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METTL3 and METTL14-mediated N6-methyladenosine modification promotes cell proliferation and invasion in endometriosis

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Abstract

Research question: Could METTL3 and METTL14-mediated N6-methyladenosine modification play possible cooperative roles in pathogenesis and progression of endometriosis?

Design: We aimed to investigate the m^6A methylation profiles and the roles of METTL3 and METTL14 in the m^6A regulation and pathogenesis of endometriosis. The m^6A methylation and mRNA levels in paired ectopic endometrium (EC) and eutopic endometrium (EU) were measured using m^6A–mRNA epitranscriptomic microarrays. The functions of m^6A methylation in mRNAs were predicted using bioinformatics analysis. The levels of m^6A writers were detected using qPCR. The role of METTL3 and METTL14 in endometriosis was explored using EU stromal cells.

Results: The m^6A methylation levels were decreased in 1,312 mRNAs and increased in 518 mRNAs, and 1,797 mRNAs were increased and 2,580 mRNAs were reduced in the EC compared with the EU. Pathway analysis found that the genes with hypo-methylated m^6A were significantly associated with important pathways in endometriosis, including estrogen, Hippo, and PI3K–Akt signaling and cell–cell adhesion. Furthermore, METTL3 and METTL14 were downregulated in

the EC compared with the EU. *METTL3* and *METTL14* knockdown caused cell proliferation and invasion.

Conclusion: Taken together, these data reveal a differential m^6A epitranscriptomic pattern in endometriosis; and the reduction of *METTL3* and *METTL14* expression indicates its role in endometriosis pathogenesis and progression. Thus, METTL3 and METTL14 may be a novel treatment target of the disease.

Keywords: Endometriosis; METTL3; METTL14; N6-methyladenosine; mRNAs

INTRODUCTION

Endometriosis is a common gynecological disorder defined by the presence of a functional endometrium outside of the uterine cavity. Up to 10% of women of reproductive age suffer from endometriosis; the incidence rate is rising over time (Eisenberg et al., 2018; Taylor et al., 2021). Endometriosis affects women's quality of life and family harmony because of pelvic pain, pelvic lumps, low fertility, high treatment cost, and unsatisfactory long-term treatment effect (Eisenberg et al., 2018; Taylor et al., 2021). The pathogenesis of endometriosis is complicated, and the underlying mechanisms remain to be clarified. Though the retrograde menstruation theory has been widely accepted for the onset of endometriosis, there is discrepancy between the high rate of retrograde menstruation and the low disease incidence (Wang et al., 2020). Thus, there is research on other mechanisms, including dysregulation of local estrogen production, immune dysfunction, and genetic and epigenetic factors, that may help explain the phenomenon (Wang et al., 2020).

The post-transcriptional modifications of RNA, one of the most important epigenetic mechanisms regulating gene expression, affect protein production and generate different phenotypes (Liu et al., 2021). For example, N6-methyladenosine (m^6A) modification, the most prevalent post-transcriptional modification, regulates the fate decisions of m^6A-containing mRNAs via RNA splicing, destabilization, degradation, and translation (Fu et al., 2014; Wang et al., 2014;Wang et al., 2015; Zaccara et al., 2019). The level of m^6A modification varies in DNA damage, cell proliferation, and differentiation (Liu et al., 2022; Jiang et al., 2021). Research has revealed the important functions of m^6A in multiple biological processes, including cancer and organ development (Liu et al., 2022; Livneh et al., 2020; Wiener and Schwartz, 2021). The levels of m^6A

modification in biological processes are primarily determined by three types of enzymes, methyltransferases (also called writers), demethylases (erasers), and binding proteins (readers) (Yang et al., 2018; Wiener and Schwartz, 2021). The m^6A writers comprise methyltransferase that catalyzes m^6A modification on RNAs, including methyltransferase Like 3 (METTL3), METTL14, KIAA1429, Wilms Tumor 1 Associated Protein (WTAP), RNA Binding Motif Protein 15 (RBM15), and RBM15B. Abnormal activities of m^6A writers affect m^6A modification levels, leading to aberrant gene expression and protein synthesis (Fu et al., 2014). METTL3 plays a critical role in m6A catalytic process, and METTL14 acts as a coactivator during m6A deposition on nuclear RNAs which increases catalytic efficacy (Jiang et al., 2021). The dysregulation of METTL3/METTL14 complex exerts a significant effect on immune responses and cancer progression (Jiang et al., 2020; Huang et al., 2021). Moreover, METTL3/METTL14 deficiencies have been reported to be correlated with the cell functions in tumor growth and immunosurveillance (Dong et al., 2021; Liu et al., 2021).

Despite the substantial progress in disease research, the METTL3/METTL14 regulatory patterns and potential roles of m^6A methylation in endometriosis remain unknown. Thus, in the present study, we investigated the differential m^6A methylation levels in paired ectopic and eutopic endometria and predicted the functions of m^6A methylation using bioinformatics analysis. Furthermore, we detected the expression of m^6A regulator genes and explored the function of METTL3/METTL14 in endometrial cells. Our findings will help provide new insights into the pathogenesis of endometriosis.

MATERIALS AND METHODS

Ethical approval and informed consent

This study was approved on 24 October 2019 by the Medical Ethics Committee of Xiangya Hospital, Central South University, no. 201910255, and written informed consent was obtained from all subjects.

Sample collection

Eighteen female patients with ovarian endometriomas between the age of 23 and 47 were enrolled

(Table 1). The average body mass index was 20.56 ± 2.31 kg/m^2, and all the participants had and regular menstrual cycles. They were received gynecological surgery for detected ovarian cyst range from 0.2 to 7 years, and 8 cases were diagnosed of bilateral ovarian endometriomas and 10 of unilateral endometriomas by surgical exploration. None of them took steroid hormone medications in latest 3 months. Subsequently, paired ectopic endometrium (EC) and eutopic endometrium (EU) were collected in the proliferative phase from each patient. Endometriosis was pathologically diagnosed and staged at stage III–IV using the revised American Fertility Society staging system, with 10 cases in at stage III and 8 at stage IV.

