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Research Article

Superovulation with human chorionic gonadotropin (hCG) trigger and gonadotropin releasing hormone agonist (GnRHa) trigger differentially alter essential angiogenic factors in the endometrium in a mouse ART model†

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Abstract

Gonadotropin-releasing hormone agonists (GnRHa) are used as an alternative to human chorionic gonadotropin (hCG) to trigger ovulation and decrease the risk of ovarian hyperstimulation syndrome. GnRHa is less potent at inducing ovarian vascular endothelial growth factor (VEGF), but may also affect endometrial angiogenesis and early placental development. In this study, we explore the effect of superovulation on endometrial angiogenesis during critical periods of gestation in a mouse model. We assigned female mice to three groups: natural mating or mating following injection with equine chorionic gonadotropin and trigger with GnRHa or hCG trigger. Females were killed prior to implantation (E3.5), post-implantation (E7.5), and at midgestation (E10.5), and maternal serum, uterus, and ovaries were collected. During peri-implantation, endometrial *Vegfr1* and *Vegfr2* mRNA were significantly increased in the GnRHa trigger group ($P < 0.02$) relative to the hCG group. *Vegfr1* is highly expressed in the endometrial lining and secretory glands immediately prior to implantation. At E7.5, the ectoplacental cone expression of *Vegfa* and its receptor, *Vegfr2*, was significantly higher in the hCG trigger group compared to the GnRHa group ($P < 0.05$). Soluble VEGFR1 and free VEGFA were much higher in the serum of mice exposed to the hCG trigger compared to GnRHa group. At midgestation, there was significantly more local *Vegfa* expression in the placenta of mice triggered with hCG. GnRHa and hCG triggers differentially disrupt the endometrial expression of key angiogenic factors during critical periods

of mouse gestation. These results may have significant implications for placental development and neonatal outcomes following human in vitro fertilization.

Summary Sentence

The gonadotropin-releasing hormone agonists and human chorionic gonadotropin agents used to trigger ovulation before in vitro fertilization differentially modulate levels of key angiogenic factors during critical periods of implantation, trophoblast invasion, and placental development in a mouse model.

Key words: gonadotropin-releasing hormone agonist, human chorionic gonadotropin, assisted reproductive technology, superovulation, vascular endothelial growth factor, implantation, placental development

Introduction

Successful implantation requires synchronized communication between the blastocyst and endometrium, which is tightly regulated by several hormonal, immunologic, and pro-angiogenic processes. During human in vitro fertilization (IVF), the hormonal milieu of the endometrial–embryo environment is altered by gonadotropin administration for follicular growth followed by a luteinizing hormone (LH) analogue to induce the resumption of meiosis. This results in supraphysiological levels of ovarian steroids and excess production of vascular endothelial growth factor (VEGF) [1], a critical mediator of angiogenesis and implantation [2]. Its effects are mediated by two tyrosine kinase receptors, VEGFR1 also called FLT1 (fms-like tyrosine kinase) and VEGFR2 [3]. Studies of VEGFA, VEGFR1, or VEGFR2 homozygous knockout mice result in embryonic death [4, 5]. Even heterozygous mutation of VEGFA is lethal for mice [6, 7]. Interestingly, having too much activation of soluble VEGFR1, also called sFLT1, has been implicated in preeclampsia [8]. In IVF, VEGF is believed to be responsible for complications such as ovarian hyperstimulation syndrome (OHSS) [9]. Patients undergoing IVF and specifically patients with OHSS are known to be at an increased risk for disorders of placentation and lower birth weight [10–12].

Traditionally final oocyte maturation has been triggered using human chorionic gonadotropin (hCG) to mimic the preovulatory LH surge. In recent years, however, the hCG trigger has been replaced by GnRH agonist either alone or mixed with a small amount of hCG in a subset of patients [13]. Unlike hCG which has a long half-life lasting 24 h and efficacy of 5–7 days, gonadotropin-releasing hormone (GnRH) agonists are short acting (12–24 h) and therefore reduce the risk of OHSS [14]. This is believed to be due to lower VEGF production by the ovaries and is supported by a rodent study looking at the effect of superovulation followed by the GnRHa trigger on ovarian production of *Vegf*. In this study, Miller et al. found that the hCG and GnRHa triggering inversely modulated *Vegf* mRNA in rat and human granulosa cells [15].

Chronic exposure to hCG has also been found to downregulate LH receptors in the endometrium of baboons and humans leading to decreased endometrial receptivity [16]. Evans et al. investigated the effect of the hCG trigger on the endometrium and found enlarged spiral artery formation in superovulated women compared to natural cycle endometrium [17], suggesting an effect of superovulation on the vasculogenesis of the endometrium during the preimplantation period. However, there is little data on the effects of the GnRH agonist on endometrial receptivity and placentation. GnRH and LH receptors have been reported in human and rodent endometrium [18]. Several clinical studies have suggested decreased pregnancy rates related to inadequate luteal support following GnRH agonist trigger [19]. This is the first study to assess the impact of the GnRH trigger on uterine angiogenesis in a mouse model.

Therefore in this study we examine the effect of hCG or GnRH agonist trigger on endometrial angiogenesis, implantation, and placental vasculogenesis in a mouse model. Our objective is to determine the impact of superovulation (with final oocyte maturation induced with hCG or GnRH agonist) on endometrial angiogenesis during critical periods of murine gestation. We hypothesize that the GnRH agonist will differentially alter endometrial *Vegf* production, and impact implantation leading to aberrant placental development and function, which may have clinical implications in human IVF.

Materials and methods

Ethics

All experiments with mice were conducted in accordance with the SSR's specific guidelines and standards.

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Female CF1 mice (6–10-weeks old, Envigo) and adult B6D2F1/J males (Jackson Labs) were housed at 25 °C on a 14/10-h light (6 AM/8 PM) schedule. All mice were fed with breeder diet of irradiated 9F sterilizable rodent diet (24% crude protein, 9.3% fat, 36% carbohydrate) from Harlan laboratories (#7960, Teklad diets, Madison, WI, USA). All mice were maintained in accordance with the National Institute for Health Guide for Care and Use of Laboratory Animals.

Superovulation, tissue, and serum collection

Female mice were assigned to three different treatment protocols:

- GnRH agonist trigger group: 5 IU PMSG (G5270, MilliporeSigma, Burlington, MA, USA) was given via intraperitoneal (IP) injection, followed 48 h later by an IP injection of 3.5 μg GnRH agonist (leuprolide acetate, Sandoz Inc., Princeton, NJ, USA) as previously described [15].
- Human chorionic gonadotropin (hCG) trigger group: IP injection of 5 IU PMSG, followed 48 h later by IP injection of 5 IU hCG (Pregnyl, Merck & Co., Inc., Whitehouse Station, NJ, USA) [20].
- Control group: no injections.

