The direct and indirect effects of kisspeptin-54 on granulosa lutein cell function

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Short running title

Kiss-54 on ovarian gene expression/steroidogenesis

Abstract

Study question: What are the *in vivo* and *in vitro* actions of kisspeptin-54 on the expression of genes involved in ovarian reproductive function, steroidogenesis and ovarian hyperstimulation syndrome (OHSS) in granulosa lutein (GL) cells when compared with traditional triggers of maturation?

Summary answer: The use of kisspeptin-54 as an oocyte maturation trigger augmented expression of genes involved in ovarian steroidogenesis in human GL cells including, FSH receptor (*FSHR*), LHCG receptor (*LHCGR*), steroid acute regulatory protein (*STAR*), aromatase, estrogen receptors alpha and beta (*ESR1, ESR2*), 3-beta-hydroxysteroid dehydrogenase type 2 (*3BHSD2*) and inhibin A, when compared to traditional maturation triggers, but did not alter markers of OHSS.

What is known already: Human chorionic gonadotropin (hCG) is the most widely used trigger of oocyte maturation, but is associated with an increased risk of OHSS. The use of gonadotropin releasing hormone (GnRH) agonists to trigger oocyte maturation is a safer alternative to hCG. More recently, kisspeptin-54 has emerged as a novel therapeutic option that safely triggers oocyte maturation even in women at high risk of OHSS. Kisspeptin indirectly stimulates gonadotropin secretion by acting on hypothalamic gonadotropin releasing hormone (GnRH) neurons. Kisspeptin and its receptor are also expressed in the human ovary, but there is limited data on the direct action of kisspeptin on the ovary.

Study design, size, duration: Forty-eight women undergoing IVF treatment for infertility consented to kisspeptin-54 triggering and/or granulosa cell collection and were included in the study. Twelve women received hCG, 12 received GnRH agonist and 24 received kisspeptin-54 to trigger oocyte maturation. In the kisspeptin-54 group, 12 received one injection of kisseptin-54 (9.6nmol/kg) and 12 received two injections of kisspeptin-54 at a 10hr interval (9.6nmol/kg x2).

Participants/materials, setting, methods: Follicular fluid was aspirated and pooled from follicles during the retrieval of oocytes for IVF/ICSI. GL cells were isolated and either RNA extracted immediately or cultured *in vitro* +/- kisspeptin or hCG.

Main results and the role of chance: GL cells from women who received kisspeptin-54 trigger *in vivo* had a higher gene expression of; *FSHR* ($p=0.0001$), *LHCGR*, ($p<0.05$), *STAR* ($p<0.05$),

3BHSD2, ($p<0.05$), aromatase (*CYP19*, $p<0.05$), inhibins A ($p<0.05$) and B ($p<0.05$), *ESR1* ($p<0.05$) and *ESR2* ($p<0.05$) compared to other triggers. Markers of vascular permeability and oocyte growth factors were unchanged (*VEGFA*, *SERPINF1, CDH5*, amphiregulin, epiregulin). Gene expression of kisspeptin receptor was unchanged. Whereas GL cells treated *in vitro* with hCG induced steroidogenic gene expression, kisspeptin-54 had no significant direct effects on either OHSS genes or steroidogenic genes.

Limitations, reasons for caution: Most women in the study had PCOS, which may limit applicability to other patient groups. For the analysis of the *in vitro* effects of kisspeptin-54, it is important to note that granulosa lutein cells had already been exposed *in vivo* to an alternate maturation trigger.

Wider implications of the findings: The profile of serum gonadotropins seen with kisspeptin administration compared to other triggers more closely resemble that of the natural cycle as compared with hCG. Thus, kisspeptin could potentially permit an ovarian environment augmented for steroidogenesis, in particular progesterone synthesis, which is required for embryo implantation.

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Introduction

Infertility is estimated to affect one in seven couples and the number of couples receiving fertility treatment is increasing each year (1). The most dangerous complication of assisted reproductive technology (ART) is ovarian hyperstimulation syndrome (OHSS), which can cause ovarian enlargement, ascites, hydrothorax, renal failure, acute respiratory distress syndrome, and can even be life-threatening (2, 3). Severe OHSS occurs in approximately 2-8.9% of cycles and moderate OHSS in a further 10.2-15.6% (1, 4). Moreover, the risk of severe OHSS is further increased in women with polycystic ovaries to ~12-15% (5-7).

Human chorionic gonadotropin (hCG) has traditionally been used to trigger oocyte maturation, but due to a prolonged duration of action on ovarian *LHCGR*, it can be complicated by OHSS (8-10). Gonadotropin releasing hormone agonists (GnRHa) have a shorter duration of action than hCG, and thus are associated with a lower risk of inducing OHSS (11-13). However, GnRHa are often used as second line agents due to concerns regarding implantation rates (14, 15). Kisspeptin is a recently discovered neuropeptide that is obligate for puberty and reproduction (16, 17). It regulates the hypothalamo-pituitary–gonadal (HPG) axis by acting on kisspeptin receptors (*KISS1R*) on GnRH neurons to stimulate GnRH, and consequently gonadotropin release (25, 26). We have recently demonstrated that Kisspeptin-54 triggers oocyte maturation in a study of 60 women at high risk of OHSS (18). Across all doses tested, kisspeptin-54 resulted in oocyte maturation in 95% of patients with biochemical pregnancy, clinical pregnancy, and live birth rates per transfer of 63%, 53%, and 45%, respectively, with no clinically significant OHSS.

Several studies have compared alterations in gene expression, gonadotropin levels, vascular endothelial markers and growth factor profiles in serum, follicular fluid and granulosa lutein (GL) cells between hCG and GnRH agonist triggered IVF protocols (21, 17, 18, 23). Levels of vascular endothelial growth factor (*VEGF*) (19-22), Cadherin 5 (*CDH5*) (23) and platelet endothelial derived factor (*PEDF*, also known as *SERPINF1*) (24) are putative markers of OHSS and have been shown to be different between hCG and GnRHa triggered cycles (19, 20, 25). Microarray analysis of cumulus and mural granulosa cells has shown differences in genes involved in lipid and steroid metabolism, and platelet degranulation (26). In addition, Haas et al 2014 studied the

differential effects on gene expression in granulosa cells from women who were triggered with hCG or GnRH agonists (23). They found that granulosa cells from women who had received GnRHa as a trigger had lower expression of genes related to steroidogenesis and gonadotropin regulation (*CYP19A1, CYP11A1, 3BHSD, FSHR,* inhibin B and amphiregulin) (23). These changes may reflect the different hormonal profiles and likelihood to induce OHSS between the two triggers.

It is known that both kisspeptin (*KISS1*) and its G-protein-coupled kisspeptin receptor (*KISS1R*) are expressed in the human ovary (27). However, the direct effects of kisspeptin on the ovary have yet to be fully elucidated. No study has examined the effects of triggering oocyte maturation using kisspeptin-54 on expression of genes involved in OHSS and steroidogenesis in the ovary. Thus, the aims of this study were: 1. To assess the *in vivo* effects of kisspeptin-54 on gene expression markers of ovarian function/steroidogenesis and OHSS in human GL cells, in comparison to traditional triggers of oocyte maturation (hCG, GnRHa). 2. To determine whether the effects of kisspeptin-54 treatment are via the hypothalamic-pituitary-gondal axis and/or via direct actions on human GL cells. This is the first study to address the impact of triggering oocyte maturation with kisspeptin-54 on human granulosa cell function.