RNA Extraction

First, eighteen paired EC and EU tissues were homogenized in 1 ml of TRIzol (Invitrogen, USA), and total RNA was isolated with 0.2 ml of chloroform. Then, RNA was precipitated with 0.5 ml of isopropanol, washed with 75% ethanol, and dissolved in an appropriate volume of RNase-free water. Finally, RNA concentration and purity were determined using a NanoVue Plus spectrophotometer (Healthcare Bio-Science AB, Uppsala, Sweden), and RNA integrity was accessed using denaturing agarose electrophoresis.

m^{6}A-mRNA Epitranscriptomic Microarray

Paired EC and EU tissues for microarray were collected from 3 patients who had the disease duration of 1 to 7 years, with two at stage III and one at stage IV, two bilateral endometrioma and one unilateral endometrioma. Total RNA was incubated with an anti-m^{6}A antibody (Synaptic Systems). The m^{6}A-methylated RNAs were eluted from the immunoprecipitated magnetic beads (the IP fraction), and unmodified RNAs were obtained from the supernatant (the Sup fraction). The IP and Sup RNAs were amplified as cRNAs and labeled with Cy5 and Cy3, respectively, using an Arraystar Super RNA Labeling Kit. The labeled cRNAs were hybridized onto an Arraystar Human mRNA&lncRNA Epitranscriptomic Microarray (8 × 60K; Arraystar, USA) at 65°C for 17 h in an Agilent Hybridization Oven. After washing, the arrays were scanned with an Agilent Scanner G2505C.

Bioinformatic analysis

Acquired array images were analyzed using the Agilent Feature Extraction software (version 11.0.1.1). The raw intensity of the Cy5-labeled IP cRNAs and the Cy3-labeled Sup cRNAs was normalized to the average log2-scaled intensity of the spiked-in RNA. Then, the probe signals with a present (P) or marginal (M) QC flags in at least 1 out of the three paired samples were reserved for further analysis. The percentage of m^6A modification was calculated as the m^6A methylation level based on the normalized intensities of the IP and Sup, and the m^6A quantity was counted based on the normalized intensities of IP. The RNA level was calculated based on the total IP (Cy5-labelled) and Sup (Cy3-labelled) normalized intensities, and an additional quantile normalization method of limma package was used to normalize the RNA expression level between arrays before probes flag screening. The hierarchical clustering analysis was performed using the R software on all detected RNA based on the similarities of their m6A methylation quality or mRNA expression level, of which the closeness of their relationships was displayed on top of the heatmaps. The differentially m^6A-methylated or expressed RNAs in the EC and EU were determined by filtering with the fold change and statistical thresholds. Gene ontology (GO) analyzed differentially m6A-methylated or expressed mRNAs using topGO package in R environment. KEGG pathway analysis were performed to explore the potential biological pathways of differentially m6A-methylated or expressed mRNAs by fisher's exact test.

Evaluation of candidate mRNA with qRT-PCR

Total RNA from eighteen paired EC and EU was extracted from paired EC and EU tissues using TRIzol and quantified using a NanoVue Plus spectrophotometer (Healthcare Bio-Science AB). The reverse transcriptional reaction was done using a reverse transcriptase kit (TaKaRa, China), and PCR was done using the SYBR Green qPCR Mix (Bio-Rad, USA) and specific primer sets (Table 2) on an Applied Biosystems 7900 Real-Time PCR system. The experiments were performed in triplicates, and relative mRNA levels were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical staining

Eighteen paired EC and EU tissue sections were dewaxed and rehydrated. Antigen retrieval was conducted in sodium citrate solution (0.01 M) at 98℃ for 15 min, endogenous peroxidase was

blocked with hydrogen peroxide (3%) at room temperature for 20 min, and then the sections were blocked in fetal bovine serum (10%) for 1 hour. Subsequently, the slices were incubated with primary antibody against MMP2 (1:200; Poteintech), NCOA1 (1:200; ABclonal), PPP2R2D (1:200; Biosss), YWHAZ (1:200; Proteintech), METTL3 (1:500; Abcam), and METTL14 (1:200; ABclonal) at 4°C overnight. After rewarm at 37°C, secondary antibody was added and incubated at room temperature for 1 hour. Finally, the sections were colored with dimethylaminoazobenzene (DAB) and counterstained with hematoxylin. Five fields were observed under high power from every immunostained section and the positive signals were counted.

MeRIP-qPCR

First, 1–3 μg of total RNA from eighteen paired EC and EU and a spiked-in control mixture for m^6A were added to 300 μl of IP mixture containing 3 μl of RNase inhibitor (Enzymatics) and 2 μl of anti-m^6A rabbit polyclonal antibody (Synaptic Systems). After the reaction was incubated at 4°C for 2 h, 20 μl of Dynabeads™ M-280 sheep anti-rabbit IgG (Invitrogen) per sample with 0.5% BSA was added to incubate at 4°C for 2 h, washed with 300 μl of IP buffer twice, re-suspended in the anti-m^6A antibody mixture prepared above, and incubated overnight. After the EP tube was placed on a magnetic bead stand for 10 min to allow the beads to attach to the tube wall, the beads were washed with 500 μl of IP buffer three times and eluted with 200 μl of elution buffer at 50°C for 1 h. Then, the isolated co-precipitated RNA was used for qPCR as described above with the primers for three m^6A-enriched genes *MMP2 (forward primer 5′-GGATGATGCCTTTGCTCG-3′ and reverse primer 5′-ATCGGCGTTCCCATACTT-3′), NCOA1 (5′-CAGCTTCACTTCAGTCCGC-3′ and 5′-CTTTGCCACTAAGGAAGGATA-3′),* and *TLR4 (5′-CAGGATGAGGACTGGGTAAGGA-3′ and 5′-ATGGAGGCACCCCTTCTTCTA-3′).*

Cell culture and transfection

Eutopic endometrial tissues for cell culture were collected from 3 patients who had the disease duration of 1 to 2 years, with two at stage III and one at stage IV, one bilateral endometrioma and two unilateral endometrioma. Tissues were cut into pieces and digested with collagenase and then DNase I. After filtration through a 70-μm cell strainer, endometrial

stromal cells were collected and cultured in F12/DMEM with 10% fetal bovine serum in an incubator with 5% CO_2 at 37°C. siRNA-targeted human *METTTL3* or *METTL14* (RiboBio, Guangzhou, China) was transfected into the cells using Lipofectamine 2000 (Invitrogen, USA). The siRNA-targeted human *METTTL3* included METTL3 siRNA-1(5′-CAAGTATGTTCACTATGAA-3′), siRNA-2(5′-GACTGCTCTTTCCTTAATA-3′) and siRNA-3(5′-GGACTCGACTACAGTAGCT-3′). The siRNA-targeted human *METTTL14* included METTL14 siRNA-1 (5′-GGATGAAGGAGAGACAGAT-3′), siRNA-2 (5′-TATCGGCTTGTAAGTACAT-3′), and siRNA-3 (5′-GGACCAACGCTTACAAATA-3′). After 48 h, the cells were then harvested for subsequent experiments. All experiments were performed in three biological replicates, with three technical replicates in each experiment.

