Profile genome-wide DNA methylation of human granulosa cells in primary ovarian insufficiency

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Abstract

Background

Premature ovarian failure which leads to loss of fertility and low level of estrogen have serlously affected women's physical and mental health, especially, women of child-bearing age. The etiology remains unclear. DNA methylation, as a key epigenetic modification might play an importan role in primary ovarian insufficiency (POI). We explore the whole DNA methylation of POI by whole genome bisulfite sequencing (WGBS), and find the pattern of DNA methylation in POI and analyze the differentially methylated regions (DMRs) genes related POI.

Methods

According to clinical and experimental standards, follicular fluid samples were collected from three women with POI and three women with normal ovarian reserve (NOR). Ovarian granulosa cells were purified from each follicular fluid sample. Whole genomic DNA methylation sequencing of the granulosa cells was performed by WGBS.

Results

We obtained whole-genome methylation maps of human granulosa cells. The average percentages of methylated cytosines in CG contexts exceeded 99%, in CHG and CHH contexts was less than 1%. The average methylation level in CG was about 80%, in CHG and CHH was less than 0.4%. there was no significant difference in CpG methylation level between NOR and POI. But there were DMRs between two groups. Distribution of DMRs was enriched in promoter and exon. Differentially methylated genes were used to perform Gene ontology(GO) analysis and enriched in GO items of cellular process, biological regulation, metabolic process, developmental process,binding and catalytic activities.

Conclusion

DNA methylation plays a critical role in POI and serve as resources for further investigation in the future.

Background

Primary ovarian insufficiency (POI) is defined as a significant reduction in the primordial follicle pool of oocytes that is intrinsic to the ovary, and induction of amenorrhea prior to age 40[1]. This is associated with decreased estrogen and hypergonadotropic levels in the blood. POI affects 0.1% of women under the age of 35 years and 1% of women under the age of 40 years [2]. POI generates two types of consequence. One is premature hypoestrogenism, which in turn causes the premature aging of several tissues, for example, osteoporosis, cardiovascular diseases, or neurodegenerative diseases. The second

consequence is infertility. Fertility is markedly decreased though infertility of POI isn't absolute. The etiology of POI which is highly heterogeneous includes genetic, endocrine, autoimmune, metabolic, infectious, and iatrogenic factors [3].But about 90% of POI is considered idiopathic without known cause[4]. This had seriously hindered the clinical application in POI. The exact aetiology of POI still needs to be explored.

Epigenietics is defined as 'molecular factors and processes around the DNA that regulate genome activity independent of DNA sequence, and that are mitotically stable' [5]. Epigenetic changes include DNA methylation, histone modifications, expression of noncoding RNA (ncRNA), RNA methylation, and alterations in chromatin structure [6]. They can be induced by environmental factors such as nutrition, toxicants, ionizing radiation, and so on. Observations had shown that environmental exposure is a key factor in ovarian disease [7]. So epigenetics, in contrast to genetics, can regulate gene expression in response to enviromental stimulation. DNA methylation is known to an epigenetic modification that play an important role in controlling gene expression during mammalian development.

POI is diminished ovarian function, which is generally attributed to accelerated depletion of oocytes and their surrounding granulosa cells [8]. Granulosa cell can afford growth factors, nutrients and survival factors to oocytes. Granulosa cells have been demonstrated to play a major role in fate of follicles, such as follicular growth and follicular atresia [9, 10]. The granulosa cells also protects oocytes from oxidative stress damage in vitro[11]. There were potential regulators and markers in granulosa cells which are involved in oocyte maturation[12]. So granulosa cells can provide important information about the development and maturation of oocytes though granulosa cells are discarded in the process of human assisted reproductive technology. Meanwhile the latest tools, such as WGBS, microarrays have provided new insights to explore interactions with the oocyte and granulosa cells.

In our study the aim is to explore the whole DNA methylation of POI by whole genome bisulfite sequencing, find the pattern of DNA methylation in POI and analyze the DMRs genes related POI.

Results

1. Methylome of human ovarian granulosa cells

Genomic DNA methylation patterns in ovarian granulosa cells were compared between NOR and POI. A total of 571.9 Gb of sequencing data averaging 95.3Gb per sample were obtained for the whole genome bisulfite sequencing of human ovarian granulosa cells. A statistical summary of various sequencing result parameters was reported. Clean reads sum up to average of 614 million read pairs. 0.3% of the cytosines were read as C instead of T. This indicated that the bisulphite conversion efficiency is 99.7%. The mapping efficiency for each library is 91.3%, 92.5%, 92.1%, 92.7%, 84.8% and 83.1% for C001, C002, C003, S001, S002 and S003 libraries, respectively. In our study the average of depth per strand was 13. The average of clean Q30 bases rate was 93%. An average of 582,490,035 cytosine methylation positions was called for each sample. DNA methylate cytosines can be classified into three types, CG, CHG, and

CHH (H = A, G or T). The percentages of methylated cytosines in CG, CHG and CHH contexts were 99.15%, 0.19% and 0.65%, respectively for NOR and 99.24%, 0.15% and 0.58%, respectively for POI (Fig. 1). The average methylation level in CG, CHG and CHH were 80.14%, 0.4%, and 0.37% for NOR and 79.54%, 0.33%, and 0.31% for POI (Fig. 2). Genome-wide CpG methylation and CpG methylation level are generally similar between NOR and POI. We present Pearson correlation coefficient (PCC) to quantify the degree of colocalization between the six samples studied in this report (Fig. 3). There was a much greater similarity of DNA methylation pattern in NOR than in POI.

