Excessive ovarian stimulation upregulates the Wnt-signaling molecule DKK1 in human endometrium and may affect implantation: an in vitro co-culture study

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BACKGROUND: High serum estradiol (E2) levels following ovarian stimulation lead to reduced implantation and pregnancy rates, yet the underlying mechanisms remain unknown. We investigated if aberrant expression of genes in the Wnt-signaling pathway may be involved.

METHODS: Microarray and real-time PCR analysis were performed to analyze gene expression profiles of endometrial samples taken at day hCG + 7 in stimulated cycles, and days LH + 7 and LH + 10 in natural cycles. Expression of several Wnt-signaling transcripts, including Dickkopf homolog 1 (DKK1), DKK2 and secreted frizzled-related protein 4 (sFRP4), was analyzed throughout the menstrual cycle. JAr spheroid/Ishikawa endometrial cell co-culture experiments were established to study effects of DKK1 on spheroid attachment in vitro.

RESULTS: We identified 351 differentially expressed genes. Endometrial samples taken at hCG + 7 had similar expression profiles to those at LH + 7. DKK1 transcripts were up-regulated and DKK2 and sFRP4 were down-regulated in the stimulated compared with LH + 7 group (all P < 0.05). DKK1 transcripts were low in proliferative phase (PS) and increased in late-secretory phase (LS, P < 0.05), although DKK2 peaked in mid-secretory phase (P < 0.05). sFRP4 transcripts were high in PS. Treatment of spheroid with recombinant human DKK-1 protein dose-dependently suppressed (P < 0.05 versus control) spheroids attachment onto endometrial cells (associated with decreased β-catenin protein): this suppression was nullified by anti-DKK1 antibody.

CONCLUSION: Gene expression patterns in stimulated cycles resembled those of LS in natural cycles, when the implantation window is about to close, suggesting high serum E2 and/or progesterone concentrations may advance endometrial development, altering the implantation window and possibly decreasing pregnancy rate. Aberrant expression of DKK1 might impair embryo attachment and implantation vivo.

Key words: ovarian stimulation / endometrium / Wnt-signaling / implantation / spheroids

Introduction

IVF is an effective treatment for various causes of infertility. Ovarian stimulation is used in a majority of assisted reproduction units in order to improve the pregnancy rate by increasing the number of oocytes and thus embryos available for transfer. Recruitment and development of multiple follicles in response to gonadotrophin stimulation are key factors leading to successful IVF treatment. GnRH agonists are commonly used during ovarian stimulation to prevent a premature LH surge. Several recent studies have compared the endometrial gene expression profiles in the luteal phase of natural and controlled ovarian stimulated cycles (Mirkin et al., 2004; Horcajadas et al.,...
2005, 2008; Simon et al., 2005; Macklon et al., 2008; Liu et al., 2008; Haouzi et al., 2009a). Yet, only a few differentially expressed genes were found in these studies, suggesting that the current controlled ovarian stimulation protocols could induce endometrial responses similar to those in natural cycles. Excessive ovarian responses, however, may lead to an increased risk of ovarian hyperstimulation syndrome (OHSS) and adverse outcomes in IVF treatment (Simon et al., 1995, 1998; Pellicer et al., 1996; Ng et al., 2000; Macklon and Fauser, 2000). We previously reported that women with a serum E2 concentration >20 000 pmol/l on the day of hCG administration showed decreased implantation and pregnancy rates in IVF cycles (Ng et al., 2000). These excessive response cases have dysynchronous development of the endometrium with delayed glandular maturation and advanced stromal morphology, as determined by endometrial morphometric analysis (Basir et al., 2001). These women also have a significantly higher endometrial vascularity on Day 7 after hCG injection (hCG + 7) (Ng et al., 2004) and a discrete mRNA expression profile (Liu et al., 2008) when compared with those with lower E2 levels. Yet, the embryo (Ng et al., 2000) and oocyte (Ng et al., 2003) quality is not affected by the high serum E2 level.

Implantation is a critical step in the establishment of a pregnancy. There are a few studies (Carson et al., 2002; Kao et al., 2002; Borthwick et al., 2003; Riesiewijk et al., 2003; Mirkin et al., 2005; Talbi et al., 2006; Haouzi et al., 2009b; Tseng et al., 2009) comparing the gene expression profiles of endometria during the implantation window with those in the proliferative or early luteal phases of the cycle. In each of these studies, differential expression of a large number of genes was found. However, only a few of these differentially expressed genes were common to most of these studies, indicating the complexity and variability of endometrial development (Horcajadas et al., 2004; Mirkin et al., 2008).

The Wnt-signaling pathway is crucial to estrogen-mediated uterine growth (Hou et al., 2004) and implantation (Mohamed et al., 2005; Xie et al., 2008) in mice. Dickkopf homolog 1 (DKK1) is a Wnt-signaling antagonist (Kawano and Kypta, 2003) which is up-regulated in the implantation window (Kao et al., 2002; Tulac et al., 2003). DKK1 is expressed in preimplantation embryos and the stromal cells of the mouse uterus (Li et al., 2008). Injection of DKK1 antisense oligonucleotide into the mouse uterus on Day 3 of a pregnancy inhibits embryo implantation (Li et al., 2008). DKK1 secreted from decidual cells also plays a role in trophoblast cell invasion and outgrowth (Peng et al., 2008). Yet, mice deficient in DKK1 died at birth with morphological defects, including lack of anterior head structures and duplications and fusions of the limb digits (Mukhopadhyay et al., 2001).

