Recombinant human follicle-stimulating hormone alters maternal ovarian hormone concentrations and the uterus and perturbs fetal development in mice

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Kelley, Rebecca L., Karen L. Kind, Michelle Lane, Rebecca L. Robker, Jeremy G. Thompson, and Lisa J. Edwards. Recombinant human follicle-stimulating hormone alters maternal ovarian hormone concentrations and the uterus and perturbs fetal development in mice. *Am J Physiol Endocrinol Metab* 291:*E761–E770, 2006. First published May 23, 2006; doi:10.1152/ajpendo.00079.2006.—Gonadotropins are routinely administered to produce multiple oocytes for clinical in vitro fertilization (IVF) treatment, laboratory research, and livestock industries. Studies in mice have shown gonadotropin stimulation using equine chorionic gonadotropin (eCG) affects the endometrium, implantation, and fetal development. Evidence from clinical studies also indicates that stimulation with recombinant human follicle-stimulating hormone (rhFSH) may be detrimental to the endometrium and implantation rates. We investigated the effect of rhFSH in mice on maternal plasma hormone concentrations and uterine gene and protein expression and the effect of a stimulated maternal environment on pregnancy. Adult females were stimulated with rhFSH or eCG, followed by human chorionic gonadotropin (hCG). On day 4 of pseudopregnancy, mice either had embryos transferred to the uterus or were killed, and blood and uterine samples were collected. Pregnancy outcomes were examined on day 15. Gonadotropin stimulation increased plasma progesterone concentrations on day 4 compared with controls, whereas estradiol concentrations were unaffected. Stimulation also reduced uterine leukemia inhibitory factor (*Lif*) mRNA, but the expression of estrogen and progesterone receptors (*Esr1* and *Pgr*), homeobox gene *Hoxa10*, and *Vegf* mRNA were unchanged. Furthermore, distribution of uterine PGR protein expression was altered by stimulation, but LIF protein was unchanged. Stimulated embryo transfer recipients had lower pregnancy rates than controls, and fetuses from the rhFSH group had reduced weight, length, and maturity. These results demonstrate that gonadotropin stimulation with rhFSH or eCG alters the preimplantation maternal environment, which results in reduced pregnancy rates and fetal development in the mouse.

endometrium; equine chorionic gonadotropin; ovarian stimulation; gonadotropin; mouse

GONADOTROPI C HORMONES ARE routinely administered during in vitro fertilization (IVF) treatment to stimulate the ovaries to produce high numbers of oocytes. Typically, exogenous follicle-stimulating hormone (FSH) is used to mature multiple follicles, then human chorionic gonadotropin (hCG) mimics luteinizing hormone (LH) by inducing ovulation. Although this procedure is widely used in human IVF, laboratory research and livestock industries, the possible consequences of gonadotropin stimulation are largely unknown. Evidence from some studies indicates that stimulation before IVF causes endometrial abnormalities and may reduce implantation rates (6, 13, 33, 34). Studies in mice have revealed that exogenous gonadotropins perturb embryo and oocyte development (15–18, 50) and cause changes to the mother (40, 44, 45), both of which contribute to reduced implantation rate, fetal development rate, and fetal weight (16, 18, 21, 51). Preparations of exogenous gonadotropins can vary, and to date the majority of animal studies have used equine chorionic gonadotropin (eCG) as a source of exogenous FSH. In this study, we have investigated the effect of recombinant human (rh)FSH, which is commonly used in clinical IVF, on the endometrium and fetal development in mice.

Although it is possible that changes to the endometrium are induced directly by exogenous gonadotropins (42), the cyclic development of the endometrium is controlled by the ovarian steroid hormones estrogen and progesterone (8). It has been demonstrated in several species that endogenous ovarian hormone concentrations are altered by gonadotropin stimulation (17, 28, 31, 32), so it is likely that stimulation influences the uterus indirectly by altering estradiol and progesterone production. Changes in steroid hormone concentrations can also influence implantation rates and fetal development in the rat and sheep (3, 26). Changes in the expression of steroid hormone receptors [which have been observed after gonadotropin stimulation in women (20, 23, 33, 36)] may also influence endometrial receptivity, because they mediate cellular responses to those hormones. Implantation also requires the correct expression of uterine cytokines. Many of these, such as leukemia inhibitory factor (*LIF*), vascular endothelial growth factor (*VEGF*), and homeobox A10 (*Hoxa10*), are hormonally regulated, and their expression can indicate the receptive state of the endometrium. In the present study, therefore, we have examined the effect of gonadotropin stimulation on the uterine expression of *LIF*, *VEGF*, and *Hoxa10*.

