



## Genome-wide DNA Methylation Profiles of Small Intestine and Liver in Fast-growing and Slow-growing Weaning Piglets

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**ABSTRACT:** Although growth rate is one of the main economic traits of concern in pig production, there is limited knowledge on its epigenetic regulation, such as DNA methylation. In this study, we conducted methyl-CpG binding domain protein-enriched genome sequencing (MBD-seq) to compare genome-wide DNA methylation profile of small intestine and liver tissue between fast- and slow-growing weaning piglets. The genome-wide methylation pattern between the two different growing groups showed similar proportion of CpG (regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence) coverage, genomic regions, and gene regions. Differentially methylated regions and genes were also identified for downstream analysis. In canonical pathway analysis using differentially methylated genes, pathways (triacylglycerol pathway, some cell cycle related pathways, and insulin receptor signaling pathway) expected to be related to growth rate were enriched in the two organ tissues. Differentially methylated genes were also organized in gene networks related to the cellular development, growth, and carbohydrate metabolism. Even though further study is required, the result of this study may contribute to the understanding of epigenetic regulation in pig growth. (**Key Words:** DNA Methylation, MBD-seq, Epigenetic Profile, Weaning Piglet, Genome-wide Methylation Profile)

### INTRODUCTION

Pigs are an important economic animal and their growth rate is one of the main concerns of farmers, companies and scientists. Growth rate is known to be affected by the interaction of genetic and environmental factors in many animals including pigs (Hazel et al., 1943; Martorell et al., 1977; Bourdon and Brinks, 1982). In order to identify the genetic factors that underlie growth rate in pigs, many genome-wide association studies have been conducted. A number of different genes, variants and genetic regions

were reported to have important effects on the growth rate (Lo et al., 1992; Mrode and Kennedy, 1993; Andersson et al., 1994; Estelle et al., 2008; Rubin et al., 2012).

While the genetic information based on the genome sequence can provide some insights, it has not been able to fully explain into the correlation between genome sequences and phenotypic traits. In light of this problem, epigenetic studies such as the ENCODE (encyclopedia of DNA element) project (The ENCODE Project Consortium, 2004; The ENCODE Project Consortium, 2011) have received attention because many phenotypic traits including growth rate could be influenced by epigenetic factors such as DNA methylation. Previous studies have shown that DNA methylation can affect gene regulation, phenotypic trait, genomic imprinting, and disease development (Li et al., 1993; Siegfried et al., 1999; Bird, 2002; Dolinoy, 2008). In spite of the importance of DNA methylation, only a small number of studies have been conducted to identify genome-wide methylation profiles of economic animals (Li et al., 2011; Walker, 2012; Hu et al., 2013; Su et al., 2014). In

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particular, there has yet to be a study on genome-wide methylation and its relationship to economic traits such as growth rate in pigs.

In this study, we conducted methyl-CpG binding domain protein-enriched genome sequencing (MBD-seq) and bioinformatics analysis to assay the genome-wide DNA methylation pattern in the small intestine and liver samples of 21 day old weaning piglets with different growth rates. These two organs are important for digestion and absorption of nutrients. Besides, these organs undergo morphological changes and functional maturation during the postnatal period (Xu, 1996). For example, profound growth of organ tissue and the epithelial modification including the loss of ability of absorbing macromolecules occur in small intestine. The gene expression in liver is involved in metabolizing nutrients and generating growth factors like (Insulin-like growth factor 1 (*IGF-1*)). By comparing the genome-wide methylation pattern of two groups exhibiting different growth performances, we identified differentially methylated regions (DMRs) and genes related with these regions. Pathway and network analysis of these differentially methylated genes identified a number of candidate genes which may affect growth performance in pigs. This is the first study for the genome-wide DNA methylation distribution of weaning piglets using next generation sequencing and the results of this study may contribute to the improvement of epigenetic understanding of growth rate in pigs.

