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ORIGINAL ARTICLE

Human fallopian tube epithelium co-culture with murine ovarian follicles reveals crosstalk in the reproductive cycle

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STUDY QUESTION: Do interactions between human fallopian tube epithelium and murine follicles occur during an artificial reproductive cycle in a co-culture system *in vitro*?

SUMMARY ANSWER: In a co-culture system, human fallopian tissues responded to the menstrual cycle mimetic by changes in morphology and levels of secreted factors, and increasing murine corpus luteum progesterone secretion.

WHAT IS KNOWN ALREADY: The entire fallopian tube epithelium, including ciliated and secretory cells, can be regulated in the reproductive cycle. Currently, there are no *in vitro* culture models that can monitor fallopian tissues in real time in response to factors produced by the ovary. In addition, there are no reports on the impact of fallopian tissue on ovarian function during the menstrual cycle.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: Human fallopian tissue (n = 24) was obtained by routine hysterectomies from women (aged 26–50 years, mean age = 43.6) who had not undergone exogenous hormonal treatment for at least 3 months prior to surgery. CD1 female mice were used for ovarian follicle isolation. The human fallopian epithelium layers were either co-cultured with five murine multilayer secondary follicles (150–180 µm follicles, encapsulated in one alginate gel bead) for 15 days or received stepwise steroid hormone additions for 13 days. The fallopian tissue morphology and cilia beating rate, as measured by an Andor Spinning Disk Confocal, were investigated. Oviduct-specific glycoprotein 1 (OVGP1), human insulin-like growth factor 1 (hIGF1), vascular endothelial growth factor A (VEGF-A) and interleukin 8 (IL8) as biological functional markers were measured either by ELISA or western blot to indicate dynamic changes in the fallopian epithelium during the reproductive cycle generated by mouse follicles or by stepwise steroid hormone induction. Three or four patients in each experiment were recruited for replicates. Data were presented as mean ± SD and further analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

MAIN RESULTS AND THE ROLE OF CHANCE: The cultured fallopian tube epithelium responded to exogenous steroid hormone stimulation, as demonstrated by enhanced cilia beating rate (~25% increase, P = 0.04) and an increase in OVGP1 secretion (P = 0.02) in response to 1 nM estradiol (E_2) treatment when compared with 0.1 nM E_2. Conversely, 10 nM progesterone plus 1 nM E_2 suppressed cilia beating rate by ~30% (P = 0.008), while OVGP1 secretion was suppressed by 0.1 nM E_2 plus 50 nM progesterone (P = 0.002 versus 1 nM E_2 alone). Human fallopian tube epithelium was co-cultured with murine secondary follicles to mimic the human menstrual cycle. OVGP1 and VEGF-A secretion from fallopian tissue was similar with stepwise hormone treatment and when cultured with murine follicles. However, the secretion patterns of hIGF1 and IL8 differed in the luteal phase when comparing steroid treatment with follicle co-culture. In co-culture, hIGF1 secretion was suppressed in the luteal versus follicular phase (P = 0.005) but stepwise hormone treatment had no effect on hIGF1. In co-culture, IL8 secretion was also suppressed on luteal phase day 15 (P = 0.013) versus follicular phase day 7, but IL8 secretion increased continuously under high E_2/progesterone treatment (P = 0.003 for D13 versus D3). In the co-culture system, the corpus luteum continuously produced progesterone in the presence of fallopian tube tissue until Day 18 while, without fallopian tissue, progesterone started to drop from Day 13.

LIMITATIONS, REASONS FOR CAUTION: One limitation of this study is that murine follicles were used to mimic the human menstrual cycle. However, although secretion patterns of peptide hormones such as inhibins and activins differ in mice and humans, the co-culture system used here did reveal interactions between the tissues that govern reproductive function.

WIDER IMPLICATIONS OF THE FINDINGS: *In vitro* co-culture models of fallopian reproductive tissues with ovarian follicles can provide an important tool for understanding fertility and for uncovering the mechanisms responsible for reduced fertility. In addition, the role of oviductal secretions and how they influence ovarian function, such as the production of progesterone during the menstrual cycle, can be uncovered using this model.

LARGE-SCALE DATA: None.

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Key words: fallopian tube / follicle / *in vitro* culture / menstrual cycle / luteal deficiency / co-culture

Introduction

The human fallopian tube (named oviduct in non-primate species) is a critical organ in the female reproductive tract that naturally facilitates the transport of the cumulus–oocyte complex [COC], sperm and embryos (Hunter, 2005). The fallopian tube consists of distinct segments, including the fimbriated ends that lie adjacent to the ovary, the ampulla, the ampulla–isthmus junction and the isthmus portion (Croxatto, 2002). The entire length of the fallopian tube is lined with ciliated and secretory epithelial cells. The ciliated cells are most abundant at the fimbriated/ampulla segment. The fimbriae wrap around the ovary and transport the COC into the ampulla portion (Croxatto, 2002). The secretory cells, which produce vast numbers of growth factors and glycoproteins that act in both an autocrine and paracrine manner to regulate fallopian tube cells, sperm and embryo movement and development, are mostly found in the ampulla and the ampulla–isthmus junction (Buhi *et al.*, 2000).

The entire epithelium, including ciliated and secretory cells, can be regulated by hormones, cytokines, gametes and the embryos that interact with it (Abe *et al.*, 1999; Bauersachs *et al.*, 2004). The beating of cilia is critical to facilitate the movement of eggs, sperm and the embryo. Estrogen and progesterone can regulate the ciliary beating rate in the reproductive cycle, which ultimately controls the location of fertilization in the fallopian tube and later guides embryo transfer into the uterus (Mahmood *et al.*, 1998; Nakahari *et al.*, 2011). Oviduct-specific glycoprotein 1 [OVGP1], produced by fallopian secretory cells, positively influences the capacitation of sperm and its motility prior to fertilization (Erickson-Lawrence *et al.*, 1989; Verhage *et al.*, 1997). Insulin-like growth factor 1 [IGF1] is one of the growth factors produced by the secretory epithelium cells, and cyclic changes in IGF1 concentration during the menstrual cycle are critical to embryonic development (Carlsson *et al.*, 1993; Schmidt *et al.*, 1994; Stevenson and Wathes, 1996; Pushpakumara *et al.*, 2002; Swangchan-Uthai *et al.*, 2011). Vascular endothelial growth factor A [VEGF-A] is a potent mitogen for vascular endothelium and can stimulate vascular permeability. The mRNA expression of VEGF-A in the human fallopian tube epithelium has been reported to be highest during the periovulatory stage (Lam *et al.*, 2003; Lam and Haines, 2005; Lam *et al.*, 2005). However, VEGF-A expression in the fallopian tube may correlate with gonadotrophin expression rather than with estradiol [E_2] levels (Lam *et al.*, 2003; Lam *et al.*, 2005). Interleukin 8 [IL8] is expressed by fallopian tube cells and its expression is highest prior to ovulation, which correlates with peaks in cell height, secretion and ciliation (Palter *et al.*, 2001). In addition, IL8 has a pro-inflammatory role in the fallopian tube during tubal infections and subsequent hydrosalpinges (accumulation of inflammatory fluid within the lumen) (Balasubramaniam *et al.*, 2012). Many of these changes in signaling molecules and hormones facilitate proper fertilization and embryo development in the fallopian tube and can serve as surrogate measurements of normal fallopian tube function (Carlsson *et al.*, 1993).

