In silico, in vitro and in vivo analysis identifies a potential role for steroid hormone regulation of FOXD3 in endometriosis-associated genes

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STUDY QUESTION: Can bioinformatics analysis of publically available microarray datasets be utilized in identifying potentially important transcription factors (TF) in the hormonal regulation of the endometrium?

SUMMARY ANSWER: Systems integration and analysis of existing complex (published) datasets, predicted a role for the novel transcription factor, Forkhead Box D3 (FOXD3) in healthy endometrium and in endometriosis, which was followed by the demonstration of decreased levels of the protein upon decidualisation of normal human endometrial stromal cells in vitro and differential endometrial expression in the stroma in endometriosis.

WHAT IS KNOWN ALREADY: The reported endometriosis-associated endometrial aberrations are most pronounced in the progesterone-dominant secretory phase and progesterone resistance is a proposed causative factor.

STUDY DESIGN, SIZE, DURATION: The study was initially an ‘in silico’ study, with confirmatory ‘wet lab’ data from western blotting (WB), qPCR and Immunohistochemistry (IHC) on endometrial biopsies obtained from 142 women undergoing gynaecological surgery.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study was conducted at a University Research Institute. Bioinformatic analysis of selected published microarray datasets identified differentially regulated genes for the early and mid-secretory phases relative to the proliferative phase. Diseases and Functions categories were identified with Ingenuity (IPA) ‘core analysis’ software. The key transcription factors controlling secretory phase gene changes were revealed with oPOSSUM software. FOXD3 expression levels were examined in human endometrial samples from women aged 18–55 years by WB, IHC, and qPCR. The progesterone regulation of endometrial FOXD3 levels was examined in vivo and in cultured primary human endometrial stromal cells in vitro.

MAIN RESULTS AND THE ROLE OF CHANCE: Initial data mining and subsequent bioinformatics analysis of human endometrial micro-array datasets identified FOXD3 to be a key regulator of gene expression specific to secretory phase/endometriosis. FOXD3 was dynamically expressed in healthy endometrium and differentially expressed in endometriosis. In vitro decidualisation of primary endometrial stromal cells significantly decreased FOXD3 protein ($P = 0.0005$) and progestagen (Levonorgestrel) treatment also reduced the high endometrial FOXD3 protein ($P = 0.0001$) and mRNA levels ($P = 0.04$) seen in untreated women with endometriosis, with a shift of FOXD3 from the nucleus to the cytoplasm.

LIMITATIONS, REASONS FOR CAUTION: The quality of Bioinformatics analysis and results depends on the published micro-array data.

WIDER IMPLICATIONS OF THE FINDINGS: An in depth analysis of FOXD3 function and its relationship with estrogen and progesterone might provide insights into its potential deregulation in proliferative disorders of the endometrium including endometrial cancer where its expression is also deregulated. Further, FOX transcription factors are increasingly seen as novel therapeutic targets in disease.

† The first two authors contributed equally.
Introduction

Endometriosis is one of the most common chronic gynaecological conditions, characterized by the presence of endometrial-like tissue which undergoes proliferation, bleeding and regeneration outside the uterine cavity. It is both a source of distress and an economic burden affecting over 15% of young women during their reproductive life, yet the pathogenesis remains unknown (Sourial et al., 2014). The definitive diagnosis requires invasive surgery and available treatment modalities are neither curative nor universally acceptable due to their permanent or reversible contraceptive effect. The lack of understanding regarding the aetiology and pathogenesis of endometriosis has resulted in a deficit of novel and effective treatments.

Endometriosis is characterized by a dependence on estrogen and an apparent resistance to progesterone (Bulun et al., 2006). Estrogen and progesterone work together to regulate the function of normal endometrium by controlling the expression of hundreds of genes during the various stages of the menstrual cycle (Kao et al., 2003). There are a plethora of studies demonstrating differential expression of a variety of genes and their products in the eutopic endometrium of women with endometriosis compared with healthy, fertile control women (Hapangama et al., 2008; Meola et al., 2010; Vouk et al., 2011; Fassbender et al., 2012; Hwang et al., 2014). These differences are proposed to be responsible for subfertility, pain, menstrual dysfunction and the initiation and propagation of ectopic endometriotic lesions (Sourial et al., 2014). However, Fassbender et al. found no differential gene expression between samples from endometriosis patients compared with control samples regardless of cycle stage or severity of endometriosis (Fassbender et al., 2012). The majority of reported aberrations are confined to the progesterone dominant secretory phase of the cycle and consequently relative progesterone resistance in endometrial tissues is proposed to be a fundamental causative factor (Al-Sabbagh et al., 2012). We therefore hypothesized that differential gene expression in the secretory phase endometrium relative to the proliferative phase may identify a specific gene set that is involved in the molecular abnormalities underpinning the pathological endometrium associated with endometriosis.

