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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Histone demethylase KDM4A and KDM4B expression in granulosa cells from women undergoing in vitro fertilization

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Abstract

Purpose To assess expression of the histone demethylases *KDM4A* and *KDM4B* in granulosa collected from women undergoing oocyte retrieval and to determine if expression was related to pregnancy outcome.

Methods Cumulus and mural granulosa cells were obtained from women undergoing oocyte retrieval. *KDM4A* and *KDM4B* mRNA expression was determined by qRT-PCR. KDM4A and KDM4B proteins were immunohistochemically localized in ovarian tissue sections obtained from archival specimens.

Results KDM4A and KDM4B protein was localized to oocytes, granulosa cells, and theca and luteal cells in ovaries from reproductive-aged women. KDM4A and KDM4B mRNA expression was overall higher in cumulus compared to mural granulosa. When comparing granulosa demethylase gene expression, *KDM4A* and *KDM4B* mRNA expression was higher in both cumulus and mural granulosa from not pregnant patients compared to patients in the pregnant-live birth group.

Conclusions Histone demethylases *KDM4A* and *KDM4B* mRNA are differentially expressed in cumulus and mural granulosa. Expression of both *KDM4A* and *KDM4B* mRNA was lower in cumulus granulosa and mural granulosa from pregnant compared to not pregnant patients. These findings suggest that altered expression of histone demethylases may impact epigenetic changes in granulosa cells associated with pregnancy.

Keywords Ovary · Granulosa · KDM4 · Histone demethylase · Pregnancy · IVF

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Introduction

The dynamics of ovarian follicular development and ovulation are tightly regulated events occurring in response to changing patterns of gene expression [1–3]. The importance of some genes within this complex network is understood and is evidenced by altered follicle development and/or loss of ovulation and luteal function when disrupted [4]. However, the trend towards delayed fertility in Westernized societies has resulted in a significant growth in the number of patients requiring assisted reproductive technologies. A more complete understanding of the processes governing follicle maturation and ovulation is essential for improving outcomes of in vitro fertility treatments.

One mechanism controlling gene expression occurs at the level of histones that function in packaging DNA. The nucleosome, considered the building block of chromatin, is composed of eight histones, two each H2A, H2B, H3, and H4. Histones regulate the availability of DNA to the transcriptional machinery through post-translational modifications of histone

tails which can include phosphorylation, acetylation, ubiquitination, and methylation [5, 6]. Histone lysine methylation is a dynamic process that can both regulate transcriptional activation and repression and has been shown to play important roles in normal development, and transformation and progression of cancer [7]. The methylation state of lysine residues is controlled by enzymes, methyltransferases, and demethylases that function in the addition and removal of methylation marks. Two classes of methyltransferase, a family of SET-domain-containing enzymes and DotL1/Dot1p, mediate the transfer of a methyl group from *S*-adenosyl-L-methionine to histone lysine residues [8, 9]. Demethylation is carried out by two families of demethylases, the amine oxidases (lysine-specific demethylase, LSD/KDM1A, and LSD2/KDM1B) which remove mono- and di-methyl histone lysine marks and Jumonji C domain-containing iron-dependent dioxygenases which remove mono-, di-, and tri-methyl lysine residues (JmjC-KDMs). The methylation state of different lysine residues of histone 3 and 4 is associated with transcriptional activation (H3K4, H3K36, H3K79) or repression (H3K9, H3K27, H3K56, H4K20) [7]. Although the role of histone modifications in controlling gene expression has recently been an area of intense investigation, thus far there has been little attention given to this process as it may relate to follicle development and ovulation.

The JmjC-containing family of histone demethylases consists of approximately 24 genes that can be categorized into seven functionally divergent subfamilies. The highly conserved KDM4 subfamily (KDM4A-E) demethylates H3K9 and H3K36 di- and tri-methyl marks [10]. Diverse roles for KDM4 demethylases have been described, ranging from directing differentiation during embryogenesis, to promoting tumor progression, to maintaining an undifferentiated pattern of gene expression in stem cells [11]. A recent study reported a meta-analysis of next-generation sequencing profiles using the RNA-Seq Atlas and GENT databases. That study indicated robust expression of KDM4 subfamily members in the ovary [11]. Although discrete roles for KDM4 members in ovarian biology have yet to be described, many functions already ascribed to KDM4 have a correlation to processes occurring in the ovary.

Both KDM4A and KDM4B have roles in nuclear hormone receptor function. KDM4B is induced in an estrogen-dependent manner in breast cancer cells and can bind to the estrogen receptor and facilitate expression of estrogen-dependent genes [12–14]. Estrogen receptors are essential for normal ovarian follicular development, ovulation, and luteal function [15–20]. In prostate cancer, KDM4B stabilizes the androgen receptor (AR) and interacts to induce AR-mediated transcription [21, 22]. KDM4A and KDM4B both promote endometrial cancer progression by regulating AR and subsequent androgen-dependent expression of c-myc and p27kip1 [23]. Androgen receptor in the ovary functions in normal follicle development [24–26]. Inactivation of the AR in women is associated with premature ovarian failure [25], and AR polymorphisms have been associated with polycystic ovary syndrome [27–29] substantiating the importance of AR-mediated processes in normal ovarian biology. Progesterone receptor (PR) is another steroid receptor essential for normal follicle development, ovulation, and luteal function [30–33], and a role for Jumanji family histone demethylase KDM5A/B in PR-mediated gene expression has been demonstrated [34–36]. Most recently, a role for KDM4A as a maternal effect gene has been described in a murine knockout model [37].

