Clinical Epigenetics

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Effects of multisuperovulation on the transcription and genomic methylation of oocytes and offspring

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Abstract

Background Controlled ovarian stimulation is a common skill of assisted reproductive technologies (ARTs). In the clinic, some females would undergo more than one controlled ovarian stimulation cycle. However, few studies have focused on the influence of multi-superovulation on oocytes and offspring.

Results Here, we found that multi-superovulation disrupted the transcriptome of oocytes and that the differentially expressed genes (DEGs) were associated mainly with metabolism and fertilization. The disruption of mRNA degradation via poly (A) size and metabolism might be a reason for the reduced oocyte maturation rate induced by repeated superovulation. Multi-superovulation results in hypo-genomic methylation in oocytes. However, there was an increase in the methylation level of CGIs. The DMRs are not randomly distributed in genome elements. Genes with differentially methylated regions (DMRs) in promoters are enriched in metabolic pathways. With increasing of superovulation cycles, the glucose and insulin tolerance of offspring is also disturbed.

Conclusions These results suggest that multi-superovulation has adverse effects on oocyte quality and offspring health.

Keywords Oocyte, Offspring, Multi-superovulation, DNA methylation, Metabolic disorder, Transcriptome

Background

Infertility accounts for ~ 15% of reproductive-aged couples worldwide, and has reached ~ 30% in some regions of the world [1, 2]. Although infertility is associated with many factors, the most effective treatment for infertility is assisted reproductive technologies (ARTs) including in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo culture in vitro, and embryo transfer. Currently, ~ 8 million children are produced via IVF [3]. Approximately 1–3% of births are born via IVF in Western countries, and approximately 1.7–5% of births are born via IVF in China, Japan, and other countries [4]. Many studies have confirmed that ARTs may be associated with a greater risk of chronic diseases, such as metabolic disorders, obesity, diabetes, cardiovascular diseases, and malignancies [5]. For example, ART offspring have a higher fasting blood glucose and serum insulin levels, and the HOMA-B level is lower than that of natural offspring at the age of 6–10 years [4, 6]. Twins produced via ARTs also have a higher risk of cardiovascular diseases than do natural twins [7]. Flow-mediated dilation of the artery is lower in ART offspring. The association between ARTs and the risk of cardiovascular diseases has also been confirmed by other studies [6]. These

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findings indicate that ARTs may have negative effects on children's health in the long-term.

The hypothesis that adult diseases, such as cardiovascular diseases and metabolic diseases, originate from germ cells and fetuses has been confirmed by previous studies, and might be associated with the altered epigenetic modifications [8–10]. In vitro maturation alters the methylation profile of mouse oocytes [11]. Superovulation changes the global methylation of oocytes at high dosages of gonadotropins in mice [12]. In humans, hormonal hyperstimulation alters the DNA methylation levels of imprinted genes, such as H19, PEG1 and KvDMR1, in GV and MI oocytes [13–15]. These findings indicate that ARTs may have a negative influence on DNA methylation in oocytes.

Controlled ovarian stimulation is a common treatment for obtaining more oocytes via ARTs, and in the clinic, many couples would undergo several assisted reproductive cycles to obtain a live baby. Therefore, these women have to have more than one ovarian stimulation cycle. Nevertheless, few studies have focused on the effects of multi-ovarian stimulation on the oocyte quality. Previous studies suggest that ovarian stimulation at a lower dosage of gonadotropins has limited effects on the DNA methylation of mouse oocytes [11, 12, 16]. Our previous studies revealed that repeated superovulation has adverse effects on the mitochondrial function of cumulus cells, follicular development, the oocyte maturation rate, and histone modifications during early embryo development [16–18]. These findings suggest that repeated superovulation has negative effects on oocyte maturation. Oocyte maturation includes cytoplasmic and nuclear maturation, which are regulated by metabolism, and mRNA degradation by deadenylating poly (A) tails [19–21]. In the present study, we used a lower dosage of gonadotropins (7.5 IU) to establish a mouse model to investigate the influence of multi-superovulation on the transcription and DNA methylome of oocytes.

Materials and methods

Animals

Female ICR mice (CD-1) were purchased from Jinan Pengyue Company (Jinan, China) and fed under controlled conditions at 23 °C and a light:dark cycle of 12 h:12 h. All the procedures and protocols were supported by the Animal Ethics Committee of Qingdao Agricultural University.

Animal models, oocyte retrieval, and producing offspring production

Four-week-old mice were randomly divided into three groups, and animal models were produced as shown in Fig. 1A. Briefly, each mouse was intraperitoneally injected with 7.5 IU PMSG (Pregnant Mare Serum Gonadotropin, Ningbo Hormone Product Co. Ltd., China), and 7.5 IU hCG (Human Chorionic Gonadotropin, Ningbo Hormone Product Co. Ltd., China) 46–48 h later after PMSG. The interval between two superovulation cycles was seven days. According to the super-ovulated cycles, the groups were, respectively, marked as R1 (one super-ovulated cycle), R3 (three super-ovulated cycles), and R5 (five super-ovulated cycles). At the last injection of hCG, mice were mated with normal male mice immediately to produce offspring, or used to retrieve metaphase II (MII) oocytes 13–14 h later. Ovulated cumulus-oocyte complexes (COCs) were collected from the oviduct ampulla. The surrounding cumulus cells were removed by incubating COCs with 0.1% hyaluronidase. The cleaned oocytes were collected and stored at − 80 °C immediately.

For germinal vesicle oocytes (GV) oocytes, COCs were collected from ovaries at 46–48 h after the last injection of PMSG. The cumulus cells were removed via capillary pipetting.

Transcriptome analysis of oocytes

Smart-seq was used to investigate the transcriptome of the oocytes and was performed by Gene Denovo Biotechnology Co. (Guangzhou, China). Fifty MII oocytes retrieved from ≥ five female mice were pooled together for each sample, and each group contained three replicates. Total RNA was purified using a TRIzol reagent kit (Invitrogen, USA) according to the handling book. During the purification of total RNA, genomic DNA was removed using DNase. RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and gel electrophoresis. The purified RNA was reversely transcribed into cDNA using the NEBNext Ultra RNA library Prep Kit for Illumina (NEB, #7530, USA). The cDNA library was sequenced using the Illumina Novaseq 6000 platform. Raw reads were filtered by fastp (version 0.18.0). Clean data were mapped to the reference genome using HISAT2.2.4 with "-rna-standness RF" and other parameters set to defaults. The mapped reads were assembled by StringTie v1.3.1 in a reference-based approach. The expression abundance and variation in transcription regions were quantified by the fragment per kilobase of transcript per million mapped reads (FPKM) value, which was calculated using RSEM software. Differential expression analysis was performed with DESeq2 software [22] via reads count, and differentially expressed genes (DEGs) with false discovery rates (FDR) ≤ 0.05 and absolute fold changes ≥ two were identified. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using Omicsmart platform (https://www.omicsmart.com/). Trend analysis was performed by ShortTime-series