The majority of research into gonadotropin stimulation in animals has been carried out using eCG, which has both FSH- and LH-like activity (1, 35). Few studies have investigated the effects of rhFSH, which is a pure form of the hormone produced by Chinese hamster ovary cells (25). The dual actions of eCG may affect ovarian follicle development differently from rhFSH, thereby causing different ovarian steroid hormone production and ultimately different endometrial responses. Until very recently, virtually all research in animal

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models has used eCG, rather than the rhFSH administered for clinical IVF. Utilizing the rhFSH stimulation protocol for mice recently developed by Edwards et al. (15), this study aimed to investigate the effects of rhFSH stimulation on preimplantation ovarian hormone concentrations, endometrial gene and protein expression, and the influence of the stimulated maternal environment on implantation and fetal development in mice.

MATERIALS AND METHODS

Animals. All procedures were approved by The University of Adelaide Animal Ethics Committee and The North Western Adelaide Health Services Animal Ethics Committee. Mice were housed under a 14:10-h light-dark regimen and had free access to standard pellet food and water. All mice used were F1 hybrid CBA × C57Bl/6.

Unless otherwise specified, all chemicals are from Sigma Chemical (Castle Hill, NSW, Australia).

Gonadotropic hormone treatments. Adult virgin female mice (8–11 wk old) were divided into four groups for gonadotropin stimulation. Mice received 2.5 or 10 IU of rhFSH (Puregon; Organon, Oss, The Netherlands) given as two injections, 24 h apart, or 5 IU eCG (Folligon serum gonadotropin; Intervet Australia, Victoria, Australia) as one injection of hormone plus one control injection of saline, 24 h apart. To induce ovulation, 5 IU hCG (Pregnyl; Organon) were given 24 h after the second injection. Injections were 0.1 ml intraperitoneal in 0.9% saline, and control animals received saline only injections. Doses of rhFSH were selected from an earlier study that showed 2.5 IU induced minor increases in the number of ovulations and that 10 IU induced the maximum response (15). The gonadotropin stimulation protocol was synchronized to the mouse’s estrus cycle [determined by daily vaginal smears (43a)] such that the hCG injection was given when the mouse would naturally be in estrus. Immediately after the third injection, mice were caged individually with a vasectomized male of proven fertility overnight. The following morning was deemed day 1 of pseudopregnancy if a copulatory plug was observed. On day 4 of pseudopregnancy, either an embryo transfer was performed or mice were humanely killed and uterus and blood samples were collected for analysis.

Day 4 pseudopregnancy. On day 4 of pseudopregnancy [the time of preimplantation endometrial receptivity (8, 14)], the effect of gonadotropin administration on steroid hormone concentrations and uterine characteristics were examined. Mice were anesthetized using 2% ketamine administration on steroid hormone concentrations and uterine characteristics were examined. Mice were anesthetized using 2% ketamine hydrochloride (Ketalar; Parke-Davis, Morris Plains, NJ) and 1% xylazine (Rompun; Bayer, Shawneetown, IL) in 0.9% saline injected intraperitoneally. Mice were anesthetized using 2% avertin (65:35 mixture of 2.5% tribromoethanol and N,N,N′,N′-tetrabromoethylenediamine; Pax Ravon Pharmaceuticals, Raleigh, NC). Uterine horn was cut into sections and fixed in 4% paraformaldehyde for protein expression analysis or frozen in liquid nitrogen for gene expression analysis. RNA extraction. Uterine samples were thawed and homogenized in Tri Reagent using an Ultra-Turrax homogenizer (Ika-Werk; Janke & Kunkel, Staufen, Germany). Total RNA was extracted using Tri Reagent according to the manufacturer’s instructions, as previously described (15). Briefly, the samples were centrifuged, and the supernatant was pipetted into a fresh tube. Chloroform (0.2 ml) was added, and then the samples were shaken and incubated at room temperature for 10 min. Samples were then centrifuged, and the upper aqueous phase was transferred to fresh tube. Isopropyl alcohol (0.5 ml) was added to precipitate RNA at −80°C overnight. Samples were centrifuged, and the RNA pellet was washed with ice-cold 75% ethanol, shaken, and centrifuged. The pellet was air dried and resuspended in 40 μl molecular-grade water. DNA contamination was excluded by treating samples with DNase (DNase-free; Ambion, Austin, TX) in buffer (Ambion) for 30 min at 37°C. DNase was inactivated by 5 µl DNase inactivation reagent (Ambion), and then samples were centrifuged and the RNA supernatant was transferred to fresh tube.

