Arginine methyltransferases mediate an epigenetic ovarian response to endometriosis

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Abstract

Endometriosis is associated with infertility and debilitating chronic pain. Abnormal epigenetic modifications in the human endometrium have recently been implicated in the pathogenesis of this condition. However, whether an altered epigenetic landscape contributes to pathological changes in the ovary is unknown. Using an established baboon endometriosis model, early-, and late-stage epigenetic changes in the ovary were investigated. Transcript profiling of key chromatin-modifying enzymes using pathway-focused PCR arrays on ovarian tissue from healthy control animals and at 3 and 15 months of endometriosis revealed dramatic changes in gene expression in a disease duration-dependent manner. Ingenuity Pathway Analysis indicated that transcripts for chromatin-remodeling enzymes associated with reproductive system disease and cancer development were abnormally regulated, most prominently the arginine methyltransferases CARM1, PRMT2, and PRMT8. Downregulation of CARM1 protein expression was also detected in the ovary, fully-grown oocytes and eutopic endometrium following 15 months of endometriosis. Sodium bisulfite sequencing revealed DNA hypermethylation within the PRMT8 promoter, suggesting that deregulated CpG methylation may play a role in transcriptional repression of this gene. These results demonstrate that endometriosis is associated with changes of epigenetic profiles in the primate ovary and suggest that arginine methyltransferases play a prominent role in mediating the ovarian response to endometriosis. Owing to the critical role of CARM1 in nuclear receptor-mediated transcription and maintenance of pluripotency in the cleavage stage embryo, our results suggest that epigenetic alterations in the ovary may have functional consequences for oocyte quality and the etiology of infertility associated with endometriosis.

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Introduction

Endometriosis is a chronic inflammatory disease that affects 5–10% of women of reproductive age in the USA (Goldstein *et al.* 1980, Eskenazi & Warner 1997). It is defined by the presence of ectopic, estrogen-sensitive, endometrium-like tissue lesions primarily in the pelvic peritoneum and ovaries. These lesions cause inflammatory responses associated with angiogenesis, scarring, neuronal infiltration, adhesions, and fibrosis (Berkley *et al.* 2005, Tokushige *et al.* 2006). Endometriosis is diagnosed as the underlying condition in 50–60% of women and teenage girls with pelvic pain (Eskenazi & Warner 1997, Alvarez *et al.* 2012) and dramatically impairs the quality of life. Retrograde menstruation has historically been considered a primary risk factor for the

development of the disease (Sampson 1927, Rock *et al.* 1982, Bulun 2009). However, reports also suggest that other etiological factors, such as *in utero* exposure to diethylstilbestrol, environmental exposure to endocrine-disrupting agents, low birth weight and dietary choices may play significant roles in the development of endometriosis (Missmer *et al.* 2004, Bulun 2009, Diamanti-Kandarakis *et al.* 2009, Missmer *et al.* 2010). Accumulating evidence indicates that endometriosis is associated with aberrant transcriptional profiles in the eutopic endometrium of women and baboons resulting in dysregulation of critical signaling pathways (Kao *et al.* 2003, Burney *et al.* 2007, Afshar *et al.* 2013). Importantly, in some cases, abnormal gene expression patterns and altered epigenetic modifications, such as hypomethylation of CpG islands within promoter regions

of steroid hormone receptors, have also been identified in endometriotic lesions (Wu *et al.* 2005, Kim *et al.* 2007, Xue *et al.* 2007a,b).

Endometriosis causes infertility in up to 50% of patients and affects 20–40% of women seeking infertility evaluation (Berube *et al.* 1998, Giudice 2010). The disease is associated with decreased pregnancy rates following IVF and recurrent pregnancy loss (Vercammen & D'Hooghe 2000, Barnhart *et al.* 2002, De Hondt *et al.* 2005). However, the specific mechanisms involved in sub-fertility associated with this condition are not known. Importantly, the effects of short- and long-term endometriosis on ovarian physiology remain to be determined. Here, we present new evidence indicating that endometriosis is linked with altered patterns of ovarian gene expression and demonstrate that a prominent epigenetic pathway associated with oocyte quality and developmental potential is severely disrupted in primate females with induced endometriosis.

Both structural and genomic similarities between baboon and human chromatin domains highlight the significance of the non-human primate model for the analysis of epigenetic modifications in this disease (Foret *et al.* 2014). Using an established baboon endometriosis induction model, we demonstrate changes in the ovarian epigenetic landscape in response to early and late stages of this condition. Comparative transcript profiling in ovaries from healthy control animals and following endometriosis induction revealed dramatic changes in the expression levels of key chromatin-modifying enzymes, suggesting a specific epigenetic response to endometriosis in the ovary. Importantly, our results indicate that reduced expression of arginine methyltransferases in oocytes and granulosa cells may be a major contributing factor to the etiology of endometriosis-associated infertility.

Materials and methods

Animal husbandry and tissue collection

Adult baboons were obtained from the Southwest National Primate Center (San Antonio, TX, USA) and housed in individual cages in the Biological Research Laboratories of the University of Illinois. The animals consisted of normally cycling females ranging in age from 5 to 15 years and weighing between 12 and 20 kg. Endometriosis was experimentally induced in females of reproductive age by i.p. inoculation with menstrual endometrium on day 1 or 2 of two consecutive menstrual cycles as described previously (Fazleabas *et al.* 2002, 2003). Disease progression was monitored in each animal by consecutive laparoscopies at 3, 6–7, 9–10, 12, and 15–16 months after inoculation. Laparoscopy and laparotomies were performed between days 9 and 11 post-ovulation, the mid-luteal stage of the baboon menstrual cycle, which coincides with the window of uterine receptivity (Fazleabas *et al.* 1999). The stage of the cycle was determined by measuring estradiol levels in daily blood draws to determine

the day of ovulation as previously reported (Fazleabas *et al.* 1999). At each laparoscopy, the number of lesions was counted and changes within the pelvic cavity were documented by video recording (Fazleabas *et al.* 2003, Hastings *et al.* 2006). Three months following disease induction, significantly more red lesions were observed, while at 6 months following disease induction, significantly more blue lesions were present. During subsequent disease progression, similar levels of red, blue, chocolate, white, and mixed lesions were seen, although a trend towards more red lesions indicated that the disease was still active (Hastings *et al.* 2006). Following each laparoscopy, a laparotomy was performed and endometrial tissue (ranging from 0.5 to 1 g) was obtained using an endometriectomy procedure (Fazleabas *et al.* 1999). Ovaries were collected post-mortem from healthy control animals and females with endometriosis that did not present ovarian endometrioma. All experimental procedures were approved by the Animal Care Committee of the University of Illinois, Chicago, IL, USA.