Materials & methods:

Study approval

As previously described in Abbara et al, JCEM 2015 (18), the kisspeptin study was carried out in the IVF Unit at Hammersmith Hospital under a license from the United Kingdom Human Fertilization and Embryology Authority. Written informed consent was obtained from all subjects prior to inclusion in the study. Approval of this study as a Clinical Trial of an Investigative Medicinal Product was granted by the Medicines and Healthcare Products Regulatory Agency, UK. The study was registered on the National Institutes of Health Clinical Trials database (ClinicalTrials.gov NCT01667406). The study was performed in accordance with the Declaration of Helsinki. Written informed consent was also obtained from all subjects prior to collection of granulosa lutein cells. The Hammersmith and Queen Charlotte's Research Ethics Committee, London, United Kingdom, approved both studies (Reference 08/H0707/152, 10/H0707/2).

Study Participants

Forty-eight women undergoing IVF treatment for infertility at Hammersmith hospital consented to use of kisspeptin-54 maturation trigger use and/or granulosa cell collection and were included in the study. Twelve women received hCG, 12 received GnRH agonist and 24 received kisspeptin-54 to trigger oocyte maturation. In the kisspeptin-54 group, 12 received one injection of kisseptin-54 (9.6nmol/kg) and 12 received two injections of kisspeptin-54 at a 10hr interval (9.6nmol/kg x2). The indications for IVF were infertility for PCOS, tubal problems, male factor infertility or infertility of unknown cause. In addition, these women met the NHS criteria for assisted reproduction, i.e. had a body mass index <30kg/m^2, were aged <40yrs and were non-smokers. Polycystic ovaries (PCO) were defined sonographically as the presence of 12 or more follicles in each ovary measuring 2 to 9 mm in diameter and/or increased ovarian volume (>10 mL). Polycystic ovary syndrome (PCOS) was defined according to the Rotterdam diagnostic criteria (i.e. 2 or more of the following: oligo- or anovulation, hyperandrogenism and polycystic ovaries). Multicystic ovaries were defined sonographically as the presence of multiple bilateral cysts >10mm

in ovaries with normal stroma. The inclusion criteria for kisspeptin-54 triggered patients have been detailed in Abbara et al (18) and included: serum AMH ≥40 pmol/L (≥5.6 ng/mL) or total AFC ≥23, age 18–34 years, early follicular phase serum FSH ≤12 mIU/mL, and both ovaries intact. Exclusion criteria were moderate/severe endometriosis or poor response to more than one previous cycle of IVF treatment.

Study outcomes

We measured gene expression of factors involved in ovarian reproductive function and steroidogenesis; *LHCGR*, *FSHR*, *CYP19A1*, cholesterol side-chain cleavage enzyme (CYP11A1), *3BHSD2*, *STAR*, kisspeptin (*KISS1*), kisspeptin receptor (*KISS1R*), *ESR1, ESR2*. We also measured gene expression of putative markers of OHSS; vascular endothelial growth factor (*VEGFA*), cadherin 5 (*CDH5*), Serpin Family F Member 1 (*SERPINF1*, also known as *PEDF*, pigment endothelial-derived factor) and growth factors involved in regulating oocyte growth; amphiregulin (*AREG*) epiregulin (*EREG*), inhibin A and B (*INHBA, INHBB*). These were measured from granulosa lutein cells extracted at the time of oocyte retrieval and from cells cultured *in vitro* and treated with either hCG or kisspeptin-54. For *in vivo* treated samples, a separate analysis compared those women who received one or two kisspeptin-54 injections. Women who received hCG and were either 'control' or PCO were also analyzed separately to remove the confounding effect of PCO. Intracellular signaling responses phospho-ERK, phospho-AKT and calcium signal generation in response to *in vitro* kisspeptin-54 treatment were also measured.

Ovarian stimulation protocol

Participants who did not receive kisspeptin-54 underwent cycles of In Vitro Fertilization (IVF) under two distinct stimulation protocols: Long day 21 (LD-21) or antagonist. In a LD-21 cycle gonadotropin-releasing hormone agonist (Buserelin 0.5 mg, Sanofi, Guildford UK) was used to suppress the pituitary production of gonadotropins for two weeks starting from day 21 of the menstrual cycle. Ovarian suppression was confirmed with an ultrasound scan and stimulation was

started using recombinant follicular stimulating hormone. FSH dose varies based on ovarian reserve and can range between 112.5 to 450 units. Human chorionic gonadotropin (hCG) 5000 units was administered once at least three follicles reached ≥18mm in diameter; oocyte retrieval followed 36 hours later. The antagonist cycle was more commonly used in PCO cases to reduce the risk of OHSS. Ovarian stimulation was started on the second day of the menstrual cycle, a GnRH antagonist (Cetrorelix or Ganirelix 0.25mg daily , Merck Serono, Feltham, UK) was used from day 5 to prevent a premature LH surge. Human chorionic gonadotropin (hCG) (Ovitrelle and Pregnyl, Merck Serono) or a GnRHa (Buserelin 2mg) was administered when at least three follicles reached ≥18mm in diameter, and transvaginal oocyte retrieval was carried out 36 hours thereafter. The IVF protocol for the kisspeptin-54 (Bachem, Bubendorf, Switzerland) trial has previously been described in detail, see Abbara et al, JCEM 2015, (18).

Granulosa-lutein cell collection and isolation

Follicular fluid was aspirated and pooled from follicles during the retrieval of oocytes for IVF/ICSI. The GL cells were extracted by centrifugation at 1000rpm for 5 min to separate the fluid from cells, as previously described (28). The cell pellets were re-suspended in M199 Thermo fisher Scientific, Waltham, MA USA) and layered onto Percoll (GE healthcare, Chicago, USA)(28) gradient and then centrifuged at 1600rpm for 30 min to separate red blood cells. The GL cells at the interface were collected and washed with Dulbecco's phosphate buffered saline (DPBS, Thermo Fisher Scientific). The cells were then either frozen and stored at -80^{o}C or RNA was extracted immediately.

Quantitative Real-Time PCR (RT-qPCR)

RNA was extracted from cells using RNeasy® plus Mini Kit (Qiagen Inc., CA, USA) according to manufacturer's instructions. The RNA samples were stored at −80 °C until all samples were collected. The quantity, quality and integrity of samples was analysed using Agilent Technologies 2200 Tapestation (Agilent, CA, USA). Samples with an RNA integrity number greater than 5 were included. RNA was reverse transcribed into cDNA using Invitrogen superscript IV First Strand synthesis system.

Quantitative, real time PCR (QPCR) was carried out on 384 well plates using POWER SYBR Green (Applied Biosystems, CA, USA) according to the manufacturer's instructions on an Applied Biosystems 7900 HT instrument. Primer sequences are listed in supplementary table 1. The relative mRNA expression of each gene was calculated using the housekeeping genes β-actin and β2-microglobulin as reference genes. A geNorm kit (Primer Design, Chandler's Ford, UK) was used to select the best candidate reference genes. Placental cDNA was used as positive control tissue for KISS1 and KISS1R analysis.

Granulosa cell culture & *in vitro* treatment with hCG/kisspeptin

GL cells isolated from pooled follicular fluid were cultured in 6-well plates ($1x10^6$ cells per well) in serum-free culture medium (supplemented DMEM/F12 Ham mixture, Thermo Fisher Scientific). The media was completely refreshed every 48 hours. After 72 hrs in culture, cells were treated with either 1μM kisspeptin-54 dissolved in 0.9% saline or 10nM hCG (Harbor UCLA Medical Centre, USA) dissolved in PBS for 48 hrs, to mimic the *in vivo* oocyte maturation trigger protocol. After this 36 hr period, cells were lysed, collected for RNA extraction and qPCR, as described above. The cells were cultured for 72 hrs prior to treatment as we have found that the number and activity of gonadotropin receptors (and the response of steroidogenic enzymes to gonadotropins) increase with time in culture (supplementary materials figure 1), despite having been subjected to high doses of FSH and/or hCG *in vivo*.

