

# Kisspeptin-10 inhibits OHSS by suppressing VEGF secretion

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## Abstract

The aim of the present study was to elucidate the effects of kisspeptin-10 (Kp-10) on ovarian hyperstimulation syndrome (OHSS) and its related mechanism in OHSS rat models, human umbilical vein endothelial cells (HUVECs) and human luteinized granulosa cells. OHSS is a systemic disorder with high vascular permeability (VP) and ovarian enlargement. KISS1R (KISS1 receptor) is the specific receptor of kisspeptin. The kisspeptin/KISS1R system inhibits the expression of vascular endothelial growth factor (VEGF), which is the main regulator of VP. In our study, decreased expression of Kiss1r was observed in both ovaries and lung tissue of OHSS rats. Injection of exogenous Kp-10 inhibited the increase of VP and VEGF while promoting the expression of Kiss1r in both the ovarian and lung tissue of OHSS rats. Using HUVECs, we revealed that a high level of 17- $\beta$  estradiol ( $E_2$ ), a feature of OHSS, suppressed the expression of KISS1R and increased VEGF and nitric oxide (NO) through estrogen receptors (ESR2). Furthermore, KISS1R mRNA also decreased in the luteinized human granulosa cells of high-risk OHSS patients, and was consistent with the results in rat models and HUVECs. In conclusion, Kp-10 prevents the increased VP of OHSS by the activation of KISS1R and the inhibition of VEGF.

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## Introduction

Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic, serious and potentially fatal complication of ovarian stimulation. It contains a broad spectrum of clinical manifestations including massive ovarian enlargement, high level of 17- $\beta$  estradiol ( $E_2$ ), high vascular permeability (VP), ascites, pleural effusion, reduced renal perfusion, thrombotic complications and possibly death (Delvigne & Rozenberg 2002, Garcia-Velasco & Pellicer 2003). Mild OHSS occurs in 32% of IVF cycles while 10–15% of IVF patients develop moderate OHSS and 5–8% of the patients are diagnosed with severe OHSS (Humaidan *et al.* 2010, Toftager *et al.* 2016). Despite being a potentially life-threatening condition, the pathogenesis of OHSS is still unclear due to its elusive pathophysiology.

The symptoms of OHSS are due to high VP, and the most important mediator is vascular endothelium growth factor (VEGF). VEGF makes local capillaries leaky by binding to and phosphorylating VEGF receptor 2 (VEGFR2) in endothelial cells (Gille *et al.* 2001). In addition, VEGF is a VP enhancer whose potency is 5000 times stronger than histamine and plays an important role in the incidence of OHSS (Yan *et al.* 1993,

Neulen *et al.* 1995, Wang *et al.* 2002). Thus, VEGF promotes OHSS onset by regulating nitric oxide (NO) and junction proteins and thereby increasing VP (Farkkila *et al.* 2011, Wang *et al.* 2015). Apart from being a powerful mediator of VP, VEGF also participates in the formation of the corpus luteum by mediating vascularization (Aboulghar & Mansour 2003, Harada *et al.* 2010).

To date, discontinuing gonadotropin therapy (coasting) and reducing human chorionic gonadotropin (hCG) usage are common approaches in the prevention of OHSS. Some practitioners prescribe gonadotropin releasing hormone agonist (GnRHa) instead of hCG for final oocyte maturation in order to prevent OHSS (Stadtmauer *et al.* 2011). However OHSS may still occur in the very high risk individual using GnRHa (Fatemi *et al.* 2014, Ling *et al.* 2014). Another common strategy used in clinics is embryo cryopreservation. Although it is useful for avoiding the late form of OHSS caused by endogenous hCG, embryo cryopreservation cannot prevent early OHSS development caused by exogenous hCG administration (Braat *et al.* 2010, Naredi *et al.* 2014). Therefore OHSS is a potentially lethal condition with an unclear mechanism and it is necessary for us to investigate the new mechanisms involved in mediating OHSS.

Kisspeptin (Kp) is a polypeptide encoded by the *KISS1* gene and its specific receptor is KISS1R (KISS1 receptor) (Muir *et al.* 2001). The Kp precursor contains 145 amino acids and can be hydrolyzed into Kp-54, Kp-14, Kp-13 and Kp-10. Kp-10, the shortest subtype with high activity, plays a vital role in many tissues. The Kp/KISS1R system participates in the female reproductive endocrine system by directly regulating each part of the hypothalamo-pituitary-ovarian axis (HPOA) (Novaira *et al.* 2009, Millar & Newton 2013). Kisspeptin is the GnRH secretagogue and exogenously administered kisspeptin could induce a dose-dependent release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. In addition, Kp and KISS1R are expressed in the ovaries, where Kp is involved in ovarian follicular development and participates in progesterone regulation without affecting  $E_2$  production (Peng *et al.* 2013, Jayasena *et al.* 2014, Fernandois *et al.* 2016). Kp-10 inhibits VEGF expression in human umbilical vein endothelial cells (HUVECs) through KISS1R, while VEGF is an important mediator of OHSS onset (Cho *et al.* 2009). Moreover, Kp-54, another member of the Kp family, can be used effectively and safely to trigger oocyte maturation for IVF patients at a high risk of OHSS (Abbara *et al.* 2015). Thus, we suggested that Kp/KISS1R system could prevent OHSS by inhibiting VEGF.

