Endometrial DNA damage response is modulated in endometriosis

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STUDY QUESTION: Is the DNA damage response (DDR) dysregulated in the eutopic endometrium of women with endometriosis?

SUMMARY ANSWER: Endometrial expression of genes involved in DDR is modulated in women with endometriosis, compared to those without the disease.

WHAT IS KNOWN ALREADY: Ectopic endometriotic lesions are reported to harbour somatic mutations, thereby hinting at dysregulation of DDR and DNA repair pathways. However, it remains inconclusive whether the eutopic endometrium also manifests dysregulated DDR in endometriosis.

STUDY DESIGN, SIZE, DURATION: For this case–control study conducted between 2015 and 2019, eutopic endometrial (E) samples (EE- from women with endometriosis, CE- from women without endometriosis) were collected in either mid-proliferative (EE-MP, n = 23; CE-MP, n = 17) or mid-secretory (EE-MS, n = 17; CE-MS, n = 9) phases of the menstrual cycle. This study compares: (i) DNA damage marker localization, (ii) expression of DDR genes and (iii) expression of DNA repair genes in eutopic endometrial samples from women with and without endometriosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study included (i) 40 women (aged 31.9 ± 0.81 years) with endometriosis and (ii) 26 control women (aged 31.4 ± 1.02 years) without endometriosis. Eutopic endometrial samples from the two groups were divided into different parts for histological analysis, immunohistochemistry, RNA extraction, protein extraction and comet assays. Eighty-four genes of relevance in the DNA damage signalling pathway were evaluated for their expression in eutopic endometrial samples, using RT2 Profiler PCR arrays. Validations of the expression of two GADD (Growth Arrest DNA Damage Inducible) proteins - GADD45A and GADD45G were carried out by immunoblotting. DNA damage was assessed by immunohistochemical localization of γ-H2AFX (a phosphorylated variant of histone H2AX) and 8-OHdG (8-hydroxy-2’-deoxyguanosine). RNA sequencing data from mid-proliferative (EE-MP, n = 4; CE-MP, n = 4; CE-MS, n = 9 each) and mid-secretory phase (EE-MS and CE-MS, n = 4 each) endometrial samples were scanned to compare the expression status of all the genes implicated in human DNA repair. PCNA (Proliferating Cell Nuclear Antigen) expression was determined to assess endometrial proliferation. Residual DNA damage in primary endometrial cells was checked by comet assays. Public datasets were also scanned for the expression of DDR and DNA repair genes as our RNASeq data were limited by small sample size. All the comparisons were made between phase-matched endometrial samples from women with and without endometriosis.

MAIN RESULTS AND THE ROLE OF CHANCE: Endometrial expression of DDR genes and intensity of immunolocalized γ-H2AFX were significantly (P < 0.05) higher in EE, compared to CE samples. DDR proteins, especially those belonging to the GADD family, were found to be differentially abundant in EE, as compared to CE. These patterns were evident in both mid-proliferative and mid-secretory phases. Intriguingly, higher DDR was associated with increased cell proliferation in EE-MP, compared to CE-MP. Furthermore, among...
Introduction

Endometriosis, an estrogen-dependent disease, is characterized by the extra-uterine growth of endometrium-like tissue or endometriotic lesions. The disease contributes to chronic pelvic pain, dysmenorrhea, dyspareunia, subfertility or infertility and thereby adversely affects the quality of life in ~10–15% of women in their pre-menopausal years (Macer and Taylor, 2012). Molecular mechanisms that enable endometriotic lesions to thrive at extra-uterine sites have been investigated extensively. Collectively, these investigations have revealed that endometriotic lesions manifest the features typical of a malignant tumour such as invasion, adhesion, recurrence and neo-angiogenesis (Siufi Neto et al., 2014). Akin to cancer cells, endometriotic lesions show higher susceptibility to DNA damage due to high oxidative stress (Yamaguchi et al., 2008; Ngo et al., 2009; Dai et al., 2019) and, consequently, accumulate somatic mutations in various genes such as ARID1A, PTEN, TP53, PIK3CA, KRAS or PPP2R1A (Sato et al., 2000; Bischoff et al., 2002; Wiegand et al., 2010; Govatati et al., 2014; Anglesio et al., 2017). Cancer-driver somatic mutations have been reported in the endometriotic lesions from a considerable number of women with iatrogenically induced endometriosis and deep-infiltrating endometriosis (DIE) (Lac et al., 2019a). Interestingly, such mutations were detected even in histologically normal eutopic endometrial samples (Lac et al., 2019b). Although the frequencies of mutated alleles were higher in endometriotic lesions, a remarkable overlap was observed in the mutational landscapes of the epithelial cells from endometriotic lesions and normal eutopic endometrium (Suda et al., 2018). These investigations suggest that certain sub-clonal mutations may confer a growth advantage to eutopic endometrial cells at ectopic sites. Collectively, these studies also indirectly hint at compromised DNA damage response (DDR) and DNA repair pathways in ectopic lesions and eutopic endometrium. While some evidence exists for dysregulated DDR and DNA repair in ectopic lesions (Fuseya et al., 2012; Grassi et al., 2015), eutopic endometrium has not been extensively investigated for its basal DDR and DNA repair capacity in endometriosis. Such studies are relevant, considering that the eutopic endometrium is the most likely source of cells that form endometriotic lesions at ectopic sites.

The few investigations undertaken to probe the DDR in eutopic endometrium have been inconclusive. Carvalho et al. (2013) reported higher DNA damage and lower DNA repair capacity in the eutopic endometrium of women with endometriosis (EE), as compared with tubal cells of women without endometriosis. However, this study did not include eutopic endometrial cells from women without endometriosis (CE). Other investigations on the levels of specific DNA mismatch repair proteins included endometriotic ectopic lesions and CE, but not EE samples in their study designs (Fuseya et al., 2012; Grassi et al., 2015). A recent study demonstrated reduced expression of genes involved in double-strand break repair (DSBR) proteins such as Rad51 and BRCA1, thereby indicating dysfunctional DNA repair capacity in EE samples (Choi et al., 2018). However, Hapangama et al. reported that the DNA damage surveillance is bypassed in the EE during the proliferative as well as secretory phases of the menstrual cycle (Hapangama et al., 2008). Thus, it warrants more investigations to resolve whether the eutopic endometrial cells bypass the DDR pathway or have compromised DDR in endometriosis.

