Inc-MAP3K13-7:1 Inhibits Ovarian GC Proliferation in PCOS via DNMT1 Downregulation-Mediated CDKN1A Promoter Hypomethylation

Xueying Geng,1,2 Jun Zhao,1,2 Jiayu Huang,1,2 Shang Li,1,2 Weiwei Chu,1,2 Wang-sheng Wang,1,2 Zi-Jiang Chen,1,2,3,4,5 and Yanzhi Du1,2

1Center for Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200135, China; 2Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Shanghai 200135, China; 3Center for Reproductive Medicine, Cheeckog College of Medicine, Shandong University, Jinan, Shandong 250012, China; 4National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, Jinan, Shandong 250012, China; 5Key Laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, Jinan, Shandong 250012, China

Polycystic ovary syndrome (PCOS) is an endocrine-related disease and global cause of infertility that is associated with abnormal folliculogenesis. Inhibited granulosa cell (GC) proliferation is recognized as a key factor that underlies aberrant follicle maturation. Many epigenetic landscape modifications have been characterized in PCOS patients. However, the epigenetic regulation pathways in follicular dysplasia are not completely understood. In this study, we reported a novel mechanism of DNA hypomethylation induced by long non-coding RNAs (lncRNAs) and its function in cell cycle progression. We observed that Inc-MAP3K13-7:1 was highly expressed in GCs from patients with PCOS, with concomitant global DNA hypomethylation, decreased DNA methyltransferase 1 (DNMT1) expression, and increased cyclin-dependent kinase inhibitor 1A (CDKN1A, p21) expression. In KGN cells, Inc-MAP3K13-7:1 overexpression resulted in cell cycle arrest in the G0/G1 phase, as well as the molecular inhibition and genetic silencing of DNMT1. Mechanistically, Inc-MAP3K13-7:1 inhibited DNMT1 expression by acting as a protein-binding scaffold and inducing ubiquitin-mediated DNMT1 protein degradation. Moreover, DNMT1-dependent CDKN1A promoter hypomethylation increased CDKN1A transcription, resulting in attenuated GC growth. Our work uncovered a novel and essential mechanism through which Inc-MAP3K13-7:1-dependent DNMT1 inhibition regulates CDKN1A/p21 expression and inhibits GC proliferation.

INTRODUCTION
Polycystic ovary syndrome (PCOS) is a common and heterogeneous endocrine disorder in reproductive-aged women. Its overall prevalence according to the criteria of Rotterdam is approximately 8%–15%.1–3 Women with PCOS experience ovarian defects such as arrested follicular development,4,5 anovulation and poor oocyte quality,6,7 which adversely affect their reproductive health. The pathogenesis of PCOS is largely unknown. The follicles of patients with PCOS are arrested in the small antral follicular stage,2 and they cannot be recruited to develop into mature follicles, thereby halting ovulation. The granulosa cells (GCs) in the follicles provide nutrients and growth regulators for the development of oocytes, which are essential for follicular development and ovulation.8,9 Therefore, delays in the proliferation of small antral follicle GCs can delay follicular development, which likely plays an important role in the pathogenesis of PCOS.

Although genetic variations and environmental factors are vital to the development and progression of PCOS,11,12 emerging studies suggest that epigenetics, especially DNA methylation, plays a key role in the pathogenesis of PCOS.13,14 Increased methylation is usually associated with gene silencing or reduced gene expression.15 Persistent changes in the ovarian epigenome lead to altered gene expression, which subsequently affects ovulation,16 steroid hormone synthesis,17 and lipid metabolism18 in ovarian GCs. Previous studies revealed a reduction in the global DNA methylation of ovarian GCs in women with PCOS compared with the findings in healthy controls;18 however, in-depth mechanistic research is needed to elucidate the role of DNA methylation in the pathogenesis of PCOS.

Long non-coding RNAs (lncRNAs) are defined as transcripts longer than 200 nt without coding potential19 that mediate a variety of cellular functions, including chromatin modification and transcriptional and post-transcriptional regulation.20 Despite recent studies demonstrating aberrant lncRNA expression in human GCs from women with and without PCOS,21–23 only a few of these differentially expressed lncRNAs have been carefully characterized concerning their functional roles in the development of PCOS. Based on available reports, lncRNAs are involved in oocyte development, GC
proliferation, and steroidogenesis. The preliminary work of our group identified novel IncRNAs that were differentially expressed in luteinized GCs obtained from women with and without PCOS using microarray analysis and characterized that one such IncRNA, named LINC-01572:28, inhibited GC growth by decreasing p27 (Kip1) degradation. Despite these findings, the functions of other novel IncRNAs that we found in PCOS are indistinct.

Previous studies uncovered a novel regulatory mechanism of gene methylation governed by IncRNAs, specifically that DNA methyltransferases (DNMTs) may themselves be targets of IncRNAs. In accordance with this idea, recent studies have identified IncRNAs that interact with DNMTs to reprogram global DNA methylation, thereby regulating multiple cellular activities. However, the mechanism by which novel IncRNAs could interact with DNMTs in ovarian GC cells is unclear.

In this study, we identified Inc-MAP3K13-7:1 as a functional IncRNA that could directly bind to DNMT1 and increase its degradation. Inc-MAP3K13-7:1 controlled the expression of the proliferation inhibitor p21 (Waf1/Cip1) by regulating the methylation of its gene promoter region, thereby preventing G1/S cell cycle progression, inhibiting cell proliferation, and ultimately creating a GC growth arrest phenotype. Our study provided novel insights into the mechanisms by which IncRNAs regulate cell proliferation via epigenetic modulation, identifying a novel site of epigenetic interpretation in the pathogenesis of PCOS, and led us to propose that Inc-MAP3K13-7:1 might be an important target for mediating GC function.

RESULTS

Inc-MAP3K13-7:1 Expression Was Upregulated in PCOS, whereas the DNA Methylation Level Was Decreased

Given the prior findings of differentially expressed IncRNAs in microarray analysis, we identified a novel IncRNA, dubbed Inc-MAP3K13-7:1 (Figure S1A), and further confirmed this finding using the lncRNA databases LNCipedia and NONCODE (https://lncipedia.org/db/transcript/lnc-MAP3K13-7-1, http://www.noncode.org/show_rna.php?id=NONHSAT093752&version=2&utd=1#). To identify the full-length of the Inc-MAP3K13-7:1 transcript, 5’ and 3’ rapid amplification of cDNA ends (RACE) studies were performed, and the PCR product was validated via sequencing. We confirmed that the Inc-MAP3K13-7:1 transcript was 1,483 nt in length, with an additional 4 nt at the 3’ poly(A) tail compared to the sequence in LNCipedia (Figures S1B–S1D). Inc-MAP3K13-7:1 was identified as a sense intronic IncRNA within an intron of the coding gene VPS8 with no overlap of its exonic sequence, and the IncRNA was found to be highly conserved in humans and fruit flies with no similar sequences found in zebrafish, mice, and apes (Figures S1A and S1E). Analysis of its coding potential strongly indicated that Inc-MAP3K13-7:1 lacked protein-coding capacity (Figure S1F). Consistently, a cellular fractionation assay revealed that Inc-MAP3K13-7:1 transcripts were predominantly localized in the nuclear extract (Figure S1G).