Reverse transcription. Reverse transcription was carried out using Superscript II RNase H-Reverse Transcriptase (RT) (Invitrogen). Two micrograms of RNA in a total volume of 11 µl were incubated with random primers (250 ng; Roche Applied Science, Castle Hill, NSW, Australia) at 70°C for 10 min. Samples were made up to a volume of 19 µl containing 1× reaction buffer (Invitrogen), 10 mM dithiothe- til (Invitrogen), and 0.5 mM dNTPs (Applied Biosystems, Foster City, CA). Reactions were incubated at 25°C for 10 min and then at 42°C for 2 min. For a final volume of 20 µl, 200 U Superscript II RT (Invitrogen) were added and incubated at 42°C for 50 min, followed by 70°C for 15 min to inactivate the enzyme. The cDNA was stored at −20°C.

Real-time RT-PCR and primers. Real-time PCR was performed in a 5700 GeneAmp sequence detection system (Applied Biosystems). The thermal cycling program was 2 min at 50°C and then 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A cycle threshold value was calculated by GeneAmp 5700 software v1.3. Oligonucleotide primers were designed using Primer express software v2.0 (Applied Biosystems) and were synthesized by Gene-works (Hindmarsh, SA, Australia) (Table 1). Standard curves were generated using eight dilutions in water of uterine cDNA to give concentration ranges equivalent to cDNA generated from 0.005 to 50

Groups of ~15 morphologically normal presumptive zygotes were cultured in 20-μl drops of mKSOM overlaid with oil at 37°C in 5% O2-6% CO2-89% N2. After ~90 h in culture, embryos at blastocyst stage were transferred to recipients anesthetized with 2% avertin. The uterus was located and punctured with a sharp, finely drawn glass pipette (Total Quality Manufacturing, Marino, SA, Australia) containing 6 blastocysts. The blastocysts and <5 μl of medium were expelled into the uterine lumen, and the procedure was repeated on the other uterine horn. Twelve blastocysts were transferred to each mouse. The outer incision was closed using Michel suture clips (Fine Science Tools, Vancouver, BC, Canada).

Assessment of implantation rate and postimplantation development. On day 15 of pregnancy, embryo transfer recipients were killed by cervical dislocation, and their uterine contents were examined. The number of fetuses and resorptions was recorded. Fetuses were weighed and crown-rump length measured, and their maturity was assessed using a scoring system based on the external development of skin, limbs, eyes, and ears (30, 52). Placentas were also weighed, and the surface area was measured. Hormone concentrations. Plasma estradiol and progesterone concentrations were determined by RIA using RIA kits from Diagnostic Systems Laboratories (DSL; Houston, TX). According to the manufacturer, the DSL-4400 estradiol RIA kit has 100% specificity for 17β-estradiol and the sensitivity is 4.7 pg/ml, and the DSL-3400 progesterone kit has 100% specificity for progesterone and the sensitivity is 0.10 ng/ml. The interassay coefficient of variation was ~<15%, and the intra-assay coefficient of variation was ~<10% for both assays.
Table 1. Primer sequences used for real time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank Accession No.</th>
<th>Primer Sequence</th>
<th>Amplion Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lif</td>
<td>NM_008501</td>
<td>Forward CGCGTGTTCAGACCTTGC</td>
<td>100 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse CTATGTTTCAGGCTTTCTT</td>
<td></td>
</tr>
<tr>
<td>Vegf</td>
<td>NM_009505</td>
<td>Forward CCACGTCAAGAGAAGCAATCA</td>
<td>75 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse TCATTCTCTCTATGGCTGGTCTTT</td>
<td></td>
</tr>
<tr>
<td>Hoxa10</td>
<td>NM_008263</td>
<td>Forward TGAGGTCATAGTGCAAGGA</td>
<td>78 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse CCTGGACTCCTCGATAAACAAC</td>
<td></td>
</tr>
<tr>
<td>Esr1</td>
<td>NM_007956</td>
<td>Forward AGGCAGCTACGGAAGAC</td>
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<tr>
<td></td>
<td></td>
<td>Reverse CTCTGCGGCTGCAATCA</td>
<td></td>
</tr>
<tr>
<td>Pgr</td>
<td>NM_008829</td>
<td>Forward TCTAGGAGGCAGAAGACAA</td>
<td>79 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse TGGCTCTCTGAATGCTGTTA</td>
<td></td>
</tr>
<tr>
<td>18s rRNA</td>
<td>AF176811</td>
<td>Forward AGAAGGGGTACACATCA</td>
<td>91 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse CCGTGTATTATTTGCTACTACCT</td>
<td></td>
</tr>
</tbody>
</table>

