Molecular Human Reproduction, Vol.16, No.5 pp. 347–360, 2010
Advanced Access publication on March 4, 2010 doi:10.1093/molehr/gaq012

MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

Do GnRH analogues directly affect human endometrial epithelial cell gene expression?

Xiaomei Zhang[1,3] Silvina Bocca[1], Anahi Franchi[1,4] Sandra Anderson[1], Mandeep Kaur[2], Vladimir B. Bajic[2], and Sergio Oehninger[1,*]

[1]Department of Obstetrics and Gynecology, The Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, Norfolk, VA 23507, USA [2]Computational Bioscience Research Center, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia [3]Present address: Department of Obstetrics and Gynecology, Reproductive Medicine Center, Subei People's Hospital, Yangzhou University, Yangzhou, Jiangsu Province 225001, P.R. China [4]Present address: Laboratorio de Bioquímica y Biología Reproductiva, Instituto de Ciencia y Tecnología de Alimentos, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Córdoba, Argentina

*Correspondence address. E-mail: oehninsc@evms.edu

Submitted on December 17, 2009; resubmitted on January 25, 2010; accepted on February 10, 2010

ABSTRACT: We examined whether Gonadotrophin-releasing hormone (GnRH) analogues [leuprolide acetate (LA) and ganirelix acetate (GA)] modulate gene expression in Ishikawa cells used as surrogate for human endometrial epithelial cells *in vitro*. The specific aims were: (i) to study the modulatory effect of GnRH analogues by RT–PCR [in the absence and presence of E_2 and P4, and cyclic adenosine monophosphate (cAMP)] on mRNA expression of genes modulated during the window of implantation in GnRH analogues/rFSH-treated assisted reproductive technology cycles including *OPTINEURIN* (*OPTN*), *CHROMATIN MODIFYING PROTEIN* (*CHMP1A*), *PROSAPOSIN* (*PSAP*), *IGFBP-5* and *SORTING NEXIN 7* (*SNX7*), and (ii) to analyze the 5′-flanking regions of such genes for the presence of putative steroid-response elements [estrogen-response elements (EREs) and P4-response element (PREs)]. Ishikawa cells were cytokeratin+/vimentin− and expressed ERα, ERβ, PR and GnRH-R proteins. At 6 and 24 h, neither LA nor GA alone had an effect on gene expression. GnRH analogues alone or following E_2 and/or P4 co-incubation for 24 h also had no effect on gene expression, but P4 significantly increased expression of *CHMP1A*. E_2 + P4 treatment for 4 days, alone or followed by GA, had no effect, but E_2 + P4 treatment followed by LA significantly decreased *IGFBP-5* expression. The addition of 8-Br cAMP did not modify gene expression, with the exception of *IGFBP-5* that was significantly increased. The GnRH analogues did not modify intracellular cAMP levels. We identified conserved EREs for *OPN, CHMP1A, SNX7* and *PSAP* and PREs for *SNX7*. We conclude that GnRH analogues appear not to have major direct effects on gene expression of human endometrial epithelial cells *in vitro*.

Key words: endometrium / EREs/PREs identification / gene expression / GnRH / Ishikawa cells

Introduction

Gonadotrophin-releasing hormone (GnRH) is a decapeptide with a critical role in reproduction. A second GnRH type (GnRH-II) is expressed in the midbrain, hippocampus and other areas of the hypothalamus (Cui *et al*., 2000; Millar, 2003; Cheng and Leung, 2005; Wu *et al*., 2009a and b). Numerous studies have demonstrated that GnRH receptors (GnRH-R) exist in different reproductive tissues such as the ovary, placenta, endometrium, and also in endometrial cancer cells (Millar *et al*., 2001, 2003; Ramakrishnappa *et al*., 2005; Cheung and Wong, 2008). GnRH and GnRH-II receptors are present in the endometrium, with highest levels reported in the secretory phase followed by a decline in the decidua (Dong *et al*., 1998; Raga *et al*., 1998; Takeuchi *et al*., 1998; Borroni *et al*., 2000; Gründker *et al*., 2001; Shemesh, 2001). It has been reported that GnRH can act in an autocrine and paracrine manner to suppress cell proliferation and activate apoptosis in the endometrium as well as in endometrial cancer cells (Hsueh and Jones, 1981; Cheung and Wong, 2008). Both GnRH and GnRH-II exhibit regulatory roles in tissue remodeling during decidualization, embryo invasion and placentation (Cheon *et al*., 2001; Paria *et al*., 2002; Chou *et al*., 2003).

The GnRH analogues are commonly used as adjuvants to gonadotrophins in controlled ovarian hyperstimulation (COH) cycles in women undergoing assisted reproductive technologies (ARTs) to prevent a premature LH surge. In addition to the established GnRH central action, the endometrial expression of GnRH and its receptor implies an additional site of action for GnRH analogues. It has been speculated that COH as performed during *in vitro* fertilization (IVF)

therapy may negatively affect embryo implantation (Garcia et al., 1984; Gordon, 2001; Horcajadas et al., 2008). Many questions remain about a controversial negative impact of GnRH antagonists on endometrial receptivity (Borthwick et al., 2003; Bourgain and Devroey, 2003; Al-Inany et al., 2007; Huang, 2008; Oehninger, 2008).

We recently reported on a clinical study designed to investigate gene expression profiles of the human endometrium during the window of implantation of GnRH analogue-recombinant FSH-treated COH cycles in oocyte donors compared with temporally matched natural cycles (Mirkin et al., 2004). Microarray results demonstrated significant variations in the expression of the following five genes: *OPTINEURIN* (*OPTN*) and *PROCOLLAGEN TYPE III N-ENDOPEPTIDASE* (or *CHROMATIN MODIFYING PROTEIN 1A, CHMP1A*), which were up-regulated in GnRH agonist-treated cycles versus natural cycle controls, *PROSAPOSIN* (*PSAP*) and *IGF-BINDING PROTEIN-5* (*IGFBP-5*), which were up-regulated in GnRH antagonist-treated cycles versus natural cycle controls, and *SORTING NEXIN 7* (*SNX7*), which was up-regulated in GnRH agonist cycles versus antagonist cycles (Mirkin et al., 2004). The three genes that contributed the best discriminating expression profile (highest variations) by linear discriminant analysis (LDA) were *OPTN*, *CHMP1A* and *SNX7*, all of them significantly up-regulated in GnRH agonist-treated cycles.

On the other hand, estrogens and progesterone (P4) exert tissue-specific actions through interaction with their respective receptor subtypes, and following the interaction of these ligand–receptor complexes with effectors, which include different DNA-response elements and important co-regulator proteins (Jabbour et al., 2006). The two isoforms of estrogen receptor, ERα and ERβ, have been identified in mammals, exerting its action genomically through estrogen-response elements (EREs) in a ligand-dependent fashion (Kato et al., 1995; Kuiper et al., 1997; Diel, 2002). The identification of the palindromic sequence that defines EREs and P4-response elements (PREs) allows for the *in silico* discovery of putative hormone receptor targets in the genome.

Activation of the cyclic adenosine monophosphate (cAMP) pathway is an obligatory event that starts the critical process of endometrial decidualization (Gellersen et al., 2007). Decidualization of endometrial stromal cells occurs *in vivo* in response to P4 and involves activation of the protein kinase A (PKA) pathway (Tierney et al., 2003). After ovulation, the endometrium is increasingly exposed to a variety of local and endocrine factors that are capable of stimulating cAMP production in stromal cells, and adenylate cyclase activity in the human endometrium increases during the secretory phase (Gellersen et al., 2007).

On the other hand, few reports have postulated regulation of endometrial epithelial cell functions by cAMP. Chan et al. (1999) were the first to provide direct evidence of a cAMP-activated Cl^- conductance, presumably CFTR, in murine endometrial epithelial cells (Zheng et al., 2004). On the other hand, Zhou et al. (1994) reported that hCG treatment increased expression of the COX-2 gene in human endometrial gland epithelial cells, using a cAMP/type I PKA signaling pathway.

Within this context, the overall objective of this study was to examine the modulatory effects of GnRH analogues [leuprolide acetate (LA), a GnRH agonist, and ganirelix acetate (GA), a GnRH antagonist] on gene expression of human endometrial epithelial cells. To accomplish this goal, we used Ishikawa cells as a surrogate for human endometrial epithelium, a known and established *in vitro* cell culture model, originated from an endometrial adenocarcinoma and known to display ER and PR in culture (Nishida, 2002; Navarro et al., 2003; Bocca and Archer, 2005; Uchida et al., 2005).

The specific aims were: (i) to characterize Ishikawa cells by immunofluorescence and immunocytochemistry [to confirm the epithelial cell origin and purity (cytokeratin+/vimentin−) and the protein expression of ERα, ERβ, PR and GnRH-R] and by RT–PCR (for mRNA expression of *ERα, ERβ, PR, GnRH-R* and *GnRH-II-R*); (ii) to study the *in vitro* modulatory effect of GnRH analogues (in the absence and presence of E_2 and P4 on mRNA expression of *OPTN, CHMP1A, PSAP, IGFBP-5* and *SNX7*, endometrial genes previously shown to be modified *in vivo* by GnRH analogues in recombinant FSH-treated ART cycles (Mirkin et al., 2004) using quantitative, real-time RT–PCR and (iii) to analyze the 5′-flanking regions of such genes for the presence of putative EREs and PREs (Mirkin et al., 2005).

