Original Article
Effect of high ovarian response on the expression of endocrine gland-derived vascular endothelial growth factor (EG-VEGF) in peri-implantation endometrium in IVF women

Li-Zhen Xu[1,2], Min-Zhi Gao[2], Li-Hua Yao[2], A-Juan Liang[2], Xiao-Ming Zhao[2], Zhao-Gui Sun[3]

[1]Center for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University, Jinan 250021, China; [2]Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Center for Reproductive Medicine, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200135, China; [3]Shanghai Institution of Planned Parenthood Research, Shanghai 200032, China

Abstract: Objective: To investigate the effect of ovarian stimulation on the expression of EG-VEGF mRNA and protein in peri-implantation endometrium in women undergoing IVF and its relation with endometrial receptivity (ER). Design: Prospective laboratory study. Setting: University hospital. Patients: Eighteen women in stimulated cycles (SC) as study subjects and 18 women in natural cycles (NC) as controls. Women in SC group were classified with two subgroups, high ovarian response (SC1, n=9) with peak serum E_2>5,000 pg/mL and moderate ovarian response (SC2, n=9) with peak serum E_2 1,000-5,000 pg/mL. Intervention(s): Endometrial biopsies were collected 6 days after ovulation in NC or after oocyte retrieval in SC. Main outcome measure(s): Endometrium histological dating was observed with HE staining. EG-VEGF mRNA expression levels determined by real-time polymerase chain reaction analysis, and protein levels by immunohistochemistry. Results: All endometrial samples were in the secretory phase. The endometrial development in SC1 was 1 to 2 days advanced to NC, and with dyssynchrony between glandular and stromal tissue. Immunohistochemistry analysis showed that EG-VEGF protein was predominantly expressed in the glandular epithelial cells and endothelial cells of vessels, and also presented in the stroma. The image analysis confirmed that both the gland and stroma of endometrium in SC1 had a significantly lower EG-VEGF protein expression than that in SC2 and NC endometrium. Moreover, EG-VEGF mRNA levels were significantly lower in SC1 than in NC. Both EG-VEGF protein and mRNA levels had no significant difference between SC2 and NC. Conclusion: Decreased expression of EG-VEGF in the peri-implantation is associated with high ovarian response, which may account for the impaired ER and lower implantation rate in IVF cycles.

Keywords: EG-VEGF, high ovarian response, peri-implantation, IVF-ET, endometrial receptivity

Introduction

In reproductive woman, the uterine endometrium undergoes dynamic change of proliferation, differentiation and shedding in response to ovarian hormone. During the window of implantation (WOI), the morphology and function of endometrium is transformed to adapt to embryo implantation, and then endometrial receptivity (ER) is established [1]. Besides embryo quality, the impaired ER is the main cause of implantation failure [2, 3].

During the routine cycle of in vitro-fertilization and embryo transfer (IVF-ET), controlled ovarian hyperstimulation (COH) is often applied to stimulate multiple follicular development. In this process, the endometrium is exposed to the environment of supra-physiological level of steroid hormone, which may result to the change of endometrial development, such as asynchronous endometrial development with delayed glandular maturation and advanced stromal morphology transformation [4, 5]. Studies have previously shown that COH caused changed expression of some ER biomarkers in endometrial glandular epithelium [4, 6-8]. Moreover, other studies reported that the expression and secretion of some growth factors were changed during COH period [8, 9].

These studies suggested that high level of estradiol (E_2) and progesterone (P_4) produced after COH may change the endometrial development and the expression of some ER associated molecules, which are detrimental to ER and can affect embryo implantation. The association of high ovarian response with low pregnancy rate has already been reported [10]. As E_2 act more lastingly and fluctuate more sharply than P_4 during the whole menstrual cycle, so its role to impact on the ER in COH cycles was focused on in many studies.

The physiological angiogenesis is an important process for endometrial remodeling, and during which some vasotropic factors play key roles on vascular development, function and degeneration. Endocrine gland-derived vascular endothelial growth factor (EG-VEGF), also termed as prokineticin (PK1), is first found as an angiogenesis factor with reproductive tissue specificity. Being the mitogen of endothelial cells in the microvessel bed of endocrine gland, EG-VEGF is functioned to regulate and modulate the growth, structure and function of endothelial cells, and to induce new angiogenesis [11-13]. However, its function in female genital tract has not been totally elucidated. Different from VEGF, EG-VEGF is only expressed in the endometrium of reproductive women and is highest expressed in the mid-luteal phase of WOI [14, 15]. Similar to this, another study found by gene microarray chip screening and quantitive PCR that EG-VEGF gene was significantly up-regulated in the mid-secretory phase, when compared with in the early-secretory phase within the same natural cycle [16]. More recently, the in-vitro effect of EG-VEGF on cultured human uterine microvascular endothelial cell (UtMVEC-Myo) have also been reported, as proliferation and tube formation was observed [17]. These studies imply that EG-VEGF may play an important role in vascular development in peri-implantation endometrium and may be a biomarker of ER. Furthermore, in vitro studies also confirmed that endometrial EG-VEGF expression was stimulated by both hCG and steroid hormones [14, 18], suggesting its expression in endometrium is highly depend on hormones. So it is presumable that COH may change its expression and function, as study has already speculated that EG-VEGF may function in glandular-stromal dyssynchrony in peri-implantation endometrium [15].

