



Title	Excessive ovarian stimulation up-regulates the Wnt-signaling molecule DKK1 in human endometrium and may affect implantation: An in vitro co-culture study
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**Excessive Ovarian Stimulation up-regulates DKK1 of Wnt-signaling
Pathway in Human endometrium affects Implantation: An in vitro
Co-culture study**

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Short title: Wnt-signaling molecules in endometrium

Abbreviations: hCG, human chorionic gonadotrophin; HMG, human menopausal
gonadotrophin; IVF, in vitro fertilization; LH, luteinizing hormone.

Abstract

BACKGROUND: High serum estradiol levels following ovarian stimulation affects endometrial development and gene expression profiles leading to reduced implantation and pregnancy rates. Yet, the underlying molecular mechanism leading to these changes
5 remains unknown. Accumulating data suggested that aberrant expression of a number of genes in the Wnt-signaling pathway may be involved in these processes.

METHODS: In this study, microarray and real-time PCR analysis were performed to analyze the gene expression profile of endometrial samples taken at hCG+7 in stimulated cycles and LH+10 in the natural cycles and the expression of several Wnt-signaling
10 transcripts including DKK1, DKK2 and sFRP4 were analyzed throughout the menstrual cycle. The JAr spheroid-endometrial cells co-culture experiment was established to study the effect of DKK1 on spheroid attachment in vitro.

RESULTS: Endometrial samples taken at hCG+7 in stimulated cycle shared similar gene expression profiles at LH+10 in the natural cycle. The transcripts of several Wnt-signaling
15 molecules including DKK1, DKK2 and sFRP4 were aberrantly expressed in the stimulated cycles. Treatment of spheroid with recombinant human DKK-1 protein dose-dependently suppressed spheroid attachment onto the endometrial Ishikawa cell line. The suppressive effect of recombinant DKK-1 protein was nullified with the anti-DKK1 antibody; while the anti-DKK1 antibody alone had no effect on spheroid attachment. Furthermore, the decrease
20 in spheroid attachment was associated with down-regulation of β -catenin expression in the DKK-1 treated JAr cells.

CONCLUSION: High serum estradiol and/or progesterone concentrations advanced the gene expression profiles of peri-implantation endometrium in patients following ovarian stimulation. Aberrant expression of the Wnt-signaling molecule might impair embryo
25 attachment and implantation in vivo.

Introduction

In vitro fertilization (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 is a key factor leading to successful IVF treatment. Gonadotrophin releasing hormone agonists are commonly used during ovarian stimulation to prevent a premature luteinizing hormone (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 similar endometrial responses as 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 serum E₂ concentration on the day of hCG administration >20,000 pmol/L were associated with decreased implantation and pregnancy rates in IVF cycles (Ng et al., 2000). These excessive response cases have dys-synchronous development of the endometrium with delayed glandular maturation and advanced stromal morphology as determined by endometrial morphometric analysis (Basir et al., 2001). They also have a significantly higher endometrial vascularity on day 7 after injection of human chorionic gonadotrophin (hCG+7) (Ng et al., 2004) and a discrete mRNA expression profile (Liu et al., 2008) when compared to 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; Riesewijk 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 in most of these studies, indicating the complexity and variability of endometrial development (Horcajadas et al., 2004; Mirkin et al., 2005).

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 et al., 2003) which is up-regulated in the implantation window (Kao et al., 2002; Tulac et al., 2003). It 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 uterine horns 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). However, the effects of super-physiological concentration of the steroid hormone on endometrial development at molecular level remain

unknown. In this study, the expression of Wnt-signaling molecules (e.g. DKK1, DKK2 and 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 Dkk-1 on embryo attachment was analyzed using a spheroid-endometrial co-culture model.

Materials and Methods

Human Subjects

Thirty-seven infertile women (median = 33, range = 27 to 39 year) undergoing IVF
5 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 intra-uterine 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
10 two 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-proliferate phase (EPMP, n=8), late proliferate phase
15 (LP, n=8), early secretory phase (ES, n=8), middle secretory phase (MS, n=15) and late
secretory phase (LS, n=6). They had no significant differences ($p<0.05$) in terms of age and
duration of infertility. Informed written consent prior to participation in the study was
obtained, and was approved by the Institutional Review Board of the University of Hong
Kong and Hospital Authority Hong Kong, West Cluster.

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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 LH surge, which was defined as the day when the serum
25 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 gonadotrophin-releasing hormone 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) injection was administered after confirmation of pituitary down-regulation. Human chorionic gonadotrophin (Profasi, Serono, Geneva, Switzerland) 10,000 IU was administered when the leading follicle reached 18 mm in diameter and there were at least three follicles >15 mm in diameter. Serum E₂ concentration was measured on the day of the ovulatory hCG injection and the patients were classified into either moderate (serum E₂ ≤ 20,000 pmol/L) or excessive (serum E₂ > 20,000pmol/L) responders as described previously (Ng et al., 2000).

15 ***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 cDNA synthesis kit (Amersham, Uppsala, 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) as described (Lee et al., 2005). Primers were designed using Primer Premier v5.0 (Premier Biosoft International, Palo Alto, CA). 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 minutes; 40 cycles at 95°C for 15 seconds, 60°C for 35 seconds and 72°C for 45 seconds. The $2^{-\Delta\Delta CT}$ 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.