The present study was undertaken to investigate (i) whether the eutopic endometrium in endometriosis shows signs of DNA damage, (ii) whether the EE has the competence to respond to DNA damage or elicit DDR and (iii) whether the expression of DNA repair genes is modulated in EE, compared to CE.

Materials and methods

Ethical statement

The study was approved by the National Institute for Research in Reproductive Health (NIHRH) Ethics Committee for Clinical Studies (258/2014). All the study participants gave written informed consent.

Collection of samples

Eutopic endometrial samples included in the ‘Endometriosis group’ were collected using a pipelle endometrial sampler from women (aged
31.9 ± 0.81 years) undergoing laparoscopic surgery for endometriosis. Histological analyses of lesions excised from ovaries or peritoneum or Pouch of Douglas and uterosacral sites were carried out to confirm the diagnosis. Patients with endometriosis who had coexisting leiomomas, adenomyosis, polyps, polycystic ovarian syndrome or gynaecological malignancies were excluded. Saging of endometriosis (Stages I–IV) was carried out following the criteria of revised American Society for Reproductive Medicine (1997). The menstrual cycle phase of the samples was assessed based on histological dating by an expert histopathologist (G.F.) as per Noyes criteria (Noyes et al., 1975) and the last menstrual period. Of the eutopic endometrial samples collected from women with endometriosis, 23 were histologically dated as ‘mid-proliferative’ (EE-MP) and 17 as ‘mid-secretory’ (EE-MS) phase. Details of participant characteristics are provided in Supplementary Tables SI and SII. ‘Control’ eutopic endometrial samples were collected from women aged 31.4 ± 1.02 years undergoing laparoscopy for either interval tubal ligation or experiencing infertility because of male factors. Women in this group were found to have no evidence of endometriosis on laparoscopy or any other gynaecological pathology such as leiomyomas, adenomyosis, polyps, polycystic ovarian syndrome or malignancies. In the control group, 17 samples were histologically dated as ‘mid-proliferative’ (CE-MP) and 9 as ‘mid-secretory’ (CE-MS) phase. Although detailed information on the duration and type of contraceptives used throughout the life of participants was not recorded, it was ensured that women who were on any medication or oral contraceptives in the past 3 months are not included in both the groups.

Tissue processing

Endometrial tissues were thoroughly rinsed with saline to get rid of any red blood cell contamination. Tissues were then divided into three parts, one part fixed in 10% neutral buffered formalin [10% (v/v) formaldehyde prepared in phosphate-buffered saline (PBS) containing 46 mM disodium hydrogen phosphate, 29 mM sodium dihydrogen phosphate, pH 7.4] solution for histological and immunohistochemical analyses; the second part in TRIzol reagent for RNA isolation and the remaining part in cell lysis buffer [9.0 M urea, 4% (w/v) CHAPS, 80 mM Tris base] for immunoblotting. Tissues kept in TRIzol reagent (Ambion, USA) or cell lysis buffer were stored at −80°C till further processing. A part of the endometrial tissue from some samples was digested with collagenase type 1A (as detailed in the section on comet assay) to prepare a single-cell suspension. These primary endometrial cells were stored in cell-freezing medium [80% (v/v) DMEM/F12, 10% (v/v) newborn calf serum and 10% (v/v) dimethyl sulfoxide (DMSO)] at −196°C.

Histological and immunocytochemical analyses

After fixation, the tissues were transferred to 70% (v/v) alcohol for 24 h and then passed through ascending grades of 70–100% (v/v) alcohol for 30 min in each grade. This was followed by incubation of tissues in xylool (xylene:alcohol 1:1) and then in 100% xylene with a change given every 15 min. Tissues were incubated in molten wax at 56°C for 15 min and allowed to solidify overnight at room temperature (RT). Next day, the wax was replenished with fresh molten wax and paraffin blocks were made. After solidification of the wax, the paraffin blocks were removed out of the moulds and tissues were sectioned at 5-μm thickness using a microtome (Leica Biosystems, USA) on poly-L-lysine (Sigma-Aldrich, USA)-coated glass slides.

Histological dating

Tissue sections were deparaffinized in 100% xylene solution twice, for 15 min each, and then rehydrated using descending grades of alcohol for 50–100% (v/v) alcohol and finally in water, for 5 min each. Sections were stained with haematoxylin for 1 min and then incubated in water for 10 min. Following this, sections were passed through ascending grades of alcohol from 30% (v/v) to 70% (v/v) and then immersed in Eosin Y solution. Sections were dehydrated through 90% (v/v) and 100% alcohol for 5 min each and then kept in 100% xylene overnight. Next day, the sections were mounted using distyrene, a plasticizer, and xylene (DPX) mountant (Fisher Scientific, India). Tissues were dated for the menstrual cycle phase following the Noyes criteria of histological dating (Noyes et al., 1975).

Immunohistochemical analysis

Tissue sections were deparaffinized in xylene for 15 min twice and then rehydrated using descending grades of alcohol. All solutions (other than those used for dehydration and rehydration) used for immunostaining of Proliferating Cell Nuclear Antigen (PCNA), AT-Rich Interactive Domain-containing Protein 1A (ARID1A) and 8-hydroxy-2’-deoxyguanosine (8-OHdG) were prepared in 1× PBS and for immunostaining of a phosphorylated variant of histone H2AX (γ-H2AX/γ-H2AXF), in 1× Tris-buffered saline. For antigen retrieval, sections were heated at 97°C for 30 min for the detection of γ-H2AX or 96°C for 5 min for 8-OHdG or 95°C for 20 min for PCNA or autoclaved for 10 min for ARID1A, in sodium citrate buffer (pH 6.0) supplemented with 0.05% (v/v) Tween-20. Sections were allowed to cool naturally to RT and incubated for 10 min in 0.1% (v/v) Triton X-100 for nuclear permeabilization. Endogenous peroxidase was inactivated by incubating in 0.3–3% H2O2 prepared in 100% alcohol. Sections blocked in 1% (v/v) normal serum were incubated with antibodies (details in Supplementary Table SIII) against the antigen of interest overnight at 4°C. Sections were incubated with respective biotinylated secondary antibodies (Vector Laboratories, USA) at 37°C for 1 h and avidin–biotin complex in dark at RT for 30 min. Diaminobenzidine at 1 mg/ml was used as a chromogen and 0.03% (v/v) H2O2 as a substrate. Sections were counterstained with haematoxylin for 35 s and mounted using DPX. On average, 30–40 fields were screened per section and images were captured using Cell Sens software (Olympus Corporation, Japan). ImageJ (version 1.46r, National Institutes of Health, USA) software was used for immunohistochemical analyses.

