Transcriptome-wide N6-methyladenine methylation in granulosa cells of women with decreased ovarian reserve

Chang Liu1†, Linshuang Li2†, Bo Yang3, Yiqing Zhao2, Xiyuan Dong2, Lixia Zhu2, Xinling Ren2, Bo Huang2, Jing Yue2, Lei Jin2, Hanwang Zhang2 and Lan Wang2*

Abstract

Background: The emerging epitranscriptome plays an essential role in female fertility. As the most prevalent internal mRNA modification, N6-methyladenine (m6A) methylation regulate mRNA fate and translational efficiency. However, whether m6A methylation was involved in the aging-related ovarian reserve decline has not been investigated. Herein, we performed m6A transcriptome-wide profiling in the ovarian granulosa cells of younger women (younger group) and older women (older group).

Results: m6A methylation distribution was highly conserved and enriched in the CDS and 3'UTR region. Besides, an increased number of m6A methylated genes were identified in the older group. Bioinformatics analysis indicated that m6A methylated genes were enriched in the FoxO signaling pathway, adherens junction, and regulation of actin cytoskeleton. A total of 435 genes were differently expressed in the older group, moreover, 58 of them were modified by m6A. Several specific genes, including BUB1B, PHC2, TOP2A, DDR2, KLF13, and RYR2 which were differently expressed and modified by m6A, were validated using qRT-PCR and might be involved in the decreased ovarian functions in the aging ovary.

Conclusions: Hence, our finding revealed the transcriptional significance of m6A modifications and provide potential therapeutic targets to promote fertility reservation for aging women.

Keywords: Granulosa cell, N6-methyladenosine, RNA modification, Aging, Ovary

Introduction

Ovary is a critical regulator of female fertility, serving as the source of oocytes and a major supplier of steroid sex hormones [1]. As a reproductive organ, ovary exhibits a rate of aging that is much faster than other somatic organs [2]. According to the human biologic clock, female fertility starts at puberty and decreases after the age of 30, with a steep decrease after 35, culminating in the menopause at 50 years of age [3, 4]. With the modern tendency of delaying childbearing, ovarian aging has become an age-related disease that entails careful considerations for reproductive care systems [5, 6].

Ovarian functional decline with aging is generally characterized by the gradual decrease in both the quantity and quality of ovarian follicles [2, 7, 8]. Multiple factors influence oocyte quality during aging [9]. Granulosa cells, which form multiple layers surrounding the oocyte and communicate with oocytes via direct contact or paracrine pathways, play critical roles in regulating oocyte competence [10]. Besides, granulosa cells also provide
m6A modifications are essential for folliculogenesis, oocyte development, and maturation [19–21]. Moreover, aged granulosa cells may inhibit oocyte potential and maturation, especially N6-methyladenine (m6A), is one of the most prevalent epigenetic modifications affecting the regulation of gene expression [18]. It has been well established that m6A modifications are essential for folliculogenesis, oocyte development, and maturation [19–21]. Besides, recent evidence indicated that m6A demethylase was decreased in the aged ovaries, suggesting that m6A methylation might participate in the process of ovarian aging [22]. However, whether m6A methylation is associated with the diminished ovarian reserve in the aged ovaries, has not been clarified.

Therefore, in the present study, we aim to characterize the m6A epigenetic profiles and their functions in the granulosa cells of naturally aging women. Granulosa cells collected from older women and younger women were used for m6A-targeted antibody coupled with high-throughput sequencing (MeRIP-Seq) and RNA sequencing (RNA-Seq). The potential functions of the modified genes were analyzed and validated using quantitative real-time polymerase chain reaction (qRT-PCR).

**Material and methods**

**Study population**

A total of twelve women were recruited in the study. Since ovarian function was gradually decreased from the mid to late 30s [23], women aged 25-30 years and received IVF treatment with GnRH antagonist protocols. Granulosa cells were collected at the time of oocyte retrieval. Women were totally informed of the procedures, and signed informed consent was obtained from all participants. This study was approved by the Institutional Review Board of Tongji Hospital (TJ-IRB20210213).

**Granulosa cell collection**

Follicular fluid aspirated from follicles of individuals during oocyte retrieval was pooled and considered as independent samples. Granulosa cells were isolated from the follicular fluid as previously described [26]. Briefly, follicular fluid was centrifuged at 1200 rpm for 10 min to precipitate cells. The cell pellets were resuspended in PBS and layered onto a 50% Percoll Reagent (Sigma-Aldrich, MO, USA) and centrifugated at 1200 rpm for 30 min to separate granulosa cells from blood cells. Granulosa cells at the interface were harvested and washed with PBS and red blood cell lysis buffer (Servicebio, Wuhan, China). After centrifugation at 1500 rpm for 15 min, granulosa cells were collected and used for RNA extraction.