Preovulatory oocytes were collected from primate ovaries by follicle puncture post-mortem and cultured in minimal essential medium (MEM) supplemented with 3 mg/ml BSA (Sigma–Aldrich) and 10 μ M of the phosphodiesterase inhibitor milrinone (Sigma–Aldrich) to prevent germinal vesicle breakdown. Surrounding granulosa cells were gently removed by continuous pipetting. *In vitro*-matured oocytes were obtained following culture in fresh MEM/BSA medium supplemented with 5% fetal bovine serum (Hyclone, Logan, UT, USA) for 16 h under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂ at 37 °C before transfer to a 2% paraformaldehyde (PFA), 0.1% Triton X-100 fixative solution for 20 min at room temperature and blocking in 1 mg/ml BSA, 0.01% Triton X-100 in PBS overnight at 4 °C as described previously (De La Fuente *et al.* 2004).

Immunohistochemistry

Endogenous protein was detected by immunohistochemistry on paraffin-embedded ovarian and uterine tissue sections obtained from animals following 15 months of endometriosis as well as from disease-free controls. Briefly, tissues were fixed in 4% PFA for 24 h before dehydration and paraffin embedding. Tissue sections (5 μ M) were placed on glass slides, deparaffinized and rehydrated through xylene (twice for 10 min) and serial ethanol washes (2 min each in 100, 95, 70, 50, and 30% ethanol). Epitopes were retrieved by incubation in boiling 10 mM sodium citrate supplemented with 0.05% Tween 20 (Sigma–Aldrich) for 30 min. Auto-fluorescence and unspecific antibody binding were inhibited by quenching in 0.1 M glycine (Sigma–Aldrich) in double distilled water for 30 min and blocking in PBS supplemented with 5% adult bovine serum (Hyclone) and 0.1% Triton X-100 (Sigma–Aldrich) for 50 min at room temperature. Primary antibodies were diluted in blocking buffer and slides were incubated overnight at 4 °C before washing in PBS, 0.05% Tween 20, and secondary antibody exposure. Optimal primary and secondary antibody dilutions were determined empirically for each marker and applied as follows: a mouse monoclonal anti-CARM1 [3H2] antibody (Abcam Cambridge, MA, USA) at a dilution of 1:200, a polyclonal rabbit anti-histone H3 trimethylated at lysine 9 (H3K9me3, Abcam) at a dilution of 1:200, a rabbit polyclonal anti-DNA methyltransferase 1 (DNMT1)

antibody (1:500, pATH52, a kind gift of Dr T Bestor), a goat polyclonal anti-poly(ADP-ribose) polymerase 1 (PARP1) antibody at a dilution of 1:400 (R&D Systems, Minneapolis, MN, USA) and a mouse anti- β -tubulin antibody (1:500, Sigma-Aldrich). Secondary antibodies, Alexa-Fluor 488 or 555 conjugated goat anti-mouse IgG and Alexa-Fluor 488 or 555 goat anti-rabbit IgG, were purchased from Molecular Probes, Inc. (Eugene, OR, USA) and used at a 1:1000 dilution for 1 h at room temperature.

Immunocytochemistry of whole-mount baboon oocytes was performed on PFA-fixed ova by incubation in primary antibody (H3K9me3, 1:500; DNMT1, 1:500; and PARP1, 1:400) in 1 mg/ml BSA in PBS, 0.01% Triton X-100 (blocking buffer) at 4 °C overnight, followed by three 20-min washes in blocking buffer and immunological detection with the appropriate secondary antibodies at a dilution of 1:1000 for 1 h at room temperature. Slides were mounted in anti-fading medium supplemented with DAPI (Vectashield; Vector Laboratories, Burlingame, CA, USA), to counterstain chromatin and were examined on a Leica DMRE fluorescence microscope equipped with epifluorescence (Leica Microsystems, Inc.). Micrographs were captured with a Leica DFC 350F camera using Openlab 3.1.7 Image Analysis Software.

RNA extraction and pathway-focused transcript profiling

Gene expression analyses were conducted using pathway-focused human PCR Arrays for Epigenetic Chromatin-Modifying Enzymes (SABiosciences, Valencia, CA, USA; Qiagen) allowing the simultaneous quantification of a panel of 84 mRNA transcripts encoding for key chromatin-modifying enzymes known to establish genomic DNA methylation (including *de novo* and maintenance DNMTs) as well as enzymes that regulate essential histone post-translational modifications, including histone acetylation, methylation, phosphorylation, and ubiquitination. mRNA was isolated from flash-frozen ovarian tissues obtained at the mid-secretory phase of the estrous cycle from control animals ($n=2$) and female baboons following 3 months ($n=2$) and 15 months ($n=2$) of endometriosis using the Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen). cDNA synthesis by reverse transcription was conducted using the RT² PreAMP cDNA Synthesis Kit using primer pairs specific to this pathway. Quantitative real-time PCR was performed on a Roche Light Cycler 480 System using an instrument-specific RT² qPCR Master Mix (SABiosciences, Qiagen) according to manufacturer's instructions. RT efficiency, qPCR performance, and cDNA quality were confirmed in array-integrate control reactions. Expression data were normalized to a series of internal housekeeping genes (Hprt1, Rpl13A, Gapdh, and β -actin). Data points were excluded from the analysis when expression levels of genes were below detectable limits. In addition, unspecific amplification as indicated by multiple peaks in melting curves due to lack of sequence homology between individual human and primate genes were observed for Aurora kinase B, CIITA, Dnmt3B, HDAC1, HDAC2, KDM4C, KAT2B, PRMT7, SETD6, and the housekeeping control B2M and were thus excluded from the analysis. Raw threshold cycle data between control ovaries and ovaries obtained from females with

induced endometriosis were compared using RT² Profiler PCR Array Data Analysis Software (SABiosciences, Qiagen) version 3.5 to conduct all $\Delta\Delta C_t$ -based fold-change calculations.