2.Human ovarian granulosa cells gene body methylation patterns

We analyzed the methylation levels of the promoters (2kb upstream), genes, Transcriptional Termination Region(TTR) (2kb downstream)(Fig. 4). The CpG methylation level approaching the TSS was a sharp drop. The CpG methylation increased rapidly after TSS and reached a plateau.There was no difference in trends but exited different methylation level in promoters between NOR and POI. To further investigate the CpG methylation patterns of human ovarian granulosa cells around the gene bodies, we extracted upstream, transcriptional start sites(TSS), first exon, first intron, internal exon, internal intron, last exon and downstream. A gene structure methylation distribution is shown in Fig. 5. Distribution of almost differentially methylated regions was in the context of upstream, TSS, first exon and internal exon.

3.Differentially methylated regions (DMRs) exited between NOR and POI

There was no significant difference in genome-wide CpG methylation between two groups. But there exited differentially methylated regions. We further analyzed distribution of DMRs across variou genomic elements such as the promoters, 5'-Untranslated Regions (5'-UTR), exon, Introns, 3'-Untranslated Regions (3'-UTR), CGI (Fig. 6). There was an obvious enrichment of methylation in exon. 5'-UTR had the minimum numbers. There exits correlation between methylation preference and sequence context in some plant [13]. In order to study a similar relationship in human granulosa cells, the distribution of methylated bases were calculated the standard deviation and mean of bases targeted by MTase motifs. Methylation percentage of 9-mer sequences in which the methylated cytosine was in the fifth position (allowing an analysis of three nucleotides upstream and four nucleotides downstream of CG, CHG, and CHH methylation) was calculated. The result showed that there was no obvious sequence context specificity in human granulosa cells, indicating that there is no correlation between sequence context and methylation preference (Fig. 7). So methylation preference couldn’t explain the formation of DMRs. In order to explore the biological function of differentially methylated genes related POI, we further performed the Gene Ontology (GO) Enrichment analysis. GO analysis was performed based on biological processes (BP), cellular components (CC), and molecular functions (MF). For BP, differentially methylated genes (sample VS control) tended to be enriched in the GO terms of cellular process, single organism process, biological

regulation, metabolic process, developmental process. For CC, differentially methylated genes tended to be enriched in the GO terms of cell part, organelle part, membrane. For MF, differentially methylated genes tended to be enriched in the GO terms of binding and catalytic (Fig. 8).

Discussion

POI results in significantly impaired function of ovary in women before their age 40 [1, 14]. When ovaries fail, the patients cannot produce enough hormones or release eggs regularly, and thus suffer from infertility accompanied by menopause syndrome [15, 16]. On clinic, POI is a heterogeneous endocrine disorder with a wide spectrum of causes and high complexity. Due to the rare resource of samples from patients, scientists have limited opportunities to dig out the underlying mechanism of POI. Over the past decades, numerous studies have uncovered single factor-caused POI on genetic level [17, 18], which expand our understanding on molecular biology of POI. However, few of the genetic factors could be applied in clinical diagnosis and treatment. Genome wide methylation identificates functional regulations beyond single factor analysis, providing an effective approach to investigate this disease. DNA methylation is an important epigenomic modification for transcriptional regulation, which is the most studied aspect of the epigenetic code. POI is a complex and highly heterogeneous women endocrine disorder and is associated with DNA methylation [19]. Previous studies almost focus on DNA methylation of genes related POI, for example, Tet1, FMR1[20, 21]. DNA methylation in POI granulosa cell remains unclear. We performed WGBS to study DNA methylation in human granulosa cells. We systematically investigated DNA methylation level and pattern between POI and donors healthy.

So far WGBS is considered a comprehensive and unbiased method for DNA methylation studies. In our study the average of clean reads was 614 million read pairs. Thus, sequencing data was 95.3Gb per sample. This ensured the accuracy of the sequencing. Bisulphite conversion efficiency is 99.7%, the average of depth was 13 for the whole genome, and the average of clean Q30 bases rate was 93%.These indicated a high conversion rate and reliable data.

According to our mapping results, average percentages of methylated cytosines in CpG context was 99%. CHG and CHH context were methylated less than 1%. These trends were similar in vertebrates[22] CHH and CHG methylation is high in plant methylomes [23]. Consistent with the results from previous reports, most differences is non-CpG high methylation in plants [24].The percentages of methylated cytosines in CHG and CHH context was 17.3% and 7.2% in human embryonic stem cells [25]. This indicate there are notable inter-species differences.

POI has been subdivided into three consecutive and progressive stages: occult, biochemical, and overt ovarian insufficiency based on different FSH levels, fecundity, and menstrual status [26]. Accordinng to clinical staging standards experimental samples were collected. There was different among three POI and similar among three NOR in the Pearson Correlation coefficient. It seems that DNA methylation play a role in the process of POI. For more accurate staging of POI, whether it is necessary to introduce DNA methylation in the future remains to be further studied.

In our study we further explored the distribution of DMRs and found that most DMRs were in promoter regions and exon regions. These indicated that there was an obvious enrichment of methylation in transcription regions, which would affect gene regulation. Except for some specific genes, levels of DNA methylation at a promoter-associated CpG island are generally negative correlation with gene expression[27, 28, 29]. Differentially methylated regions both in promoter and gene body regions contribute to consecutive phenotypic effect[30]. Differentially methylated genes were analyzed and enrich in different GO items. GO subcategories, such as cellular process, binding activities and catalytic activities, which response to stimulus and cell death[24], suggested that methylation mediated these pathways in POI. Meanwhile these provide a reference direction for further research on the mechanism of POI.

Conclusions

Our data reveal the role of DNA methylation during the development of of POI. These will provide new theoretical basis for the accurate diagnosis of POI.