All three groups (n = 10 per group) were mated to fertile B6D2F1/J males. The presence of a vaginal plug confirmed mating

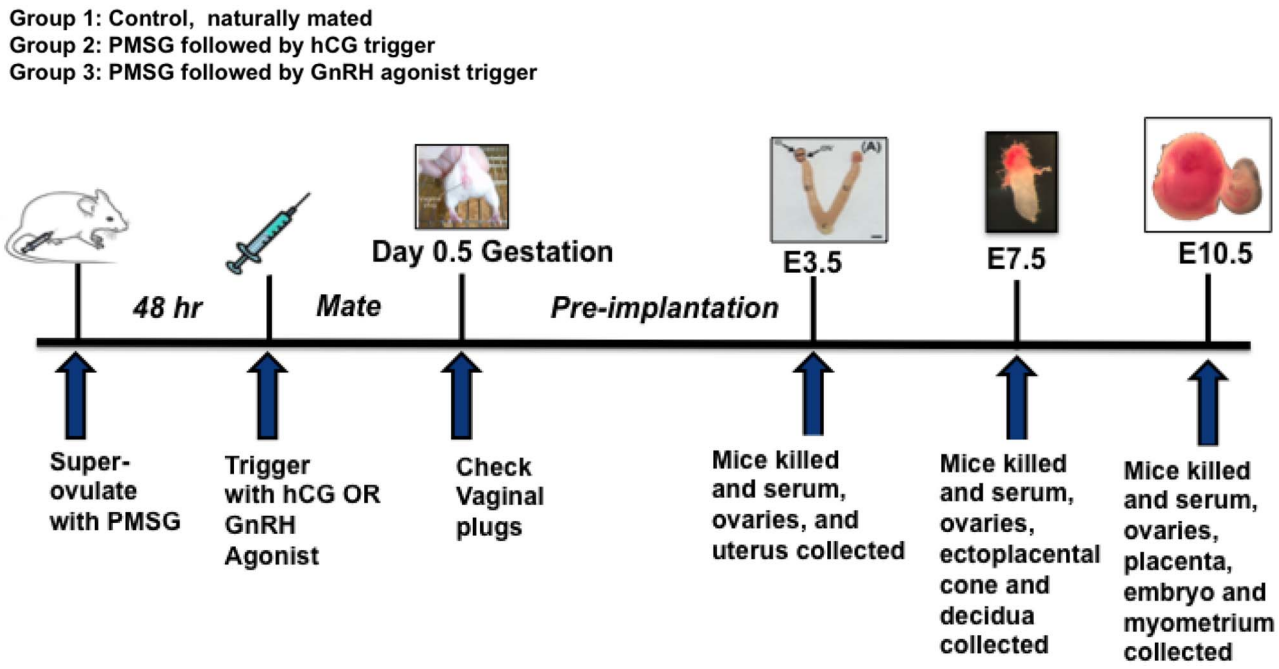


Figure 1. Experimental design: female mice were superovulated and ovulation was induced with either hCG or GnRHa trigger followed by mating with fertile males. A control group was also mated naturally. Vaginal plugs were checked the following day, embryonic day 0.5 (E0.5). Females were then killed at E3.5, E7.5, and E10.5 generating three groups at each gestational time point. Tissues and serum were collected for analysis. PMSG, pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin; GnRHa, gonadotropin releasing hormone agonist.

and was considered embryonic day (E) 0.5. The females were then separated from the males after copulation (Figure 1).

To assess the peri-implantation uterine microenvironment, pregnant mice were anesthetized using isoflurane (Butler Animal Health Supply, Dublin, OH, USA) and killed by cervical dislocation on embryonic day 3.5 (E3.5), and the ovaries and uterus were removed. Whole blood was collected using cardiac puncture for steroid hormone quantification. Serum was isolated and stored at −80 °C and analyzed for progesterone, VEGFA, and sVEGFR1 levels using Progesterone ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA), Quantikine Mouse VEGF Immunoassay (R&D Systems, Minneapolis, MN, USA), and Quantikine ELISA Mouse VEGFR1/Flt-1 Immunoassay (R&D Systems, Minneapolis, MN, USA), respectively.

Uterine tissue was fixed overnight in 4% paraformaldehyde in Phosphate buffered saline (PBS) for histologic analysis and snap frozen and stored at −80 °C for molecular analysis; total RNA was extracted from the whole mouse uterus and ovaries for quantitative reverse transcription polymerase chain reaction (qRT-PCR). To assess early placentation, a second set of female mice were mated with B6D2F1/J males in one of the three treatment groups as above and killed on E7.5. The number of implantation sites was recorded. The implantation sites were then dissected to collect ectoplacental cones and decidua, which were snap frozen and stored at −80 °C for molecular analysis. Pooled RNA from the ectoplacental cone was analyzed via qRT-PCR for expression of genes regulating trophoblast differentiation including *Hand1*, *Mash2*, *Stra13*, and *Tpbpa*.

Another set of mice ($n = 10$) were killed at E10.5 to assess resorption rate at midgestation. Placentas were dissected, snap frozen, and stored at −80 °C for molecular analysis. A subset of placentas and embryos were photographed using the Leica MZFLIII fluorescence stereo microscope (Leica Microsystems, Wetzlar, Germany) and then placed in formalin for histologic analysis and fixed overnight at 4 °C.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from frozen mouse tissue using the Total RNA Isolation/NucleoSpin RNA II Kit (Macherey-Nagel, Bethlehem, PA, USA), treated with DNase (DNA-free, Life Technologies), and quantified by absorbance at 260 nM. RNA (300 ng) was reverse-transcribed with random primers using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). RNA quality was assessed using the RNA ScreenTape Analysis which uses an RNA integrity number equivalent (RINe) to provide user-independent quality score for total RNA (Agilent Technologies, Inc., Germany). Gene primer sequences for *Vegfa*, *Vegfr1*, *Vegfr2*, *Lhr*, *Tpbpa*, *Stra13*, *Mash2*, *Hand1*, and *Gapdh* are shown in Supplementary Table S1. PCR was performed on an ABI PRISM 7500 sequence detector (Thermo Fisher Scientific, Waltham, MA, USA) with SYBR Green1 (Thermo Fisher Scientific). Each sample was run in triplicate, and the reactions were carried out in 40 cycles. Abundance of mRNA relative to reference gene *Gapdh* was calculated using the ΔC_T method [relative mRNA abundance $= 2^{-(\text{CT gene of interest} - \text{CT GAPDH})}$].

Immunofluorescence

Uterine tissue was fixed overnight at 4 °C in 4% paraformaldehyde in PBS, dehydrated, and paraffin embedded. Serial tissue sections of 4 µm thickness were cut and mounted on glass slides. Tissues were deparaffinized and rehydrated. Antigen retrieval was performed using citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0). Nonspecific antibody was blocked by incubation with 1% bovine serum albumin in Tris-buffered saline (TBS; 50 mM Tris base, 9% NaCl, pH 8.4). The primary antibody for VEGFR1 (ab32152, Abcam, Cambridge, MA, USA) diluted 1:400 in immunofluorescence antibody dilution buffer (Cell Signaling Technology; cat #12378) was applied and incubated overnight at 4 °C. Sections were then washed sequentially in TBS containing 0.5% Tween 20 (TBST; pH 8.4) and TBS followed by incubation at room temperature for 30 min