Materials and Methods

In vitro culture of Ishikawa cells

Ishikawa cells were kindly provided by Dr Paul Web (University of California, San Francisco, CA, USA). Ishikawa cells were cultured in DMEM/F-12 1:1 medium (Dulbecco's modified Eagle's medium/Ham's F-12; Gibco, Grand Island, NY, USA/Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) containing antibiotics (penicillin/streptomycin 1% vol./vol., Gibco), and 5 pg/ml insulin (Sigma) at 37°C in a 95% air–5% CO_2 atmosphere. According to the individual experiment, once 50–80% confluence was reached, Ishikawa cells were cultured for an additional 24-h-period in DMEM supplemented with charcoal-stripped and dextran-treated 2% FBS (Hyclone, Logan, UT, USA) and then subjected to different treatments as shown in Experiments 1–4. Cell structural integrity was assessed by inverted microscopy (Navarro et al., 2003). The GnRH analogues studied were LA (Abott Laboratories, North Chicago, IL, USA) and GA (Organon, Roseland, NJ, USA).

Immunofluorescence and immunocytochemistry

The epithelial origin and purity of Ishikawa cells were assessed by immunofluorescent analysis of cytokeratin and vimentin, specific markers for epithelial and stromal cells, respectively (specific antibodies obtained from Abcam, Cambridge, MA, USA); anti-cytokeratin antibody was used in 1:100 dilution anti-vimentin antibody in 1:50 dilution. Immunofluorescence was also used for the detection of GnRH-R with a mouse monoclonal anti-GnRH-R antibody (1:25 dilution, Abcam). Mouse non-immune IgG (isotype control, eBioscience, San Diego, CA, USA) was used as the negative control, and goat anti-mouse bound to FITC in a 1:150 dilution was used as the secondary antibody. DAPI was used to counterstain the nuclei. Slides were mounted with anti-fading medium (Vector Laboratories, Burlingame, CA, USA) and analyzed under fluorescence microscopy (Nikon Eclipse E600) equipped with a SPOT-RT Slider digital camera (Diagnostic Instruments Inc., Sterling, MI, USA).

Immunohistochemistry was performed for ERα, ERβ and PR on cultured Ishikawa cells. Briefly, endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min, and non-specific binding sites were blocked with 2% normal goat serum (NGS) for 60 min at room temperature. The primary antibodies for ERα, ERβ and PR (mouse monoclonal, Abcam) were serially diluted in a solution of PBS-2% NGS to optimize sensitivity and specificity and used at a dilution of 1:25. After primary antibody incubation, sections were washed three times with PBS and incubated with biotinylated goat anti-mouse secondary antibody (Abcam) at a dilution of

Table I Oligonucleotides used for real-time PCR.

Gene name	Primer sequence	Annealing temperature	Product size
OPTN	Fw: 5′-CACTGGCACGGCATTGTCTAA Rv: 5′-ACCTTCTGATTCCCTTCCCTT	58°C	123 bp
CHMP1A	Fw: 5′-GTGTATGCCGAGAACGCCAT Rv: 5′-CCTGCTGCTCGAACCTGTC	58°C	217 bp
SNX7	FW: 5′-AAAGCGGATGTCTGGACTCTC Rv: 5′-TCAGGGCATTATTAGCACATTC	58°C	221 bp
PROSAPOSIN	Fw: 5′-TGTGCTCTGCTCTCAACCTCT Rv: 5′-GCCACCACCTCAGTCATGT	58°C	115 bp
IGFBP-5	FW: 5′-TGTGACCGCAAAGGATTCTAC Rv: 5′-GCAGCTTCATCCCGTACTTG	58°C	100 bp
ERα	Fw: 5′-GGGAATGATGAAAGGTGGGAT Rv: 5′-GGCTGTTCTTCTTAGAGCGTT	58°C	173 bp
ERβ	Fw: 5′-GTTCTGGACAGGGATGAGG Rv: 5′-TCACGGCGTTCAGCAAGTG	59°C	220 bp
PR	Fw: 5′-AGCCCTAAGCCAGAGATTCA Rv: 5′-TAGGATCTCCATCCTAGACC	58°C	303 bp
GnRH-R	Fw: 5′-ACCCCCACGAACTACAACTGA Rv: 5′-TGATTTACTGGGTCTGACAACCT	60°C	184 bp
GnRH-R-II	Fw: 5′-AACCTCACAAATACACATGC Rv: 5′-TTTCGTTCACATAAGCCTCT	60°C	225 bp
GAPDH	Fw: 5′-GAGTCAACGGATTTGGTCGT Rv: 5′-CGTAGCAAGGCACAGATCAG	58°C	225 bp

1:120 for 30 min at room temperature. After rinsing with PBS, the immunoreactive antigen was visualized by incubating with avidin-biotinylated horseradish peroxidase (1:100) complex for 30 min and 3,3′-diaminobenzidine (0.5 mg/ml) as chromagen for 3 min to complete the reaction. Negative controls included sections that were treated with the omission of the primary antibody. Slides were counterstained with Mayer's hematoxylin (Sigma) followed by dehydration in a graded series of ethanol, cleared in xylene, and mounted with mounting media. Representative fields were photographed at ×200 and ×400 magnification with an Olympus microscope (Olympus Corp., Tokyo, Japan) using an Olympus Q-color 3 camera.

Quantitative real-time RT–PCR

We investigated mRNA expression of *ERα, ERβ, PR, GnRH-R* and *GnRH-II-R* (to further characterize Ishikawa cells) and *OPTN, CHMP1A, PSAP, IGFBP-5* and *SNX7* (to study the possible modulatory effects of GnRH analogues). After incubations, cells were washed, scraped and total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality of total RNA extracted was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and quantification of total RNA was performed on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA).

cDNA was generated from 300 ng of total RNA in a total volume of 20 μl containing: 2.5 μM random hexamers, 2.5 U/μl murine leukemia virus reverse transcriptase, 1U/μl RNase inhibitor, 1x PCR buffer, 1 mM each deoxy-NTP, 5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA, USA). RT parameters were as follows: 23°C for 10 min, 42°C for 15 min (RT reaction), 99°C for 5 min (transcriptase deactivation) and 5°C for 5 min in an iCycler thermal cycler (BioRad, Hercules, CA, USA). cDNA solutions were then stored at −20°C. Preparations without reverse transcriptase were used as negative controls, in which the absence of PCR products indicated a complete lack of contaminating genomic DNA.

Quantitative real-time PCR was performed using a Lightcycler Fastart DNA Master Plus SYBR green I and a Lightcycler 2.0 instrument (Roche Applied Science, Indianapolis, IN, USA) in a 20-μl total reaction volume, containing 2-μl cDNA and 0.5 μM of each sense and antisense primers (except GADPH which was used at 0.3 μM).

Before amplification, samples were denatured at 95°C for 10 min. The template was amplified by 45 cycles of denaturation at 95°C for 10 s, annealing of primers at the specific temperature as given in Table I for 5 s and extension at 72°C for 10 s, followed by a final extension at 72°C for 10 min. The melting protocol consisted of heating the samples to 95°C followed by cooling to 65°C for 15 s and slowly heating at 0.1°C per second to 95°C while monitoring fluorescence. Melting curve analysis was performed after each run to verify specific amplification. Negative control consisted of PCR water replacing the cDNA solution (no template control). All PCR products exhibited a single peak in melting curves and were identified as single bands of the appropriate size on ethidium bromide-stained 3% agarose gel electrophoresis. In addition, amplification specificity was confirmed by sequencing all PCR products, after purification using QIAquick kit (Qiagen), performed by UC Davis, CA, USA (http://dnaseq.ucdavis.edu/SoftwareDownloads.html). All PCR products demonstrated 97–99% homology with the respective human sequences. cDNA levels were determined using a standard curve and the values obtained were normalized to those found for GADPH (housekeeping gene) to account for differing amounts of starting material (Franchi *et al.*, 2008). Table I presents oligonucleotides (primer sequences) used for real-time PCR, annealing temperatures and expected amplicon/PCR product sizes.

In silico promoter analysis for EREs and PREs

For *in silico* identification of EREs and PREs, we used two tools, DEREF (Dragon ERE Finder) (Bajic *et al.*, 2003) and (Dragon PRE Locator,

http://apps.sanbi.ac.za/PRE/index.php). DEREF has been designed to achieve high sensitivity and high specificity on promiscuous ERE sequences. DEREF has been designed to achieve high sensitivity and high specificity. It makes, on an average, 1 prediction in 13 300 nucleotides in two stranded search at a sensitivity of 83%, which makes it very useful for selective predictions of EREs. For the present analysis, search for EREs is performed using a sensitivity of 87%. DPREL model uses dinucleotide position-weight matrices and is designed on the same principles, but achieves much better performance of 96% sensitivity while making one prediction in 67 780 nucleotides in the randomly selected human DNA (204 358 000 nucleotides). It is used with its default setting. It should be noted that DEREF and DPREL differ in the way how they report position of the motif on the complementary strand. DEREF reports the actual position where the motif starts (counted from the 5′ end of the forward strand), and the motif spreads towards the 5′ end of the forward strand. DPREL, however, reports the position that corresponds to the 3′ end of the motif on the complementary strand. The reason for this difference is that it is easier to observe when the palindromic patterns are predicted on both strands.

Promoters

For the genes of interest, we extracted promoter regions that correspond to 3000 bp upstream and 200 bp downstream (−3000, +200) relative to the 5′ end of Exon1. The ortholog promoter sequences of 11 mammalian species (including human) were extracted using TOUCAN 2 (Aerts *et al.*, 2005). The ortholog species included were human, cow, dog, hedgehog, elephant, opossum, monkey, mouse, rabbit, chimp and rat.