We next confirmed the expression level of Inc-MAP3K13-7:1 in a cohort of 42 women with PCOS and in 42 controls. Inc-MAP3K13-7:1 expression was significantly elevated in GCs from patients with PCOS (Figure 1A). We characterized global DNA hypomethylation changes in another cohort of women with PCOS and controls by assessing 5-methylcytosine (5-mC) levels in the genomic DNA of GCs via ELISA with antibody specific to 5-mC. A significant reduction in the 5-mC level was noted in GCs from women with PCOS (Figure 1B).

We further explored the expression of DNMTs in GCs between the two groups. The classic DNMTs DNMT1 and DNMT3A were both downregulated at the mRNA level in PCOS (Figures 1C and 1D), whereas DNMT3B mRNA expression remained unchanged (Figure S2A). In addition, DNMT1 protein levels were significantly decreased in another cohort of patients with PCOS versus the controls (Figure 1E), whereas DNMT3A expression did not differ between the groups (Figure S2B). To understand the clinical significance of Inc-MAP3K13-7:1 levels in PCOS, we performed a correlation study and found that Inc-MAP3K13-7:1 expression was positively correlated with serum luteinizing hormone (LH) levels in both patients with PCOS and controls (Figure S3).

Inc-MAP3K13-7:1 Reduced DNMT1 Expression and Prevented Cell Proliferation In Vitro

To explore the molecular mechanisms and functions of Inc-MAP3K13-7:1, we stably overexpressed Inc-MAP3K13-7:1 in KGN cells using a lentiviral system (Figure 2A). To clarify whether Inc-MAP3K13-7:1 could regulate the expression of its neighboring gene VPS8, we first demonstrated that Inc-MAP3K13-7:1 overexpression did not affect VPS8 expression (Figures S4A and S4B). In line with the findings of decreased DNA methylation and DNMT expression in GC cells from patients with PCOS (Figure 1), global DNA methylation levels tended to be lower in KGN cells after Inc-MAP3K13-7:1 transfection (Figure 2B). Real-time PCR demonstrated that Inc-MAP3K13-7:1 overexpression did not change DNMT1 or DNMT3A mRNA expression (Figure 2A). However, western blotting revealed that DNMT1 protein expression, but not DNMT3A protein expression, was decreased by Inc-MAP3K13-7:1 overexpression (Figure 2C).

DNMT1 is known to be involved in activation of the cell cycle and DNA replication, and p21 (Waf1/Cip1) and DNMT1 are potential antagonists. Therefore, we compared the mRNA levels of the cell cycle inhibitor cyclin-dependent kinase inhibitor 1A (CDKN1A, p21) in GCs between patients with PCOS and controls, observing a significant increase in CDKN1A expression in the PCOS group (Figure 2D). These findings prompted us to explore whether Inc-MAP3K13-7:1 induced DNMT1 silencing and affected cell proliferation. Cell Counting Kit-8 (CCK-8) experiments illustrated that the proliferation of KGN cells was greatly inhibited by Inc-MAP3K13-7:1 overexpression (Figure 2E). Using flow cytometry, we observed that Inc-MAP3K13-7:1 overexpression in KGN cells increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase (Figure 2F). The expression of proliferating cell nuclear antigen (PCNA),
CDKN1A (p21), and CDKN1B (p27) was evaluated using real-time PCR and western blotting. The results confirmed that the levels of the positive regulators of the G1-to-S transition such as phosphorylated retinoblastoma protein (p-Rb) and phosphorylated cyclin-dependent kinase 2 (p-CDK2) were decreased by lnc-MAP3K13-7:1 overexpression, similarly as those of PCNA. Conversely, the mRNA and protein levels of the negative G1-to-S regulator p21 (Waf1/Cip1) were decreased by lnc-MAP3K13-7:1 overexpression, whereas CDKN1B mRNA levels were unchanged (Figures 2G, 2H, and S4C). Moreover, annexin V/7-aminoactinomycin D (7-AAD) flow cytometry illustrated that lnc-MAP3K13-7:1 overexpression did not increase apoptosis (Figure S4D).

### Inc-MAP3K13-7:1 Suppressed G1/S Cell Cycle Progression Partly through Silencing DNMT1
To confirm that cell cycle progression was regulated by DNA methylation in GCs, we treated KGN cells with the DNMT inhibitor 5-aza-2′-deoxycytidine (5-Aza), which could incorporate into DNA and inhibit DNMT activity by forming covalent complexes with its catalytic domains of DNMTs, leading to their depletion. As expected, we found that DNMT1 levels decreased as the 5-Aza concentration was increased (Figure 3A), and cell cycle progression was mainly blocked in the G0/G1 phase (Figure 3B). Meanwhile, 5-Aza treatment prevented cell proliferation as confirmed using the CCK-8 assay (Figure 3C). We uncovered that some proliferation-related markers such as p-Rb, PCNA, and p-CDK2 were gradually silenced by increasing concentrations of the inhibitor, whereas inhibitors of proliferation such as p21 (Waf1/Cip1) were gradually upregulated (Figures 3D and S5A). To further confirm that DNMT1 directly repressed cell proliferation, we silenced its expression in KGN cells (Figure 3E). The small interfering RNA (siRNA)-mediated silencing of DNMT1 indeed affected both cell cycle progression and cell proliferation (Figures 3F and 3G). The findings of western blotting also supported this result (Figures 3H and S5B).