5 ***RNA Isolation and Affymetrix Microarray***

Total RNA from 38 samples were 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 7 endometrial samples from natural cycling women (LH+7, n=3; LH+10, n=4) and 3 endometrial samples on Day hCG+7 from women with excessive response (hCG+7(E), n=3) was checked by an Agilent 2100 bioanalyzer (Waldboronn, 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 GenChips HG_U133A microarray (Affymetrix Inc., Santa Clara, CA) which composed of 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

25 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
5 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 statistically analyzed by One Way ANOVA with the p-value set at 0.05 or less. Genes that were differentially expressed
10 ($p < 0.05$, 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- and down-regulated genes. Each differentially expressed gene was
15 individually annotated with GeneCards terms (<http://thr.cit.nih.gov/cards/index.shtml>) and GeneSpring Gene Ontology (GO) 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 largest values were selected to generate a two-dimension scatter plot to
20 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 the gene expression profile. Differentially expressed genes were separated and clustered into up-regulated and
25 down-regulated groups.

Western blotting

Proteins from the spheroid and Ishikawa cells were extracted with RIPA buffer (1 ml) containing protease inhibitor and the mixtures were then centrifuged at 10,000 g 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 1X SDS loading buffer. After boiling, the samples were resolved in 12% SDS-PAGE and were subjected to Western blotting. Antibodies against β -catenin (1:5000, BD Transduction Laboratory), GSK3- β (1:1000, BD Transduction Laboratory) and b-actin (1:5000, Sigma) were obtained from different commercial sources. Horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:5000, GE Healthcare) was used, and specific signal was visualized by the enhanced chemiluminescence (ECL) method.

Spheroid-endometrial cell attachment assay

Choriocarcinoma cell (JAR, ATCC HTB-144) and endometrial adenocarcinoma (Ishikawa, ECACC 99040201) were cultured at 37°C in a humid atmosphere with 5% CO₂. JAR cells were maintained in RPMI 1640 (Sigma), supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 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 μ M dibutyryl-cAMP (dbcAMP), 5 μ M methotrexate (MTX), 0.1-10 μ g/ml human recombinant DKK-1 (hrDKK-1) or 1 μ g/ml bovine serum albumin (BSA) for 2 days. Anti-DKK1 antibody (5-fold excess) was used to

neutralize the hrDKK-1 protein at 4°C for 24 hours before being used for JAr treatment. Multi-cellular spheroids were generated by shaking the trypsinized cells at 70 rpm for 24 hours. 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 hour at 37°C in a humidified atmosphere with 5% CO₂. Non-adherent spheroids were removed by centrifugation at low g-force (120 rpm) for 10 minutes. 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).

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Statistical analysis

All results were expressed as means \pm S.E.M. Statistical comparisons were performed by One Way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls or Mann-Whitney U test where appropriate. A probability of $p < 0.05$ was used to indicate a significant difference.

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Results

Changes of differentially expressed genes in menstrual cycle

Previously, we reported that excessive ovarian stimulation affected the expression of a number of genes in the implantation window when compared with the natural cycle. A number of genes were up-regulated such as AOX1, NNMT, GPx3 and PAEP/glycodelin; while others such as SLC26A2, MMP26, SLC7A4 and PTGS1/COX1 were down-regulated in the excessive responders of hCG+7 samples (hCG+7(E)) when compared with that in the LH+7 samples in the natural cycles. The temporal expressions of the 8 differentially expressed genes above were further evaluated in this study using endometrial samples taken from different phases (early-, mid- and late-proliferative and secretory phases) of natural cycles. The expressions of AOX1, NNMT, GPx3 and PAEP were high in the late-secretory (LS) phase of the menstrual cycle (Fig. 1A). On the other hand, the expressions of SLC26A2, MMP26, SLC7A4 and PTGS1/COX1 were high in the mid-secretory (MS) phase but 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

To confirm that excessive ovarian stimulation resulting in advanced endometrial gene expression, we compared the gene expression profiles of hCG+7(E) and LH+7 with that of LH+10. The demographic data of the subjects are shown in Table 1. No significant differences in age and duration of infertility were found between the three groups of subjects. The serum estradiol and progesterone concentrations in the stimulated group (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 (LH+7, LH+10 in natural cycles and hCG+7(E) in stimulated cycle) were extracted and subjected to Affymetrix HG_U133A analysis. Gene clustering and principal component analyses (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 together in one region, while 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 together 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 is 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 5 up-regulated and 3 down-regulated genes were identified (Fig. 2C and D). Only 3 genes were found to be differentially up-regulated among the three groups of endometrial samples (Table 2).

Differential expression of Wnt-signaling molecules in endometrial samples

We previously reported aberrant expression of Wnt-signaling molecules including DKK1, DKK2 and sFRP4 in the endometria of excessive responders in microarray analysis

(Liu et al., 2008). Real-time RT-PCR analysis of DKK1, DKK2 and sFRP4 confirmed that DKK1 was up-regulated and DKK2 and sFRP4 were down-regulated in the moderate (hCG+7-M, n=15) and excessive (hCG+7(E), n=17) responders of the stimulated cycle group when compared to patients in the natural (LH+7, n=15) group (Fig. 3). Interestingly, the expression levels of DKK1, DKK2 and sFRP4 transcripts in LH+10 and hCG+7(E) were similar (Fig. 3). The expression levels of the DKK1, DKK2 and sFRP4 proteins in the human endometria were studied by Western blotting. DKK1 showed an up-regulation in protein expression in the stimulated (M and E) and LH+10 samples, but not for DKK2 and sFRP4 proteins.

