Altered m<sup>6</sup>A modification is involved in up-regulated expression of FOXO3 in luteinized granulosa cells of non-obese polycystic ovary syndrome patients

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Abstract
The pathophysiology of polycystic ovary syndrome (PCOS) is characterized by granulosa cell (GC) dysfunction. m<sup>6</sup>A modification affects GC function in patients with premature ovarian insufficiency (POI), but the role of m<sup>6</sup>A modification in PCOS is unknown. The purpose of the prospective comparative study was to analyse the m<sup>6</sup>A profile of the luteinized GCs from normovulatory women and non-obese PCOS patients following controlled ovarian hyperstimulation. RNA m<sup>6</sup>A methylation levels were measured by m<sup>6</sup>A quantification assay in the luteinized GCs of the controls and PCOS patients. Then, m<sup>6</sup>A profiles were analysed by methylated RNA immunoprecipitation sequencing (MeRIP-seq). We reported that the m<sup>6</sup>A level was increased in the luteinized GCs of PCOS patients. Comparative analysis revealed differences between the m<sup>6</sup>A profiles from the luteinized GC of the controls and PCOS patients. We identified FOXO3 mRNA with reduced m<sup>6</sup>A modification in the luteinized GCs of PCOS patients. Selectively knocking down m<sup>6</sup>A methyltransferases or demethylases altered expression of FOXO3 in the luteinized GCs from the controls, but did not in PCOS patients. These suggested an absence of m<sup>6</sup>A-mediated transcription of FOXO3 in the luteinized GCs of PCOS patients. Furthermore, we demonstrated that the involvement of m<sup>6</sup>A in the stability of the FOXO3 mRNA that is regulated via a putative methylation site in the 3'-UTR only in the luteinized GCs of the controls. In summary, our findings showed that altered m<sup>6</sup>A modification was involved in up-regulated expression of FOXO3 mRNA in the luteinized GCs from non-obese PCOS patients following controlled ovarian hyperstimulation.

KEYWORDS
FOXO3, luteinized granulosa cells, mRNA decay, N<sup>6</sup>-methyladenosine, polycystic ovary syndrome
1 | INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder and a common cause of female infertility in women of reproductive age. One of the main characteristics of the syndrome is granulosa cell (GC) dysfunction, which largely contributes to hyperandrogenism, abnormal follicle development and anti-Müllerian hormone excess. Aberrant gene expression profile was found in GCs of PCOS patients. Multiple differential expressed genes were highly related to the pathogenesis of PCOS. However, the regulation mechanisms of these genes were largely unknown.

Forkhead Box O3 (FOXO3) plays important roles in diverse cellular processes including apoptosis, metabolism, cell proliferation and cell survival. FOXO3 is regulated at several mechanistic levels, such as transcriptional activity, cellular localization, mRNA expression and protein stability. Oxidative stress induces FOXO activation and nuclear translocation by c-Jun N-terminal kinase (JNK) or mammalian Ste20-like kinase 3 (MST1) activation despite phosphorylation by protein kinase B (Akt). After energy deprivation, the increased AMP/ATP ratio leads to AMP-activated protein kinase (AMPK) activation. AMPK activates FOXO3 activity by phosphorylation at six different residues. Insulin-like growth factor-1 (IGF-I)/insulin and phosphoinositide 3-kinase (PI3K)/Akt signalling pathway inactivate FOXOs by phosphorylation resulting in FOXOs nuclear exclusion. Zhao et al reported that elevated wnt family member 5A (WNT5A) activates PI3K/Akt signalling in GCs of PCOS patients. By contrast, Rice and colleagues showed metabolic insulin resistance in GCs of PCOS patients, suggesting that PI3K/Akt signalling is impaired. While many studies have been focused on the regulation of FOXO3 activity by post-translational modifications, the regulation of FOXO3 expression is largely unknown. FOXO3 is one of the differential expressed genes which have higher expression in PCOS. Its overexpression in GCs is associated with higher apoptosis in PCOS patients. To date, the factors that up-regulate and activate FOXO3 in GCs of PCOS patients are still unclear.