GL cells were also cultured for 24 hours in similar conditions, then treated with 0.1, 1 or 10 μM kisspeptin-54 for 0, 5, 10, 15, 30, 60 mins, with or without LH 10nM (Harbor UCLA Medical

Centre, USA). After treatment, the cells were harvested and rinsed with PBS and then lysed with 40µL RIPA buffer per well (Thermo Fisher Scientific). Cell lysates were centrifuged at 12,000 × *g* for 10 mins and the supernatant removed. Protein was quantified using the BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific). Each sample was denatured at 70 °C for 10 mins, loaded to a Bolt 4-12% Bis-Tris Plus Gels (Invitrogen, CA, USA), and then transferred to a polyvinylidene difluoride membrane (Thermo Fisher Scientific). The membranes were washed 3 times for 15 mins in TBST (Tris-buffered saline, 0.1% Tween 20) and blocked with 5% BSA for 1 hr. Membranes were incubated with primary antibodies (phospho-p42/44 MAPK, Phospho-Akt (Ser473) Antibody, Cell Signaling Technology, MA, USA) at 4 °C overnight. Membranes were then incubated with horseradish peroxidase- conjugated anti-rabbit IgG secondary antibody (Thermo Fisher Scientific) for 1 hr at room temperature. The immunoblots were visualized by enhanced chemiluminescence detection (Thermo Fisher Scientific) and quantified by densitometric scanning. The band densities were normalized with that of GAPDH. GL cells cultured for 24 hrs as described above and were also pre-treated with a Fluo-4 calcium indicator dye (Invitrogen) for 1 hr to detect calcium flux in response to ligand administration at live imaging, then kisspeptin-54 10µM was administered and cell fluorescence emission recorded, as per manufacturer's instructions.

Statistical analysis

One-way ANOVA or students t-test was used to compare means between 3 or 2 parametric groups. Mann Whitney U and Kruskal Wallis tests were used for comparing medians of non-parametric data. A Tukey's correction for multiple comparisons was carried out. Pearson correlation coefficient was used to correlate gene expression results with FSH dose, age and antral follicle count. A power calculation was used to determine that 12 samples would be required for *in vitro* treatment with kisspeptin-54 based on the *in vivo* gene expression data (beta 80%; alpha 5%). A p-value of <0.05 was considered to indicate statistically significance. Statistical analysis was performed using GraphPad, Prism, version 6.

Results

Baseline Characteristics

Women who received kisspeptin-54 to trigger oocyte maturation were significantly younger than those who received hCG or GnRH agonist (29 v 33 years, p=0.001) (see Table 1). Mean body mass index was similar in the three groups. Baseline antral follicle count (AFC) was lower in women who were triggered with hCG than with GnRH agonist or kisspeptin, as half of this group did not have PCO/PCOS (p<0.001). AFC was not significantly different between the GnRH agonist and kisspeptin groups. The mean dose of recombinant FSH received as part of IVF stimulation protocol was higher in women without PCOS who received hCG compared to those who had PCOS or had a GnRH agonist (p=0.01) or kisspeptin (p=0.002) trigger. All the women in the GnRH agonist or kisspeptin groups had polycystic ovaries or multicystic ovaries. Half of the women in the hCG group had PCO/PCOS and half had IVF for male factor infertility, tubal problems or unexplained infertility.

Gene expression- Gonadotropin receptors / Kisspeptin receptor

GL cells from women who had received kisspeptin-54 had a 14-fold (p<0.001) and 8-fold (p<0.01) higher expression of FSH receptor gene than GL cells from women who had received hCG or GnRH agonist, respectively (see Figure 1). The mean rise in serum FSH level 4 hrs after administration of kisspeptin-54 was 2.4 ± 1.8mIU/ml. Given that women in the kisspeptin-54 group were more likely to have polycystic ovaries and higher antral follicle counts and that *FSHR* expression is known to be higher in GL cells from women with PCOS, we correlated *FSHR* to antral follicle count to show that the higher *FSHR* expression was not confounded (see Figure 1b).

GL cells from women who had received kisspeptin-54 had a 2-fold (NS) and 2.5-fold (p<0.05) higher expression of LH receptor gene than GL cells from women who had received hCG or GnRH agonist, respectively (see Figure 1D). *LHCGR* expression was significantly higher in women who received 2 injections of kisspeptin-54 10 hours apart when compared with women who had received only one (p<0.05). The mean rise in serum LH level 4 hours after administration of kisspeptin was 55.4 ± 30.7mIU/ml (1c). There was no correlation between *LHCGR* expression and

baseline AFC. Women with a higher peak levels of serum LH appeared to have lower GL cell *LHCGR* gene expression and vice versa (see Figure 1F, NS). There was no correlation between age and LHCGR and FSHR gene expression, or baseline serum LH or FSH level. Kisspeptin gene (*KISS1*) expression was not detected in GL cells. Kisspeptin receptor (*KISS1R*) was expressed, but was similar in GL cells from women who received hCG, GnRHa or kisspeptin-54 (see Figure 1G). Excluding the control women from the hCG group did not change any of the above results.

Gene expression- ovarian steroidogenic enzymes

CYP19A1 (aromatase) expression was 3.6-fold ($p<0.05$) and 4.5-fold ($p<0.05$) higher in GL cells from women who had received kisspeptin-54 than hCG or GnRHa respectively (see Figure 2). *CYP11A1* expression was similar amongst the 3 groups. Expression of *STAR* was 3.4-fold higher ($p<0.01$) and 1.8-fold higher ($p<0.05$) in women who had received kisspeptin-54 than PCO women in hCG group and GnRHa group respectively. *STAR* expression was higher in those who had received 2 injections of kisspeptin-54 to trigger oocyte maturation than those who had received one ($p=0.04$). 3 β -Hydroxysteroid dehydrogenase 2 (*HSD3B2*) expression was 7.5- ($p<0.01$) and 2.5-fold higher ($p<0.05$) in kisspeptin group than those who had received hCG (PCO only) and GnRHa, respectively. *ESR1* and *ESR2* both had showed 3-fold higher expression in cells from kisspeptin treated than GnRHa treated women. Inhibin A gene expression was 2.5-fold higher from GL cells from women who had received kisspeptin-54 than hCG or GnRHa ($p<0.01$). Inhibin A gene expression did not relate to pretreatment antral follicle count. Inhibin B expression was similar amongst the 3 groups.

Gene expression- markers of ovarian hyperstimulation syndrome

There was no significant difference, amongst the three maturation triggers, between the gene expression of genes that are involved in vascular permeability and integrity; *VEGFA, SERPINF1* and *CHD5*, nor in expression of growth factors amphiregulin and epiregulin, which have been previously shown to be potential markers of ovarian hyperstimulation syndrome (see Figure 3).

In vitro effects of kisspeptin-54

Granulosa lutein cells (n=12) cultured and treated *in vitro* with kisspeptin-54 alone for 48 hrs did not elicit the same changes in gene expression as those *in vivo.* Gene expression of *LHCGR, FSHR, CYP19A1, CYP11A1, HSD3B2, STAR, KISS1R, ESR1, ESR2, VEGF, SERPINF1, AREG, EREG, INHBA, INHBB* were similar in kisspeptin-54 treated GL cells compared to untreated cells (supplementary figure 2). GL cells treated with hCG had significantly higher expression *of LHCGR, STAR, CYP11A1, INHBA* and lower expression of *INHBB* and *ESR2* compared to untreated cells or cells treated with kisspeptin-54 (supplementary Figure 2). GL cells cultured and treated with kisspeptin-54 did not stimulate production of phospho-ERK, phospho-AKT or elicit an intracellular calcium signaling response.

