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Research Article

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

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Abstract: Given that ovarian stimulation is vital for assisted reproductive technology (ART) and results in elevated serum estrogen levels, exploring the impact of elevated estrogen exposure on oocytes and embryos is necessary. We investigated the effects of various ovarian stimulation treatments on oocyte and embryo morphology and gene expression using a mouse model and estrogen-treated mouse embryonic stem cells (mESCs). Female C57BL/6J mice were subjected to two types of conventional ovarian stimulation and ovarian hyperstimulation; mice treated with only normal saline served as controls. Hyperstimulation resulted in high serum estrogen levels, enlarged ovaries, an increased number of aberrant oocytes, and decreased embryo formation. The messenger RNA (mRNA)-sequencing of oocytes revealed the dysregulated expression of lysine-specific demethylase 6b (*Kdm6b*), which may be a key factor indicating hyperstimulation-induced aberrant oocytes and embryos. In vitro, Kdm6b expression was downregulated in mESCs treated with high-dose estrogen; treatment with an estrogen receptor antagonist could reverse this downregulated expression level. Furthermore, treatment with high-dose estrogen resulted in the upregulated expression of histone H3 lysine 27 trimethylation (H3K27me3) and phosphorylated H2A histone family member X (γ-H2AX). Notably, knockdown of *Kdm6b* and high estrogen levels hindered the formation of embryoid bodies, with a concomitant increase in the expression of H3K27me3 and γ-H2AX. Collectively, our findings revealed that hyperstimulation-induced high-dose estrogen could impair the demethylation of H3K27me3 by reducing Kdm6b expression. Accordingly, Kdm6b could be a promising marker for clinically predicting ART outcomes in patients with ovarian hyperstimulation syndrome.

Key words: Ovarian stimulation; Histone methylation; Assisted reproductive technology (ART)

1 Introduction

Since the genesis of in vitro fertilization and embryo transfer, over nine million babies have been born through assisted reproductive technology (ART) (Berntsen et al., 2019). Although ART remains a crucial tool in the clinical management of infertility, its manipulations may impact fertilization, cleavage, and embryonic development. Indeed, ART offspring exhibit substantially higher pregnancy complications than those conceived naturally, accompanied by an increased incidence of chromosomal aneuploidy (Pinborg et al., 2013; Qin et al., 2016). These findings highlight the potential adverse effects of ART manipulations.

The initial phase of ART involves ovarian stimulation, wherein women with infertility are administered exogenous gonadotropins to obtain multiple oocytes (Venetis et al., 2023). In clinical settings, conventional ovarian stimulation treatments include the gonadotropin

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(Gn) alone protocol and the gonadotropin-releasing hormone (GnRH) agonist long protocol, which can markedly enhance serum estrogen levels (Filicori et al., 2005; Diamond et al., 2015; Beebeejaun et al., 2021). In humans, serum estrogen levels following ovarian stimulation are 10–15 times higher than those during natural ovulation (Cahill et al., 2000). Irrespective of the ovarian stimulation treatment employed, excessive drug dosage or inadequate monitoring of follicle development may lead to the occurrence of ovarian hyperstimulation syndrome (OHSS) (Practice Committee of the American Society for Reproductive Medicine, 2016). In cases of OHSS, serum estrogen levels were found to be 50% higher than those in patients undergoing routine ART (Xu et al., 2014). Compared with frozen embryo transfer, fresh embryo transfer maintains high serum estradiol levels throughout pregnancy, which is most notable in patients with OHSS (Shi et al., 2018; Bortoletto et al., 2022). Using a progesterone-treated delayed implantation mouse model, Ma et al. (2003) demonstrated the adverse effects of high estrogen exposure on the synchronous development of the embryo and endometrium. Although the "freeze-all" strategy mitigates the adverse effects of high estrogen on endometrial receptivity, its impact on oocyte and embryo quality persists (Shi et al., 2018). Therefore, assessing the potential influence of elevated estrogen exposure on oocytes and embryos is crucial.

Genome reprogramming involves changes in chromatin configuration, with histone methylation modification as a crucial determinant. Exogenous stimuli or environmental changes may alter histone methylation patterns (Wang and Dey, 2006; Zhang et al., 2009; Tang et al., 2019). Maekawa et al. (2016) demonstrated that the expression of histone modification enzymes was altered in granulosa cells derived from superovulated mice. Moreover, markedly enhanced histone H3 lysine 27 (H3K27) trimethylation (H3K27me3) can be observed during embryogenesis following repeated superovulation (Tang et al., 2019). H3K27me3 is crucial for gene silencing (Zhou et al., 2011; Liu et al., 2016). The accumulation of H3K27me3 on the promoter region was shown to dysregulate gene expression (Bogliotti and Ross, 2012). In vivo, H3K27me3 accumulation is reportedly mediated by histone methyltransferases and demethyltransferases, and is associated with a deficiency in the DNA damage repair function (Chen et al., 2012; Gardner et al., 2017).

Lysine-specific demethylase 6B (Kdm6b) specifically catalyzes H3K27 demethylation and converts H3K27me3 to H3K27 monomethylation (H3K27me1) (Lee et al., 2007). The absence of *Kdm6b* is primarily responsible for aberrant H3K27me3 expression (Agger et al., 2007). A recent study has revealed that the loss of *Kdm6b* disturbs P53 pathway-mediated activity and results in cell differentiation defects in mice (Guo et al., 2022). These aforementioned studies have directed our attention toward the potential involvement of *Kdm6b* in oocyte and embryonic development during ovarian stimulation.

In the present study, we aimed to investigate the modulation of *Kdm6b* expression in oocytes and embryos following various ovarian stimulation treatments using a mouse model. Altered Kdm6b expression and its downstream signaling were verified using estrogen-treated mouse embryonic stem cells (mESCs). Furthermore, we explored the effect of *Kdm6b* knockdown.

2 Results

2.1 Effects of ovarian stimulation on ovarian follicles and hormone levels

Following different types of ovarian stimulation in mice (Fig. 1a), ovarian weight and its ratio to body weight were significantly increased in the OHSS group (Table S1; Figs. 1b and 1c), with the ovaries appearing notably hyperemic (Fig. 1d). Consistent with the changes in serum hormone levels, hyperstimulation significantly increased the serum levels of estradiol, progesterone, and prolactin. However, no significant differences were observed among the natural ovulation control (NC), pregnant mare serum gonadotropin (PMSG), and GnRH groups (Figs. 1e–1g). Caspase-3 and proliferating cell nuclear antigen (PCNA) are related to apoptosis and proliferation, whereas phosphorylated H2A histone family member X (γ-H2AX) is a specific DNA damage marker that detects double-strand breaks (Trakarnphornsombat and Kimura, 2023). As shown in Fig. 1h, hyperstimulation significantly increased the percentages of Caspase-3-positive and γ-H2AX-positive cells (Figs. 1i and 1k), and decreased the percentage of PCNA-positive cells (Fig. 1j). Although Caspase-3 was also elevated in the GnRH group, there were no differences in PCNA or γ-H2AX between the NC, PMSG, and GnRH groups. These findings indicate that ovarian

