Genetic risk factors for endometriosis near estrogen receptor I and coexpression of genes in this region in endometrium

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ABSTRACT: The etiology and pathogenesis of endometriosis are complex with both genetic and environmental factors contributing to disease risk. Genome-wide association studies (GWAS) have identified multiple signals in the estrogen receptor I (*ESR1*) region associated with endometriosis and other reproductive traits and diseases. In addition, candidate gene association studies identified signals in the *ESR1* region associated with endometriosis risk suggesting genetic regulation of genes in this region may be important for reproductive health. This study aimed to investigate hormonal and genetic regulation of genes in the *ESR1* region in human endometrium. Changes in serum oestradiol and progesterone concentrations and expression of hormone receptors *ESR1* and progesterone receptor (*PGR*) were assessed in endometrial samples from 135 women collected at various stages of the menstrual cycle. Correlation between hormone concentrations, receptor expression and expression of genes in the *ESR1* locus was investigated. The effect of endometriosis risk variants on expression of genes in the region was analyzed to identify gene targets. Hormone concentrations and receptor expression varied significantly across the menstrual cycle. Expression of genes in the *ESR1* region correlated with progesterone concentration; however, they were more strongly correlated with expression of *ESR1* and *PGR* suggesting coregulation of genes. There was no evidence that endometriosis risk variants directly regulated expression of genes in the region. Limited sample size and cellular heterogeneity in endometrial tissue may impact the ability to detect significant genetic effects on gene expression. Effects of these variants should be validated in a larger dataset and in relevant individual cell types.

Key words: endometrium / oestradiol / progesterone / *ESR1* / gene expression

Introduction

Endometriosis, defined as the growth of endometrial-like tissue (glands/stroma) outside the uterine cavity (Giudice and Kao, 2004) affects 6–10% of reproductive age women and 35–50% of women with pain, infertility or both (Giudice and Kao, 2004). Endometriotic lesions are believed to stem from cells in the endometrium that are refluxed into the peritoneal cavity during retrograde menstruation. Once in the peritoneal cavity these cells attach, establish lesions and proliferate. Inherent variation in endometrial cells is believed to

predispose some women to developing the disease. What these differences are is under investigation.

Endometriosis pathogenesis has a clear genetic component. Genome-wide association studies (GWAS) have to date, identified 27 genomic regions strongly associated with endometriosis risk (Nilufer et al., 2018). All regions, however, are intronic or intergenic, not specifically linked to protein coding of individual genes and their likely mechanistic contribution to endometriosis risk is subtle. In depth analysis is required to understand the influence of genetic variants on disease risk.

Genetic variants are major regulators of gene expression (Wright et al., 2014), accounting for 15–100% of variation in gene expression through their interaction with promoters of proximal genes (Powell et al., 2012). This interaction is most likely to occur in genes within 1 megabase (Mb) of the causal variant (Fung et al., 2017). Identifying the causal variants and understanding their influence on proximal gene regulation will be vital in determining genetic influence on disease.

The most recently published meta-analysis of endometriosis GWAS (Sapkota et al., 2017) identified four independent genetic risk variants within the chromosome 6q25.1–6q25.2 region, all of which flank the estrogen receptor 1 (*ESR1*) gene. Endometriosis is an oestrogen-dependent disease (Bulun et al., 2000). Biological effects of oestrogen are mediated by oestrogen receptors encoded by *ESR1* and estrogen receptor 2 (*ESR2*). While both receptors are expressed in endometrium oestrogenic activity is dominated by *ESR1* activation (Matsuzaki et al., 2001). In addition variants in this region have also been independently associated with other reproductive traits and diseases including age at menarche (Perry et al., 2014), age at first birth (Barban et al., 2016), breast cancer (Michailidou et al., 2017) and uterine leiomyomata (Gallagher et al., 2019) suggesting genetic regulation in this region significantly influences endometrial function and reproductive health.

The endometrium is a complex tissue that undergoes cyclical breakdown and regeneration across the menstrual cycle. Changes in endometrial composition occur in response to oestrogen and progesterone concentrations and are accompanied by significant changes in genes expression (Ponnampalam et al., 2004; Fung et al., 2018; Mortlock et al., 2020). As the endometrium is the likely source of initiating cells of endometriotic lesions understanding the hormonal and genetic risk factors on gene transcription in endometrium and any differences in endometriosis patients will be important in understanding potential mechanisms of disease pathogenesis.

Therefore, given the importance of oestrogen activity in endometrial function and reproductive traits and the identification of multiple, independent signals of genetic association in the region of *ESR1*, we analyzed genes expression in the *ESR1* region, as well as their relationships between hormones, their receptors and genetic variants. We identify the expression for a set of genes in this region strongly correlated with *ESR1* expression suggestive of significant coregulation of these genes that may have an impact on endometrial function. We also found no direct association between the genetic variants in this region and an influence on gene expression, although we did find further support for the genetic overlap between endometriosis and uterine fibroids.

