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ORIGINAL ARTICLE

Reduced expression of intestinal *N*-acetylglutamate synthase in suckling piglets: a novel molecular mechanism for arginine as a nutritionally essential amino acid for neonates

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Abstract The objective of this study was to determine developmental changes in mRNA and protein levels for *N*-acetylglutamate synthase (NAGS; a key enzyme in synthesis of citrulline and arginine from glutamine/glutamate and proline) in the small intestine of suckling piglets. The porcine NAGS gene was cloned using the real-time polymerase-chain reaction (RT-PCR) method. The porcine NAGS gene encoded 368 amino acid residues and had a high degree of sequence similarity to the "conserved domain" of human and mouse NAGS genes. The porcine NAGS gene was expressed in *E. coli* BL21 and a polyclonal antibody against the porcine NAGS protein was developed. Real-time RT-PCR and western-blot analyses were performed to quantify NAGS mRNA and protein, respectively, in the jejunum and ileum of 1- to 28-day-old pigs. Results indicated that intestinal NAGS mRNA levels were lower in 7- to 28-day-old than in 1-day-old pigs. Immunochemical analysis revealed that NAGS protein was localized in enterocytes of the gut. Notably, intestinal NAGS protein abundance declined progressively during the 28-day suckling period. The postnatal decrease in NAGS protein levels was consistent with the previous report of reduced NAGS enzymatic activity as well as reduced synthesis of citrulline and arginine in the small intestine of 7- to 28-day-old pigs. Collectively, these results suggest that intestinal NAGS expression is regulated primarily at the post-transcriptional level. The findings also provide a new molecular basis to explain that endogenous synthesis of arginine is impaired in sow-reared piglets and arginine is a nutritionally essential amino acid for the neonates.

Keywords *N*-Acetylglutamate synthase · Arginine · Development · Piglets

Abbreviations

Arg	Arginine
CPSI	Carbamylphosphate synthetase I
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IPTG	Isopropyl-β-D-thiogalactopyranoside
MTS	Mitochondrial targeting signal
NAG	*N*-acetylglutamate
NAGS	*N*-acetylglutamate synthase
RT-PCR	Real-time polymerase-chain reaction
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

Introduction

N-acetylglutamate synthase (NAGS) catalyzes the formation of *N*-acetylglutamate (NAG) from acetyl-coenzyme A and L-glutamate in mitochondria (Morizono et al. 2004).

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NAG is an activator of both Δ^1-pyrroline-5-carboxylate (P5C) synthase and carbamylphosphate synthetase I (CPSI) (Wu et al. 2004a), therefore playing a crucial role in regulating in vivo synthesis of citrulline and arginine by the small intestine (Caldovic et al. 2002a, b; Wu et al. 2009). NAGS is expressed primarily in the mitochondrial matrix of the liver and absorptive epithelial cells of the small intestine (enterocytes) (Wu and Morris 1998). This protein has a key regulatory role in hepatic and intestinal ureagenesis by supplying NAG to modulate CPSI activity (Schmidt et al. 2005; Wu et al. 2009). Inherited defects in the NAGS gene cause hyperammonaemia due to inactivation of CPSI (Morizono et al. 2004).

Piglets have a particularly high requirement of arginine for growth and metabolic function (Rhoads and Wu 2009; Tan et al. 2009). Although sow's milk was traditionally thought to provide adequate amino acids for supporting piglet growth (Wu 2010), recent studies identified submaximal growth of suckling piglets (Kim and Wu 2009; Wu et al. 2000a, 2004a). Notably, arginine is remarkably deficient in sow's milk, which provides at most 40% of arginine requirements by the 1-week-old piglets (Kim and Wu 2004). Available evidence shows that a low concentration of mitochondrial NAG is responsible for the striking decline in the intestinal synthesis of citrulline and arginine during the suckling period (Wu et al. 2009). Thus, the metabolic activation of intestinal citrulline and arginine synthesis by *N*-carbamoylglutamate (a metabolically stable analogue of NAG) provides a novel, effective strategy to increase endogenous arginine provision (Wu et al. 2004a; 2010a). These findings indicate that increasing NAG availability stimulates intestinal synthesis of citrulline and arginine, thereby enhancing endogenous arginine provision.

The molecular basis for age-dependent change in the activity of intestinal NAGS is not known. Thus, the present study was conducted to determine NAGS expression at both mRNA and protein levels in the small intestine of suckling piglets.

Materials and methods

Animal housing and sample collection

A total of 24 newborn Landrace × Yorkshire piglets (12 males and 12 females) were selected from 6 litters for this study. All piglets were freely nursed by sows. There were six piglets (3 males and 3 females) per age group. The number of piglets were chosen on the basis of their known variation (Li et al. 2009b; Wang et al. 2009a; Yin et al. 2004, 2009; Tang et al. 2005; Hou et al. 2008) and the statistical power calculation (Fu et al. 2010; Li et al. 2008; Yin et al. 2008). This study was carried out in accordance with the Chinese guidelines for animal welfare and experimental protocol (He et al. 2009; Kong et al. 2009; Wu et al. 2010a).

On days 1, 7, 21, and 28 of age, piglets were anesthetized with an overdose intraperitoneal injection of 10% sodium pentobarbital before being kiled (Deng et al. 2009; Tan et al. 2009; Fang et al. 2009). The entire small intestine was then rapidly removed from the piglets (Haynes et al. 2009; Yin et al. 2001; Yang et al. 2005). The jejunum and ileum were rapidly separated and cleaned several times with ice-cold phosphate-buffered solution (PBS) (Chen et al. 2009; Huang et al. 2005; Kong et al. 2007). The isolated intestinal segments were immediately frozen in liquid nitrogen and stored in a freezer at −70°C.

RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg liquid nitrogen pulverized tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen, USA) according to the manufacturer's instructions. The RNA quality was checked by 1% agarose gel electrophoresis and stained with 10 μg/ml ethidium bromide (Deng et al. 2007; Li et al. 2009a; Wang et al. 2009a, b; Tan et al. 2010). The RNA had an OD260: OD280 ratio between 1.8 and 2.0. Synthesis of the first strand cDNA was performed with oligo (dT) 20 and Superscript II reverse transcriptase (Invitrogen) (Wence et al. 2009).

