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Engineering the ovarian cycle using *in vitro* follicle culture

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STUDY QUESTION: Can cultured follicles model the ovarian cycle, including follicular- and luteal-phase hormone synthesis patterns and ovulation?

SUMMARY ANSWER: Under gonadotrophin stimulation, murine follicles grown in an encapsulated three-dimensional system ovulate *in vitro* and murine and human follicle hormone synthesis mimics follicular and luteal phases expected *in vivo*.

WHAT IS KNOWN ALREADY: Studies of the human ovary and follicle function are limited by the availability of human tissue and lack of *in vitro* models. We developed an encapsulated *in vitro* follicle growth (eIVFG) culture system, which preserves 3D follicular structure. Thus far, the alginate system has supported the culture of follicles from mice, dog, rhesus macaque, baboon and human. These studies have shown that cultured follicles synthesize steroid hormones similar to those observed during the follicular phase *in vivo*.

STUDY DESIGN, SIZE, DURATION: Cultured murine follicles were treated with human chorionic gonadotrophin (hCG) and epidermal growth factor (EGF) and either assayed for luteinization or removed from alginate beads and assayed for ovulation. Human follicles were also cultured, treated with follicle-stimulating hormone (FSH), hCG and EGF to mimic gonadotrophin changes throughout the ovarian cycle, and culture medium was assayed for hormone production.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Murine and human follicles were cultured in alginate hydrogel and hormone production [17β-estradiol, progesterone, inhibin A, inhibin B, activin A and anti-Müllerian hormone (AMH)] was quantified in medium by enzyme-linked immuno assay (ELISA). Human ovarian tissue was acquired from females between 6 and 34 years of age with a cancer diagnosis. These participants were undergoing ovarian tissue cryopreservation at National Physicians Cooperative sites as part of the Oncofertility Consortium.

MAIN RESULTS AND THE ROLE OF CHANCE: When grown in this system, 96% of mouse follicles ovulated in response to hCG and released meiotically competent eggs. Ovulated follicles recapitulated transcriptional, morphologic and hormone synthesis patterns post-luteinizing hormone (LH/hCG). In addition to rodent follicles, individual human follicles secreted steroid and peptide hormones that mimicked the patterns of serum hormones observed during the menstrual cycle.

LIMITATIONS, REASONS FOR CAUTION: This was a descriptive study of an *in vitro* model of ovulation and the ovarian hormone cycle. The ovulation studies were limited to murine tissue and further studies are needed to optimize conditions using other species.

WIDER IMPLICATIONS OF THE FINDINGS: The eIVFG system reliably phenocopies the *in vivo* ovarian cycle and provides a new tool to study human follicle biology and the influence of cycling female hormones on other tissue systems *in vitro*.

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Key words: *in vitro* follicle growth / ovulation / luteinization / ovarian cycle / alginate

Introduction

Biomedical research of the female reproductive system is limited by ethical considerations related to the acquisition and use of healthy ovarian tissue (Kim *et al.*, 2010; Tingen *et al.*, 2010). There are few models to study the human ovary and no *in vitro* models exist that replicate a complete ovarian cycle. New research tools and techniques are needed, not only for fundamental research of ovarian follicle biology, but also for preclinical testing and toxicity screening. Moreover, there remains a gap in our knowledge about the environment in which the female gamete, the oocyte, develops. The broader goal of *in vitro* follicle growth is to develop methods that support maturation of a competent human egg for use by cancer survivors. Our hypothesis is that the development of systems that support the full repertoire of reproductive hormones provides new information about follicle development that can aid in this goal.

Follicle development underlies each ovarian cycle producing steroid and peptide hormones essential for female reproduction. As the basic unit of the ovary, the follicle is compartmentalized with interdependent somatic cells (granulosa and theca cells) and a central oocyte. Once a follicle is selected to resume growth, rising follicle-stimulating hormone (FSH) causes follicular-phase hormone synthesis, including estrogen and inhibin A and B. These hormones support oocyte and endometrial growth and provide feedback to the hypothalamus and pituitary. The mid-cycle luteinizing hormone (LH) surge sets the timing of female fertility by triggering ovulation. In addition to expulsion of a mature oocyte, ovulation leads to the terminal differentiation of the follicular somatic cells into the progesterone-producing corpus luteum (CL). The luteal-phase hormones facilitate eventual implantation or menstruation. Furthermore, the pattern of follicular and luteal hormones determines female fecundity and influences multiple organ systems.

Different approaches exist to study follicle development including ovarian hormone production and gamete maturation. The process of ovulation poses a unique investigative challenge and studies have been limited to *in vivo* or *in vitro*-perfused ovary models (Cajander *et al.*, 1984; Brannstrom *et al.*, 1987). Systems that support *in vitro* ovulation have been described with follicular rupture rates ranging from 7 to 80% (Boland *et al.*, 1994; Hartshorne *et al.*, 1994; Johnson *et al.*, 1995; Rose *et al.*, 1999). The development of a robust *in vitro* model of ovulation would provide a new tool to examine basic follicle biology and develop follicle-targeted contraceptives. In addition to ovulation, studies of hormone production require more than cell culture approaches. Patterns of follicular-phase hormone synthesis have been described in 3D follicle culture systems (Picton and Gosden 2000; Xu *et al.*, 2006b, 2011b, 2013b; Kedem *et al.*, 2011; Sanchez *et al.*, 2012; Telfer and Zelinski, 2013), while *in vitro* investigations of luteinization have remained in 2D primary cell cultures (Oonk *et al.*, 1989; Meidan *et al.*, 1990; Engelhardt *et al.*, 1991). Although much has been learned from these studies, granulosa or theca cells luteinized *in vitro* differ from those luteinized *in vivo*. Richards *et al.* (1986) showed that cells luteinized *in vitro* required LH/cAMP to maintain progesterone synthesis. In contrast, cells luteinized *in vivo* synthesized progesterone independent of gonadotrophin stimulation (Richards *et al.*, 1986; Oonk *et al.*, 1989). Moreover, a model supportive of complete luteinization *in vitro* would provide a more efficient approach to study the second half of the ovarian cycle.