Hypoxia and HIF-1α stimulate KDM4B which in turn functions to support HIF-1α-dependent gene expression to mediate the hypoxic response [38]. KDM4B expression in ovarian cancer correlated with expression of the hypoxic marker CA-IX and facilitated the expression of metastatic gene expression in hypoxic growth conditions [39]. The ovary receives a robust vascular supply yet the degree of hypoxia and the significance of hypoxia within the avascular granulosal compartment of the follicle continues to be debated [40]. Recent studies have demonstrated a role for HIF-1α within the ovary during follicle development, ovulation, and establishment of the corpus luteum [41–45]. KDM4A protein is induced independently of HIF stabilization [46] but can also regulate the expression of HIF-1α mRNA [47]. The potential interaction between hormone signaling, hypoxia, and KDM4 expression therefore provides rationale to investigate their contributions to fertility in human patients.

The current study examines the presence of *KDM4A* and *KDM4B* in cumulus and mural granulosa cells collected from women undergoing oocyte retrieval and seeks to determine if *KDM4A*/*B* expression is related to pregnancy outcome.

Materials and methods

Patients

Patients undergoing IVF by a single provider were recruited and informed consent was obtained. Ovarian follicle development was stimulated in patients using standard microdose flare or antagonist protocols and monitored by serial ultrasound and estradiol levels. Briefly, participants underwent controlled ovarian stimulation with recombinant FSH and human menopausal gonadotropins. When at least two follicles reached ≥ 18 mm in size, human chorionic gonadotropin was administered and 34–36 h later transvaginal ultrasound-guided oocyte retrieval was performed. This study was approved by the Institutional Review Board (#13637) at the University of Kansas Medical Center. All patients were consented for collection of granulosa cells prior to oocyte retrieval.

Granulosa cell preparation

Granulosa cells were collected and prepared as previously described with minor modification [48]. Mural granulosa from all follicles of an individual patient were pooled after removal of cumulus-oocyte complexes. Mural granulosa were enriched by sequential centrifugation over 75% percoll followed by 35% percoll and were washed in Hanks Balanced Salt Solution (HBSS) prior to RNA isolation. Cumulus-oocyte complexes were transferred to a clean dish where cumulus granulosa were cut from oocytes. Cumulus cells were pooled from a single patient and washed three times in HBSS prior to RNA isolation. Contamination by red blood cells was reduced by incubation of the cell fractions in ACK lysis buffer. This preparation method results in enriched cell populations. Any contamination with non-granulosa such as white blood cells or cross contamination of mural with cumulus or cumulus with mural cells was not specifically measured.

RNA isolation and PCR

Total RNA was isolated using Tri-reagent (ThermoFisher) according to manufacturer's instructions, and integrity was confirmed by analysis of 18S and 28S RNA. cDNA was synthesized using total RNA (3 μg) from each sample and SuperScript II reverse transcriptase (Invitrogen). The resulting cDNA was diluted 10-fold in sterile water, and aliquots were subjected to qRT-PCR to quantify mRNA levels. Primers were designed using Roche Primer design tool and tested using NCBI Primer-BLAST/Primer3 [39, 49]. Primer sequences were as follows: 18S rRNA forward gcccgaagcgtttactttga and reverse tccattattcctagctgcggtatc, *hKDM4A* forward gccgctagaagtttcagtgag and reverse gcgtcccttggacttcttatt, *hKDM4B* forward ggactgacggcaacctctac and reverse cgtcctcaaactccacctg, hPGR forward ctctcaggctggcatggt and reverse ccactggctgtgggagag, and hStAR forward ccacccctagcacgtggat and reverse tcctggtcactgtagagagtctcttc. Primers were assessed by melt-curve analysis and generated only a single peak. Real-time PCR was carried out using SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA) and specific primers (250 nM each). Amplification and fluorescence detection were carried out using the ABI Prism 7500 Real Time PCR System (Applied Biosystems) and the following cycling conditions: initial hold step (95 °C for 10 min), 40 cycles of two-step PCR (92 °C for 15 s, then 60 °C for 1 min), and a dissociation step (95 °C for 15 s, 60 °C for 15 s, and a sequential increase to 95 °C). The comparative cycle threshold method was used for relative quantification of the amount of mRNA for each sample normalized to 18S RNA (ΔCT). Data are presented as fold change ($2^{\Delta\Delta Ct}$) in expression relative to the pregnant-live birth cohort.

