Physiol Genomics 50: 714–723, 2018.
First published May 18, 2018; doi:10.1152/physiolgenomics.00052.2017.

RESEARCH ARTICLE | *Genome-wide Association Studies and Function*

Genome-wide DNA methylation profiles of porcine ovaries in estrus and proestrus

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Submitted 22 June 2017; accepted in final form 11 May 2018

Zhou X, Yang S, Yan F, He K, Zhao A. Genome-wide DNA methylation profiles of porcine ovaries in estrus and proestrus. *Physiol Genomics* 50: 714–723, 2018. First published May 18, 2018; doi: 10.1152/physiolgenomics.00052.2017.—DNA methylation is an important epigenetic modification involved in the estrous cycle and the regulation of reproduction. Here, we investigated the genome-wide profiles of DNA methylation in porcine ovaries in proestrus and estrus using methylated DNA immunoprecipitation sequencing. The results showed that DNA methylation was enriched in intergenic and intron regions. The methylation levels of coding regions were higher than those of the 5′- and 3′-flanking regions of genes. There were 4,813 differentially methylated regions (DMRs) of CpG islands in the estrus vs. proestrus ovarian genomes. Additionally, 3,651 differentially methylated genes (DMGs) were identified in pigs in estrus and proestrus. The DMGs were significantly enriched in biological processes and pathways related to reproduction and hormone regulation. We identified 90 DMGs associated with regulating reproduction in pigs. Our findings can serve as resources for DNA methylome research focused on porcine ovaries and further our understanding of epigenetically regulated reproduction in mammals.

DNA methylation; estrus vs. proestrus; MeDIP-Seq; porcine ovary

INTRODUCTION

DNA methylation is an important epigenetic modification in eukaryotes (10) and is often associated with chromatin condensation, histone deacetylation, genomic imprinting, and disease development (48, 51, 54). DNA methylation in promoter regions leads to stable gene silencing (7, 20, 33). Recent studies have demonstrated that DNA methylation in gene bodies can also affect gene expression (69). DNA methylation often occurs at the 5-position of cytosine and has been found in every vertebrate examined. In somatic cells, DNA methylation typically occurs in CpG dinucleotides (CpGs). Non-CpG methylation involving CpT, CpA, and CpC is prevalent in embryonic stem cells and neural cells (4, 15, 37, 38). The majority of CpGs are methylated, whereas unmethylated CpGs are often grouped in clusters called CpG islands (CGIs), which are present in the 5′-regulatory regions of many genes. Overall, 60% of the CGIs of all gene promoters are unmethylated (64).

The domestic pig (*Sus scrofa*) is an important farm animal that is used as a food source. Pigs are also used for biomedical research and comparative genome studies because of their physiological, metabolic, and genomic similarities to humans (11, 34, 55, 70). Transcriptomes analysis of mRNA and miRNA are widely studied to investigate the molecular mechanisms of phenotype differences in pigs, such as variations in skeletal muscle growth or fatness (17, 61, 75). However, more studies are needed to gain insight on the methylomes of pigs. Artificial selection for high prolificacy and meat production has transformed the DNA methylation pattern in pigs, resulting in associated genotypic and phenotypic changes, such as in the Landrace, Rongchang, and Tibetan pig breeds. In Europe, the Landrace breed has been selected for more than 100 yr for its propensity toward having less adipose tissue, whereas the Rongchang breed has been selected for its abundant adipose tissue, and the Tibetan breed is essentially a wild breed that has undergone very little artificial selection. During the transformation process, the DNA methylomes of these pig breeds also had variable patterns in different anatomic tissues (11, 34), leading to changes in chromatin structure and gene transcription. In females, ovaries are critical regulators of reproduction and breeding, and DNA methylation plays a crucial role in the estrous cycle and follicular maturation in the ovaries (18, 39, 40, 45, 73). Several whole-genome studies have revealed that DNA methylation is associated with polycystic ovary disease, and there is increasing evidence that DNA methylation is crucial in ovary development and maturation (50, 66, 67, 71).

Many technologies have been developed to analyze genome-wide DNA methylation profiles, including methylated DNA binding domain sequencing (MBD-Seq), methylated DNA immunoprecipitation sequencing (MeDIP-Seq), whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS) (1, 38, 46, 60). The resolutions of MBD-Seq and MeDIP-Seq are ~200 bp via the enrichment of methylated DNA (8, 16), whereas WGBS and RRBS achieve single-base resolution through bisulfite conversion. Although the resolution and genomic coverage of MeDIP-Seq is lower than that of WGBS and RRBS, it is a cost-effective approach for comparative methylome analysis. MeDIP has already been widely used in genome-wide methylation analysis of many animals (12, 35, 52, 59, 65).

Here, we used MeDIP-Seq to obtain genome-wide DNA methylation profiles of porcine ovaries from proestrus and estrus pigs to analyze their methylomes. To obtain detailed DNA methylation profiles, the methylation levels of porcine ovaries and the methylation status of intergenic and coding regions were analyzed. We obtained DNA methylation profiles for pigs in different stages of the estrous cycle and identified differentially methylated genes (DMGs) related to the reproductive process and the regulation of hormones that might

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contribute to reproductive regulation within the estrous cycle. Our findings will advance our understanding of the methylome of the porcine ovary.

MATERIALS AND METHODS

Ethics statement. Tissue collection was conducted at slaughter according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004). Tissue collection procedures were approved by the Animal Care and Use Committee of Zhejiang Agriculture and Forestry University (Lin'an, Zhejiang, China), to ensure compliance with international guidelines for animal welfare.

Animals. Ovarian samples were collected respectively from three estrus and three proestrus multiparous sows (Landrace, 28 mo old, fourth parity). The animals were reared in the same environment and were fed the same diet ad libitum. All animals were inspected daily for health status, and no symptoms of diseases were displayed. The estrus pigs were humanely killed within 24 h of exhibiting standing reflex; the proestrus pigs were humanely killed 16 days after exhibiting standing reflex. The lutea were removed, and ovarian samples were quickly frozen in liquid nitrogen before storage at −80°C.