Experiments

Experiment 1

Objective: to determine whether the GnRH agonist LA modulates expression of *OPTN, CHMP1A* and *SNX7*. Following initial culture as depicted earlier, and after 80% confluence, Ishikawa cells were treated for 6 h with three GnRH agonist concentrations (0.01, 0.1 and 1 μM) versus untreated controls.

Rationale: the selection of these three genes was based upon the application of LDA to the clinical data mentioned earlier (Mirkin *et al.*, 2004). The examined concentrations of LA were based on the therapeutic plasma serum levels after subcutaneous administration in the clinical setting (Klemmt *et al.*, 2009). Studies have previously shown modulation of gene expression in endometrial cells as early as 1–6 h of stimulation (Tierney *et al.*, 2003).

Experiment 2

Objective: to examine the impact of short-time E_2 and/or P4 co-incubation (24 h) on the effect of GnRH analogues on gene expression (examined genes: *OPTN, CHMP1A* and *SNX7* for GnRH agonist, and *IGFBP-5* and *PSAP* for GnRH antagonist). The examined conditions were: untreated controls, E_2 (10 nM for 24 h) (Sigma), P4 (10 nM for 24 h) (Sigma), LA (1 μM for 24 h), GA (1 μM for 24 h), E_2 (10 nM for 12 h) followed by P4 (10 nM for 12 h) and E_2 (10 nM) plus LA or GA (1 μM for 12 h) followed by P4 (10 nM for 12 h).

Rationale: E_2 and P4 modulate the effect of GnRH analogues on gene expression.

Experiment 3

Objective: to examine the impact of higher dose and longer time E_2- and P4-priming conditions on the effect of GnRH analogues on gene expression (*OPTN, CHMP1A, SNX7, IGFBP-5* and *PSAP*). The examined conditions were: untreated controls, E_2 (30 nM) plus P4 (1 μM) for 4 days, E_2 (30 nM) plus P4 (1 μM) for 4 days followed by the GnRH agonist LA (1 μM for 6 h) and E_2 (30 nM) plus P4 (1 μM) for 4 days followed by the GnRH antagonist GA (1 μM for 6 h).

Rationale: the longer treatment mimics the time needed for *in vitro* decidualization of stromal cells, which has been shown to peak at Day 3 of culture (Klemmt *et al.*, 2009); as such, we speculated that longer sex steroid priming of epithelial cells might result in a more robust gene regulation.

Experiment 4

Objective: to examine the impact of a cAMP analogue (8-bromo-cAMP, Sigma) on the effect of GnRH analogues on gene expression. The examined conditions were: untreated controls, cAMP (1 mM for 24 h), cAMP (1 mM for 24 h) followed by the GnRH agonist LA (1 μM for 6 h) and cAMP (1 mM for 24 h), followed by the GnRH antagonist GA (1 μM for 6 h).

Rationale: the effect of GnRH analogues on endometrial epithelial cells gene expression may be modulated by cAMP. It has been reported that cAMP analogues modulate endometrial (stromal) cell gene expression during decidualization. These effects were observed at short treatment times (0–6 h, cell cycle regulation), intermediate times (12–24 h, cellular differentiation including genes regulating cell morphology and secretory patterns) and later periods (24–48 h, immunomodulatory genes) (Tierney *et al.*, 2008).

Additionally, GnRH has been shown to induce production of cAMP in the pituitary gonadotrophs, resulting in increased gene expression and release of newly synthesized LH (reviewed in Counis *et al.*, 2005). As such, we also tested whether GnRH analogues resulted in the modification of intracellular cAMP levels in Ishikawa cells, using a cAMP enzyme immunoassay kit, following manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA). Intracellular cAMP concentrations were measured after GnRH analogues treatments (0.1, 1 and 10 μM) in a 30-min incubation period. The known activator of adenylate cyclase Forskolin (30 μM, Sigma) was used as a positive control.

Statistical analysis

Three different (independent) experiments were performed for each GnRH analogue, dose, time and E_2, P4 and cAMP treatment condition. Gene expression levels (relative gene expression to GADPH) were compared using one-way analysis of variance. The Holm–Sidak and Dunnet *post hoc* tests for pair-wise multiple comparisons were used as appropriate. $P < 0.05$ values were considered statistically significant. Data are presented as mean ± standard error of the mean.

Results

Characterization of Ishikawa cells

Figure 1A presents immunofluorescence results confirming the expected presence of GnRH-R protein in Ishikawa cells, as well as purity of epithelial origin by cytokeratin+/vimentin− immunostaining. Figure 1B presents results of cultured Ishikawa cells demonstrating the presence of ERα, ERβ and PR protein by immunohistochemistry.

Figure 2 presents RT–PCR results of Ishikawa cells demonstrating mRNA expression of *GnRH-R* (184 bp) and *GnRH-II-R* (225 bp), *OPTN* (123 bp), *CHMP1A* (217 bp), *PSAP* (115 bp), *IGFBP-5* (100 bp), *SNX7* (221 bp), *ERα* (173 bp), *ERβ* (220 bp) and *PR* (303 bp), as well as the housekeeping gene *GADPH* (225 bp).

Treatments results

Experiment 1

There was no significant effect of the GnRH agonist LA on the expression of the examined genes (*OPTN, CHMP1A* and *SNX7*) at

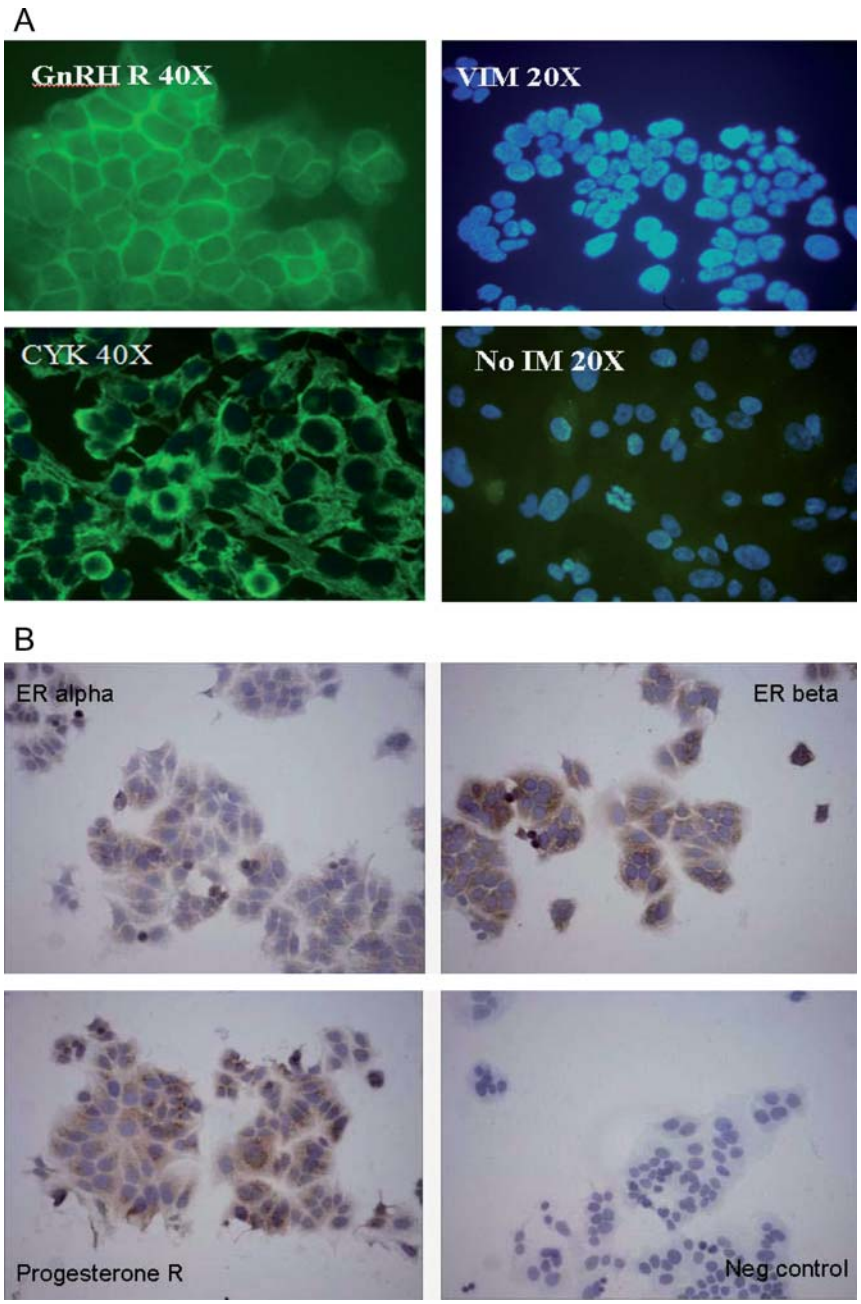

Figure 1 (**A**) Immunofluorescent microscopy (green) of Ishikawa cells confirming epithelial purity [cytokeratin (+) and vimentin (−)] and demonstration of GnRH-R (+) presence. Mouse non-immune IgG was used as the negative control. Nuclei are stained in blue (DAPI). (**B**) Immunocytochemistry of Ishikawa cells demonstrating positive staining (brown) for ERα, ERβ and PR protein. Negative controls included sections that were treated with omission of the primary antibody.

any concentration tested (0.01, 0.1 and 1 μM) after 6 h of incubation ($n = 3$ different experiments for each gene, $P > 0.1$, Fig. 3).