**m6A-targeted antibody coupled with high-throughput sequencing (MeRIP-Seq) and RNA sequencing (RNA-Seq)**

Three pairs of samples from the two groups were used for MeRIP-Seq and RNA-Seq, while other samples were used for validation. The MeRIP-Seq was performed in accordance with the published procedure with slight modifications. Briefly, the total RNA of granulosa cells was extracted using RNA-easy Isolation Reagent (Vazyme, Nanjing, China) following the manufacturer’s instructions. The quality of extracted RNA was determined using Nanodrop spectrophotometer for RNA purity (A260/A280) and standard denaturing agarose gel electrophoresis for RNA integrity. RNA was randomly fragmented to 200 nt and purified with the RNA clean and concentrator kit (Zymo Research, CA, USA). A total of 1 μg fragmented mRNA was used for RNA-Seq, while 500 μg of fragmented RNA was incubated with anti-m6A polyclonal antibody (ABE572, Sigma-Aldrich, MO, USA), followed by immunoprecipitation with protein-G beads (10002D, Invitrogen, CA, USA) and protein-G
beads (10004D, Invitrogen, CA, USA). Bound RNA was extracted with TRIzol reagent (15596018, Invitrogen, CA, USA). Both the m^6^A immunoprecipitated samples and the input samples were used for library construction with Illumina NovaSeq 6000 sequencer (Illumina, CA, USA).

**Data analysis**

The quality of the raw reads was determined by FastQC. Trimming was performed for known Illumina TruSeq adapter sequences, poor reads, and ribosomal RNA reads. Then, clean reads were aligned to the human reference genome with the use of Hisat2 software (v2.0.4). Methylated sites on RNAs were identified by MACS software. Cutadapt (v2.5) was used to trim adapters and filter for sequences, remaining reads were then aligned to the human Ensemble genome GRCh38 (mouse Ensemble genome GRCm38) using Hisat2 aligner (v2.1.0) under parameters: “-rna-strandness RF”. M^6^A peaks were identified using exomePeak R package (v2.13.2) under parameters: “PEAK_CUTOFF_PVALUE = 0.05, PEAK_CUTOFF_FDR = NA, FRAGMENT_LENGTH = 200". Differential m^6^A peak were identified using exomePeak R package under parameters: “PEAK_CUTOFF_PVALUE = 0.05, PEAK_CUTOFF_FDR = NA, FRAGMENT_LENGTH = 200". Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed using clusterprofile R package (v3.6.0) [27–29]. m^6^A-RNA-related genomic features were visualized using Guitar R package (v1.16.0). Identified m^6^A peaks which adjusted p value <0.05 were chosen for the de novo motif analysis using homer (v4.10.4) under parameters: “-len 6 -rn”. The reads mapped the genome were calculated using feature-Counts (v1.6.3). Differential gene expression analysis was performed using the DESeq2 R-package. Differentially methylated sites with a |fold change| cutoff of >2 and an adjusted p value of <0.05 were identified by diffReps. Gene set enrichment analysis (GSEA) [30] was performed to further explore signaling pathways and cellular functions in the process of female reproductive aging.

**RNA extraction and qRT-PCR**

RNA extraction and qRT-PCR were performed following the published procedures with minor modifications [31, 32]. Briefly, RNA of granulosa cells was extracted using RNA-easy Isolation Reagent (Vazyme, Nanjing, China) following the manufacturer’s instructions. Then, 500 ng RNA was used for cDNA synthesis with the use of PrimeScript™ RT Master Mix (RR036A, Takara, Japan). qRT-PCR was performed with SYBR® Premix Ex Taq™ II (RR420A, Takara, Japan) and Light Cycler 96 instrument (Roche, Mannheim, Germany). Each sample was detected in triplicate and the relative gene expression was normalized to GAPDH. The oligonucleotide sequences of primers used in the experiments were listed in Table 5.

**Statistical analysis**

Quantitative variables were expressed as mean ± standard deviation (SD), and Student’s t-test was performed to evaluate statistical significance. Statistical significance was set at the adjusted p-value < 0.05.

**Results**

**Clinical characteristics of participants**

The clinical characteristics of samples that used for MeRIP-Seq was listed in Table 1. Besides, the detailed controlled ovarian stimulation protocols for individuals were displayed in the Supplemental Table 1. As data indicated, the age of the younger group was significantly different from that in the older group (p<0.001). Besides, AFC was significantly decreased in the older group compared to the younger group (p=0.001). No statistical significance was found in the menstrual cycle and basal hormonal levels between the two groups.

**Overview of m^6^A methylation in the granulosa cell mRNA**

Granulosa cells collected from older and younger women were used for MeRIP-Seq to obtain the transcriptome-wide m^6^A map. In the younger group, 6689, 4765, 5719 m^6^A peaks were identified in each sample, respectively. Meanwhile, there were 5243, 4703, and

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**Table 1  Clinical characteristics of participants.**

| Variables | younger (n = 3) | older (n = 3) | p-value |
|-----------|----------------|--------------|---------|
| Age (years) | 26.33±1.15 | 43.67±1.15 | <0.001 |
| Menstrual cycle (days) | 30.33±2.52 | 22.61±1.51 | 0.10 |
| FSH (mIU/mL) | 9.04±3.11 | 7.26±4.04 | 0.58 |
| LH (mIU/mL) | 6.13±3.01 | 3.75±2.30 | 0.34 |
| AMH (ng/mL) | 2.85±1.66 | 0.72±0.57 | 0.10 |
| AFC | 13.33±2.08 | 3.00±1.00 | 0.001 |

Data were presented as mean ± standard deviation (SD)

AFC antral follicle count
Fig. 1 Overview of m^6^A methylation in the granulosa cell mRNA. A The number of m^6^A peaks in each sample. B The number of m^6^A peaks in each chromosome. C Venn diagram of m^6^A peaks in younger groups. D Venn diagram of m^6^A peaks in older group. YWJL, YWYQ, YQTT, OZH, OZJM, OYDX are labels of the six participants in the both groups. Y stands for the younger group and O stands for the older group. The rest letters are initials of each participant.