Lif, leukemia inhibitory factor; Vegf, vascular endothelial growth factor; Hoxa10, homeobox A10; Esr1, estrogen receptor 1; Pgr, progesterone receptor.

ng RNA for Esr1 (also known as estrogen receptor-α), from 0.05 to 100 ng for Hoxa10 and Vegf, and from 0.05 to 200 ng for progesterone receptor (Pgr), and Lif. Curves were plotted as log starting RNA concentration vs. cycle threshold for each standard, and expression in each experimental sample, relative to the level of expression in the standard, was calculated from the standard curve. PCR was performed in a total volume of 20 μl containing 1× SYBR green master mix (Applied Biosystems), forward and reverse primers, cDNA, and molecular-grade water. The primer concentration for 18s rRNA (housekeeper), Pgr, and Lif was 500 nM; for Esr1 and Hoxa10 it was 100 nM; and for Vegf it was 50 nM. The amount of cDNA per reaction was equivalent to that generated from 4 ng of RNA for Esr1 and Vegf, 1 ng for 18s rRNA, 50 ng for Hoxa10, and 75 ng for Pgr and Lif. Dissociation curves, to detect nonspecific amplification, were generated for all reactions, and the presence of a single amplified product was also confirmed by gel electrophoresis. Results were normalized against those obtained for 18s rRNA.

Immunohistochemistry. Rabbit anti-human progesterone receptor primary antibody (DakoCytomation, Glostrup, Denmark) was used at a 1:500 dilution. Goat anti-mouse Lif primary antibody (R&D Systems, Minneapolis, MN) was used at 1:100. Goat anti-rabbit and rabbit anti-goat biotinylated secondary antibodies were used at 1:500 (Vector Laboratories, Burlingame, CA). All incubations were carried out at room temperature in a humidified container. Uterine samples were fixed in 4% paraformaldehyde for 24 h at 4°C followed by two washes in PBS for 24 h each, and then they were stored in 70% ethanol and embedded in paraffin wax. Cross sections were cut at 6 μm onto poly-l-lysine-coated slides (Polysine; Menzel-Glaser/H11003). Tissue sections were placed in 70% ethanol and embedded in paraffin wax. Cross sections were cut at 6 μm onto poly-l-lysine-coated slides (Polysine; Menzel-Glaser/H11003). Sections were then dewaxed in ascending grades of ethanol and Histosolve (Thermo-Shandon, Pittsburgh, PA), air-dried, and then mounted using DPX (BDH Chemicals).

Statistical analysis. For all data a level of 5% (P < 0.05) was taken as statistically significant, and SPSS 13.0 for Windows was used for statistical tests. Gene expression from real time RT-PCR is expressed as arbitrary units relative to 18s rRNA and relative to the control group designated as 1. Hormone and gene data were analyzed using a one-way ANOVA test followed by a Duncan's post hoc test to determine significant difference between treatment groups. Pregnancy rates from embryo transfers were analyzed using a χ² test, and nonpregnant animals were excluded from all subsequent analyses. Implantation and fetal development rate were analyzed using ANOVA (nonviable fetuses were not included in the calculation of fetal development rate). Fetal and placental data were analyzed using a linear mixed-model repeated-measures test, using mothers as subjects and litter size as a covariate.

RESULTS

Tissue weights and ovulation rates. A total of 44 mice were examined on day 4 of pseudopregnancy (10–12 in each treatment group). Gonadotropin stimulation increased the ovulation rate, represented by the number of corpora lutea on the ovaries (2.5 IU rhFSH 24.3 ± 3.1; 10 IU rhFSH 32.8 ± 1.3; 5 IU eCG 28.3 ± 2) compared with the control group (11.1 ± 0.7) (P < 0.001, total n = 26). Stimulation with 10 IU rhFSH produced significantly more corpora lutea than 2.5 IU (P < 0.05), but eCG was not different from either rhFSH treatment. Ovarian weight was increased significantly by stimulation (2.5 IU rhFSH 17.8 ± 0.9 mg; 10 IU rhFSH 19.6 ± 0.7 mg; 5 IU eCG 20.8 ± 1.1 mg) compared with the control (13.9 ± 0.9 mg) (P < 0.001, total n = 44). Stimulation with eCG produced significantly heavier ovaries than 2.5 IU rhFSH (P < 0.05), but 10 IU rhFSH was not different from the other gonadotropin treatments. Uterine weight was not affected by gonadotropin stimulation (control 87.7 ± 4.3 mg; 2.5 IU rhFSH 80.4 ± 3.5 mg; 10 IU rhFSH 86.2 ± 1.8 mg; 5 IU eCG 88.7 ± 2.4 mg; total n = 42).