Techniques in the emerging field of *in vitro* follicle growth include several culture environments supporting individual follicle development (Gutierrez *et al.*, 2000; Telfer *et al.*, 2000; Wu *et al.*, 2001; Xu *et al.*, 2006a; Dunning *et al.*, 2011; Kedem *et al.*, 2011; Vanacker *et al.*, 2012). We developed an encapsulated *in vitro* follicle growth (eIVFG) culture system, which preserves 3D follicular structure with paracrine signaling maintained between oocyte, granulosa and theca cells (Kreeger *et al.*, 2006; Tingen *et al.*, 2011; Tagler *et al.*, 2012; Hornick *et al.*, 2013). In addition to murine follicles, success to date in the culture of larger mammalian species has been reported in the alginate system including follicles from dog, rhesus macaque, goat, baboon and human (Xu *et al.*, 2009a,b, 2010, 2011b,c, 2013a; Songsasen *et al.*, 2011; Camboni *et al.*, 2013; Fisher *et al.*, 2013; Araujo *et al.*, 2014; Silva *et al.*, 2014; Wang *et al.*, 2014). Further, eIVFG has facilitated studies of inter- and intra-follicular mechanisms governing follicular-phase processes including antrum formation, oocyte maturation and granulosa cell proliferation (Kreeger *et al.*, 2006; West-Farrell *et al.*, 2009; Hornick *et al.*, 2013).

The goal of this study was to develop and characterize an *in vitro* model that would recapitulate the hormone patterns and mid-cycle ovulation associated with the ovarian cycle. We hypothesized that eIVFG could support not only follicular-phase processes, but also the transformation associated with ovulation and luteinization. Such a model would provide a valuable tool to study follicle-targeted agents and human follicle dynamics. It would also provide a preclinical tool to assess the effects of drugs or toxins on reproductive function.

Materials and Methods

Animals

CD1 mice were housed in a temperature- and light-controlled environment (14 h light:10 h dark) and provided with food and water *ad libitum*. Animals were fed Teklad Global irradiated 2919 low-phytoestrogen chow (Harlan Laboratories, Indianapolis, IN, USA). To minimize differences in nutrient availability, eight females were housed per dam (if necessary pups were sacrificed at birth).

Murine follicle culture and *in vitro* ovulation and luteinization

Multi-layered secondary follicles (180–210 μm in diameter) were mechanically isolated from ovaries of 18-day-old mice and individually encapsulated in 0.5% (w/v) alginate (FMC BioPolymers, Philadelphia, PA, USA) as previously described (Kreeger *et al.*, 2006). Alginate-encapsulated follicles were placed in individual wells of a 96-well plate containing 100 μl of growth medium [alpha minimum essential medium (alphaMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10 mIU/ml recombinant FSH (Organon, Roseland, NJ, USA), 3 mg/ml bovine serum albumin (BSA, MP Biomedicals, Irvine, CA, USA), 1 mg/ml bovine fetuin (Sigma-Aldrich, St. Louis, MO, USA), 5 μg/ml insulin (Sigma-Aldrich), 5 μg/ml transferrin (Sigma-Aldrich) and 5 ng/ml selenium (Sigma-Aldrich)] and cultured for 4 days in a 5% CO_2:21% O_2 atmosphere. Half of the culture medium was exchanged on day 2 and conditioned medium stored at −80°C. To remove them from alginate, follicles were transferred into 1 ml of Liebovitz L-15 medium (Invitrogen) containing 10 U/ml alginate lyase (Sigma-Aldrich) for 20 min at 37°C. Follicular rupture was induced by incubating follicles in 100 μl maturation medium [alphaMEM with 1.5 IU/mL human chorionic gonadotrophin (hCG; Sigma-Aldrich), 5 ng/mL epidermal growth factor (EGF; BD Biosciences, Franklin Lakes, NJ, USA), 3 mg/ml BSA, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium] for 14–16 h in an ultra-low attachment 96-well plate (Corning, Tewksbury, MA, USA). Rupture

was assayed visually and via dissection ($n = 70$); unruptured follicles were those that required mechanical isolation of the cumulus oocyte complex (COC). Oocytes were isolated from a subset of follicles ($n = 36$) and scored visually [metaphase II (MII), germinal vesicle breakdown (GVBD) or degenerated]. For RNA or protein analysis, follicles were aspirated, transferred into microcentrifuge tubes, flash frozen in liquid nitrogen and stored at −80°C. For each protease inhibitor, dose–response curves were made and the concentration at which inhibition of ovulation occurred without cell death was used. The following compounds were used: 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 500 μM (Sigma-Aldrich); SB-3CT, 250 μM (Santa Cruz, Dallas, TX, USA); urokinase plasminogen activator (uPA) inhibitor, 50 μM (Santa Cruz); matrix metalloproteinase-2 (MMP-2) IV, 250 μM (EMD Millipore, Billerica, MA, USA); mifepristone (RU-486), 100 μM (Sigma-Aldrich). Ulipristal acetate (UPA, 50 μM; also referred to as CDB-2914 or VA2914) was a gift from HRA Pharma (Paris, France). In the luteinization experiments, on day 10 half of the growth medium was replaced with maturation medium (as above) and follicles remained encapsulated in alginate. Follicles were matured for 16 h at 37°C and 5% CO_2 at which point 50 μl of medium was replaced with growth medium (as above). Cultures were continued for four additional days, with medium exchanged and images taken every other day.