Fig. 2 Different m^6^A methylation of genes in the granulosa cells of older women. A The distribution of m^6^A peaks across the length of mRNAs were compared in the two groups. B The distribution of m6A peaks in the older group. C The distribution of m^6^A peaks in the younger group. D Volcano plots display the different m^6^A methylation peaks with statistical significance (|fold change|>2 and adjusted p < 0.05). Red dots represent the hypermethylated transcripts and blue dots represent the hypomethylated transcripts. E The top 20 significantly enriched pathways for the differently methylated genes in the older group.
Fig. 2 (See legend on previous page.)
5449 detected m6A peaks in the samples of the older group (Fig. 1A). m6A methylation distribution at different chromosome loci was shown (Fig. 1B). Data showed that the most m6A methylation were chromosome 1 with 799 m6A peaks and chromosome 19 with 671 m6A peaks. The Venn diagram was provided in Fig. 1C-D. In the Venn diagram, we displayed the number of m6A methylated genes of each sample. In the older group, there were 5147 peaks with read counts more than 0 in sample OYDX and OZH, 4902 peaks with read counts more than 0 in sample OZH and OZJM, 6242 peaks with read counts more than 0 in sample OYDX and OZJM, altogether 4829 peaks with read counts more than 0 in the three samples. In the younger group, there were 5562 peaks with read counts more than 0 in sample YQTT and YWJL, 6739 peaks with reads counts more than 0 in sample YQTT and YWYQ, 5878 peaks with reads counts more than 0 in sample YWJL and YWYQ, altogether 5465 peaks with reads counts more than 0 in the three samples. Averagely, there were 1846 reads in each peak for the older group and 2149 reads in each peak for the younger group, indicating that the number of m6A methylation sites was higher in the younger group. Whereas the number of m6A methylated genes was higher in the older group (5147 genes) compared to the younger group (3248 genes; See Dataset 1 in the related files).

**Different m6A methylation of genes in the granulosa cells of older women**

The metagene profiles of different m6A peaks for the whole transcriptome visualized m6A methylated regions in the two groups. Interestingly, m6A peak distributions were similar between the two groups (Fig. 2A). Further analysis calculated the preferential localization of m6A on RNA transcripts by dividing m6A peaks into six nonoverlapping genomic regions: 5' untranslated region (UTR), transcription start site (TSS), start codon, coding sequence (CDS), stop codon, and 3' UTR (Fig. 2B-C). Data showed that the most m6A methylated peaks were concentrated in the CDS (45.1%-45.7%) and 3' UTR (27%-27.4%), followed by stop codon (21.7%-21.8%), start codon (3.7%), 5'UTR (1.5%-1.7%), and TSS (0.3%-0.4%). These results showed the highly consistent topological patterns of m6A methylation, indicating the conservation of motif recognition of m6A methylation in the granulosa cells. Here the motif refers to “GGAC” (Suppl Fig. 1).

The differently m6A methylated peaks in the older group were analyzed with the criteria of |fold change|>2 and adjusted p-value <0.05 (Fig. 2D). A total of 3005 genes with different m6A methylation in the older group compared to the younger group, with 1297 hypermethylated and 1708 hypomethylated. The top 10 hypermethylated and hypomethylated genes in the older group were listed in Table 2. The function of these differently methylated genes was annotated with GO and KEGG pathway