Cloning of the NAGS cDNA and construction of recombinant expression plasmid

Primers for the porcine NAGS cDNA sequence were designed with Primer 5.0 based on the human and mouse NAGS cDNA. The primers used in this study were 5′-CG CGCGAATTCAGGAGGAATTTAAAATGAGAGGATC GCATCACCATCACCATCACGACATGAAGCCTCTGG TGGTTCTGGGGCTG-3′ (forward) and 5′-CTG CAG GTCGACTCATTCAGCTGCCTGGGTCAGAAGCCG-3′ (reverse). The expected sizes of the PCR product of the NAGS gene was 1464 bp (corresponding to the human NAGS sequence, GenBank accession number NM153006). PCR conditions were as follows: denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 10 min. The PCR products were separated by electrophoresis on a 2% agarose gel in Tris-borate-EDTA buffer and visualized by staining with ethidium bromide (Kang et al. 2010; Wang et al. 2009a, b; Wu et al. 2010b, c). The purified PCR product was digested with *Sal*I and *Eco*RI and subcloned into pLM1 vector, which was digested with the same endonucleases. The recombinant pLM1-NAGS plasmid

was identified by double restriction digestion with *Sal*I and *Eco*RI, followed with sequencing at Sangon Biotechnology, Inc. (Shanghai, China).

Expression of recombinant NAGS protein in *E. coli*

The following procedure was used to express the NAGS protein in *E. coli*. Normally, the *E. coli* strain BL21 (DE3) cells with the pLM1-NAGS plasmid was grown at 37°C in 500 ml of LB medium containing 100 mg/l ampicillin until a desirable cell density ($OD_{600} = 0.4$) was reached. At this point, the cells were incubated at room temperature with the addition of 0.2 mM IPTG overnight with shaking at 180 rpm. The cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate buffer (pH 8.0) (containing 300 mM NaCl, 10 mM imidazole, 0.1% Triton X-100, 15% glycerol, and 10 mM β-mercaptoethanol) incubated with 5 mg lysozyme at room temperature for 30 min, and then lysed by sonication four times for 30 s each time using an Ultrasonic sonicator equipped with a 8-mm-diameter tip. After removal of cell debris, the protein was purified using Hi-Trap column (Amersham, Piscataway, NJ, USA) according to manufacturer's instructions (Zeng et al. 2007; Tang et al. 2010). The protein content was determined using method of Bradford (Bradford 1976) with bovine serum albumin (BSA) as the standard. The eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide (Laemmli 1970). The purified enzyme fractions were combined and dialyzed for 24 h against a solution [50 mM potassium phosphate buffer (pH 8.0), 5% glycerol, and 5 mM β-mercaptoethanol] and then stored at −70°C until use (Kang et al. 2008; Tang et al. 2009).

Preparation of a polyclonal antibody against the porcine NAGS

A New Zealand rabbit at 3 months of age was subcutaneously injected with 100 μg of purified porcine NAGS protein. Immunizations were performed three times in a 14-day interval and the sampling of blood started on day 14 after the first injection. Blood samples (~10 ml) were collected weekly. Anti-serum was tested by Dot-ELISA, using the crude antiserum diluted at 1:5,000, 1:10,000, 1:40,000, 1:80,000, and 1:160, 000 (v/v) in 0.5 × PBS.

Real-time RT-PCR analysis of mRNA

Primers for NAGS and GAPDH were designed with Primer 5.0 based on the porcine NAGS cDNA sequence (Table 1) to produce an amplification product (Table 1). GAPDH was used as a housekeeping gene to normalize the target gene. Real-time RT-PCR was performed using SYBR Green PCR Mix, which contained $MgCl_2$, dNTP, and Hotstar Taq polymerase (Dekaney et al. 2008; Wang et al. 2009a). A cDNA template solution (2 μl) was added to 12.5 μl SYBR Green mix, as well as 1 μmol/l of each forward and reverse primers (total volume in the assay mixture = 25 μl). The following protocol was used for the PCR analysis in this study: (i) pre-denaturation program (10 s at 95°C); (ii) amplification and quantification program (5 s at 95°C, 20 s at 60°C, repeated 40 cycles); and (iii) melting curve program (60–99°C with heating rate of 0.1°C/s and fluorescence measurement). The identity of each product was confirmed by dideoxy-mediated chain termination sequencing at Sangon Biotechnology, Inc. The relative expression ratio (R) of mRNA was calculated by $R = 2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Real-time RT-PCR efficiencies were acquired by the amplification of dilution series of cDNA according to equation $10^{(-1/slope)}$ and were consistent between target gene and the reference gene (GAPDH). Results are expressed as the relative values to those for the control group (Livak and Schmittgen 2001; Yang et al. 2003, 2004; Liu et al. 2009). Negative controls were performed in which cDNA was substituted for water.

Table 1 Real-time RT–PCR primers used in this study

Gene	Primers	
NAGS	Sense:	5′-GTGAGCACCAAAGAACGGC-3′
	Antisense:	5′-GCCAGATAGTCGTCCCGCA-3′
GAPDH	Sense:	5′-AAGGAGTAAGAGCCCCTGGA-3′
	Antisense:	5′-TCTGGGATGGAAACTGGAA-3′

Immunochemical analysis of NAGS protein in the small intestine

The localization of NAGS protein in the small intestine was identified using immunohistochemical analysis (Wu et al. 2010a). The samples of jejunum and ileum were fixed in 10% formalin, dehydrated, and embedded in paraffin wax, incubated with 3% H_2O_2 for 15 min at room temperature, and then washed with DD-H_2O and PBS for 5 min. After blocking with 5% BSA for 30 min, the samples were incubated with a polyclonal rabbit anti-porcine NAGS antibody diluted at 1:800 in PBS overnight at 4°C. Negative controls were incubated with PBS. After washing with PBS for 5 min, all the samples were incubated with Biotin-labeled goat anti-rabbit IgG (1:200, booster), stained with BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) and NBT (nitro-blue tetrazolium chloride) substrates, re-stained with Nuclear Fast Red, and mounted with water-soluble mounting medium. Immunochemical staining sections were photographed using a Leica DFC

320 digital camera (Leica Microsystems, Wetzlar, Germany). The cells that expressed NAGS protein had a brown-yellow color, while this color did not appear in cells containing no NAGS or in the negative control samples.

