GnRH antagonist alters the migration of endometrial epithelial cells by reducing CKB

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Abstract

Some studies have demonstrated that the implantation rate of fresh transfer cycles is lower in the gonadotropin-releasing hormone antagonist (GnRH-ant) protocol than in the GnRH agonist (GnRH-a) protocol during *in vitro* fertilization (IVF). This effect may be related to endometrial receptivity. However, the mechanisms are unclear. Here, endometrial tissues obtained from the mid-secretory phase of patients treated with GnRH-a or GnRH-ant protocols and from patients on their natural cycle were assessed. Endometrial expression of B-type creatine kinase (CKB), which plays important roles in the implantation phase, was significantly reduced in the GnRH-ant group. At the same time, expression of the endometrial receptivity marker HOXA10 was considerably reduced in the GnRH-ant group. GnRH-ant exposure in endometrial epithelial cells (EECs) *in vitro* decreased CKB expression and ATP generation and blocked polymerization of actin. Furthermore, *in vitro* GnRH-ant-exposed Ishikawa cells showed enhanced F-actin depolymerization, and these effects were rescued by CKB overexpression. Similar effects were observed after CKB knockdown, and these effects were rescued by CKB overexpression. Moreover, cell migration was decreased in CKB-knockdown Ishikawa cells compared with that in control cells, and this effect was also rescued by CKB overexpression. Overall, these findings showed that GnRH-ant affected CKB expression in EECs, resulting in cytoskeletal damage and migration failure. These results provide insight into the roles and molecular mechanisms of GnRH-ant treatment in the endometrium.

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Introduction

Gonadotropin-releasing hormone antagonist (GnRH-ant) and GnRH agonist (GnRH-a) protocols are the two main clinical protocols for controlled ovarian stimulation (COS) in *in vitro* fertilization-embryo transfer (IVF-ET) (Devroey *et al.* 2009). Although still unclear, many studies and meta-analyses have suggested that the implantation and pregnancy rates of fresh transfer cycles are lower in the GnRH-ant protocol than in the GnRH-a protocol (Al-Inany *et al.* 2007, Orvieto & Patrizio 2013, Lambalk *et al.* 2017). In a previous study conducted at our center, the clinical pregnancy rate (PR) in the GnRH-ant protocol was about 20% less than that in the GnRH-a protocol (32 vs 51%) (Xu *et al.* 2018). Further investigations have suggested that this observation may be related to endometrial receptivity. For example, although top-quality embryo and blastocyst formation rates achieved

using the GnRH-ant protocol have been shown to be equivalent to those using the GnRH-a protocol, the overall pregnancy rate is reduced when using the GnRH-ant protocol (Bukulmez *et al.* 2008). Interestingly, this tendency is not observed when IVF-ET is performed using frozen embryos (Eftekhar *et al.* 2012). Similarly, a previously conducted donor-egg cycle analysis indicated that the recipient implantation rate (IR) and PR were not significantly different in patients receiving GnRH-ant- or GnRH-a-cycle-derived embryos (Prapas *et al.* 2005), and the results of mouse experiments have suggested that GnRH-ant can reduce the expression of endometrial receptivity markers, such as HOXA10, LIF, and ITGB3 (Ruan *et al.* 2006, Li *et al.* 2015). Immunohistochemical analysis of human endometrial samples also showed that HOXA10 protein expression decreased significantly during the window of implantation (WOI) in the GnRH-ant cycle (Rackow *et al.* 2008). Collectively, these

results suggested that the negative effects of GnRH-ant on pregnancy outcomes were likely to occur at the endometrial rather than the ovular or embryonic level (Hernandez 2000, Tarlatzis & Bili 2003) and that GnRH-ant may adversely affect endometrial function and subsequently prevent embryo implantation; however, the specific mechanism is still unclear.

Key biological mechanisms, including energy metabolism and cytoskeleton remodeling, underlie human endometrial receptivity (Chen *et al.* 2015). Additionally, dynamic changes in the endometrium, which occur during the menstrual cycle, have high metabolic demand (Kim & Moley 2009). Previous studies demonstrated that the 'brain' or 'B'-type creatine kinase (CKB) is upregulated dramatically during the receptive phase (Chen *et al.* 2015), thereby mediating energy metabolism. The important role of CKB in metabolite regulation has been demonstrated in malignant cells (Loo *et al.* 2015). Creatine is phosphorylated by CKB, yielding phosphocreatine, which acts as a metabolic kinase to promote cancer progression. However, few reports have described the role of CKB in endometrial receptivity.