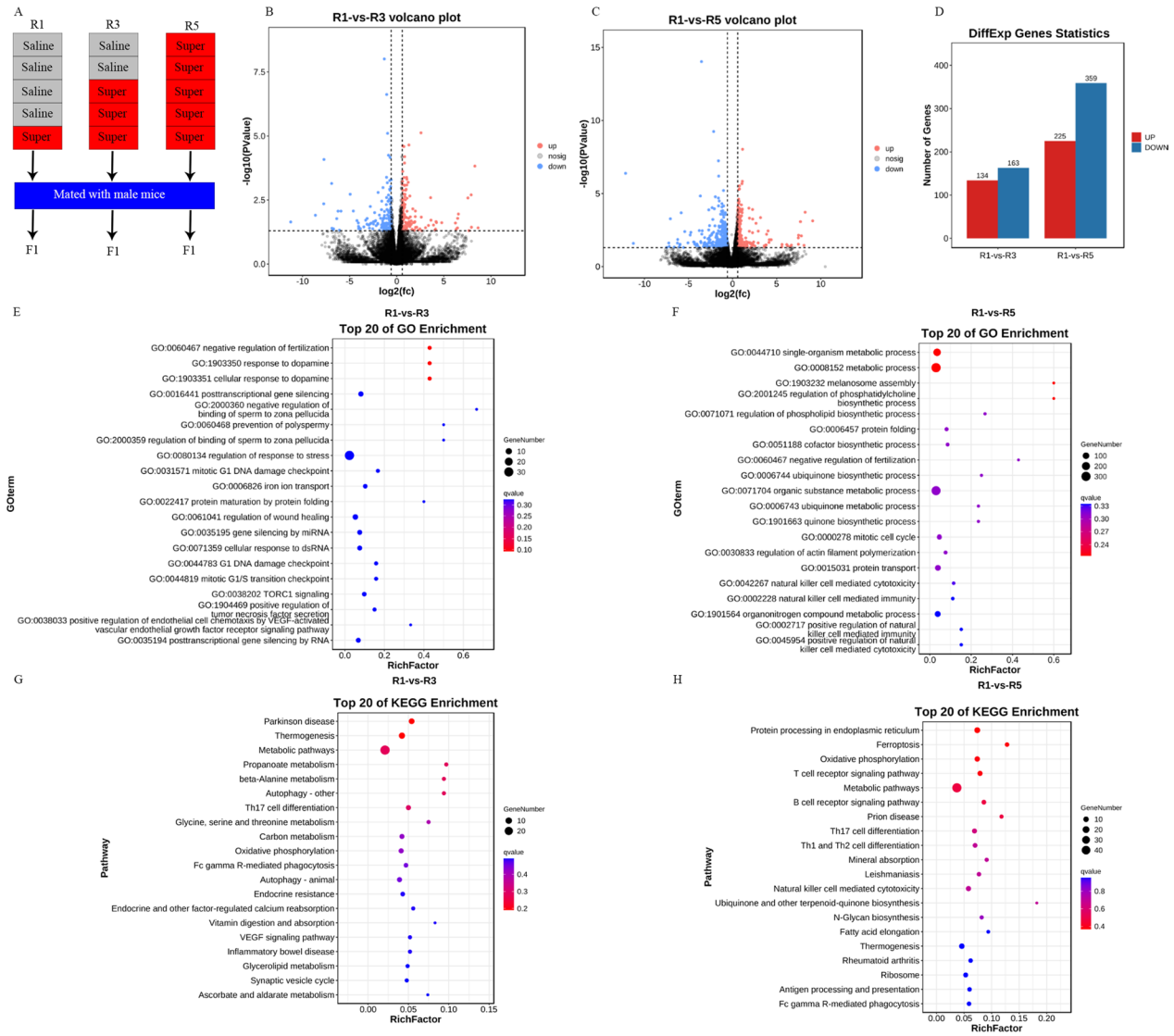

Fig. 1 Multi-superovulation has adverse effects on the transcriptome of oocytes. A schedule of repeated superovulation and producing offspring; **B** and **C** differential expression genes (DEGs) are identified in R3 and R5; red, up-regulated genes; blue, down-regulated genes; **D** numbers of DEGs in R3 and R5; **E** and **F** GO analysis of DEGs in R3 and R5; (G and H) KEGG analysis of DEGs in R3 and R5

Expression Miner software with the parameters (-pro 20 -ratio 1.0000 [log2(2) = 1, log2(1.5) = 0.5849625, log2(1.2) = 0.2630344]).

Poly (A) tail assay

A poly (A) tail assay (PAT) was performed according to a previous study [19, 21] with minor modifications. Briefly, total RNA was purified from 100 oocytes using the RNAprep Pure Micro Kit (Tiangen, #DP420, Beijing, China). Adaptor P1 (5'-P-GGTCACCTTGATCTG AAGC-NH2–3') was anchored at the 3' end with T4 RNA ligase (NEB). Then, it was used for cDNA synthesis using Hifair III 1st Strand cDNA Synthesis Kit (Yeasen, #11139ES60, Shanghai, China) with the P1-antisense primer P2 (5'-GCTTCAGATCAAGGTGACCTTTTT -3'). The product was used as template to amplify specific genes with gene-specific primer and P2 primer at the following conditions: 30 s at 94 °C, 30 s at 58 °C, and 40 s at 72 °C for 35 cycles. Then, the products were subsequently examined using 2.5% agarose gel electrophoresis. The primers used are shown in Table S1.

Whole-genome bisulfite sequencing (WGBS-seq) for small samples

To investigate the whole-genomic DNA methylation in oocytes, WGBS-seq for small samples was used [23].

After hCG injection, the mice were killed by cervical dislocation and the metaphase II (MII) oocytes were collected from the ampulla of fallopian tube. 100 MII oocytes (mouse number: R1:R3:R5 = 10:20:40) were pooled together for each sample. The samples were subsequently lysed with lysis buffer for 1 h at 37 °C. Bisulfite treatment was performed on the lysed samples using EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer's instructions. The treated DNA was ligated with adapters, and then it was used as a template to construct a sequencing library. The quality of the library was evaluated using a Qubit 3.0 fluorometer, Agilent 2100 Bioanalyzer, and StepOnePlus™ Real-Time PCR System. The library was sequenced using a Hiseq 2500, which was performed by ANOROAD.

To obtain clean data, the raw data were filtered via Trimmomatic(v0.36) software to remove the low quality and adapter sequences. The clean data were mapped to the mouse reference genome (GRCm38) using Bismark(v0.16.3) in paired mode with Bowtie2. The unique mapped reads were used for further analysis. CGIs (CpG islands) and repeat elements were extracted from the UCSC Genome browser. The CGI shore was defined as a 2k region around the CGI, and promoter was defined as the upstream 2k region from transcriptional start site. Differentially methylated regions (DMRs) were identified using DSS software based on β-binomial distribution model and tested by Wald method. DMRs were recognized as fragment length ≥ 200 bp, absolute difference of methylation between groups ≥ 0.25, CpG sites ≥ 5, and absolute Areastat value ≥ 100. KEGG analysis was performed using the online tools (https://www.omicshare.com/tools/Home/Soft/pathwaygsea).

Bisulfite treatment and sequencing

Bisulfite treatment of the oocytes was performed as described in our previous study [24]. Briefly, MII oocytes were retrieved from the ampulla of fallopian tube. Each sample included five oocytes. The samples were incubated with lysis buffer for 37 min, and then treated with 3 M NaOH for 15min. After that, the mixture was embedded in 2% low melting agarose beads. Beads treated with bisulfite for 4 h were washed with TE (Tris–HCl and EDTA), 0.3 M NaOH, and water, respectively. Then, beads with DNA were used as template for nest PCR. PCR products from at least 20 samples were pooled together, and then cloned to a T-vector for sequencing. Sequencing was repeated more than four times for each target sequence. At least ten available clones were used for each target sequence. The methylation level was analyzed using BiQ Analyzer which could filtered the possible repeated sequences.

