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Estradiol induces heparanase-1 expression and heparan sulphate proteoglycan degradation in human endometrium

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BACKGROUND: This study seeks to determine whether estrogen is able to regulate the expression of heparanase-1 (HPR1) in human endometrium. METHODS: HPR1 expression and heparan sulphate (HS) deposition in the endometrium collected in various menstrual phases were analysed by immunohistochemical and immunofluorescence staining, respectively. HPR1 expression in the endometrial cells unexposed or exposed to estradiol was analysed by using RT-PCR and luciferase reporter assay. HPR1 activity was analysed by using a novel enzyme-linked immunosorbent assay (ELISA). Cell surface HS levels were analysed by flow cytometry. Serum HPR1 activity in women receiving follicle-stimulating hormone (FSH) for IVF was measured by ELISA. RESULTS: HPR1 expression was rarely detected in the endometrium in the early and mid-proliferative phases but was increased in the late proliferative phase and in the secretory phases. HPR1 expression was negatively associated with HS in the basement membrane (BM) of the endometrial glands. HPR1 gene expression, HPR1 promoter activity and HPR1 enzymatic activity were increased in the endometrial cells when exposed to 17β-estradiol (E_2), whereas cell surface HS levels showed a decrease which could be blocked by PI-88, an HPR1 inhibitor. Serum HPR1 levels were increased in women with moderately elevated blood estrogen levels after receiving FSH. CONCLUSIONS: HPR1 is differentially expressed in the endometrium in different menstrual phases. Estrogen plays an important role in inducing HPR1 expression, subsequently leading to HS degradation on the endometrial cell surface and in the BM of the endometrium.

Key words: endometrium/estradiol/heparanase/heparan sulphate/ovarian hyperstimulation syndrome

Introduction

Heparan sulphate proteoglyeans (HSPGs) are important components of the cell surface, the extracellular matrix (ECM) and the basement membrane (BM). At least 13 *HSPG* genes from 5 distinct classes have been identified. They include three pericellular HSPGs (perlecan, agrin and the hybrid HSPG/collagen type XVIII), four integral membrane syndecans and six glycosyl-phosphatidylionosito-anchored glypicans (Perrimon and Bernfield, 2000). HSPGs comprise a core protein that is covalently attached to a unique glycosaminoglycan chain characterized by a linear array of alternating disaccharide units (Stringer and Gallagher, 1997; Perrimon and Bernfield, 2000; Esko and Lindahl, 2001; Iozzo, 2001). Previous studies have shown that the HSPG gene expression and the presence of HS on HSPG protein cores can be regulated by steroid hormones in the endometrium. For example, Russo *et al.* (2001) reported that administration of 17β-estradiol (E_2) leads to increased expression of syndecan-3 in the uterus of ovariectomized rats. Syndecan-3 is largely located in the epithelial cells of glands and in the endometrial stroma as well as in the smooth muscle cells of the myometrium. Potter and Morris reported that syndecan-1 expression is decreased in the basolateral epithelial cells but is increased in the basal epithelial cells as the cycle progresses from metestrus toward estrus (Potter and Morris, 1992). Paradoxically, Morris *et al.* (1988) showed that E_2 can accelerate the turnover of HSPGs in the lysosomes of murine endometrial epithelial cells. Though the significance of HSPG expression and regulation in the endometrium remain to be defined, it appears that the regulation of HSPG expression and the status of HS in HSPGs by estrogen may have important physiological functions.

Heparanase-1 (HPR1) is an endoglycosidase that specifically degrades HSPGs (Hulett *et al.*, 1999; Vlodavsky *et al.*, 1999; Parish *et al.*, 2001; Vlodavsky and Friedmann, 2001). HPR1

expression is up-regulated in a variety of malignancies and plays an important role in tumour angiogenesis and metastasis. HPR1 stimulates tumour angiogenesis by releasing the growth factors such as fibroblast growth factor and vascular endothelial growth factor (VEGF) stored in the ECM (Elkin *et al.*, 2001; Goldshmidt *et al.*, 2002; Vlodavsky *et al.*, 2002; Elkin *et al.*, 2003; Edovitsky *et al.*, 2004; Mikami *et al.*, 2004; Cohen *et al.*, 2005; Zcharia *et al.*, 2005) and by inducing the expression of VEGF (Zetser *et al.*, 2006) and cyclooxygenase-2 (Okawa *et al.*, 2005). HPR1 promotes tumour metastasis by degrading HSPGs in the ECM and BM, allowing tumour cells to spread to a distant site (Vlodavsky *et al.*, 1999, 2002; Marchetti and Nicolson, 2001; Vlodavsky and Friedmann, 2001; Edovitsky *et al.*, 2004; Cohen *et al.*, 2005). In addition, HPR1 can function as an adhesion molecule (Goldshmidt *et al.*, 2003; Zetser *et al.*, 2003) and promote endothelial cell migration (Gingis-Velitski *et al.*, 2004a). Recent studies suggest that HPR1 is involved in the pathogenesis of many other diseases such as diabetic nephropathy (Maxhimer *et al.*, 2005) and delayed type hypersensitivity (Edovitsky *et al.*, 2005).

HPR1 expression is also detected in several reproductive cell types and may play an important role in tissue remodelling. For example, HPR1 expression is detected in the extravillous trophoblasts invading the decidua and in the endothelium of fetal capillaries (Haimov-Kochman *et al.*, 2002). HPR1 expression in these cells may facilitate embryo implantation by promoting trophoblast cell invasion and tissue remodelling. Indeed, Zcharia *et al*. (2004) demonstrated that transgenic mice overexpressing HPR1 in all tissues under the control of a chicken β-actin promoter have a significantly higher embryo implantation rate than control mice. Further studies by these investigators showed that pretreatment of mouse embryos with recombinant HPR1 *in vitro* is able to increase the implantation rate (Revel *et al.*, 2005). It was not clear whether HPR1 is also expressed in the endometrium and whether HPR1 is involved in endometrial tissue remodelling and in preparing the endometrium for embryo implantation and subsequent angiogenesis. On the basis of a prior study (Elkin *et al.*, 2003) demonstrating the ability of estrogen to induce HPR1 expression in breast cancer, we hypothesize that HPR1 may be differentially expressed in different menstrual phases in the endometrium, due to regulation by steroid hormones. In the present study, we report that HPR1 expression is increased in the endometrium in the late-proliferative phase (LP) and plate secretory phases (LS), and that estrogen is able to induce HPR1 expression and HS degradation on the cell surface and in the BM of the endometrium.