Steroid hormone levels. Gonadotropin stimulation did not significantly alter mean plasma estradiol concentrations compared with controls (Fig. 1). Plasma progesterone concentrations, however, were increased by all gonadotropin treatments compared with the control group (Fig. 1). Furthermore, progesterone concentrations were higher in the 10 IU rhFSH-treated group compared with 2.5 IU, whereas eCG was not different from the other treatments. Mean progesterone-to-estradiol ratio was increased in both rhFSH treatment groups compared with the control, but the eCG
group was not significantly different from either the control or rhFSH groups (Fig. 1). The correlation between plasma progesterone concentrations and both ovarian weight ($R^2 = 0.334$, $n = 43$) and numbers of corpora lutea ($R^2 = 0.625$, $n = 14$) was significant ($P < 0.001$; data not shown).

**Uterine gene expression.** Expression of five genes regulated by estradiol and progesterone known to be important in implantation and indicative of uterine receptivity were measured with real-time RT-PCR (Fig. 2). Uterine gene expression was examined in five randomly selected mice from each treatment group. The expression of *Lif* mRNA was reduced in all treatment groups compared with controls. There was no effect, however, of rhFSH or eCG on the expression of *Vegf, Hoxa10, Esr1*, or *Pgr* genes in the uterus on day 4 of pseudopregnancy.

**Uterine protein expression.** Uterine PGR protein expression was assessed by immunohistochemistry in seven to nine mice per group. The pattern of immunostaining was categorized into four groups. Nuclear staining for PGR in the luminal epithelium (LE), much of the stroma, and not the glandular epithelium (GE; Fig. 3A) is typical of mice on day 4 of pregnancy (49). In some mice, immunostaining for PGR was observed in the stroma and only part of the LE (Fig. 3B) or in none of the LE (Fig. 3C). A fourth group showed immunostaining only in cytoplasmic areas of the GE (Fig. 3D), which to our knowledge has not been previously reported in the mouse. Controls treated with serum instead of the primary antibody were blank, indicating that the immunostaining was specific for the anti-PGR antibody. Numbers of mice in each category of staining is shown in Table 2. Normal PGR distribution was seen in 75% of controls, with the remainder showing partial LE staining. No rhFSH-treated mice showed presence of PGR in the full LE, and only 27% showed staining in the LE at all. Of the eCG-stimulated mice, 57% had immunostaining for PGR in the LE. Only rhFSH-treated mice (27%) stained exclusively in the GE.

LIF protein expression in the uterus was also assessed by immunohistochemistry in 20 mice. Typically, LIF was expressed predominantly in the apical cytoplasm of LE cells, the cytoplasm of GE cells, and the myometrium (Fig. 4). LIF was also sometimes observed in the stroma, but the immunostaining was generally less intense. These observations match those reported previously for mice on day 4 of pregnancy (8, 55). No differences in the spatial expression of LIF protein were observed between control and treatment mice.

**Embryo transfers.** Gonadotropin stimulation with 10 IU rhFSH or 5 IU eCG significantly decreased pregnancy rates compared with saline-injected control mice (Table 3). In pregnant mice, there was no significant difference between treatments in the number of implantations or the percentage of implanted embryos that became fetuses. Stimulation with 10 IU rhFSH decreased fetal weight, length, and maturity when compared with the saline-injected control (Table 4). Placental weight and area were not significantly affected by rhFSH (Table 4). Only two pregnancies were achieved after stimulation with eCG, resulting in only three fetuses from one dam (with a mean fetal length: 12.37 ± 0.17 mm; mean weight: 175.0 ± 2.69 mg; mean maturity: 14.67 ± 0.08 days). One placenta was fused (weight 173.7 mg), and the other weighed 64.8 mg.

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**Fig. 1.** Effect of gonadotropin stimulation on plasma estradiol (A) and progesterone (B) concentrations and progesterone-to-estradiol concentration ratio (C) on day 4 of pseudopregnancy. Adult female mice were injected with 2.5 IU recombinant human follicle-stimulating hormone (rhFSH), 10 IU rhFSH, or 5 IU equine chorionic gonadotropin (eCG), followed by 5 IU human chorionic gonadotropin (hCG). Controls received saline injections. Hormone concentrations were determined by radioimmunoassay. Values are means ± SE; $n = 10–12$ animals per treatment. Different superscripts indicate a significant difference ($P < 0.05$).
DISCUSSION

This study has demonstrated that gonadotropin stimulation of female mice with either rhFSH or eCG alters the preimplantation hormonal and uterine environment. The numbers of corpora lutea and consequent plasma progesterone concentrations were increased in response to exogenous gonadotropins. In addition, uterine Lif gene expression was decreased and the distribution of PGR protein in the endometrium was altered. Embryo transfer experiments also demonstrated that changes to the mother caused by gonadotropin stimulation decreased pregnancy rates, fetal weight, and fetal maturity.

