Supplementary information

High-dose estrogen impairs demethylation of H3K27me3 by decreasing Kdm6b expression during ovarian hyperstimulation in mice

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Materials and methods

Mouse model

Collection of oocytes and embryos

C57BL/6J female and male mice aged 6–8 weeks were purchased from the Zhejiang Chinese Medical University Laboratory Animal Research Center and were fed under controlled temperature and humidity conditions with a 12 h dark–light cycle. Food and water were provided ad libitum.

We randomly divided 136 female mice into four groups of 34 mice per group as follows: (a) the natural ovulation control group (NC), administered consecutive intraperitoneal injections of normal saline; (b) the gonadotropin-releasing hormone (GnRH) agonist group, administered an intraperitoneal injection of triptorelin acetate with 1.5 μg/100 g of normal saline at 9:00 am for nine days, followed by injections of 10 IU pregnant mare serum gonadotropin (PMSG; Ningbo Hormone Production Co. Ltd., China) and 10 IU human chorionic gonadotropin (HCG; Ningbo Hormone Production Co. Ltd.) at 5:00 pm on days 9 and 11, respectively (Ruan et al., 2006); (c) the PMSG group, administered an intraperitoneal injection of normal saline at 9:00 am for nine days, followed by injections of 10 IU PMSG and 10 IU HCG at 5:00 pm on days 9 and 11, respectively (Ruan et al., 2006); and (d) the OHSS group, administered an intraperitoneal injection of 20 IU PMSG at 5:00 pm for three days, followed by a 5 IU HCG injection at 5:00 pm on day 4 (Fainaru et al., 2009). The PMSG and GnRH groups represent conventional ovarian stimulation treatments, such as the Gn alone protocol and the GnRH agonist long protocol. The OHSS group simulated ovarian hyperstimulation induced by overdose stimulation in clinical settings.

Cumulus-oocyte complexes were isolated from the fallopian tube ampulla and placed in M2 medium (Sigma-Aldrich, St. Louis, MO, USA) 16–18 h after HCG administration, followed by dissociation using 0.3 mg/mL hyaluronidase (Sigma-Aldrich) (n=10). After the last HCG administration, mice were allowed to mate. The following morning, females with vaginal plugs were selected, and the time point was designated as day 0.5 (i.e., 0.5 days post-coitus (dpc)). Zygotes, 2-cell embryos, and blastocysts were collected at 0.5 dpc (n=6), 1.5 dpc (n=6), and 3.5 dpc (n=6), respectively.

Serum analysis

Prior to dissection, female mice (n=6) were anesthetized with pentobarbital sodium (30 mg/kg) 2 h after HCG treatment. Blood samples were obtained via cardiac puncture and were centrifuged at 3000 r/min for 15 min. Serum levels of estradiol, progesterone, and prolactin in the supernatant were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO) following the manufacturer's instructions. The experiment was performed in triplicate for each serum sample, and the mean value was calculated.

Immunohistochemical analysis

Mice were dissected after the collection of blood samples. The ovaries were fixed in 4% paraformaldehyde (Biosharp), embedded in paraffin, sectioned into serial slices (5 μm thick), deparaffinized, and boiled in 1× citric acid (pH 6.0) for antigen repair. After cooling to room temperature, the slices were immersed in a 3% H_2O_2 solution for 20 min. Subsequently, 10% goat serum was added, and the sections were further incubated at 37 °C for 30 min. After discarding the blocking buffer, the slices were incubated with antibodies against Caspase-3, proliferating cell nuclear antigen (PCNA), and phosphorylated H2A histone family member X (γ-H2AX) overnight at 4 °C, followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at 37 °C. Subsequently, 3,3'-diaminobenzidine (Sigma-Aldrich) was added to the slices for visualization under a microscope (3DHISTECH, The Digital Pathology Company, Budapest, Hungary). The positively stained brown area was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The positive ratio was calculated as the percentage of the number of positive cells to the total number of cells.