Bioinformatics is used to collate an extensive amount of information on gene expression, gene function, gene products and cellular function to identify the key players in a disease process to predict suitable targets for treatment and has been recently used to improve the diagnosis of Hepatocellular Carcinoma (Zhang et al., 2011), lymphoma (O’Neill and Song, 2003) and oral cancer (Singaraju et al., 2012).

In this study, we first mined publicly available human endometrial microarray data. Our bioinformatics analysis of selected data sets revealed Forkhead Box D3 (FOXD3) to be a potentially important transcription factor in the hormonal regulation of the endometrium and in the regulation of the aberrantly expressed genes associated with endometriosis. We subsequently characterized the expression of FOXD3 protein across the menstrual cycle in healthy endometrium. We also interrogated its differential expression in the endometrium of women with endometriosis in vivo and studied the role of hormone regulation of FOXD3 in endometrial tissue of women on hormonal therapy and in primary human endometrial stromal cells in vitro.

Materials and Methods

Bioinformatics methods

In silico analysis of endometrial datasets and identification of differentially regulated genes in early and mid-secretory phases

Microarray data (GDS2737, reference series GSE6364) were recovered from the Gene Expression Omnibus according to strict selection criteria (Supplementary Table SI). Methods of collection and validation of endometrial samples have been previously described in the original study (Talbi et al., 2006). One ‘normal’ sample from the published data was excluded due to the presence of adenomyosis. In order to investigate fold change, Log2 transformation was performed on expression data with mean ratios of expression calculated for all samples at either early secretory (ES) or mid-secretory (MS) phase relative to a mean of all proliferative phase samples (n = 5) (PROL). A fold change of ±2 was considered significant for analysis after t-test and adjusted P-value < 0.05 filtering to correct for false discovery rate. The ‘R’ statistics package version 2.15.1 (http://www.r-project.org/) and multtest package (http://www.bioconductor.org/) were used to identify differentially regulated genes for the ES and MS phases relative to the PROL Phase. Resulting gene lists were uploaded into the ‘Core analysis’ tool of the Ingenuity (IPA) software and a functional enrichment analysis was performed for Diseases and Functions categories of IPA.

Identification of key transcription factors

Identifiers of up- and down-regulated genes provided by the analysis above were entered into oPOSSUM 3.0 (http://www.cisreg.ca/opossum/) to identify enriched potential transcription factor binding sites (TFBS) in their promoter regions. Human Single Site Analysis (SSA) was implemented on vertebrate-specific TFBS. A concise list of relevant TFs regulating the genes close to the promoter region was obtained by using 2000/0 on the upstream/downstream score. The most highly conserved regions (cut-off 0.6) were chosen with matrix score threshold set to 80%. The TFs were sorted by Z-Score which expresses the divergence of the value. Use of oPOSSUM software allowed the identification of conserved sequences across the species hence offered a wider-scale in silico detection of the majority of relevant TFBS across the human genome.

Considering the Fisher scores and the large number of genes included in this analysis, in order to ensure that the results were not due to chance, we performed a contrasting analysis entering 2 random sets of 2000 genes that we found not to be differentially regulated in secretory phase into oPOSSUM.

Patients and tissues

Ethical approval was obtained from the Liverpool Adult Local Research Ethics committee (LREC 04/Q1505/112) and informed written consent was
obtained from all participants. Endometrial biopsies were obtained from 142 women (18–55 years) undergoing gynaecological surgery (Hapangama et al., 2009). Group 1 consisted of 72 fertile women without endometriosis undergoing female sterilization or hysterectomy for non-endometrial pathology. Group 2 consisted of 35 women with surgically diagnosed active peritoneal endometriosis. No women in either group were on hormonal contraceptives and all had regular menstrual periods. All endometrial samples were assigned to a cycle stage based on histological assessment and the date of last menstrual period. Eutopic endometrial biopsies were taken during the proliferative phase (PROL, cycle days 5–12; n = 30 for group 1, n = 10 for group 2), MS (cycle days 18–24 days; n = 23 for group 1, n = 14 for group 2) and late-secretory phase (LS, cycle days 25–28; n = 19 for group 1, n = 11 for group 2) of the cycle. Group 3 included 11 women undergoing excision of endometriosis and ectopic peritoneal endometriotic lesions were collected in addition to eutopic endometrium. Group 4 included endometrial biopsies from 24 women treated with hormones (levonorgestrel releasing hormone analogues (GnRHas, leuprorelin acetate, anti-FSH, anti-LH)) and agonadotrophin-releasing hormone analogues (GnRHas, leuprorelin acetate, n = 11) for endometriosis-associated pelvic pain. Samples were divided and (i) fixed (≥ 24 h in 10% (v/v) buffered formalin) and paraffin embedded for immunohistochemical staining; (ii) immediately snap frozen and stored at −80°C for protein extraction and (iii) immediately placed into RNA later® (Sigma, Dorset, UK) for extraction of total RNA. Not all samples were used in all experiments.