Our previous microarray data showed that the endometrial gene expression profile on hCG + 7 in women with excessive response was different to that of natural cycling women on Day 7 after a LH surge (LH + 7), and that the former group of women had aberrant expression of Wnt-signaling molecules (Liu et al., 2008). A number of genes were up-regulated, such as aldehyde oxidase 1 (AOX1), nicotinamide N-methyltransferase (NNMT), glutathione peroxidase 3 (GPx3) and glycodelin (PAEP) although others, such as solute carrier family 26 (sulfate transporter), member 2 (SLC26A2), metalloproteinase 26 (MMP26), solute carrier family 7, member 4 (SLC7A4) and prostaglandin-endoperoxide synthase 1 (PTGS1/COX1), were down-regulated in the excessive responders of hCG + 7 samples (hCG + 7(E)) when compared with LH + 7 samples in the natural cycles (Liu et al., 2008). The temporal expression of these eight differentially expressed genes was further evaluated in this study using endometrial samples taken from different phases of natural cycles. The expression of Wnt-signaling molecules [e.g. DKK1, DKK2 and secreted frizzled-related protein 4 (sFRP4)] in the human endometrium from patients receiving IVF treatment was examined and compared with the endometrial gene expression profiles of patients from the natural (LH + 7 and LH + 10) and stimulated cycle (hCG + 7). Furthermore, the role of recombinant human Dkk-1 on embryo attachment was analyzed using a spheroid-endometrial co-culture model.

Materials and Methods

Human subjects

Thirty-eight infertile women (median = 33, range = 27–39 year) undergoing IVF treatment at the Assisted Reproduction Unit at the Department of Obstetrics and Gynaecology, Queen Mary Hospital, Hong Kong were recruited for the study. They had regular menstrual cycles, normal uterus and no significant intrauterine or ovarian abnormalities as determined by transvaginal ultrasonography. Only women with regular menstrual cycles and male factor infertility who had not received any steroidal hormones for 2 months or more prior to the study and also agreed to the use of condoms for contraception during the study cycle were recruited for endometrial biopsies in the natural cycles. Another 45 human endometrial biopsies were taken from patients who visited our IVF clinic for infertility treatment. The samples were taken in different phases of the menstrual cycle including early-/mid-proliferative phase (EP/MP, n = 8), late proliferative phase (LP, n = 8), early secretory phase (ES, n = 8), middle secretory phase (MS, n = 15) and late secretory phase (LS, n = 6). The women were not significantly different (P < 0.05) in terms of age and duration of infertility. Informed written consent was obtained prior to participation in the study, which was approved by the Institutional Review Board of the University of Hong Kong and Hospital Authority Hong Kong, West Cluster.

Tissue collection

Endometrial biopsies were taken either on LH + 7 or LH + 10 from 21 patients in natural cycles. Blood was taken daily for serum E2 and LH concentration, starting 18 days before the next expected menstruation until the LH surge, which was defined as the day when serum LH level was more than double the mean of the preceding readings. For the stimulated cycles, the subjects were recruited from those who did not have embryo transfer after IVF treatment because of either failure of fertilization or risk of OHSS. Ovarian stimulations were carried out as described previously using the long protocol (Ng et al., 2000). Briefly, the subjects were pretreated with GnRH analogue, buserelin (Suprecur, Hoechst, Frankfurt, Germany) nasal spray 150 μg four times a day from the mid-luteal phase of the cycle proceeding the treatment cycle. The human menopausal gonadotrophin (hMG, Menogon, Ferring GmbH, Kiel, Germany) nasal spray 150 μg four times a day from the mid-luteal phase of the cycle proceeding the treatment cycle. The human menopausal gonadotrophin (hMG, Menogon, Ferring GmbH, Kiel, Germany) injection was administered after confirmation of pituitary down-regulation. hCG (Profasi, Serono, Geneva, Switzerland) 10 000 IU was administered when the leading follicle reached 18 mm diameter and there were at least three follicles >15 mm in diameter. Serum E2 concentration was measured on the day of the oculatory hCG injection and the patients were classified into either moderate (serum E2 ≤ 20 000 pmol/l) or excessive (serum E2 > 20 000 pmol/l) responders as described previously (Ng et al., 2000).
RNA isolation and real-time PCR

Total RNA from endometrial samples was purified using the Absolutely RNA RT–PCR miniprep kit (Stratagene, La Jolla, CA, USA) as described previously (Lee et al., 2006). One microgram of total RNA was reverse transcribed using the first strand complementary DNA (cDNA) synthesis kit (Amersham, Upplasa, Sweden). The cDNA product was diluted with distilled water to a final volume of 50 μl. Real-time PCR was performed in an iCycler (Bio-Rad, Hercules, CA, USA) as described (Lee et al., 2005). Primers for AOX1, NNMT, GPx3, PAEP, SLC26A2, MMP26, SLC7A4 and PTGS1/COX1 were designed using Primer Premier v5.0 (Premier Biosoft International, Palo Alto, CA, USA). All real-time PCR assays were performed with TaqMan PCR Master Mix (Applied Biosystems, Warrington, UK). A standard PCR cycling protocol was performed: 1 cycle at 95°C for 10 min; 40 cycles at 95°C for 15 s, 60°C for 35 s and 72°C for 45 s. The 2−ΔΔCT relative quantification method was performed to analyze the data from real-time PCR experiment as described before (Livak and Schmittgen, 2001). The house-keeping 18S gene was chosen as the internal control for sample normalization.