Experiment 2

This experiment examined whether short co-incubation time (24 h) with E2 and P4 had an impact on the effect of the GnRH analogues to modify gene expression ($n = 3$ different experiments for each GnRH analogue and each gene studied). Neither E_2 (10 nM), LA (1 μM), GA (1 μM) alone, nor the $E_2 + P4$ co-treatment (with or without each analogue) had a significant effect on gene expression versus control levels ($P > 0.1$, data not shown). The only exception was the finding that P4 alone resulted in significantly higher gene expression of *CHMP1A* ($P = 0.03$) than control levels (Fig. 4A).

Experiment 3

This experiment investigated the impact of higher dose and longer time (4 days) of E_2 (30 nM) and P4 (1 μM)-priming on the effect of GnRH

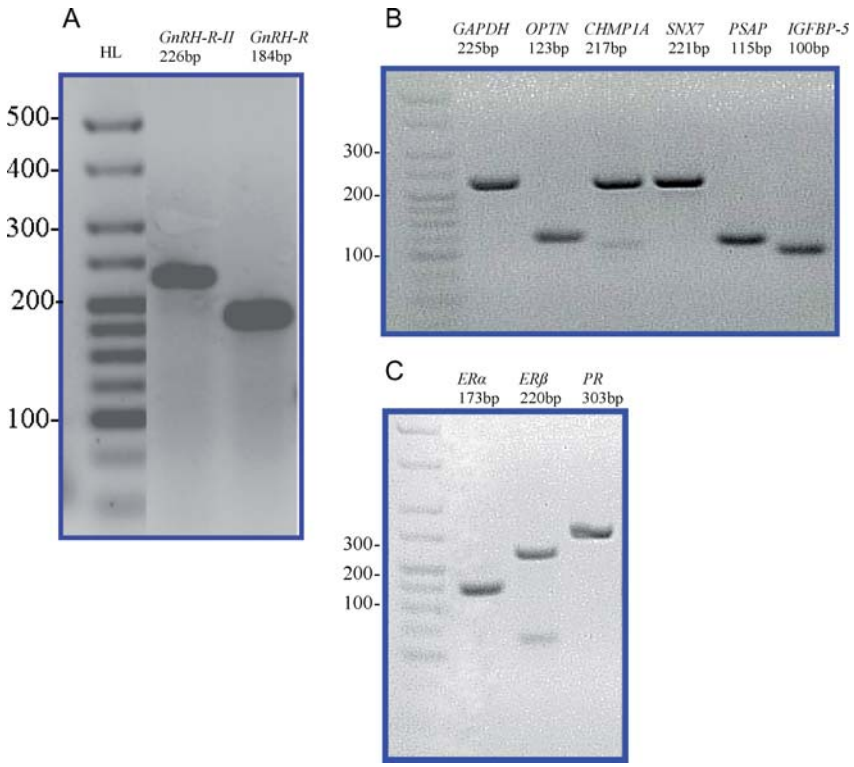

Figure 2 Results of RT–PCR of Ishikawa cells demonstrating bands that correspond to (**A**) GnRH-R (184 bp) and GnRH-II-R (225 bp); (**B**) OPTN (123 bp), CHMP1A (217 bp), PSAP (115 bp), IGFBP-5 (100 bp) and SNX7 (221 bp) and (**C**) ERα (173 bp), ERβ (220 bp) and PR (303 bp). HL, molecular marker with molecular weights shown as base pairs.

analogues (after 6 h of incubation) on gene expression ($n = 3$ different experiments for each GnRH analogue and each gene studied).

For *OPTN, CHMP1A, SNX7* and *PSAP* genes: E_2+ P4 alone for 4 days, or followed by the GnRH agonist LA or the GnRH antagonist GA, had no effect on the relative expression levels of any of these genes ($P > 0.1$, data not shown). On the other hand, for *IGFBP-5* gene: E_2 + P4 treatment for 4 days followed by LA (but not GA) after 6 h significantly decreased mRNA expression levels versus control ($P = 0.03$) (Fig. 4B).

Experiment 4

This study examined the impact of pretreatment with the cAMP analogue (8-bromo-cAMP) for 24 h on the effect of GnRH analogues on Ishikawa cell gene expression ($n = 3$ different experiments for each GnRH analogue and each gene studied). For *OPTN, CHMP1A, SNX7* and *PSAP* genes, none of the treatments (cAMP alone, and with a GnRH agonist or GnRH antagonist) affected gene expression levels ($P > 0.1$, data not shown). For *IGFBP-5* gene, expression levels were significantly increased by cAMP alone ($P = 0.002$), as well as by cAMP with the GnRH agonist LA ($P = 0.0007$) and the GnRH antagonist GA ($P = 0.0003$). Neither LA nor GA significantly augmented the effect observed with cAMP alone (Fig. 4C).

In addition, neither GnRH analogue (at 0.1, 1 and 10 μM for 30 min incubation) resulted in significant changes of cAMP levels as measured by an enzyme immunoassay ($P > 0.1$ versus controls), whereas forskolin resulted in a significantly increased production, $P < 0.05$, Fig. 4D).

Identification of EREs and PREs in the genes studied

Prediction of EREs

EREs of length 17 bases were searched for on ortholog promoters of each gene (*OPTN, CHMP1A, SNX7, PSAP* and *IGFBP-5*). Results are presented in Table II.

OPTN: at a sensitivity of 87%, we were able to predict 12 EREs on promoters of 6 species including human, monkey, mouse, rabbit, chimp and rat. We found the element TT-GGCCA-GGC-TGGTC-TC (shown in red) fully conserved in human, monkey and chimp promoters.

CHMP1A: at a sensitivity of 87%, we were able to predict two EREs on promoters of two species. The identified EREs were conserved between human and chimp, with one mismatch in the spacing nucleotide and another in the most 3′-flanking nucleotide.

SNX7: we identified seven different EREs on the promoters of six orthologous species. Two EREs TT-GGCCA-GAT-TGGCC-AA and TT-GGCCA-ATC-TGGCC-AA were fully conserved in the promoters of human and chimp (shown in red and pink, respectively) and a conservation of 16 nucleotides was observed in monkey for both EREs, with the only 1 nucleotide mismatch in each case, either as the most 5′-flanking position or the most 3′-flanking position. Elephant and rabbit promoters contain fully conserved element AA-GGACT-AGC-TAACC-AC.

PSAP: DEREF predicted EREs on promoters of eight orthologous species. Human and chimp promoters have completely conserved

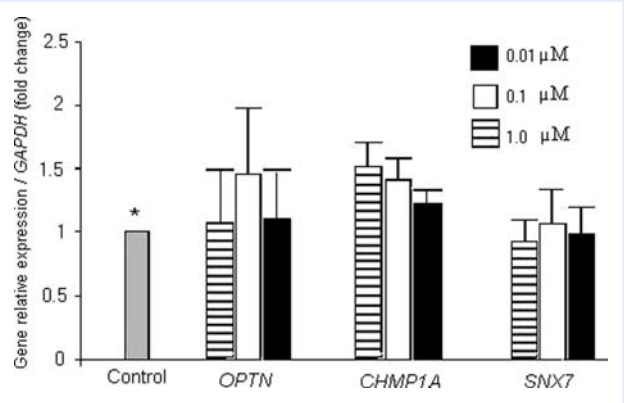

Figure 3 Experiment 1: lack of the effect of the GnRH agonist LA (three different concentrations) on mRNA expression of OPTN, CHMP1A and SNX7 in Ishikawa cells (*$P > 0.1$, controls versus all treatment conditions).

17 bp sequence of EREs (shown in red). Human and mouse have 14 bp conserved sequences of EREs (shown as bold black), with one mismatch in the 3-nucleotide spacer and mismatched flanking nucleotides on the 5′ end, respectively. Human and monkey have 15 bp that are conserved (shown in blue).

IGFBP5: no EREs were identified in human promoters. The identified EREs were not conserved between opossum and rabbit.

Prediction of PREs

PREs of length 15 bases were searched for on 10 ortholog promoters of each of *OPTN, CHMP1A, SNX7, PSAP* and *IGFBP-5* genes. We were not able to predict PREs on the promoters of four of these genes, *OPTN, CHMP1A, PSAP* and *IGFBP-5* for any of the species we tested.

SNX7: two different PREs were identified on the promoters of *SNX7* genes in three species (Table II). The element AGGACATGGTGTTCT was identified in the promoters of human and chimp species (shown in red). The results suggest that only *SNX7* is likely controlled by P4, whereas the other three genes are not.

Discussion

Ovarian stimulation for IVF is known to affect luteal phase function. Questions about the impact of the use of GnRH analogues, particularly antagonists, have been raised. A recent Cochrane review (Al-Inany *et al*., 2007) described lower pregnancy rates in women treated with a GnRH antagonist, compared with those using an agonist, despite the fact that GnRH antagonist is a more effective repressor of LH. The endometrium in IVF cycles is subjected to an altered endocrinological environment (particularly hyperestrogenism secondary to gonadotrophin stimulation, but also to high levels of P4 secondary to the presence of multiple functioning corpora lutea and/or exogenous supplementation). In addition, there is also a plausible direct effect of the GnRH analogues on the endometrium. The question still remains whether the previously reported differences in endometrial gene expression in COH cycles performed with GnRH analogues and gonadotrophins result in endometrial changes having a major functional impact on embryo implantation (Bourgain and Devroey, 2003; Martínez-Conejero *et al*., 2007; Huang, 2008; Oehninger, 2008).