Body weight, and glucose (GTT) and insulin tolerance test (ITT)

Body weight (g) was tested at the age of birth, three weeks, six weeks, eight weeks, and 12 weeks, respectively. GTT and ITT were performed at eight weeks of age as described in previous studies [24]. Briefly, glucose at a dose of 2 g/kg body weight was given by intraperitoneal injection after 16 h fasting. Blood glucose levels were tested at 0, 30, 60, 90, and 120 min after injection, respectively. ITT was carried out after 4 h fasting. Insulin (Actrapid®, Novo Nordisk) was injected at 10 IU/kg body weight, and blood glucose levels were tested at 0, 30, 60, 90, and 120 min after injection, respectively.

Statistical analysis

The data are shown as the means ± SD or percentages. Statistical comparisons were performed using GraphPad Prism (version 9.0) and an online tool (https://www.socscistatistics.com/tests/). The statistical significance of the data was tested with two-tailed Student's t tests, chi-square tests, one-way ANOVA, or Fisher's exact tests, as appropriate. Statistical significance was recognized as $p < 0.05$.

Results

Repeated superovulation disturbs the transcriptome of oocytes

Our previous studies have demonstrated that multi-superovulation has adverse effects on follicular development, cumulus cell function, oocyte maturation and early embryo development. Here we generated a mouse model (Fig. 1A), and examined the influence of repeated superovulation on the transcriptome of oocytes using smart-seq. The results of Pearson correlation analysis and principal component analysis (PCA) of the samples are shown in Fig. S1, which revealed that the transcriptomes of groups were distinguishable. We obtained a total of 405 million clean reads, the mapping rate was ≥ 93.74%, and ≥ 57.51% of the 22,186 genes were covered. We found that gene expression in R3 and R5 oocytes was affected (Fig. 1B, C). Compared with those in R1, 297 differentially expressed genes (DEGs) were identified in R3 including 134 upregulated and 163 downregulated DEGs, and 584 DEGs were identified in R5 including 225 upregulated and 359 downregulated DEGs (Fig. 1B, C). With increasing of superovulation times, more DEGs were identified in the oocytes (Fig. 1D). A total of 93 DEGs were overlapped between R3 and R5 (Fig. S2). To understand the functions of the DEGs, we analyzed their enrichment. GO analysis revealed that DEGs were enriched in fertilization and metabolic processes

in R3 and R5 (Fig. 1E, F). KEGG analysis showed that the DEGs enriched in metabolic pathways in R3 and R5 (Fig. 1G, H). The 93 overlapping DEGs were mainly associated with cellular and metabolic processes, and were significantly enriched in the fertilization process (Fig. S3). These results suggest that repeated superovulation affects the transcriptome of oocytes, which may reduce oocyte quality.

Expression trend analysis

To further understand the effects of the number of superovulation cycles on gene expression, we analyzed the expression trends of genes using ShortTime-series Expression Miner [25]. A total of 455 of 937 genes were filtered (fold change ≥ 2 and correlation between repeats > 0). A total of 482 genes were included in the trend analysis. The genes were clustered into 8 profiles (Fig. 2A–H), and the expression of genes in profiles 0,

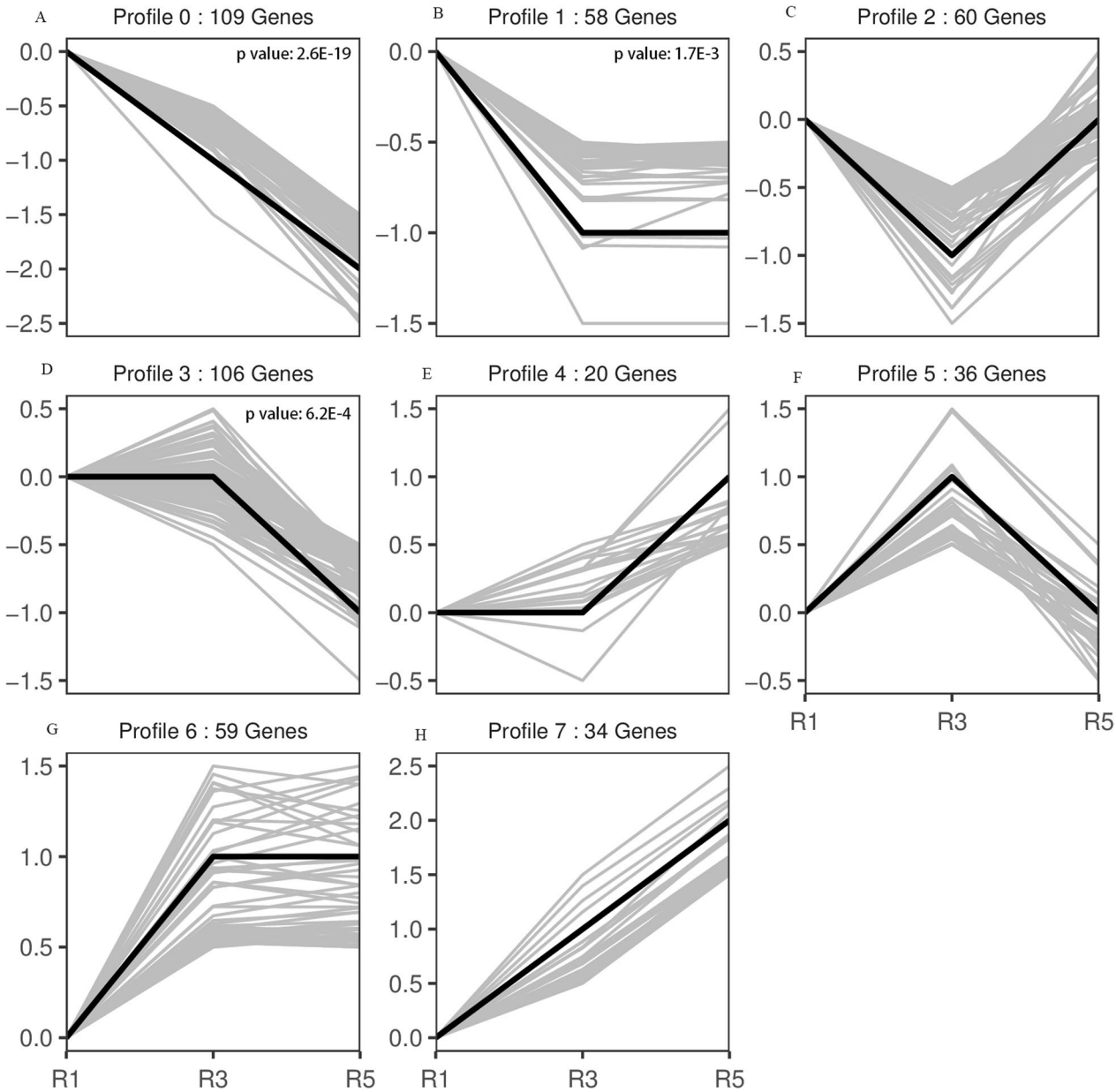

Fig. 2 Trend analysis of the groups. The expression trends in R1, R3 and R5 were analyzed using ShortTime-series Expression Miner software. Genes were divided into eight profiles (**A**–**H**). With the increase of superovulation time, the expression of genes was significantly reduced in profiles 0, 1, and 3 (**A**, **B** and **D**)

1 and 3 was significantly reduced with the increase of superovulation times (Fig. 2A, B and D). We further analyzed the enrichment of genes in profiles 0, 1, and 3. GO analysis showed that genes in profile 0 were enriched in metabolic and reproductive processes including prevention of polyspermy, negative regulation of fertilization, and isocitrate metabolism (Fig. S4A). The genes in profile 1 were enriched in protein maturation and glycosylation (Fig. S4B), and the genes in profile 3 were enriched in osteoblast differentiation, and catalytic processes (Fig. S4C). KEGG analysis showed that genes in profile 0 were enriched in protein processing, and acid metabolism (Fig. S4D), genes in profile 1 were enriched in glycan biosynthesis, and ECM-receptor interaction (Fig. S4E), and genes in profile 3 were enriched in the B-cell receptor signaling pathway, and histone metabolism (Fig. S4F). These results suggest that there is a decreasing trend in the expression of genes in oocytes induced by multi-superovulation.

