

In vitro grown human ovarian follicles from cancer patients support oocyte growth

Min Xu^{1,2}, Susan L. Barrett^{1,2}, Erin West-Farrell^{2,3},
Laxmi A. Kondapalli^{1,2}, Sarah E. Kiesewetter^{1,2},
Lonnie D. Shea^{2,3,4}, and Teresa K. Woodruff^{1,2,4,5}

¹Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA ²Member of the Oncofertility Consortium, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA ³Department of Chemical and Biological Engineering, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA ⁴Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

⁵Correspondence address. The Thomas J. Watkins Professor of Obstetrics and Gynecology, Northwestern University, Feinberg School of Medicine, 250 E. Superior Street, Suite 10-119, Chicago, IL 60611, USA. E-mail: tkw@northwestern.edu

BACKGROUND: Young female adult and adolescent cancer patients facing life-preserving but fertility-threatening chemo- or radiation-therapy are increasingly seeking options to protect their reproductive potential. Ovarian tissue cryopreservation with transplantation is a promising technique to safeguard fertility in cancer patients. However, this method may risk re-introduction of the original cancer to the survivor of the disease. Thus, developing a method for *in vitro* growth of immature follicles may provide a method for fertility restoration in the future.

METHODS: Human secondary follicles were isolated from ovarian tissues obtained from cancer patients and grown *in vitro* within a bio-engineered culture system for 30 days.

RESULTS: Human ovarian follicles became steroidogenically active, and developed from the early secondary to antral stage *in vitro*. The follicles contained healthy, growing oocytes that were connected by transzonal projections between the somatic cells and oocyte.

CONCLUSIONS: Our data support the notion that human follicle development can be achieved *in vitro* in a bio-engineered culture system. More studies are required to investigate whether the fully sized oocytes obtained from *in vitro* grown follicle are competent to resume meiosis and be fertilized.

Key words: human / ovarian follicle / *in vitro* growth / oocyte

Introduction

Young women facing a new cancer diagnosis are left with difficult choices, not only in how to manage their cancer, but also to decide whether to take fertility-protective measures. Several chemotherapeutics used in anti-cancer regimens unintentionally threaten fertility by destroying the immature follicle pool. The elimination of the primordial follicle pool results in either acute ovarian failure or a chronic ovarian insufficiency, resulting in an early menopause and associated loss of reproductive capacity (Agarwal and Chang, 2007; Cvancarova *et al.*, 2009; Jeruss and Woodruff, 2009; Schover, 2009). For female adolescents and young adults, the increasing probability of surviving a cancer diagnosis combined with the expectation and desire for reproductive options following cancer remission is driving the intense need for fertility sparing techniques.

Currently, there are several methods available to women hoping to preserve their fertility before cancer therapy. As it has been used most often, traditional hormone stimulation and *in vitro* fertilization (IVF) followed by embryo cryopreservation is the most successful approach to preserve fertility (Oktay *et al.*, 2003, 2005; Rao *et al.*, 2004; Juretzka *et al.*, 2005; Lee *et al.*, 2006). Recently, live births have also been achieved with cryopreserved oocytes harvested before cancer treatment (Yang *et al.*, 2007; Porcu *et al.*, 2008). However, both methods require a delay in cancer treatment and hormonal stimulation that are not prudent for some patients. In contrast, ovarian tissue cryopreservation followed by transplant is a promising fertility preservation approach that can usually be performed immediately without hormonal stimulation. Transplantation of ovarian cortical strips has resulted in viable offspring in sheep (from cryopreserved tissue) (Gosden *et al.*, 1994; Salle *et al.*, 2002) and monkeys (from fresh

tissue) (Lee et al., 2004). In the human, ovarian function can be restored within 3 months after transplanting thawed tissue into a woman with ovarian failure (Oktay et al., 2004; Schmidt et al., 2005; Demeestere et al., 2006; Rosendahl et al., 2006). Furthermore, live births in humans have been achieved by transplanting fresh ovarian tissue (Silber et al., 2005, 2008; Silber and Gosden, 2007). Worldwide, five live births have been reported thus far as a result of auto-transplanting frozen/thawed ovarian tissues (Donnez et al., 2004; Meirow et al., 2005; Demeestere et al., 2007; Andersen et al., 2008). Despite these promising findings, transplantation of cryopreserved tissue carries the risk of re-introducing cancer cells into the patient (Shaw and Trounson, 1997; Meirow et al., 1998, 2008). Thus, the *in vitro* growth of immature follicles derived from ovaries collected prior to commencement of cancer treatment is an important direction for research.