Cell viability assay

The primary eutopic stromal cells seeded in 96-well culture plates at 10,000 cells/well were transfected with 50 nm si-METTL3 shRNA, si-METTL14 shRNA or shNC (Guangzhou Ruibo Biotechnology Inc., Guangzhou, China) and 1×10^{-7} mmol/L estradiol. After incubation for 48 h, the cells were treated with 10 μl of Cell Counting Kit-8 (CCK-8) solution (Med Chem Express, NJ, USA). The OD value of each well was measured at 450 nm and recorded by Infinite M200 PRO (Tecan, Grödig, Austria).

Transwell migration and Matrigel invasion assays

Transwell migration and Matrigel invasion assays were performed using Transwell chambers and 8mm polycarbonate filters (Discovery Labware Inc., Bedford, MA, USA). The eutopic stromal cells were transfected with *si-METTL3* shRNA, *si-METTL14* shRNA or shNC for 48 h and suspended in 200 μl of serum-free DMEM/F-12 (Gibco) at 2×10^4 and 2×10^5 cells/l for the transwell migration and Matrigel invasion assay, respectively. Each experimental parameter was set up in duplicates. Afterward, the suspended cells were seeded into the top chambers with or without a Matrigel coating (BD Biosciences, USA). Meanwhile, 700 μl of DMEM/F-12 with 10% FBS (Gibco) was placed into the bottom chambers.

After incubation at 37°C for 24 h(migration assay) or 48 h (invasion assay), the cells remaining on the membrane surface were removed. Subsequently, the cells were washed with PBS

twice, and methanol was added to the upper and lower chambers for 20 min. Next, the cells were stained with 0.1% crystal violet for 15 min and examined under an optical microscope (200× magnification). Finally, the cells migrating through the membrane and the cells invading the Matrigel were randomly selected from each chamber and counted in five non-overlapping 200× fields under a light microscope using ImageJ software.

***In vitro* scratch assay**

The eutopic stromal cells at 1×10^5/well were seeded into 6-well plates. After 24 h, an incision was made on the cell plates with a 200-μl sterile pipette tip, and the cells were treated with si-METTL3 shRNA, si-METTL14 shRNA or shNC. The migration of the cells to the incision was observed under an inverted microscope after 48 h. The distances between the incision areas were measured using ImageJ.

Western blot

The GAPDH antibody (WL01114; WanleiBio) (1:500) was used for normalization, as multiple laboratories have shown that GAPDH has been securely expressed in endometrial stromal cells. The polyclonal rabbit IgG antibody against human NCOA1 (51114-1-AP; ProteinTech), polyclonal rabbit IgG antibody against human MMP2 (10373-2-AP; ProteinTech), polyclonal rabbit antibody against human PPP2R2D (bs-19968P; Biosss), polyclonal rabbit IgG antibody against human YWHAZ (14881-1-AP; Proteintech), were diluted at 1:1000. The secondary antibody (M21007; Abmart) was applied at 1:5,000.

Statistical analysis

Statistical analysis was done using *SPSS* 25.0 and GraphPad Prism 9.0. The data were presented as mean ± standard deviation. Two-tailed Student's *t*-tests were used for pairwise comparisons, and one-way ANOVA analysis for comparisons among multiple groups. Statistical significance was set at a *P*-value (two-sided) of <0.05.

Results

Differential profiles and potential roles of m^{6}A methylation in endometriosis

We performed m^{6}A–mRNA epitranscriptomic microarray with the tissues from paired EC and EU. Hierarchical clustering showed differential mRNA methylation levels in the EC and EU tissues (Figure 1A). In addition, volcano plots displayed mRNAs with differentially methylated levels (Figure 1B). The methylation levels were downregulated in 1,312 mRNAs and upregulated in 518 mRNAs in the EC compared with the EU; thus, there were more hypo-methylated genes in the EC.

Moreover, GO and KEGG pathway analyses were performed to investigate the biological function of m^{6}A modification in endometriosis. The top 10 enriched biological processes, cellular components, and molecular function genes with hyper-methylated and hypo-methylated m^{6}A were identified (Figure 1C and D, respectively). The top clusters of biological functions were mainly related to stem cell differentiation and the cell cycle. The pathways of the genes with hyper-methylated and hypo-methylated m^{6}A in endometriosis were revealed (Figure 1E and F, respectively). The genes with hyper-methylated m^{6}A were enriched in the pathways related to the transcriptional dysregulation in cancer and ether lipid metabolism. Meanwhile, the genes with hypo-methylated m^{6}A were significantly associated with the estrogen signaling pathway, Hippo signaling pathway, PI3K–Akt signaling pathway and adherens junction pathway in endometriosis. These data revealed distinct differences in the m^{6}A methylation profiles between the EC and EU, suggesting that the genes with hypo-methylated m^{6}A might be related to the pathogenesis of endometriosis.

Conjoint analysis of the m^{6}A methylation profile and mRNA transcription

Hierarchical clustering uncovered differentially expressed mRNAs in the EC and EU (Figure 2A). The volcano plot exhibited 1,797 upregulated mRNAs and 2,580 downregulated mRNAs in the EC compared to the EU (Figure 2B), indicating that more genes were downregulated in the EC. In addition, we conducted a conjoint analysis of the m6A methylation and mRNA levels. There is a significant enrichment of methylated RNA in differentially expressed genes by chi square test (P<0.001). The four-quadrant plot displayed the distribution of differential genes in both mRNA and m6A methylation levels (Figure 2C); 154 upregulated mRNAs were m6A hyper-methylated (hyper-up, upper right, orange), and 659 upregulated mRNAs were m6A hypo-methylated

(hypo-up, lower right, pink), whereas 220 downregulated mRNAs were m6A hyper-methylated (hyper-down, upper left, green), and 697 downregulated mRNAs were m6A hypo-methylated (hypo-down, lower left, blue). The conjoint analysis results indicated that there were more hypo-methylated and upregulated (hypo-up) genes (659) than hyper-methylated and downregulated (hyper-down) genes (220) in the EC.