## MATERIAL AND METHODS

### Animals

Samples used in this study were a multicross hybrid breed of Yorkshire, Landrace, and Duroc. From three sows, the pigs with the highest and lowest average daily gain were selected 21 days after birth totaling six pigs. The average weight of the fast growing and slow growing pigs were 8.15 kg and 5.53 kg respectively. Liver and small intestine tissues were sampled from each pig for sequencing.

### Sequencing

Before a genomic DNA library construction, methylated DNA fragments were captured using the Methycap kit (Diagenode, Denville, NJ, USA). TruSeq™ DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) was used following the manufacturer's protocol. To generate raw data for each sample library, 51 cycle single-end sequencing using HiSeq 2000 was carried out. To obtain enough read for analysis, three samples were multiplexed within one lane of HiSeq 2000 sequencing lanes.

### Bioinformatics analysis

FastQC (Andrews, 2010) was used to check the quality

of the raw reads. Read bases with unnecessary sequences (adaptor, index, primer sequence of illumina platform) or low quality scores (under Q20 of phred scale) were filtered using Trimmomatic (Bolger et al., 2014). Filtered read were aligned to the pig reference genome (*Sus scrofa* 10.2, *susScr3*) using Bowtie2 (Langmead and Salzberg, 2012) with the default option parameters. Removal of duplicated read from polymerase chain reaction and alignment information sorting were conducted using Picard tools (<http://picard.sourceforge.net>) and Samtools (Li et al., 2009). To identify the DMR, methylated DNA immunoprecipitation sequencing data analysis (MEDIPS) (Chavez et al., 2013) and BSgenome (Pages, 2009) libraries of pig were used. The parameter setting of MEDIPS is as follows: `uniq = TRUE`, `extend = 300`, `shift = 0`, `window size = 100`. EdgeR method was used to identify DMR regions and false discovery rate (FDR) was used for multiple test correction. Regions shown to be significant ( $FDR < 0.05$ ) were used for downstream analysis. After identifying genomic windows that showed significant differential coverage between conditions, neighboring significant windows were merged into a large continuous region. The DMR regions were annotated using Peak analyzer v1.4 (Salmon-Divon et al., 2010) with three options: overlap, nearest downstream gene, and nearest transcription starting site). To calculate the proportion of uniquely mapped bases, various information (CpGs, repeats, genes) was downloaded from University of California, Santa Cruz (UCSC) table browser (Meyer et al., 2013). Canonical pathway and network analyses were conducted using Ingenuity Pathway Analysis (<http://www.ingenuity.com>, Fisher's exact test  $p < 0.05$ ).

## RESULTS

### Genome-wide methylation profile and differentially methylated regions

Produced and trimmed raw read data information from liver and small intestine samples of six pigs is described in Supplementary Table 1. The average alignment rate of the 12 samples was 71.28%, and on average 36.08% of reads was uniquely mapped to the reference genome. The details of the mapping rate of 12 samples are described in Table 1. The Pearson's correlation values of all samples were close to one in a saturation analysis using MEDIPs and the read coverage was enough to conduct downstream analysis. The saturation plots of the 12 samples are shown in Supplementary Figure 1. Supplementary Figure 2 shows the proportion of covered CpG region using pie chart. The covered CpG pattern for samples of the same organ showed similar patterns between high and low average daily gain groups. The sequence pattern coverage histogram (Supplementary Figure 3) also showed similar shape