RNA isolation and affymetrix microarray

Total RNA from 38 samples was isolated using the RNeasy Mini Kit (Qiagen, Crawley, Sussex, UK) according to the supplier’s protocol. The total RNA bound to the column was eluted in RNase-free water. RNA quality control, sample labeling, GeneChip hybridization and data acquisition were performed at the Genome Research Centre, The University of Hong Kong. In brief, the quality of total RNA of seven endometrial samples from natural cycling women (LH + 7, n = 3; LH + 10, n = 4) and three endometrial samples on hCG + 7 from women with excessive response [hCG + 7(E), n = 3] was checked by an Agilent 2100 bioanalyzer (Waldbronn, Germany). The RNA was then amplified and labeled with the MessageAmp II-Biotin Enhanced Single Round cRNA Amplification Kit (Ambion Inc., Texas). Double-stranded cDNA was generated by reverse transcription from 1 μg total RNA with an oligo(dT) primer bearing a T7 promoter. The double strand cDNA was used as a template for in vitro transcription to generate biotin-labeled cRNA. After fragmentation, 15 μg of cRNA was hybridized to the GeneChips HG_U133A microarray (Affymetrix Inc., Santa Clara, CA, USA) which comprised more than 22,000 probe sets that analyzed over 18,000 human transcripts and variants. The GeneChips were washed and stained using the GeneChip Fluidics Station 450 (Affymetrix Inc.) and then scanned with the GeneChip Scanner 3000 7G (Affymetrix Inc.).

Microarray analysis

GeneSpring 7.2 software (Agilent Technologies, Palo Alto, CA, USA) was used to analyze the microarray data. Per-Chip normalization was carried out first with the Robust Multi-chip Average (RMA) analysis algorithm based on the expression values of all genes. After RMA preprocessor analysis, the expression values on each microarray chip were normalized to the 50th percentile, and all the positive data were returned. The Per-Gene normalization was performed next. The expression values of each gene on all microarray chips were normalized to the median, and the cutoff value was set as 0.05. Genes with expression values lower than the cutoff were not analyzed further.

The normalized expression values of all genes were first analyzed using the one way analysis of variance (ANOVA) statistical test, with the P-value set at 0.05 or less. Genes that were differentially expressed (P < 0.05, One Way ANOVA) between sample sets were identified. The genes with ≥2-fold difference in pair-wise comparisons among LH + 7 day and LH + 10 day in natural cycles and hCG + 7 day of excessive responders in stimulated cycles were selected.

All differentially expressed genes (≥2-fold and P < 0.05) were represented in a Venn diagram as up-regulated and down-regulated genes. Each differentially expressed gene was individually annotated with GeneCards terms (http://www.genecards.org/index.shtml) and GeneSpring Gene Ontology annotations for categorizing into functional groups. Principal component analysis (PCA) was performed to detect variable components in all 10 samples based on the normalized microarray data of all genes. The two principal variable components with highest values were selected to generate a two-dimension scatter plot to visualize the difference in gene expression profiles between samples.

Unsupervised clustering was employed to analyze the difference in gene expression profiles among 10 samples based on the normalized microarray data of all genes. Ten samples were clustered into subgroups according to their similarity in gene expression profile. Differentially expressed genes were separated and clustered into up-regulated and down-regulated groups.

Spheroids-endometrial cell attachment assay

Choriocarcinoma cells (JAr, ATCC HTB-144) and endometrial adenocarcinoma (Ishikawa, ECACC 99040201) were cultured at 37°C in a humidified atmosphere with 5% CO2. JAr cells were maintained in RPMI 1640 (Sigma), supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine and penicillin/streptomycin (100 U/ml and 0.1 mg/ml, Gibco). Minimal essential medium (MEM, Sigma) supplemented with 10% FBS, L-glutamine and penicillin/streptomycin was used for Ishikawa cells. Adhesion of the choriocarcinoma cells to endometrial cells was quantified using an adhesion assay as described (Hohn et al., 2000; Uchida et al., 2007) with modifications. Briefly, JAr cells were cultured in RPMI 1640 medium with or without 10 μM dibutyryl-cAMP (dbcAMP), 5 μM methotrexate (MTX), 0.1–10 μg/ml recombinant human DKK-1 (rhDKK-1) or 1 μg/ml bovine serum albumin (BSA) for 2 days. Anti-DKK1 antibody (5-fold excess) was used to neutralize the hDKK-1 protein at 4°C for 24 h before being used for JAr treatment. Multi-cellular spheroids were generated by shaking the trypsinized JAr cells at 4 g for 24 h. The spheroids were transferred onto the surface of a confluent monolayer of an endometrial Ishikawa cell line. The cultures were maintained in MEM medium with supplements for 1 h at 37°C in a humidified atmosphere with 5% CO2. Non-adherent spheroids were removed by centrifugation at 10 g for 10 min. Attached spheroids were counted under a light microscope, and expressed as the percentage of the total number of spheroids used (% adhesion). Photographs of cultures were taken with a Nikon Eclipse TE300 inverted microscope (Nikon, Japan).