Validation of the m^{6}A methylation level and expression of genes

Validation was performed on *MMP2*, *NCOA1*, *TLR4*, *PPP2R2D* and *YWHAZ*, five genes included in the conjoint analysis of hypo-methylated m^{6}A and upregulated gene expression. *MMP2*, *NCOA1*, *TLR4 were* enriched in estrogen signaling, while*PPP2R2D* and *YWHAZ* were enriched both in Hippo and PI3K-Akt signaling pathways. The m^{6}A methylation levels were examined using MeRIP-qPCR (Figure 3A). There was no significant difference in the m^{6}A methylation of the *TLR4* mRNA in the EC and EU. However, the m^{6}A methylation of the *MMP2* mRNA significantly decreased by 38.5% ($P < 0.05$), and that of the *NCOA1* mRNA decreased by 59.6% ($P < 0.05$) in the EC compared with the EU. The mRNA levels were measured using qPCR (Figure 3B). Compared with those in the EU, the mRNA levels of *MMP2* and *NCOA1* in the EC were significantly upregulated by 2.82- and 2.98-fold, respectively ($P < 0.05$); while that of *TLR4* had no significant difference between two groups. MMP2 was positive mainly in the nucleus, with higher expression in EC compared to paired EU. NCOA1 was positive mainly in the cytoplasm, with a higher level in EC than that in paired EU. And PPP2R2D and YWHAZ were both positive in the cytoplasm and nucleus, with a higher level in EC than that in paired EU (Figure 3C).

Differential expression of METTL3 and METTL14 in the ectopic endometrium and eutopic endometrium

The expression of *METTL3*, *METTL14*, *WTAP* and *RBM15* was detected using qPCR. The expression of *METTL3* and *METTL14* significantly decreased by 70.6% and 63.2%, respectively, in the EC compared to the EU (Figure 4A). However, there were no significant differences in the expression of *WTAP* and *RBM15*. By immunohistochemistry, both METTL3 and METTL14 protein were positive mainly in the nucleus, with a higher level in EC than that in paired EU (Figure 4B).

Downregulation of *METTL3/METTL14* promotes cell proliferation, migration and invasion

Next, we investigated the role of *METTL3* and *METTL14* in cell proliferation and invasion. We treated endometrial stromal cells with si-*METTL3* and si-*METTL14*. qPCR and Western blotting assays demonstrated that the expression of *NCOA1, MMP2, PPP2R2D* and *YWHAZ* was increased after *METTL3* and *METTL14* knockdown (Figure 5 A, B). CCK-8 assay showed that *METTL3* or *METTL14* knockdown promoted cell proliferation; this result was enhanced by both *METTL3* and *METTL14* knockdown (Figure 5C). Similarly, cell migration was increased after *METTL3* or *METTL14* knockdown and enhanced by both *METTL3* and *METTL14* knockdown. The migration ability of endometrial stromal cells was further examined using the starch assay (Figure 5D). Lastly, Trans-well assay results indicated that *METTL3* or *METTL14* knockdown promoted the invasion and migration of endometrial stromal cells; this result was reinforced by both *METTL3* and *METTL14* knockdown (Figure 5E).

Discussion

Studies of endometriosis have suggested several important models for the induction of endometriotic growth in the pelvis. For example, because of increased local E2 concentrations in the EC, the activation of the estrogen signaling pathway has been thought to play a crucial role in endometriosis progression. The m^6A modification, one of the most crucial modifications on eukaryote mRNAs, may regulate the endometriotic progression. However, the m^6A–mRNA epitranscriptomic profile and the exact molecular mechanism requires clarification.

In the present study, we performed m^6A–mRNA epitranscriptomic microarray and found more hypo-methylated genes in the EC than in the EU. In addition, the m^6A methylation and mRNA transcription conjoint analysis identified more hypo-methylated with upregulated (hypo-up) genes in the EC. Moreover, pathway analysis revealed that the genes with hypo-methylated m^6A were significantly associated with the estrogen signaling pathway, Hippo signaling pathway, PI3K–Akt signaling pathway, CAMs, and adherens junction. These results are consistent with the reported association between estrogen signaling dysregulation and endometriosis (Han et al., 2015; Marquardt et al., 2019; Vercellini et al., 2014; Zhou et al., 2016). In addition, the functions of steroid receptors are regulated by steroid receptor coactivators (SRCs), comprising SRC-1, SRC-2,

and SRC-3 (Marquardt et al., 2019). SRC-1 and SRC-2, encoded by *NCOA1* and *NCOA2*, respectively, are the most significant regulators in the endometrium; SRC-1 is mainly involved in E_2 signaling, and SRC-2 in progesterone functions (Shi et al., 2014; Marquardt et al., 2019). Moreover, the activation of PI3K–Akt signaling promotes endometriotic lesion growth by upregulating Erβ (Zhou et al., 2016). In addition, Hippo signaling enhances cell proliferation and apoptosis; inhibiting the pathway decreases cell proliferation and apoptosis and reduces endometriotic lesions in a nude mice endometriosis model (Song et al., 2016). Meanwhile, aberrant cell *adhesion* is thought to be a crucial initiation of the growth of the EC (Tsai et al., 2018). Our results suggested that m^6A methylation might play a role in endometriosis via the post-transcriptional regulation of those critical pathways.

We further verified the hypomethylation of *NCOA1*, *MMP2*, *PPP2R2D* and *YWHAZ* mRNA, corresponding with the upregulation of their mRNA levels. Matrix metallopeptidase 2 (MMP2) is related to high estrogen levels and promotes cell invasion and metastasis in breast carcinoma (Di et al., 2005). In addition, *MMP2* expression in the endometrium is increased under estrogen stimulation (Li et al., 2018; Li et al., 2019) and involved in the migration and invasion of endometrial cells in endometriosis (Li et al., 2018). Increased expression of YWHAZ in endometriosis enhances cell proliferation (Joshi et al., 2015). Upregulated PPP2R2D in gastric cancer promoted cell proliferation and migration, and is positively correlated with tumor stage (Yu et al., 2018). Thus, *NCOA1*, *MMP2*, *PPP2R2D* and *YWHAZ* which would be activated by m^6A hypomethylation may contribute to the pathogenesis of endometriosis.