Here, we used Ishikawa cells as surrogate of human endometrial epithelial/glandular cells. The functional nature of Ishikawa cells was validated through the characterization of epithelial cell origin and purity (cytokeratin+/vimentin−), confirmation of protein expression of ERα, ERβ, PR and GnRH-R and presence of mRNA transcripts of *ERα, ERβ, PR, GnRH-R* and *GnRH-II-R*. We elected to study five endometrial genes that were significantly altered (up-regulated) in the luteal phase of IVF cycles (using gonadotrophins and GnRH analogues) as evidenced by our previous microarray analysis (Mirkin *et al*., 2004). Other authors have reported similar and other altered gene expression profiles using parallel approaches (Carson *et al*., 2002; Kao *et al*., 2002; Riesewijk *et al*., 2003; Horcajadas *et al*., 2008; Macklon *et al*., 2008).

Two of these five genes (*PSAP* and *IGFBP-5*) have functions previously linked to endometrial physiology. The previously recorded presence of PSAP in the endometrium (epithelial cells) suggests roles for this protein in glycosphingolipid metabolism or transport in the uterine environment, and these functions appear to be steroid dependent (Spencer *et al*., 1995). On the other hand, the IGF system plays a fundamental role in endometrial biology, acting via autocrine and/or paracrine mechanisms (Zhou *et al*., 1994). IGFBPs regulate the mitogenic and metabolic actions of IGFs by inhibiting or, in some cases, enhancing the receptor binding of IGF. IGFBPs have an important role in the regulation of endometrial cellular mitosis, stroma-glandular relation and embryo-endometrial communication (Zhou *et al*., 1994; Mohan and Baylink, 2002; White *et al*., 2005).

OPTN is a 74-kDa protein implicated in the signal transduction of the tumor necrosis factor pathway and has been implicated in membrane traffic regulation, cellular morphogenesis and apoptosis (Hattula and Peränen, 2000). SNX7 contains a phox domain, which is a phosphoinositide-binding domain, with a fundamental role in orchestrating the formation of protein complexes that are involved in endosomal sorting and signaling (Cullen, 2008). Similarly, the CHMP1A gene encodes a member of the CHMP/Chmp family of proteins which are involved in multivesicular body sorting of proteins to the interiors of lysosomes. The initial prediction of the protein sequence encoded by this gene suggested that the encoded protein was a metallopeptidase. The nomenclature has been updated recently to reflect the correct biological function of this encoded protein (Li *et al*., 2008).

Our results demonstrated that the GnRH agonist LA, in the absence of sex steroids, did not have a direct effect on the expression levels of *OPTN, CHMP1A* and *SNX7* (the three main target genes identified by LDA analysis in our previous analysis of COH cycles) (Mirkin *et al*., 2004) in Ishikawa cells. We examined different doses of the agonist (range of 0.1–1 μM) at both short (6 h) and longer (24 h) incubation times (Experiments 1 and 2). In addition, the GnRH antagonist GA did not modify expression of *PSAP* or *IGFBP-5* at 24 h, in the absence or presence of 24-h sex steroids treatment (Experiment 2). The GnRH analogues LA and GA also resulted in unmodified levels of intracellular cAMP, a known second messenger resulting from receptor activation by its specific ligand in the pituitary gonadotrophs (Experiment 4).

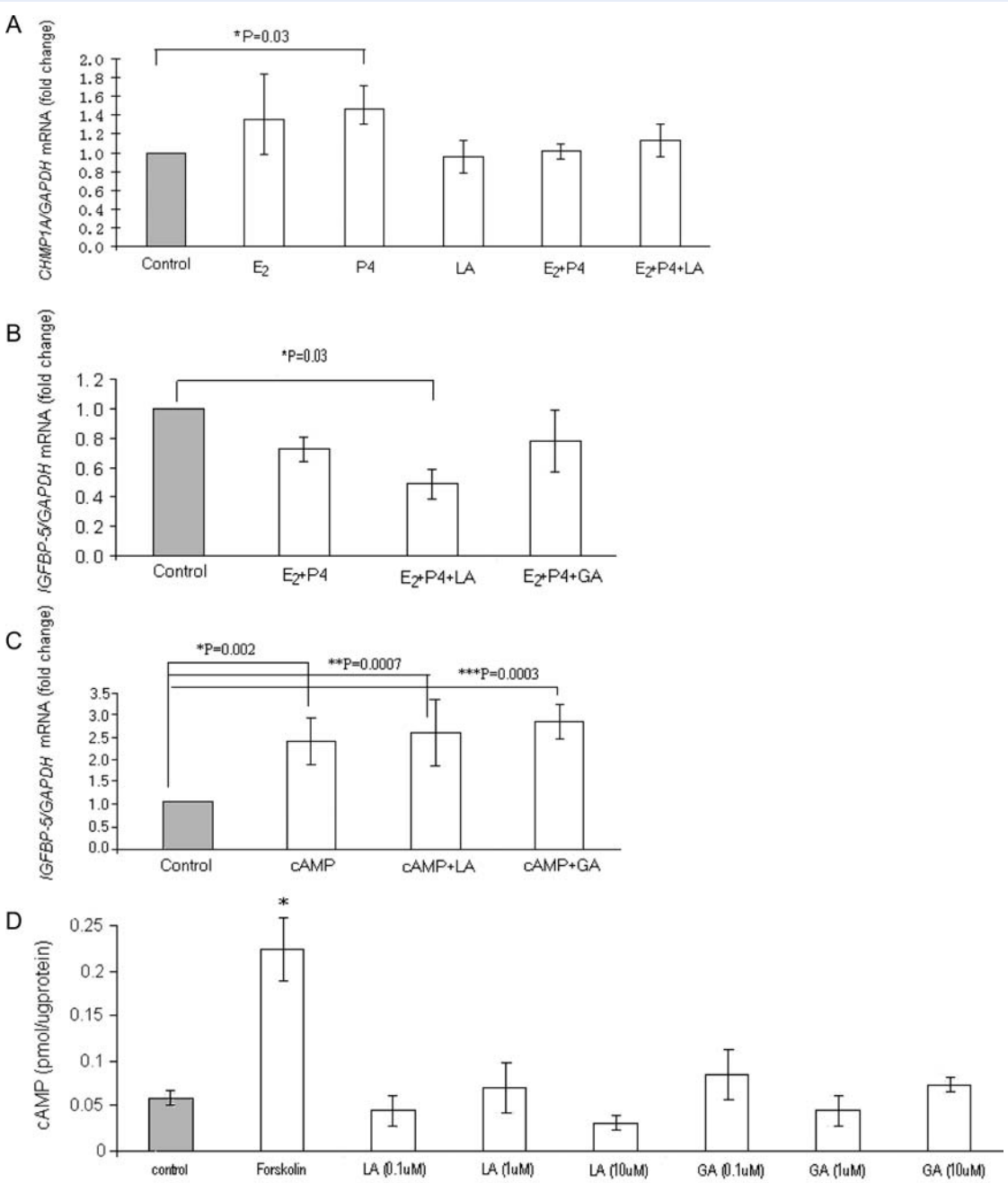

Figure 4 (**A**) Experiment 2: although the GnRH agonist LA and E_2 did not affect gene expression, P4 alone (at 24 h) resulted in significantly higher gene expression of CHMP1A compared with control levels ($P = 0.03$). (**B**) Experiment 3a: analysis of IGFBP-5 gene expression levels in Ishikawa cells subjected to long-term (4 days) pretreatment with E_2 and P4 showed that steroid treatment followed by LA resulted in significantly decreased mRNA expression versus controls ($P = 0.03$). (**C**) Experiment 3: impact of cAMP analogue (8-Br cAMP) pretreatment for 24 h on gene expression. *Control versus cAMP, $P = 0.0003$; **control versus cAMP + LA, $P = 0.0007$; ***control versus cAMP + GA, $P = 0.003$. (**D**) Experiment 4: measurement of intracellular cAMP concentrations in Ishikawa cells: lack of effect of GnRH analogues treatment (incubation time: 30 min) (*control versus Forskolin, $P = 0.03$; control versus all other conditions, $P > 0.1$).

On the other hand, sex steroid treatment affected expression of some of the genes studied. P4 alone (24-h incubation) resulted in significantly higher gene expression of *CHMP1A* than control conditions. Furthermore, for *IGFBP-5*, E_2 + P4 pretreatment for 4 days followed by LA (but not GA) after 6 h significantly decreased mRNA expression levels versus control (Experiment 3). Because sex steroid hormones, whether under physiological or supraphysiological conditions as seen in COH cycles, may act through specific response elements, we investigated the presence of putative EREs and PREs. On its own, the presence of hormone-response elements (HREs) in the promoter region

Table II Predicted EREs and PREs on ortholog promoters at a sensitivity 87%.