Acquisition of human ovarian tissue

Human ovarian tissue was obtained from three females with a cancer diagnosis (Supplementary data, Table SI) between 6 and 34 years of age. These participants were undergoing ovarian tissue cryopreservation at National Physicians Cooperative (NPC) sites as part of the Oncofertility Consortium. Following the surgical removal of ovarian tissue, 80% was cryopreserved for future clinical use and up to 20% was designated for research. The research tissue was transported to the laboratory in SAGE OFC holding Media (Cooper Surgical, Trumbull, CT, USA) at 4°C for 14–24 h. In all cases, the ovarian tissue was processed using a standard technique in ovarian tissue cryopreservation in which the ovarian cortex is separated from the medulla using a Thomas Stadie-Riggs Tissue Slicer (http://oncofertility.northwestern.edu/media/dissection-human-ovary-preparation-cryopreservation). For each of the described experiments, five follicles were used from three women (Supplementary data, Table SI).

Human follicle culture and *in vitro* luteinization

Human follicles were isolated and cultured as multiple follicles using a modified method as described previously (Abir *et al.*, 1999; Hornick *et al.*, 2013). Briefly, ovarian cortical strips were cut into 1 mm^3 pieces in alpha-MEM Glutamax (Invitrogen) supplemented with 1% Pen-Strep, 1% Serum Protein Substitute (SPS, Cooper Surgical, Trumbull, CT, USA) and 1.5 IU/ml hCG. The tissue was then enzymatically digested by supplementing alpha-MEM Glutamax with 1% Liberase TM (Roche, Indianapolis, IN, USA) and 0.1% DNase (Worthington, Lakewood, NJ, USA) for 30 min at 37°C. After rinsing the cortex three times with fresh SAGE OFC holding medium (Cooper Surgical), follicles were then mechanically isolated from the cortex using insulin-gauge needles and encapsulated in 0.3% alginate (NovaMatrix, Philadelphia, PA, USA). Encapsulated human follicles were transferred to a 96-well plate containing 100 μl of growth media [alpha-MEM Glutamax (Invitrogen) supplemented with 0.5 mg/ml fetuin, 0.3% human serum albumin (Cooper Surgical), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium (Sigma-Aldrich) and 5 mIU recombinant FSH (A.F. Parlow, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, USA)], and placed in an incubator at 37°C and 5% CO_2. Half of the culture medium was exchanged every other day and stored at −20°C. Follicles were imaged at each medium change using a Leica DM IRB inverted microscope equipped with 4× and 20× objectives to assess growth and survival. Follicles that contained an oocyte surrounded completely by somatic cells and continued to increase in diameter were defined as live follicles. Dead follicles were defined by unhealthy oocytes, dark granulosa cells or lost structural integrity. Luteal conversion was triggered by hCG administration for 36 h and the follicles were cultured for an additional 15 days. After 36 h incubation in maturation media [alpha-MEM Glutamax (Invitrogen) supplemented with 20 IU/ml hCG, 5 ng/ml EGF, 0.3% human serum albumin (Cooper Surgical), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium (Sigma-Aldrich)] half of the medium was exchanged for growth medium without FSH.

Immunohistochemistry

In alginate beads follicles were fixed overnight at 48°C in solution containing 4% paraformaldehyde (Sigma-Aldrich), 0.1 M sodium cacodylate (Sigma-Aldrich), 0.1 M sucrose (Sigma-Aldrich) and 10 mM $CaCl_2$ (Sigma-Aldrich) to prevent alginate degradation. Following fixation, follicles were dehydrated and embedded in paraffin. The follicles were sectioned at 5 μm and adhered to glass slides and stained with hematoxylin–eosin.

Hormone assays

17β-estradiol, progesterone, inhibin A, inhibin B, activin A and anti-Müllerian hormone (AMH) were measured in medium by commercially available enzyme-linked immuno assay (ELISA) kits [estradiol and progesterone (Calbiotech, Spring Valley, CA, USA); AMH and inhibin B, inhibin A (Beckman Coulter, Pasadena, CA, USA); activin A (Ansh Labs, Webster, TX, USA)]. The limits of sensitivity for estradiol, progesterone, inhibin A, inhibin B, activin A and AMH were 3.94 pg/ml, 0.22 ng/ml, <5.0 pg/ml, 7 pg/ml, 0.065 ng/ml, 0.006 ng/ml, respectively. Assays for mouse estradiol and progesterone were performed at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

Ethical approval

Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the established Institutional Animal Care and Use Committee (IACUC) protocol at Northwestern University. Human ovarian tissue was obtained following informed consent under Northwestern University Institutional Review Board-approved protocols.

Statistical analysis

All experiments were independently performed at least three times, unless otherwise noted. For comparisons between groups, a one-way ANOVA followed by the Bonferroni post hoc test was performed (Prism4; Graph Pad Software).