Considering of above, ER may be impaired during COH cycle and EG-VEGF was found to have important effect on ER by laboratory work, the clinical observational studies should be performed to determine the possible significance of EG-VEGF during COH cycles. In this study, peri-implantation endometrial samples were collected from IVF women in stimulated cycles (SC), with samples in natural cycles (NC) as control, to detect endometrial EG-VEGF expression at both the morphological and molecular levels, with aim to elucidate the effect of high ovarian response on endometrial EG-VEGF expression and its association with ER.

Materials and methods

Patients selection

A total of 36 infertile women undergoing assisted reproductive treatments because of tubal and/or male factors were recruited from the Department of Reproductive Medicine at Renji hospital (School of Medicine, Shanghai Jiaotong University). All women had normal BMI (body mass index, BMI) (18.5-23.9 kg/m^2)and regular menstrual cycles while endocrine examination confirmed their day 3 serum FSH, LH, E_2 and corpus luteum midterm level of serum concentration of P was in the normal range. All cases have no ovarian surgery. Normal ovulation and the presence of a normal uterine cavity were verified by ultrasound examination. None had accepted exogenous hormone therapy in the 3 months preceding the study. The study was approved by the institutional ethics committee of Renji Hospital and all subjects provided written informed consent.

There were 18 women in the SC and NC group, separately. In the SC group, subjects were subgrouped to high ovarian response (SC1, E_2>5,000 pg/mL, n=9) and moderate ovarian response (SC2, E_2 1,000-5,000 pg/mL, n=9) according to E_2 level on hCG day. All undergone COH and oocytes retrieval (OR), but ET was not performed in order to prevent ovarian hyperstimulation syndrome (OHSS) or because of poor quality embryo or other personal factors. In the NC group, all subjects were infertile women caused by male factor. They were monitored by vaginal ultrasound examination and serum E_2 and P_4 to confirm the day of ovulation.

Controlled ovarian hyperstimulation protocol

COH was used as long protocol of GnRHa combined with gonadotrophin (Gn). Briefly, 0.05 mg GnRHa (triptorelin [Decapeptyl]; Ferring, Kiel, Germany) was initiated daily from 7 days after ovulation and changed to 0.025 mg daily after the pituitary is down-regulated, then Gn (Gonal-F, Merck Serono, Geneva, Switzerland) was added until day of hCG. The initiative dose of Gn were 100-225 IU, then be adjusted with ovarian response. The patients were monitored with Doppler ultrasonography (SSD-1400; Aloka, Tokyo, Japan) for follicle size and endometrial thickness and morphology during COH treatment. When the leading follicle reached 18 mm or two follicles reached 17 mm, or both, and serum E_2 levels corresponded to the number of follicles larger than 14 mm in size, 5,000 IU hCG (Lizhu, Zhuhai, China) was injected. Oocyte retrieval (OR) was conducted under vaginal ultrasound guidance 34-36 hours later. Due to ET cancel, the patients did not accept luteal phase support treatment.

Hormone assays

Serum FSH, E_2 and P_4 concentrations were measured by chemiluminescence analysis (Immulite 1000; Siemens, Erlangen, Germany). Serum levels of E_2 were assayed in the preovulatory phase and on the day of endometrial biopsy. Reference levels were 34-400 pg/mL in the preovulatory phase and 27-246 pg/mL in the luteal phase. The inter-assay variation was 4-8%. Serum levels of progesterone were assayed on the day of endometrial biopsy. Reference levels were 0.48-1.72 ng/mL in the follicular phase and 0.95-21 ng/mL in the luteal phase. The inter-assay variation was 8-10% in the follicular phase and 3-4% in the luteal phase.

Endometrial biopsies and histological evaluation

Endometrial biopsy was taken on day 6 after OR and ovulation in SC and NC, separately. Samples were collected with an endometrial straw (LILYCLEANER, Shanghai Jiabao medical healthcare science and technology Ltd. China).