Materials and methods

Study participants and sample collection

A total of 151 women of European ancestry were recruited at the Royal Women's Hospital, Melbourne with the study protocols reported in detail previously. Participants were reproductive-aged women who had not received hormone treatment in the 3 months prior to surgery and all gave written informed consent prior to participation. A total of 109 women were surgically diagnosed with endometriosis and the remaining 42 women had no evidence of endometriosis at surgery. Endometrial tissue samples, collected by uterine curettage

at the time of surgery were divided into two portions and either stored in RNAlater (Life Technologies, Grand Island, NY, USA) at -80°C until RNA extraction, or formalin fixed for routine pathology assessment. Stage of the menstrual cycle was determined by an experienced pathologist from histological assessment of endometrial biopsy samples (Noyes et al., 1950) and assigned to one of the seven stages (1 M = menstrual, 2 EP = early-proliferative, 3 MP = mid-proliferative, 4 LP = late-proliferative, 5 ES = early-secretory, 6 MS = mid-secretory, 7 LS = late-secretory). Samples were excluded if the histologic stage of the menstrual cycle could not be determined. The study protocol was approved by the Human Research Ethics Committees of the Royal Women's Hospital, Melbourne (#11/24 and 16/43), the QIMR Berghofer Medical Research Institute and The University of Queensland.

Oestrogen and progesterone hormone assays

Serum oestradiol concentrations were measured in duplicate by radioimmunoassay (Human Estradiol Coated Tube Radio Immuno Assay Kit, Catalog No. 07238102, MP Biomedicals LLC, Solon, OH, USA). The functional sensitivity of the assay was 10 pg/ml. The interassay and intraassay coefficients of variation were 7.2 and 6.6, respectively.

Serum concentrations of progesterone were determined in duplicate by the IMMULITE 2000 immunoassay for human progesterone (Catalogue No. L2KPW2/10381181, Siemens Healthcare Diagnostics, Los Angeles, CA, USA). The minimum detectable serum concentration was 0.1 ng/ml. The interassay coefficient of variation was 3.9 and the intraassay coefficient of variation was 6.8. These tests were conducted at the Ligand Assay and Analysis Core Laboratory at the University of Virginia Center for Research in Reproduction.

Raw progesterone concentrations (ng/ml) were compared with histological dating to confirm menstrual cycle stage (proliferative = 1.3 ng/ml, secretory = >3.03 ng/ml, etc.). Samples with progesterone concentrations outside the expected range for each menstrual stage, identified by histological dating, were removed from the analysis ($n = 16$). We retained 135 samples with cycle stages classified into M ($n = 9$), EP ($n = 5$), MP ($n = 51$), LP ($n = 9$), ES ($n = 14$), MS ($n = 27$) and LS ($n = 20$) phases. The participants were aged between 18 and 39 years (median = 31) with BMI between 15 and 45 (median = 24). Clinical data for the 135 women are listed in [Supplementary Table S1](#). Prior to statistical analysis hormone concentrations were log transformed for normality.

Gene expression measurement and normalization

Total RNA was extracted from endometrial tissues using RNA lysis solution (RLT buffer) and AllPrep DNA/RNA mini kit (QIAGEN, Valencia, CA, USA). RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). All samples had RNA Integrity Number (RIN) > 8 . RNA concentrations were measured using the NanoDropND-6000 as described previously (Fung et al., 2018). Total RNA was amplified using an Ambion Illumina TotalPrep RNA amplification kit (Ambion) and RNA was converted to biotinylated cRNA. Expression profiles were obtained by hybridizing 750 ng of cRNA to Illumina Human HT-12 v4.0 Beadchips (Illumina

Table 1 Four independent single nucleotide polymorphisms (SNPs) on chromosome 6 associated with endometriosis risk identified by GWAS (Sapkota et al., 2017).

SNPs	Position (hg19)	GWAS P-value
rs1971256	151816011	3.74×10^{-08}
rs2206949	152037556	2.73×10^{-7}
rs17803970	152553718	7.04×10^{-8}
rs71575922	152554014	2.02×10^{-8}

GWAS, genome-wide association studies.

Inc., San Diego, USA), as described previously (Fung et al., 2017). Samples were scanned (Illumina iScan Reader) and randomized across arrays and array positions.

Gene expression normalization was performed as described by Fung et al. (2018). To achieve a stabilized distribution across average expression levels, preprocessed expression levels were transformed using a quantile adjustment across individuals, followed by scaling to log2. Further normalization was performed to allow expression levels to be compared across chips and genes.