Protein extraction and western blotting

Proteins were extracted from endometrial samples suspended in cell lysis buffer using a sample grinding kit (Amersham Biosciences, UK). Protein samples (8–12 μg) were resolved on SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Merck Millipore, USA). After transfer, the blots were blocked for 1 h at RT with 5% (w/v) bovine serum albumin (Sigma-Aldrich, USA) prepared in 1×
TBS containing 0.05% (v/v) Tween-20 (TBST) or 2% (w/v) non-fat dried milk powder prepared in 1 × PBS containing 0.05% (v/v) Tween-20 (PBST). This was followed by overnight incubation at 4°C of the blots with antibodies against the protein of interest (details are in Supplementary Table SIII). The blots were washed with either TBST or PBST and incubated with secondary antibody conjugated to horseradish peroxidase (Dako, Denmark) for 1 h at RT. The blots were thoroughly washed with TBST or PBST. Finally, the blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA) and chemiluminescent signals were captured using ChemiDoc™ MP Imaging System (Biorad, USA). Since there exists a possibility of a modulation in the endometrial levels of housekeeping proteins (such as GAPDH) in endometriosis (Joseph and Mahale, 2019), normalization of the protein of interest across different samples was carried out using the total protein load for each sample (quantified using ImageQuant TL software), instead of considering a single housekeeping protein. After developing, the blots were stained with Coomassie Brilliant Blue R-250 for densitometric analysis using Image Quant (IQLT) software (GE Healthcare Biosciences, USA) to compare the total protein load across different samples.

RNA extraction

For total RNA extraction, endometrial tissues suspended in TRIzol reagent were homogenized. Homogenates were centrifuged to pellet down debris if any. The supernatants mixed with the equal volume of chloroform were spun at 13500g at 4°C for 15 min. RNA in the aqueous phase was precipitated using 100% isopropanol. After incubation at RT for 45 min, RNA was pelleted at 13500g at 4°C for 40 min and washed twice with 80% chilled ethanol. The pellet was resuspended in 30–50 μl nuclease-free water and treated with RNase free DNase (1 U/μg of DNA) at 37°C for 30 min. Inactivation of DNase was carried out by repeating all the steps from the addition of TRIzol to isopropanol precipitation. The purity and concentration of total RNA were determined using a microplate reader.

RT² profiler PCR array

Total RNA (1.0 μg) was converted into cDNA using the RT² First Strand Kit (Qiagen). The cDNA diluted with 91 μl of nuclease-free water was added to SYBR green Master Mix to obtain a final volume of 2.7 ml (Qiagen). The reaction mix (25 μl) was dispensed into each well of the RT2 profiler array (Qiagen). The array had 84 genes known to be involved in DNA damage signalling pathway and five housekeeping genes namely β-actin, β2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phospho-riboisyltransferase 1 and ribosomal protein large P0; one well to test genomic DNA contamination; three wells each for reverse transcription control and positive PCR control reactions. All the steps were performed according to the manufacturer’s protocol using the Applied Biosystems Quant Studio 5 real-time PCR system. The cycling conditions were pre-incubation at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, for the acquisition of fluorescence data.

RNA sequencing

RNA sequencing of eutopic endometrial samples from seven controls (mid-proliferative, n = 3 and mid-secretory, n = 4) and eight endometriosis cases (n = 4 each for mid-proliferative and mid-secretory phase samples) was carried out at Genotypic Technology Pvt. Ltd. (India). All the samples had RNA integrity number above 6.0, as revealed by bioanalyzer profiles (Agilent RNA Bioanalyzer chip, USA).

For library preparation, 1.0 μg of Qubit quantified total RNA was enriched for poly A using oligo dT microparticles. The sequencing library was constructed using the SureSelect Strand-Specific RNA Library Prep Kit. Briefly, fragmentation of mRNA was performed for 4 min at 94°C in the presence of divalent cations and the first-strand cDNA was synthesized. HighPrep™ PCR Clean-up System (Magbio, USA) was used to clean up the single-stranded cDNA. The second-strand cDNA was synthesized and end-repaired using Second Strand Synthesis + End Repair mix. The strand-specificity was retained by the addition of dUTP. The cDNA was cleaned up using HighPrep™ PCR Clean-up System. Ligation of adapters to the cDNA molecules was performed after the addition of ‘A’ base. HighPrep clean-up was performed post-ligation. The library was indexed and adapter-ligated fragments were enriched using 10 cycles of PCR. Qubit was used to quantify the prepared library, and its quality was validated by running an aliquot on the D1000 Tape Station Kit (Agilent, USA). Finally, the adapter-positive fragments were quantified using the KAPA qPCR quantification kit (KAPA Biosystems, USA).

Reference-based transcriptome analysis

For RNA sequencing of eutopic endometrial samples, Illumina NextSeq500 platform was used. The Tuxedo pipeline was used for RNAseq data analysis. On average, 28 million raw reads per sample were generated. Low-quality bases with Phred score <30 were removed and ~26 million processed reads as FASTQ files were used for subsequent analyses. FASTQ files were aligned to the Homo sapiens GRCh38.p10 build genome (downloaded from the Ensemble database) using HISAT2. Approximately 93% of the processed reads were aligned to the reference genome. The aligned reads were then processed using Cufflinks to generate transcript assemblies. Cuffmerge, a script included in Cufflinks package, was used to merge several transcript assemblies to generate an assembly Gene Transfer Format (GTF) file. Transcript abundance was calculated in the form of normalized read count, i.e. FPKM (fragments per kilobase transcript per million mapped reads) counts. This GTF file was used for identifying differentially expressed transcripts (DETs) between control and endometriosis groups using Cuffdiff. These transcripts were categorized into either up-regulated or down-regulated with a cut-off of 1.5-fold based on log2-transformed fold change values. All the transcripts which were differentially regulated by at least 1.5-fold in eutopic endometrial samples in control and endometriosis groups were used for ingenuity pathway analysis (IPA) (Qiagen, USA). The datasets generated by RNA sequencing of mid-proliferative (accession number GSE153739) and mid-secretory phase (accession number GSE153740) eutopic endometrial samples from women with and without endometriosis were submitted to the Gene Expression Omnibus (GEO) database.
Cross-comparison with public datasets on endometriosis