Gonadotropin stimulation increased the numbers of corpora lutea on the ovaries, which resulted in an increased plasma progesterone concentration on day 4 of pseudopregnancy. Plasma estradiol concentrations, however, were not affected by gonadotropin stimulation. In mice, the progesterone-to-estradiol ratio may be more critical to implantation rates than concentrations of each hormone (22, 39), because estrogen and progesterone generally have antagonistic actions in the endometrium. Both rhFSH and eCG raised the plasma progesterone-to-estradiol ratio when compared with controls, but the increase was not significant after eCG. This contrasts with a...
previous study, in which mice were administered a higher dose of eCG (10 IU) without regard to the estrus cycle (17).

Progesterone is essential in preparing the endometrium for implantation; thus correct expression of PGR is vital for the endometrium to respond to endocrine signals correctly and become receptive (12). Our study shows that gonadotropin stimulation caused a change in the spatial distribution of PGR protein on day 4 of pseudopregnancy. In control mice, PGR was predominantly located in the nucleus of stromal and LE cells, in agreement with previous work (49). In a high percentage of rhFSH- or eCG-treated mice, however, PGR was absent from the LE. This distribution of PGR protein is equivalent to reports from mice on day 5 of pregnancy (49), suggesting that gonadotropin administration may accelerate the maturation of

Fig. 3. Immunohistochemistry for the progesterone receptor (PGR) protein in the uterus on day 4 of pseudopregnancy. Shown are typical representatives of normal expression of PGR (A), partial expression of PGR in the luminal epithelium (LE; B), PGR absent from LE (C), and PGR immunostaining in the glands only (D). Distribution of treatment groups into these immunostaining categories is shown in Table 2. Brown stain indicates presence of PGR protein. GE, glandular epithelium; S, stroma.
the endometrium. Because PGR is regulated by estrogen and progesterone, it is likely that changes in concentrations of these hormones are responsible for altered PGR expression. Interestingly, stimulation had no effect on progesterone receptor (PGR) expression, although previous studies indicate that we should see no change in PGR expression in glands only. No differences between control and accelerated endometrial expression were observed.

Table 2. Summary of immunohistochemistry for PGR in the uterus on day 4 of pseudopregnancy

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>2.5 IU rhFSH (n = 8)</th>
<th>10 IU rhFSH (n = 7)</th>
<th>5 IU eCG (n = 7)</th>
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<tr>
<td>Present in full LE</td>
<td>6 (75)</td>
<td>0</td>
<td>0</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Present in partial LE</td>
<td>2 (25)</td>
<td>2 (25)</td>
<td>2 (28.5)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Absent from LE</td>
<td>0</td>
<td>5 (62.5)</td>
<td>2 (28.5)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Present in glands only</td>
<td>0</td>
<td>1 (12.5)</td>
<td>3 (43)</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are no. of mice exhibiting pattern of immunostaining for the PGR with % of treatment group in parentheses; n, total no. of mice in each group. LE, luminal epithelium. See Fig. 3 for photos of immunostaining. Adult female mice were administered 2.5 IU recombinant human follicle-stimulating hormone (rhFSH), 10 IU rhFSH, or 5 IU equine chorionic gonadotropin (eCG). Controls were injected with saline.

In this study, we have observed that gonadotropin stimulation decreases Lif gene expression in the uterus on day 4 of pseudopregnancy. LIF is essential for implantation in the mouse, because the murine endometrium is unresponsive to the attaching blastocyst in the absence of LIF. LIF is expressed in the endometrium of the uterus on day 4 of pregnancy and falls on day 5 (4, 43). In this study, we have observed that gonadotropin stimulation decreases Lif gene expression in the uterus on day 4 of pseudopregnancy. LIF is essential for implantation in the mouse, because the murine endometrium is unresponsive to the attaching blastocyst in the absence of LIF. LIF is a member of the interleukin-6 cytokine family, and expression in the murine endometrium is induced by estrogen.

