Single-base-resolution methylome of giant panda's brain, liver and pancreatic tissue

Jianying Ren[1,*], Fujun Shen[2,*], Liang Zhang[2], Jie Sun[1], Miao Yang[1], Mingyu Yang[1], Rong Hou[2], Bisong Yue[1] and Xiuyue Zhang[1]

[1] Key Laboratory of Bio-resources and Eco-environment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu, China
[2] Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, Chengdu Research Base of Giant Panda Breeding, Chengdu, China
* These authors contributed equally to this work.

ABSTRACT

The giant panda (*Ailuropoda melanoleuca*) is one of the most endangered mammals, and its conservation has significant ecosystem and cultural service value. Cytosine DNA methylation (5mC) is a stable epigenetic modification to the genome and has multiple functions such as gene regulation. However, DNA methylome of giant panda and its function have not been reported as of yet. Bisulfite sequencing was performed on a 4-day-old male giant panda's brain, liver and pancreatic tissues. We found that the whole genome methylation level was about 0.05% based on reads normalization and mitochondrial DNA was not methylated. Three tissues showed similar methylation tendency in the protein-coding genes of their genomes, but the brain genome had a higher count of methylated genes. We obtained 467 and 1,013 different methylation regions (DMR) genes in brain vs. pancreas and liver, while only 260 DMR genes were obtained in liver vs pancreas. Some lncRNA were also DMR genes, indicating that methylation may affect biological processes by regulating other epigenetic factors. Gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis indicated that low methylated promoter, high methylated promoter and DMR genes were enriched at some important and tissue-specific items and pathways, like neurogenesis, metabolism and immunity. DNA methylation may drive or maintain tissue specificity and organic functions and it could be a crucial regulating factor for the development of newborn cubs. Our study offers the first insight into giant panda's DNA methylome, laying a foundation for further exploration of the giant panda's epigenetics.

INTRODUCTION

Epigenetics study heritable changes in gene expression that do not involve altering the DNA sequence (*Fazzari & Greally, 2004*). DNA methylation, which is one of the most studied epigenetic modifications, is common in animals, plants and even fungi. It has been proven to play important roles, like cell development, complex diseases, phenotype change and evolutionary process (*Martienssen & Colot, 2001*; *Ziller et al., 2013*). Basically, DNA methylation, which is the addition of a methyl group (CH3) covalently to cytosine, most

Corresponding author
Xiuyue Zhang,
zhangxiuyue@scu.edu.cn

commonly at the C5-methylcytosine (5mC), is transmitted with high fidelity over many cell generations through mitosis and transgenerational through meiosis (*Bock & Lengauer, 2008*; *Heard & Martienssen, 2014*). It is now recognized that DNA methylation, in concert with other regulators, is a major epigenetic factor influencing gene activities (*Gibney & Nolan, 2010*; *Jaenisch & Bird, 2003*).

Unique to Carnivora, giant pandas are specialized herbivores with an almost exclusive bamboo diet, but it retains a typical carnivore's digestive tract. Moreover, giant panda possesses elusive reproductive traits such as giving birth to most altricial neonate (*Jin et al., 2011*; *Li et al., 2010*; *Wei et al., 2015*). All these features have attracted the extensive attention of geneticists and evolutionary biologists. Considering the function of DNA methylation in rapid adaptation to changing environment and heritable nature, it may also be reflected in some biological processes of giant panda (*Baker-Andresen, Ratnu & Bredy, 2013*; *Dimond & Roberts, 2016*; *Heyn et al., 2013*; *Sevane, Martínez & Bruford, 2019*). Meanwhile, it has been demonstrated that epigenetic mechanisms regulate gene expression as potential candidates to confer tissue-specific gene expression and the epigenetic alterations are also increasingly implicated in metastasis (*Chatterjee et al., 2017*; *Mathers, Strathdee & Relton, 2010*). The absence of comprehensive genome-wide profiling and functional analysis of DNA methylation has hindered our understanding of epigenetic regulation of the giant panda.

Earlier analyses on DNA methylation often focused on promoter regions or CpG islands (CGI) through microarray and hence only partial DNA methylation status was observed (*Deaton & Bird, 2011*; *Jones, 2012*; *Martienssen & Colot, 2001*). Currently, several methods falling into three major assay types are utilized to study DNA methylation. Among these assays, whole-genome bisulfite sequencing is the gold standard (*Ziller et al., 2016*). Differentiated cells develop a stable and unique DNA methylation pattern that regulates tissue-specific gene transcription (*Moore, Le & Fan, 2013*). The pancreas and liver tissues are involved in digestion and metabolism, and the brain has obviously multi-functional features, as its regulatory function to multiple organs, including digestive organ (*D'Aldebert et al., 2009*; *Douris & Maratos–Flier, 2013*; *Myers & Olson, 2012*; *Peters et al., 2011*). In this study, we performed bisulfite sequencing (BS-seq) on the three tissues of a 4-day-old captive giant panda simultaneously which is a critical period in the development of a giant panda. We surveyed the methylation status at single-base scale, calculated methylation level of promoter and conducted pairwise comparison. Our goal was to obtain the tissue-specific influence of DNA methylation on the newborn cub of giant panda.

MATERIALS AND METHODS

Material and sequencing

Sample collection and utility protocols were approved by the Ethics Committee, College of Life Sciences, Sichuan University (Grant No: 20190506001). Our experimental procedures complied with the current laws on animal welfare and research in China. The brain, liver and pancreatic tissues were collected from a 4-day-old giant panda which was the progeny of "Aibang" (http://www.panda.org.cn/english/news/events/2014-08-20/4185.html). This newborn baby had been fed by his mother and subjected to 24-h onsite alternate care by

the base nursery staff since his birth. Nothing abnormal about the cub was detected in terms of body temperature, urination, defecation and breast milk intake in the first 4 days after his birth, and his weight has increased from 145 g at birth to 172 g. The giant panda cub died due to asphyxia and all visceral organs appeared to be normal (http://www.panda.org.cn/english/news/news/2016-05-12/5649.html) (*Peng et al., 2018*). All methods were performed in accordance with the relevant guidelines and regulations by professional veterinarians. The fresh tissue samples were immediately stored at −80 °C. Genomic DNA of the tissues was extracted following the Qiagen DNeasy Blood & Tissue Kit. DNA purity was checked using the Nano Photometer spectrophotometer (IMPLEN, Westlake Village, CA, USA). DNA concentration was measured using Qubit DNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA). Library construction and sequencing was performed using the same protocols employed by a previous study (*Xu et al., 2018*).

Raw reads QC and BS-seq data alignment

At first, the raw bisulfite-converted paired-end reads were quality checked using FastQC v.0.11.5 (Simon Andrews, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), adapter and low quality reads were trimmed by using Trim Galore! v.0.4.4 (Felix Krueger, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with the parameter -q 20 –length 20 and it was then rechecked using FastQC. Bismark v.0.18.2 was utilized to align the trimmed sequence reads to each of four bisulfite converted reference genomes, which were derived from the conversion of each strand and the corresponding complement using Bowtie2 v.2.3.2 with –N 1 –score-min L,0,-0.2 –ignore-quals –no-mixed –no-discordant –maxins 1000, with the other parameters set as default (*Krueger & Andrews, 2011*). After deduplication (Picard-tools v2.9.0-1-SNAPSHOT) and removing non-conversion bases, methylation was subsequently called for each covered cytosine and the summary statistics were calculated using the bismark_methylation_extractor script.