Western blotting

Proteins from the spheroids and Ishikawa cells were extracted with 1 ml RIPA buffer [1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing protease inhibitor and the mixtures were then centrifuged at 10,000g for 10 min to remove the cell debris as described (Lee et al., 2008). The supernatants were collected and denatured at 95°C for 5 min in 1× SDS loading buffer. After boiling, the samples were resolved by electrophoresis on a 12% SDS-polyacrylamide gel and were subjected to western blotting. Antibodies against β-catenin (1:5000, BD Transduction Laboratory), glycogen synthase kinase-3 (GSK3-β, 1:1000, BD Transduction Laboratory) and β-actin (1:5000, Sigma) were obtained from different commercial sources. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:5000, GE Healthcare) was used, and specific signal was visualized by the enhanced chemiluminescence method.
Statistical analysis

All results were expressed as means ± SEM. Statistical comparisons were performed by one way ANOVA followed by the Student–Newman–Keuls or Mann–Whitney U-test where appropriate. A probability of \( P < 0.05 \) was used to indicate significant difference.

Results

Changes in differentially expressed genes in menstrual cycle

The expression of AOX1, NNMT, GPx3 and PAEP was high in the LS phase of the menstrual cycle (Fig. 1A). On the other hand, the expression of SLC26A2, MMP26, SLC7A4 and PTGS1/COX1 was highest in the MS phase and decreased in the late-secretory phase (Fig. 1B). These observations suggest that ovarian stimulation advanced the gene expression profiles in patients with excessive ovarian responses.

Demographic data

The demographic data of the subjects are shown in Table I. No significant differences in age and duration of infertility were found between the three groups of subjects. The serum estradiol (E2) and progesterone concentrations in the stimulated group \([\text{hCG} + 7(E)]\) were significantly higher \( (P < 0.05) \) than that of LH + 7 and LH + 10 groups in the natural cycles.

Microarray analysis

The total RNA from 10 endometrial samples \([\text{LH} + 7, \text{LH} + 10 \text{ in natural cycles and hCG} + 7(E) \text{ in stimulated cycle}]\) were extracted and subjected to Affymetrix HG_U133A analysis. Gene clustering and PCA were used to group and generate the two-dimensional representations of the 10 samples based on their gene expression patterns (Fig. 2A and B). Interestingly, two hCG + 7(E) samples from the excessive responders and one LH + 10 sample from natural cycling women were clustered in one region, although the remaining one hCG + 7(E) sample and three other LH + 10 samples were clustered in another region. The three LH + 7 samples were grouped as a cluster distinct from the other samples (Fig. 2A), indicating that the endometrial gene expression profiles of hCG + 7(E) samples were more similar to the LH + 10 samples. Similar findings were observed when PCA was performed (Fig. 2B).

The number of genes that were differentially expressed in the endometrial samples among the three groups are summarized in the Venn diagrams (Fig. 2C and D). Altogether, 351 differentially expressed (259 up-regulated and 92 down-regulated) genes were identified. There were 146 up-regulated and 46 down-regulated genes identified in both hCG + 7(E) and LH + 10 samples when compared with the LH + 7 samples. The endometrial gene expression profiles in the LH + 10 and the hCG + 7(E) samples were very similar; only five up-regulated and three down-regulated genes were identified (Fig. 2C and D). Only three genes were found to be differentially up-regulated among the three groups of endometrial samples (Table II).

Differential expression of Wnt-signaling molecules in endometrial samples

Real-time RT–PCR analysis of DKK1, DKK2 and sFRP4 showed that DKK1 was up-regulated and DKK2 and sFRP4 were down-regulated in the moderate \([\text{hCG} + 7-M, n = 15]\) and excessive \([\text{hCG} + 7(E), n = 17]\) responders of the stimulated cycle group when compared...
Table I Demographic data of the 38 subjects

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>Natural cycle LH + 7 (n = 11)</th>
<th>LH + 10 (n = 10)</th>
<th>Stimulated cycle hCG + 7 (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.9 ± 3.0 (27, 36)</td>
<td>35.0 ± 1.8 (33, 39)</td>
<td>32.6 ± 3.0 (27, 38)</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>6.3 ± 2.9 (3, 13)</td>
<td>5.9 ± 3.7 (2, 15)</td>
<td>5.1 ± 1.9 (2, 8)</td>
</tr>
<tr>
<td>Estradiol on LH/hCG day (pmol/l)</td>
<td>789 ± 315 (442, 1313)</td>
<td>524 ± 158 (257, 673)</td>
<td>13 222 ± 6833 (3536, 28 900)</td>
</tr>
<tr>
<td>Progesterone on LH+/hCG day 7 (nmol/l)</td>
<td>55.7 ± 26.2 (11.3, 88.6)</td>
<td>63.8 ± 19.6 (29.2, 89.6)</td>
<td>25.9 ± 6.9 (16, 45)</td>
</tr>
<tr>
<td>hMG dosage (ampoule)</td>
<td>NA</td>
<td>NA</td>
<td>128 ± 8.9 (0, 29)</td>
</tr>
<tr>
<td>hMG duration (day)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Number of oocytes aspirated</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Number of oocytes fertilized</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD (range), NA: not applicable; NS: not significant.
+– Denotes significant difference between groups.