Discussion

To our knowledge this is the first study to examine both *in vivo* and *in vitro* effects of kisspeptin-54 on human ovarian granulosa lutein cells (GL cells). The data contributes to the current literature elucidating the direct and indirect ovarian effects of kisspeptin-54. As the therapeutic role for kisspeptin continues to grow, it is important that its mechanism of action and effects on target organs are comprehensively examined. We have demonstrated that kisspeptin, when given as a trigger for oocyte maturation in IVF, alters granulosa lutein gene expression considerably when compared to traditional triggers of maturation. It results in a higher expression of FSH and LH receptor, and higher expression of genes involved in ovarian steroid production and action; steroidogenic acute regulatory protein, 3β-hydroxysteroid dehydrogenase, aromatase, inhibin A, estrogen receptors 1 and 2. These changes occur in the absence of changes in the expression of kisspeptin receptor (and with no evidence of kisspeptin gene expression in these cells). These alterations in gene expression are not seen in GL cells treated *in vitro* with kisspeptin-54 alone and are therefore more consistent with an indirect effect of kisspeptin-induced changes in gonadotropin secretion. GL cells from women who received kisspeptin-54 do not have changes in markers of

ovarian hyperstimulation *VEGF, PEDF*, Cadherin 5, amphiregulin and epiregulin, despite these women having a high baseline risk of OHSS.

Kisspeptin-54, the major circulating isoform of kisspeptin in humans, has been shown to effectively trigger egg maturation in women undergoing IVF treatment (29), with no clinically significant OHSS (18). It is known that hypothalamic kisspeptin affects ovarian activity indirectly via stimulating gonadotropin secretion. Both kisspeptin and G-protein-coupled kisspeptin receptor (KISS1R) are expressed in the human ovary (30). *In-vitro* treatment of cultured rat luteal cells with kisspeptin-10 increased basal and hCG-induced progesterone levels but not estradiol production, with concomitant increases in levels of key steroidogenic enzymes *STAR, CYP11A*, and *HSD3B2* and increased the phosphorylation of Erk1/2 mitogen-activated protein kinase (30). A study in pre-ovulatory chicken granulosa cells treated with kisspeptin-10 found an increase in progesterone secretion and steroidogenic gene expression (31). These studies suggest a direct effect of kisspeptin-10, however evidence for this direct effect is contradicted by the observation that rodent models with GnRH neuron-specific deletions of *KISS1R* are infertile mice and fail to go through puberty (32). They subsequently knocked in the *KISS1R* to the GnRH neurons to the model, resulting restoration of normal pubertal onset and fertility suggesting that it is the GnRH neuron that is the key site of kisspeptin signaling (33).

Kisspeptin-54 administration stimulates a rise in both LH and FSH but with a relatively greater effect on LH, a similar pattern to what has been shown previously (33). Doses of GnRH agonist used in current IVF treatment are likely to be at the high end of the dose-response curve, resulting in a higher amplitude of FSH and LH secretion than observed with kisspeptin-54 (34). Kisspeptin stimulates GnRH neurons to secrete endogenous GnRH, whereas a GnRH agonist can also stimulate GnRH receptors which are not contiguous with GnRH neurons resulting in a higher amplitude of gonadotropin secretion at equimolar doses (29). The lower amplitude of gonadotropin secretion with kisspeptin-54, compared with GnRHa or hCG may explain the significantly higher expression of *FSHR* and *LHCGR* (35-37). In fact, the excursions in serum gonadotropins are closer to that of the peri-ovulatory changes in gonadotropins in the natural unstimulated cycle (38). FSH and LH have been shown to downregulate expression of their own receptors (39-41). However, a

study by Sasson et al (42) showed that in luteinised granulosa cells, LH administration resulted in increased density of *LHCGR* transcripts, although these cells responded inefficiently to gonadotropin stimulation. The rise in FSH receptor expression is not related to dose of recombinant FSH administration during the stimulation phase of the IVF cycles, as women triggered with kisspeptin-54 or GnRHa received similar doses of recombinant FSH, and less than those who received hCG. It also did not correlate with the higher antral follicle count that the women in the kisspeptin-54 group had, as PCOS is known to be associated with increased GL cell FSHR expression (43).

The relatively high *FSHR* gene expression after kisspeptin-54 administration and subsequent augmentation of downstream signaling, could in turn augment enzymes involved in progesterone synthesis (*STAR, HSD3B2*), androgen synthesis (*HSD3B2*), and aromatization of androgens to estrogens (*CYP19*A1). Estradiol has been shown to upregulate expression of its own receptor and the increased production of estradiol may be an explanation for the increase in ER gene expression that we observe here (44, 45). Enhanced production and/or action may in turn contribute to upregulation of FSH and LH receptor expression (46). We also saw a higher expression of inhibin A in granulosa cells from kisspeptin treated women than hCG or GnRH agonists. Inhibins are produced by granulosa cells and negatively feedback to inhibit FSH secretion by the pituitary. Inhibin A is the predominant form produced during the late follicular phase and it has been shown previously that inhibin A, in addition to its feedback on the pituitary, can act directly on the ovaries and can suppress *FSHR* gene expression in granulosa cells (47). It is possible that the upregulation of inhibin A may be a compensatory response to the higher *FSHR* expression.

There was no significant difference seen in markers of potential ovarian hyperstimulation syndrome (OHSS) between the 3 groups, despite reports in the literature showing that triggering maturation with hCG results in an increased likelihood of OHSS, and studies showing alterations in these markers in hCG treated women (19, 20, 25, 26). The likely explanation for this is that the hCG group in this study had lower baseline risk of OHSS with a lower baseline antral follicle count and older age, and hence also did not have any evidence of changes associated with OHSS. The kisspeptin trial specifically targeted women at high risk of OHSS. In addition, we know that no

women in this study actually developed clinically significant OHSS. Recently it has been shown in a rat OHSS model that kisspeptin-10 prevented the increased vascular permeability of OHSS probably by the activation of KISS1R and the inhibition of *VEGF* (48). Thus, kisspeptin triggering could therefore additionally prevent OHSS through a direct ovarian action, thus indicating as to why these women at high risk of OHSS did not have higher *VEGF* expression.

We treated granulosa lutein cells *in vitro* with kisspeptin-54 or hCG and measured steroidogenic and gonadotropin receptor gene expression. Kisspeptin treatment alone did not alter steroidogenic or gonadotropin receptor gene expression. This is consistent with kisspeptin's action being predominantly at the level of the hypothalamus and pituitary as opposed to direct action at the ovary. Alternatively, kisspeptin may require the presence of other gonadotropins to directly alter steroidogenesis, or perhaps an alternative isoform e.g. Kiss-10 (31, 49) may have differing receptor affinity for ovarian *KISS1R*.

There are several limitations to our study. 1. Nearly all the women in our study had polycystic ovaries. We have dealt with this confounder by completing the analyses with and without the women who do not have PCOS, to interrogate this as a confounder. 2. For the analysis of *in vitro* effects of kisspeptin-54, it is important to note that granulosa lutein cells have already been exposed *in vivo* to an alternate maturation trigger, which could confound the results. However, we have shown GL cells maintain gonadotropin receptor expression and capacity for steroidogenesis in culture, suggesting that they are still useful for *in vitro* measurement of steroidogenesis. 3. As part of the selection process for inclusion in the kisspeptin trial women had to be 34 years of age or younger, as they were pre-selecting women at higher risk of OHSS. Younger women are at higher risk of OHSS and this may have cofounded the data relating to markers of ovarian hyperstimulation.