Western blot analysis of NAGS protein

The frozen samples of the jejunum and ileum were rapidly transferred into ice-cold K-Hepes buffer (200 mM mannitol, 80 mM K-Hepes, 41 mM KOH, pH 7.5) containing pepstatin, leupeptin, K-EDTA, and phenylmethylsulphonyl fluoride (PMSF) as protease inhibitors (Hou et al. 2010). After homogenizing with a tip sonicator, the homogenate was centrifuged at 10,000 *g* for 20 min at 4°C and the supernatant fluid was saved at −70°C. After measurement of the total protein concentration (Bio-Rad Protein Kit) in the supernatant fluid, 50 μg protein were incubated in the Laemmli's buffer, and SDS-PAGE was performed on 5–12% polyacrylamide gels. For western blot analysis, proteins were transferred electrophoretically from unstained gels to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, USA) (Tan et al. 2010). After blocking with 5% milk powder in Tris-buffered saline-0.1% Tween-20 (TBST) for 2 h, the blots were incubated with the primary antibody (porcine NAGS, affinity-purified antibody, 200 μg/ml, 1:800; *β*-tubulin, 1:1000; Santa Cruz, CA, USA) for 2 h at room temperature. After washing with TBST, blots were incubated with secondary antibody at 1:2500 (HRP-labeled goat IgG, Santa Cruz, USA; horseradish peroxidase-linked anti-rabbit IgG, Abcam, MA, USA) for 2 h at room temperature. PVDF membranes were developed using ECL™ Western blot detection reagents (Amersham Biosciences). Equal volumes of ECL™ reagents A and B were added to the PVDF membrane and incubated for 1 min. Finally, the PVDF membranes were exposed to Alpha FluorChem FC2 (Cell Biosciences, Santa Clara, CA, USA), and the amounts of proteins were quantified by scanning densitometry. The levels of NAGS protein were normalized to those for *β*-tubulin and results are expressed as the relative values to those for the control group.

Bioinformatics and statistical analysis

BLAST was used to identify homologous sequences in the GenBank databases. Sequences were aligned by the multiple alignment program CLUSTAL X (Thompson et al. 1997). All data, expressed as means ± SEM, were subjected to ANOVA analysis using the SAS (SAS Institute, Cary, NC). Differences among group means were compared using the Tukey multiple comparison test. A *P*-value of less than 0.05 was taken to indicate statistical significance.

Results

Sequence analysis of the porcine NAGS gene

Results of gene sequencing indicated that the porcine NAGS gene (NM_001097520.1) was 1,107 bp and coded for a protein consisting of 368 amino acids (NP_001090989.1). The amino acid sequence of the porcine NAGS protein shared 77.95% and 76.43% of identity to the human NAGS (NP_694551.1) and mouse NAGS (CAM23258.1), respectively. Based on the alignment of the conserved domain (highlighted in grey) in NAGS protein, the porcine protein had 93.2% and 90.76% of similarity to the human and mouse NAGS, respectively (Fig. 1).

Expression of recombinant porcine NAGS protein in *E. coli*

Expression of the porcine NAGS protein in *E. coli* BL21 (DE3) was carried out at different temperatures and IPTG concentrations, and the NAGS protein was obtained under all the tested conditions. It was found that the porcine NAGS protein was expressed better at 0.2 mM IPTG because this experimental condition resulted in more soluble protein at room temperature (25°C) than at 37°C. A nickel metal-affinity resin column was used for single-step purification of NAGS protein. The purity of the protein was examined by SDS-PAGE and a single band corresponding to 40 KDa was detected (Fig. 2). This molecular weight was similar to the software-computed value of 40.3226 KDa.

Immunochemistry of recombinant porcine NAGS

The polyclonal antibody obtained from the present study reacted specifically with the NAGS protein of the pig small intestine on the western blot analysis (Fig. 2). The porcine NAGS protein could be recognized by the antibody even at a dilution of 1:40,000. Immunochemical analysis revealed that NAGS protein was localized in enterocytes of the gut (Fig. 3). Other cell types of the pig small intestine did not contain NAGS protein.

mRNA levels for intestinal NAGS in suckling piglets

GAPDH mRNA was measured in all samples as the internal reference to the NAGS mRNA. In all small-intestinal segments, NAGS mRNA levels did not differ ($P > 0.05$) between male and female piglets (data not shown). NAGS mRNA levels declined ($P < 0.05$) in the jejunum and ileum with increasing age during the suckling period (Fig. 4). Specifically, NAGS mRNA levels were lower in 7- to 28-day-old than in 1-day-old pigs. Of note, NAGS mRNA levels in the jejunum posterior were