DNA extraction and preparation for MeDIP. The ovaries from each pig was were placed into a mortar containing liquid nitrogen. DNA was extracted from the homogenized mixed powder using a Genomic DNA clean and concentrator Kit (D4011, Zymo) according to the manufacturer's instructions. Qubit DNA BR assay kit (Q32850, Thermo) was used for DNA quantification. For MeDIP, genomic DNA was sonicated to produce DNA fragments ranging in size from 150 to 500 bp using a Covarias sonication system. After end repair, base addition at the 3′-end and adapter ligation was performed using a NEXTflex methyl-seq 1 kit (5118-01, Bioo) and a NEXTflex DNA barcode-6 kit (514101, Bioo). Double-stranded DNA was denatured for 10 min at 95°C to obtain single-stranded DNA, which was immunoprecipitated using a Methylated DNA IP Kit (D5101, Zymo).

MeDIP library preparation and sequencing. MeDIP methyl enriched DNA was purified with a ZYMO DNA Clean and Concentrator kit following the manufacturer's instructions and amplified using a NEXTflex methyl-seq 1 kit (5118-01, Bioo). After excising amplified DNA between 220 and 320 bp in length on a 2% agarose gel, we purified the DNA with a Minelute Gel Extraction Kit (28604, Qiagen). The quality and quantity of the amplified DNA were evaluated using an Agilent 2100 Bioanalyzer High Sensitivity DNA chip. The quantification of library molarity was done using a Kapa RT-qPCR Kit (KK4602, KAPA Biosystem). The qualified libraries were subjected to high-throughput sequencing using an Illumina HISeq 2500 (Illumina) to generate 49 bp paired-end reads for methylation profiles analysis by the Beijing CapitalBio (Beijing, China).

Data analysis. Raw reads containing adapters, unknown, or low-quality bases were first filtered out using the NGS QC Toolkit software, and the purified reads were used for subsequent analyses. The purified reads were then aligned to *S. scrofa* reference genome downloaded from UCSC (http://genome.ucsc.edu/) with Burrows-Wheeler alignment. Uniquely mapped reads with high mapping quality (score ≧ 10) were analyzed using the MEDIPS software package. MEDIPS is used to conduct genome-wide differential coverage analysis of sequencing data derived from DNA enrichment experiments. Methylation level estimation and differential methylation analysis were performed with MEDIPS for all consecutive 250 bp windows across the genome. Annotation information from the porcine reference genome was derived from TxDb.Sscrofa.UCSC.susScr3.refGene and org.Ss.eg.db using the R Bioconductor software package. The genomic regions 2 kb upstream and 500 bp downstream of the TSS were considered proximal promoter regions. Annotation information on CPIs was derived from files downloaded from UCSC (http://hgdownload.cse.ucsc.edu/goldenPath/susScr3/database/). Differentially methylated regions (DMRs) were identified using edger Bio-conductor package following the exact test for a negative binomial distribution. Genes related to DMRs that exhibited a greater than twofold difference in the number of reads between different samples and a P value < 0.01 were identified as DMGs. Gene ontology (GO) and KEGG pathway enrichment analyses of the DMGs were performed using KOBAS 2.0.

MeDIP assay. Genomic DNA was extracted by overnight proteinase K digestion in lysis buffer (50 mM Tris·HCl pH 8.0, 10 mM EDTA pH 8.0, 0.5% SDS) before phenol-chloroform extraction, ethanol precipitation, and RNaseA digestion. Genomic DNA was sonicated to produce DNA fragments ranging in size from 300 to 800 bp. The fragmented DNA (5 μg for MeDIP) was denatured for 10 min at 95°C and immunoprecipitated overnight at 4°C with 2 μg mouse monoclonal antibody for 5-methylcytosine (5mC) (ab10805, Abcam) in a final volume of 500 μl immunoprecipitation (IP) buffer (10 mM sodium phosphate pH 7.0, 140 mM NaCl, 0.5% Triton X-100). We incubated the mixture was incubated with 60 μl protein A/G agarose (SC2003, Santa Cruz Biotechnology) for 2 h before washing all unbound fragments three times with 1 ml IP buffer. Washed beads were then resuspended in 250 μl of lysis buffer and incubated with proteinase K for 2 h at 50°C. Immunoprecipitated DNA fragments were then purified using DNA purification columns (28104, QIAGEN, Germany) and eluting into 20 μl TE. For quantitative (q)PCR analysis, MeDIP products were validated by real-time quantitative PCR (RT-qPCR) using SYBR premix Ex tag II (RR820A, Takara) for qPCR analysis, 10 μl were diluted in 100 μl TE with each qPCR reaction using 2 μl of diluted DNA. DNA copies in immunoprecipitation samples were normalized to input DNA control samples. Primers and parameters information for MeDIP is available in the supplemental material (Supplemental Table S1). (The online version of this article contains supplemental material.)

Statistics. Data are presented as means ± SE. Significant differences were analyzed by the Mann-Whitney test or one-way ANOVA using SPSS software (ver., 20.0, SPAA). P values < 0.05 were considered to be statistically significant.

