





# Genetic and epigenetic changes in the eutopic endometrium of women with endometriosis: association with decreased endometrial $\alpha_v\beta_3$ integrin expression

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**ABSTRACT:** About 40% of women with infertility and 70% of women with pelvic pain suffer from endometriosis. The pregnancy rate in women undergoing IVF with low endometrial integrin  $\alpha_v\beta_3$  (LEI) expression is significantly lower compared to the women with high endometrial integrin  $\alpha_v\beta_3$  (HEI). Mid-secretory eutopic endometrial biopsies were obtained from healthy controls (C; n=3), and women with HEI (n=4) and LEI (n=4) and endometriosis. Changes in gene expression were assessed using human gene arrays and DNA methylation data were derived using 385K Two-Array Promoter Arrays. Transcriptional analysis revealed that LEI and C groups clustered separately with 396 differentially expressed genes (DEGs) ( $P<0.01$ : 275 up and 121 down) demonstrating that transcriptional and epigenetic changes are distinct in the LEI eutopic endometrium compared to the C and HEI group. In contrast, HEI vs C and HEI vs LEI comparisons only identified 83 and 45 DEGs, respectively. The methylation promoter array identified 1304 differentially methylated regions in the LEI vs C comparison. The overlap of gene and methylation array data identified 14 epigenetically dysregulated genes and quantitative RT-PCR analysis validated the transcriptomic findings. The analysis also revealed that aryl hydrocarbon receptor (AHR) was hypomethylated and significantly overexpressed in LEI samples compared to C. Further analysis validated that AHR transcript and protein expression are significantly ( $P<0.05$ ) increased in LEI women compared to C. The increase in AHR, together with the altered methylation status of the 14 additional genes, may provide a diagnostic tool to identify the subset of women who have endometriosis-associated infertility.

**Key words:** integrin  $\alpha_v\beta_3$  / endometriosis / epigenetics / gene expression / infertility / eutopic endometrium / aryl hydrocarbon receptor / methylation

## Introduction

Endometriosis is estimated to affect 10–15% of reproductive-aged women. Up to 40% of women with infertility have endometriosis, and over 70% of women with chronic pelvic pain suffer from this condition (Practice Committee of the American Society for Reproductive Medicine, 2006; Bulun, 2009). The predominant mechanism for the

development of endometriosis is thought to be the result of retrograde menstruation in susceptible women (Halme *et al.*, 1984). Although 90% of women undergo retrograde menstruation, there is a need to better understand which genetic, epigenetic, and/or environmental factors predispose some women to develop endometriosis. More importantly, endometriosis itself has significant implications for a woman's fertility as well as chronic pelvic pain or dysmenorrhea

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(Miller et al., 2012, 2017; Tanbo and Fedorcsak, 2017). Assessment of endometrial histology and expression of receptivity markers, such as integrin status (Ceydeli et al., 2006; Chen et al., 2016; Dorostghoal, 2017), may be useful to identify women with defects in uterine receptivity and have the added utility to predict which patients could benefit from medical or surgical intervention (Lessey et al., 1995; Lessey, 2000; Germeyer et al., 2014). Spatial and temporal expression of integrins, which are heterodimeric glycoproteins, have been observed to undergo a dynamic change in the endometrium during the menstrual cycle in women and non-human primates along with extracellular matrix remodeling (Fazleabas et al., 1997; Lessey, 1998). Integrins are a group of cell surface receptors that play critical roles in cell-matrix adhesion and, upon ligand binding, transduces external signals to the cytoskeleton (Giancotti and Ruoslahti, 1999; Salvatori et al., 2014). The pregnancy rate in women undergoing IVF with low  $\alpha_v\beta_3$  integrin expression was significantly lower compared to the women with higher  $\alpha_v\beta_3$  expression (Miller et al., 2012). It is possible that low integrin  $\beta_3$  levels might be related to the histologic delay in the endometrium, as it does not appear until Day 20 of the menstrual cycle. This phenomenon is also referred to as a Type I defect (Lessey et al., 1995; Lessey, 1998). The lower expression of integrin  $\beta_3$  and 'in phase' glandular histology (Day 20 and beyond) in the eutopic endometrium of women with endometriosis is referred to as Type II endometrial defect (Lessey et al., 1994; Lessey, 2000).

Epigenetic changes are reversible changes that do not alter the nucleotide sequence but still regulate gene expression and act as a bridge between the environmental signals and genetics (Zhang and Ho, 2011). It is very well established that epigenetic mechanisms regulate female reproductive tract functions and contribute to the non-malignant and malignant pathogenesis of gynecological disorders, including endometriosis (Kim et al., 2007; Izawa et al., 2013; Jones et al., 2013; Dyson et al., 2014; Naqvi et al., 2014; Houshdaran et al., 2016; Kim et al., 2019; Houshdaran et al., 2020). One of the earliest reports related to the aberrant methylation changes in endometriosis focused on the methylation of CpG islands within the 5' untranslated region of the HOXA10 gene (Wu et al., 2005). HOXA10, a homeobox transcription factor, is critical for normal uterine functions and is hypermethylated in the endometrium of baboons and women with endometriosis (Wu et al., 2005; Kim et al., 2007). Subsequent studies have attempted to analyze the epigenetic alterations between: the endometrium of women with and without endometriosis (Houshdaran et al., 2016; Akter et al., 2019); isolated endometrial stromal cells from eutopic and ectopic endometrium (Yamagata et al., 2014); isolated endometrial stromal cells from women with and without endometriosis (Yotova et al., 2017); and *in vitro* decidualized endometrial stromal cells from normal or diseased endometrium (Logan et al., 2012; Maekawa et al., 2019). These comparisons are very valuable and reveal the extent of epigenetic alterations on phenotypic changes in the endometrium of women with endometriosis (Izawa et al., 2013). However, one of the outstanding questions has been to determine the molecular differences between the subset of women who can get pregnant with endometriosis compared to those who cannot. The opportunity for surgical intervention exists such that women who are operated upon for endometriosis see an improvement in their fertility regardless of the amount of disease at the time of surgery (Marcoux et al., 1997; Joshi et al., 2017). These observations further support the fact that epigenetic changes play a critical role in the

pathophysiology of endometriosis and could be reversed by surgical and/or therapeutic interventions. However, there is a significant need to understand the epigenetic regulations in the context of endometriosis-associated infertility.

This study aims to investigate the transcriptomic and epigenetic changes in the eutopic endometrium during the window of implantation in women with endometriosis and low  $\alpha_v\beta_3$  endometrial expression compared to disease-free controls. We hypothesize that women with endometriosis together with low  $\alpha_v\beta_3$  endometrial expression have a distinct transcriptomic signature, which is influenced by epigenetic modification that may contribute to the observed infertility in a subset of women with endometriosis.

## Materials and methods

### Human sample acquisition

Endometrial biopsies were obtained from women undergoing a workup for infertility at Greenville Health System (Division of Reproductive Endocrinology and Infertility) and the University of North Carolina, per IRB (Pro00013885) protocols approved by both institutions. Samples included timed endometrial biopsies in the mid-secretory phase as well as endometrium obtained in the proliferative phase. None of the subjects had been on hormone therapy for the preceding 3 months. The control samples represent endometrium obtained at laparoscopy or timed to the LH surge in the clinic. In the treatment group, endometriosis was diagnosed at laparoscopy by experienced gynecological surgeons and confirmed by pathology when present. Endometrial biopsies from these patients were saved for immunohistochemistry, and RNA and DNA isolation. The endometrial biopsies were histologically assessed for  $\alpha_v\beta_3$  expression. For protein isolation (western blots), human endometrial samples were obtained through the Michigan State University's Center for Women's Health Research Female Reproductive Tract Biorepository and Spectrum Health Universal Biorepository (Grand Rapids, MI, USA) (IRB- 2010-085). Participants were 18–45 years of age and had regular menstrual cycles. The details of the samples utilized in this study are provided in the [Supplementary Tables SI and SII](#).