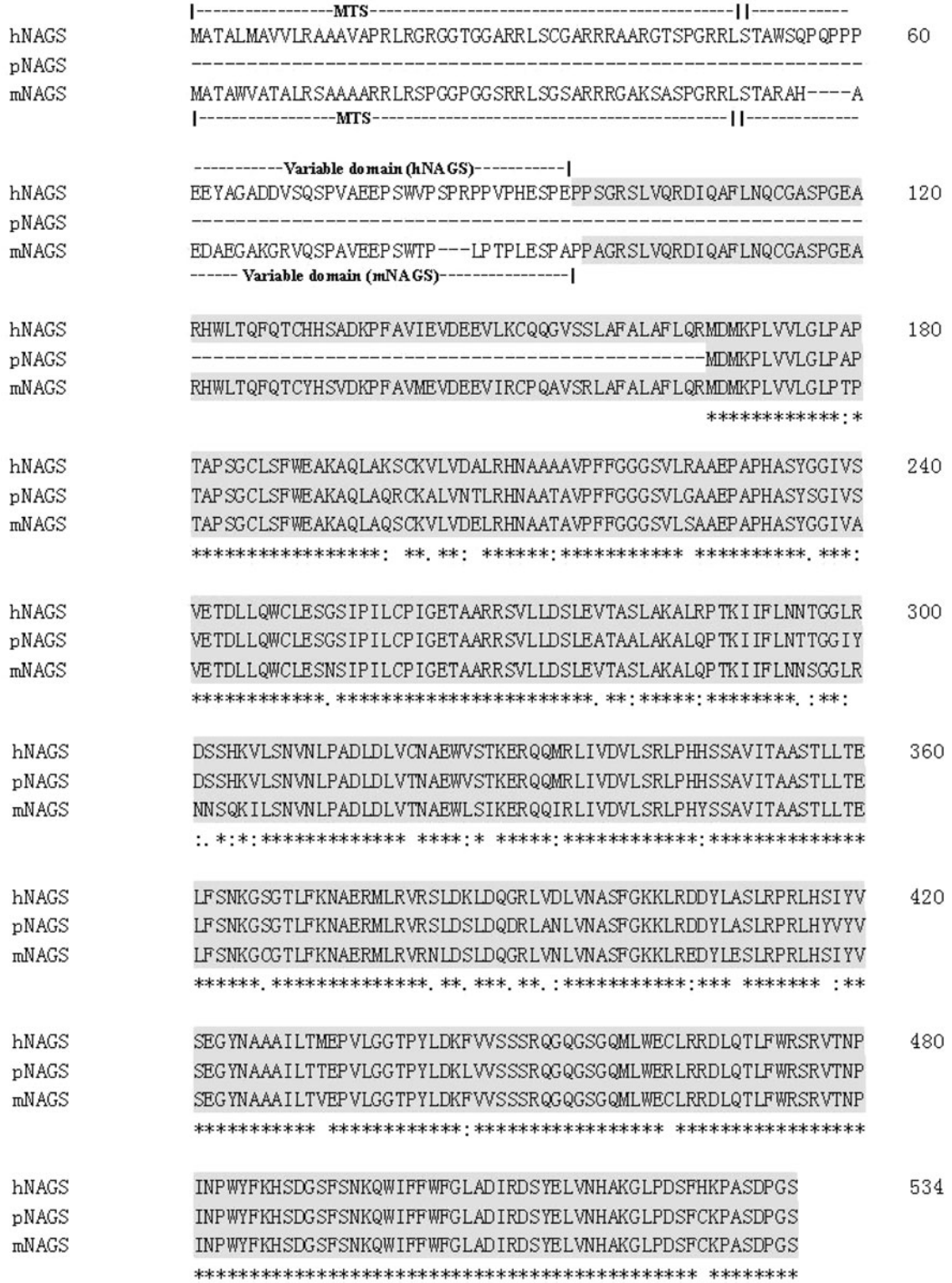

Fig. 1 Alignment of amino acid sequence of NAGS for humans (NP_694551.1), mice (CAM23258.1) and pigs (NP_001090989.1). Conserved domain of NAGS protein in human, mouse, and pig are highlighted in *grey*, residues conserved in all sequences are marked with "*"; residues not conserved in all sequences but conserved in some sequences are marked with "or"

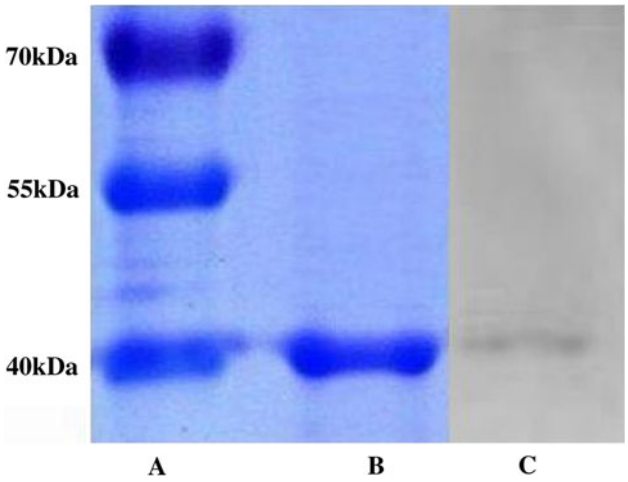

Fig. 2 SDS-PAGE of NAGS protein. **a** Marker, **b** purified NAGS protein, **c** western blot of NAGS

approximately 15% higher in 21- and 28-day-old pigs than in 7-day-old pigs.

Protein levels for intestinal NAGS in suckling piglets

β-Tubulin protein was measured in all samples as the internal reference to the NAGS protein. NAGS protein was readily detected in the piglet small intestine. In all small-intestinal segments, NAGS protein levels did not differ ($P > 0.05$) between male and female piglets (data not shown). The abundance of NAGS protein declined ($P < 0.05$) progressively in the jejunum and ileum as the age of piglets increased from 1 to 28 days (Fig. 5).

Discussion

Arginine is an essential amino acid for maximal growth of young mammals (Flynn et al. 2002; Wu et al. 2007), not only due to its abundance in tissue proteins (Wu et al. 2009) but also because of its key role in regulation of gene expression (Jobgen et al. 2006, 2009; McKnight et al. 2010; Palii et al. 2009) and cell signaling (Li et al. 2009c; Rhoads and Wu 2009). In addition, there is evidence that arginine is deficient in the milk of most studied mammals, including pigs (Yao et al. 2008; Wu and Knabe 1995; Wu et al. 1995) and humans (Davis et al. 1994). Thus, endogenous synthesis of arginine is expected to play a crucial role in maintaining arginine homeostasis in neonates fed enterally or parenterally (Flynn and Wu 1996; Wu 2009, 2010). Based on daily weight gain and arginine catabolism, endogenous synthesis of arginine has been estimated to provide at least 60% of total daily arginine requirements in 7-day-old piglets (Wu et al. 2004a). However, intestinal synthesis of arginine from glutamine and glutamate decreases by 70–73% in 7-day-old suckling piglets in comparison with newborn piglets and declines further in 14- to 28-day-old piglets (Wu et al. 1994, 1995). The postnatal decline in endogenous synthesis of arginine may result from decreases in cellular activities of both P5C synthase and NAGS (Wu et al. 2004a; Wu et al. 1995).