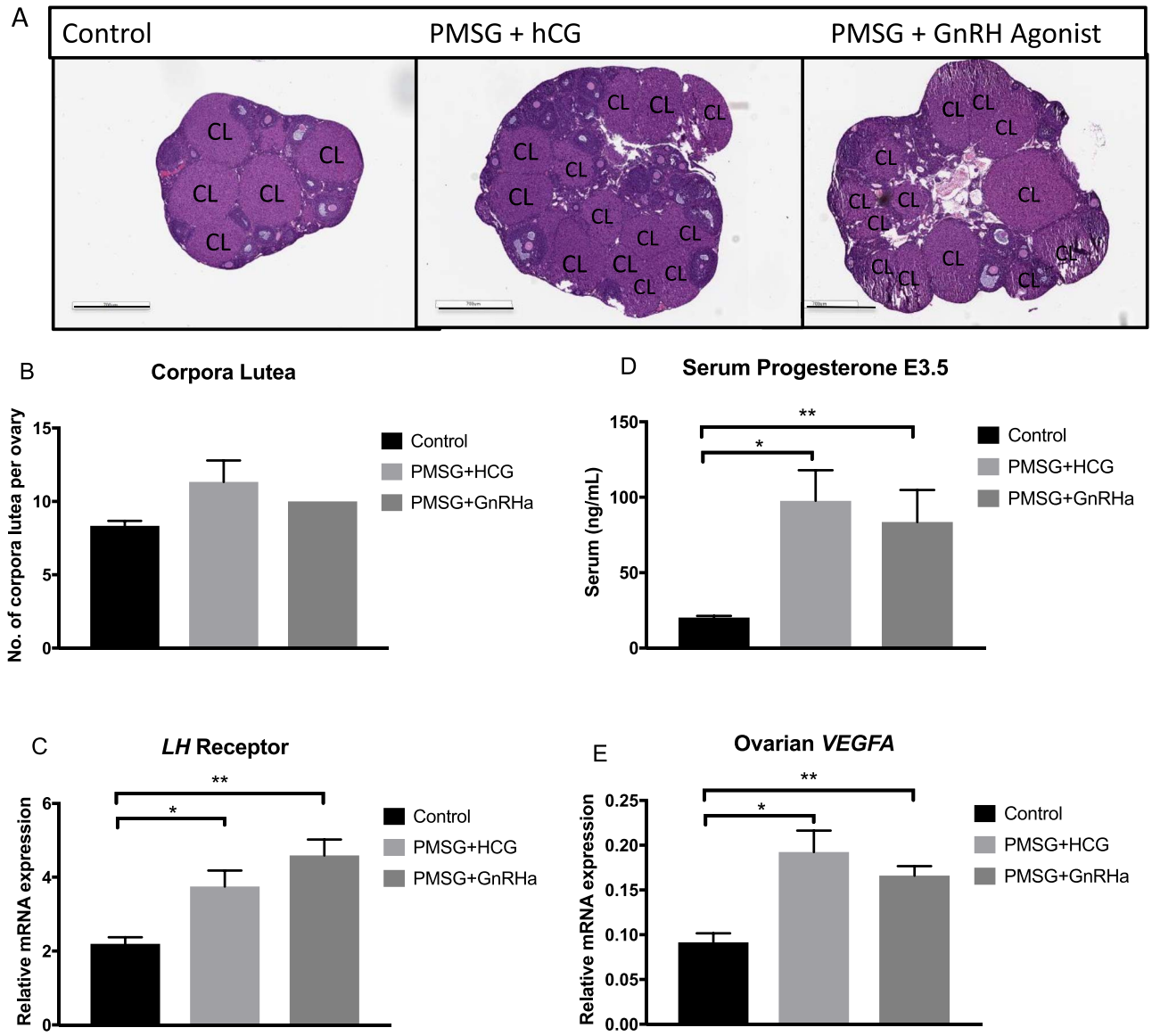


Figure 2. The ovarian response to superovulation induced with hCG and the GnRHa trigger on E3.5. (A) Representative H&E ovaries of superovulated mice compared to control. Scale bar: 700 μm. (B) Superovulation increased the number of corpora lutea in the superovulated groups compared to controls ($n = 4$–5 per group). (C) Ovarian *Lhr* mRNA expression was significantly higher in superovulated groups compared to naturally mated mice at E3.5 ($n = 4$–5 mice per group, $P < 0.02$). (D) Production of progesterone was elevated in the serum of superovulated mice compared to control ($n = 3$, $P < 0.05$). (E) Ovarian *Vegfa* mRNA expression was higher in the superovulated groups compared to control ($n = 4$–5 mice per group, $P < 0.01$). Mean ± SEM reported.

with anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (goat anti-rabbit, Thermo Fisher Scientific, Rockford, IL, USA cat# A-11008) diluted 1:500 in immunofluorescence antibody dilution buffer. The sections were then washed with TBS, and coverslip was mounted with a mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA; cat #H-1200). Sections were photographed using the DeltaVision fluorescent microscope imaging system (GE Life Sciences, Pittsburgh, PA, USA).

Histologic analysis

Ovaries and placentas were fixed overnight at 4 °C in 4% paraformaldehyde in PBS, dehydrated, and paraffin embedded. Placentas were oriented vertically so that cross-sections could be obtained. Serial tissue sections of 4 μm thickness were cut and mounted on glass slides by the Case Western Reserve Core (University Hospitals, Cleveland, OH, USA). Tissues were deparaffinized using citrate buffer and stained with hematoxylin and eosin (H&E). Corpora lutea were counted manually using the widest representative section. For the microvessel analysis, placentas were deparaffinized and stained with monoclonal antibody to PLVAP (MECA-32) (Bio-Rad, Raleigh, NC, USA). Quantification of vessel density was performed using Spectrum and ImageJ software [21].

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, La Jolla, CA, USA). All experiments were performed in triplicate or greater. Preliminary