### DNA and RNA isolation

Mid-secretory eutopic endometrial biopsies (25 mg) were processed for genomic and DNA and RNA isolation. Genomic DNA was extracted using the QIAGEN DNA isolation kit following the manufacturer's protocol (QIAGEN, Valencia, CA, USA). Total RNA was isolated using Trizol reagent (Invitrogen, Waltham, MA, USA) as previously described (Joshi et al., 2017), and an RNA quality check was performed using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA).

### DNA methylation analysis

DNA methylation data from low endometrial Integrin  $\alpha_v\beta_3$  (LEI) (n=4), high endometrial integrin  $\alpha_v\beta_3$  (HEI) (n=4), and controls (C, n=3) groups were obtained using the Roche Nimblegen 385 K Two-Array DNA Methylation Promoter Arrays. Using the NimbleGen Dual-Color DNA Labeling Kit, labeling of the immunoprecipitated and input DNA samples with Cy5 and Cy3 was performed. Differential DNA methylation analysis was performed in four steps using statistical

software R (<http://www.R-project.org/>, Vienna, Austria). In the first step, raw data from GPR files were imported and normalized using a LOESS-like method. In the second step, a linear model with Group as the only parameter was fitted for the  $M$  value (ratio of IP/Input in log<sub>2</sub> scale) of each probe separately using limma (Smyth, 2004). Appropriate contrasts were then used to calculate the difference between HEI vs C, LEI vs C, and LEI vs HEI. In the third step, a sliding window approach was used to identify the enriched differentially methylated regions (DMRs). Specifically, a RandomSet (Newton *et al.*, 2007) analysis was performed on probe scores ( $-\log_{10}$  transformed  $P$ -value derived in step two) for each sliding window of 750 bps. The sliding windows with  $<5$  probes were ignored. In the last step, a gene-level differential methylation result was generated where each the gene was summarized by the most significant differentially methylated window in the  $-3.5$  k to  $+0.75$  k promoter region in each comparison.

## Expression analysis

RNA expression data from the same samples used for DNA methylation analysis were derived using Affymetrix Human Gene 1.0 ST Arrays, which covers 32 020 well-established annotated RefSeq coding transcripts. The data were aligned to human reference genome hg18 and analyzed using R, and Bioconductor packages with custom CDF downloaded (Dai *et al.*, 2005). Data were preprocessed using Robust Multiarray Analysis and quality assessed using arrayQualityMetrics. Differential expression analysis was performed using limma (Smyth, 2004). Functional categories from Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) associated with differential expression and methylation were discovered using clusterProfiler (Sartor *et al.*, 2009).

The typical limitation of probe-based technologies, such as a microarray, is the small number of observations per gene. This produces low power for statistical tests like Student's  $t$ -test, the most widely used statistical method in genetic studies for comparing two groups, owing to instability in estimating gene-specific variances (Tusher *et al.*, 2001; Smyth *et al.*, 2003). When sample sizes are small, it would be even harder to observe low  $P$ -values. In this study, the limited sample size combined with individual genetic variations among patients resulted in low statistical power, which was inflated after correction for multiplicity. As a result, we were unable to identify differentially expressed genes (DEGs) that were significantly changed when applying a false discovery rate (FDR)  $=0.05$ . In order not to compromise the biological context of this research and to avoid weakening the biological relevance of the data, unadjusted  $P$ -values are reported for the list of the DEGs. However, in this study, we tested and validated most candidate genes by quantitative RT-PCR (qRT-PCR) analysis to confirm changes in expression.

## qRT-PCR techniques

qRT-PCR was performed as previously described (Joshi *et al.*, 2017). Briefly, for mRNA analysis, total RNA (1000 ng) was reverse transcribed using a High-Capacity cDNA synthesis kit (ThermoFisher Scientific, Waltham, MA, USA). After cDNA synthesis, the qRT-PCR reaction was carried out for genes using specific primers (Supplementary Table SIII) for nine genes listed in Table I, including aryl hydrocarbon receptor (AHR) and 18S (internal control) with SyBrGn PCR master mix (ThermoFisher Scientific, Waltham, MA, USA). All quantitative real-time PCR (RT-PCR) reactions were run for

40 cycles, and fold change was calculated using  $\Delta\Delta C_t$  method (Livak and Schmittgen, 2001).

## Immunohistochemistry and H-SCORE analysis

Immunostaining for beta 3 integrin and AHR proteins was performed on formalin-fixed eutopic endometrial sections from women with and without endometriosis, as previously described (Germeyer *et al.*, 2014). Briefly, paraffin-embedded sections of each sample (5  $\mu$ m) were deparaffinized in xylene and rehydrated. Non-specific binding sites were blocked with 2% normal goat serum for 30 min at room temperature followed by overnight incubation at 4°C with either the mouse monoclonal SSA6 antibody specific to the beta 3 integrin (1:2000) or 1:400 a-AHR receptor (H-211) sc5579 (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Subsequently, sections were washed with PBS and incubated with biotinylated goat anti-mouse secondary antibody (Vectastain Elite ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) at a dilution of 1:200 for 30 min at room temperature. After rinsing with PBS, 3,3'-diaminobenzidine was used as a chromagen, and slides were counterstained with toluidine blue followed by dehydration in a graded series of ethanol, cleared in xylene, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). The resulting staining was evaluated by a single-blinded observer, and images were captured using the Spot Insight 4 Camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) mounted on a Nikon Eclipse E600 Microscope (Nikon Inc., Japan) (Joshi *et al.*, 2017). H-SCORE was calculated using the following equation:  $H-SCORE = \sum P_i (i+1)$ , where 'i' is the intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and  $P_i$  is the percentage of stained epithelial cells for each intensity, varying from 0% to 100%. Low intra-observer ( $r=0.983$ ;  $P<0.0001$ ) and inter-observer ( $r=0.994$ ;  $P<0.0001$ ) differences for H-SCORE in uterine tissues have been reported previously using this technique (Detre *et al.*, 1995; Jackson *et al.*, 2007). A low integrins H-SCORE was defined as  $\leq 0.7$  (Lessey *et al.*, 1994; Franasiak *et al.*, 2014).

## Western blot analysis

Endometrial tissues were lysed with lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4)), 2.5 mM EDTA, 0.125% Nonidet P-40 (vol/vol)) with both a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and a phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). A total of 20  $\mu$ g of total protein lysates were electrophoresed using SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). Casein (0.5% v/v) was used to block the membrane before exposure to antibodies against AHR and beta-actin overnight. Immunoreactivity was visualized by incubation with a horse-radish peroxidase-linked secondary antibody followed by exposure to enhanced chemiluminescence reagents according to manufacturer's instructions (GE Healthcare Biosciences, Piscataway, NJ, USA).

## Statistical analysis

Differences in gene expression were compared following normalization against the 18 s internal control gene. One-way ANOVA was used to test the null hypothesis of group differences, followed by Tukey's *post-hoc* test. For qRT-PCR analysis, gene expression was normalized

**Table 1** Genes that are differentially methylated and differentially expressed in the low endometrial  $\alpha_v\beta_3$  integrin expression vs healthy control comparison.