Results

An ovulatory stimulus elicits follicular rupture *in vitro*

The encapsulated alginate system has been shown to support the development of secondary murine follicles, yielding fertilizable and developmentally competent oocytes (Xu *et al.*, 2006a). To determine whether follicles grown in the alginate system ruptured and released a COC in response to an ovulatory stimulus, secondary murine follicles were mechanically isolated and encapsulated. After four days of culture, follicles grew from 203 ± 2 to 317 ± 3 μm (Fig. 1A) and were removed from alginate to permit COC extrusion. Follicles were treated with hCG and EGF for 14 h to mimic the LH surge and then assayed for rupture (Fig. 1B). The meiotic stage of the extruded gametes was also scored. After hCG administration, 96% (67/70) of follicles ruptured. Analysis

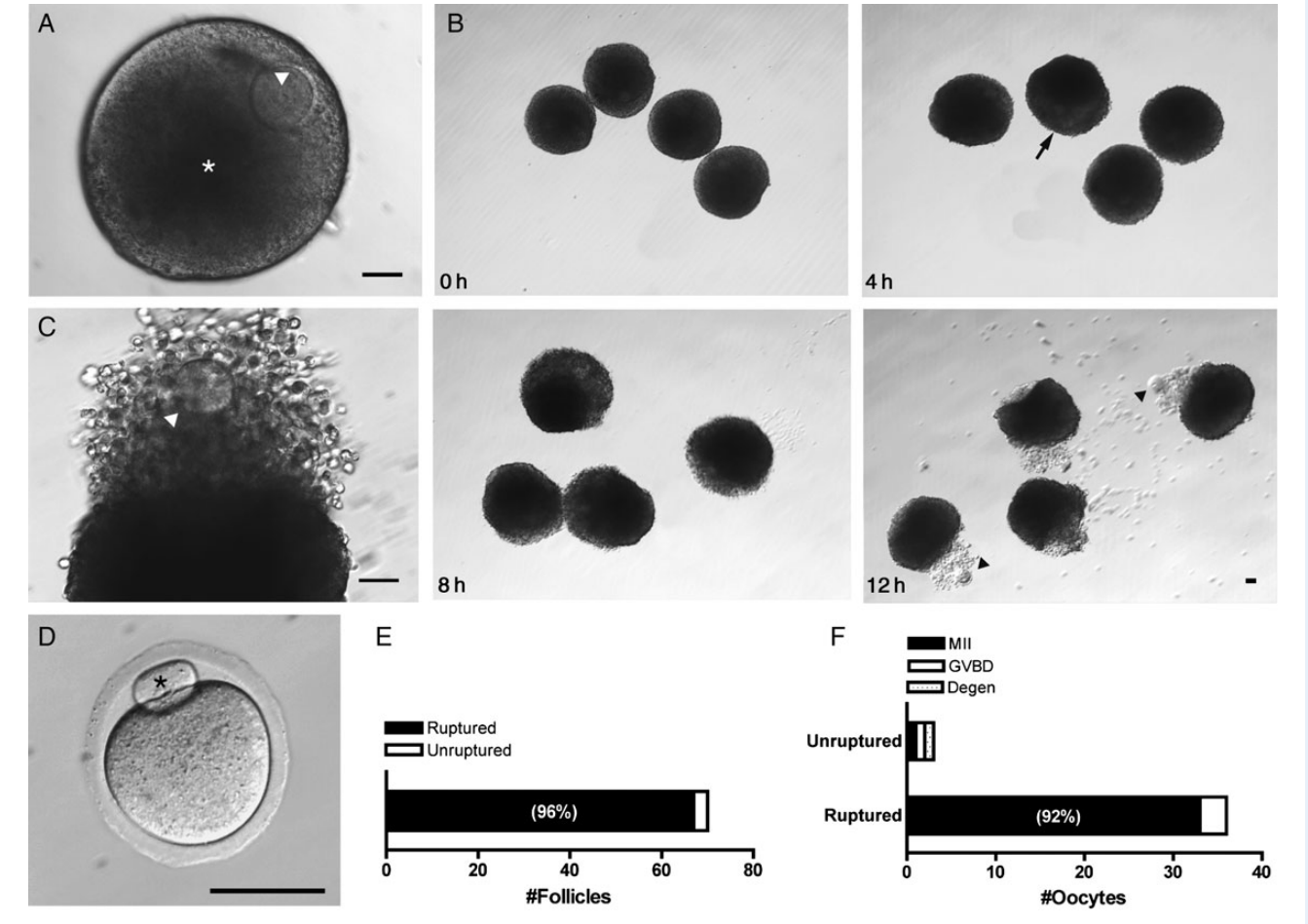

Figure 1 An ovulatory stimulus elicits follicular rupture *in vitro*. Murine follicles cultured in alginate rupture and release metaphase II (MII) eggs in response to hCG. (**A**) On the day of hCG administration, follicles contained an eccentric germinal vesicle (GV)-stage oocyte (arrowhead) and fluid-filled antrum (asterisk); scale bar = 50 μm. (**B**) Shown are representative images of follicles in the hours following hCG treatment (0, 4, 8 and 12 h). In some follicles, cumulus expansion was visible by 4 h (arrow). Extruded oocytes (arrowheads) and cumulus cells were visualized 12 h post-hCG; scale bar = 50 μm. (**C**) Image of a representative ruptured follicle 14 h post-hCG. The oocyte (arrowhead) is seen within the expanded cumulus matrix; scale bar = 50 μm. (**D**) Image of a representative MII egg collected from ruptured follicles with its extruded first polar body (asterisk); scale bar = 50 μm. (**E**) Overall, 96% of all follicles ruptured ($n = 70$). (**F**) Of the ruptured follicles ($n = 36$), 92% released MII stage eggs, while 8% were in the GVBD stage. Unruptured follicles were those that required mechanical isolation of the COC.

of a subset of ruptured follicles showed that 92% (33/36) released mature MII eggs (Fig. 1D–F). The small fraction (4%) of follicles that did not rupture contained an equal distribution of MII, GVBD and degenerated oocytes (Fig. 1F). Cumulus expansion was visible by 4 h and extruded COCs visualized at 12 h for most follicles (Fig. 1B). Follicular rupture was highly localized to the area of COC expulsion and the remainder of the follicular structure remained intact (Fig. 1C). This morphology was conserved across ruptured follicles (Supplementary data, Fig. S1). Thus, *in vitro* grown follicles ruptured in response to hCG and recapitulated *in vivo* morphological changes.