| gene   | pattern | chromosome | peak region | peak start | peak end | log2.FC | lg.p  |
|--------|---------|------------|-------------|------------|----------|---------|-------|
| PRPF6  | Hyper   | 20         | CDS         | 63984943   | 63994934 | 6.78    | -46.7 |
| KPNB1  | Hyper   | 17         | 3'UTR       | 47683285   | 47683436 | 5.82    | -208  |
| QKI    | Hyper   | 6          | CDS         | 163478842  | 163535115| 5.72    | -116  |
| LARP7  | Hyper   | 4          | CDS         | 112647817  | 112650501| 5.07    | -321  |
| ANP32E | Hyper   | 1          | CDS         | 150220739  | 150229200| 4.72    | -35.8 |
| ARID4B | Hyper   | 1          | CDS         | 235213858  | 235219954| 4.67    | -65.6 |
| ICE1   | Hyper   | 5          | CDS         | 5462879    | 5463030  | 4.6     | -11.8 |
| SCAPER | Hyper   | 15         | Exon        | 76705922   | 7674969  | 4.49    | -256  |
| ABCF1  | Hyper   | 6          | CDS         | 30579931   | 30583093 | 4.41    | -46.5 |
| YLPML  | Hyper   | 14         | CDS         | 74797722   | 74798023 | 4.37    | -20.2 |
| PCNT   | Hypo    | 21         | CDS         | 46357108   | 46363666 | -5.22   | -30.2 |
| ALB    | Hypo    | 4          | CDS         | 73413418   | 73415297 | -5.17   | -11.8 |
| RAB35  | Hypo    | 12         | 3'UTR       | 120097090  | 120097299| -5.11   | -6.55 |
| PLLP   | Hypo    | 16         | 3'UTR       | 57252248   | 57252549 | -4.75   | -9.84 |
| PEAK1  | Hypo    | 15         | CDS         | 77180157   | 77180548 | -4.63   | -7.46 |
| GNPAT  | Hypo    | 1          | CDS         | 231267739  | 231269870| -4.46   | -27.6 |
| CAM5AP1| Hypo    | 9          | CDS         | 135822676  | 135823006| -4.1    | -5.91 |
| FKBP11 | Hypo    | 12         | exon        | 48923428   | 48923668 | -4.04   | -14.3 |
| INS-IGF2| Hypo   | 11         | 3'UTR       | 21323217   | 2132527  | -3.95   | -19.9 |
| FKBP11 | Hypo    | 12         | exon        | 48923420   | 48923660 | -3.93   | -13.8 |
Fig. 3 Different mRNA expression profiles in the granulosa cells of older women. A Hierarchical cluster analysis of differentially expressed genes between the two groups. B The top 20 significantly enriched pathways for the differently expressed genes in the older group. C The top 20 enriched GO terms of differentially expressed genes in the older group. GSEA analysis of G2M checkpoint D and hedgehog pathways E.
analysis. The top 20 enriched Go terms and top 20 pathway enrichments of the differently m^6^A methylated genes were shown. Results indicated that the differently methylated genes were enriched in the regulation of transcription, cellular response to DNA damage stimulus, and apoptosis (Suppl Fig. 2). Moreover, the associated pathway of the differently methylated genes including FoxO signaling pathway, adherens junction, and regulation of actin cytoskeleton (Fig. 2E).

### Different mRNA expression profiles in the granulosa cells of older women

RNA-Seq revealed a significantly different mRNA expression profile in the granulosa cells of older women compared to younger women. The hierarchical clustering of the mRNA expression was shown (Fig. 3A). A total of 435 differently expressed genes ([fold change] >2 and adjusted \( p < 0.05 \)) were identified, with 212 genes up-regulated and 223 genes down-regulated in the older group. The top 10 up-regulated and down-regulated genes in the older group were listed in Table 3. The functions of the differently expressed genes were analyzed using GO and KEGG pathway analysis. Results showed that the most relevant pathways were complement and coagulation cascades, and cell cycle pathway (Fig. 3B). Moreover, GO enrichment terms indicated the differently expressed genes were enriched in mitotic nuclear division and mitotic cell cycle (Fig. 3C). GSEA analysis of the entire expression profiles indicates various cellular processes and structures affected by female reproductive aging. We found G2M checkpoint pathway (adjusted \( p \)-value<0.001) and hedgehog signaling pathway (adjusted \( p \)-value=0.084) were significantly enriched in ovarian granulosa cells of older women (Fig. 3D-E). The detailed information and the collection of genes of core enrichment in each pathway were listed in the supplemental materials (See Dataset 2 in the related files).

### Relationship between m^6^A methylation and mRNA expression

A conjoint analysis was conducted for m^6^A-seq and RNA-Seq data. We found 58 genes that commonly had different m^6^A methylation levels and differently expressed mRNA levels (Table 4). Among them, 11 genes were up-regulated with hypermethylation, 15 genes were down-regulated with hypermethylation, 21 genes were up-regulated with hypomethylation, and 11 genes down-regulated and hypomethylated.

The conjoint analysis of m^6^A peaks and differently expressed mRNAs were visualized, and the top 10 genes were indicated (Fig. 4A). Cumulative distribution analysis indicated that m^6^A methylated mRNAs were significantly increased in the older group compared to the younger group (Fig. 4B).

### Validation of the related mRNA

Among the 58 genes that had different m^6^A methylation levels and differently expressed mRNA levels, six of them (BUB1B, TOP2A, PHC2, DDR2, KLF13, and RYR2), were validated in another set of samples using qRT-PCR. Data showed that the expression of BUB1B and TOP2A were significantly up-regulated in the older group, whereas PHC2, DDR2, KLF13 and RYR2 displayed a decreased expression in this group (Figs. 5-6). The expression of these genes was consistent with the sequencing results. Integrative genomics viewer (IGV) plots of the m^6^A methylation abundances and expression abundances of these genes in the two groups were shown (Figs. 5-6).

### Discussion

m^6^A methylation is the most prevalent type of RNA methylation in eukaryotic mRNAs and plays an important role in modulating gene expression [33]. It has been well established that m^6^A methylation is essential for human fertility and is involved in the folliculogenesis and oocyte maturation [19, 34, 35]. However, the role of m^6^A methylation in ovarian aging has not been investigated. Thus, in this study, we performed MeRIP seq and RNA-Seq to characterize the transcriptome-wide m^6^A methylation in granulosa cells of older women.