Immunohistochemical staining

Ovaries from eight individual women were obtained through the University of Kansas Cancer Center Biospecimen Repository. Ovaries examined in the present study were removed for reasons unrelated to ovarian pathology such as cyst on the contralateral ovary, fallopian tube rupture due to ectopic pregnancy, oophorectomy and hysterectomy following pregnancy, and prophylactic oophorectomy due to history of breast cancer. The ovaries were histopathologically assessed as having no diagnostic abnormalities. Ovaries were from women of reproductive age (age 29–35 years) and were otherwise obtained de-identified. Immunostaining was carried out using standard peroxidase/DAB methods and hematoxylin counterstain [39]. Rabbit monoclonal KDM4A (#5328, Cell Signaling) and KDM4B antibody (#8638, Cell Signaling) were each used at dilutions of 1:50 and 1:100, respectively. Rabbit monoclonal IgG (#3900S, Cell Signaling) at the same concentration was used as a control for staining. Immunostaining was carried out on a single representative section from each ovary. Representative images were captured using an Olympus BX40 upright microscope equipped with an Olympus DP72 camera and CellSens software version 1.4 (Olympus, with permission from Dr. Mary Zelinski, ONPRC).

Statistical analysis

Patient demographics and cycle characteristics in pregnant and not pregnant groups were analyzed using a Mann-Whitney test to determine if population medians differed. qRT-PCR was carried out on each sample and run in duplicate or triplicate for each primer pair. The mean ΔCT for each primer pair (specific gene–18S) was determined for all patient samples in cumulus and mural samples individually. The median fold change between pregnant-live birth and not pregnant groups ($2^{\Delta\Delta Ct}$) was determined and compared by Mann-Whitney test (GraphPad Prism7); $p < 0.05$ was considered different. Correlation analysis was determined using Spearman's rank correlation coefficient (GraphPad Prism7). Spearman's correlation (r_s) = 1.0–0.8 very strong, 0.79–0.6 strong, 0.59–0.4 moderate, 0.39–0.2 weak, 0.19–0 very weak correlation.

Results

Immunostaining for KDM4B in ovarian follicles was initially noted during the analysis of human ovarian surface epithelial cells in a related study [39]. This observation prompted a more thorough investigation. Cellular localization of KDM4A and KDM4B was examined by immunohistochemical staining of normal ovaries collected from reproductive-aged women.

Intense immunostaining for KDM4B was observed in theca, granulosa cells, corpora lutea, and oocytes. KDM4B staining in granulosa was apparent in follicles of all developmental stages from squamous granulosa of primordial follicles to both mural and cumulus granulosa of large antral follicles (Fig. 1). KDM4A immunostaining appeared diffuse across the ovary with specific localization to oocytes, granulosa cells, and luteal cells (Fig. 1). In all cases, immunostaining appeared to be confined to the nucleus.

Patient demographics and cycle characteristics of the patients included in the current analysis are described in Table 1. A total of 84 patients undergoing oocyte retrieval were categorized based on pregnancy outcome. Groups included patients undergoing embryo transfer that became pregnant and resulted in a live birth (pregnant-live birth) and patients that did not become pregnant following embryo transfer and who did not demonstrate a chemical pregnancy (not pregnant). Women in the pregnant-live birth group were younger, had higher anti-mullerian hormone (AMH) levels, were administered less FSH, achieved higher peak estradiol levels, had more oocytes at retrieval, and had greater numbers of two pronuclei (2PN) embryos compared to women in the not pregnant group (Table 1).

Expression of mRNA-coding histone demethylase *KDM4A* and *KDM4B* in cumulus and mural granulosa was examined for all patients. In general, expression of both histone demethylases was higher in cumulus granulosa cells compared to mural granulosa. Expression of *KDM4A* mRNA was approximately 5-fold higher and expression of *KDM4B* mRNA was approximately 2-fold higher in cumulus granulosa compared to mural granulosa cells.

Analysis of histone demethylase mRNA revealed differential expression in granulosa from women pregnant-live birth and women not pregnant. *KDM4A* mRNA expression level was higher in both cumulus granulosa and mural granulosa from women in the not pregnant group compared to the pregnant-live birth group. In cumulus cells, *KDM4A* mRNA was 12.82-fold higher in the not pregnant group compared to the pregnant-live birth group ($p = 0.0022$; Fig. 2). In mural granulosa cells, *KDM4A* mRNA levels were 5.34-fold higher in the not pregnant group compared to the pregnant-live birth group ($p = 0.0332$; Fig. 2).

Similar to *KDM4A*, *KDM4B* mRNA expression level was higher in both cumulus and mural granulosa from women in the not pregnant group compared to the pregnant-live birth group (Fig. 2). In cumulus cells, *KMD4B* mRNA levels were 5.07-fold higher and in mural granulosa 1.32-fold higher in the not pregnant group compared to the pregnant-live birth group ($p = 0.0012$ cumulus and $p = 0.04$ mural; Fig. 2).

Spearman's correlation was run to determine the general relationship between cumulus and mural granulosal expression of the KDM4 histone demethylases within each experimental group. Expression levels of *KDM4A* and *KDM4B* mRNA were very strongly correlated in cumulus cells and in mural cells from patients in the not pregnant group. In comparison, the correlation of *KDM4A* and *KDM4B* mRNA expression levels was weaker in cumulus and mural cells from patients in the pregnant-live birth group (Fig. 3).