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) was performed as described earlier (Zelenko *et al.* 2012). Briefly, gene symbols and fold change values of up- and downregulated genes were imported into IPA Software and the top resulting significantly regulated molecular and biological networks were identified followed by functional analysis of the data set and canonical pathway analysis.

Sodium bisulfite genomic sequencing

Sodium bisulfite genomic sequencing was conducted as described previously (Wang *et al.* 2009). Briefly, genomic DNA was isolated from ovarian samples of control animals ($n=6$) and animals following 15 months of endometriosis ($n=5$) using the DNeasy Blood and Tissue Kit (Qiagen) before bisulfite conversion (DNA methylation Gold Kit, Zymo Research, Irvine, CA, USA) and nested PCR amplification (two rounds of 35 cycles each) using the following primer pairs: 'outerF' 5'-TTATTYGGTT-TAGTTTTAGGAGAGA-3'; 'outerR' 5'-TCCAACCCAACCCC-TCAACTAAAC-3'; and 'innerF' 5'-GGTAGAAGTTGAGAGGA GTTG-3'; 'innerR' 5'-ATTTTCCTCCCTTCTCATCTACTC-3'. Amplicons of two independent nested PCRs were mixed and subcloned via TOPO-TA Cloning Technology (Invitrogen). Twelve clones per sample were analyzed by genomic sequencing of the PRMT8 promoter region located -38 to +186 bp relative to the transcription start site. The average level of DNA methylation of each individual animal was considered a biological repeat and used for statistical analysis. Data were analyzed using an online Quantification Tool for Methylation Analysis (<http://quma.cdb.riken.jp/>) with open and filled circles describing unmethylated and methylated CpG sites respectively.

Statistical analysis

All experiments were conducted in three biological replicates, with the exception of PCR arrays, which were performed in duplicate biological replicates. Representative examples are shown. Gene expression levels in ovarian tissues were compared using SABiosciences' Software module to perform all $\Delta\Delta C_t$ -based fold-change calculations from raw threshold cycle data. ANOVA and pair-wise comparison (*t*-test) between groups of experimental replicates was conducted to define the fold up or downregulation and statistically significant thresholds ($P<0.05$). *t*-Test analysis was also used to determine statistically significant differences in DNA methylation patterns by bisulfite genomic sequencing. Differences were considered significant when $P<0.05$.

Results

Transcriptionally repressive marks at centromeric heterochromatin are highly conserved in the primate oocyte genome

Aberrant epigenetic regulation of chromatin structure and function is increasingly recognized as a major contributor to the development of male and female

infertility. However, little is known regarding epigenetic pathways and regulators that are potentially involved in the etiology of infertility during endometriosis. Interestingly, histone post-translational modifications show a remarkable degree of conservation among mammals (Cowell *et al.* 2002, Woo & Li 2012). Yet, the patterns of expression and chromosomal localization of H3K9me3, a histone modification essential for heterochromatin formation and genome stability, in primate oocytes are not known. Therefore, we initially determined whether the centromeric localization of this prominent histone mark is conserved in the baboon ovary. In primordial, primary as well as early pre-antral follicles, surrounding ovarian granulosa cells exhibit prominent nuclear staining while the emerging theca cell layer exhibits only basal fluorescence (Fig. 1A and B, arrows). Notably, H3K9me3 is highly enriched in the ovarian surface epithelium (Fig. 1A, arrowhead). Analysis of H3K9me3 localization at different stages of oocyte growth and differentiation revealed the presence of prominent nuclear foci associated with bright DAPI staining in the majority of oocytes from pre-antral follicles. Notably, several H3K9me3 foci were found in close apposition with the nucleolus (Fig. 2A, arrow). To determine whether H3K9me3 is associated with heterochromatin domains, we compared the distribution of this chromatin mark in pre-ovulatory oocytes from adult females (Fig. 2B). Consistent with our previous observations, H3K9me3 exhibits a focal localization to heterochromatin domains in oocytes at the non-surrounded GV (NSN-GV) stage, exhibiting decondensed chromatin (Fig. 2B, top panel). Notably, the perinucleolar heterochromatin rim of the condensing nucleolus in surrounded nucleolus GV stage oocytes (SN-GV) presents bright H3K9me3 associated with the nascent karyosphere (Fig. 2B, middle panel). In addition, this histone modification is prominent in the centromeres of condensing chromosomes following germinal

vesicle breakdown (Fig. 2B, bottom panel). Together, our results indicate that H3K9me3 association with condensed heterochromatin is conserved between mouse and primate ovarian somatic cells and oocytes. These results also demonstrate that H3K9me3 is a prominent epigenetic mark in granulosa cells of primary and early pre-antral follicles in the baboon ovary at both the follicular and luteal stage of the estrous cycle.

Endometriosis is associated with global changes in gene expression of chromatin-modifying enzymes in the ovary

Altered gene expression profiles in the eutopic endometrium and endometrial lesions in patients with endometriosis as well as in animal models of this disease (Gui *et al.* 1999, Hapangama *et al.* 2010, Colon-Diaz *et al.* 2012, Khan *et al.* 2012, Afshar *et al.* 2013) have been linked to altered epigenetic chromatin modifications such as DNA methylation at critical promoter regions (Nasu *et al.* 2011) and changes in the patterns of specific histone modifications (Kawano *et al.* 2011, Monteiro *et al.* 2014). However, whether endometriosis impacts the epigenetic landscape in the ovary, resulting in dysregulation of gene expression, is unclear. In addition, little is known regarding the underlying molecular mechanisms eliciting altered epigenetic modifications in endometriosis. To determine the mechanisms involved in the establishment of abnormal epigenetic profiles we compared the patterns of gene expression of key chromatin remodeling enzymes in ovaries of healthy control baboons and animals with endometriosis using human pathway-focused PCR arrays. Transcript profiling of mRNA extracted from baboon ovaries during the luteal phase of the estrous cycle from controls and following 3 and 15 months following endometriosis induction, allowed the simultaneous analysis of 75 epigenetic chromatin-modifying enzymes. Pair-wise comparison of

Figure 1 Prominent expression patterns of histone H3 trimethylated at lysine 9 (H3K9me3) in primate ovarian follicles. (A) H3K9 trimethylation is strongly detectable in granulosa cells of primary follicles (arrow) and the ovarian surface epithelium (arrowhead). (B) H3K9me3 immunofluorescence intensity declines during follicular growth in pre-antral (arrow) and antral follicles (not shown). Scale bar = 10 μ m.