Evidence exists that disruption of fallopian tube epithelial cell integrity, and thus disruption of normal fallopian tube function, may lead to alterations in the menstrual cycle. For example, although tubal sterilization is a common, effective and safe contraception method, one potential complication is post-tubal ligation syndrome. Post-tubal ligation syndrome is characterized by one or more sequelae post-surgery, including abnormal bleeding or pain, changes in sexual behavior or emotional health, exacerbation of premenstrual symptoms, menstrual disturbances and early menopause (Williams *et al.*, 1951; Muldoon, 1972; Stock, 1978; Cooper, 1983; Gentile *et al.*, 1998; Grynnerup *et al.*, 2013; Ye *et al.*, 2015). A few groups have reported luteal deficiency or a reduction in progesterone production after tubal ligation (Donnez *et al.*, 1980; Alvarez-Sanchez *et al.*, 1981; Corson *et al.*, 1981; Donnez *et al.*, 1981; Helm and Sjöberg, 1986). Disruptions in normal fallopian tube function caused by tissue destruction may lead to menstrual cycle alterations (Donnez *et al.*, 1981). However, luteal deficiency after tubal ligation surgery is controversial, and the underlying mechanisms for the deficiency remain unclear.

In this study, murine follicles were cultured *in vitro* where they were matured and luteinized to mimic the human menstrual cycle (Skory *et al.*, 2015). A unique system was developed to monitor the interaction between the follicle and the fallopian tissue throughout both the follicular and luteal phases of the menstrual cycle in real time. Using this system, the changes that were observed in both human fallopian tube morphology and the production of OVGP1, IGF1, VEGFA and IL8 were consistent with *in vivo* observations. Interestingly, the presence of fallopian tube tissue increased progesterone production by the corpus luteum. Co-culture provides a tool to explore the interaction between the fallopian tube and the ovarian follicle across the menstrual cycle and revealed that the fallopian tube does indeed impact ovarian biology.

Materials and Methods

Chemicals and reagents

17β-estradiol [E_2], progesterone [P_4] and hCG were purchased from Sigma (St. Louis, MO, USA). rhFSH was a gift from Dr Mary Zelinski at the Oregon Primary Center. Human fallopian tissue (n = 24) was obtained from routine hysterectomies in women (ranged from 26 to 50 years, mean age = 43.6) who had not undergone exogenous hormonal treatment for at least 3 months prior to surgery. Written informed consent was obtained from each patient and approval granted by the ethics committee of Northwestern University. CD1 mice were purchased from Harlan Laboratories (Madison, WI, USA). All procedures involving mice were approved by the Northwestern University Animal Care and Use Committee. Mice were housed and bred in a barrier facility (Chicago, IL, USA) and were provided with food and water *ad libitum*. Temperature, humidity and photoperiod (14 L:10D) were kept constant during the study.

Human fallopian tissue culture

The human fallopian tissue was kept in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium with 10% fetal bovine serum (FBS) on ice and processed within a 24-h period. The tissue was washed two times with warm phosphate-buffered saline (PBS) and transferred into warm dissection medium (Liebowitz 15 with 0.5% penicillin/streptomycin (P/S) and 1% FBS) (Invitrogen, Grand Island, NY, USA). The fallopian tube was cut open, and the inner layer was mechanically dissected using forceps. The layer, primarily composed of epithelium and underlying stroma, was transferred to alpha minimum essential medium (MEM) with 0.3% bovine serum albumin (BSA), 0.5 mg/ml Fetuin B (Sigma-Aldrich, St. Louis, MO, USA), 1% P/S and Insulin-Transferrin-Selenium (ITS) medium (5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium) (Invitrogen, Grand Island, NY, USA) and cut into 2 × 2 mm pieces for culture. The tissue was cultured on 0.4 μm pore Millicell inserts (EMD Millipore Co, Billerica, MA, USA) in a 12-well plate at 37°C and 5% CO_2 for 14 days with E_2 0.1 nM supplemented. The tissue was fixed for the histological analysis. There were three treatment plans for the tissues: (i) the tissue was treated with steroids in different combinations, those being E_2 0.1 nM, E_2 1 nM, E_2 1 nM + P_4 10 nM or E_2 0.1 nM + P_4 50 nM for 7 days without rhFSH and hCG: (ii) the fallopian tissues were co-cultured with mouse multilayered secondary follicles for 15 or 18 days with rhFSH for the first 7 days, on Day 7 the hCG was added for 16 h and then no rhFSH and hCG was added from Day 8: (iii) the fallopian tissues were treated with stepwise steroid hormones of five stages consisting of E_2 0.1 nM (3 days), E_2 1 nM (3 days following), on Day 6 the hCG was given for 16 h, E_2 1 nM + P_4 10 nM (3 days following from Day 7) and E_2 0.1 nM + P_4 50 nM (3 days following from day 10) for 13 days in total to mimic the different stages of the menstrual cycle. In addition, rhFSH was added for the first 6 days, and no rhFSH and hCG were added from Day 7.

Histology

Cultured primary fallopian tissue on Days 7 and 15 or corpus luteal tissue on Day 18 were fixed with Modified Davison's fixative (Electron Microscopy Science Inc., Hatfield, PA, USA) for 24 h at 4°C and then processed and embedded in paraffin. Hematoxylin and eosin staining were performed using standard methods. Immunofluorescence staining [IF] was performed and visualized as previously described (Kim *et al.*, 2013). The primary antibodies used for the IF were as follows: estrogen receptor [ER] alpha (Abgent, Pittsburgh, PA, USA) at a 1:70 dilution, progesterone receptor [PR] (Dako, Carpinteria, CA, USA) at a 1:200 dilution, and OVGP1 (LsBio, Seattle, WA, USA) at a 1:100 dilution. The tissue stained without adding primary antibody was considered a negative control.

Immunoblot analysis

The cultured human fallopian tissue was homogenized in ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 137mM NaCl, 10% glycerol, 1% NP-40 and 2 mM EDTA) with proteinase inhibitor. Equal amounts of protein, measured by the BCA protein assay kit (Thermo Scientific, MA, USA), were loaded into precast NuPAGE 4–12% gradient Bis-Tris gels and electrophoresis was performed. Proteins were dry transferred using an iBlot system (Invitrogen, NY, USA) to a nitrocellulose membrane (Life Technologies, Carlsbad, CA, USA). The blots were probed with a polyclonal anti-OVGP1 antibody (LsBio, Cambridge, MA, USA) overnight at 4°C followed by anti-rabbit secondary antibody conjugated to horseradish peroxidase (Zymed, San Francisco, CA, USA). Proteins were detected by enhanced chemiluminescence primer (GE HealthCare Life Sciences, Pittsburgh, PA, USA) and exposed using a FluorChem® HD machine (Alpha Innotech, San Jose, CA, USA). The same blot was stripped with buffer (Thermo Scientific, Hanover Park, IL, USA) and reprobed with a monoclonal anti-α-tubulin antibody (Sigma, St. Louis, MO, USA) followed by an anti-mouse secondary antibody conjugated to horseradish peroxidase. Protein quantification was performed using densitometry analyses on ImageJ Imaging Software (National Institutes of Health, USA). The conditioned medium was collected and probed via western blot analyses (as above) using a polyclonal anti-hIGF1 antibody (Abcam, Cat: ab9672, Cambridge, MA, USA).