Gene symbol	Gene name	Gene expression data		Methylation array data	
		log2FC	RNA expression	PEAK	DNA methylation
<b>RNPC3</b>	RNA binding region (RNPI, RRM) containing 3	0.673	Up	-0.629	Decreased
<b>SLC18A2</b>	Solute carrier family 18 member A2	1.159	Up	-0.445	Decreased
SMCHD1	Structural maintenance of chromosomes flexible hinge domain containing 1	0.733	Up	-0.508	Decreased
SNORD58A	Small nucleolar RNA, C/D box 58A	1.353	Up	-0.518	Decreased
<b>PGLYRP2</b>	Peptidoglycan recognition protein 2	-0.629	Down	0.504	Increased
SNORD82	Small nucleolar RNA, C/D box 82	0.596	Up	-0.506	Decreased
<b>ANXA3</b>	Annexin A3	0.930	Up	-0.362	Decreased
<b>HIST1H4F</b>	H4 clustered histone 6	-0.657	Down	0.664	Increased
HIST1H3I	H3 clustered histone 11	-0.656	Down	0.928	Increased
<b>PGK2</b>	Phosphoglycerate kinase 2	1.073	Up	-0.449	Decreased
<b>AHR</b>	Aryl hydrocarbon receptor	0.834	Up	-0.545	Decreased
<b>CCDC146</b>	Coiled-coil domain containing 146	0.628	Up	-0.487	Decreased
AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5	0.586	Up	-0.828	Decreased
<b>PLEKHF2</b>	Pleckstrin homology and FYVE domain containing 2	1.117	Up	-0.507	Decreased

The genes highlighted in bold, and *italics* were validated by quantitative RT-PCR analysis to confirm the gene array results.

against 18 s followed by the Student's *t*-test for pair-wise comparison at a 95% CI ( $P < 0.05$ ) between negative control and endometriosis. The qRT-PCR data were analyzed for the *Q*-value using the equation:  $q_i = p_i \times N / i$ , where  $p_i$  is the  $i$ -th smallest *P*-value out of *N* total *P*-values of the experiment (Benjamini and Hochberg, 1995) for the correction of multiple testing. The data were analyzed using Graphpad Prism 8.0 statistical software (GraphPad Software, San Diego, CA, USA).

## Results

### Classification of endometrial samples based on integrin $\alpha_v\beta_3$ expression

Immunohistochemical evaluation of integrin  $\alpha_v\beta_3$  expression categorized endometrial biopsies into mid-secretory eutopic endometrium from disease-free control (C;  $n=3$ ), HEI expression and LEI expression women with endometriosis ( $n=4$ /group). H-score analysis for  $\alpha_v\beta_3$  expression revealed that LEI expression women with endometriosis had significantly reduced expression of  $\alpha_v\beta_3$  compared to disease-free control ( $P=0.0006$ ) and HEI expression women with endometriosis ( $P=0.0075$ ), as shown in Fig. 1A.

### The transcriptomic signature of eutopic endometrium is unique between controls and women with endometriosis

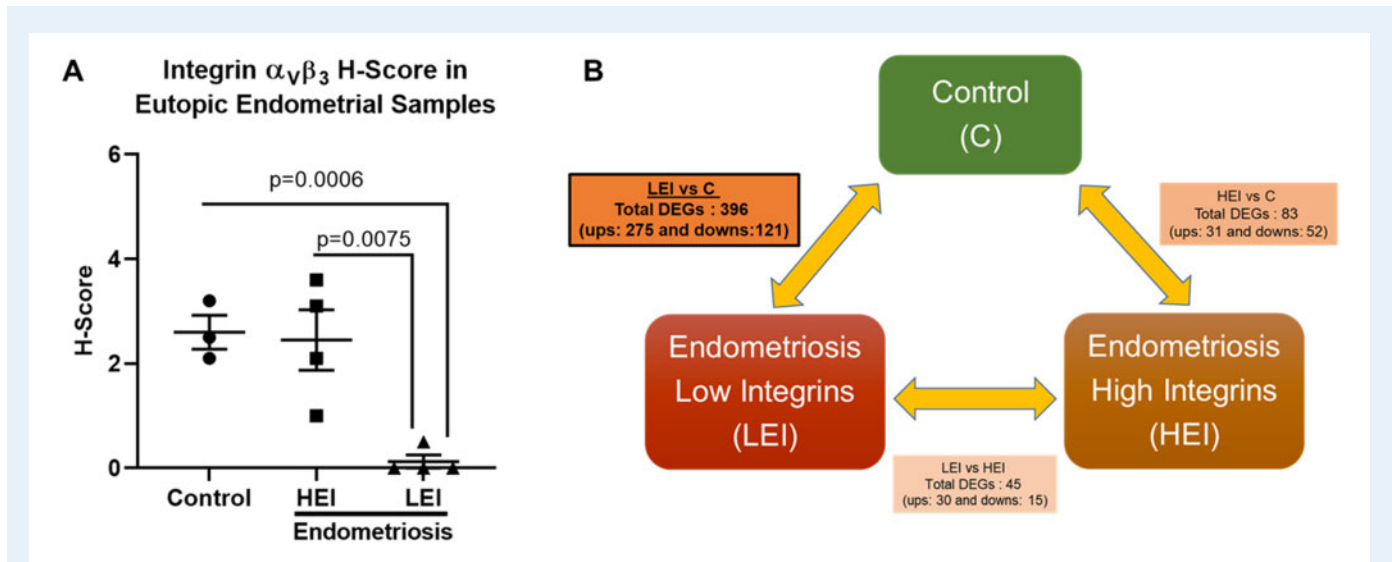
Eutopic endometrial biopsies from all three groups were analyzed for global gene expression profiles and genome-wide changes in promoter methylation patterns in the same samples to identify the influence of an altered methylation signature on gene expression patterns. The data

obtained from the gene expression arrays identified 396 DEGs (275 up and 121 down;  $P < 0.05$ , with  $\pm 1.5$ -fold change) in the LEI vs C group comparison. In contrast, 83 DEGs (31 up and 52 down) in the HEI vs C and only 45 (30 up and 15 down) DEGs in the LEI vs HEI comparisons were observed. We observed the highest gene expression changes in the eutopic endometrium between the low integrin endometriosis group vs the control group, suggesting that the gene expression signature of women with low  $\alpha_v\beta_3$  is unique compared to control or women with endometriosis with higher integrin  $\alpha_v\beta_3$  expression (Fig. 1B).

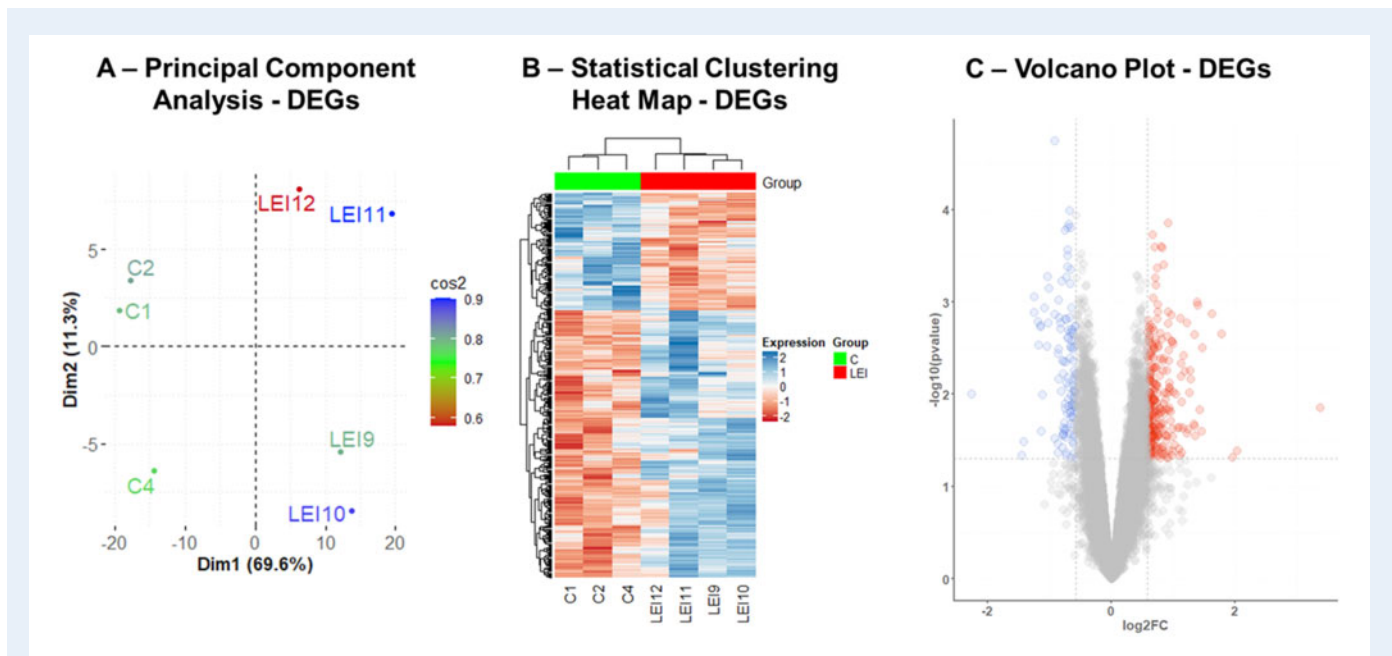
Principal component analysis (PCA) separated the LEI and control endometrial biopsy samples (Fig. 2A). Additionally, the hierarchical clustering of DEGs further supported the PCA analysis, and the LEI and C group samples clustered distinctly differently, as evident by the heatmap in Fig. 2B. The comparative analysis of the eutopic endometrial gene expression between the LEI and C group is represented in the Volcano plot (Fig. 2C). The PCA analysis, statistical clustering heatmap, and volcano plots for HEI vs C and LEI vs HEI comparisons are illustrated in Supplementary Figs S1A–C and S2A–C, respectively. In summary, these data demonstrated that a subset of women with endometriosis has significantly decreased integrin  $\alpha_v\beta_3$  expression along with a higher number of DEGs compared to controls, which might be the primary reason for observed infertility in this subset of women. These observations made a compelling case to primarily focus on the LEI vs C comparison in this study.

