Aberrant expression of metastasis-inducing proteins in ectopic and matched eutopic endometrium of women with endometriosis: implications for the pathogenesis of endometriosis

D.K. Hapangama1,2,*, R.S. Raju1, A.J. Valentijn1, D. Barraclough3, A. Hart4, M.A. Turner1,2, A. Platt-Higgins5, R. Barraclough5, and P.S. Rudland5

1Department for Women’s and Children’s Health, Institute of Translational Medicine, University of Liverpool, Liverpool, UK 2Liverpool Women’s Hospital NHS Foundation Trust, Liverpool, UK 3Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK 4University of Lancaster, Lancaster, UK 5Institute of Integrative Biology, University of Liverpool, Liverpool, UK

*Correspondence address. E-mail: dharani.hapangama@liverpool.ac.uk

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BACKGROUND: Endometriosis is a metastatic disease without obvious tumorigenesis. Expression of S100P, S100A4, osteopontin (OPN) or anterior gradient homologue 2 (AGR2) proteins can induce metastasis but fail to induce tumorigenesis per se. We now explore whether this group of metastasis-inducing proteins (MIPs) are associated with the pathogenesis of endometriosis.

METHODS: Eutopic endometrial biopsies were taken from 73 women (35 fertile women without endometriosis and 38 women with surgically diagnosed endometriosis). Ectopic endometriotic lesions were collected from eight of the women with endometriosis. The expression of MIPs at the cellular level was evaluated by immunohistochemistry and the presence of these proteins in the endometrial tissues was verified by western blotting and their gene expression was confirmed by RT–PCR.

RESULTS: All four MIPs were immunolocated in the endometrium of control women and S100P, AGR2 and OPN showed a cyclical variation. Progesterone phase eutopic endometrium of both groups showed a similar staining pattern for all MIPs, whereas secretory phase endometrium showed a differential expression between controls and cases. The secretory phase endometrial immunostaining showed weak stromal and perivascular AGR2, and decreased stromal and glandular S100P. In contrast, immunostaining for all MIPs was increased in the late secretory endometrial samples of women with endometriosis and intense immunostaining was seen for S100A4 in the stroma (P < 0.05) and for S100P (P < 0.001) and AGR2 (P < 0.0001) in both glands and stroma (P < 0.001). All active peritoneal endometriotic lesions showed strong immunostaining for each of the MIPs studied.

CONCLUSIONS: We propose that these MIPs enhance endometrial cell invasiveness and contribute to the establishment of ectopic endometriotic deposits after retrograde menstruation.

Key words: endometriosis / S100P / AGR2 / S100A4 / osteopontin

Introduction

Endometriosis is a common benign gynaecological condition with a huge personal and economic burden (Bulun, 2009). The aetiology and pathogenesis remain uncertain. Therefore, studies investigating the pathogenesis of endometriosis are important to provide targets for novel, more effective and universally acceptable treatments. Endometriosis is defined as the implantation of endometrial tissue at...
ectopic sites beyond the uterine cavity. Retrograde menstruation remains the most prominent of the various theories put forward to explain the pathogenesis of endometriosis (Sampson, 1927). However, retrograde menstruation occurs in most women (Halm et al., 1984), while endometriosis occurs in only ~10% (Eskens and Warner, 1997; Cramer and Missmer, 2002). This suggests that other factors are involved in the establishment of endometriosis.

Endometriosis is characterized by the acquisition of some of the properties of malignant tissues such as increased cell proliferation, invasion and the induction of metastasis, but it does not exhibit other properties of neoplastic cells such as their uncontrolled proliferation. We have recently shown that the eutopic endometrial cells of women with endometriosis show increased cell proliferation which may contribute to their survival in the peritoneal cavity (Hapangama et al., 2008a, 2009). In a baboon model of endometriosis, we observed that initiation of the disease in the peritoneal cavity can induce telomerase and other pro-proliferative proteins in the eutopic endometrial cells (Hapangama et al., 2010). These observations support the following model for the pathogenesis of endometriosis. Firstly, an increase in expression of genes and proteins (Gashaw et al., 2006) that promote angiogenesis and adhesion occurs in endometrial tissue that has undergone retrograde menstruation. This initiates the production of ectopic endometriotic deposits. Secondly, the ectopic endometriotic deposits induce a local inflammatory response and secrete various cytokines (Minici et al., 2007). Thirdly, the cytokines (or other substances) act on the eutopic endometrium to induce the pro-proliferative markers such as telomerase, nucleolin and proliferating cell nuclear antigen (Hapangama et al., 2008a, 2009, 2010). Fourthly, the induced eutopic endometrial cells adopt the pro-proliferative, apoptosis-resistant phenotype (Taniguchi et al., 2011) which has a survival advantage in the peritoneal cavity. Finally, retrograde menstruation of endometrium, with the pro-proliferative phenotype together with expression of other genes that also promote cell survival (Hastings and Fazleabas, 2006), gives rise to further endometriotic deposits and maintains the disease. This model, however, does not explain how the endometriotic cells invade the peritoneum and surrounding tissue.