Quantification of fallopian epithelia cilia beating

The fallopian tissues were cultured in E_2 0.1 nM, E_2 1 nM, E_2 1 nM + P_4 10 nM, E_2 0.1 nM + P_4 50 nM for 7 days. The movies of cilia beating were acquired using an Andor Spinning Disk Confocal with 5-ms exposure time and 5-ms readout time. Each movie was taken in 100 individual frames. Five movies in different areas of the sample were taken. The Multiple Kymograph as a plugin function in the NIH ImageJ software was used to quantify the cilia beating. In each movie, eight clusters of cilia were picked for the cilia beating quantification and 4–5 movies were measured.

Follicle co-culture with fallopian epithelia layer

CD1 mice (14 days old) had the ovaries removed, and 150–180 μm size follicles were isolated from the ovaries. Five follicles were encapsulated into one 0.5% (w/v) alginate bead for further culture (Xiao *et al.*, 2015). The alginate bead was placed in 1 well of a 12-well plate, and the fallopian tube epithelial layer tissue was placed on the 0.4-μm insert membrane. The insert containing the fallopian tube culture was placed into the same well as the follicles in the alginate bead. The medium in the 12-well co-culture system consisted of follicle culture medium, which was made from alpha MEM with 0.5 mg/ml fetuin (protein for supporting follicle growth and maintaining integrity of the zona pellucida) (Sigma-Aldrich, St. Louis, MO, USA), 0.3% BSA, ITS and 10 mIU/ml rhFSH as a supplement for the first 7 days of culture. On Day 7, the follicles were supplemented with *in vitro* maturation (IVM) medium, which contained 1.5 IU/ml hCG, and 10 mIU/ml rhFSH in the follicle growth medium for 16 h. After 16 h, the medium was changed back to follicle growth medium without rhFSH and hCG. The follicles, which were luteinized due to exposure to hCG, were continually cultured for another 7 days. Every other day, 350 uL of the medium was collected for the E_2, P_4, VEGF-A and IL8 ELISA test, as well as western blot analysis for hIGF1. Pictures were taken every other day in order to monitor the follicle growth. The fallopian tissues were fixed on Days 7 and 15 for histological analyses.

Hormone ELISA

E_2 and P_4 in the follicle culture medium or co-culture medium were detected by ELISA kits (Calbiotech, CA, USA) using the manufacturer's protocol. The sensitivity for the E_2 and P_4 assay is 3.94 pg/ml and 0.22 ng/ml, respectively. The intra-assay and inter-assay coefficients of variation (CV) for the E_2 and P_4 assay are all <10%. Human VEGF-A and IL8 in the fallopian-follicle co-culture medium were detected by the human VEGF-A ELISA kit (Thermo Scientific, CA, USA) and human IL8 ELISA kit (Life Technologies, Carlsbad, CA, USA) using the manufacturer's protocol. The sensitivity for the human VEGF-A kit and human IL8 kit is 5 and 4.4 pg/ml, respectively. The intra-assay and inter-assay CV for the human VEGF-A kit and human IL8 kit are all <10%. Each sample was run in duplicate in every ELISA.

Stepwise exogenous steroid hormone treatment

In order to compare the co-culture system with exogenous steroid treatment, exogenous E_2 and P_4 were added to the fallopian tissue culture medium in a stepwise protocol. In the first 3 days, the fallopian tissue was treated with E_2 0.1 nM, and then in the next 3 days, it was increased to 10 nM to match E_2 levels in the co-culture system. In the first 6 days, the growth medium was supplemented with 10 mIU/ml rhFSH to mimic the follicular phase. On Day 6, the tissues were treated with 1.5 IU/ml hCG together with rhFSH 10 mIU/ml and E_2 10 nM to mimic ovulation for 16 h. On Day 7, the culture medium was changed to remove hCG and rhFSH, but it contained E_2 (10 nM) and P_4 (10 nM) for 3 days. On Day 10, the tissue was treated with E_2 0.1 nM and P_4 50 nM for another 3 days. The conditioned medium was collected on Days 3, 6, 10 and 13 for hIGF1 western blot, VEGF-A and IL8 ELISA. The fallopian tissues were snap frozen on Days 3, 6, 10 and 13 for the OVGP1 western blot.

Statistical analyses

For the cilia beating quantification, western blot band densitometry quantification, and VEGF-A and IL8 ELISA, data were presented as mean ± SD. The data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. A $P < 0.05$ was considered statistically significant by Prism 6 software (GraphPad Software, Inc, CA, USA).

Results

Human fallopian tube epithelial tissue *in vitro* culture

A microporous insert culture system was selected for the current study of fallopian tissue (Fig. 1A). The fallopian epithelial layers after 14 days of culture in low E_2 treatment were viable (Fig. 1B), but the cultured fallopian epithelium tissue gradually became flat after a few days in culture on the transwell membrane, and this caused the cultured tissue to present with fewer folds as compared with uncultured fallopian tissue (Fig. 1A). The epithelial structure was maintained along with ERα and PR expression for 14 days (Fig. 1B). OVGP1, a steroid responsive protein expressed *in vivo* in the fallopian tube epithelium, also could be detected in the cultured fallopian epithelium layers after 14 days, although after culture more protein accumulated in the cytoplasm, possibly due to a change in polarization as compared with OVGP1 expression in the uncultured fallopian tissue (Fig. 1B). Therefore, culture of human fallopian tube epithelium on microporous membrane inserts maintains tissue structure, steroid receptor expression and hormone responsiveness over 14 days.

The human fallopian epithelial layer responds to exogenous steroid hormone treatment

In order to know whether the cultured tissue can respond to exogenous steroid hormone treatment, the 7-day steroid hormone treatments, E_2 0.1 nM, E_2 1 nM, E_2 1 nM + P_4 10 nM and E_2 0.1 nM + P_4 50 nM, were designed to monitor cilia beating (representing ciliated

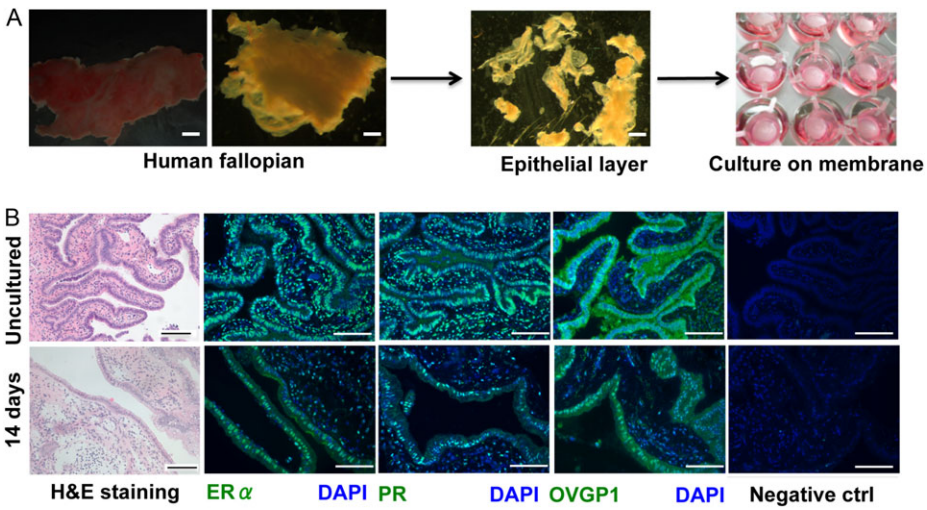

Figure 1 Human fallopian tube *in vitro* culture on the insert membrane. (**A**) The human fallopian tube was cut open and the epithelial layer was mechanically isolated. The epithelial layer was cut into 2 × 2 mm pieces and cultured on the insert membrane. Scale bar: 1 mm. (**B**) The epithelial layer was treated with low dose of estradiol (E_2) 0.1 nM for 14 days. Hematoxylin and eosin staining was used to evaluate the morphology of human fallopian tube epithelial layer after 14 days in culture compared with uncultured tissue. ERα, PR and oviduct-specific glycoprotein 1 (OVGP1) immunofluorescent staining was performed to characterize the human fallopian epithelial culture system. The scale bar indicated 100 μm. ER, estrogen receptor; PR, progesterone receptor.