High level of  $E_2$  is another characteristic of OHSS, which is closely related to the number of follicles and accounts for the increase of serum VEGF and VP to a large extent.  $E_2$  promotes the expression of VEGF in several cells, depending on the presence of ESR1 (Bogin & Degani 2002, Garvin *et al.* 2006). Thus,  $E_2$  is not only a predictor but also a regulator of OHSS onset. Serum  $E_2$  plays an indispensable role in OHSS onset, and some  $E_2$  inhibition drugs such as letrozole have been used in clinic to induce ovulation as well as prevent OHSS (He *et al.* 2014, Sahin *et al.* 2016). Moreover, the *KISS1R* promoter contains an estrogen response element (ERE), the binding site of estrogen receptors, which may play a negative role in *KISS1R* transcription in pituitary cells (DeFino *et al.* 2010a). Thus, the Kp/KISS1R system may be regulated by  $E_2$  and may participate in the pathogenesis of OHSS. Therefore our study investigated the function of Kp/KISS1R system in OHSS prevention and the regulation of high  $E_2$  to Kp/KISS1R system.

## Materials and methods

### Animal models

A total of 18 immature 22-day-old female Wistar rats were fed *ad libitum* with a 12-h light and 12-h darkness schedule. The rats were randomly divided into three groups: OHSS group, the OHSS+Kp-10 group and the control group. In the OHSS

group, rats were subcutaneously injected with 10IU PMSG (PROSPEC, East Brunswick, USA) for 4 consecutive days to promote follicular development. They were then given 30IU hCG (s.c.) (Livzon Group, Guangdong, China) on the fifth day (Ferrero *et al.* 2014). In the OHSS+Kp-10 group, the management was same as the OHSS group except that the animals were given 30IU hCG and 40 nmol Kp-10 (intravenous) (GenScript, Nanjing, China) together on day 5. In the control group, the 24-day-old rats were pretreated with 10IU PMSG. Two days later, 10IU hCG was injected into the control group to mimic a routine ovarian stimulation (Ferrero *et al.* 2014).

All the rats were killed by decapitation after hCG administration for 48 h.

Protocols for animal handling were performed in accordance with UK legal requirements and approved by the animal ethics committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University.

### OHSS indicators measurement

#### VP detection

All the rats were anesthetized by pentobarbital 48 h after hCG injection. First, 0.2 mL methylene blue was injected into the caudal vein of the rats, and the peritoneal cavity was filled with 5 mL 0.9% saline 30 min after methylene blue injection. After the rats were massaged for 30 s, the fluid was extracted from the peritoneal cavity and centrifuged. The methylene blue concentration highlighting the abdominal vascular permeability (VP) was measured at 600 nm by microplate spectrophotometer (Ferrero *et al.* 2014).

#### Sample collection and processing

The whole blood was collected from the orbit before hCG administration and the ovaries were removed and weighted immediately after the rats were killed. The ovaries and serum were stored at  $-80^\circ\text{C}$  for further experiments.

### Patients and subjects

Patients with high level of  $E_2$  (serum  $E_2$  level higher than 6000 pg/mL on the day of hCG administration) or with more than 25 dominant follicles were identified as being at high risk of OHSS in IVF cycles. Follicles whose diameter was larger than 1.4 cm on the ovum retrieval day during IVF cycles were identified as dominant follicles. Human granulosa-lutein cells were recovered from the follicular fluid as described previously (Iwase *et al.* 2009) from the Center for Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. The granulosa cells were purified with Ficoll-Paque (GE-HealthCare Bio-Science, Uppsala, Sweden) and stored at  $-80^\circ\text{C}$ . Cases with a low level of  $E_2$  (<4000 pg/mL) and fewer dominant follicles (<15) in IVF cycles were chosen as the control group. Age, BMI, protocol and hormonal status were matched, and endocrine diseases such as primary ovarian insufficiency (POI) and polycystic ovary syndrome (PCOS) were excluded. The menstrual cycles of all patients are within 26–32 days.

All procedures were reviewed and approved by the Institutional Review Board of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University.

### **E<sub>2</sub> concentration measurement**

The E<sub>2</sub> concentration of rat serum before hCG administration was detected using an Estradiol ELISA Kit (Cayman Chemical).