Statistical analysis of serum hormone and endometrial gene expression

Comparison across menstrual cycle stage

Statistical analysis of the data was carried out using R software (Khan, 2013). A two-way ANOVA was performed to test for differences in mean oestradiol and progesterone serum levels between women at different menstrual cycle stages. Differences in *ESR1* and *PGR* mRNA expression in endometrial tissue across the menstrual cycle were also assessed using a two-way ANOVA. A *P*-value <0.05 was considered statistically significant. Subsequent pair-wise *t*-tests were performed between menstrual cycle stages.

Correlation between target genes in the *ESR1* region and reproductive hormones

We defined target genes of the *ESR1* region as genes within ± 1 Mb of the independent genomic loci associated with endometriosis risk on chromosome 6 (Table 1) and that showed positive expression in endometrial tissue. This range spanned from 1 Mb upstream from the most proximal signal to 1 Mb downstream from the most distal signal (range: 150816011–153554014) (Fig. 1).

To investigate possible hormonal regulation of target genes, we performed a Spearman's rank correlation test with both oestrogen and progesterone concentration and target gene mRNA expression. A correlation analysis was also performed between the respective hormone receptors *ESR1* and *PGR*. Pair-wise correlations were calculated along with the correlation between each gene and oestrogen and progesterone concentrations. The resulting *P*-values were corrected for multiple testing using the Bonferroni method and *P*-value <0.05 was considered statistically significant. The significance of correlations was also considered controlling for genome-wide false discovery rate (FDR) using the Benjamini–Hochberg method, an FDR <0.05 was considered significant.

Influence of genetic variants on target gene expression

Identification of genetic variants within the *ESR1* region

In addition to the large-scale GWAS studies, other targeted candidate gene studies have assessed genetic variants in the *ESR1* region and their relationship to endometriosis. To include these variants in the analysis of target gene expression, we conducted a literature search for scientific articles reporting genetic association between endometriosis risk and polymorphisms in the *ESR1* gene. The search was performed in electronic databases PubMed, EMBASE and Web of Science and used the following keywords 'endometriosis', 'polymorphism', 'ESR1', 'estrogen receptor alpha', 'ER alpha'. No restrictions were set on sample size or ethnicity. Additional relevant manuscripts were obtained from the reference lists. Studies with the following criteria were included: (i) full-text articles; (ii) studies that evaluated the relationship between the *ESR1* polymorphisms and the risk of endometriosis; (iii) case–control studies, where patients were diagnosed with endometriosis, while controls were patients not affected by endometriosis. The exclusion criteria were: (i) meta-analyses, reviews, editorial articles or publications where the number of cases and/or controls in the analysis was not clearly stated; (ii) publications that did not report allele frequencies separately; (iii) studies where no statistically significant associations with endometriosis risk were reported.

Genetic variants in the *ESR1* regions and endometrial eQTLs

Expression quantitative trait loci (eQTLs) are genetic variants that influence gene expression. We assessed the overlap between genetic variants associated with endometriosis in the *ESR1* region and endometrial eQTL data generated by Fung et al. (2018). For this analysis, we restricted the data to probes/genes expressed in >90% of samples which included a total of 45 923 *cis*-eQTLs for 417 unique genes and 2968 *trans*-eQTLs for 82 unique genes. Genetic variants associated with endometriosis were classified as overlapping an eQTL if the risk variant, or variants in high linkage disequilibrium (LD) ($r^2 > 0.8$) with the risk variant, corresponded to the eQTL SNP (eSNP) significantly associated with gene expression.

Summary data-based Mendelian randomization assessment of association

A summary data-based Mendelian randomization (SMR) analysis can assess if any variants in the *ESR1* region have a causal or pleiotropic association with the expression of nearby genes and endometriosis risk. Using previously generated SMR analysis data available from Fung et al. (2018) we extracted information on the predefined target genes. The SMR was conducted using GWA meta-analysis summary data from Sapkota et al. (2017) consisting of 7 899 416 SNPs genotyped across >12 000 cases together with endometrial eQTL data from Fung et al. (2018).

Association with other reproductive traits

Variants in the *ESR1* locus associated with other reproductive traits, including breast cancer (Dunning et al., 2016; Michailidou et al., 2017), age at menarche (Perry et al., 2014), age at first birth (Barban et al., 2016) and uterine fibroids (Gallagher et al., 2019), were also tested for correlation with endometriosis risk variants to assess if any variants were shared between traits. Correlation was assessed by LD between genetic variants, estimated using LDlink tools (DOI: 10.1093/

Figure 1 Genes surrounding the estrogen receptor 1 (*ESR1*) region on chromosome 6 and relative positions of independent signals from GWAS with reported significant associations for age at first birth (Barban et al., 2016), age at menarche (Perry et al., 2014), breast cancer (Zheng et al., 2009; Cai et al., 2011; Michailidou et al., 2017), uterine fibroids (Gallagher et al., 2019) and endometriosis (Sapkota et al., 2017). Endometriosis candidate SNPs refers to coding variants in *ESR1* reported to be associated with endometriosis risk (Georgiou et al., 1999; Choi et al., 2001; Kitawaki et al., 2001; Hsieh et al., 2007; Govindan et al., 2009; Xie et al., 2009; Wang et al., 2013).

bioinformatics/btv402) and reference haplotypes from Phase 3 of the 1000 Genomes Project European population. We analyzed pair-wise LD between these variants and the independent association signals for endometriosis risk. A threshold of $r^2 < 0.8$ was applied to classify variants in strong LD with the endometriosis associated SNP.