A spheral-endometrial co-culture assay was used to study the effect of Wnt-signaling molecules on attachment of trophoblast cells to human endometrial cells. The expression of Wnt-signaling molecules in human endometrial samples was studied using real-time PCR (Fig. 4). DKK1 transcript was low in the proliferative phase, but increased significantly in the secretory phase of the cycle. Interestingly, the expression levels of DKK1, DKK2 and sFRP4 transcripts in LH+/hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7 and hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4).

In conclusion, the inhibition of spheroid attachment by rhDKK-1 protein and MTX was not mediated by an increase in GSK-3β activity, as the expression of DKK1, DKK2 and sFRP4 proteins in the human endometrium was not regulated by an increased GSK-3β expression. Instead, the expression levels of DKK1, DKK2 and sFRP4 proteins were studied using real-time PCR (Fig. 4). DKK1 transcript was low in the proliferative phase, but increased significantly in the secretory phase of the cycle. Interestingly, the expression levels of DKK1, DKK2 and sFRP4 transcripts in LH+7 (E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4). DKK1, DKK2 and sFRP4 transcripts in LH+7, hCG + 7(E) were similar (Fig. 4).
Discussion

Excessive ovarian stimulation in patients undergoing IVF treatment affects normal endometrial development and is manifested as asynchronous stromal and epithelial cell maturation (Basir et al., 2001). In line with our previous study, real-time PCR analysis confirmed advancement of gene expression patterns during the menstrual cycle following ovarian stimulation in the excessive responders. This was further confirmed by

Figure 2  Microarray analysis of human endometrial samples from LH + 7, LH + 10 and hCG + 7.

Normalized expression data of all genes in each microarray chip were used in the clustering and principal component analysis (PCA) analyses. (A) Hierarchical clustering analysis of 10 human endometrial samples taken from LH + 7, LH + 10 and hCG + 7 (excessive responders with serum estradiol >20,000 pmol/l on hCG day). The up-regulated (red) and down-regulated (green) genes of 10 endometrial samples were clustered into two major groups. (B) PCA was used to group samples with similar gene expression patterns. Two principal variables in the gene expression profile data were presented in a two-dimension system between the natural cycles (closed circle, LH + 7; closed circle LH + 10) and excessive responders (closed triangle, hCG + 7) of stimulated cycles. The percentages of variances for the PCA1 and PCA2 were 27.65 and 20.16%, respectively. (C and D) Venn diagram representation of differentially expressed genes in the three groups of samples. All differentially expressed genes (≥2-fold; one way analysis of variance (ANOVA), P < 0.05) in pair-wise comparisons among the natural cycles (LH + 7, LH + 10) and excessive responders (hCG + 7) of stimulated cycles were shown. Similar gene expression patterns found in multiple pair-wise comparisons are shown in the overlapping areas. Venn diagrams show the (C) 259 up-regulated and (D) 92 down-regulated genes among the three groups of samples.

Table II  Genes that are differentially expressed among three groups of samples (≥2-fold; one way analysis of variance, P< 0.05), assessed by microarray analysis

<table>
<thead>
<tr>
<th>Affymetrix code</th>
<th>Gene name</th>
<th>Fold change hCG + 7 versus LH + 7</th>
<th>Fold change LH + 10 versus LH + 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>220196_at</td>
<td>Mucin 16, cell surface associated/CA125</td>
<td>2.1</td>
<td>7.5</td>
</tr>
<tr>
<td>212992_at</td>
<td>AHNAK2 nucleoprotein 2/C14orf78</td>
<td>2.8</td>
<td>5.7</td>
</tr>
<tr>
<td>208161_s_at</td>
<td>ATP-binding cassette, sub-family C (CFTR/MRP)</td>
<td>2.5</td>
<td>5.1</td>
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</table>
similar endometrial gene expression profiles between LH + 10 and hCG + 7(E) samples by microarray analysis. We previously reported aberrant expression of Wnt-signaling molecules (Dickkopf homolog 1 (DKK1), DKK2 and secreted frizzled-related protein 4 (sFRP4)) in natural (LH + 7 and LH + 10), stimulated (hCG + 7) samples (moderate and excessive responders, M and E, respectively). The fold-change in the mRNA expression levels of these genes among natural cycles at LH + 7 (LH + 7, n = 15 and LH + 10, n = 10), and stimulated samples (moderate, n = 15 and excessive, n = 17) were studied. A value of one was set for the natural group (LH + 7). Representative western blotting of DKK1, DKK2 and sFRP4 proteins in the endometrial samples taken from natural cycle at LH + 7, LH + 10 and in the stimulated cycle at hCG + 7 are shown below. a–b denotes significant difference at $P < 0.05$. Data are mean ± SEM.