Further, GnRH-ant may adversely affect endometrial function by regulating the expression of CKB, and this may be the potential mechanism underlying its antagonist effects. Accordingly, the present study investigated CKB expression and ATP changes in the mid-secretory phase endometrium in IVF cycles to provide insight into defects that could contribute to implantation failure.

Materials and methods

Patients

This study was conducted in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all patients for their participation in this study, which was approved by the Institutional Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine (no. 2012-57). Women aged 25–32 years who received IVF treatment for tubal obstruction at the Reproductive Medical Center of Ruijin Hospital and who gave up fresh embryo transfers owing to personal reasons were recruited. The inclusion and exclusion criteria were described previously (Chen *et al.* 2015). Furthermore, all the women included in these analyses had given birth to live babies after subsequent frozen embryo transfer (FET) treatments with natural cycle.

Study design and groups

Patients were classified into three groups. In the first group (GnRH-ant group), patients received controlled ovarian stimulation via administration of 150–225 IU/day of recombinant follicle-stimulating hormone (rFSH; Gonaf-F; Merck Serono) beginning on the second day of their menstrual cycle when their estradiol (E_2) level was less than 80 pg/mL and no ultrasound follicles with a maximum diameter of greater than 10 mm were detected. After 5 days of stimulation,

or when at least one follicle with a diameter of greater than or equal to 14 mm was visualized, patients were subcutaneously administered 0.25 mg/day GnRH antagonist (Cetrotide; Merck Serono) until human chorionic gonadotropin (hCG)-mediated ovulation induction. The second group (GnRH-a group) included patients whose pituitary function was reduced via s.c. administration of 0.1 mg/day GnRH-a (Decapeptyl; Ferring, Kiel, Germany) during the middle luteal phase of their preceding cycle. The above described rFSH injections were initiated once patient pituitary function was confirmed to be suppressed (as indicated by E_2 levels of less than 50 pg/mL, with no cysts or ultrasound follicles greater than 10 mm) and continued until the day of hCG-mediated ovulation induction.

The follicular growth in patients in the GnRH-ant and GnRH-a groups was evaluated using transvaginal ultrasound, and plasma E_2 , LH, and progesterone levels were measured using a commercially available immunoassay kit (Beckman-Coulter, CA, USA) to allow adjustment of the initial ovulation-stimulation dose of rFSH. Specifically, 5000 IU hCG (LiZhu, Zhuhai, China) was administered to patients upon detection of at least two follicles with a diameter greater than 18 mm, and oocyte retrieval was performed 36 h later by transvaginal ultrasound-guided follicle aspiration. Patients who elected to have all embryos cryopreserved for personal reasons were included in this study. These patients received luteal support with progesterone vaginal gel 600 mg/day (Crinone, Merck Serono) initiated on day 1 after oocyte retrieval until endometrial collection (on day 7 after oocyte retrieval), similar to the regimen used for patients who underwent fresh-embryo-transfer.

Patients assigned to the control group were assessed during their natural cycles via transvaginal ultrasound scan every second day on days 7–9 of their menstrual cycle until detection of a dominant follicle with a diameter greater than 15 mm. Further, the patients underwent daily ultrasonography and plasma E_2 /LH/progesterone measurement. Follicular disappearance and fluid appearance in the posterior *cul-de-sac* were confirmed by ultrasonography. Ovulation was also confirmed with the rise in LH, drop in E_2 , and subsequent rise in progesterone levels ($P > 6.0$ ng/mL) (Prior *et al.* 2015). Endometrial collection was performed on the day 7 after ovulation.

Human endometrial tissue biopsy

Human endometrial tissue samples were obtained during the mid-secretory phase, on the seventh day after ovulation ($n=6$) or oocyte retrieval (GnRH-ant: $n=6$, GnRH-a: $n=6$), with single-use S-type endometrial biopsy tubes (TY-C3.1/30-1S; TianYi, Zhejiang, China). The samples were washed immediately in normal saline to completely remove residual blood and were divided into three parts. The first part was placed into 10% buffered formalin for paraffin embedding, the second was used for endometrial epithelial cell (EEC) isolation and actin polymerization assays, and the third was placed in liquid nitrogen until further use.