### Functional analysis of DEGs (LEI vs C) in eutopic endometrium of low integrin endometriosis women compared to controls

It has been reported that a subset of women with endometriosis and unexplained infertility have low expression of integrin  $\alpha_v\beta_3$  in the



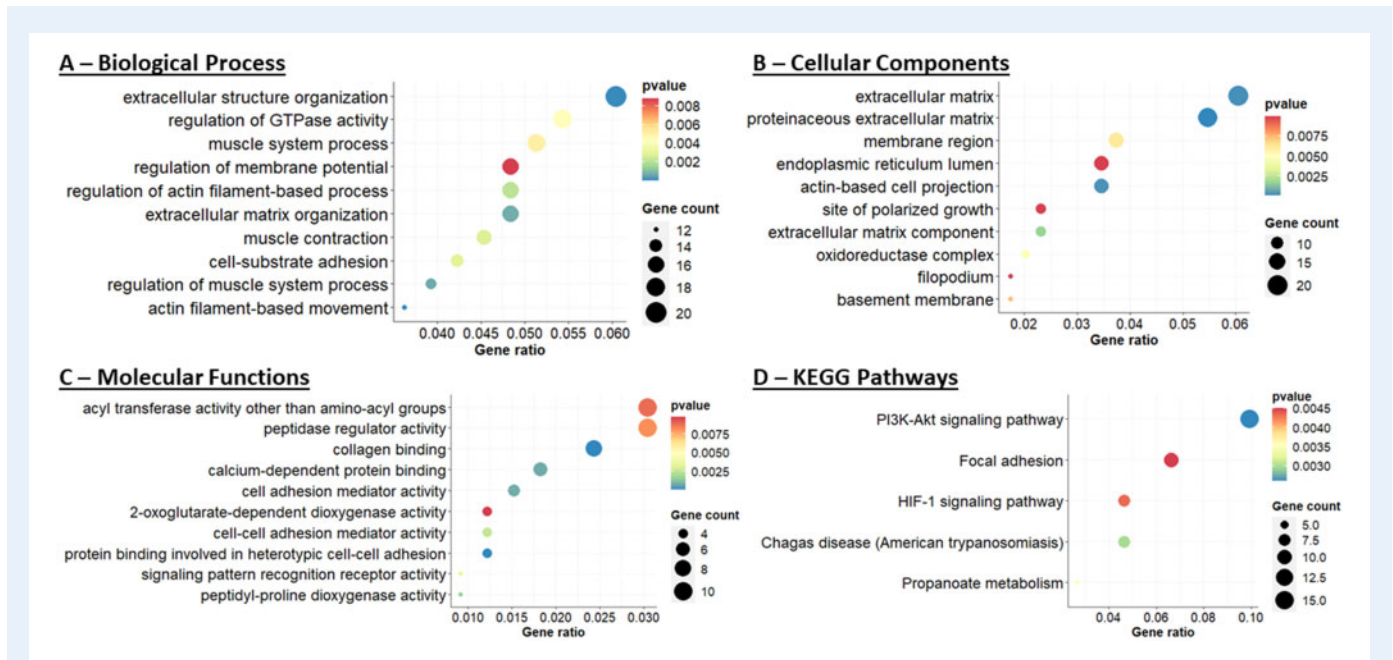
**Figure 1. Integrin  $\alpha_v\beta_3$  expression is significantly decreased in the subset of women with endometriosis compared to controls.** H-score analysis shows that integrin  $\alpha_v\beta_3$  expression is significantly decreased in the subset of women with endometriosis compared to controls (A). Microarray performed on mid-secretory eutopic endometrial samples from three groups of women demonstrates that the highest number of DEGs was observed in the low endometrial integrin  $\alpha_v\beta_3$  (LEI) vs controls (C) comparison (B). Data are displayed as mean  $\pm$  SD and analyzed by One-Way ANOVA followed by Tukey's multiple comparisons test. HEI, high endometrial integrin  $\alpha_v\beta_3$ .



**Figure 2. Gene array analysis identified 396 DEGs in the LEI vs control comparisons.** PCA for 396 DEGs identified in the LEI vs C comparisons (A). Heatmap of DEGs shows that the two groups (LEI and C) cluster separately. Each column represents an individual sample, and each row represents the individual DEG in LEI vs C comparisons (B). The volcano plot shows the statistical significance (Y-axis) against the fold change (X-axis) between the LEI and C groups (C). Each blue dot represents the significantly decreased expression of an individual gene, and each red dot the significantly increased expression of a particular gene on the Volcano Plot. Dotted horizontal and vertical lines represent the cut-off fold change ( $\pm 1.5$ -fold) and statistical significance ( $P < 0.05$ ), respectively.

eutopic endometrium and have poor pregnancy outcomes following IVF procedures (Miller *et al.*, 2012). However, aberrant molecular functions and pathways associated with this defect are not known in

detail. To explore the potentially altered biological functions in the eutopic endometrium of women with endometriosis (LEI) compared to controls (C), we used the R clusterProfiler package to conduct pathway