The ability to invade surrounding tissue and disseminate to ectopic sites is also acquired by malignant metastatic cells in many commonly occurring cancers (Mendoza and Khanna, 2009). One prototypical group of proteins that can induce invasion and metastasis are the metastasis-inducing proteins (MIPs). MIPs have been characterized by transfection of a benign neoplastic rat mammary model of breast cancer (Davies et al., 1993; Liu et al., 2005; Wang et al., 2006). The functional relevance of the MIPs is indicated by reports that high expression of MIPs is associated with shorter survival times among breast cancer patients (Rudland et al., 2002; Wang et al., 2006) known to express the four MIPs studied was used as external positive controls. All samples were split into two parts; one was fixed for 24 h at 4 °C in 4% (v/v) buffered formalin, rinsed and routinely embedded in paraffin wax and was used for immunohistochemistry staining and for extracting RNA for RT–PCR, as previously described (Hapangama et al., 2008a; Bohmann et al., 2009). The other part was immediately snap frozen with liquid nitrogen and was kept at −80°C for protein extraction.

**Materials and Methods**

**Patients and specimens**

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 prior to inclusion. A total of 73 premenopausal women aged 18–45 years were recruited; all had regular menstrual cycles (26–30 days), were not on any hormonal treatments or using an intrauterine contraceptive device. The fertile control (FC) group of 35 women was recruited prior to laparoscopy while undergoing female sterilization and had no detectable endometriosis and no history of symptoms or prior diagnosis of the condition. The endometriosis group consisted of 38 women with surgically diagnosed endometriosis who either underwent diagnostic laparoscopy or excision surgery. A pipelined endometrial sampler (Promidem, Neuilly-en-Thelle, France) was used to obtain a sample of eutopic endometrium and all endometrial samples were assigned to cycle a stage based on assessment of histological criteria by two experienced pathologists according to the criteria described by Noyes et al. (1950), the date of last menstrual period and profile of circulating hormones. Only the samples that were qualified by all three criteria were included in the analysis. Eutopic endometrial biopsies were taken during the proliferative phase (PROL, cycle days 5–12; FC n = 5: Endo n = 9), window of implantation (WOI, cycle days 21 ± 2 days; FC n = 18: Endo n = 17) and in the late secretory phase (LS, cycle days 26 ± 2 FC n = 12: Endo n = 12) of the cycle in both groups.

In addition to the endometrium, 13 active, ectopic peritoneal red and blue endometriotic lesions were collected from eight of the women in the endometriosis group who underwent excision surgery for removal of endometriosis. A blood sample was taken at the time of the endometrial biopsy for the assessment of serum estradiol and progesterone levels. Breast carcinoma tissue (Rudland et al., 2002; Wang et al., 2006) known to express the four MIPs studied was used as external positive controls. All samples were split into two parts; one was fixed for 24 h at 4°C in 4% (v/v) buffered formalin, rinsed and routinely embedded in paraffin wax and was used for immunohistochemistry staining and for extracting RNA for RT–PCR, as previously described (Hapangama et al., 2008a; Bohmann et al., 2009). The other part was immediately snap frozen with liquid nitrogen and was kept at −80°C for protein extraction.

**Tissue extraction for western blotting**

Endometrial tissue (20 mg wet weight tissue/0.1 ml buffer) was minced with a scalpel blade prior to extraction in RIPA buffer ([50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/o) sodium deoxycholate and 0.1% (w/o) sodium dodecyl sulphate (SDS)] supplemented with a protease inhibitor cocktail (#P8340, Sigma) by sonication (Ultrasonic Generator, Type 7535A, Dawes, output 40 with microtip) on ice by several pulses over 30 s. After incubating on ice for 30 min, the lysates were cleared by centrifugation at 14 000g, 20 min, 4°C and the supernatants stored at −20°C.
Immunohistochemistry

Histological sections were cut at 5 μm on APES coated slides, dewaxed in xylene and rehydrated through graded ethanols to water as previously described by Warburton et al. (1982). Immunohistochemical staining was carried out using an indirect ABComplex System (Dako Ltd., Ely, UK) (Hsu et al., 1991) for AGR2 and S100A4 antibodies or by using a commercially available enhanced horse-radish peroxidise (HRP) labelled polymer system, the DAKO EnVision + System, peroxidise (3,3-diaminobenzidine, DAB) (Dako Ltd) (Heras et al., 1995), for OPN and S100P antibodies. Endogenous peroxidase activity in the tissue sections was blocked either by immersing the slides in 100% methanol containing 0.05% (v/v) H2O2 for 20 min at room temperature (Streefkerk et al., 1972) or by using the reagent supplied in the EnVision + kit.