**Table 1.** Summary of read alignment rate of each sample using bowtie2 with default option (%)

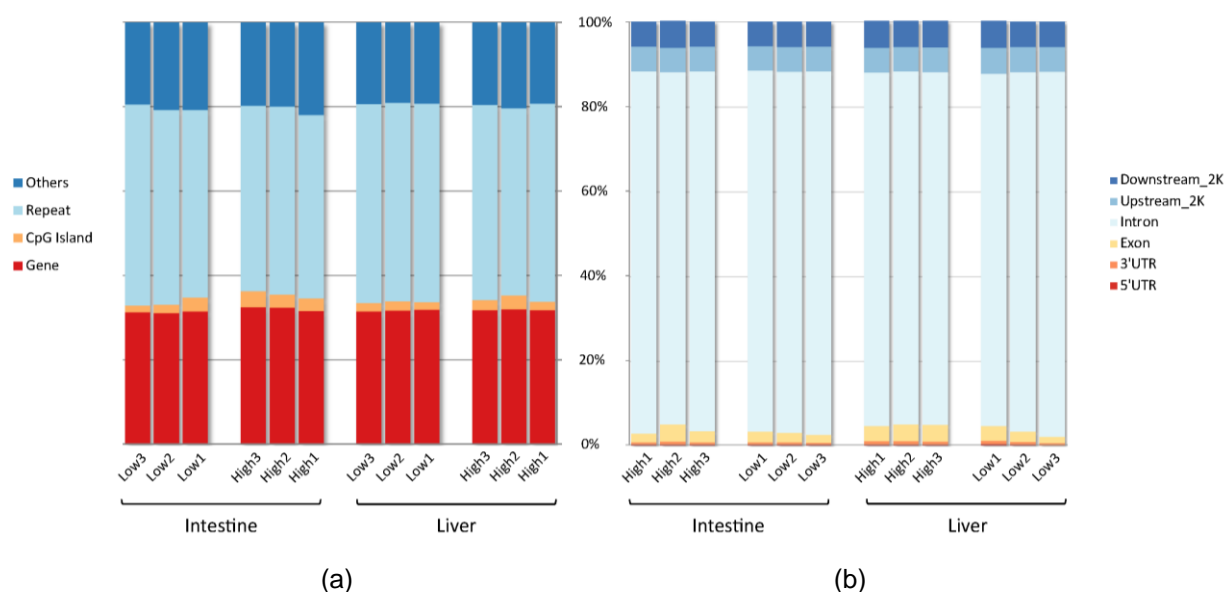
	Intestine_high1	Intestine_high2	Intestine_high3	Intestine_low1	Intestine_low2	Intestine_low3
<b>Intestine</b>						
Overall alignment rate	71.02	73.65	75.50	71.33	66.50	72.00
Aligned exactly 1 time	32.33	40.75	35.71	38.28	31.67	33.92
Aligned over 1 time	38.69	32.89	39.78	33.28	34.83	38.08
Unaligned	28.98	26.35	24.50	28.67	33.50	28.00
<b>Liver</b>						
Overall alignment rate	75.11	73.75	71.05	63.06	68.71	73.74
Aligned exactly 1 time	40.00	37.46	36.33	33.97	37.31	35.31
Aligned over 1 time	35.10	36.30	34.72	29.10	31.40	38.43
Unaligned	24.89	26.25	28.95	36.94	31.29	26.26

between the two groups. On average, 0.27% of mapped reads did not cover any CpG region. Also, average enrichment score was 4.515 in the small intestine samples and 4.296 in the liver samples. This shows that MBD-seq used in this study properly captured the methylated DNA. Supplementary Table 2 shows the details of the enrichment score for each sample. The pairwise correlation plots of the genome wide coverage profiles between all samples used in this study were summarized in Supplementary Figure 4. Same as the CpG coverage pattern, there was no distinct difference between high and low average daily gain groups. Uniquely mapped bases were categorized by regions of the genome (gene, CpGs, repeats and others) and regions of the gene (exon, intron, 5'-untranslated region [UTR], 3'-UTR, upstream-2k and downstream-2K). Figure 1 shows the proportion of uniquely mapped bases for each sample. Most of the bases were located in repeat regions (45.74%) and gene regions (31.77%). On average CpG island regions