RESULTS

Mapping and statistical analysis of MeDIP-Seq reads in chromosomes. To understand the effects of DNA methylation during the pig estrous cycle, we performed MeDIP-Seq analysis of the ovaries of estrus and proestrus pigs. Ovarian DNA was extracted from three estrus ovaries (es-ovaries) and three proestrus ovaries (proes-ovaries). Then, we performed genome-wide DNA methylation profiling of each ovary sample using MeDIP-Seq. After filtering out raw reads containing adapters, unknown, and low quality bases, we obtained ~39–55 million clean reads for each library. In the three estrus pigs, the rates of clean reads were 92.53, 92.41, and 94.37%. In the three proestrus pigs, the rates of clean reads were 94.7, 95.06, and 94.44% (Table 1). In the estrus samples 80.14, 80.03, and 81.98% of the reads were mapped to the *S. scrofa* genome assembly ver. 10.2. In the proestrus samples 82.75, 81.86, and 82.45% of the reads were mapped to the *S.*

Table 1. *Characteristics of the reads in estrus and proestrus porcine ovaries*

Sample	Raw Reads	Clean Reads	Clean Rate, %	Q20	Q30
Estrus1	46121192	42677154	92.53	96.97	92.16
Estrus2	42412856	39192198	92.41	96.95	92.10
Estrus3	45795310	43216154	94.37	97.48	93.31
Proestrus1	56376974	53389146	94.70	97.58	93.55
Proestrus2	55709524	52957542	95.06	97.69	93.85
Proestrus3	59010814	55731656	94.44	97.48	93.32

Table 2. *Mapping results of MeDIP-Seq*

Sample	Total Reads	Mapped Reads	Mapped Rate, %
Estrus1	42677154	34201272	80.14
Estrus2	39192198	31364392	80.03
Estrus3	43216154	35426686	81.98
Proestrus1	53389146	44177378	82.75
Proestrus2	52957542	43350580	81.86
Proestrus3	55731656	45952302	82.45

scrofa genome assembly ver. 10.2 (Table 2). MeDIP-Seq reads were detected in all porcine chromosomes (SSC1–18, mitochondria and the X chromosome). In chromosomes 1, 2, 13, and 15 of estrus pigs, the distributions of reads in the *S. scrofa* genome were significantly decreased compared with those in proestrus pigs. By contrast, in chromosomes 3, 6, 7, 10, 11, and 12 of estrus pigs, the distributions of reads in the *S. scrofa* genome were significantly increased compared with those of proestrus pigs (Fig. 1*A*) (raw data in Supplemental Table S2). In estrus pigs, 21–30% and 31–40% of the GC contents of the reads were significantly decreased compared with those of reads in proestrus pigs, and 51–60% of the GC contents of the reads were significantly increased (Fig. 1*B*) (raw data in Supplemental Table S2).

Next, we evaluated the methylation profiles around the genome. An analysis of reads distributions in different elements of the ovary genome region showed that reads were present primarily in intergenic and intron regions. The proportions of MeDIP-Seq reads mapping to promoter elements, exon elements and intron elements in proes-ovaries were 0.51, 0.58, and 5.97%, respectively, whereas they were 0.54, 0.68, and 6.06%, respectively, in es-ovaries. The reads distributions in the promoter and exon regions were significantly increased compared with those of proestrus pigs (Fig. 2*A*) (raw data in Supplemental Table S2). We also analyzed the distributions of reads in 2,000 bp regions upstream from transcription start sites (TSS), in coding regions and in the 3,000 bp regions downstream from transcription ending site (TES). DNA methylation around the TSS showed low peaks in both estrus and proestrus pigs; after a sharp increase in DNA methylation in the 5′-region of the coding region, the methylation level remained high until the TES, whereas DNA hypomethylation was present at the 3′-ends of genes. The methylation level was higher in the coding regions of estrus than in those of proestrus pigs. We also found two spikes in coding regions, which suggests some common regions in genes are modified (Fig. 2*B*).

Comparison of DMRs between estrus and proestrus pigs. We detected 80,207 hypomethylated DMRs (Supplemental Table S3) and 32,059 hypermethylated DMRs (Supplemental Table S4) in the estrus vs. proestrus comparison. To compare the DNA methylation profiles of the porcine ovaries, the MEDIPS package was used to identify DMRs (P value < 0.01 and $|\log_2 FC| \geq 1$) across groups of samples (Fig. 3*A*). After merging significant neighboring windows, we found that the majority of DMRs were located in intron (4,732 hypomethylated and 1,954 hypermethylated regions, detailed list of DMRs in Supplemental Tables S5 and S6) and intergenic (74,116 hypomethylated and 29,426 hypermethylated regions) elements. The comparisons between estrus vs. proestrus ovarian genomes identified 4,813 (4.29%) DMRs of CGIs. The CGI elements of the coding and 5′- and 3′-flanking regions included 228 hypomethylated and 274 hypermethylated DMRs (detailed list of DMRs in Supplemental Tables S7 and S8). Promoter elements contained 529 hypomethylated and 223 hypermethylated DMRs (detailed list of DMRs in Supplemental Tables S9 and S10). Exon elements contained 413 hypomethylated and 306 hypermethylated DMRs (detailed list of DMRs in Supplemental Tables S11 and S12) (Fig. 3*B*). Interestingly, we identified 17 hypomethylated DMRs of miRNAs: ssc-MIR182, ssc-MIR9793, ssc-MIR181C, ssc-MIR143, ssc-MIR145, ssc-MIR339-1, ssc-MIR9795, ssc-MIR9819, ssc-MIR9790, ssc-MIR328, ssc-MIR125A, ssc-MIR429, ssc-MIR493, ssc-MIR9846, ssc-MIR29B-2, ssc-MIR9788-2, and ssc- MIR202 (details on their positions are listed in Supplemental Table S3). We also identified 16 hypermethylated DMRs of miRNAs: ssc-MIR9796, ssc-MIR365-1, ssc-MIR196A-2, ssc-MIR769, ssc-MIR9841, ssc-MIR127, ssc-MIR95, ssc-MIR9846, ssc-MIR326, ssc-MIR708, ssc-MIR9814, ssc-MIR9788-2, ssc-MIR142, ssc-MIR10B, ssc-MIR218B, and ssc-MIR504 (details on their positions are listed in Supplemental Table S4). Most of the DMRs of miRNAs were spread randomly in the genome. The physical positions of the