Immunohistochemical analysis

Paraffin-embedded sections were deparaffinized with xylene before being rehydrated via exposure to an ethanol series.

Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in methanol, and the sections were then incubated overnight with anti-CKB or anti-HOXA10 antibodies (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article) in a humidified chamber at 4°C. Secondary biotinylated antibodies and peroxidase-conjugated streptavidin were used in accordance with the manufacturer's instructions, and diaminobenzidine was used to produce a brown-colored deposit.

Protein extraction and western blotting

Endometrial tissue samples were lysed using RIPA lysis buffer (Thermo Fisher Scientific), treated with protease inhibitors, homogenized on ice, and centrifuged (12,000g, 10 min, 4°C) to facilitate protein collection. The extracted protein samples (40 µg/well) were then separated via SDS-PAGE using 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked (1 h, room temperature) with Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 and 5% non-fat milk and immunoblotted (overnight, 4°C) with the primary antibody (Supplementary Table 1). After washing with TBS, the membranes were incubated (1 h, room temperature) with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; product no. 7074; Cell Signaling Technology). The resulting signals were detected via enhanced chemiluminescence (Millipore), according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Primary cell culture and treatment

EECs were isolated from endometrial curettage samples ($n=6$) collected using standard protocols following a hysteroscopic tubal patency assessment. EECs were then isolated from the collected cells according to a previously described protocol with some modifications (Zhang *et al.* 2012). Fresh endometrial tissues were minced prior to digestion via incubation with shaking for 1 h at 37°C in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 0.25% type II collagenase (Gibco). The dispersed endometrial cells were then separated via filtration through a 40-µm nylon cell strainer. Unfiltered glands were rinsed with 30 mL PBS and removed from the strainer. After centrifugation, EECs were initially seeded in 35-mm dishes in DMEM/F-12 medium and purified via transfer to new dishes. The cells were used after reaching 70–80% confluence. EECs were treated for 48 h with different concentrations (10^{-5} mol/L, 10^{-8} mol/L) (Öztürk *et al.* 2010, Britten *et al.* 2012) of GnRH-a (Decapeptyl; Ferring), GnRH-ant (Cetrotide; Serono), or a solvent (control). Cells were then collected for quantitative PCR (qPCR), Western blotting, and/or actin polymerization analysis.

Immunofluorescence colocalization

EEC climbing films were fixed in 4% paraformaldehyde for 20 min at 4°C. Slides were blocked (30 min) with 5% goat serum (Thermo Fisher Scientific) before being incubated overnight at

4°C with primary antibodies, including anti-GnRH-receptor (1:200) and anti-cytokeratin 7 (CK7) antibodies (1:200). After washing three times with PBS, the samples were incubated for 1 h at room temperature with appropriate fluorescently labeled secondary antibodies; 4',6-Diamidino-2-phenylindole (DAPI; 1:1000 dilution; product no. D9542; Sigma-Aldrich, Inc.) was employed to label cellular nuclei. The resulting fluorescence was analyzed using a laser-scanning confocal microscope (Carl Zeiss LSM-510).

Ishikawa cell culture, treatment, and transfection with overexpression plasmids

Ishikawa cells were seeded (0.5×10^5 /well) in 24-well plates, incubated overnight, and supplied with fresh DMEM/F-12 medium (without penicillin/streptomycin). Cells were then transfected with vectors with or without CKB-specific sequences (Genechem, Shanghai, China) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After 72 h of transfection, the cells were treated with different drugs for the indicated times and at the indicated concentrations.

Lentiviral silencing of CKB

Lentiviral plasmids containing shRNA sequences targeting CKB (CKB-sh) or negative control (NC) were produced by Hanbio Company (Shanghai, China) and their transfection was performed according to the manufacturer's instructions. Briefly, Ishikawa cells (30% confluence in six-well plates) were transfected with lentiviral plasmids. The culture medium was then replaced with transduction-enhancing solution containing lentivirus and 10 µg/mL polybrene. After 12 h, the medium was replaced with complete medium, and the cells were cultured for 72 h. The cells were then selected with 1 µg/mL puromycin/streptomycin and harvested for subsequent studies. The target sequences for CKB shRNA are listed in Table 1.