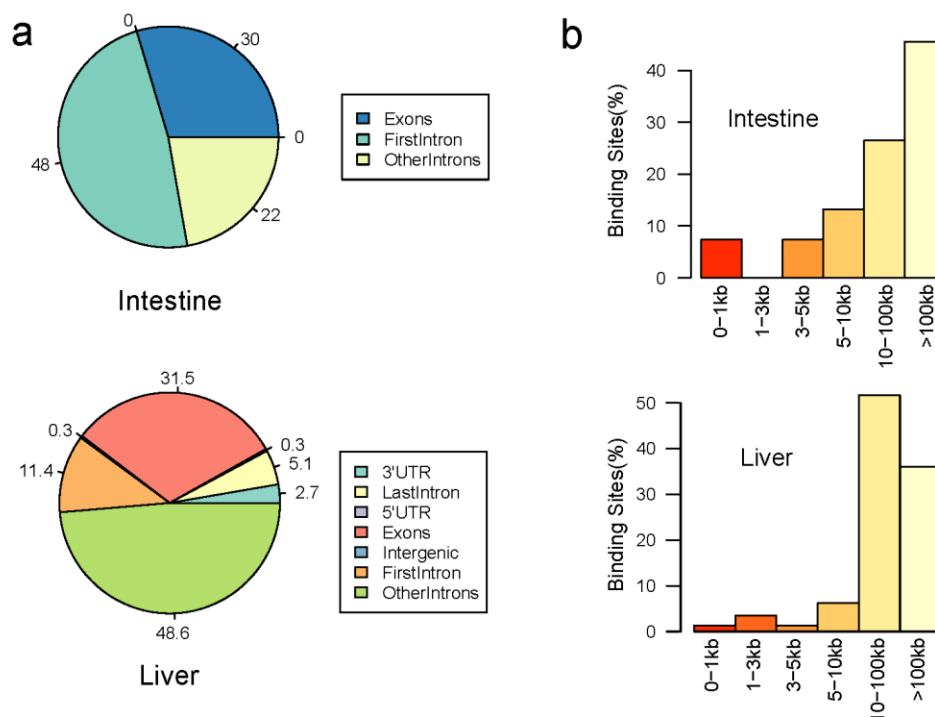
(2.54%) made up a smaller proportion in comparison to other regions. Within the gene region, the average proportion of intron region was 84.50%. Upstream\_2K region and downstream\_2K region of genes were 5.92% and 6.10%, respectively. The sum proportion of Exon and UTR region was 3.76% on average. Same as the coverage of CpGs using pie chart and histogram, there was no distinct difference in the proportion of genome-wide methylation profile based on the genomic regions between two groups. After binomial test using MEDIPs, the number of identified and merged DMR in high average daily gain group was 30 (5 down, 25 up) in the small intestine tissue and 258 (3 down, 255 up) in the liver tissue compared to the low average daily gain group.

#### Differentially methylated region annotation and pathway, network analysis

Annotation results of DMR using peak analyzer with



**Figure 1.** Genomic distribution of the uniquely mapped bases in two organ. (a) All uniquely mapped bases were classified into four type: bases uniquely mapped to genes (red), CpG Island (orange), repeats (light blue), others (blue). (b) Uniquely mapped bases in gene region were classified into 6 regions: bases uniquely mapped to 5'-UTR (red), 3'-UTR (orange), Exon (yellow), Intron (sky blue), Upstream\_2K (light blue), Downstream\_2K (Blue).



**Figure 2.** Annotation result of differentially methylated genes using Peak Analyzer. (a) Location and proportion of differentially methylated genes in two samples. (b) Distance distribution of nearest downstream genes from differentially methylated regions.

the Ensembl genome information is shown in (Figure 2). In the small intestine, 30% of DMR was located in exon region and 70% of DMR was located in intron region. In liver, 3%, 31.5%, and 64.5% of DMR were located in UTR, exon and intron region, respectively. Distribution of the distances to the nearest downstream gene from DMR showed that most of DMRs were more than 5 kb from the downstream genes. The list of genes that overlapped with