All endometrial samples were routinely stained with Hematoxylin-eosin (H-E) staining for patho-logically diagnosis and histological dating. The main steps of H-E were performed as follows: each sample was immediately washed in phosphate-buffered saline (PBS) and fixed in para-formaldehyde for 24 h, embedded in paraffin and later cut into 3 µm-thick sections. After dewaxing, the sections were stained with hematoxylin for 10 minutes, differentiated with 1% hydrochloric acid for 30 seconds, and became blue after being flushed with water. Then the sections were stained with eosin for 1 minute, dehydrated and sealed with transparent neutral gum. The result showed that the nucleolus was dyed to be blue and cytoplasm to be red. All slides were blindly evaluated by a single experienced observer for histological dating according to the Noyes's criteria [19].

Immunohistochemistry and image analysis

Localization of EG-VEGF was performed by immunohistochemistry in 3 µm paraffin-embedded sections using commercially available rabbit anti-human polyclonal antibody (Abcam, USA). Briefly, sections were dewaxed in xylene and then rehydrated using decreasing grades of ethanol. Antigen retrieval was performed by microwave heating in 0.01 M sodium citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was quenched with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min at room temperature. After treating with protein blocking solution containing 10% goat serum to block nonspecific binding, the sections were incubated overnight at 4°C with the primary antibody at a dilution of 1:200. A goat anti-rabbit biotinylated secondary antibody was applied for 30 min at room temperature. An avidin-biotin peroxidase detection reagent kit (Proteintech Group, USA) with 3,30-diaminobenzidine tetra-hydrochloride (DAB) was employed for signal detection. Negative controls were performed using 10% goat serum instead of primary antibody. The positive results showed brown granules appeared in nucleolus or cytoplasm of tissue cells.

Sections after immunohistochemistry were observed under light microscope (Axioplan 2 microscope, Zeiss, Jena, Germany). Photographs were transformed to digitalized image after video scanned (AxioCam, Zeiss), and expression level of EG-VEGF protein was quantitated by computerized image analysis using

Table 1. Comparison of demographic data, endometrial thickness and hormone levels among the three groups

	SC1 group (n=9)	SC2 group (n=9)	NC group (n=18)
Age (year)	29.89±4.70	28.22±3.19	30.67±3.36
Infertility duration (year)	5.33±3.43	3.78±2.73	4.39±2.68
Type of infertility			
Primary infertility	7	6	14
Secondary infertility	2	3	4
Gn dosage (IU)	1227.78±174.19	1258.33±231.17	-
Gn duration (day)	9.00±0.87	9.11±0.60	-
Peak E_2 (pg/mL)[#]	8579.89±1476.03	3511.89±928.88[a,**]	263.83±61.43[a,**,b,**]
Endometrium thickness (mm)	10.44±1.51	10.22±1.79	9.74±1.89
Steroid levels on the day of endometrial biopsy			
E_2 (pg/mL)[#]	2515.22±1493.10	1484.50±76.61[a,*]	114.76±50.91[a,**,b,**]
P_4 (ng/mL)[#]	43.74±16.33	27.30±18.57[a,**]	13.37±4.53[a,**,b,*]

#, have difference among three groups; a, compared with SC1 group; b, compared with SC2 group. *, $P < 0.05$; **, $P < 0.01$.
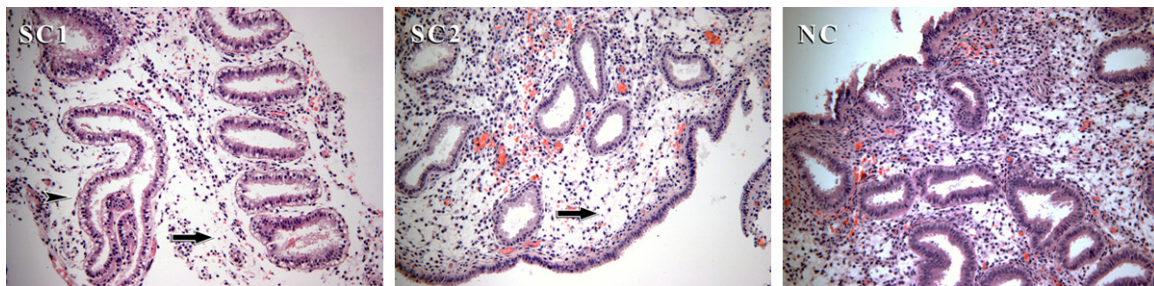
Figure 1. Photomicrographs of uterine endometrial tissue sections stained with H-E method for histological dating. The tissues were identified as in the secretory phase. Endometrial development was found 1 to 2 d in advance in SC1 group, not that in SC2 and NC groups. In the two SC groups, gland expansion and tortuous (short arrow) and edema in stroma (long arrow) were indicated. SC, stimulated cycles; NC, natural cycles. Original magnification ×200.

photodensitometry (KS400 Version 3.0, Zeiss, Germany). Staining was analyzed as the percentage of brown area occupied.