Differential methylation analysis

The detection of different methylation regions (DMR) is a prerequisite for characterizing different epigenetic status. DMRs were predicted using metilene in de-novo mode among sites with at least 10× coverage. The metilene software uses a binary segmentation algorithm combined with a two-dimensional statistical test to calculate the DMR. Whether in the framework of international consortia with dozens of samples per group, or even without biological replicates, it produces highly significant and reliable results (*Jühling et al., 2016*). We then used the metilene_output.pl script to filter the output file using the criterion of at least 10 CpGs within a DMR and q-value at 0.05.

Metaplot analysis

For metagene plot, gene body regions were divided proportionally with 20 bins. Upstream 2,000 bp or downstream 2,000 bp regions were divided into 20 bins (100 bp in each bin). The average methylation level of each bin was calculated for each gene and plotted by Python.

GO and KEGG analyses

Gene ontology (GO) enrichment analysis of DMR-overlapping genes were performed using the R package clusterProfiler (v3.6.0) (*Yu et al., 2012*). GO terms with *q*-value less than 0.05 were considered statistically significant. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DMR-overlapping genes uses KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/) (*Wu et al., 2006*).

All other calculations and picture plotting in this study used in-house Python (v2.7.13) or R (*R Core Team, 2018*) scripts.

RESULTS

DNA methylation whole-genome bisulfite sequencing, quality control and reads mapping

We performed whole genome BS-seq of brain, liver and pancreatic tissues obtained from the male giant panda. Approximately 988 million (64.5×) BS-seq raw reads were generated from brain, 1,079 million (70.4×) from liver and 703 million (45.9×) from pancreas (*Ziller et al., 2015*). The raw reads are available from the NCBI SRA browser (Bioproject accession number, PRJNA482275). The BS-seq conversion rate was 99.67%, 99.60% and 99.69%, respectively. Almost 99.99% of the raw reads passed the QC but with different read length.

Generally, DNA methylome studies are based on genome, hence, a better assembled genome and good annotation is important. To be prudent, we chose the NCBI Refseq edition (genome build accession: GCF_000004335.2), due to its annotation strategies and more extensive feature annotation, and the mapping efficiency was 50.4% (brain), 73.91% (liver) and 70.5% (pancreas) (*Li et al., 2010*). Vertebrate CGI are short interspersed DNA sequences that deviate significantly from the average genomic pattern by being CpG-rich and approximately 70% of annotated gene promoters are associated with a CGI (*Deaton & Bird, 2011*). We used newcpgplot, which is a part of EMBOSS toolkit (http://emboss.sourceforge.net/), to determine the genome's CpG island with default parameters and got 72,965 CGI. We chose 1,000 bp upstream of the first exon as promoter and at least one CpG island within it is called high CpG content (HCG), otherwise, it is a low CpG content (LCG). We got 11,431 HCG and 3,351 LCG—with only 58.6% being HCG (75% in human) (*Saxonov, Berg & Brutlag, 2006*).

5mC calling and whole genome methylation level analysis

The whole genome methylation level is about 0.05% according to reads normalization (Figs. S1–S3). In the three tissues, about 249, 398 and 247 million paired-end reads are uniquely mapped. There are 14, 21 and 13 billion cytosine analyzed in total with 76.2%, 68.7% and 70.4% cytosines methylated in the CpG context. Due to breakage of the genome DNA randomly while building the sequencing library, we checked the genome coverage of the reads to reveal the coverage of reads. The result showed that about 80% cytosine loci were at least 5× coverage (Figs. S4–S6). In total, 95.24%, 96.44% and 94.77% cytosine sites in the reference genome were covered by sequencing datasets. All these results reflect that the library construction and sequencing is pretty good. The CpG methylation distribution in tissues follows a bimodal distribution (Figs. S7 and S8). This

means that most CpGs are either fully methylated or fully unmethylated (*Akalin et al., 2012*).

There are 6,518 bp cytosines in the panda's mitochondrial DNA (mtDNA). As an example, in brain and pancreas, 6,410 and 6,428 cytosine loci are detected in the BS-data but the mC was rarely discovered even at the 751× and 773× on average, correspondingly. We can infer that the cytosine loci in the giant panda's mitochondrial genome are not methylated and might play any function just like in human beings (*Hong et al., 2013*). Nevertheless, it indicates good consistency with the non-conversion rate.

The proportion of reads that supported methylation of covering depth at a specific site was generally defined as the methyl cytosine methylation level. To better depict the whole genome methylation level, we constructed a genome, which has 21 pseudo chromosomes, through a concatenation operation of the 81,466 scaffolds (mitochondrial genome not included). We then calculated the methylation level of 1,000 bp window size and 1,000 bp step size to get the whole-genome scale view of the three tissues' methylome (Figs. S9–S11). The low methylation level in the end of pseudo chromosome 21 may be caused by too many short length scaffolds and unplaced contigs. At the whole-genome scale, most parts of the genome share common methylation status. Methylated cytosine always functions in a continuous way, therefore we calculated the methylation level in CpG, CHG and CHH context at 5× coverage separately within 10,000 bp window and the methylation level of cytosine in CHG, CHH context (H = A, T or C) is almost close to 0 (Fig. S12).

The methylated cytosines in different gene elements have different functions (*Arechederra et al., 2018*). We calculated the mean methylation level in promoters (we chose the upper 1,000 bp of the first exon as promoter according to other studies), exons, introns, genes and intergenic non-coding regions (IGN) (*Akalin et al., 2012*). The average methylation level of different elements is similar in the three tissues at 5× coverage. The promoter regions have the lowest methylation at 0.43, 0.39 and 0.39 respectively (Fig. 1A) (*Saxonov, Berg & Brutlag, 2006*). In addition to that, the methylation level in exons is slightly higher than that in intron and the IGN is a bit higher than in gene bodies. The methylation patterns of genic regions were similar to those of other species, showing increased CG methylation in gene bodies and flanking regions but reduced methylation in transcriptional start/end sites (*Jones, 2012*; *Lister et al., 2009*; *Zhang et al., 2017*). Meanwhile, CHH and CHG methylation was depleted in all regions (*Jones, 2012*). We also did the protein-coding genes' metaplot analysis to better reflect the DNA methylation tendency across the gene body, upstream and downstream. CG methylation is increased in gene bodies and flanking regions but reduced in gene body start/end sites (Fig. 1D). Furthermore, CpG methylation in brain protein-coding gene is a little higher than liver and pancreas just as the mean methylation analysis.