### **Total RNA extraction and quantitative real-time PCR**

The total RNA of cells and tissues was extracted using an animal total RNA isolation kit (FOREGENE, Chengdu, China), and was then reverse transcribed into cDNA (TAKARA). The expression of the selected genes including *VEGF*, *KISS1R*, *KISS1*, *VE-cadherin*, *ESR2* and  $\beta$ -*ACTIN* was estimated by quantitative real-time PCR (qRT-PCR) using SYBR (Toyobo, Osaka, Japan). The expression of these genes was normalized to the housekeeping gene  $\beta$ -*ACTIN* using  $\Delta\Delta$ Ct method. The sequences for the primers of target genes are presented in Table 1.

### **Western blot**

30  $\mu$ g protein from tissue or cells lysed with a RIPA buffer was loaded onto 8–10% SDS gel coupled with loading buffer, transferred to a nitrocellulose (NC) membrane. The nonspecific binding sites were then blocked using 5% non-fat dry milk and incubated with diluted anti-VEGF antibody (Santa Cruz Biotechnology) (1:200), anti-KISS1R antibody (Santa Cruz Biotechnology) (1:200), anti-ESR1 antibody (Cell Signaling) (1:1000) and anti-ESR2 antibody (Sigma, Chemical) (1:200) at 4°C for overnight. This step was followed by incubation for 1.5 h with diluted peroxidase-conjugated secondary antibodies at room temperature. Finally, the protein signals were detected using ECL Western blotting substrate, and the bands obtained were quantified by densitometry and normalized to the GAPDH.

### **Cell culture**

HUVECs were maintained in DMEM/F-12 medium (Gibco), containing 10% fetal bovine serum (Gibco) and 1% Penicillin–Streptomycin–Neomycin (Gibco). The cultures were incubated

at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were passaged every 3 days to obtain exponential growth.

### **Small interfering (si) RNA knocking down**

2 × 10<sup>5</sup> HUVECs were seeded on six-well plates for 24 h. The culture medium was changed and a mixture of siRNA (50 pmol) and RNAiMAX (Invitrogen) (9  $\mu$ L) in OPTI-MEM (250  $\mu$ L) was added into each well. Cells were further cultivated for 48 h before being treated with E<sub>2</sub> (Sigma, Chemical) (5000 pg/mL) for 24 h. The specific sequences targeting *ESR2* were as follows:

*ESR2* siRNA, sense, 5'-CCAGCCAUGACAUUCUAUATT-3,  
anti-sense, 5'-UUAUAGAAUGUCAUGGCUGGTT-3.  
Scrambled siRNA (NC), sense, 5'-UUCUCCGAACGUGUCACGUTT-3,  
anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3.

### **NO concentration measurement**

HUVECs were lysed using a cell and tissue lysis buffer (Beyotime, Jiangsu, China) for NO assay and then the NO concentration of lysate was detected using a Griess Reagent Kit (Beyotime).

### **Statistical analysis**

Results were expressed as the mean value  $\pm$  standard deviation (s.d.). The differences between the experimental and the control groups were analyzed using a one-way ANOVA and unpaired Student's *t* test. Significance was defined as *P* < 0.05. All analyses were conducted using SPSS 21.0 for Windows (IBM).

## **Results**

### **Reduced expression of *Kiss1r* was observed in OHSS rats while *Kp-10* injection inhibited the increase of VP and VEGF of OHSS rats by *Kiss1r* activation**