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Expression of Wnt-signaling molecules in endometrium in menstrual cycle

The expression of five Wnt-signaling molecules including DKK1, DKK2, sFRP4, Wnt5B and FZD5 in human endometrial samples taken on early-/mid-proliferative (EP/MP), late-proliferative (LP), early-secretory (ES), mid-secretory (MS) and late-secretory (LS) phases were studied using real-time PCR (Fig. 4). The expression of DKK1 transcript was low in the proliferative phase, but increased significantly in the late-secretory phase; while DKK2 transcript increased from the proliferative phase, peaked in the mid-secretory phase and decreased in the late-secretory phase. On the other hand, the expression of sFRP4 was high in the proliferative phase, but was significantly down-regulated in the secretory phase of the cycle. Interestingly, Wnt5B decreased from the proliferative phase to the mid-secretory phase but increased significantly in the late-secretory phase of the cycle. There was no significant change in the expression of FZD5 throughout the menstrual cycle.

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Wnt-signaling in Spheroid-endometrial cell attachment assay

A spheroid-endometrial co-culture assay was used to study the effect of

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Wnt-signaling molecules on attachment of trophoblast cells onto human endometrial cells in vitro. The spheroid was generated by shaking the trypsinized JAr cells at 70 rpm for 24 hrs at 37°C. JAr spheroid of diameters 60-300 µm were used for attachment of the JAr cells onto the endometrial Ishikawa cells (Figure 5A). To select the most effective chemicals that

5 inhibit spheroid attachment, we treated the JAr cells with 10 µM dbcAMP or 5 µM MTX before spheroid generation. It was found that both dbcAMP and MTX suppressed the attachment of the spheroid onto endometrial Ishikawa cells effectively (Figure 5B). Treatment of JAr with recombinant human DKK-1 protein (rhDKK-1, 0.1-10 µg/ml) dose-dependently suppressed spheroid attachment onto Ishikawa cells (Figure 5C). The

10 percentage of attachment decreased from 69.2% to 43.5% at 0.1 to 10 µg/ml DKK-1 protein used. The decrease in attachment was significantly different ($p < 0.05$) from the control at a DKK-1 concentration of 0.1 to 10 µg/ml. No significant change in attachment was observed when BSA was compared with the untreated control (82.8% vs 86.0 %, respectively). In all the treatment groups, the average viability of JAr cells was 88-95% as

15 determined by Trypan Blue staining (data not shown).

The specific action of recombinant DKK-1 protein was further confirmed with anti-DKK1 antibody in the co-culture experiment. Anti-DKK1 antibody (5-fold molar excess) successfully neutralized the inhibitory effect of rhDKK-1 protein from 64.1% to 87.6% attachment rate of JAr spheroid (Figure 5D). The anti-DKK1 antibody alone had no

20 effect on JAr spheroid attachment when compared with the untreated control (93.0% vs 95.7%). It was observed that DKK-1 down-regulates β -catenin expression in the JAr spheroid (Fig. 5E), but not the Ishikawa cells (data not shown). Interestingly, this inhibition was not mediated by an increased GSK-3 β expression in the spheroid after DKK-1 treatment.

Discussion

Excessive ovarian stimulation in patients undergoing IVF treatment affects normal endometrial development and manifests 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 following ovarian stimulation in the excessive responders. This was further confirmed by similar endometrial gene expression profiles between LH+10 and hCG+7(E) samples by microarray analysis. Our previous study suggested that aberrant expression of Wnt-signaling molecules in hCG+7 samples (Liu et al., 2008) may alter the development of the endometrium and thereby lower the receptivity of patients. This in vitro functional study using a spheroid-endometrial cell co-culture model demonstrates that spheroid treated with a Wnt-signaling molecule (DKK1) lowered the attachment rate of JAr cells on Ishikawa cells when compared with untreated or BSA control.

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 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 3 summarizes recent microarray studies on human endometrial receptivity between natural and stimulated cycles. By using real-time RT-PCR, we demonstrated that the mRNA expression of 4 genes (AOX1, NNMT, GPx3 and PAEP) up-regulated in excessive responders were high only in the late-secretory phase of a natural cycle, whereas that of another 4 genes down-regulated in excessive responders were low in

the late-secretory phase. These observations suggest that excessive ovarian stimulation advanced endometrial development and shortened 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 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 late-secretory 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 the speculation, endometrium taken on the day of oocyte retrieval after GnRH antagonist/rec-FSH cycles showed an advanced maturation for 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 discrepancy of the results obtained from different studies could be due to differences in the stimulation protocols used (buserelin/HMG vs leuprolide/HMG+FSH) and in estrogen levels (≥ 20000 pmol/L vs 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). This pathway 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 glycogen synthase kinase-3 and affects the implantation process (Mohamed et al., 2005).

DKK1 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 expressions 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 dys-regulation 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 by adding recombinant DKK1 in the JAr/spheroid-Ishikawa cell attachment experiment (Hohn et al., 2000). The endometrial epithelial carcinoma cell line Ishikawa was selected as the model for receptive endometrium since the Ishikawa cells express various well known receptivity- and implantation-related molecules like integrins (Castelbaum et al., 1997), cell surface, extra cellular matrix (ECM), and cell adhesion molecules (Hannan et al., in press), as well as estrogen and progesterone receptors (Nishida et al., 1985; Lessey et al., 1996; Nishida et al., 1996; Nishida, 2002) responsible for hormonal stimulation. The trophoblastic choriocarcinoma cell line JAr displays cytotrophoblastic characteristics (Apps et al., 2009) and an ability to attach to the Ishikawa cells in vitro (Heneweer et al. 2005).