Low/High methylated promoter genes

DNA methylation in the promoter region is often associated with gene silencing (*Deaton & Bird, 2011*). We classified the promoter into low methylated promoter (LMP) and high methylated promoter (HMP), with these two categories using the mean methylation level 0.2 and 0.8 respectively (*Eckhardt et al., 2006*). Overall, the number of
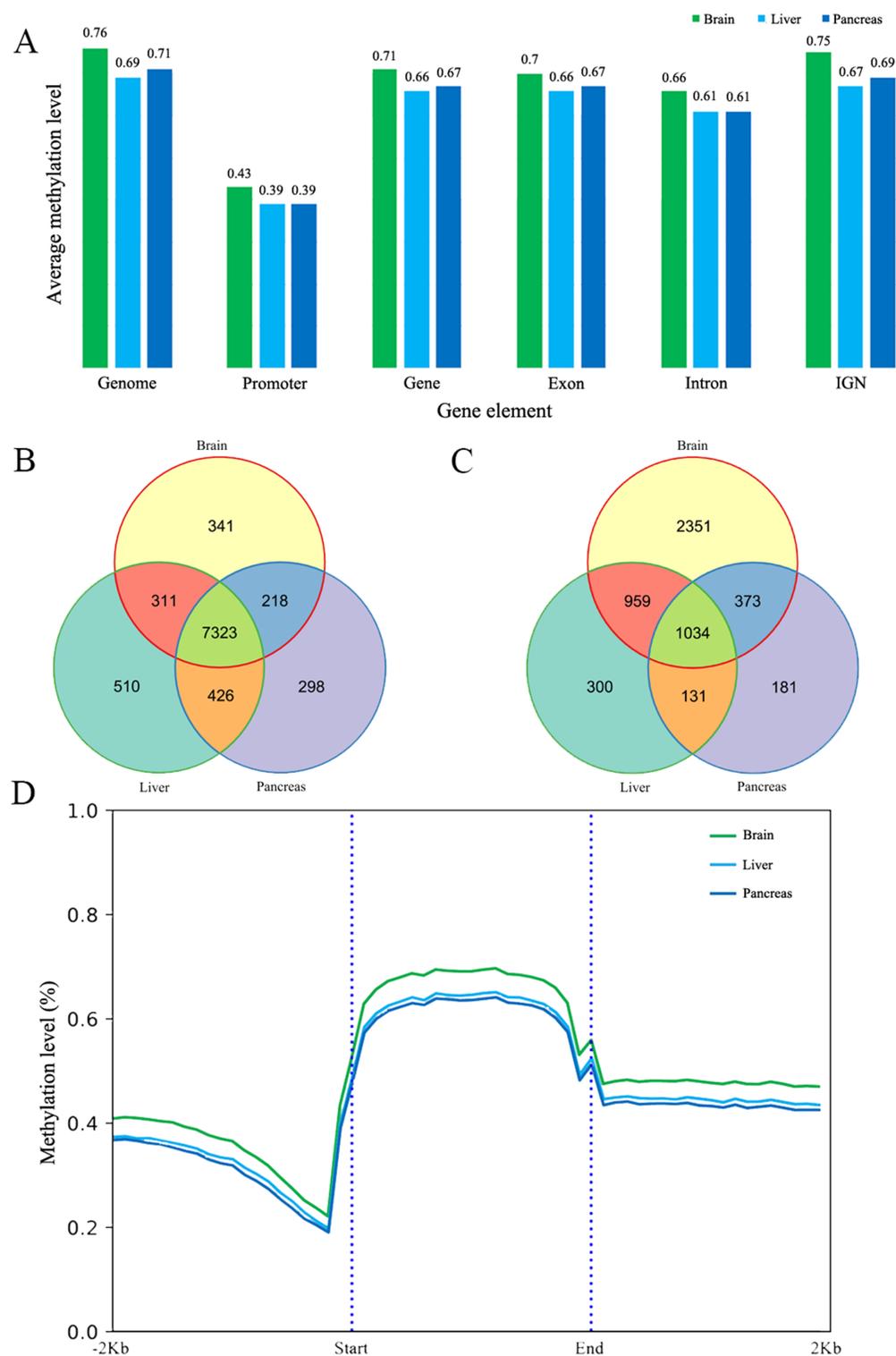
Figure 1 The DNA methylation level and patterns. (A) The mean methylation level in different gene elements including promoter, gene, exon, intron and intergenic region in three tissues; (B) and (C) The Venn diagram of low (B) and high (C) methylated promoter gene number of three tissues; (D) The methylation patterns diagram of protein-coding genes in brain, liver and pancreas. Start and end denotes the start and end locus of the protein-coding genes.

LMP genes is much more than HMP genes in the three tissues. It was found that the number of LMP is nearly equal in liver and pancreas whereas the brain possesses more methylated promoters than liver and pancreas (Figs. 1B and 1C). Based on the results of GO analysis, most of LMP genes' GO items are about housekeeping functions but differently distributed in the three tissues studied. LMP genes in brain are specifically distributed at Wnt-related signaling pathway, DNA replication, regulation of axon genesis, pallium development, ncRNA processing and intracellular protein transport. The LMP genes between liver and pancreas are differentially distributed in peptide biosynthetic process, RNA metabolic process, DNA metabolic, regulation of cell cycle and protein transport and targeting. Additionally, HMP genes in brain are enriched at inflammatory and defense response. Based on KEGG analysis results, LMP genes are differently distributed at melanogenesis, Wnt signaling pathway, glioma and thermogenesis in brain. It's worth pointing out that central melanin-concentrating hormone influences liver and adipose metabolism (Imbernon et al., 2013). The brain and pancreas have endocrine resistance and ErbB signaling pathway. Longevity regulation pathway is only distributed in liver. HMP genes in brain are enriched at cytokine-cytokine receptor interaction, platelet activation, JAK-STAT signaling pathway and Fc epsilon RI signaling pathway. However, in the liver it is enriched at necroptosis. Brain and pancreas both have JAK-STAT signaling pathway (Figs. S13 and S14). In combination with the tissues' functions, all these GO and KEGG results reflect that DNA methylation, no matter low or high, might lead to common and different tissue functions even at this stage.

