Postnatal ontogeny of small intestinal histomorphology, crypt cell proliferation and peptide transporter 1 gene expression in piglets

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Abstract

Ontogeny of the morphology, cell proliferation, and peptide transporters in each region of the small intestine support optimal nutritional status and growth of pigs from early to later life. To assess these, growth of piglets was studied at birth (d 0), suckling (d 7), weaning (d 21) and post-weaning (d 28, 49 and 77) days. Hematology, small intestinal histomorphology, crypt cell proliferation, and peptide transporter 1 (PepT1) mRNA expression were measured from the animals at newborn, and at 7, 21, 49 and 77 days old. Among the three age groups (newborn to 21, 21 to 28 and 21 to 77 days old), all animals progressively improved body weight (BW) and body weight gain (BWG) from the early suckling stage to the post-weaning stage (p<0.0001) but their BWG declined during one week after weaning (d 21 to d 28). In the youngest age group (d 0 to d 7), the villus height, ratio of villus height to crypt depth, proliferation, and turnover rate of enterocytes at distinct regions along the small intestine, particularly the jejunum, reached maximal value and then dramatically decreased by the day the animals were weaned (d 21) and thereafter (p<0.05). The PepT1 mRNA expression was found to be distributed along the small intestine. The relative expression of PepT1 mRNA in the suckling pigs paralleled that in the post-weaning pigs. These data suggest that ontogenetic adaptation of the histomorphology, crypt cell number, and turnover rate as well as relative expression of PepT1 mRNA in the small intestine may be useful during early suckling state, especially the first week, and efficiently promotes diet transition post-weaning.

Keywords: growth, morphology, peptide transporter, piglet, small intestine

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Introduction

During the suckling to post-weaning period, piglets are exposed to a variety of stresses. Stress factors can affect growth, morphological changes and maturation of the gastrointestinal tract (GIT) (Plaske et al., 1997), which may bring about critical functional consequences to the growing pigs. However, optimum development of the GIT accompanying both cellular adaptation and molecular regulation in the small intestine is not fully understood.

There are some evidences indicating that ontogeny of the small intestinal morphology and peptide transporter 1 (PepT1) expression may affect gut health to improve piglet growth during early suckling to post-weaning periods (Xu et al., 1992; Nosworthy et al., 2013). The small intestine of mammals is where the majority of nutrient absorption occurs to support the rapid development in the early stage of life. This corresponds with our previous findings that proliferation and turnover rates of enterocytes in the small intestine are reflected in the development and function of normal and low birth weight piglets (Wiyaporn et al., 2013). Interestingly in neonatal guts, a high proportion of amino acids was present as small peptides due to significantly lower proteolytic enzymes compared to adults (Henderson et al., 2001). Thus, the capacity for peptide transport may support optimal nutritive status and growth since PepT1 is localized to the apical membrane of intestinal villi to access di- and tripeptides passing through the intestinal lumen (Ganapathy et al., 1994; Wang et al., 2009). Therefore, the present study aimed to evaluate piglet growth by primarily focusing on the adaptation of histomorphology and relative expression of PepT1 mRNA in three segments of the small intestine from birth to suckling, and from weaning to post-weaning stages. Understanding these ontogenetic events helps clarify the patterns of small intestinal development and function for not only piglets but also human infants due to parallels between pig and human GITs (Shulman, 1993).