It was found that DKK1 dose-dependently inhibited the attachment process and that this inhibitory effect could be neutralized by anti-DKK1 antibody. Interestingly, DKK1

suppressed the attachment process associated with down-regulation of β -catenin expression in the spheroid but not the Ishikawa cells. But, no observable changes in GSK-3 β and β -actin expression were found. Although results from the present co-culture study support the importance of Wnt-signaling on 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 & 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. In line with this, ectoplacental cones migration 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 suggest that Wnt-signaling plays a significant role during mouse pre-implantation development (Kemp et al., 2005, Lloyd et al., 2003, Mohamed et al., 2004, Na et al., 2007) and blastocyst activation (Mohamed et al., 2004; De Vries et al., 2004). It has also been showed that activation of Wnt-signaling in 293T cells itself increases the secretion of DKK-1 (Niida et al., 2004) to the medium providing a direct evidence for the presence of a feedback loop to regulate the Wnt- signaling activation at the cellular level. However, to definitively establish the existence of such feedback loop in endometrium and embryo further investigations are needed.

In summary, high serum estradiol and/or progesterone concentrations affect the development and the gene expression profiles of peri-implantation endometrium in humans. Aberrant expression of Wnt-signaling molecules (e.g. DKK1) caused by high serum estradiol/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.

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Figure Legend

Figure 1

Quantitative RT-PCR of differentially expressed genes in menstrual cycle. (A) Real-time RT-PCR analysis of AOX1, NNMT, GPx3 and PAEP/glycodelin genes which were up-regulated in hCG+7 samples and (B) real-time RT-PCR analysis of Slc26A2, MMP26, SLC7A4 and PTGS1/COX1 genes which were down-regulated in hCG+7 samples when compared with LH+7 samples. The fold-change (mean \pm S.E.M.) in the expression levels of these genes among the menstrual cycle at early proliferative (EP), mid-proliferative (MP), late-proliferative (LP), early-secretory (ES), mid-secretory (MS) and late-secretory (LS) were determined (n=3 to 5 samples).

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 PCA analyses. (A) Hierarchical clustering analysis of 10 human endometrial samples taken from LH+7, LH+10 and hCG+7 (excessive responders with serum E2 > 20,000pmol/L on hCG day). The up-regulated (red) and down-regulated (green) genes (rows) of 10 endometrial samples (columns) were clustered into 2 major groups. (B) Principal component analysis (PCA) was used to group samples with similar gene expression patterns. Two principal variables in the gene expression profile data were presented in a 2-dimension system between the natural cycles (\bullet , LH+7; \circ LH+10) and excessive responders (\blacktriangle , 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 diagrams representation of differentially expressed genes in three groups of samples. All differentially expressed genes (≥ 2 -fold; one

way ANOVA, $p < 0.05$) in pair-wise comparisons among the natural cycles (LH+7, LH+10) and excessive responder (hCG+7) of stimulated cycles were shown. Similar gene expression patterns found in multiple pair-wise comparisons were shown in the overlapped areas. Venn diagrams showed the (C) 259 up-regulated and (D) 92 down-regulated genes among the 3 groups of samples.

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 (DKK1, DKK2 and 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 expression levels of these genes among natural cycle 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 were shown at the bottom. a-b denotes significant different at $p < 0.05$.

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, $n=8$), late-proliferate phase (LP, $n=8$), early-secretory phase (ES, $n=8$), mid-secretory phase (MS, $n=15$), late-secretory phase (LS, $n=6$). The fold-change (mean \pm S.E.M.) of the gene is relative to EPMP which was arbitrary

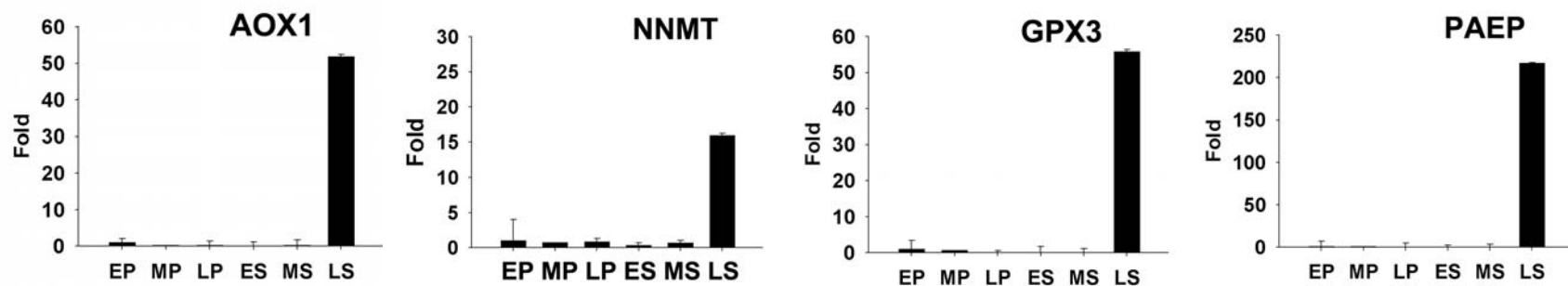
set to 1. a-b, a-c and b-c denotes statistically different at $p < 0.05$ by one way ANOVA.

Figure 5

Effect of DKK-1 treatment on *in vitro* attachment between JAr spheroids and Ishikawa cells. (A) Spheroids of 60-200 μ m in size were prepared from JAr cells for co-culture study (left), and a spheroid was attached to Ishikawa monolayer (right) after 1 hour of co-culture. Scale bar = 100 μ m. (B) Effect of 10 μ M dbcAMP and 5 μ M MTX on the attachment of spheroid onto Ishikawa cells. (C) Effect of DKK-1 recombinant protein and BSA on the attachment of JAr spheroid onto endometrial Ishikawa cells. The results were from 3 or more pooled experiments and the total number of spheroid used is 1022. DKK-1 dose-dependently suppressed JAr spheroid attachment from 0.1-10 μ g/ml. BSA at 1 μ g/ml did not suppress spheroid attachment under the same culture condition. (D) Neutralization effect of DKK-1 antibody on JAr spheroid attachment. DKK-1 antibody neutralized the suppressive effect of rhDKK-1 on spheroid attachment study. Different letter denotes significantly different ($p < 0.05$) from the control by Mann-Whitney test. (E) Western blotting of JAr cells treated with DKK-1. The expression of β -catenin, GSK-3 β and β -actin proteins were shown.