Table 3. Pregnancies, implantations, and viable fetuses assessed on day 15 of pregnancy after embryo transfer to recipients

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 16)</th>
<th>10 IU rhFSH (n = 23)</th>
<th>5 IU eCG (n = 19)</th>
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<tr>
<td>No. of pregnancies</td>
<td>12</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Pregnancy rate, %</td>
<td>75</td>
<td>35*</td>
<td>11*</td>
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<tr>
<td>No. of implantations</td>
<td>8.7±0.7</td>
<td>6.3±1.2</td>
<td>3, 3</td>
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<tr>
<td>Implantation rate, %</td>
<td>72.2±5.7</td>
<td>52.1±9.6</td>
<td>25, 25</td>
</tr>
<tr>
<td>No. of viable fetuses</td>
<td>3.4±0.7</td>
<td>2.6±0.8</td>
<td>3, 0</td>
</tr>
<tr>
<td>Viable fetuses/embryos implanted, %</td>
<td>43.4±9.6</td>
<td>36.0±9.5</td>
<td>100, 0</td>
</tr>
</tbody>
</table>

Values are means ± SE, except for the 2 pregnancies in eCG group, where values for each pregnancy are shown; n, no. of embryo transfer recipients. Controls were injected with saline. Mice treated with 2.5 IU rhFSH were not included in embryo transfer experiments. Implantation data excludes nonpregnant mice. Implantation rate, no. of fetuses and resorptions/no. of embryos transferred per mouse; Viable fetuses/embryos implanted, percentage of implanted embryos that developed into viable fetuses per mouse. *Significant differences between control and treatment groups, P < 0.05.

In this study, we have observed that gonadotropin stimulation decreases Lif gene expression in the uterus on day 4 of pseudopregnancy. LIF is essential for implantation in the mouse, because the murine endometrium is unresponsive to the attaching blastocyst in the absence of LIF. LIF is a member of the interleukin-6 cytokine family, and expression in the murine endometrium is induced by estrogen. Normally gene expression peaks on day 4 of pregnancy and falls on day 5 (4, 43); thus our observation of decreased Lif expression is compatible with the suggestion that exogenous gonadotropins accelerate the maturation of the endometrium. Immunohistochemical assessment showed no effect of gonadotropin stimulation on the spatial distribution of LIF protein in the uterus. A previous study using eCG-stimulated mice determined that LIF protein distribution decreases on day 5 of pregnancy compared with days 3 and 4 (55). Therefore, in the present study, using stimulated mice on day 4 of pregnancy, we would not expect a change in LIF protein distribution compared with nonstimulated controls if gonadotropins cause accelerated endometrial development.

Table 4. Fetal and placental outcomes assessed on day 15 of pregnancy after embryo transfer to recipients

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 41)</th>
<th>10 IU rhFSH (n = 16)</th>
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<tbody>
<tr>
<td>Fetal weight, mg</td>
<td>185.2±5.6</td>
<td>81.7±9.8*</td>
</tr>
<tr>
<td>Fetal length, mm</td>
<td>12.2±0.1</td>
<td>10.7±0.2*</td>
</tr>
<tr>
<td>Fetal maturity, days</td>
<td>14.7±0.1</td>
<td>14.1±0.1*</td>
</tr>
<tr>
<td>Placental weight, mg</td>
<td>83.3±3.4</td>
<td>76.3±5.9</td>
</tr>
<tr>
<td>Placental area, mm²</td>
<td>57.6±1.4</td>
<td>52.2±2.3</td>
</tr>
<tr>
<td>Fetal-to-placental weight ratio</td>
<td>2.3±0.1</td>
<td>1.10±0.2*</td>
</tr>
</tbody>
</table>

Values are estimated marginal mean ± SE, adjusted for litter size; n, no. of fetuses from 10 control mothers and 5 rhFSH-treated mothers. Controls were injected with saline. Data from pregnancies of mice administered eCG are not included, because only 1 pregnancy produced fetuses. *Significant differences between control and treatment groups, P < 0.001. 

Fig. 4. Immunohistochemistry for leukemia inhibitory factor (LIF) protein in a representative section from the uterus of a control mouse on day 4 of pseudopregnancy. Brown stain indicates presence of LIF protein. M, myometrium; B, blood vessel.
Both LIF and PR are essential for implantation, so it is possible that the gonadotropin-induced abnormalities in uterine LIF and PGR expression alone are responsible for the lower pregnancy rates after embryo transfer. We believe, however, that their aberrant expression is an indicator that the window of receptivity has occurred early. LIF and PGR, rather than being expressed abnormally throughout pregnancy, were probably expressed normally during a period of uterine receptivity before day 4; however, this remains to be determined.