Effects of protease inhibitors and emergency contraceptives on ovulation *in vitro*

To further verify that our ovulation model mimicked the process of follicular rupture *in vivo*, we analyzed protease expression and action *in vitro*. First, we analyzed transcripts known to be regulated by LH/cAMP and important to ovulation: progesterone receptor (*Pgr*), tumor necrosis factor, alpha-induced protein 6 (*Tnfaip6*), prostaglandin-endoperoxidase protein synthase 2 (*Ptgs2*), plasminogen activator, urokinase (*Plau*) and a disintegrin and metalloproteinase with thrombospondin type 1 motif 1 (*Adamts-1*) (Supplementary data, Fig. S2). Importantly, temporal gene expression within hCG-treated follicles phenocopied expression observed in whole ovaries *in vivo* (Canipari *et al.*, 1987; Camp *et al.*, 1991; Park and Mayo, 1991; Espey *et al.*, 2000; Yoshioka *et al.*, 2000). We then treated follicles with protease inhibitors to examine their actions during *in vitro* ovulation. Follicles treated with either a serine protease inhibitor (AEBSF), uPA inhibitor, MMP-2/9 inhibitor (SB-3CT) or MMP-2 inhibitor ruptured at significantly lower rates when compared with those treated with vehicle alone (60%, $P < 0.05$; 56%, $P < 0.01$; 55%, $P < 0.01$; 58%, $P < 0.05$, respectively; vehicle, 94%) (Fig. 2A and B). These results are consistent with studies

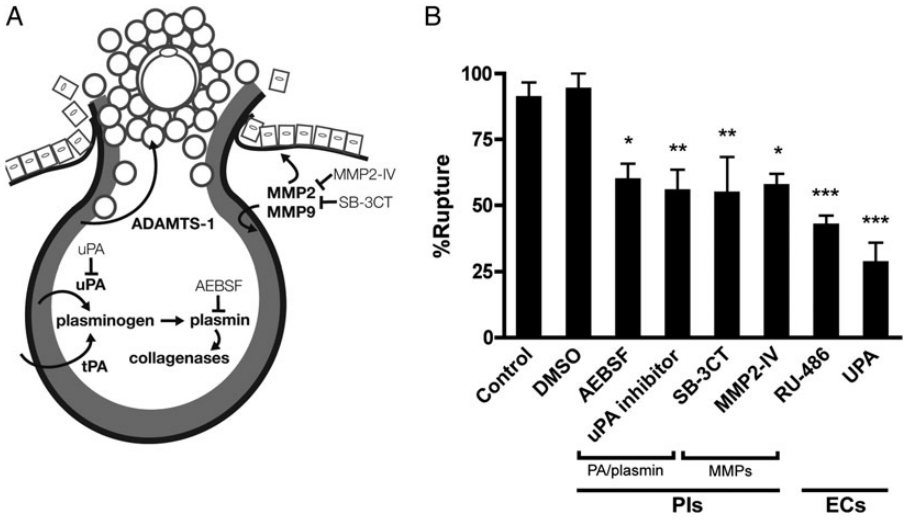

Figure 2 Effects of protease inhibitors and emergency contraceptives on ovulation *in vitro*. To assess the effects of the matrix metalloproteinases (MMPs) and PA/plasmin system on follicular rupture, inhibitors were added concomitant to hCG and rupture rates were determined. Similarly, the effects of emergency contraceptives (RU-486 and UPA) were assayed. (**A**) Schematic representation of putative proteases involved in ovulation and targeted action of protease inhibitors, see Materials and Methods. (**B**) Rupture rates of follicles treated with hCG and protease inhibitors (PIs) or emergency contraceptives (ECs). Control follicles are those treated with hCG only. Data are expressed as mean ± SEM, $n = 3–4$ cultures, 30–56 follicles; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ according to one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

of knock-out models (uPA$^{-/-}$/tPA$^{-/-}$, MMP-2$^{-/-}$, MMP-9$^{-/-}$) showing that a reduction, but not complete suppression, of ovulation occurred with inhibition of the gelatinases or PA/plasmin (Leonardsson *et al.*, 1995; Itoh *et al.*, 1997; Vu *et al.*, 1998).

We next assayed the effects of RU-486 and UPA on follicular rupture to validate the required role of progesterone receptor (PR) in the present ovulation model. Further, we aimed to investigate whether PR-targeted emergency contraceptives act directly on the follicle to inhibit ovulation in addition to their effects on the hypothalamic–pituitary–gonadotrophin axis. When follicles were treated with either progesterone agent, follicular rupture was significantly inhibited compared with vehicle-treated follicles (RU-486, 43%; UPA, 29%; $P < 0.001$) (Fig. 2B). Thus, these results validate the fidelity of our *in vitro* ovulation model as well as identify the direct effects PR emergency contraceptives may have on the follicle itself.