Patients receiving ovarian stimulation for IVF treatment have about 5% chance of developing OHSS (Delvigne and Rozenberg, 2002), which is manifested in terms of abdominal bloating, feeling of fullness, nausea and diarrhea. Our previous clinical study showed that excessive ovarian stimulation was also associated with a significant reduction in the pregnancy rate (Ng et al., 2000) and abnormal endometrial

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Figure 3 Quantitative RT–PCR and western blotting of differentially expressed genes in Wnt-signaling pathway.

Real-time RT–PCR experiments were performed to analyze the gene expression patterns of three differentially expressed Wnt-signaling molecules (Dickkopf homolog 1 (DKK1), DKK2 and secreted frizzled-related protein 4 (sFRP4)) in natural (LH + 7 and LH + 10), stimulated (hCG + 7) samples (moderate and excessive responders, M and E, respectively). The fold-change in the mRNA expression levels of these genes among natural cycles at LH + 7 (LH + 7, n = 15 and LH + 10, n = 10), and stimulated samples (moderate, n = 15 and excessive, n = 17) were studied. A value of one was set for the natural group (LH + 7). Representative western blotting of DKK1, DKK2 and sFRP4 proteins in the endometrial samples taken from natural cycle at LH + 7, LH + 10 and in the stimulated cycle at hCG + 7 are shown below. a–b denotes significant difference at $P < 0.05$. Data are mean ± SEM.

Figure 4 The expression Wnt-signaling molecules Dkk-1, Dkk-2, sFRP4, Wnt5B and FZD5 in menstrual cycle.

Real-time RT–PCR analysis was performed on human endometrial biopsies at early-/mid-proliferate phase (EPMP, n = 8), LP (n = 8), ES (n = 8), MS (n = 15), LS (n = 6). The fold-change (mean ± SEM) of the gene is relative to EPMP which was arbitrarily set to 1. a–b, a–c and b–c denotes significant difference at $P < 0.05$ by one way ANOVA.

Wnt-signaling molecules in endometrium

(DKK1) lowered the attachment rate of JAr spheroids on Ishikawa cells when compared with untreated or BSA control.
development (Basir et al., 2001). It is hypothesized that high serum estrogen and progesterone concentrations following excessive ovarian stimulation change the endometrial gene expression profiles (Liu et al., 2008) resulting in advancement of endometrial development, not conducive to embryo implantation. Table III summarizes recent microarray studies on human endometrial receptivity for natural and stimulated cycles. By using real-time RT–PCR, we demonstrated that the mRNA expression of four genes (AOX1, NNMT, GPx3 and PAEP) which were up-regulated in excessive responders were high only in the LS phase of a natural cycle, whereas mRNAs for another four genes that were down-regulated in excessive responders were high in the MS and low in the LS phase. These observations suggest that excessive ovarian stimulation advanced endometrial development, thereby probably shortening the implantation window for embryo attachment.

This hypothesis was further supported by microarray analysis of 10 endometrial samples using gene clustering and PCA analyses. Our data...
## Table III  Recent microarray studies on human endometrial gene expression profiles