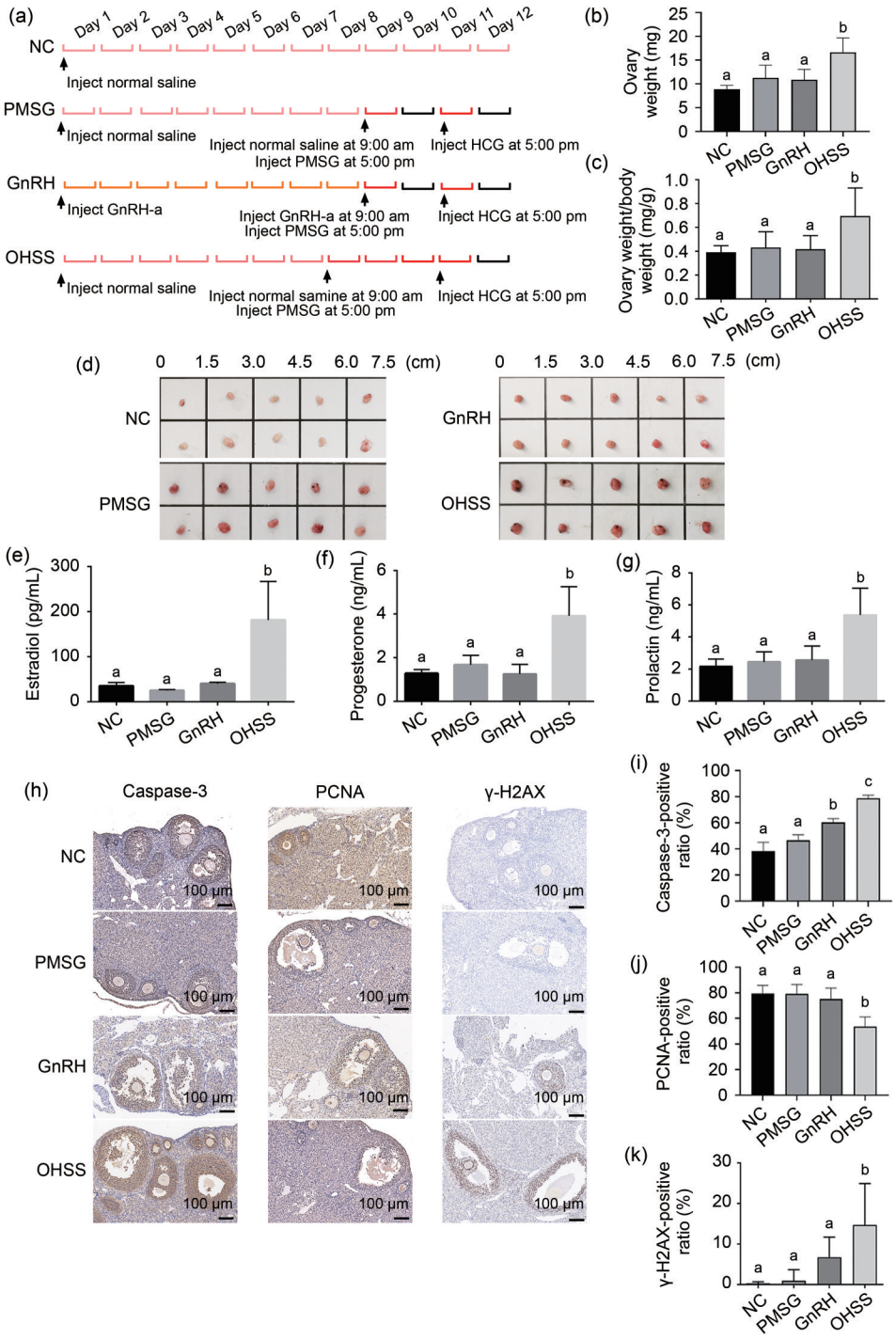


Fig. 1 Effects of ovarian stimulation on ovary morphology and hormone levels. (a) Schedule of different ovarian stimulation treatments. (b, c) Effects of different ovarian stimulation treatments on ovary weight (*n*=10) (b) and ovary/body weight ratio (*n*=10) (c). (d) Effects of different ovarian stimulation treatments on the morphology of ovaries (*n*=10). (e–g) Serum levels of estradiol (e), progesterone (f), and prolactin (g) in the four groups (*n*=6). (h) Immunohistochemical analyses of Caspase-3, PCNA, and γ-H2AX in ovaries from different ovarian stimulation-treated mice. (i–k) Effects of different ovarian stimulation treatments on Caspase-3 (i), PCNA (j), and γ-H2AX (k) expression (*n*=6). Data are presented as mean±standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA); different letters indicate significant differences (*P*<0.05) between groups. NC: natural ovulation control; PMSG: pregnant mare serum gonadotropin; GnRH: gonadotropin-releasing hormone; OHSS: ovarian hyperstimulation syndrome; HCG: human chorionic gonadotropin; PCNA: proliferating cell nuclear antigen; γ-H2AX: phosphorylated H2A histone family member X.

hyperstimulation could enhance serum estrogen levels and impact the expression of apoptosis and proliferation-related genes in ovarian follicles.

2.2 Effects of ovarian hyperstimulation on oocyte and embryo development

To evaluate the impact of ovarian stimulation on oocyte and embryo development, oocytes, 2-cell embryos, and blastocysts were collected from each group of mice (Fig. 2a). As shown in Table S2, ovarian stimulation significantly increased the number of retrieved oocytes (Fig. 2b), although a high number of heteromorphic oocytes were detected in the OHSS group (Fig. 2c). Consistently, the counts of 2-cell embryos and blastocysts were higher in the PMSG and GnRH groups than in the NC group, while they were significantly