The aberrant m^6A methylation levels are related to the aberrant expression of genes encoding m^6A regulators. Additionally, m^6A is produced by the methyltransferase complex, which is in the nucleus and composed of METTL3, METTL14, WTAP, and RBM15 (Zaccara et al., 2019; Huang et al., 2021). Furthermore, METTL3 and METTL14 form a heterodimeric complex and cooperatively function as the central m6A writers. METTL14 recognizes and binds to RNA substrates, which structurally activates the catalytical activity of METTL3. (Zaccara et al., 2019; Huang et al., 2021). Thus, we measured the expression level of both METTL3 and METTL14 in paired EC and EU tissues. METTL3 and *METTL14* was validated to be significantly downregulated in EC Compared with the EU, consistent with a previous database analysis (Jiang et al., 2020).

METTL3 acts as the core catalytic enzyme in m^6A modification; whereas METTL14, with a high binding affinity to target RNAs, acts as an activator of METTL3 (Zaccara et al., 2019). Dysregulation of METTL3 and METTL14 may plays an important role in tumorigenic processes via epitranscriptomic mechanisms (Liu et al., 2021). However, the function of METTL3 and METTL14 in endometriosis remains unknown.

In our study, downregulated *METTL3* and *METTL14* promoted stromal cell proliferation, and migration and invasion in the EU. Thus, the data reflected the biological functions of m^6A writers in endometriosis. For example, the expression of *NCOA1*, *MMP2*, *PPP2R2D* and *YWHAZ* was found to be significantly increased after the knockdown of *METTL3* and *METTL14*, implying that *METTL3* and *METTL14* played a vital role in endometriosis by modulating the m^6A methylation of related genes. Additionally, SRC-1, encoded by *NCOA1*, enhances the invasion of endometriotic epithelial cells via its cooperative interactions with Erβ to enable the implantation of endometriotic lesions (Han et al., 2015). Moreover, MMP2 is involved in the estrogen-related ectopic growth of the endometrium (Wang and Ma, 2012). *PPP2R2D* and *YWHAZ* are implicated in cell proliferation and migration (Joshi et al., 2015; Yu et al., 2018). These data indicated that downregulation of *METTL3* and *METTL14* promoted the growth and infiltration of endometriotic lesions into extra uterine organs by modulating m^6A methylation levels of several genes associated with the estrogen signaling pathway. Further investigations are needed to clarify the specific mechanism in modulating the target genes in endometrial cells in endometriosis.

In summary, the present study profiled the m^6A methylation pattern and the expression of m^6A regulator genes in endometriosis. We found more hypo-methylated and upregulated genes in the EC. Bioinformatic analysis indicated a role of potential post-transcriptional modulation in the pathogenesis of endometriosis. Furthermore, we observed that the downregulation of *METTL3* and *METTL14* contributed to the proliferation, invasion, and migration of endometrial stromal cells in endometriosis. The main limitation of the study is the small specimen number with no normal endometrium as a control. And on the other hand, we only enrolled specimens from ovarian endometriosis in proliferative phase; thus, the results cannot be extrapolated to other types of endometriosis or those in secretory phase. A larger sample size with a wide range of types endometriosis would help to improve the validity for subsequent analysis and verification. In addition, further exploration the mechanism and regulatory pattern of m^6A methylation in an

animal model could be a productive issue for future research in endometriosis.

Data availability statement

All data generated or analysed during this study are included in this published article, or they are available from the corresponding author on reasonable request.

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Authors' roles

YWY and CZ performed the experiments and analyzed the data. YWY wrote the manuscript. YZ conceived and supervised the study. LCS obtained funding, designed the research plan, and revised the manuscript. All authors read and approved the final manuscript.

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KEY MESSAGE

A total of 1312 mRNAs were identified dysregulating the m6A methylation levels.Pathway analysis found the genes were significantly associated with estrogen, Hippo, and PI3K–Akt signaling and cell–cell adhesion. METTL3 and METTL14 knockdown caused cell proliferation and invasion in the pathogenesis and progression of endometriosis.

Table 1. Clinical characteristics of 18 patients with endometriosis

Items	**Patient variables**
Age	32.56±7.55
BMI	20.56±2.51
Disease course	0.2-7 years
Phase of menstrual cycle	proliferation
Location of endometriosis	
unilateral ovarian endometriomas	10
bilateral ovarian endometriomas	8
Stage	
III	10
IV	8

Table 2. Primers for qRT-PCR

Primers	Sequence (5' - 3')
METTL3-F	CTGGGCACTTGGATTTAAGGAA
METTL3-R	TGAGAGGTGGTGTAGCAACTT
METTL14-F	GAGCTGAGAGTGCGGATAGC
METTL14-R	GCACATGTATCATAGGAAGCCC
WTAP-F	TTGTAATGCGACTAGCAACCAA
WTAP-R	GCTGGGTCTACCATTGTTGATCT
RBM15-F	GTGAGGACTCGACTTCCCG
RBM15-R	GCCGCTATCGGTCTTTCCG
MMP2-F	GATACCCCTTTGACGGTAAGGA
MMP2-R	CCTTCTCCCAAGGTCCATAGC
NCOA1-F	AATGAATACGAGCGTCTACAGC
NCOA1-R	TTTCGTCGTGTTGCCTCTTGA
TLR4-F	TGCCTCTCTTGCATCTGGCTGG
TLR4-R	CTGTCAGTACCAAGGTTGAGAGCTGG
PPP2R2D-F	GTCAAGGACAGGGCAGACTTC
PPP2R2D-R	AGCTGTTCTCAGCTGTTCTATCA
YWHAZ-F	CTCTCTTGCAAAGACAGCTTTT
YWHAZ-R	GTCCACAATGTCAAGTTGTCTC
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGGACTGTGGTCATGAG