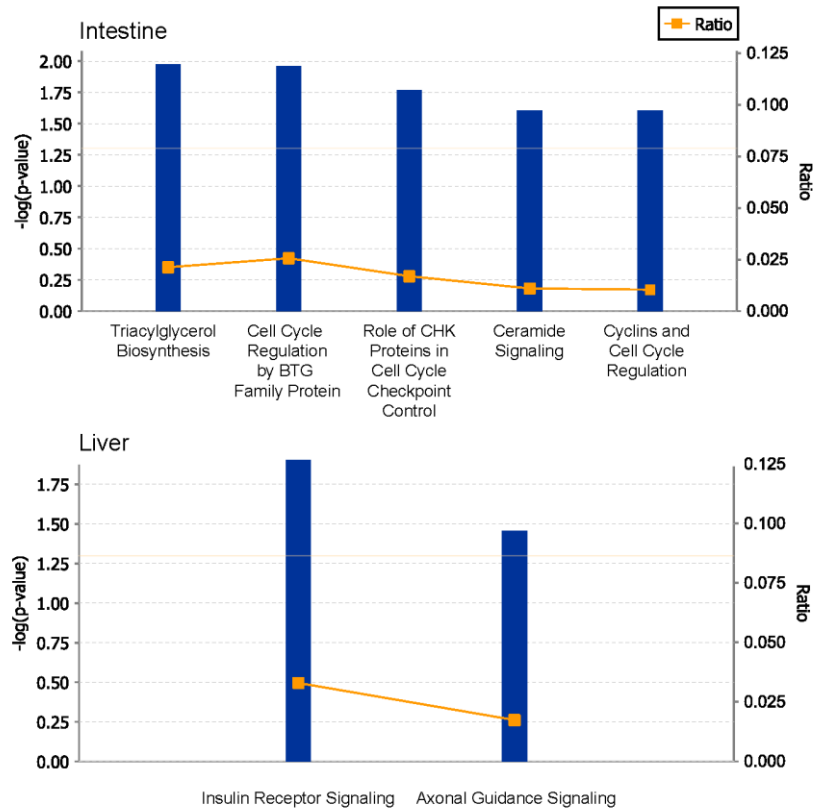
DMRs, which were used in pathway and network analysis, is summarized in Table 2 and the full differentially methylated genes are listed in supplementary Table 3. Genes of nearest downstream gene and nearest transcription starting site are described in the supplementary Table 4.

Figure 3 shows the enrichment canonical pathway related to growth and cell cycle using ingenuity pathway analysis in the small intestine and liver tissues. The first

**Table 2.** Differentially methylated genes list using MEDIPs and peak analyzer (FDR<0.05)

Organ	Ensembl gene ID	Gene symbol	Gene description
Small intestine	ENSSSCG00000012939	<i>BRMS1-201</i>	Breast cancer metastasis suppressor 1
	ENSSSCG00000013658	<i>S1PR2-201</i>	Sphingosine-1-phosphate receptor 2
	ENSSSCG00000026161	<i>E2F6-201</i>	E2F transcription factor 6
	ENSSSCG00000001025	<i>DSP-201</i>	Desmoplakin
	ENSSSCG00000014802	<i>NUMA1-201</i>	Nuclear mitotic apparatus protein 1
	ENSSSCG00000016912	<i>PPAP2A-201</i>	Phosphatidic acid phosphatase type 2A
Liver	ENSSSCG00000004012	<i>THBS2-201</i>	Thrombospondin 2
	ENSSSCG00000005719	<i>RAPGEF1-201</i>	Rap guanine nucleotide exchange factor (GEF) 1
	ENSSSCG00000007602	<i>BAIAP2L1-201</i>	BAI1-associated protein 2-like 1
	ENSSSCG00000008040	<i>TSC2-201</i>	Tuberous sclerosis 2
	ENSSSCG00000010140	<i>RTN4R-201</i>	Reticulon 4 receptor
	ENSSSCG00000010756	<i>MGMT-201</i>	O-6-methylguanine-DNA methyltransferase
	ENSSSCG00000011241	<i>DCLK3-201</i>	Doublecortin-like kinase 3
	ENSSSCG00000014811	<i>INPPL1-201</i>	Inositol polyphosphate phosphatase-like 1
	ENSSSCG00000023677	<i>CHMP1A-201</i>	Charged multivesicular body protein 1A
	ENSSSCG00000026412	<i>PLXNA2-201</i>	Plexin A2

MEDIPs, methylated DNA immunoprecipitation sequencing data analysis; FDR, false discovery rate.