Methods

1. Sample collection and cell purification

Follicular fluid samples were collected from the center of assisted reproduction. After the oocytes were collected for continued ART treatment per standard ART laboratory protocol, the remaining follicular fluid was collected rather than being discarded. Follicular fluid samples were collected anonymously from two groups of women: oocytes donors (n = 3) whose average age were 27.67 years and had robust response to ovarian stimulation during assisted reproductive technology (ART) and POI patients (n = 3) whose average age were 27.33 years and had at least 6 months of amenorrhea, high FSH plasma levels, low E2 plasma levels and a normal 46, XX karyotype. POI was collected according to European Society of Human Reproduction and Embryology Guideline Group on POI[31]. Women who had past histories of anticancer treatment, pelvic surgery, ovarian infection, and/ or autoimmune disease were excluded from the study.

Ovarian granulosa cells were purified from each follicular fluid sample with similar methods as previously described[32]. Briefly, sample were placed on Ficoll-paque gradient and centrifuged for 20 min at 900×g at room temperature. The interphase was collected, and washed in DPBS. Collagenase was added for 4 minutes to disperse the cell clumps. After the cells were washed again with DPBS, CD45 labeled Dynabeads was used to deplete leukocyte contaminations. The purity of each granulosa cell preparation was confirmed with flow cytometry using anti-CD45 antibody. Over 95% purity was achieved for all granulosa cell samples before being used in subsequent experiments.

2. Methylated DNA analysis by whole genome bisulfite sequencing

Genomic DNA was extracted from purified granulosa cells. DNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). DNA concentration was measured using Qubit® DNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). Methylated DNA analysis by whole genome bisulfite sequencing was performed as described by Lina Wang et al[33].

3. Data analysis

Results were analyzed using Student's T-test and data were expressed as mean ± s.d. P values less than 0.05 were considered statistically significant.

Abbreviations

Primary ovarian insufficiency (POI); Whole genome bisulfite sequencing (WGBS); Differentially methylated regions (DMRs); Normal ovarian reserve (NOR); Gene ontology(GO); Noncoding RNA (ncRNA); Pearson correlation coefficient (PCC); Transcriptional Termination Region(TTR); Transcriptional start sites(TSS); Biological processes (BP); Cellular components (CC); Molecular functions (MF)

Declarations

Ethics approval and consent to participate

All human materials used in this study were received under approval of the office of Human Subjects Research at the third hospital of Peking University(2016S2-007).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

We have no competing interests, and all authors have approved the manuscript for submission.

Funding declaration

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Authors' contributions

Xiumei Zhen, Huafang Gao, Cuilian.Zhang and Baoli Yin designed research; Lina Wang, Baoxia Gu, Yan Zhao, Cuiling Lu, Yahui Hu and Rui Ma performed research and analyzed data; Xiumei Zhen, Huafang Gao, Cuilian Zhang and Baoli Yin wrote the paper; all authors approved the final version of the manuscript.

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Figures

Fig1

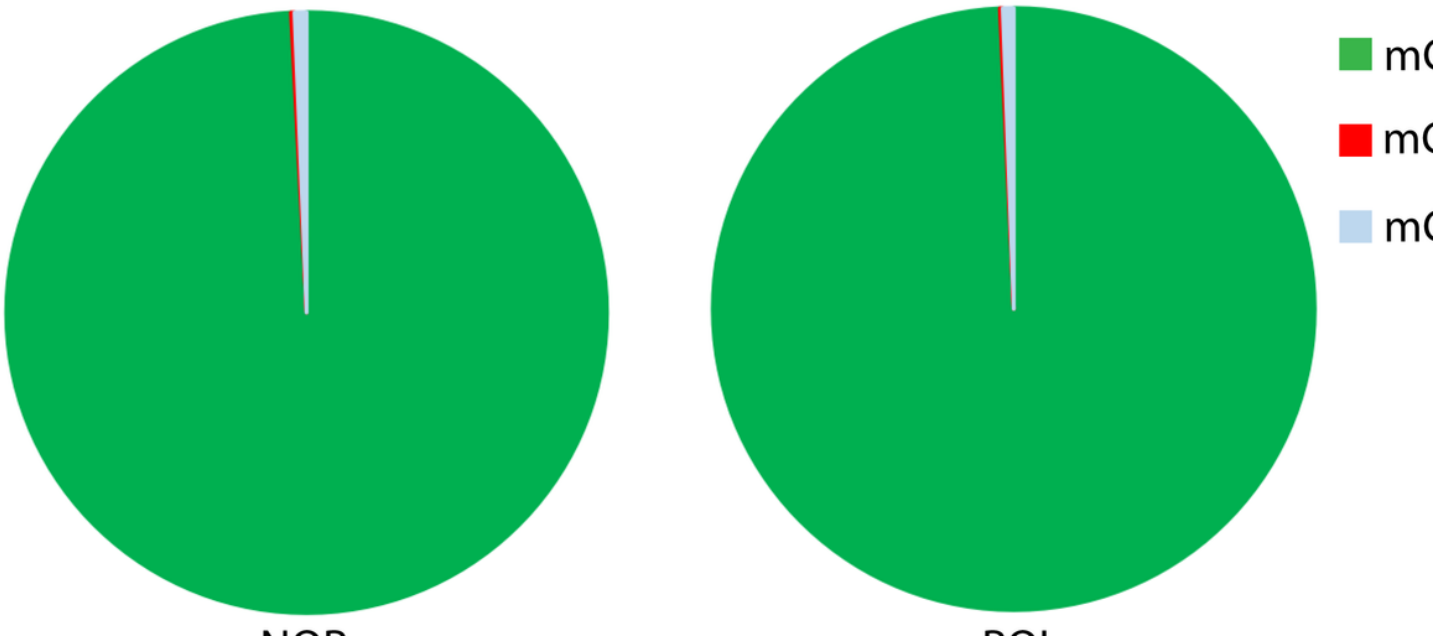

Figure 1

Proportion of Methylated Cytosine Relative percentages of methylcytosines(mCs) in the contexts of CG, CHG(H=A, C or T) and CHH.