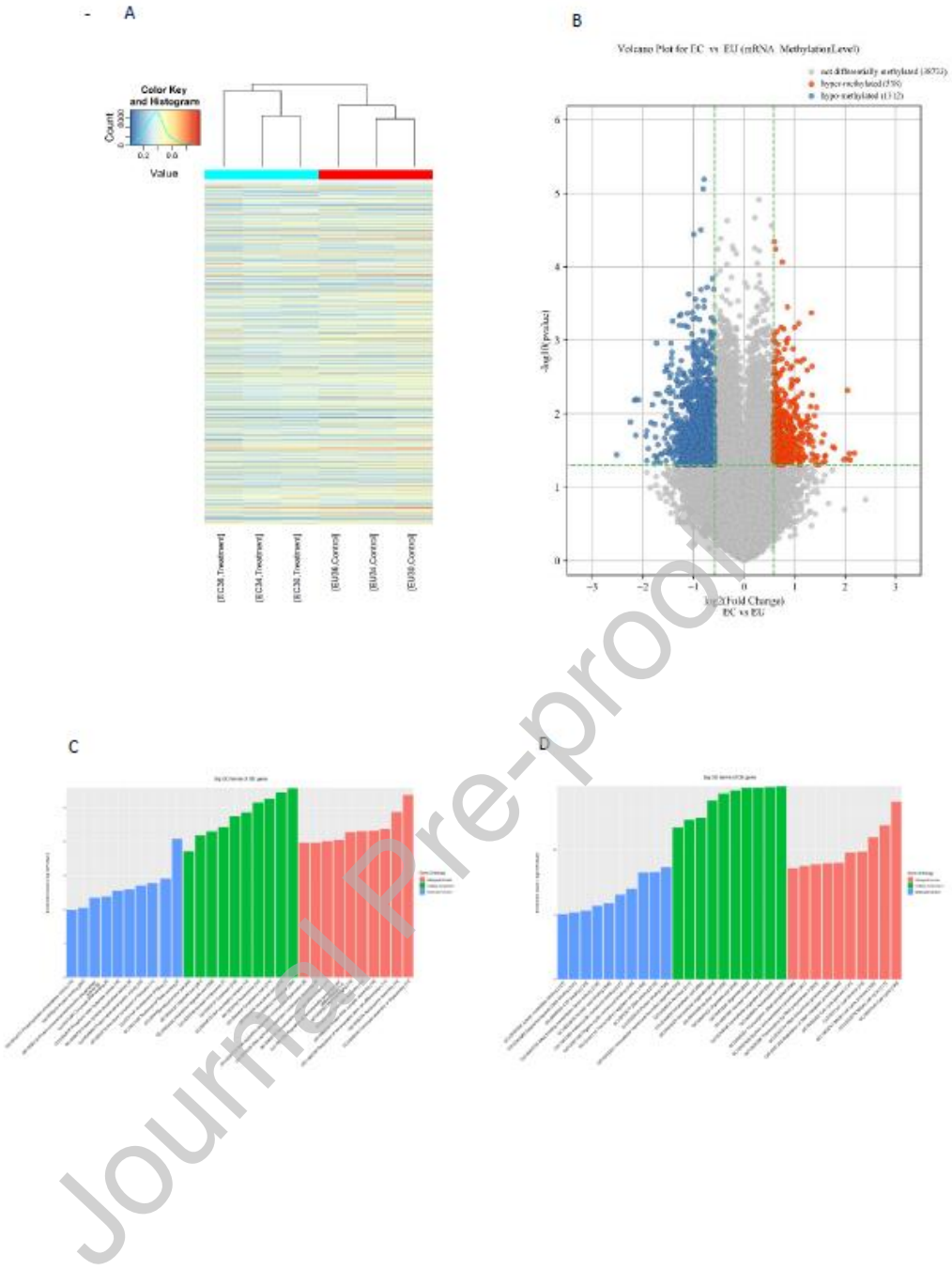


Figure 1. Characterization and bioinformatic analysis of m^{6}A methylation in endometriosis. (A) The hierarchical clustering of the mRNA methylation levels in paired ectopic endometrium (EC) and eutopic endometrium (EU). (B) The volcano plots of the 1,312 mRNAs with downregulated methylation levels and 518 mRNAs with upregulated methylation levels in the EC compared with the EU. The red and blue plots indicate statistically significant change in the mRNA methylation level based on |FC| ² 1.5 and *P*-values ¢ 0.05. (C) The top 10 GO terms of genes with hyper-m^{6}A

methylation levels. (D) The top 10 GO terms of genes with hypo-m^{6}A methylation levels. (E) The KEGG pathways of genes with hyper-m^{6}A methylation levels. (F) The KEGG pathways of genes with hypo-m^{6}A methylation levels. All samples were assayed in triplicate.

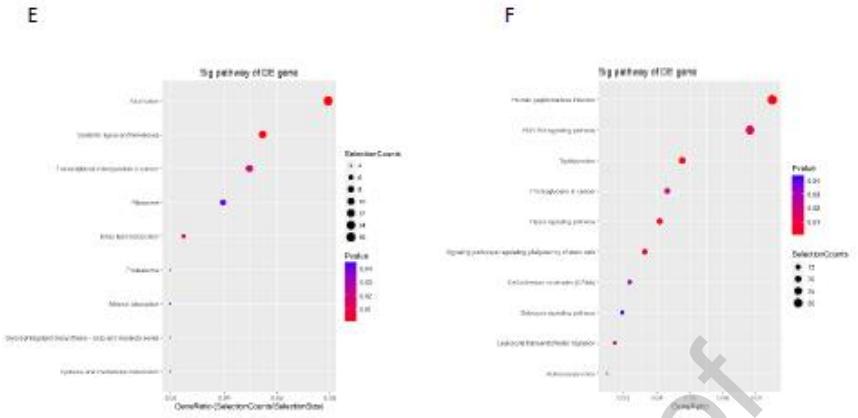
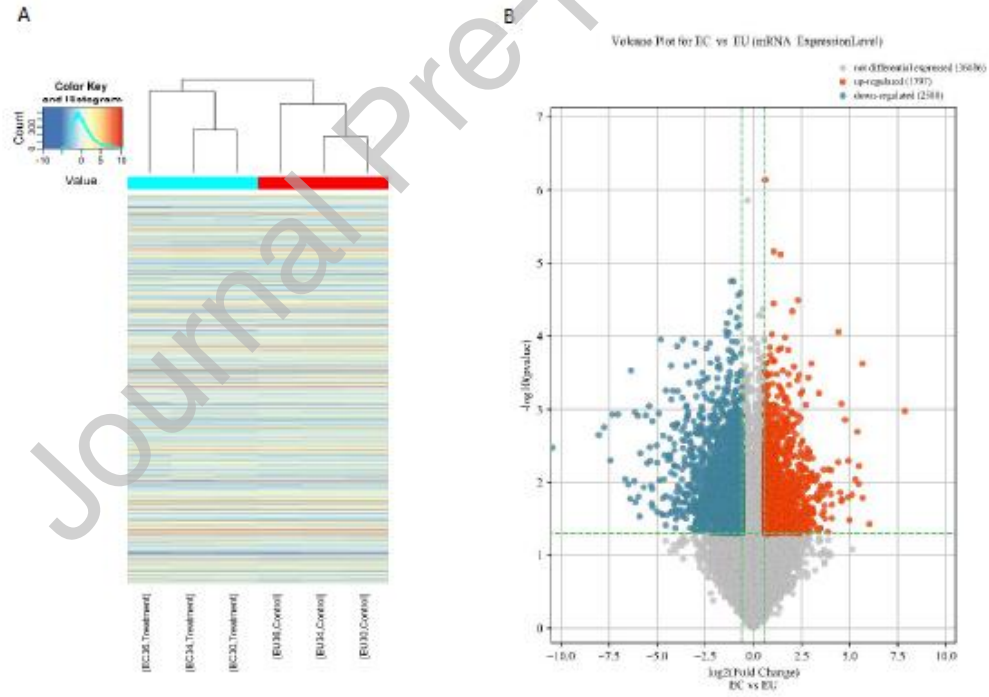