Materials and methods

Human study

The use of human tissues was approved by the Rush University Medical Center Institutional Review Board. Women with regular menstrual cycles (28–30 days) of the reproductive ages were recruited by the senior author (W.P.) from an IVF clinic as part of an infertility evaluation. During laparoscopy, pelvic organs were examined for the presence and extent of endometriosis. Women with pelvic inflammatory disease, endometriosis and adhesions were not included in the study. Samples of the uterine endometrium from 33 women (mean age ± SD = 32.7 ± 7.2 years old, median age, 34 years old; range 21–44 years old) were obtained with Novak's curette from the uterine fundus. No hormonal therapies were used in these 33 women during the cycle. The menstrual phases were classified as early-proliferative (EP), mid-proliferative (MP), late-secretory (LP), early-secretory (ES), mid-secretory (MS) and LS phases, on the basis of the histological morphology and the date of menses as previously described (Noyes and Haman, 1953; Dmowski *et al.*, 2001). Part of each specimen was fixed immediately in 4% formaldehyde and embedded in paraffin within 48 h in a single pathology laboratory. Paraffin blocks of the uterine endometrial specimens were retrieved for the study from the pathology laboratory repository.

To study the effect of estrogen in regulating HPR1 expression *in vivo*, we tested whether increased blood estradiol levels correlated with increased serum HPR1 levels. To address this, we analysed HPR1 activity in the serum samples from seven women undergoing controlled ovarian stimulation with follicle-stimulating hormone FSH for IVF. The diagnoses for these infertile patients were endometriosis (three), ovarian factor (two), male factor (one) and tubal factor (one). The patients were treated with 150 to 600 units of recombinant FSH (Follistim, Organon USA or GonalF, Serono, USA) by s.c. injection until oocyte retrieval. The blood samples were collected at various time points according to each individual's response. The dose of FSH was adjusted according to the ovarian response to FSH stimulation, e.g. serum E_2 levels and the follicular growth. The former was quantitfized by using an Immulite kit (Diagnostic Products Corporation, LA, CA, USA); the later was monitored by ultrasonography. After the hormonal assay, serum samples were stored in a −80ºC freezer until assayed for HPR1 activity. Serum samples collected at multiple times during the IVF cycle were used in this study.

Immunohistochemistry

Sections of endometrial specimens and an HPR1-positive pancreatic adenocarcinoma that was included as a positive control (PC) were de-waxed with xylene and rehydrated. Slides were heat-inactivated in 10 mM sodium citrate (pH 6.0) in a microwave for 3 min. Cooled slides were rinsed with PBS and then incubated with 1% H_2O_2 in methanol for 30 min at room temperature. Sections were blocked with 5% normal goat serum in phosphate-buffered saline (PBS) for 30 min at room temperature, followed by 1 h incubation with an anti-HPR1 rabbit antiserum (1:500 dilution) in PBS. This antiserum was raised by immunizing a rabbit with a peptide containing the amino acid residues from 273 to 290 of the 50 kDa HPR1 subunit (Fairbanks *et al.*, 1999). Normal rabbit serum was included as a negative control (NC). Slides were washed and then incubated with goat anti-rabbit antibody–biotin conjugate (PharMingen, San Diego, CA, USA) diluted at 1:300 in PBS with 5% human serum. Strepavidin–horse-radish conjugate (Zymed, San Francisco, CA, USA) diluted at 1:200 in PBS with 5% normal human serum was added and incubated for 45 min at room temperature. Colour development was done with diaminobenzidine (DAB) substrate (Sigma, St. Louis, MO, USA) followed by DAB enhancer (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with Mayer's haemotoxylin for 2-min, dehydrated and mounted. HPR1 expression was graded in a blinded fashion by two investigators (J.S. and X.X.) in this study. Negative HPR1 expression was defined as no HPR1 or weak signal detected in <10% of the stromal or glandular cells. Positive HPR1 expression was defined as HPR1 signal in at least 10% of cells with moderate or strong intensity in either the stromal or glandular cells.

Immunofluorescence analysis of HS deposition

The sections were de-waxed, rehydrated and fixed with 1% paraformaldehyde. The slides were incubated with an anti-HS mAb (Clone HepSS, Seikagaku Corp., Chuo-ku, Tokyo, Japan) at a concentration of 5 μg ml^{-1} at room temperature for 30 min. The same concentration of normal mouse IgM was used as a negative control. The slides were washed three times with PBS, followed by incubation with a goat anti-mouse IgM conjugated with FITC (1:50) (ICN Biomedicals, Aurora, OH, USA) and then washed and incubated with rabbit anti-goat IgG conjugated with FITC (1:50) (ICN Biomedical, Aurora, OH). The slides were washed and sealed with 50% glycerin in PBS containing anti-fade reagent 1,4-diazabicyclo(2,2,2)octane (DABCO) (25 mg ml^{-1}). HS expression was examined under fluorescent microscopy. Negative HS staining was defined as the absence of fluorescence signal in the BM in >90% of the glands. Positive HS staining was defined as the presence of fluorescence signal in the BM in >10% of the glands. The pictures were taken with a digital camera attached to a Nikon Eclipse TE200 fluorescence microscope.

Endometrial cell culture and stimulation with E_2

Approximately 200 mg of fresh tissue in the LP or ES phase were minced into small pieces (1–2 mm^3) and washed in fresh medium to remove mucus or debris. Tissue fragments were then digested in the medium with collagenase III (100 μg ml^{-1}) and DNase (200 units ml^{-1}) at 37°C for 45 min by stirring. At the end of incubation, cell clumps were further mechanically dispersed by aspiration with a Pasteur pipette. The mixture of single stromal cells and the large clumps of epithelium were washed twice with Hank's balanced salt solution (Pharmacia, Piscataway, NJ, USA). The stromal and epithelial cells were not further separated, since our immunohistochemical (IHC) analysis revealed that HPR1 expression was detected in both cell types. Primary endometrial cells were grown in RPMI 1640 supplemented with 5% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, Utah). After incubation for 24 h, the unattached float cells were removed. In some experiments, the medium was replaced with phenol red-free RPMI 1640 medium containing 5% charcoal/dextran-treated fetal bovine serum. There was no significant difference in the experimental results conducted with the medium containing phenol red or no phenol red. The monolayers of endometrial cells from a 72-h primary culture were trypsinized and seeded in 6-well plates. Cells were left unstimulated or stimulated with various concentrations of E_2. After incubation for 48 h, the conditioned media were collected and spun down at 4°C, 15 000 *g* for 15 min. The supernatants were collected and stored at −80°C until use. The cells were washed three times with PBS and then detached by incubation with 5 mM EDTA in PBS for 5 min. The cells were lysed in HPR1 assay buffer [0.1 M sodium acetate, pH 5.0, 0.1 mg ml^{-1} bovine serum albumin (BSA), 0.01% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 10 μg ml^{-1} leupeptin and aprotinin each] followed by three quick freeze and thaw cycles. The cell lysates were spun down at 4°C, 15 000 *g* for 15 min. The supernatants were collected and analysed for protein concentration by using a Protein Assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates were analysed for HPR1 activity by using an enzyme-linked immunosorbent assay (ELISA) method as described below.