Materials and Methods

Animals and experimental design: Cross-bred (Large White x Landrace) pregnant sows were raised at a local commercial pig farm with a commercially available complete feed. After parturition, pre-weaning piglets (Large White x Landrace x Duroc) were allowed to suckle until 21 days of age from sows that were fed a common lactation diet (17.5% crude protein, 5.4% crude fat, 5.0% crude fiber, 0.8% calcium, 0.5% phosphorus with metabolizable energy (ME) of 3.2 Mcal/kg). Weaned pigs were fed a starter diet based on corn, soybean meal and fish meal (22.6% crude protein, 4.2% crude fat, 3.4% crude fiber, 0.8% calcium, 0.7% phosphorus with ME of 3.3 Mcal/kg). The diet was provided as a mash formulated to meet or exceed the National Research Council (2012) recommendations for post-weaners. All pigs had free access to drinking water throughout the study. The piglets were weighed within 3 h after birth and before suckling, and only piglets with normal birth weight (1.4 to 1.6 kg) (Morise et al., 2008) were used in this study. All piglets from each litter remained with their dams for the duration of suckling. At the beginning, the experiment was carried out on 25 female piglets. During 11 weeks of study, five female pigs per age group were weighed at birth (d 0), days 7, 21, 28, 49 and 77. Their body weight gain (BWG) was determined for each specific time: newborn to 21 days old, 21 to 28 days old and 21 to 77 days old. Signs of diarrhea, morbidity, abnormal behavior and mortality were also monitored daily by attentive veterinarians throughout the experimental period. At the age of 0, 7, 21, 49 and 77 days, four pigs from each age group were sedated with azaperone (6 mg/kg BW, intramuscular) and euthanized with an overdose of sodium pentobarbital (50 mg/kg BW, intravenous or 200 mg/kg BW, intraperitoneal) for sample collection. All procedures described in this manuscript were approved by the Animal Care and Use Committee of Faculty of Veterinary Science, Chulalongkorn University.

Sampling and sample processing procedures: Blood samples via puncture of the anterior vena cava were collected from 4 pigs per age group into vacutainer tubes coated with anticoagulant (heparin) for piglets aged 0, 7, 21, 49 and 77 days. Then, small intestinal tissues sampled from the duodenum, jejunum and ileum were collected to determine histomorphology, immunohistochemistry, and PepT1 gene expression. To ensure consistency between pigs, the tissue samples were divided into duodenum, jejunum and ileum using the posterior pylorus, ligament of Treitz and the ileo-cecal junction as landmarks and a 5 cm length was excised from the middle of each part. These sections were cut longitudinally and opened out flat. A second 1.5 cm length was also removed from each part and left as a ring. All the tissues were thoroughly washed in cold phosphate buffer saline (PBS) and the rings were placed in 10% buffered formalin at room temperature for later histomorphological analysis and immunohistochemistry. The mucosa from each segment of the small intestine was scraped with a glass slide, placed in a vial containing RNA later solution (Applied Biosystems, USA) and stored at -20ºC pending RNA extraction.

Hematology measurement: White blood cells, red blood cells and lymphocyte counts in the whole blood were analyzed using an automatic blood analyzer (Coulter 1890, Diamond diagnostic inc. Holliston, USA).

Small intestinal sample analyses

Histomorphological analysis: To determine histomorphological changes in the small intestines of piglets, the preserved tissue segments were prepared using standard paraffin embedding techniques and processed through tissue sections. The slides were stained with hematoxylin and eosin and then villus height and crypt depth were measured under 200x magnification using Scion image software (Scion image; Scion Corporation, Frederick, MD). Each histological section (one each for duodenum, jejunum and ileum) was divided into 4 quadrants and at least 4 villi or crypts were measured per quadrant. Villus height (in micrometers) was measured from its tip to base excluding the intestinal crypt, while crypt depth
was taken as the invagination depth between adjacent villi as described by Martin-Rodrigues et al. (2007). An average of villus height or crypt depth from each quadrant was expressed as mean villus height and crypt depth. Then, an average of these 4 quadrants per 1 section was expressed as mean villus height and crypt depth for each pig.