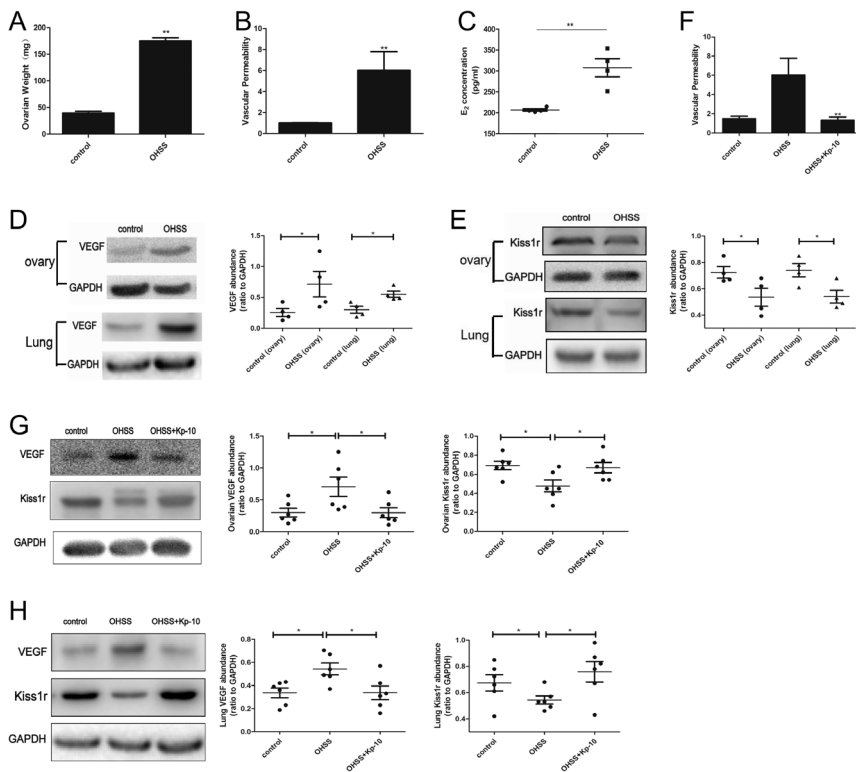
In order to investigate the relationship between Kp/Kiss1r system and OHSS onset, we built OHSS rat models at first. The significantly higher ovarian weight, abdominal VP, serum E<sub>2</sub> concentration and VEGF abundance in ovaries and lung tissue of the OHSS group (Fig. 1A, B, C and D) demonstrated that our OHSS models were successful. Lung tissue consists of numerous capillaries and venules, which play a vital role in the regulation of VP. The expression of *Kiss1r* significantly decreased both in ovaries and lung tissue of OHSS rats (Fig. 1E) without affecting ovarian *kiss1* mRNA (data not shown).

Exogenous Kp-10 was then injected into OHSS rats to verify the effect of Kp/Kiss1r system on OHSS onset. The exogenous Kp-10 injection inhibited the increase of abdominal VP of the OHSS group almost to the level of the control group (Fig. 1F). Meanwhile, Kp-10 also

**Table 1** Sequences for the primers of target genes in the real-time PCR.

Genes	Sequence of primers
<i>KISS1R</i>	Forward: 5'-ATCTACGTCATCTGCCGCCAC-3' Reverse: 5'-TCACGTACCAGCGGTCCACAC-3'
$\beta$ - <i>Actin</i>	Forward: 5'-GGGAAATCGTGCGTGACATTAAG-3' Reverse: 5'-TGTGTTGGCGTACAGGTCTTTG-3'
<i>VEGF</i>	Forward: 5'-TCACCAAGGCCAGCACATAG-3' Reverse: 5'-TTAACTCAAGCTGCCTCGCC-3'
<i>KISS1</i>	Forward: 5'-TGAATCACTGTTTCTTGCC-3' Reverse: 5'-CGAAGGAGTTCAGTTGTAGT-3'
<i>VE-cadherin</i>	Forward: 5'-GCGACTACCAGGACGCTTTCA-3' Reverse: 5'-CATGTATCGGAGGTCGATGGTG-3'
<i>ESR2</i>	Forward: 5'-GCAGACCACAAGCCAAATG-3' Reverse: 5'-AGCGATCTTGCTTCACACCA-3'





**Figure 1** Reduced expression of KISS1R was observed in ovaries and lung tissue of OHSS rats while the increase of VP and VEGF was inhibited by exogenous Kp-10 injection in OHSS rat models. (A, B, C and D) Ovarian weight, abdominal VP, serum E<sub>2</sub> concentration and VEGF expression of ovaries and lung tissue of the control and OHSS group.  $n=4$ . The left panel shows the representative images of Western blot assays while the right panel clarifies the protein levels of all animals. The OD value was used to represent the abdominal VP of rats and this figure shows the ratio of OHSS group to control group. (E) The expression of Kiss1r of the control and OHSS groups in both ovaries and lung tissue.  $n=4$ . (F) Abdominal VP of the control, OHSS and OHSS + Kp-10 group.  $n=6$ . (G) The expression of ovarian VEGF and Kiss1r in the control, OHSS and OHSS + Kp-10 groups.  $n=6$ . (H) The expression of VEGF and Kiss1r in lung tissue of the control, OHSS and OHSS + Kp-10 groups.  $n=6$ . \* $P < 0.05$ , \*\* $P < 0.01$ . Western blots show the representative images.

significantly enhanced the expression of Kiss1r as well as inhibited VEGF in the ovaries (Fig. 1G) and lung tissue (Fig. 1H) of OHSS models. Thus, Kp/Kiss1r system has an effect on OHSS prevention by inhibiting VEGF expression.