cell function) and OVGP1 expression (representing secretory cell function) (Verhage *et al.*, 1990; Mahmood *et al.*, 1998; Shao *et al.*, 2007). Cilia beating frequency [CBF] significantly increased with E_2 treatment and decreased upon the addition of P_4 (Fig. 2A). CBF increased to 32.9 ± 4.56 Hz in response to 1 nM E_2, which is almost a 25% increase compared to 0.1 nM E_2 treatment (25.9 ± 2.75 Hz). The decrease in CBF to 23.1 ± 3.94 Hz (~30% decrease comparing with E_2 1 nM treatment) in the tissue treated with E_2 1 nM + P_4 10 nM represents the impact of progesterone on cilia beating during the early luteal phase. Similarly, high progesterone 50 nM combined with E_2 0.1 nM treatment repressed the CBF to 27.7 ± 1.97 Hz, which is similar to 0.1 nM E_2 treatment levels, representing the later luteal phase stage. In order to confirm induction of estrogen-regulated gene products, OVGP1 protein was measured after culture with hormone treatments. OVGP1 was induced by 1 nM E_2 treatment, and the level slightly decreased in the tissue treated with the addition of 10 nM P_4. The OVGP1 level in the fallopian tissue treated with 50 nM P_4 + 0.1 nM E_2 was similar to the tissue treated with 0.1 nM E_2 only (Fig. 2B).

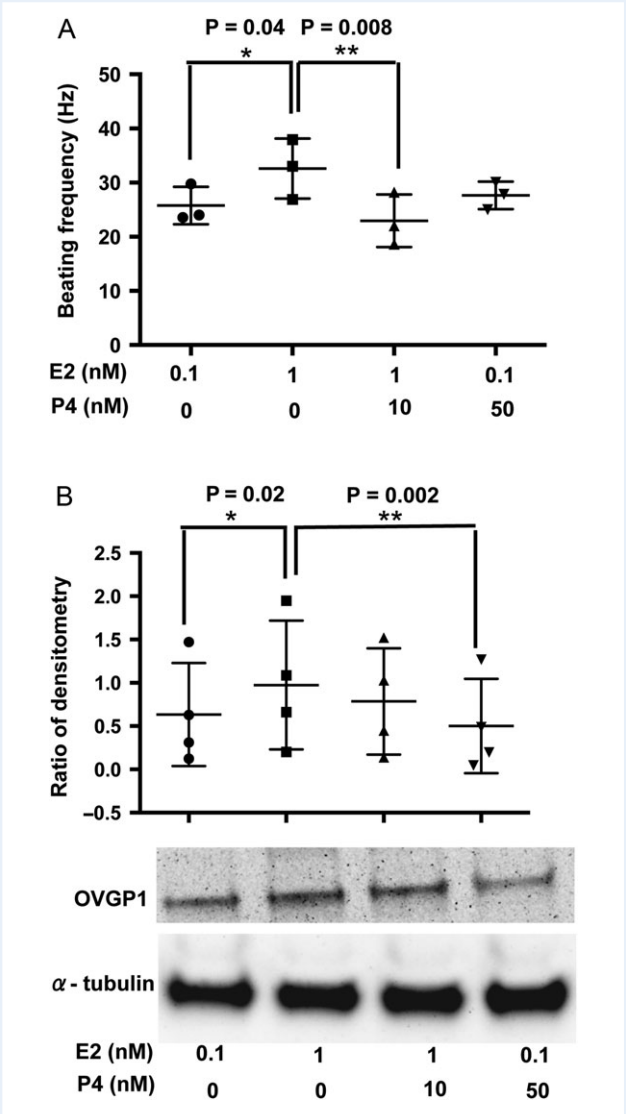

Figure 2 Functional response of human fallopian tube epithelium cultured in the presence of E_2 and P_4 for 7 days. The human epithelial layers were treated with E_2 0.1 nM, E_2 1 nM, E_2 1 nM + P_4 10 nM or E_2 0.1 nM + P_4 50 nM for 7 days to mimic different stages of menstrual cycle. (**A**) After 7 days of culture in the indicated treatments, cilia beating frequency was measured using an Andor spin disk microscope with a 100 × objective and a 5-ms exposure time and 5-ms readout time. Quantification of cilia beating frequency for $n = 3$ fallopian endometrium cultures from 3 individual patients. (**B**) Immunoblot of OVGP1 in cultured fallopian epithelium after treatment described above for 7 days. Bar graph represents relative band density using α-tubulin as the loading control for $n = 4$ cultured fallopian endometrium lysates from four patients. '*'statistically significant differences between groups. The data are mean ± SD. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis, and $P < 0.05$ was considered statistically significant.

In-vitro co-culture system establishment for mouse multilayer secondary follicles with human fallopian epithelial layer

While the fallopian tube has been studied in response to the menstrual cycle, these studies are typically accomplished by either collecting tissues at different phases of the cycle or culturing with purified steroid hormones (Mahmood *et al.*, 1998; Lyons *et al.*, 2002a). The murine follicles were previously shown to mimic the human follicular and luteal phase in terms of steroid hormone and peptide hormone production (Skory *et al.*, 2015). Five mouse secondary (150–180 µm) follicles were encapsulated into one three-dimensional alginate gel bead (Xu *et al.*, 2006, 2007; Xiao *et al.*, 2015). The follicles inside the bead were placed in the bottom of a 12-well plate, and the fallopian epithelial layer was placed on the transwell membrane. The follicles and the fallopian tubes were co-cultured for 15 days to represent a compressed reproductive cycle (Fig 3A, details in Materials and Methods). E_2 continually increased as the follicle grew with rhFSH stimulation until Day 7 (Fig. 3B), at which time 1.5 IU/ml hCG was administered to simulate maturation *in vitro*. E_2 then slowly dropped to baseline levels in the luteal phase. P_4 increased after hCG administration and plateaued from Days 10 to 15 (Fig. 3B). The thickness of the fallopian tube epithelium appeared greater than in tissues cultured without follicles (Fig. 3C). By Day 15, the thickness declined to basal levels (Fig. 3C).