This is the first study to examine the effects of kisspeptin-54, when used as an oocyte maturation trigger for IVF treatment, on granulosa cell gene expression. We have shown that kisspeptin-54 *in vivo* augments gonadotropin receptor gene expression and steroidogenic enzymes, when compared with traditional oocyte maturation triggers hCG and GnRHa. In addition, we have studied the *in*

vitro effects of kisspeptin-54 on granulosa lutein cell gene expression and steroidogenesis, overall adding to the limited knowledge base on the direct and indirect effects of kisspeptin in the human ovary. The excursions in serum gonadotropins are closer to that of the physiological menstrual cycle than other triggers and the expression of receptors and steroidogenic enzymes potentially creates an ovarian environment augmented for progesterone synthesis and luteal phase implantation. This mirrors what has been shown in animal models treated with kisspeptin (30, 31). As the therapeutic uses of kisspeptin continue to expand knowledge of its effects on targets organs are critical. In conclusion, it appears that changes in markers of ovarian steroidogenesis seen in those who had received kisspeptin-54 are related to the altered gonadotropin profile seen with kisspeptin, as opposed to being related to direct ovarian stimulation by kisspeptin.

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Author contributions

1. Owens L is the first author. She was involved in concept, data acquisition, analysis, manuscript writing and final approval.
2. Abbara A was involved in study design, data interpretation, article drafting and revision.
3. Lerner A was involved in study design, sample acquisition, lab experiments, analysis and drafting the manuscript.
4. O'Floinn S was involved in study design, lab experiments, statistical analysis and drafting the manuscript.
5. Christopoulos G was involved in study design, patient consent, sample collection, manuscript revision and approval.
6. Khanjani S was involved in study design, patient consent, sample collection, manuscript revision and approval.

7. Islam R was involved in study design, patient consent, sample collection, manuscript revision and approval.
8. Hardy K was involved in study design, data analysis/interpretation, drafting and revising the manuscript.
9. Hanyaloglu A was involved in study design, data analysis/interpretation, writing and revising the manuscript.
10. Lavery SA was involved in study design, sample collection, writing and revising the manuscript.
11. Dhillo WS was involved in study conception, data analysis, writing and revising the manuscript.
12. Franks S is the senior author and was involved in study conception, data analysis, manuscript writing and revision.

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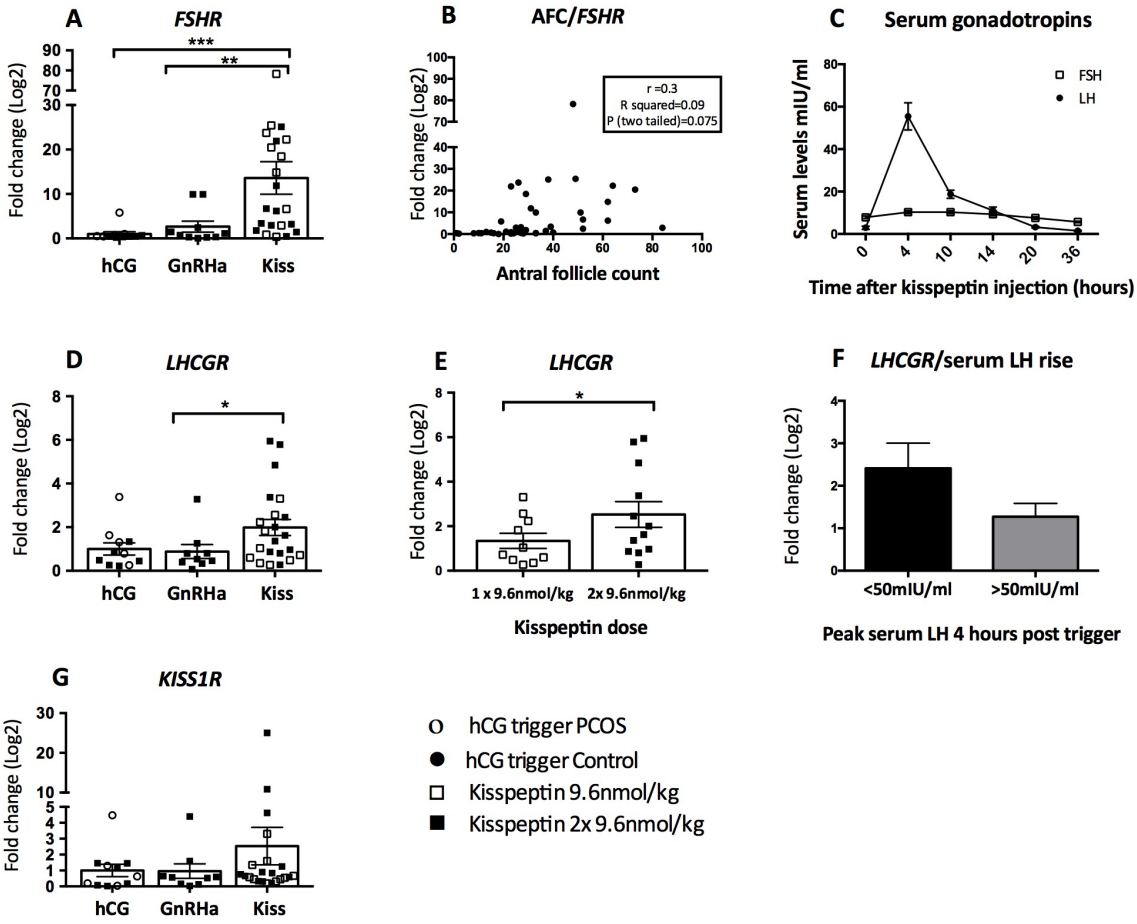
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Table 1. Baseline Characteristics

	hCG trigger N=12	**GnRH agonist trigger N=12**	**Kisspeptin trigger N=24**	**ANOVA p-value**
Mean Age (yrs) (±SD)	33.62 ±4.9	33.4 ±3.8	29.5 ±2.8	0.001
Body Mass index (kg/m^2) (±SD)	24.5 ±3.7	23.8 ±3.5	25.3 ±3.3	ns
Dose of FSH (iU) (mean±SD)	PCO 1804 ± 511 Non-PCO 3403±1301	1785 ±836	1607 ±605	0.0011
Antral follicle count (mean±SD)	14 ±8	34 ±12	41 ±17	<0.0001
Ovarian morphology	50% PCO/PCOS 50% normal ovaries	80% PCO/PCOS 20% multicystic ovaries	92% PCO/PCOS 8% multicystic ovaries	