m6A is the most prevalent modification of mRNA in higher eukaryotes. The modifications are reversible and dynamically regulated by the m6A modulators. m6A modulators consist of the ‘writers’, the ‘erasers’ and the ‘readers’. Briefly, m6A modifications are installed by the ‘writers’ (Methyltransferase like 3 (METTL3) and Methyltransferase like 14 (METTL14)), removed by the ‘erasers’ (Fat mass and obesity-associated protein (FTO) and alkB homolog 5 (ALKBH5)), and recognized by the ‘readers’ (YTH domain-containing proteins and Eukaryotic initiation factor 3 (eIF3)). m6A modification has diverse biological functions such as nuclear RNA export, RNA splicing, protein translation regulation and RNA decay. In the YTHDF2-mediated decay pathway, mRNAs with increased m6A abundance in 3’-UTR are down-regulated due to reduced RNA stability.

Several human diseases are associated with altered m6A modification. m6A levels of sperm RNA are elevated in patients with asthenozoospermia. Increased m6A levels in the granulosas were reported in patients with premature ovarian insufficiency (POI), affecting apoptosis and cell proliferation in GCs. Aberrant m6A modification, through the effects on RNA metabolism, plays critical roles in a variety of cancers. However, whether m6A modification plays a role in the pathogenesis of PCOS is unknown.

Given that m6A modification affects the GC function in patients with POI, we suggested alteration of the m6A profile in the luteinized GCs of PCOS patients, which may account for the dysregulation of certain key genes for PCOS. Here, we showed the differences of m6A distribution between the luteinized GCs of normovulatory women and PCOS patients following controlled ovarian hyperstimulation. We identified FOXO3 mRNA with differential m6A peaks, targeted for decay by YTHDF2. Our results indicated that hypomethylated FOXO3 mRNA caused the dysregulation of FOXO3 in luteinized GCs from PCOS patients following controlled ovarian hyperstimulation.

2 | SUBJECTS AND METHODS

2.1 | Subjects

 Forty-three control patients with tubal factor infertility or male infertility and 36 PCOS patients were recruited in the Reproductive Medicine Center at the First Affiliated Hospital of Wenzhou Medical University between December 2017 and August 2019. Inclusion criteria for control patients were aged between 25 and 35 years, having a regular menstrual cycle, serum testosterone (T) level < 2 nmol/L, 18.5 < body mass index (BMI) < 27, having a normal ovarian reserve and a normal uterus. PCOS was diagnosed according to the Rotterdam revised criteria. The inclusion criteria for PCOS patients were aged between 25 and 35 years, having oligo- or anovulation, serum testosterone level > 2 nmol/L, 18.5 < BMI < 27, anti-Müllerian hormone (AMH) > 7 ng/mL and having a normal uterus. The exclusion criteria were smoking, systemic diseases, endometriosis, abnormal serum level of prolactin, dysfunctional thyroid or having previous long-term medication use.

This study was approved by the Ethics Committees of the First Affiliated Hospital of Wenzhou Medical (Approved number: YS2016-063 and YS2019-046). Written informed consent was obtained from all the participants.

2.2 | Measurement of hormones

 Plasma levels of AMH, luteinizing hormone (LH), follicle-stimulating hormone (FSH), T and estradiol (E2) were collected and measured by chemiluminescence immunoassay (CLIA) between day 3 and day 5 of the menstrual cycle before the controlled ovarian stimulation. Serum fasting glucose levels and fasting insulin levels were measured by an oxidase-peroxidase method and a CLIA method, respectively. The inter-assay coefficients of variation were 6.1% for AMH, 7.2% for LH, 5.3% for FSH, 10.0% for T, 8.9% for E2, 4.3% for glucose and 5.4% for insulin. The intra-assay coefficients of variation were 3.8% for AMH, 5.3% for LH, 4.6% for FSH, 8.1% for T, 6.8% for E2, 2.1% for glucose and 2.9% for insulin.
Insulin in follicle fluids was detected by enzyme-linked immunosorbent assay (ELISA, R&D Systems). HOMA-IR was calculated by the formula (HOMA-IR = fasting insulin (mU/L) × fasting glucose (mmol/L)/ 22.5).²⁷