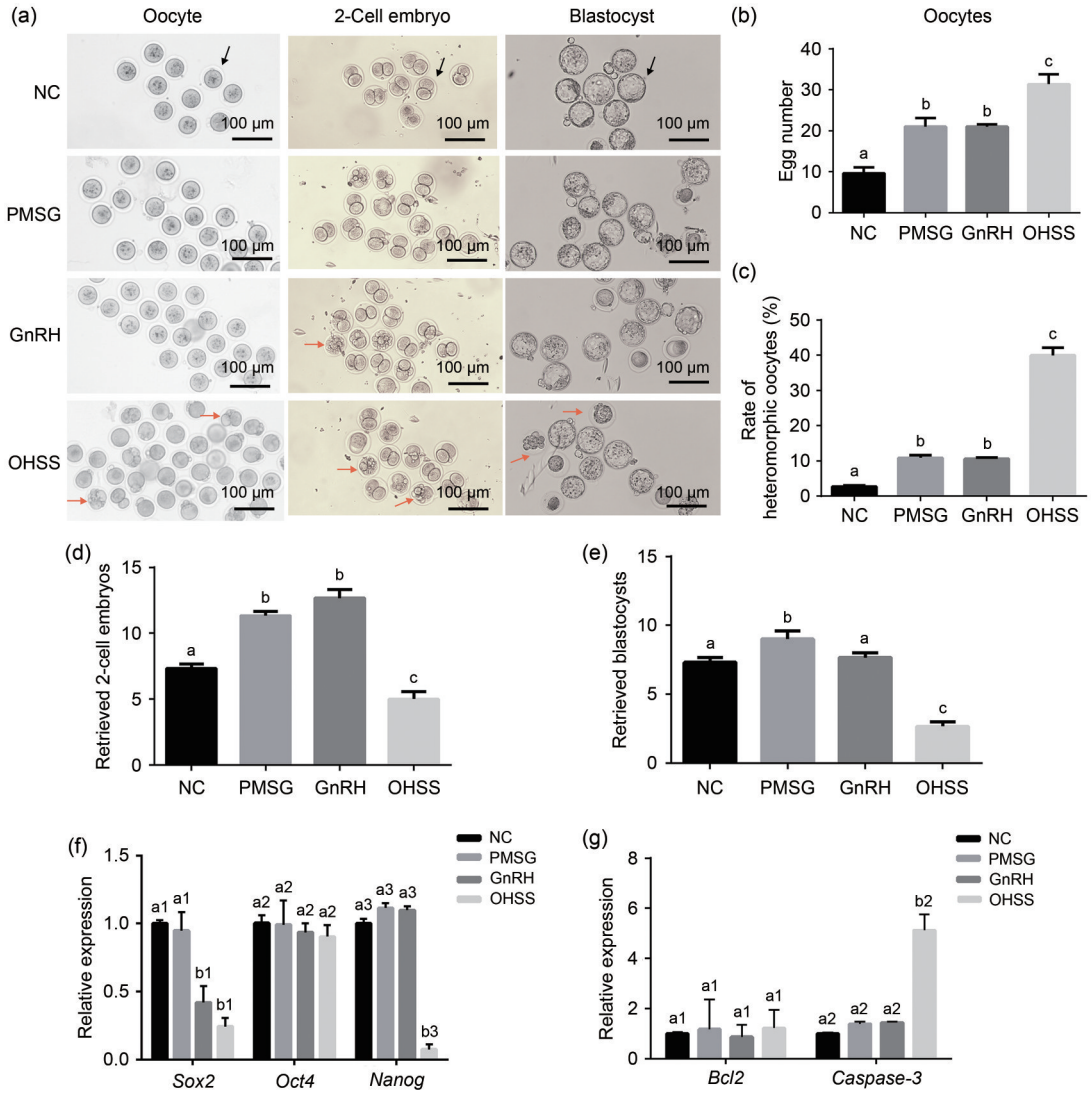


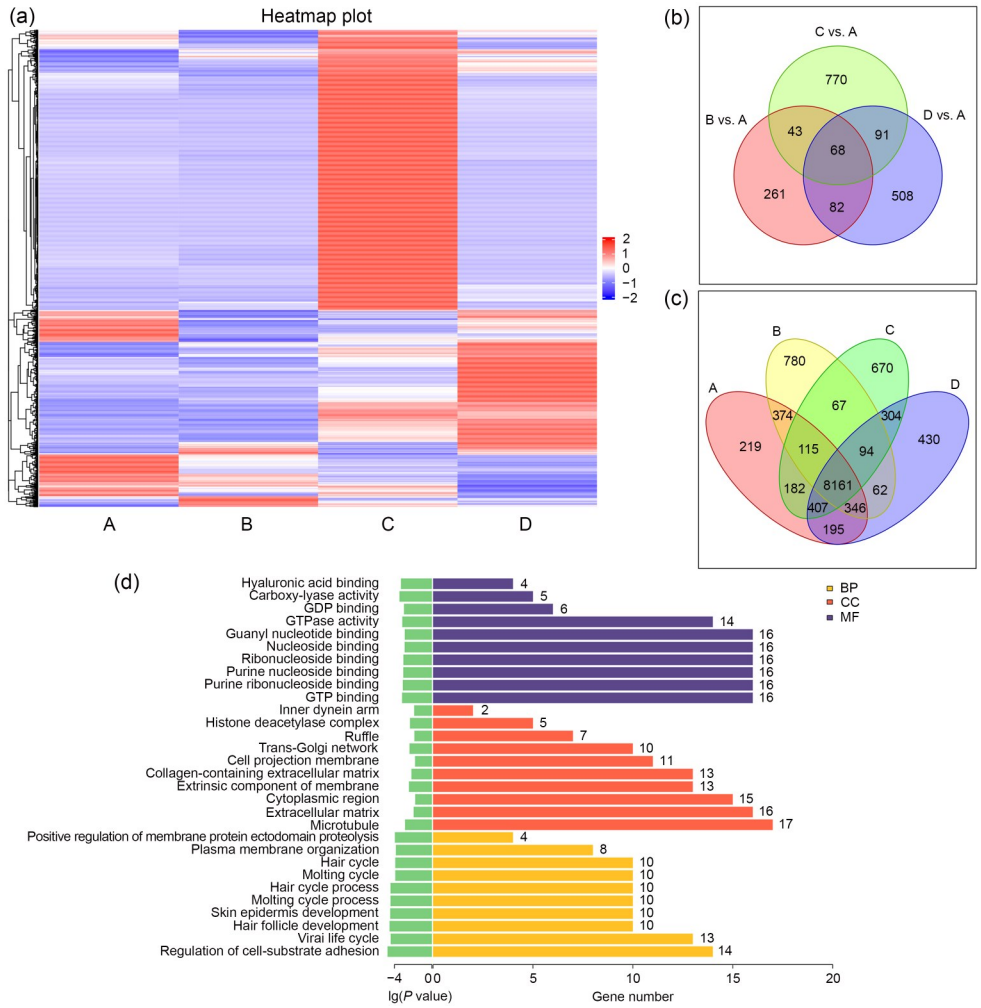
Fig. 2 Effects of ovarian stimulation on oocytes and embryos. (a) Morphology images of retrieved oocytes and early embryos in vivo. Samples were photographed promptly after their removal (n=10). The black arrows indicate normal oocytes and embryos, while the red arrows indicate aberrant oocytes and embryos. (b) The average number of eggs per mouse retrieved after 12–16 h of human chorionic gonadotropin (HCG) administration (n=10). (c) Proportion of heteromorphic oocytes retrieved from 10 mice (n=10). (d) The number of 2-cell embryos retrieved from each group (n=6). (e) The number of blastocysts retrieved from each group (n=6). (f) Expression of embryonic development potential-related genes in zygotes of each group (n=6). (g) Expression of apoptosis-related genes in oocytes of each group (n=6). Data are presented as mean± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA); different letters indicate significant differences (P<0.05) between groups. NC: natural ovulation control; PMSG: pregnant mare serum gonadotropin; GnRH: gonadotropin-releasing hormone; OHSS: ovarian hyperstimulation syndrome.

reduced in the OHSS group (Figs. 2d and 2e). These results revealed that ovarian hyperstimulation could increase the probability of aberrant oocytes and embryos.

Octamer-binding transcription factor 4 (Oct4) is a crucial regulator of pluripotency and self-renewal in oocytes and embryos, interacting with SRY (sex determining region Y)-box 2 (Sox2) to regulate Nanog expression and maintain the robustness of pluripotent stem cells (Fleming et al., 2004). We further clarified the adverse effects of hyperstimulation on oocytes and embryos, revealing that *Sox2* expression was reduced in the GnRH and OHSS groups, with *Nanog* expression substantially decreased only in the OHSS group in zygotes (Fig. 2f). No significant alteration in B-cell lymphoma 2 (*Bcl2*) expression was observed; however, the expression of *Caspase-3* was increased in the OHSS group (Fig. 2g). These findings suggest that ovarian hyperstimulation could induce oocyte apoptosis and impair the embryonic potential.

2.3 Effects of different ovarian stimulation treatments on oocyte gene transcription

To elucidate the mechanism underlying oocyte impairment, we conducted a comprehensive transcriptomic analysis of 100 oocytes obtained from each group. Based on the heatmap and Venn diagram, among 12 407 genes detected, 8161 were co-expressed and 284 differentially expressed genes (DEGs) were identified in the four groups (Figs. 3a–3c). Table S3 summarizes the Gene Ontology (GO) analysis of the functions with significant enrichment (P<0.05) (Figs. 3d–3f). Genes related to cell cycle regulation (*Wnt4*, *Sox4*, *Cdk4*, and *Kmt2a*), histone methylation and acetylation (*Kmt2a*, *Prdm9*, *Hdac2*, *Chd5*, *Prdm12*, and *Suv39h2*), and chromatin remodeling (*Hdac2* and *Kdm6b*) were altered in the ovarian stimulation groups. Next, we verified the expression of *Kdm6b*, *Kmt2a*, and *Hdac2* in oocytes from the four groups by performing reverse transcription-quantitative



