCCAAG 1667
                                     * **** ***** ** * * * * *

Pig      DQ231578      CACATCAGCCGCTTCATTCTGCCCATTGGTGCCACTGTCAACATGGACGG 362
Human    NM_005628      CACATCAGCCGTTTCATCCTGCCCATCGGCGCCACCGTCAACATGGACGG 1786
Mouse    NM_009201      CACATCAGCCGTTTCATCCTACCCATCGGCGCCACCGTCAACATGGACGG 1763
Rabbit   NM_001082378    CACATCAGCCGCTTCGTGCTGCCCATCGGGGCCACGGTCAACATGGACGG 1717
                                     ***** ** * * * * * *****

Pig      DQ231578      GGCCGCGCTCTTCCAGTGCCTGGCCGCGGTGTTTCATTGCACAGCTCAACC 412
Human    NM_005628      TGCCGCGCTCTTCCAGTGCCTGGCCGCGAGTGTTCATTGCACAGCTCAGCC 1836
Mouse    NM_009201      GGCCGCGCTGTTCAGTGTGTGGCCGCGAGTGTTCATCGCACAACTAAACG 1813
Rabbit   NM_001082378    CGCCGCGCTGTTCAGTGCCTGGCCGCGGTGTTTCATTGCGCAGCTCAACC 1767
                                     ** ***** ***** ** * * * * *

Pig      DQ231578      AGCGGTCCTTGGACTTTGTGAACATTATCACTATCCTGGTCACCGCCACG 462
Human    NM_005628      AGCAGTCCTTGGACTTCGTAAAGATCATCACCATCCTGGTCACGGCCACA 1886
Mouse    NM_009201      GGGTGTCCCTGGACTTCGTGAAGATCATCACCATCCTGGTCACAGCCACA 1863

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