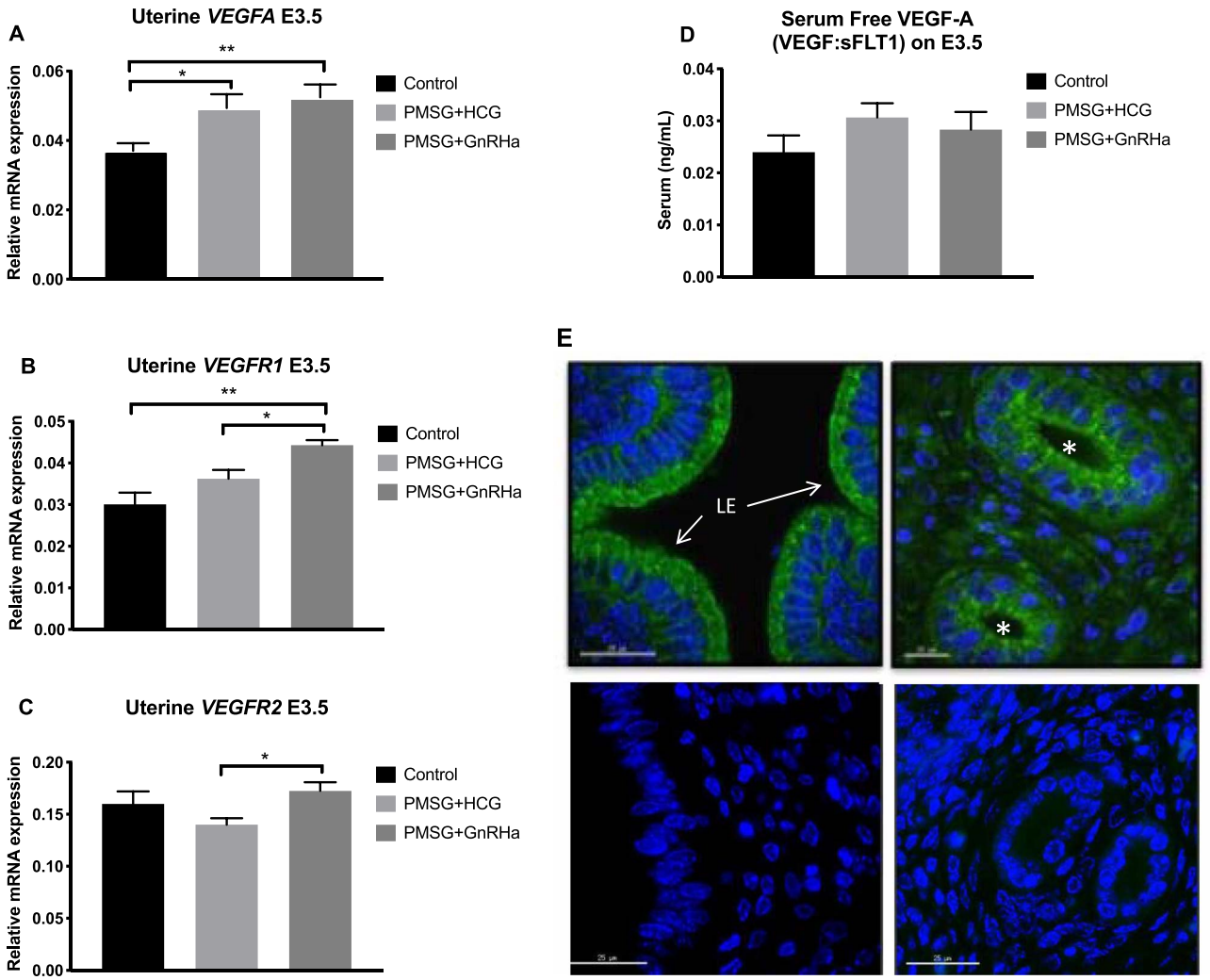


Figure 3. VEGFA and its receptors mRNA expression in the uterus after superovulation during the preimplantation period (E3.5). Adult female mice superovulated and triggered with hCG or the GnRH agonist had significantly higher uterine expression of (A) *Vegfa* at day 3.5 of gestation ($P < 0.05$). The GnRHa trigger increased (B) uterine *Vegfr1* and (C) *Vegfr2* compared to the hCG trigger ($P < 0.02$, $n = 4$–5 mice per group) ($n = 4$–5 mice per group). (D) Serum free VEGF levels were similar between the groups ($n = 3$ mice per group, $P = 0.6$). Data are presented as mean ± SEM. (E) VEGFR1 (stained green) is highly expressed in the uteri of E3.5 luminal epithelium lining (arrows) and secretory glands (stars). Negative controls in lower panels show no specific staining with secondary antibody alone. Scale bars: 15–25 µm.

power calculation demonstrated that 10 mice per group would provide 80% power at $\alpha = 0.05$, to detect an 8% difference in VEGF expression. One-way ANOVA followed by post hoc multiple comparisons testing was used to assess the differences in serum markers and gene expression between the three groups at E3.5. Only the hCG trigger group and GnRHa were compared at E7.5 and E10.5 using Student *t*-test. Control groups were not included in the statistical analyses at these time points due to the lower number of implantation sites in control compared to experimental groups. Controls were shown as a reference of "normal" values of the multiple variables. Differences were considered statistically significant when $P < 0.05$.

Results

Angiogenic signaling during early pregnancy

In order to confirm that our model of superovulation was successful, we examined the ovaries of mice superovulated with PMSG followed by the hCG or GnRH agonist trigger. We found more corpora lutea in the superovulated groups compared to the control group on day 3.5 of gestation (Figure 2A and B). Females exposed to superovulation had elevated ovarian mRNA expression of LH receptors and elevated serum progesterone levels ($P < 0.02$; Figure 2C and D). In addition, the superovulated groups had significantly higher ovarian *Vegfa* expression compared to naturally mated controls ($P < 0.01$; Figure 2E). There were no significant differences in the number of corpora lutea or serum progesterone levels between the two superovulated groups.

To investigate the mechanisms of how superovulation followed by the hCG and GnRH agonist trigger affects angiogenesis, we examined the uterus of pregnant mice on E3.5. We found increased expression of uterine *Vegfa* mRNA in both superovulated groups compared to controls (Figure 3A). Interestingly, mice triggered with the GnRHa had higher levels of uterine *Vegr1* and *Vegfr2* compared to the hCG trigger group (Figure 3B and C). However, serum levels of free VEGFA were similar regardless of the trigger agent (Figure 3D, Supplementary Figure S1). Using fluorescent immunohistochemistry, VEGFR1 was highly localized to the uterine

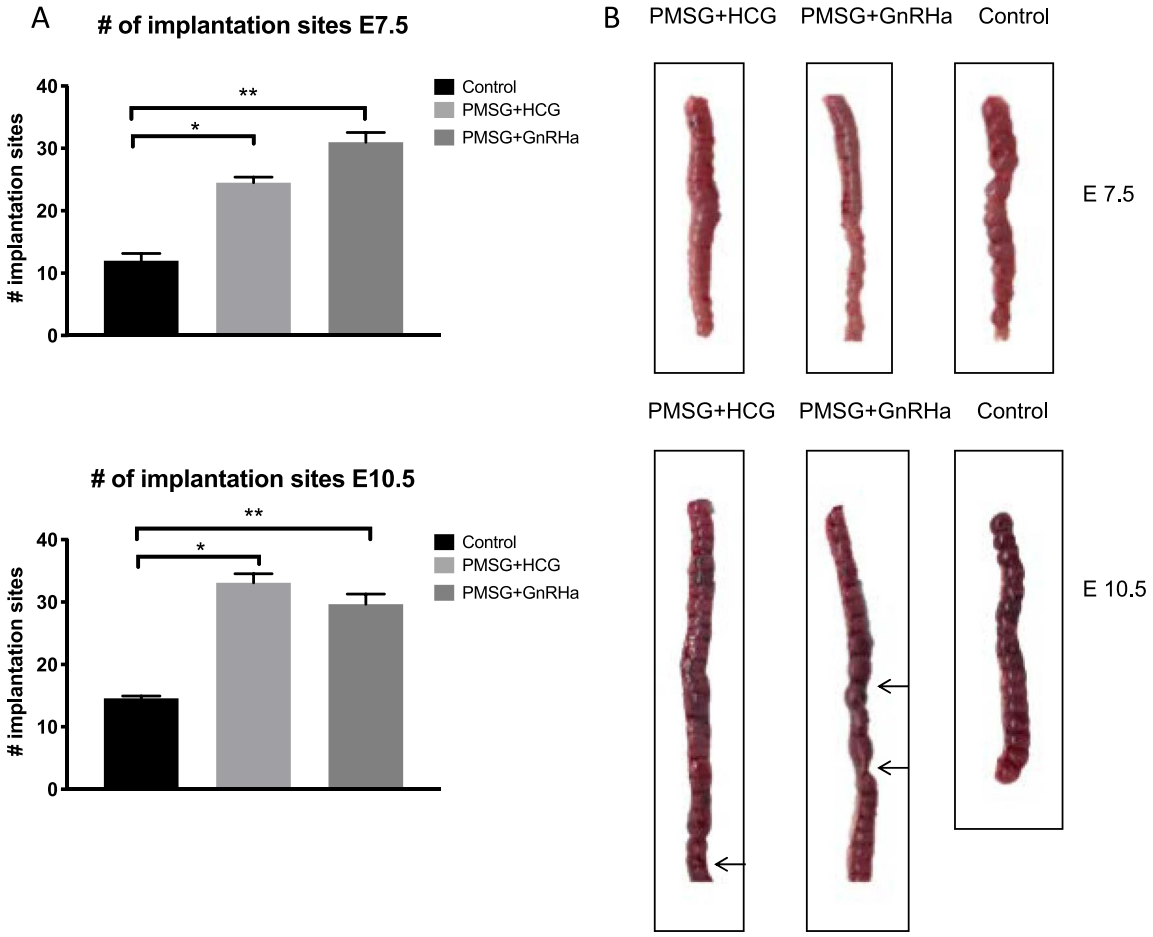


Figure 4. The impact of superovulation (induced with hCG and GnRHa triggers) on the post-implantation uterus. (A) The number of implantation sites in the superovulated groups were significantly higher relative to naturally mated controls ($P < 0.0001$, $n = 6$–10 mice per group). Data are represented as mean ± SEM. (B) Gross morphologic appearance of pregnant uteri at E7.5 (top) and E10.5 (bottom). Marked implantation sites at midgestation (E10.5) are resorption sites (black arrows).

secretory glands and endometrial lining during the preimplantation period (Figure 3E). This data suggest an important regulatory role of VEGFA/VEGFR1 during implantation.