**Proliferative cell marker (Ki-67) by immunohistochemistry:** Immunohistochemistry was performed to analyze Ki-67 proliferative cell marker. The paraffin-embedded tissues were sectioned at 4 μm thickness, placed on silane-coated slides. The sections were then deparaffinized and heated at 95°C for 10 min in 10 mM sodium citrate buffer (pH 6.0). Steps were performed in an immunostainer using the LSAB staining method. Nonspecific background was reduced by incubating the sections with 10% bovine serum albumin (BSA) for 30 min at room temperature. Endogenous peroxidase was inactivated by treatment with 3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were subsequently incubated at 4°C overnight with a primary antibody, the monoclonal anti-mouse Ki-67 (Clone MB-1, DAKO, Denmark), at a dilution of 1:200. Subsequently, the sections were incubated in secondary antibody detection system (Envision, DAKO, Denmark; 1:400) at room temperature for 45 min. Peroxidase activity was developed using diaminobenzidine (DAB) as chromogen. The immunostained slides were counterstained with hematoxylin for 30 s and rinsed with tap water. Finally, the sections were dehydrated and mounted (DAKO). The immunostained slides were examined under bright field microscopy at 400x magnification (Nikon, Japan) and images were captured with a Hamamatsu digital camera (C4742-95, Japan). Crypt cell nuclei with clear positive brown staining were counted. Each transverse section was divided into 4 quadrants and a total number of 500 cells within the crypt area were counted. Ratio between the labeled Ki-67-positive and total cell count was expressed as a percentage for small intestine segments.

**Real-time RT-PCR analysis:** Relative expressions of porcine PepT1 mRNA in duodenal, jejunal, and ileal mucosa were determined by real-time quantitative PCR, where 18S ribosomal RNA was used as a reference gene. The primer set used for porcine PepT1 was AGC ATC TTC TTC ATC GTG GTC AA, and GTC TTG AAC TTC CCC AGC CA, and for 18S rRNA was CCG CGG TTC TAT TTT GGT GTT TTT, and CGG GCC GGG TGA GGF TTC. The target size of PepT1 and 18S rRNA was 206 and 399 base pair, respectively. Total RNA from the mucosa of the duodenum, jejunum and ileum was extracted using Aurum total RNA fatty and fibrous kit and reverse-transcribed into complementary DNA (cDNA) by iScript Reverse transcription supermix for RT-qPCR kit (Bio-Rad Laboratory, Hercules, USA) in accordance with the manufacturer’s instructions. One microgram of the total RNA samples was reverse-transcribed into first strand cDNA using random primers. All final sample products were adjusted to the same concentration using nuclease-free water. Quantitative real-time PCR was carried out using an ABI 7300 Real-time PCR system (Applied Biosystem, USA). The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, followed by annealing and extension at 58°C for 60 s. A melt curve was performed at the end to confirm specificity of the resulting PCR products. Each sample was done in duplicate, and relative expression of PepT1 was calculated in relation to the 18S rRNA using the formula 2-ΔΔCt (Schmittgen and Livak, 2008).

**Statistical analysis:** Data were expressed as means ± standard errors of the mean (SEM). For overall growth, small intestinal histomorphology, Ki-67 proliferative cell index and PepT1 mRNA relative expression, the data were analyzed by ANOVA with Tukey’s test. Correlations between the ratio of villus height to crypt depth and the percentage of Ki-67 proliferative cell index of the small intestine were determined by a proc reg procedure. Statistical significance was taken as p<0.05, while 0.05<p<0.10 values were considered as a statistical trend (SAS institute, Cary, NC, USA).

**Results**

**Growth and health of piglets:** The body weight (BW) from birth to 77 days old, and the body weight gain (BWG) of all age groups (newborn to 21, 21 to 28 and 21 to 77 days old) are shown in Figure 1. There were significant differences in BW among the age groups (p<0.0001) with the exception of the first week after birth and at one week old after weaning (d 21 to d 28) as shown in Figure 1A. During the suckling phase (newborn to 21 days old), and both 21 to 28- and 21 to 77-day-old post-weaning phases, the BWG values were 3.76 ± 0.55, 0.95 ± 0.46 and 28.63 ± 1.77 kg, respectively (Figure 1B), similar to the BW values. Thus, the post-weaners which gained weight month by month had more vigorous performance when compared with the suckling piglets (p<0.0001). Throughout the study, all the piglets appeared in good health (data not shown) according to the hematological parameters (Egeli et al., 1998).