Semi-quantitative RT–PCR

Cells were seeded in 6-well plates and stimulated with the indicated concentrations of E_2 for 24 h. Cells were lysed in 1 ml TRIzol and RNA was extracted by following the manufacturer's instruction (Life Technologies). RNA concentration was quantified by ultraviolet absorption. After reverse transcription of 500 ng total RNA with oligo(dT) priming, the resulting single stranded cDNA was amplified using Taq DNA polymerase (Life Technologies). Oligonucleotides HPR-3 (5′-TTCGATCCCAAGAAGG-AATCAAC-3′) and HPR-4 (5′GTAG TGATGCCATGTAACTGAAT-C-3′) were used for amplifying a 587 bp heparanase cDNA fragment. Oligonucleotides 5′TGAAGGTC GGAGTCAACGGATTTGGTC-3′ and 5′ATGGACTGTGGTCATG AG-TCCTTCCACG-3′ were used to amplify a 527 bp GAPDH cDNA fragment. The PCR conditions were an initial denaturation of 4 min at 94°C and subsequent denaturation for 45 s at 94°C, annealing for 1 min at 55°C and extension for 1 min at 72°C (32 cycles). Aliquots of 10 μl PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Luciferase reporter gene expression

Primary endometrial cells were prepared and grown in RPMI 1640 supplemented with 5% charcoal/dextran-treated fetal bovine serum in a T-75 flask. Cells were harvested and washed twice with PBS. Cells (5×10^6 cells per sample) were transfected with 8 μg of plasmid containing the luciferase reporter gene driven by a 0.3- or 3.5-kb HPR1 promoter fragment (pGL3/HPR-0.3 and pGL3/HPR-3.5) and 2 μg of pCMV/SPORT containing the β-galactosidase gene as an internal control by electroporation with 300 V and 900 μCi in a Gene Pulser II (Bio-Rad, Hercules, CA, USA). Luciferase reporter constructs have been previously described (Jiang *et al.*, 2002). A plasmid containing the luciferase reporter gene without an HPR1 promoter (pGL3/Basic) was included as an NC. Cells were then washed and seeded in a 24-well plates in the absence or presence of the indicated concentrations of E_2. After incubation for 48 h, cells were harvested, and cell lysates were prepared. In some experiments, cells were also transfected with FuGENE6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA), following the manufacturer's instruction. Similar results were obtained. The luciferase activity was quantified by using the luciferin substrate and read in a TECAN plate reader (Phenix Research Products, Hayward, CA, USA). The relative light unit in each sample was normalized against the β-galactosidase activity measured by a colorimetric assay, as previously reported (Eustice *et al.*, 1991). The means of data in triplicate from two representative experiments were presented.

Flow cytometric analysis

Endometrial cells from the primary cell culture in T-75 flasks were seeded in 6-well plates and stimulated with E_2 (1×10^{-8} or 2×10^{-7} M) for 24 h in the absence or presence of PI-88 (50 μg ml^{-1}). Single cell suspensions of endometrial cells were prepared by using Cell Dissociation Solution. Cells (5×10^5 per sample) were stained by incubation for 30 min at 4°C with an anti-HS mAb (0.5 μg per sample) (clone HepSS, Seikagaku Corp., Chuo-ku, Tokyo, Japan) or mouse IgM as an isotype control. Cells were incubated with FITC-labelled goat anti-mouse IgM (5 μl per sample) for 30 min at 4°C, followed by rabbit FITC-labelled anti-goat IgG (5 μl per sample) for 30 min at 4°C. Cell surface HS expression was analysed in a Becton Dickson flow cytometer.

HPR1 activity assay

HPR1 activity in the serum samples, cell lysates and supernatants of endometrial cell cultures was measured by using a novel ELISA, as previously described (Quiros *et al.*, 2006). Briefly, Matrigel (BD Biosciences, San Diego, CA, USA), an artificial BM which contains abundant HSPGS, was dissolved in ice-cold PBS (0.1 M, pH 7.4)/carbonate-buffered saline (0.1 M, pH 9.6) (volume:volume, 50:50) at a concentration of 20 μg ml^{-1} and used to coat ELISA plates (25 μl per well) at 4°C overnight. The plates were then washed three times with PBS containing 0.05% Tween-20 and blocked with 5% BSA in

PBS at room temperature for 1 h. Serum samples were diluted at 1:5 in HPR1 assay buffer (0.1 M sodium acetate, pH 5.0, 0.1 mg ml^{-1} BSA, 0.01% Triton X-100, 0.5 mM PMSF, and 10 μg ml^{-1} leupeptin and aprotinin each). The supernatants of cultured endometrial cells were premixed with 10× HPR1 assay buffer. Diluted serum samples, supernatant and cell lysates (25 μl per well) were added to each well and incubated at 37°C overnight. After a wash, anti-HS-specific mAb (clone HepSS, Seikagaku Corp., Chuo-ku, Tokyo, Japan) (1:1000, diluted in PBS containing 5% BSA) was added and incubated at room temperature for 1 h. After washing, horse-radish peroxidase-conjugated goat anti-mouse IgM antibody (1:2000 diluted in PBS with 5% BSA) was added and incubated at room temperature for 1 h, followed by addition of 50 μl 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid substrate. The OD405 absorbance was read in an ELISA plate reader (Bio-Rad, Hercules, CA, USA). HPR1 activity in serum samples was calculated on the basis of a standard curve of serially diluted purified platelet HPR1 (starting at 1:200) at a concentration of 1 μl HPR1 with the activity of degrading 0.133 μg HS per hour at 37°C in HPR1 buffer. HPR1 purification and characterization from human platelets were conducted, as previously reported (Ihrcke *et al.*, 1998). HPR1 activity was designated as per 100 units capable of degrading 1 ng HS at 37°C h^{-1} in HPR1 buffer.

Statistical analysis

χ^2 test was used to analyse the significance of a difference in the frequency of HPR1 expression in the proliferative and secretory phases. Fisher's exact test was used to analyse the relationship of HPR1 expression with the deposition of HS in the BM. Spearman rank order correlation test was used to analyse the correlation between serum HPR1 activity and blood E_2 levels. A P-value < 0.05 was considered as statistically significant. All statistics were conducted by using the SigmaStat3 software (Richmond, CA, USA).

Results

IHC analysis of HPR1 expression in the endometrium

We first conducted IHC analysis to determine whether HPR1 expression differed in the endometrial tissue at the different phases of the menstrual cycle. As shown in Figure 1, HPR1 was neither expressed in glandular epithelial cells nor in the stromal cells in two endometrial specimens collected in the EP and MP phases. However, HPR1 was detected in the stromal and glandular cells in the endometrial specimens collected in the LP and ES, MS and LS phases. Approximately 80% of the specimens expressed HPR1 in both the stromal cells and glandular epithelia in the functionalis layers with comparable intensity. A normal rabbit serum was used as a NC; non-specific signals were not observed in this endometrial specimen collected in the LP phase. A HPR1-positive pancreatic adenocarcinoma was included as a PC; strong signals were observed in the tumour cells.