In vitro decidualisation of endometrial stromal cells and cell fractionation
Stromal cells were isolated from normal healthy proliferative phase endometrial biopsies (n = 7) by mechanical and enzymatic digestion (Valentijn et al., 2013). Stromal cells were plated in Dulbecco’s modified Eagle’s Medium (DMEM)/F12 (phenol-red free; Life Technologies, Paisley, UK), 10% (v/v) fetal bovine serum (FBS) (Sigma, Dorset, UK), Primocin® (antimicrobial; Source Bioscience, Nottingham, UK) for 24 h. Next they were split 1/3 and maintained in DMEM/F12 (phenol-red free) with 2% (v/v) charcoal stripped FBS (Sigma, Dorset, UK), Primocin® (antimicrobial; Source Bioscience, Nottingham, UK) for at least 24 h. Decidualisation was induced according to Vasquez et al. (2015). Briefly, cells were exposed to 10 nM 17β-estradiol, 100 nM medroxyprogesterone acetate (MPA) (both from Sigma, Dorset, UK) and prepared as 1 mg/ml stocks in ethanol and 1 mM 2‘-O-dibutyryladenosine-3’, cAMP (DO627; Sigma, Dorset, UK), in DMEM/F12 (phenol-red free; Life Technologies, Paisley, UK) with 2% (v/v) charcoal stripped FBS (Sigma, Dorset, Uk), Primocin® (antimicrobial; Source Bioscience, Nottingham, UK), for 3 days. Control was medium alone with ethanol vehicle. Cells were fractionated using a kit (#9038; New England Bio Labs, Hertfordshire, UK) according to the manufacturer’s instructions.

Western blotting
Endometrial tissue and cultured stromal cells were extracted in RIPA buffer [(50 mM Tris-Cl, pH8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) sodium dodecyl sulphate (SDS)] supplemented with protease (P8340, Sigma, Dorset, UK) and phosphatase (PhosSTOP, Roche Diagnostics, West Sussex, UK) inhibitors. Protein was estimated using (50 mM Tris-Cl, pH8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) sodium dodecyl sulphate (SDS)) supplemented with protease (P8340, Sigma, Dorset, UK) and phosphatase (PhosSTOP, Roche Diagnostics, West Sussex, UK) inhibitors. Western blotting was performed using Bio-Rad CFX Manager (Bio-Rad, Hertfordshire, UK). The data from in vivo and in vitro studies were analysed across the groups with Kruskal–Wallis test (IHC); in different groups with Mann–Whitney U-test (Westerns, qPCR, IHC) and the treatment effects with a paired t-test in cell culture experiments (Westerns) using GraphPad Prism5 (GraphPad Software San Diego California, USA, www.graphpad.com).

Immunohistochemistry (IHC)
3 µm-thick paraffin sections were stained with rabbit polyclonal anti-human FOXD3 (Poly6317, BioLegend, London, UK) applied at a concentration of 3 µg/ml in 0.5% BSA/tris buffered saline and incubated overnight at 4°C after antigen retrieval at pH 6 (Valentijn et al., 2013). Detection was with ImmPRESS anti-rabbit polymer and visualization with ImmPRESS DAB (Vector Laboratories, Peterborough, UK). Sections were lightly counterstained with haematoxylin (Thermo Shandon, Runcorn, UK) dehydrated, cleared and mounted. Non-immune rabbit IgG (Vector Laboratories, Peterborough, UK) at 3 µg/ml replaced the primary antibody as a negative control, with human placenta as a tissue positive control.

Semi-quantitative scoring of the IHC staining
Each immunostained section was analysed semi-quantitatively (considering the whole section area) using a modified Quickscore method (Valentijn et al., 2013). Nuclear staining was assessed separately for glands, stroma and luminal epithelium. Sections were double scored blind. Where scores differed by 3 or more, these were analysed by a third observer and agreement obtained.

RNA extraction, cDNA synthesis and qPCR
Total RNA from tissue samples and cultured Ishikawa cells were extracted using TRIzol® Plus RNA Purification System (Life Technologies, Paisley, UK), and quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). Total RNA was reverse transcribed using AMV First Strand cDNA synthesis kit (New England Bio Labs, Hertfordshire, UK) after DNase treatment (DNase I (#M0303), New England Bio Labs, Hertfordshire, UK). The manufacturer’s protocol. cDNA was preamplified for FOXD3 using PrimePCR PreAmp assay and SsoAdvanced™ PreAmp supermix and amplified by qPCR using iTaq Universal SYBR Green supermix and the CFX Connect Real-Time System (Bio-Rad, Hertfordshire, UK). Primers and reaction conditions are listed in Supplementary Table SII. Relative transcript expression was calculated by the △△Ct method, normalized to the reference gene Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta (YWHAZ) (Vestergaard et al., 2011) using Bio-Rad CFX Manager (Bio-Rad, Hertfordshire, UK).