<table>
<thead>
<tr>
<th>Study</th>
<th>First day (sample number)</th>
<th>Second day (sample number, drug used)</th>
<th>Fold change (P-value)</th>
<th>Gene</th>
<th>Microarray</th>
<th>Probe set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Up</td>
<td>Down</td>
<td></td>
</tr>
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<td>Natural cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carson et al. (2002)</td>
<td>LH + 2–4 (n = 3)</td>
<td>LH + 7–9 (n = 3)</td>
<td>≥2 (&lt;0.05)</td>
<td>323</td>
<td>370</td>
<td>HG-U95A</td>
</tr>
<tr>
<td>Kao et al. (2002)</td>
<td></td>
<td></td>
<td>≥2 (&lt;0.05)</td>
<td>156</td>
<td>377</td>
<td>HG-U95A</td>
</tr>
<tr>
<td>Riesewijk et al. (2003)</td>
<td></td>
<td></td>
<td>≥3 (n/a)</td>
<td>153</td>
<td>58</td>
<td>HG-U95A</td>
</tr>
<tr>
<td>Mirkin et al. (2005)</td>
<td>LH + 3 (n = 3)</td>
<td>LH + 8 (n = 5)</td>
<td>≥2 (n/a)</td>
<td>49</td>
<td>50</td>
<td>HG-U95A</td>
</tr>
<tr>
<td>Talbi et al. (2006)</td>
<td>Early Secretory phase (n = 3)</td>
<td>Mid-secretory phase (n = 8)</td>
<td>≥1.5 (&lt;0.05)</td>
<td>1415</td>
<td>1463</td>
<td>HG-U133 Plus 2.0</td>
</tr>
<tr>
<td>Haouzi et al. (2009b)</td>
<td>LH + 2 (n = 31)</td>
<td>LH + 7 (n = 31)</td>
<td>≥2 (0.05)</td>
<td>945</td>
<td>67</td>
<td>HG-U133 Plus 2.0</td>
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<tr>
<td>Tseng et al. (2009)</td>
<td>Mid-secretory phase (n = 28)</td>
<td>Late-secretory phase (n = 28)</td>
<td>n/a</td>
<td>126</td>
<td>n/a</td>
<td>HG-U133 Plus 2.0</td>
</tr>
<tr>
<td>Current study</td>
<td>LH + 7 (n = 4)</td>
<td>LH + 10 (n = 3)</td>
<td>≥2 (&lt;0.05)</td>
<td>182</td>
<td>70</td>
<td>HG-U133A</td>
</tr>
<tr>
<td>Natural versus stimulated cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mirkin et al. (2004)</td>
<td>LH + 8 (n = 5)</td>
<td>hCG + 9 (n = 5, Follistim/ganirelix/Gonal-F/cetrotrelax, 0.25 mg/day)</td>
<td>≤1.19 (n/a)</td>
<td>6</td>
<td>6</td>
<td>HG-U95Av2</td>
</tr>
<tr>
<td></td>
<td>LH + 8 (n = 5)</td>
<td>hCG + 9 (n = 5, Leuproide acetate, 0.25 ~ 0.5 mg/day)</td>
<td>≥1.2 (n/a)</td>
<td>5</td>
<td>1</td>
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<tr>
<td>Horcajadas et al. (2005)</td>
<td>LH + 7 (n = 14)</td>
<td>hCG + 7 (n = 5, Leuproide acetate, 1 mg/day)</td>
<td>≥3 (&lt;0.01)</td>
<td>281</td>
<td>277</td>
<td>HG-U133A</td>
</tr>
<tr>
<td>Simon et al. (2005)</td>
<td>LH + 7 (n = 14)</td>
<td>hCG + 7 (n = 4, Ganirelix, 0.25 mg/day)</td>
<td>≥2 (n/a)</td>
<td>22</td>
<td>69</td>
<td>HG-U133A</td>
</tr>
<tr>
<td></td>
<td>LH + 7 (n = 14)</td>
<td>hCG + 7 (n = 4, Ganirelix, 2 mg/day)</td>
<td>≥2 (n/a)</td>
<td>88</td>
<td>24</td>
<td>HG-U133A</td>
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<tr>
<td></td>
<td>LH + 7 (n = 14)</td>
<td>hCG + 7 (n = 4, buserelin, 0.6 mg/day)</td>
<td>≥2 (n/a)</td>
<td>22</td>
<td>100</td>
<td>HG-U133A</td>
</tr>
<tr>
<td>Macklon et al. (2008)</td>
<td>LH + 5 (n = 4)</td>
<td>hCG + 5 (n = 4, Orgalutran, 0.25 mg/day)</td>
<td>≥2 (&lt;0.05)</td>
<td>142</td>
<td>98</td>
<td>HG-U133 Plus 2.0</td>
</tr>
<tr>
<td>Horcajadas et al. (2008)</td>
<td>LH + 7 (n = 5)</td>
<td>hCG + 7 (n = 5, Leuproide acetate, 1 mg/day)</td>
<td>n/a (&lt;0.01)</td>
<td>69</td>
<td>73</td>
<td>HG-U133A</td>
</tr>
<tr>
<td>Liu et al. (2008)</td>
<td>LH + 7 (n = 5)</td>
<td>hCG + 7 (n = 8, buserelin, 0.6 mg/day)</td>
<td>≥2 (&lt;0.01)</td>
<td>249</td>
<td>161</td>
<td>HG-U133A</td>
</tr>
<tr>
<td>Haouzi et al. (2009a)</td>
<td>LH + 2 (n = 21)</td>
<td>hCG + 2 (n = 21, n/a)</td>
<td>≥2 (&lt;0.05)</td>
<td>321</td>
<td>4</td>
<td>HG-U133 Plus 2.0</td>
</tr>
<tr>
<td></td>
<td>LH + 7 (n = 21)</td>
<td>hCG + 5 (n = 21, n/a)</td>
<td>≥2 (&lt;0.05)</td>
<td>657</td>
<td>0</td>
<td>HG-U133A</td>
</tr>
<tr>
<td>Current study</td>
<td>LH + 10 (n = 4)</td>
<td>hCG + 7 (n = 3, buserelin 0.6 mg/day)</td>
<td>≥2 (&lt;0.05)</td>
<td>3</td>
<td>5</td>
<td>HG-U133A</td>
</tr>
</tbody>
</table>

EST: expressed sequence tag.
showed that the three hCG + 7(E) samples and the four LH + 10 samples were more closely related than the three LH + 7 samples, demonstrating that the endometrial gene expression patterns of hCG + 7(E) resembled that of the LS phase (LH + 10) in natural cycles, when the implantation window is about to be closed (Wilcox et al., 1999; Norwitz et al., 2001). Therefore, it is speculated that excessive ovarian stimulation advances endometrial development which leads to early closure of the implantation window and therefore a decrease in the pregnancy rate. In line with this speculation, endometrium taken on the day of oocyte retrieval after GnRH antagonist/recombinant FSH cycles showed an advanced maturation of 2–3 days (Noyes’ criteria) for patients with pregnancies (Van Vaerenbergh et al., 2009). On the other hand, a delay of gene expression was observed in endometria from leuprolide/HMG + FSH cycles and natural cycles. A delay of 2 days was found between samples collected on hCG + 7 and on LH + 7 (Horcajadas et al., 2008). The discrepancies among the results obtained from different studies could arise from differences in the stimulation protocols used (buserelin/HMG versus leuprolide/HMG + FSH) and in estrogen levels (≥20 000 pmol/l versus unselected) of the recruited subjects.