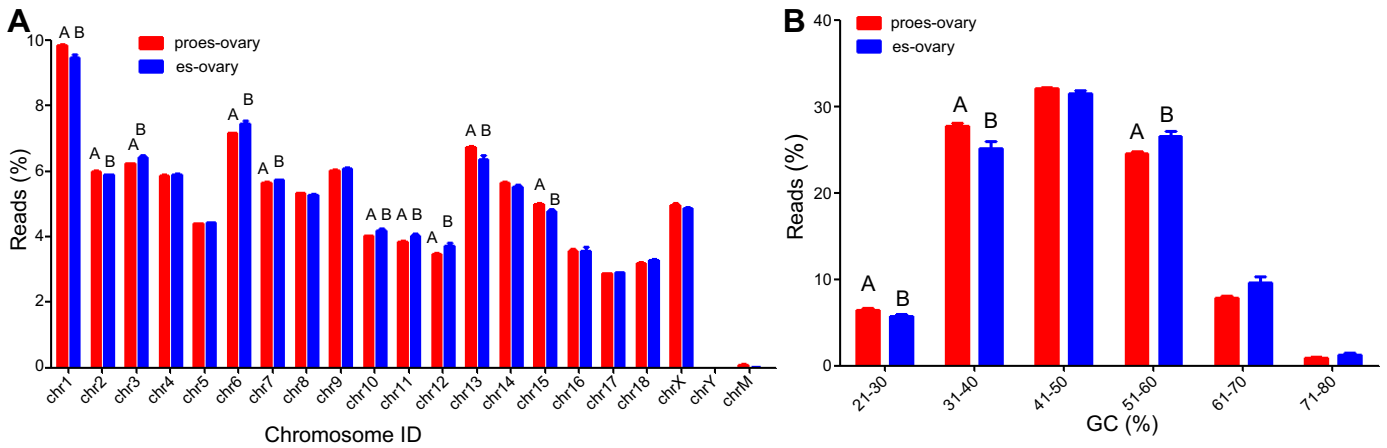

Fig. 1. Read distributions in pig genomes. *A*: chromosome distributions of reads in estrus (es-ovary) compared with proestrus ovaries (proes-ovary). *B*: read distributions of GC content in the estrus vs. proestrus ovary genomes. Significant differences were assessed by the Mann-Whitney test. Letters denote significant ($P < 0.05$) differences between values.

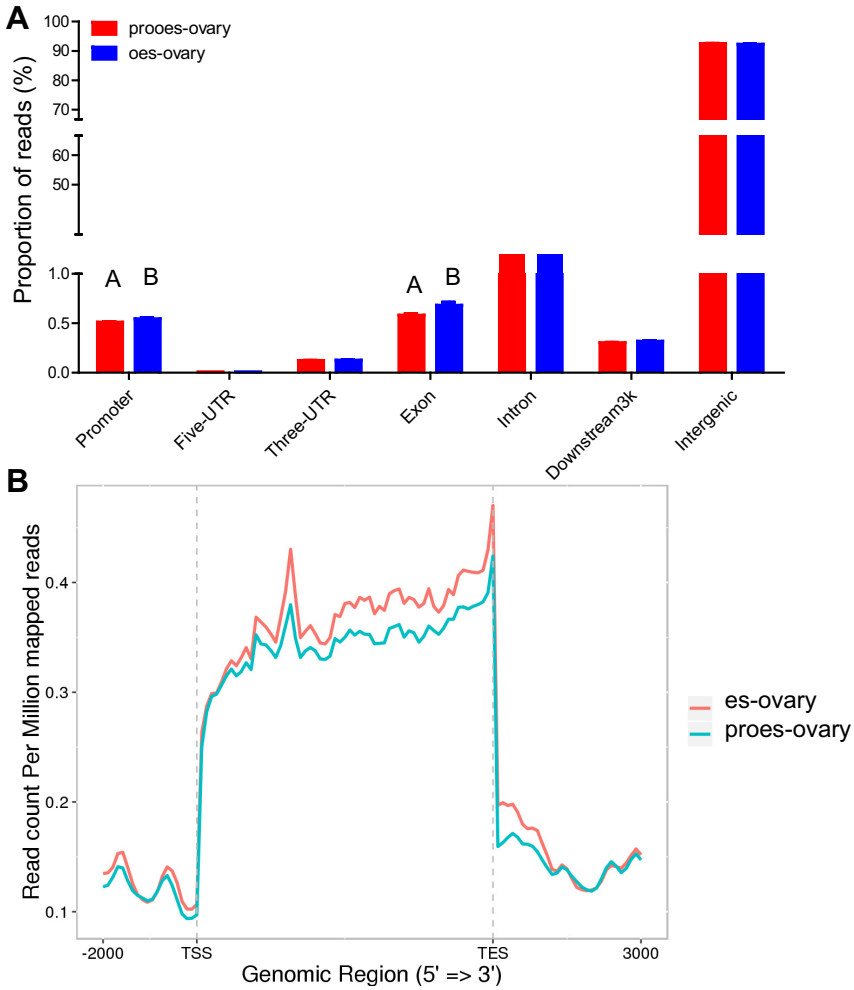

Fig. 2. Read distributions in different elements of the genomes from estrus and proestrus ovaries. *A*: the *x*-axis shows the different components of the genome. The *y*-axis is the proportion of reads. Significant differences were assessed by the Mann-Whitney test. Letters denote significant ($P < 0.05$) differences between values. *B*: methylation distributions in the coding and flanking regions. In coding regions, each gene was split into 60 equal windows. The 2 kb regions upstream and downstream were split into 20 nonoverlapping windows. The number of reads per million mapped reads was calculated for each window. UTR, untranslated region; TSS, transcription start site.