A public dataset GSE51981 [submitted by Tamaresis et al. (2014) to the GEO database] containing global gene expression data from eutopic endometrial samples from women with (n = 75) and without endometriosis (n = 34) was screened for the expression profiles of eutopic endometrial samples in proliferative and mid-secretory phases of menstrual cycle. Forty-four proliferative phase and 35 mid-secretory phase samples included in the dataset were further analysed to compare the expression status of DDR/DNA repair genes in phase-matched control and endometriosis groups. The GSE51981 dataset had also been also used by Bakhtiari-zadeh et al. (2018) to construct a weighted gene co-expression networks in endometriosis. Raw CEL files containing microarray data from GPL750 Affymetrix Human Genome U133 plus 2.0 array platform were normalized using R package and expression values (normalized signal intensities generated by hybridized probe sets in the microarray) were log2-transformed. t-Statistics from the Bioconductor package LIMMA was used to identify differentially expressed genes (DEGs) with >1.5-fold change and false discovery rate <0.05 (Tamaresis et al., 2014). For the present study, normalized expression data of DEGs in 28 women without endometriosis (n = 20 in proliferative phase and 8 in mid-secretory phase) and 51 women with endometriosis (n = 24 in proliferative phase and 27 in mid-secretory phase) were considered to determine whether there exists a concordance with the expression patterns of DDR/DNA repair genes seen in our RNASeq-based investigations.

Comet assay

A single-cell suspension was prepared from endometrial samples, according to the protocol reported earlier (Zhang et al., 1995). Briefly, endometrial tissues were digested in DMEM/F12 Ham (Invitrogen, USA) supplemented with 10% newborn calf serum (Invitrogen) and collagenase type IA (1 mg/ml) (Sigma-Aldrich, USA) at 37°C for 1 h with gentle shaking. The homogenate was first passed through a 250-μm sieve and then through a 40-μm sieve. The filtrate known to have enriched stromal cell fraction was centrifuged at 260g at RT for 10 min. The stromal cell pellet was washed with complete DMEM to get rid of blood cells. Undigested endometrial glands retained on 40-μm sieve were backwashed with 0.1% PBSA [1 × PBS with 0.1% (w/v) BSA] and spun at 262g at RT for 10 min to get a pellet of epithelial cells. Trypsin-EDTA [0.025% (v/v), Invitrogen] made in PBSA was added to the pellet to obtain a single-cell suspension. Complete DMEM/F12 was added to the cell suspension and spun at 262g at RT for 10 min.

For comet assay, both epithelial and stromal cell populations from the same sample were combined and ~20 000 cells were mixed with 0.5% (w/v) low melting point agarose (LMPA) and overlaid uniformly on a glass slide [pre-coated with 1% (w/v) normal melting point agarose for 16 h] using a coverslip. These slides were kept on an ice pack for the solidification of LMPA containing cells. The coverslip was removed gently, and the slides were incubated in freshly prepared chilled lysis buffer [2.5 M NaCl, 100 mM EDTA, 100 mM Tris base with freshly added 1% (v/v) Triton X 100 and 1% (v/v) DMSO just before use] for 1 h at 4°C. The slides were placed in freshly prepared electrophoresis buffer (200 mM EDTA, 10 N NaOH, pH 13) for 15 min to allow unwinding of DNA and electrophoresed at 25 V for 20 min.Slides were then incubated with neutralization buffer (0.4 M Tris base, pH 7.5) and stained with ethidium bromide. Cells were viewed under a fluorescent microscope (Zeiss) and AxioVision, release 4.8.2 software (Germany). More than 500 cells were randomly counted using OpenComet (Gyorii et al., 2014) plugin of ImageJ software for determination of the percentage of cells with residual damaged DNA and mean tail length.

Statistical analyses

SPSS software (V.25) was used for statistical analyses. To test the normality of the distribution, the Shapiro–Wilk test was used. Student’s unpaired t-test was used for data that followed a normal distribution. In the case of unequal variance, Student’s unpaired t-test with Welch’s correction was used. Mann–Whitney U test, a nonparametric test, was used for all immunohistochemical analyses. Analyses of the array data were carried out using the ΔΔCt method. Relative fold change was calculated using 2−ΔΔCt method and P-values were calculated using the Student’s t-test of ΔΔCt data for each gene. For RNASeq data, Benjamini–Hochberg multiple testing correction was used to calculate the false discovery rate. FPKMs (mean ± SEM) for the transcript of interest were compared between control and endometriosis groups and the statistical significance of the difference between mean FPKMs was calculated by the Student’s unpaired t-test. As our RNASeq data were derived from a small sample size, the GSE51981 dataset, a comparatively large dataset (Tamaresis et al., 2014) processed using R package and with false discovery rate (FDR) <0.05, was analysed for the relative expression of DDR/DNA repair genes. Data were presented as mean ± SEM and P-value <0.05 was considered statistically significant.

Results

DDR and DNA repair in the eutopic endometrium of women with endometriosis during the mid-proliferative phase of the menstrual cycle

Endometrial DDR in endometriosis

Five DDR genes [DDIT3 (P < 0.01), GADD45A (P < 0.05), GADD45G (P < 0.001), MCH1 (P < 0.05), PP1R15A (P < 0.05)] were found significantly up-regulated at the transcript level by at least 1.5-fold in mid-proliferative phase (MP) eutopic endometrial samples from women with endometriosis (EE-MP), compared to those from women without endometriosis (CE-MP), as revealed by the RT2 Profiler PCR array analysis of 84 DDR genes. XRCC3, ERCC1, MAPP12, MDC1, MU3, RAD9A and TP73 genes also showed a trend towards higher expression in EE-MP, compared to CE-MP (Fig. 1A). Analysis of the GSE51981 dataset available in the GEO database revealed significant up-regulation of MAPK12, MDC1, PP1R15A, RAD9A, XRCC3 and ERCC1 genes in the eutopic endometrial samples of women with endometriosis (n = 24), compared to those from women without the disease (n = 20), as shown in Supplementary Fig. S1. Interestingly, endometrial expression of GADD45B, another GADD45 gene, was significantly up-regulated in women with endometriosis, compared to women without the disease and this pattern was evident in both

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Figure 1. Mid-proliferative phase eutopic endometrial samples from women with endometriosis show higher DNA damage, higher DNA damage response and higher proliferation. (A) Volcano plot to represent relative expression of DNA damage signalling-associated genes in eutopic endometrium of women with endometriosis (EE, n = 6), compared to those without endometriosis (CE, n = 6). △ indicates a gene displaying differential expression by at least 1.5-fold in endometriosis, compared to control and △ in green indicates a gene displaying differential expression by at least 1.5-fold with P-value <0.05. Red dotted line indicates –log2(P-value), P = 0.05. (B–D and E–G) Immunodetection of GADD45A and GADD45G proteins, respectively, in EE (EE1–EE3) and CE (CE1–CE3) samples. (B and E) Representative images indicating chemiluminescent detection of endometrial GADD45A and GADD45G proteins, respectively. (C and F) Coomassie blue-stained
proliferative and mid-secretory phase EE samples included in the GSE51981 dataset.