Fig2

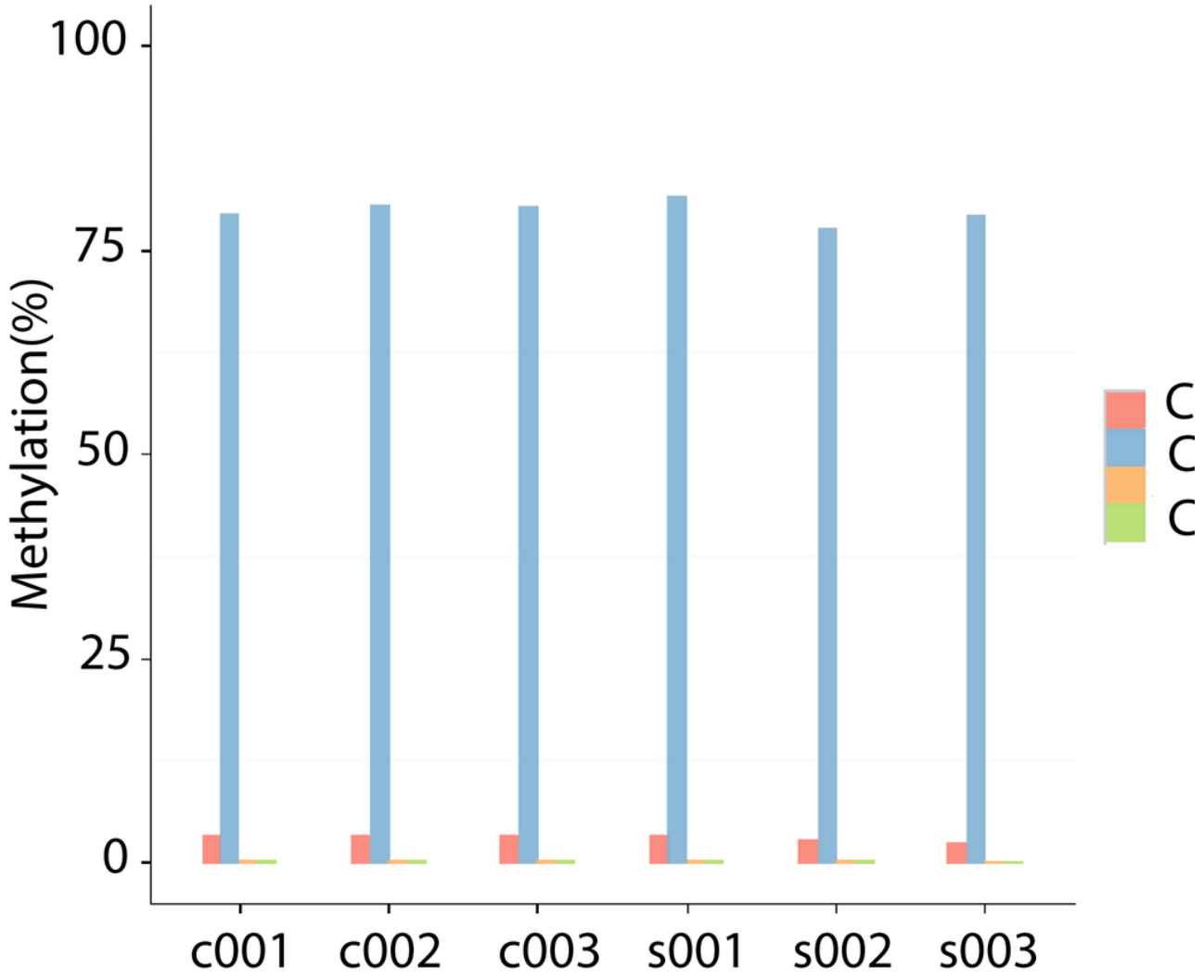

Figure 2

Whole Genomic Methylation Methylation level of mCs in six samples. The x-axis was defined as samples name. The y-axis was defined as methylation level.