ATP assay

Total levels of cellular ATP were measured using a Luminescent ATP Detection Assay Kit (Abcam) according to the manufacturer's instructions. Briefly, EECs were seeded in 96-well plates, treated for 48 h with GnRH-a or GnRH-ant (final concentration 10^{-5} or 10^{-8} mol/L), and incubated (700 rpm, 5 min) with detergent (50 µL/well). After the addition of reconstitution substrate solution (50 µL/well), EECs were further incubated for 5 min with shaking at 700 rpm, and cellular ATP was measured using a luminescence spectrometer. Ishikawa cells transduced with negative or CKB knockdown lentivirus with or without CKB overexpression plasmid were evaluated to determine cellular ATP levels.

Table 1 Target sequences for CKB shRNA.

ShRNA	Sequence (5'–3')
CKB sh1	TCGACGACCACTTCCTCTTCGACAA
CKB sh2	CATGAGAAGTTCCTCGGAGGTGCTTA
CKB sh3	CCTACGAAGTGTCAAGGATCTCTT

Actin polymerization assay

EEC or Ishikawa cell monolayers treated with or without specified stimuli were fixed with 4% formaldehyde, scraped, and loaded into 96-well plates. The cells were then permeabilized (5 min) with 0.5% Triton X-100 and stained with Phalloidin (1:1000, 1 h, room temperature; Sigma). The mean F-actin staining fluorescence intensity was analyzed using a laser-scanning confocal microscope (Carl Zeiss LSM-510).

qPCR

Total RNA was extracted from cultured Ishikawa cells using a spin column (RNeasy Mini Kit; Qiagen) according to the manufacturer's instructions, and reverse-transcription (RT) was conducted using RT Master Mix (Takara). qPCR was performed using an Applied Biosystems 7500 real-time PCR System (ABI, Singapore), SYBR Green Master Mix (Takara), and appropriate primers (Supplementary Table 2), according to the manufacturer's instructions. Generated data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Migration assay

For the wound-healing experiments, the transfected cells were seeded separately into six-well plates (1×10^6 cells/well) and cultured overnight. Once a monolayer of cells had formed, a 200- μ L pipette tip was used to scratch the cell layer. Cell debris was removed carefully with PBS, and cells were then cultured in serum-free medium to permit wound healing. Images were acquired at the same position under an inverted microscope at 0 or 24 h after scratching. Three independent experiments were conducted.

Cell adhesion assay

Cell adhesion was examined using *CKB* shRNA-transduced cells or cells transfected with a *CKB* overexpression plasmid. Ishikawa cells (1×10^6 cells) were seeded into six-well plates and cultured for 24 h. JAR cells were stained with PKH26 according to the manufacturer's protocol (Sigma) and then

added to confluent Ishikawa monolayers in RPMI-1640 medium. These cocultured cells were cultured at 37°C in an incubator for 1 h and subjected to vigorous shaking at 140 rpm for 5 min before refreshing the medium. Finally, cells were imaged using an Olympus BX53M fluorescence microscope (Olympus). The number of counted cells in 4–5 randomly chosen areas in each well was used for statistical analysis. The experiment was repeated three times.

Statistical analysis

Statistical analyses were conducted using SAS 8.2 software (SAS Institute Inc.), and all data were expressed as means $\pm$ standard errors of the means (S.E.M.s). Student's *t*-test was used to compare two groups. One-way ANOVA was used to compare three groups if normality (and homogeneity of variance) assumptions were satisfied, otherwise the Kruskal–Wallis test followed by Mann–Whitney *U* test was used for analyzing the data. Differences in *P* values of less than 0.05 were considered statistically significant.

Results

Demographic characteristics

Among the three groups, there were no significant differences in demographic characteristics. The average age, BMI, basal hormonal levels, and other parameters during COS of the antagonist and agonist groups were similar (Table 2).

CKB and *HOXA10* expression levels varied in endometrial tissues from patients in the different groups

CKB production and localization were examined in endometrial tissues by immunohistochemical and Western blot analyses. *CKB* protein was strongly localized to the apical side of EECs during the receptive phase. However, *CKB* protein levels were obviously

Table 2 Clinical parameters of the patients. Data are presented as mean $\pm$ s.d.