### Table 3

| gene   | pattern | log2.FC | lg.p  |
|--------|---------|---------|-------|
| C2     | Up      | 9.01    | -5.72 |
| DNAJC22| Up      | 8.99    | -5.95 |
| GABBR2 | Up      | 8.94    | -5.44 |
| HSPB6  | Up      | 8.86    | -4.76 |
| NPC1L1 | Up      | 8.80    | -5.27 |
| GLP2R  | Up      | 8.78    | -3.85 |
| FAM83D | Up      | 8.42    | -3.21 |
| CENPA  | Up      | 8.26    | -2.94 |
| GPR21  | Up      | 8.19    | -2.80 |
| RASGRF1| Up      | 8.17    | -2.78 |
| CDH13  | Down    | -8.79   | -4.07 |
| NRG1   | Down    | -8.60   | -4.71 |
| SRSF12 | Down    | -8.35   | -3.26 |
| FBX1L13| Down    | -8.33   | -3.86 |
| ADM2   | Down    | -8.32   | -3.22 |
| SMIM10L2B| Down  | -8.27   | -3.03 |
| SLC16A2| Down    | -8.17   | -3.96 |
| BPIFB2 | Down    | -8.02   | -2.75 |
| F12    | Down    | -7.90   | -2.55 |
| DFYS4L | Down    | -7.90   | -2.56 |
Table 4  List of genes that exhibit changes in both m\textsuperscript{6}A methylation and mRNA level in the older group compared to younger group