Figure 2 Conserved epigenetic heterochromatin marks in primate ovaries. (A) Immunolocalization of histone H3 lysine 9 trimethylation (H3K9me3, green) in baboon ovaries. Similar to the mouse, H3K9 trimethylation is distinctly detectable at heterochromatin domains within the oocyte nucleus (arrow). In addition, H3K9me3 is a prominent marker of ovarian granulosa cells in this species. β -tubulin immunodetection (red) and the DNA dye DAPI (blue) were used as counterstains. Scale bar=20 μ m. (B) High-resolution micrographs of (top panel) NSN- and (middle panel) SN-GV stage oocyte nucleus of the baboon. Heterochromatin domains are immunolabeled with H3K9me3 antibodies (green, arrows). The position of the nucleolus is indicated by an asterisk (middle panel). (Lower panel) Centromeric heterochromatin of condensing chromosomes in baboon oocytes is detectable by H3K9me3 immunolabeling (arrows). Scale bar=10 μ m.

normalized, $\Delta\Delta$ Ct-based fold-change calculations between groups of experimental replicates was used to define the individual fold up- or downregulation in gene expression (Fig. 3A). No significant changes in gene expression between experimental groups were detected within the panel of housekeeping genes included in the arrays (*ACTINB*, *GUSB*, *GAPDH*, and *HSP90AB1*). However, this analysis consistently revealed a trend towards a global decrease in the expression of chromatin-modifying enzymes in ovaries after 3 months of endometriosis compared to controls. Importantly, transcript downregulation was further exacerbated after 15 months of disease (Fig. 3A and B). Changes in gene expression greater than or equal to twofold were considered biologically significant and revealed a panel of six chromatin-modifying enzymes with significant ($P<0.05$) downregulation after 3 months of endometriosis (ranging -2.1 - to -4.4 -fold; Table 1; highlighted in bold), while decreased transcript levels were detectable in 20 enzymes in samples obtained following

15 months of the disease (ranging -2.5 - to -17.8 -fold, Fig. 3A and Table 1 (highlighted in bold). Differences and similarities in transcript profiles among experimental groups are represented in the form of a Venn diagram (Fig. 3B). While transcript levels were unchanged for 69 of 75 genes between the control and the 3 months endometriosis group, altered transcript levels were detectable in 53 of 75 genes in the 15 months endometriosis group compared to the controls (Table 1 and Supplemental Table 1, see section on supplementary data given at the end of this article). At both experimental time points, the most prominent changes associated with endometriosis were evident in transcripts encoding protein arginine methyltransferases (Table 1). Specifically, 3 months following endometriosis induction, a significantly lower transcription level of PRMT8 (-4.4 -fold) was noted in the baboon ovary, which was further reduced to -17.8 -fold after 15 months of the disease. A striking decline in gene expression was also detectable for PRMT2 (-5.8 -fold) and the coactivator-associated

Figure 3 Endometriosis alters the gene expression levels of epigenetic chromatin remodeling enzymes in the baboon ovary. (A) Quantitative transcript profiling using pathway-focused PCR arrays revealed striking global changes in gene expression levels in response to 3 months of endometriosis in the majority of the 75 candidate genes tested (blue bars) as compared to disease-free control animals (normalization control). Differences in gene expression were found to be further exacerbated following a 15-month disease period (red bars). (B) Venn diagram indicating differences and similarities in transcript levels between experimental groups and controls of a total of 75 genes analyzed.

arginine methyltransferase CARM1 (−4.2-fold) after 15 months of disease. Collectively, these results provide evidence that the presence of endometriotic lesions correlates with alterations in gene expression in the ovary and suggest a critical role of arginine methyltransferases in mediating this epigenetic ovarian response. IPA revealed gene expression, cell cycle, DNA replication, recombination, and DNA repair as the most significantly altered networks with an IPA score of 40. The top significantly regulated bio functions and associated diseases/disorders for the 3 months endometriosis group were cancer, reproductive system disease, hematological disease, endocrine system disorders, and gastrointestinal disease, while samples obtained 15 months following endometriosis induction revealed gene expression changes commonly involved in cancer, reproductive system disease, hematological disease, dermatological disease, and inflammatory disease (Table 2). These results highlight critical molecular changes in the ovary, which may contribute to the characteristic physiological changes associated with infertility, cancer predisposition and inflammatory disease in women with endometriosis.

Endometriosis alters the DNA methylation pattern within the promoter region of the arginine methyltransferase PRMT8 gene

DNA methylation at promoter sites plays an essential role in the regulation of gene expression. While

hypomethylation is generally associated with a relaxed chromatin configuration, transcript overexpression and genome instability, DNA hypermethylation is known to result in transcriptional silencing through acquisition of epigenetic repressors and inhibition of transcription factor binding (Suzuki & Bird 2008). To gain mechanistic insight into the differential expression patterns of arginine methyltransferases in response to endometriosis, we conducted sodium bisulfite sequencing of genomic DNA extracted from ovarian samples from controls ($n=6$) and baboons following 15 months of endometriosis induction ($n=5$). A specific promoter region containing 23 CpG dinucleotides (−38 to +186 relative to the transcription start point of PRMT8) was investigated and significant ($P<0.05$) hypermethylation was detectable in CpG dinucleotide 8 in samples collected from animals with endometriosis (Fig. 4) compared to controls. These data provide critical insight into the potential molecular basis of transcriptional repression of PRMT8 by an epigenetic mechanism involving PRMT8 promoter hypermethylation in ovaries from baboons with endometriosis.