RT-PCR

The endometrial samples were put into liquid nitrogen and total RNA was extracted using Trizol reagent (Invitrogen Trizol Reagent, Cat No. 15596-026, Lot No. 1140375). Two mg RNA was reverse transcribed to cDNA in a 20 µl system. PCR reactions were carried out in a 25 µl reaction system using specified primer for 35 cycles. According to Genebank, primers of EG-VEGF (forward: 5'-AAATGCAAATGGTGGTGG-TT-3', reverse: 5'-TCTGAATCAGTGCCCCTTTC-3') and β-actin (forward: 5'-GGACTTCGAGCAAGAG-ATGG-3', reverse: 5'-AGCACTGTGTTGGCGTACAG-3') were designed using Primer Premier 3.0

software and synthesized by Shanghai Sangon Biotech Co., Ltd. PCR products were electro-phoresed with 2% TAE. Five µl of D2000 DNA Marker and 10 µl of samples were spotted and electrophoresed in 400 V electrophoresis cell for 40 min. Electrophoresis strips were recorded by Gel scanner imaging system. The estimated products of EG-VEGF and β-actin are 200 and 234 bp, respectively.

EG-VEGF mRNA expression levels were arrayed by SYBR Grenn relative quantitative method. Gene expression of each sample was internal referenced by β-actin, and negative referenced by empty tube without cDNA. Real-Time PCR was conducted in a 25 µl system (40 cycles). The reactive condition includes: pre-denatural-ization at 95°C for 1 min, denaturalization at 95°C for 15 sec, annealing at 60°C for 15 sec,
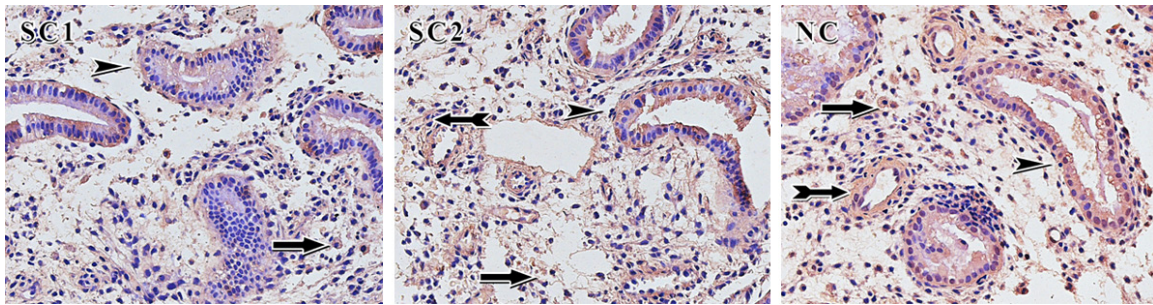
Figure 2. Immunohistochemical studies were undertaken on sections of endometrium to determine the cellular distribution of EG-VEGF. Immunohistochemical staining for EG-VEGF was detected in all samples. EG-VEGF was mainly expressed in the glandular epithelium of endometrium, and also in the stroma and vessels. Lower intensity of staining was detected in SC1 compared with SC2 and NC group. SC, stimulated cycles; NC, natural cycles. Original magnification ×200.

extension at 72°C for 45 sec, collected by SYBR fluorescent light, and for 40 cycles. Results were calculated relative to β-actin and differences quantified using the $2^{-\Delta\Delta Ct}$ method as described previously [20, 21].

Statistical analysis

The expressive level of protein and gene in samples were assayed by relative quantitative method. All data were analyzed by SPSS 18.0 software (SPSS Inc., Chicago, IL). The values are shown as the mean ± S.D. when applicable. All data were checked for their normal distribution by submission to the Kolmogrov-Smirnov test and if significant, non-parametric statistical analysis was applied. Differences among the groups were tested for significance using ANOVA, Kruskal Wallis, Mann-Whitney *U*, Student's t-test in parametric variables and chi-square test in categorical variables, where appropriate. *P*<0.05 was considered as statistical difference, *P*<0.01 was considered as significantly statistical difference.

Results

Comparison of basic clinical characteristics

There were no significant differences in average age, infertility duration, infertility type and endometrial thickness among SC1, SC2 and NC groups (*P*>0.05). Likewise, the average Gn dosage and duration was similar between SC1 and SC2 groups. However, as was expected, all hormone parameters including the peak E_2, and the average E_2 and P_4 levels on the day of endometrial biopsy were significantly higher in SC groups (including both SC1 and SC2) than in NC group (*P*<0.01), and higher in SC1 than in SC2 group (*P*<0.01) (**Table 1**).