We next investigated whether DNMT1 mediated the inhibitory effects of Inc-MAP3K13-7:1 on cellular growth. First, we examined the effect of DNMT1 overexpression on the proliferation of KGN cells. As indicated in Figures 4A–4C, cell growth was stimulated when DNMT1 was overexpressed. This evidence supported the findings in Figure 3 and more firmly demonstrated the correlation between DNMT1 and the proliferative phenotype in ovarian GCs. Based on these data, we next conducted a rescue assay by transfecting DNMT1 overexpression plasmids into cells stably overexpressing
Figure 2. Inc-MAP3K13-7:1 Upregulation Decreased DNMT1 Protein Levels and Inhibited GC Proliferation In Vitro

(A) Quantitative real-time PCR analysis of Inc-MAP3K13-7:1 overexpression using lentiviral vectors in KGN cells. Expression of Inc-MAP3K13-7:1 in stably transfected cells in comparison with that in empty vector-transfected cells (left, n = 9, p < 0.0001). Quantitative real-time PCR analysis of DNMT1 and DNMT3A expression after Inc-MAP3K13-7:1 overexpression (middle and right, DNMT1, n = 9, p = 0.614; DNMT3A, n = 3, p = 0.433). (B) Global DNA methylation of KGN cells after Inc-MAP3K13-7:1 overexpression compared with that in empty vector-transfected cells (n = 3, p = 0.0864, Mann-Whitney U test). (C) Western blot analysis of DNMT1 and DNMT3A protein expression in KGN cells after Inc-MAP3K13-7:1 overexpression. The quantification of protein levels is presented at the bottom (n = 3 for each group; DNMT1, p < 0.0001; DNMT3A, p = 0.682).

(D) Quantitative real-time PCR detection of cyclin-dependent kinase inhibitor 1A (CDKN1A) expression in GCs from 42 patients with PCOS and 42 normal controls (p = 0.0004). (E and F) Effects of Inc-MAP3K13-7:1 overexpression on KGN cell growth measured using the Cell Counting Kit-8 assay (E, n = 3, p < 0.0001) and cell cycle distribution determined using flow cytometry (F, n = 4, p < 0.0001 for each group).

(G) Quantitative real-time PCR analysis of changes in proliferation-related marker expression (proliferating cell nuclear antigen [PCNA] as a positive regulator and CDKN1A and CDKN1B as negative regulators) 72 h after Inc-MAP3K13-7:1 lentivirus transfection (n = 6; PCNA, p = 0.019; CDKN1A, p = 0.004; CDKN1B, p = 0.339). (H) Western blot analysis of changes in proliferation-related marker expression (PCNA, p21, cyclin E1, CDK2, p-CDK2 [Thr160], retinoblastoma protein [Rb], and p-Rb [Ser807/Ser811]) normalized to β-actin protein expression in Inc-MAP3K13-7:1-overexpressing KGN cells. The quantification of protein levels is presented on the right (n = 3; Rb, p = 0.156; p-Rb, p = 0.003; cyclin E1, p = 0.923; PCNA, p = 0.003; CDK2, p = 0.663; p-CDK2, p < 0.0001; p21, p = 0.007). In (A)–(C) and (E)–(H), mean ± SD. In (A) and (C)–(H), *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t test).
Inc-MAP3K13-7:1. In Inc-MAP3K13-7:1-overexpressing cells transfected with the control vector, the cell cycle was mainly arrested in the G0/G1 phase. These effects were rescued by DNMT1 overexpression, which resulted in a lower percentage of cells in the G1 phase and a higher percentage of cells in the S phase (Figure 4B). The cell growth curves indicated that Inc-MAP3K13-7:1-induced cell growth repression could be rescued by DNMT1 overexpression (Figure 4C). The western blotting results were consistent with the aforementioned findings, as p-CDK2 and PCNA expression was suppressed by Inc-MAP3K13-7:1 overexpression, and this suppressive effect was rescued by DNMT1 overexpression (Figure 4D).

**DNMT1 Suppression Led to CDKN1A Upregulation through Decreased Promoter Methylation**

Given that DNMT1 could inhibit cell cycle progression, we sought to identify the downstream targets of DNMT1 in cell cycle progression. Previous studies extensively revealed that DNMT1 could regulate the methylation level of gene promoter regions through direct binding, thereby regulating the transcription levels of genes and ultimately affecting cell activity. CDKN1A expression was significantly increased in KGN cells by DNMT1 silencing, whereas CKDN1B and TP53 expression did not obviously change (Figure 5A). DNMT1 mRNA levels were decreased after 12 h of 5-Aza exposure before returning to normal by 48 h. Unexpectedly, CDKN1A mRNA levels did not change in first 12 h but surged after 48 h (Figure 5B). This result encouraged us to investigate whether these treatments affected the methylation of the CDKN1A promoter region. Using pyrosequencing, we clarified that DNMT1 silencing or 5-Aza treatment induced a demethylation trend in the CDKN1A promoter region and some significant methylation reductions at specific CpG sites (Figures 5C–5E).

**Inc-MAP3K13-7:1 Catalyzed the Ubiquitination of DNMT1 through Direct Binding**

As we previously demonstrated, Inc-MAP3K13-7:1 downregulated DNMT1 through a post-transcriptional mechanism. We next explored the mechanism by which Inc-MAP3K13-7:1 silences DNMT1 expression. Considering the foremost localization of Inc-MAP3K13-7:1 in the nucleus, we wondered whether Inc-MAP3K13-7:1 could regulate DNMT1 mRNA localization or, in other words, prevent DNMT1 mRNA from leaving the nucleus and entering the cytoplasm for translation. We performed an RNA cellular fractionation assay and found that the distribution of DNMT1 mRNA was not significantly changed by Inc-MAP3K13-7:1 overexpression (Figure 6A). Based on this finding, we further considered whether Inc-MAP3K13-7:1 influenced DNMT1 protein degradation. Immunofluorescence staining and protein subcellular fractionation assays demonstrated that Inc-MAP3K13-7:1 overexpression promoted DNMT1 protein from initially aggregating in the nucleus to disperse into the cytoplasm (Figures 6B and 6C). Because DNMT1 mainly functions as a DNMT in the nucleus, it is primary degraded by the ubiquitin-proteasome pathway. Thus, we speculated that Inc-MAP3K13-7:1 could facilitate DNMT1 degradation. We treated KGN cells with carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) to block the degradation of ubiquitinated proteins by proteasomes and found that Inc-MAP3K13-7:1-induced DNMT1 downregulation could be blocked by the addition of MG132 (Figure 6D). Meanwhile, protein stability analysis revealed a significantly reduced half-life of DNMT1 in Inc-MAP3K13-7:1-upregulated cells (Figure 6E). These results suggested that Inc-MAP3K13-7:1 was involved in proteasome-mediated DNMT1 degradation. To further verify our hypothesis, the interaction between ubiquitin and DNMT1 was assessed using co-immunoprecipitation (coIP) assays, and increased Inc-MAP3K13-7:1 expression resulted in increased DNMT1 ubiquitination (Figure 6F). Furthermore, RNA immunoprecipitation (RIP) assays illustrated that Inc-MAP3K13-7:1 could directly bind to DNMT1 (Figure 6G).