Effects of endometriosis on histone and DNMT enzymes in the endometrium and ovary

The arginine methyltransferase CARM1 is implicated in the regulation of gene expression and is thought to play a critical role in mammalian development, cell proliferation, and differentiation (Chen *et al.* 1999, Wysocka *et al.* 2006, Torres-Padilla *et al.* 2007). CARM1 levels in the baboon endometrium (Fig. 5A, green) and ovary (Fig. 5B, green) were examined by immunohistochemistry using specific antibodies against CARM1. CARM1 protein labeling (green) varied prominently in samples collected from animals at different stages of the estrous cycle in both tissue types. Strong immunoreactivity was detectable in samples collected from animals at the late proliferative/follicular and mid-secretory/mid-luteal phase (top and middle panels), while CARM1 was notably reduced in samples of the late secretory/late luteal phase (lower panels, Fig. 5A and B). H3K9me3 immunostaining (red) served as experimental control and DNA was counterstained with DAPI. A distinctive staining pattern was observed in the endometrium where CARM1 was enriched in the cytoplasm of endometrial glands cells (Fig. 5A, arrows). In contrast, CARM1 showed ubiquitous staining and was detectable in the cytoplasm of fully-grown oocytes as well as in surrounding granulosa cells in the ovary (Fig. 5B, arrows). CARM1 also showed prominent staining of associated endothelial cells (Fig. 5B, arrowheads).

Next, we set out to test whether CARM1 transcript downregulation during endometriosis, as detected by PCR arrays, correlates with a reduction in CARM1 protein expression. Endometrial and ovarian sections from control animals and following 15 months of

Table 1 Significant fold up/downregulation in gene expression of chromatin-modifying enzymes following 3 and 15 months of endometriosis.

Synonyms	Gene	Control vs 3 months	P value (control vs 3 months)	Control vs 15 months	P value (control vs 15 months)
CREBP1, CREB2, HB16, MGC111558, TREB7	ATF2	−2.6	0.0365	−2.6	0.0495
PRMT4	CARM1	−0.3	0.5532	−4.2	0.0025
FLJ13327, FLJ57977, FLJ58022, FLJ58223, KIAA0675, UURF2, hRUL138	DZIP3	−1.9	0.0318	−3	0.0078
BAT8, C6orf30, DKFZp686H08213, FLJ35547, G9A, KMT1C, NG36, NG36, G9a	EHMT2	−0.1	0.6066	−3.5	0.0002
KAT1	HAT1	−2.1	0.0474	−1.7	0.5445
HA6116, HD4, HDACA, HDACA, KIAA0288	HDAC4	−1.7	0.0177	−2.8	0.0192
HDACL1, RPD3	HDAC8	−1.9	0.0716	−4.1	0.0172
2ADUB, 2ADUB, DKFZp779J1554, DKFZp779J1721, KIAA1915, RP4-592A1.1	MYSM1	−2.3	0.0417	−3.1	0.0066
KAT6A, MGC167033, MOZ, RUNXBP2, ZNF220	MYST3	−1.7	0.0006	−3	0.0328
ACTR, AIB-1, AIB1, CAGH16, CTG26, KAT13B, MGC141848, RAC3, SRC-3, SRC3, TNRC14, TNRC16, TRAM-1	NCOA3	−1.7	0.0173	−2.5	0.0148
SID6-1512	NEK6	−0.2	0.4369	−3.2	0.0158
ANM1, HCP1, HRMT1L2, IR1B4	PRMT1	0	0.6785	−3.7	0.0463
HRMT1L1, MGC111373	PRMT2	−1.8	0.0211	−5.8	0.0147
HRMT1L3, HRMT1L4	PRMT8	−4.4	0.0444	−17.8	0.0012
MGC1911, MSK1, MSPK1, RLPK	RPS6KA5	−2.2	0.0773	−2.8	0.0293
KIAA0339, KMT2F, Set1, Set1A	SETD1A	−0.3	0.4835	−3.2	0.0016
FLJ16420, FLJ22472, FLJ23184, FLJ45883, FLJ46217, HIF-1, HSPC069, HYPB, KIAA1732, KMT3A, SET2	SETD2	−0.7	0.431	−3	0.0106
C21orf18, C21orf27	SETD4	−1.9	0.009	−3.5	0.0064
KMT5A, PR-Set7, SET07, SET8	SETD8	−0.4	0.5172	−3.8	0.0001
C13orf4, CLLD8, CLLL8, DKFZp586I0123, DKFZp761J1217, KMT1F	SETDB2	−2.2	0.0113	−3.5	0.0018
FLJ21080, MGC104324, ZMYND1, ZNFN3A1, ba74P14.1	SMYD3	−2.1	0.0426	−0.6	0.7016
CGI-85, CGI85, KMT5B, MGC118906, MGC118909, MGC21161, MGC703	SUV420-H1	−1.9	0.0619	−3.2	0.0109
KIAA1063, USP3L	USP22	−2.5	0.1012	−3.6	0.0423
FLJ23286, KIAA1090, MGC176638, MMSET, NSD2, REIIBP, TRX5, WHS	WHSC1	−0.7	0.4557	−3.7	0.0027

endometriosis were immunolabeled using CARM1 antibodies (green, Fig. 6). A striking downregulation of CARM1 was evident in the endometrium (Fig. 6A) and ovary (Fig. 6B) 15 months following endometriosis induction, while control tissues showed expression levels of CARM1 consistent with the mid-secretory/luteal stage of the estrous cycle. These results suggest that expression of CARM1 may be regulated in an estrous cycle-dependent manner. Importantly, these findings substantiate the notion that endometriosis is associated with a significant downregulation of CARM1 transcripts and protein.