Tissue-specific DMR profiling

Genes are methylated either within their promoters or within their transcribed regions and gene methylation is highly correlated with transcription levels. Moreover, the function of gene body methylation (GbM) seems bigger than what we previously thought (Arechederra et al., 2018). GbM genes often compose the bulk of methylated genes within angiosperm genomes and are enriched for housekeeping functions (Bewick & Schmitz, 2017). Consequently, to eliminate uncertainties, we inspected the DMRs not only in promoters but also in whole gene bodies and got the DMR overlapping genes within upstream (1,000 bp), gene bodies and downstream (1,000 bp) (Akalin et al., 2012). With 10× coverage, a p-adjust value cutoff of 0.05 and at least 10 CpGs contained, we got 1,018 DMRs (421 hyper and 597 hypo) in brain vs pancreas (see Supplement File). On the intersection of at least 50% length of DMR, we got 467 DMRs overlapping genes. There are 67 genes including more than one DMRs and GLI3 overlaps with six DMRs (Fig. 2A). Gene GLI3 encodes C2H2-type zinc finger proteins that are subclass of the Gli family. They are characterized as DNA-binding transcription factors and function as both a repressor and activator of transcription which is specifically involved in the development of pancreas (Nielsen et al., 2008). Additionally, we found that DMRs not only overlap mRNA but also lncRNA (22), and even a tRNA (Nielsen et al., 2008; Zhao, Sun & Wang, 2016). Furthermore, it's interesting that multi-DMRs also appear in lncRNA. Using the same criteria, we got 2,598 DMRs (755 hyper and 1,843 hypo) and 1,013 DMR genes in brain vs liver. There are 445 DMRs (371 hyper and 74 hypo) and 260 DMR genes in
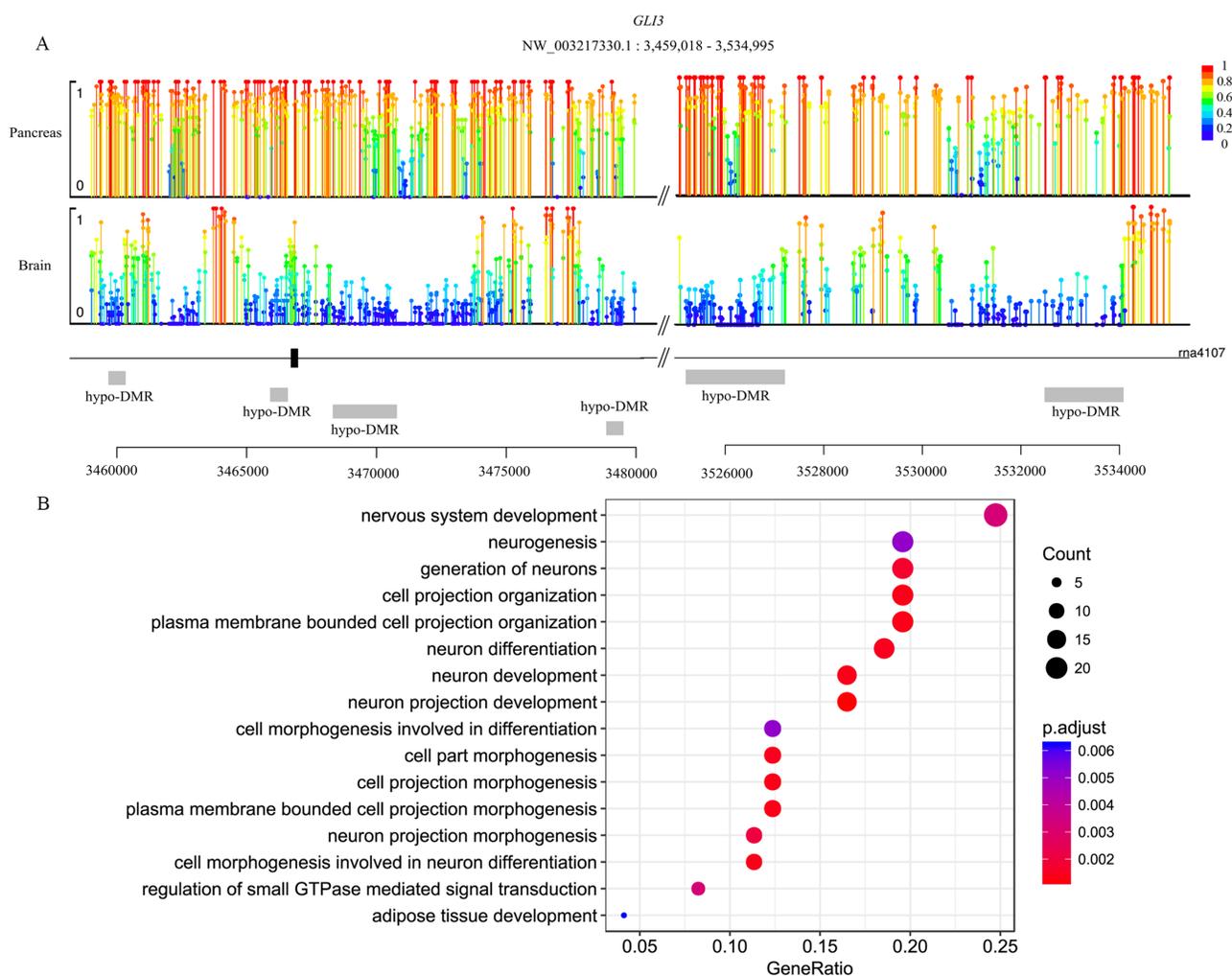
Figure 2 Lollipop plot of DNA methylation level in gene *GLI3* and DMR gene's GO enrichment result in brain and pancreas comparison. (A) There are six DMRs that are all hypo with different length in *GLI3* gene. Mean methylation level (red to deep blue color), DMR status and DMR relative location are all labeled; (B) DMR genes' GO enrichment result in the brain and pancreas comparison.

liver vs pancreas and the least DMR gene number probably indicates the tissue function similarity.

Functional analysis of DMR overlapping genes

In the comparison of brain and pancreas, 23 genes show functions related to binding, seven genes are involved in activation/coactivation, 12 genes function as transcription factors and 11 genes are zinc finger proteins (see Supplement File supp.tables.xlsx sheet 4). These regulatory genes may cause extensive and complex consequences under DNA methylation. Different tissues manifest different energy requirements. Although DNA methylation is absent in mtDNA, we found that 10 DMR genes have a functional relationship with mitochondria. In addition, we found that *HDAC1*, *HDAC4* and *HDAC9* are also DMR genes. They are all histone deacetylases and responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). This means

Table 1 Statistically significant enriched KEGG items of DMR genes in brain and pancreas comparison (Partial).

Term	ID	Input number	Background number	p-value
Hippo signaling pathway—multiple species	aml04392	5	29	0.000286698
cAMP signaling pathway	aml04024	12	197	0.000321699
Axon guidance	aml04360	11	173	0.000402346
Fatty acid biosynthesis	aml00061	3	12	0.002039626
Propanoate metabolism	aml00640	4	32	0.003433939
Hedgehog signaling pathway	aml04340	4	44	0.009636186
Glucagon signaling pathway	aml04922	6	99	0.010197961
Insulin signaling pathway	aml04910	7	138	0.013726705
Valine, leucine and isoleucine degradation	aml00280	4	51	0.015366193
Pancreatic secretion	aml04972	5	89	0.024286973
Pantothenate and CoA biosynthesis	aml00770	2	17	0.042526108

that DNA methylation may also have influences on histone (*Zhang & Reinberg, 2001*). Recent findings have suggested a more general role for the central nervous system in metabolic control (*Myers & Olson, 2012*). Neuronal systems can regulate energy intake, energy expenditure, and endogenous glucose production sense and respond to input from hormonal and nutrient-related signals (*Schwartz & Daniel, 2005*). Here, we found that some DMR genes are obesity genes or related to the former which have been studied by genomic and genome-wide association study (GWAS) methods, for example, *FTO, PCSK5, PCSK6, NTRK2, ABCC4, TXNIP* and *RPS6KA2* (*Al Muftah et al., 2016*; *D'Angelo & Koiffmann, 2012*; *Dina et al., 2007*; *Milagro et al., 2013*). The above mentioned genes are hypo-methylated in brain but hyper-methylated in liver and pancreas except *RPS6KA2* which is hyper-methylated in brain and pancreas while hypo-methylated in liver.