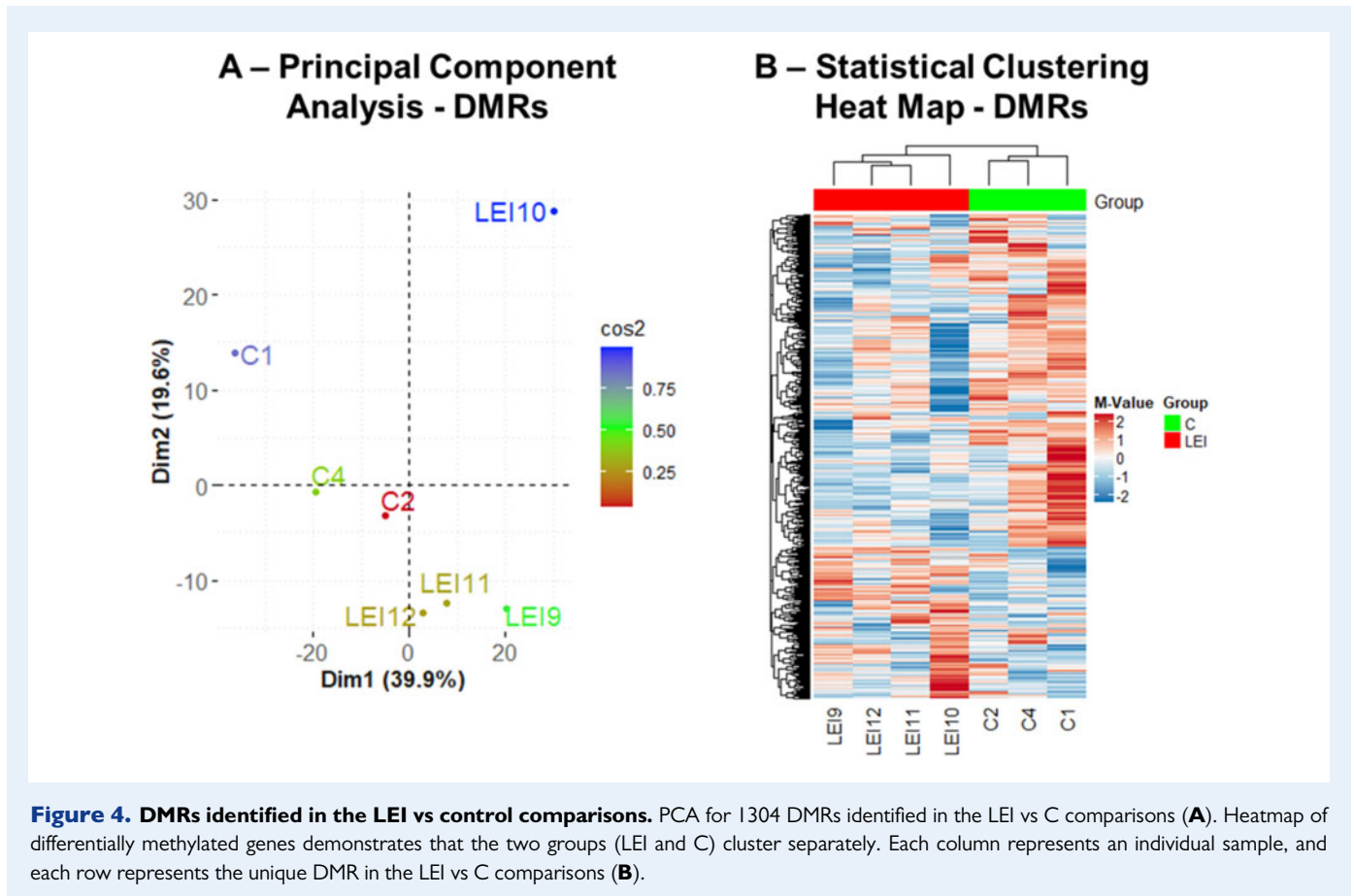
**Figure 3. Downstream molecular and functional analysis of 396 DEGs in the LEI vs control comparisons.** Altered biological process (A), molecular functions (B), cellular component (C), and KEGG pathways analysis (D). Color (red to blue) gradient bar represents the degree of P-values for statistical significance, and the size of the circle (smaller to bigger) represents the number of genes involved in each of the molecular or functional pathways.

enrichment analysis for 396 DEGs (Yu et al., 2012). This analysis revealed that biological processes (Fig. 3A and Supplementary Table SIV) associated with extracellular structure organization, GTPase activity, actin-filament-based movement, cell-substrate adhesion, and regulation of membrane potential were among the top 10 significantly ( $P < 0.01$ ) altered biological processes in the LEI group compared to controls. Analysis of the cellular component (Fig. 3B) ( $P < 0.01$ ; Supplementary Table SV) affected in the LEI vs C comparison identified extracellular matrix, actin-based cell projection, proteinaceous extracellular matrix, endoplasmic reticulum lumen, oxidoreductase complex, and filopodium as the major affected cellular components. The top 10 significantly ( $P < 0.01$ ) altered molecular function analysis (Fig. 3C and Supplementary Table SVI) resulted in the compromised activity of peptidase regulator, cell adhesion mediator, cell-cell adhesion mediator, protein binding involved in heterotypic cell-cell adhesion, and collagen binding. In addition, both phosphatidylinositol-3-kinase and protein kinase B (PI3K-AKT) and hypoxia-inducible factor signaling pathways are reported to be altered in women with endometriosis (Wu et al., 2011; Yin et al., 2012; Kim et al., 2014, 2015; Matsuzaki and Darcha, 2015; McKinnon et al., 2016; Wu et al., 2019). The KEGG analysis also revealed that the DEGs were mostly enriched in PI3K-AKT signaling, focal adhesion, propanoate metabolism, and HIF-1 signaling were the major affected pathways in the LEI women with endometriosis (Fig. 3D) ( $P < 0.01$ ; Supplementary Table SVII). Together with our comprehensive functional pathway analysis, we reveal that the eutopic endometrium with low integrin expression results in markedly altered biological, cellular and molecular responses that could

contribute to the compromised implantation and pregnancy outcomes in the subset of women with endometriosis.

### Altered methylation profile influences the eutopic endometrial gene expression in a subgroup of women with endometriosis

Altered methylation changes in the eutopic endometrium of women with and without endometriosis have been reported previously (Aghajanova and Giudice, 2011; Dyson et al., 2014; Houshdaran et al., 2016, 2020; Liu et al., 2020). However, to the best of our knowledge, aberrant methylation changes specifically in a subset of women with endometriosis-associated infertility and lower eutopic endometrial integrin  $\alpha_3\beta_3$  expression have never been studied. To achieve this objective in this study, the eutopic endometrial samples from LEI and C groups were analyzed on Roche Nimblegen 385 K Two-Array DNA Methylation Promoter Arrays. Differential DNA methylation analysis resulted in substantial changes in global methylation patterns associated with endometriosis. We observed 1304 DMRs, identified by 5 or more probes (FDR  $< 0.006$ ) between LEI compared to controls. PCA and statistical clustering performed on DMRs demonstrated that LEI and C endometrial tissue samples have distinct methylation profiles and segregated the samples from the two groups (Fig. 4A and B). This study also observed 1325 DMRs between HEI vs C (Supplementary Fig. S3A and B) and 1233 DMRs between LEI vs HEI comparisons (Supplementary Fig. S4A and B). However, considering that the highest gene expression changes were observed between LEI and C groups and because of the clinical relevance of endometrial low integrin