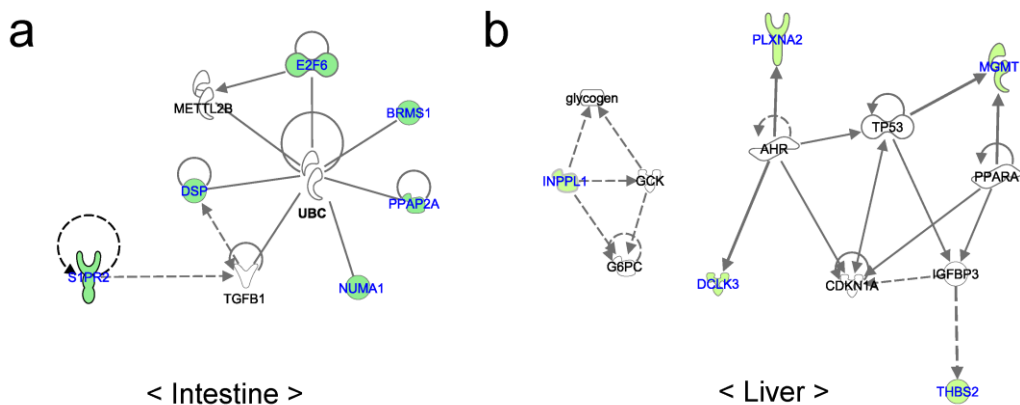


**Figure 3.** Enriched canonical pathways of differentially methylated genes using ingenuity pathway analysis (IPA). P-value of Fisher’s exact test for enriched pathway, ratio: proportion of genes in the pathway, gray line: cut off line for transformed p-value 0.05.

enriched canonical pathway of small intestine was the triacylglycerol biosynthesis pathway. And there were four enriched pathways related with cell cycle and cell growth (cell cycle regulation by B-cell translocation gene family protein, role of checkpoint kinase proteins in cell cycle checkpoint control, cyclins and cell cycle regulation, ceramide signaling). Phosphatidic acid phosphatase type 2A (*PPAP2A*), *E2F6*, and *SIPr2* genes were related to the enriched pathway. In liver, insulin receptor signaling and

axonal guidance signaling pathway were enriched. *RAPGEF1*, *TSC2*, *INPPL1*, *BAIAP2*, *PLXNA2*, *CHMP1A*, and *RTN4R* were related to the two pathway enriched in liver.

Figure 4 shows the networks of genes overlapped with DMR in the two organs. In the small intestine, all genes (*BRMS1*, *DSP*, *E2F6*, *NUMA1*, and *PPAP2A*) except *SIPR2* were directly connected with ubiquitin C to form a network concerned with cancer, cell to cell signaling and



**Figure 4.** Identified gene networks using differentially methylated genes. Networks of potentially interacting proteins were placed into node. Nodes with green color indicate the identified differentially methylated genes in this study.

interaction and cellular growth and proliferation. Seven genes (*DCLK3*, *MGMT*, *PLXNA2*, *THBS2*, *TSPAN4*, *INPPL1*, and *TSC2*) overlapping with DMR regions the liver were associated with four networks. Among these networks, two networks were related to cellular growth and carbohydrate metabolism.