Statistical analyses
Z score and one-tailed Fisher Exact score were calculated to analyse over-representation of TFBS using oPOSSUM 3.0 (http://www.cisreg.ca/oPOSSUM/). Right-tailed Fishers Exact statistical tests were used to calculate whether the likelihood of associations between a set of focus genes and a category is due to random chance. This enabled evaluation of enriched conditions in a pathway or higher order ontological categories and provides a hypergeometric distribution based network score and P-Values conveyed as the − log (Fisher’s Exact Test).

The data from in vivo and in vitro studies were analysed across the groups with Kruskal–Wallis test (IHC); in different groups with Mann–Whitney U-test (Westerns, qPCR, IHC) and the treatment effects with a paired t-test in cell culture experiments (Westerns) using GraphPad Prism5 (GraphPad Software San Diego California, USA, www.graphpad.com).
Results

Published normal endometrial microarray datasets identify cycle phase specific differentially regulated genes

There were 1299 and 777 differentially regulated genes identified in the ES and MS phase respectively relative to the PROL phase in normal endometrial biopsies (Supplementary Table SII). All of the 10 top most differentially regulated genes in the MS phase (Table I A) have been reported to be associated with endometriosis in previous publications and as expected the enrichment analysis revealed ‘endometriosis’ to be the top Diseases and Functions category in the MS phase (Table I B).

Identification of key transcription factors controlling secretory phase changes

Table II shows the most enriched transcription factor binding sites (TFBS) ranked by Z-score (full gPOSSUM output in Supplementary Table SIV). The top scoring transcription factors identified in the ES and MS phase endometrial samples were FOXD3, ARID3A, FOXA1, SRY, FOXI1, Nkx2-5 and FOXq1. The significance of this result was supported by analysis of 2 sets of 2000 randomly selected genes which were not differentially expressed where FOXD3 was placed 109th and 114th by Z-score (data not shown).

Human eutopic endometrium, ectopic endometriotic lesions and endometrial cell lines express FOXD3 protein and mRNA

FOXD3 protein (western blotting) was detected in proliferative and early- to mid-secretory phase human endometrium and the FOXD3 transcript (qPCR) across the menstrual cycle (Fig. 1A). There was a significant increase in FOXD3 transcript expression levels from proliferative to mid-late secretory phase (P = 0.01, Kruskal–Wallis test). FOXD3 transcript and protein were also present in the endometrial cancer cell lines Ishikawa, HEC1A and MFE280 (Supplementary Fig. S1C). Furthermore, we demonstrated nuclear and cytoplasmic FOXD3 immunoreactivity in all endometrial cell types including epithelial, stromal, endothelial and immune cells in eutopic and ectopic endometrial tissue by IHC (Figs 1B and 2A–C, F and G). Interestingly, ectopic peritoneal lesions and endometrial basalis layer both showed minimal stromal FOXD3 immunoreactivity (Fig. 2F and H and Supplementary Fig. S1D).

Nuclear FOXD3 is expressed in the normal human endometrium and is differentially expressed in endometriosis

In fertile control women, there was a significant increase in stromal nuclear FOXD3 immunoreactivity in the LS phase (P < 0.008, Fig. 1B–C), which was not observed in the endometriosis group. Whilst there was no significant change across the cycle in glandular epithelial FOXD3 expression in normal fertile women, there was an apparent increase in FOXD3 immunoreactivity in glandular epithelium in the endometriosis group (P < 0.07, Fig. 1Cii). FOXD3 mRNA levels were similar in homogenized endometrial tissue collected in the secretory phase from women with endometriosis compared with that of the control women (Fig. 1Ciii).

FOXD3 is down-regulated in decidualising human endometrial stromal cells

The immunostaining Quickscores for FOXD3 in stromal cells were significantly lower in the MS phase normal samples compared with the LS

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Mean log fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL14</td>
<td>8.571</td>
</tr>
<tr>
<td>MFSD4</td>
<td>8.142</td>
</tr>
<tr>
<td>C2CD4A</td>
<td>7.812</td>
</tr>
<tr>
<td>100P</td>
<td>7.285</td>
</tr>
<tr>
<td>ANGPTLI</td>
<td>6.869</td>
</tr>
<tr>
<td>C2CD4B</td>
<td>6.715</td>
</tr>
<tr>
<td>SCARAS</td>
<td>6.585</td>
</tr>
<tr>
<td>MIT1M</td>
<td>6.039</td>
</tr>
<tr>
<td>CRISP3</td>
<td>5.799</td>
</tr>
<tr>
<td>CXCL13</td>
<td>5.566</td>
</tr>
</tbody>
</table>