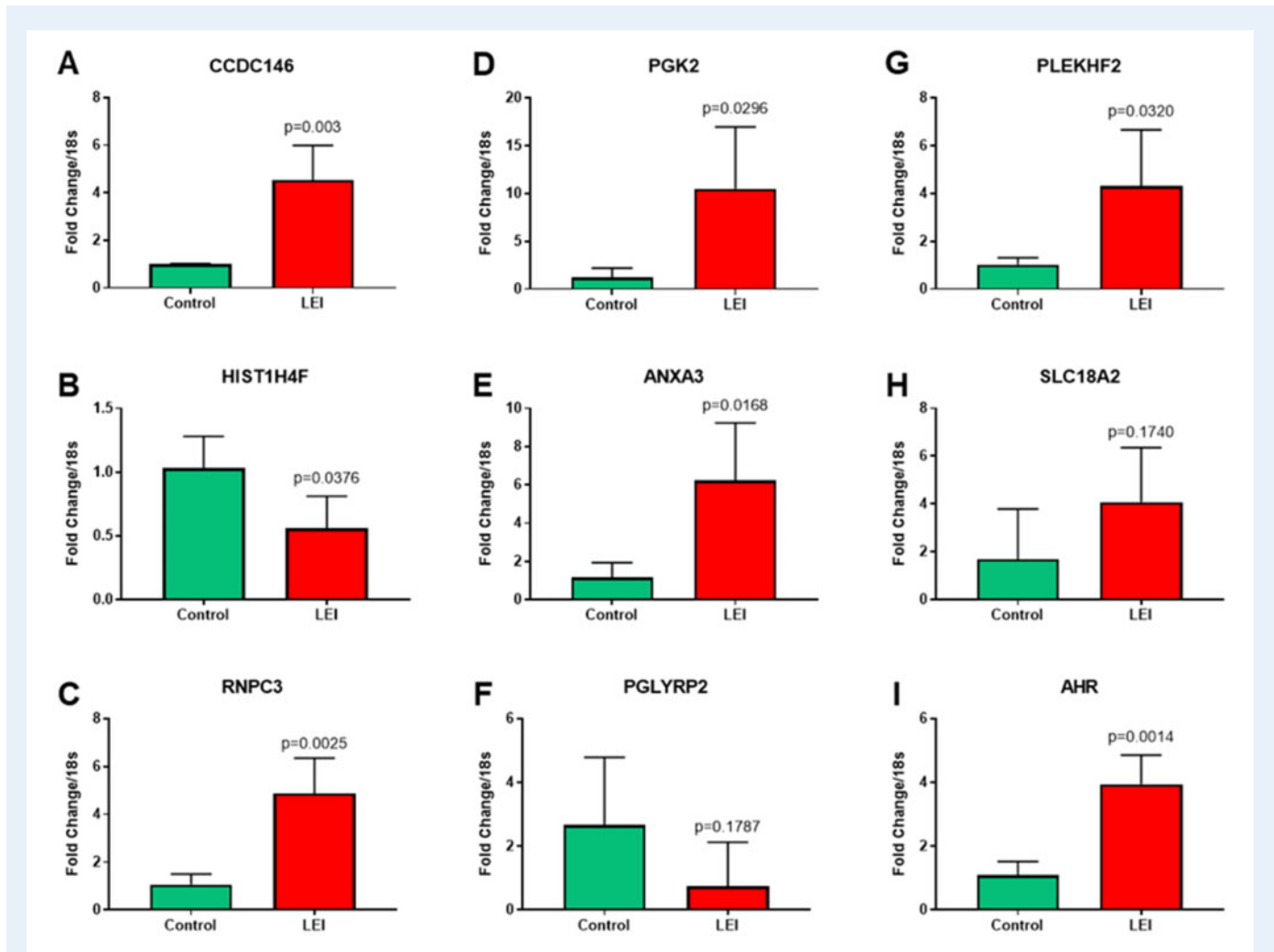
expression and infertility, we focused on the LEI vs C comparison for further downstream analysis. To identify the changes in the gene expression due to an altered methylome in LEI vs C comparison, we did overlap the DEGs and DMRs data. We created a list of genes that had an opposite pattern in gene expression analysis and methylation status. This analysis identified 14 differentially (Table I) expressed transcripts ( $P < 0.01$ : 11 up and 3 down), which showed inverse changes in promoter methylation status. The details of these 14 gene symbols and their known function from the Pubmed Gene database are provided in Supplementary Table SVIII. Further, building upon NCBI Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) search engine, we performed an extensive *in silico* research to compare and verify the findings of this study with previous similar datasets and explore any possible overlap with reported expression quantitative trait loci (eQTLs) (Mortlock *et al.*, 2020) and/or methylation quantitative trait loci (mQTLs) (Mortlock *et al.*, 2019) datasets (Supplementary Table SVIII). The *in silico* data obtained from the methylation array and gene array overlap analysis was validated by performing qRT-PCR analysis for nine genes randomly selected from Table I. qRT-PCR data were in agreement with the methylation and gene expression analysis. They suggested that seven genes were significantly ( $P < 0.05$ ) altered in the eutopic endometrium of the LEI group compared to controls (Fig. 5). The expression pattern of the other two genes also matched the array data; however, owing to higher variation, SLC18A2 ( $P = 0.174$ ) and PGLYRP2 ( $P = 0.1787$ ) were not statistically significant (Fig. 5).  $Q$ -values

analysis for the correction of multiple testing of qRT-PCR data suggested that PGK2, PGLYRP2, and SLC18A2 had  $Q$ -values  $> 0.05$  threshold (Benjamini and Hochberg, 1995).

Taken together, these data suggest that differential methylation of the promoter regulated the subset of DEG in LEI women. We confirmed these previously unreported epigenetically regulated genes by the qRT-PCR analysis, which may serve as a diagnostic marker for observed infertility in a subset of women with endometriosis. Identifying these key transcripts may serve as a valuable tool for appropriate decision making for both clinicians and patients undergoing IVF.

### Hypomethylation of the AHR promoter region is associated with increased AHR expression in the eutopic endometrium of LEI women with endometriosis compared to controls

AHR expression is elevated in the eutopic endometrium of LEI women with endometriosis compared to disease-free controls. AHR is reported to be altered in women with endometriosis (Khorram *et al.*, 2002; Mariuzzi *et al.*, 2016); however, the exact mechanism regulating AHR expression in endometriosis is not known. The data from this study for the first time suggested that AHR is epigenetically regulated, and hypomethylation of AHR promoter may be the reason for its increased expression (Table I) in eutopic endometrium of women with



**Figure 5. qRT-PCR validation of genes that are differentially methylated and differentially expressed in the LEI vs control comparison.** qRT-PCR validation of genes, which are differentially methylated and differentially expressed in the LEI (n=4) vs C (n=3) comparison. The green bar represents the control samples, and the red bar represents the LEI samples. Data displayed as mean  $\pm$  SD and analyzed by performing an unpaired Student's *t*-test. CCDC146 - coiled-coil domain containing 146, HIST1H4F - H4 clustered histone 6, RNPC3 - RNA binding region (RNPI, RRM) containing 3, PGK2 - phosphoglycerate kinase 2, ANXA3 - annexin A3, PGLYRP2 - peptidoglycan recognition protein 2, PLEKHF2 - pleckstrin homology and FYVE domain containing 2, SLC18A2 - solute carrier family 18 member A2, AHR - aryl hydrocarbon receptor.

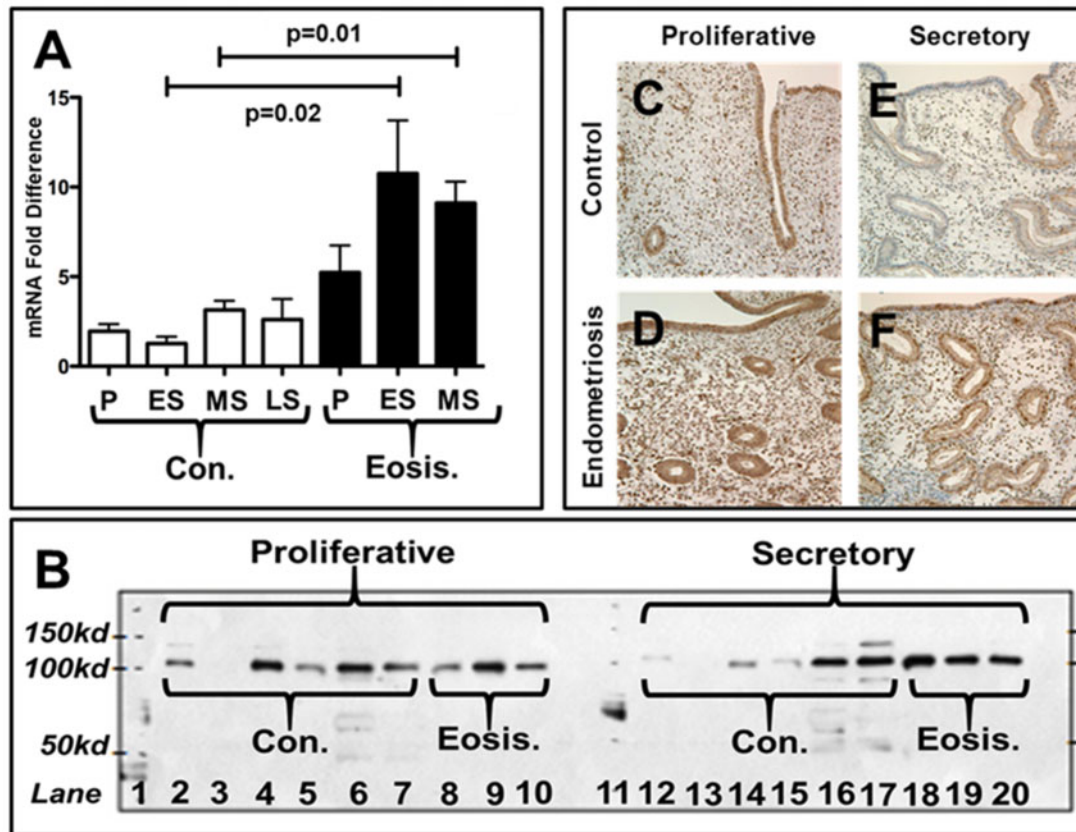
endometriosis. In addition, qRT-PCR analysis data strongly suggested that AHR is significantly ( $P=0.0014$ ) increased in the LEI group compared to controls (Fig. 5). Further, by qRT-PCR, AHR mRNA expression was noted to be significantly higher in endometriosis samples compared to controls during the early ( $P=0.02$ ) and mid-secretory phases ( $P=0.01$ ) (Fig. 6A). Western blots were generated from eutopic endometrial samples from normal controls (n=6) and women with endometriosis (n=3) from the proliferative and secretory phases (Fig. 6B). While proliferative expression was similar in control and endometriosis samples after correcting for beta-actin (not shown), an increased AHR protein level was noted in the secretory phase of women with endometriosis. Immunohistochemistry (Fig. 6C-F) illustrates the appearance and distribution of AHR in normal proliferative and secretory endometrium and differences in AHR staining in the