Oocyte mRNA-seq data analysis

Total RNA was extracted from oocytes collected from each group using the RNeasy Kit (Qiagen) with DNase I treatment (Thermo Fisher Scientific) according to standard protocols. RNA concentration and integrity were assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Samples with an RNA integrity number > 7.0 were used to construct the RNA-seq library using the Smart-seq2 system (Clontech, San Jose, CA, USA). The quality of the final libraries was assessed using BioAnalyzer and Qubit (Agilent). All libraries were sequenced on an Illumina NovaSeq (150 bp, paired-end) platform (Illumina, San Diego, CA, USA). After quality control, the sequence data were processed using STAR v2.7.7a (https://github.com/alexdobin/STAR) to generate read alignments against the mm10 reference sequence. Raw read counts for annotated genes were obtained through feature counts using default settings, which were subsequently normalized and analyzed using DEseq2 (https://support.bioconductor.org/tag/DESeq2/). Genes were designated as differentially expressed genes (DEGs) based on P-values <0.05 and $|\log_2(\text{fold change})|>1$. Gene ontology analysis was used to classify the DEGs according to biological processes, cellular components, and molecular functions.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from oocytes and embryos using a WTA Amplification Efficiency Kit (Qiagen). RNA was reverse transcribed to obtain a sufficient quantity of cDNA, followed by amplification in accordance with the manufacturer's protocol. cDNA was diluted at 1:100 before use and was then either subjected to RT-PCR analysis or stored at −80 °C until further use. RT-qPCR was performed using 10 μL of 5× TB Green buffer (TaKaRa), 0.4 μL of each forward and reverse primer, 0.4 μL of ROX reference dye, 2 μL of cDNA, and 6.8 μL of DEPC H_2O. Subsequently, *Kdm6b*, *Kmt2a*, and *Hdac2* were selected to verify the mRNA-seq results. Primer sequences are listed in Table 1. The cycle threshold (C_t) value of individual genes was normalized to that of *Gapdh*. The relative expression of target genes was calculated using the $2^{-\Delta\Delta C_t}$ method.

In vitro assay

Cell culture and transfection

Briefly, mESCs were cultured in high-glucose Dulbecco's modified Eagle medium (06-1055-57-1ACS; Biological Industries) containing 15% StemSure® serum replacement (191-18375; Wako), 1× MEM non-essential amino acids (N1250; Solarbio), 0.1 mmol/L β-mercaptoethanol (21985-023; Gibco), and 1000 IU/mL leukemia inhibitory factor (ESG1107; Sigma-Aldrich) on a 0.1% gelatin-coated dish (01-944-1B; Biological Industries). Estrogen was used at concentrations of 1×10^{-10} mol/L, 1×10^{-9} mol/L (physiological concentrations to mimic the NC group), 1×10^{-7} mol/L (medium concentration to mimic routine ovulation), and 1×10^{-6} mol/L (high concentration to mimic hyperstimulation), with dimethyl sulfoxide (DMSO) as the negative control group. To eliminate the effects of estrogen, cells were cultured in complete medium containing 0.1% estrogen receptor antagonist, ICI 182780 (ab120131; Abcam) for 1 h prior to estrogen treatment. Small interfering RNAs (siRNAs) were synthesized from GenePharma (si-Kdm6b sense 5'–3': GGGACAAGGAGACCUUUAUTT, antisense 5'–3': AUAAAGGUCUCCUUGUCCCTT; negative control: sense 5'–3': UUCUCCGAACGUGUCACGUTT, and antisense 5'–3': ACGUGACACGUUCGGAGAATT), followed by transfection into mESCs using lipofectamine RNAiMAX (Invitrogen), according to the product manual, to downregulate Kdm6b expression. The transfection efficiency was analyzed by performing RT-qPCR 48 h post-transfection; protein expression analysis was examined after 72 h of transfection. Twenty-four hours post-transfection, 10^{-6} mol/L estrogen was added to the medium, followed by incubation for 48 h to verify the effects of high estrogen exposure.

Embryoid body preparation

Briefly, mESCs were cultured and transfected as described above. After 48 h of transfection, the mESCs were harvested and then diluted to 1×10^{5} cell/mL using mESC-completed medium without LIF. Using a pipette, 20 μL droplets of the cell suspension were placed within a 10 cm plastic dish lid. Then, 10 mL of phosphate-buffered saline (PBS) was added to the bottom of the dish. In one fluid motion, the lid with droplets was inverted and placed onto the base of the Petri dish containing PBS; the dish was subsequently placed in a 5% CO_2 incubator at 37 ℃. After 72 h in droplets, embryoid bodies were collected and photographed using an inverted microscope.