DMRs in the pig chromosomes of estrus and proestrus pigs were also compared (SSC1-18, mitochondria and the X chromosome; Fig. 3*C*).

DMGs. The DMGs were identified using DMR data. We detected 3,134 hypermethylated and 3,570 hypomethylated DMGs in the estrus and proestrus pigs (Fig. 4*A*). After filtering out the DMGs with DMRs only located in intergenic regions, we identified 1,090 hypermethylated and 1,892 hypomethylated DMGs in estrus and proestrus pigs. In estrus vs. proestrus pigs, there were 161 hypermethylated and 389 hypomethylated DMGs in the promoter regions (Fig. 4*B*), 217 hypermethylated and 318 hypomethylated DMGs in the exon regions (Fig. 4*C*), and 783 hypermethylated and 1,399 hypomethylated DMGs in the intron regions (Fig. 4*D*). The methylation details of the DMGs are listed in Supplemental Tables S13 and S14; highlighted lines indicate the same genes with different DMRs.

Validation of MeDIP-Seq data via MeDIP and RT-qRCR analysis. To confirm the reliability of the MeDIP-Seq results, three gene (*IRF7, NNAT*, and *COX17*) promoter regions showing hypermethylation in es-ovaries vs. proes-ovaries and three gene (*DMRT1, PDLIM4*, and *FKBP10*) promoter regions showing hypomethylation in es-ovaries vs. proes-ovaries were randomly selected for validation using MeDIP and RT-qPCR analysis. MeDIP-qPCR analysis shows that the 5mC levels of CGIs in the *IRF7, NNAT*, and *COX17* promoters were significantly increased in estrus pigs compared with proestrus pigs. In the *DMRT1, PDLIM4*, and *FKBP10* promoters, the 5mC levels of CGIs were significantly decreased in estrus pigs compared with proestrus pigs. These results are consistent with the MeDIP-Seq results (Fig. 5).

GO analysis of DMGs. GO analysis was performed for the DMGs detected in both estrus and proestrus pigs. We identified 3,651 DMGs, a total of 2,038 DMGs were annotated in three categories: biological process, cellular component, and molecular functions. The DMGs that involved biological processes include the following enriched terms: metabolic process (1,259 DMGs), response to stimulus (833 DMGs), multicellular organismal process (621 DMGs), localization (601 DMGs), reproduction process (142 DMGs), biological adhesion (137 DMGs), and reproduction (107 DMGs) (Fig. 6*A*). The DMGs involved in cellular components include the following enriched terms: catalytic activity (641 DMGs), molecular transducer activity (201 DMGs), molecular function regulator (121 DMGs), enzyme regulator activity (106 DMGs), and structural molecule activity (88 DMGs) (Fig. 6*B*). The DMGs that were involved in molecular function include the following enriched terms: cell part (1,485 DMGs), extracellular region (606 DMGs), membrane part (603 DMGs), organelle part (572 DMGs), and extracellular region part (483 DMGs) (Fig. 6*C*). The GO enrichment analysis was performed to gain insight into the biological processes, cellular components, and