Factors secreted from the fallopian tube exhibit cyclic changes in the co-culture system

The *in vitro* menstrual cycle generated by the follicle influenced several factors secreted from the fallopian tube. OVGP1 significantly increased on Day 7 before ovulation as compared with OVGP1 levels in the fallopian tissue culture only with rhFSH present in the culture medium (Fig. 4A). By Day 15, after hCG stimulation on Day 7, the OVGP1 returned to a level similar to the fallopian culture only levels (Fig. 4A). hIGF1 was elevated before IVM when estrogen levels were high and then significantly declined after IVM (Fig. 4B), which was not seen in samples cultured without follicles (Supplementary Figure 1). VEGF-A secreted by the human fallopian tube cells continuously increased until Day 15 (Fig. 4C). Previous reports suggested that E_2 could not

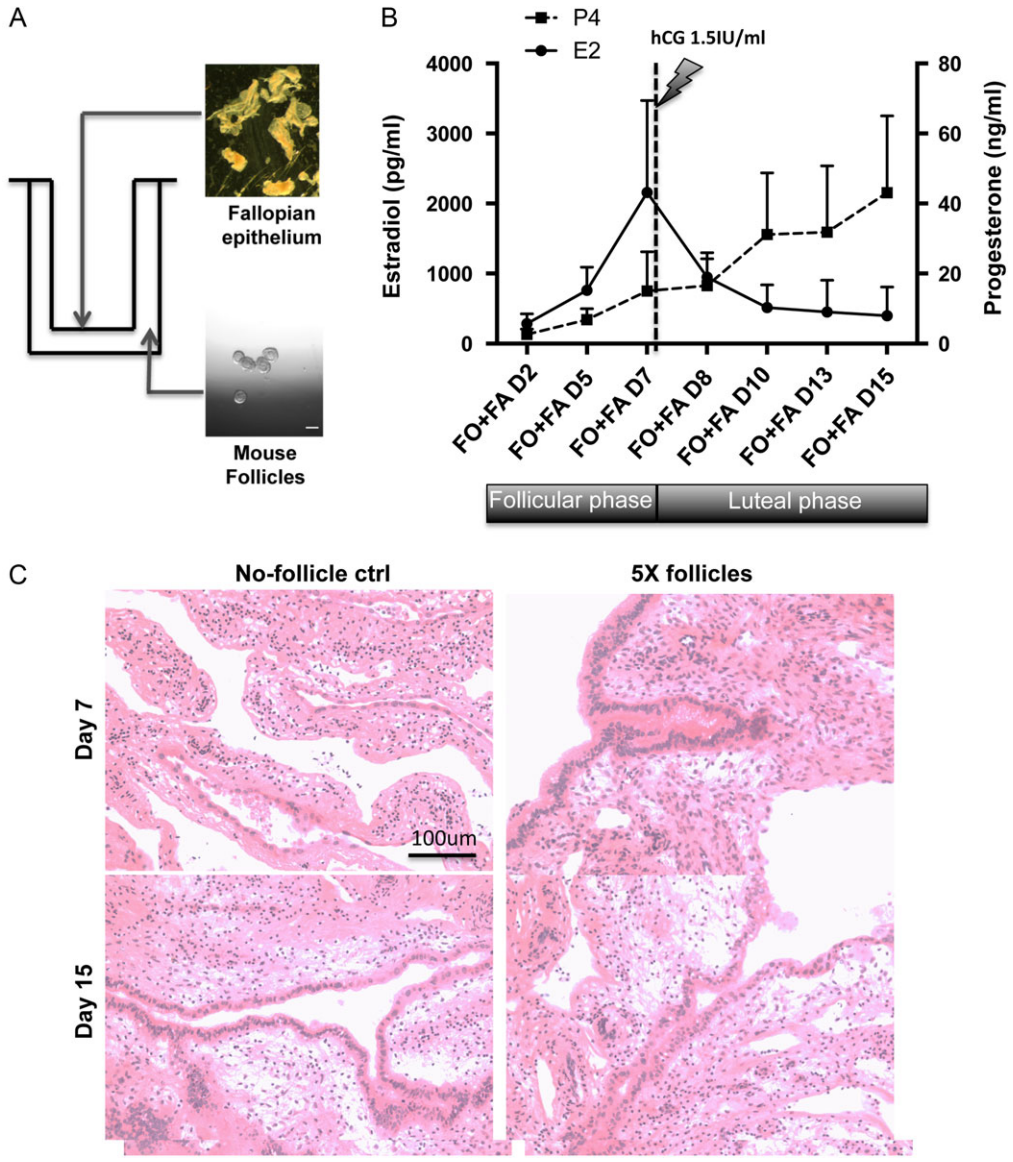

Figure 3 Human fallopian epithelium and murine follicle co-culture system mimic the menstrual cycle *in vitro*. (**A**) Schematic of follicle and fallopian epithelium co-culture model. Five secondary follicles (150–180 μm) were encapsulated into a single 0.5% alginate bead, which was placed in the bottom of each well. Fallopian epithelium was cultured on a 0.4-μm insert membrane, which was placed into the 12-well plate containing encapsulated follicles. (**B**) Steroid secretion pattern in the follicle and fallopian co-culture system. E_2 and P_4 levels were measured in co-culture medium at different time points. The co-cultures were maintained in growth medium supplemented with 10 mIU/ml recombinant human FSH (rhFSH) for first 7 days. After 7 days, the follicles were treated with 1.5 IU/ml hCG for 16 h to induce IVM. The luteinized follicles were then cultured for another 7 days without rhFSH and hCG. 'FO' represented 'follicle' and 'FA' represented 'fallopian'. Three patients' tissue was included in this co-culture system. (**C**) Morphology of the fallopian epithelial tissue cultured alone or co-cultured with follicles for 7 days and 15 days. Scale bar: 100 μm in size.

enhance VEGF-A, but rather VEGF-A was induced by gonadotrophin hormones, such as rhFSH or hCG (Lam *et al.*, 2003; Lam *et al.*, 2005). To test this, the fallopian tissue was cultured with either rhFSH or hCG for 6 days compared with the vehicle control, and the VEGF-A expression did not change in either of the treatments (Supplementary Figure 2). IL8 expression peaked on day 7 before hCG stimulation, which correlated with high E_2 secretion from the follicle, and then significantly decreased during the luteal phase (Fig. 4D), when low E_2 and high P_4 levels are present.

Fallopian tubes respond to stepwise exogenous steroid hormone treatment

In order to know whether cyclic changes in OVGP1, hIGF1, VEGF-A and IL8 from fallopian tube cells in the co-culture system are due to the steroid hormones or due to other factors produced by the follicle, a stepwise exogenous steroid hormone treatment was used (Fig. 5A). OVGP1 was induced by a high dose of E_2 (10 nM) on Day 6, and high levels were maintained until Day 10 when the tissues were exposed to