**DISCUSSION**

Inc-MAP3K13-7:1 represents one of the few lncRNAs that regulate DNA methylation, and it is the first recognized lncRNA to modulate ovarian GC proliferation through interactions with DNMTs. Recent studies identified lncRNAs as novel players in the regulation of DNA methylation. lncRNAs can act in a cis (at the site of transcription) or a trans (far from the transcription sites) manner. For example, HOTAIR silences the HoxD locus in a cis manner by recruiting polycomb repressive complex 2 to modify the chromatin structure. Conversely, H19 can inhibit SAHH in a trans manner, thereby blocking DNMT3B-mediated methylation at numerous genomic loci. This is an intriguing model for regulating key epigenetic modification events in specific diseases. Previous studies of the epigenetic pathogenesis of PCOS only assessed the role of DNA methylation or lncRNA alone, and we innovatively found the connection between the two. Our study first described the low expression of DNMT1 in the GCs of women with PCOS, and it might be the key to...
the DNA hypomethylation in PCOS. The reason for the inconsistent behaviors in mRNA level and protein level of DNMT3A may be that DNMT3A plays a crucial role in oogenesis and the establishment of genomic imprinting, and therefore its expression is relatively conserved. Our findings suggested that the lncRNA could participate in the establishment of genomic methylation patterns by interacting with DNMT1 and cause aberrant DNA methylation though site-specific adjustments. This proposal will provide a broader perspective for epigenetic research in PCOS.

DNA methylation is the clearest and most classic epigenetic modification in mammals. The only known chemical modification of genomic DNA in mammals is the addition of a methyl group at position C5 in CpG dinucleotides by DNMTs. DNMT1 participates in the maintenance of genome methylation by adding methyl groups to hemi-methylated DNA strands during DNA replication. This protein is highly expressed during the S phase, and it interacts with PCNA. The relationship between DNA methylation and cell growth was previously established. Rhee et al. found that the disruption of DNMT1 and DNMT3B in HCT116 colon cancer cells reduced 5-mC content by 95% and slowed cell growth. Conversely, DKO8 cells retained higher methylation levels and grew more rapidly following disruption of these genes. Meanwhile, Egger et al. confirmed that DNMT1 helps maintain DNA methylation, and it is essential for cell proliferation. Accumulating evidence suggests that DNMT1 is associated with the expression of p21 (Waf1/Cip1). Of particular note, previous studies did not emphasize the specific mechanism by which DNMT1 influences cell proliferation. Our findings provided the first evidence that DNMT1 silencing could epigenetically increase Cdkn1a expression by decreasing its promoter methylation in KGN cells. Because of time and technical constraints, we only tested a few candidate targets of DNMT1, and only Cdkn1a expression was altered when DNMT1 was overexpressed or silenced. However, we think that the Cdkn1a gene is not the only target of DNMT1 involved in cell growth, and dynamic intracellular methylation changes must affect other candidate genes and related pathways that are worthy of further research.

In the physiological state, the membrane protrusions of GCs in the preantral follicles can pass through the zona pellucida and form a gap connection with the cell membrane of oocytes. The contact of these cell membranes provides a channel for bidirectional communication and nutrition support. The proliferation of GCs in the primary follicles increases the cell layers and impels the follicles to become secondary follicles. GCs continue to develop after the recruitment of estrogen and follicle-stimulating hormone (FSH), and the follicles with the lowest FSH threshold become dominant follicles. Cdkn1a expression is altered in different follicle developmental stages. A prior study suggested that Cdkn1a mRNA expression is significantly higher in GCs from estrogen-inactive dominant follicles and pre-ovulatory follicles than in those from other follicles. After the ovulatory trigger, Cdkn1a is upregulated to inhibit GC proliferation, and it is essential for the initial decline in GC mitosis. The aforementioned results indicate that during follicular development, Cdkn1a expression is decreased in GCs to actively stimulate their growth, allowing them to develop synchronously with follicles. However, more ovarian follicular arrests, fewer mature follicles, and more frequent anovulation are observed in patients with PCOS compared with the findings in healthy controls. The available data suggest that GC dysfunction may contribute to the abnormal folliculogenesis and anovulation observed in PCOS, as weakened GC proliferation resulted in insufficient nutrition and communication for oocyte development. In rat experiments, high-fat diet-induced obesity triggered the arrest of GCs by inducing Cdkn1a expression, thereby causing abnormal follicular development. Earlier studies by our group also demonstrated that PCNA levels in the GCs of patients with PCOS are significantly decreased, corresponding with significantly increased p27 protein levels and suggesting abnormally low GC proliferation in patients with PCOS. Two different studies focusing on miRNA functions in PCOS both mentioned that the Cdkn1a levels in patients with PCOS are increased and regulated by PTEN or MST4. Our findings of elevated Cdkn1a expression in patients with PCOS are consistent with these prior results. However, some controversy remains concerning Cdkn1a levels in GCs from patients with PCOS. Two studies using ovarian cortices suggested that Cdkn1a levels were reduced in patients with PCOS. It is noteworthy that the former two studies used luteinized GCs for sequencing, while the latter two used excised ovarian cortices. When the non-coding RNA sequencing results obtained from these two types of samples were verified on KGN cells, the target gene Cdkn1a changed oppositely. It is not enough to explain this only with the small numbers of participants or the complex cell components in the ovarian cortex. As we mentioned before, the expression of Cdkn1a in GCs from pre-ovulatory follicles and after ovulation is higher than that of GCs during the follicular phase. It could mean that the basic Cdkn1a expression of the collecting luteinized GCs is relatively high. Therefore, when elucidating the mechanism of follicular arrest, neither ovarian cortices nor luteinized GCs are the most ideal.
Figure 5. DNMT1 Silencing or 5-Aza Treatment Upregulated CDKN1A Expression by Decreasing Its Promoter Methylation Levels