Species	Start	Strand	Pattern
Predicted EREs on ortholog promoters of *OPTN* gene			
Human	2472	+	TT-GGCCA-GGC-TGGTC-TC
Monkey	1539	+	TT-GGCCA-GGC-TGGTC-TC
	2867	+	GG-GGTGG-GCG-GGGCC-TG
	939	−	GA-GAACA-AAA-CAACC-CT
Mouse	1259	+	CA-GGGCA-TAT-TGGCC-AA
	1275	−	TT-GGCCA-ATA-TGCCC-TG
Rabbit	58	−	GT-AATCA-TGG-CAGCC-TT
Chimp	1831	+	TT-GGCCA-GGC-TGGTC-TC
	1235	−	GA-GAACA-AAA-CAACC-CT
Rat	1050	+	GT-AATCA-GAC-TGGCC-TT
	2610	+	AA-GATCA-TGA-TGGCC-TT
	2888	+	CG-GGTGG-GCG-TGGTC-TG
	2626	−	AA-GGCCA-TCA-TGATC-TT
	173	−	TA-GATCA-GGC-TAGCC-TG
Predicted EREs on ortholog promoters of *CHMP1A* gene			
Human	1891	−	TT-GGCCA-GGC-TGGTC-TT
Chimp	2196	−	TT-GGCCA-GGA-TGGTC-TC
Predicted EREs on ortholog promoters of *SNX7* gene			
Human	1219	+	TT-GGCCA-GAT-TGGCC-AA
	1235	−	TT-GGCCA-ATC-TGGCC-AA
	671	−	TT-GGCCA-GAA-TGGTC-TC
Hedgehog	652	−	CT-GGTGG-AAC-TAACC-CC
Elephant	1023	−	AA-GGACT-AGC-TAACC-AC
Monkey	1201	+	GT-GGCCA-GAT-TGGCC-AA
	1217	−	TT-GGCCA-ATC-TGGCC-AC
Mouse	2115	+	AA-AATCA-TAC-TGATC-CT
Rabbit	1023	−	AA-GGACT-AGC-TAACC-AC
Chimp	1224	+	TT-GGCCA-GAT-TGGCC-AA
	1240	−	TT-GGCCA-ATC-TGGCC-AA
	676	−	TT-GGCCA-GAA-TGGTC-TC
Predicted EREs on ortholog promoters of *PSAP* gene			
Human	2448	+	TT-GGCCA-GGC-TGGTC-TT
Cow	253	+	CG-GGCCA-AGG-TGCCC-GA
	1376	−	CA-GGAGG-CCC-TGCCC-CA
Elephant	160	+	CT-GGTGG-GGA-GGCCC-TG
	310	−	AA-GGACC-CAA-TGCCC-CA
Opossum	1154	+	GG-AGTCT-TCC-TGACT-CA
Monkey	8	+	GA-GAACA-GCC-TGGCC-AA
	2335	+	TT-GGTCA-GGC-TGGTC-TC
	1091	−	AA-GGCCT-GGG-TGGCC-CA
Mouse	2591	+	AA-GGCCA-TAG-TGACT-CG
	2607	−	CG-AGTCA-CTA-TGGCC-TT
	896	−	AA-GGCCA-GCC-TGGTC-TT
Rabbit	1286	+	CG-GGTGG-GTG-TAGCC-TG
	1170	−	CA-GGGCC-ACG-TGGCC-GG
Chimp	1296	+	TT-GGCCA-GGC-TGGTC-TT

Continued

Table II *Continued*

Species	Start	Strand	Pattern
Predicted EREs on ortholog promoters of *IGFBP-5* gene			
Opossum	509	−	AG-AGTCT-CCC-TGACT-CC
Rabbit	73	−	AT-GGCCA-CAA-CGGCC-TG
	1426	−	GG-GGTCT-GCC-TGCCC-CT
Predicted PREs on ortholog promoters of *SNX7* gene			
Human	2830	+	AGGACATGGTGTTCT
Dog	240	+	GGGAAATAATGTTCT
Chimp	2724	+	AGGACATGGTGTTCT

Scores for human, dog, chimp are 0.701900, 0.702296, 0.701900, respectively.

of a gene is not evidence of the hormonal control of expression of that gene. However, the presence of HREs in a promoter suggests that such HRE sites could be used by activated hormone receptors and consequently could affect gene expression. The supporting evidence for this could be strengthened if similar HREs could be identified in the promoters of orthologous genes. This would suggest evolutionary conservation of HRE sites across various species, implying potential importance of the presence of such sites. Our analysis covered these aspects.

The analysis of the predicted EREs on the promoters of the target genes showed that there is a consistent conservation of patterns of EREs in the promoters of the closely related orthologous species. This conservation might be an indicator of the control of these genes through the EREs. In cases where there is conservation of the ERE across human, chimp and monkey, such as for *OPTN* and *SNX7*, we can hypothesize that the EREs conserved between them are the active ERE sites in primates. These conclusions are derived on the basis of considering individual target genes and their respective orthologs.

Further evidence indicative of the importance of the predicted EREs would be the conservation of EREs across different human target genes. Table III represents the conservation of EREs predicted at a sensitivity of 87% in the promoters of all the genes under analysis. EREs (shown in red) are present in the promoters of four of the human genes under study. For *OPTN, CHMP1A* and *PSAP*, the ERE pattern is preserved in 16 out of 17 nucleotides, with mismatch only on the most 3′-flanking nucleotide. The pattern is 100% preserved in *CHMP1A* and *PSAP* genes. Moreover, in four target genes, both half-sites are fully conserved. This strongly suggests that these EREs could be active sites. On the basis of these results, we conclude that all four target genes have a potential to be controlled by estrogen.

In summary, the *in silico* prediction of EREs and PREs in the promoters of the five target genes and their orthologs from other mammalian species suggest that most of these genes (*OPTN, CHMP1A SNX7* and *PSAP*) have support for their potential control by E_2 and one gene (*SNX7*) by P4. Conversely, we did not identify EREs or PREs for *IGFBP-5* (Table IV).

Notwithstanding the presence of putative EREs and PREs and their likely significance for steroid hormone *in vivo* regulation, the *in vitro* treatment of Ishikawa cells with $E_2 + P4$ (for 24 h or 4 days) did not affect *per se* the expression of most studied genes. In fact, although EREs were identified for *OPTN, CHMP1A, SNX7* and *PSAP*, and PREs were identified for *SNX7*, gene expression was not modulated by sex steroid treatment under the Ishikawa cell culture conditions studied herein, with the exceptions of *CHMP1A* and *IGFBP-5*. It remains to be determined whether higher doses and/or longer pre-incubation times (as possibly seen in COH cycles) might affect gene expression patterns as we described previously in the *in vivo* studies (Mirkin *et al*., 2004), either directly or through effects of stromal products acting on a paracrine fashion.

Progesterone alone enhanced expression of *CHMP1A* at 24 h, in spite of the fact that we could not identity PREs for this gene,

Table III EREs at a sensitivity of 87% predicted in promoters of target human genes.

Species	Start	Strand	Pattern	Genes
Human	2472	+	**TT-GGCCA-GGC-TGGTC-TC**	*OPTN*
Human	1891	−	**TT-GGCCA-GGC-TGGTC-T**T	*CHMP1A*
Human	2448	+	**TT-GGCCA-GGC-TGGTC-TT**	*PSAP*
Human	1219	+	**TT-GGCCA-GAT-TGGCC-AA**	*SNX7*
	1235	−	**TT-GGCCA-ATC-TGGCC-AA**	
	671	−	**TT-GGCCA-GAA-TGGTC-TC**	

Table IV Summary of identified HREs in the promoters of the target human genes.

Gene	ERE	PRE
IGFBP-5	−	−
PSAP	+	−
OPTN	+	−
CHMP1A	+	−
SNX7	+	+

suggesting alternative, non-classical mechanisms of gene activation (O'Brien *et al*., 2006). Moreover, the GnRH agonist LA, but only after $E_2 + P4$ pretreatment for 4 days, significantly decreased mRNA expression levels of *IGFBP-5*. The lack of identification of EREs and PREs for this gene also suggests sex steroid priming through other non-classical pathways. This is supported by the fact that there are several pathways for the activation of estrogen-controlled genes that are not based on the binding of estrogen receptor to EREs (Nilsson and Gustafsson, 2000; Harrington *et al*., 2006).

At least two microarray studies have shown that *IGFBP-5* was up-regulated in COH cycles where GnRH antagonists were used in combination with gonadotrophins, with (Mirkin *et al*., 2004) or without P4 supplementation of the luteal phase (Macklon *et al*., 2008). It has been reported that *IGFBP-5* gene expression is inhibited by P4 during the natural cycle (Giudice *et al*., 1991; Zhou *et al*., 1994) and is down-regulated *in vitro* in decidualized endometrial stromal cells by cAMP acting through PKA activation (Tierney *et al*., 2003). In the study of Mirkin *et al*. (2004), there were no differences in the serum steroid levels on the day the endometrial biopsies were performed (matched cycle Day 21) between COH cycles accomplished with GnRH antagonist with P4 supplementation, GnRH antagonist without P4 supplementation or GnRH agonist with P4 supplementation. According to Macklon *et al*. (2008), up-regulation of *IGFBP-5* in the P4 non-supplemented cycles was unanticipated, as this IGFBP is the only one whose expression is suppressed in the secretory phase, compared with the proliferative phase. As such, Macklon *et al*. (2008) also concluded that dysregulation of the endometrial transcriptome in the stimulated cycle is not fully attributable to supraphysiological sex steroid levels at the folliculo-luteal transition.

Our results using Ishikawa cells, however, did not demonstrate an effect of GnRH antagonist on *IGFBP5*; rather, the GnRH agonist modulated expression of *IGFBP-5*, and this effect resulted in a significant down-regulation. The fact that modulation of gene expression by the GnRH agonist was observed after a 4-day $E_2 + P4$ priming period suggests interaction between sex steroid modulation and the agonist. Since we were not able to identify EREs or PREs for this gene, it can be speculated that the sex steroids might regulate the epithelial cell through non-classic mechanisms. O'Brien *et al*. (2006) demonstrated that estrogen-induced responses in the uterus can be divided into classical and non-classical pathways by using genetic models that selectively discriminate between these mechanisms. The non-classical pathway, which is ERE independent and functions probably via protein–protein interactions with transcription factors and/or signals via rapid, non-genomic pathways originating at the cell membrane, mediates the estrogen-dependent epithelial cell proliferation and growth responses, whereas the classical pathway, where the ER binds directly to an ERE to activate or repress gene expression, is required for other responses, such as hyperemia and water imbibition.