	Control group (n = 6)	GnRH-ant group (n = 6)	GnRH-a group (n = 6)	<i>P</i> value
Age (year)	29.5 $\pm$ 1.9	28.2 $\pm$ 1.7	29.7 $\pm$ 2.1	0.407
BMI	21.4 $\pm$ 1.2	21.2 $\pm$ 1.7	21.1 $\pm$ 1.6	0.914
basal FSH (mIU/mL)	6.4 $\pm$ 0.7	6.8 $\pm$ 0.9	6.7 $\pm$ 1.1	0.780
basal LH (mIU/mL)	5.4 $\pm$ 1.0	5.9 $\pm$ 1.1	6.0 $\pm$ 0.6	0.588
basal E ₂ (pg/mL)	43.8 $\pm$ 11.7	51.5 $\pm$ 8.7	47.3 $\pm$ 8.4	0.479
Baseline antral follicle count	–	9.7 $\pm$ 2.1	10.3 $\pm$ 1.4	0.570
Dosage of gonadotrophin (IU)	–	2187.5 $\pm$ 333.5	2287.5 $\pm$ 412.8	0.654
Duration of stimulation (days)	–	9.8 $\pm$ 0.69	10.8 $\pm$ 0.9	0.076
No. of oocytes retrieved	–	11.3 $\pm$ 2.4	12.2 $\pm$ 2.4	0.593
No. of mature follicles on the day of trigger (diameter >13 mm)	–	8.7 $\pm$ 1.5	9.7 $\pm$ 1.4	0.296
E ₂ on the day when hCG was injected (pg/mL)	–	4919.7 $\pm$ 699.6	4590.5 $\pm$ 417.2	0.387
P on the day when hCG was injected (ng/mL)	–	1.10 $\pm$ 0.21	1.08 $\pm$ 0.18	0.855
Thickness of endometrium before ovulation (mm)	8.8 $\pm$ 0.4	9.3 $\pm$ 0.9	9.0 $\pm$ 0.6	0.505

FSH, follicle-stimulating hormone; LH, luteinizing hormone; P, progesterone.

reduced in GnRH-ant-treated endometrial samples compared with those in natural and GnRH-a-treated samples (Fig. 1A, B and C). In addition, mRNA and protein levels of the molecular marker of endometrial receptivity HOXA10 in the analyzed endometrial tissues were assessed via qPCR, immunohistochemical staining, and Western blotting, respectively. Notably, HOXA10 mRNA and protein expression levels were significantly reduced in endometrium samples isolated from patients of the GnRH-a and GnRH-ant groups compared to those isolated from the control group. Furthermore,

HOXA10 mRNA and protein expression was lower in the GnRH-ant than in the GnRH-a group (Fig. 1D, E, F and G). Interestingly, EECs isolated from GnRH-ant-treated endometrial samples showed reduced F-actin expression (Supplementary Fig. 1).

GnRH-receptor expression on isolated EECs

CK7 is a molecular marker of human EECs. Colocalization analyses showed that GnRH receptor was expressed in human EECs (Fig. 2).

Figure 1 CKB and HOXA10 expression levels varied in endometrial tissues in patients from different groups. (A) Immunohistochemical staining showing CKB levels in the natural (control), GnRH-ant, and GnRH-a patient groups. Scale bar = 50 μ m. (B) Western blotting analysis of CKB expression levels. GAPDH (loading control) levels were used as a control for protein loading. (C) Densitometric measurement of CKB levels relative GAPDH expression. (D) Immunohistochemical staining of HOXA10 in the control, GnRH-ant, and GnRH-a patient groups. Scale bar = 50 μ m. (E) Western blotting analysis of HOXA10 expression levels. (F) Densitometric measure of HOXA10 levels after normalization to GAPDH. (G) Quantitative real-time PCR analysis of HOXA10 mRNA expression after being normalized to that of GAPDH. * P < 0.05, ** P < 0.01.

Figure 2 Immunofluorescence analysis showing CK7 and GnRH receptor localization in endometrial epithelial cells (EECs). Primary cultured EECs climbing films were stained for CK7 (red), GnRH receptor (green), and DAPI (blue). Scale bar=20 μ m.