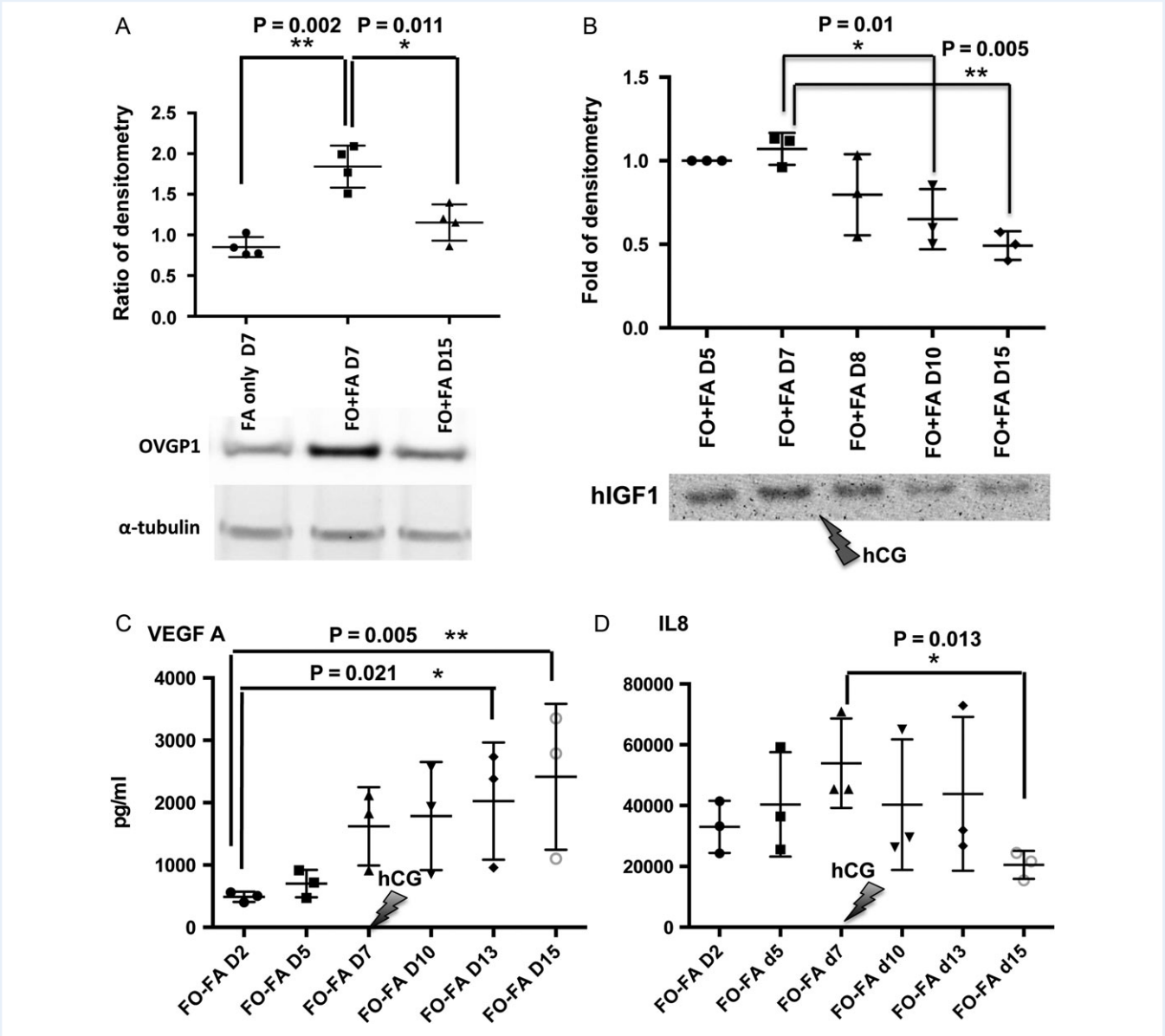

Figure 4 Factors from the human fallopian epithelium undergo dynamic changes in the co-culture system. (**A**) Immunoblot of OVGP1 level in cultured fallopian epithelium in the co-culture system or fallopian culture only. The first 7 days for both co-culture and fallopian culture only has rhFSH present in the culture medium. On Day 7, the hCG was added in culture media for the co-culture system. On Day 8, the media for the co-culture was changed to the media without hCG and rhFSH. The fallopian tissue was harvested on Days 7 and 15. The tissue lysate was used for the OVGP1 western blot. The alpha-tubulin was used as loading control. The immunoblot represents the tissue status from one patient. Bar graph represents relative band density using α-tubulin as the loading control for $n = 4$ cultured fallopian epithelium lysates from four patients. '*' corresponded to statistically significant differences between groups. The data are mean ± SD. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis, and $P < 0.05$ was considered statistically significant. (**B**) Human insulin-like growth factor 1 (hIGF1) levels were measured in the conditioned medium of the co-culture system at different time points by immunoblot analysis. Bar graph represents relative band density compared to culture day 5. Three individual experiments were performed using fallopian tissues from three patients. (**C**) Human vascular endothelial growth factor A (hVEGF-A) level and (**D**) human interleukin 8 (IL8) were measured in the conditioned medium of the co-culture system at different time points by human VEGF-A ELISA kit or human IL8 ELISA kit. Three patients were recruited for the experiments and two independent cultures for one experiment. * and ** are statistically significant differences between groups. The data are mean ± SD. One-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis, $P < 0.05$ was considered statistically significant. 'FO' represented 'follicle' and 'FA' represented 'fallopian' in all of the panels.

a high dose of E_2 10 nM and a low dose of P_4 10 nM. The OVGP1 level was significantly suppressed when the steroid hormone treatment changed to a high dose of progesterone 50 nM and a low dose of E_2 0.1 nM on Day 13 (Fig. 5B). In contrast to the follicle co-culture system, steroid hormone treatment with E_2 and P_4 did not significantly modify hIGF1 levels (Fig. 5C). VEGF-A continuously increased in response to P_4 treatment, which was similar to the co-culture system (Fig. 5D). Lastly, IL8 was increased by E_2 (10 nM), but P_4 at 50 nM