As described, several differences were noted in the pregnant-live birth group and not pregnant group including age, AMH level, and peak estradiol levels (Table 1). Correlation of these parameters and *KDM4A* or *KDM4B* mRNA expression was analyzed. No correlation was observed between *KDM4A* or *KDM4B* mRNA expression within each cell type and pregnancy status and age, BMI, AMH, peak estradiol, FSH administered, number of oocytes retrieved, or number of 2PN embryos (Fig. 4 and Supplementary Figures 1–6).

Discussion

The present studies are the first to demonstrate expression of mRNA for the histone demethylases *KDM4A* and *KDM4B* in granulosa cells. Compared to patients who become pregnant following ART, expression of both histone demethylases was higher in the patients who did not become pregnant, indicating that these epigenetic regulators may play a role in directing proper granulosal function around the time of ovulation and luteal formation.

Ongoing studies are exploring demethylase activity and specific genes affected by *KDM4A* and *KDM4B* in cumulus and mural granulosa to correlate mRNA expression with biological function. However, the current findings provide some evidence to suggest differential expression of histone demethylases may impact epigenetic changes and gene expression in granulosa associated with pregnancy.

As an initial approach to examine the potential significance of histone demethylase expression in granulosa cell function, patients were categorized based on pregnancy outcome and expression of *KDM4A* and *KDM4B* mRNA in each group was compared. The general demographics of the patients segregating into the pregnant-live birth and not pregnant groups was similar to many previous reports. As anticipated, women that became pregnant following embryo transfer were younger and exhibited higher levels of AMH compared to women that did not become pregnant [50–52]. Age and AMH data extended to anticipated outcomes of less FSH administered, higher peak estradiol, more oocytes retrieved, and greater numbers of 2PN embryos in the pregnant-live birth group [53–55]. It is interesting to note there was no segregation of BMI in the two groups. Several studies have indicated increased BMI inversely correlates with pregnancy outcome [56, 57]. Mean BMI for patients in the pregnant-live birth group was 25.59 (range 19.15–44.8) and was not different from the not pregnant group, 26.85 (range 17.14–40.59); however, both groups were