Figure 1

A



B

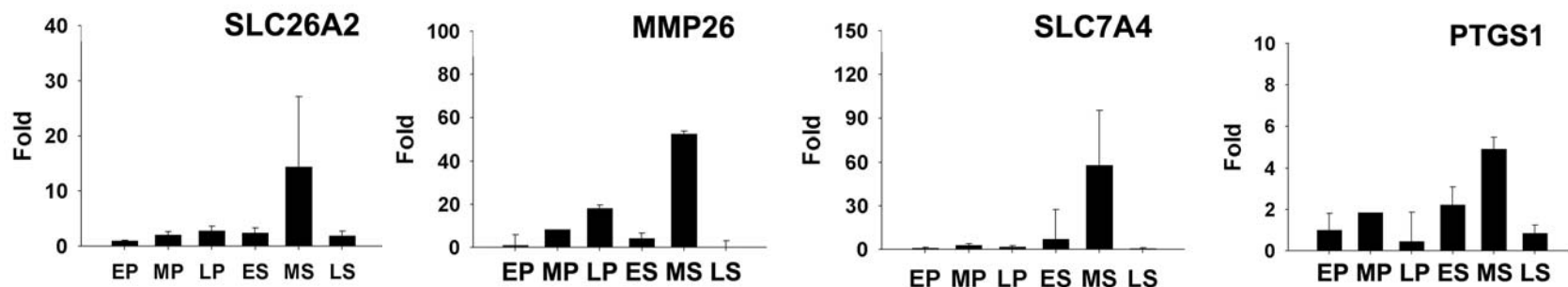
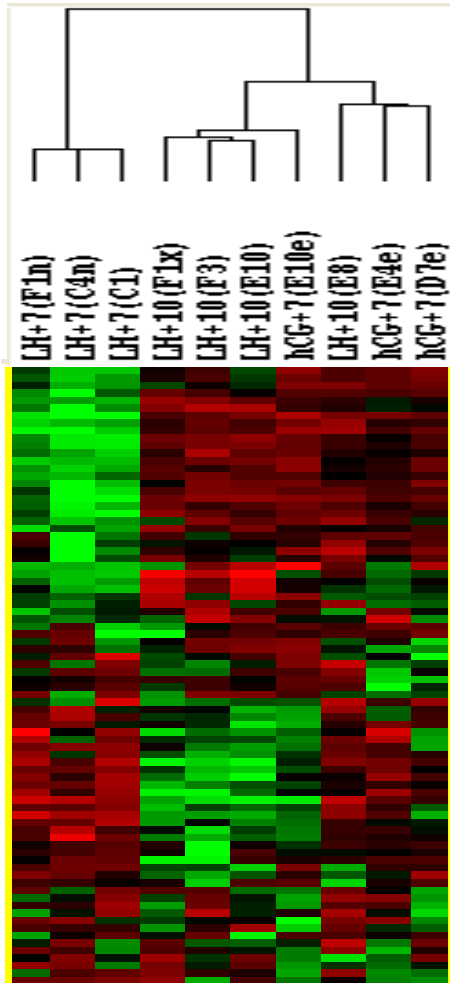
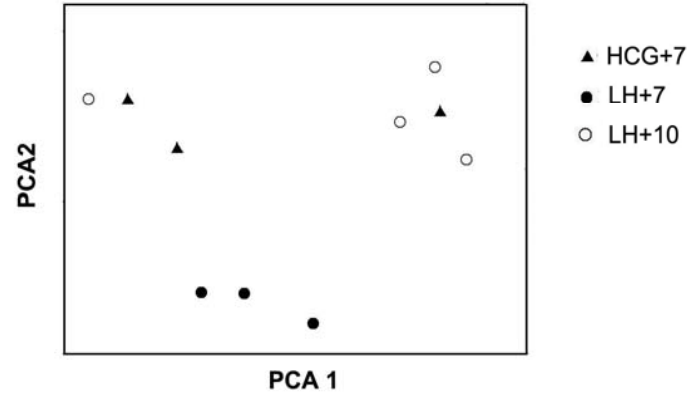