We also found that *IGFBP-5* was the only gene modulated by cAMP analogue treatment. *IGFBP-5* gene expression levels were significantly increased by cAMP alone as well as by cAMP with the GnRH agonist LA and the GnRH antagonist GA, but neither LA nor GA significantly augmented the effect observed with cAMP alone (Experiment 4). The cAMP analogue significantly up-regulated *IGFBP-5* expression upon 24 h of incubation, an effect that was not subjected to further modulation by either of the GnRH analogues. It appears, therefore, as though *IGFBP-5* is a potential target gene of several regulatory mechanisms (steroids, GnRH and cAMP) in endometrial epithelial cells. The observed effect of cAMP on epithelial cells differs from the stromal compartment where *IGFBP-5* was down-regulated *in vitro* by decidualized stromal cells by cAMP acting through PKA activation (Tierney *et al*., 2003).

Reports from several other laboratories have established that GnRH analogues can modulate the expression of other genes, particularly in endometrial stromal cells *in vitro*. In fact, TIMP-1 and -3 (tissue inhibitors of matrix metalloproteinases) mRNA expression and protein secretion into the medium were significantly decreased by a GnRH agonist compared with control groups (Raga *et al*., 1999), whereas IL-1B mRNA expression and protein expression were significantly enhanced by a GnRH agonist (Raga *et al*., 2008). Other studies have demonstrated that GnRH analogues can induce apoptotic cell death in endometrial epithelial cells and endometriotic cells *in vitro* (Meresman *et al*., 2003, Bilotas *et al*., 2007). Moreover, GnRH agonists have been shown to act directly on isolated stromal cells (primary cultures) and in epithelial cell lines altering the expression and activation of *SMADS*, intracellular signals activated by transforming growth factor-β (Luo *et al*., 2003). Nevertheless, the results of our study do not demonstrate a direct effect of GnRH analogues on the genes studied in the Ishikawa epithelial cell model.

Klemmt *et al*. (2009) recently analyzed the *in vitro* effects of GnRH analogues on the decidualizing endometrium and blastocyst invasion. The concentrations of the GnRH analogues they used were also based on the physiological plasma serum levels after systemic administration, while the time of incubation of 72 h was selected to allow for *in vitro* decidualization. The authors demonstrated that GnRH analogues did not significantly influence the extent of decidualization of endometrial stromal cells *in vitro*. In addition, no adverse effects of GnRH analogues were seen on human blastocyst invasion. They concluded that GnRH analogues affect neither the capacity of the endometrium to support invasion nor the invasive potential of the blastocyst in the early stages of implantation. Our results are similar as related to endometrial epithelial cells, since at the concentrations and time periods used in our studies we did not detect any direct actions of the GnRH antagonists on the tested genes, except for the $E_2 + P4$-primed down-regulation of *IGFB-5* by the GnRH agonist. The results of these two studies appear to be consistent with the outcome of a clinical report showing no impact on pregnancy, following the use of a GnRH antagonist administered throughout the estrogenic phase in recipients of donor eggs (Prapas *et al*., 2009).

In summary, in the Ishikawa cell model used and under the experimental conditions tested: (i) GnRH antagonist treatment did not affect

gene expression; (ii) GnRH agonist down-regulated *IGFBP-5* following long-term sex steroid priming, but *IGFBP-5* lacked ERE and PRE; we speculate that this effect is mediated through the identified GnRH-R and via a cAMP-independent mechanism; (iii) cAMP up-regulated *IGFBP-5* expression, and more studies are needed to examine the pathways and significance of cAMP-derived actions and (iv) most target genes demonstrated the presence of conserved EREs, posing the question as to estrogen regulation under other experimental conditions or in the *in vivo* scenario. The observed down-regulation of *IGFBP-5* gene expression *in vitro* by the GnRH agonist coincides with patterns seen in the natural cycle, and as such it may not have a significant functional effect at the endometrial level. We conclude, based on the experimental conditions described in Ishikawa cells, that GnRH analogues appear not to have major direct effects on gene expression of human endometrial epithelial cells *in vitro*.

Authors' roles

X.Z. performed the cell culture experiments and the RT–PCR. S.B. participated in the design, statistical analysis and writing of the paper. A.F. performed the immunofluorescence experiments and participated in cAMP measurement studies. S.A. participated in the RT–PCR. M.K. and V.B.B. performed the *in silico* analysis and participated in writing of the paper. S.O. participated in the design and writing of the paper. All authors had substantial contributions to the conception and design, or acquisition of data, or analysis and interpretation of data, and gave final approval of the version to be published.

Funding

Howard & Georgeanna Jones Foundation for Reproductive Medicine and Eastern Virginia Medical School.

References

Aerts S, Van Loo P, Thijs G, Mayer H, de Martin R, Moreau Y, De Moor B. TOUCAN 2: the all-inclusive open source workbench for regulatory sequence analysis. *Nucleic Acids Res* 2005;**33**:W393–W396.

Al-Inany HG, Abou-Setta AM, Aboulghar M. Gonadotrophin-releasing hormone antagonists for assisted conception: a Cochrane review. *Reprod Biomed Online* 2007;**14**:640–649.

Bajic VB, Tan SL, Chong A, Tang S, Ström A, Gustafsson JA, Lin CY, Liu ET. Dragon ERE Finder ver.2: a tool for accurate detection and analysis of estrogen response elements in vertebrate genomes. *Nucleic Acids Res* 2003;**31**:3605–3607.

Bilotas M, Barañao RI, Buquet R, Sueldo C, Tesone M, Meresman G. Effect of GnRH analogues on apoptosis and expression of Bcl-2, Bax, Fas and FasL proteins in endometrial epithelial cell cultures from patients with endometriosis and controls. *Hum Reprod* 2007;**22**:644–653.

Bocca SM, Archer DF. Raloxifene (Rlx) has antiestrogenic effect on vascular endothelial growth factor (VEGF) mRNA and protein while down-regulating progesterone receptors in a human endometrial cell line in vitro. *Fertil Steril* 2005;**84;**:S119–S120.

Borroni R, Di Blasio AM, Gaffuri B, Santorsola R, Busacca M, Viganò P, Vignali M. Expression of GnRH receptor gene in human ectopic endometrial cells and inhibition of their proliferation by leuprolide acetate. *Mol Cell Endocrinol* 2000;**159**:37–43.

Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC, Smith SK. Determination of the transcript profile of human endometrium. *Mol Hum Reprod* 2003;**9**:19–33.

Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. *Hum Reprod Update* 2003;**9**:515–522.

Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M, Yuan L, Fritz MA, Lessey B. Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Mol Hum Reprod* 2002;**8**:871–879.

Chan LN, Chung YW, Leung PS, Liu CQ, Chan HC. Activation of an adenosine 3′,5′-cyclic monophosphate-dependent Cl- conductance in response to neurohormonal stimuli in mouse endometrial epithelial cells: the role of cystic fibrosis transmembrane conductance regulator. *Biol Reprod* 1999;**60**:374–380.

Cheng CK, Leung PC. Molecular biology of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and their receptors in humans. *Endocr Rev* 2005;**26**:283–306.

Cheon KW, Lee HS, Parhar IS, Kang IS. Expression of the second isoform of gonadotrophin-releasing hormone (GnRH-II) in human endometrium throughout the menstrual cycle. *Mol Hum Reprod* 2001;**7**:447–452.

Cheung LW, Wong AS. Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues. *FEBS J* 2008;**275;**:5479–5495.

Chou CS, Zhu H, MacCalman CD, Leung PC. Regulatory effects of gonadotropin-releasing hormone (GnRH) I and GnRH II on the levels of matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitor of metalloproteinases-1 in primary cultures of human extravillous cytotrophoblasts. *J Clin Endocrinol Metab* 2003;**88**:4781–4790.

Counis R, Laverrière JN, Garrel G, Bleux C, Cohen-Tannoudji J, Lerrant Y, Kottler ML, Magre S. Gonadotropin-releasing hormone and the control of gonadotrope function. *Reprod Nutr Dev* 2005;**45**:243–254.

Cui J, Smith RG, Mount GR, Lo JL, Yu J, Walsh TF, Singh SB, DeVita RJ, Goulet MT, Schaeffer JM *et al.* Identification of Phe313 of the gonadotropin-releasing hormone (GnRH) receptor as a site critical for the binding of nonpeptide GnRH antagonists. *Mol Endocrinol* 2000;**14**:671–681.

Cullen PJ. Endosomal sorting and signalling: an emerging role for sorting nexins. *Nat Rev Mol Cell Biol* 2008;**9**:574–582.

Diel P. Tissue-specific estrogenic response and molecular mechanisms. *Toxicol Lett* 2002;**127**:217–224.

Dong KW, Marcelin K, Hsu MI, Chiang CM, Hoffman G, Roberts JL. Expression of gonadotropin-releasing hormone (GnRH) gene in human uterine endometrial tissue. *Mol Hum Reprod* 1998;**4**:893–898.

Franchi A, Zaret J, Zhang X, Bocca S, Oehninger S. Expression of immunomodulatory genes, their protein products and specific ligands/receptors during the window of implantation in the human endometrium. *Mol Hum Reprod* 2008;**14**:413–421.

Garcia JE, Acosta AA, Hsiu JG, Jones HW Jr. Advanced endometrial maturation after ovulation induction with human menopausal gonadotropin/human chorionic gonadotropin for in vitro fertilization. *Fertil Steril* 1984;**41**:31–35.

Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med* 2007;**25**:445–453.

Giudice LC, Milkowski DA, Lamson G, Rosenfeld RG, Irwin JC. Insulin-like growth factor binding proteins in human endometrium: steroid-dependent messenger ribonucleic acid expression and protein synthesis. *J Clin Endocrinol Metab* 1991;**72**:779–787.

Gordon K. Gonadotropin-releasing hormone antagonists implications for oocyte quality and uterine receptivity. *Ann NY Acad Sci* 2001;**943**:49–54.

Gründker C, Völker P, Emons G. Antiproliferative signaling of luteinizing hormone-releasing hormone in human endometrial and ovarian

cancer cells through G protein alpha(I)-mediated activation of phosphotyrosine phosphatase. *Endocrinology* 2001;**142**:2369–2380.

Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA, Katzenellenbogen BS. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. *Mol Endocrinol* 2006;**20**:491–502.

Hattula K, Peränen J. FIP-2, a coiled-coil protein, links Huntingtin to Rab8 and modulates cellular morphogenesis. *Curr Biol* 2000;**10**:1603–1606.

Horcajadas JA, Mínguez P, Dopazo J, Esteban FJ, Domínguez F, Giudice LC, Pellicer A, Simón C. Controlled ovarian stimulation induces a functional genomic delay of the endometrium with potential clinical implications. *J Clin Endocrinol Metab* 2008;**93**:4500–4510.

Hsueh AJ, Jones PB. Extrapituitary actions of gonadotropin-releasing hormone. *Endocr Rev* 1981;**2**:437–461.

Huang SJ. Tug of war between ovarian stimulation and endometrial receptivity. *Reprod Sci* 2008;**15**:335.

Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. *Endocr Rev* 2006;**27**:17–46.

Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA, Giudice LC. Global gene profiling in human endometrium during the window of implantation. *Endocrinology* 2002; **143**:2119–2138.

Kato S, Sasaki H, Suzawa M, Masushige S, Tora L, Chambon P, Gronemeyer H. Widely spaced, directly repeated PuGGTCA elements act as promiscuous enhancers for different classes of nuclear receptors. *Mol Cell Biol* 1995;**15**:5858–5867.

Klemmt PA, Liu F, Carver JG, Jones C, Brosi D, Adamson J, Mardon HJ, McVeigh E. Effects of gonadotrophin releasing hormone analogues on human endometrial stromal cells and embryo invasion in vitro. *Hum Reprod* 2009;**24**:2187–2192.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 1997;**138**:863–870.

Li J, Belogortseva N, Porter D, Park M. Chmp1A functions as a novel tumor suppressor gene in human embryonic kidney and ductal pancreatic tumor cells. *Cell Cycle* 2008;**7**:2886–2893.

Luo X, Xu J, Chegini N. Gonadotropin releasing hormone analogue (GnRHa) alters the expression and activation of Smad in human endometrial epithelial and stromal cells. *Reprod Biol Endocrinol* 2003;**1**:125.

Macklon NS, van der Gaast MH, Hamilton A, Fauser BC, Giudice LC. The impact of ovarian stimulation with recombinant FSH in combination with GnRH antagonist on the endometrial transcriptome in the window of implantation. *Reprod Sci* 2008;**15**:357–365.

Martínez-Conejero JA, Simón C, Pellicer A, Horcajadas JA. Is ovarian stimulation detrimental to the endometrium? *Reprod Biomed Online* 2007;**15**:45–50.

Meresman GF, Bilotas MA, Lombardi E, Tesone M, Sueldo C, Barañao RI. Effect of GnRH analogues on apoptosis and release of interleukin-1beta and vascular endothelial growth factor in endometrial cell cultures from patients with endometriosis. *Hum Reprod* 2003;**18**:1767–1771.

Millar R, Lowe S, Conklin D, Pawson A, Maudsley S, Troskie B, Ott T, Millar M, Lincoln G, Sellar R *et al.* A novel mammalian receptor for the evolutionarily conserved type II GnRH. *Proc Natl Acad Sci USA* 2001;**98**:9636–9641.

Millar RP. GnRH II and type II GnRH receptors. *Trends Endocrinol Metab* 2003;**14**:35–43.

Mirkin S, Nikas G, Hsiu JG, Díaz J, Oehninger S. Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. *J Clin Endocrinol Metab* 2004;**89**:5742–5752.

Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S, Oehninger S. In search of candidate genes critically expressed in the human endometrium during the window of implantation. *Hum Reprod* 2005;**20**:2104–2117.

Mohan S, Baylink DJ. IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. *J Endocrinol* 2002; **175**:19–31.

Navarro FJ, Mirkin S, Archer DF. Effect of raloxifene, 17beta-estradiol, and progesterone on mRNA for vascular endothelial growth factor isoforms 121 and 165 and thrombospondin-1 in Ishikawa cells. *Fertil Steril* 2003; **79**:1409–1415.

Nilsson S, Gustafsson JA. Estrogen receptor transcription and transactivation: basic aspects of estrogen action. *Breast Cancer Res* 2000;**2**:360–366.

Nishida M. The Ishikawa cells from birth to the present. *Hum Cell* 2002; **15**:104–117.

O'Brien JE, Peterson TJ, Tong MH, Lee EJ, Pfaff LE, Hewitt SC, Korach KS, Weiss J, Jameson JL. Estrogen-induced proliferation of uterine epithelial cells is independent of estrogen receptor alpha binding to classical estrogen response elements. *J Biol Chem* 2006;**281**:26683–26692.

Oehninger S. Revealing the enigmas of implantation: what is the true impact of ovarian hyperstimulation? *Fertil Steril* 2008;**89**:27–30.

Paria BC, Reese J, Das SK, Dey SK. Deciphering the cross-talk of implantation: advances and challenges. *Science* 2002;**296**:2185–2188.

Prapas N, Tavaniotou A, Panagiotidis Y, Prapa S, Kasapi E, Goudakou M, Papatheodorou A, Prapas Y. GnRH antagonists and endometrial receptivity in oocyte recipients: a prospective randomized trial. *Reprod Biomed Online* 2009;**8**:276–281.

Raga F, Casañ EM, Kruessel JS, Wen Y, Huang HY, Nezhat C, Polan ML. Quantitative gonadotropin-releasing hormone gene expression and immunohistochemical localization in human endometrium throughout the menstrual cycle. *Biol Reprod* 1998;**59**:661–669.

Raga F, Casañ EM, Wen Y, Huang HY, Bonilla-Musoles F, Polan ML. Independent regulation of matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-3 in human endometrial stromal cells by gonadotropin-releasing hormone: implications in early human implantation. *J Clin Endocrinol Metab* 1999;**84**:636–642.

Raga F, Casañ EM, Bonilla-Musoles F. Gonadotropin-releasing hormone (GnRH)-I regulation of interleukin (IL)-1b and IL-1 receptor antagonist expression in cultured human endometrial stromal cells. *J Obstet Gynaecol Res* 2008;**34**:464–472.

Ramakrishnappa N, Rajamahendran R, Lin YM, Leung PC. GnRH in non-hypothalamic reproductive tissues. *Anim Reprod Sci* 2005;**88**:95–113.

Riesewijk A, Martín J, van Os R, Horcajadas JA, Polman J, Pellicer A, Mosselman S, Simón C. Gene expression profiling of human endometrial receptivity on days LH + 2 versus LH + 7 by microarray technology. *Mol Hum Reprod* 2003;**9**:253–264.

Shemesh M. Actions of gonadotrophins on the uterus. *Reproduction* 2001; **121**:835–842.

Spencer TE, Graf GH, Bazer FW. Sulfated glycoprotein-1 (SGP-1) expression in ovine endometrium during the oestrous cycle and early pregnancy. *Reprod Fertil Dev* 1995;**7**:1053–1060.

Takeuchi S, Futamura N, Minoura H, Toyoda N. Possible direct effect of gonadotropin releasing hormone on human endometrium and decidua. *Life Sci* 1998;**62**:1187–1194.

Tierney EP, Tulac S, Huang ST, Giudice LC. Activation of the protein kinase A pathway in human endometrial stromal cells reveals sequential categorical gene regulation. *Physiol Genomics* 2003;**16**:47–66.

Uchida H, Maruyama T, Nagashima T, Asada H, Yoshimura Y. Histone deacetylase inhibitors induce differentiation of human endometrial adenocarcinoma cells through up-regulation of glycodelin. *Endocrinology* 2005;**146**:5365–5373.

White CA, Dimitriadis E, Sharkey AM, Salamonsen LA. Interleukin-11 inhibits expression of insulin-like growth factor binding protein-5 mRNA in decidualizing human endometrial stromal cells. *Mol Hum Reprod* 2005;**11**:649–658.

Wu HM, Cheng JC, Wang HS, Huang HY, MacCalman CD, Leung PC. Gonadotropin-releasing hormone type II induces apoptosis of human endometrial cancer cells by activating GADD45alpha. *Cancer Res* 2009a;**69**:4202–4208.

Wu HM, Wang HS, Huang HY, Soong YK, MacCalman CD, Leung PC. GnRH signaling in intrauterine tissues. *Reproduction* 2009b;**137**:769–777.

Zheng XY, Chen GA, Wang HY. Expression of cystic fibrosis transmembrane conductance regulator in human endometrium. *Hum Reprod* 2004;**19**:2933–2941.

Zhou J, Dsupin BA, Giudice LC, Bondy CA. Insulin-like growth factor system gene expression in human endometrium during the menstrual cycle. *J Clin Endocrinol Metab* 1994;**79**:1723–1734.