Alginate supports murine follicle luteinization

Once we established that cultured murine follicles could ovulate *in vitro*, we next assessed the capacity of these follicles to luteinize *in vitro*. Secondary follicles (153 ± 0.8 μm, $n = 116$) were isolated from mice and encapsulated in 0.5% alginate. Follicles were cultured for 10 days, treated with hCG and cultured for an additional 5 days. Medium was changed every other day, thus tapering the hCG concentration 16 h post-administration. Following hCG, follicles showed morphological signs of luteinization, marked by an increased granulosa cell cytoplasmic-to-nuclear ratio (Fig. 3A). Both steroid and peptide hormones were quantified to assess luteinization. As shown in Fig. 3B, progesterone rose steadily for 3 days post-hCG and progesterone synthesis remained elevated at the time of culture termination 5 days post-hCG. Estradiol synthesis sharply decreased upon hCG administration, with levels reaching nadir at the end of culture. For peptide synthesis, follicles produced increasing amounts of both inhibin A and inhibin B and as would be expected *in vivo*. Further, hCG decreased expression of both inhibin A and B by the end of culture.

To further validate the luteal phenotype of follicles *in vitro*, changes in key transcripts before and after hCG treatment were assessed including: steroidogenic acute regulatory protein (*Star*), cholesterol side-chain cleavage cytochrome P450 (*Cyp11a1*), 3β-hydroxysteroid dehydrogenase (*Hsd3b1*), 20α-hydroxysteroid dehydrogenase (*20αHSD*), 17α-hydroxylase (*Cyp17a1*), aromatase (*Cyp19a1*), follicle-stimulating hormone receptor (*Fshr*) and luteinizing hormone/choriogonadotrophin receptor (*Lhcgr*). Changes in these transcripts matched those observed *in vivo* with the up-regulation of steroidogenic enzymes *Star*, *Cyp11a1* and *20αHSD* and the down-regulation of *Cyp17a1* and *Cyp19a1* (Kidwell *et al.*, 1966; Park and Mayo 1991; Kaynard *et al.*, 1992) (Supplementary data, Fig. S3). While we observed a transient increase in *Lhcgr* expression 1 day post-hCG, it remained down-regulated 3 and 5 days post-treatment. *Fshr* was down-regulated at all time points post-hCG (Supplementary data, Fig. S3). Thus, cultured follicles luteinized *in vitro* as demonstrated by changes in hormone synthesis. Further, progesterone production was independent of constant gonadotrophin stimulation.

eIVFG supports the complete hormone cycle in human follicles

Combined with prior reports (Xu *et al.*, 2006b; West-Farrell *et al.*, 2009), we have shown that murine follicles cultured in an eIVFG system could provide a complete *in vitro* model of the ovarian cycle. We then sought to model the entire human ovarian cycle. To do so, primary to secondary stage human follicles (74–260 μm) were isolated from ovarian tissue (Supplemental data, Table SI and Fig. S4), encapsulated in alginate and monitored throughout growth via light microscopy. Follicle growth and