Next, RT² Profiler PCR array data were validated at the protein level. GADD45S protein levels were significantly ($P < 0.05$) higher in EE-MP, compared to CE-MP (Fig. 1E and G). This was in congruence with the array data indicating a higher level of GADD45S transcripts in EE-MP, compared to CE-MP. GADD45A protein levels were also found higher in EE-MP, compared to CE-MP samples, though the difference failed to reach statistical significance (Fig. 1B and D). Higher levels of GADD45 proteins indicated a possibility of endometrial samples encountering higher DNA damage in endometriosis.

Localization of DNA damage markers in endometriosis

Immunolocalization studies were carried out to detect 8-OHdG (8-hydroxy-2'-deoxyguanosine), a marker of oxidative stress-induced DNA damage and γ-H2AFX (a phosphorylated variant of histone H2A) indicating double-stranded or single-stranded DNA breaks.

8-OHdG was detected in the nuclei and cytoplasmic compartments of both epithelial and stromal cells (Supplementary Fig. S2). An apparent but not statistically significant modest increase was observed in the stromal levels of 8-OHdG in EE-MP, compared to CE-MP samples (Supplementary Fig. S2C).

γ+H2AFX-positive foci in the nuclei were found to be predominantly localized in glandular epithelial cells (Fig. 1H). γ-H2AFX foci in the glandular epithelial cells were significantly ($P < 0.05$) higher in EE-MP, compared to CE-MP (Fig. 1I and Supplementary Fig. S3). This suggests that as compared to CE-MP, EE-MP encounters higher DNA damage.

Endometrial DNA repair and cell proliferation in endometriosis

Next, RNASeq data from the mid-proliferative phase endometrial samples (GEO accession no GSE153739) were scanned to determine if the endometrial expression of other genes related to DNA damage and related pathways is dysregulated in endometriosis. Although the RNASeq data suggested differential expression of 24.8% of the total transcripts by at least 1.5-fold in women with endometriosis, compared to control women, its major limitation was the small sample size and hence it did not reveal significant number of differentially expressed genes with low FDR after multiple testing correction. Therefore, the data were validated for the expression of genes previously reported for their dysregulation in endometriosis and also compared with public datasets.

ARID1A (AT-Rich Interactive Domain-Containing Protein 1A) was selected as there exist several reports indicating a down-regulation in its endometrial expression in endometriosis (Kim et al., 2015; Xie et al., 2017). Our RNASeq data also indicated a down-regulation in the levels of ARID1A transcripts in EE-MP, compared to CE-MP samples. A decrease in the endometrial ARID1A expression was also observed at the protein level (Supplementary Fig. S4A–C). Further in situ localization indicated a significant down-regulation ($P < 0.05$) in the expression of ARID1A protein in the glandular epithelial compartment of EE-MP, compared to that of CE-MP (Supplementary Fig. S4D–F).

IPA revealed enrichment of DETs in canonical pathways such as ‘GADD45 signalling’, ‘p53 signalling’, ‘ATM signalling’, ‘G1/S checkpoint regulation’ and ‘G2/M DNA damage checkpoint regulation’. This corroborated our data indicating an up-regulation in the endometrial DDR in women with endometriosis, compared to control women (Supplementary Fig. S5A). Interestingly, among diseases and disorders, ‘cancer’ was found to be one of the most enriched terms accounting for 3921 DETs in EE-MP (Supplementary Fig. S5B). Also, molecular and cellular functions such as ‘cellular growth and proliferation’ and ‘cell death and survival’ were found to be enriched by the DETs in EE-MP, compared to CE-MP (Supplementary Fig. S5C). The transcripts encoding various activators of functions, namely ‘cell proliferation’ and ‘repair of DNA’ were also up-regulated, whereas those encoding the inhibitors of these functions showed down-regulation in EE-MP, compared to CE-MP (Supplementary Fig. S5D).

To investigate whether the proliferation in eutopic endometrium is indeed higher in endometriosis, the expression of PCNA (a proliferative marker) was compared in EE-MP and CE-MP samples. EE-MP samples were found to have significantly ($P < 0.05$) higher expression of PCNA, compared to CE-MP (Fig. 1N–P). The increase was restricted to the stromal compartment, as revealed by the immunohistochemical localization (Fig. 1M and Supplementary Fig. S6A). A higher stromal cell count per unit area, compared to that in CE-MP, reaffirmed a higher proliferative index in EE-MP (Supplementary Fig. S7). Thus, both cell proliferation and DDR were found higher in EE-MP, compared to CE-MP.

Next, the levels (FPKMs) of transcripts encoded by all the human DNA repair genes [enlisted in Wood et al. (2005) and Iyama and Wilson (2013)] were compared between EE and CE samples (Fig. 2A and Supplementary Fig. S8). A majority of these genes showed a trend towards higher expression in EE, compared to CE. Interestingly, the levels of transcripts related to non-homologous end joining (NHEJ) repair pathway were found higher in EE, compared to CE samples. These included PNP (Polynucleotide Kinase 3'-Phosphatase) ($P < 0.05$), POLM (DNA Polymerase Mu) ($P = 0.07$) and POLL (DNA Polymerase Lambda) ($P = 0.05$).