GnRH-ant decreased CKB expression, ATP production, and actin polymerization in EECs

Cultured EECs were treated with two concentrations of GnRH-ant (10^{-8} and 10^{-5} mol/L) (Oztürk *et al.* 2010, Britten *et al.* 2012) to determine the dosage required to inhibit ATP generation. The results show that when EECs were exposed to the GnRH analog at 10^{-8} mol/L, there was little difference in ATP production (Supplementary Fig. 2). However, exposure of EECs to the GnRH analog at 10^{-5} mol/L significantly inhibited ATP generation, and this effect was notably greater in the GnRH-ant group than in the GnRH-a group (Fig. 3A). CKB mRNA and protein expression was decreased *in vitro* after GnRH-ant treatment (Fig. 3C and D). At the same time, F-actin levels were reduced, and cell appeared shrunken (Fig. 3B).

GnRH-ant decreased actin polymerization in vitro in Ishikawa cells, and overexpression of CKB rescued this effect

Ishikawa cells were used to examine the influence of GnRH-ant on F-actin. Cells shrank and exhibited reduced F-actin levels after treatment with the GnRH analogs. Notably, these morphological changes were more obvious after exposure to GnRH-ant (Fig. 4B). Subsequent transfection of the Ishikawa cells with a CKB overexpression vector led to a significant increase in CKB mRNA levels by 48 h after transfection (Fig. 4A) and alleviation of F-actin inhibition in the GnRH-ant group (Fig. 4B and C).

CKB knockdown/overexpression affected actin polymerization, ATP levels, and migration of Ishikawa cells

The effects of CKB knockdown and overexpression in Ishikawa were examined using RT-qPCR and Western blotting (Fig. 5A, B, C, D, E and F). Ishikawa cells shrank and exhibited reduced F-actin levels after CKB shRNA transfection; this effect was rescued by CKB

overexpression (Fig. 5G). CKB shRNA also significantly inhibited ATP generation, and this effect was rescued after transfection with a CKB overexpression plasmid (Fig. 5H). The fluorescence intensity of fluorescence-labeled JAR cells adherent on CKB-knockdown or -overexpression Ishikawa cells was not obviously changed (Fig. 5I and J). The migration rate was decreased in CKB shRNA-transduced Ishikawa cells compared with that in mock-transduced Ishikawa cells, and this effect could be rescued by CKB overexpression (Fig. 5K and L).

Discussion

CKB regulates the reservoir of rapidly mobilized high-energy phosphates in tissues such as the brain by reversibly catalyzing the transfer of the high-energy phosphate group between ATP and various phosphorylated molecules, yielding phosphocreatine (Wyss & Kaddurah-Daouk 2000). CKB levels are significantly increased in endometrial WOI (Parmar *et al.*

Figure 3 GnRH-ant decreased CKB expression, ATP production, and actin polymerization in EECs. (A) *In vitro* ATP levels in primary cultured EECs ($n=6$). (B) Confocal analysis of F-actin content in EECs *in vitro* (F-actin, red; DAPI, blue). Scale bar=20 μ m. (C) Western blotting analysis of *in vitro* CKB levels in EECs. (D) Densitometric measurement of CKB levels relative to GAPDH expression. * $P<0.05$, ** $P<0.01$.

Figure 4 GnRH-ant decreased actin polymerization *in vitro* in Ishikawa cells, and overexpression of CKB rescued this effect. (A) RT-qPCR analysis of the efficiency achieved during transfection of Ishikawa cells with CKB expression plasmids *in vitro*. (B) Confocal analysis of F-actin content in Ishikawa cells *in vitro* (F-actin, red and DAPI, blue). Scale bar = 20 μm . (C) Fluorescent intensity/cell = mean fluorescent intensity $\times$ area of the cell. * $P < 0.05$, ** $P < 0.01$.