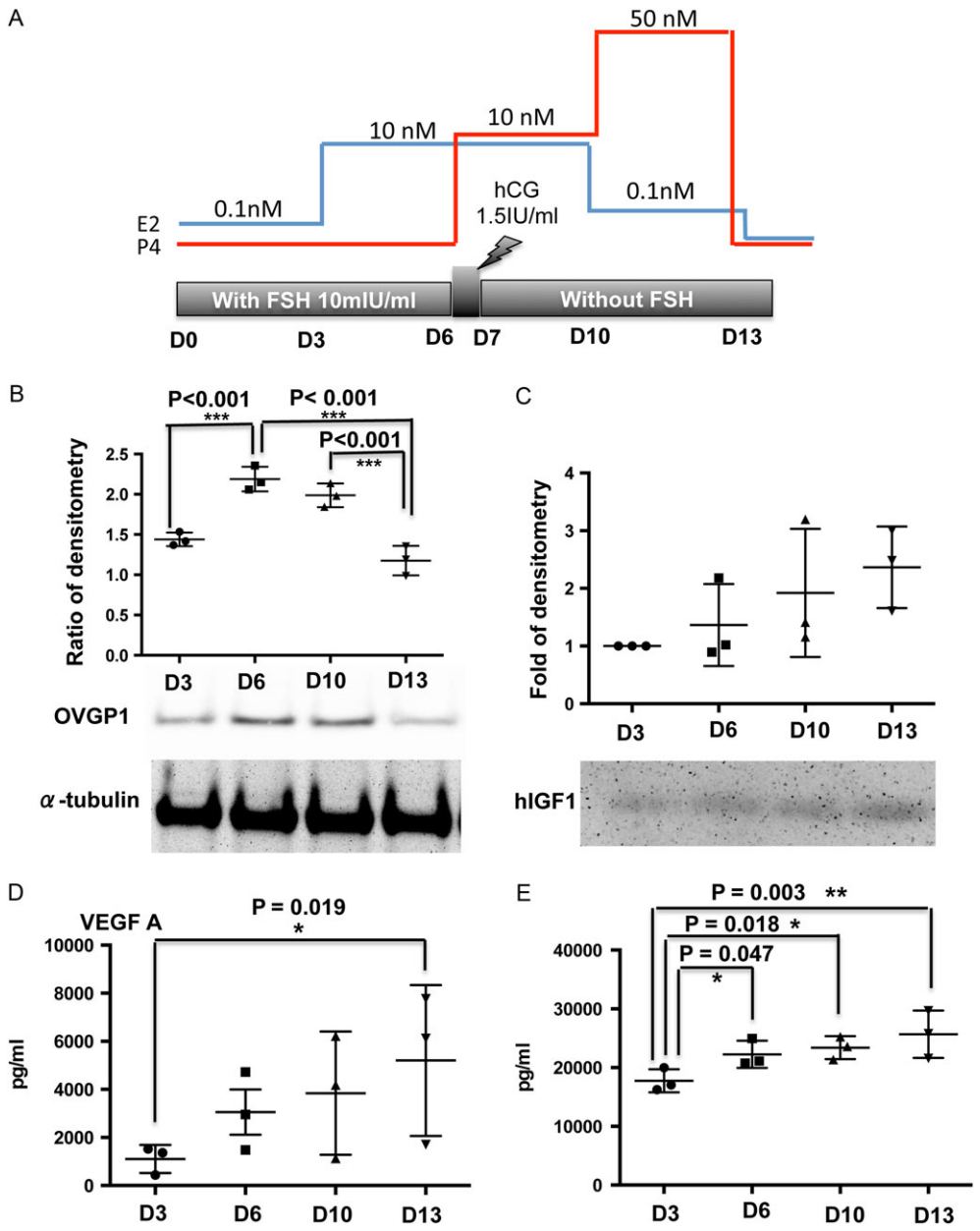

Figure 5 Fallopian tube response to stepwise exogenous steroid hormone treatment. (**A**) Schematic of the stepwise exogenous steroid hormone treatment regimen. For the first 3 days, the fallopian tissue was treated with E_2 0.1 nM followed by the increasing E_2 concentration 10 nM to match the E_2 level in the co-culture system for another 3 days. For the first 6 days, the growth medium was supplemented with 10 mIU/ml rhFSH to mimic the follicular phase. On Day 6, the tissues were treated with 1.5 IU/ml hCG to mimic the ovulation stage for 16 h, on Day 7 the culture medium changed into the growth medium without rhFSH and hCG, but with E_2 10 nM and P_4 10 nM for 3 days. On Day 10, the tissue was treated with E_2 0.1 nM and P_4 50 nM for another 3 days. (**B**) Immunoblot of OVGP1 in cultured fallopian epithelium in the stepwise steroid hormone treatment regimen. The fallopian tissue was harvested on Days 3, 6, 10 and 13. The tissue lysate was used for the OVGP1 western blot. The alpha-tubulin was used as loading control. The immunoblot represents one patient's tissue status. Bar graph represents relative band density using α-tubulin as the loading control for $n = 3$ cultured fallopian epithelium lysates from three women. The data are mean ± SD. '*' Corresponded to statistically significant differences between groups. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis, and $P < 0.05$ was considered statistically significant. (**C**) hIGF1 levels were measured in the conditioned medium of the stepwise steroid hormone treatment at different time points by immunoblot analysis. Bar graph represents relative band density compared to culture day 3. Three individual experiments were performed using fallopian tissues from three patients. (**D**) hVEGF-A and (**E**) hIL8 were measured in the conditioned medium of stepwise steroid hormone treatment regimen at different time points using a human VEGF-A ELISA kit or human IL8 ELISA kit. Three individual experiments were performed using fallopian tissues from three patients, and two individual cultures for each experiment. The data are mean ± SD. '*' corresponded to statistically significant differences between groups. One-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis, $P < 0.05$ was considered statistically significant.

and low E_2 at 0.1 nM did not suppress IL8, as was seen in the luteal phase of the co-culture (Fig. 5E).

Human fallopian epithelial tissue affects corpus luteal function

Due to a lack of knowledge about the influence of the fallopian tube on the ovary, the ability of the human fallopian tube tissue to affect *in vitro* murine follicles was investigated in our co-culture system. In the co-culture system and in the follicle-only system, E_2 production was similar. In both studies, E_2 was the highest on Day 7, which was just before ovulation, and then it gradually declined back to basal levels (Fig. 6A). Interestingly, the progesterone profile was significantly different in the presence of the fallopian tube. The progesterone level continuously increased in the co-culture system up to 18 days (Fig. 6B). The peak concentration of P_4 in the co-culture system was ~90 ng/ml. However, in the follicle-only culture, the progesterone levels peaked on Day 13 and declined to basal levels by Day 18 (Fig. 6B). The peak concentration of P_4 in the follicle culture only system was ~25 ng/ml. On Day 18, tissues from both culture systems were fixed for histology. The corpus luteum in the co-culture system maintained morphological signs of luteinization, based on the granulosa cell cytoplasmic–nuclear ratio (Fig. 6C). The corpus luteum in the follicle-only culture on Day 18 was filled with dense fibrous tissue (Fig. 6D).

Discussion

The fallopian tube is an important reproductive tissue to facilitate the transport of the COC, sperm and embryos and to support early embryo development. *In vitro* cultured murine follicles were engineered to secrete hormones in a manner that phenocopied the reproductive cycle (Skory *et al.*, 2015) and were used to stimulate a physiological response in primary human fallopian tube tissue. In the co-culture system, the factors produced by the follicles during growth, ovulation and luteinization impacted fallopian tissue morphology and protein secretion, such as OVGP1, IGF1, VEGF-A and IL8, producing similar results to *in vivo* observations (Palter *et al.*, 2001; Pushpakumara *et al.*, 2002; Lam *et al.*, 2003; Briton-Jones *et al.*, 2004). In order to understand whether the dynamic changes of these factors in the reproductive cycle generated by the murine follicle were due to the steroid hormones, stepwise exogenous estrogen and progesterone were used to mimic the menstrual cycle steroid hormone patterns in our co-culture system. In the co-culture system and in response to stepwise exogenous estrogen or progesterone with the same pattern of gonadotrophin hormones, such as FSH and hCG exposure in the treatment, the OVGP1 regulation in the fallopian tissue showed a similar trend in both the co-culture system and with the stepwise steroid hormone treatment, which confirmed that the OVGP1 level is tightly dependent on estrogen regulation. The regulation of hIGF1 and IL8 was different in the steroid-