Analysis of the possible reasons for the reduced oocyte maturation rate

Our previous studies report that the increase of superovulation times reduces the oocyte maturation rate and early embryo development [16, 18]. To explore the possible reasons for the reduced oocyte maturation rate, we analyzed DEGs involved in oocyte meiosis. We identified 2 DEGs in R3 including anaphase-promoting complex subunit 6 (*Apc6*) and mitogen-activated protein kinase 3 (*Mapk3*), and 6 DEGs in R5, including *Apc5/8*, *Mapk3*, RING-box protein 1 (*Rbx1*), serine/threonine-protein phosphatase 2B catalytic subunit (*Cna*), and F-box protein 5 (*Fbxo5*, also known as *Emi1*). As shown in Fig. 3A, the MAPK signaling pathway is crucial for oocyte maturation because it regulates the activation of APC [26]. Activated APC drives the degradation of cyclin B1 and securing, which activates separase to regulate chromosome separation. EMI1 can inhibit the function of APC to regulate oocyte maturation [27]. Although the increased expression of *Rbx1* and decreased expression of *Emi1* and *Mapk3* may activate APC in R5 oocytes, the expression of APC was reduced (Fig. 3B, D). These findings suggest that the altered expression of these genes might not be the main reason for the reduced oocyte maturation rate in R5.

Disruption of metabolism in oocytes has adverse effects on oocyte quality [21]. In the present study, KEGG analysis showed that DEGs were enriched in metabolic pathways. We identified 28 DEGs in R3 and 49 DEGs in R5, which were associated with energy metabolism, lipid metabolism, carbohydrate metabolism, and amino acid metabolism (Table S2, and Table S3). Li et al. reported that FAO was enhanced in the meiotic resumption of oocytes [21]. Here, we found that the expression of aldehyde dehydrogenase family 7 member A1 (*Aldh7A1*), which is related to fatty acid degradation, was decreased in R3 and R5 (Fig. 3E). During oocyte maturation, a reduction in PUFAs is crucial. PUFA is catalyzed by phospholipase A2 (PLA2) [21]. Transcriptomic analysis revealed that the expression of PLA2 receptor 1 (*Pla2r1*) was increased in R5 oocytes (Fig. 3E). These findings suggest that repeated superovulation may induce PUFAs accumulation in R5 oocytes. High levels of PUFAs may reduce the oocyte maturation rate via decreased the expression of NFKB activating protein (NKAP), which inhibits mRNA degradation by decreasing BTG4 [21]. We analyzed the transcriptomic data and found that the expression of *Nkap* was respectively reduced by approximately 25% and 20% in R3 and R5, respectively, although the difference was not statistically significant (Fig. 3E). Degradation of transcripts is crucial for meiotic resumption, and BTG4 is an important component that triggers oocyte mRNA decay via the deadenylation of poly(A) tails [19, 20]. However, the expression of *Btg4* was similar among the groups (Fig. 3F). Nevertheless, we found that the expression of the mRNA surveillance pathway genes, poly(A) binding protein nuclear 1 (*Pabpn1*) in R3 oocytes and *Pabpn1*, RNA binding protein with serine rich domain 1 (*Rnps1*), regulator of nonsense transcripts 1 (*Upf1*), cleavage stimulation factor subunit 2 (*Cstf2*) and RNA-binding protein Musashi (*Msi*) in R5 oocytes, was altered (Fig. 3E-G). During nuclear polyadenylation, *Cstf2* plays a key role in initiating polyadenylation and *Pabpn1* contributes to limiting the length of the poly(A) tail at 200–250 nt [28]. Here we found that the expression of *Cstf2* was increased and that of *Pabpn1* was decreased in R5 oocytes (Fig. 3E). Transcriptomic analysis also showed that the expression of *Msi2* [29], which mediates cytoplasmic polyadenylation was decreased and that the expression of *Upf1* [30] mediating mRNA decay was increased in R5 oocytes (Fig. 3E, G). These results suggest that the poly(A) tail sizes of mRNAs in oocytes may be affected by repeated superovulation via polyadenylation and deadenylation. To address this question, we examined the polyadenylation length of several transcripts in oocytes (*Padi6*, *Cdk1*, *Eif6*, *Pabpc11*, and *Cops3*) using the poly(A) tail (PAT) assay (Fig. 3H, I). Consistent with the findings of a previous study, poly (A) tails were shorter in control MII oocytes than in GV oocytes; but the poly (A) size of *Pabpc11* in R3 and R5 GV oocyte was greater than that in R1, and it was shorter for *Cdk1* in R3 and R5 GV oocytes compared with R1. The deadenylation of *Eif6*, *Cdk1*, and *Cops3* in R3 and R5 MII oocytes may