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Figure 2. Mid-proliferative phase eutopic endometrial samples from women with endometriosis show higher expression of select DNA repair genes. (A) Relative levels of the transcripts encoded by DNA repair genes implicated in various repair pathways such as mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), Fanconi anaemia (FA) and non-homologous end joining (NHEJ) in EE and CE samples. Fragment per kilobase per million reads (FPKMs) of only the full length (for genes encoding more than one full-length transcript, the one that showed higher FPKM values was considered) or the second-longest (in cases where full-length transcript was not detected) transcripts of interest were compared in EE ($n = 4$) and CE ($n = 3$) samples. (B–D) Residual DNA damage in eutopic endometrial cells from women with and without endometriosis, as assessed by alkaline comet assay. (B) Images (a and c) and respective zoomed areas (b and d) of DNA comets of primary endometrial cells. Scale bar in (B) indicates 100 µm size. (C and D) Percentage of damaged cells and mean tail length of comets, respectively, in CE-MP ($n = 3$) and EE-MP ($n = 3$) samples (*$P < 0.05$).
Analysis of the expression data from proliferative phase eutopic endometrial samples from women without ($n = 20$) and with endometriosis ($n = 24$) in GSE51981 reaffirmed the trend observed in our RNASeq data (Supplementary Fig. S9). Endometrial PNKP, POLM and POLL were significantly up-regulated in women with endometriosis, compared to those without the disease. This hinted at higher activation of NHEJ, an error-prone repair pathway, in the eutopic endometrium of women with endometriosis, compared to those without endometriosis.

Among the down-regulated genes, transcript levels of only RPA2 (Replication Protein A2) were significantly different between EE-MP and CE-MP (Fig. 2A). This was in agreement with the trend observed for RPA2 in the GSE51981 dataset (Supplementary Fig. S9).

Interestingly, the percentage of cells with residual DNA damage was comparable in mid-proliferative phase EE and CE samples (Fig. 2D). Although derived from a small sample size ($n = 3$ in each group) (Supplementary Fig. S10), these observations suggest that higher expression of DDR and DNA repair genes may help in counteracting higher DNA damage encountered by the eutopic endometrium in women with endometriosis.

Overall, results suggest that eutopic endometrial cells in endometriosis, compared to their counterparts in women without endometriosis, encounter higher level of DNA damage (as indicated by significantly higher intensity of immunolocalized $\gamma$H2AFX) and consequently evoke greater DDR (as indicated by the up-regulation of DDR genes) to repair their genome (as revealed by the up-regulation of DNA repair genes and comparable percent of cells with damaged DNA).

**DDR and DNA repair in the eutopic endometrium of women with endometriosis during the mid-secretory phase of the menstrual cycle**

**Endometrial DDR in endometriosis**

Mid-secretory phase eutopic endometrial samples from women with endometriosis (EE-MS), compared to phase-matched eutopic endometrial samples from women without endometriosis (CE-MS), showed significant ($P < 0.05$) up-regulation in the expression of PRKDC at the transcript level in EE-MS, compared to CE-MS (Fig. 3A). A trend towards higher expression, albeit non-significant, was observed for ATR, ATRIP, CDKN1A, DDIT3, FANCA, GADD45A, MAPK12, NTHL1, TP73, XRCC3, MLH3 and PMS1 transcripts in EE-MS, compared to CE-MS. Interestingly, as observed in the mid-proliferative phase comparisons of EE and CE, mid-secretary phase EE also showed a trend towards higher expression of DDIT3, GADD45A, MAPK12, TP73 and XRCC3, compared to phase-matched CE.

MAPK12, XRCC3, CDKN1A and FANCA were among the significantly up-regulated genes in the mid-secretory phase endometrial samples from women with endometriosis ($n = 27$) compared to those without endometriosis ($n = 8$), as revealed by the analysis of GSE51981 dataset. The expression of NTHL1 was also found up-regulated in the eutopic endometrial samples from women with endometriosis, compared to those without the disease (Supplementary Fig. S11).

RT$^2$ Profiler PCR array data for the mid-secretory phase EE and CE were validated at the protein level. EE-MS samples were found to have significantly ($P < 0.05$) higher expression of GADD45A, compared to CE-MS samples (Fig. 3B and D). On the other hand, the endometrial expression of GADD45G protein was significantly ($P < 0.05$) lower in EE-MS, compared to CE-MS (Fig. 3E and G). This was in contrast to the significant up-regulation observed in the GADD45G levels in the mid-proliferative phase comparisons.

**Localization of DNA damage markers in endometriosis**

Next, we investigated whether the mid-secretory phase eutopic endometrium also shows signs of DNA damage. Immunohistochemical analysis revealed a significant ($P < 0.05$) increase in $\gamma$H2AFX-positive foci in the glandular epithelial cells of EE-MS, compared to those of CE-MS (Fig. 3H and Supplementary Fig. S3B). Unlike the mid-proliferative phase EE, wherein $\gamma$H2AFX positive foci were predominantly localized in the glandular epithelial compartment, mid-secretory phase EE showed immunoreactivity for $\gamma$H2AFX in both glandular epithelial and stromal compartments. Further in both the compartments, immunostaining scores for $\gamma$H2AFX were significantly ($P < 0.05$) higher in EE-MS, compared to their counterparts in CE-MS (Fig. 3I and J).

**Endometrial DNA repair and cell proliferation in endometriosis**

Analysis of RNASeq data from the mid-secretory phase samples (accession no-GSE153740) revealed enrichment of canonical pathways such as ‘cyclins and cell cycle regulation’, ‘G1/S checkpoint regulation’, ‘p53 signaling’, ‘ATM signaling’ and ‘role of BRCA1 in DNA damage response’ by the DETs in EE-MS (Supplementary Fig. S12A). As also revealed in the comparative analysis of the mid-proliferative phase samples, ‘cancer’ emerged as one of the most enriched diseases by the transcripts displaying differential expression in EE-MS, compared to CE-MS (Supplementary Fig. S12B). Molecular and cellular functions known to be involved in ‘cellular growth’ and ‘cell death and survival’ were enriched by the transcripts displaying differential expression in EE-MS, compared to CE-MS (Supplementary Fig. S12C). However, in contrast to the conclusions derived from the mid-proliferative phase analysis of RNASeq data, ‘cell proliferation’ and ‘repair of DNA’ were predicted to be inhibited in EE samples, compared with their counterparts in women without endometriosis during the mid-secretary phase (Supplementary Fig. S12D). ‘Inhibition of cell proliferation’ was indicated by reduced expression of PCNA in EE-MS, compared to phase-matched CE-MS (Supplementary Fig. S6B and Fig. 3K–P). A reduction in the expression of PCNA, though statistically non-significant, was evident in the stromal compartment (Fig. 3M). Immunoblotting analysis also revealed a decrease in endometrial PCNA expression in EE-MS, compared to CE-MS samples (Fig. 3N–P).