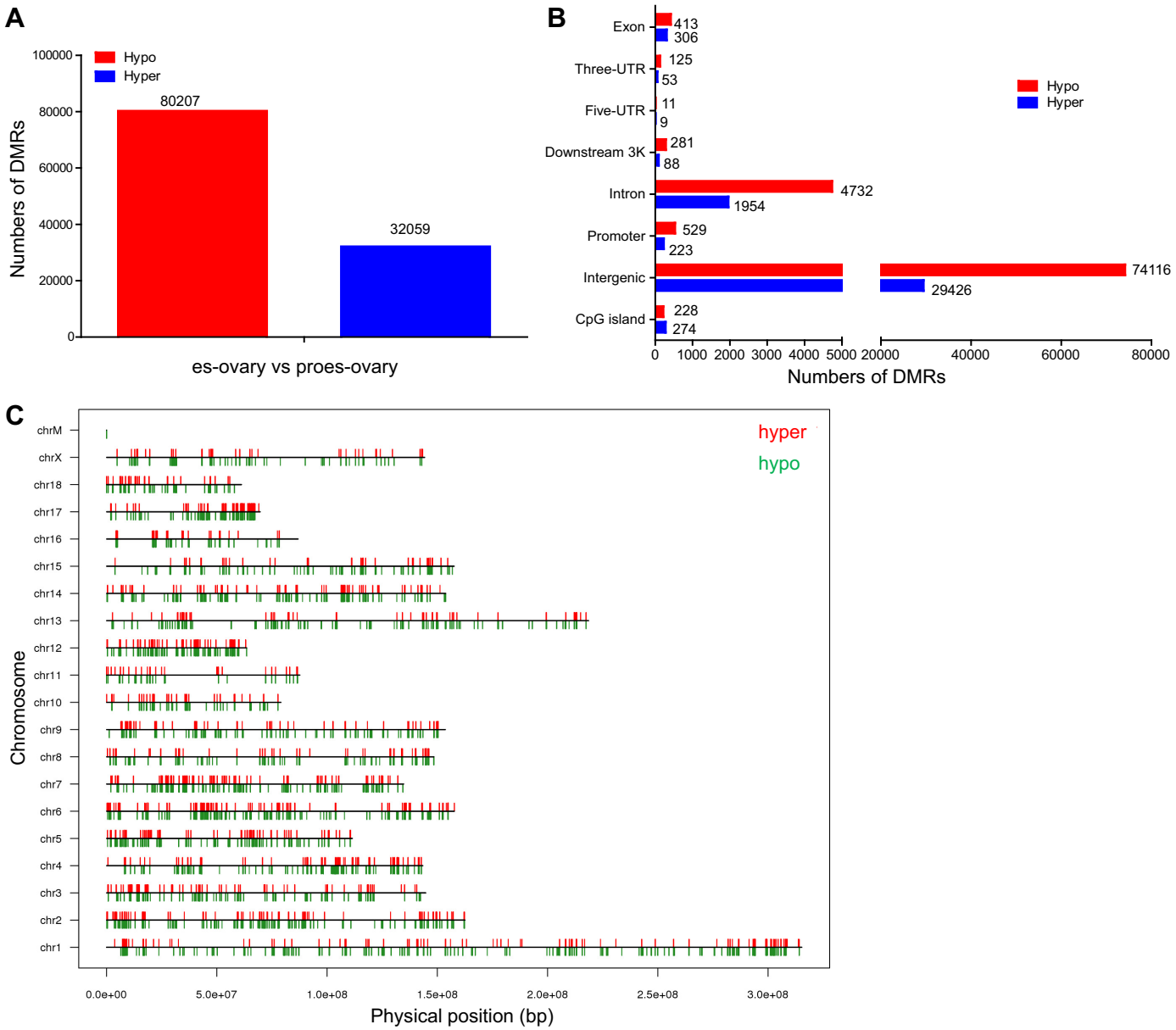

Fig. 3. Distributions of differentially methylation regions (DMRs) in estrus vs. proestrus pigs. *A*: total hypermethylated and hypomethylated DMRs in estrus and proestrus pigs. *B*: distributions of DMRs in different elements of the porcine genome. *C*: physical positions of DMRs in chromosomes.

molecular functions in which the DMGs might be involved. The significantly enriched GO terms of DMGs are listed in Fig. 6*D* (false discovery rate < 0.05). In the estrus pigs vs. proestrus pigs, the most significantly enriched terms were hormone activity, immune response, positive regulation of immune system process, response to hormone, receptor binding, steroid metabolic process, steroid metabolic process, and cell cholesterol metabolic process. KEGG pathway analysis was performed to investigate the pathways in which the DMGs are involved. The estrus pigs vs. proestrus DMGs were significantly enriched in the PPAR, AMPK, Toll-like receptor, Prolactin, and NF-κB signaling pathways.

Candidate DMGs associated with reproduction regulation. DMGs associated with reproductive process and hormone regulation were identified. In coding regions and 5′- and 3′-flanking regions, we found 67 DMGs in estrus and proestrus pigs that were involved in reproductive processes (DMG details are listed in Supplemental Table S15); 23 DMGs involved in hormone regulation were also identified (DMG details are listed in Supplemental Table S16). We retrieved DMGs according to the GO enrichment analysis (details of GO enrichment are shown in Supplemental Table S17).

DISCUSSION

In females, the ovary is the ovum producing reproductive organ that is responsible for controlling reproductive processes. Studies have demonstrated that DNA methylation plays an important role in the maturation of the ovary and the estrous cycle (28, 32, 39–41). In this study, we determined genome-wide DNA methylation profiles using MeDIP-Seq, to investigate the differences in the methylome in estrus and proestrus

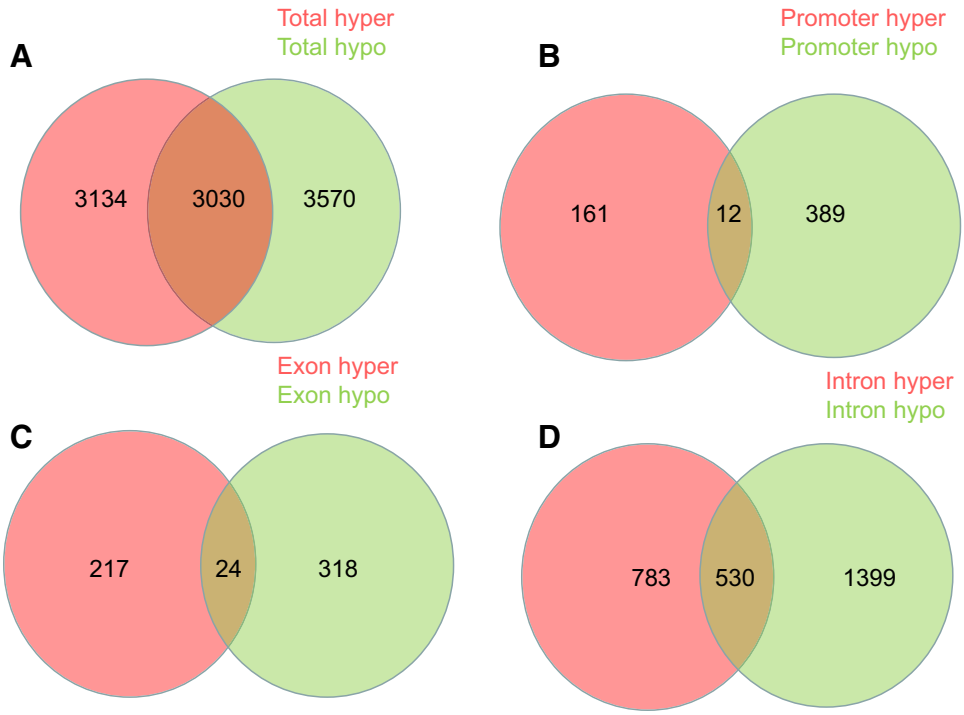

Fig. 4. Distributions of differentially methylation genes (DMGs) in estrus vs. proestrus pigs. *A*: DMGs that were unique or shared between hypermethylated and hypomethylated genes. *B*: DMGs that were unique or shared between promoter-hypermethylated and promoter-hypomethylated genes. *C*: DMGs that were unique or shared between exon-hypermethylated and exon-hypomethylated genes. *D*: DMG that were unique or shared between intron-hypermethylated and intron-hypomethylated genes.

porcine ovaries. We compared the whole-genome methylation profiles of ovaries from three estrus and three proestrus pigs and identified a number of DMGs that might affect the reproductive process and hormone regulation during the estrous cycle.