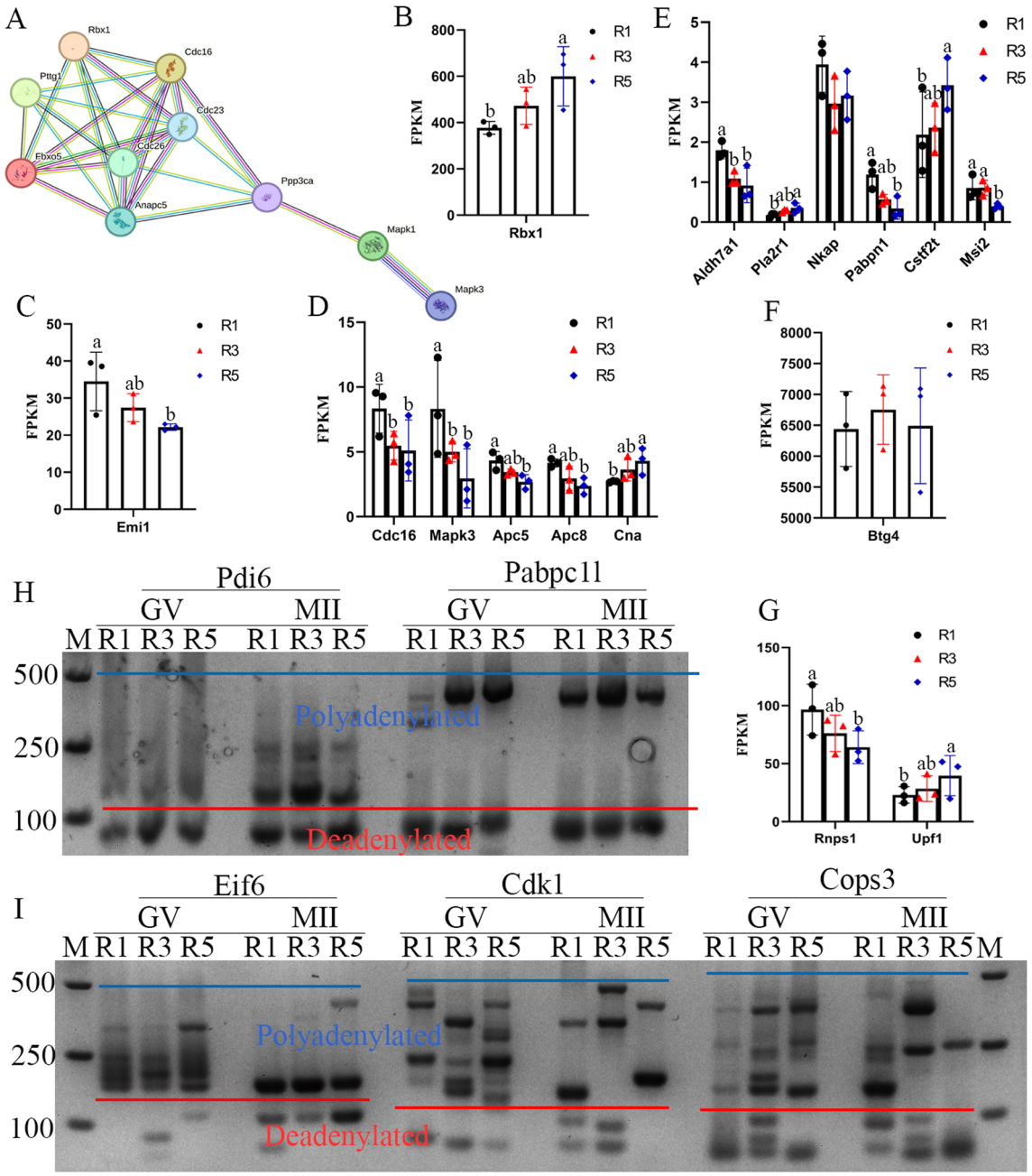

Fig. 3 Analysis of DEGs associated with oocyte maturation. **A** Protein–protein interaction network of several key genes involved in meiotic resumption; colored nodes, query proteins and first shell of interactors; empty nodes, proteins of unknown 3D structure; filled nodes, a 3D structure is known or predicted; color line, known or predicted interaction and others between proteins; **B**–**G** expression of related genes is analyzed using PFKM; same letter, there is no significant difference between groups; different letter, there is significantly statistical difference between groups; **H**, **I** poly (A) tail size analysis of several genes in GV and MII oocytes; M, DNA marker; numbers on the left of the panel, size of the band (bp)

be blocked compared with those in R1 (Fig. 3H). These data indicate that disturbed metabolism and mRNA degradation might explain the decreased oocyte maturation rate induced by repeated superovulation.

Multi-superovulation alters whole-genome methylation of oocytes

A previous study reported that superovulation at a lower dose of hormones (< 10 IU per mouse) doesn't affect

the methylation patterns of imprinted genes [11]. Nevertheless, the effects of multi-superovulation on global genomic methylation in oocytes are still obscure. Here, we investigated the influence of repeat superovulation on the methylation of oocytes at a base-resolution using a whole-genome bisulfite sequencing (WGBS-seq) approach for small samples. 100 MII oocytes from at least 10 mice were pooled together for each sample. We obtained 700 million clean reads, the Q30 value was 90.89%, and the percentage of clean GCs proportion was 25.39%. Clean reads were mapped to the reference genome mm10 using Bismark (v0.16.3), and the mapped ratio and unique mapped ratio were 34.82% and 32.05%, respectively. The genomic methylation levels of mapped C in R3 and R5 was lower compared with those in R1 (Fig. 4A). According to the context, Cs are classed into three types, CG, CHG, and CHH (H = A, T, or G). Compared with R1, there was a slight increase in the genomic CG methylation level in R3 oocytes, but there was a slight decrease in the CG methylation level in R5 oocytes (Fig. 4B). The methylation levels of CHG and CHH was decreased with the multi-superovulation (Fig. 4B). After that, we further analyzed the methylation level of CG in the genome regions, and the results revealed that there was an increase in the methylation level in the 3'-UTRs of R3 and R5 oocytes (Fig. 4C). The methylation level of CGIs in R3 and R5 oocytes was increased compared with those in R1 (Fig. 4D). The methylation levels of repeat regions in R5 oocytes were also lower compared with R1 oocytes (Fig. 4E). These results indicate that multi-superovulation alters the whole genome DNA methylation of oocytes.

Differentially methylated region (DMR) analysis

To better evaluate the effect of multi-superovulation on the epigenetic landscape, we analyzed the DMRs in oocytes using DSS software. Compared with R1, we respectively identified 2006 DMRs for R3 including 1085 (54.09%) hyper-DMRs and 921 (45.91%) hypo-DMRs (Fig. 5A), and 2208 DMRs for R5 including 1178