DNMTs are responsible for establishment and maintenance of CpG methylation at regulatory sequences and play important roles in both genome stability and transcriptional mechanisms (Suzuki & Bird 2008, Ha et al. 2011). Ectopic endometriotic lesions in women have been reported to show DNMT1, DNMT3A, and DNMT3B overexpression in comparison with the eutopic endometrium (Wu et al. 2006). In addition, genomic DNA methylation patterns may also be affected

by the loss of enzymatic activity of DNMT1 following non-covalent interactions with the DNA damage response protein PARP1 (Caiafa et al. 2009). PARP1 is a ubiquitously expressed nuclear protein (Doherty et al. 2002, Ratnam et al. 2002, Birt et al. 2013) involved in the maintenance of genome stability and cellular DNA damage response through proper poly(ADP-ribosylation) of histone molecules and other proteins (Ame et al. 2004, Vassena et al. 2005, Wu et al. 2006). Importantly, poly(ADP)-ribosylation can also be modulated in response to developmental or environmental stressors in order to regulate global chromatin remodeling processes (Kim & Fazleabas 2004, Nezhat et al. 2014). Whether DNMTs and PARPs play a role in the ovarian epigenetic response to endometriosis is unknown. Therefore, ovarian sections obtained from controls and animals 3 and 15 months after disease induction were immunolabeled using specific antibodies against DNMT1 (green) and PARP1 (red, Fig. 7A). DNMT1 was expressed at very low levels in the cytoplasm of both primordial and primary oocytes from control ovaries

Table 2 Significant bio functions.

Diseases and disorders	P value	No. of molecules
Three months endometriosis		
Cancer	2.07×10^{-4} – 4.00×10^{-2}	6
Reproductive system disease	3.27×10^{-4} – 3.30×10^{-2}	6
Hematological disease	3.73×10^{-4} – 2.15×10^{-2}	7
Endocrine system disorders	4.28×10^{-4} – 4.28×10^{-4}	6
Gastrointestinal disease	4.28×10^{-4} – 2.31×10^{-2}	8
15 months endometriosis		
Cancer	1.84×10^{-14} – 4.71×10^{-2}	22
Reproductive system disease	2.15×10^{-14} – 4.52×10^{-2}	16
Hematological disease	4.48×10^{-14} – 1.22×10^{-2}	18
Dermatological diseases	3.99×10^{-13} – 1.84×10^{-2}	10
Inflammatory disease	3.99×10^{-13} – 1.85×10^{-6}	9

with some primordial follicles also exhibiting nuclear and nucleolar DNMT1 staining (arrowheads). However, no detectable differences were observed in the oocytes from ovaries with endometriosis. In contrast, prominent PARP1 staining was observed in the oocyte nucleus (arrowheads), nucleolus and the cytoplasm during the early stages of follicular growth and differentiation in primordial and primary oocytes as well as in follicular granulosa cells in control sections. Interestingly, induction of endometriosis resulted in increased PARP1 intensity in primordial and primary follicles following 15 months of disease (Fig. 7A).

We also compared the patterns of PARP1 and DNMT1 protein expression in fully-grown oocytes aspirated from ovarian follicles from control animals and following 15 months of endometriosis (Fig. 7B). At this stage of oocyte growth, DNMT1 is no longer detectable in the nucleus of baboon oocytes, but localizes diffusely to the cytoplasm, which is consistent with expression patterns in pre-ovulatory mouse oocytes (Doherty *et al.* 2002, Ratnam *et al.* 2002). DNMT1 expression was noticeably increased in pre-ovulatory oocytes of animals following 15 months of endometriosis (lower panel). A similar increase in cytoplasmic PARP1 staining was observed in pre-ovulatory oocytes after 15 months of endometriosis. These data suggest an endometriosis-dependent upregulation of DNMT1 and PARP1 proteins in baboon oocytes and provide additional evidence for a complex epigenetic response to this condition in the female gonad.

Discussion

Endometriosis is a complex gynecological condition that develops spontaneously only in humans and non-human primates. The baboon model of induced endometriosis is a well-established animal model for the study of critical pathophysiological disease parameters in an environmentally controlled and replicable manner (De Hondt *et al.* 2005, Fazleabas 2006). Moreover,

(Woo & Li 2012) this model system has provided valuable insight into disease aspects that are both remarkably similar and directly applicable to the study of endometriosis in women. For example, experimental induction of endometriosis in baboons leads to the development of endometriotic lesions that are morphologically and histologically indistinguishable from those found in women (Fazleabas *et al.* 2002, Harirchian *et al.* 2012). Both species exhibit identical disease characteristics, including subfertility (D’Hooghe *et al.* 1996, Fazleabas *et al.* 2002); and molecular studies in women and baboons with endometriosis suggest a strong conservation of the molecular pathways at play (Gui *et al.* 1999, Kim & Fazleabas 2004, Burney *et al.* 2007, Afshar *et al.* 2013). However, little is known regarding changes in the ovarian epigenetic landscape in response to endometriosis, effects on oocyte quality, and any potential risks for transgenerational inheritance of epigenetic modifications induced by this condition in germ cells.

Figure 4 Alterations in DNA methylation patterns within the Prmt8 promoter region in response to endometriosis. (A) Analysis of Prmt8 promoter methylation patterns revealed a significant ($P < 0.05$) hypermethylation of CpG8 (asterisk) within the -38 to $+186$ bp region relative to the transcription start site following endometriosis (filled bars) compared to controls (open bars). Sodium bisulfite genomic sequencing spanning 23 CpG dinucleotides within the primate Prmt8 promoter was conducted on controls (B) and ovaries following 15 months of endometriosis (C). Open circles indicate lack of DNA methylation while filled circles denote presence of DNA methylation. Each row represents an individual clone and clones are grouped according to samples from individual animals.

Figure 5 The arginine methyltransferase CARM1 levels vary in different estrous cycle stages in the baboon ovary and endometrium. Immunohistochemistry revealed strong immunoreactivity of CARM1 (green) in tissues at the late proliferative/follicular and mid-secretory/mid-luteal stage in (A) endometrial as well as in ovarian sections (B). CARM1 is, in contrast, barely detectable in samples obtained at the late secretory/late luteal stage in the ovary and the endometrium (lower panels). H3K9me3 immunodetection was conducted as internal control and DNA was counterstained with DAPI. Scale bar = 100 µm.