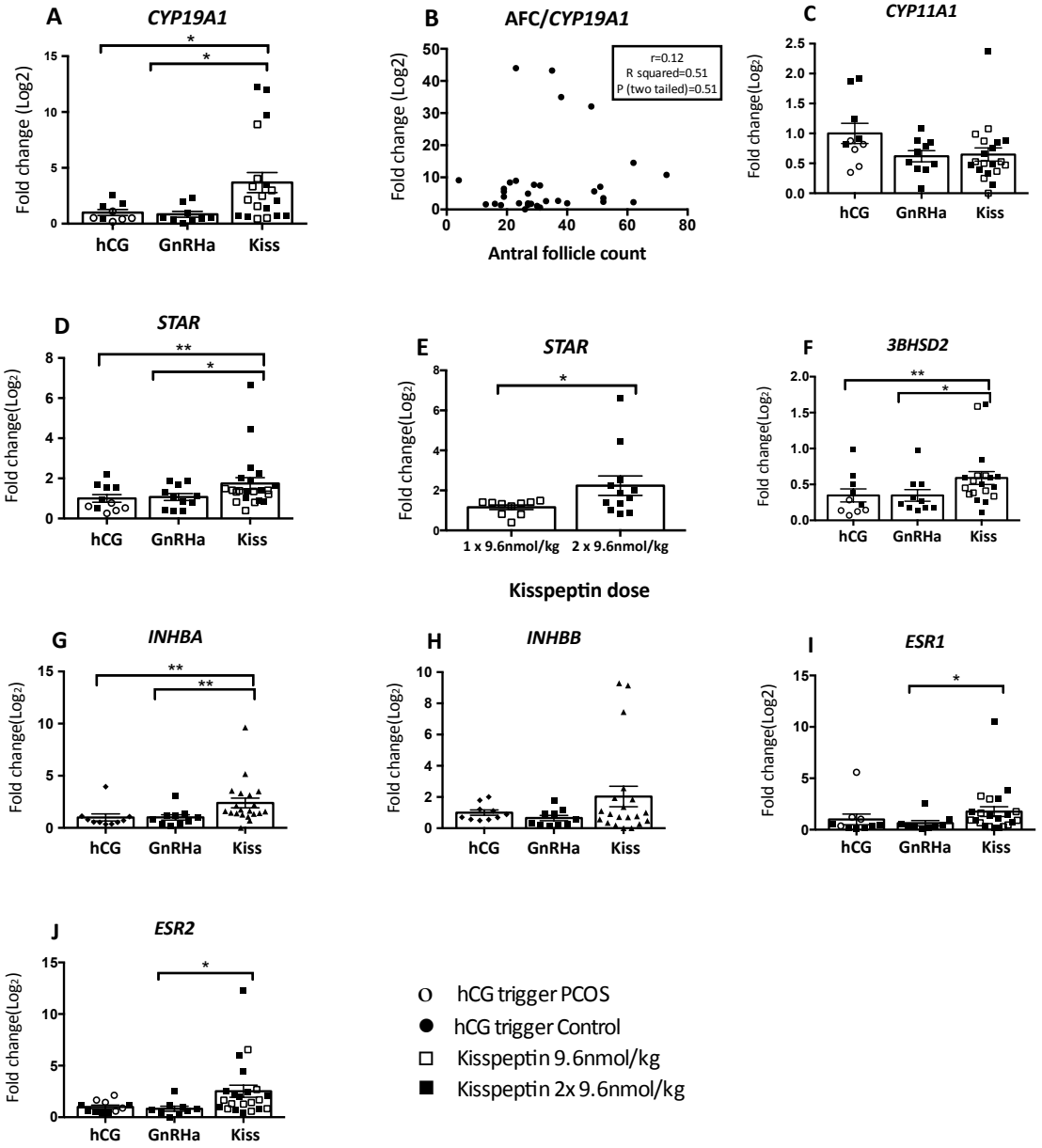
Figure 1.


A
FSHR
***
**
Fold change (Log2)
hCG
GnRHa
Kiss
B
AFC/FSHR
r =0.3
R squared=0.09
P (two tailed)=0.075
Fold change (Log2)
Antral follicle count
C
Serum gonadotropins
FSH
LH
Serum levels mIU/ml
Time after kisspeptin injection (hours)
D
LHCGR
*
Fold change (Log2)
hCG
GnRHa
Kiss
E
LHCGR
*
Fold change (Log2)
1 x 9.6nmol/kg
2x 9.6nmol/kg
Kisspeptin dose
F
LHCGR/serum LH rise
Fold change (Log2)
<50mIU/ml
>50mIU/ml
Peak serum LH 4 hours post trigger
G
KISS1R
Fold change (Log2)
hCG
GnRHa
Kiss
hCG trigger PCOS
hCG trigger Control
Kisspeptin 9.6nmol/kg
Kisspeptin 2x 9.6nmol/kg

Gene expression of gonadotropin and kisspeptin receptor genes in human granulosa cells after oocyte maturation with hCG, GnRH agonist or Kisspeptin-54, and correlation of gene expression with antral follicle count and serum gonadotropins. hCG group (n=12), GnRH agonist (GnRHa) (n=12) or Kisspeptin-54 (Kiss) (n=24). Kisspeptin-54 (9.6 nmol/kg) was given as 1 or 2 doses at a 10hr interval and these groups are delineated separately. The hCG group were analyzed with and without the control (non-PCO women). Data is presented as the mean + SEM and scattergram of individual gene expression values.

(A) Follicle stimulating hormone receptor (*FSHR*) gene expression is higher in GL cells from women who received kisspeptin-54 as maturation trigger than hCG ($p \leq 0.001$) or GnRH agonist ($p \leq 0.01$). (B) *FSHR* gene expression does not correlate with baseline antral follicle count, r= 0.3, R squared= 0.09, P=0.075. (C) Mean ± SEM change in serum FSH and LH levels from 0-36 hours after kisspeptin-54 administration. (D) Luteinizing hormone gene expression (*LHCGR*) is higher in GL cells from women who received kisspeptin-54 as maturation trigger than GnRH agonist ($p<0.05$) but is not higher than hCG. (e) *LHCGR* gene expression is higher in GL cells from women who received two kisspeptin-54 injections to trigger maturation (2 x 9.6nmol/kg 10 hours apart, n=12) than women who received one injection (9.6nmol/kg), p=0.038. (F) Correlating *LHCGR* gene expression with changes in serum LH after kisspeptin injection. Women who had a rise in serum LH of >50 mIU/ml 4 hours post trigger had a non-significantly lower gene expression of LHR than women who had a rise in serum LH of <50 mIU/ml 4 hrs post trigger. (g) kisspeptin receptor gene (*KISS1R*) expression was similar in granulosa cells from women who received hCG, GnRH agonist or kisspeptin to trigger oocyte maturation.