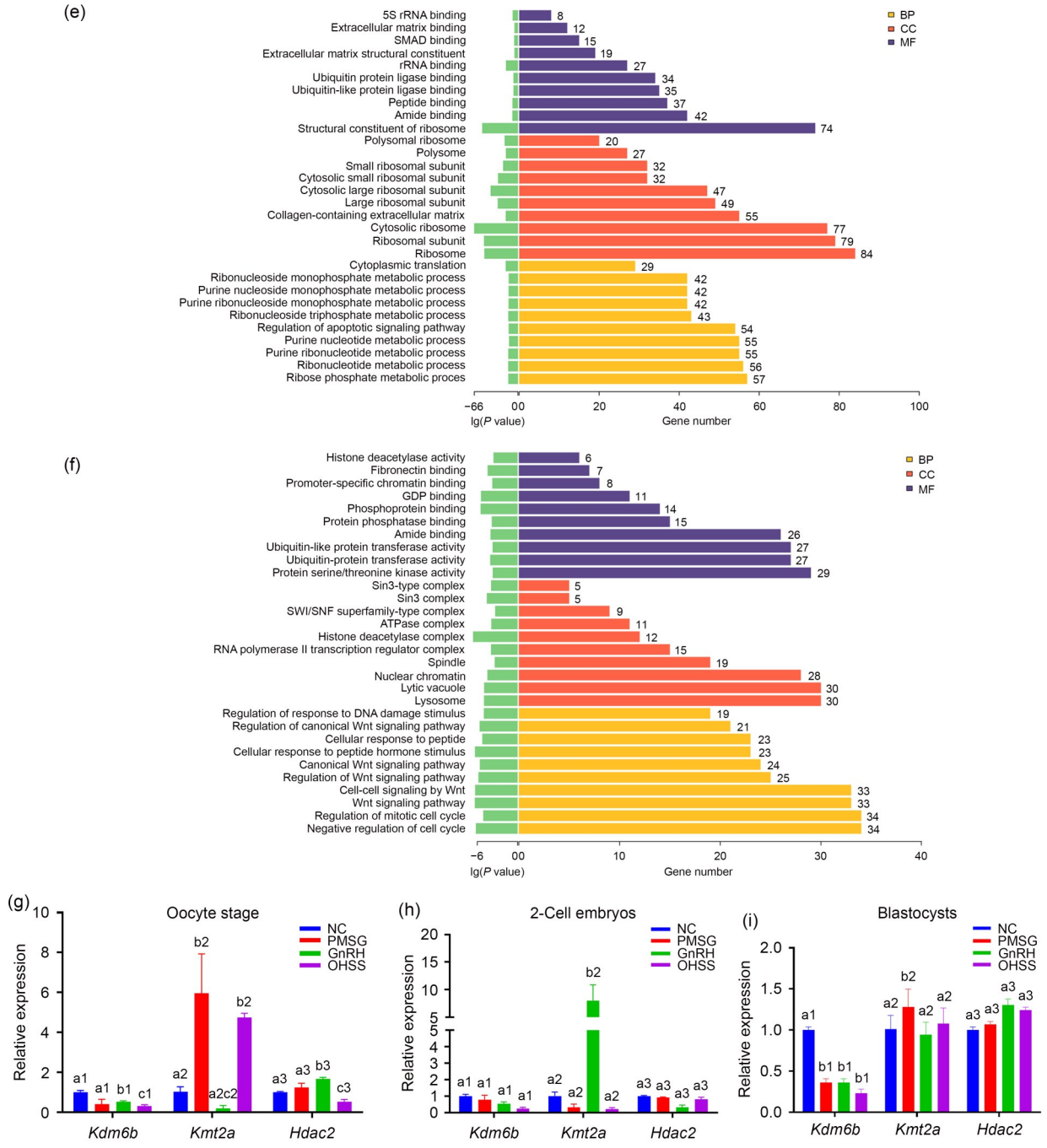


Fig. 3 Analysis of differentially expressed genes (DEGs) of oocytes obtained from different ovarian stimulation treatments. Groups A, B, C, and D represent the natural ovulation control (NC), gonadotropin-releasing hormone (GnRH), pregnant mare serum gonadotropin (PMSG), and ovarian hyperstimulation syndrome (OHSS) groups, respectively. (a) Heatmap of DEGs. (b) Venn diagram of DEGs of oocytes. (c) Venn diagram of oocyte gene expression. (d) Gene ontological classification of DEGs in the NC and GnRH groups. (e) Gene ontological classification of DEGs in the NC and PMSG groups. (f) Gene ontological classification of DEGs in the NC and OHSS groups. (g) RT-qPCR analyses of *Kdm6b*, *Kmt2a*, and *Hdac2* selected from mRNA-seq in the oocytes of each group (100 oocytes, n=10). (h) mRNA expression levels of *Kdm6b*, *Kmt2a*, and *Hdac2* in the 2-cell embryos of each group (40 embryos, n=6). (i) mRNA expression levels of *Kdm6b*, *Kmt2a*, and *Hdac2* in the blastocysts of each group (20 embryos, n=6). (g–i) Data are presented as mean±standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA); different letters indicate significant differences (P<0.05) between groups. BP: biological process; CC: cellular component; MF: molecular function; GDP: guanosine diphosphate; GPT: guanosine triphosphate; rRNA: ribosomal RNA; SMAD: mothers against decapentaplegic homolog; SWI/SNF: switch/sucrose non-fermentable.

polymerase chain reaction (RT-qPCR) analysis, revealing patterns consistent with those found in the messenger RNA-sequencing (mRNA-seq) results (Fig. 3g). Based on these findings, ovarian stimulation, especially hyperstimulation, could impact the gene transcriptional levels in oocytes. We further assessed the mRNA expression of *Kdm6b*, *Kmt2a*, and *Hdac2* in early embryos. At the 2-cell embryo stage, the expression of *Kdm6b* or *Hdac2* did not differ significantly among the four groups; however, *Kmt2a* expression was upregulated in the GnRH group (Fig. 3h). Until the blastocyst stage, the expression of *Kdm6b* was significantly reduced following three ovarian stimulation treatments, and the expression of *Kmt2a* was increased in the PMSG group (Fig. 3i). In blastocysts, *Hdac2* expression did not vary significantly between the four groups (Fig. 3i). The reduced *Kdm6b* expression was consistent in oocytes and blastocysts after hyperstimulation, thereby indicating that *Kdm6b* may play a crucial role during hyperstimulation.

2.4 Effects of ovarian hyperstimulation on Kdm6b expression

Based on both mRNA-seq and RT-qPCR results, the expression of the gene encoding Kdm6b, a key member of the H3K27me3 demethylase family, was significantly reduced in oocytes derived from the OHSS group (Fig. 3g). Hyperstimulation-induced aberrant oocytes could be attributed to decreased *Kdm6b* expression. We performed immunofluorescence to investigate the protein expression of Kdm6b in oocytes derived from the four groups. The OHSS group exhibited significantly lower fluorescence intensity than the NC, PMSG, and GnRH groups (Figs. 4a and 4b).

Kdm6b plays a crucial role in H3K27 modification and participates in transcriptional activation during embryonic development (Agger et al., 2007; Burchfield et al., 2015; Huang et al., 2018). After examining the altered *Kdm6b* expression in oocytes, we conducted an immunofluorescent analysis to validate the expression level of H3K27me3. The OHSS group displayed a significantly higher fluorescence intensity than the NC, PMSG, and GnRH groups (Figs. 4c and 4d). Meanwhile, we found that the change in DNA damage marker γ-H2AX was consistent with that of H3K27me3, demonstrating a significantly higher intensity level in the OHSS group (Figs. 4c and 4e). Based on these findings, ovarian hyperstimulation could reduce the expression of Kdm6b and increase the H3K27me3 level in oocytes.

2.5 Detrimental effects of high estrogen levels on mESC development

Typically, patients with OHSS exhibit markedly elevated estrogen levels (D'Angelo et al., 2004). Consistently, our enzyme-linked immunosorbent assay (ELISA) results revealed that the serum estradiol level in the OHSS group was 10 times higher than that in the NC group (Fig. 1e). To determine the adverse effects of high estrogen exposure, mESCs were exposed to varying estrogen concentrations. mESCs formed flat colonies on gelatin-coated dishes (Fig. 5a). The cytoplasmic localization of purplish-black alkaline phosphatase and the expression levels of pluripotent genes were consistent across all experimental groups, indicating that the utilized mESCs could undergo differentiation and that estrogen did not impact the pluripotency of mESCs (Fig. 5c). The cell counting kit-8 (CCK-8) assay results revealed that the cytoactivity of mESCs decreased within 24 h of high estrogen treatment. Moreover, the treatment with 1×10^{-7} and 1×10^{-6} mol/L estrogen suppressed mESC proliferation for up to 48 and 72 h (Fig. 5b). These findings suggest that high-dose estrogen exposure could inhibit mESC proliferation.

2.6 Effects of high-dose estrogen exposure on the expression of Kdm6b and H3K27me3

Herein, *Kdm6b* expression was downregulated in mouse oocytes and blastocysts obtained from the OHSS group. To investigate whether the altered expression could be attributed to estrogen exposure, we cultured mESCs with estrogen at different concentrations or dimethyl sulfoxide (DMSO; control) for 48 h. We discovered that *Kdm6b* mRNA expression was increased in cells treated with 1×10^{-10} mol/L estrogen; however, the expression was then significantly decreased in cells treated with 1×10^{-7} and 1×10^{-6} mol/L estrogen, compared with 1×10^{-10} mol/L estrogen (Fig. 5d). Despite these changes in *Kdm6b* mRNA expression following treatment with 1×10^{-9} or 1×10^{-7} mol/L estrogen, no significant differences in Kdm6b protein expression were observed under these conditions (Figs. 5e and 5f). However, consistent with the altered mRNA level, Kdm6b protein expression was downregulated following high-dose estrogen treatment of mESCs (Figs. 5e and 5f).