**Histomorphology of small intestine:** As shown in Figure 2, there were significant differences among the age groups in villus height (Figure 2A), crypt depth (Figure 2B), and villus height to crypt depth ratio (Figure 2C) of the small intestinal segments (p<0.05). From birth up to day 77 of life, each of the regions of the small intestine demonstrated that the villus height and the ratio of villus height to crypt depth of the jejunum were significantly higher in the youngest age group (d 0 to d 7) compared with the weaned and post-weaned pigs (p<0.0001). Remarkably different development of the villi was observed in the jejunum on day 7 of life. The length of jejunal villi increased from newborn up to day 7 of life, reaching a maximum of 1475 μm, whereas the ratio of villus height to crypt depth at d 0 and d 7 appeared to be about 8.6 and less than 8.0, respectively. Subsequently, the villus height and the ratio of villus height to crypt depth of the post-weaners did not exceed 365 μm and 2.98, respectively, and no difference was observed thereafter. When compared among the three segments of the small intestine within the same day, the villus heights of...
jejenum on day 7 and day 77 of life were significantly higher than those of the duodenum and ileum ($p<0.01$). The ratio of villus height to crypt depth, however, was higher only on day 7 of life ($p=0.068$). Significant deepening in the crypt depth of the jejunum and ileum was found in the first week after birth, reaching a maximum of 200 $\mu$m, when compared with the weaned day ($p<0.05$). Among the three segments of the small intestine taken within the same day, the crypt depth of the duodenum was significantly deeper than the jejunum and ileum on day 21 of life ($p=0.004$), whereas the jejunal crypt depth was significantly deeper than the ileal crypt depth only on day 77 of life ($p=0.014$).

Figure 1  Piglet body weight (A) on days 0, 7, 21, 28, 49 and 77 of life, and body weight gain (B) in the three age groups: 0 to 21, 21 to 28 and 21 to 77 days old (n=5 per group). Values are presented as means $\pm$ standard error of the means. $a,b,c,d$ indicate that means with different superscript letters differ significantly ($p<0.0001$) between age groups.

**Ki-67 proliferative cell index:** In all tested animals, there was clearly active cell division as indicated by the Ki-67 immuno-positive staining. Crypt cell nuclei with clear brown staining (arrow) are shown in Figure 3A. As shown in Figures 3A-E, the sections of pig jejunum on days 0 (A), 7 (B), 21 (C), 49 (D) and 77 (E) of life were immunostained and visualized. The percentages of Ki-67 proliferative cell index of the small intestinal segments in all age groups are presented in Figure 4. Focusing on the jejunum and ileum, there were significant differences in the percentage of Ki-67 positive crypt cells in the youngest pigs (d 0 to d 7) compared with the weaned and post-weaned pigs ($p=0.007$) with a value of about 1.7-fold greater than the older pigs. Meanwhile, the duodenum tended to increase these positive cell numbers in the newborn pigs compared with the weaned and post-weaned pigs ($p=0.052$). Both the jejunum and ileum decreased rapidly and remained relatively constant with age from 21 to 77 days old. Moreover, when compared across all three segments of the small intestine within the same day, the percentage of Ki-67 positive cells of the ileum was significantly higher than that of the duodenum on the day of birth ($p=0.004$), but there was no significant difference in the percentage of Ki-67 positive cells between the jejunum and duodenum or the jejunum and ileum on day 0. In addition, a significant positive correlation between the ratio of villus height to crypt depth and the percentage of Ki-67 positive cells of the small intestine was found regardless of segments and ages ($r^2=0.4766$, SER=12.66 and $p<0.001$).