Sections were incubated in a moisture chamber with antibodies as follows: polyclonal affinity-purified anti-AGR2 (Liu et al., 2005) was used at a dilution of 1:100 in phosphate-buffered saline (PBS) containing 2% (w/v) bovine serum albumen (BSA) pH7.4 and left overnight, following an initial pre-incubation with 2% (w/v) BSA in PBS for 1 h at room temperature. Polyclonal anti-S100A4 (Dako Ltd) was applied at a dilution of 1:2000 in 0.5% (w/v) BSA in PBS overnight. Monoclonal anti-OPN (MP111B10, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used at a dilution of 1:300 in 0.5% (w/v) BSA in PBS overnight. Monoclonal anti-S100P (BD Transduction Laboratories, Cowley, Oxford) was used at a dilution of 1:120 in 1% BSA (w/v) in PBS for 2 h, following an initial pre-incubation with 1% (w/v) BSA in PBS for 30 min at room temperature. Sections were then thoroughly washed in three changes of PBS.

For anti-AGR2 and anti-S100A4, biotinylated donkey anti-rabbit immunoglobulin G (IgG; Amersham, Bucks) was applied at a dilution of 1:100 in phosphate-buffered saline (PBS) containing 0.05% (v/v) H2O2 for 20 min at room temperature. For anti-OPN and anti-S100P, indirect immunohistochemical staining was carried out using the EnVision + reagents, prepared according to the manufacturer’s instructions (Dako Ltd). All sections were then thoroughly washed in three changes of PBS.

Semi-quantitative analysis of immunohistochemical staining

All slides were coded and scored by two independent observers prior to breaking the code. Intensity of brown positive immunostaining for S100P, S100A4, AGR2 and OPN within the different cell types in the endometrium (glandular and luminal epithelium, stroma, perivascular and endothelial cellular compartments) was estimated in a light microscope using x 400 magnification and a standard semi-quantitative scoring system. This scoring system has previously been demonstrated to show a high correlation with staining measured by image analysis in previous studies (Wang et al., 1998; Hapangama et al., 2002, 2008a). This method employs a four point semi-quantitative scoring system based on a global assessment of all slides relating to each participant. Staining was classified as negative/no staining = 0, weak = ±, strong = + and very strong = + +, as previously described (Hapangama et al., 2002, 2008a,b, 2009, 2010).

Table 1 A List of PCR primer used in RT–PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Sequences (5’ &gt; 3’)</th>
<th>Size</th>
<th>Tm (°C)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
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<tr>
<td>Human AGR2</td>
<td>NM_006408</td>
<td>Fwd 5’217</td>
<td>5’ GCT CCT TGT GGC CCT CTC CTA CAC 3’</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 3’570</td>
<td>5’ ATC CTG GGG ACA TAC TGG CCA TCA G 3’</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>Human GPDH</td>
<td>NM_002046</td>
<td>Fwd 5’628</td>
<td>5’ ACC ACA GTC CAT GCC ATC AC 3’</td>
<td>20</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 3’1079</td>
<td>5’ TCC ACC ACC CTG TTG CTG TA 3’</td>
<td>20</td>
<td>67</td>
</tr>
<tr>
<td>Human OPN</td>
<td>NM_001040058</td>
<td>Fwd 5’562</td>
<td>5’ GTC ACT GAT TTT CCC ACG GAC CTG CC 3’</td>
<td>26</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 3’843</td>
<td>5’ TCC ATA ACT GTC CTT CCC ACG GCT GT 3’</td>
<td>26</td>
<td>73</td>
</tr>
<tr>
<td>Human S100A4</td>
<td>NM_002961</td>
<td>Fwd 5’70</td>
<td>5’ ATG GGC TGC CCT CTG GAG AAG G 3’</td>
<td>22</td>
<td>74</td>
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<td></td>
<td>Rev 3’375</td>
<td>5’ TCA TTT CCT CTT GGG CTG CTT A 3’</td>
<td>22</td>
<td>67</td>
</tr>
<tr>
<td>Human S100P</td>
<td>NM_005980</td>
<td>Fwd 5’211</td>
<td>5’ GGA GCT ACC AGG CCT GCC GAG TGG 3’</td>
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<td>76</td>
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<tr>
<td></td>
<td></td>
<td>Rev 3’390</td>
<td>5’ CCA GGG CAT CAT TTT AGT CCT GCC 3’</td>
<td>24</td>
<td>75</td>
</tr>
</tbody>
</table>

SDS–PAGE and immunoblotting

Equal volumes of lysate were analysed by SDS–PAGE on precast 4–15% gradient gels (Mini-PROTEAN TGX, Bio-Rad) and transferred to polyvinylidene difluoride membrane (Millipore). Non-specific binding was blocked by incubation with PBST (PBS, 0.05% (v/v) Tween 20) plus 5% (w/o) non-fat milk (MPBST). Membranes were incubated with primary antibody diluted in MPBST overnight at 4°C. Primary antibodies used: AGR2 (in-house, Liu et al., 2005) 1:500; OPN (ab8448, Abcam) 1:1000; S100A4 (AS114 Dako) 1:1000; S100P (#610306 BD Transduction Labs) 1:250. Membranes were also probed with an antibody to pan cytokeratin (C2562, Sigma) 1:1000 and actin (A4700, Sigma) 1:1000 to ensure equal loading. After washing the membrane in PBST, HRP-linked rabbit antimouse (P0260, Dako) or goat anti-rabbit (A0545, Sigma) secondary antibodies (1:10,000 in MPBST) were added at room temperature for 60 min. After washing in PBST, signal detection was performed using SuperSignal West or Pico Chemiluminescence (ThermoScientific) and CL-Xposure film (ThermoScientific).