Results

Hormone concentrations across the menstrual cycle

Oestradiol concentrations across the menstrual cycle ranged from 10 to 300 pg/ml (Fig. 2a and Supplementary Fig. S1a log concentrations). Oestradiol concentrations increased during the proliferative phase of the cycle followed by a decline in the secretory phase (Fig. 2a).

Differences in oestradiol concentrations across the menstrual cycle were consistent in women with and without endometriosis and samples were analyzed together to increase power in subsequent analyses (Supplementary Fig. S2). There was a significant difference in the oestradiol concentration between menstrual cycle stages ($P < 2.5 \times 10^{-3}$). Pair-wise analysis identified significant differences between menstrual stage and an increase in oestradiol concentration in late-proliferative ($P = 0.009$), early-secretory ($P = 0.008$) and mid-secretory ($P = 0.023$) stages (Supplementary Table SII).

Progesterone concentrations ranged from 0.1 to 21.3 ng/ml across the menstrual cycle (Fig. 2b and Supplementary Fig. S1b, log concentrations). Progesterone concentrations were low during the proliferative phase and increased in the early-secretory phase, followed by decline in the late-secretory phase of the cycle (Fig. 2b). These differences in progesterone concentrations between stages of the menstrual cycle were highly significant ($P < 2 \times 10^{-16}$). Pair-wise analysis results

Figure 2 Raw concentrations of (a) oestradiol and (b) progesterone across the menstrual cycle in serum samples from 135 women and gene expression changes of (c) *ESR1* and (d) progesterone receptor (*PGR*). X-axis represents stages of menstrual cycle (M, menstrual; EP, early-proliferative; MP, mid-proliferative; LP, late-proliferative; ES, early-secretory; MS, mid-secretory; LS, late-secretory) and Y-axis represents raw concentrations of oestradiol, progesterone and normalized gene expression of *ESR1* and *PGR*. Boxplots represents two central quartiles with the mean shown by the horizontal line. Dots represent the individual data points.

showed significantly higher progesterone concentrations in the secretory stages compared with proliferative stages (Supplementary Table SIII).

Hormone receptor mRNA expression across the menstrual cycle

There were no significant differences in hormone receptor mRNA expression between women with and without endometriosis and differences in expression across the menstrual cycle were consistent regardless of endometriosis diagnosis. Samples were analyzed together to increase power in subsequent analyses (Supplementary Fig. S3). *ESR1* mRNA expression was significantly higher ($P < 2.2 \times 10^{-16}$) in the proliferative stage than in the secretory stage of the menstrual cycle (Fig. 2c). Subsequent pair-wise analysis indicated significant differences between all proliferative stages with menstrual and secretory stages (Supplementary Table SIV). In the secretory stage, oestrogen

receptor gene expression was inversely related with progesterone concentration across the menstrual cycle (Fig. 2c and b).

Similarly, *PGR* mRNA expression was higher in the proliferative compared with the secretory stage of the menstrual cycle. *PGR* expression increased from the proliferative to early-secretory stages of the menstrual cycle (Fig. 2d) in the opposite direction to progesterone concentrations (Fig. 2b). Variation in *PGR* expression across the menstrual cycle was highly significant ($P < 2.2 \times 10^{-16}$). Subsequent pair-wise analysis indicated significant differences between all proliferative stages and secretory stages (Supplementary Table SV).

Correlation between target genes in the *ESR1* risk region

Given the multiple independent signals of genetic association in the region of *ESR1* with many reproductive traits and diseases and the potential these variants lead to a coordinated influence on gene

Figure 3 Correlation matrix plot showing the correlation coefficients for the level of expression of pairs of genes as they appear on chromosome 6q25.1–6q25.2 within the *ESR1* region. Gene expression was measured in endometrial samples from women collected across the menstrual cycle. Red color denotes genes showing positive correlations and blue denotes genes showing negative correlations.

expression in the *ESR1* region we analyzed the relationship between these genes. From the expression arrays, we identified 22 probes representing 15 genes within 1 Mb of genetic markers significantly associated with endometriosis risk (Fig. 1). Following Bonferroni correction for multiple testing, 16 out of the 22 probes showed significant pairwise correlations of mRNA expression in several groups (Fig. 3). The expression of *RMND1*, *ARMT1*, *CCDC170*, *ZBTB2* and *FBXO5* were all positively correlated with *ESR1* expression ($r > 0.4$) (Table II). Expression of *MTHFD1L* ($r = -0.47$), *AKAP12* ($r = -0.40$) and *MTRF1L* ($r = -0.32$) were negatively correlated with *ESR1* expression (Table II). There were no data for *SYNE1*, the gene immediately downstream of *ESR1*, as the probes for *SYNE1* on the HT12 chips did not detect expression of this gene.