To gain further insight into the functional role of DMRs, we examined the enrichment of GO and KEGG pathways for the DMR-overlapping genes. In the comparison of brain and pancreas, these genes were significantly enriched in several developmental processes and signal transduction, including cell part morphogenesis (GO:0032990), adipose tissue development (GO:0060612), nervous system development (GO:0007399) and regulation of small GTPase mediated signal transduction (GO:0051056) (Fig. 2B). In KEGG pathway analysis, pathways that were identified are involved in many biological processes, like hormone regulation (aml04910, aml04922), metabolization (aml00061, aml00640), development (aml04392, aml04340) and brain functional pathways (aml04360, aml04530) (Table 1). Interestingly, the most significantly enriched pathway is the hippo signaling pathway (aml04392) which controls diverse aspects of cell proliferation, survival, and morphogenesis in eukaryotes and the core organization of these networks is conserved over a billion years of evolution. The cAMP signaling pathway is also significantly enriched. It's a well-known signal pathway which uses cAMP as second messenger, responds to hormones and stimulates pivotal physiologic progresses including metabolism, secretion, gene transcription etc. In a nutshell, DNA methylation

Table 2 Enriched KEGG items of DMR genes in brain and liver comparison (Partial).

Term	ID	Input number	Background number	p-value
PI3K-Akt signaling pathway	aml04151	31	331	1.62891939132e-05
Hippo signaling pathway	aml04390	17	150	0.000172197945661
cAMP signaling pathway	aml04024	17	197	0.002799888959
Axon guidance	aml04360	22	173	3.93969253235e-06
Fatty acid elongation	aml00062	4	23	0.0168841307934
Propanoate metabolism	aml00640	4	32	0.0436637692321
Hedgehog signaling pathway	aml04340	8	44	0.000626603087941
Glucagon signaling pathway	aml04922	10	99	0.00747421215857
Valine, leucine and isoleucine degradation	aml00280	7	51	0.00555857761228
Pantothenate and CoA biosynthesis	aml00770	5	17	0.0010940947287
Natural killer cell mediated cytotoxicity	aml04650	10	92	0.0047030370925
Fc gamma R-mediated phagocytosis	aml04666	10	82	0.00221616313219
Fc epsilon RI signaling pathway	aml04664	7	65	0.017469871196
B cell receptor signaling pathway	aml04662	6	71	0.0668349466587

exhibits influence on these key pathways to show a more extensive and complex function. Although the whole genome methylation level is only 0.05, it influences key pathways and can have an extensive range of functions.

The GO enrichment results of brain and liver comparison shows that there are some enriched items which are related to immunity, for example, T cell differentiation (GO:0030217), alpha-beta T cell activation (GO:0046631) and alpha-beta T cell differentiation (GO:0046632) (Fig. S15). This means that DNA methylation influences the immunity functions of liver. This situation is also reflected in the KEGG enrichment analysis, for example, B cell receptor signaling pathway (aml04662), natural killer (NK) cell mediated cytotoxicity (aml04650) and Fc gamma R-mediated phagocytosis (aml104666) (Table 2). Additionally, the PI3K-Akt signaling pathway (aml04151), which can be activated by many types of cellular stimuli or toxic insults, and regulates fundamental cellular functions such as transcription, growth and survival, is also significantly enriched. Furthermore, we also found that some DMR genes are lncRNAs. A recent study of 5,641 lncRNA transcripts in rhesus macaques found a tight association with epigenetic markers and CGIs (Chen et al., 2015). The relationship of DNA methylation and lncRNA may be worth a more detailed study. In addition to this, DNA methylation also has different influences on liver and pancreas and this is reflected in GO and KEGG annotation, such as some metabolic processes, leukocyte activation and migration, T cell selection, PI3K-Akt signaling pathway and cAMP signaling pathway (Fig. S16 and S17).

DISCUSSION

The various types of omics studies in the giant panda are helpful to understand the species at a system biological level and to unravel some biological mysteries—like the feed switch. The increasing number of recent studies have confirmed that epigenetics has a tight
relationship with gene expression (*Gibney & Nolan, 2010*; *Jaenisch & Bird, 2003*). Moreover, studies have shown that adult tissues exhibit highly distinct genome-wide DNA methylation signatures, hypermethylation is highly tissue-specific and located within genes that are involved in tissue-specific processes (*Byun et al., 2009*; *Rakyan et al., 2008*; *Slieker et al., 2015*; *Ziller et al., 2013*).

Some fatty acids are major components of neuronal tissues, directly involved in processes of neuronal differentiation and survival and are considered to be crucial for brain growth and development (*Glade & Smith, 2015*; *Salem et al., 2001*; *Zhang et al., 2016*). Adipose tissue development (GO:0060612) and fatty acid biosynthesis (aml00061) are also significantly enriched, and some key genes are hypomethylated, like *ACACA* and *ACSL1*. Moreover, *ACACB*, which may be involved in the regulation of fatty acid oxidation rather than fatty acid biosynthesis, is hyper-methylated. All these findings indicate that fatty acids are prone to biosynthesis under the influence of DNA methylation for brain development. Pancreas is an organ, having both an endocrine and a digestive exocrine function. Some tissue-specific functions are enriched, such as pancreatic secretion, glucagon and insulin signaling pathways and fatty acid biosynthesis (Table 1; Fig. S13). The various functions of the liver are carried out by hepatocytes, like carbohydrate, lipid, protein, bile acid and bilirubin metabolism. In the comparison of brain and liver, our results show that the DMR genes are enriched not only in metabolism but also in some immunity related pathways, like NK cell mediated cytotoxicity (aml04650), Fc gamma R-mediated phagocytosis (aml04666) and Fc epsilon RI signaling pathway (aml04664). NK cells are lymphocytes of the innate immune system and equipped with various receptors whose engagement allows them to discriminate between target and nontarget cells (*Cheent & Khakoo, 2009*). Activating receptors bind ligands on the target cell surface and trigger NK cell activation and target cell lysis. The *CD48* gene which encodes a immunoglobulin-like receptor in the CD2 subfamily and is found on the surface of lymphocytes and other immune cells like *ICAM2* (which mediates adhesive interactions), *TNFSF10* (which induces apoptosis) are all important for NK-cell mediated clearance. They are all hypomethylated in liver, but the *FYN* gene, which encodes a membrane-associated tyrosine kinase that regulates immune response, was hypermethylation in liver. Considering the fact that *FYN* plays a role in many biological processes like axon guidance (hypomethylation in brain), we suspect that there may exist some other mechanism to make a compensation for its seemingly abnormal high methylation (*Thude et al., 2015*). In addition, studies have shown that normal postnatal hepatic development and maturation involves extensive epigenetic remodeling of the genome, adapting to the marked changes in the nutritional environment (*Ehara et al., 2015*; *Cannon et al., 2016*). In a word, it is easy to get a conclusion that DNA methylation has a prevalent influence on tissue-specific and organic functions. Unique to Carnivora, giant pandas are specialized herbivores. After weaning, giant pandas also have a large change in diet. To meet this alteration, whether existing epigenetic reprogramming for these important organs involved in digestion and metabolism, such as liver and pancreas, needs further study.