| Gene         | pattern | chromosome | m\textsuperscript{6}A methylation | mRNA level |
|--------------|---------|------------|-----------------------------------|------------|
|              |         |            | Peak region | Peak start | Peak end | strand | Log2FC | p-value |
| FBXO16       | Hyper-up | 8          | 3'UTR       | 28348525   | 28348824 | -       | 7.88   | 0.00    |
| BUB1B        | Hyper-up | 15         | CDS         | 40196615   | 40199635 | +       | 2.26   | 0.01    |
| GCOM1        | Hyper-up | 15         | CDS         | 57708755   | 57709055 | +       | 2.19   | 0.05    |
| ALG11        | Hyper-up | 13         | exon        | 52024458   | 52024758 | +       | 1.62   | 0.02    |
| AC118553.2   | Hyper-up | 1          | 3'UTR       | 100082036  | 100082246 | +       | 1.57   | 0.05    |
| TOP2A        | Hyper-up | 17         | CDS         | 40392617   | 40398613 | -       | 1.56   | 0.01    |
| ENCl         | Hyper-up | 5          | 3'UTR       | 74629497   | 74629647 | -       | 1.52   | 0.00    |
| TTYH3        | Hyper-up | 7          | 3'UTR       | 2663994    | 2664114  | +       | 1.30   | 0.01    |
| VSIG4        | Hyper-up | X          | 3'UTR       | 66021796   | 66022093 | -       | 1.25   | 0.03    |
| GBP4         | Hyper-up | 1          | CDS         | 89186522   | 89188595 | -       | 1.23   | 0.03    |
| NAV1         | Hyper-up | 1          | 3'UTR       | 201820403  | 201820614 | +       | 1.21   | 0.05    |
| FOXO6        | Hyper-down | 1     | CDS         | 41361981   | 41362132 | +       | -4.16  | 0.00    |
| TMEM60       | Hyper-down | 7     | CDS         | 77793902   | 77794195 | -       | -4.12  | 0.00    |
| CARD6        | Hyper-down | 5     | CDS         | 40843357   | 40843538 | +       | -2.02  | 0.03    |
| CHKB         | Hyper-down | 20 | CDS         | 5923505    | 5923834  | +       | -1.37  | 0.00    |
| FAHD1        | Hyper-down | 16 | CDS         | 1827774    | 1827954  | +       | -1.13  | 0.04    |
| GRAMD4       | Hyper-down | 22 | exon        | 46677152   | 46677333 | +       | -1.07  | 0.05    |
| SWTI         | Hyper-down | 1   | CDS         | 185174473  | 18517495 | +       | -1.01  | 0.01    |
| KIAA1143     | Hyper-down | 3   | CDS         | 44753476   | 44761513 | -       | -0.96  | 0.02    |
| PHC2         | Hyper-down | 1   | CDS         | 33324821   | 33325001 | -       | -0.84  | 0.01    |
| ERUN2        | Hyper-down | 8   | CDS         | 37751710   | 37754250 | +       | -0.82  | 0.03    |
| NUDT16       | Hyper-down | 3   | CDS         | 131383177  | 131383447 | +      | -0.75  | 0.03    |
| DDR2         | Hyper-down | 1   | 3'UTR       | 162783533  | 162783714 | +       | -0.70  | 0.02    |
| KLF13        | Hyper-down | 15 | 3'UTR       | 31373135   | 31373525 | +       | -0.66  | 0.02    |
| SPG7         | Hyper-down | 16 | 3'UTR       | 89557527   | 89557738 | +       | -0.65  | 0.03    |
| ZC3H13       | Hyper-down | 13 | CDS         | 45970418   | 45979928 | -       | -0.60  | 0.03    |
| PDF          | Hypo-up   | 16 | 3'UTR       | 69328650   | 69329155 | -       | 0.89   | 0.02    |
| ZNF100       | Hypo-up   | 19 | exon        | 21727731   | 21727971 | -       | 1.54   | 0.03    |
| ARL13B       | Hypo-up   | 3   | 3'UTR       | 94053277   | 94053487 | +       | 1.48   | 0.01    |
| PYGO2        | Hypo-up   | 1   | 3'UTR       | 154958495  | 154958766 | -       | 1.14   | 0.02    |
| FCGR3A       | Hypo-up   | 1   | CDS         | 161542833  | 161544753 | -       | 1.08   | 0.01    |
| SGMS2        | Hypo-up   | 4   | CDS         | 107895406  | 107895886 | +       | 1.05   | 0.02    |
| ARL2BP       | Hypo-up   | 16  | 3'UTR       | 57252258   | 57252528 | +       | 1.03   | 0.03    |
| NCKI         | Hypo-up   | 3   | CDS         | 136946219  | 136948511 | +       | 0.96   | 0.04    |
| Gene | pattern | chromosome | M^2A methylation | mRNA level |
|------|---------|------------|-------------------|------------|
|      |         |            | Peak region | Peak start | Peak end | strand | Log2FC | p-value |
| SC5D | Hypo-up | 11 | CDS | 121307317 | 121307467 | + | 0.95 | 0.01 |
| A2M  | Hypo-up | 12 | CDS | 9112466 | 9113419 | - | 0.91 | 0.00 |
| BMI1 | Hypo-up | 10 | 3'UTR | 22329597 | 22329807 | + | 0.88 | 0.04 |
| USP1 | Hypo-up | 1  | CDS  | 62444767 | 62445155 | + | 0.86 | 0.03 |
| EID3 | Hypo-up | 12 | CDS  | 104304157 | 104304696 | + | 0.84 | 0.01 |
| TCF4 | Hypo-up | 18 | 3'UTR | 55224249 | 55224400 | - | 0.69 | 0.01 |
| Dyrk2| Hypo-up | 12 | CDS  | 67658128 | 67658728 | + | 0.68 | 0.02 |
| KLHL15| Hypo-up | 15 | CDS  | 24006321 | 24006650 | - | 0.64 | 0.04 |
| DNAJB6| Hypo-up | 7  | 3'UTR | 157416030 | 157416211 | + | 0.62 | 0.02 |
| ABI2 | Hypo-up | 2  | 3'UTR | 203439386 | 203439806 | + | 0.62 | 0.02 |
| FAM208B| Hypo-up | 10 | CDS  | 5730657 | 5730808 | + | 0.60 | 0.01 |
| TMF1 | Hypo-up | 3  | CDS  | 69047933 | 69048203 | - | 0.59 | 0.02 |
| APO8 | Hypo-up | 2  | CDS  | 21026792 | 21026912 | - | 0.52 | 0.04 |
| PTMD1| Hypo-down| 11 | exon | 47571495 | 47571824 | + | -3.59 | 0.01 |
| TNC  | Hypo-down| 9  | CDS  | 115085879 | 115087046 | - | -2.68 | 0.01 |
| KLF12| Hypo-down| 13 | 3'UTR | 73694467 | 73694737 | - | -1.59 | 0.00 |
| RYR2 | Hypo-down| 1  | CDS  | 237784213 | 237784394 | + | -1.28 | 0.03 |
| ZNF592| Hypo-down| 15 | CDS  | 84782922 | 84783103 | + | -0.85 | 0.01 |
| DHC17| Hypo-down| 11 | 3'UTR | 71435159 | 71435340 | - | -0.84 | 0.02 |
| DNMBP| Hypo-down| 10 | CDS  | 99880023 | 99880203 | - | -0.77 | 0.03 |
| LATS2| Hypo-down| 13 | 3'UTR | 20974400 | 20974711 | - | -0.69 | 0.01 |
| HMGCS1| Hypo-down| 5  | CDS  | 43298614 | 43298885 | - | -0.68 | 0.05 |
| FOXP1| Hypo-down| 3  | 3'UTR | 70958795 | 70959066 | - | -0.57 | 0.03 |
| AMOTL1| Hypo-down| 11 | 3'UTR | 94869367 | 94871242 | + | -0.44 | 0.03 |
RNAs can be methylated by Methyltransferase-like 3 (METTL3), Methyltransferase-like 14 (METTL14) ("writers"), and demethylated by the fat mass- and obesity-associated (FTO) protein and the α-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) protein ("erasers") [36]. There are also a bunch of "readers" of m6A which regulate the stability of RNA methylation, such as YTH domain proteins (YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2) [36]. Among those proteins above, many were reported to be associated with mammalian ovarian function and aging. Previous study reported that knocking down METTL3 in female oocytes inhibits its maturation and the maternal-to-zygotic transition by decreasing mRNA translation efficiency [21]. Inactivation of METTL3 in mice oocytes also leads to DNA damage accumulation in oocytes, defective follicle development, and abnormal ovulation [37]. Among readers of m6A methylation, studies confirmed that the protein level of FTO, but not ALKBH5, was significantly decreased in human aged ovaries and in ovaries of women diagnosed with premature ovarian insufficiency [38, 39]. Knocking down FTO, rather than ALKBH5, resulted in a decreased proliferation rate, increased apoptosis rate, and decreased cell viability in human ovarian granulosa cells [39]. YTHDC1 is required in oocyte growth and maturation and YTHDC1-deficient oocytes are blocked at the primary follicle stage [35]. YTHDC2 and YTHDF2 are also essential for the meiotic initiation and progression of female germ cells [34, 40]. In the current study, we found the number of m6A methylated genes was higher in the granulosa cells of older women with decreased ovarian reserve, [41] which was consistent with increased m6A levels that Sun et al reported [22]. Besides, our cumulative analysis also supported this point. Interestingly, the younger group displayed increased m6A peaks compared to the older group, indicating that granulosa cells of younger women have more m6A methylation sites. The overall m6A modification level was primarily determined by the m6A modified genes, as well as the level of modification of each transcript [42]. Therefore, the increased number of m6A peaks in the younger group does not contradict the relative lower m6A level in the ovaries of younger women that previous
Fig. 5 (See legend on previous page.)
According to our results, m⁶A methylation sites were globally enriched within the CDS and 3'UTR region in both the younger and older group. This phenomenon was in agreement with previous studies, showing CDS and 3'UTR regions were the most m⁶A modified regions in other cells and tissues [42, 43], indicating the conserveness of m⁶A methylation. A total of 3005 differently m⁶A methylated peaks were detected in the older group compared to the younger group, including 1297 hypermethylated and 1708 hypomethylated peaks. Bioinformatics analysis indicated that the most relative pathway that these dys-methylated genes enriched in was FoxO signaling pathway. Besides, several other pathways, such as adherens junction and regulation of actin cytoskeleton were also interfered with.