Histological staging of endometrium in COH cycles

All endometrial samples were approved in secretory phases by histological dating, consistent with the stage of COH cycles. There were 0, 4 and 5 cases in early, mid and late secretory phases in SC1 group, while there were 2, 4, 3 and 4, 8, 6 cases in SC2 and NC group, respectively. Endometrial development was 1 to 2 d in advance in SC1 group, and not that in SC2 and NC groups. Endometria in SC1 showed an obvious glandular-stromal dyssynchrony, with persistent subnuclear vacuoles in the glands, and glandular development lag behind stroma. Besides, glandular expansion and tortuous, and edema in stroma were observed in the two SC groups, more severe depending on the degree of ovarian response (**Figure 1**).

Localization and quantification of EG-VEGF protein in human peri-implantation endometrium

To determine the cellular distribution of EG-VEGF protein, immunohistochemical staining for EG-VEGF was performed on all samples. EG-VEGF was showed mainly present in the glandular epithelium and microvascular endothelial cells in the endometria, and less intensity of staining was detected in the stroma (**Figure 2**). Although there were some individual

Table 2. Immunohistochemical comparison of EG-VEGF protein levels between SC and NC group

	SC1 group (n=9)	SC2 group (n=9)	NC group (n=18)
EG-VEGF			
Glandular compartment#	2.26±1.03[a,**,b,**]	4.82±1.25	5.69±1.40
Stromal compartment#	0.54±0.23[a,**,b,*]	1.13±0.38	1.35±0.64

#, have difference among three groups; a, compared with NC group; b, compared with SC2 group. *, $P<0.05$; **, $P<0.01$.
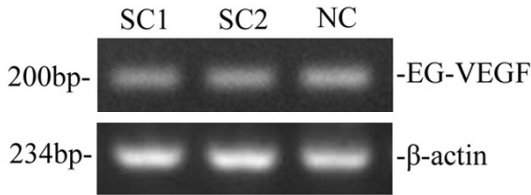
Figure 3. Typical samples for electrophoretic bands of RT-PCR products from EG-VEGF mRNA in peri-implantation endometria. SC, stimulated cycles; NC, natural cycles.

variations, but generally both glandular and stromal EG-VEGF expression were significantly lower in SC1 group when compared with SC2 and NC group ($P<0.01$, $P<0.01$; $P<0.05$, $P<0.01$). EG-VEGF expression in the glandular epithelium and stroma was also lower in SC2 group than in NC group, but did not reach statistical significance ($P>0.05$) (**Table 2**).

EG-VEGF mRNA levels in human peri-implantation endometrial

RT-PCR showed that products of EG-VEGF mRNA levels in SC group were obviously lower than in NC group (**Figure 3**). Real-time PCR validated that EG-VEGF mRNA levels were 0.67±0.21, 0.87±0.43 and 1.17±0.69 in SC1, SC2 and NC group, respectively. The expression levels of EG-VEGF mRNA in SC1 were significanly lower than in NC group ($P<0.05$). The expression levels of EG-VEGF mRNA in SC2 were also lower than in NC group, but they did not reach a statistical significance ($P>0.05$) (**Figure 4**).

Discussion

Appropriate range of E_2 is necessary for successful pregnancy in IVF cycle, high level of E_2 in high ovarian responders was demonstrated to have negative impact on IVF pregnancy rate

[22]. Meanwhile, the pregnancy rate of high ovarian responder in frozen embryo transfer cycle was higher than in fresh embryo transfer cycle, suggesting that high E_2 may affect ER rather than embryo quality [10, 23]. The uterine **endometrium** is the direct target organ functioned by estrogen and progesterone. Appropriate levels of estrogen and progesterone are important regulators to stimulate the endometrium develop from pre-implantation to receptive status. Small dose of estrogen can make the endometrium accept and transport the message from the blastocyst. On the basis of estrogen function, progesterone action is added to stimulate endometrial stromal cells differentiation and promote decidual reaction [24].

During the COH cycle, premature secretory change of endometrium after OR and in early luteal phase was observed, and followed by obvious glandular-stromal dyssynchrony in mid-luteal phase subsequently [4]. When the endometrial development advanced more than 3 days on the day of OR, no pregnancy was acquired after ET [25]. Our study of peri-implantation endometrium showed that serum E_2 and P_4 levels in peri-implantation phase in high and moderate ovarian responders were significantly higher than in NC controls. High ovarian responders had significantly asynchronous endometrial development with delayed glandular maturation and advanced stromal morphology, when compared with moderate ovarian responders and NC controls. These results suggested that different hormone levels come from different ovarian responses have different influences on the endometrial histological development. The supraphysiological status of high E_2 and abnormal E_2/P_4 ratio resulting from excessive ovarian responses may be one cause for endometrial asynchrony, and once this change accumulates to certain degree, it may compromise the ER.