Phenocopying the complex interplay of growth factors and hormone signals required for the coordinated developmental process of follicle development and oocyte maturation *in vitro* is challenging; yet follicle culture systems for mouse (Eppig and Schroeder, 1989; Spears et al., 1994; Cortvrindt et al., 1996; Eppig and O'Brien, 1996; O'Brien et al., 2003; Xu et al., 2006a, b), large animals (Newton et al., 1999; Gutierrez et al., 2000; Telfer et al., 2000; Wu et al., 2001; Picton et al., 2003; Thomas et al., 2007) and the human (Roy and Treacy, 1993; Abir et al., 1997, 1999, 2001, 2006; Hovatta et al., 1997; Wright et al., 1999; Scott et al., 2004a, b; Telfer et al., 2008; Amorim et al., 2009) have been developed in both 2-dimensional (2D) and 3-dimensional (3D) formats. These systems have been successful in supporting the development of mouse secondary follicles, but have not yet been adapted for routine use in large animals and the human. The purpose of the *in vitro* human follicle growth (IVFG) system is to mimic the *in vivo* process by providing follicles with appropriate growth factors and hormones, in the correct amount, at the right time, which allows growth of the follicle and oocyte whereas maintaining the essential

connections between somatic cells and the oocyte called transzonal projections (TZPs) (Anderson and Albertini, 1976; Albertini and Barrett, 2003).

The aim of the present study was to investigate *in vitro* human secondary follicle growth and survival in a long-term 3D culture system. We examined the ability of these follicles to form an antrum, secrete hormones and support oocyte development.

Materials and Methods

Ovary collection

Ovarian tissues were obtained from 14 female cancer patients (Table I), ages 16–39 years (mean \pm SD, 27.3 \pm 8.1) following informed consent under an Institutional Review Board-approved protocol at Northwestern University. These patients were seeking fertility-sparing options in our Oncofertility program, which provided them with a full-range of fertility-sparing options. Ovaries were removed by laparoscopic and transported to the laboratory in <2 h in cold (4°C) MOPS-HTF Medium (CooperSurgical, Trumbull, CT, USA).

Follicle isolation

All follicles included in this study were isolated from fresh tissue using a modified method as described previously (Abir et al., 1999). Briefly, ovarian cortical strips were cut into \sim 1 mm³ pieces in dissection media (Liebovitz L-15 media, Invitrogen, Carlsbad, CA, USA) supplemented with 1% Serum Protein Substitute (SPS, CooperSurgical, Trumbull, CT, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The tissue was then enzymatically digested by transferring to incubation media, made by supplementing dissection media (above) with 0.2% collagenase and 0.02% DNase (Worthington Biochemical, Lakewood, NJ, USA), for 1.5 h at 37°C in a shaking water bath. In most cases, this short-term digestion was not enough to completely free follicles from the ovarian cortex because of the toughness of human cortex; however, the enzymatic digestion facilitated the following mechanical isolation. After rinsing cortex twice with fresh dissecting media, follicles

Table I Secondary follicle isolation

Patient	Age	Diagnosis	FL-Mgel (n)	FL-ALG (n)	Total (n)
Pt1	36	Breast Cancer	2	0	2
Pt2	39	Breast Cancer	0	4	4
Pt3*	36	Breast Cancer	0	0	0*
Pt4*	36	Breast Cancer	0	0	0*
Pt5	29	Cervical Cancer	6	0	6
Pt6	19	Ewing's Sarcoma	3	0	3
Pt7*	16	Ewing's Sarcoma	0	0	0*
Pt8*	26	Hodgkins Lymphoma	2	3	5*
Pt9	16	Non-Hodgkins Lymphoma	4	0	4
Pt10	33	Ovarian Cancer	0	0	0
Pt11	27	Ovarian Mass	1	0	1
Pt12	27	Ovarian Mass	0	0	0
Pt13	16	Penioblastoma	2	4	6
Pt14	26	Rectal Cancer	2	7	9

Note: FL-Mgel, follicle cultured in Matrigel; FL-ALG, follicle cultured in 0.5% alginate.