Effect of superovulation with the GnRHa and hCG trigger on angiogenesis-related gene expression during early trophoblast invasion

On E7.5 and E10.5, there were significantly more implantation sites in both superovulated groups compared to the natural mating group. There was no difference in the number of implantation sites between the trigger groups (Figure 4). During this critical period in mouse gestation, the trophoblast of the ectoplacental cone begins to invade the decidua and differentiate into trophoblastic giant cells. Superovulation followed by the hCG trigger led to significantly higher expression of local *Vegfa* and its receptor, *Vegfr2*, in the ectoplacental cone as compared to the GnRHa trigger group (Figure 5). This suggests that hCG adversely affects angiogenic processes at the time of placental development. In addition, soluble *Vegfr1* (also called *sflt1*), known to be implicated in preeclampsia, is elevated in the hCG trigger group, which leads to less bioavailable VEGFA (Figure 6B and C). Total *Vegfa* (which includes bound and unbound VEGFA) was similar between the groups (Figure 6A).

During the post-implantation period, there are robust angiogenesis and invasion of the trophoblast into the decidua [22]. Markers of trophoblast differentiation, *Mash2* and *Hand1*, were similar between the superovulated groups. In contrast, *Stra13*, essential for the final step of trophoblast giant cell differentiation, was significantly higher in the hCG trigger group (Figure 7). This aberrant expression may account for some of the gross morphological abnormalities observed in superovulated placentas (Figure 8B).

Effect of superovulation on placental development

Midgestational (E10.5) embryos and uteri of naturally mated and superovulated mice were dissected and captured on the microscope. The embryos of superovulated mice were smaller compared to naturally mated embryos (Figure 8A). In addition, the corresponding placentas of superovulated mice were more fragmented and smaller compared to control mice (Figure 8B). A critical factor for maternal spiral artery formation, *Tpbpa*, was significantly lower in the placenta from the superovulated groups (Figure 8C). Finally, the number of resorptions was significantly higher in the superovulated groups comparedto controls (Figure 8D).

At midgestation (E10.5), serum levels of free VEGFA were most elevated in the hCG trigger group (Figure 9) corresponding to low levels of serum sVEGFR1. When analyzing the placenta, the hCG trigger group had the highest levels of local *Vegfa*

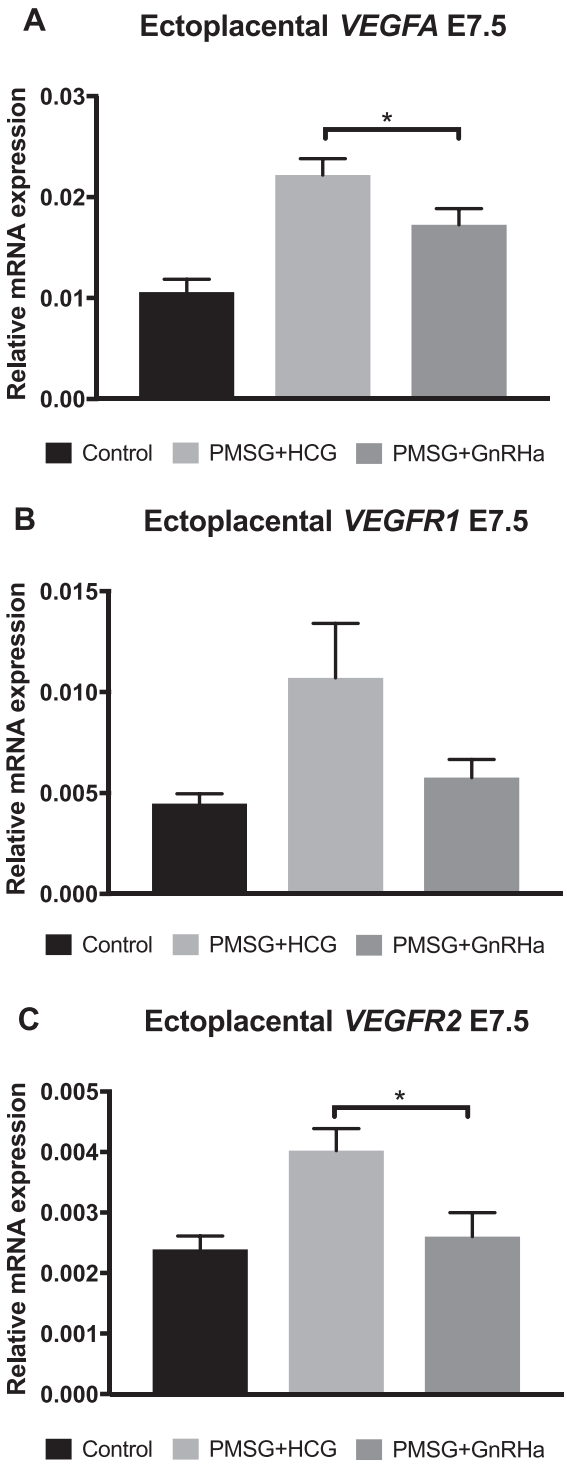


Figure 5. Superovulation followed by the hCG trigger differentially alters levels of VEGFA and its activating receptor VEGFR2 at E7.5. (A) During trophoblast development, the hCG trigger group increases ectoplacental cone expression of *Vegfa* relative to the GnRH trigger group ($P = 0.05$). (B) *Vegfr1* is not significantly higher ($P = 0.09$). (C) *Vegfr2* mRNA expression locally in the ectoplacental cone is significantly higher in the hCG triggered group ($P < 0.02$, $n = 6$ mice per group). Data are represented as mean ± SEM. Note: statistical analysis was only performed between superovulated groups (Student *t*-test). Controls (black) are shown as a reference.

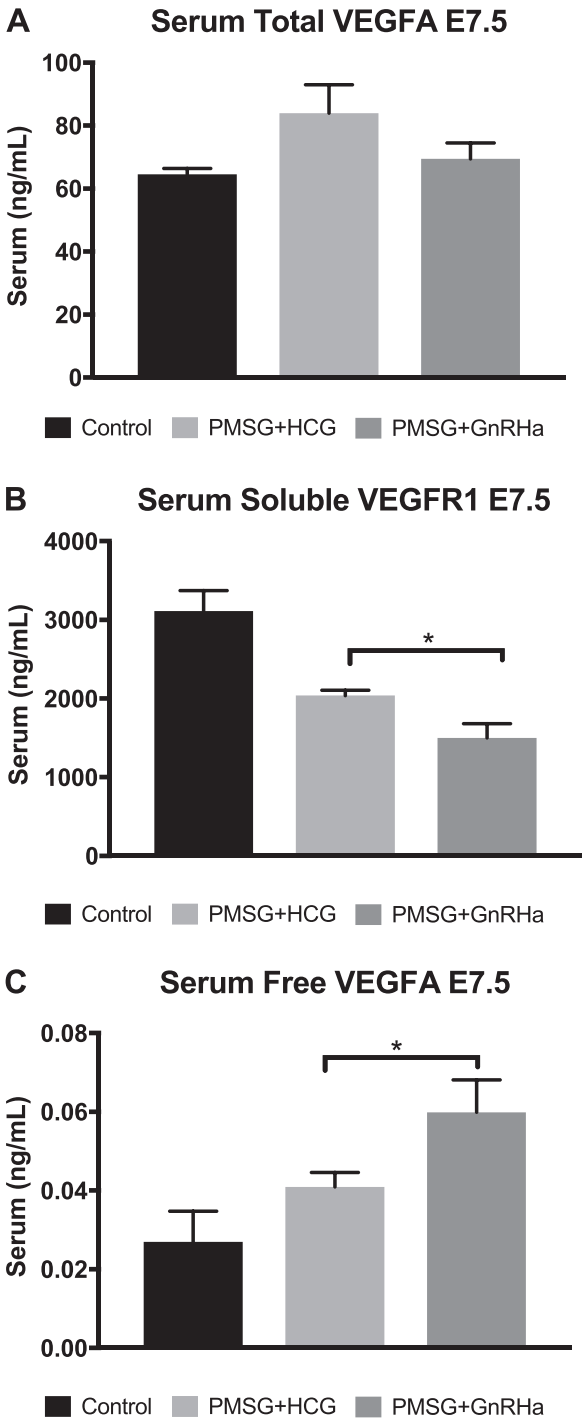


Figure 6. Post-implantation (E7.5) serum levels of total VEGF, soluble VEGFR1, and free VEGFA. (A) Total bound and unbound VEGF is similar between the groups ($P = 0.3$). (B, C) Soluble VEGFR1 is higher in the hCG group leading to less bioavailable VEGFA compared to the GnRH agonist group ($P < 0.05$, $n = 4$–6 mice per group). Mean ± SEM reported. Note: statistical analysis was only performed between superovulated groups (Student *t*-test). Controls (black) are shown as a reference.