Reverse transcription polymerase chain reaction

RT–PCR of the endometrial samples was carried out as previously described (Liu et al., 2005; Bohmann et al., 2009). Briefly, total RNA...
(2 μg) was extracted from 12 formalin-fixed, paraffin-embedded (FFPE) tissue samples using QuickExtract FFPE RNA Extraction Kit (Epicentre Biotechnologies, Cambridge, UK) and was reverse-transcribed in 10 μl with 200 units SuperScript reverse transcriptase III (Invitrogen, Paisley, UK). Subsequently, the first-strand cDNA reaction mixture (1 μl) was amplified by PCR with Taq DNA polymerase (Qiagen) as previously described (Liu et al., 2005). Genbank accession numbers, primer position and sequence, and product sizes are presented in Table I. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to provide a normalization control. PCR was carried out as follows: 94°C for 3 min followed by 26 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1.5 min. Following agarose gel electrophoresis, PCR products were visualized with SYBR Safe (Invitrogen, Paisley, UK). PCR product identity was confirmed by a correct sized band by agarose gel electrophoresis and DNA sequence analysis of the PCR product.

Statistical analysis of semi-quantitative immunohistochemical staining

The Statistical Package for the Social Sciences (SPSS) version 15.0 for Windows was used for all statistical calculations. Semi-quantitative scores from different groups were compared by non-parametric tests (Mann–Whitney U (MWU) or Kruskal–Wallis (KW) test as appropriate). The joint effects of endometriosis and the effect of the timing of the biopsy in the menstrual cycle on endometrial staining scores for MIPs were examined for the various endometrial cellular compartments by employing two-way analysis of variance (ANOVA) including the interactions (Univariate linear modelling). Since the data are ordinal with heteroscedasticity the P-values from this method are less precise. We therefore interpret only the low P-values (<0.01) as statistically significant. Furthermore, the conclusions were checked by a non-parametric analysis; the Box method of Akritas et al. (1997) implemented in Intercooled Stata 11 software package. The semi-quantitative staining scores between matched ectopic and eutopic endometrial samples from the same patients were compared with Wilcoxon’s signed-rank (WSR) test. A value of P < 0.05 was considered significant.

Results

Demographics

Patient characteristics of the women included have been previously described (Hapangama et al., 2009) and are detailed in Table II. Circulating estrogen and progesterone levels did not show a statistically significant differences between the two patient groups (P > 0.05) (Hapangama et al., 2009). Among the endometriosis patients, there were three, eight and six fertile women having had at least one full term delivery, respectively, in the proliferative phase, WOI and LS phase groups and the remainder of the women with endometriosis had never been pregnant.

External positive controls for the MIPs

Paraffin sections of human breast carcinoma, known to express AGR2 (Fig. 1ia), S100P (Fig. 1ia), S100A4 (Fig. 1iiia) and OPN (Fig. 1iva) were used as an external positive controls and specific brown staining for the corresponding protein was seen. Mouse or rabbit IgG (Dako, USA) was used as the negative control, as appropriate, and there was no staining identified in the negative control sections.
Figure 1  Immunolocalization of the MIPs across the menstrual cycle in human eutopic endometrium. Photomicrographs are representatives of the immunostaining for AGR2 (i), S100A4 (ii), S100P (iii) and OPN (iv); any positive cytoplasmic and nuclear staining is brown; (a) External positive breast carcinoma control tissue (with inserts illustrating the negative control sections); (b) proliferative phase (PROL) endometrium of FCs, (c) PROL endometrium of endometriosis group (Endo), (d) FC endometrium of WOI, (e) Endo WOI, (f) FC LS endometrium (LS) and (g) Endo LS. Scale bar = 100 μm (i, v and e) applicable to all panels. In proliferative phase, both groups show some positive staining for all the markers studied (i–iv, b and c). Note the decrease of the metastatic inducers AGR2 and S100P in the FC WOI and LS, and the increased staining for OPN in the epithelial fraction of the FC WOI (i–iv, d and f). In endometriosis samples, the immunostaining for the metastasis-inducers is increased in the WOI and LS (i–iv, e and g).
Immunohistochemical staining of eutopic endometrium

Anterior gradient homologue 2

FC women showed weak positive immunoreactivity to AGR2 during the proliferative phase endometrium in all cellular compartments except in the endothelial cells. Thereafter the endometrium of FC women showed a significantly greater staining for AGR2 localized to the luminal epithelium of the WOI (MWU test, $P = 0.0001$) and some stromal staining of the LS samples (mean score 0.31, MWU test $P = 0.01$), while all other cellular compartments showed virtually no AGR2 immunoreactivity (KW test $P < 0.05$, Figs 1 and 2, Table III). Samples of endometrial tissue taken during the secretory phase from women suffering from endometriosis on the other hand showed significantly greater immunoreactivity to AGR2 in all tissue compartments studied compared with the secretory phase endometrium of FC women: WOI and LS (MWU test $P < 0.01$ and $P < 0.005$, respectively, Table III).