**Quantification of PepT1 mRNA in small intestine:** The relative expression of PepT1 mRNA in all small intestinal segments of all animals tested at various ages is presented in Figure 5. The expression of PepT1 was found to be highly variable and no significant difference was found between the age groups or within the three segments of the small intestine.

**Discussion**

This current study demonstrated the adaptation of histomorphological changes and PepT1 gene expression in the small intestine of piglets from newborn up to post-weaning periods (77 days old). This study focused on four target groups of different
ages, newborn (d 0), suckling (d 0 to 21), weaning (d 21) and post-weaning (d 21 to 77) in pigs. Paredes et al. (2012) demonstrated that the BW of piglets at the end of the study was affected by birth weight, weaning weight of life. However, piglets aged one week after weaning (d 21 and d 28) had a non-significant change in growth, according to the result of Gancarcikova et al. (2009). Thus, this result indicates that piglets do not adapt or catch up with weight gain for some time, as demonstrated by the remarkably different BWG, due to early life stress that has a durable impact on growth and small intestinal function in pig neonates.

**Figure 2** Villus height (A), crypt depth (B), and ratio of villus height to crypt depth (C) of the small intestine: duodenum, jejunum and ileum in piglets on days 0, 7, 21, 49 and 77 of life (n=4 per age group). Values are presented as means ± standard error of the means of histomorphological measurements. Significant effects of age and intestinal location were observed. Lines represent significant difference between intestinal sections within an age group (* p<0.05, ** p<0.01, † p<0.10). a,b,c indicate that means with different superscript letters differ significantly (p<0.05) within each specific intestinal site compared between age groups.

**Figure 3** Immunohistochemical staining of Ki-67 depicted from pig jejunum on days 0 (A), 7 (B), 21 (C), 49 (D) and 77 (E) of life. Positive cells are indicated by brown nuclear stain (arrow); the inset showed staining with on primary antibody (scale bar 50 μm).
Changes in histomorphology and cytoproliferation of the small intestine among the age groups indicate age-dependent alteration of the villus height, crypt depth, ratio of villus height to crypt depth, and Ki-67 proliferative cell index. The limited number of investigations into the ontogeny of porcine intestinal morphology and their variable results are questioned. However, it is well accepted that many factors, both intrinsic and extrinsic (Klein, 1989; Lalles et al., 2007), could affect the alteration of small intestinal morphology and play a regulatory role in the digestive tract. Throughout this study period, the height of the villi and the ratio of the villus height to crypt depth were highest in the jejunum during the suckling period, whereas the crypt depth was variable or consistently changed with age. The increase in villus height because of greater nutrient absorption appears to be the case, particularly in the duodenum and jejunum, in the piglets aged 1 week after birth. The increased villus height with animal age may reflect the
nutrient absorption and availability from sow colostrums and milk. Gancarcikova et al. (2009) reported that the nucleus in enterocytes in the finger-like villi of the jejunum of germ-free piglets was apically located at 3 h after birth. The deeper crypts, identified as the villus factory, indicate fast villus turnover rate for renewing of the villi. This massive turnover involves and continues proliferation of cells in the crypt base with subsequent migration along the intestinal crypt-villus axis. Gancarcikova et al. (2009) suggested that the overall mass of the small intestine of conventional control pigs, when compared with germ-free pigs, was increased and the villi were usually uniform in shape and appeared with crypts. Cell division in crypts is a response to enterocyte turnover rate. The increase in the number of Ki-67 proliferative cell index at birth and 7 days old indicates significant increase in turnover rate in the jejunum and ileum. Additionally, the remarkably significant positive correlation between ratio of villus height to crypt depth and Ki-67 proliferative cell index of the small intestine supported the idea. This finding is in agreement with Wiyaporn et al. (2013). Thus, it is possible that the suckling pigs had higher epithelial cell turnover than the weaned pigs and post-weaners. Based on the data mentioned above, whether the developmental pattern of PepT1 mRNA may be regulated by the age of piglets raised in a commercial farm was continuously evaluated in this study.