2009, Chen *et al.* 2015), suggesting that CKB may be required to achieve the endometrial receptive state. These findings are supported by the observation that CKB is predominantly expressed on the luminal surface of EECs, which are the first uterine cells to contact the embryo. Correct embryo implantation is necessary for the success of a pregnancy and is related to metabolic changes in uterine cells, such as glycogen accumulation (Frolova & Moley 2011). From the late proliferative phase to the secretion phase, glycogen accumulates in human uterine glandular and luminal epithelial cells in a manner likely required to regulate energy homeostasis

during embryo implantation (Verma 1983, Cornillie *et al.* 1985, Spornitz 1992). Given that hypoxic conditions are maintained during normal embryonic development and endometrial differentiation (Hustin *et al.* 1990, Jauniaux *et al.* 2000), normal increases in CKB levels, similar to accumulated glycogen, during the WOI may represent an adaptive response to maintain the energy balance. Creatine kinase transforms phosphocreatine into ATP and creatine to meet increased cellular energy requirements in a low ATP state (Wallimann *et al.* 2011, Chen *et al.* 2013), allowing the endometrium to maintain its energy balance under hypoxic conditions occurring during early pregnancy. In this study, decreased CKB expression was found in biopsy tissues from the GnRH-ant group. In addition, expression of the endometrial receptivity marker HOXA10, whose expression is a prerequisite for endometrial receptivity (Bagot *et al.* 2000, Taylor 2000), was considerably reduced in the GnRH-ant group. Consistent with the present study, other studies have previously suggested that the GnRH-ant likely reduces HOXA10 expression during the endometrial WOI (Rackow *et al.* 2008, Li *et al.* 2015). Thus, the GnRH-ant reduces CKB expression, which may be related to insufficient endometrial receptivity. Subsequently, *in vitro* analyses revealed that treatment of EECs with GnRH analogs for 48 h at 10^{-5} mol/L inhibited ATP generation and that this effect was more obvious after treatment with GnRH-ant than with GnRH-a. Moreover, CKB expression in EECs decreased significantly after treatment with GnRH-ant. Therefore, the inhibition of ATP generation and reduced CKB levels observed in the present study suggested that GnRH-ant treatment may disrupt cellular energy balance during formation of the endometrial receptive state.

EECs isolated from GnRH-ant-treated endometrial samples showed reduced F-actin expression. In addition, *in vitro* analyses showed that GnRH-ant treatment reduced F-actin expression in both EECs and Ishikawa cells. The abnormal F-actin expression observed was suggestive of actin fiber depolymerization, which may directly affect the basic functions of cells. Interestingly, the GnRH-ant-induced depolymerization of F-actin was rescued partly via CKB overexpression, suggesting that the energy metabolism status is likely related to F-actin polymerization. Cytoskeletal polymerization and depolymerization processes require energy; thus, insufficient energy reserves may lead to imbalances in these processes. Most functions of EECs during WOI are closely related to changes in the cytoskeleton (Demir *et al.* 2002, Png & Murphy 2002, Hosie *et al.* 2008); thus, the presence of an abnormal cytoskeleton is expected to disrupt endometrial receptivity. Notably, in this study, GnRH-ant treatment was found to disrupt the cellular energy balance and directly induce cytoskeleton damage, which may further affect endometrial receptivity. Thus, endometrial receptivity is likely dependent on the maintenance of correct

Figure 5 CKB knockdown/overexpression affected actin polymerization, ATP levels, and migration in Ishikawa cells. (A, B and C) CKB expression levels after CKB silencing in Ishikawa cells using RT-qPCR and western blotting. (D, E and F) Ishikawa cells were transfected with control shRNA, CKB shRNA, or a combination of mock and CKB plasmids. CKB expression levels were detected using RT-qPCR and Western blotting. (G) F-actin content in control and treated Ishikawa cells. (H) *In vitro* ATP levels in control and treated Ishikawa cells. (I and J) Analysis of JAR spheroid adhesion on Ishikawa cells after CKB shRNA or CKB plasmid transfection. (K) Wound healing assay in Ishikawa cells transfected with negative shRNA, CKB shRNA, or mock and CKB plasmids. Wound width was measured at 0 and 24 h after wounding. (L) Histograms showing quantification of the results (wound closure). All data are presented as means $\pm$ S.E.M.S. * P < 0.05; ** P < 0.01; *** P < 0.001.

energy metabolism, necessitating normal cytoskeleton structure.