### High level of E<sub>2</sub> suppressed KISS1R and increased VEGF in HUVECs

OHSS is a systemic disorder with high VP and high VEGF expression while vascular endothelial cells are mainly responsible for the regulation of VP with high expression of VEGF (Kang *et al.* 2016). Thus, HUVECs were chosen as the *in vitro* cell line. Furthermore, a high level of E<sub>2</sub> is a characteristic of OHSS and E<sub>2</sub> may play a negative role in the expression of KISS1R in previous studies (DeFino *et al.* 2010a). Thus, HUVECs were treated with E<sub>2</sub> to investigate the mechanism underlying the decreased KISS1R in OHSS. Treatment of HUVECs with high E<sub>2</sub> for 48 h reduced KISS1R mRNA and protein levels in a concentration-independent manner (Fig. 2A). Meanwhile, high E<sub>2</sub> (>5000 pg/mL) also increased VEGF mRNA and NO synthesis ( $t=-3.044$ ,  $P=0.038$ ) (Fig. 2B and C) in HUVECs, which both represent the increase of VP. Therefore a high level of E<sub>2</sub> inhibited KISS1R and promoted the increase of VEGF and VP.

In addition, HUVECs were treated with both Kp-10 and E<sub>2</sub> to verify the effect of Kp-10 on the promotion of E<sub>2</sub> to VEGF. E<sub>2</sub> promoted VEGF mRNA significantly in HUVECs while Kp-10 treatment inhibited the increase of VEGF induced by E<sub>2</sub> (Fig. 2D). This result was consistent with rat models and demonstrated that high E<sub>2</sub> could

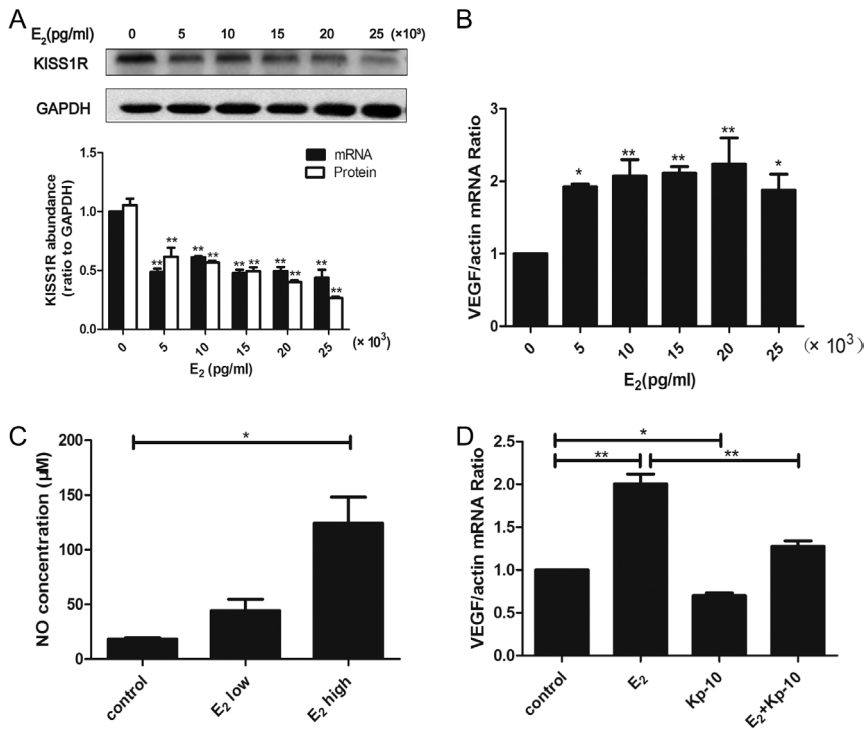
promote the expression of VEGF partially by inhibiting Kp/KISS1R system.

### E<sub>2</sub> suppressed KISS1R and increased VP in HUVECs through the activation of ESR2

E<sub>2</sub> is activated when it combines with estrogen receptors (ERs), including ESR1 and ESR2. ESR1 is almost absent while ESR2 is present in HUVECs (Fig. 3A). Specific ESR2 siRNA was transferred into HUVECs, and ESR2 was reduced to less than 10% (Fig. 3B). The reduction of KISS1R after E<sub>2</sub> treatment was partially reversed both in mRNA and protein levels after ESR2 knockdown, which suggested that ESR2 participated in the negative regulation of E<sub>2</sub> to KISS1R (Fig. 3C). Furthermore, the expression of VEGF decreased both in mRNA and protein levels after ESR2 knockdown (Fig. 3D). The downstream factors of VEGF also changed with ESR2 reduction. *VE-cadherin* mRNA increased and NO was reduced significantly ( $t=7.39$ ,  $P=0.002$ ) (Fig. 3E and F), which could mediate a decrease in VP. Thus, E<sub>2</sub> inhibited KISS1R and promoted VEGF through binding to ESR2, resulting in a high VP.