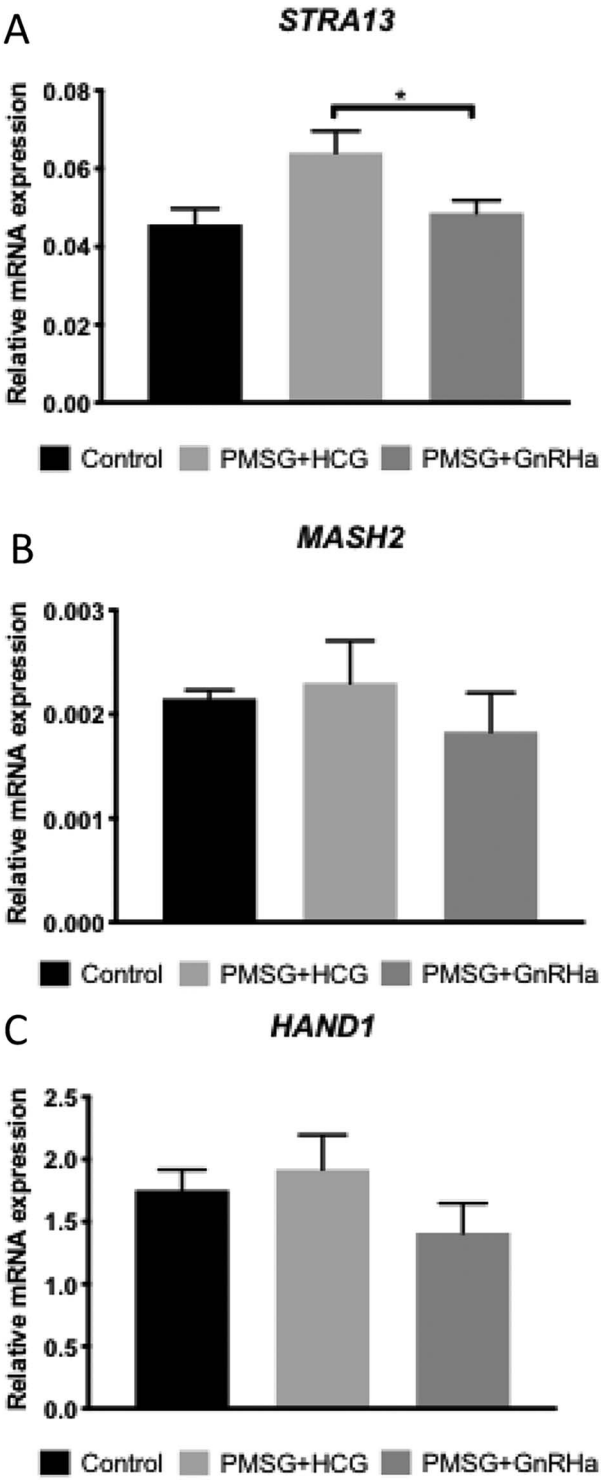


Figure 7. RT-qPCR analysis of trophoblast markers in the pooled ectoplacental cones of the three treatment groups. (A) *Stra13* is higher in the hCG trigger group compared to GnRH agonist and the control group ($P < 0.03$, $n = 6$ mice per group). (B, C) There were no differences in expression for *Mash2* and *Hand1* between the superovulated groups. Data displayed as mean ± SEM. Note: statistical analysis was only performed between superovulated groups (Student *t*-test). Controls (black) are shown as a reference.

expression (Figure 10A). Of note, the *Vegfa* receptors, membrane-bound *Vegfr1* and *Vegfr2*, were similar among the trigger groups (Figure 10B and C). There were no differences in placental microvessel density between the groups (Figure 10D). This suggests that elevated levels of bioavailable VEGFA at midgestation may stimulate placental *Vegfa* production.

Plasma progesterone, VEGFA, and soluble VEGFR1 concentrations

Circulating progesterone levels were significantly higher in both superovulated groups compared to natural mating at E3.5 ($P < 0.05$; Figure 2D). Levels of free VEGFA were lowest in the hCG trigger group on E7.5 and then found to be highest on E10.5. Free VEGFA levels were inversely related to soluble VEGFR1 (also called sFLT1), with the hCG trigger groups having the highest levels of sVEGFR1 on E7.5 and lowest levels on E10.5 (Supplementary Figure S1). This important negative regulator of VEGFA is associated with the development of preeclampsia [23], and levels were altered by superovulation.

Discussion

In this study, we used a novel mouse model involving superovulation and GnRHa trigger to examine early pregnancy and placental development. This is the first study to compare the GnRHa and hCG trigger on angiogenic processes of early pregnancy and placental development in a mouse model. Our results indicate that the GnRHa and hCG triggers differentially modulate levels of local and systemic VEGF and its receptors throughout pregnancy, which may have significant implications for the development of preeclampsia and intrauterine growth restriction. During early pregnancy, mice triggered with the GnRHa had increased expression of endometrial *Vegfr1* and *Vegfr2* compared to the hCG trigger group on E3.5. Importantly, VEGFR1 was localized to the luminal epithelium where the blastocyst is implanting, suggesting a possible regulatory role for *Vegfa/Vegfr1* during embryo implantation. During the critical period of trophoblast invasion and differentiation (E7.5), we found an increased production of *Vegfa* and *Vegfr2* in the ectoplacental cones of mice triggered with hCG. There was a trend toward increased membrane-bound *Vegfr1* in the ectoplacental cone ($P = 0.09$). Most clinically significant was the increased systemic levels of soluble VEGFR1 in mice triggered with hCG, similar to the animal models used to induce preeclampsia [24]. At midgestation, we found local placental *Vegfa* production to be highest in the hCG trigger group and serum levels of soluble VEGFR1 (also called sFLT1) highest in the GnRHa trigger group. This again mimics the animal models of preeclampsia induced by excess placental expression of *Vegfa*. We found higher VEGF levels at E7.5, while the HCG group showed high VEGF levels at E10.5. One possible explanation for these findings may be related to the half-life of HCG compared to the GnRH agonist. In humans, we know the half-life of hCG is 24 h with an efficacy of 5–7 days compared to GnRH agonists which are metabolized within 12–24 h. Perhaps this phenomenon is also occurring in the mouse where the GnRH agonist is causing an initial surge in free VEGFA levels at E7.5 and then it drops off by E10.5. However, the hCG continues its effects leading to a steady rise in free VEGFA levels at E10.5.