Table I Top 10 up-regulated genes and diseases and functions categories identified in the mid-secretory phase of the menstrual cycle by analysing published normal endometrial microarray datasets. IA: Mid-secretory (MS) phase, top 10 up-regulated Genes by Fold Change; IB: MS phase, the top 10 Diseases or Functions categories.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Disease or Functions annotation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organismal Injury and Abnormalities, Reproductive System Disease</td>
<td>Endometriosis</td>
<td>1.60E-27</td>
</tr>
<tr>
<td>Cancer, Organismal Injury and Abnormalities, Reproductive System Disease</td>
<td>Female genital tract serous cancer</td>
<td>4.74E-23</td>
</tr>
<tr>
<td>Cancer, Organismal Injury and Abnormalities, Reproductive System Disease</td>
<td>Uterine serous papillary cancer</td>
<td>1.41E-21</td>
</tr>
<tr>
<td>Cell Death and Survival</td>
<td>Necrosis</td>
<td>6.29E-18</td>
</tr>
<tr>
<td>Cell Death and Survival</td>
<td>Cell death</td>
<td>3.88E-17</td>
</tr>
<tr>
<td>Cell Death and Survival</td>
<td>Apoptosis</td>
<td>6.30E-15</td>
</tr>
<tr>
<td>Cell Death and Survival</td>
<td>Cell death of tumor cell lines</td>
<td>7.29E-15</td>
</tr>
<tr>
<td>Cancer, Organismal Injury and Abnormalities</td>
<td>Serous neoplasm</td>
<td>1.61E-14</td>
</tr>
<tr>
<td>Cancer, Organismal Injury and Abnormalities</td>
<td>Breast or colorectal cancer</td>
<td>4.75E-13</td>
</tr>
<tr>
<td>Cancer, Organismal Injury and Abnormalities</td>
<td>Breast or ovarian cancer</td>
<td>2.12E-12</td>
</tr>
</tbody>
</table>
suggesting post-translational modification. Upon decidualisation, nuclear FOXD3 was present as a doublet, levels were low/undetectable in the nucleus (refer to long exposure). In decidualised stromal cells, FOXD3 (Fig. 3C). In decidualised stromal cells, FOXD3 levels were low/undetectable in the nucleus (refer to long exposure). In the nuclear fraction of control cells FOXD3 was present as a doublet, suggesting post-translational modification. Upon decidualisation, nuclear FOXO1A was increased (Fig. 3C).

**Common endometriosis treatments modulate FOXD3 protein and mRNA**

We next assessed the effect of LNG-IUS and GnRH-as on FOXD3 expression in endometrial biopsies. Both treatments were associated with a significant reduction in nuclear FOXD3 immunoreactivity ($P = 0.0001$ and $P = 0.007$ respectively, Fig. 2D) and mRNA levels ($P = 0.04$. Secretory Group 2 versus LNG Group 4, Fig. 2E) compared with MS phase endometrial samples from women with untreated endometriosis, with a shift from the nucleus to the cytoplasm observed in the glandular epithelia (Fig. 2A–C). Furthermore, ectopic endometriotic lesions collected from a woman on GnRH analogue treatment showed weak/absent nuclear FOXD3 immunoreactivity in both epithelial and stromal cells (Fig. 2G). Stromal cell expression of FOXD3 was significantly lower in ectopic lesions compared with the corresponding eutopic endometrium ($P = 0.01$, Wilcoxon test, Fig. 2H).

**Discussion**

Endometriosis creates a significant clinical and economic burden on young women and society. Therefore, the utility of resources and research need to be maximised to enhance our understanding of the disease to develop novel and effective treatments. In this context, mining of publically available data sets together with bioinformatics identified FOXD3 as a potentially important transcription factor in the secretory endometrium. In vivo data also suggested that it was important in endometriosis. Although FOXD3 transcripts are detectable in human endometrium (Li et al., 2013), evidence of expression at the protein level was lacking. We now demonstrate the expression of FOXD3 protein in the human endometrium for the first time with differential expression in the mid-secretory phase of the menstrual cycle, suggesting a potential role for progesterone signalling in its regulation.

FOXD3 belongs to the Forkhead Box (FOX) family of transcription factors which are known to mediate cell cycle progression, survival and differentiation (reviewed (Lam et al., 2013)). FOXD3 expression was originally discovered in embryonic stem cells and their malignant equivalent (Sutton et al., 1996) and is critical for maintaining the stem cell pool (a FOXD3 knockout is embryonically lethal) (Tompers et al., 2005). Western blot analysis of human endometrial tissue for FOXD3 showed that it was expressed in the proliferative and secretory phases (analysis predominantly of mid-secretory). Although it was apparently increased in the secretory phase, this was not significant. The multiple immunoreactive bands on western blotting presumably reflect the cellular heterogeneity of the endometrium and are also suggestive of post-translational modification. The lower molecular weight band for FOXD3 detected by western blotting, predominantly in the secretory phase endometrium could be nonspecific as this band was not detected in the stromal cell cultures or the cancer cell lines (Supplementary data).