Proliferation and pluripotency analysi

The mESCs were then cultured with 1×10^{-10}, 1×10^{-9}, 1×10^{-7}, and 1×10^{-6} mol/L estrogen and DMSO for 24, 48, and 72 h. Subsequently, the cells were seeded in 96-well plates, and 10 μL CCK-8 solution (Yeasen) was added to each well for 90 min to detect cell proliferation using a Thermo Scientific Microplate Reader (VARIOSKAN FLASH). After estrogen treatment for 48 h, mESCs were rinsed with PBS and immersed in 4% paraformaldehyde (Biosharp) for 1 h at room temperature. An alkaline phosphatase staining solution kit (Yeasen) was prepared in accordance with the manufacturer's protocol. After washing thrice with PBS, the cells were stained using the prepared staining solution for 30 min. Subsequently, the stained cells were washed with distilled water to stop staining, and staining patterns were observed using an inverted microscope (Leica, Wetzlar, Germany).

RNA isolation and RT-qPCR

RNA was extracted from mESCs using a RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. To obtain sufficient cDNA, RNAs were reverse transcribed using the PrimeScript™ RT reagent kit (Takara), followed by RT-qPCR as described above. The primer sequences are listed in Table 1.

Western blotting

Briefly, mESCs were lysed on ice with 120 μL RIPA buffer (FD009, Fude Biological

Technology) for 30 min. Cell lysates were centrifuged at 12000×*g* for 15 min. The protein was heated at 95 °C for 15 min and resolved using 4%–12% sodium dodecyl sulfate-polyacrylamide gels. The resolved proteins were then transferred to polyvinylidene fluoride membranes (BS-PVDF-22; Biosharp), which were then blocked with 5% bovine serum albumin (9048-46-8; Solarbio) at room temperature for 90 min. Subsequently, the membranes were incubated with the primary antibodies at 4 °C overnight, followed by incubation with secondary antibodies for 1 h at room temperature. The antibodies used are listed in Table 2. Finally, the membranes were washed with 1× Tris-buffered saline with Tween and were visualized using enhanced chemiluminescence (FD8000, Fude Biological Technology).

Immunofluorescence staining

Estrogen-treated mESCs were immersed in 4% paraformaldehyde (Biosharp) for 1 h at room temperature, followed by permeabilization using 0.2% Triton X-100 (Biosharp) for 1 h at 4 °C and blocking in 3% bovine serum albumin (9048-46-8; Solarbio) for 30 min at room temperature. Subsequently, the samples were incubated with the primary antibodies at 4 °C overnight and then incubated with secondary antibodies the following day for 1 h at room temperature. The antibodies used are listed in Table 2. After incubation, D9542, 4',6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) was added to the immunofluorescence dish for 15 min to visualize DNA. Finally, the samples were scanned using an FV1200-0SR microscope (OLYMPUS, Tokyo, Japan).

Statistical analysis

Data are presented as mean±standard deviation. Data analyses were performed using GraphPad Prism 9.0 software (www.graphpad-prism.cn; GraphPad Software, Inc., Boston, MA, USA). Two-tailed unpaired *t*-tests and analysis of variance were used to compare two sets and more than three sets of data, respectively. Statistical significance was set at $P<0.05$.

Table 1 Primer sequences

Gene	Forward	Reverse
Kdm6b	GACGAGCCTGCCTACTAC	TGCCATTCTCACTTGTAACG
Kmt2a	AGGGAAGCTCCAAATAGGAAGG	GGGTCTTTATCCGTTCTGTGG
Hdac2	CCAGAACACTCCAGAATA	CATCTCCACTGTCTTCAT
Oct4	GGCTTCAGACTTCGCCTCC	AACCTGAGGTCCACAGTATGC
Sox2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Nanog	TCTTCCTGGTCCCCACAGTTT	GCAAGAATAGTTCTCGGGATGAA
Caspase3	ATGGAGAACAACAAAACCTCAGT	TTGCTCCCATGTATGGTCTTTAC
Bcl2	ATGATAACCGGGAGATCGTG	GACGGTAGCGACGAGAGAAG
Gapdh	GGGAAACTGTGGCGTGAT	AAGGTGGAGGAGTGGGTGT

Table 2 Antibodies

Name	Company	Cat. No	WB	IF/IHC
Kdm6b	ABclonal	A17382	1:1000	
H3K27me3	Diagenode	C15410096 (pAb-069-050)	1:1000	1:200
Phospho-Histone H2A.X (Ser139)	Cell Signaling	#9718	1:1000	1:200
β-actin	Cell Signaling	#4967	1:1000	
Tubulin	Cell Signaling	#2128	1:1000	
Caspase3	Abcam	ab184787		1:500
PCNA	Boster	BM0104		1:200
CoraLite594–conjugated Goat Anti-Rabbit IgG(H+L)	Prointech	SA00013-4		1:200
CoraLite488-conjugated Goat Anti-Mouse IgG(H+L)	Prointech	SA00013-1		1:200
Secondary antibody	Signalway Antibody	#L3032; #L3012	1:5000	1:200

IHC, immunohistochemistry; IF, immunofluorescence; WB, western blotting.