Figure 2

A



B



C



D

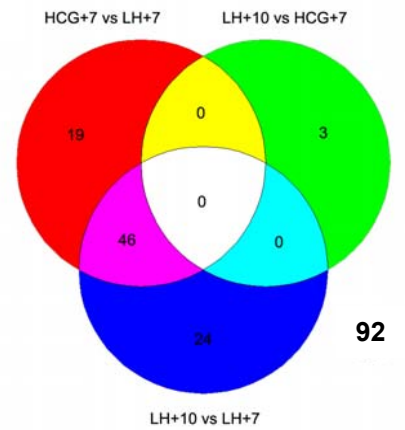


Figure 3

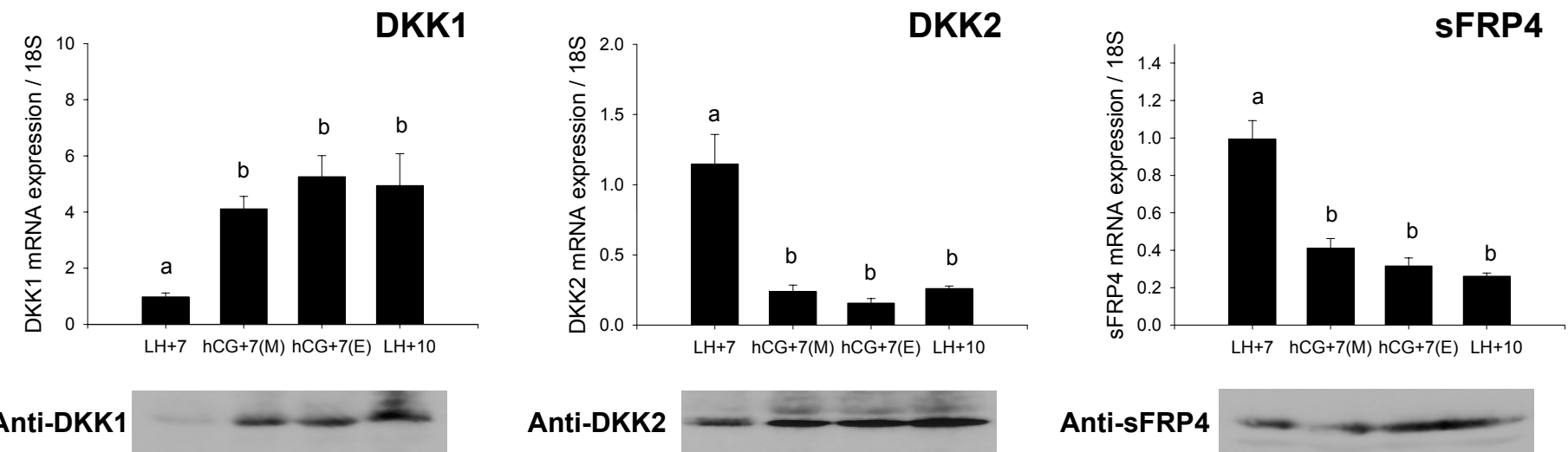


Figure 4

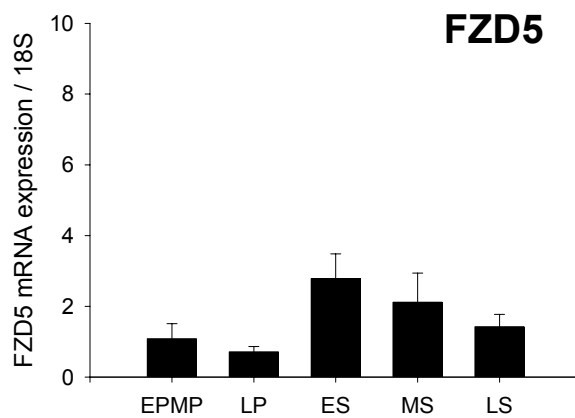
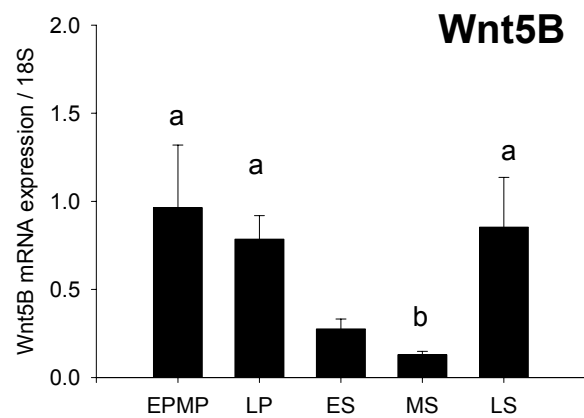
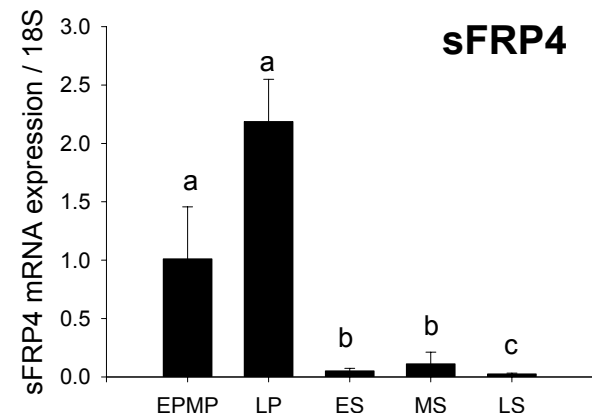
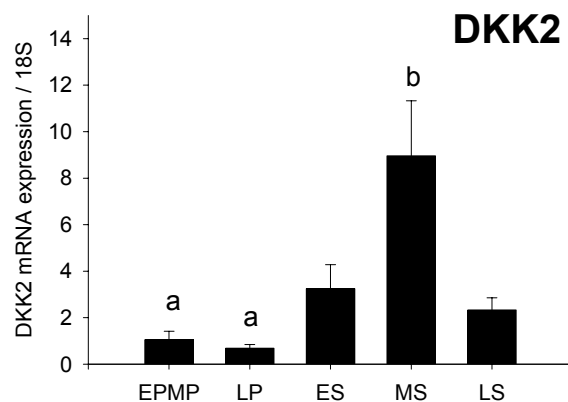
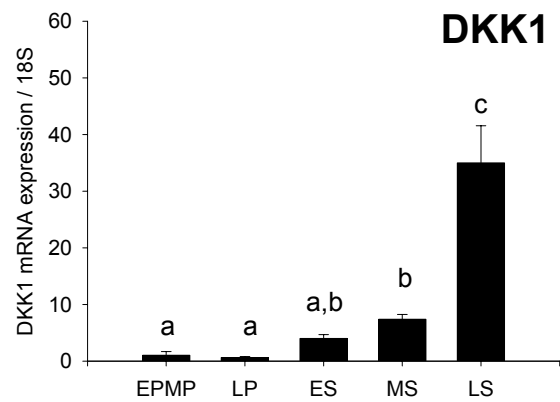