Our previous microarray analyses showed that DKK1 was up-regulated by >3-fold and DKK2 was down-regulated by >2-fold in excessive responders (Liu et al., 2008). These molecules regulate the canonical Wnt-signal pathway in Xenopus embryos (Wu et al., 2000), a pathway that is critical for estrogen-mediated uterine growth (Hou et al., 2004) and implantation in mice (Mohamed et al., 2005; Xie et al., 2008). The suppression of Wnt-signaling pathway results in accumulation of phosphorylated β-catenin by active GSK-3 and affects the implantation process (Mohamed et al., 2005).

DDK1 is up-regulated in the human endometrium during the implantation window in natural cycles (Kao et al., 2002; Tulac et al., 2003). Ovarian stimulation further increased DKK1, but suppressed DKK2 and sFRP4 expression in our hCG + 7(E) endometrial samples. The expression levels of these molecules were similar between hCG + 7(E) and LH + 10 samples, suggesting that dysregulation of Wnt-signaling molecules in hCG + 7 samples might contribute to a sub-optimal environment for implantation. In relation to this, the temporal changes of Wnt-signaling molecules (DKK1, DKK2, sFRP4, Wnt5B and FZD5) throughout the menstrual cycle confirmed our hypothesis that an advancement of endometrial gene expression patterns was found.

Since the expression of DKK1 transcript and protein were significantly reduced in the stimulated and LH + 10 samples, we further hypothesized that an aberrant increase of DKK1 expression might affect embryo attachment, an initial step of implantation. The hypothesis was tested in vitro by adding rhDKK1 in the JAR spheroids-Ishikawa cell attachment experiment (Hohn et al., 2000). The endometrial epithelial carcinoma Ishikawa cell line was selected as the model for receptive endometrium since the Ishikawa cells express various well known receptivity- and implantation-related molecules, such as integrins (Castelbaum et al., 1997), cell surface, extra cellular matrix and cell adhesion molecules (Hannan et al., 2009), as well as estrogen and progesterone receptors (Nishida et al., 1985, 1996; Lessey et al., 1996; Nishida, 2002) responsible for hormonal stimulation. The trophoblastic chorionicarcinoma cell line JAR displays cytotrophoblastic characteristics (Apps et al., 2009) and an ability to attach to the Ishikawa cells in vitro (Henewee, 2005).

It was found that DKK1 dose-dependently inhibited the attachment process and that this inhibitory effect could be abolished by anti-DKK1 antibody. Interestingly, DKK1 suppressed the attachment process associated with down-regulation of β-catenin expression in the spheroids but not the Ishikawa cells, but no observable changes in GSK-3β and β-actin protein were found. Although results from the present co-culture study support the importance of Wnt-signaling in implantation, extrapolating these results to the in vivo context should be treated with caution. In fact, this and other in vitro co-culture models using human embryos or endometrial samples have their limitations, including the limited availability of donated human embryos and variation in biological responses between patients’ samples (Teklenburg and Macklon, 2009). Yet, inactivation of nuclear Wnt-β-catenin signaling limits blastocyst competency for implantation (Xie et al., 2008; Chen et al., 2009). Moreover, DKK1 secreted from decidual cells plays an important role in trophoblast cell invasion (Peng et al., 2008). In line with this, migration of ectoplacental cones was inhibited when DKK1 was suppressed by anti-sense DKK1 oligonucleotide but stimulated by anti-sense β-catenin oligonucleotide (Peng et al., 2008).

Accumulating evidence suggests that Wnt-signaling plays a significant role during mouse preimplantation development (Lloyd et al., 2003; Mohamed et al., 2004; Kemp et al., 2005; Na et al., 2007) and blastocyst activation (De Vries et al., 2004; Mohamed et al., 2004). It has also been shown that activation of Wnt-signaling in 293T cells increases the secretion of DKK-1 into the medium (Niida et al., 2004), providing direct evidence for the presence of a feedback loop to regulate Wnt-signaling activation at the cellular level. However, to definitively establish the existence of such a feedback loop between endometrium and the embryo, further investigations are needed.

In summary, high serum E2 and/or progesterone concentrations affect the development and gene expression profile of peri-implantation endometrium in humans. Aberrant expression of Wnt-signaling molecules (e.g. DKK1) caused by high serum E2/progesterone levels may be associated with sub-optimal endometrial development, not conducive to embryo attachment in vivo. Further functional studies on the differentially expressed genes using primary endometrial cells and human embryos may provide valuable insights to our understanding of endometrial receptivity and embryo implantation in vivo.

Acknowledgements

We are also grateful to Dr Harshana Rambukwella, School of English, The University of Hong Kong for commenting on our manuscript.

Funding

This work was supported in part by grants from the Committee on Research and Conference Grant, The University of Hong Kong to K.F.L. and Hong Kong Research Grant Council to P.C.H. (HKU7514/05M).

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Submitted on June 29, 2009; resubmitted on October 31, 2009; accepted on November 5, 2009