References

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Ruan HC, Zhu XM, Luo Q, et al., 2006. Ovarian stimulation with GnRH agonist, but not GnRH antagonist, partially restores the expression of endometrial integrin β3 and leukaemia-inhibitory factor and improves uterine receptivity in mice. *Hum Reprod*, 21(10):2521-2529. https://doi.org/10.1093/humrep/del215

Table S1 Ovarian weight of each mouse

Group	Ovarian weight (mg)									
	1	2	3	4	5	6	7	8	9	10
NC	8	8.4	9.9	8.4	9.2	8.2	7.9	10.2	9.7	8.7
PMSG	14.6	7.9	12.4	9.8	15.1	9.3	8.9	12.9	8.5	13.3
GnRH	8.4	7.8	10.7	10.2	11.5	10.9	14.3	8.8	13.8	12.3
OHSS	21.3	16.5	18.2	13.9	13	18.1	21	15.9	12.6	15.9

GnRH: gonadotropin-releasing hormone; NC: natural ovulation control; OHSS: ovarian hyperstimulation syndrome; PMSG: pregnant mare serum gonadotropin.

Table S2 Average numbers of retrieved oocytes, 2-cell embryos, and blastocysts from the four groups

Group	NC	PMSG	GnRH	OHSS
Average number of retrieved oocytes (*n*=10)	8.70 ± 1.83	22.40 ± 2.50	23.30 ± 2.79	33.20 ± 3.32
The rate of heteromorphic oocytes (%)	2.71 ± 0.13%	10.87 ± 1.30%	10.06 ± 0.74%	40.0 ± 3.81%
Retrieved 2-cell embryos number (*n*=6)	7.33 ± 0.82	11.50 ± 1.05	12.00 ± 1.41	5.33 ± 1.03
Retrieved blastocysts number (*n*=6)	7.00 ± 0.85	9.67 ± 1.03	8.16 ± 1.17	3.00 ± 1.09

Data are presented as mean±standard deviation. GnRH: gonadotropin-releasing hormone; NC: natural ovulation control; OHSS: ovarian hyperstimulation syndrome; PMSG: pregnant mare serum gonadotropin.

Table S3 Differentially expressed genes associated with gene stability

Description	Gene symbol
Positive regulation of cell cycle	*Wnt4/Id2/Cdkn1a/Dusp3/Uhrf2/Mif/Gipc1/Rpl23/Anxa1/Pbx1/Ccnd2/Sox4/Hnrnpu/App/Myc/Cdk4/Ctgf/Eif4ebp1/Sh2b1/Prkca*
Regulation of G1 / S mitotic cycle	*Id2/Rhou/Zpr1/Cdkn1a/Jade1/Inhba/Anxa1/Ccnd2/Sox4/E2f5/Psme1/Cdk4/Rps6/Eif4ebp1/Camk2d/Anp32b/Ezh2/Dbf4/E2f5/Psme3/Rdx/Rbm38*
Mitotic cell cycle phase transitions	*Nop53/Id2/Psmg2/Rhou/Zpr1/Cdkn1a/Jade1/Inhba/Cdk5rap3/Rpl24/Anxa1/Pbx1/Ccnd2/Sox4/E2f5/App/Psme1/Cdk4/Rps6/Eif4ebp1/Tpd52l1*
Positive regulation of G2/M mitotic cycle	*Pbx1/App/Cdk4*
Cell cycle arrest	*Id2/Skil/Cdkn1a/Uhrf2/Mif/Inhba/Rpl23/Thbs1/Sox4/Myc/Gas1/Ing4/Cdk4/Ilk*
Positive regulation of cell cycle arrest	*Id2/Cdkn1a/Uhrf2/Rpl23/Sox4*
Negative regulation of cell cycle arrest	*Mif/Rpl23/Cdk4*
Chromatin remodeling	*Hdac2/Anp32b/Kdm6b/Rbbp7/Smarcc1*
Mitotic spindle assembly	*Rhoa/Mybl2/Hnrnpu*
DNA modification	*Dppa3/Ezh2/H1foo/Gnas/Bmi1/Kmt2a*