We considered a set of eight genes which correlated significantly with *ESR1* mRNA expression to further investigate their pattern of expression relative to serum progesterone and oestrogen concentrations and their receptors (Fig. 3 and Table II). Correlations with oestradiol concentrations were low and not significant while correlations with

progesterone were high and significant for only *RMND1*, *ARMT1*, *CCDC170*, *ESR1*, *FBXO5*. Expression of these genes had a higher correlation with the changes in the endometrial expression of receptor genes (*ESR1* and *PGR*) (Supplementary Tables SVI and SVII) than with hormone concentrations (oestradiol and progesterone) (Supplementary Tables SVIII and SIX), suggesting likely coregulation between this set of genes and *ESR1*.

Analysis of genetic influence on gene expression

Association between genetic variants in the *ESR1* region

Endometriosis GWAS report significant associations between endometriosis risk and four independent SNPs in the *ESR1* region (Table I). Two independent signals were identified in the genes *CCDC170* (rs1971256, $P = 3.74 \times 10^{-8}$) and *SYNE1* (rs71575922, $P = 2.20 \times 10^{-8}$). Further analysis by Sapkota et al. (2017),

Table II Genes significantly correlated with *ESR1* expression in endometrium and their correlation with serum oestradiol and progesterone concentrations and expression of *PGR* in samples from 135 women.

Genes	Gene names	<i>ESR1</i>	<i>PGR</i>	Oestradiol	Progesterone
<i>MTHFD1L</i>	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) I like	−0.47*	−0.45*	−0.17	0.16
<i>AKAP12</i>	A-kinase anchoring protein 12	−0.40*	−0.49*	−0.17	0.24
<i>ZBTB2</i>	Zinc finger and BTB domain containing 2	0.40*	0.23	−0.26*	−0.31*
<i>RMND1</i>	Required for meiotic nuclear division I homolog	0.57*	0.59*	−0.24	−0.39*
<i>ARMT1</i>	Acidic residue methyltransferase I	0.38*	0.51*	−0.21	−0.33*
<i>CCDC170</i>	Coiled-coil domain containing 170	0.71*	0.80*	−0.07	−0.52*
<i>ESR1</i>	Estrogen receptor I	1.00*	0.83*	−0.15	−0.59*
<i>FBXO5</i>	F-box protein 5	0.59*	0.76*	0.07	−0.41*
<i>MTRF1L</i>	Mitochondrial translational release factor I like	−0.32*	−0.36*	0.08	−0.17

*Correlation is significant following Bonferroni correction.

Table III List of studies that evaluated the relationship between *ESR1* gene polymorphisms and endometriosis risk.

Variant	Alleles	Ethnicity	Cases	Controls	P-value	References
rs2234693	C > T	Asian (Indian)	110	117	<0.05	Govindan <i>et al.</i> (2009)
		Greek	57	57	<0.05	Georgiou <i>et al.</i> (1999)
		Asian (Japanese)	109	27	<0.0005	Kitawaki <i>et al.</i> (2001)
		Asian (Taiwanese)	112	110	<0.005	Hsieh <i>et al.</i> (2007)
		Asian (South Korean)	77	67	<0.05	Choi <i>et al.</i> (2001)
rs9340799	A > T	Asian (Taiwanese)	112	110	<0.005	Hsieh <i>et al.</i> (2007)
		Asian (Chinese)	214	160	<0.002	Xie <i>et al.</i> (2009)
rs3798573	A > G	Asian (Chinese)	312	357	<0.01	Wang <i>et al.</i> (2013)

conditioning on these original signals, identified two further independent signals in the *ESR1* region (Table I and Supplementary Fig. S4).

A literature search for candidate gene studies identified a further three SNPs with reported association with endometriosis risk (Table III; smallest *P*-value rs2234693, *P* < 0.005). Two variants, rs2234693 and rs9340799, are located in intron 1 and the variant rs3798573 is located in intron 6 of the *ESR1* gene. We found no correlation between these three candidate *ESR1* variants with the variants identified in the GWAS studies (LD estimates r^2 < 0.1). Using the large-scale data from the GWAS meta-analysis, we found no evidence of an association with endometriosis for these three candidate SNPs (rs2234693, *P* = 0.38; rs9340799, *P* = 0.92; rs3798573, *P* = 0.2).