Among 33 endometrial specimens analysed, we found that HPR1 expression was detected in one of seven endometrial specimens (14%) in the EP and MP phases each. HPR1 was detected in three of six specimens taken in the LP phases and in 10 of 13 specimens during the secretory phases (Table I). Fisher's exact test revealed that HPR1 expression in the secretory phases (HPR1 positive in 10 of 13 specimens) was significantly higher than in the LP phases (HPR1 positive in

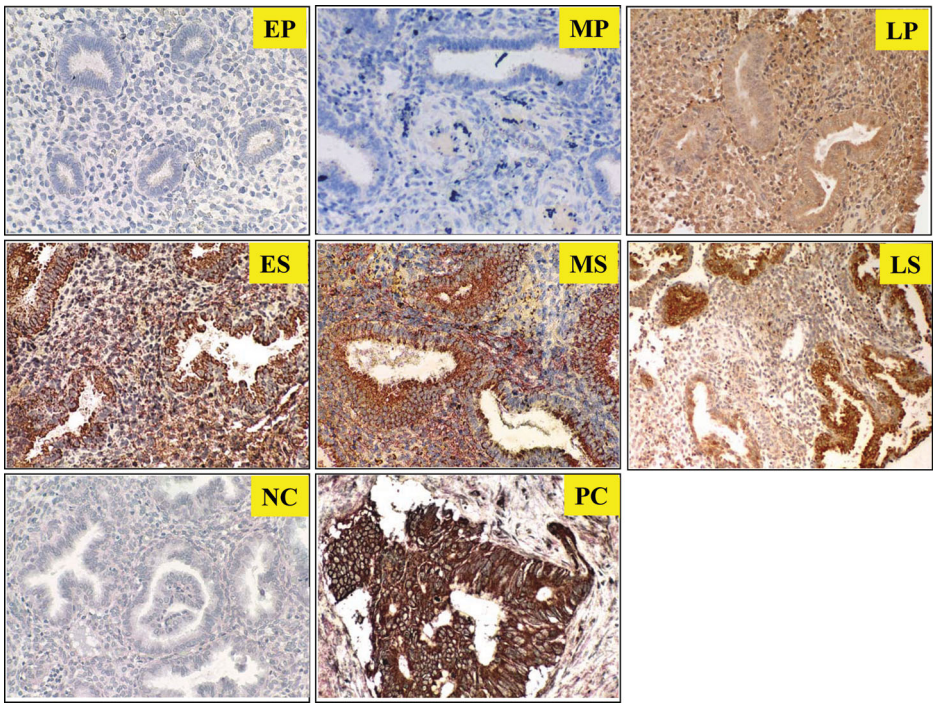

Figure 1. Immunohistochemical staining (IHC) of heparanase-1 (HPR1) expression. HPR1 expression was not present in endometrium in the early- and mid-proliferative (EP and MP) phase, but was strongly present in both the stromal and glandular cells in the endometrium in the late proliferative (LP), and early- mid- and late-secretory (ES, MS and LS) phases. Normal rabbit serum was included as a negative control (NC). A HPR1-positive pancreatic adenocarcinoma was included as a positive control (PC) for IHC staining.

Table I. Heparanase-1 (HPR1) expression in the endometrium during the menstrual cycle

Proliferative phase	Number of cases	HPR1 positive	Secretory phase	Number of cases	HPR1 positive
EP	7	1 (14%)	ES	6	4 (67%)
MP	7	1 (14%)	MS	3	2 (66%)
LP	6	3 (50%)	LS	4	4 (100%)

EP, early-proliferative; MP, mid-proliferative; LP, late-proliferative; ES, early-secretory; MS, mid-secretory; LS, late-secretory. HPR1 expression in the proliferative phase versus the secretory phase, $P = 0.005$.

2 of 14 specimens) ($P = 0.002$), but was not significantly higher than in the LP phase (HPR1 positive in three of six specimens) ($P = 0.32$).

Detection of heparan sulphate proteoglycan in the BM

We next tested whether increased HPR1 expression in the endometrium led to the degradation of HS in the BM of the endometrial glands. HSPG expression in the BM of HPR1-positive and HPR1-negative endometrial tissues was analysed by using immunofluorescence (IF) staining with a monoclonal antibody specific for HS. As shown in Figure 2, HS deposition was present in the BM of an HPR1-negative endometrial specimen collected in the MP phase (A) but was absent in the BM of a HPR1-positive endometrium collected in the MS phase (B). In some specimens, HS signal was also present in the nuclear membrane. Normal mouse IgM was included as an NC and no signal was observed (C). We analysed the relationship between HPR1 expression and the deposition of HS. As shown in Table II, 7 of 10 HPR1-positive endometrial specimens did not exhibit HS deposition in the BM, whereas 10 of 12 HPR1-negative endometrial specimens had intact HS deposition. Statistical analysis revealed that HPR1 expression was negatively associated with the presence of HSGP in the endometrium ($P = 0.036$), suggesting that HPR1 expression is responsible for the degradation of HS in the BM of the endometrium.

Induction of HPR1 expression in endometrial cells by estrogen

Recently, Elkin *et al*. (2003) reported that E_2 is able to induce HPR1 expression in MCF7 cells, a breast cancer cell line. We hypothesized that increased HPR1 expression in the LP and LS phases may be due to the rising estrogen levels. We first conducted a semi-quantative RT–PCR to test whether E_2 was able to induce HPR1 mRNA expression in endometrial cells. As shown in Figure 3A, E_2 at the concentration of 10^{-9} M dramatically induced HPR1 mRNA expression, whereas continued increase in E_2 concentration was relatively less effective in inducing HPR1 expression. To further test whether E_2-induced HPR1 gene expression was due to increased promoter activation, we conducted luciferase reporter gene assays in primary endometrial cells using four luciferase reporter constructs. As shown in Figure 3B, E_2 had no effect on the luciferase activity in the cells tranfected with the empty vector or the vector containing a 0.3-kb HPR1 promoter fragment with the luciferase gene (pGL3/HPR1-0.3). However, in comparison with untreated cells, E_2 at the concentration of 1×10^{-9} M consistently increased luciferase activity by ~60% in the cells transfected with plasmids containing the luciferase reporter gene driven by a 3.5-kb HPR1 promoter fragment (pGL3/HPR1-3.5). We next tested whether induction of HPR1 promoter activity by E_2 corresponded to the induction of HPR1 mRNA expression. As shown in Figure 3C, E_2 effectively induced expression of a 3.5-kb HPR1 promoter-driven luciferase gene, however at the concentration of 1×10^{-9} M, E_2 was slightly more effective in inducing HPR1 promoter activity than when it was used at 1×10^{-8} and 1×10^{-7} M.

To further confirm the ability of E_2 to induce HPR1 expression, we conducted an enzymatic assay to test whether HPR1 activity in the cell lysates and in the supernatant of the endometrial cells exposed to E_2 was increased. As shown in Figure 3D, HPR1 activity was increased in the cell lysates of the endometrial cells treated with E_2 at 10^{-10}, 10^{-9}, 10^{-8} or 1×10^{-7} M by 35, 48, 63, and 31%, respectively. HPR1 activity was increased in the supernatants of the endometrial cells treated with E_2 at 10^{-10}, 10^{-9}, 10^{-8} or 1×10^{-7} M by 34, 43, 77, and 41%, respectively. E_2 used at the concentration 1×10^{-8} M gave rise to the optimal induction of HPR1 activity in both supernatant and cell lysates. The above observations consistently indicate that E_2 used at very high concentrations ($>1 \times 10^{-8}$ M) was less effective in inducing HPR1 expression.