S100A4

Weakly positive S100A4 staining was seen in the endometrial stromal, perivascular and endothelial cells of FC women without a cyclical variation in the staining pattern (Table III, KW test $P > 0.05$). When the secretory phase endometrium was compared with the proliferative phase endometrium of women with endometriosis, significantly more intense staining for S100A4 was seen in stromal (KW test $P < 0.02$), perivascular ($P < 0.001$) and endothelial ($P < 0.007$) compartments, with the strongest staining seen in the LS phase (Figs 1 and 2). Furthermore, when the secretory phase endometrium of women with endometriosis was compared with that of FC women, women with endometriosis showed a significantly more intense staining in stromal (MWU test $P = 0.0001$), perivascular ($P = 0.0001$) and endothelial ($P = 0.0001$) compartments (Table III). The luminal epithelial cells of FC women and the glandular epithelium of all women did not show positive immunostaining for S100A4 (Table III, Fig. 2).

S100P

Positive S100P staining was seen in both epithelial and stromal fractions of FC endometrium and there was significantly reduced stromal staining during the WOI (KW test $P = 0.02$, Table III, Figs 1 and 2). The proliferative phase endometrial samples from both groups showed similar weak S100P immunoreactivity in all tissue compartments studied, except the endothelial cells, and there were no statistically significant differences between the two groups (MWU test, $P > 0.05$). The eutopic endometrial stromal cells, however, showed more intense staining for S100P in women with endometriosis in the WOI (MWU test, $P = 0.006$) when compared with the FC group. Furthermore, when compared with the FCs, the women with endometriosis in the LS phase showed significantly more intense S100P immunoreactivity in all eutopic endometrial compartments (MWU test, $P < 0.001$), except for the endothelial cells which remained negative for S100P staining in all women.

Osteopontin

All women showed either weak or no OPN immunostaining in the proliferative phase. In FC women, the glandular and luminal epithelial staining for OPN increased from proliferative phase to secretory phase with the highest levels in the WOI phase (Table III, KW test, $P < 0.002$). Furthermore, the pattern of epithelial staining for OPN was similar in the eutopic endometrium of the women with endometriosis and of FCs (MWU test, $P > 0.05$) during the WOI. In the LS eutopic endometrium of women with endometriosis, there was a significantly greater epithelial and stromal (MWU test, $P < 0.002$ and $P = 0.007$, respectively) OPN immunoreactivity compared with the FC (Table III, Figs 1 and 2).

The interaction between the patient groups and phase of the menstrual cycle at the time of the biopsy showed a strong statistical significance for S100P staining in glands and stroma (Fig. 2, two-way ANOVA (Univariate general linear modelling), $P = 0.0001$), for AGR2 staining in epithelium ($P < 0.003$), and for staining for OPN in epithelial and stromal cells ($P < 0.01$). This reinforces our finding that endometriosis is associated with a specific pattern of MIP immunoreactivity at specific points in the menstrual cycle. Although there is evidence of a similar trend for S100A4 immunostaining in the epithelial compartment, it did not attain statistical significance ($P = 0.052$, Fig. 2).

Immunohistochemistry of ectopic endometrium

All red and blue peritoneal lesions showed strong immunoreactivity for all four MIPs studied. The superficial red lesions showed moderate immunoreactivity, whereas strong immunostaining was seen in more established blue lesions (Fig. 3). Furthermore, significantly increased staining was seen in ectopic endometrial glandular cells for S100P (mean $\pm$ SD 1.4 $\pm$ 0.6 versus 0.25 $\pm$ 0.4, WSR test $P = 0.03$), AGR2 (mean $\pm$ SD 2.08 $\pm$ 0.6 versus 1.03 $\pm$ 0.5, WSR $P = 0.04$) and S100A4 (mean $\pm$ SD 1.6 $\pm$ 0.5 versus 0 $\pm$ 0, WSR $P = 0.02$) and in endometrial cells for S100P (mean $\pm$ SD 1.29 $\pm$ 0.5 versus 0 $\pm$ 0, WSR $P = 0.02$), AGR2 (mean $\pm$ SD 1.25 $\pm$ 0.4 versus 0.4 $\pm$ 0.3, WSR $P = 0.03$) and S100A4 (mean $\pm$ SD 2.67 $\pm$ 0.4 versus 0.6 $\pm$ 0.6, WSR $P = 0.02$) when compared with the matched eutopic endometrial samples from the same patient.