In addition to the four independent, GWAS-identified, variants for endometriosis risk there are also additional variants associated with breast cancer (Zheng *et al.*, 2009; Cai *et al.*, 2011; Dunning *et al.*, 2016), age at first birth (Barban *et al.*, 2016), age at menarche (Perry *et al.*, 2014) and uterine fibroids (Gallagher *et al.*, 2019) (Fig. 1) within the *ESR1* region. We found that none of the SNPs associated with age at first birth, age at menarche or breast cancer were in strong LD with the endometriosis associated SNP (r^2 < 0.7). The uterine fibroid SNP (rs58415480), however, was in strong LD (r^2 = 0.9) with the endometriosis SNP (rs71575922), located in the *SYNE1* region.

Genetic regulation of gene expression in the *ESR1* region and their relationship with endometriosis risk

We investigated previously published endometrial eQTLs (Fung *et al.*, 2018) to determine whether they influence expression of the 15 target genes in the region. There were significant *cis*-eQTLs for three genes (*ZBTB2*, *ARMT1*, *MTHFD1L*) and *trans*-eQTLs for 10 genes (*RMND1*, *CCDC170*, *ARMT1*, *FBXO5*, *PLEKHG1*, *MTHFD1L*, *AKAP12*, *LOC442270*, *MTRF1L*, *RGS17*). We restricted our analysis to consider only *cis*-eQTLs, resulting in 38 SNPs that influence *ARMT1* expression, 16 SNPs that influence *MTHFD1L* expression and 22 SNPs that influence *ZBTB2* expression. Signals for genetic regulation of *MTHFD1L* and *ZBTB2* expression did not overlap with the GWAS signals for genetic association with endometriosis. Analysis of LD between the endometrial *cis*-eQTLs and the lead SNPs from the four independent *ESR1* GWAS association signals (listed in Table I) identified 20 *cis*-eQTLs for *ARMT1* in LD (r^2 > 0.8) with the rs2206949 GWAS-identified risk SNP in the *ESR1* region.

Overlap between the genetic regions that regulate *ARMT1* expression and the endometriosis risk SNPs in the *ESR1* region was tested using the SMR analysis. The SMR test employs statistical modeling that can be used to infer causal relationships (Zhu *et al.*, 2016). Results of the SMR analysis showed no evidence of a causal or

pleiotropic association between the variants regulating *ARMT1* expression and endometriosis risk, suggesting the endometrial eQTL for *ARMT1* and endometriosis risk SNPs in the *ESR1* region are independent. We also tested for an association between the eQTLs found to influence other genes in the *ESR1* region and endometriosis risk and found no evidence for a causal relationship between these genetic variants.

We also tested whether the lead SNPs associated with age at menarche, age at first birth, breast cancer and uterine leiomyomata in the *ESR1* region were in LD ($r^2 > 0.8$) with endometrial eQTLs. None of the trait associated SNPs in this region showed evidence of regulation of gene expression in endometrium therefore no further analysis of causality with the SMR test was performed.

Discussion

In recent years, GWAS studies have made large inroads into identifying the genetic basis of many diseases. The mechanistic relationship between genetic risk factors and disease, however, is proving difficult to elucidate, as the gene or genes they influence is not clear. Understanding the regulation of genes in risk regions will help prioritize gene targets for future functional studies. GWAS studies have reported multiple independent risk variants for endometriosis, breast cancer and other reproductive traits near the *ESR1* locus on chromosome 6. An influence on *ESR1*, or coregulated genes could contribute to disease risk. Endometrium, the likely source of cells initiating endometriotic lesions, is significantly regulated by hormone concentrations. We analyzed potential target genes around the *ESR1* loci, expressed in endometrium and determined their relationship to both hormones and receptor concentrations and identified a set of genes coregulated with *ESR1*. We also assessed the association between relevant genetic variants in this region from other reproductive traits or candidate gene studies and found no association with endometriosis or expression of these genes.

Sex hormones have a significant role in the regulation of biological systems. Concentrations of oestrogen and progesterone (Draper et al., 2018) and expression of genes in the endometrium vary across the menstrual cycle in association with the changing histology and structure of the endometrium (Ponnampalam et al., 2004; Talbi et al., 2006; Fung et al., 2018; Mortlock et al., 2020). The protein expression patterns of steroid receptors ESR and PGR also vary across the cycle (Lessey et al., 2006; Critchley and Saunders, 2009). Results of our studies showed that *ESR1* and *PGR* in endometrium gradually decreased from the proliferative to secretory phases. These changes in expression pattern reflect cyclic changes in oestradiol and progesterone concentrations in the circulation. Our results showed that oestradiol concentrations increased throughout the proliferative phase followed by a decline in the secretory phase whereas, the progesterone concentrations remained low during the proliferative phase and increased in the early-secretory phase, followed by decline in the late-secretory phase of the cycle.