Repression of Kdm6 activity during mESC differentiation leads to DNA damage owing to elevated H3K27me3 levels (Hofstetter et al., 2016). Considering

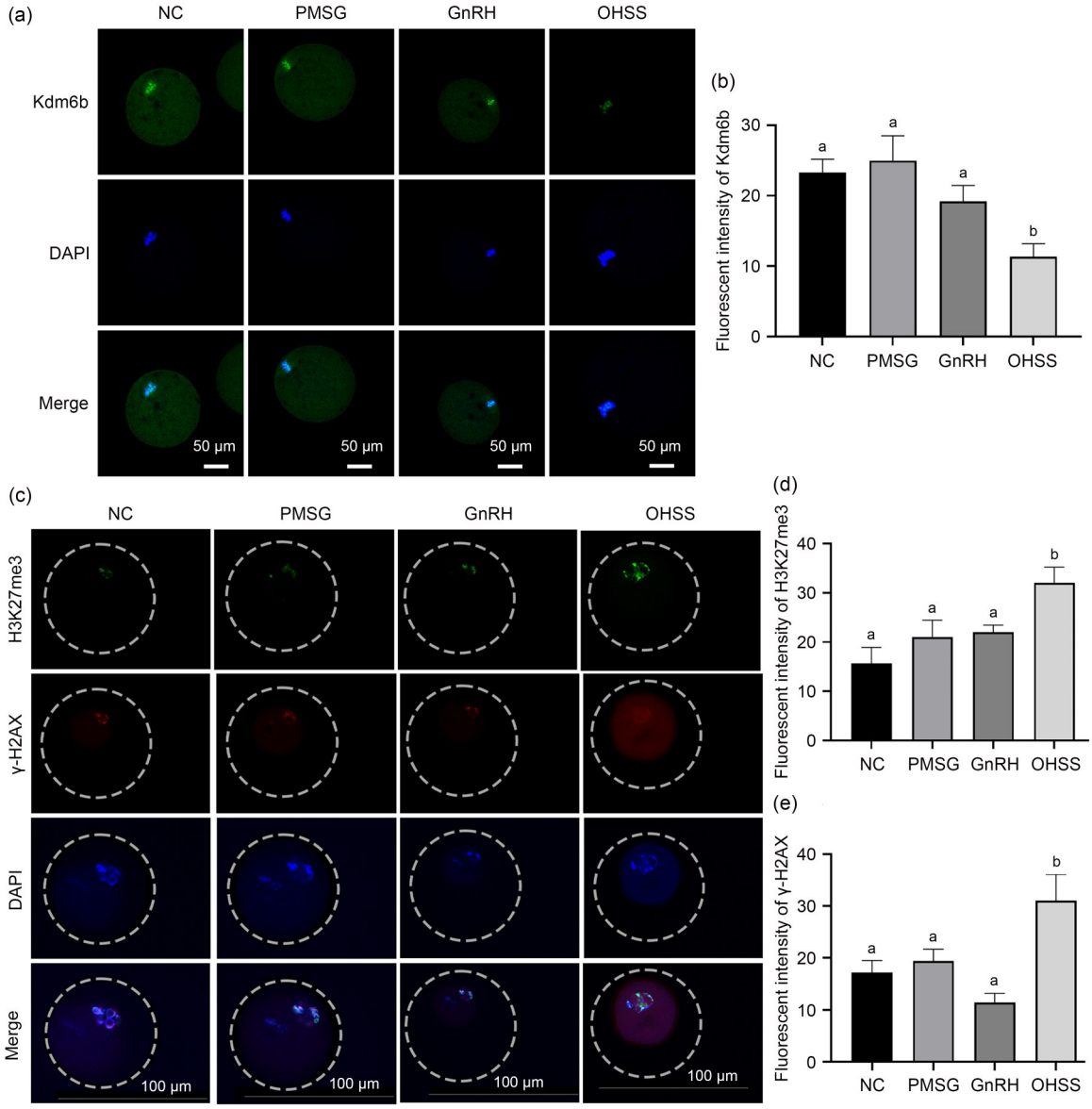


Fig. 4 Expression of Kdm6b at early embryo stages and H3K27 methylation levels. (a) Immunofluorescence analyses of Kdm6b in oocytes of each group. (b) Fluorescence intensity of Kdm6b (60 oocytes, *n*=6). (c) Immunofluorescence analyses of H3K27me3 and γ-H2AX in oocytes of each group. (d, e) Fluorescence intensities of H3K27me3 and γ-H2AX (60 oocytes, *n*=6). Data are presented as mean±standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA); different letters indicate significant differences (*P*<0.05) between groups. NC: natural ovulation control; PMSG: pregnant mare serum gonadotropin; GnRH: gonadotropin-releasing hormone; OHSS: ovarian hyperstimulation syndrome; DAPI: 4',6-diamidino-2-phenylindole.

the altered expression of Kdm6b in mESCs following exposure to high estrogen levels, we next examined the protein expression levels of H3K27me3 and γ-H2AX using western blot analysis in mESCs following estrogen treatment. H3K27me3 expression increased following estrogen treatment, with a significant increase observed in the 1×10^{-6} mol/L group (Figs. 5g and 5h). Moreover, γ-H2AX expression exhibited a similar trend to that of H3K27me3, with increasing estrogen concentrations resulting in an upward trend in expression (Figs. 5g and 5i). We further quantified the immunofluorescence intensities of H3K27me3 and γ-H2AX to validate their expression levels. Consistently, the fluorescence intensities of H3K27me3 and γ-H2AX were significantly elevated in the estrogen-treated mESCs, particularly in the 1×10^{-6} mol/L group (Figs. 5j–5l).