Induction of cell surface heparan sulphate degradation by E_2

We next tested whether induction of HPR1 expression by E_2 in endometrial cells led to the degradation of cell surface HS. Endometrial cells were left unstimulated or stimulated with

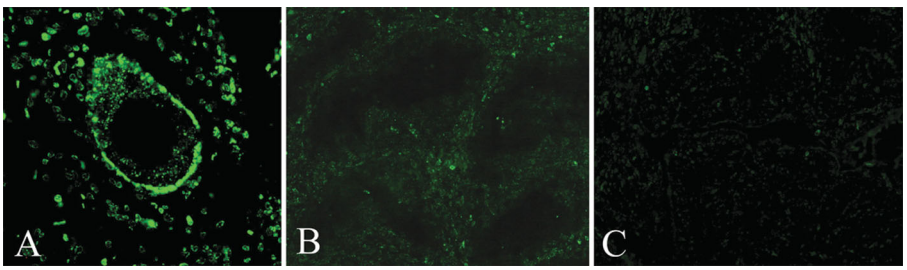

Figure 2. Immunodetection of heparan sulphate proteoglycan (HSPG) in the endometrial tissue (**A**) HS deposition was present in the basement membrane (BM) of an endometrial gland from an HPR1-negative endometrium specimen collected in the EP phase. (**B**) No HS signal was present in the BM of the endometrial glands in an HPR1-positive specimen collected in the MS phase. (**C**) Mouse IgM was included as isotype control, showing no non-specific staining.

Table II. HPR1 expression and heparan sulphate (HS) deposition in the basement membrane (BM)

	Number	HS Positive (%)
HPR1 positive	10	3 (30%)
Proliferative phase	2	0
Secretory phase	8	3
HPR1 negative	12	10 (83%)
Proliferative phase	10	9
Secretory phase	2	1

HS positivity in HPR1-positive specimens versus HS positivity in HPR1-negative endometrium, $P = 0.036$.

E_2 (1×10^{-8} or 2×10^{-7} M) in the absence or presence of PI-88 (50 μg ml^{-1}) for 48 h. As shown in Figure 4A, cell surface HS was detected at modest levels in untreated cells. However, cell surface HS levels were slightly decreased in the endometrial cells exposed to E_2 at 2×10^{-7} M (Figure 4B). Reduction of cell surface HS levels was much more pronounced when the endometrial cells were treated with E_2 at 1×10^{-8} M (Figure 4C). To confirm that E_2-induced cell surface HS degradation was mediated by increased HPR1 expression, we tested whether PI-88, a novel HPR1 inhibitor, was able to restore cell surface HS levels in E_2-treated cells. As shown in Figure 4E, PI-88 (50 μg ml^{-1}) dramatically increased cell surface HS levels in E_2-treated endometrial cells compared with those in E_2-treated cells without PI-88 (Figure 4C). PI-88 also slightly increased cell surface HS levels in untreated cells (compare Figure 4D and A). These observations strongly suggest that the decrease in cell surface HS levels in E_2-treated glandular cells is mediated by accelerated degradation due to increased HPR1 expression.

Serum heparanase-1 levels in in vitro *fertilization patients*

We next tested whether moderately increased E_2 levels led to an optimal increase of serum HPR1 activity, whereas excessive E_2 levels in blood led to a lesser increase of serum HPR1 activity. To address this, we analysed serum HPR1 levels in seven women treated with FSH for IVF. As shown in Figure 5A–G, continued FSH treatment led to a linear increase of blood E_2 levels in all five of seven patients. Interestingly, serum HPR1 levels were maximally increased in all seven patients when their blood E_2 levels ranged between 300 and 900 pg ml^{-1} ($1.1–3.3 \times 10^{-9}$ M). It should be noted that E_2 concentration in the uterus is ~10-fold higher, e.g. ~at 10^{-8} M (Bulun *et al.*, 2005). Further increases in blood E_2 levels (>900 pg ml^{-1}) did not lead to further increase in serum HPR1 activity, but rather a decrease in serum HPR1 activity in all patients, compared with that in the samples with optimal blood E_2 levels. To further examine the role of estrogen in regulating HPR1 expression, we analysed the relationship between serum HPR1 activity and estradiol levels in 18 samples whose serum estradial levels were <900 pg ml^{-1}. As shown in Figure 5H, serum HPR1 activity was very low in 10 samples whose serum estradiol levels were <215 pg ml^{-1} but was dramatically increased in eight samples whose serum estradiol levels were <215 pg ml^{-1}. The spearman rank order correlation test revealed that serum HPR1 activity in 18 samples from 7 patients correlated well with blood E_2 levels ($P < 0.001$).

Discussion

Our present study provides several lines of evidence that estrogen is able to regulate HPR1 expression in human endometrium *in vitro* and *in vivo*: (i) E_2 was able to activate the HPR1 promoter and to induce HPR1 gene expression in the endometrial cells; (ii) Up-regulation of HPR1 expression by E_2 led to accelerated degradation of cell surface HS, which was blocked by a HPR1 inhibitor; (iii) Serum HPR1 levels were increased when blood estrogen levels were moderately elevated after FSH administration; (iv) HPR1 expression was increased in the endometrial specimens taken in the LP phase when blood estrogen levels are increased. It should be noted that because of the small number of samples, we were unable to find that HPR1 expression was significantly higher in the late than in the EP and MP phases in normal endometrium, but it was indeed significantly increased in the endometrium from women with endometriosis (X. Xu, unpublished data). The ability of E_2 to induce HPR1 expression in the endometrial cells is in agreement with a prior observation made by Elkin *et al*. (2003) in MCF-7 cells, a breast cancer cell line. Intriguingly, the optimal concentrations of E_2 to induce HPR1 expression in both the breast cancer cell line and the endometrial cells were approximately between 10^{-8} and 10^{-9} M. Higher E_2 concentrations (>10^{-8} M) were less effective in inducing HPR1 expression and subsequent cell surface HS degradation. Consistent with these *in-vitro* observations, our clinical study showed that serum HPR1 levels were increased in the IVF patients with moderately increased blood E_2 levels (up to 900 pg ml^{-1} or 3.3×10^{-9} M); however, a further increase of blood E_2 levels did not lead to a further increase of serum HPR1 activity.