*The patients received chemo/radiations therapy before tissue donation.

were then mechanically isolated from the cortex using 25-gauge needles in dissection media, transferred to maintenance media (α MEM, (Invitrogen, Carlsbad, CA, USA), supplemented with 1% SPS, 100 IU/ml penicillin and 100 μ g/ml streptomycin), and placed in an incubator at 37°C and 5% CO₂. The mechanical isolation was carried out for 2–6 h prior to encapsulation. Only immature follicles (Class 1 and 2) (Gougeon, 1996) that contained a clear, visible, centrally-located oocyte, healthy granulosa cells and no signs of antrum formation were encapsulated and cultured (Fig. 1A). A few small antral follicles were fixed in 4% paraformaldehyde (PFA) as a control for confocal analysis of follicle structure, oocyte-somatic cell interactions as well as germinal vesicle (GV) stage.

Matrix preparation

Sodium alginate (55–65% guluronic acid) was obtained from FMC BioPolymers (Philadelphia, PA, USA). A 1% (w/v) solution of sodium alginate was stripped using activated charcoal (0.5 g charcoal/g alginate) to remove organic impurities, sterile filtered using 0.22 μ m filters, and lyophilized within Steriflip conical tubes (Millipore, Billerica, MA, USA). Sodium alginate was reconstituted to 0.5% (w/v) alginate in 1 \times phosphate buffered saline (PBS) for use (Xu *et al.*, 2006a, b). Matrigel (BD Cat 354234, Bedford, MA, USA) was thawed on ice overnight, and diluted 1:3 with cold α MEM (Scott *et al.*, 2004a, b).

Follicle encapsulation and culture

Selected follicles were encapsulated into either a 0.5% alginate bead (Xu *et al.*, 2006a, b) or embedded into diluted Matrigel matrix as previously described (Scott *et al.*, 2004a, b) with slight modification. Briefly, single follicles were transferred to individual droplets of alginate (\sim 5 μ l) on a

polypropylene mesh (0.1 mm opening, McMaster-Carr, Atlanta, GA, USA). The mesh was then inverted and gently tapped over a cross-linking solution (50 mM CaCl₂ and 140 mM NaCl), causing the droplets to fall into the solution (Xu, *et al.*, 2009). The beads were allowed to cross-link for two minutes, removed, and rinsed in maintenance media. Beads were then transferred to individual wells of a 96-well plate containing 100 μ l of culture media (Xu *et al.*, 2006a, b) [α MEM supplemented with 6% SPS (v/v), 1 mg/ml bovine fetuin (Sigma-Aldrich, St. Louis, MO, USA), 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml sodium selenite (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 IU/ml FSH (gift from Organon, Roseland, NJ, USA)]. SPS was used here to replace the bovine serum albumin used for murine follicle culture. To embed a follicle into the Matrigel, the Millicell insert (Millipore, Billerica, MA, USA) was filled with 100 μ l diluted Matrigel and permitted to pre-gel for 30 min. The individual follicle was then transferred into the middle of the Matrigel and allowed to gel in the incubator for an additional 30 min. The insert was then transferred to one well of a 24-well plate containing 400 μ l of the culture media described above. Follicles, either encapsulated within 0.5% alginate or embedded within Matrigel were cultured at 37°C with 5% CO₂ for up to 30 days. At different time points, some follicles were fixed or frozen for histological examination or gene expression analysis (unpublished data). Ten follicles from each treatment group were grown for 30 days. Every 2 days, half of the culture media was exchanged and stored at –80°C for hormonal measurements. Fresh culture media was prepared weekly.

Follicle and oocyte measurement

Photographs of each follicle (during the culture period) were captured using a Leica DM IL light microscope (Leica, Wetzlar, Germany) equipped