NAGS is a mitochondrial protein required for intestinal arginine synthesis, because the product (NAG) of this enzyme is an allosteric activator of CPSI (Wu and Morris 1998) and P5C synthase (Wu et al. 2004a). Wakabayashi et al. (1991) reported a profound decrease in NAGS

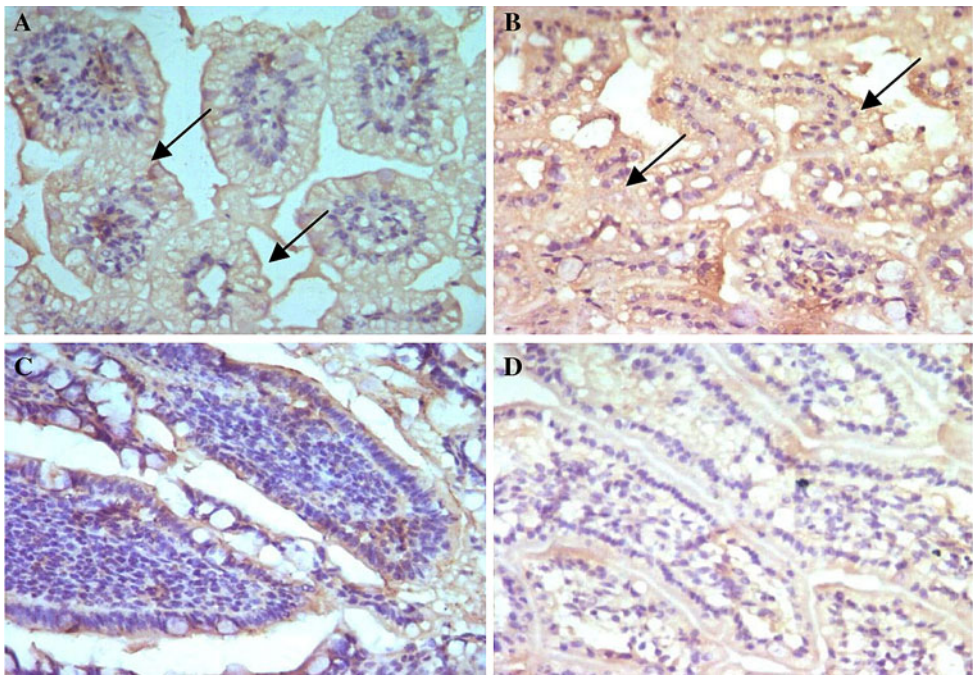

Fig. 3 Immunohistochemical analysis of intestinal NAGS protein in suckling piglets. **a** ileum; **b** jejunum; **c** ileum, negative control, **d** ileum, negative control. (400×)

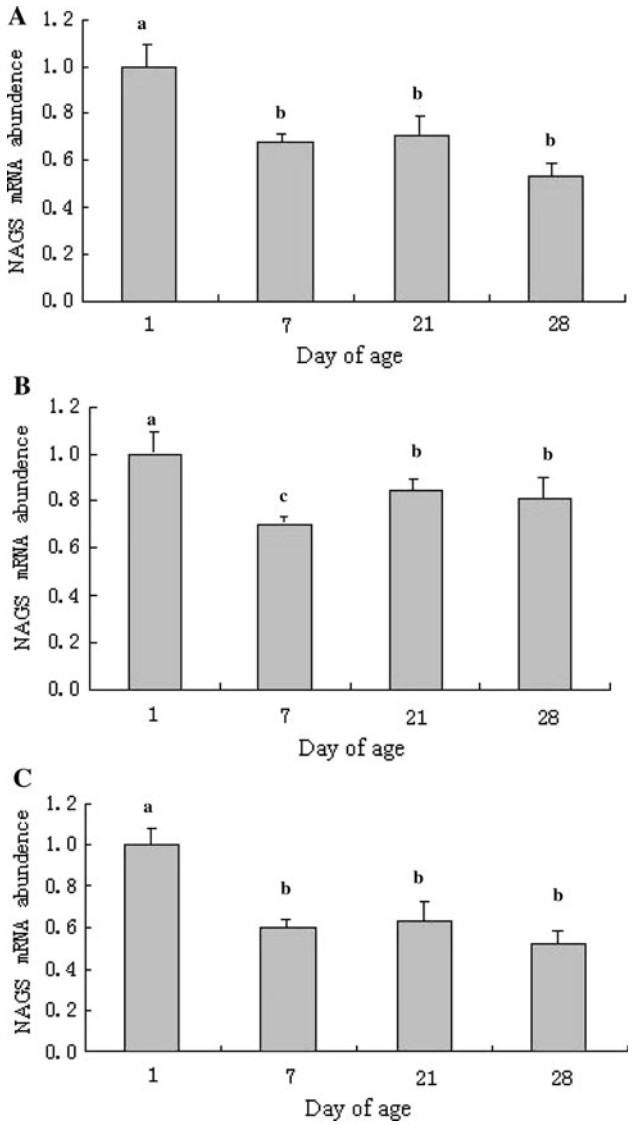

Fig. 4 NAGS mRNA abundance in the jejunum anterior (**A**), jejunum posterior (**B**) and ileum (**C**) of suckling piglets from day 1 to 28 of age. *a–c* Means with different superscripts in a row differ ($P < 0.05$). $n = 6$