### KISS1R was reduced in the granulosa cells of patients at high risk of OHSS with no significant change observed in KISS1

The expression of the Kp-10/KISS1R system was detected in clinic samples to verify the results in rat models and HUVECs. The granulosa cells of patients at high risk



**Figure 2** E<sub>2</sub> inhibited KISS1R and promoted VEGF in HUVECs. (A) Expression of KISS1R mRNA and protein after treating with E<sub>2</sub> (5000, 10,000, 15,000, 20,000 and 25,000 pg/mL) for 48 h in HUVECs. (B) VEGF mRNA expression after treating with E<sub>2</sub> for 24 h in HUVECs. (C) NO concentration of HUVECs lysate after treating with E<sub>2</sub> (low: 5000 pg/mL, high: 10,000 pg/mL) for 24 h. Control: 18.48 ± 1.57 μM, E<sub>2</sub> low: 44.4 ± 14.6 μM, E<sub>2</sub> high: 124.3 ± 33.4 μM. (D) VEGF mRNA level of HUVECs after treating with E<sub>2</sub> (5000 pg/mL), Kp-10 (10 μM), both E<sub>2</sub> and Kp-10. In the E<sub>2</sub> + Kp-10 group, HUVECs were pretreated with E<sub>2</sub> for 24 h and then 10 μM Kp-10 for another 24 h. \**P* < 0.05, \*\**P* < 0.01, *n* = 3 separate experiments. Western blots show the representative images.

of OHSS showed a significantly decreased expression of *KISS1R* mRNA (*n* = 13) while no significant change was identified in *KISS1* mRNA between the OHSS and control groups (Fig. 4A and B). The clinical information of patients is presented in Table 2. The results of clinic samples were consistent with rat models and HUVECs.

## Discussion

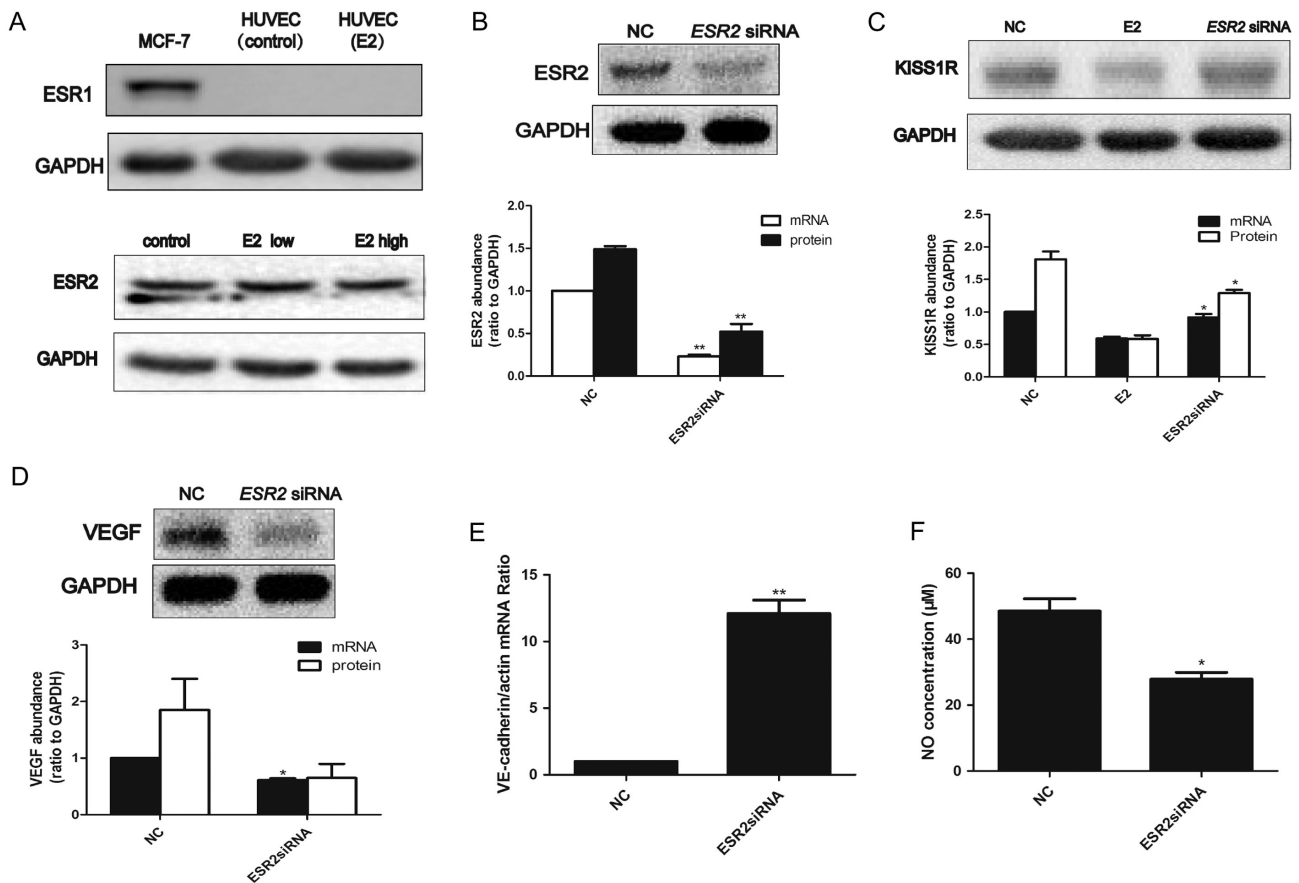
The Kp/KISS1R system is an acknowledged VEGF inhibitor, and VEGF promotes the incidence of OHSS (MacLean *et al.* 2014, Golzar & Javanmard 2015). Thus, we hypothesized that Kp/KISS1R system played a role in the pathogenesis of OHSS. In order to investigate the role of Kp/KISS1R in the incidence of OHSS, we measured the expression of Kp/KISS1R in ovaries and lung tissue of OHSS models, which were built according to an established method (Ferrero *et al.* 2014). The ovaries are the origin of OHSS onset, while lung tissue is abundant with vessels which are closely related to VP regulation and pleural effusion. Thus, the high VEGF and decreased Kiss1r both in ovaries and lung tissue had an effect on the pathogenesis of OHSS.