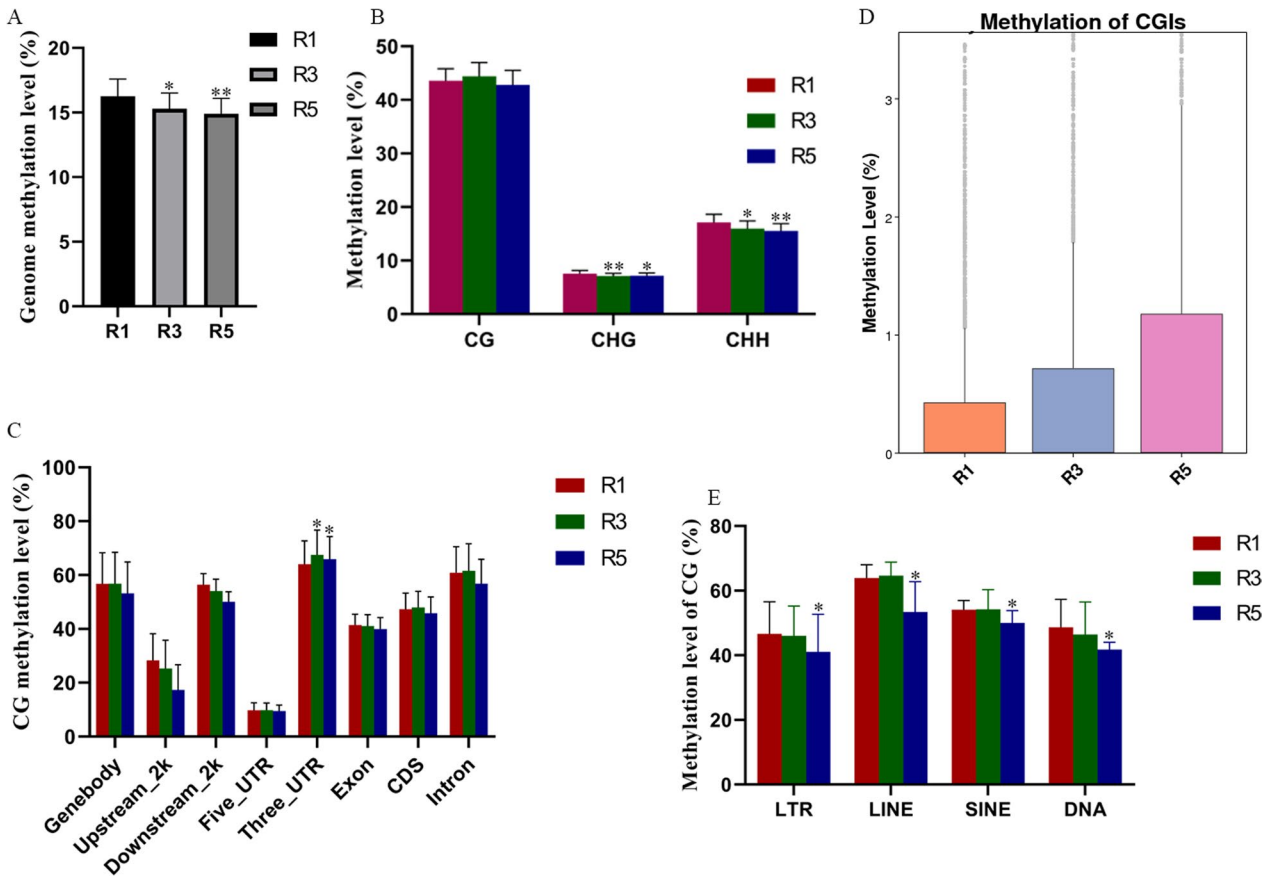

Fig. 4 Whole-genome DNA methylation in oocytes. **A** Whole-genome methylation level of all mapped cytosine; **B** there are three types of C (cytosine) including CG, CHG, and CHH (H = A, T, or G), and the whole-genome methylation of these types of C are presented; **C** whole-genomic methylation level of CG in the genomic regions of genes; **D** the genomic methylation level of CpG islands (CGIs) is analyzed; **E** the CG methylation levels of repeat regions. *$p < 0.05$, **$p < 0.01$

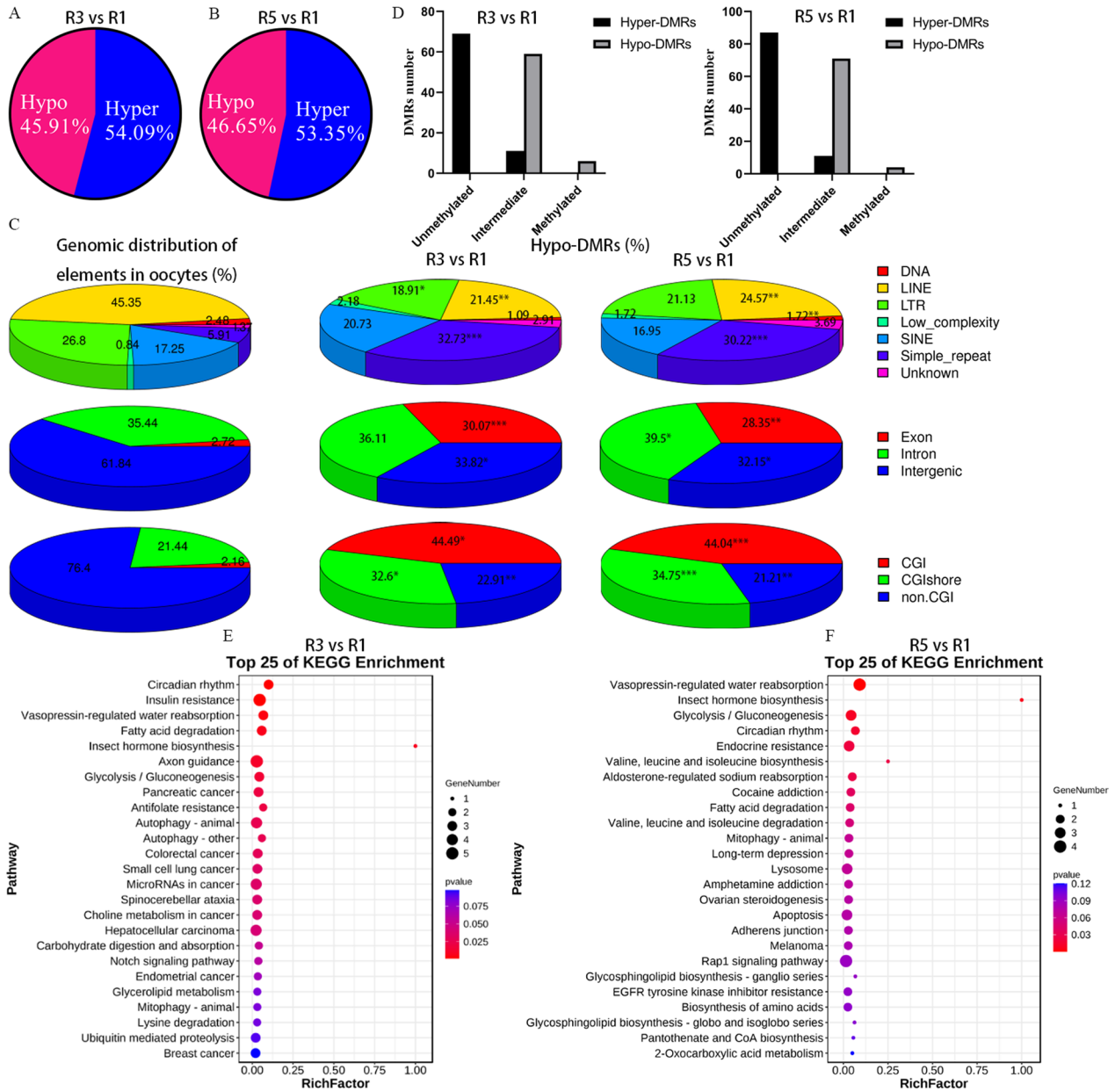

Fig. 5 Differentially methylated region analysis. **A** and **B** Compared with R1, the differentially methylated regions (DMRs) are identified in R3 and R5; hyper, hyper-DMRs; hypo, hypo-DMRs; **C** the distribution of DMRs in genomic regions is analyzed; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **D** according to the methylation level, CGIs are divided CGIs into three classes: unmethylated (methylation < 20%), intermediate (20% ≤ methylation ≤ 80%), and methylated (methylation > 80%). Hyper-DMRs are mainly distributed at unmethylated CGIs, and hypo-DMRs are mainly distributed at intermediate CGIs; **E** and F the KEGG enrichment of genes with DMRs at promoters is analyzed

(53.35%) hyper-DMRs and 1030 (46.65%) hypo-DMRs (Fig. 5B). We further examined the distribution of DMRs at genomic elements. For R3 and R5, hyper-DMRs were significantly enriched in simple repeats, exons, CGIs and CGI shores, but depleted from LINEs (long interspersed nuclear elements), LTRs (long terminal repeats), intergenic regions, and non-CGIs (Fig. S5). Notably, hypo-DMRs of R3 were enriched in repeats, exons, CGIs and CGI shores, but depleted from LINEs, LTRs, intergenic regions, and non-CGIs (Fig. 5C). For R5, the hypo-DMRs were significantly depleted from DNA transposons, LINEs, intergenic regions and non-CGIs, but enriched in repeats, exons, introns, CGIs and CGI shores (Fig. 5C). These results suggest that DMRs are not

randomly distributed throughout the genome in R3 and R5 oocytes.