The giant panda not only gives birth to unusually immature and most altricial neonates with the smallest neonate-maternal weight ratio (about 1/1,000) among eutherian mammals, but it also exhibits the shortest known gestation among Ursidae (*Zhu et al., 2001*). In humans, some data strongly show that preterm infants frequently exhibit some abnormal features such as suboptimal nutrient intake in early postnatal life, growth failure within the neonatal intensive care unit (NICU), higher levels of insulin resistance, abnormal partitioning of fat deposition, with the brain being exquisitely vulnerable to undernutrition and later cognitive attainment being permanently affected by suboptimal nutrient intakes (*Embleton, 2013*). Therefore, the rapid development and large requirement of nutrition at the early postnatal stage might be vital for premature newborn cubs (*Zhu et al., 2001*). A prolonged transition time between colostrum and mature milk for the supply of immune protection and nutrition might be a compensatory strategy for unusually immature neonates (*Griffiths et al., 2015*). However, the rapid establishment of the neonates' own physiological, metabolic and defensive systems should be a better guarantee to survive this critical period. For inducing these establishments, in the external environmental side, the miRNAs related to basic metabolism, neuron development and immunity were found to be horizontally transferred to the cubs from the giant panda's breast milk (*Ma et al., 2017*). At individual itself side, based on the result of GO and KEGG analyses, these LMP genes have more items and their functions are mainly involved in basic metabolism and development, like cell cycle, DNA replication, translation, RNA transport, metabolic processes etc. According to the canonical view about the negative relationship of gene expression and DNA methylation, these LMP genes might be associated with the active expression of these genes and were also important for the development of the preterm newborn cubs. On the other hand, HMP genes have a smaller number of GO or KEGG items. All these results imply that the DNA methylation may be a crucial regulator for the development of giant panda newborn cubs.

CONCLUSION

We have carried out, to our knowledge, the first DNA methylation study of giant panda. LMP genes might be associated with the active expression of these genes for organic functions and were important for the development of the delicate panda cubs. There were 1,018, 2,598 and 445 tissue specific DMRs discovered through three tissues' pairwise comparison and it seems that similar functional tissues have the least number DMRs. GO and KEGG analyses results demonstrated that DNA methylation has a relationship with tissue-specific functions or biological processes and may be a crucial regulator for the development of giant panda newborn cubs. As the first step to elucidate the biological role of DNA methylation, this study reports the characteristics of DNA methylation of key growth stages and supplies important information for further insight into the regulatory functions of DNA methylation in the giant panda.

ACKNOWLEDGEMENTS

We thank Jake George James and Dr. Megan Price for revising our manuscript, Jinnan Ma, Dr. Chaochao Yan and Dr. Zhenxin Fan for their suggestions.

Funding

This research was supported by the National Natural Science Foundation of China (No. 31770574 and No. 31570534). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
National Natural Science Foundation of China: 31770574 and 31570534.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Jianying Ren performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Fujun Shen performed the experiments, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Liang Zhang performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Jie Sun analyzed the data, prepared figures and/or tables, approved the final draft.
- Miao Yang analyzed the data, prepared figures and/or tables, approved the final draft.
- Mingyu Yang analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, approved the final draft.
- Rong Hou conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Bisong Yue conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Xiuyue Zhang conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

This research was approved by the Ethics Committee, College of Life Sciences, Sichuan University (Grant No: 20190506001).

Data Availability

The raw NGS data is available at NCBI Bioproject under accession number PRJNA482275.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7847#supplemental-information.

REFERENCES

Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, Mason CE. 2012. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biology* **13(10)**:R87 DOI 10.1186/gb-2012-13-10-r87.

Al Muftah WA, Al-Shafai M, Zaghlool SB, Visconti A, Tsai P-C, Kumar P, Spector T, Bell J, Falchi M, Suhre K. 2016. Epigenetic associations of type 2 diabetes and BMI in an Arab population. *Clinical Epigenetics* **8(1)**:13 DOI 10.1186/s13148-016-0177-6.

Arechederra M, Daian F, Yim A, Bazai SK, Richelme S, Dono R, Saurin AJ, Habermann BH, Maina F. 2018. Hypermethylation of gene body CpG islands predicts high dosage of functional oncogenes in liver cancer. *Nature Communications* **9(1)**:3164 DOI 10.1038/s41467-018-05550-5.

Baker-Andresen D, Ratnu VS, Bredy TW. 2013. Dynamic DNA methylation: a prime candidate for genomic metaplasticity and behavioral adaptation. *Trends in Neurosciences* **36(1)**:3–13 DOI 10.1016/j.tins.2012.09.003.

Bewick AJ, Schmitz RJ. 2017. Gene body DNA methylation in plants. *Current Opinion in Plant Biology* **36**:103–110 DOI 10.1016/j.pbi.2016.12.007.

Bock C, Lengauer T. 2008. Computational epigenetics. *Bioinformatics* **24(1)**:1–10 DOI 10.1093/bioinformatics/btm546.

Byun H-M, Siegmund KD, Pan F, Weisenberger DJ, Kanel G, Laird PW, Yang AS. 2009. Epigenetic profiling of somatic tissues from human autopsy specimens identifies tissue- and individual-specific DNA methylation patterns. *Human Molecular Genetics* **18(24)**:4808–4817 DOI 10.1093/hmg/ddp445.

Cannon MV, Pilarowski G, Liu X, Serre D. 2016. Extensive epigenetic changes accompany terminal differentiation of mouse hepatocytes after birth. *G3: Genes|Genomes|Genetics* **6(11)**:3701–3709 DOI 10.1534/g3.116.034785.

Chatterjee A, Stockwell PA, Ahn A, Rodger EJ, Leichter AL, Eccles MR. 2017. Genome-wide methylation sequencing of paired primary and metastatic cell lines identifies common DNA methylation changes and a role for EBF3 as a candidate epigenetic driver of melanoma metastasis. *Oncotarget* **8(4)**:6085–6101 DOI 10.18632/oncotarget.14042.

Cheent K, Khakoo SI. 2009. Natural killer cells: integrating diversity with function. *Immunology* **126(4)**:449–457 DOI 10.1111/j.1365-2567.2009.03045.x.

Chen J-Y, Shen QS, Zhou W-Z, Peng J, He BZ, Li Y, Liu C-J, Luan X, Ding W, Li S, Chen C, Tan BC-M, Zhang YE, He A, Li C-Y. 2015. Emergence, retention and selection: a trilogy of origination for functional de novo proteins from ancestral LncRNAs in primates. *PLOS Genetics* **11(7)**:e1005391 DOI 10.1371/journal.pgen.1005391.

D'Aldebert E, Biyeyeme Bi Mve M-J, Mergey M, Wendum D, Firrincieli D, Coilly A, Fouassier L, Corpechot C, Poupon R, Housset C, Chignard N. 2009. Bile salts control the antimicrobial peptide cathelicidin through nuclear receptors in the human biliary epithelium. *Gastroenterology* **136(4)**:1435–1443 DOI 10.1053/j.gastro.2008.12.040.

D'Angelo CS, Koiffmann CP. 2012. Copy number variants in obesity-related syndromes: review and perspectives on novel molecular approaches. *Journal of Obesity* **2012**:845480 DOI 10.1155/2012/845480.

Deaton AM, Bird A. 2011. CpG islands and the regulation of transcription. *Genes & Development* **25(10)**:1010–1022 DOI 10.1101/gad.2037511.

Dimond JL, Roberts SB. 2016. Germline DNA methylation in reef corals: patterns and potential roles in response to environmental change. *Molecular Ecology* **25(8)**:1895–1904 DOI 10.1111/mec.13414.