Kisspeptin polypeptide can be hydrolyzed into Kp-54, 14, 13 and 10. We chose Kp-10 instead of other subtypes to investigate the role of Kp in OHSS prevention because previous studies have indicated that Kp-10 inhibited the expression of VEGF in HUVECs, the main inducer of high VP. Moreover, Kp-10 also promotes follicle maturation, ovulation and progesterone production (Pineda *et al.* 2010, Sebert *et al.* 2010, Xiao *et al.* 2011, Ni *et al.* 2012). Exogenous Kp-10 injection decreased VP and VEGF by

enhancing Kiss1r in OHSS rats without affecting ovulation. Therefore Kp-10 injection is a promising approach to effectively and safely prevent OHSS. Kp-54 injection also induces egg maturation and reduces the incidence of OHSS in women at high risk (Abbara *et al.* 2015). Kp-54 is the primary fragment of kisspeptin also found circulating during human pregnancy. Therefore other subtypes of kisspeptin may also have effects on OHSS prevention, which will require further investigation.

Since decreased KISS1R was involved in OHSS onset, we further investigated the mechanism. Serum E<sub>2</sub> on the day of hCG administration is an important indicator of OHSS prediction (Papanikolaou *et al.* 2006, Delvigne 2009). Moreover, E<sub>2</sub> also inhibits the transcription of *KISS1R* in the pituitary cell line. Therefore we treated HUVECs with a high level of E<sub>2</sub> (>5000 pg/mL) to investigate the relationship among E<sub>2</sub>, KISS1R and OHSS onset. E<sub>2</sub> inhibited the expression of KISS1R and promoted VEGF and NO in HUVECs. Thus, a high level of E<sub>2</sub> boosted OHSS by inhibiting KISS1R, which resulted in increased VEGF and high VP, inducing edema, ovarian enlargement and pleural effusion.

E<sub>2</sub> not only suppresses KISS1R in endothelial cells but also regulates the expression of KISS1 in the arcuate nucleus (ARC) by negative feedback (Smith *et al.* 2005). This is consistent with the hypothesis that high E<sub>2</sub> inhibits the KISS1/KISS1R system to induce the incidence of OHSS. However it is contrary to the result demonstrating no significant change in ovarian *KISS1* mRNA and serum kisspeptin concentration (data not shown) between the OHSS and control groups. Different organs react differently to high E<sub>2</sub> (Millar *et al.* 2010). Thus, E<sub>2</sub> might