In contrast to the trend revealed by mid-proliferative phase endometrial samples, mid-secretary phase samples demonstrated reduced expression of a majority of transcripts encoded by DNA repair genes. MSH6 ($P = 0.047$) of mismatch repair (MMR) pathway; PARP2, UNG ($P = 0.03$) and TDG ($P = 0.04$) of base excision repair (BER) pathway; GEN1 and RAD51 ($P = 0.04$) of homologous recombination (HR) pathway; and FAPP2 ($P = 0.02$) of the FA pathway showed down-regulation in EE-MS compared to CE-MS (Fig. 4). Among the DETs,
Figure 3. Mid-secretory phase eutopic endometrial samples from women with endometriosis also show signs of higher DNA damage and increased (though insignificant) expression of select DNA damage response genes. (A) Volcano plot to represent the relative expression of DNA damage signalling-associated genes in eutopic endometrium of women with endometriosis (EE, n = 7), compared to those without endometriosis (CE, n = 8). △ indicates a gene displaying differential expression by at least 1.5-fold in endometriosis, compared to control and Δ in green indicates a gene displaying differential expression by at least 1.5-fold with P-value < 0.05. Red dotted line indicates −log2(P-value), P = 0.05. (B–D) Immunodetection of GADD45A and GADD45G proteins, respectively, in EE (EE1–EE3) and CE (CE1–CE3) samples. (B and E) Representative images indicating chemiluminescent detection of endometrial GADD45A and GADD45G proteins. (C and F) Coomassie
only REV1 (P < 0.05) and POLN (P < 0.01) showed a significant up-regulation in EE-MS, compared to CE-MS samples (Supplementary Fig. S13).

Analysis of GSE51981 dataset revealed the same trends, i.e. reduced expression of MSH6 (P < 0.001), TDG (P < 0.001), PARP2 (P < 0.01) and GEN1 (P < 0.01) in the mid-secretory phase eutopic endometrium of women with endometriosis (n = 27), compared to those without endometriosis (n = 8). Other DDR transcripts such as RIF1, DCLRE1A and DUT were also found to be down-regulated in EE-MS, compared to CE-MS in our study (Supplementary Fig. S13), as well as in GSE51981 (Supplementary Fig. S14).

Although more investigations on larger sample size are warranted, it is likely that the DNA repair capacity in the eutopic endometrial cells of women with endometriosis tapers off from the mid-proliferative-to-mid-secretory phase in the menstrual cycle.
Discussion

According to one estimate, eukaryotic DNA spontaneously accumulates 10^8 lesions per day due to misincorporation, deamination, depurination, methylation and oxidation of DNA bases (Hoeijmakers, 2009). Eukaryotic cells repair these lesions through different pathways such as MMR, BER, nucleotide excision repair (NER), intra-strand crosslink repair, single-strand break repair, DSBR, NHEJ and HR.

DDR pathway is one of the earliest events that occur in response to DNA damage. The pathway involves the activation of various DDR proteins and their recruitment to the site of damage (Podhorecka et al., 2010). Transcriptional changes, especially those mediated by p53, also ensue in response to DNA damage (Riley et al., 2008). Collectively, these processes ensure genomic integrity either by repairing the damage or eliminating the cells with damaged DNA.

The ability to elicit a response to damaged DNA is likely to be modulated in cells that are chronically exposed to environmental toxicants, mutagens or dysregulated levels of endogenous biomolecules. We hypothesized that the DDR in the eutopic endometrial cells is modulated in endometriosis. The hypothesis partly stemmed from the unequivocally established role of estrogens in the pathophysiology of endometriosis (Zondervan et al., 2018). Estrogen and its metabolites have been recognized as genotoxic mutagens (Liehr, 2000). Evidence derived from animal studies implies a causal relationship between exposure to environmental agents such as dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin and endometriosis (Rier et al., 1999). However, human data in this direction remain inconclusive (Guo, 2004). Therefore, it remains to be established whether environmental exposure to toxicants modulates endometrial DDR in endometriosis. On the other hand, there are ample data that link higher oxidative stress and inflammation with endometriosis (Cheong et al., 2002; May et al., 2010; Kitajima et al., 2014; Da Broi and Navarro, 2016). Thus, there is a strong possibility of these two events modulating the endometrial DDR in endometriosis. Also, an innate dysregulation in the DNA repair machinery may affect the endometrial DDR in endometriosis. Taiwanese women carrying single-nucleotide polymorphisms in Excision Repair Cross Complementation group 1 genes (ERCC1, ERCC2 and ERCC6) involved in the NER pathway were found to have a higher risk of developing endometriosis (Shen et al., 2019). Likewise, polymorphisms in other DNA repair genes such as XRCC4 (Hsieh et al., 2008); XRCC1 (Bau et al., 2007; Hsieh et al., 2012), KCNQ2, ATIR and hOGGI (Hsieh et al., 2012) are reported to be associated with endometriosis.

The present study reports differential expression of DDR genes in the eutopic endometrium of women with endometriosis, compared to women without endometriosis. Interestingly, GADD45A, DDIT3 and MAPK12, which have all been reported to be involved in cell cycle arrest in response to stress stimuli, showed a similar expression pattern, i.e. higher expression in EE compared to CE, during the mid-proliferative as well as mid-secretory phases of the menstrual cycle. Furthermore, significantly higher immunolocalization of γ-H2AFX foci in EE, compared to CE, was evident in both mid-proliferative and mid-secretory phases of the cycle. This was suggestive of the persistence of stimuli that induce DNA damage in the eutopic endometrium of women with endometriosis.

It was intriguing to note that the higher DNA damage did not affect the proliferative status of mid-proliferative phase eutopic endometrium in endometriosis. A significant increase was observed in the stromal cell count and expression of PCNA (a proliferation marker), in EE-MP, compared to CE-MP. This paradox raised two possibilities. The first possibility is that DNA damage occurs only in the epithelial cells and, hence, stromal cells continue to proliferate. This possibility was negated by a higher level of base oxidation product 8-OHdG observed in the stromal compartment of EE-MP, compared to CE-MP. The second possibility is reduced sensitivity of EE cells to DNA damage-induced quiescence. It is likely that the damage induced is of low grade and proliferation-inducing stimuli outweigh the DNA damage-inducing stimuli (Heldt et al., 2018).