Large epidemiological studies have shown that singletons conceived from fresh embryo transfer had an increased risk of low birth weight, preterm delivery, and preeclampsia as compared to spontaneous conception [25–27]. Many human and animal studies have shown these adverse outcomes are due to exposure to supraphysiological hormone levels from ovarian stimulation [28,

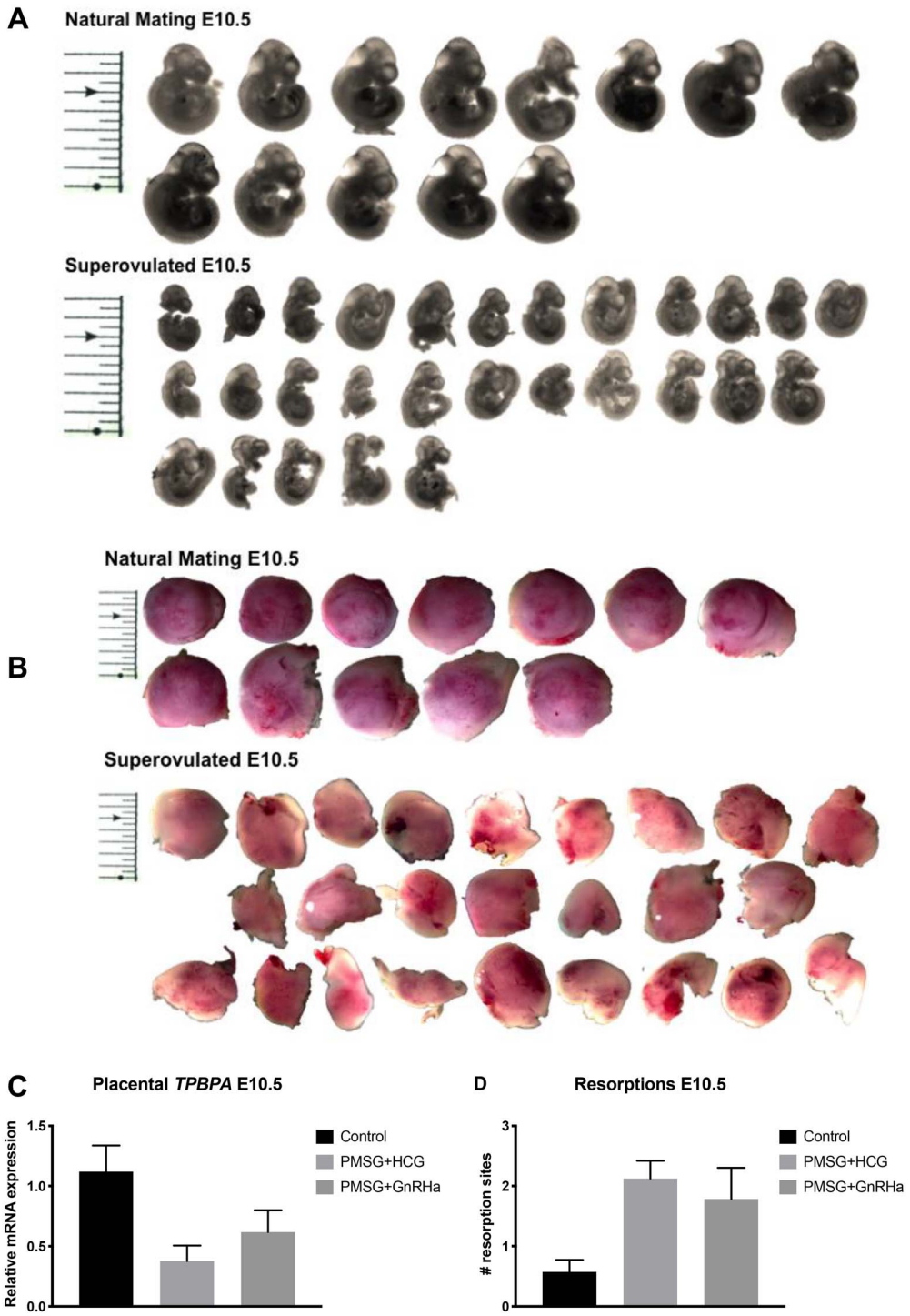
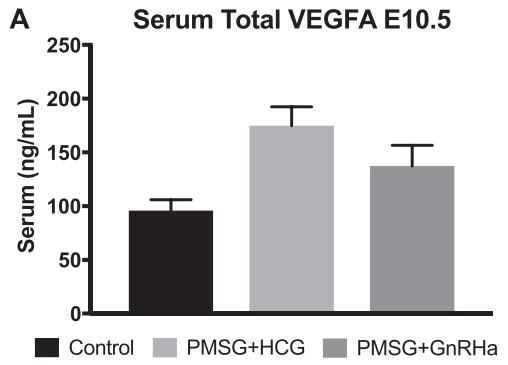
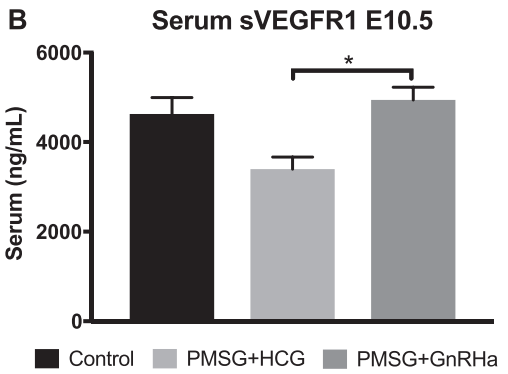
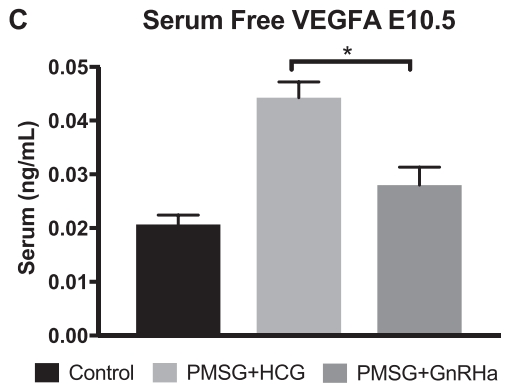


Figure 8. Impact of superovulation on embryonic and placental development. (A) Gross morphological appearance of E10.5 embryos developed in superovulated mice triggered with hCG and GnRHa. (B) Gross morphological appearance of E10.5 placentas from superovulated and control mice. Scale bars: 1 mm. (C) qRT-PCR of placental *Tpbpa*, essential for maternal spiral artery formation, is similarly low in both superovulated groups. (D) The number of resorbed sites was similar in the superovulated groups regardless of trigger agent, but significantly higher than controls ($n = 7$–10 mice per group). Mean ± SEM reported. Note: statistical analysis was only performed between superovulated groups. Controls (black) are shown as a reference.

29]. It is unknown if the adverse pregnancy outcomes associated with IVF have their origins during the preimplantation environment, which then translate into abnormal trophoblast differentiation and eventually placental formation. Human studies have attempted to understand this critical period of development and found that IVF pregnancies had significantly lower placental volumes and lower perfusion of the endometrium during the first trimester when compared to natural conception, with fresh embryo pregnancies having