Oestrogenic effects act via the oestrogen receptors (ER) localized in epithelial, stromal and vascular cells. Of the two isoforms, the expression of *ESR1* mRNA (Parl et al., 1987; Koji and Brenner, 1993) and protein (ER α) (Press and Greene, 1984; Bergeron et al., 1988; Lessey et al., 1988) has been the most extensively studied in the uterus. ER α

protein is expressed in the epithelial glands and in the stroma of both the functional and basal layers of the endometrium. In response to progesterone, the expression of ER α is downregulated in the stromal and epithelial cells of endometrium during the secretory phase (Lessey et al., 1988; Young, 2013).

Steroid receptor expression in the endometrium may influence gynecological pathologies. Uterine receptivity defects such as luteal phase defect, endometriosis and polycystic ovarian syndrome have been related to ER α downregulation in endometrial epithelial cells during the mid-secretory phase (Lessey et al., 2006). It was reported that ER β mRNA was present in glandular epithelial cells and stromal cells throughout the menstrual cycle with predominant expression of ER β in glandular epithelial cells (Matsuzaki et al., 1999). ER β protein (Lecce et al., 2001) has been studied in human endometrial vascular, stromal and epithelial compartments with highest levels of ER β expression observed during periovulatory period in epithelial cells. In our data, *ESR2* was not detected on the array chip.

PGR is expressed as two functionally distinct isoforms, PR-A and PR-B acting as transcriptional regulators of progesterone responsive genes (Kastner et al., 1990). PGR expression in healthy cycling women exhibits well-described temporal and locational expression patterns (Lessey et al., 1988; Snijders et al., 1992). Studies on protein expression in human endometrium shows that the predominant form during the secretory phase is the PR-A isoform, whereas the PR-B isoform has been reported to decline in both stromal and glandular cells during the latter half of the menstrual cycle (Brosens et al., 1999; Mote et al., 2001). Reduced PGR expression in the endometrium of women with endometriosis has been proposed, however this evidence has limitations (McKinnon et al., 2018). Reduced expression has been shown in endometriotic lesions (Attia et al., 2000). In this study, we found no significant differences in *ESR1* or *PGR* expression in eutopic endometrium of women with and without endometriosis, supporting our previously published analyses (Fung et al., 2018). It is possible subtle differences in the timing of changes in hormones, their receptors or cellular content between women with and without endometriosis may contribute to varied expression.

We assessed patterns of expression for 15 genes within 2 Mb of the *ESR1* locus in endometrium. We identified a set of genes that show correlated changes in expression indicative of coregulation with *ESR1*. The set included genes immediately upstream of *ESR1* (*RMND1*, *ARMT1*, *CCDC170*) and a gene (*FBXO5*) downstream of *ESR1* whose expression was significantly positively correlated with *ESR1* expression ($r > 0.4$). Two genes located upstream of *ESR1* (*MTHFD1L* and *AKAP12*) and *MTRF1*, located downstream of *ESR1* were significantly negatively correlated with *ESR1*. Patterns of expression for these genes across the menstrual cycle were more strongly correlated with receptor gene expression (*ESR1* and *PGR*) than with hormone concentrations. Neighboring genes in close proximity in humans can be expressed concurrently (Hurst et al., 2002; Takai and Jones, 2004; Trinklein et al., 2004). There is also evidence that *RMND1*, *CCDC170* and *ARMT1* expression, immediately upstream of the *ESR1* are correlated with *ESR1* expression in tumor biopsies taken from postmenopausal women with stage I to IIIB ER+ breast cancer (Dunbier et al., 2011). Therefore, genes in the *ESR1* region may be coregulated and not just menstrual cycle-dependent.

Genetic variants may also influence gene expression, contributing to gynecological pathologies. Four independent genetic risk variants for

endometriosis around *ESR1* have been identified using GWAS, although the causal variant, the variant that directly contributes to endometriosis risk, has not yet been confirmed. Candidate gene association studies based on selected genes with inferred biological function have also identified additional genetic variants in the *ESR1* region that may be related to endometriosis risk. In the literature search we identified three candidate SNPs (Choi *et al.*, 2001; Kitawaki *et al.*, 2001; Hsieh *et al.*, 2007; Govindan *et al.*, 2009; Xie *et al.*, 2009; Wang *et al.*, 2013) that were located in intronic regions of *ESR1* that have been proposed as causal variants. Investigating these SNPs in the endometriosis GWAS meta-analysis data (Sapkota *et al.*, 2017), we found no evidence of an association with endometriosis (smallest *P*-value rs3798573, *P*=0.2), nor was there a correlation with genome-wide significant GWAS signals in the *ESR1* region. These results suggest the true signals in the region of *ESR1* associated with endometriosis risk are noncoding variants located in intergenic regions which have subtle influences on gene regulation in the region.