Subcellular fractionation and subsequent western blotting of human endometrial stromal cells showed that FOXD3 is predominantly cytoplasmic and that the nuclear fraction undergoes post-translational modification, hence the doublet. Nuclear-cytoplasmic switching of FOXO TFs is controlled by post-translational modification (Eijkelenboom and Burgering, 2013) and we anticipate a similar regulation for FOXD3 in the endometrium as was observed in the glandular epithelia of the treatment group. In support of this, FOXD3 has been reported to shift sub-cellular location in the islet cells of the pancreas in a rat model of diabetes (Perera et al., 2006). The transcript for FOXD3 was significantly increased in whole endometrium from proliferative to mid-late secretory phase and this might be due to the resident leucocytes that increase substantially through the secretory phases comprising as much as 40% of all stromal cells at the onset of menstruation (Starkey et al., 1991). Leucocytes are reported to express the transcript for FOXD3 (http://www.genecards.org).

In the normal human endometrium, nuclear FOXD3 protein was constitutively expressed in the glands and did not change across the menstrual cycle. The lowest FOXD3 expression levels in the endometrial stroma was seen in the MS phase, at a time when progesterone is maximal, suggests a role for progesterone signalling in FOXD3 regulation.

Human decidualisation commences during the mid-luteal phase of the menstrual cycle, independent of embryo implantation and is driven by luteal progesterone and the convergence of signalling pathways involving cyclic adenosine monophosphate (cAMP), and estrogen (Ramathal et al., 2010). When isolated healthy primary endometrial stromal cells were induced to decidualise in vitro, FOXD3 protein levels were significantly reduced and this was associated with a loss in nuclear expression of FOXD3. The cyclic AMP-inducible FOXO1A (Christian et al., 2002; Buzzio et al., 2006; Labied et al., 2006) and IGFBP1, a hallmark of samples, suggesting that FOXD3 expression might be modulated by decidualisation. Human endometrial stromal cells were induced to decidualise in culture for 3 days and subsequently analysed by western blotting (Fig. 3Bi). The expression of FOXD3 was significantly down-regulated ($P = 0.0005$, Fig. 3Bi), while FOXO1A and insulin-like growth factor binding protein-1 (IGFBP-1), markers of decidualisation, were both significantly up-regulated ($P = 0.007$ and $P = 0.017$ respectively, Fig. 3Bi). Fractionation of control cells showed that while FOXD3 was detected in the nucleus, it was predominantly cytoplasmic (Fig. 3C). In decidualised stromal cells, FOXD3 levels were low/undetectable in the nucleus (refer to long exposure). In the nuclear fraction of control cells FOXD3 was present as a doublet, suggesting post-translational modification. Upon decidualisation, nuclear FOXO1A was increased (Fig. 3C).

**Table II Top 10 enriched transcription factor binding sites (TFBS) by oPOSSUM analysis ordered by Z score for genes differentially expressed in the mid-secretory phase when compared with the proliferative phase in endometrial biopsies.**

<table>
<thead>
<tr>
<th>Transcription factor binding site</th>
<th>Z-score</th>
</tr>
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<tbody>
<tr>
<td>Foxd3</td>
<td>25.13</td>
</tr>
<tr>
<td>ARID3A</td>
<td>23.48</td>
</tr>
<tr>
<td>FOXA1</td>
<td>23.04</td>
</tr>
<tr>
<td>SRY</td>
<td>22.58</td>
</tr>
<tr>
<td>Foxq1</td>
<td>22.17</td>
</tr>
<tr>
<td>Nkx2-5</td>
<td>22.16</td>
</tr>
<tr>
<td>TBP</td>
<td>22.00</td>
</tr>
<tr>
<td>Sox5</td>
<td>21.10</td>
</tr>
<tr>
<td>FOX1I</td>
<td>20.01</td>
</tr>
<tr>
<td>Pdx1</td>
<td>19.63</td>
</tr>
</tbody>
</table>