In this study, ATP generation was found to be reduced with CKB knockdown. Additionally, actin polymerization decreased, and cell migration also decreased. However, there were no significant changes in cell adhesion. The opposite results were observed when CKB was overexpressed. Accordingly, CKB was involved in the regulation of actin polymerization/depolymerization and could ultimately affect the migration of EECs. Importantly, many studies have shown that changes in the cytoskeleton can directly affect cell migration (Flamini *et al.* 2011, Fife *et al.* 2014, Tang & Gerlach 2017). Thus, the effects of the antagonists on changes in CKB and the cytoskeleton may lead to changes in epithelial cell migration.

CKB has been shown to have a role in cancer cell migration (Christofk *et al.* 2008, Dang *et al.* 2009, Cairns *et al.* 2011, Kaelin & McKnight 2013) and tumor progression. Indeed, combination therapy including CKB inhibition significantly suppresses colon cancer metastasis (Loo *et al.* 2015). Migration is also a key step during implantation. However, the role of CKB in embryo implantation has not been reported. During embryo implantation, blastocysts attach to and then invade the epithelium to enter the underlying stroma. During the early stage of this process, the EEC barrier is disrupted. To achieve embryonic penetration, EECs migrate away from the implantation site, and EEC migration and invasion can facilitate the restructuring of the disrupted endometrial epithelium. The embryo is then covered with epithelial cells, resulting in restoration of the EEC barrier (Cui *et al.* 2018). The dysfunction of epithelial migration affects endometrial receptivity. In this study, CKB silencing/overexpression was found to alter endometrial cell migration and invasion, further suggesting that CKB was closely related to the establishment of endometrial receptivity. GnRH-ant can lead to insufficient production of CKB and ATP, thereby affecting the migration of epithelial cells. This may be a fundamental reason for the decline in endometrial receptivity caused by GnRH-ant.

In peripheral blood circulation, endogenous GnRH levels were very low. Therefore, the biological effects of the combination of GnRH and GnRH receptor outside the hypophysis gland are extremely low (Ortmann & Diedrich 1999). However, the affinity of the GnRH-ant (Cetrorelix) to the GnRH receptor was 20 and 2 times higher than those of natural GnRH and GnRH-a (buserelin), respectively (Reissmann *et al.* 2000). Therefore, the GnRH-ant is more likely to combine with the GnRH receptor outside the hypophysis gland, including the endometrium (Ramakrishnapa *et al.* 2005). In this study, the identification of the GnRH receptor in EECs further confirmed this possibility. Several human malignant tumors, including cancers of the endometrium, ovary, breast, prostate, and urinary

bladder, express GnRH and its receptor as part of an autocrine system that regulates cell proliferation (Grundker *et al.* 2002). Various investigators have observed time- and dose-dependent growth inhibitory effects of GnRH-ant in cell lines derived from these cancers (Emons *et al.* 2003, Dondi *et al.* 2006). Although the precise mechanisms remain unclear, it is believed that the well-known signaling mechanisms present in pituitary gonadotrophs of the GnRH receptor are not involved in forwarding the anti-metastatic and antiproliferative effects of GnRH analogs in cancer cells. Instead, GnRH analogs may inhibit cancer cell metastasis and proliferation by reducing the synthesis of local growth factors in a dose-dependent manner (Grundker & Emons 2017). However, there are few reports assessing the effects of the GnRH-ant on normal uterine epithelial cells. Despite advancements in this study, the molecular mechanisms through which CKB reduced and inhibited endometrial cell migration in women undergoing the GnRH-ant protocol were not clarified. GnRH-ant may also affect the expression of CKB by changing the concentration of local growth factors, leading to a series of biological effects, but this has yet to be confirmed. Therefore, more in-depth studies are required and are currently underway.

Conclusions

In summary, GnRH-ant affected CKB expression in EECs. These effects could damage the cytoskeleton, disrupt cell migration, and alter endometrial receptivity. These findings established CKB as a new therapeutic target for improving endometrial receptivity in the clinical setting and indicated that although the GnRH-ant protocol may effectively induce multifollicle ovulation, its negative effects should also be considered and managed to facilitate the design of improved clinical therapeutic regimens.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0572>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Q C and C X designed the study. Q C, Y F, and X W Z performed the study. Z Y and Y P K participated in parts of the experiments and analysis of the data. Q C, A J Z, and C X wrote the paper. All authors read and approved the final manuscript.

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