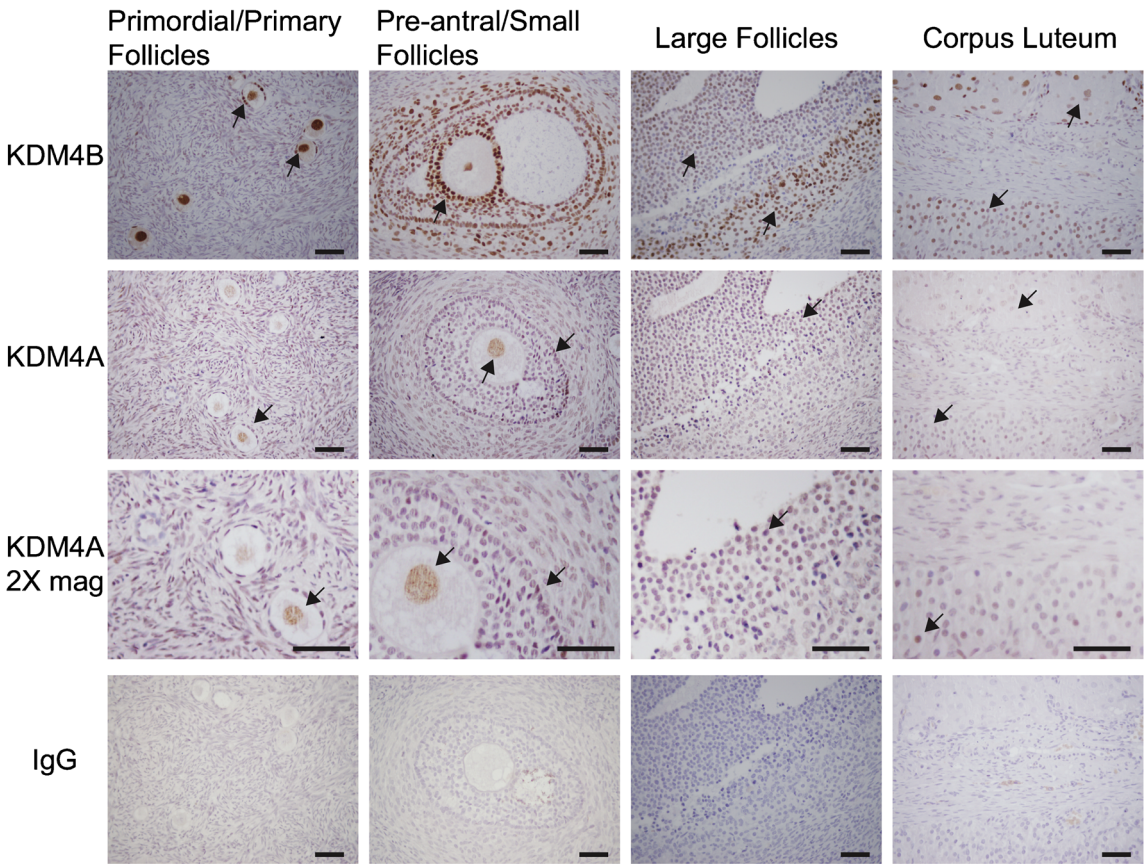

Fig. 1 Immunolocalization of KDM4A and KDM4B in human ovaries. KDM4B was immunolocalized to granulosa cells and theca in large and small follicles, corpora lutea, and granulosa and oocyte in primordial and primary follicles. In all cells, localization of KDM4B was confined to the nucleus. KDM4A immunostaining was localized to granulosa and luteal cells and was weakly and diffusely noted throughout the ovary. Due to the weaker level of staining, ×2 magnified images are presented for KDM4A. Control immunostaining using IgG-stained sections are presented. The figure includes results from four different ovaries and is representative of eight ovaries analyzed. Black arrowheads denote staining for KDM4B and KDM4A. Bar indicates 50 μm

within the overweight category as defined by the World Health Organization (WHO. Obesity and overweight. Fact sheet; Updated June 2016 http://www.who.int/mediacentre/factsheets/fs311/en/). The diagnosed cause of infertility for the current study group is indicated in Table 1. Due to the relatively small sample size of this study, the significance of *KDM4A* or *KDM4B* expression as an indicator of infertility within a specific etiology cannot be assessed. However, it will be of future interest to determine, for example, if ovulatory dysfunction is associated with altered expression of *KDM4A* and or *KDM4B*.

The ovulatory surge of LH initiates significant change in the expression patterns of many genes within the ovary leading to ovulation and establishment of the corpus luteum. The importance of many of these genes has been further demonstrated by knock-out and knock-down studies in rodents [18, 33, 58–65] and through the identification of mutations associated with infertility in women [66–72]. Recent studies have now begun to tie the expression of several of these critical genes to methylation state providing greater insight to the mechanisms controlling the final maturation of the follicle and ovulation. Increased expression of CYP11A1 in rat granulosa following an ovulatory dose of hCG was associated with increased H3K4me3 and decreased H3K9me3 and H3K23me3 [73]. Similarly, H3K9me3 marks in the StAR promoter were decreased after hCG [74]. These data indicate rapid changes in histone methylation following the ovulatory surge occurring with rapid changes in gene expression. Additional studies highlight altered patterns in methylation of critical genes found in pathological conditions associated with altered fertility. Cumulus granulosa from women with endometriosis have lower CYP19 gene expression compared to cumulus from women without endometriosis. Hypermethylation of H3K9 in the CYP19 promoter was associated with the lower expression [75]. CYP19 expression is also reduced in women with PCOS [76–78]. Studies have demonstrated differences of methylation state in PCOS ovarian tissue compared to controls where some genes were hypermethylated (such as IGFBP2, CYP19A1, AMHR2) and others hypomethylated (INSR, AMH) in PCOS ovaries [79]. Ovarian function and fertility can be impacted by exposure to chemicals in the environment and these effects have also been linked to altered DNA methylation of key genes. Exposure to

Table 1 Patient demographics

	Pregnant-live birth[a]	Not pregnant[b]
Number	31	53
Age, mean (range)	32.2 (27–46) *	34.6 (24–44)
BMI, mean ± sem	25.59 ± 0.96	26.85 ± 0.69
AMH, ng/ml mean ± sem	4.27 ± 0.64 **	2.83 ± 0.50
Estradiol, peak pg/ml mean ± sem	3107 ± 291 *	2285 ± 161
FSH, IU administered mean ± sem	3468 ± 351.6 *	4640 ± 255.7
Number oocytes retrieved, mean ± sem	17.83 ± 1.63 **	12.34 ± 0.79
Number 2PN embryos, mean ± sem	12.17 ± 1.11 **	6.53 ± 0.58
Infertility etiology		
Male factor	13	20
Combined male/female factor	3	11
Female factor	18	33
Endometriosis	2	8
PCOS	7	4
DOR	3	12
Tubal	5	22
Tubal ligation	2	1
Uterine	0	2
Other	2	10
Unexplained	3	5

PCOS polycystic ovarian syndrome, *DOR* diminished ovarian reserve, *BMI* body mass index, *AMH* anti-mullerian hormone, *2PN* two pronuclei

[a] Pregnant live birth includes patients undergoing embryo transfer and confirmed pregnancy and live birth. Includes 25 singleton and 6 twin births

[b] Not pregnant includes patients undergoing embryo transfer and not pregnant, no biochemical pregnancy, and no spontaneous abortion