2.3 | Transvaginal ultrasonography

Ultrasound examination was performed on the 3rd-5th day of the menstrual cycle with a 7 MHz transvaginal transducer (LOGIC 400, General Electric Medical Systems) to calculate follicle number. The basal antral follicle count (AFC) was assessed as the sum of all follicles of 2-10 mm in diameter. The polycystic ovary was defined as 12 or more AFC in each ovary.

2.4 | Ovarian stimulation and GCs isolation

All participants underwent controlled ovarian stimulation with gonadotrophin-releasing hormone (GnRH) agonist long protocol. Briefly, patients received 0.1 mg/day of GnRH agonist (Decapeptyl, Ferring, Germany) from day 20 of a spontaneous menstrual cycle until the day of human chorionic gonadotrophin (hCG) injection. When pituitary down-regulation was achieved (usually after 14 days of GnRH agonist injection), ovarian stimulation was initiated with a minimum of 150 IU/day of recombinant human FSH (rFSH, Gonal F, Merck Serono). The dosages of rFSH were adjusted according to serum E2 levels and follicle growth of the patients. 250 µg of recombinant hCG (Ovidrel, Merck Serono) was administered, when at least three follicles were >17 mm in diameter. Transvaginal aspiration was performed 34-36 hours later to retrieve oocytes and follicular fluids. The follicular fluids from follicles >14 mm in diameter and without obvious blood contamination were collected. The follicular fluids were centrifuged at 340 g for 8 minutes to pellet the GCs. The cell pellets were resuspended in 1x PBS solution (Gibco), overlaid on 40%/80% gradient solution (PureCeption, SAGE) to pellet the GCs. The pellet was centrifuged at 320 g for 20 minutes. GCs in the interface were collected and washed with 1x PBS solution.

2.5 | RNA m6A quantification

Total RNA was isolated using the Total RNA Kit II Kit (Omega Bio-Tek). Polyadenylated RNA was purified from total RNA using the GenElute mRNA Miniprep Kit (Sigma). 500 ng total RNA or polyadenylated RNA was used to determine the RNA m6A methylation levels using the Epigentek m6A RNA Methylation Quantification Kit (Epigentek). Briefly, a standard curve was prepared according to the manufacturer’s instruction. The RNA samples were coated on the strip wells, followed by incubation with capture antibody. After washes with washing buffer, detection antibody and enhance solution were added separately. Then, the signals were developed by the detection solution. The RNA m6A levels were quantified by the absorbance at 450 nm, and the m6A contents were calculated based on the standard curve.

2.6 | Methylated RNA immunoprecipitation sequencing (MeRIP-seq) and MeRIP-qPCR

MeRIP was performed using Magna MeRIP m6A Kit (Millipore, Billerica, MA, USA) following the manufacturer’s instruction. Briefly, 50 µg of total RNA was precipitated using 2.5 volumes of 100% ethanol, one-tenth volumes of 3 mol/L sodium acetate and 1 mg/mL glycogen. Then, the RNA was resuspended, fragmented and immunoprecipitated with anti-m6A antibody or normal mouse IgG. 10% of fragmented RNA was keep as input. The immunoprecipitated RNA was eluted by competition with m6A sodium salt and recovered using miRNeasy Mini Kit (QiAGEN, Hilden, Germany).