## DISCUSSION

### Methylation profiles in small intestine and liver

This is the first study to compare the genome-wide methylation profile of two organ tissues (small intestine and liver) between two groups of weaning period piglets, which show differential performance in growth. Small intestine and liver are important organs for piglets that absorb and metabolize the nutrition in breast milk. As it is well known that DNA methylation of promoter region and gene body region can affect the gene expression via chromatin structure changes or transcription efficiency (Lorincz et al., 2004; Klose and Bird, 2006; Suzuki and Bird, 2008), the object of this study was to compare the genome-wide methylation pattern and identify methylated genes that could affect the growth rate of piglets. Bases of uniquely mapped reads in our study were enriched in the repeat and gene regions and this was similar to the results of a previous study on chickens (Li et al., 2011; Hu et al., 2013). However, the intron region comprised a large proportion of methylated genes (>80%) compared to the other analysis. There was no big difference in the DNA methylation pattern (CpGs coverage and genomic regions) between two groups using enriched read location. A small number of differentially methylated genes were identified in the small intestine sample and a higher number of genes were methylated in the liver compared to the small intestine. The small intestine sampled showed no DMR located in UTR and only a small proportion of DMR of the liver were located in UTR. The distances of DMRs from the nearest downstream genes and transcription starting site under 1 kb was a small proportion of the overall distribution. This showed that the different epigenetical regulation between two groups may come from chromatin structure change, not from repress regulation in promoter regions.

### Potential genes and pathways related to the piglet growth rate

Small intestine is the most important organ for piglet's absorption of nutrients. The breast milk which provide nutrients for the piglets has a high fat content. This fat in the breast milk is broken down by lipase and bile into monoacylglycerol and free fatty acid. In these forms, it is absorbed into enterocytes, which is then rebuilt into tryacylglycerol and secreted as chylomicron before being

used by the body. Enriched pathways and related genes in the small intestine were closely related to cell cycle regulation and lipid metabolism. Sphingosine-1-phosphate receptor 2 (*SIPR2*) is a member of G protein-coupled receptor, and it is related to the sphingosine-1-phosphate-induced cell proliferation (An et al., 2000; Adada et al., 2013). E2F transcription factor 6 (*E2F6*) is a member of transcription factors that play a crucial role in the cell cycle control (Trimarchi et al., 2001). The protein of nuclear mitotic apparatus protein 1 (*NUMA1*) gene interacts with microtubules and organizes the mitotic spindle during cell division (Purohit et al., 1999). These genes are thought to be associated with the formation of villi of newborn, and villi play an important role in absorption of nutrients by expanding the surface of small intestine. *PPAP2A* is an integral membrane glycoprotein that conducts hydrolysis and uptake of lipids from extracellular space (Roberts et al., 1998). Therefore, these differentially methylated genes and related pathways are associated with digestion and absorption of breast milk of which a major nutrient is fat. This different nutrient absorption is thus thought to be related with the different growth rate of piglet before weaning.

Pathway analysis of the liver, which plays a key role in lipid and energy metabolism, showed that insulin receptor signaling and axonal guidance signaling pathway were enriched. Differentially methylated genes (*INPPL1*, *PLXNA2*, *MGMT*, *DCLK3*, and *THBS2*) formed networks such as carbohydrate metabolism, cellular growth and proliferation. Inositol polyphosphate phosphatase-like 1 (*INPPL1*) encodes an SH2-containing 5'-inositol phosphatase that is associated with the regulation of insulin function, and the functions of the liver are primarily controlled by the hormone insulin (Saltiel and Kahn, 2001; Fritsche et al., 2008). Previous study using *INPPL1* knockout mouse showed that the absence of *INPPL1* confers the high resistance to the weight gain from high-fat diet (Sleeman et al., 2005). Hence, *INPPL1* and enriched pathways related with the differentially methylated genes are thought to be closely related to the weight gain and growth rate of piglets.

Even though further study is required to identify the epigenetic effect of these genes, results of analysis of pathways and networks on growth rate of piglets may provide some clues to the epigenetic mechanism of piglet growth rate.

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