The chromosome 6q25.1 region is a well replicated risk locus for breast cancer in Europeans and Asians (Cai *et al.*, 2011). In our analysis, none of the breast cancer associated signals correlated with the endometriosis risk SNPs in the *ESR1* region. Similarly, SNPs in the *ESR1* region that were also reported to be associated with age at menarche (rs6933660) and age at first birth (rs726281, rs67229052) were not correlated with the endometriosis risk SNPs and were not associated with endometriosis. Thus, although there is a known relationship between age at menarche, age at first birth, breast cancer and endometriosis, these phenotypes do not appear to share the same candidate causal variants in the *ESR1* region. Evidence of independent genetic risk factors for these related traits and diseases together with correlated expression of genes linked to *ESR1* expression suggest complex regulation of genes in this region in different tissue types.

Women with endometriosis are two times more likely to have a uterine fibroid diagnosis (Gallagher *et al.*, 2019). GWAS of uterine fibroids also identified one signal in the *ESR1* region (Gallagher *et al.*, 2019). The uterine fibroids associated signal (rs58415480) is in strong LD with one of the endometriosis signals (rs71575922). Biological overlap/coexistence between these two gynecological diseases has long been suspected due to the similarities in molecular mechanisms and progenitor cells (Uimari *et al.*, 2011; Tanmahasamut *et al.*, 2014; Gallagher *et al.*, 2019). Gallagher *et al.* (2019) report a significant moderate genetic correlation between uterine fibroids and endometriosis ($r=0.39$, $P=9.77 \times 10^{-13}$) and evidence of a causal relationship. Our findings support the presence of genetic overlap between uterine leiomyomata and endometriosis. Further studies are necessary to discover the underlying biological relationships in development of these two diseases.

Annotation of risk SNPs identified by GWAS with gene expression data can expand our ability to understand the genetic basis of complex traits. We therefore annotated trait associated GWAS SNPs to endometrium specific eQTLs. We found that none of the trait associated lead SNPs, or correlated SNPs (estimates for LD $r^2 > 0.8$) in the *ESR1* region, showed evidence regulating gene expression for this set of target genes in endometrium. Variants located in regulatory elements might activate promoters affecting more than one gene. Variants associated with breast cancer have been reported to regulate *ESR1* in reporter assays and may regulate other genes *RMND1*, *CCDC170* and *ARMT1* supporting coregulation of genes in this region (Dunbier *et al.*, 2011; Dunning *et al.*, 2016). Other approaches will be required to

understand how independent genetic risk factors in the region of *ESR1* increase risk for multiple diseases and traits.

A limitation in our study was that the power to detect tissue specific eQTLs was limited by the sample size. Other limitations include the use of endometrial tissue consisting of multiple cell types (including stroma and epithelium) undergoing changes in cellular composition and cell activity across the menstrual cycle. Genetic regulation of genes in the *ESR1* region may be cell-type specific and the effects of risk variants in specific cell types remains to be investigated. Statistical methods developed to predict cell composition in whole blood without cell sorting require validated reference sets that are not yet available for endometrium. New techniques such as single-cell RNA-seq provide the expression profiles of individual cells and are a promising approach to provide additional insights into cell-type-specific gene expression. However, single-cell seq is expensive and can also introduce new practical, economic and computational challenges.

In summary, GWAS results show strong association between genetic risk variants and endometriosis risk on chromosome 6q25. The *ESR1* gene in this region is a strong candidate, but the signals are intergenic and genetic studies show multiple independent genetic signals associated with endometriosis and other reproductive traits and diseases. *ESR1* expression was correlated with the expression of seven other genes across the region with strongest correlated expression for *CCDC170* providing strong evidence of coregulation of genes in this region. We found no evidence that genetic markers for endometriosis risk regulate the expression of *ESR1* or other genes from this region in endometrium. These SNPs may have more subtle effects on gene expression or regulate genes in cell-specific manner. Other studies will be necessary to identify the functional effects of these variants on endometriosis risk. The roles of other genes on chromosome 6q25 that we show have correlated expression with *ESR1* should also be considered.

Supplementary data

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

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

Su.M., Sa.M. and G.W.M. designed the study with input from the other authors. Su.M., Sa.M., J.N.F., S.H., S.J.H. and J.G. coordinated data

collection and quality control of data with support, input and oversight from S.H., P.A.W.R., L.C.G., B.M. and G.W.M. Data analysis was performed by Su.M. and Sa.M., which was interpreted by all authors. Su.M., Sa.M. and G.W.M. drafted the report with input from all other authors. The final manuscript has been critically revised and approved by all authors.

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Conflict of interest

None declared.

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