Dina C, Meyre D, Gallina S, Durand E, Körner A, Jacobson P, Carlsson LMS, Kiess W, Vatin V, Lecoeur C, Delplanque J, Vaillant E, Pattou F, Ruiz J, Weill J, Levy-Marchal C, Horber F, Potoczna N, Hercberg S, Le Stunff C, Bougnères P, Kovacs P, Marre M, Balkau B, Cauchi S, Chèvre J-C, Froguel P. 2007. Variation in FTO contributes to childhood obesity and severe adult obesity. *Nature Genetics* **39(6)**:724–726 DOI 10.1038/ng2048.

Douris N, Maratos–Flier E. 2013. Two paths diverge in the brain: melanin-concentrating hormone controls hepatic and adipose metabolism. *Gastroenterology* **144(3)**:501–504 DOI 10.1053/j.gastro.2013.01.029.

Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S. 2006. DNA methylation profiling of human chromosomes 6, 20 and 22. *Nature Genetics* **38(12)**:1378–1385 DOI 10.1038/ng1909.

Ehara T, Kamei Y, Yuan X, Takahashi M, Kanai S, Tamura E, Tsujimoto K, Tamiya T, Nakagawa Y, Shimano H, Takai-Igarashi T, Hatada I, Suganami T, Hashimoto K, Ogawa Y. 2015. Ligand-activated PPARα-dependent DNA demethylation regulates the fatty acid β-oxidation genes in the postnatal liver. *Diabetes* **64(3)**:775–784 DOI 10.2337/db14-0158.

Embleton ND. 2013. Early nutrition and later outcomes in preterm infants. In: Shamir R, Turck D, Phillip M, eds. *Nutrition and Growth*. Basel: Karger Publishers, 26–32.

Fazzari MJ, Greally JM. 2004. Epigenomics: beyond CpG islands. *Nature Reviews Genetics* **5(6)**:446–455 DOI 10.1038/nrg1349.

Gibney ER, Nolan CM. 2010. Epigenetics and gene expression. *Heredity* **105(1)**:4–13 DOI 10.1038/hdy.2010.54.

Glade MJ, Smith K. 2015. Phosphatidylserine and the human brain. *Nutrition* **31(6)**:781–786 DOI 10.1016/j.nut.2014.10.014.

Griffiths K, Hou R, Wang H, Zhang Z, Zhang L, Zhang T, Watson DG, Burchmore RJS, Loeffler IK, Kennedy MW. 2015. Prolonged transition time between colostrum and mature milk in a bear, the giant panda, *Ailuropoda melanoleuca*. *Royal Society Open Science* **2(10)**:150395 DOI 10.1098/rsos.150395.

Heard E, Martienssen RA. 2014. Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* **157(1)**:95–109 DOI 10.1016/j.cell.2014.02.045.

Heyn H, Moran S, Hernando-Herraez I, Sayols S, Gomez A, Sandoval J, Monk D, Hata K, Marques-Bonet T, Wang L, Esteller M. 2013. DNA methylation contributes to natural human variation. *Genome Research* **23(9)**:1363–1372 DOI 10.1101/gr.154187.112.

Hong EE, Okitsu CY, Smith AD, Hsieh C-L. 2013. Regionally specific and genome-wide analyses conclusively demonstrate the absence of CpG methylation in human mitochondrial DNA. *Molecular and Cellular Biology* **33(14)**:2683–2690 DOI 10.1128/MCB.00220-13.

Imbernon M, Beiroa D, Vázquez MJ, Morgan DA, Veyrat-Durebex C, Porteiro B, Díaz-Arteaga A, Senra A, Busquets S, Velásquez DA, Al-Massadi O, Varela L, Gándara M, López-Soriano F-J, Gallego R, Seoane LM, Argiles JM, López M, Davis RJ, Sabio G, Rohner-Jeanrenaud F, Rahmouni K, Dieguez C, Nogueiras R. 2013. Central melanin-concentrating hormone influences liver and adipose metabolism via specific hypothalamic

nuclei and efferent autonomic/JNK1 pathways. *Gastroenterology* **144(3)**:636–649.e6 DOI 10.1053/j.gastro.2012.10.051.

Jaenisch R, Bird A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics* **33(S3)**:245–254 DOI 10.1038/ng1089.

Jin K, Xue C, Wu X, Qian J, Zhu Y, Yang Z, Yonezawa T, Crabbe MJC, Cao Y, Hasegawa M, Zhong Y, Zheng Y. 2011. Why does the giant panda eat bamboo? A comparative analysis of appetite-reward-related genes among mammals. *PLOS ONE* **6(7)**:e22602 DOI 10.1371/journal.pone.0022602.

Jones PA. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics* **13(7)**:484–492 DOI 10.1038/nrg3230.

Jühling F, Kretzmer H, Bernhart SH, Otto C, Stadler PF, Hoffmann S. 2016. metilene: fast and sensitive calling of differentially methylated regions from bisulfite sequencing data. *Genome Research* **26(2)**:256–262 DOI 10.1101/gr.196394.115.

Krueger F, Andrews SR. 2011. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* **27(11)**:1571–1572 DOI 10.1093/bioinformatics/btr167.

Li R, Fan W, Tian G, Zhu H, He L, Cai J, Huang Q, Cai Q, Li B, Bai Y, Zhang Z, Zhang Y, Wang W, Li J, Wei F, Li H, Jian M, Li J, Zhang Z, Nielsen R, Li D, Gu W, Yang Z, Xuan Z, Ryder OA, Leung FC-C, Zhou Y, Cao J, Sun X, Fu Y, Fang X, Guo X, Wang B, Hou R, Shen F, Mu B, Ni P, Lin R, Qian W, Wang G, Yu C, Nie W, Wang J, Wu Z, Liang H, Min J, Wu Q, Cheng S, Ruan J, Wang M, Shi Z, Wen M, Liu B, Ren X, Zheng H, Dong D, Cook K, Shan G, Zhang H, Kosiol C, Xie X, Lu Z, Zheng H, Li Y, Steiner CC, Lam TT-Y, Lin S, Zhang Q, Li G, Tian J, Gong T, Liu H, Zhang D, Fang L, Ye C, Zhang J, Hu W, Xu A, Ren Y, Zhang G, Bruford MW, Li Q, Ma L, Guo Y, An N, Hu Y, Zheng Y, Shi Y, Li Z, Liu Q, Chen Y, Zhao J, Qu N, Zhao S, Tian F, Wang X, Wang H, Xu L, Liu X, Vinar T, Wang Y, Lam T-W, Yiu S-M, Liu S, Zhang H, Li D, Huang Y, Wang X, Yang G, Jiang Z, Wang J, Qin N, Li L, Li J, Bolund L, Kristiansen K, Wong GK-S, Olson M, Zhang X, Li S, Yang H, Wang J, Wang J. 2010. The sequence and de novo assembly of the giant panda genome. *Nature* **463(7279)**:311–317 DOI 10.1038/nature08696.

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo Q-M, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR. 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* **462(7271)**:315–322 DOI 10.1038/nature08514.

Ma J, Wang C, Long K, Zhang H, Zhang J, Jin L, Tang Q, Jiang A, Wang X, Tian S, Chen L, He D, Li D, Huang S, Jiang Z, Li M. 2017. Exosomal microRNAs in giant panda (*Ailuropoda melanoleuca*) breast milk: potential maternal regulators for the development of newborn cubs. *Scientific Reports* **7(1)**:3507 DOI 10.1038/s41598-017-03707-8.