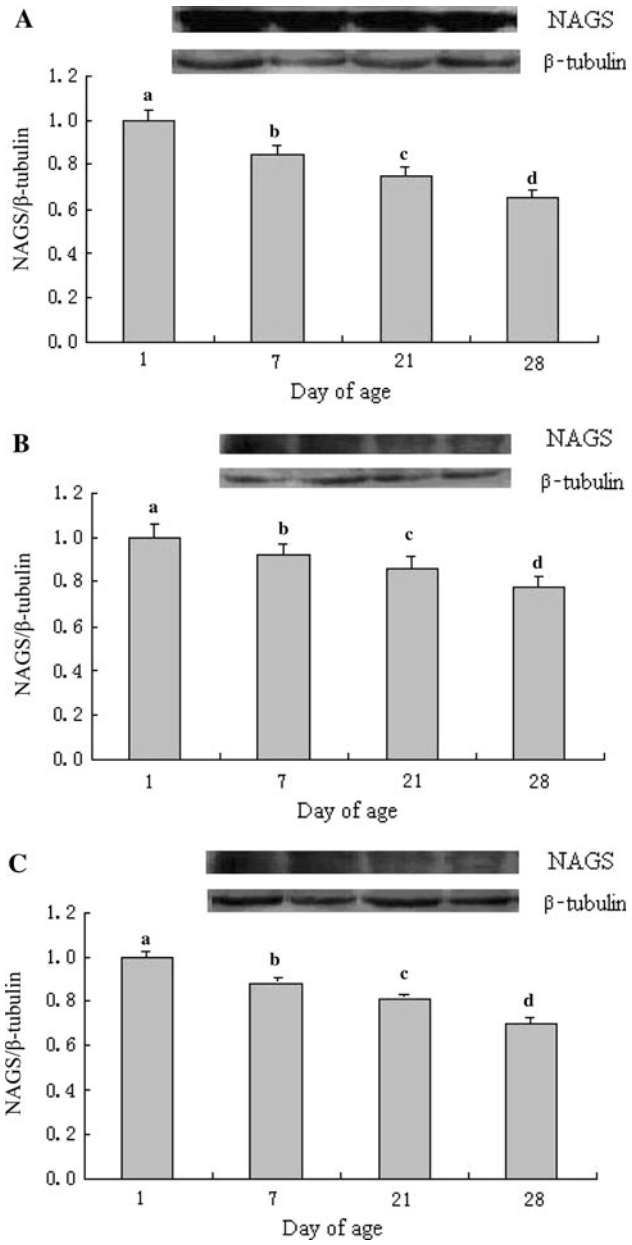

Fig. 5 Protein amounts of NAGS in the jejunum anterior (**A**), jejunum posterior (**B**) and ileum (**C**) of suckling piglets from day 1 to 28 of age. *a–d* Means with different superscripts in a row differ ($P < 0.05$). $n = 6$

activity in the small intestinal mucosa of 3-day-old rats compared with the newborn rats. The NAGS activity was also found to decrease progressively in enterocytes of 7- to 28-day-old piglets compared with 1-day-old pigs (Wu et al. 2004b). Therefore, it has been suggested that the reduced activity of intestinal NAGS may be responsible for the impaired synthesis of arginine by enterocytes in suckling pigs (Wu et al. 2004a, 2007). However, molecular evidence for developmental changes in intestinal NAGS gene expression has previously been lacking.

The amino acid sequences of NAGS in human and mouse consist of three regions with different degrees of conservation: the mitochondrial targeting signal (MTS), the variable domain, and the conserved domain (Schmidt et al. 2005; Caldovic et al. 2006). Our results of alignment by the clustal X showed that the porcine NAGS gene had a high degree of similarity to the "conserved domain" of the human and mouse NAGS. In order to study the regulation of intestinal NAGS expression by hormones and nutrients, we successfully purified sufficient amounts of NAGS protein from *E. coli* after the porcine NAGS gene was expressed in the bacteria. To our knowledge, this is the first report of expression of the porcine NAGS gene in *E. coli*.

The pig is a useful model to study developmental expression pattern of NAGS in the small intestine (Wu et al. 2004a). Our results indicated that mRNA and protein levels for NAGS decreased in the jejunum and ileum of sow-reared piglets with increasing age from 1 to 28 days (Fig. 4). Interestingly, there was a modest increase in NAGS mRNA abundance in the jejunum posterior between 7 and 21 days of age, which was apparently not consistent with the data on NAGS enzymatic activity (Wu et al. 2004a). Note that NAGS mRNA levels in the jejunum and ileum of 7- to 28-day-old piglets were still lower than those for 1-day-old pigs (Fig. 4), as we observed for the intestinal NAGS protein (Fig. 5). Similarly, circulating levels of cortisol in piglets decrease progressively during the suckling period (Wu et al. 2000b, c). Thus, it is possible that the transcription of the NAGS gene may be regulated by cortisol, as reported for other arginine-metabolic enzymes (Flynn et al. 2009).

Caldovic et al. (2002a) reported that the enzymic activity of mouse NAGS increased after removal of its predicted MTS. To explain a higher level of jejunal NAGS mRNA in 14- to 28-day-old than in 7-day-old pigs (Fig. 4) but an opposite observation for NAGS protein (Fig. 5), we suggest that translation of the intestinal NAGS gene into a biologically active protein and its post-translational modifications may be impaired in the 14- to 28-day-old suckling pigs compared with newborn piglets, as reported for global protein synthesis in piglet skeletal muscle (Suryawan et al. 2007, 2009). Alternatively, degradation of intestinal NAGS protein may be progressively enhanced in piglets during the suckling period. Further studies are warranted to test these novel hypotheses. Such work can be greatly facilitated by our successful development of the porcine NAGS antibody.

At present, little is known about the regulation of NAGS activity by covalent modifications (e.g., phosphorylation and acetylation). This possibility should be examined in future research. However, the data on postnatal changes in both NAGS mRNA and protein levels in the piglet small intestine suggest that expression of the NAGS gene is regulated primarily at the post-transcriptional level. Additionally, it is evident that studies of intestinal NAGS expression must involve quantification of NAGS protein or determination of NAGS activity and should not be limited solely to the measurement of NAGS mRNA levels. Collectively, results of our current and previous work (Wu et al. 2004a) indicate that NAGS enzymatic activity is a valid indicator of NAGS protein expression in the pig small intestine.

In summary, intestinal NAGS expression in young pigs is age-dependent and declines during the suckling period. The postnatal decrease in the levels of intestinal NAGS protein is consistent with the previous report of reduced synthesis of citrulline and arginine from glutamine/glutamate and proline in enterocytes of 7- to 21-day-old pigs compared with newborn pigs. These results provide a novel molecular basis to explain that endogenous synthesis of arginine is impaired in sow-reared piglets and that arginine is a nutritionally essential amino acid for the neonates. Regulation of NAGS expression may provide a new attractive strategy to improve arginine nutrition and growth in suckling piglets.