The recovered RNA was treated with DNase I (0.1 U/µL, Thermo Scientific, Waltham, MA, USA) and reverse-transcribed by random hexamer primer and RevertAid Reverse Transcriptase (Thermo Scientific). Subsequently, second strand cDNA was synthesized using DNA Polymerase I and RNase H (Thermo Scientific). Sequencing library was constructed using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB) and purified using AMPure XP system (Beckman Coulter). Library quality was assessed on a Bioanalyzer 2100 (Agilent). The clustering of the index-coded samples was generated on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Library preparations were sequenced on a Hiseq 4000 Platform (Illumina).

Real-time quantitative RT-PCR analysis (qRT-PCR) was carried out with the samples with anti-m6A antibody, the samples with normal IgG, and input samples in triplicates. Ct of the samples with anti-m6A antibody were normalized to input by subtracting the Ct of input from the Ct of IP samples: \( \Delta C_{t} = C_{t_{\text{IP}}} - C_{t_{\text{input}}} \). Then, the per cent of input for each IP sample was calculated: \( \% \text{Input} = 2^{-\Delta C_{t}} \times 100 \). The primers for MeRIP-qPCR were listed in Table S1.

2.7 | MeRIP-seq data analysis

Sequencing data were mapped to the reference genome (ftp://ftp.ensembl.org/pub/release-94/fasta/homo_sapiens/dna/) using BWA mem (version 0.7.12). m6A peaks were identified by peak finding algorithm in MACS2 (version 2.1.0). The threshold of enrichment was set at \( q < 0.05 \). Differential peak analysis was based on the fold enrichment of peaks between the PCOS group and the control group. When the odds ratio > 2, a differential peak was determined. The statistics of pathway enrichment of genes with differential peaks in KEGG pathway was tested using KOBAS software.

2.8 | Primary cell culture and RNA interference

The purified luteinized GCs were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% foetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified incubator under 5% CO2. The siRNAs against
YTHDF2, METTL3, METTL14, FTO and ALKBH5 were obtained from GenePharma Corporation (China). When the confluence reached 80%, the cells were dissociated and seeded in 24-well plates (10^5 cells/well). One day later, the cells were transfected with siRNA (GenePharma) targeting negative control, YTHDF2, METTL3, METTL14, FTO or ALKBH5 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. The sequences of siRNA were listed in Table S2.

2.9 Real-time quantitative RT-PCR analysis

For the GCs collected from follicular fluids, total RNA was extracted using the Total RNA Kit II Kit (Omega Bio-Tek). For the GCs cultured in 24-well plates, total RNA in each well was extracted using MicroElute Total RNA Kit (Omega Bio-Tek). The total RNA was reverse-transcribed by the PrimeScript RT reagent Kit with gDNA Eraser (Takara) according to the manufacturer’s instruction. Real-time quantitative RT-PCR analysis (qRT-PCR) was performed on a Corbett Rotor Gene 6000 Real-time Cycler using TB Green Premix Ex Taq II (Takara). Each sample was analysed in triplicates to obtain the threshold cycle number. GAPDH mRNA was used as an internal control for normalization. Relative expression levels were quantified using comparative 2^ΔΔCt method and expressed as fold changes relative to the controls. The sequences of the primers were listed in Table S1.

2.10 Western blot analysis

The total protein was extracted from the cultured GCs using RIPA buffer (Beyotime) containing 1mM PMSF (Beyotime). The concentration of the protein was measured using BCA protein assay kit (Solarbio). The cell extracts were separated on a 12% SDS-PAGE Gel (ExpressPlus PAGE Gel, GenScript) and transferred to methanol-activated polyvinylidenefluoride membranes. The membranes were blocked with 5% non-fat milk powder for 1 hour and incubated with the primary antibody for 2 hour at room temperature. The mouse anti-FOXO3 (Proteintech, 66428-1-lg) or mouse anti-Beta-actin (Proteintech, 60008-1-lg) antibody was used as primary antibody. Then, the membranes were incubated with HRP-conjugated affinipure goat anti-mouse IgG (H + L) (Biosharp, BL1001A) for 1 hour at room temperature. The signals were developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific).