A large number of DNA repair genes showed a trend towards higher expression in the mid-proliferative phase EE samples compared to the phase-matched CE samples. Interestingly, analyses of our RNAseq data and the GSE51981 dataset revealed higher expression of the repair genes associated with NHEJ in EE-MP, compared to CE-MP. It may be added here that NHEJ is an error-prone DNA repair pathway. Detailed investigations are warranted to determine whether an up-regulated NHEJ pathway leads to genomic alterations in the eutopic endometrial cells. It will be of interest to investigate whether the cells with a modified genome have a higher propensity to form endometriotic lesions. Indeed there exist reports to suggest the presence of sub-clonal mutations in the epithelial cells of normal endometrium (Lac et al., 2019b). However, it remains to be established whether eutopic endometrial cells from women with endometriosis, compared to their healthy counterparts, have a higher frequency of sub-clonal mutations.

In the mid-secretory phase EE, expression of several DNA repair genes was found to be reduced, compared to the phase-matched CE samples. Mid-secretory phase endometrial cells with their differentiated phenotype are likely to have reduced DNA repair efficiency, compared to proliferative phase endometrial cells. It is postulated that terminally differentiated cells invest their DNA repair machinery only for the repair of expressed genes (Nouspikel and Hanavalt, 2002). Down-regulation in the expression of DNA repair genes in differentiated EE-MS cells is likely to affect the fidelity of transcription and consequently the activity or expression of translated proteins. It will be of interest to determine whether the transcription-coupled repair is active or dysfunctional in the mid-secretory phase EE and if this has any implications on the survival and proliferation of endometrium at ectopic sites.

To the best of our knowledge, there are only three reports (Hapangama et al., 2008; Choi et al., 2018; Dai et al., 2019) on investigations of DNA damage in the eutopic endometrium of women with endometriosis. The present study reaffirms some of these observations and presents some novel inferences. We observed significantly higher proliferation in the stromal compartment of mid-proliferative phase endometrial samples from women with endometriosis, as previously reported by Hapangama et al. (2008). However, in contrast to their other findings, we did not observe sustenance of the proliferation in the mid-secretory phase endometrial samples from women with endometriosis. This could be attributed to the different clinical characteristics of the patients in our study. While Hapangama et al. (2008) investigated eutopic endometrial samples from women with peritoneal endometriosis, a majority of our study participants had ovarian endometriosis, akin to the study by Choi et al. (2018). Our observations corroborate the inference drawn by Choi et al. (2018) indicating a higher level of γ-H2AFX in the eutopic endometrium of
women with endometriosis, compared to its counterparts in women without endometriosis. Dai et al. have recently reported lower levels of BARD1 (a component of BRCA1 complex) and BRCA1 in the eutopic endometrium from women with DIE, compared to the samples from women without endometriosis (Dai et al., 2019). This study indirectly hints at an aberrant DDR in the eutopic endometrium in women with DIE. It is likely that the basal DDR and DNA repair capacity differ in the eutopic endometrium of women presenting with different subtypes of endometriosis.

A major limitation of the present study is its small sample size, especially for RNASeq investigations. To gain higher confidence, we scouted public domain for the datasets on gene expression profiling of eutopic endometrium in endometriosis derived using a larger sample size. Only one dataset (GSE134056) (Akter et al., 2019) in the GEO database was derived using RNASeq. However, this dataset did not provide participant details (menstrual cycle phase/clinical features). Among the microarray-based datasets (GSE120103, GSE25628, GSE6364, GSE1981), GSE1981 was the largest dataset with gene expression profiles from well-characterized histologically dated eutopic endometrial samples from women with and without endometriosis (Tamaresis et al., 2014). A cross-comparison of our observations with this dataset revealed concordance for a sizeable number of DDR/DNA repair genes in terms of their endometrial expression in endometriosis. Interestingly, this dataset used for the construction of weighted gene co-expression network showed enrichment of a major functional module with DNA repair genes in endometriosis (Bakhtianazadeh et al., 2018). This reiterates that aberrations in DDR and DNA repair pathways in the eutopic endometrium are major pathobiological events in endometriosis.

Although age and BMI were not significantly different between the two groups, the endometriosis cohort had a sizeable number of infertile women (31/40). This raises a possibility that the effects observed on the expression of DDR and DNA repair genes in the endometriosis group were caused by infertility rather than by the disease per se. This possibility could have been ruled out only by investigating eutopic endometrial samples from infertile women without endometriosis or any other coexisting uterine or pelvic pathology.

In brief, the present study demonstrates that eutopic endometrial cells in women with endometriosis experience higher genomic insults but are capable of mounting a DDR and repair their genome, at least in the proliferative phase of the menstrual cycle. Furthermore, increased expression of the DDR genes was not associated with a stall in the proliferation of EE cells. Higher expression of DNA repair genes in the mid-proliferative phase probably helps in countering higher DNA damage encountered by eutopic endometrial cells, as revealed by comparable residual damage observed in control and endometriosis groups. However, in the mid-secretory phase, endometrial expression of DNA repair genes is reduced in women with endometriosis, compared to control women. Thus, menstrual cycle phase-dependent alterations were observed in the endometrial DDR in women with endometriosis. It will be worthwhile to explore the effect of dysregulated DDR and DNA repair in the uterine endometrium on lesion formation/progression in endometriosis.

Supplementary data
Supplementary data are available at Human Reproduction online.

Data availability
The data underlying this article (accession numbers GSE153739 and GSE153740) are available in the Gene Expression Omnibus (GEO) database.

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Authors’ roles
K.B. carried out execution of the major experiments such as RNA isolation, qRT-PCR, immunoblotting, RNA Seq data analysis, data compilation, and manuscript preparation. J.D., D.S., S.K. and P.C. were involved in the collection of samples, sample processing and immunohistochemical analyses. R.R.K. carried out histological and immunohistochemistry experiments. G.F. classified endometrial samples based on histological dating. R.S., U.D., N.W. and A.C. were involved in participant recruitments, consent administrations, clinical categorization and collection of endometrial samples. U.C. provided scientific inputs and assisted in statistical analyses and manuscript editing. R.G. provided clinical insights and scientific inputs and also coordinated the study. G.S. was involved in designing the study, planning and execution of experiments, data interpretation and manuscript writing.

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Conflict of interest
Nothing to disclose.

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