Figure 1-



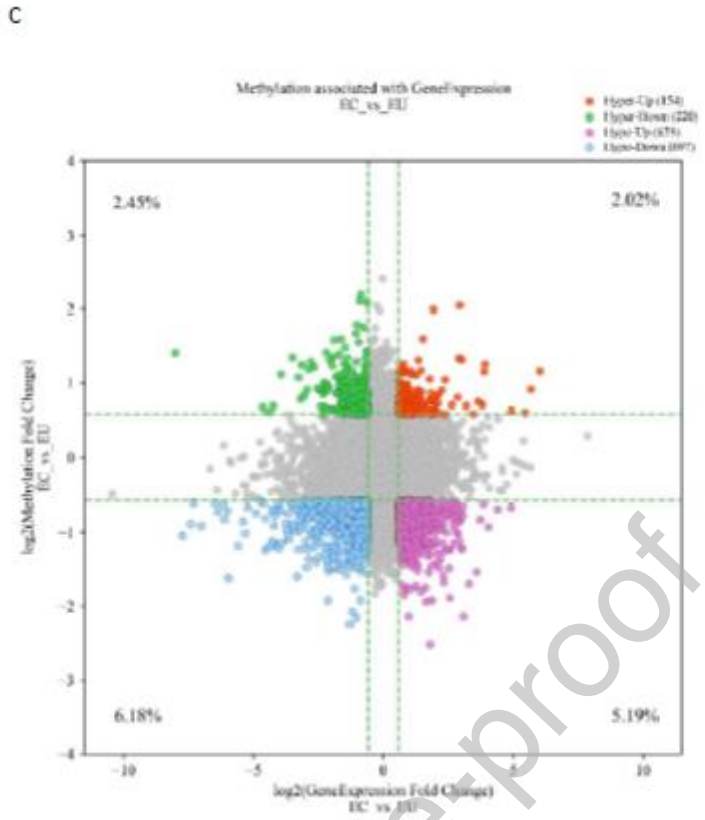


Figure 2. Conjoint analysis of m^6A methylation levels and mRNA transcription. (A) The hierarchical clustering of the mRNA levels in EC and EU. (B) The volcano plots of 1,797 upregulated mRNAs and 2,580 downregulated mRNAs in the EC compared with the EU. The red and blue plots indicate statistically significant change in the mRNA level based on |FC| ² 1.5 and *P*-values ¢ 0.05. (C) The four-quadrant plots of the distribution of genes with significant changes in both mRNA and m^6A methylation levels**.** Upper right quadrant: hyper-up, orange; lower right quadrant: hypo-up, pink; upper left quadrant: hyper-down, green; lower left quadrant: hypo-down, blue. All samples were assayed in triplicate.