Although our present study and the study by Elkin *et al.* (2003) have provided strong evidence that HPR1 expression can be regulated by estrogen, the underlying molecular mechanisms are not fully understood. RT–PCR and luciferase reporter gene assays suggest that E_2-induced HPR1 expression is at the transcriptional level. Numerous putative estrogen-responsive elements (EREs) are found to be present in the HPR1 promoter (Elkin *et al.*, 2003). However, it is not clear whether HPR1 gene transactivation is mediated by direct binding of estrogen receptors (ERs) to these ERE sites. Previous studies have shown that estrogen can regulate the expression of low-density lipoprotein receptor and several others genes with a GC-rich promoter, including VEGF, retinoic receptor α, TGF-α and progesterone receptor (PR), through the interaction of ER and the transcription factor Sp1 (see review by Bjornstrom and Sjoberg, 2005). Moreover, estrogen can activate Raf-1 kinase in an ER-independent manner (Singh *et al.*, 2000). Raf kinase activation induces the expression of Egr-1 transcription factor (Pratt *et al.*, 1998) and activates the Ets family transcription factors such as Ets-1 (Lincoln *et al.*, 2003). Interestingly, all of these

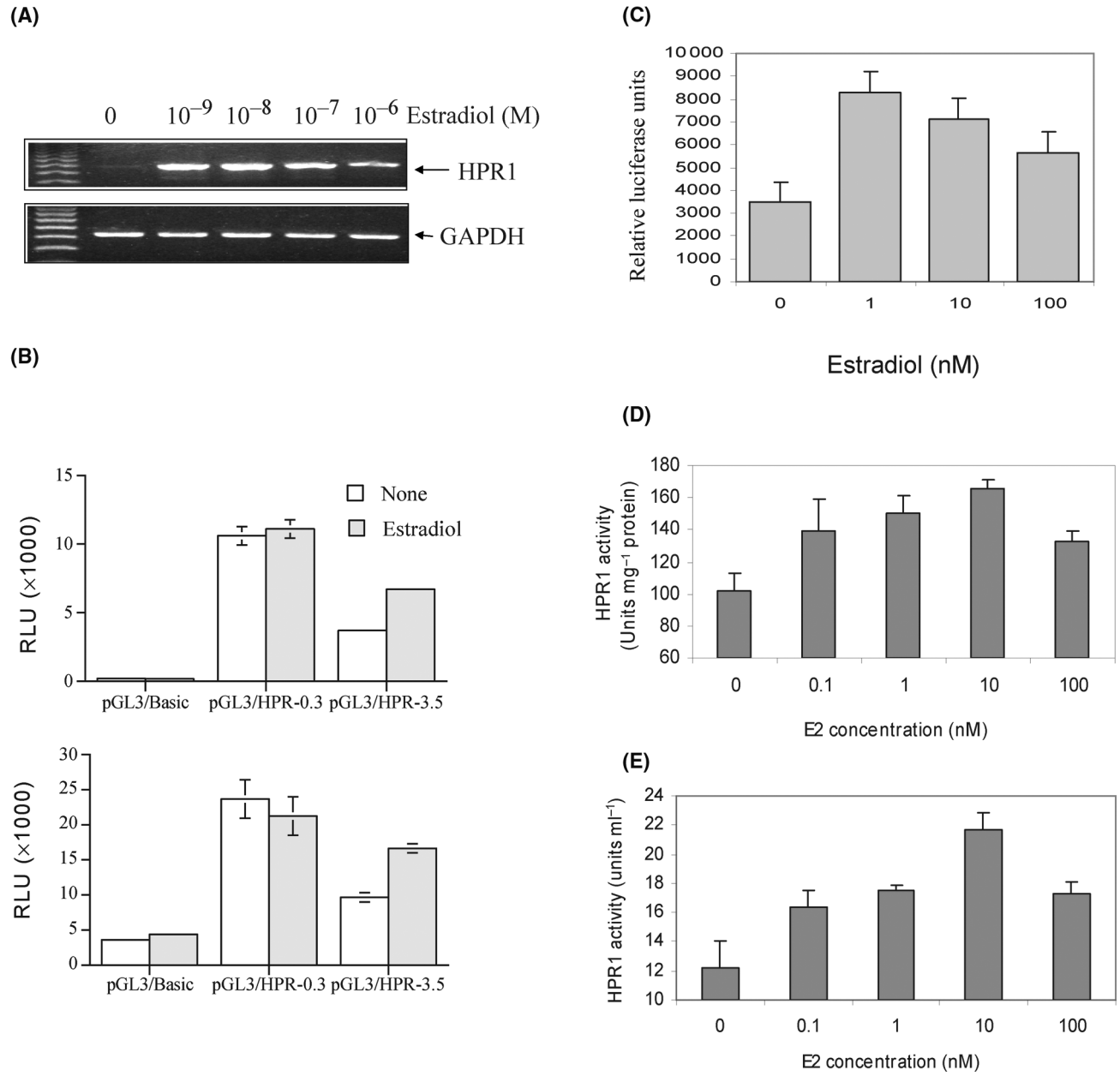

Figure 3. Induction of HPR1 expression by E_2. (**A**) Induction of HPR1 mRNA by E_2. Second passage endometrial cells were allowed to reach 80% confluence and then stimulated with the indicated concentration of E_2. After 24 h, the cells were analysed for HPR1 expression by RT–PCR. (**B**) Activation of HPR1 promoter activity by E_2. Second passage endometrial cells were transfected with HPR1 promoter-driven luciferase reporter plasmids and after 48 h, analysed for luciferase activity. The results from two independent experiments are shown as the mean ± SD of triplicate test in each experiment. (**C**) Dose–response of HPR1 promoter-driven luciferase reporter gene expression. Second passage endometrial cells were transfected with a 3.5-kb HPR1 promoter-driven luciferase reporter construct and then left unstimulated or stimulated with indicated concentrations of E_2. After 48 h, cells were analysed for luciferase activity. (**D** and **E**) Increased HPR1 activity in the cell lysates and supernatants of endometrial cells exposed to E_2. Endometrial cellswere treated with the indicated concentrations of E_2 for 48 h. HPR1 activity in the cell lysates (**D**) and supernatants (**E**) were then measured by enzyme-linked immunosorbent assay (ELISA).

three families of transcription factors, Sp1, Egr-1 and Ets, have been shown to play an important role in regulating HPR1 gene expression (Jiang *et al.*, 2002; de Mestre *et al.*, 2003, 2005; Lu *et al.*, 2003; Ogishima *et al.*, 2005). Therefore, it is likely that these transcription factors may act in concert with ER to mediate estrogen–induced HPR1 expression.