We demonstrate here that aberrant gene expression is not restricted to ectopic endometrial lesions or the eutopic endometrium, but is extensively detectable in the ovaries of animals at different stages of the disease. Moreover, our data provide the first evidence that endometriosis is associated with significantly dysregulated expression patterns of key epigenetic factors in the ovary, which might contribute to the etiology and pathophysiology of endometriosis-associated infertility. A striking reduction in histone arginine methyltransferases PRMT2 and PRMT8 as well as CARM1 signal the early and long-term ovarian response to disease duration. Notably, the 17.8-fold downregulation in ovarian PRMT8 transcript levels is associated with hypermethylation of promoter sequences. In addition, both transcript and protein levels of CARM1 were significantly reduced in granulosa cells and pre-ovulatory oocytes after 15 months of endometriosis. Our findings provide critical insight into the potential mechanisms involved

in the establishment of abnormal epigenetic modifications in both oocytes and granulosa cells during endometriosis. For example, arginine methyltransferases contribute to histone methylation, transcriptional regulation, and DNA repair (Bedford 2007). CARM1 plays a critical role in estrogen-dependent nuclear receptor mediated transcription in somatic cells (Bedford 2007) and maintenance of pluripotency in the mammalian pre-implantation embryo (Torres-Padilla *et al.* 2007). Thus, disruption of the ovarian epigenetic landscape may have functional consequences for oocyte quality and the etiology of infertility associated with endometriosis.

A number of epigenetic aberrations, such as DNA hypermethylation of promoter regions (Wu *et al.* 2005, 2006, Nasu *et al.* 2011) as well as altered patterns of histone modifications (Kawano *et al.* 2011, Monteiro *et al.* 2014) have recently been identified in the eutopic and ectopic endometrium of women with endometriosis and provide critical insight into the role of epigenetic modifications in the regulation of aberrant gene expression in this disease. We extend upon these findings by demonstrating differential expression of an array of epigenetic chromatin-modifying enzymes in the baboon ovary following endometriosis induction. Increasing changes in transcript levels correlated with the duration of the disease indicate that gene expression changes during endometriosis may develop gradually,

Figure 6 Endometriosis alters CARM1 levels in the ovary and endometrium. (A) CARM1 (green) is clearly detectable in mid-secretory endometrial sections obtained from control animals. By comparison, 15 months of endometriosis induce CARM1 downregulation and lead to reduced immunoreactivity (lower panel). (B) CARM1 is barely detectable in ovarian sections from animals following 15 months of endometriosis (lower panel) as compared to disease-free controls (upper panel). DNA is counterstained with DAPI. Scale bar = 50 µm.

Figure 7 Global patterns of DNA methyltransferase (DNMT) DNMT1 and PARP1 in ovaries from animals with endometriosis. (A) Immunohistochemistry on paraffin sections obtained from control females (upper panel) and animals following 3 and 15 months of endometriosis (lower panels) indicates expression of the DNMTs DNMT1 (green) in the oocytes of primary and secondary follicles. PARP1 immunolabeling was prominently detected in nucleoli and nuclei as well as the granulosa cells of primary and early pre-antral follicles. Endometriosis burden for 15 months resulted in increased PARP1 labeling intensity. (B) Immunostaining of whole-mount oocytes with anti-DNMT1 and anti-PARP1 antibodies indicates diffuse cytoplasmic staining and an increase in DNMT1 in response to endometriosis compared to oocytes obtained from control animals. DNA is counterstained with DAPI (blue).

and show distinctive, stage-specific transcript profiles. Recent chronological expression data in the eutopic endometrium of baboons obtained by microarray analysis revealed an early disease burden and a transitory dominance of an estrogenic phenotype followed by a stable progesterone-resistant phenotype (Afshar *et al.* 2013), which is also found in human endometriosis (Burney *et al.* 2007). Intriguingly, the endometrial transcriptome 3 months following induction

of disease showed a remarkable lack of differential gene expression (Afshar *et al.* 2013) similar to relatively limited gene expression changes detectable in the ovary at 3 months of endometriosis in our present study. Whether the ovarian transcriptome undergoes analogous stage-specific transitions remains to be investigated. However, evidence indicates that aberrant gene expression patterns in the eutopic endometrium are secondary to the disease and directly and acutely affected by endometriotic lesions rather than representing endometrium-inherent prerequisites for endometriosis (Afshar *et al.* 2013). Pathophysiological changes associated with the disease may likely also be responsible for abnormal gene expression in endometriotic ovaries. Thus, the identification of the precise molecular mechanisms that cause such striking epigenetic changes in the ovary will be a step of major importance.

In this study, members of the protein arginine N-methyltransferase (PRMT) family of chromatin-modifying enzymes were among the most prominently altered genes identified by pathway-focused PCR arrays in ovaries of baboons at both 3 and 15 months following induction of endometriosis. Downregulation of CARM1 protein was confirmed by immunohistochemistry in the endometrium and ovary and revealed dynamic expression patterns according to the stage of the menstrual cycle with higher protein expression during the late proliferative and mid-secretory phase and negligible expression at the late secretory phase, suggesting regulatory effects of a cyclically changing hormonal environment consistent with CARM1's role in regulating estrogen receptor alpha (ER α) mediated gene pathways in response to ER α activation.