Martienssen RA, Colot V. 2001. DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science* **293(5532)**:1070–1074 DOI 10.1126/science.293.5532.1070.

Mathers JC, Strathdee G, Relton CL. 2010. Induction of epigenetic alterations by dietary and other environmental factors. *Advances in Genetics* **71**:3–39 DOI 10.1016/B978-0-12-380864-6.00001-8.

Milagro FI, Mansego ML, De Miguel C, Martínez JA. 2013. Dietary factors, epigenetic modifications and obesity outcomes: progresses and perspectives. *Molecular Aspects of Medicine* **34(4)**:782–812 DOI 10.1016/j.mam.2012.06.010.

Moore LD, Le T, Fan G. 2013. DNA methylation and its basic function. *Neuropsychopharmacology* **38(1)**:23–38 DOI 10.1038/npp.2012.112.

Myers MG, Olson DP. 2012. Central nervous system control of metabolism. *Nature* **491(7424)**:357–363 DOI 10.1038/nature11705.

Nielsen SK, Møllgård K, Clement CA, Veland IR, Awan A, Yoder BK, Novak I, Christensen ST. 2008. Characterization of primary cilia and Hedgehog signaling during development of the human pancreas and in human pancreatic duct cancer cell lines. *Developmental Dynamics* **237(8)**:2039–2052 DOI 10.1002/dvdy.21610.

Peng R, Liu Y, Cai Z, Shen F, Chen J, Hou R, Zou F. 2018. Characterization and analysis of whole transcriptome of giant panda spleens: implying critical roles of long non-coding RNAs in immunity. *Cellular Physiology and Biochemistry* **46(3)**:1065–1077 DOI 10.1159/000488837.

Peters A, Kubera B, Hubold C, Langemann D. 2011. The selfish brain: stress and eating behavior. *Frontiers in Neuroscience* **5**:74 DOI 10.3389/fnins.2011.00074.

R Core Team. 2018. *R: a language and environment for statistical computing.* Version 3.4.4. Vienna: R Foundation for Statistical Computing. *Available at https://www.R-project.org/*.

Rakyan VK, Down TA, Thorne NP, Flicek P, Kulesha E, Gräf S, Tomazou EM, Bäckdahl L, Johnson N, Herberth M, Howe KL, Jackson DK, Miretti MM, Fiegler H, Marioni JC, Birney E, Hubbard TJP, Carter NP, Tavaré S, Beck S. 2008. An integrated resource for genome-wide identification and analysis of human tissue-specific differentially methylated regions (tDMRs). *Genome Research* **18(9)**:1518–1529 DOI 10.1101/gr.077479.108.

Salem N Jr, Litman B, Kim H-Y, Gawrisch K. 2001. Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* **36(9)**:945–959 DOI 10.1007/s11745-001-0805-6.

Saxonov S, Berg P, Brutlag DL. 2006. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proceedings of the National Academy of Sciences of the United States of America* **103(5)**:1412–1417 DOI 10.1073/pnas.0510310103.

Schwartz MW, Daniel P Jr. 2005. Diabetes, obesity, and the brain. *Science* **307(5708)**:375–379 DOI 10.1126/science.1104344.

Sevane N, Martínez R, Bruford MW. 2019. Genome-wide differential DNA methylation in tropically adapted Creole cattle and their Iberian ancestors. *Animal Genetics* **50(1)**:15–26 DOI 10.1111/age.12731.

Slieker RC, Roost MS, Van Iperen L, Suchiman HED, Tobi EW, Carlotti F, De Koning EJP, Slagboom PE, Heijmans BT, Chuva De Sousa Lopes SM. 2015. DNA methylation landscapes of human fetal development. *PLOS Genetics* **11(10)**:e1005583 DOI 10.1371/journal.pgen.1005583.

Thude H, Kramer K, Peine S, Sterneck M, Nashan B, Koch M. 2015. Role of the Fyn −93A>G polymorphism (rs706895) in acute rejection after liver transplantation. *Human Immunology* **76(9)**:657–662 DOI 10.1016/j.humimm.2015.09.013.

Wei F, Hu Y, Yan L, Nie Y, Wu Q, Zhang Z. 2015. Giant pandas are not an evolutionary cul-de-sac: evidence from multidisciplinary research. *Molecular Biology and Evolution* **32(1)**:4–12 DOI 10.1093/molbev/msu278.

Wu J, Mao X, Cai T, Luo J, Wei L. 2006. KOBAS server: a web-based platform for automated annotation and pathway identification. *Nucleic Acids Research* **34(Web Server)**:W720–W724 DOI 10.1093/nar/gkl167.

Xu J, Zhou S, Gong X, Song Y, Van Nocker S, Ma F, Guan Q. 2018. Single-base methylome analysis reveals dynamic epigenomic differences associated with water deficit in apple. *Plant Biotechnology Journal* **16(2)**:672–687 DOI 10.1111/pbi.12820.

Yu G, Wang L-G, Han Y, He Q-Y. 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS: A Journal of Integrative Biology* **16(5)**:284–287 DOI 10.1089/omi.2011.0118.

Zhang Y, Li F, Feng X, Yang H, Zhu A, Pang J, Han L, Zhang T, Yao X, Wang F. 2017. Genome-wide analysis of DNA Methylation profiles on sheep ovaries associated with prolificacy using whole-genome Bisulfite sequencing. *BMC Genomics* **18(1)**:759 DOI 10.1186/s12864-017-4068-9.

Zhang Y, Reinberg D. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes & Development* **15(18)**:2343–2360 DOI 10.1101/gad.927301.

Zhang T, Watson DG, Zhang R, Hou R, Loeffler IK, Kennedy MW. 2016. Changeover from signalling to energy-provisioning lipids during transition from colostrum to mature milk in the giant panda (*Ailuropoda melanoleuca*). *Scientific Reports* **6(1)**:36141 DOI 10.1038/srep36141.

Zhao Y, Sun H, Wang H. 2016. Long noncoding RNAs in DNA methylation: new players stepping into the old game. *Cell & Bioscience* **6(1)**:45 DOI 10.1186/s13578-016-0109-3.

Zhu X, Lindburg DG, Pan W, Forney KA, Wang D. 2001. The reproductive strategy of giant pandas (*Ailuropoda melanoleuca*): infant growth and development and mother–infant relationships. *Journal of Zoology* **253(2)**:141–155 DOI 10.1017/S0952836901000139.

Ziller MJ, Gu H, Müller F, Donaghey J, Tsai LTY, Kohlbacher O, Jager PL, Rosen ED, Bennett DA, Bernstein BE, Gnirke A, Meissner A. 2013. Charting a dynamic DNA methylation landscape of the human genome. *Nature* **500(7463)**:477–481 DOI 10.1038/nature12433.

Ziller MJ, Hansen KD, Meissner A, Aryee MJ. 2015. Coverage recommendations for methylation analysis by whole-genome bisulfite sequencing. *Nature methods* **12(3)**:230–232 DOI 10.1038/nmeth.3152.

Ziller MJ, Stamenova EK, Gu H, Gnirke A, Meissner A. 2016. Targeted bisulfite sequencing of the dynamic DNA methylome. *Epigenetics & Chromatin* **9(1)**:55 DOI 10.1186/s13072-016-0105-1.