(A and B) Quantitative real-time PCR analysis of DNMT1 and negative proliferation marker levels (CDKN1A, CDKN1B, TP53) after DNMT1 silencing using two different siRNAs (A, n = 6 for each group; DNMT1, p < 0.0001; CDKN1A: si-NC versus si-DNMT1-1, p = 0.0004; si-NC versus si-DNMT1-2, p = 0.003; CDKN1B: si-NC versus si-DNMT1-2, p = 0.0012) or after 5-Aza treatment (5 µM) for different durations (B, n = 3 for each group; DNMT1: 12 h, p = 0.0022, 24 h, p = 0.0057; CDKN1A: 24 h, p = 0.0035, 48 h, p < 0.0001; CDKN1B: 24 h, p = 0.0145; TP53: 24 h, p = 0.0324, 48 h, p = 0.0179). (C) The selected pyrosequencing sequence was located upstream of CDKN1A exon 1 and had 16 CpG sites named pos1–pos16. CpG island enrichment in the CDKN1A promoter region was predicted by the MethPrimer website, which was located in the promoter and first exon regions of CDKN1A. (D and E) Methylation status of the CDKN1A promoter CpG island after DNMT1 silencing using two different siRNAs (D, n = 6, pos2, p = 0.0073) or after 5-Aza treatment (5 µM) for different durations (E, n = 5, pos9, p = 0.0166). Pos, CpG site in the CDKN1A promoter; Meth, methylation level. Mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t test).
In the study, we used human luteinized GCs (hLGCs) derived from women with and without PCOS to validate the microarray results, and performed proliferation-related functional and mechanism verification in KGN cells. It is noteworthy that hLGCs are collected after gonadotropin stimulation and induction of ovulatory process, so-called collecting luteinized GCs. KGN cells are from a patient with invasive ovarian GC carcinoma, which can basally secrete progesterone and also have high aromatase activity to produce estrogen in the presence of substrate androgen. These make KGN cells have some of the characteristics of luteinized GCs. The internal pattern of luteinized GCs is different from that of GCs in the follicular phase. Specifically, the transition from GCs to luteal cells leads to a rapid shift from estrogen production to predominantly progesterone production. There were not mutually exclusive between luteinization and proliferation, luteinized GCs still had the capability to proliferate. Therefore, although not mutually exclusive between luteinization and proliferation, luteinized GCs are the most ideal cell model, but we cannot obtain them due to the limitations of current technology. Another approach is to isolate mice follicles and in vitro culture of the follicles. Due to the species conservation of Inc-MAP3K13-7:1, we could not perform this experiment on rodents. According to our results, the regulation of DNA methylation by IncRNAs could be an essential mechanism for the regulation of gene expression in PCOS. Because no homologous non-coding RNA was found in experimental animals such as zebrafish and mice, we were unable to perform in vivo functional experiments of Inc-MAP3K13-7:1. However, methyl donor supplementation animal models are worthy of consideration. Several lines of evidence illustrated that methyl donor supplementation improves multiple phenotypes associated with PCOS through epigenetic alterations. First, the maternal intake of methyl group donors can affect the DNA methylation of metabolic genes in the offspring. Subsequently, methyl donor supplementation can reverse hepatic triglyceride accumulation induced by high-fat/sucrose diets and the transgenerational amplification of obesity prevalence and severity can be prevented by methyl donor supplementation. Second, it has been reported that patients with type 2 diabetes have decreased circulating folate levels. Additionally, animal experiments and methylated DNA immunoprecipitation/high-throughput sequencing have also confirmed that folate supplementation reduced fat mass and serum glucose levels and improved insulin resistance in high-fat diet-fed mice by inducing DNA methylation and expression changes in genes associated with obesity and insulin secretion. In addition, the lack of choline in the maternal diet causes changes in the DNA methylation level of genes involved in cell cycling in the fetal brain. Third, an observational study involving 18,555 women revealed that folate supplementation reduced the risk of ovulatory infertility. Furthermore, in a mouse model, moderate-dose folate supplementation was associated with a decrease in the proportion of developmentally delayed embryos following assisted reproduction in mice and resolved the female-biased aberrant imprinted gene methylation. It is thought that the dietary manipulation of methyl donors (either supplementation or deficiency) can induce stable changes in gene methylation, altering gene expression and potentially affecting reproductive outcome. Therefore, further exploration of methyl group donor supplementation in vivo will lead to a more comprehensive understanding of the role of DNA methylation in GC proliferation and validate its utility as a new treatment for PCOS, thereby providing new evidence supporting the use of folate supplementation before pregnancy.
In this study, we unraveled the function of Inc-MAP3K13-7:1 in regulating GC proliferation. We demonstrated that Inc-MAP3K13-7:1 prevented cell cycle progression by silencing DNMT1 and upregulating CDKN1A/p21. Mechanistically, Inc-MAP3K13-7:1 directly bound to DNMT1 and induced its ubiquitination-dependent degradation, thereby suppressing the DNA methylation of the CDKN1A promoter, inhibiting GC proliferation, and promoting the development of PCOS (Figure S6). Therefore, Inc-MAP3K13-7:1 might be a potential marker for future epigenetic diagnosis for PCOS.

MATERIALS AND METHODS

Clinical Samples
Ovarian GCs were collected from patients with and without PCOS in the Center for Reproductive Medicine in Ren Ji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine between March 2017 and November 2019. The Medical Ethics Committee of Ren Ji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine approved this study process (no. 2017041411), and all participants provided written informed consent. The main reference for the diagnostic criteria of PCOS was the Rotterdam Consensus for PCOS revised in 2003. Basic and PCOS-related anthropometric variables, such as age, body mass index, and levels of FSH, LH, testosterone, estradiol, and anti-Müllerian hormone, are presented in Tables S1–S3. Human ovarian GCs were obtained from patients’ follicular fluid as previously described.

Cell Culture and Treatments
The human GC line KGN was cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (DMEM/F12) (Gibco, Grand Island, NY, USA) containing 10% charcoal-stripped fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotics (mixture of penicillin, streptomycin, and neomycin; Gibco) in a 37°C, 5% CO2 incubator (Thermo Fisher Scientific, Waltham, MA, USA). Cells were passaged every 3 days. KGN cells were authenticated via short tandem repeat PCR profiling, and no cross-contamination by other human cells was observed in March 2019.

For 5-Aza treatment, cells were seeded into six-well plates. Twenty-four hours later, cells were washed and replaced with fresh medium containing 5-Aza (50 nM to 5 μM, Sigma-Aldrich, St. Louis, MO, USA). Then, cells were collected at the indicated time for use in various experiments. For MG132 treatment, cells were harvested after 8 h of treatment (1–100 μM, Selleck Chemicals, Houston, TX, USA). In addition, cells were treated with 100 μM cycloheximide (CHX, Cell Signaling Technology, Beverly, MA, USA) and collected every 2 h to assess DNMT1 protein stability.