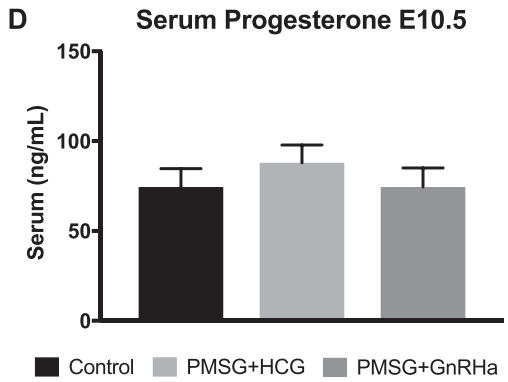
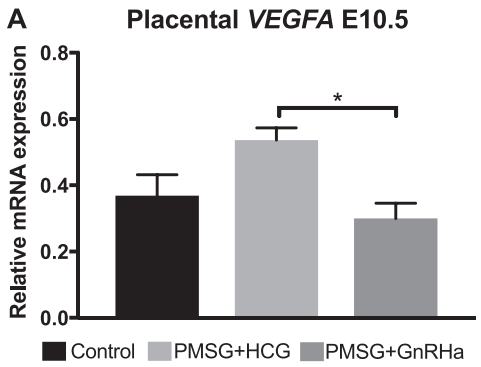
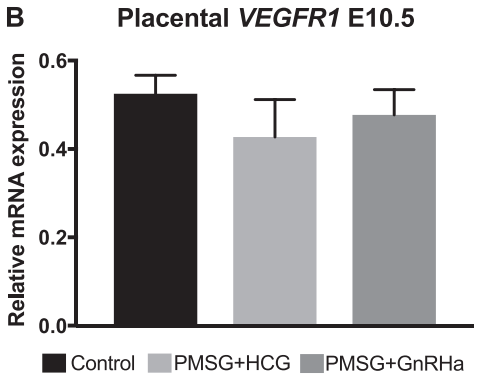
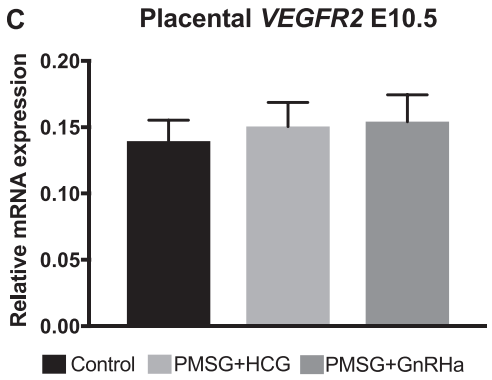


Figure 9. Serum levels of critical angiogenic factors at midgestation. (A) Both trigger groups had similar total serum VEGFA. (B) The hCG trigger group had lower levels of soluble VEGFR1 ($P < 0.02$) leading to (C) elevated free bioavailable VEFA ($P < 0.02$). (D) Progesterone levels at midgestation were similar in the superovulated groups. Mean ± SEM reported ($n = 3$–6 per group). Note: statistical analysis was only performed between superovulated groups. Controls (black) are shown as a reference.







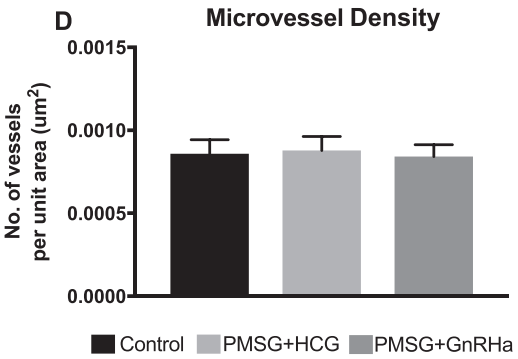


Figure 10. Superovulation with hCG trigger leads to overexpression of placental VEGFA at midgestation. (A) Local placental *Vegfa* production was higher in the hCG group compared to the GnRHa trigger group ($P < 0.003$). (B, C) *Vegf* receptors were similar in the trigger groups ($n = 8$–10 placentas). (D) Placental microvessel density was similar between all groups ($n = 7$–11 placentas). Note: statistical analysis was only performed between superovulated groups. Controls (black) are shown as a reference.

the lowest placental volume compared to frozen-thawed embryos [30, 31]. Indeed, in our assisted reproductive technology (ART) mouse model, we found altered levels of *Vegfa* and its receptors in the endometrium, developing trophoblast and placentas of mice exposed to supraphysiological hormones and further modulated by the ovulation trigger agent. This may be part of the mechanism for the impaired implantation and abnormal placentation observed in superovulated mice [11].

Clinical studies have suggested elevated levels of sVEGFR1 (also called sFLT1) to be correlated with the development of preeclampsia, usually increasing 5 weeks before development of symptoms [32, 33]. These studies collected serum levels of sVEGFR1 in the early third trimester in humans and animals [32, 34]. Very few studies have actually looked at the origins of preeclampsia from the early stages of trophoblast invasion. One animal study attempted to understand the mechanism of elevated sVEGFR1 in pregnancies complicated by preeclampsia. By overexpressing *Vegf* specifically in the mouse endometrium, they found that endometrial *Vegf* induced placental *sVegfr1* production and elevated maternal sVEGFR1 serum levels which led to miscarriage, placental dysfunction, and preeclampsia [24]. Our model is consistent with these findings, as we also observed elevated local production of *Vegf* in the hCG trigger group at E7.5 and E10.5, elevated serum levels of VEGFR1 in both trigger groups, and consequently lower bioavailable VEGF leading to adverse pregnancy outcomes. Interestingly, at midgestation (E10.5), we found elevated levels of sVEGFR1 in the GnRHa trigger group. Regardless of the trigger agent, superovulation altered the levels of endometrial-specific *Vegf* leading to abnormal serum concentrations of VEGFR1 and bioavailable VEGFA, mimicking the current working model used to induce preeclampsia.

Mechanisms for abnormal placental vasculogenesis may originate with abnormal trophoblast differentiation and invasion. We found significantly higher *Stra13* and a trend toward increased levels of *Mash2* and *Hand2* mRNA expression in the ectoplacental cones of mice triggered with hCG (Figure 7). Stra13 has been shown to induce arrest of cell cycle proliferation and stimulate trophoblast giant cell formation [35]. These transcription factors are critical for differentiation of the trophoblast [36], and aberrant expression of Stra13 may account for the dysfunction observed in placentas from IVF pregnancies. Future studies could pool more ectoplacental cones after an embryo transfer to see if there is an effect of the trigger agent or superovulation on trophoblast invasion compared to natural pregnancy. Finally, at E10.5, *Tpbpa*, a transcription factor critical for maternal spiral artery remodeling and placental function [37], was found to be abnormally low in both superovulated groups. This could explain in part the decreased placental size in the superovulated groups. However, when we analyzed microvessel density between the groups, we found no differences. Although we found no changes in microvessel density, this only sheds light on placental vasculogenesis. If there were changes in uterine arterial angiogenesis, which could directly impact blood flow to the fetus, these would not be seen in the microvessel density. Measuring changes in uterine blood flow by Doppler ultrasound studies could be a target of future work. Recent findings have also suggested an association between the immune response of the endometrium and endometrial angiogenesis (extension of existing blood vessels) and placental vasculogenesis (de novo formation of new blood vessels) [38, 39]. Future studies could analyze immune response in the placenta as a marker of aberrant angiogenesis, including expression of natural killer cells [40], which are also involved in the regulation of angiogenesis [41].

Our data on pregnancy outcomes are limited by the lack of an embryo transfer. We mitigated this by limiting our comparisons at E7.5 and E10.5 to the experimental groups only, where litter size was similar, and excluding controls, which had a smaller litter size. It is possible that overcrowding may have skewed placental size, embryo health, and production of the measured outcomes. However, since both trigger groups were similarly superovulated, any differences could be attributed to the trigger agent and not the uterine overcrowding. Future studies involving embryo transfer would be useful to assess later pregnancy outcomes and to directly compare to a natural environment.

The implications of our pilot study indicate that the trigger agent has the potential to alter levels of critical angiogenic factors involved in early pregnancy establishment and placental development following human IVF. More studies are needed to characterize the effect of the GnRH agonist trigger on embryonic growth, pregnancy, and neonatal outcomes as its use is increasing to mitigate the risk of OHSS.

Author's contributions

R.W. was involved in study concept and design, statistical analysis, and critical revision of the manuscript and figures. S.M. was involved in study concept, design, and data interpretation and he also provided the laboratory space and equipment to complete the study. T.R.S. was involved in study concept and design, performing all experiments, drafting the manuscript, and responsible for the final content submitted for publication. J.G. was involved in study concept and design and revision of manuscript. M.M. was involved in study concept and design and data interpretation. Y.S.P., G.P., J.W., P.A., and T.R.S. were involved in performing all experiments and obtaining the data. All authors were involved in critical revision of the manuscript and the approval of the final manuscript.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest

The authors have declared that no conflict of interest exists.

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