2.11 Plasmid construction and dual-luciferase reporter assay

The proximal 3'-UTR of FOXO3 was amplified and inserted into 3'-downstream of the Renilla luciferase gene of the psiCHECK™-2 Vector between Xhol and NotI restriction sites. The motif of m^6A binding was mutated by fusion PCR with a pair of complementary primers containing the A-to-T mutation. The primary culture human GCs were transfected with wild-type m^6A motif reporter plasmid or mutant m^6A motif reporter plasmid using Lipofectamine 2000. The Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) 48 hours later. The Renilla luciferase activities were normalized to Firefly luciferase activities.

2.12 Statistical analysis

Data are expressed as the means ± SD. The normality and homogeneity of variance of the data were assessed. Differences between groups were determined by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test using SPSS 16.0 software package. Significance was set at P < 0.05.

3 RESULTS

3.1 m^6A levels in the luteinized GCs of the controls and PCOS patients

To investigate the role of m^6A modification in the luteinized GCs of PCOS patients, we recruited normovulatory controls and PCOS
patients in our reproductive centre (Table S3). The insulin-glucose parameters indicated that the PCOS patients had insulin resistance in the present study. We examined m^6^A levels in the total RNA of the luteinized GCs collected following ovarian hyperstimulation by RNA m^6^A quantification. m^6^A levels were twofold higher in the luteinized GCs of PCOS patients compared with the controls (Figure 1A). We further isolated polyadenylated RNA from the total RNA and examined the m^6^A levels. Consistently, the m^6^A levels of polyadenylated RNA were increased in the luteinized GCs of PCOS patients (Figure 1B).

3.2 Differential m^6^A modification in the luteinized GCs of the controls and PCOS patients

We conducted MeRIP-seq to analyse the transcriptome-wide distribution of m^6^A modification in GCs. 2764 and 3405 m^6^A peaks were identified in the controls and PCOS patients, respectively (Table S4). We selected 8 transcripts with m^6^A peaks to verify the sequencing data by MeRIP-qPCR. The qRT-PCR results were in agreement with the sequencing data (Figure S1). We analysed the differential peaks based on the fold enrichment of peaks of the two groups. 1719 and 2195 peaks were distinct in the controls and PCOS patients, respectively, while 996 peaks were overlapped in both groups (Figure 2A and Table S5).

Similar to the other studies, m^6^A peaks were strongly enriched around the stop codon in the controls (Figure 2B,D).^2^8^-^3^0^ However, m^6^A peaks in PCOS patients showed a less prominent enrichment around the stop codon and increased locations to the CDS (coding sequence) and the TSS (transcription start sites) regions (Figure 2C,E).

3.3 m^6^A modification targets the FOXO3 transcript in the luteinized GCs of the controls but not PCOS patients

We suggested that m^6^A modification plays a role in dysregulation of PCOS-associated genes. We examined the expression of AMH, AR, FOXO1 and FOXO3 in our study. Consistent with the previous studies,^2^3^,^3^1^ the expression of AMH, AR, FOXO1 and FOXO3 were up-regulated in the luteinized GCs of PCOS patients compared with the controls (Figure 3A). To screen potential candidates, we compared the expression of m^6^A methyltransferases and demethylases in the luteinized GCs of the controls and PCOS patients. The expression of METTL3, METTL14, FTO and ALKBH5 were all elevated in PCOS patients compared with the controls (Figure 3A). We selectively knocked down the expression of METTL3, METTL14, FTO or ALKBH5 in the luteinized GCs of the controls and PCOS patients, respectively. The knockdown efficiency was confirmed by qRT-PCR analysis (Figure S2). Due to differential m^6^A peaks identified in FOXO1 and FOXO3 transcripts, we examined the expression of FOXO1 and FOXO3 in these cells. In the controls, depletion of METTL3 or METTL14 increased the expression of FOXO3, and silencing of FTO suppressed the expression of FOXO3, suggesting m^6^A modification regulated FOXO3 mRNA degradation (Figure 3C,E,G). However, depletion of the m^6^A methyltransferases or demethylases did not affect the expression of FOXO3 in PCOS patients (Figure 3D,F,H), which indicated that effects of m^6^A modification on FOXO3 mRNA were cell-specific. Our results also showed that knockdown of methyltransferases or demethylases did not affect the expression of FOXO1 in GCs (Figure 3C-J). The common target gene SBK1 was used as a positive control to confirm the results. Collectively, these results demonstrated that selectively knocking down m^6^A methyltransferases or demethylases did not alter the expression of FOXO3 in the luteinized GCs of PCOS patients, but did so in the controls, suggesting an absence of m^6^A-regulated transcription of FOXO3 in the luteinized GCs of PCOS patients.
3.4 | m^6A modification regulates the stability of the FOXO3 transcript via the YTHDF2-mediated decay pathway in the luteinized GCs of the controls