CGIs are common at gene promoters in the vertebrate genome, and are typically in an unmethylated state. Hyper-methylated CGIs would lead to gene silencing by inhibiting the binding of transcription factors with promoters [31]. Therefore, we divided CGIs into three classes: unmethylated (methylation < 20%), intermediate (20% ≤ methylation ≤ 80%), and methylated (methylation > 80%). We further analyzed the distribution of DMRs in CGIs, and found that hyper-DMRs in R3 and R5 oocytes were distributed mostly in unmethylated CGIs and hypo-DMRs were distributed mainly in intermediate CGIs (Fig. 5D). These results indicate that unmethylated and intermediate CGIs are prone to be affected by multi-superovulation in oocytes.

To better understand the potential biological role, we analyzed the enrichment of genes with DMRs in promoters in KEGG pathways. The results revealed that genes with DMRs in promoters were enriched in insulin resistance, endocrine resistance, and metabolic pathways (such as fatty acid degradation and glycolysis/gluconeogenesis) in R3 and R5 oocytes (Fig. 5E and F) which suggests that the offspring might have a high risk of metabolic disorders.

To further confirm the methylation of DMRs, four DMRs were analyzed using bisulfite sequencing (BS) as described in a previous study [32]: DMR1, DMR2 and DMR3 were hyper-DMRs and respectively located at Chr11: 72,095,768–72,096,262, Chr18: 10,553,105–10,553,724, and Chr1: 85,832,423–85,833,055. DMR4 is a hypo-DMR and located at Chr5: 124,262,048–124,263,233. Compared with R1 oocytes, DMR1 methylation level was significantly increased in R3, but it was similar between R1 and R5 oocytes (Fig. S6). The methylation of DMR2 was not significantly affected by multi-superovulation in oocytes (Fig. S6). In R3 and R5 oocytes, the methylation level of DMR3 was significantly higher than that in R1 oocytes (Fig. S6). The methylation level of DMR4 in R3 and R5 oocytes was significantly higher than that in R1 oocytes (Fig. S6). These results suggest that multi-superovulation has adverse influence on DNA methylation of oocytes.

Offspring metabolism is disturbed by multi-superovulation

Children produced via assisted reproductive technologies have a higher risk of metabolic dysfunction, which may be associated with glucose metabolism and cardiovascular functions [4, 6]. KEGG analysis also showed that genes with DMRs in promoter regions in R3 and R5 oocytes were enriched in metabolic pathways (Fig. 5E–F). Therefore, we examined the influence of multi-superovulation on offspring, and we found that the mean number of pups per litter in R5 was significantly lower than that in R1 and R3 (Fig. 6A). The average body weight of female offspring at 12 weeks of age was similar in R1, R3, and R5, but it was higher in R3 at six and eight weeks of age than that in R1 (Fig. 6B). For male offspring, the average body weight in R3 and R5 was significantly higher than that in R1 at six weeks of age, but it was similar in groups at 8 and 12 weeks of age (Fig. 6C). We also examined the glucose and insulin tolerance (GTT and ITT) in offspring. For females, the blood glucose level of the GTT at 0 min was significantly higher in R5 than in R1, and the blood glucose level of the ITT in R5 was significantly higher than that in R1(Fig. 6D and E). The GTT and ITT in R3 were not significantly different from that in R1 (Fig. 6D and E). The GTT and ITT in R5 male offspring were also significantly altered compared with those in R1, and they were similar between R1 and R3 (Fig. 6F and G). These results suggest that multi-superovulation has adverse effects on the metabolism of offspring.

Discussion

Controlled ovarian stimulation is widely used in assisted reproductive technologies (ARTs). ARTs have become an effective method to treat infertility and subfertility in the clinic. Some women would undergo several controlled ovarian stimulation cycles to obtain a baby. In domestic animals, superovulation is also widely used. Currently, the ovum-pick-up and in vitro production (OPU-IVP) is used to produce bovine and porcine embryos, but exogenous FSH is still commonly used. However, few studies have focused on the influence of repeated superovulation on oocyte quality and offspring health. In the present study, we found that multi-superovulation altered the transcriptome and disrupted whole-genome DNA methylation in oocytes, and that the metabolism of offspring was also influenced.

To increase the number of oocytes harvested from human and domestic animals, superovulation is widely used, and some females will undergo repeated superovulation in their life. However, superovulation disturbs the proteome of oocytes in mice [33], and has an adverse influence on oocyte maturation in cattle [34]. A high dose of FSH reduces ATP levels in rabbit oocytes [35], and abnormal microfilament formation is detected in hamster oocytes after superovulation [36]. Repeated superovulation induces ovarian dysfunction and decreases oocyte quality [37, 38]. Our previous studies reported that multi-superovulation accelerated primordial germ cell activation, increased follicular atresia, induced mitochondrial dysfunction in granulosa cells, reduced oocyte harvest and maturation rates, and disrupted histone modifications in early embryos [16–18]. However, the mechanisms underlying the lower oocyte maturation rate