Of 117 TFBS FOXD3 was placed the top TFBS by Z-score in the mid-secretory phase.
Figure 1 Forkhead Box D3 (FOXD3) expression in human endometrium. (A) Expression of FOXD3 protein (i and ii) and mRNA (iii) in total tissue extracts from human endometrium as determined by western blot (WB) and qPCR. Samples for WB were extracted from full thickness endometrium recovered during the proliferative (PROL; \( n = 7 \)) and secretory (SECRET; \( n = 7 \)) phases with beta actin used as a loading control (i and ii). E = early secretory phase; M = mid-secretory phase. We believe the lower molecular weight band* denotes a non-specific band for FOXD3 and possibly relates to the degree of blood contamination in the biopsy. Samples for qPCR were extracted from 4 proliferative phase, 7 mid-secretory phase and 5 late secretory phase endometrial biopsies with YWHAZ as a housekeeping gene. FOXD3 expression changed significantly across the menstrual cycle (\( P = 0.01 \), Kruskal–Wallis test) with significant increases in transcript expression between proliferative phase (PROL) and both mid- and late-secretory phase (MSP and LSP) samples (\( P = 0.02 \)) (iii). Graphs show mean and SEM (ii and iii), Mann–Whitney U test and Kruskal–Wallis test were used to calculate statistical significance. (B) Representative micrographs demonstrating immunoreactivity of FOXD3 in human endometrium. i: Group 1 (fertile women without endometriosis), proliferative phase; ii: Group 2 (women with surgically diagnosed endometriosis), proliferative phase; iii: Group 1, mid-secretory phase; iv: Group 2, mid-secretory phase; v: Group 1, late-secretory phase; vi: Group 2, late-secretory phase. Non-immune rabbit IgG replaced the primary antibody as a negative control, with human placenta as a tissue positive control (Supplementary Fig. S1). (C) i–ii: Summary of immunohistochemistry quickscore data. Nuclear staining only was scored for intensity and proportion of positively stained cells. i–ii: Cycling endometrium from 76 patients, box and whisker plots. \( N = 10 \) for proliferative phase (PROL) in group 1, \( N = 14 \) for PROL in group 2, \( N = 16 \) each for group 1 at mid-secretory (MS) and late-secretory (LS) phases, \( N = 10 \) each for group 2 at MS and LS. (i) Stromal compartment demonstrated an increase in nuclear staining in group 1 from the MS to LS phase (\( P < 0.008 \)). (ii) Functionalis glands demonstrated apparently higher nuclear reactivity in Group 2 in the progesterone dominant mid-secretory phase compared with group 1 (\( P < 0.07 \)). (iii) Expression of FOXD3 mRNA in total tissue extracts from full thickness endometrium recovered during the secretory phase from healthy control (Group 1, \( n = 12 \)) and endometriosis (Group 2, \( n = 9 \)) as determined by qPCR (mean ± SEM). YWHAZ was used as a housekeeping gene.
Figure 2  Hormonal regulation of Forkhead Box D3 (FOXD3) in vivo. (A–C) Representative micrographs demonstrating immunoreactivity of FOXD3 in human endometrium. (A) Mid-secretory phase untreated endometriosis sample showing strong nuclear staining in stromal and epithelial cells, (B) Levonorgestrel releasing-Intrauterine system LNG-IUS treatment showing stromal decidualisation of eutopic endometrium and increased cytoplasmic staining, (C) GnRH agonist (GnRHα) treatment showing weak glandular staining and almost absent stromal staining. (D–E) Summary data showing the effect of commonly used endometriosis treatments on FOXD3 protein and transcript (patients treated with hormones prior to biopsy). (D) Box and whisker plot of IHC quickscore data showing the effect of LNG-IUS and GnRHα on nuclear expression of FOXD3 in functional glands during the mid-secretory (MS) phase. Group 2, n = 10; Group 4 LNG-IUS, n = 10; Group 4 GnRHα, n = 10. LNG-IUS and GnRHα significantly reduced glandular epithelial nuclear FOXD3 compared with MS phase Group 2 (P = 0.0001 and P = 0.007 respectively). (E) Expression of FOXD3 mRNA in total tissue extracts from human endometrium as determined by qPCR shown as mean (± SEM). Samples homogenized from endometrium recovered during the secretory (SECRET) phase from women with endometriosis (n = 9) expressed significantly more FOXD3 transcript than those recovered whilst being treated with LNG-IUS (n = 3, P = 0.04) or GnRHα (n = 3, N/S) in Group 4. We were unable to detect FOXD3 mRNA in 2/3 of the LNG-IUS treated biopsies. (F–G) Representative micrographs demonstrating FOXD3 immunoreactivity in ectopic endometriotic lesions. (F) Ectopic lesion showing intense nuclear staining of epithelial cells and largely absent stromal staining; (G) Ectopic lesion from GnRHα treated patient showing almost exclusively cytoplasmic epithelial staining and largely negative stromal staining. (H) Summary of IHC quickscore data showing matched eutopic and ectopic endometrial stromal scores (n = 11). Individual data points and median are shown. Wilcoxon matched pairs signed rank test showed significantly lower stromal FOXD3 protein expression in the ectopic lesions (P = 0.01).
decidualisation (Gao et al., 1999), were significantly up-regulated in decidualised stromal cells.