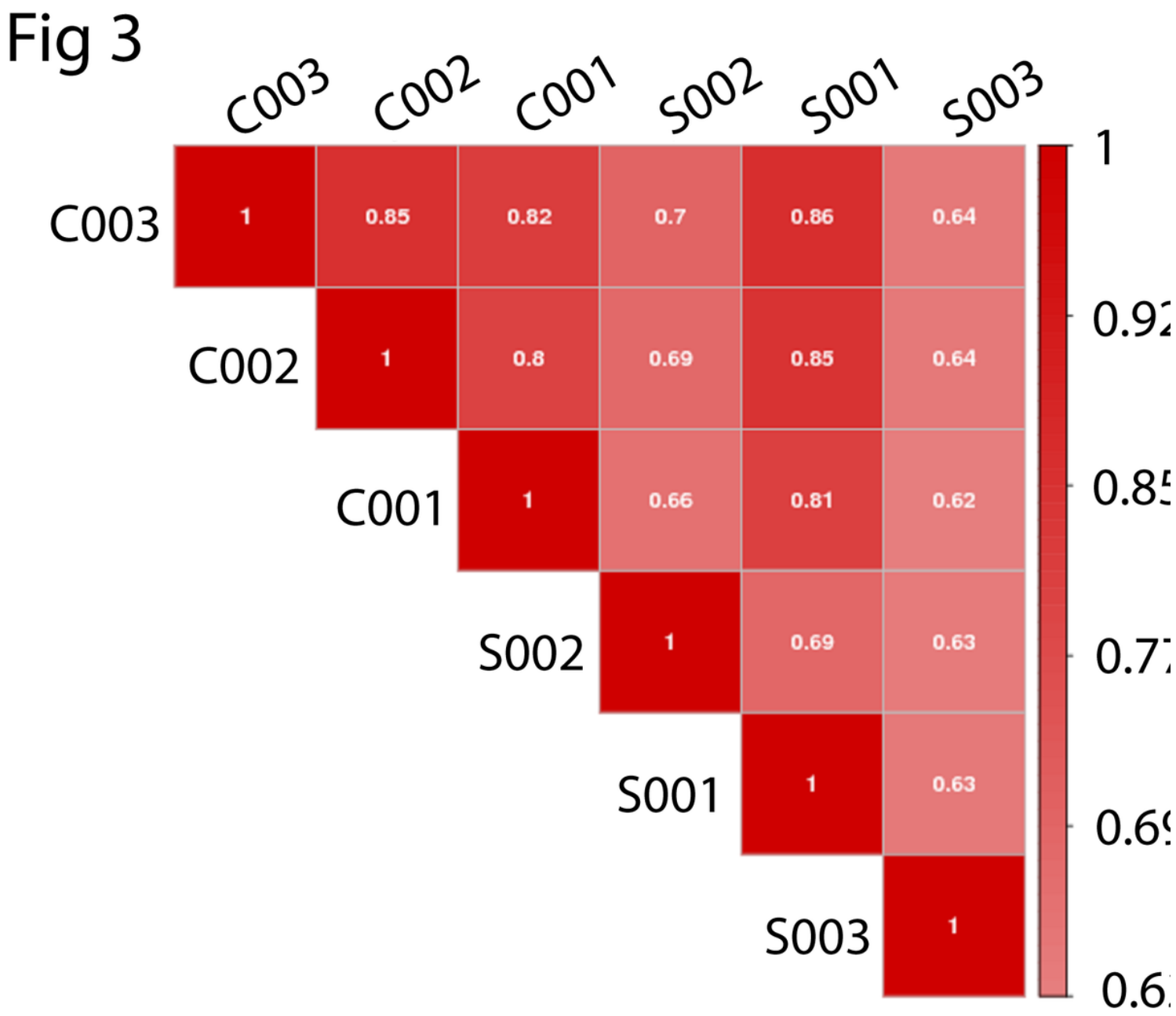

Figure 3

Pearson correlation Pearson correlation coefficient of DNA methylation pattern in six samples. Colored boxes represent a significant correlation coefficient.