The expression of Pgr, Esr1, Vegf, and Hoxa10, which are hormonally regulated genes involved in implantation, were not different in the uterus of gonadotropin-stimulated mice compared with controls. That the expression of these genes in the uterus is normal after stimulation is somewhat surprising because the estrogen and progesterone concentrations that control these genes were altered by stimulation. These results, however, are compatible with the suggestion of gonadotropin-induced endometrial acceleration, because uterine mRNA expression of these genes is normally, not different on days 4 and 5 of pregnancy (24, 40a, 49).

Embryo transfer recipients stimulated with 10 IU rhFSH produced fetuses with a mean weight 66% lighter than control fetuses. Mean fetal length and maturity were also decreased by rhFSH. In agreement with previous studies using eCG (18, 21), administration of 10 IU rhFSH or 5 IU eCG to recipients reduced pregnancy rates: only 2 pregnancies resulted from embryo transfers to 19 eCG-stimulated mice compared with 12 pregnancies from 16 transfers to control mice. The implantation rate in pregnant stimulated recipients was not different from pregnant controls, indicating that, whereas stimulated mice had reduced chances of establishing a pregnancy, those able to become pregnant implanted normal numbers of embryos. The reduction in fetal growth was not due to variation in litter size. Similarly, embryo quality was not responsible, because embryos were obtained from the same pool of donor mice, culture and transfer conditions were the same, and the embryos were randomly allocated to recipients. It is well established that preimplantation changes in the maternal environment can affect fetal development and implantation rates, such as asynchrony between the endometrium and embryo (2, 47, 53) or changes to the hormonal milieu (3, 26). Our results show that rhFSH alters the maternal environment so that pregnancy rates are reduced and fetal development perturbed.

Although we did observe subtle differences in the effects of rhFSH and eCG treatments on several outcome measures, these were not statistically significant. These minor differences may be due to the LH-like activity of eCG, which can influence the development and hormonal secretion of ovarian follicles, thereby indirectly influencing the endometrium. Alternatively, eCG may have a direct effect through endometrial LH receptors (37, 56). Clinical studies have produced conflicting results when comparing the effects of rhFSH and human menopausal gonadotropin, which also has FSH and LH activity (11). In mice, Edwards et al. (15) did not observe any differences between eCG and rhFSH in their effects on embryo quality or embryo gene expression. In addition, we did not observe a difference between high (10 IU) and low (2.5 IU) stimulation with rhFSH, except that 10 IU produced more corpora lutea and consequently higher progesterone levels.

Results from this study show changes in the preimplantation endometrium that suggest that gonadotropin stimulation may accelerate the endometrial maturation. This may be the mechanism by which exogenous gonadotropins reduce the likelihood of a successful pregnancy as described in the present study. Accelerated endometrial development after stimulation has been previously reported in rabbits (29) and humans (6, 13, 38). Several suggestions have been made regarding the mechanisms behind this, one of which is that high FSH or estrogen may increase LH receptivity of granulosa cells to cause luteinization at low LH levels, leading to an early rise in progesterone, which causes early endometrial maturation (36). High progesterone in IVF cycles has been associated with lower pregnancy rates (9, 19, 41). Alternatively, high LH at the start of gonadotropin administration may cause early estrogen production by follicles, inducing early expression of PGR in the endometrium and thus increased sensitivity to progesterone and early endometrial maturation (27, 46). Interestingly, Sibug et al. (44) have suggested that asynchrony between the endometrium and embryo only occurs when urinary rather than recombinant gonadotropins are used.

In conclusion, this study has demonstrated that gonadotropin stimulation with rhFSH or eCG alters the preimplantation maternal environment in the mouse and subsequently causes reduced pregnancy rates and perturbed fetal development. The changes in uterine LIF, and PGR expression suggest that gonadotropin stimulation may accelerate the maturation of the endometrium. Embryo transfer experiments demonstrated that gonadotropin-induced changes to the mother reduce the pregnancy rate, fetal weight, and fetal maturity. Thus this study has shown that the consequences of administering exogenous gonadotropins to produce high numbers of oocytes are apparent long after ovulation, and they are mediated at least in part by perturbations of the maternal hormonal and uterine environment. These results have implications for research in laboratory mice, because it is apparent that a variety of parameters in the gonadotropin-stimulated mouse, using rhFSH or eCG, may be different from naturally cycling mice. There can be no direct extrapolation into the clinical situation, but these results should encourage further research into the consequences of inducing multiple ovulations with exogenous gonadotropins in IVF.

GRANTS
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REFERENCES
EFFECT OF rhFSH ON PREGNANCY IN THE MOUSE