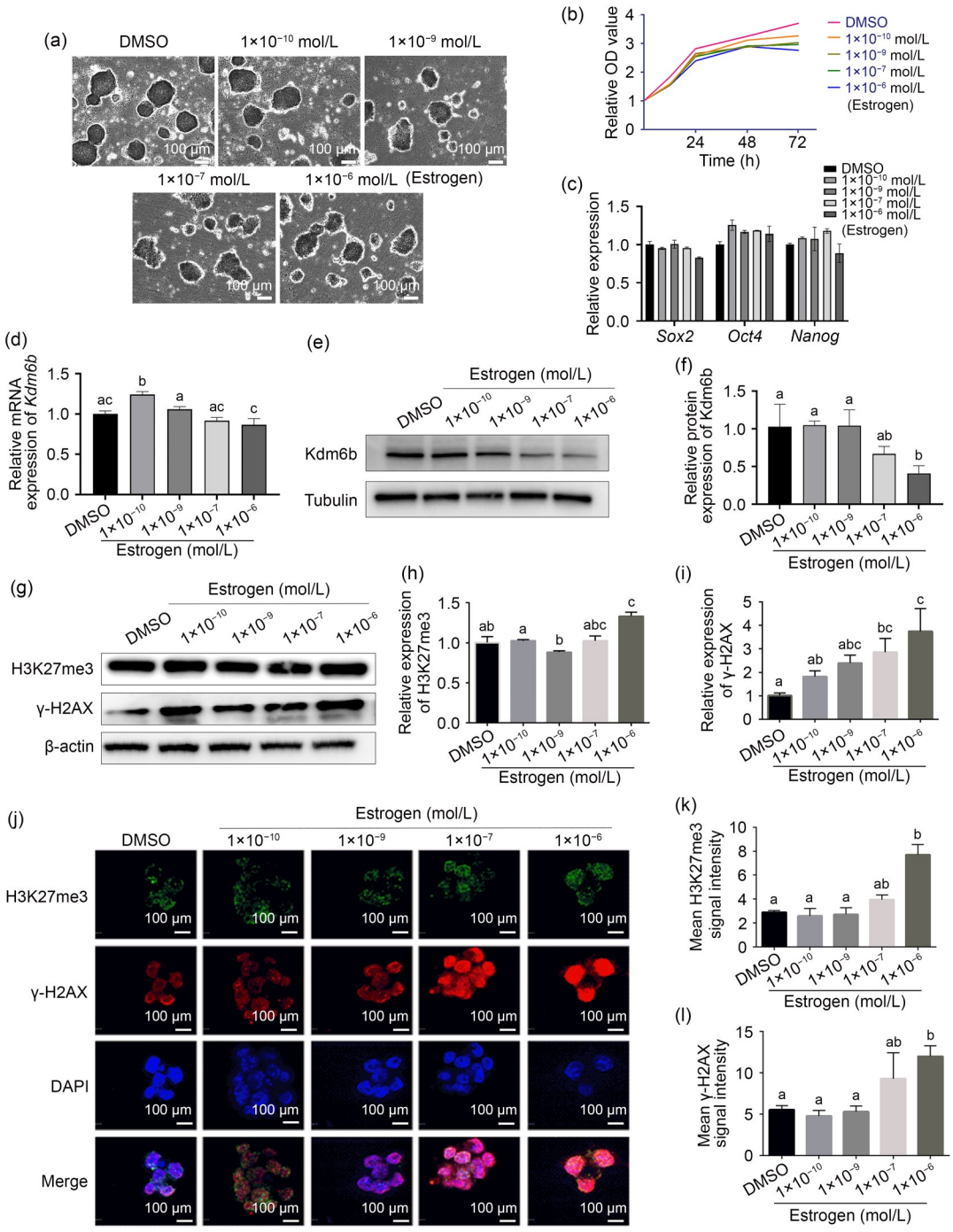


Fig. 5 Effects of high estrogen concentrations on growth and histone methylation levels of mouse embryonic stem cells (mESCs). (a) Alkaline-phosphatase activation test in mESCs. (b) Growth curves of mESCs treated with estrogen at different concentrations for 24, 48, and 72 h. (c) mRNA expression of pluripotent genes in estrogen-treated mESCs. (d) Comparison of the mRNA expression of *Kdm6b* in mESCs cultured in estrogen at different concentrations. (e) Western blot analysis of Kdm6b in mESCs cultured in estrogen at different concentrations for 48 h. Membranes were incubated with antibodies specific for Kdm6b and Tubulin. (f) Average gray values of the protein bands. (g) Western blot analyses of H3K27me3 and γ-H2AX in mESCs cultured with estrogen at different concentrations. (h, i) Gray scale densitometry analyses of H3K27me3 and γ-H2AX. (j) Immunofluorescence analyses of H3K27me3 and γ-H2AX in mESCs cultured with estrogen at different concentrations. (k, l) Fluorescence intensities of H3K27me3 and γ-H2AX in mESCs. Data are presented as mean±standard deviation (SD), *n*=3, except those in (b). Statistical significance was determined using one-way analysis of variance (ANOVA); different letters indicate significant differences (*P*<0.05) between groups. DMSO: dimethyl sulfoxide; DAPI: 4',6-diamidino-2-phenylindole; OD: optical density.

These results suggest that a high dose of estrogen decreases the expression of Kdm6b and impairs the histone methylation of H3K27.

2.7 Correlation of downregulated Kdm6b expression with aberrant expression of H3K27me3 and γ-H2AX

To verify whether estrogen can regulate Kdm6b expression, we treated mESCs with the estrogen receptor antagonist ICI 182780 prior to treatment with 1×10^{-6} mol/L estrogen. Based on RT-qPCR analysis, the treatment with estrogen downregulated *Kdm6b* expression, and this reduced expression was reversed following treatment with ICI 182780 (Fig. 6a). To analyze whether the effects of estrogen on gene expression resulted from *Kdm6b* inhibition, we knocked down *Kdm6b* in mESCs. The efficiency of small interfering RNA (siRNA) knockdown was confirmed using RT-qPCR 48 h after transfection, as *Kdm6b* expression was significantly downregulated following siRNA-*Kdm6b* (si-*Kdm6b*) treatment (Fig. 6b). Subsequently, we evaluated alterations in H3K27me3 and γ-H2AX expression under estrogen treatment and in si-*Kdm6b*-transfected cells. We treated mESCs with DMSO+si-NC, DMSO+si-*Kdm6b*, 1×10^{-6} mol/L estrogen+si-NC, or 1×10^{-6} mol/L estrogen+si-*Kdm6b*. Untreated cells served as the blank control. In contrast to DMSO+si-NC, we found that 1×10^{-6} mol/L estrogen and *Kdm6b* knockdown moderately affected embryoid body formation. Combined treatment with estrogen and si-*Kdm6b* diminished embryoid body formation (Fig. 6d). Kdm6b, H3K27me3, and γ-H2AX protein levels were unaltered in the DMSO+si-NC group when compared with those in the blank control group, indicating that DMSO and si-NC did not affect Kdm6b, H3K27me3, or γ-H2AX expression level (Figs. 6c and 6e–6g). However, we found that 1×10^{-6} mol/L estrogen treatment significantly reduced Kdm6b expression and upregulated H3K27me3 and γ-H2AX expression, which was consistent with the effects of si-*Kdm6b* treatment (Figs. 6c and 6e–6g). There were no significant differences in the expression of H3K27me3 or γ-H2AX between the groups treated with only 1×10^{-6} mol/L estrogen and only si-*Kdm6b* (Figs. 6e–6g). Next, we verified the expression levels of H3K27me3 and γ-H2AX using immunofluorescence. Consistent with the results of the western blot analysis, the treatment with 1×10^{-6} mol/L estrogen or si-*Kdm6b*, either alone or in combination, upregulated the expression of H3K27me3 and γ-H2AX, with the strongest effect observed in the combined treatment group (Figs. 6h–6j). These results suggest that Kdm6b is a potential regulator of estrogen-mediated adverse effects on H3K27 methylation levels. Estrogen-induced Kdm6b inhibition weakens H3K27me3 demethylation, thereby resulting in H3K27me3 accumulation and the incidence of DNA damage.

3 Discussion

In the current study, we observed that, while conventional ovarian stimulation minimally impacted oocytes and embryos, hyperstimulation can be detrimental to the development of ovarian follicles, oocytes, and embryos. Furthermore, ovarian hyperstimulation enhanced the serum estrogen level and reduced *Kdm6b* expression in oocytes and blastocysts. Consistently, upon exposure to high-dose estrogen, oocytes displayed downregulated *Kdm6b* expression and H3K27me3 accumulation. Furthermore, we observed that high-dose estrogen negatively regulated *Kdm6b* expression, which was consistent with *Kdm6b* knockdown, resulting in H3K27me3 accumulation, and increased γ-H2AX expression and embryoid formation defects in mESCs. Collectively, our findings unequivocally suggest that ovarian hyperstimulation-induced exposure to high-dose estrogen could impair the demethylation of H3K27me3 by reducing the expression of Kdm6b. This discovery highlights the potential mechanism underlying hyperstimulation-induced adverse effects on oocytes and embryos.

Ovarian stimulation is an initial and pivotal step in ART. In clinical practice, women with infertility undergo different types of ovarian stimulation to obtain numerous oocytes. However, the global ART pregnancy rate has remained stable at approximately 50% (Chambers et al., 2021). Furthermore, conventional ovarian stimulation was found to increase the aneuploidy rate when compared with mild ovarian stimulation (Baart et al., 2007). However, the impact of ovarian stimulation on gene expression in oocytes and embryos, as well as the underlying mechanisms, warrants further investigation. Herein, we established mouse models of different ovarian stimulation treatments and estrogen-treated mESC models to analyze the effects of high-dose estrogen following ovarian stimulation on gene expression in oocytes and embryos.

Ovarian size is a predictor of the response to ovarian stimulation (Syrop et al., 1995). Reportedly, the enlargement of mouse ovaries during ovarian stimulation