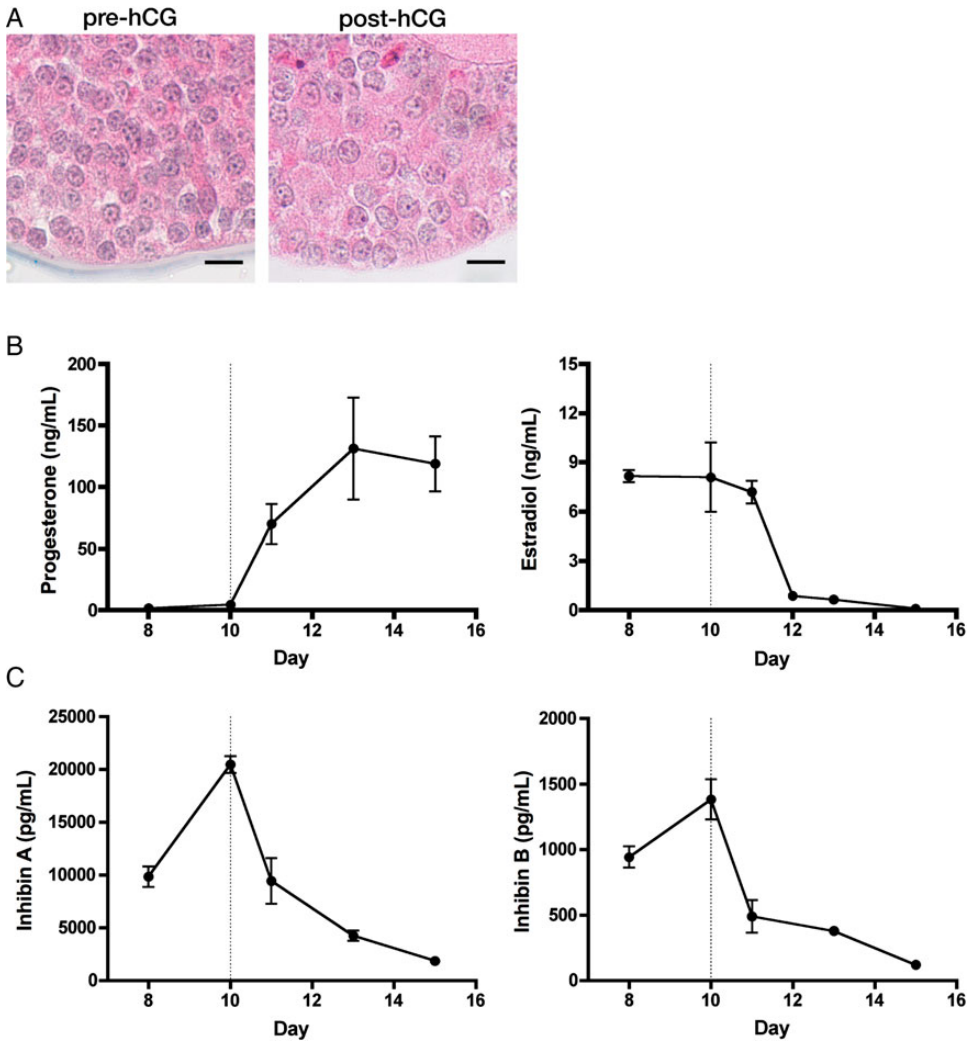

Figure 3 eIVFG supports murine follicle luteinization. Murine secondary follicles were cultured in alginate for 10 days, luteinized by hCG administration and cultured for an additional 5 days. (**A**) Follicles were fixed and stained with hematoxylin–eosin before and after hCG treatment (culture days 10 and 13, respectively). Follicles showed morphological signs of luteinization, marked by an increased granulosa cell cytoplasmic to nuclear ratio (scale bars = 10 μm). (**B**) Progesterone and estradiol and (**C**) inhibin A and inhibin B were quantified in the medium by enzyme-linked immuno assay (ELISA) ($n = 3$ cultures). Data are expressed as mean ± SEM.

morphology were used to assess viability (as described in Materials and Methods) and levels of estradiol, progesterone, inhibin A, inhibin B, AMH and activin A were measured in the medium throughout culture. As human follicle maturity cannot be precisely identified *in vitro*, follicle growth, morphology and estrogen synthesis were used as markers of terminal follicle development. Once estradiol levels plateaued, luteinization was triggered with the addition of hCG and EGF. The *in vitro* luteal phase was monitored for approximately 15 days post-hCG (similar in duration to the human *in vivo* luteal phase), resulting in total culture times ranging from 40 to 65 days.

The patterns of follicle hormone secretion mimicked those observed in serum levels *in vivo*. As the follicles matured, inhibin A and estradiol levels progressively increased as would be expected during the follicular phase *in vivo* (Fig. 4A and C). Inhibin B was the predominant form of inhibin produced by small antral follicles, known to rise in the early to mid-follicular phase and decline in the later follicular phase (Fig. 4D). Post-hCG, progesterone and activin A levels increased (Fig. 4B and F), mimicking patterns found in human serum (Muttukrishna *et al.*, 1996). Inhibin A levels dropped transiently after addition of hCG, but then rose and remained elevated throughout the luteal phase (Fig. 4C). In contrast, inhibin B levels remained low during the luteal phase (Fig. 4D). Moreover, cultured follicles replicated the luteal phase discordance of inhibin A and inhibin B unique to primates (Klein *et al.*, 2004; Welt *et al.*, 2005).

AMH, a hormone used clinically as a surrogate marker of small follicles, reached its peak in the early follicular phase and continued to decline through the luteal phase (Fig. 4E). Human serum AMH levels remain constant throughout the 28-day cycle, as a result of the steady recruitment of immature follicles into the growing pool (Durlinger *et al.*, 2002; Weenen *et al.*, 2004). For individual follicles, however, it has been shown that non-human primate follicles synthesize AMH through the time of antrum formation (Xu *et al.*, 2010). Our *in vitro* results are consistent with this