*$p < 0.02$

**$p < 0.006$ pregnant live birth vs. not pregnant

methoxychlor (MXC), a synthetic insecticide, during critical phases of ovary development during the late prenatal and early postnatal period results in altered ovarian function and reduced fertility in adult mice [80, 81]. Recent studies indicate altered DNA methylation as a mechanism leading to MCX effects. Following MCX dosing up to postnatal day 7, hypermethylation of many genes involved in normal folliculogenesis was found. In addition, altered methylation was observed at postnatal day 60, long after the final dose of MXC [82]. Therefore, patterns of DNA and histone methylation in genes critical for follicular development, ovulation, and luteal formation change as part of normal biology. Methylation patterns different from that observed in normal ovarian tissues have been associated with conditions such as PCOS and altered patterns of methylation in the ovary occur following exposure to environmental reproductive toxins. The current data suggest that the histone demethylase KDM4A and KDM4B may play a role in mediating some of these events. Future studies focused on demonstrating KDM4A and KDM4B demethylase activity on specific target genes in ovarian cell types will substantiate this finding.

Although specific roles for KDM4A and KDM4B in granulosa cell function are yet to be determined, an importance may be inferred from the expression data observed in this study. The correlation in expression level of the histone demethylase *KDM4A* and *KDM4B* mRNA was very strong in both cumulus and mural granulosa cells in the not pregnant group. It is interesting to note the correlation in expression was weaker in cells from patients in the pregnant-live birth group. This observation indicates a differential expression of these histone demethylases occurring in cells associated with successful pregnancy compared to cells associated with no pregnancy. Additional analysis was carried out to determine if other characteristics differentiating the pregnant and not pregnant group correlated to KDM4 gene expression. For example, age was significantly lower in the pregnant-live birth group, and therefore, it is possible that KMD4 mRNA expression may correlate with age. However, no strong correlation was observed in KDM4 gene expression and age (Fig. 4) or other parameters assessed including BMI, AMH, peak estradiol, FSH administered, number of oocyte retrieved, or

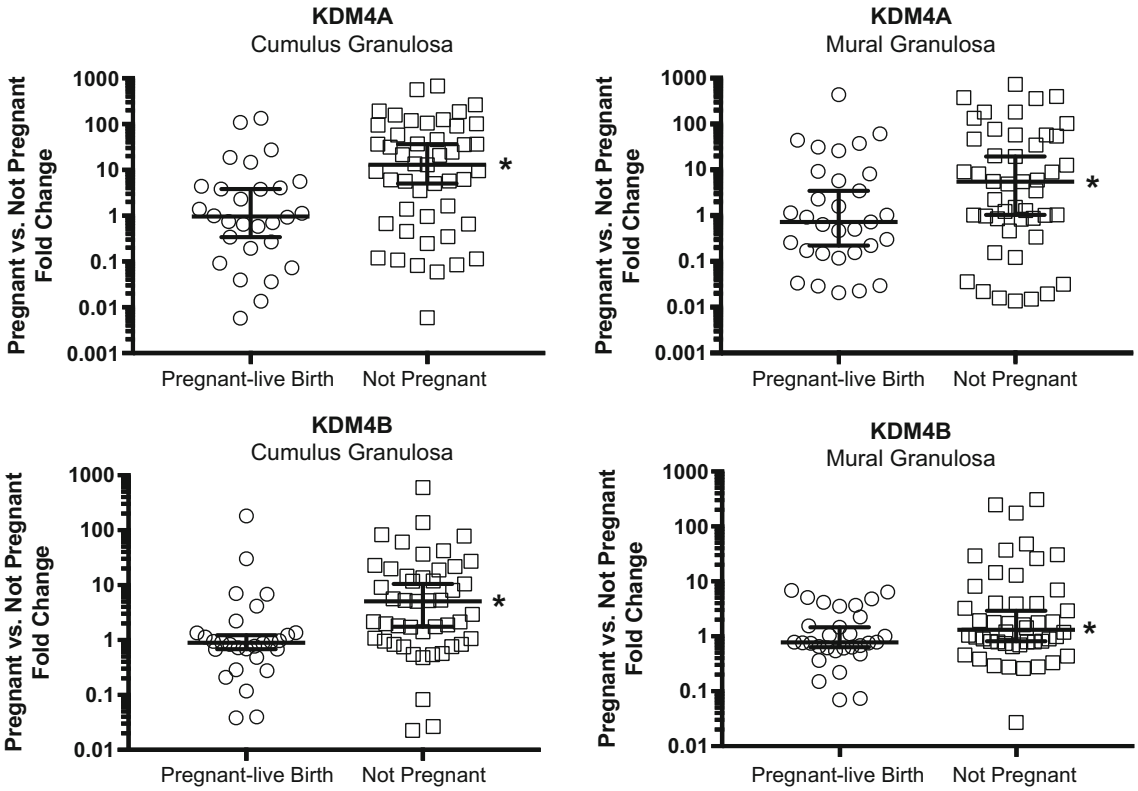

Fig. 2 Expression of KDM4A and KDM4B mRNA in cumulus and mural granulosa. Granulosa cells were collected at the time of oocyte retrieval; RNA was isolated and expression of KDM4A and KDM4B was assessed by qRT-PCR. Normalized data (ΔCT) is expressed as fold change from the mean of the pregnant cohort. The median line ± 95% confidence intervals are shown. Pregnant-live birth ($n = 31$) versus not pregnant ($n = 53$) were compared by Mann-Whitney. *$p < 0.04$ pregnant-live birth versus not pregnant

number of 2PN embryos (Supplementary Figures 1–6). Together, these data suggest changes in the expression of *KDM4A* and *KDM4B* are not related to factors such as age but may be related to specific cellular functions associated with successful pregnancy.