Effect of fertility status or stage of endometriosis on MIPs staining

When we compared fertile and infertile women during each time point in the cycle regardless of whether they had endometriosis or not, we saw a significant difference only in the LS phase. There was increased staining seen in the endometria of the six infertile women (all of which whom had endometriosis) for AGR2 in the glands (MWU test $P = 0.02$), epithelium (MWU test $P = 0.01$) and stroma (MWU test $P = 0.007$) compared to the six fertile women (all of whom did not have endometriosis).
**Discussion**

This study is the first to examine the expression of AGR2 protein in human eutopic endometrium and the first to investigate the involvement of AGR2, S100P and S100A4 in endometriosis. We have located all three markers in the endometria from healthy fertile women using IHC and we have confirmed the presence of these proteins and their gene expression in the endometrial tissue by western blotting and by RT–PCR. Our endometrial staining results for OPN were consistent with previous reports (Lessey, 2002; Allan et al., 2003; Cho et al., 2009). FC women show changes in the staining pattern in various endometrial cellular compartments for AGR2, S100P and for OPN according to the ovarian cycle, with no cyclical variation for S100A4. The expression of all four MIPs is different in women with endometriosis and these proteins are abundantly present in the ectopic endometrial lesions. We have used immunoblotting to confirm the immunostaining. It is however, difficult to use protein extracted from the whole endometrial tissue sample to assess the level of expression of these protein which may be expressed in only single cell type. Therefore, apart from showing the presence or absence of the protein; whole tissue immunoblotting may not be entirely appropriate in quantitative analysis. Therefore, to confirm the differences between the two groups, we carried out RT–PCR extracting RNA message from the same paraffin blocks where IHC staining was performed. Our results demonstrate how

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**Table III** Mean semi-quantitative scores for MIPs staining in various eutopic endometrial cellular compartments for the groups studied.

<table>
<thead>
<tr>
<th></th>
<th>Glandular epithelium mean (± SD)</th>
<th>Stroma mean (± SD)</th>
<th>Luminal epithelium mean (± SD)</th>
<th>Perivascular mean (± SD)</th>
<th>Endothelial cells mean (± SD)</th>
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<tbody>
<tr>
<td>PROL FC (n = 5)</td>
<td></td>
<td></td>
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<tr>
<td>AGR2 0.8 (± 0.5)</td>
<td>0.2 (± 0.4)</td>
<td>0.9 (± 0.6)</td>
<td>0.2 (± 0.4)</td>
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<tr>
<td>S100A4 0 (± 0.0)</td>
<td>0.5 (± 0.5)</td>
<td>0 (± 0)</td>
<td>1.3 (± 0.3)</td>
<td>1.3 (± 0.2)</td>
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<tr>
<td>S100P 0.2 (± 0.2)</td>
<td>0.6 (± 0.5)</td>
<td>0.1 (± 0.4)</td>
<td>0.2 (± 0.2)</td>
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<tr>
<td>OPN 0 (± 0)</td>
<td>0.1 (± 0.2)</td>
<td>0 (± 0)</td>
<td>0 (± 0)</td>
<td>0 (± 0)</td>
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<td>PROL Endo (n = 9)</td>
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<tr>
<td>AGR2 0.9 (± 0.8)</td>
<td>0.5 (± 0.7)</td>
<td>1.4 (± 0.8)</td>
<td>0.6 (± 0.8)</td>
<td>1.1 (± 0.9)</td>
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<td>S100A4 0 (± 0)</td>
<td>1.3 (± 0.7)</td>
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<td>1.8 (± 0.8)</td>
<td>1.3 (± 0.2)</td>
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<tr>
<td>S100P 0 (± 0)</td>
<td>0.2 (± 0.3)</td>
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<td>0.1 (± 0.3)</td>
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<td>OPN 0.3 (± 0.4)</td>
<td>0.1 (± 0.2)</td>
<td>0.3 (± 0.4)</td>
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<td>WOI FC (n = 18)</td>
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<tr>
<td>AGR2 0.5 (± 0.5)</td>
<td>0 (± 0)</td>
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<tr>
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PROL FC, fertile control group biopsies collected during the proliferative phase of the cycle; PROL Endo, endometriosis group biopsies collected during the proliferative phase of the cycle; WOI FC, fertile control group biopsies collected during the window of implantation of the cycle; WOI FC, endometriosis group biopsies collected during the window of implantation of the cycle; LS FC, fertile control group biopsies collected during the LS phase of the cycle; LS Endo, endometriosis group biopsies collected during the LS phase of the cycle.
MIPs vary during the menstrual cycle in women with and without endometriosis.

The upper functional layer of endometrium is shed during the menses and a new upper layer is regenerated from the lower basal layer. The exact origin of the cells of the basal layer that contribute to the upper layer is still debated, although it is likely to originate from the pluripotent endometrial stem cells that are characteristically slow-cycling and quiescent (Gargett and Chan, 2006). S100A4 in particular has been reported to be preferentially expressed in cancer stem/progenitor cells (Harris et al., 2008). Women with endometriosis shed more cells from the basalis layer at menstruation where a large number of putative endometrial stem/progenitor cells reside (Leyendecker et al., 2002). The non-cyclical expression pattern of S100P at the protein level in the endometrium (Tong et al., 2010) examined a group of women with regular menstrual periods without surgical exclusion data (i.e. whether they had endometriosis or not or if they were on hormonal treatments). This report examined the endometrium at different points in the secretory phase of the cycle and showed that in addition to the S100P protein, the S100P mRNA level was increased at LH + 7 compared with LH + 12 and also reported that there was no significant change in mRNA expression for S100A4. Since the expression of mRNA does not necessarily reflect that of the corresponding protein and because of the above differences in experimental design, the results are not comparable.