5’ and 3’ RACE
Total RNA was extracted from KGN cells using a TRIzol Plus RNA purification kit (Invitrogen, Carlsbad, CA, USA). The 5’ RACE and 3’ RACE experiments were performed using a GeneRacer kit (Invitrogen) according to the manufacturer’s instructions. The RACE primers targeting Inc-MAP3K13-7:1 used for the PCR are listed in Table S4. RACE PCR products were cloned into the pGM-T vector (Sangon Biotech, Shanghai, China) and sequenced.

Cell Transfection
The DNMT1 (NM_001130823.3) sequence was cloned into pCMV3 plasmids (Transheep, Shanghai, China) to overexpress DNMT1. Three independent siRNAs against DNMT1 (Table S5) were obtained from Biotrend (China). Plasmids and/or siRNAs were transfected into KGN cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Full-length Inc-MAP3K13-7:1 expression lentiviral vectors and blank vectors were obtained from GeneChem (Shanghai, China). After transduction, cells were cultured for 48 or 72 h before further treatment.

CCK-8 assay
The CCK-8 assay was performed using a commercial kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol. Briefly, 100 μL of a suspension containing 1 × 10^5 KGN cells was added to each well of a 96-well plate. After treatment for 24, 48, 72, or 96 h, 10 μL of CCK-8 solution was added to each well. Cell viability was measured according to the absorbance at 450 nm.

Cell Cycle Analysis
KGN cells were incubated for 24 h in six-well plates and harvested via trypsinization 24, 48, or 72 h after drug treatment or transfection with siRNAs and/or plasmid DNA. The cells were washed with pre-cooled phosphate-buffered saline (PBS), and then the cells were centrifuged, re-suspended in 70% pre-cooled (−20°C) ethanol for cell fixation, and incubated at 4°C for 24 h. The fixed cells were centrifuged and washed with PBS. Then, 500 μL of propidium iodide/RNase staining buffer (BD Pharmingen, San Diego, CA, USA) was added for cell re-suspension. After incubation in the dark for 15 min, cells were filtered using 400-mesh sieves. Cell cycle analysis was performed using a flow cytometer (BD FACSCalibur, BD Biosciences, CA, USA).

Cell Apoptosis Assay
Cells were seeded, incubated, and treated in six-well plates. A cell apoptosis assay was performed using flow cytometry. Briefly, 1 × 10^5 cells were re-suspended in binding buffer, incubated with 5 μL of phycoerythrin (PE)-annexin V and 5 μL of 7-AAD for 15 min in the dark at room temperature and analyzed using flow cytometry within 1 h.

DNA Isolation and Quantification of 5-mC
Total DNA was extracted from cells using a QIAGEN DNA mini kit (QIAGEN, Hilden, Germany), and DNA levels were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Global methylation of GCs was determined using a MethylFlash methylated DNA quantification kit (EpiGentek, NY, USA). Briefly, the same amount of genomic DNA from GCs was bound to the assay plate, and capture antibody was added into the wells, followed by a detection antibody, enhancer solution, and color development solution. The methylated fraction of DNA was quantified colorimetrically by
reading the absorbance at 405 nm. The amount of methylated DNA was proportional to the optical density (OD).

**RNA Extraction and Real-Time PCR**
Total RNA was extracted from cells using TRIzol (Invitrogen), and RNA levels were measured using agarose gel electrophoresis. An RNA cellular fractionation assay was performed using a Paris kit (Invitrogen) to isolate nuclear and cytoplasmic RNA from cultured cells according to the instruction manual. Subsequently, the isolated RNA was reverse transcribed into cDNA (PrimeScript RT master mix, Takara, Dalian, China). Target gene expression was measured by real-time PCR using SYBR Premix Ex Taq (Takara). The $2^{-\Delta\Delta Ct}$ method was applied to analyze mRNA expression levels normalized to those of β-actin (ACTB). The primer sequences of the tested genes are listed in Table S6.

**Western Blot Assay**
Cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer (Shenggong, Shanghai, China) supplemented with additional protease and phosphatase inhibitors (Roche, Basel, Switzerland). Proteins from nuclear and cytoplasmic compartments were extracted using a ProteoExtract subcellular proteome extraction kit (Millipore, Burlington, MA, USA) according to the manufacturer’s instructions. Equal amounts of proteins were denatured at 100°C for 10 min and then electrophoresed on a 10%–15% SDS-polyacrylamide gel. The bands were wet transferred to a nitrocellulose membrane before being blocked with 5% non-fat milk. After primary and secondary antibody incubation, a horseradish peroxidase detection system supplied by Syngene was used to detect the blots. Relative protein expression was calculated using ImageJ and normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase expression. The relative phosphorylation of target proteins was calculated as the ratios of phosphorylated proteins to target proteins. The related antibody information is summarized in Table S7.

**Immunofluorescence**
For immunofluorescence, cells were seeded onto microscope slides (Millipore). After culture and treatment, cells were fixed with −20°C pre-cooled acetone for 15 min at room temperature. After three washes with PBS, the slides were permeated using 0.5% Triton X-100 in PBS for 20 min at room temperature, followed by blocking in 10% normal goat serum for 30 min at room temperature. A sufficient amount of diluted primary antibody was added to each slide, which was then incubated in a wet box at 4°C overnight. After blocking with a fluorescent secondary antibody, the slides were then counterstained with DAPI and imaged under a fluorescence microscope (Zeiss, Jena, Germany).

**CoIP**
CoIP was performed as described previously. Briefly, whole KGN cell extracts (100 μg of total protein) were incubated with DNMT1 antibody or normal rabbit immunoglobulin G (IgG) at 4°C overnight. Then, the protein-antibody complex was incubated under agitation with 15 μL of magnetic protein A/G beads for 1 h at 4°C. The proteins in the complex were eluted by adding 20 μL of SDS loading buffer and incubated for 10 min at 100°C before separation on a SDS-PAGE gel. The samples were transferred to nitrocellulose membranes, blocked with 5% milk, and probed with a monoclonal antibody against ubiquitin (P4D1).

**RIP Assay**
An RIP assay was performed using a Magna Nuclear RIP (cross-linked) nuclear RNA-binding protein immunoprecipitation kit (Millipore, Burlington, MA, USA) according to the manufacturer’s protocol. DNMT1, EZH2, and normal IgG antibodies were used for this assay. IgG was used as negative IP controls, and EZH2 was used as positive IP controls. Additionally, two pairs of Inc-MAP3K13-7:1 primers, a uridine-rich 1 small nuclear RNA primer (NEAT1), and a nuclear paraspeckle assembly transcript 1 primer (U1) were used for real-time PCR. NEAT1 primer was used as a negative control and U1 was used as a positive control.