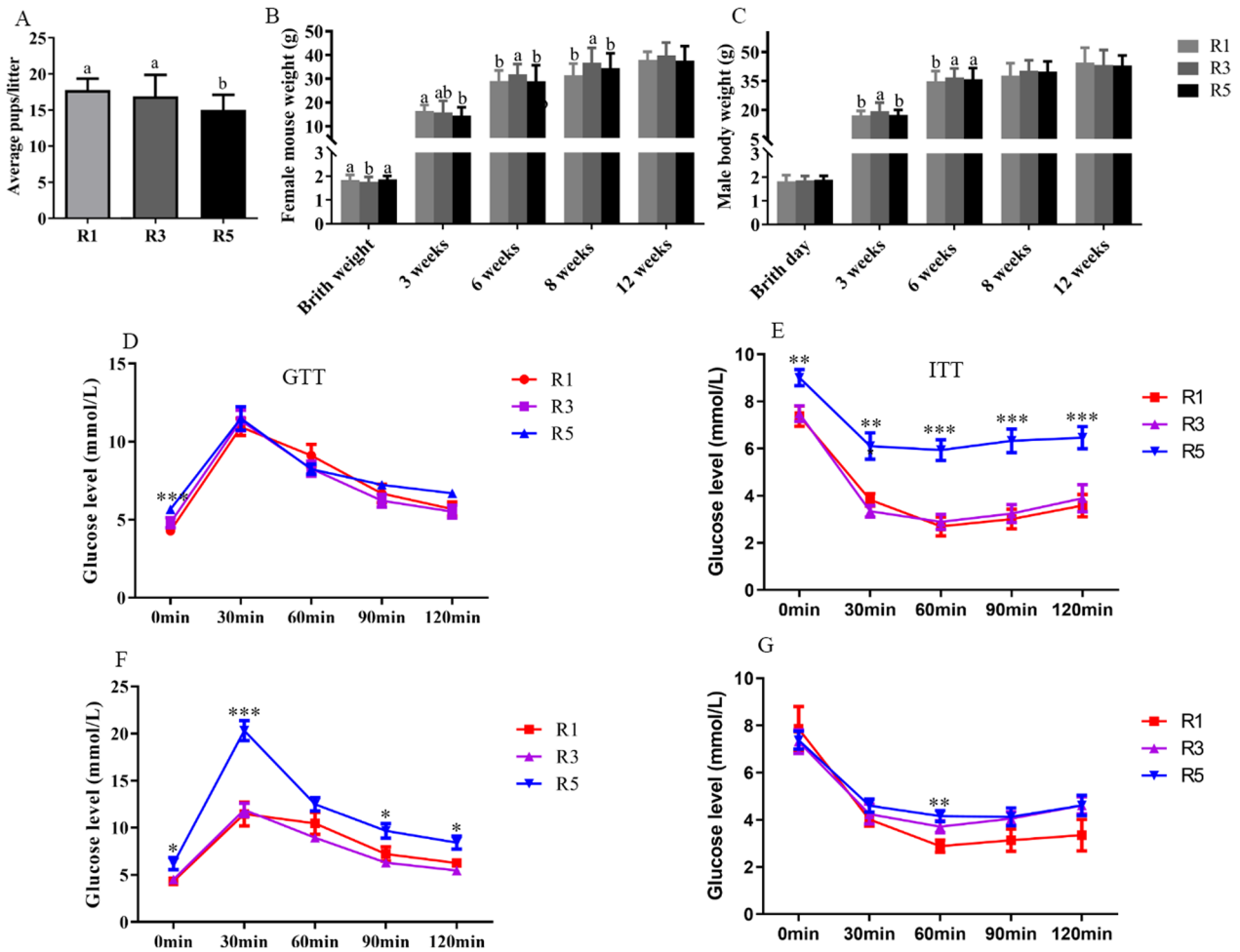

Fig. 6 Effects of repeated superovulation on offspring metabolism. **A** With the increase of superovulation times, the average number of pups per litter were reduced; the same letter means no significant difference between groups, and different letters mean significant difference between groups; **B** and **C** trends of bodyweight changes at different ages for female and male offspring; **D** and **E** effects of multi-superovulation on female offspring glucose and insulin tolerance at eight weeks of age; **F** and **G** effects of multi-superovulation on male offspring glucose and insulin tolerance at eight weeks of age; the same letter means no significant difference between groups, and different letter means significant difference between groups; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

induced by repeated superovulation are not well understood. In the present study, we find multi-superovulation disturbs transcriptome of oocytes. The DEGs in oocytes induced by repeated superovulation are associated with metabolism, and fertilization. These findings suggest that the disturbed gene expression in oocytes might be an important reason for the low oocyte quality induced by repeated superovulation.

There is a dynamic of metabolism during oocyte maturation, such as increased utilization of fatty acids in the metaphase II stage, a reduction of bile acids and their derivatives, and a decrease in polyunsaturated fatty acids (PUFAs) [21]. The reduce of PUFAs such as arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is crucial for oocyte maturation. High levels of PUFA may reduce the oocyte maturation rate via decreased NFKB activating protein (NKAP) expression, which disrupts spindle organization and chromosome congression, and inhibits mRNA degradation by decreased BTG4 [21]. BTG4 is an important component to trigger oocyte mRNAs decay by deadenylation of their poly(A) tails [19, 20]. PUFAs are released from the phospholipid membrane catalyzed by phospholipase A2 (PLA2), which is translocated from the cytoplasm to the endoplasmic reticulum, Golgi apparatus, or nuclear membrane. PUFA catabolism is catalyzed by cyclooxygenase enzymes (COX1/2) and lipoxygenase (LOX). COX and LOX inhibition reduce the oocyte maturation rate, and more PUFAs block oocytes at meiosis I stage [21]. We found that the expression of *Pla2ra* in R5 oocytes was

increased. The expression of genes involved in mRNA surveillance pathways and polyadenylation, such as *Cstf2*, *Pabpn1*, *Msi2,* and *Upf1*, is altered in R5 oocytes. These results indicate that disrupted metabolism and mRNA degradation may be a reason for the reduced oocyte maturation rate induced by repeated superovulation.

DNA re-methylation is an essential event during oogenesis. Abnormal DNA methylation state in oocytes is associated with fetal development abnormalities and a high risk of metabolic diseases in offspring. But the DNA re-methylation process is prone to be disturbed by the female environment. For example, maternal diabetes and obesity result in abnormal DNA methylation in oocytes [32, 39]. Environmental pollutants also have adverse effects on the DNA methylation of oocytes [40, 41]. Lopes et al. reported that superovulation has no significant influence on genomic methylation of bovine oocytes, but the DNA methylation level is altered at some imprinted genes [42]. Low doses of eCG and hCG have no significant effects on methylation in zygotes and imprinted genes in mouse oocytes [13]. Whereas, contrary results have also been reported by studies [13]. These indicate that there are debates on the effects of superovulation on the DNA methylation of oocytes. In the present study, we found that the whole DNA methylation of oocytes is disrupted by multi-superovulation, and with the increase of superovulation times, more DMRs are identified. Genes with DMRs in promoter regions are enriched in metabolic pathways and endocrine resistance. These suggest that the disturbed DNA methylation in oocytes induced by multi-superovulation might result in metabolic disorders in offspring. The association between DNA methylation and metabolic diseases has been demonstrated by studies, and the intergenerational inheritance of abnormal DNA methylation via oocytes is also demonstrated [24, 43]. Thus, we hypothesize that the disturbed DNA methylation in oocytes induced by repeated superovulation might be a reason for the metabolic disorders in R5 offspring. However, the direct evidence is not detected in the present study, and the effects of the uterine environment on offspring health were not excluded. Therefore, more studies are needed in the future.

Although we find multi-superovulation disturbs the transcriptome and whole-genomic DNA methylation of oocytes, there are many limitations to the present study. In the present study, we pooled oocytes together for transcriptome and methylome analysis, which cannot represent a single oocyte. Abnormal gene expression might be an important reason for the low oocyte quality induced by repeated superovulation, but the key genes and molecular mechanisms involved have not been elucidated. The underlying mechanisms of disturbed DNA methylation in oocytes are still obscure, and more studies are necessary to explore the long-term effects of repeated superovulation on offspring health. Nevertheless, it is important to explore how to alleviate the adverse influence of multi-superovulation on offspring health in the future.

Abbreviations

DEG	Differentially expressed gene
DMR	Differentially methylated region
R1	Superovulation one time
R3	Superovulation three times
R5	Superovulation five times
GTT	Glucose tolerance test
ITT	Insulin tolerance test
scBS-seq	Single-cell bisulfite sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-024-01746-3.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
We acknowledge the use of Center Experimental Platform of Qingdao Agricultural University and College of Life Science and thank Tao Xu for his invaluable help; we thank all the other people in the labs for their invaluable help.

Author contributions
Xie JK performed the animal experiments, analyzed the data, and revised the manuscript; Wang Q, Chen YH, Tang SB, and Sun HY performed the animal experiments; Ge ZJ and Zhang CL designed, supervised, analyzed the data, wrote, and revised the manuscript.

Funding
This work is supported by the Breeding Plan of Shandong Provincial Qingchuang Research Team (Innovation Team of Farm Animal Cloning 012–1622001), the Major Projects in Provincial and National Union Construction of Henan Medical Science Research Plan (SBGJ202001002), and the Doctor Foundation of Qingdao Agricultural University (6631116008).

Availability data and materials
The datasets generated and/or analyzed during the current study are available in the database BIG Sub with No. CRA012358 (https://ngdc.cncb.ac.cn/gsub/submit/gsa/subCRA019478/finishedOverview).

Declarations

Ethics approval and consent to participate
All the procedures and protocols were supported by the Animal Ethics Committee of Qingdao Agricultural University.

Consent for publication
Not applicable.

Competing interests
There are no conflicts of interest to declare.

Received: 24 April 2024 Accepted: 15 September 2024
Published online: 28 September 2024

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