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References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Caldovic L, Morizono H, Yu XL et al (2002a) Identification, cloning and expression of the mouse *N*-acetylglutamate synthase gene. Biochem J 364:825–831

Caldovic L, Morizono H, Panglao MG et al (2002b) Cloning and expression of the human *N*-acetylglutamate synthase gene. Biochem Biophys Res Commun 299:581–586

Caldovic L, Lopez GY, Haskins N et al (2006) Biochemical properties of recombinant human and mouse *N*-acetylglutamate synthase. Mol Genet Metab 87:226–232

Chen LX, Li P, Wang JJ et al (2009) Catabolism of nutritionally essential amino acids in developing porcine enterocytes. Amino Acids 37:143–152

Davis TA, Nguyen HV, Garciaa-Bravo R et al (1994) Amino acid composition of human milk is not unique. J Nutr 124:1126–1132

Dekaney CM, Wu GY, Yin YL et al (2008) Regulation of ornithine aminotransferase gene expression and activity by all-transretinoic acid in Caco-2 intestinal epithelial cells. J Nutr Biochem 19:674–681

Deng ZY, Zhang JW, Wu GY et al (2007) Dietary supplementation with polysaccharides from Semen cassiae enhances immunoglobulin production and interleukin gene expression in early-weaned piglets. J Sci Food Agric 87:1868–1873

Deng D, Yin YL, Chu WY et al (2009) Impaired translation initiation activation and reduced protein synthesis in weaned piglets fed a low-protein diet. J Nutr Biochem 20:544–552

Fang J, Yan FY, Kong XF et al (2009) Dietary supplementation with Acanthopanax senticosus extract enhances gut health in weanling piglets. Livest Sci 123:268–275

Flynn NE, Wu GY (1996) An important role for endogenous synthesis of arginine in maintaining arginine homeostasis in neonatal pigs. Am J Physiol Regul Integr Comp Physiol 271:R1149–R1155

Flynn NE, Meininger CJ, Haynes TE et al (2002) The metabolic basis of arginine nutrition and pharmacotherapy. Biomed Pharmacother 56:427–438

Flynn NE, Bird JG, Guthrie AS (2009) Glucocorticoid regulation of amino acid and polyamine metabolism in the small intestine. Amino Acids 37:123–129

Fu WJ, Stromberg AJ, Viele K et al (2010) Statistics and bioinformatics in nutritional sciences: analysis of complex data in the era of systems biology. J Nutr Biochem 21:561–572

Haynes TE TE, Li P, Li XL et al (2009) L-Glutamine or L-alanyl-L-glutamine prevents oxidant- or endotoxin-induced death of neonatal enterocytes. Amino Acids 37:131–142

He QH, Kong XF, Wu GY et al (2009) Metabolomic analysis of the response of growing pigs to dietary L-arginine supplementation. Amino Acids 37:199–208

Hou ZP, Yin YL, Huang RL et al (2008) Rice protein concentrate partially replaces dried whey in the diet for early-weaned piglets and improves their growth performance. J Sci Food Agric 88:1187–1193

Hou YQ, Wang L, Ding BY et al (2010) Dietary α-ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. Amino Acids 39:555–564

Huang RL, Yin YL, Wu GY et al (2005) Effect of dietary oligochitosan supplementation on ileal digestibility of nutrients and performance in broilers. Poult Sci 84:1383–1388

Jobgen WS, Fried SK, Fu WJ et al (2006) Regulatory role for the arginine-nitric oxide pathway in energy-substrate metabolism. J Nutr Biochem 17:571–588

Jobgen WS, Fu WJ, Gao H et al (2009) High fat feeding and dietary L-arginine supplementation differentially regulate gene expression in rat white adipose tissue. Amino Acids 37:187–198

Kang P, Yin YL, Ruan Z et al (2008) Effect of replacement of lactose with partially hydrolysed rice syrup on small intestine development in weaned pigs from 7 to 21 days. J Sci Food Agric 88:1932–1938

Kang P, Toms D, Yin YL et al (2010) Epidermal growth factor-expressing Lactococcus lactis enhances intestinal development of early-weaned pigs. J Nutr 140:806–811

Kim SW, Wu GY (2004) Dietary arginine supplementation enhances the growth of milk-fed young pigs. J Nutr 134:625–630

Kim SW, Wu G (2009) Regulatory role for amino acids in mammary gland growth and milk synthesis. Amino Acids 37:89–95

Kong XF, Wu GY, Liao YP et al (2007) Effects of Chinese herbal ultra-fine powder as a dietary additive on growth performance, serum metabolites and intestinal health in early-weaned piglets. Livest Sci 108:272–275

Kong XF, Yin YL, He QH et al (2009) Dietary supplementation with Chinese herbal powder enhances ileal digestibilities and serum concentrations of amino acids in young pigs. Amino acids 37:573–582

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Li TJ, Da QZ, Yin YL et al (2008) Dietary starch sources affect net portal appearance of amino acids and glucose in growing pigs. Animal 2:723–729

Li LL, Hou ZP, Zhang B et al (2009a) The effect of dietary addition of a polysaccharide from *atractylodes macrophala koidz* on growth performance, immunoglobulin concentration and IL-1β expression in weaned pigs. J Agric Sci 147:625–631

Li P, Kim SW, Li XL et al (2009b) Dietary supplementation with cholesterol and docosahexaenoic acid affects concentrations of amino acids in tissues of young pigs. Amino Acids 37:709–716

Li XL, Bazer FW, Gao HJ et al (2009c) Amino acids and gaseous signaling. Amino Acids 37:65–78

Liu YL, Shi JX, Lu J et al (2009) Activation of peroxisome proliferator-activated receptor-c potentiates pro-inflammatory cytokine production, and adrenal and somatotropic changes of weaned pigs after Escherichia coli lipopolysaccharide challenge. Innate Immun 15:169–178

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

McKnight JR, Satterfield MC, Jobgen WS et al (2010) Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. Amino Acids 39:349–357

Morizono H, Caldovic L, Shi DS et al (2004) Mammalian *N*-acetylglutamate synthase. Mol Genet Metab 81:S4–S11

Palii SS, Kays CE, Deval C et al (2009) Specificity of amino acid regulated gene expression: analysis of gene subjected to either complete or single amino acid deprivation. Amino Acids 37:79–88

Rhoads JM, Wu G (2009) Glutamine, arginine, and leucine signaling in the intestine. Amino Acids 37:111–122

Schmidt E, Nuoffer J, Haberle J et al (2005) Identification of novel mutations of the human *N*-acetylglutamate synthase gene and their functional investigation by expression studies. Biochim Biophys Acta 1740:54–59

Suryawan A, Orellana R, Nguyen H et al (2007) Activation by insulin and amino acids of signaling components leading to translation initiation in skeletal muscle of neonatal pigs is developmentally regulated. Am J Physiol Endocrinol Metab 293:E1597–E1605

Suryawan A, O'Connor PMJ, Bush JA et al (2009) Differential regulation of protein synthesis by amino acids and insulin in peripheral and visceral tissues of neonatal pigs. Amino Acids 37:97–110

Tan BE, Li XG, Kong XF et al (2009) Dietary L-arginine supplementation enhances the immune status in early-weaned piglets. Amino Acids 37:323–331