The WOI phase is the limited period during which the uterus possesses the biggest receptivity for embryo implantation. During this phase, the endometrium secretes some ER associated proteins, which undergo a cascade
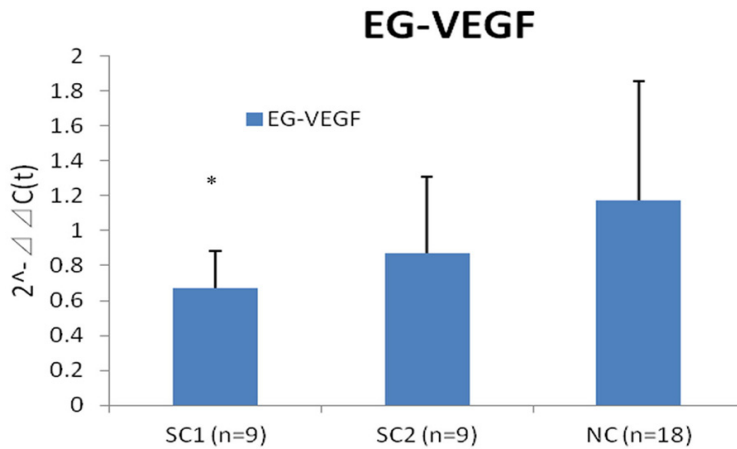
EG-VEGF

Figure 4. Quantification of EG-VEGF mRNA levels in peri-implanation endometria by Real-time PCR. (*, *P*<0.05; compared with NC group).

of functional changes triggered by steroid hormone, so as to adapt to and facilitate ER building and embryo invasion. Increased vascular permeability (VP) and angiogenesis in the endometrium around the site of blastocyst invasion is the original sign of successful implantation [26]. EG-VEGF, a newly discovered angiogenic factor regulated by steroid hormone, is exclusively expressed in endometrium of reproductive women and its expression is elevated during WOI phase and early pregnancy [15], which implies it may play a pivotal role in active angiogenesis and vascular development in peri-implantation endometrium, and maybe act as a biomarker for ER. So an altered expression of EG-VEGF would be one of the several biochemical abnormalities characterizing changed ER in excessive responders.

EG-VEGF protein was previously reported to be expressed both in endometrial gland and stroma, the expression level was low in the early follicular phase, and became stronger in glandular cells in the late follicular phase, until reached peak in the mid-luteal phase [15]. In this study we observed that EG-VEGF was moderately expressed in cytoplasm of cells in glandular epithelium and microvascular endothelium, and weakly expressed in extracellular matrix. The expression level of EG-VEGF in glandular epithelium was higher than in extracellular matrix, suggesting it may synthesized by gland and secreted to the stroma, this is coincide with Battersby's study [14]. EG-VEGF

expression in microvascular endothelium implies that it can regulate multiple biological process including endothelial cell proliferation and vasculation in WOI phase and has important role in angiogenesis of decidualization in secretory phase. In vitro study also found that EG-VEGF can significantly stimulate uterine microvascular endothelial cell line (UtMVEC-Myo) proliferation and form tubal structure [17]. The localization of EG-VEGF suggests that it may play a pivotal role in ER building and implantation by inducing vascular remodeling and increasing VP through an autocrine or paracrine role.

Our study further demonstrated that excessive responders exhibited both glandular and stromal decreased expression of EG-VEGF in endometrium as determined by immunohistochemistry, when compared with moderate responders and NC controls, suggesting that increasing E_2 levels had a negative effect on EG-VEGF protein expression. We speculate that although there had a rapid decline in serum E_2 after OR, but it was still much higher in high ovarian responders than in moderate responders and NC controls in peri-implantation period, thus it can still inhibit EG-VEGF expression, the estrogen sensitive gene. In addition, RT-PCR in this study showed that on the transcription level, EG-VEGF mRNA expressed in peri-implantation endometrium was significantly decreased in high ovarian responders than in NC controls, whereas other than EG-VEGF protein, no difference was shown for EG-VEGF mRNA expression between high and moderate responders. Furthermore, although serum E_2 in moderate responders were significantly higher than in NC controls, but no difference of EG-VEGF mRNA expression was shown, suggesting the E_2 difference between the two groups is not so big enough to inhibit EG-VEGF expression as in high ovarian responders. In discordance with our results, Ngan et al found endometrial EG-VEGF mRNA was increased after hCG injection in Gn stimulated cycle [15]. The difference

may be due to patient selection, we collected endometrial samples with different ovarian response, but there had no distinguish in their study.