secretory phase of women with endometriosis compared to normal endometrium: the results confirm the qRT-PCR data (Fig. 5). This difference in AHR appeared more prominent in the nuclei of the epithelial cells. This study has demonstrated that the AHR transcript and protein levels are significantly increased in the mid-secretory eutopic endometrium of women with endometriosis, which may be epigenetically regulated in LEI women with endometriosis.

## Discussion

Endometriosis is a multifactorial disease, and many theories are proposed for its cause, including retrograde menses, genetic, epigenetic, inflammation, environmental contaminant, lifestyle, or idiopathic





**Figure 6. AHR transcript and protein expression is significantly increased in women with endometriosis.** qRT-PCR analysis of AHR transcript in the eutopic endometrial samples obtained during the menstrual cycle from women with and without endometriosis. Note the significantly increased expression of AHR mRNA during the early and mid-secretory phase of the menstrual cycle in endometrial biopsies obtained from women with endometriosis compared to controls (A). Western blot analysis of endometrial tissue proteins shows the increased AHR expression during the mid-secretory phase in women with endometriosis compared to controls (B). Immunohistochemistry shows an increased signal intensity for AHR protein in both proliferative and mid-secretory endometrial luminal epithelium, glandular epithelium, and stroma of women with endometriosis (D & F) compared to the women without disease (C & E). Data displayed as mean  $\pm$  SD and analyzed by performing an unpaired Student's *t*-test (C, control; Eosis, endometriosis; P, proliferative; ES, early secretory, MS, mid secretory, LS, late secretory).

causes (Sampson, 1927; Burney *et al.*, 2007, 2009; Hickey *et al.*, 2014; Sourial *et al.*, 2014; Houshdaran *et al.*, 2016; Powell *et al.*, 2016; Saare *et al.*, 2016; Fung *et al.*, 2018; Saare *et al.*, 2018; Poli-Neto *et al.*, 2020). Irrespective of the cause, it appears that the presence of ectopic lesions is associated with changes in the eutopic endometrium. Such changes have been noted previously and thought to represent a response to systemic inflammatory changes of the eutopic endometrial transcriptome and epigenome, which may contribute to dysregulated uterine function as evident by the observation that about 50% of the women with endometriosis have compromised fertility (Carter, 1994; Balasch *et al.*, 1996; Burney *et al.*, 2007; Meuleman *et al.*, 2009; Houshdaran *et al.*, 2016; Yoo *et al.*, 2016, 2017; Fung *et al.*, 2018; Saare *et al.*, 2018). This was further validated in our baboon model of induced disease, which demonstrated that the presence of endometriotic lesions directly impacts eutopic gene expression (Hastings, 2006; Afshar, 2013; Joshi, 2015). Hence, it is crucial to understand the

molecular and cellular changes that contribute to dysregulated endometrial function in this subset of women with endometriosis. Integrin  $\alpha_v\beta_3$ , a cell surface adhesion receptor, expression is increased owing to enhanced expression of the  $\beta_3$  subunit after Day 19 of the menstrual cycle, which is regulated by the transcription factor HOXA10 in response to progesterone (Lessey, 1998; Illera *et al.*, 2000; Daftary *et al.*, 2002; Illera *et al.*, 2003; Singh and Aplin, 2009). We and others have previously shown that integrin  $\alpha_v\beta_3$  expression is decreased in the mid-secretory endometrium of a subset of women with endometriosis with unexplained infertility (Lessey *et al.*, 1994; Lessey, 1998; Lessey and Castelbaum, 2002; Tei *et al.*, 2003; Singh and Aplin, 2009; Young and Lessey, 2010). In this study, we investigated the differential gene expression signature of eutopic endometrium in women with low  $\alpha_v\beta_3$  integrin expression and a confirmed diagnosis of endometriosis compared to the disease-free endometrium from women with higher  $\alpha_v\beta_3$  integrin expression. Further, we compared the global DNA methylation

patterns of gene promoter regions in the eutopic endometrial biopsies to investigate the impact of aberrant DNA methylation on the observed gene expression changes in women with endometriosis and LEI.

We observed the highest DEGs (total—396; 275 up and 121 down-regulated genes) in the LEI vs C comparison. In contrast, a recent RNA-seq study conducted without considering the stage of the menstrual cycle reported no DEGs in the eutopic endometrium of infertile women with endometriosis compared to the control group (Da Broi et al., 2019). However, a previous report suggests that 1286 genes were dysregulated in endometrial biopsies of patients with severe endometriosis compared to those with the mild disease during the window of implantation (Aghajanova and Giudice, 2011). These observations further support this study's objective to separate the subgroup of endometriosis patients carefully, and precisely focus on the mid-secretory phase to study the possible causes of infertility associated with endometriosis. In our analyses, there were 83 DEGs (31 up and 52 down-regulated genes) in the HEI vs C comparison and 45 DEGs (30 up and 15 down-regulated genes) in the LEI vs HEI comparison. A three-way comparative analysis for DEGs demonstrated that mid-secretory endometrium of women with endometriosis and LEI has a distinctly different gene expression profile compared to receptive endometrium from disease-free women. Further, downstream bioinformatic functional analysis was performed on 396 DEGs. Interestingly, we observed a significant enrichment of the DEGs involved in biological processes, such as extracellular matrix organization, cell adhesion, GTPase activity, and actin-filament-based movement. These biological processes are critical for embryo movement, attachment, and invasion into the endometrial stroma (Grewal et al., 2008; Margarit et al., 2009; Singh and Aplin, 2009; Grewal et al., 2010). The GTPase binding of small monomeric proteins (RHO–Ras Homology) regulates actin polymerization. Grewal et al. have reported that GTPase-dependent endometrial stromal compartment remodeling is vital for embryo implantation (Grewal et al., 2008, 2010). Cell adhesion molecules are indispensable for successful implantation during uterine receptivity (Donaghy and Lessey, 2007; Margarit et al., 2009; Singh and Aplin, 2009), and our analysis clearly showed that the cell-substrate adhesion process is significantly compromised in LEI women with endometriosis (Merviel et al., 2001). The implantation process comprises three steps—apposition, adhesion, and invasion (Bischof and Campana, 1997; Su-Mi and Jong-Soo, 2017). The cellular component analysis for the DEGs in the LEI vs C comparison identified that most of these DEGs converge to the extracellular matrix, basement membrane, proteinaceous extracellular matrix, and site of polarized growth extracellular matrix component. This finding validates our data and supports the previous studies showing that these cellular components are significantly compromised in women with endometriosis with LEI.