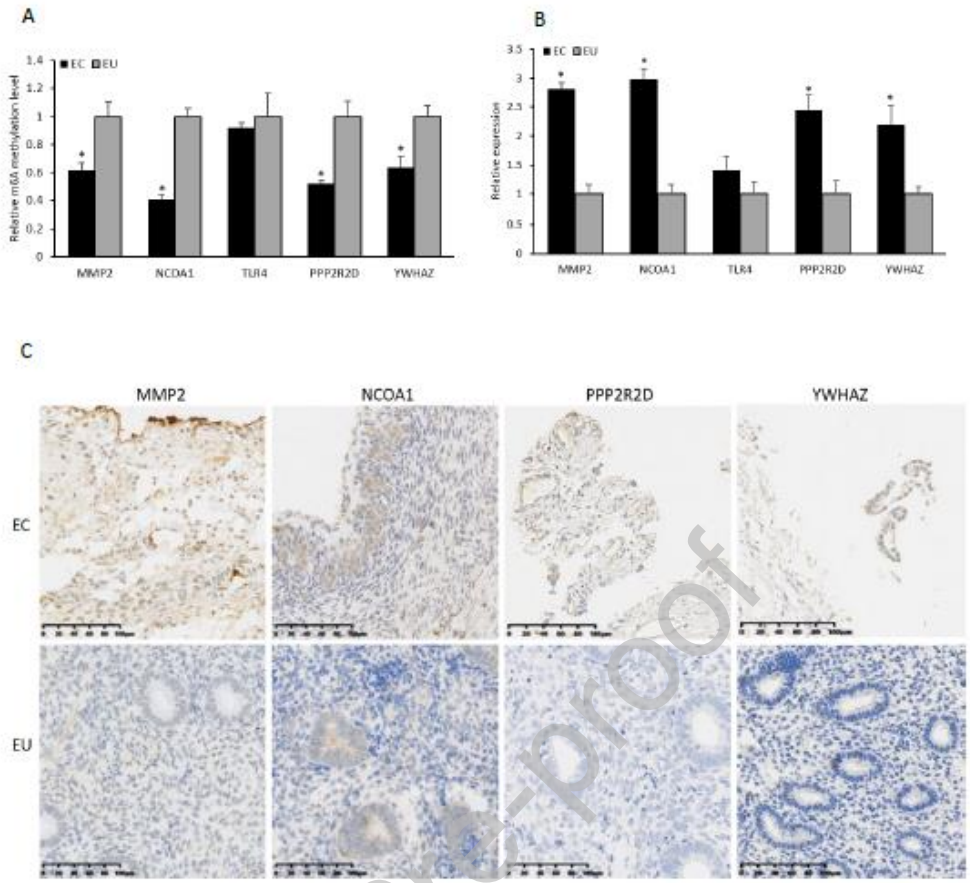


Figure 3. Validation of the m^{6}A methylation level and mRNA level of the genes enriched in estrogen signaling. (A) The m^{6}A methylation level of *MMP2*, *NCOA1*, *TLR4*, *PPP2R2D* and *YWHAZ* in the ectopic endometrium (EC, black bar) and eutopic endometrium (EU, hatched gray bar) was validated using MeRIP-qPCR. (B) The expression of *MMP2*, *NCOA1*, *TLR4*, *PPP2R2D* and *YWHAZ* in the EC (black bar) and EU (hatched gray bar) using qRT-PCR. (C) Immunohistochemistry was performed to assess the protein levels of MMP2, NCOA1, PPP2R2D and YWHAZ in the EC and EU; magnification, ×200. Two-tailed Student's t-tests were used for comparisons between EC and EU. All samples were assayed in triplicate. * indicate statistical significance (P <0.05).

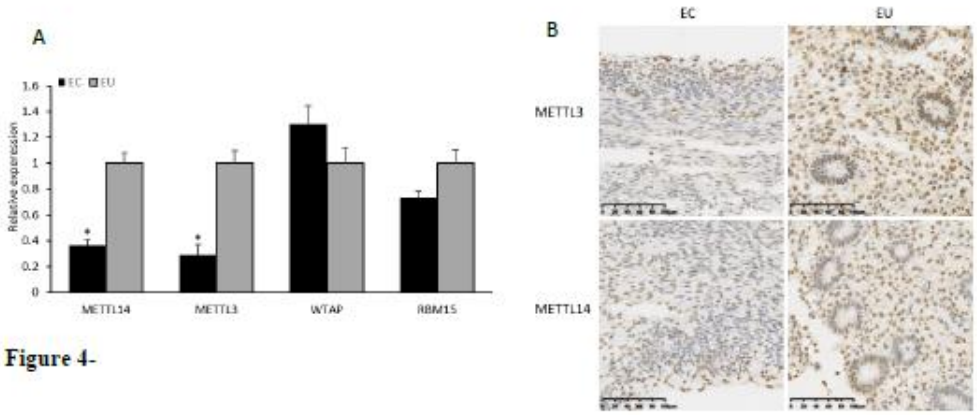


Figure 4-

Figure 4. The expression levels of m6A writers in the ectopic endometrium and eutopic endometrium. (A) The mRNA expression levels of *METTL3*, *METTL14*, *WTAP and RBM15* by qPCR. (B) The protein expression levels of METTL3 and METTL14 by Immunohistochemistry staining; magnification, ×200. Two-tailed Student's t-tests were used for comparisons between EC and EU. All samples were assayed in triplicate. * indicate statistical significance (P <0.05).

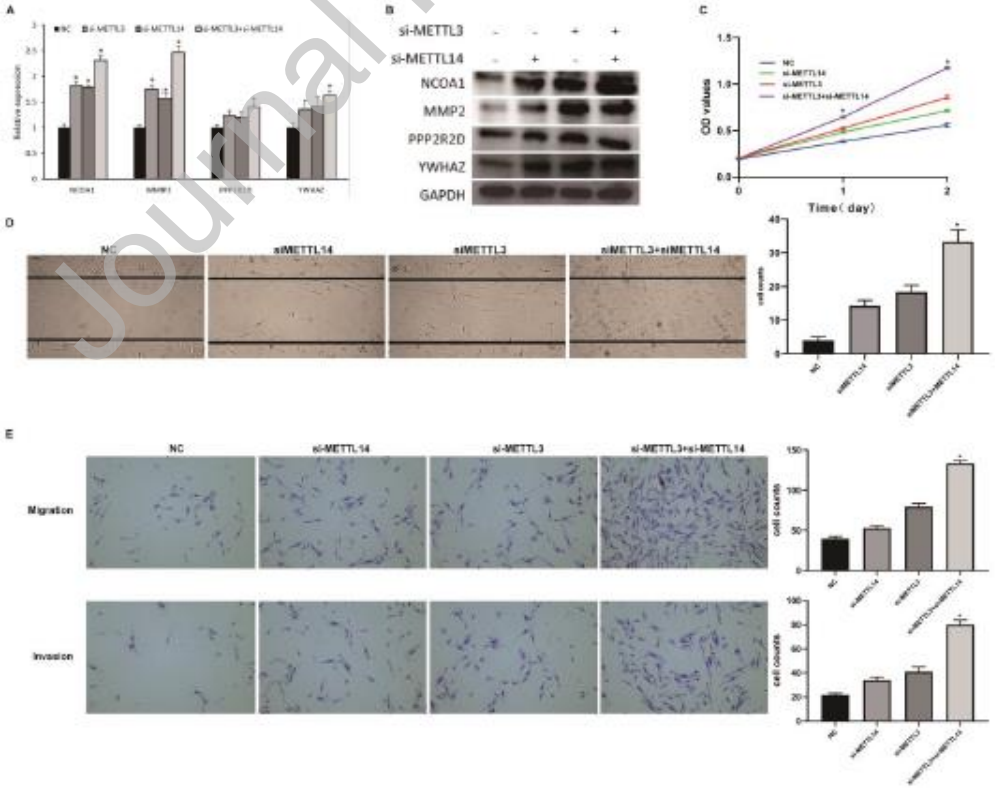


Figure 5. Knockdown of *METTL3* and（or） *METTL14* contributes to the cellular phenotype of endometriosis. (A–B) qPCR and Western blotting analysis of the expression of *NCOA1, MMP2 PPP2R2D* and *YWHAZ* after the knockdown of *METTL3* and(or) *METTL14*. Cell viability assay of the cells with *METTL3* and *METTL14* knockdown. (C) The CCK-8 assay of the cell viability after the knockdown of *METTL3* and *METTL14*. (D) The scratch assay of the cell migration after the knockdown of *METTL3* and *METTL14*. (E) Transwell assay of the effect of the *METTL3* and *METTL14* knockdown on the invasion and migration of endometrial stromal cells. NC: negative control; *si-METTL14* was transfected with *siMETTL14*; *si-METTL3* was transfected with *siMETTL3*; si-*METTL3*+*si-METTL14* were transfected with *si-METTL3* and *si-METTL14*. All samples were assayed in triplicate. * indicate statistical significance (P <0.05).