Our results show that the read distributions were highest in intergenic regions, followed by intron and exon regions; these findings are similar to the results for other species (49, 59). We found that the read distributions in chromosomes in estrus and proestrus ovaries differed significantly; during estrus the methylation or demethylation of CpGs may have occurred more frequently in chromosomes in which the read distribution changed significantly. The read distributions were also positively correlated with the GC content, which is in accordance with the results from other MeDIP-Seq studies (63, 70). The distribution difference may provide a feature for analyzing DMRs and DMGs. Our results indicate that the read distributions in exon regions were increased in estrus ovaries, potentially due to the regulatory role of DNA methylation in alternative splicing (31, 44); therefore, we speculate that alternative splicing might occur during the estrous cycle in pigs.

We found 65 hypomethylated and 42 hypermethylated DMRs in CGIs of promoter elements, implying that the expression of related genes might be up- or downregulated. Moreover, we also found 100 hypermethylated and 48 hypomethylated DMRs in CGIs located in exon elements, and 100 hypermethylated and 83 hypomethylated DMRs in CGIs located in intron elements (Supplemental Tables S13 and S14). Some studies have suggested that DNA methylation in coding regions does not block gene expression and might actually stimulate it (21, 61). Therefore, the DMRs we identified in exons and introns might be involved in regulating gene expression. In the current study, the methylation level in exons was

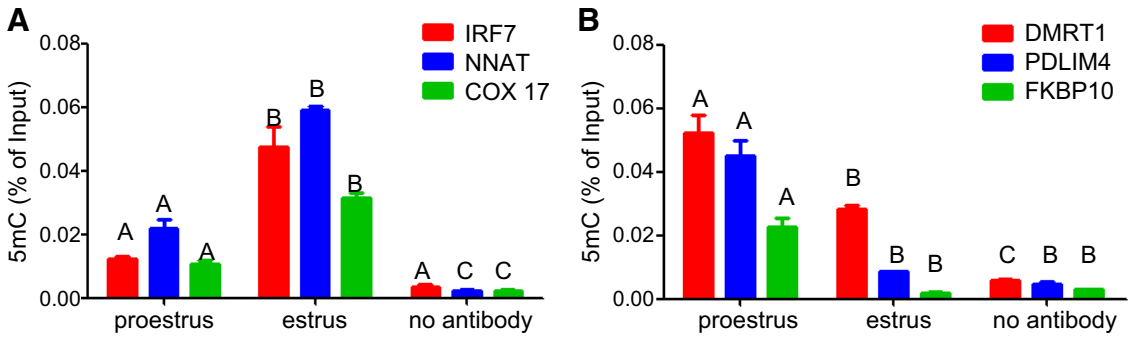

Fig. 5. Verification of DMGs by methylated DNA immunoprecipitation (MeDIP). *A*: the 5-methylcytosine (5mC) levels of the IRF7, NNAT, and COX17 promoter CpG islands in estrus vs. proestrus ovaries. *B*: the 5mC levels of the DMRT1, PDLIM4, and FKBP10 promoter CpG islands in estrus vs. proestrus ovaries. MeDIP was performed using digested chromatin from the genomics as indicated. Following immunoprecipitation with an anti-5mC antibody, the enrichment of the 5mC-containing DNA sequences was quantified by real-time PCR. Relative amounts of the 5mC-containing DNA sequences compared with the input in each group were calculated ($n = 3$/group). No antibody group was used as a negative control. Significant differences were assessed by one-way ANOVA. Letters denote significant ($P < 0.05$) differences between values.