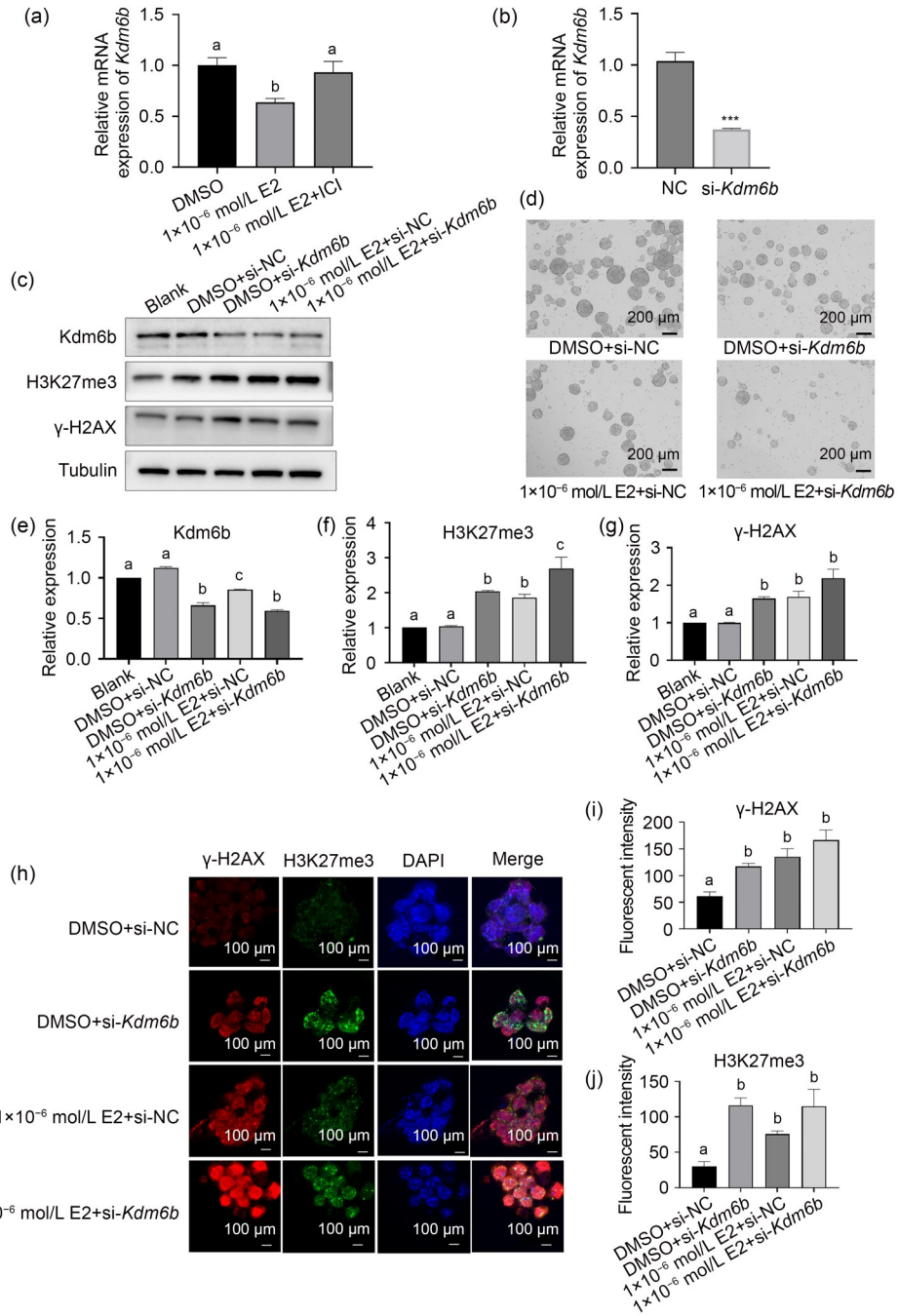


Fig. 6 Effects of *Kdm6b* inhibition on histone methylation levels and γ-H2AX expression. (a) Comparison of *Kdm6b* mRNA expression between the DMSO, 1×10^{-6} mol/L estrogen (estradiol (E2)), and 1×10^{-6} mol/L estrogen combined with ICI 182780 treatments in mESCs. (b) Comparison of *Kdm6b* mRNA expression between the NC and si-*Kdm6b* treatments in mouse embryonic stem cells (mESCs). (c) Comparison of protein levels of Kdm6b, H3K27me3, and γ-H2AX in mESCs cultured in blank control, DMSO+si-NC, DMSO+si-*Kdm6b*, 1×10^{-6} mol/L estrogen+si-NC, and 1×10^{-6} mol/L estrogen+si-*Kdm6b* media by western blot analysis. (d) Representative micrographs of embryoid body formation in the presence of DMSO+si-NC, DMSO+si-*Kdm6b*, 1×10^{-6} mol/L estrogen+si-NC, and 1×10^{-6} mol/L estrogen+si-*Kdm6b*. (e–g) Gray scale densitometry analyses of Kdm6b, H3K27me3, and γ-H2AX. (h) Immunofluorescence analyses of H3K27me3 and γ-H2AX in mESCs cultured with DMSO+si-NC, DMSO+si-*Kdm6b*, 1×10^{-6} mol/L estrogen+si-NC, and 1×10^{-6} mol/L estrogen+si-*Kdm6b* media. (i, j) Fluorescence intensities of H3K27me3 and γ-H2AX in mESCs. Data are presented as mean±standard deviation (SD), *n*=3. Statistical significance was determined using one-way analysis of variance (ANOVA); different letters indicate significant differences (*P*<0.05) between groups. DAPI: 4',6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; ICI: ICI 182780; NC: natural ovulation control; si-*Kdm6b*: siRNA-*Kdm6b*. $^{***}P$<0.001, vs. NC.

is associated primarily with follicular development and augmented blood supply (Engmann et al., 1999; Lass and Brinsden, 1999; Wakimoto et al., 2020). Our study demonstrated that OHSS could substantially increase the size of murine ovaries, although the sizes of isolated ovaries varied within each group. Given that mice exhibit inter-individual variability in their response to ovarian stimulation, it is plausible that variations in ovarian size could be attributed to individual differences. In the ovarian hyperstimulation mouse model, mice exhibited ovarian hyperemia, accompanied by elevated ovarian weight, high estrogen levels, and numerous achieved oocytes, which are consistent with the key manifestations of OHSS, proving that ovarian stimulation treatments are effective.

The process of oocyte maturation takes place within the ovarian follicles and involves the participation of cumulus-granulosa cells (Ikeda and Yamada, 2014). Disturbances in apoptosis and proliferation-related gene expression in ovarian follicles can interfere with oocyte maturation (Huang and Wells, 2010). In the present study, expression levels of Caspase-3 and γ-H2AX were increased in ovarian follicles of the OHSS group, suggesting that hyperstimulation could induce apoptosis and DNA damage in ovarian follicle development, thereby impacting oocyte quality. Morphology and development potential-related genes are associated with impaired oocyte fertility and embryo development potential (Wolf et al., 2011; Lundin and Ahlström, 2015; Whitmill et al., 2017). It has been reported that repeated ovarian stimulation can result in spindle damage, enhanced intracellular oxidative stress, and decreased *Oct4* expression in mice (Kalthur et al., 2016). Furthermore, we observed a high incidence of heteromorphic oocytes and a low rate of eumorphic 2-cell embryos and blastocysts upon ovarian hyperstimulation. We also found that zygotes from the OHSS group exhibited decreased expression of the development potential-related genes *Oct4* and *Nanog*. Therefore, our results revealed that OHSS could affect the morphology and development potential of the ovaries, oocytes, and embryos.

Histone methylation is a vital and complex process in oocyte generation and embryo development (Seki et al., 2005). In oocytes or embryos, H3K27me3 is associated with gene repression and is altered by several exogenous factors such as environmental endocrine disruptors, maternal age, and exogenous hormones (Minocherhomji et al., 2009; Liu et al., 2016). Altered histone methylation can markedly impact the developmental potential of oocytes and embryos (Yang et al., 2021). Kdm6b is recruited to the promoters of homeobox genes and bone morphogenetic proteins, which participate in follicular development and ovulation (Greene et al., 2014). The embryonic knockdown of *Kdm6b* is lethal in mice (Satoh et al., 2010). Our transcriptome analysis revealed a significant reduction in *Kdm6b* expression in oocytes derived from the OHSS group. Although the expression of *Kdm6b* was not significantly affected in 2-cell embryos, a consistent alteration was observed in blastocysts, indicating that ovarian hyperstimulation could alter the expression of *Kdm6b*, which may potentially impair its ability to demethylate H3K27me3. It is worth noting that the altered expression of the DNA damage marker γ-H2AX in oocytes was consistent with H3K27me3. The accumulation of H3K27me3 at DNA damage sites of chromosomes has been previously reported (O'Hagan et al., 2008; Chou et al., 2010). Meanwhile, we detected a decrease in Kdm6b expression, accompanied by the accumulation of H3K27me3 and γ-H2AX, in the OHSS group oocytes. Therefore, H3K27me3 accumulation caused by reduced Kdm6b expression in oocytes may increase the probability of DNA damage. Importantly, the potential mechanism through which ovarian hyperstimulation induces the abnormal development of oocytes and embryos is yet to be elucidated.