Recent in-depth studies revealed that HPR1 has multiple functions. HPR1 plays an important role not only in angiogenesis of normal and tumour tissues but also in tumour cell invasion. In addition, HPR1 can function as an adhesion molecule (Goldshmidt *et al.*, 2003; Zetser *et al.*, 2003) and contributes to cell migration (Gingis-Velitski *et al.*, 2004a). Numerous studies have shown that HPR1 is highly expressed in villous trophoblasts of both the human and bovine placenta (Dempsey *et al.*, 2000; Kizaki *et al.*, 2001, 2003; Haimov-Kochman *et al.*, 2002) and that HPR1 expression may assist trophoblastic cell invasion into the endometrium and promote angiogenesis. In support of this, Zcharia *et al*. (2004) reported that the embryo implantation

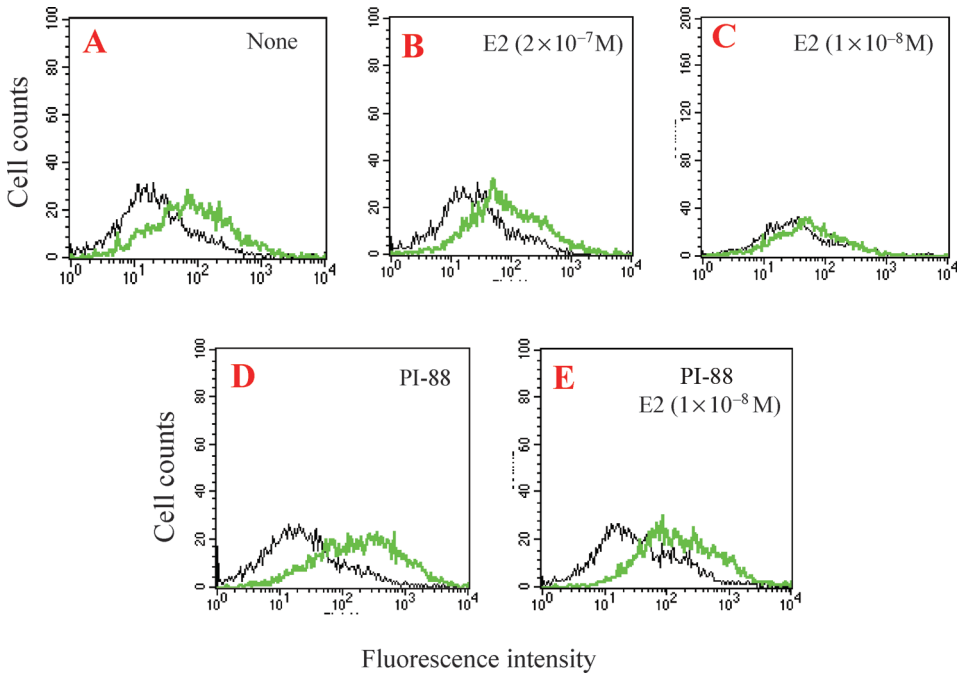

Figure 4. E_2 induces endometrial cell surface HS degradation. Endometrial cells were incubated in the absence or presence of indicated concentrations of E_2 and/or PI-88 for 48 h. Cells were then analysed for cell surface HS expression by staining with an HS-specific monoclonal antibody followed by fluorescence-activated cell sorter. The black line is a mouse IgM control; the green line is an anti-HS IgM. (**A**) Untreated control; (**B**) cells treated with E_2 (2×10^{-7} M); (**C**) cells treated with E_2 (1×10^{-8} M); (**D**) cells treated with PI-88 (50 μg ml^{-1}); (**E**) cells treated with E_2 (1×10^{-8}) plus PI-88 (50 μg ml^{-1}). The experiments were repeated twice with similar results.

rate is increased in HPR1-transgenic mice. The increased embryo implantation rate in HPR1-transgenic mice may also be in part due to the adhesion effect of HPR1 in the blastocyst. Indeed, pretreatment of mouse embryos with recombinant HPR1 *in vitro* is able to increase the embryo implantation rate (Revel *et al.*, 2005). Our present study demonstrated that HPR1 expression was increased in the endometrium in the LP phase and remained at high levels throughout the luteal phases. Though the physiological role of estrogen-induced HPR1 expression and HS degradation in the endometrium remains unclear, we speculate that HPR1 may play an important role in endometrial tissue remodelling and/or in assisting blastocyst attachment to the endometrium and facilitating trophoblast cell invasion as well as angiogenesis.

The observations that HPR1 expression can be maximally induced by an optimal concentration of estrogen may have potential physiological significance. Zcharia *et al.* (2004) reported that overexpression of HPR1 in transgenic mice leads to increased embryo implantation. However, the survival rate of implanted embryos is significantly lower in HPR1-transgenic mice than in the wild-type mice (Zcharia *et al.*, 2004), probably due to HPR1-mediated excessive angiogenesis in fetal tissues and/or in the placenta. During pregnancy, blood E_2 levels range between 1000 and 5000 pg ml^{-1} in the first trimester, 5000 and 15 000 pg ml^{-1} in the second trimester and 10 000 and 40 000 pg ml^{-1} in the third trimester. Thus, the suboptimal induction of HPR1 expression by estrogen at very high concentrations in the second and third trimesters may allow the fine-tuning of angiogenesis and avoid the detrimental effect of excessive angiogenesis in the placenta and/or in the fetal tissue.

During the preparation of this article, Kodama *et al.* (2006) reported that HPR1 expression was not detected in 11 normal endometrial specimens in the EP phase but was detected in 4 specimens in the LP phase and that HPR1 was expressed at a moderate level in the secretory phase. Similar to these findings, we found that HPR1 was rarely expressed in normal endometrial specimens in the EP and MP phases but was detected in three of six endometrial specimens in the LP phase. Because of the small number of samples, we were unable to find a significant difference in HPR1 expression in the endometrium between the EP and LP phases, and between MP and LP phases. However, we found that HPR1 expression was detected in the majority of endometrial specimens in the secretory phases. In particular, we found that HPR1 expression was detected in all four endometrial specimens in the LS phase, while blood E_2 levels should have already declined in this period. It is not clear whether there is a lapse for HPR1 turnover in the endometrial tissue. Alternatively, HPR1 expression may be regulated by other hormones. In support of this notion, our unpublished data indicate that progesterone was able to weakly induce HPR1 expression and HS degradation in endometrial cells. Progesterone may regulate HPR1 expression with a mode of action similar to estrogen, e.g. via interaction of PR with Sp1 (Owen *et al.*, 1998) and other GC-rich binding transcription factors such as basic transcription element binding protein, which are highly expressed in the endometrium (Zhang *et al.*, 2001, 2002, 2003). Alternatively, pogesterone can also activate the Src tyrosine kinase pathway (Edwards, 2005), leading to the activation of Raf kinase and Ets transcription factor, and the induction of Egr-1. Nevertheless, our