**Pyrosequencing**
Genomic DNA was extracted from treated cells and subjected to bisulfite conversion and clean-up using an EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer’s protocol. The CDKN1A (p21) promoter primers (Table S8) used for pyrosequencing were designed by PyroMark Assay Design 2.0 and synthesized by Sangon Biotech. PCR was performed to amplify samples using TaKaRa EpiTaq HS (TaKaRa). In total, 15–20 μL of PCR products was taken and used for sequencing analysis using a PyroMark Q96 RT quantitative pyrophosphate sequence analyzer (Qiagen).

**Statistical Analysis**
Data are presented as the mean ± SD. The experiment was designed to use three to five samples/independent repeats per experiment/group. Statistical analysis was performed using IBM SPSS Statistics and GraphPad Prism. Statistical significance was analyzed using Levene’s test for equality of variances followed by a two-tailed Student’s t test. Data from multiple groups were compared using one-way ANOVA with Duncan’s multiple range test. The correlation of two datasets was analyzed using Pearson’s correlation coefficient when equal variance was assumed. p <0.05 denoted statistical significance.

**SUPPLEMENTAL INFORMATION**
Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2020.11.018.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Bozdag, G., Mumusoglu, S., Zengin, D., Karabulut, E., and Yildiz, B.O. (2016). The prevalence and phenotypic features of polycystic ovary syndrome: a systematic review and meta-analysis. Hum. Reprod. 31, 2841–2855.

2. Skiba, M.A., Islam, R.M., Bell, R.J., and Davis, S.R. (2018). Understanding variation in prevalence estimates of polycystic ovary syndrome: a systematic review and meta-analysis. Hum. Reprod. Update 24, 694–709.

3. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group (2004). Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil. Steril. 81, 19–25.

4. Franks, S., Stark, J., and Hardy, K. (2008). Follicle dynamics and anovulation in polycystic ovary syndrome. Hum. Reprod. Update 14, 367–378.

5. Webber, L.J., Stubbs, S., Stark, J., Trew, G.H., Margara, R., Hardy, K., and Franks, S. (2003). Formation and early development of follicles in the polycystic ovary. Lancet 362, 1017–1021.

6. Palomba, S., D扙aio, J., and La Sala, G.B. (2017). Oocyte competence in women with polycystic ovary syndrome. Trends Endocrinol. Metab. 28, 186–198.

7. Qiao, J., and Feng, H.L. (2011). Extra- and intra-ovarian factors in polycystic ovary syndrome. Endocrinology 152, 3600–3609.

8. Huang, X., Hao, C., Bao, H., Wang, M., and Dai, H. (2016). Aberrant expression of DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21 WAF1. Science 353, 6637–6640.

9. Sen, G.L., Reuter, J.A., Webster, D.E., Zhu, L., and Khavari, P.A. (2010). DNMT1 regulates normal and ovarian steroid-induced granulosa cell differentiation. Mol. Endocrinol. 24, 2384–2396.

10. Zhao, J., Xu, J., Wang, W., Zhao, H., Liu, H., Liu, X., Liu, J., Sun, Y., Dunaif, A., Du, Y., and Chen, Z.J. (2018). Long non-coding RNA LINC01572:28 inhibits granulosa cell proliferation and enhances cell apoptosis in women with PCOS with or without hyperandrogenism. Reprod. Biomed. Online 37, 613–623.

11. Shi, Y., Zhao, H., Shi, Y., Cao, Y., Yang, D., Li, Z., Zhang, B., Liang, X., Li, T., Chen, J., and Chen, Z.J. (2014). Hypomethylation of the LH/choriogonadotropin receptor probable biomarker region is a potential mechanism underlying susceptibility to polycystic ovary syndrome. Endocrinology 155, 1445–1452.

12. Xia, Y., Shen, S., Zhang, X., Deng, Z., Xiang, Z., Wang, H., Yi, L., Gao, Q., and Wang, Y. (2015). Epigenetic pattern changes in premenopausal female Sprague-Dawley rats following exposure to androgen. Reprod. Fertil. Dev. 28, 1414–1423.

13. Pan, J.X., Tan, Y.J., Wang, P.F., Hou, N.N., Xiang, Y.Q., Zhang, J.Y., Liu, Y., Qu, F., Meng, Q., Xu, J., et al. (2018). Ablation expression and DNA methylation of lipid metabolism genes in PCOS: a new insight into its pathogenesis. Clin. Epigenetics 10, 6.

14. Sagvekar, P., Mangoli, V., Desai, S., Patil, A., and Mukherjee, S. (2017). LINE1 CpG-hypermethylation in granulosa cells and blood leukocytes is associated with PCOS and related traits. J. Clin. Endocrinol. Metab. 102, 1396–1405.

15. Jeltsch, A. (2002). Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. ChemBioChem 3, 274–293.

16. Wang, P., Zhao, H., Li, T., Zhang, W., Wu, K., Li, M., Biao, Y., Liu, H., Ning, Y., Li, G., and Chen, Z.J. (2014). Hypomethylation of the LH/choriogonadotropin receptor pro-
35. Christman, J.K. (2002). 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene 21, 5483–5495.

36. Li, W., Huang, K., Wen, F., Cui, G., Guo, H., He, Z., and Zhao, S. (2019). LINC00184 silencing inhibits glycosylation and restores mitochondrial oxidative phosphorylation in esophageal cancer through demethylation of PTEN. EBioMedicine 44, 298–310.

37. Du, Z., Song, J., Wang, Y., Zhao, Y., Guda, K., Yang, S., Kao, H.Y., Xu, Y., Willis, J., Markowitz, S.D., et al. (2010). DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitinolysis. Sci. Signal. 3, ra80.

38. Cech, T.R., and Stitz, J.A. (2014). The noncoding RNA revolution-trashing old rules to forge new ones. Cell 157, 77–94.

39. Zhou, J., Yang, Z., Zhong, T., Mueller, M., Men, Y., Zhang, N., Xie, J., Giang, K., Chung, H., Sun, X., et al. (2015). H19 IncRNA alters DNA methylation genome wide by regulating S-adenosylhomocysteine hydrolase. Nat. Commun. 6, 10221.

40. Ma, P., de Waal, E., Weaver, J.R., Bartolomei, M.S., and Schultz, R.M. (2015). A DNMT3A2-HDAC2 complex is essential for genomic imprinting and genome integrity in mouse oocytes. Cell Rep. 13, 1552–1560.

41. Holliday, R., and Pugh, J.E. (1975). DNA modification mechanisms and gene activity during development. Science 187, 226–232.