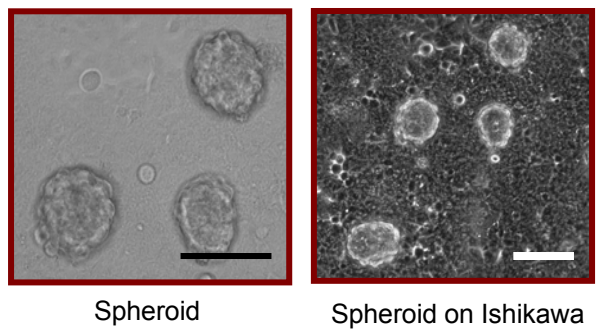
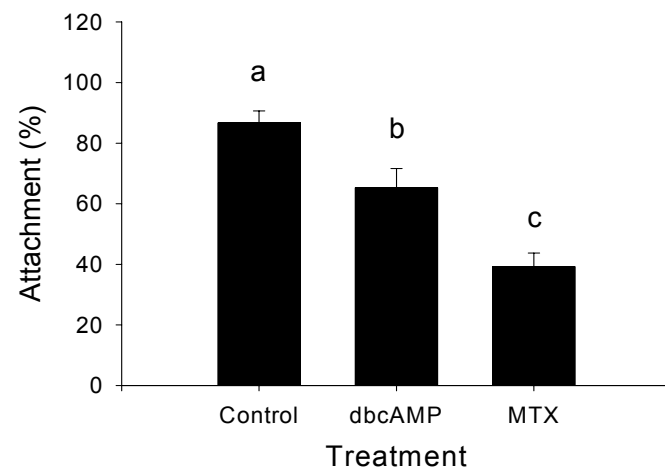
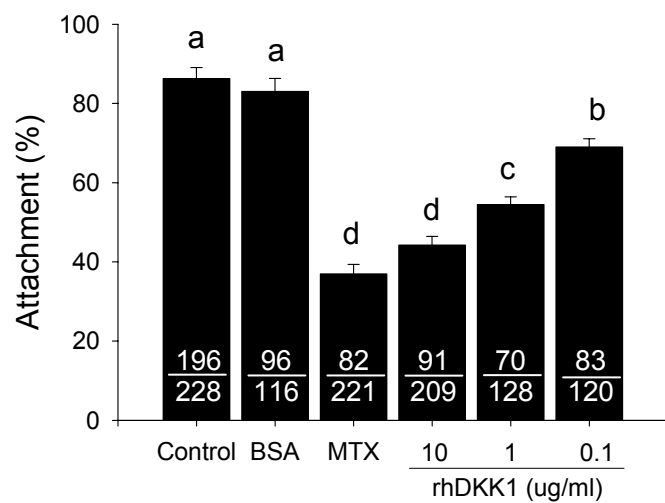
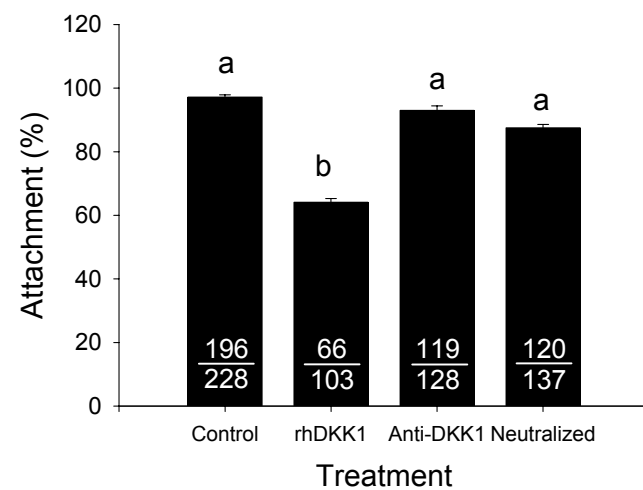
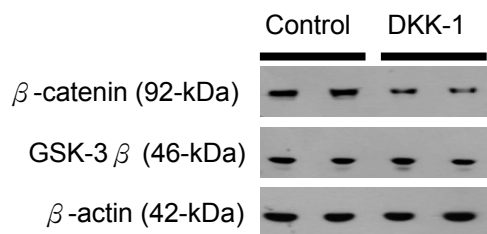
Figure 5**A****B****C****D****E**

TABLE 1 Demographic data of the 37 subjects

Demographic data	Natural cycle		Stimulated cycle	p-value
	LH+7 (n=11)	LH+10 (n=10)	hCG+7 (n=17)	
Age	31.9 ± 3.0 (27, 36)	35.0 ± 1.8 (33, 39)	32.6 ± 3.0 (27, 38)	NS
Duration of infertility (Years)	6.3 ± 2.9 (3, 13)	5.9 ± 3.7 (2, 15)	5.1 ± 1.9 (2, 8)	NS
10 Estradiol on LH/hCG day (pmol/L)	789 ± 315 ^a (442, 1313)	1060 ± 317 ^a (405, 1483)	28367 ± 10096 ^b (20600, 61608)	p<0.001
Estradiol on LH+7/+10 or hCG+7day (pmol/L)	499 ± 266 ^a (263, 1046)	524 ± 158 ^a (257, 673)	13222 ± 6833 ^b (3536, 28900)	p<0.001
15 Progesterone on LH +7/+10 or hCG+7 day (nmol/L)	55.7 ± 26.2 ^a (11.3, 88.6)	63.8 ± 19.6 ^a (29.2, 89.6)	792.8 ± 404.5 ^b (184.8, 1161.3)	p<0.001
HMG dosage (ampoule)	NA	NA	25.9 ± 6.9 (16, 45)	NA
HMG duration (day)	NA	NA	9.8 ± 1.5 (7, 13)	NA
20 Number of oocytes aspirated	NA	NA	23.9 ± 9.6 (10, 46)	NA
Number of oocytes fertilized	NA	NA	12.8 ± 8.9 (0, 29)	NA

Values are given as mean ± SD (range), NA: Not applicable; NS: Not significant.