Our MeRIP-seq showed a differential m^6A peak in 3'-UTR and near the stop codon of the FOXO3 transcript (Figure 4A). We performed MeRIP-qPCR to confirm the differential m^6A levels on the m^6A target site (Figure 4B). To determine how m^6A modification regulates the FOXO3 transcript, we analysed the sequence of the m^6A peak. A putative m^6A site was identified within the m^6A peak of 3'-UTR at position +2279 (+1 relative to the translation start site) (Figure 4C). As YTHDF2 is the main binding protein that accounts for the decay of m^6A-modified mRNAs, we next examined the effects of YTHDF2 knockdown on FOXO3 expression in human GCs. Depletion of YTHDF2 increased the amount of FOXO3 transcript, and total protein levels of FOXO3 (Figure 4D,E). The knockdown efficiency was checked by qRT-PCR (Figure S2). To assess the functionalities of the m^6A site in the 3'-UTR of FOXO3 transcript, we constructed a reporter plasmid bearing FOXO3-3'-UTR with the putative m^6A site mutated (Figure 4F). FTO-siRNA suppress the luciferase activities of the reporter plasmid with the wild-type m^6A site (Figure 4G). However, mutation of the m^6A site reversed the suppressive effects of FTO-siRNA on the luciferase activities (Figure 4G). The presence of m^6A readers in human GCs was showed by a previous study.32 To exclude the potential of lacking the m^6A readers in GCs of PCOS patients, we examined the expression of the m^6A readers in PCOS. The expression of YTHDF2 was significantly increased in the luteinized GCs of PCOS patients (Figure S3). These results indicated that m^6A modification regulated FOXO3 mRNA decay through the m^6A site in 3'-UTR in the luteinized GCs of the controls.

4 | DISCUSSION

Increasing evidence showed that m^6A modification play important roles in various physiological processes.17,33-36 Elevated or decreased
m6A level was associated with various human diseases.23-25 We showed that the level of m6A modification was higher in the luteinized GCs of PCOS patients than that in the controls. Aberrant m6A levels affect gene expression and biological functions. However, whether human diseases alter m6A profiles is largely unknown. Here, we showed that not only elevated m6A level but also altered m6A profile are associated with PCOS in the luteinized GCs.

The site selection of m6A modification is largely unknown. miRNAs regulate m6A formation at corresponding target sites.37 Altered miRNAs and other associated factors partially explain the differences of m6A profiles among cell types.37 Furthermore, m6A profile was altered when cells exposed to heat shock, ultraviolet radiation or signalling molecules.38 Acute stress regulates a fraction of genes with m6A modification in the cortex.33 The GCs of PCOS
patients present different cell characteristics. Cell survival, prolif-
eration rate and responsiveness to FSH were altered in the GCs of
PCOS patients.\textsuperscript{39,40} Differentially expressed miRNAs were also iden-
tified in the GCs of PCOS patients.\textsuperscript{41,42} Moreover, abnormal serum
AMH, androgen, activin A and follistatin were observed in PCOS pa-
tients.\textsuperscript{2,43,44} All these alterations may account for the altered m\textsuperscript{6}A
profile in PCOS. We identified 2195 differential m\textsuperscript{6}A peaks in the
luteinized GCs of PCOS patients compared to those of the controls.
KEGG analysis showed that differential peaks were enriched in met-
abolic pathway (Figure S4). Whether m\textsuperscript{6}A modification is involved in
the regulation of these transcripts with the differential m\textsuperscript{6}A modifi-
cation needs further investigation.