Regulation of gene expression is multifactorial and involves chromatin changes, transcription factor binding, non-coding RNAs, methylation, post-transcriptional modifications, and other mechanisms (Phillips, 2008). This study was extended to specifically investigate the influence of abnormal methylation on the DEGs in LEI endometrial samples compared to controls. To explore this, we performed the methylation array on the same samples utilized for the gene array. We observed 1304 DMRs in LEI compared to controls. PCA and hierarchical clustering distinctly separated the control and LEI samples emphasizing that the methylation signature is unique to both the groups in the mid-secretory phase of the menstrual cycle. Previously, Houshdaran et al.

(2016) reported the highest change in the methylome in the mid-secretory samples obtained from women with endometriosis compared to other phases of the menstrual cycle within the disease group and the disease-free controls, suggesting that the presence of ectopic lesions alters the epigenetic signature in eutopic endometrium and affects the uterine receptivity in women with the disease. A study focusing on methylation differences between ectopic lesions and eutopic tissues identified 1753 DMRs in ectopic vs control endometrium, and 2108 DMRs in ectopic vs matched eutopic endometrium (Barjaste et al., 2019). Using the machine-learning approach, Akter et al. (2019) also identified 365 DMRs among the total 2 577 382 DMRs analyzed in endometrial biopsies obtained from women with and without endometriosis.

Following the methylation signature comparison between LEI and C group, we overlapped these data with the gene array to identify the specific genes altered as a result of aberrant methylation. We identified 14 genes (RNPC3, SLC18A2, SMCHD1, SNORD58A, PGLYRP2, SNORD82, ANX3, HIST1H4F, HIST1H3I, PGK2, AHR, CCDC146, AGPAT5, and PLEKHF2), which demonstrated an inverse methylation pattern against their mRNA expression pattern in our tissue samples analyzed from the LEI and control groups. They may serve as novel candidate genes to understand infertility associated with endometriosis. We randomly selected nine genes (RNPC3, SLC18A2, PGLYRP2, ANX3, HIST1H4F, PGK2, AHR, CCDC146, and PLEKHF2) and validated their expression by RT-PCR analysis. Seven out of these nine genes were significantly ( $P < 0.05$ ) altered and matched the expression profile, and two of the genes did not follow the expression trend but were not statistically significant. These observations provide a strong basis for the experimental approach of directly comparing gene and epigenetic arrays and using *in silico* analysis to identify genes that may serve as endometrial biomarkers of endometriosis-associated infertility.

Given the potential role of AHR in the pathogenesis of endometriosis, we investigated the expression differences of AHR in the human endometrium. This is the first study to demonstrate a differential expression in AHR during the menstrual cycle in the eutopic endometrium of women with endometriosis but not in normal controls. Analysis of the AHR transcript reveals cycle stage-specific upregulation only in infertile women with endometriosis. Furthermore, the protein expression is also specifically increased in the secretory phase, as demonstrated by immunohistochemistry and western blot analysis. In contrast to our findings, a previous report indicated little AHR variation throughout the menstrual cycle (Igarashi et al., 2005), which was supported by a study from Khorram et al. (2002); however, the latter study also observed an increased expression of AHR in postmenopausal women on hormone replacement therapy, again suggesting a stimulus by ovarian sex steroids. Recent studies by Mortlock et al. identified eQTLs and mQTLs for AHR in the endometrium, suggesting that this gene's expression and methylation are also under genomic regulation (Mortlock et al., 2019, 2020). It is not clear whether low  $\alpha_v\beta_3$  expression in women with endometriosis is a cause or effect. Based on protein-protein interaction analysis, we speculate that increased AHR interacts with SPI, which in turn regulates  $\alpha_v\beta_3$  integrin expression. However, more studies are warranted to understand AHR's role in regulating integrin expression in the eutopic endometrium of women with endometriosis.

Transcriptomic and epigenomic changes are dynamic in the endometrium and are greatly influenced by ovarian steroids in a menstrual

stage-specific manner. A study conducted by Saare *et al.* (2016) found that the methylation signature was very similar between controls and endometriosis patients. They reported that only 28 DMRs were present in endometrial tissues obtained at various stages of the menstrual cycle from women with endometriosis ( $n = 31$ ) compared to controls ( $n = 24$ ). Further, in the same study, it was reported that unsupervised hierarchical clustering of DMRs segregated in endometrial tissues based on menstrual cycle stage instead of the presence or absence of endometriosis (Saare *et al.*, 2016). This observation strongly advocates the critical consideration of the menstrual cycle stage while performing disease vs control comparisons for 'Omic' studies. The major strength of our study is that all the endometrial tissue biopsies were obtained during the window of uterine receptivity and the stage of the menstrual cycle was dated carefully by the pathologist. Furthermore, endometrial biopsies were screened immunohistochemically for integrin  $\alpha_v\beta_3$  expression, and the samples were categorized based on the integrin  $\alpha_v\beta_3$  H-score into the HEI and LEI groups before they were further processed for the gene and methylation arrays. In addition, we performed an overlay of the transcriptome with the methylome to identify the transcripts specifically influenced by altered methylation status. The PCA segregated the samples in each group (C, LEI, and HEI) among various gene and methylation array comparisons. *In silico* downstream analysis of transcriptomic data defined the dysregulated cellular and molecular functions in the LEI group compared to controls. The major limitation of this study is the small sample size, which may have influenced our ability to identify DEGs that were significantly changed when applying a FDR=0.05 threshold. Another limitation was that it was not feasible to overcome the heterogeneous nature of the cell population in the samples, which is a drawback with any tissue biopsy. Despite these limitations, the results obtained have identified a previously unreported set of differentially methylated genes expressed in the mid-secretory endometrium of LEI samples from women with endometriosis. In addition, validation of the array data suggested possible epigenetic regulation was responsible for the reported increase in expression of AHR in the context of endometriosis. However, AHR has been reported to be regulated by eQTLs and mQTLs in multiple tissues (Mortlock *et al.*, 2019, 2020). Furthermore, we also identified the cellular and molecular functional pathways contributing to the observed infertility phenotype in a subset of women with endometriosis. Together this study suggests the critical involvement of epigenetic and transcriptomic alterations in the eutopic endometrium of women with endometriosis and highlights the need for future studies involving a bigger sample population to better understand the effect of epigenetic changes on the transcriptomic and proteomic functions of the endometrium during the window of uterine receptivity.

## Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

## Data availability

The data underlying this article are available in the Dryad Digital Repository, at <https://doi.org/10.5061/dryad.5tb2rbp3j>.

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## Authors' roles

N.R.J., B.A.L., and A.T.F. conceived the concept of this study and designed the experimental approach. H.-R.K.-G. performed the genomic, epigenomic data analysis, and downstream bioinformatics analysis. D.S.R. helped with bioinformatics analysis. N.R.J., K.F., E.H., J.Y.Y., and L.Y. performed the experiments. S.-M.H. assisted the epigenetic methylation array experimental design. S.L.Y. and B.A.L. provided the clinical samples, clinical insight into the study design, and edited the manuscript. J.-W.J. provided critical intellectual support, assisted with data analysis, and edited the manuscript. N.R.J., H.-R.K.-G., B.A.L., and A.T.F. wrote the manuscript. All authors contributed to the final editing and reviewed the manuscript.

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## Conflict of interest

B.A.L. has licensed technology related to alpha V/Beta 3 testing for endometrial receptivity with CiceroDx.

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