Currently, it is unclear if the elevated expression in non-pregnant patients represents aberrant induction of *KDM4A* and *KDM4B* relative to the pregnant cohort or if there is a failure to repress *KDM4A* and *KDM4B* expression at the time of ovulation. It may be that elevated expression of KDM4 in granulosa of the not pregnant group is representative of premature luteinization; KDM4A and KDM4B immunoreactivity was observed in luteal cells (Fig. 3). However, expression of *StAR* or *PGR* mRNA, two marker genes of luteinization, did

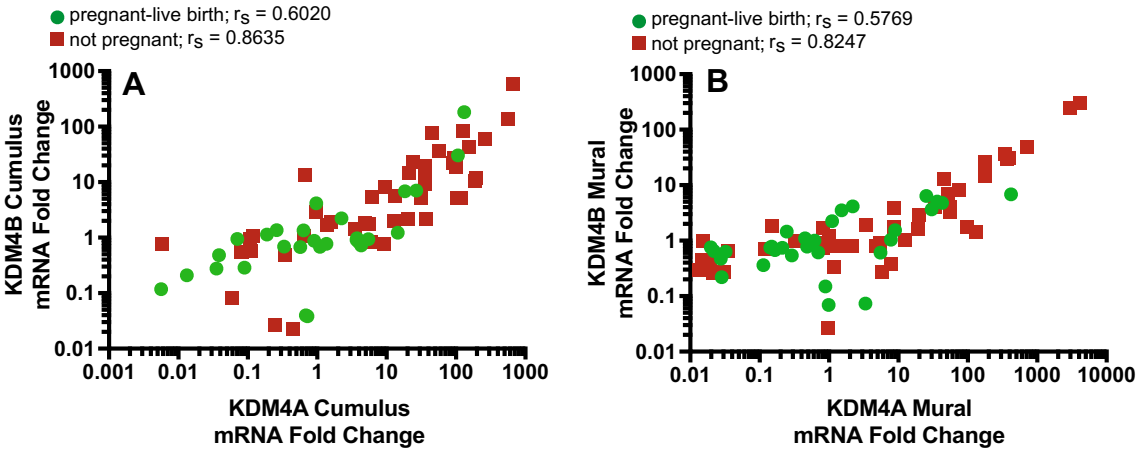

Fig. 3 Correlation of KDM4A and KDM4B mRNA expression in cumulus and mural granulosa cells. The correlation of the fold change values for expression of KDM4A and KDM4B mRNA in the cumulus granulosa (**a**) and mural granulosa (**b**) was determined for patients in the not pregnant (red) and pregnant-live birth (green) groups. Spearman's r_s values are indicated for each correlation. Spearman's correlation (r_s) = 1.0–0.8 very strong, 0.79–0.6 strong, 0.59–0.4 moderate