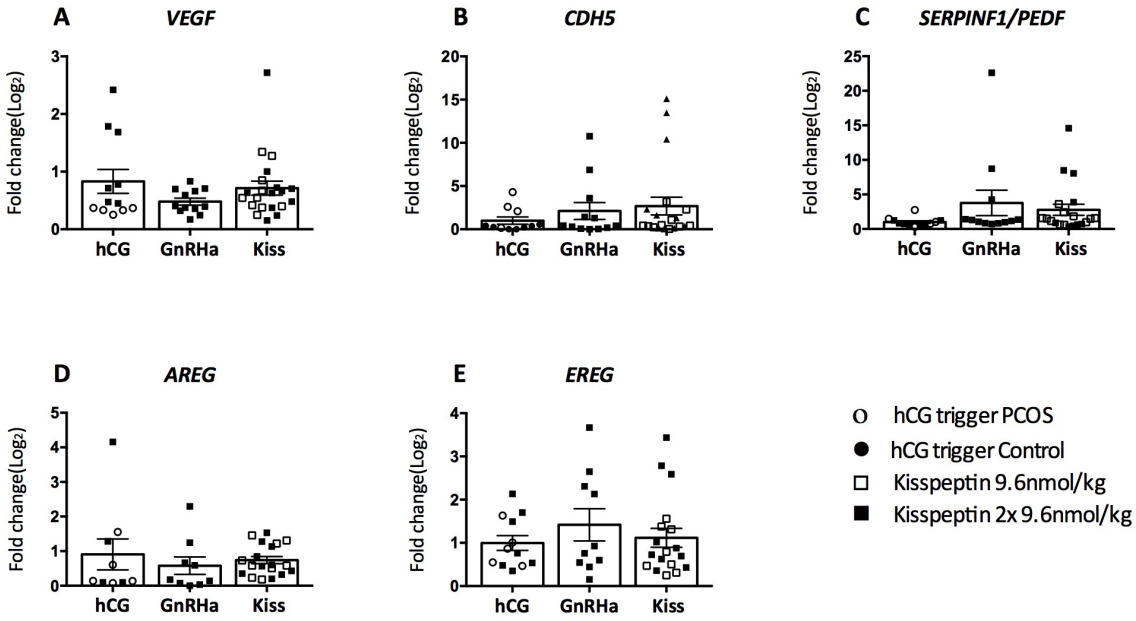
Figure 2



Gene expression of genes involved in ovarian steroidogenesis in human granulosa cells after oocyte maturation with hCG, GnRH agonist or kisspeptin-54. hCG group (n=12), GnRH agonist (GnRHa) (n=12) or Kisspeptin-54 (Kiss) (n=24). Kisspeptin-54 (9.6 nmol/kg) was given as 1 or 2 doses at a 10hr interval and these groups are delineated separately. The hCG group were analyzed with and without the control (non-PCO women). Data is presented as the mean + SEM and scattergram of individual gene expression values. (a) Aromatase (*CYP19A1*) gene expression is higher in GL cells from women who received kisspeptin as maturation trigger than hCG ($p \leq 0.05$) or GnRH agonist

(p≤0.05). (b) Aromatase gene expression does not correlate with baseline antral follicle count, r= 0.12, R squared= 0.014, P=0.514. (c) *CYP11A1* gene expression is the same in GL cells from women who received kisspeptin as maturation trigger than hCG or GnRH agonist. (d) Steroidogenic acute regulatory protein (*STAR*) gene expression is higher in GL cells from PCO women who received hCG or kisspeptin as maturation trigger than GnRH agonist (p<0.05). (e) *STAR* gene expression is higher in women who received 2 kisspeptin injections than women who received one p=0.04). (f) 3β-Hydroxysteroid dehydrogenase 2 (*HSD3B2*) gene expression is higher in GL cells from PCO women who received kisspeptin as maturation trigger than hCG (p<0.01) or GnRH agonist (p<0.05). (g) Inhibin A (*INHBA*) gene expression is higher in GL cells from women who received kisspeptin as maturation trigger than hCG (p≤0.01) or GnRH agonist p≤0.01). (i) Inhibin B (*INHBB*) gene expression is the same in GL cells from women who received hCG, GnRHa or kisspeptin as maturation trigger. (i)(j) Gene expression of estrogen receptors 1 and 2 (*ESR1/2*) is higher in GL cells from women who received kisspeptin as maturation trigger than GnRH agonist (p<0.05) but is not higher than hCG.

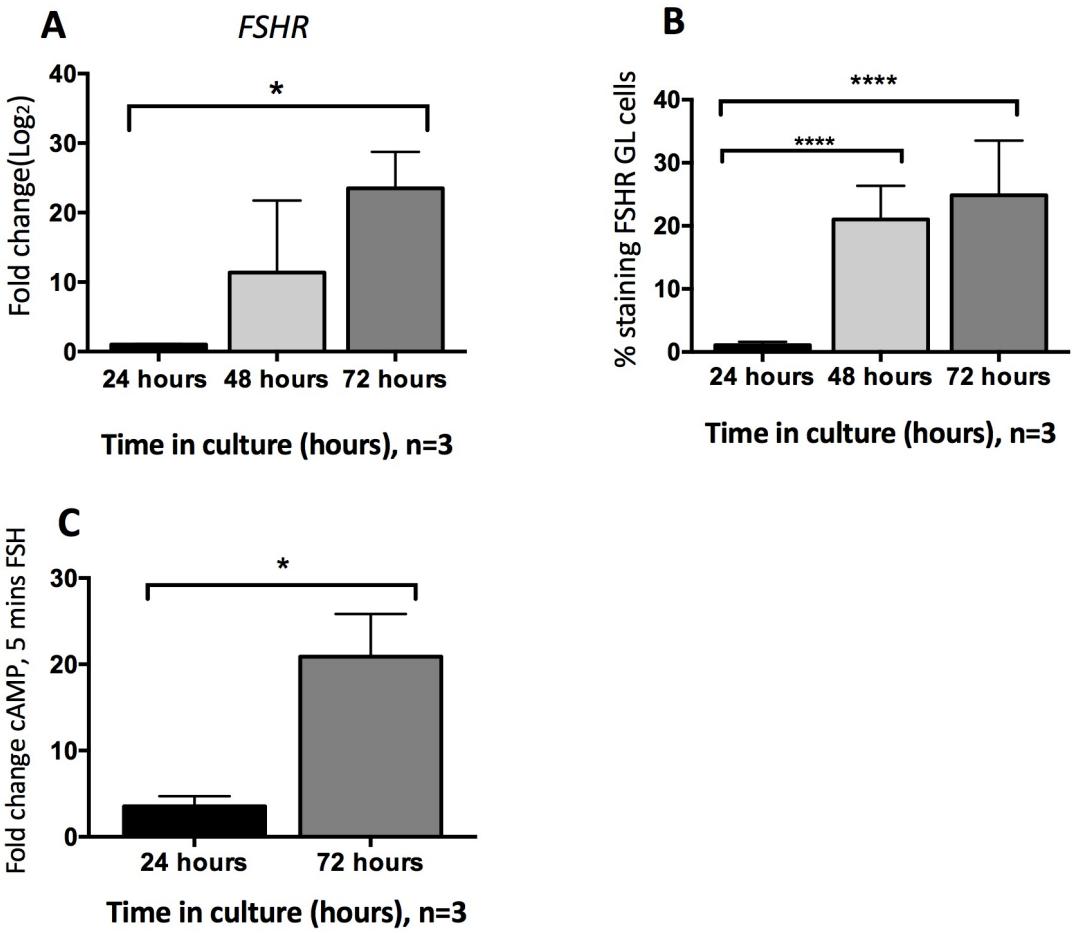
Figure 3.


A
VEGF
B
CDH5
C
SERPINF1/PEDF
D
AREG
E
EREG
Fold change(Log2)
hCG
GnRHa
Kiss
hCG trigger PCOS
hCG trigger Control
Kisspeptin 9.6nmol/kg
Kisspeptin 2x 9.6nmol/kg

Gene expression of potential markers of ovarian hyperstimulation in human luteinized granulosa cells after oocyte maturation with hCG, GnRH agonist or kisspeptin-54. hCG group (n=12), GnRH agonist (GnRHa) (n=12) or Kisspeptin-54 (Kiss) (n=24). Kisspeptin-54 (9.6 nmol/kg) was given as 1 or 2 doses at a 10hr interval and these groups are delineated separately. The hCG group were analyzed with and without the control (non-PCO women). Data is presented as the mean + SEM and scattergram of individual gene expression values. There is no significant difference between gene expression in the three groups for any of the markers (A) vacular endothelial growth factor *VEGFA*, (B) Serpin Family F Member 1 (*SERPINF1)*, (C) Cadherin-5 (*CHD5)*, (D) amphiregulin (*AREG*) and (E) epiregulin *(EREG*).