Window of implantation

Secretory phase endometrial cells of FC women exhibit reduced rates of proliferation with induction of the synthesis and release of endometrial secretory products together with neovascularization in response to the post-ovulatory high progesterone levels (Critchley and Healy, 1998 and references therein). AGR2 expression has been shown to promote tumour growth, cell migration, cellular transformation and survival (Ramachandran et al., 2008; Wang et al., 2008). An increase in S100P expression has been linked with preventing senescence and promoting cell survival in other tissues (Averboukh et al., 1996; Bertram et al., 1998). We have recently reported that cellular senescence and apoptosis may occur during the WOI of fertile healthy women as suggested by a decrease in proliferative markers and by an increase in the γ-H2AX foci in their endometrium (Hapangama et al., 2009). The significant decrease in the staining for AGR2 and S100P in specific endometrial cellular compartments during the WOI in fertile women, therefore, may reflect a decrease in proliferation, maximizing differentiation of the endometrium to optimize the cellular milieu for the implantation of a fertilized embryo. The increased OPN staining seen in the WOI when compared with the proliferative phase in the endometrial epithelial (both glandular and luminal) fraction of the FC women is in agreement with previous reports (Lessey, 2002) and further confirms a possible role of OPN in embryo—maternal dialogue (von Wolff et al., 2001). The specific increase in AGR2 immunostaining observed in the luminal epithelium of the FC women during the WOI may suggest a similar role in embryo attachment for AGR2.

Women with endometriosis show a significant increase in the endometrial immunoreactivity for AGR2, S100P and S100A4 during the WOI. We and others have previously shown that the eutopic endometrium of women with endometriosis during the WOI shows a pro-proliferative, apoptosis-resistance phenotype and that such aberrations in endometrial cell function may render the endometrium ‘hostile’ to an invading embryo (Hapangama et al., 2008a, 2009, 2010). The observed aberrant expression of AGR2 and S100P in
WOI of women with endometriosis may contribute to the proliferative phenotype (Averboukh et al., 1996; Wang et al., 2008). Zhang et al. have recently suggested that the endometrial S100P gene expression in the form of mRNA may be up-regulated during the WOI in subfertile women (Zhang et al., 2008). Therefore, an aberrant expression of S100P seen during the WOI may promote invasion, cell–cell adhesions and thus may interfere with implantation, contributing to the endometriosis-associated subfertility. Up-regulation of S100A4 as seen in endometrial cancers (Xie et al., 2009) and infiltration of adjacent host cells (fibroblasts, immune and vascular cells) of the affected regions may boost the cytokines, growth factors and MMPs in the cellular milieu (Grigorian et al., 2008 and references therein). By expressing an increased amount of S100A4 during the WOI, endometrial cells of women with endometriosis could contribute to aggravation of the pathological process by altering the cellular milieu and rendering the endometrium unsuitable for implantation. According to the literature, there is insufficient evidence for a direct causative role for endometriosis in preventing embryo-implantation causing infertility, but there is, however, epidemiological evidence to support the link between endometriosis and recurrent conception failure after assisted reproduction (Dmowski et al., 1998; Koninckx, 1998). The OPN staining pattern seen in the WOI eutopic endometrium of both FC women and women with endometriosis is consistent with previous reports (von Wolff et al., 2001; Lessey, 2002; Wai and Kuo, 2004).

LS phase

If conception fails to occur, the secretory endometrium undergoes tissue breakdown and menstrual shedding, followed by regeneration on a monthly basis. The endometrial cells in the LS endometrium of FC show increased apoptosis, senescence and a reduction in proliferation (Toki and Nakayama, 2000 and references therein; Johnson et al., 2005; Hapangama et al., 2009). AGR2 was initially described in studies exploring the differentially expressed genes in estrogen receptor-positive breast cancers (Thompson and Weigel, 1998). Overexpression of AGR2 in the rat non-metastatic breast tumour cell line is associated with increased metastasis when propagated in syngeneic rats, suggesting that AGR2 may influence tumour biology (Liu et al., 2005). Although the detected levels of staining for S100A4 do not show a cyclical variation in the endometrium of FC women, there is a significant increase in staining of the eutopic stromal, peri-vascular and endothelial cells and the ectopic endometriotic lesions of women with endometriosis. S100P and S100A4 belong to the S100 family of calcium-binding proteins which have been shown to play a fundamental role in the metastatic phase of cancer (Wang et al., 2006; Grigorian et al., 2008). There was an increase in OPN expression in the menstrual endometrium in humans (Cho et al., 2009) and in a baboon model of endometriosis (Allan et al., 2003) agreeing with our observations in the LS endometrium of women with endometriosis. There is also a report of a decrease in OPN in LS (Wei et al., 2009), but this particular study was smaller in sample number, included a control group with no surgical exclusion of endometriosis and had some inter-observer inconsistencies of OPN scores, particularly in the LS phase. Functional roles of OPN include mediating cell adhesion, chemotaxis, macrophage-directed interleukin-10 suppression, stress-dependent angiogenesis, preventing apoptosis and anchorage-independent growth of tumour cells and there has been a wealth of evidence linking OPN with the regulation of metastatic spread by tumour cells (Wai and Kuo, 2004; El-Tanani et al., 2006 and references therein). The results for women with endometriosis suggest that there is an increased signal for all four MIPS in the LS endometrium which may provide a metastatic advantage for these cells to invade the peritoneal lining and initiate endometriotic deposits following retrograde menstruation. Furthermore, by expressing S100P and AGR2, the endometrial cells
may also prevent senescence and increase their proliferative potential to survive and give rise to endometriotic deposits in the peritoneal cavity.