PRMTs play a crucial role in the dimethylation of arginine residues of many different target proteins. For instance, CARM1 and PRMT1 are co-activators of the tumor suppressor protein P53 through the specific methylation of arginine 17 in histone H3 (H3R17me2) and arginine 3 in histone 4 (H4R3me2) respectively. These histone modifications, in turn, are involved in facilitating p53-mediated transcriptional regulation (Scoumanne & Chen 2008) and contribute to the control of cellular survival and tumor suppression. In addition, loss of CARM1 function alters the turnover of key molecules involved in cell cycle control, such as cyclin A, cyclin B1, c-fos, SIRT1, and p16 (Pang *et al.* 2013). Our data demonstrate significantly reduced transcription levels of CARM1, PRMT1, PRMT2, and PRMT8 in ovaries after 15 months of disease progression, suggesting dysregulation of arginine methyltransferases might predispose to the development of benign and pre-malignant disease characteristics such as impaired control of cell proliferation, clonal expansion of abnormal cells, and genome instability (Nezhat *et al.* 2014). Notably, IPA of samples obtained at 15 months following induction of endometriosis revealed gene expression changes commonly involved in inflammatory

disease, reproductive system disease, and cancer. Taken together, these results support a hypothesis in which aberrant expression of key epigenetic chromatin-modifying enzymes may contribute to an oncogenic predisposition for specific subtypes of ovarian cancer (endometrioid and clear cell ovarian carcinoma) in individuals with endometriosis (Cheng *et al.* 2005). Although future studies will be necessary to substantiate this specific association, premalignant transformation following endometriosis is now considered a complex multifactorial process, triggered not only by genetic mutations (e.g., PTEN inactivation), but also epigenetic factors (e.g., aberrant expression of HOXA10) (Cheng *et al.* 2005) as well as environmental stimuli such as oxidative stress and pathologically elevated estrogen levels (Nezhat *et al.* 2014). Aberrant expression of PRMTs and other key epigenetic chromatin-modifying enzymes involved in nuclear receptor-mediated transcriptional regulation may be at the crossroads of these multifactorial stimuli, and thus further exacerbate a premalignant endometriotic phenotype.

Arginine methylation is also necessary for germ cell development in *Drosophila* (Anne 2010) and mice (Ancelin *et al.* 2006, Kim *et al.* 2014) and is dynamically regulated during mammalian oogenesis and preimplantation embryo development (Sarmiento *et al.* 2004, Tee *et al.* 2010). Importantly, CARM1 is known to regulate the levels of histone H3 methylation at arginine (R17) and (R26) in blastomeres destined to give rise to the inner cell mass of the mouse pre-implantation embryo, and is required for the epigenetic control of pluripotency of embryonic stem cells (Torres-Padilla *et al.* 2007, Parfitt & Zernicka-Goetz 2010). Our results demonstrate that endometriosis is associated with altered arginine methyltransferase expression and may thus interfere with PRMT-mediated ovarian estrogen-regulated gene reprogramming and proper gametogenesis. For instance, CARM1-dependent methylation of H3R17 has protective effects on histone acetylation through the release of co-repressors from chromatin (Wu *et al.* 2012) and PRMT insufficiency may, in turn, interfere with proper histone acetylation. Notably, current evidence also suggests a prominent role for CARM1 in regulating the activity of the Notch family of transmembrane receptors through methylation of intracellular arginine residues (Hein *et al.* 2015). Notch signaling is crucial for many developmental processes such as cell differentiation and proliferation and has also recently been shown to play a critical role in decidualization of both mouse and human uterine stromal cells (Su *et al.* 2015). Loss of arginine methylation reduces Notch signaling, which is in turn associated with endometriosis and impaired decidualization (Hein *et al.* 2015, Su *et al.* 2015). Downregulation of CARM1 expression as observed in this study may thus provide further insight into the pathways involved in endometriosis-induced infertility.

Immunohistochemical analysis of ovarian sections and whole-mount oocytes revealed a striking upregulation of

the maintenance methyltransferase DNMT1, which is in accordance with reports on DNMT overexpression in the ectopic endometrium in women with endometriosis (Wu *et al.* 2006). Dysregulated DNMT1 levels may alter DNA methylation at specific promoter regions influencing the transcriptional activity of target genes and could impact global chromatin methylation levels, thereby conferring susceptibility to genome instability and neoplastic transformation. Moreover, DNMT1 overexpression provides a potential link to promoter hypermethylation of the PRMT8 gene and the corresponding downregulation of PRMT8 expression following 15 months of endometriosis. Whether elevated PARP1 expression is induced in response to accumulating DNA damage in endometriotic tissues remains to be investigated in future experiments. However, IPA suggests a complex dysregulation of several canonical pathways and key molecules involved in cell cycle control, DNA replication, recombination, and repair that are commonly associated with neoplastic transformation and tumor progression as well as reproductive and endocrine system disorders in the ovary in response to endometriosis. Notably, both H3K9me3 and PARP1 were highly expressed in the granulosa cells of primary and early pre-antral follicles, suggesting that changes in epigenetic modifications may be associated with follicular growth and differentiation. Thus, our findings provide the first account of an epigenetic ovarian response to the presence of endometriotic lesions and allow critical insight into the potential mechanisms disrupting epigenetic modifications in both oocytes and granulosa cells during the course of this disease.

Exposure to peritoneal fluid obtained from patients with endometriosis leads to aberrant chromosome alignment and spindle microtubule abnormalities in mouse metaphase II oocytes (Mansour *et al.* 2010). In addition, IVF cycles conducted with donor oocytes from women with endometriosis result in significantly lower pregnancy rates compared to tubal factor control donors irrespective of the recipient status, suggesting impaired germ cell quality prior to ovulation and exposure to peritoneal fluids (Barnhart *et al.* 2002, Mansour *et al.* 2010). Importantly, our findings suggest that the establishment of abnormal epigenetic modifications to both the somatic and germ cell compartment in the ovary during the course of endometriosis is a major contributing factor in the loss of fertility associated with this condition. The resulting epigenetic instability, aberrations in gene expression and compromised chromatin structure and function may ultimately be at the core of ovarian pathophysiology, reduced oocyte quality, subfertility, and potential transgenerational transmission of an altered epigenetic landscape following endometriosis.

In summary, our results demonstrate that endometriosis is correlated with altered levels of specific epigenetic chromatin-modifying enzymes that result in changes in the chromatin environment in the oocyte genome. This mechanism might predispose to potential

transgenerational inheritance of abnormal epigenetic modifications established during oocyte growth. This notion is also supported by a recent report suggesting that endometriosis leads to heritable changes in gene expression in rat embryos over several generations (Birt *et al.* 2013). Our results demonstrate for the first time a link between endometriosis and a specific epigenetic ovarian response that may aid in identifying novel targets for epigenetic therapy to improve fertility and prevent any potential transgenerational inheritance of abnormal epigenetic modifications following endometriosis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0212>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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