In this study, both mRNA and protein level of EG-VEGF in peri-implantation endometrium of women with high ovarian response were significantly decreased than in NC controls, and the difference was more significant in protein level than in mRNA level, this imbalance maybe due to abated translation or increased protein degradation. The presence of high E_2 markedly down-regulated EG-VEGF synthesis and generated glandular-stromal dyssynchrony with glandular development being lag behind stroma in peri-implantation phase in high ovarian responders, may produce an environment that impairs the ER building and the implantation process. Because the insufficient secretion and dysfunction of EG-VEGF may affect angiogenesis in decidualization and therefore leading to suboptimal endometrial perfusion, which is consistent with the report that significantly lower endometrial blood flow was observed in SC than in NC [27], but the involved mechanism is unclear.

Studies also showed EG-VEGF and its receptors were increased in deciduas in early pregnancy, implying it may induce endometrial cells proliferation and regulate decidualization, and have important role in endometrial decidualization and ER building [28, 29]. EG-VEGF can also regulate some ER associated gene including COX2 and IL-8 expression, such as mediate fetal-maternal dialogue by regulating hCG-mediated endometrial LIF expression [15, 30, 31]. Besides, EG-VEGF may support pregnancy through VP regulation and promote VLDL transport into luteal cells to synthesize P_4 [11]. So EG-VEGF may act as a local message molecule between embryo and vessel in receptive endometrium, by targeting vascular endothelium in superficial endometrium which express its receptor, and sustain placenta development after implantation.

In summary, our study demonstrated that high E_2 produced in high ovarian responders may stimulate asynchronous endometrial development and down regulate EG-VEGF expression in peri-implantation endometrium when com-

pared with moderate ovarian responders and those in natural cycles, which may account for the impaired ER and lower pregnancy rates in the excessive responders. The involved feedback mechanism remains to be determined.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Min-Zhi Gao, Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Center for Reproductive Medicine, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200135, China. Tel: +86-21-20284570; E-mail: 328gao@sina.com

References

[1] Lessey BA, Yeh I, Castelbaum AJ, Fritz MA, Ilesanmi AO, Korzeniowski P, Sun J, Chwalisz K. Endometrial progesterone receptors and markers of uterine receptivity in the window of implantation. Fertil Steril 1996; 65: 477-83.

[2] Pierson RA. Imaging the endometrium: are there predictors of uterine receptivity? J Obstet Gynaecol Can 2003; 25: 360-8.

[3] Strowitzki T, Germeyer A, Popovici R, von Wolff M. The human endometrium as a fertility-determining factor. Hum Reprod Update 2006; 12: 617-30.

[4] Bourgain C and Devroey P. The endometrium in stimulated cycles for IVF. Hum Reprod Update 2003; 9: 515-22.

[5] Kolibianakis E, Bourgain C, Albano C, Osmanagaoglu K, Smitz J, Van Steirteghem A, Devroey P. Effect of ovarian stimulation with recombinant follicle-stimulating hormone, gonadotropin releasing hormone antagonists, and human chorionic gonadotropin on endometrial maturation on the day of oocyte pickup. Fertil Steril 2002; 78: 1025-9.

[6] Haouzi D, Assou S, Dechanet C, Anahory T, Dechaud H, De Vos J, Hamamah S. Controlled ovarian hyperstimulation for in vitro fertilization alters endometrial receptivity in humans:

protocol effects. Biol Reprod 2010; 82: 679-86.

[7] Fauser BC and Devroey P. Reproductive biology and IVF: ovarian stimulation and luteal phase consequences. Trends Endocrinol Metab 2003; 14: 236-42.

[8] Horcajadas JA, Riesewijk A, Polman J, van Os R, Pellicer A, Mosselman S, Simón C. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. Mol Hum Reprod 2005; 11: 195-205.

[9] Grondahl ML, Borup R, Lee YB, Myrhoj V, Meinertz H, Sorensen S. Differences in gene expression of granulosa cells from women undergoing controlled ovarian hyperstimulation with either recombinant follicle-stimulating hormone or highly purified human menopausal gonadotropin. Fertil Steril 2009; 91: 1820-30.

[10] Yu Ng EH, Yeung WS, Yee Lan Lau E, So WW, Ho PC. High serum oestradiol concentrations in fresh IVF cycles do not impair implantation and pregnancy rates in subsequent frozen-thawed embryo transfer cycles. Hum Reprod 2000; 15: 250-5.

[11] LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, Peale F, Gurney A, Hillan KJ, Ferrara N. Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 2001; 412: 877-84.