Since MIPs may be regulated via oestrogen receptor (ER) alpha, the observed increase of the MIPs between the WOI and LS phase in women with active peritoneal deposits may be caused by relatively unopposed estrogen action via progesterone resistance that is described in the pathogenesis of endometriosis (Bulun et al., 2010). However for the changes seen with S100P for example, progesterone resistance may not be a valid explanation as the breast cancer studies do not support S100P regulation via ER or progesterone receptor (PR) (Wang et al., 2006). Although there are some reports of S100P being associated with PR in cell lines using microarray hybridization (Bray et al., 2005), microarray experiments have not shown a significant up-regulation of S100P in association with ER/PR in primary breast cancer studies (Sorlie et al., 2001). Further functional studies are required to examine the steroid hormonal regulation specific to the MIPs in normal endometrial cells and their differential regulation in primary endometrial cells from women with endometriosis to ascertain if PR is involved in expression of MIPs. Alternatively MIPs may be induced by the pro-inflammatory cytokines present in the peritoneal fluid of women with endometriosis, that may influx into the endometrial cavity (Kyama et al., 2009; Hapangama et al., 2010). At the moment, we cannot say whether MIP expression is a causative or resultant effect of endometriosis. This question however, can only be answered in future in vitro studies using direct transfection of expression vectors for the MIPs into endometrial cells and testing for the ability of the resultant transfectants to produce endometriotic lesions in syngenic animal models such as the baboon model of endometriosis (Hapangama et al., 2010).

**Ectopic endometrium**

Endometriosis shares some characteristics with cancer in that it is a metastatic disease. However, in endometriosis, the cells that are disseminated are not neoplastic so that the condition is not fatal. Similar to cancer cells, ectopic endometriotic cells show excessive cell
division, activation of oncogenes and deficient apoptosis (Merelman et al., 2002; Harada et al., 2007; Chrobak et al., 2008). In addition to the ability to proliferate and survive in the peritoneal cavity after retrograde menstruation, the establishment of the ectopic endometriotic lesions requires attachment and invasion of the peritoneal barrier. In cancer cells, distant metastasis occurs with the expression of OPN and AGR2 which mediate host-tumour cell dialogue to initiate invasion, while S100P and S100A4 expression induce invasive properties (Moye et al., 2004; Wai and Kuo, 2004; Liu et al., 2005; Zhang et al., 2003; Wang et al., 2006; Xie et al., 2007). All active peritoneal endometriotic deposits show strong positive staining for the four MIPs studied, and the staining is significantly greater in the ectopic tissue when compared with the matched eutopic endometrial samples from the same patient. The pro-proliferative, adhesion-inducing and pro-survival roles of the MIPs may therefore encourage the invasion, expansion and survival of the ectopic lesions. The positive immunoreactivity seen in the early red endometriotic deposits and strong staining persisting in the more established blue lesions suggest an involvement of these MIPs in the pathogenesis and the progression of endometriosis. We note that the MIPs cannot induce neoplasia alone (Dunnington et al., 1983; El-Tanani et al., 2006), thus the expression of MIPs in endometriotic cells in ectopic deposits may maintain an invasive and metastatic phenotype without developing endometrial malignancy. We found that each MIP had its own pattern of expression with respect to location and influence of the menstrual cycle. This implies that during health, each MIP has its own pattern of regulation and that this divergent regulation is replaced by convergent expression during the development of disease, be it endometriosis or breast cancer. We speculate that further study of this system will uncover regulatory checkpoints that will elucidate constraints on the extent of metastasis in breast cancer and the development of endometriosis.

Conclusion

In summary, we have identified AGR2, S100P and S100A4 using immunohistochemistry in the benign premenopausal endometrium and have produced detailed information on the presence of these MIPs throughout the menstrual cycle. Eutopic endometrial cells show an increased immunoreactivity for these MIPs and are likely, therefore, to possess an invasive and metastatic advantage in the peritoneal cavity after retrograde menstruation; hence they are more likely to implant and give rise to endometriotic deposits. Their continuing presence in cells of endometriotic lesions is also likely to aid invasion and expansion, contributing to the progression of the disease. We therefore report the first immunohistochemical study with confirmatory immunoblotting and RT–PCR data suggesting a possible role of four MIPs in the pathogenesis of endometriosis.

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Authors’ roles

D.K.H. and P.S.R. were involved in conception and design of the study. All authors contributed significantly to the acquisition, analysis and interpretation of data, drafting the article and revising it critically and have approved the final version of the manuscripts to be published.

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Conflict of interest

The authors have nothing to disclose.

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