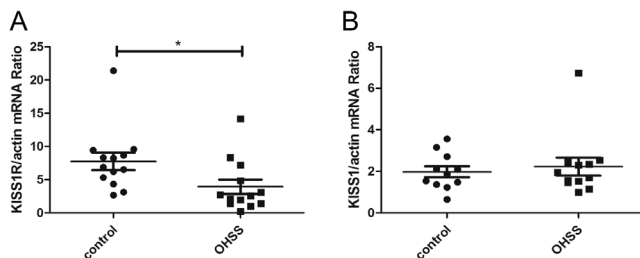


**Figure 3** E<sub>2</sub> inhibited KISS1R and promoted VP through ESR2. (A) Expression of ESR1 and ESR2 protein in HUVECs (E<sub>2</sub> low: 5000 pg/mL, E<sub>2</sub> high: 10,000 pg/mL for 24 h). (B) The efficiency of *ESR2* knockdown in HUVECs in both mRNA and protein levels after *ESR2* siRNA transfection for 48 h. (C) KISS1R mRNA and protein expression of HUVECs after *ESR2* knockdown. The gel picture is the representative image of western blot. HUVECs were treated with E<sub>2</sub> (5000 pg/mL) for 24 h after *ESR2* knockdown, and then the downstream factors were detected. (D) VEGF mRNA and protein levels after *ESR2* knockdown in HUVECs. (E and F) *VE-cadherin* mRNA level and NO concentration of HUVECs after *ESR2* knockdown. NO concentration: control group:  $48.51 \pm 5.32 \mu\text{M}$ , *ESR2* siRNA group:  $27.88 \pm 2.89 \mu\text{M}$ . \* $P < 0.05$ , \*\* $P < 0.01$ ,  $n = 3$  separate experiments. Western blots show the representative images.

have no effect on KISS1 expression of granulosa cells, or the positive and negative regulation of E<sub>2</sub> to ovarian *KISS1* mRNA offset each other and resulted in a constant expression. Moreover, intra-ovarian Kp was also regulated throughout the cycle and a surge was detected before ovulation (Castellano *et al.* 2006). We chose the

granulosa cells of ovum retrieval day to remove the difference of Kp expression between various periods.

ESR1 and ESR2 are ligand-dependent transcription factors in target tissues and have strong affinity with E<sub>2</sub> (Filardo & Thomas 2005, Prossnitz & Barton 2014). In addition, these two ERs are manifested in



**Figure 4** KISS1 and KISS1R expression in the granulosa cells of OHSS and control patients. (A) *KISS1R* mRNA expression in granulosa cells of patients at high risk of OHSS and control patients.  $n = 13$ . (B) *KISS1* mRNA expression of granulosa cells of patients at high risk of OHSS and control patients.  $n = 13$ . \* $P < 0.05$ .

**Table 2** Clinical and biological characteristics of patients involved in the present study.

	OHSS ( $n = 13$ )	Control ( $n = 13$ )
Age	$29.69 \pm 3.06$	$29 \pm 3.08$
FSH (IU/L)	$6.84 \pm 1.08$	$7.51 \pm 0.95$
LH (IU/L)	$5.66 \pm 1.49$	$5.12 \pm 1.25$
E <sub>2</sub> (pg/mL)	$44.52 \pm 17.94$	$37.43 \pm 15.31$
T (nmol/L)	$1.13 \pm 0.46$	$0.91 \pm 0.34$
AMH (ng/mL)	$6.44 \pm 0.7^*$	$4.13 \pm 0.88$
Antral follicles	$19.79 \pm 6.05$	$15.69 \pm 6.93$
hCG (IU)	$4115 \pm 1959^*$	$6076 \pm 1891$
E <sub>2</sub> of hCG day (pg/mL)	$7303 \pm 1778^{***}$	$2206 \pm 673$
Number of retrieved oocytes	$19.75 \pm 9.41^{**}$	$10.69 \pm 3.75$

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



different places and play various roles in different manners. We detected ESR1 and ESR2 in HUVECs, but only ESR2 was highly expressed in HUVECs. Moreover, the *KISS1R* promoter region contains an ERE and ESR2 is predicted to negatively regulate *KISS1R* from the sequence structure. The increased *KISS1R* due to ESR2 reduction demonstrates that  $E_2$  suppresses the transcription of *KISS1R* partially by binding to ESR2 in HUVECs. Thus,  $E_2$  inhibits *KISS1R* and promotes the increase of VP through ESR2.

Finally, the significantly decreased *KISS1R* mRNA in granulosa cells of OHSS patients demonstrates its specific role in OHSS onset. However there are also several limitations to our study. We chose human granulosa cells rather than human vascular endothelial cells to demonstrate the role of decreased *KISS1R* in OHSS because of the limitation in obtaining human endothelial cells. Further studies will be needed, and the sample size should be enlarged to reduce the overlap between the OHSS and control groups.

According to our results, a high level of  $E_2$  is a risk factor of OHSS. The development of multiple follicles during IVF cycles causes a high level of serum  $E_2$ . Then,  $E_2$  inhibits *KISS1R* expression of vascular endothelial cells and ovaries by activating ESR2. The decreased *KISS1R* in endothelial cells inhibits the suppression of endogenous Kp-10 to VEGF and facilitates OHSS onset. Exogenous Kp-10 injection promotes the activation of *KISS1R* and inhibits VEGF. Thus, the reduced VEGF and downstream factors such as NO and VE-cadherin decrease VP and inhibit the incidence of OHSS.

Our study highlights a potential mechanism of OHSS onset and provides a novel possible strategy to prevent OHSS by exogenous Kp-10 injection. In conclusion, Kp-10 prevents the increased VP of OHSS probably by the activation of *KISS1R* and the inhibition of VEGF.

## 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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