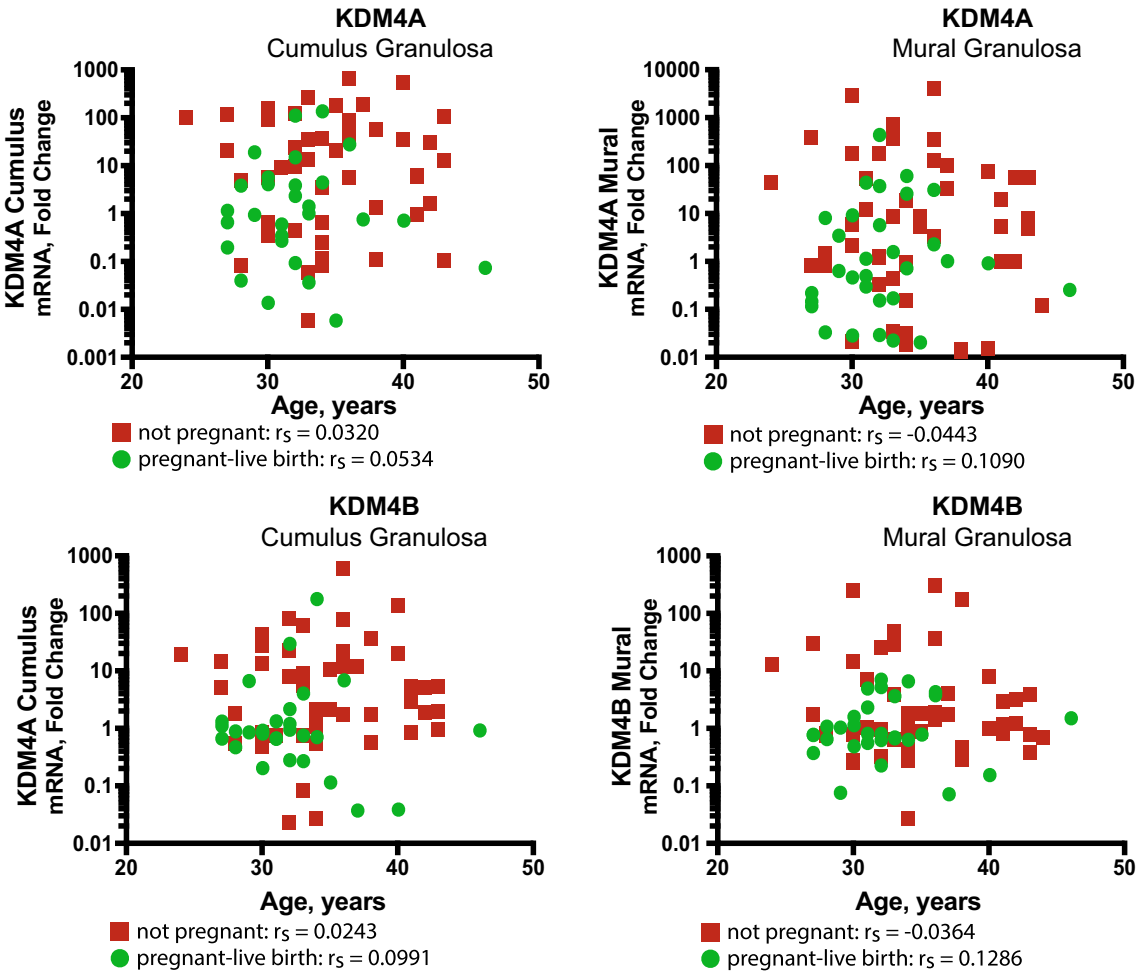

Fig. 4 Correlation of KDM4 mRNA expression in cumulus and mural granulosa cells with patient age. The fold change values for expression of KDM4A and KDM4B mRNA in the cumulus and mural granulosa in pregnant-live birth (green) and not pregnant (red) groups did not correlate with patient age. Spearman's correlation values are indicated (r_s) = 1.0–0.8 very strong, 0.79–0.6 strong, 0.59–0.4 moderate, 0.39–0.2 weak, 0.19–0 very weak correlation

not correlate with KDM4 expression in the current data set (see Supplementary Figures 7 and 8). Further, it is possible a subgroup may exist within either the non-pregnant or pregnant patient groups providing weight to the data due to unidentified pathological conditions. In addition, expression determined here may be related to infertility in general and may not reflect the expression patterns in cells from fertile women. Continued analysis of larger patient cohorts will provide insight toward these possibilities.

Immunostaining confirmed the cellular localization of KDM4A and KDM4B in specific cells within the normal human ovary. This observation is in general agreement with two other reports identifying KDM4 expression in the ovary. First, a meta-analysis of next-generation sequencing conducted on normal human tissues using RNA-Seq Atlas and GENT databases indicated KDM4A, KDM4B, and KDM4C are broadly expressed across several tissue types with high expression in the human ovary [11]. That study further described that based on reads per kilobase per million (RPKM), the expression level of KDM4A and KDM4C was higher than KDM4B in the ovary [11]. In another recent study, generation of KDM4A null mice lead to the identification of a role for KDM4A in preimplantation embryo development and in the maintenance of a uterine environment advantageous to implantation and embryo development [37]. That study further explored general expression levels in the mouse ovary where b-galactosidase staining from KMD4A, B, or C-promotor driven LacZ was illustrated from the authors data (KDM4A) and data from the International Mouse Phenotyping Consortium (KDM4B and KDM4C). In that case, both KDM4A and B expression were noted in the female reproductive tract, with greater overall KDM4A expression described and little to no expression of KDM4C observed [37]. Additionally, KDM4A was detected in the granulosa and oocytes of early stage follicles [37]. In the present study, KDM4A immunostaining was overall weak and appeared diffuse throughout the ovary. However, specific localization was observed in the oocytes, granulosa of growing follicles, and luteal cells. Expression of KDM4B was more

robust and was specifically observed in oocytes, granulosa, and theca cells. Differences in the intensity of immunostaining between KDM4A and KDM4B may be a reflection of different levels of protein expression in the human ovary or may reflect variation in the affinity of the antibodies used. Thus, the present data, although not quantitative, demonstrate KDM4A and KDM4B proteins are localized to specific cells within the human follicle and corpus luteum. Considering the newly described role of KDM4A as a maternal effect gene that contributes to the oocyte-embryo transition in the mouse [37], it is also of significant interest that the supporting granulosa cells in humans express both KDM4A and KDM4B.

The present study demonstrates the histone demethylase KMD4A and KDM4B are localized within the ovary to oocytes, granulosa cells, theca, and luteal cells. Differential expression of *KDM4A/B* mRNA was found with pregnancy in cumulus and mural granulosa cells. These data indicate KDM4A and KDM4B may have a role in control of gene expression important for function and differentiation of granulosa cells supportive of successful pregnancy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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