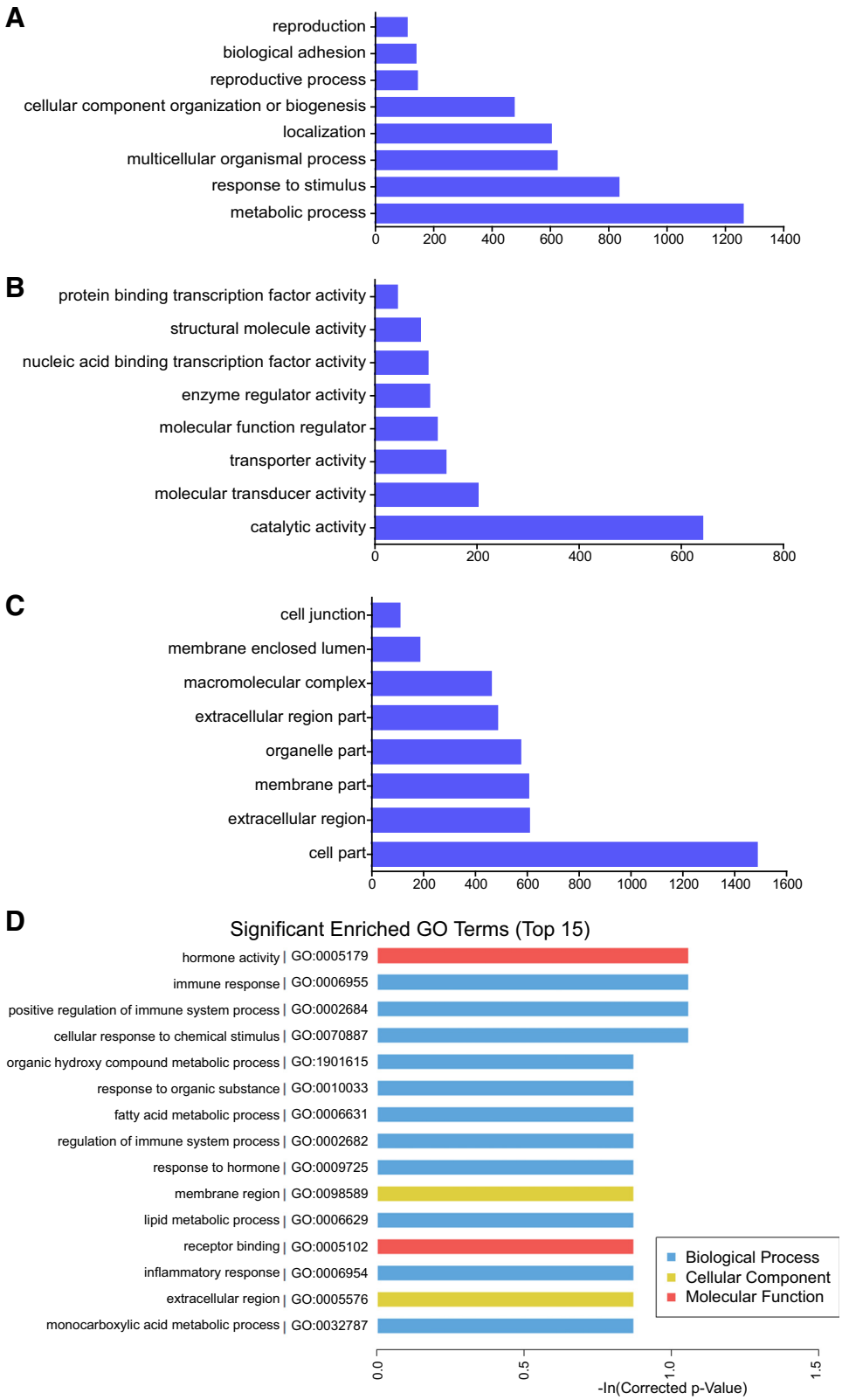

Fig. 6. Gene ontology (GO) analysis of methylated genes. *A*: biological process; *B*: cellular component; *C*: molecular function; *D*: the top 15 GO terms significantly enriched for differentially methylated genes in estrus vs. proestrus pigs.

much higher than in introns as the CpG content of exons was also greater than that of introns (53). This methylation difference might affect pre-mRNA alternative splicing (26, 44, 57). The role of altered methylation in exons and introns is still unclear, and further studies are needed to determine the effects of DNA methylation in coding regions on transcription. Most genes had several methylated regions in their promoters and coding regions. Hypomethylation and hypermethylation of the

coding regions in the pig genome might be important for regulating gene expression.

We identified 3,134 hypermethylated and 3,570 DMGs hypomethylated in estrus vs. proestrus pigs according to the DMRs data. Because the typical CGIs in promoters are more suitable for validation by MeDIP-qPCR, we randomly chose three hypermethylated genes (*IRF7, NNAT*, and *COX17*) and three hypomethylated genes (*DMRT1, PDLIM4*, and *FKBP10*) in promoters for validation by MeDIP and RT-qPCR analysis, and the results were in accordance with the MeDIP-Seq data. The DMRs with few CpGs were more difficult to analyze by MedIP-qPCR. Generally, the estrous cycle involves follicular development, ovulation, and hormone secretion in ovaries (24, 42, 58, 74). GO analysis was performed to investigate the potential functions of the DMGs responsible for regulating reproduction during the estrous cycle. After filtering out DMGs with DMRs located only in intergenic regions, we identified 67 DMGs involved in reproductive processes in estrus and proestrus pigs (Supplemental Table S15). We also found 23 DMGs involved in hormone regulation (Supplemental Table S16). For example, CYP1A1 was screened as a DMG and plays a key role in estradiol metabolism and follicular growth (2, 47). CYP19A2 and CYP19A1 are the terminal enzymes in the steroidogenic pathway and are responsible for the aromatization of androgens into estrogens in follicles, affecting granulosa cell proliferation and follicle growth in proestrus stage (5, 23). We also screened CGA as a DMG. The CGA gene is responsible for coding the alpha subunits of chorionic gonadotropin, luteinizing hormone, follicle stimulating hormone (FSH), and thyroid stimulating hormone. The alpha subunits of these hormones are identical; these hormone are crucial for follicle development (3, 14, 25, 76). BMP-7 could increase the expression of FSH receptor in human granulosa cells, affecting their growth (56). ZP3 functions as the sperm receptor and is associated with the number of piglets born alive (29, 72). The interplay between IGF1R and estrogen receptor signaling may affect female reproduction (6, 27, 68). CFTR is also an important regulator of the estrous cycle (62). Studies have demonstrated that GALP can regulate reproduction, body weight, and locomotion via interplay with insulin and leptin (13, 22). INHBA is a subunit of both activin and inhibin and plays an important role in reproductive processes, hormone activity and animal breeding (9, 19, 77). INHBA can inhibit FSH secretion and activity in granulosa cells, and INHBA gene mutations were associated with litter size in sheep 329, 77). Abnormal DNA methylation in gene promoters and coding regions could influence gene expression levels (36), although these DMGs might contribute to regulating reproduction in pigs, the role of methylation in the expression and alternative splicing of these genes during reproduction requires further study.

These results suggest that pigs have distinct processes for reproduction and hormone regulation and suggest that the differences in DNA methylation play an important role in regulating reproduction in the estrous cycle. In the current study, we provide comprehensive genome-wide DNA methylation profiles of estrus and proestrus ovaries in pigs. These DNA methylation profiles should provide new clues regarding the epigenetic regulation mechanisms in porcine ovaries.

GRANTS

This research was supported by Natural Science Foundation of China Grants 31501921 and 31600292; New species selected of Zhejiang Agriculture (livestock and poultry) major science and technology projects (2016C02054-3); and by Initial fund from Zhejiang Agriculture and Forestry University (2034020018).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

X.Z., S.Y., and A.Z. conceived and designed research; X.Z. performed experiments; X.Z., S.Y., and F.Y. analyzed data; X.Z. prepared figures; F.Y. edited and revised manuscript; K.H. and A.Z. interpreted results of experiments.

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