The average concentration of serum estradiol in patients who develop OHSS exceeds 3500 pg/mL, and the level exceeding 6000 pg/mL could serve as an indicator to predict OHSS (Delvigne, 2009; Sousa et al., 2015). High doses of exogenous hormones could impair genomic imprinting during oocyte development and the pre-implantation stage (Market-Velker et al., 2010). Based on the substantially elevated serum estradiol levels in the OHSS group, we postulate that ovarian hyperstimulation-induced genetic alterations in oocytes could be attributed to heightened estrogen levels. Moreover, mRNA-seq results suggested that ovarian hyperstimulation altered expression levels of *Kmt2a* and *Hdac2*, genes involved in histone methylation, in oocytes. Huang et al. (2018) evaluated *Kmt2a* depletion, which impaired the switch from H3K27me to H3K27ac. *Kdm6b* and *Kmt2a* are recruited to specific lysine residues on histones and are involved in the regulation of histone methylation (Klose and Zhang,

2007). Although no significant differences were observed in the expression of *Kmt2a* and *Hdac2* in embryos, these genes may collaborate to maintain the histone methylation balance in oocytes. Consequently, our focus shifted toward investigating the potential regulation of Kdm6b expression by estrogen. Owing to the limited availability of oocytes and embryos, mESCs were treated with varying estrogen concentrations to comprehensively analyze whether downregulated Kdm6b expression was affected by estrogen exposure. Herein, we found that the proliferative ability of mESCs exposed to high-dose estrogen was significantly reduced, indirectly indicating that high estrogen exposure could impair the development of mESCs.

The Jumonji C domain-containing family of lysine demethylases (KDMs) catalyzes histone demethylation, with Kdm6b specifically targeting H3K27me3-demethylase by binding to specific lysine residues on H3K27 (Klose and Zhang, 2007; Vandamme et al., 2012; Zenk et al., 2017). Estrogen receptor α (ERα) has been shown to regulate the expression of Kdm6b to demethylate the H3K27me3 during osteogenesis and in breast cancer cells (Svotelis et al., 2011; Liu et al., 2022). In the current study, *Kdm6b* expression was downregulated, whereas H3K27me3 and γ-H2AX expression levels were elevated in the high estrogen concentration group. Interestingly, physiological estrogen treatment did not significantly alter gene expression levels in mESCs. Moreover, treatment with the estrogen receptor antagonist ICI 182780 could reverse the downregulated *Kdm6b* expression induced by high-dose estrogen exposure in mESCs. These findings indicate the negative association between high-dose estrogen exposure and *Kdm6b* expression, which further impairs the histone methylation of H3K27. To verify these findings, siRNA was used to treat mESCs to achieve *Kdm6b* knockdown. Upregulated expression of H3K27me3 and γ-H2AX was observed following high estrogen exposure and *Kdm6b* knockdown, which revealed that *Kdm6b* could function as a regulator of the adverse effects of estrogen on H3K27 methylation levels.

H3K27me3 modification predominantly occurs within the promoter region of development-related genes and plays a key role in embryonic development (Liu et al., 2016). For instance, H3K27me3 accumulation in bovine embryos led to reduced embryonic development (Canovas et al., 2012). In addition, Agger et al. (2007) reported that Kdm6b plays a crucial role in regulating the expression of hexose oxidase (*HOX*) genes through the demethylation of H3K27me3 during ESC differentiation. In our study, high-dose estrogen exposure and *Kdm6b* knockdown affected embryoid body formation, which was substantially impaired following combined estrogen and si-*Kdm6b* treatment, thereby suggesting that estrogen-induced reduced Kdm6b expression may impair mESC differentiation. Kdm6b reportedly facilitates blastocyst formation and promotes differentiation by attenuating H3K27me3 modifications (Canovas et al., 2012; Kartikasari et al., 2013; Xu et al., 2013). Given the elevated estrogen concentrations during OHSS, estrogen-induced Kdm6b inhibition could weaken H3K27me3 demethylation, thereby resulting in H3K27me3 accumulation. Accordingly, H3K27me3 accumulation acts downstream of Kdm6b, ultimately resulting in abnormal oocyte and embryo development, thereby suggesting a potential mechanism for aberrant oocyte and embryonic development during OHSS.

The present study has several limitations that need to be addressed. Firstly, mice are poly-ovulators, whereas humans are mono-ovulators. Owing to ethical and clinical specimen limitations, undertaking experiments using human oocytes presents a considerable challenge. A recent GametesOmics study has revealed that numerous genes related to growth factor signaling are co-expressed in human and mouse oocytes and play key roles in gametogenesis (An et al., 2024). Moreover, several signaling pathways, ovarian physiology, and the genetic architecture involved in ovulation are shared between mice and humans (Zhang et al., 2018; Wassarman and Litscher, 2022; An et al., 2024). These similarities suggest that the effects observed in mice might also be relevant to humans. Hence, we used a mouse model, which has been widely used in similar studies, to simulate the ovarian stimulation process. Secondly, further investigations are warranted to elucidate the underlying mechanisms that govern the impact of altered H3K27me3 modifications on oocyte embryonic development. H3K27me3 plays a key role in maintaining the self-renewal capability and pluripotency of ESCs (Macrae et al., 2023). Kdm6b is a critical demethylase that specifically induces H3K27me3 demethylation to promote gene expression (Harikumar and Meshorer, 2015; Ding et al., 2021). H3K27me3 modifications regulated by Kdm6b can influence proliferation and differentiation in the stem cell culture model (Guo et al.,

2022). However, evidence supporting the involvement of Kdm6b in oocyte and embryo development during ovarian stimulation through H3K27me3 modification remains inconclusive. Future research in this area will focus on the detection of H3K27me3 modification within the promoter region of growth-related molecules to further elucidate how these observations may impact oocyte and embryo development.

The current study revealed downregulated Kdm6b expression and upregulated H3K27me3 expression in response to high-dose estrogen exposure, along with the adverse effects on oocyte and embryo development using in vivo and in vitro models. These findings have sparked further research endeavors looking into the regulatory role of Kdm6b and H3K27me3 during the process of ovarian stimulation and their functions in reproductive system development. Firstly, we demonstrated that ovarian hyperstimulation reduces *Kdm6b* expression in oocytes, which may lead to increased H3K27me3 modification. The mechanisms by which Kdm6b functions in oocyte and early embryo development, as well as its regulation of H3K27me3, will contribute to uncovering the underlying molecular mechanisms of low oocyte and embryo quality in ovarian hyperstimulation. Secondly, these findings contribute important clues to the prevention of OHSS. Notably, Kdm6b could be a predictive marker for clinical ART outcomes in patients with OHSS. Future research can focus on investigating the interaction between H3K27me3 and the estrogen signaling pathway, as well as the impact of this regulatory mechanism on ovarian development and reproductive function.

4 Conclusions

In conclusion, this study demonstrated that high-dose estrogen resulting from hyperstimulation impairs the demethylation of H3K27me3 by reducing Kdm6b expression. The ovarian stimulation mouse model revealed that *Kdm6b* expression was downregulated after hyperstimulation. Mechanistically, high estrogen levels could interfere with *Kdm6b* expression, resulting in altered H3K27me3 levels and DNA damage incidence. Thus, we report that estrogen can decrease the expression of Kdm6b, resulting in the demethylation of H3K27me3 in oocytes and embryos, which is a potential mechanism for the reduction in oocyte and embryo quality caused by hyperstimulation. These findings suggest that a personalized approach to avoiding ovarian hyperstimulation may be necessary to optimize outcomes in women undergoing ART.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

The data generated in this study are available upon request from the corresponding authors.

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Author contributions

Fan JIN and Qiongxiao HUANG initiated, conceived, and supervised the study. Quanmin KANG, Fang LE, and Ruimin ZHAO performed related experiments and statistical analysis and prepared the manuscript. Xiayuan XU and Yuanyuan ZHOU isolated oocytes and performed the mRNA-seq library construction. Lifang CHEN, Shi ZHENG, Lijun LOU, Nan JIANG, Juan SHEN, Minhao HU, and Ning WANG were involved in the study design. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Quanmin KANG, Fang LE, Xiayuan XU, Lifang CHEN, Shi ZHENG, Lijun LOU, Nan JIANG, Ruimin ZHAO, Yuanyuan ZHOU, Juan SHEN, Minhao HU, Ning WANG, Qiongxiao HUANG, and Fan JIN declare that they have no conflicts of interest.

All experiments were performed under the Guide for the Care and Use of Laboratory Animals and approval of the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (No. 11813). All institutional and national guidelines for the care and use of laboratory animals were followed.

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Supplementary information

Tables S1–S3; Materials and methods