It has been well known that m⁶A methylation affects gene expression by regulating RNA transcription, translation, and degradation. Thus, RNA-Seq was performed to determine the transcriptome of granulosa cells from older women. Data showed that a total of 435 genes were differently expressed in the older group with 212 genes up-regulated and 223 genes down-regulated. The function and related pathways of the dysregulated genes were annotated by Go and KEGG analysis. The results indicated that mitotic nuclear division, mitotic cell cycle, and cell division were the most associated Go terms. Similarly, differently expressed genes were also enriched in the cell cycle and oocyte meiosis pathway. Previous studies demonstrated that granulosa cells in the aged antral follicles exhibited a decreased quantity, lower proliferation rate, and shorter telomere length [44, 45]. Besides, granulosa cells were proved to accelerate the aging process of oocytes [8, 10]. Combined with our results, it is legitimate to assume that the differently expressed genes might interfere with the cell cycle and contribute to the decreasing viability of granulosa cells of older women.

Conjoint analysis was performed to investigate differently expressed genes that regulated by m⁶A methylation. Data showed that a bunch of genes displayed different m⁶A methylation levels and differently expressed mRNA levels (Table 4). Most of these hyper- or hypo- methylated and differently expressed genes were reported to have a role in aging of human and other mammalian species, such as NAV1, FOXO6, CARD6, FAHD1, TCF4, DNMBP, and HMGCS1 [46–52]. Some of the genes were confirmed to be enrolled in the process of ovarian tumorgenesis, such as FBXO16, TTYH3, VSIG4, ZC3H13, NCK1, BMI1, USP1, DYRK2, DNAJB6, TNC, KLF12, LAT52, and FOXP1 [53–65]. A small group of the genes were reported to be associated with physiological ovarian function including oocyte meiosis, oocyte maturation, ovarian insufficiency, ovarian sterol uptake and so on [66–70]. We picked up six of the genes associated with ovarian function and validated the mRNA expression by qPCR (BUB1B, TOP2A, PHC2, DDR2, KLF13, and RYR2).

Here, we found BUB1B, TOP2A, PHC2, DDR2 and KLF13 were hypermethylated and differently expressed in the granulosa cells of older women. BUB1B encoding BubR1 is functional in the spindle check during mitosis. Touati et al. reported a strong reduction of BubR1 in the ovaries of menopausal women and aged oocytes, hypothesizing that the gradual decline of BubR1 led to age-related aneuploidization [71]. Interestingly, our results indicated that BUB1B was up-regulated and hypermethylated in the granulosa cells of older women. We suspected that m⁶A methylation might be responsible for this phenomenon. It is well established that m⁶A modification is involved in almost all stages of RNA life cycle, including transcription, pre-mRNA splicing, mRNA export, mRNA stability, and translation [72]. Except for promoting mRNA degradation, m⁶A presence may block its accessibility to RNase, thereby enhancing the stability of mRNA [72, 73]. Meanwhile, m⁶A modification may also inhibit the combination of ribosomes and interfere with mRNA translation. Therefore, granulosa cells of older women presented with a hypermethylated and up-regulated expression of BUB1B without increasing Bub1R were reasonable (Table 5).