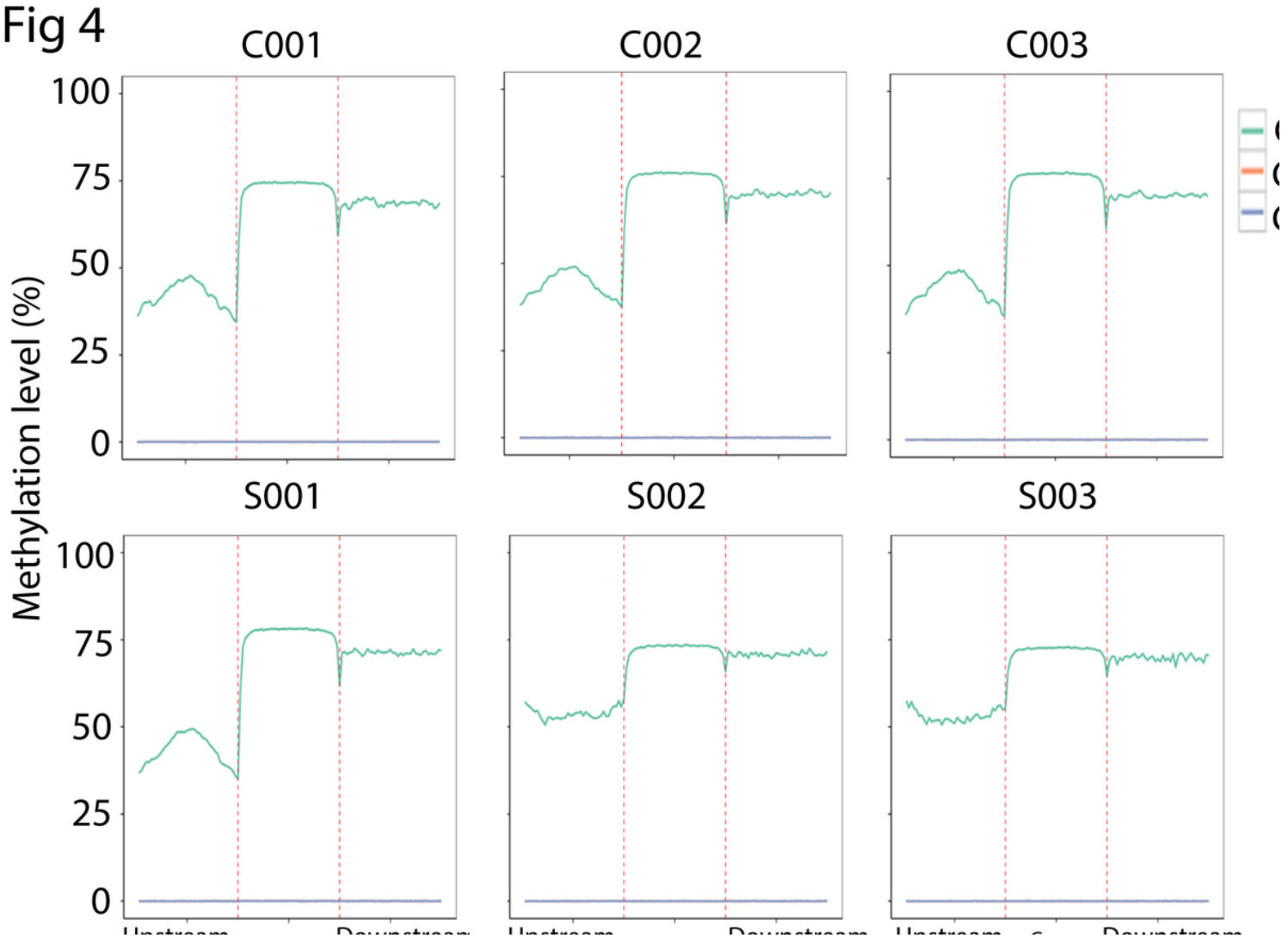

Figure 4

CpG methylation level across gene Methylation levels of the gene body, upstream 2-kb (upstream2k) and downstream 2-kb (downstream2k).

Fig 5

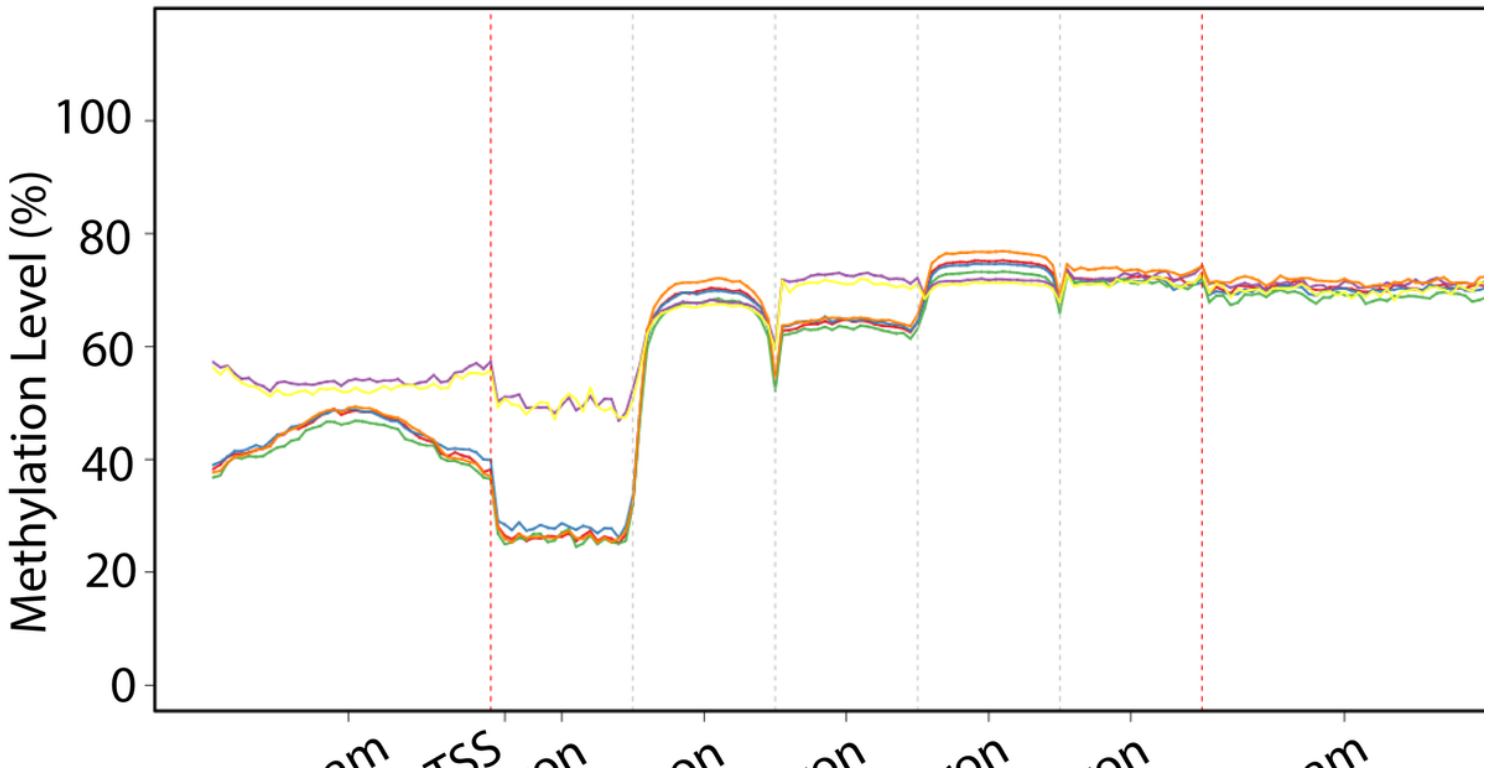

Figure 5

DNA methylation patterns in different experiments DNA methylation patterns of CpG islands located in upstream, transcriptional start sites, first exon, first intron, internal exon, internal intron, last exon and downstream in POI and NOR.