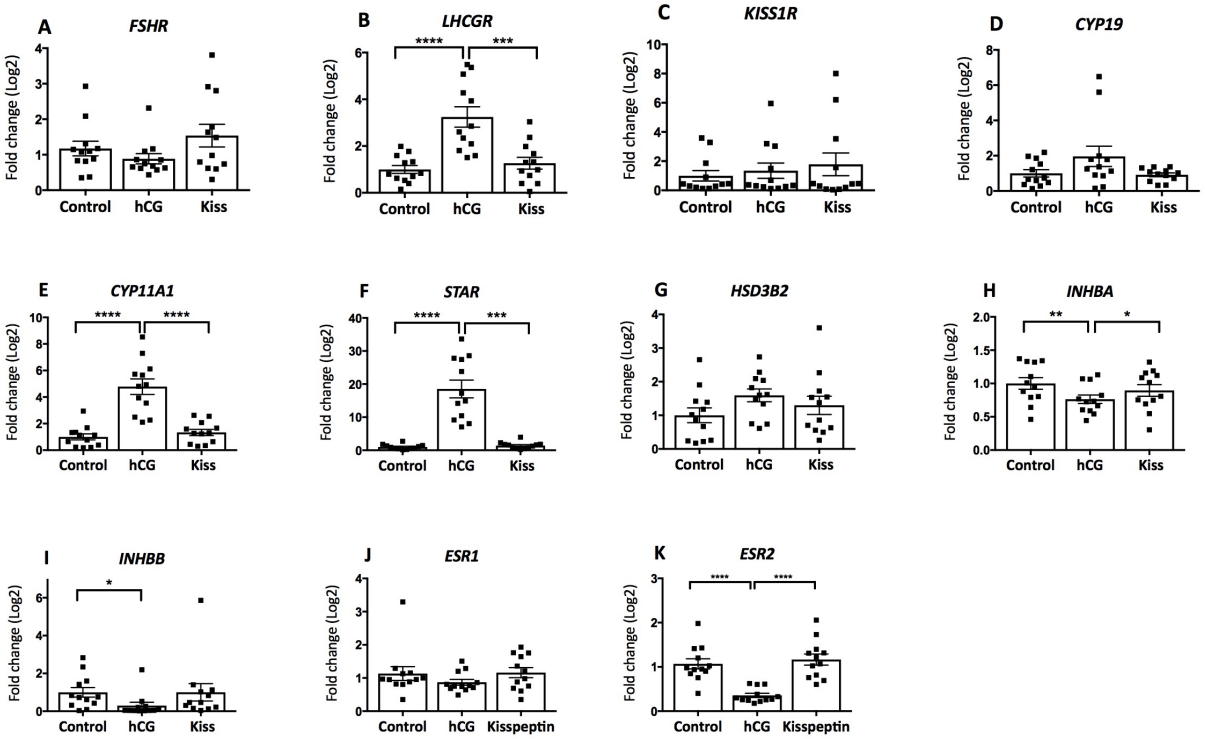
Supplementary materials

Supplemental Figure 1



FSHR expression and function resensitises over time in cell culture. (A) FSHR gene expression increases over time in cell culture of FSHR, n=3, p=0.05. (B) FSHR protein increases in time in culture, as measured by immunohistochemistry, n=3, p=<0.001. (C) cAMP generation in response to 10nM FSH administration for 5 minutes is higher at 72 hours than 24 hours, n=3, p=0.014.

Supplemental figure 2



Gene expression of genes involved in ovarian steroidogenesis in human granulosa lutein cells after in vitro treatment with hCG 10nM (n=12), kisspeptin 1uM (Kiss, n=12) or no treatment (Control, n=12). Data is presented as the mean + SEM and scattergram of individual gene expression values. (A) FSHR gene expression is not altered by in vitro treatment with hCG or kisspeptin. (B) LHCGR gene expression is higher in GLC treated with hCG than control ($P \leq 0.0001$) or those treated with kisspeptin ($P \leq 0.001$). (C) KISS1R gene expression is not altered by in vitro treatment with hCG or kisspeptin. (D) CYP19 gene expression is not altered by in vitro treatment with hCG or kisspeptin. (E) CYP11A1 gene expression is higher in GLC treated with hCG than control or those treated with kisspeptin ($P \leq 0.0001$). (F) STAR gene expression is higher in GLC treated with hCG than control ($P \leq 0.0001$) or those treated with kisspeptin ($P \leq 0.001$). (G) HSD3B2 gene expression is not altered by in vitro treatment with hCG or kisspeptin. (H) INHBA gene expression is higher in GLC treated with hCG than control ($P \leq 0.01$) or those treated with kisspeptin ($P \leq 0.05$). (I) INHBB gene expression is higher in GLC treated with hCG than control ($P \leq 0.05$). (J) ESR1 gene expression is not altered by in vitro treatment with hCG or

kisspeptin. (K) ESR2 gene expression is higher in GLC treated with hCG than control or those treated with kisspeptin ($P \leq 0.0001$).

Supplemental table 1. Primer sequences, gene ID, exon location, product size

Gene	Gene ID	Exon Location	Primer sequence (5'3')	Product size(bp)
LHCGR	3973	Fwd 5 Rev 6	Fwd GATGTTACGAAGGTCTTCTC Rev AAAAGCATTTCCTGGTATGG	90
FSHR	2492	Fwd 10 Rev 10	Fwd GCGGAACCCCAACATCGTGTC Rev TGAAGAAATCTCTGCGAAAGT	248
CYP19A1	1588	Fwd 8/9 Rev 9	Fwd GGTGAGAGAGACATAAAGATTG Rev TTCAGGATAATGTTTGTCCC	170
CYP11A1	1583	Fwd 6 Rev 7	Fwd AGCATCAAGGAGACACTAAG Rev GCAGGAATCATGTAATCTCG	95
HSD3B2	3284	Fwd 2 Rev 2/3	Fwd AAGTGTTTCCTGCTACTTTG Rev CTGGAGCTTAGAAAATTCCTC	181
STAR	6770	Fwd 4 Rev 5	Fwd GACAAAGTGATGAGTAAAGTGG Rev CAGCTCGTGAGTAATGAATG	195
KISS1	3814	Fwd 2 Rev 3	Fwd CCACTTTGGGGAGCCATTAG Rev CCAGTTGTAGTTCGGCAGGTC	295
KISS1R	84634	Fwd 1/2 Rev 2	Fwd GACCAACTTCTACATCGC Rev GATGTAGTTGACGAACTTGC	136
VEGFA	7422	Fwd 4/5 Rev 5/6	Fwd AATGTGAATGCAGACCAAAG Rev GACTTATACCGGGATTTCTTG	106
INHBA	3624	Fwd 1/2 Rev 2	Fwd GAGAATTTGCTGAAGAGGAG Rev CAAAAGTTGTTGTGATTGCC	136
INHBB	3625	Fwd 2 Rev 2	Fwd AACGTTTCAGGGTATGAATG Rev TTTACTCTACCTTCTGGGTG	82
AREG	374	Fwd 3 Rev 3/4	Fwd AAAGAAAGAAAAAGGGAGGC Rev CATTTGCATGTTACTGCTTC	142
EREG	2069	Fwd 2-3 Rev 3	Fwd GTTCAGACAGAAGACAATCC Rev GACTCATGTCCACCAGATAG	112
ESR1	2099	Fwd 6-7 Rev 7	Fwd GGAGTGTACACATTTCTGTC Rev CAAAGTGTCTGTGATCTTGTC	90
ESR2	2100	Fwd 7 Rev 7-8	Fwd AAATCTTTGACATGCTCCTG Rev AGGGTACATACTGGAATTGAG	113
CDH5	1003	Fwd 10 Rev 10-11	Fwd CGCAATAGACAAGGACATAAC Rev TATCGTGATTATCCGTGAGG	92
SERPINF1	5176	Fwd 4 Rev 4-5	Fwd GAACAGAATCCATCATTCACC Rev CTTCTTCTCAAAGACGATCC	146