PepT1 had unique developmental features in the intestine of neonatal animals (Boudry et al., 2014). The ontogeny of PepT1 is complicated and some variable results of PepT1 mRNA expression (Wang et al., 2009) dependent on distinct regions along the intestine (Terada et al., 2005; Lu and Klassen, 2006) and the days of life were reported (Shen et al., 2001). The data obtained in this study were different from those previously reported and could probably be explained by the different species and number of animal samples. The PepT1 mRNA expression is potentially important during the early life of mammals. In suckling piglets, PepT1 mRNA expression was found within the first 2 days after birth (D’Inca et al., 2011). In a rat model, Shen et al. (2001) reported that mRNA expression of this transporter was at the highest level three to five days after birth, then it declined rapidly at all locations in the small intestine during the days following the suckling period. According to this study, no significant difference in the relative expression of PepT1 mRNA was detected along the small intestine of the suckling pigs. Thus, Nosworthy et al. (2013) reported that uptake of dipeptide glycylsarcosine was relatively consistent in all locations in the small intestine during the suckling period, particularly in piglets aged 2 to 3 weeks. The relative quantity of PepT1 mRNA, which is particularly higher in the duodenum after birth than at the time of weaning in pigs, may contribute to the predominantly rapid uptake of dipeptides from sow milk protein in 1-week-old piglets (Mavromichalis et al., 2001). However, some conditions such as lactating sows fed deficient protein diet can affect amino acid or peptide transport and consequently cause them and their litters alteration of some plasma amino acid concentrations (Rungharoen et al., 2011). The jejunum or ileum may be responsible for the digestion and absorption not only of protein, but also other nutrients from a grain based diet consumed by weaned pigs. Consequently, providing a substrate, such as protein or peptide, may maintain or stimulate PepT1 regulation in this segment of the small intestine. This is in agreement with some previous findings (Adibi, 2003; Danial, 2004; Radeva et al., 2007) suggesting that transcriptional and post-transcriptional regulation of PepT1 occurs in response to alterations in the nutritional status and in disease states. Future studies characterizing the discrepancy between mRNA levels and activity of PepT1 observed in healthy piglets are warranted.

In conclusion, this study provided evidence that the ontogeny of small intestinal morphology, cell proliferation, turnover rate and PepT1 gene expression of the neonatal pigs significantly affect the growth and responsiveness of the intestine to physiological challenges later in life. Therefore, the elongated villus height, increased enterocyte number and turnover rate during suckling period as well as the relative stabilities of these parameters and PepT1 mRNA expression after weaning help in the developmental regulation during early neonatal life and respond to diet transition in post-weaners.

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บทคัดย่อ

พัฒนาการช่วงหลังคลอดของเนื้อเยื่อสัณฐาน การเพิ่มจำนวนคริปท์เซลล์และการแสดงออกของยีนตัวขนส่งเพปไทด์ 1 ในลำไส้เล็กสุกร

บุญฤทธิ์ ทองทรง 1* สุรีรัตน์ สุธงษา 2 มาสสุภา วิยาภรณ์ 3

การพัฒนาเปลี่ยนสัณฐานวิทยา การเพิ่มจำนวนเซลล์ และการขนส่งเพปไทด์ในแต่ละตำแหน่งของลำไส้เล็กสุกรน้ าหนักตัวอาจสูงสุดในช่วงที่ทารกกำลังดูดนม ทำให้การเปลี่ยนแปลงสัณฐานวิทยา favourable ต่อการเติบโตของลำไส้เล็กสุกรถูกสืบเนื่องไปเรื่อยๆ จนกระทั่งระยะหลังหย่านมและคืนไปช่วงระยะหลังดูดนม

คำสำคัญ: การเติบโต สัณฐานวิทยา ลำไส้เล็ก สุกร ลำไส้เล็กสุกร ลำไส้เล็กสุกร ลำไส้เล็กสุกร