Insulin resistance is prevalent in PCOS patients and plays a cru-
cial role in metabolic abnormalities. Although obesity increases risks
for insulin resistance, several studies have demonstrated that both
obese and non-obese PCOS patients had impaired insulin signal-
ling.\textsuperscript{45,46} In the present study, the non-obese PCOS patients had sig-
nificantly higher insulin 0, insulin 120 and HOMA-IR compared with
the controls. Moreover, the insulin levels in follicular fluid were nota-
bly higher in the non-obese PCOS patients. These indicated that the
PCOS patients in our study had insulin resistance. As FOXO proteins
mediate the effects of insulin signalling on metabolism,\textsuperscript{47} it is likely
that altered expression of FOXO3 is involved in the defects of insulin
signalling in the luteinized GCs of PCOS patients. Here, we demon-
strated an absence of m\textsuperscript{6}A-mediated FOXO3 mRNA destabilization
which altered expression of FOXO3 in the luteinized GCs of PCOS
patients. Our findings have offered a potential mechanism for insulin
resistance.

It is well known that insulin activates PI3K/Akt signalling to
induce the nuclear exclusion of FOXO3.\textsuperscript{5} However, the effects of
PI3K/Akt signalling on GCs of PCOS patients are controversial.
Zhao et al showed that WNT5a expression was elevated in GCs of
PCOS patients.\textsuperscript{10} Although they have demonstrated that WNT5a
increased inflammation and oxidative stress via PI3K/Akt/NF-κB
signalling in human GCs, they did not confirm that WNT5a acti-
vated PI3K/Akt/NF-κB signalling in GCs of PCOS patients. As
insulin resistance is attributed to defects in PI3K/Akt signalling,
metoctic insulin resistance in GCs of PCOS patients suggests im-
paired PI3K/Akt signalling.\textsuperscript{9,11} Thus, it is possible that abnormalities in
other signalling cascade rather than PI3K/Akt signalling contrib-
uted to chronic inflammation in GCs of PCOS patients. To date, the
factors that up-regulate and activate FOXO3 remain unclear in GCs
of PCOS patients. Here, we demonstrated that YTHDF2 mediated
FOXO3 mRNA decay via a putative m\textsuperscript{6}A site in the 3' UTR in the
luteinized GCs of the controls. In contrast to the findings in the
controls, although the expression of YTHDF2 was elevated in the
luteinized GCs of PCOS patients, FOXO3 mRNA was hypometh-
ylated and irresponsible to m\textsuperscript{6}A modification. Our results showed
an absence of m\textsuperscript{6}A-based regulation of FOXO3 expression in the
luteinized GCs of PCOS patients. Taken together, we inferred that
altered m\textsuperscript{6}A modification caused upregulation of FOXO3 in
the luteinized GCs of PCOS patients following controlled ovarian
hyperstimulation.

In summary, the present study provided m\textsuperscript{6}A profiles of norm-
ovulatory women and PCOS patients in luteinized GCs following
controlled ovarian hyperstimulation. We demonstrated that altered
m\textsuperscript{6}A modification disturbed the regulation of FOXO3 expression in
the luteinized GCs of PCOS patients. However, the site selection
mechanism of m\textsuperscript{6}A in PCOS needs to be explored in future studies.

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CONFLICT OF INTEREST
The authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTION
Shen Zhang: Conceptualization (lead); Data curation (lead); Funding
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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