Fig 6

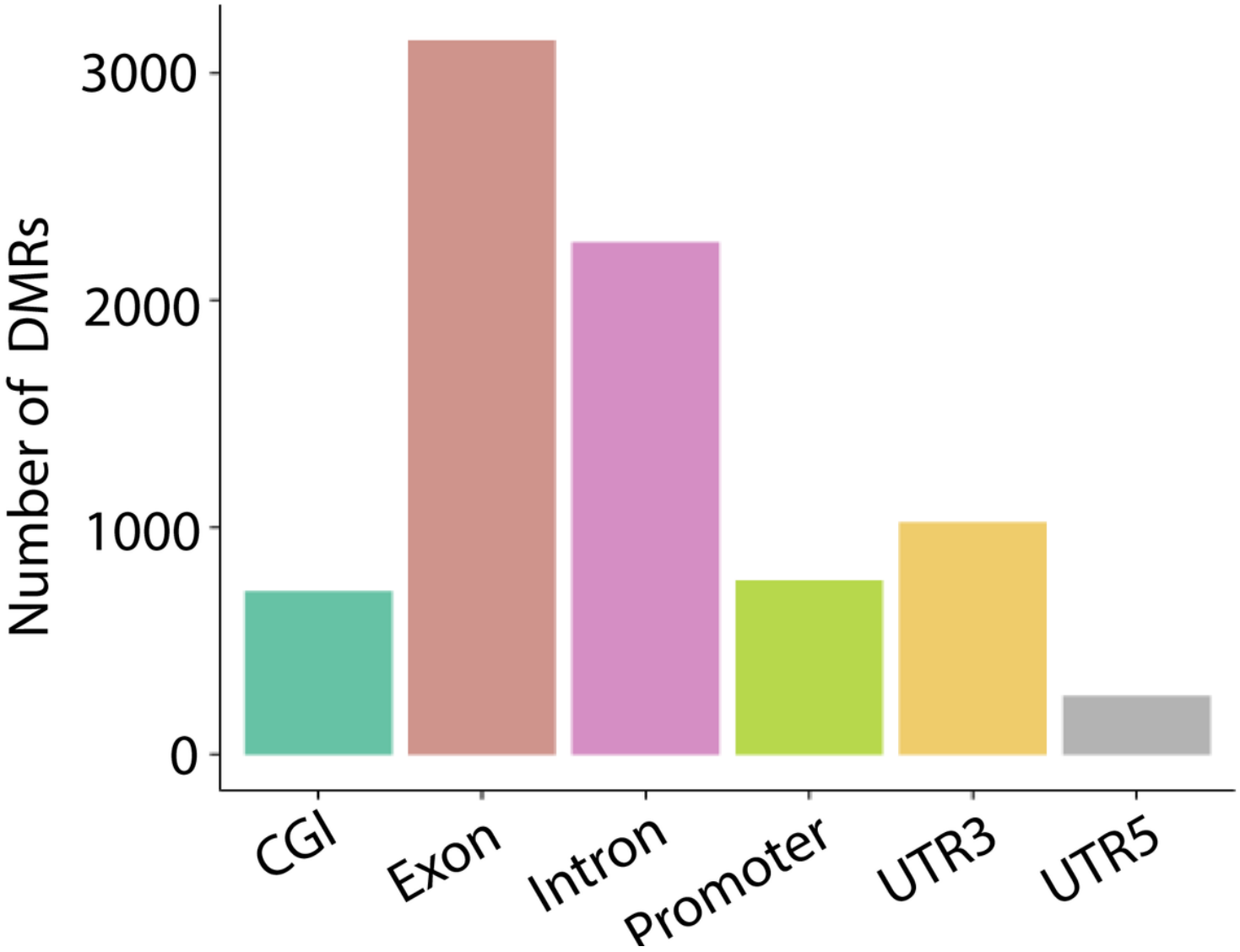

Figure 6

Number of differentially methylation regions for different genomic regions Genomic elements were divided. These elements was shown in the x-axis. The y-axis was defined as number of DMRs.