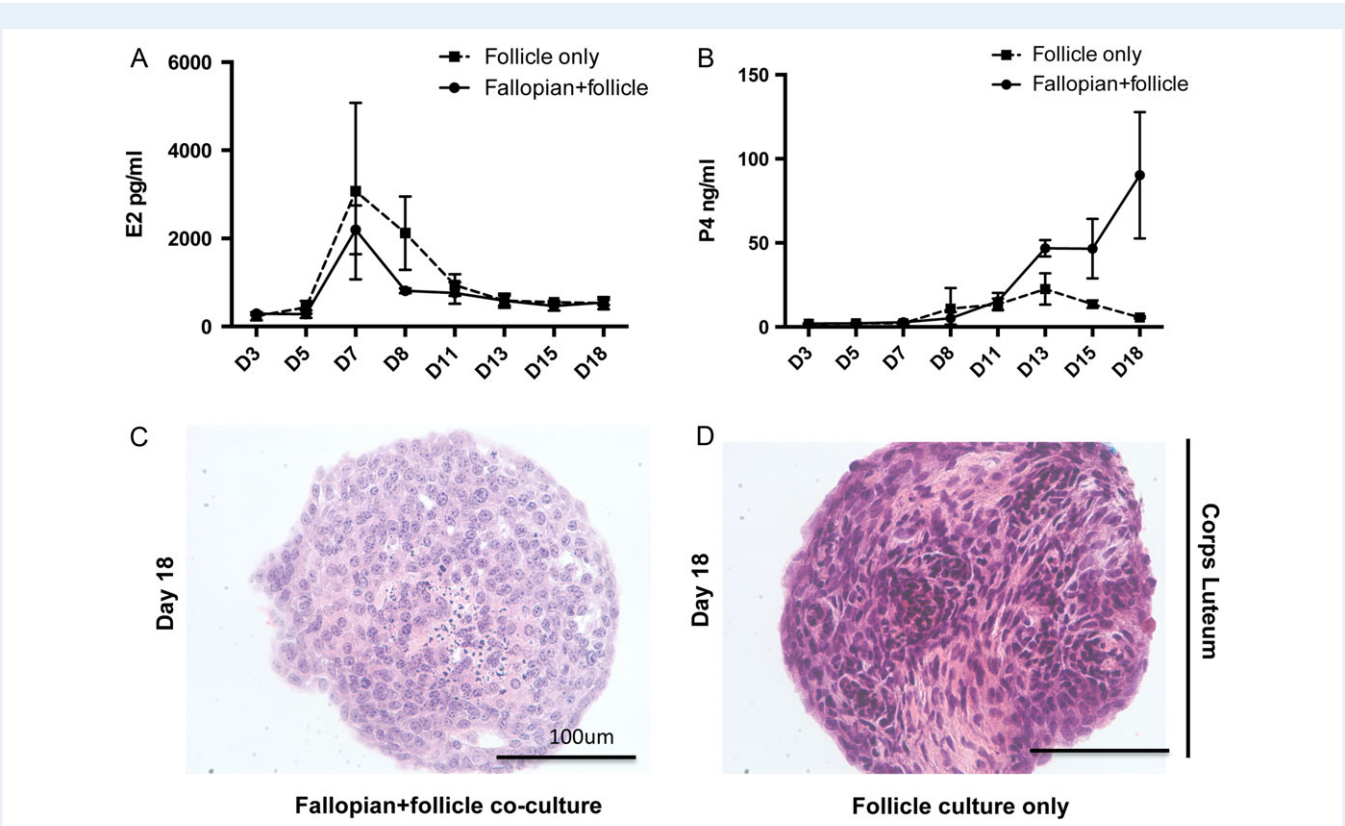

Figure 6 Human fallopian epithelium in the co-culture system affects murine follicle steroid hormone secretion. The E_2 (**A**) and P_4 (**B**) levels in the condition medium at the different time points from either the human fallopian epithelium and murine follicle co-culture system or from the murine follicle culture alone system. The corpora luteum either from co-culture or follicle-only culture after 18 days were fixed for the histological analysis (**C**, **D**). Three independent experiments were performed using tissue from three different patients. The data are mean ± SD.

only treatment and the murine follicle co-culture system in the luteal phase. Therefore, it is likely that the follicle during the ovulation stage or the corpus luteum produces factors other than progesterone that can act on human tissues to modify hIGF1 and IL8 expression in the luteal phase. Proper regulation of hIGF1 and IL8 expression in the menstrual cycle are important for fertility based on their established role in early embryo development (Palter *et al.*, 2001; Lam and Haines, 2005; Swangchan-Uthai *et al.*, 2011). The co-culture system allowed for investigation into the contribution of additional factors produced by the follicles during growth, ovulation and luteinization.

Furthermore, using our two *in vitro* culture systems, the expression of VEGF-A in the fallopian tube significantly increased in the luteal phase, which also increased in response to a high dose of progesterone. VEGF-A did not increase significantly in the follicular phase or in response to a high dose of E_2. Therefore, VEGF-A in the fallopian tissue is regulated by P_4 based on our data, but not by E_2, which confirms previous findings (Lam *et al.*, 2005). The previous report, however, speculated that the change in VEGF-A was due to gonadotrophins, which was tested directly in our *in vitro* culture system by adding 10 mIU/ml rhFSH or 1.5 IU/ml hCG, which were used in the co-culture system and in the stepwise steroid hormone treatment. Neither rhFSH nor hCG impacted VEGF-A expression in the fallopian epithelium tissue, confirming that VEGF-A expression in the fallopian tissue is dependent on P_4 simulation. The regulation of VEGF-A in the fallopian tissue is different from other reproductive tissues, such as ovary or uterus. For example, in the ovary, VEGF-A regulation is highly dependent on the gonadotrophin hormones rhFSH and hCG (Hazzard *et al.*, 1999), but in the uterus, VEGF-A regulation occurs in response to both steroid hormones (Shifren *et al.*, 1996; Sugino *et al.*, 2002). VEGF-A likely influences vascular permeability in the fallopian tissue to provide the proper environment for fertilization and early embryo development (Lam and Haines, 2005).

Co-culture of human fallopian tissue with murine ovarian follicles proved to be an effective model to study the dynamic interactions between the organs. Using the co-culture system, the fallopian tissue enhanced luteal function based on morphological changes and enhanced production of P_4. The ability of the fallopian tube to increase progesterone production is consistent with some clinical studies that suggest that tubal ligation reduces luteal function (Donnez *et al.*, 1980; Corson *et al.*, 1981; Donnez *et al.*, 1981). VEGF-A, secreted from the fallopian tissue in response to ovarian follicles, may contribute to the enhanced corpus luteal P_4 production (Tesone *et al.*, 2005; Qiu *et al.*, 2012). Using this co-culture system, the factors from fallopian tissue that benefit the corpus luteal function can be further explored. One limitation of this study is that murine follicles were used to mimic the human menstrual cycle. There are slight differences in the peptide hormones, such as inhibins and activins, between mouse and human (Matulis and Mayo, 2012). However, the steroid hormones estrogen and progesterone, along with many of the cytokine factors, are conserved across species, and the co-culture system with murine ovarian follicles was able to reveal interactions between the tissues that govern function.

The integration of multiple biological assays to define functional changes in response to murine follicles will be an important tool for monitoring the influence of pharmaceuticals and endocrine disruptors on fertility. For example, cilia beating frequency is an essential function of the fallopian tube to transport sperm, the COC, and the embryo to the uterus. The role of the cilia is critical in fertility as demonstrated by rodent studies where muscular activity of the tube was abolished without any change in tubal transport time of the ovum, and immotile cilia in women is associated with reduced fertility (McComb *et al.*, 1986; Halbert *et al.*, 1989; Lyons *et al.*, 2002b). In the current study, using digital microscopy, the rate of cilia beating was monitored in response to purified hormones mimicking the menstrual cycle. CBF rates were similar to previous studies reporting between 20 and 40 MHz (Lyons *et al.*, 2006). The current study did not evaluate if cilia movement in the culture system impacted spermatozoa capacitation or oocyte pickup. In the future, models can be designed that integrate OVGP1, IGF, VEGF-A, IL8 and cilia beating, which can be used to provide important information regarding the impact of pharmaceuticals that alter fertility.

These studies provide in vitro confirmation that cultured fallopian tube epithelium responds to a reproductive cycle mimetic generated by murine follicles. Follicles produce factors beyond just steroid hormones that influence fallopian tube secretion of IL8 and IGF1. The crosstalk of ovarian and fallopian tissue enhanced progesterone production by the corpus luteum. *In vitro* co-culture models of fallopian reproductive tissues with ovarian follicles can provide an important tool for understanding fertility and for uncovering the mechanisms responsible for reduced fertility. In addition, the role of oviductal secretions and how they influence ovarian function, such as production of progesterone during the menstrual cycle, can potentially be uncovered using this model.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/

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Authors' roles

J.Z. contributed to the conception and design of the study, acquisition of data, analysis and interpretation, drafting the article and the final approval of the version to be published. Y.X. contributed to the follicle isolation for the co-culture system. A.S.R contributed to the ELISA testing and scientific editing. M.E.P, J.J.K and T.K.W. contributed to the conception and design of the study and revised the article critically for important intellectual content. J.E.B. contributed to the conception and design of the study, acquisition of data, analysis and interpretation, drafting the article and the final approval of the version to be published.

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Conflict of interest

None declared.

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