Similarly, TOP2A, which encodes a unit of topoisomerase II, was up-regulated and hypomethylated in the older group. Topoisomerase II that controls and alters the topologic states of DNA during DNA replication, transcription, and repair [74]. It has been demonstrated that genotoxic treatment increased the DNA damage accumulation in the topoisomerase II-deficient ovarian granulosa cells, suggesting a critical role.
**Fig. 6** (See legend on previous page.)
of topoisomerase II in the ovary [75]. PHC2 encodes a component of polycomb group (PcG) multiprotein PRC-1-like complex, maintaining the transcriptionally repressive state of numerous genes and regulating cell renewal [76]. A previous study reported that PRC1 deficiency contributes to the dysregulated expression of cytoskeletal and adherens junction proteins in the ovarian follicles [77]. Considering the functions of the altered mRNA in the older group, we suspect that m6A methylation reduces the expression of PHC2 expression, relating to the dysregulated adherens junctions in the granulosa cells of older women. Moreover, the expression of TOP2A and PHC2 validated by qRT-PCR also supported the sequencing data.

DDR2, discoidin domain receptor 2, is a collagen tyrosine kinase receptor gene and was down-regulated and hyper-methylated in the older group. It was reported as an important regulator of ovarian function [68]. Silencing the DDR2 resulted in decreased chromatin maintenance, disturbed hormonal signaling pathways in ovarian granulosa cells, blocked ovulation, and infertility [68]. This is in accordance with the worse fertility outcomes of aged women of both natural conception and in-vitro fertilization [78, 79]. KLF13 encode the Kruppel-like factors which are important Sp1-like eukaryotic transcriptional proteins [80]. It is reported to take part in the sterol uptake and steroid biosynthesis in ovarian cells [69]. Similar to DDR2, KLF13 was down-regulated and hyper-methylated in the older group according to our sequencing data. This may have a relationship with the disturbed profile of steroid hormone levels in aged women [81].

Among the six genes RYR2 (ryanodine receptor-2) was hypo-methylated in the older group. It encodes the ryanodine receptor which is one of the components of the intracellular calcium channel. This gene is selectively expressed in ovarian cumulus cells right before ovulation and plays an important role in oocyte maturation and activation [82, 83]. In our conjoint study, we found RYR2 was down-regulated and hypo-methylated in older group. This is in accordance with the decreased oocyte quality in the aged women [84].

Taken together, our study revealed the transcriptome-wide m6A methylation in the aged granulosa cells, and suggested this modification might be associated with the altered mRNA expression and decreased ovarian functions during aging. Therefore, our study may provide novel insights to understand the mechanisms of ovarian aging and provide a candidate reservoir to promote fertility preservation for the aging women. However, certain limitations in the study cannot be ignored. The major one is our sample size. Given the small sizes of the study groups (three samples per group), these findings should be interpreted with great caution and confirmatory studies with larger sample size are required to validate the global m6A methylations. Further research with a larger number of samples is required to validate the global m6A methylations. Besides, clarifying the role of epigenetic enzymes that contribute to the altered methylation in the aging ovaries, should be explored in subsequent studies.

### Abbreviations
m6A: N6-methyladenine; MeRIP-Seq: m6A-targeted antibody coupled with high-throughput sequencing; RNASeq: RNA sequencing; qRT-PCR: quantitative real-time polymerase chain reaction; AFC: antral follicle count; AMH: Anti-Müllerian hormone; IVF: in vitro fertilization; BMI: body mass index; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; SD: standard deviation.

### Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12864-022-08462-3.

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| Gene name symbol | Forward primer | Reverse primer |
|------------------|----------------|---------------|
| DNA Topoisomerase II Alpha | TOP2A | GATGACAACCGGTGGTTGAG | CCACCCGATACCGATCCCT |
| Polyhomeotic Homolog 2 | PHC2 | CAGAAGCTGACTCCTCAGACA | GGGGAAACCGTGACAGTAT |
| BUB1 mitotic checkpoint kinase B | BUB1B | ACTGATAGCTGTCACCGCTGTG | GGCTTCTTGTGGCTAGGT |
| Discoidin domain receptor 2 | DDR2 | GATGACAGCAACACTCAGGG | AACACTTGGGACGGAAT |
| Kruppel-like factor 13 | KLF13 | GATTACGGGAATCTTGGCA | GCCGAACTTCTGGTCAGTC |
| Ryanodine receptor-2 | RYR2 | ATAGAAGCGCACCATAAGCA | ATGCTCAGGGCGATAAAAAC |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH | AGAAGGCTGGGGCTATTG | AGGGGGCATCCACAGCTT |

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Additional file 1.
Additional file 2.

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None.

### Authors’ contributions
The first two authors contributed equally to this work. L.W., B.Y., B.H., and H.Z. designed the experiments. L.L., L.Z., X.R., and L.J. collected the samples and conducted the experiments. C.L., X.D., and Y.Z. prepared the manuscript, which was edited by L.W. and J.Y. All authors have read and approved the manuscript.
Available datasets and materials
The datasets generated for this study could be found in the online repositories. The names of the repositories and accession number could be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181106.

Declarations

Ethics approval, accordance and consent to participate
This study was approved by the Institutional Review Board of Tongji Hospital (TJ-HB20210213). Women were totally informed of the procedures and signed informed consent was obtained from all participants. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
None.

Author details
1Reproductive Medicine Center, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, People’s Republic of China. 2Reproductive Medicine Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People’s Republic of China. 3Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People’s Republic of China.

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