In endometriosis samples however, there was no significant change in stromal FOXD3 protein observed in the secretory phase by IHC. While there was a clear change in FOXD3 transcript/protein across the menstrual cycle in the normal endometrium, we were unable to detect a similar change across the cycle in women with endometriosis. Progesterone resistance of the endometrium, proposed as a pathological feature of endometriosis may also explain the lack of cyclical changes observed in stromal FOXD3 protein expression in women with endometriosis. Moreover, stromal cells derived from endometrium from women with endometriosis have reduced capacity for decidualisation (Klemmt et al., 2006).

Development and progression of endometriosis depends on the presence of estrogen and is associated with an abnormal response to bioavailable progesterone with deregulation of several progesterone target genes, creating a condition of progesterone resistance. Progesterone resistance of the endometrium, proposed as a pathological feature of endometriosis may also explain the lack of cyclical changes observed in stromal FOXD3 protein expression in women with endometriosis. Moreover, stromal cells derived from endometrium from women with endometriosis have reduced capacity for decidualisation (Klemmt et al., 2006).

To date little is known about the physiological role of FOXD3 relative to other members of the FOX family in non-embryonic, adult cells. The limited available evidence suggests that FOXD3 acts as a transcriptional repressor in adult cells (Sutton et al., 1996). FOXD3 was down-regulated during stromal cell decidualisation in vitro, which raises the possibility that its down-regulation might allow transcription of genes associated with differentiation. A gain-of-function (overexpression) and loss-of function (small interfering RNA) approach followed by a transcriptomic profile of decidualising endometrial stromal cells in future studies should shed light on this. Furthermore, future studies should examine the role of FOXD3 in the reduced decidualisation capacity reported in women with endometriosis.

Figure 3 Forkhead Box D3 (FOXD3) is down-regulated in decidualising stroma. Stromal cells isolated from proliferative phase endometrial biopsies were induced to decidualise in vitro. (A) Micrographs of control and decidualised stromal cells. (B) i: Representative western blot of control (Ct) and decidualised (Dec) stromal cells immunoblotted for FOXD3, FOXO1A, insulin like growth factor binding protein 1 (IGFBP1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and; ii: the densitometric analysis (n = 7, mean ± SEM). (C) Control and decidualised stromal cells were fractionated: whole cell lysate (WCL), cytoplasmic fraction (Cy) and nuclear fraction (N) and immunoblotted for FOXD3 (**short exposure and *long exposure), FOXO1A, GAPDH (cytoplasmic marker) and Histone H3 (nuclear marker). A representative (n = 3) western blot is shown.
endometriosis (Klemmt et al., 2006) with subfertility and compare that to those who are fertile to unravel the mechanism preventing implantation in women with endometriosis.

The endometrial effect of progesterone is exerted via the stroma, and apart from the progesterone receptor, androgen and glucocorticoid receptors are also postulated to be involved in progesterone action in the endometrium (Henderson et al., 2003). Our observation of differential stromal and epithelial expression of FOXD3 across the cycle may indicate cell specific regulation of FOXD3 in the endometrium.

Glandular expression of FOXD3 did not appear to change according to the phases of the menstrual cycle. While there was a trend for increased glandular expression of FOXD3 in endometriosis, this was not significant. We also observed in multiple endometriotic lesions from the same patient different expression patterns for FOXD3. The majority of the endometriotic lesions we examined were from secretory phase samples and so we are unable to comment on whether these would change across the menstrual cycle. However previous studies reported that the cycle-specific changes of steroid hormone receptor expression observed in normal endometrium are not always shared by endometriotic lesions (Nisolle et al., 1994; Fujishita et al., 1997; Beliard et al., 2004). Furthermore endometriotic lesions are proposed to be phenotypically similar to the endometrial basalis layer (Leyendecker et al., 2002; Valentijn et al., 2013) which is thought to be less hormonally responsive (reviewed in (Hapangama et al., 2015)). Interestingly, we observed a shift of FOXD3 from the nucleus to the cytoplasm in the glands of LNG- and GnRHa-treated endometrium, indicating a role for steroid hormones. A loss of nuclear FOXD3 would suggest an effect on transcriptional activity.

In conclusion, further work is needed to define the molecular actions of FOXD3 and its transcriptional impact on the endometrium. An in depth analysis of FOXD3 function and its relationship with estrogen and progesterone might provide insights into its potential deregulation in proliferative disorders of the endometrium including endometrial cancer where its expression is also deregulated (Li et al., 2013). Further, FOX transcription factors are increasingly seen as novel therapeutic targets in disease, either targeted directly or the pathways they regulate (Yang and Hung, 2009).

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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**Authors’ roles**

D.K.H. conceived the study and D.K.H., A.J.V., and J.A.D. were involved in all aspects of study design, execution, analysis, manuscript drafting and critical discussion. D.M. and O.V. contributed to the bioinformatics aspect of the study as well as critical discussion. All authors had final approval of the submitted version.

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**Conflict of interest**

All authors declare no conflict of interest.

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