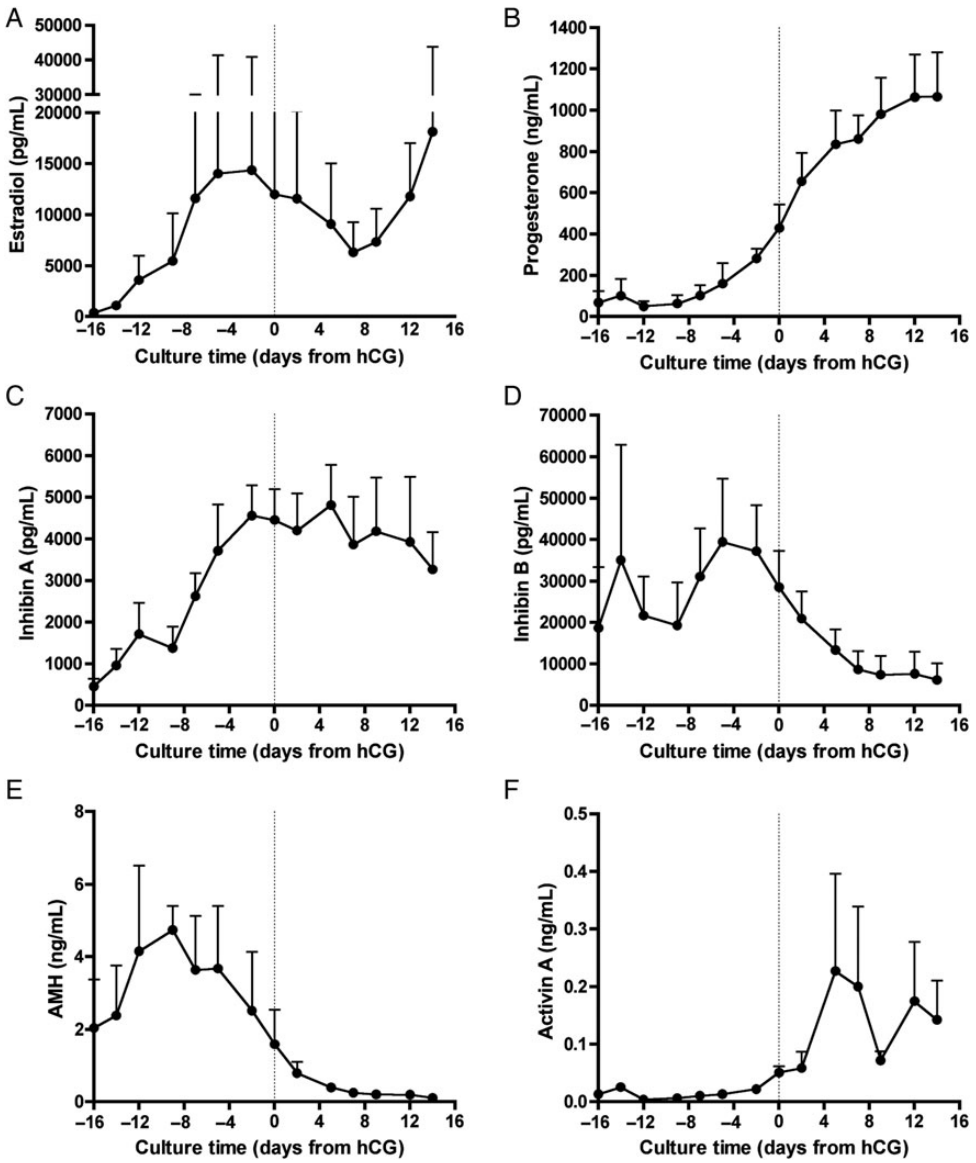

Figure 4 eIVFG supports the complete hormone cycle in human follicles. Secondary human follicles were isolated and cultured in alginate from 40 to 65 days. Steroid and peptide hormones were quantified in the medium throughout the culture period. The culture time on the *x*-axis indicates the time of hCG addition (day 0, dotted line); follicles were cultured for 14–15 days post-hCG. The concentration of (**A**) estradiol, (**B**) progesterone, (**C**) inhibin A, (**D**) inhibin B, (**E**) AMH and (**F**) activin A are reported throughout culture. Data are expressed as mean ± SD for $n = 5$ follicles. Follicles were collected from the ovaries of three women and cultured individually.

finding; individually cultured human follicles produced AMH approximately through the time of antrum formation. This finding underscores the value of *in vitro* systems to reveal aspects of human follicle biology that cannot be explored *in vivo*. Moreover, with gonadotrophin stimulation mimicking the timing of pituitary secretion, human follicles grown *in vitro* supplied a similar pattern of steroid and peptide hormone secretion observed *in vivo*.

Discussion

The ovarian cycle involves dynamic hormone changes that influence all of female physiology. Further, the mid-cycle release of a fertilizable oocyte involves transformative changes within the follicle determining the timing and rate of reproduction. The study of these events poses significant challenges due to the structural and paracrine requirements of growing follicles. Within an eIVFG system, we have shown that individual follicles can mimic the events associated with follicular growth, ovulation and luteinization—the complete ovarian cycle.

The field of *in vitro* follicle growth includes several cellular and biomaterial-based systems that support 3D follicular structure. These include approaches using V-shaped wells, droplets covered with oil, culture inserts and hydrogel encapsulation (Torrance *et al.*, 1989; Nayudu and Osborn 1992; Boland *et al.*, 1993; Hartshorne *et al.*, 1994; Abir *et al.*, 1997, 2001; Pangas *et al.*, 2003; Shea *et al.*, 2014).

Ovulation was reported in some of these systems with rupture rates between 7 and 30% (Boland *et al.*, 1994; Hartshorne *et al.*, 1994; Johnson *et al.*, 1995). With such low ovulatory rates in these early reports, *in vitro* systems could not serve as reliable ovulation models. Rose *et al.* reported that follicles grown on culture inserts had rupture rates between 15 and 80%, depending on the final diameter (Rose *et al.*, 1999). Herein we report that 96% of follicles grown in alginate ovulate. This improvement in ovulation rate may reflect higher follicle quality or improved maturation protocols. Another possibility is that removal from alginate encapsulation weakens the follicle wall. Moreover, we have shown that the morphological and transcriptional changes associated with this model are characteristic of *in vivo* ovulatory patterns. Further, this model provides a high-fidelity system to study ovulatory mechanisms independent of the ovarian surface epithelium, vasculature and inflammatory changes.

Given significant species differences in periovulatory regulation, developing improved primate models will be imperative to clinical translation. To date, luteinization studies in primates have employed primary 2D cell culture (Chaffin *et al.*, 2003; Duffy and Stouffer 2003) or *in vivo* models using stimulation protocols (Chandrasekher *et al.*, 1994; Young *et al.*, 2003; Xu *et al.*, 2011a). Herein we report that human follicles undergo luteinization when treated with hCG in the alginate culture system, which provides a new model to study primate luteinization in real-time. With minimal animal use and subject harm, individual follicles can be tracked throughout the periovulatory period. Furthermore, immature oocytes and eggs may simultaneously be collected for clinical or research purposes. This model system may thus be used to address important questions regarding primate luteinization, a necessary process to support pregnancy.

Limitations to our study include a small number of human follicles, which restricted studies to hormone analysis. Ovulation was not assessed in human follicles due to limited human tissue and loss of oocyte visibility during human follicle culture. Future studies will investigate the effects of follicle hormone dynamics on other tissues within larger co-culture and microfluidic systems.

In summary, we have shown that eIVFG is able to recapitulate the capstone events of the ovarian cycle including hormone production, follicle growth, differentiation and ovulation. Our success in culturing human and mouse follicles in a system that permits both follicular- and luteal-phase hormone synthesis yields an important investigative tool not only in reproduction, but also toxicology, endocrinology and physiology.

Supplementary data

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

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Authors' roles

R.M.S. and T.K.W. were responsible for study conception and design. R.M.S. and Y.X. carried out follicle cultures and acquired hormone data. R.M.S. prepared the manuscript with T.K.W. and L.D.S. providing critical review.

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Conflict of interest

The authors have no competing interests.

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