Fig 7

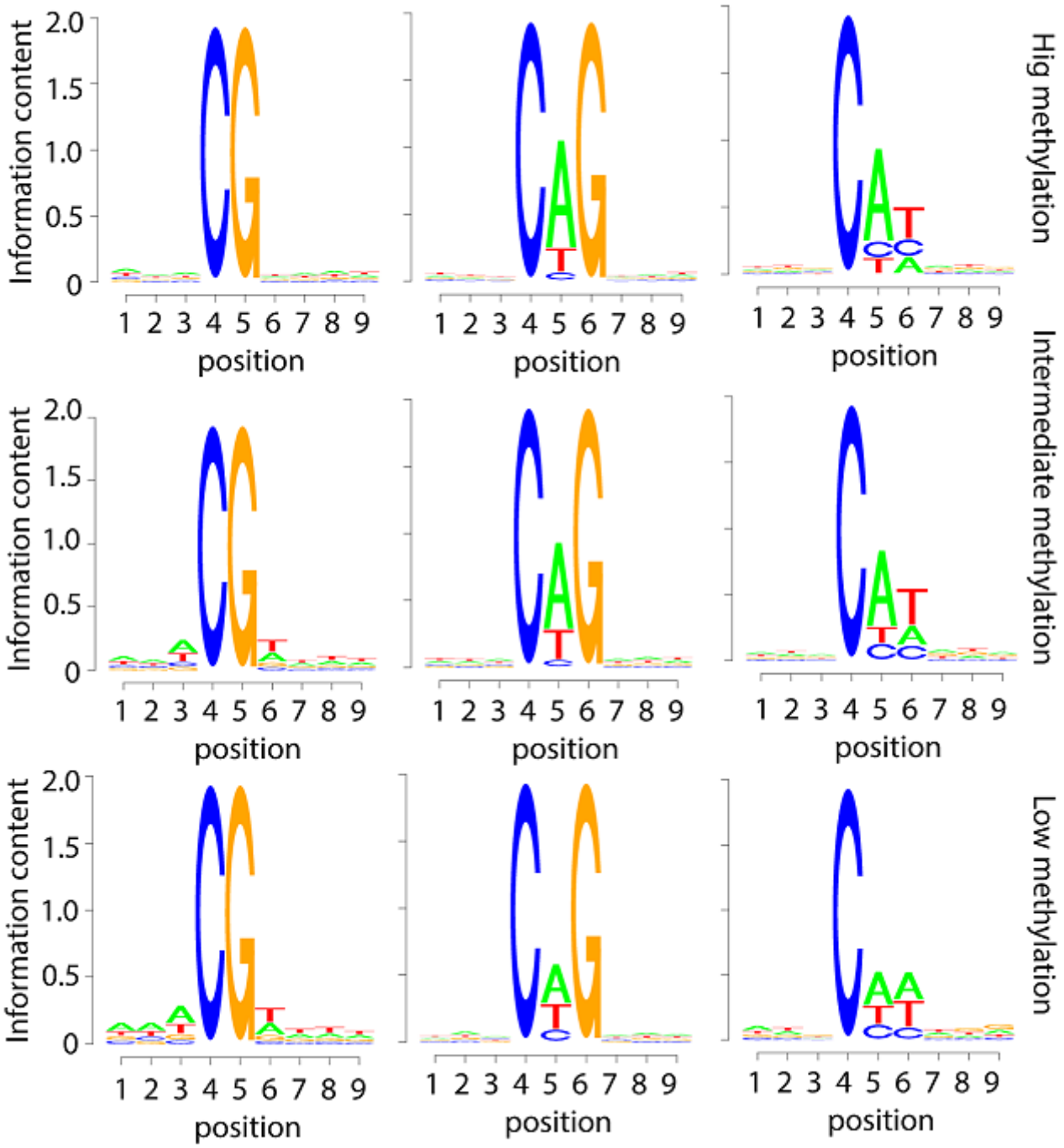

Figure 7

Sequences for methylation in CG, CHG, and CHH contexts We divided the mC site methylation level into three groups: High Methylation, Intermediate Methylation and Low Methylation for 9-mer sequences in which the methylated cytosine is in the fifth position.

Fig 8

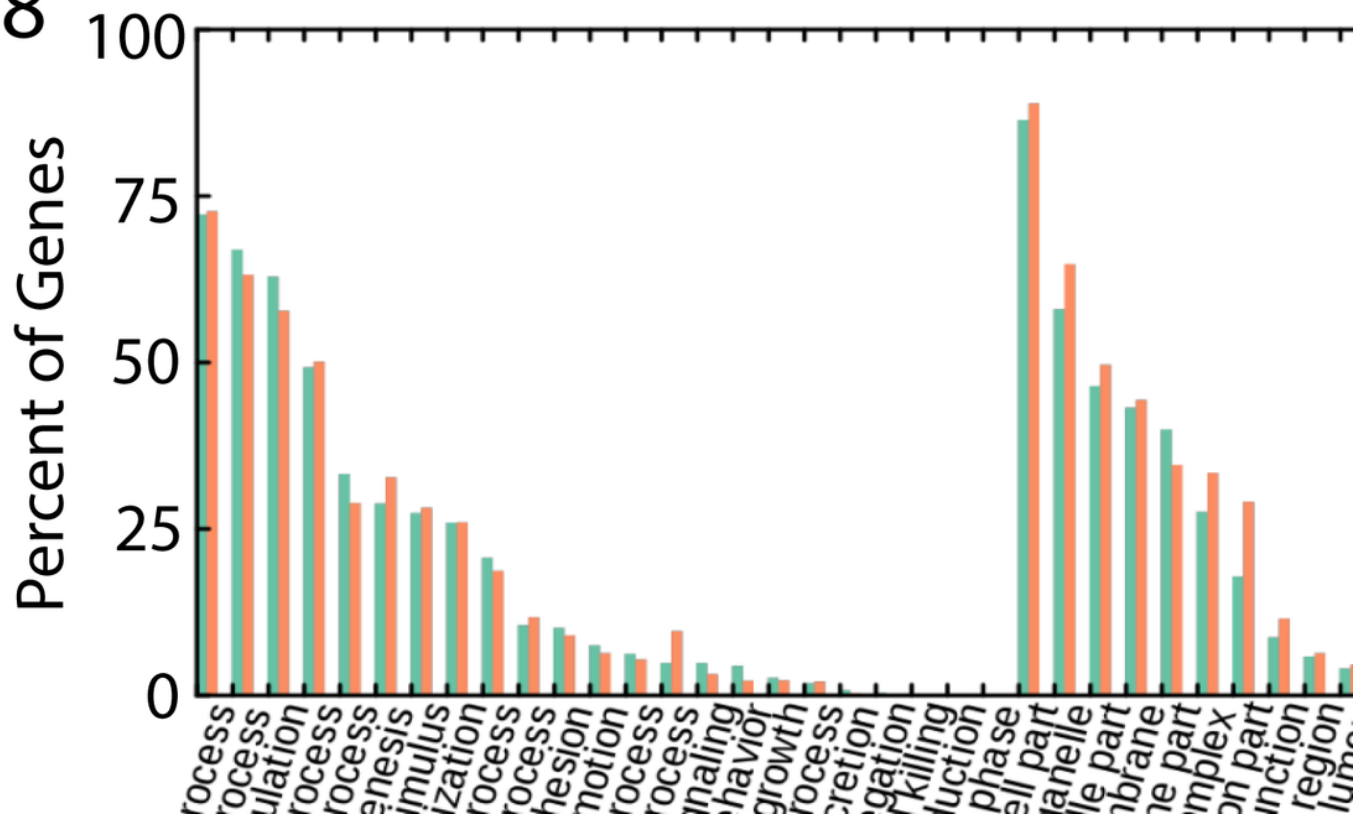

Figure 8

Gene ontology enrichment analysis of high methylated and hypo methylated genes Annotations of differentially methylated genes were grouped into three terms: biological process, cellular components and molecular function. Gene numbers and percentages were listed for each category.