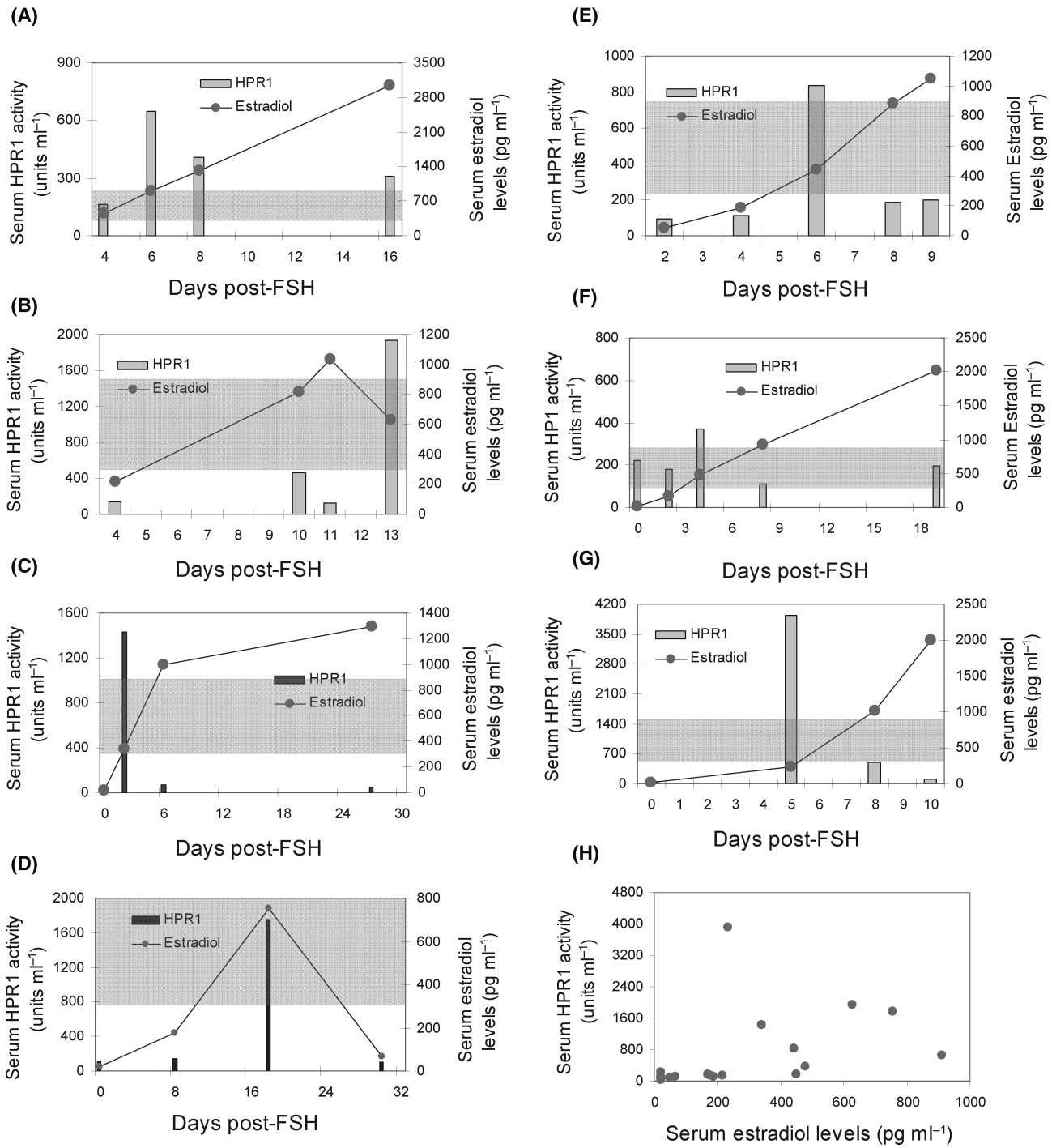

Figure 5. Comparison of serum HPR1 and estrogen levels in IVF patients receiving follicle-stimulating hormone (FSH). (**A**–**G**) Blood samples were collected from seven women (each graph represents the data from an individual patient) at the indicated days after starting FSH treatment. Serum HPR1 activity was analysed by using ELISA (left *y*-axis) and presented as a bar graph. Serum estradiol levels (right *y*-axis) were presented as a linear graph. Serum estradiol levels in the range of 300–900 pg ml^{-1} (shadowed area) produced the highest serum HPR1 activity in all seven patients. (**H**) Correlation of serum HPR1 levels with serum E_2 levels. Estrogen levels in 18 serum samples from 7 patients with serum E_2 levels $<$900 pg ml^{-1} were plotted against serum HPR1 activity. Spearman rank order test revealed that serum HPR1 activity correlated with serum E_2 levels ($P < 0.001$).

in-vitro and clinical studies, along with that of Kodama *et al.* (2006), strongly suggest that HPR1 expression in the endometrial cells can be up-regulated by E_2.

The ability of E_2 to induce HPR1 and HS degradation in the endometrial cells is in line with a prior study (Morris *et al.*, 1988) showing that estrogen is able to increase the turnover of HSPGs in the lysosomes of the murine uterine epithelial cells in which HPR1 is localized and processed to become an enzymatically active latent enzyme (Gingis-Velitski *et al.*, 2004b; Zetser *et al.*, 2004). Consistent with this, analysis of

HS revealed that HPR1 expression was associated with the lack of HS deposition in the BM of the endometrial glands, further suggesting that HPR1 plays a critical role in degrading HS. However, we found that HS deposition was detected in the BM in a few HPR1-positive endometrial specimens. This could be due to incomplete degradation of HS in the BM of the endometrial glands or due to an E_2-mediated increase of HSPG biosynthesis (Russo *et al.*, 2001), which may offset the degradation of HS side chains mediated by HPR1. Nevertheless, the observations of increased turnover of HSPGs in the endometrial epithelial cells may reflect changes associated with blastocyst attachment and invasion of the endometrium.

The ovarian hyperstimulation syndrome (OHSS) is an iatrogenic complication manifested by massive ovarian enlargement, extravascular fluid accumulation, intravascular volume depletion, renal failure and hypovolemic shock (Kaiser, 2003; Delbaere *et al.*, 2004; Budev *et al.*, 2005). This complication often occurs following the administration of FSH in women undergoing IVF. The molecular pathogenesis of this syndrome is poorly understood. Numerous studies suggest that elevated plasma VEGF levels are responsible for triggering the onset of OHSS (Geva and Jaffe, 2000). Interestingly, a recent study by Zester *et al.* (2006) demonstrated that HPR1 is able to induce the expression of VEGF. In addition, HPR1 is able to directly damage the endothelial barrier by degrading pericellular HSPGs of the endothelial cells. Edovitsky *et al.* (2005) reported that vessel permeability and extravasation of leukocytes and plasma proteins are increased at the site of delayed-type hypersensitivity-associated inflammation as a result of increased HPR1 expression in the endothelium. Consistently, Negrini *et al.* (2005) reported that an intravenous injection of a bolus of heparanase into rabbits leads to HS degradation and development of oedema in the lungs. Our present study shows that serum HPR1 levels in IVF patients were elevated particularly when blood estrogen levels were moderately increased. On the basis of these findings, we propose that the persistence of high serum HPR1 levels in women with moderately elevated estrogen levels may be a key to triggering the onset of OHSS. Elevated serum HPR1 levels may increase the vessel permeability by directly disrupting the BM of the endothelium and/or indirectly by stimulating the production of VEGF. Further studies are under the way to determine whether IVF patients who develop OHSS have persistently high serum HPR1 and/or VEGF levels.

In summary, this study provides compelling evidence that HPR1 expression in the endometrium is regulated by E_2 and that HPR1 expression leads to HS degradation on the cell surface and in the BM of the endometrial glands.

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