Tan BE, Yin Y, Kong X et al (2010) L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. Amino Acids 38:1227–1235

Tang ZR, Yin YL, Nyachoti CM et al (2005) Effect of dietary supplementation of chitosan and galacto-mannan-oligosaccharide on serum parameters and the insulin like growth factor-I mRNA expression in early-weaned piglets. Domest Anim Endocrinol 28:430–441

Tang ZR, Yin YL, Zhang YM (2009) Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin–lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d. Bri J Nutr 101:998–1005

Tang ZR, Zhang YM, Stewart AF et al (2010) High-level expression, purification and antibacterial activity of bovine lactoferricin and lactoferrampin in Photorhabdus luminescens. Protein Expr Purif. doi:10.1016/j.pep.2010.05.013

Thompson JD, Gibson TJ, Plewniak F et al (1997) The CLUSTAL_X windows interface: Xexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Wakabayashi Y, Yamada E, Hasegawa T et al (1991) Enzymological evidence for the indispensability of small intestine in the synthesis of arginine from glutamate I. Pyrroline-5-carboxylate synthase. Arch Biochem Biophys 291:1–8

Wang XQ, Ou DY, Yin JD et al (2009a) Proteomic analysis reveals altered expression of proteins related to glutathione metabolism and apoptosis in the small intestine of zinc oxide-supplemented piglets. Amino Acids 37:209–218

Wang W, Shi C, Zhang J et al (2009b) Molecular cloning, distribution and ontogenetic expression of the oligopeptide transporter PepT1 mRNA in Tibetan suckling piglets. Amino Acids 37:593–601

Wence W, Gu WT, Tang XF et al (2009) Molecular cloning, tissue distribution and ontogenetic expression of the amino acid transporter b0, +cDNA in the small intestine of Tibetan suckling piglets. Comp Biochem Physiol B 154:157–164

Wu G (2009) Amino acids: metabolism, functions, and nutrition. Amino Acids 37:1–17

Wu G (2010) Recent advances in swine amino acid nutrition. J Anim Sci Biotech 1:49–61

Wu GY, Knabe DA (1995) Arginine synthesis in enterocytes of neonatal pigs. Am J Physiol Regul Integr Comp Physiol 269:R621–R629

Wu G, Morris SM Jr (1998) Arginine metabolism: nitric oxide and beyond. Biochem J 336:1–17

Wu G, Knabe DA, Flynn NE (1994) Synthesis of citrulline from glutamine in pig enterocytes. Biochem J 299:115–121

Wu G, Knabe DA, Yan W, Flynn NE (1995) Glutamine and glucose metabolism in enterocytes of the neonatal pig. Am J Physiol Regul Integr Comp Physiol 268:R334–R342

Wu GY, Meininger CJ, Knabe DA (2000a) Arginine nutrition in development, health and disease. Curr Opin Clin Nutr Metab Care 3:59–66

Wu G, Flynn NE, Knabe DA (2000b) Enhanced intestinal synthesis of polyamines from proline in cortisol-treated piglets. Am J Physiol Endocrinol Metab 279:E395–E402

Wu G, Flynn NE, Knabe DA, Jaeger LA (2000c) A cortisol surge mediates the enhanced polyamine synthesis in porcine enterocytes during weaning. Am J Physiol Regul Integr Comp Physiol 279:R554–R559

Wu GY, Knabe DA, Kim SW (2004a) Arginine nutrition in neonatal pigs. J Nutr 134:2783S–2790S

Wu GY, Jaeger LA, Bazerb FW et al (2004b) Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications. J Nutr Biochem 15:442–451

Wu GY, Bazer FW, Davis TA et al (2007) Important roles for the arginine family of amino acids in swine nutrition and production. Livest Sci 112:8–22

Wu GY, Bazer FW, Davis TA et al (2009) Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168

Wu X, Ruan Z, Gao YL et al (2010a) Dietary supplementation with L-arginine or *N*-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. Amino Acids 39:831–839

Wu WZ, Wang XQ, Wu GY et al (2010b) Differential composition of proteomes in sowcolostrum and milk from anterior and posterior mammary glands. J Anim Sci 88:2657–2664

Wu X, Yin YL, Li TJ et al (2010c) Dietary protein, energy and arginine affect LAT1 expression in forebrain white matter differently. Animal 4(9):1518–1521

Yang CB, Jiang Y, Huang KH et al (2003) Application of real-time PCR for quantitative detection of Campylobacter jejuni in poultry, milk and environmental water. FEMS Immunol Med Microbiol FEMSIM 38:265–271

Yang CB, Jiang Y, Huang KH et al (2004) A real time PCR assay for the detection and quantitation of Campylobacter jejuni using SYBR green I and the light cycler. Yale J Biol Med 77:125–132

Yang CB, Li AK, Yin YL et al (2005) Effects of dietary supplementation of cysteamine on growth performance, carcass quality, serum hormones and gastric ulcer in finishing pigs. J Sci Food Agric 85:1947–1952

Yao K, Yin YL, Chu WY et al (2008) Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. J Nutr 138:867–872

Yin YL, Baidoo SK, Schulze H et al (2001) Effect of supplementing diets containing hulless barley varieties having different levels of non-starch polysaccharides with β-glucanase and xylanase on the physiological status of gastrointestinal tract and nutrient digestibility of weaned pigs. Livest Prod Sci 71:97–107

Yin YL, Deng ZY, Huang RL et al (2004) The effect of arabinoxylanase and protease supplementation on nutritional value of diets containing wheat bran or rice bran in growing pig. J Anim Feed Sci 13:445–461

Yin YL, Tang ZR, Sun ZH et al (2008) Effect of galacto-mannan-oligosaccharides or chitosan supplementation on cytoimmunity and humoral immunity response in early-weaned piglets. Asian-Aust J Anim Sci 21:723–731

Yin FG, Liu YL, Yin YL et al (2009) Dietary supplementation with Astragalus polysaccharide enhances ileal digestibilities and serum concentrations of amino acids in early weaned piglets. Amino Acids 37:263–270

Zeng J, Geng MM, Liu YD et al (2007) Expression, purification and molecular modelling of the Iro protein from Acidithiobacillus ferrooxidans Fe-1. Protein Expr Purif 52:146–152