[12] Brouillet S, Hoffmann P, Alfaidy N, Feige JJ. Prokineticins: new regulatory peptides in human reproduction. Med Sci (Paris) 2014; 30: 274-9.

[13] Negri L, Lattanzi R, Giannini E, Melchiorri P. Bv8/Prokineticin proteins and their receptors. Life Sci 2007; 81: 1103-16.

[14] Battersby S, Critchley HO, Morgan K, Millar RP, Jabbour HN. Expression and regulation of the prokineticins (endocrine gland-derived vascular endothelial growth factor and Bv8) and their receptors in the human endometrium across the menstrual cycle. J Clin Endocrinol Metab 2004; 89: 2463-9.

[15] Ngan ES, Lee KY, Yeung WS, Ngan HY, Ng EH, Ho PC. Endocrine gland-derived vascular endothelial growth factor is expressed in human peri-implantation endometrium, but not in endometrial carcinoma. Endocrinology 2006; 147: 88-95.

[16] Haouzi D, Mahmoud K, Fourar M, Bendhaou K, Dechaud H, De Vos J, Reme T, Dewailly D, Hamamah S. Identification of new biomarkers of human endometrial receptivity in the natural cycle. Hum Reprod 2009; 24: 198-205.

[17] Lee YL, Chan YL, Chow WN, Ng EH, Lee KF, Yeung WS, Ho PC. Endocrine gland-derived vascular endothelial growth factor stimulates proliferation and tube formation in human uterine microvascular endothelial cell through the mitogen-activated protein kinase but not through the Akt pathway. Fertil Steril 2009; 91 Suppl 5: 2163-71.

[18] Ferrara N, LeCouter J, Lin R, Peale F. EG-VEGF and Bv8: a novel family of tissue-restricted angiogenic factors. Biochim Biophys Acta 2004; 1654: 69-78.

[19] Noyes RW, Hertig AT, and Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol 1975; 122: 262-3.

[20] Liva, KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-8.

[21] Udvardi MK, Czechowski T and Scheible WR. Eleven golden rules of quantitative RT-PCR. Plant Cell 2008; 20: 1736-7.

[22] Joo BS, Park SH, An BM, Kim KS, Moon SE, Moon HS. Serum estradiol levels during controlled ovarian hyperstimulation influence the pregnancy outcome of in vitro fertilization in a concentration-dependent manner. Fertil Steril 2010; 93: 442-6.

[23] Aflatoonian A, Oskouian H, Ahmadi S, Oskouian L. Can fresh embryo transfers be replaced by cryopreserved-thawed embryo transfers in assisted reproductive cycles? A randomized controlled trial. J Assist Reprod Genet 2010; 27: 357-63.

[24] Kodaman PH and Taylor HS. Hormonal regulation of implantation. Obstet Gynecol Clin North Am 2004; 31: 745-66, ix.

[25] Ubaldi F, Bourgain C, Tournaye H, Smitz J, Van Steirteghem A, Devroey P. Endometrial evaluation by aspiration biopsy on the day of oocyte retrieval in the embryo transfer cycles in patients with serum progesterone rise during the follicular phase. Fertil Steril 1997; 67: 521-6.

[26] Welsh AO and Enders AC. Chorioallantoic placenta formation in the rat: II. Angiogenesis and maternal blood circulation in the mesometrial region of the implantation chamber prior to placenta formation. Am J Anat 1991; 192: 347-65.

[27] Ng EH, Chan CC, Tang OS, Yeung WS, Ho PC. Endometrial and subendometrial blood flow measured during early luteal phase by three-dimensional power Doppler ultrasound in excessive ovarian responders. Hum Reprod 2004; 19: 924-31.

[28] Duncan WC, Shaw JL, Burgess S, McDonald SE, Critchley HO, Horne AW. Ectopic pregnancy as a model to identify endometrial genes and signaling pathways important in decidualization and regulated by local trophoblast. PLoS One 2011; 6: e23595.

[29] Macdonald LJ, Sales KJ, Grant V, Brown P, Jabbour HN, Catalano RD. Prokineticin 1 induces Dickkopf 1 expression and regulates cell proliferation and decidualization in the human endometrium. Mol Hum Reprod 2011; 17: 626-36.

[30] Evans J, Catalano RD, Morgan K, Critchley HO, Millar RP, Jabbour HN. Prokineticin 1 signaling and gene regulation in early human pregnancy. Endocrinology 2008; 149: 2877-87.

[31] Evans J, Catalano RD, Brown P, Sherwin R, Critchley HO, Fazleabas AT, Jabbour HN. Prokineticin 1 mediates fetal-maternal dialogue regulating endometrial leukemia inhibitory factor. Faseb J 2009; 23: 2165-75.