25 ^{a-b} denotes significant difference between groups.

TABLE 2 Genes that differentially expressed between three groups of samples (≥ 2 -fold; one way ANOVA, $p < 0.05$) by microarray analysis.

Code	Gene Name	Fold change	
		hCG+7 vs LH+7	LH+10 vs LH+7
Differentially up-regulated genes in 3 groups			
220196_at	Mucin 16, cell surface associated / CA125	2.1	7.5
212992_at	AHNAK2 nucleoprotein 2 / C14orf78	2.8	5.7
208161_s_at	ATP-binding cassette, sub-family C (CFTR/MRP)	2.5	5.1

- 5 hCG+7: hCG+7 day in the stimulated cycle;
 LH+7: LH+7 day in the natural cycle;
 LH+10: LH+10 day in the natural cycle.

TABLE 3 Recent microarray studies on human endometrial gene expression profiles

Study	First Day (Sample number)	Second Day (Sample number, Drug used)	Fold change (p-value)	Gene		Microarray	Probe set
				Up	Down		
Natural Cycle							
Carson et al., 2002	LH+2~4 (n=3)	LH+7~9 (n=3)	≥ 2 (<0.05)	323	370	HG-U95A	12,686 human genes and ESTs
Kao et al., 2002	Proliferative phase (n=4)	LH+8~10 (n=7)	≥ 2 (<0.05)	156	377	HG-U95A	12,686 human genes and ESTs
Riesewijk et al., 2003	LH+2 (n=5)	LH+7 (n=5)	≥ 3 (n/a)	153	58	HG-U95A	12,686 human genes and ESTs
Mirkin et al., 2005	LH+3 (n=3)	LH+8 (n=5)	≥ 2 (n/a)	49	58	HG-U95A	12,686 human genes and ESTs
Talbi et al., 2006	Early Secretory phase (n=3)	Mid-secretory phase (n=8)	≥ 1.5 (<0.05)	1415	1463	HG-U133 Plus 2.0	>47,000 transcripts
Haouzi et al., 2009b	LH+2 (n=31)	LH+7 (n=31)	≥ 2 (0.05)	945	67	HG-U133 Plus 2.0	>47,000 transcripts
Tseng et al., 2009	Mid-secretory phase (n=28)	Late-secretory phase (n=28)	n/a	126	n/a	HG-U133 Plus 2.0	>47,000 transcripts
Current Study	LH+7 (n=4)	LH+10 (n=3)	≥ 2 (<0.05)	182	70	HG-U133A	>22,000 human DNA fragments
Natural vs Stimulated cycle							
Mirkin et al., 2004	LH+8 (n=5)	hCG+9 (n=5, Follistim/ganirelix/Gonal-F/cetrorelix, 0.25 mg/day)	≥ 1.19 (n/a)	6	6	HG-U95Av2	12,686 human genes and ESTs
	LH+8 (n=5)	hCG+9 (n=5, Leuprolide acetate, 0.25~0.5 mg/day)	≥ 1.2 (n/a)	5	1		
Horcajadas et al., 2005	LH+7 (n=14)	hCG+7 (n=5, Leuprolide acetate, 1mg/day)	≥ 3 (≤ 0.01)	281	277	HG-U133A	>22,000 human DNA fragments
Simon et al., 2005	LH+7 (n=14)	hCG+7 (n=4, Ganirelix, 0.25 mg/day)	≥ 2 (n/a)	22	69	HG-U133A	>22,000 human DNA fragments
	LH+7 (n=14)	hCG+7 (n=4, Ganirelix, 2 mg/day)	≥ 2 (n/a)	88	24		
	LH+7 (n=14)	hCG+7 (n=4, Buserelin, 0.6mg/day)	≥ 2 (n/a)	22	100		
Macklon et al., 2008	LH+5 (n=4)	hCG+5 (n=4, Orgalutran, 0.25mg/day)	≥ 2 (<0.05)	142	98	HG-U133 Plus 2.0	>47,000 transcripts
Horcajadas et al., 2008	LH+7 (n=5)	hCG+7 (n=5, Leuprolide acetate, 1mg/day)	n/a (<0.01)	69	73	HG-U133A	>22,000 human DNA fragments
Liu et al., 2008	LH+7 (n=5)	hCG+7 (n=8, Buserelin, 0.6mg/day)	≥ 2 (<0.01)	249	161	HG-U133A	>22,000 human DNA fragments
Haouzi et al., 2009a	LH+2 (n=21)	hCG+2 (n=21, n/a)	≥ 2 (<0.05)	321	4	HG-U133 Plus 2.0	>47,000 transcripts
	LH+7 (n=21)	hCG+5 (n=21, n/a)	≥ 2 (<0.05)	657	0		
Current Study	LH+10 (n=4)	hCG+7 (n=3, Buserelin 0.6mg/day)	≥ 2 (<0.05)	3	5	HG-U133A	>22,000 human DNA fragments