42. Bansal, A., and Pinney, S.E. (2017). DNA methylation and its role in the pathogenesis of diabetes. Pediatr. Diabetes 18, 167–177.

43. Bestor, T.H. (2000). The DNA methyltransferases of mammals. Hum. Mol. Genet. 9, 2395–2402.

44. Rhee, I., Bachman, K.E., Park, B.H., Jair, K.W., Yen, R.W., Schuebel, K.E., Cui, H., Feinberg, A.P., Lengauer, C., Kinzler, K.W., et al. (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416, 552–556.

45. Egger, G., Jeong, S., Escobar, S.G., Cortez, C.C., Li, T.W.H., Saito, Y., Yoo, C.B., Jones, B.C., Guda, K., Yang, S., Kao, H.Y., Xu, Y., Willis, J., Okabe, T., Goto, K., et al. (2001). Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology 142, 417–425.

46. Murphy, B.D. (2000). Models of luteinization. Biol. Reprod. 63, 2–11.

47. Yoshisaka, S., Abe, H., Sakumoto, R., and Okuda, K. (2013). Proliferation of luteal steroidogenic cells in cattle. PLoS ONE 8, e64186.

48. Gu, C., Liu, S., Wu, Q., Zhang, L., and Guo, F. (2019). Integrative single-cell analysis of transcriptome, DNA methyleome and transcriptability in mouse oocytes. Cell Res. 29, 110–123.

49. Wu, Y., Zhang, Z., Liao, X., and Wang, Z. (2015). High fat diet triggers cell cycle arrest via regulation of methyltransferases DNA MT1 and PRMT6. Gastroenterology 149, 2402–2412.

50. Escobar-Morreale, H.F. (2018). Polycystic ovary syndrome: definition, aetiology, pathogenesis, diagnosis and treatment. Nat. Rev. Endocrinol. 14, 270–284.

51. Ouyang, S., Liu, Z., and Wu, J. (2018). Folic acid supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. Genes Nutr. 8, 105–113.

52. Waterland, R.A., Trasvino, M., Tahiliani, K.G., Rached, M.T., and Mirza, S. (2008). Methyl donor supplementation prevents transgenerational amplification of obesity. Int. J. Obes. (Lond.) 32, 1373–1379.

53. Nilsson, E., Matte, A., Perlliyev, A., de Mello, V.D., Kakela, P., Pihlajamaki, J., and Ling, C. (2015). Epigenetic alterations in human liver from subjects with type 2 diabetes in parallel with reduced folate levels. J. Clin. Endocrinol. Metab. 100, E1491–E1501.

54. Li, W., Tang, R., Ma, F., Ouyang, S., Liu, Z., and Wu, J. (2014). Folic acid supplementation affects the DNA methylation profile and improves insulin resistance in high-fat-fed mice. J. Nutr. Biochem. 59, 76–83.

55. Nishi, Y., Yanase, T., Obi, K., Ichino, I., Saito, M., Nomura, M., Mukasa, C., Okabe, T., Goto, K., et al. (2001). Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology 142, 417–425.

56. Ling, C. (2015). Epigenetic alterations in human liver from subjects with type 2 diabetes in parallel with reduced folate levels. J. Clin. Endocrinol. Metab. 100, E1491–E1501.

57. Li, W., Tang, R., Ma, F., Ouyang, S., Liu, Z., and Wu, J. (2014). Folic acid supplementation affects the DNA methylation profile and improves insulin resistance in high-fat-fed mice. J. Nutr. Biochem. 59, 76–83.

58. Niculescu, M.D., Yamamoto, Y., and Zeisel, S.H. (2004). Choline availability modulates human neuroblastoma cell proliferation and alters the methylation of the promoter region of the cyclin-dependent kinase inhibitor 3 gene. J. Neurochem. 89, 1252–1259.

59. Chavarro, J.E., Rich-Edwards, J.W., Rosner, B.A., and Willett, W.C. (2008). Use of multivitamins, intake of B vitamins, and risk of ovulatory infertility. Fertil. Steril. 89, 668–676.

60. Rahimi, S., Martel, J., Karahan, G., Angle, C., Behan, N.A., Chan, D., MacFarlane, A.J., and Trayling, P. (2016). Intestinal PPARa cooperates to silence genes in human cancer cells. J. Clin. Endocrinol. Metab. 101, E1564–E1573.

61. Gu, C., Liu, S., Wu, Q., Zhang, L., and Guo, F. (2019). Integrative single-cell analysis of transcriptome, DNA methylome and transcriptability in mouse oocytes. Cell Res. 29, 110–123.

62. Pauwels, S., Ghosh, M., Duca, R.C., Bekaert, R., Freson, K., Huybrechts, J. L., Linge, S.A.S., Koppen, G., Devlieger, R., and Godderis, L. (2017). Maternal intake of methyl-group donors affects DNA methylation of metabolic genes in infants. Clin. Epigenetics 9, 16.

63. Chavarro, J.E., Rich-Edwards, J.W., Rosner, B.A., and Willett, W.C. (2008). Use of multivitamins, intake of B vitamins, and risk of ovulatory infertility. Fertil. Steril. 89, 668–676.

64. Rahimi, S., Martel, J., Karahan, G., Angle, C., Behan, N.A., Chan, D., MacFarlane, A.J., and Trayling, P. (2016). Intestinal PPARa cooperates to silence genes in human cancer cells. J. Clin. Endocrinol. Metab. 101, E1564–E1573.

65. Nilsson, E., Matte, A., Perlliyev, A., de Mello, V.D., Kakela, P., Pihlajamaki, J., and Ling, C. (2015). Epigenetic alterations in human liver from subjects with type 2 diabetes in parallel with reduced folate levels. J. Clin. Endocrinol. Metab. 100, E1491–E1501.

66. Li, W., Tang, R., Ma, F., Ouyang, S., Liu, Z., and Wu, J. (2014). Folic acid supplementation affects the DNA methylation profile and improves insulin resistance in high-fat-fed mice. J. Nutr. Biochem. 59, 76–83.

67. Chavarro, J.E., Rich-Edwards, J.W., Rosner, B.A., and Willett, W.C. (2008). Use of multivitamins, intake of B vitamins, and risk of ovulatory infertility. Fertil. Steril. 89, 668–676.

68. Rahimi, S., Martel, J., Karahan, G., Angle, C., Behan, N.A., Chan, D., MacFarlane, A.J., and Trayling, P. (2016). Intestinal PPARa cooperates to silence genes in human cancer cells. J. Clin. Endocrinol. Metab. 101, E1564–E1573.