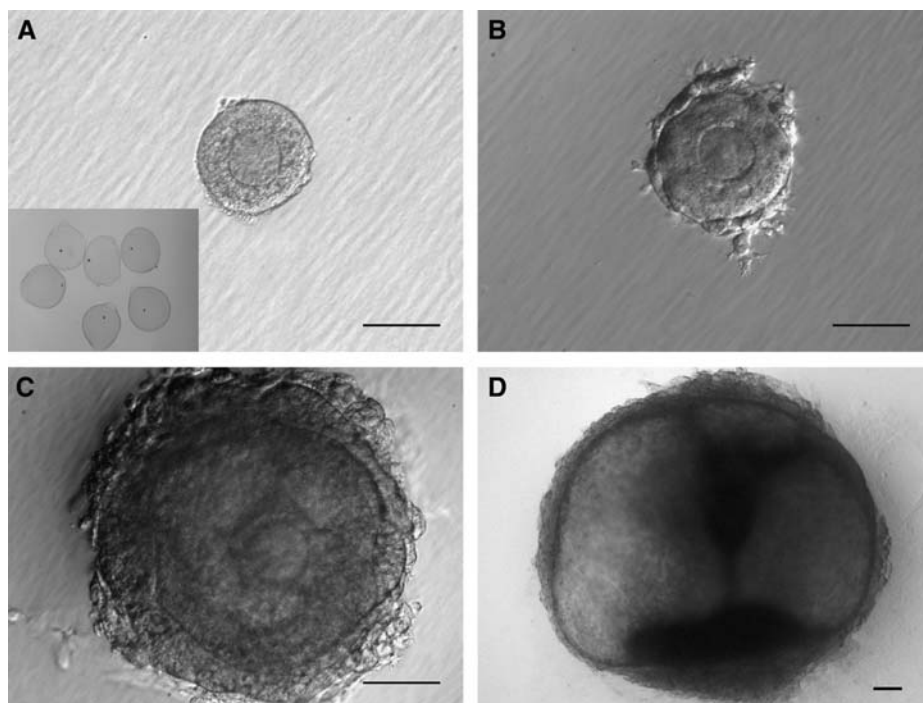


Figure 1 Characteristics of *in vitro* growth follicles.

Secondary follicles (135 μ m) (A) were isolated from the ovarian cortex and grown in a 3D environment (A, insert) for 30 days. Granulosa cells continuously proliferated. The follicles developed theca-like cells at day 5 (182 μ m) (B), form an antrum and a central COC at day 15 (345 μ m) (C), and maintained 3D structural integrity throughout the culture (1489 μ m) (D). Bar = 100 μ m.

with phase objectives, a heated stage, a Spot Insight 2 Megapixel Color Mosaic camera and Spot software (Spot Diagnostic Instruments, Sterling Heights, MI, USA). Follicle diameters were later measured using ImageJ software (National Institutes of Health, USA) as previously described (West et al., 2007; West-Farrell et al., 2009). Oocyte diameters were measured without zona pellucida.

Sectioning and staining

Follicles were fixed in 4% PFA for 30 min at 37°C and then overnight at 4°C. Fixed follicles were dehydrated in increasing concentrations of ethanol (10–100%) and embedded in paraffin using an automated tissue processor (Leica, Mannheim, Germany). Serial 5 µm sections were cut and stained with hematoxylin and eosin.

3D confocal staining and imaging

Follicles for confocal microscopy studies were fixed in 4% PFA at 37°C for 1 h, followed by 1 h in wash buffer (Barrett and Albertini, 2007). They were then stained overnight at 4°C on a shaker with Rhodamine-Phalloidin (1:50 Molecular Probes, Invitrogen, Eugene, Oregon) that labels F-actin in TZPs, rabbit anti-connexin 43 (1:100 Zymed) that labels gap junctions or affinity purified polyclonal rabbit antisera that recognize the inhibin βA-subunit (1:100) or the inhibin βB-subunit (1:100). The inhibin subunit antisera were a gift from Dr W. Vale (The Salk Institute, La Jolla, CA, USA). The primary antibodies were followed by incubation with 1:800 goat anti-rabbit Alexa 568 for inhibin βA and connexin 43, 1:800 goat anti-rabbit Alexa 488 for inhibin βB (Molecular Probes) and 1 µg/ml Hoechst 33342 (Molecular Probes) for chromatin/DNA. Follicles were mounted in 5–10 µl of a 50% glycerol/PBS solution containing 25 µg/ml sodium azide. The coverglass was placed on glass shards, to prevent the compression of the follicle. Follicles were imaged on a Zeiss LSM 510 confocal microscope using a Neofluar 40× oil objective. Overlapping 1–3 µm sections were taken throughout each follicle imaged.

Hormone assays

17β-estradiol (E2) and Progesterone (P4) concentrations in culture media were measured by specific electrochemiluminescent assay using a Roche Elecsys 2010 Analyzer (Roche, Indianapolis, IN, USA). The inter-assay variations were 6.1% for 17β-estradiol and 5.4% for progesterone. The limits of sensitivity were 5 pg/ml for 17β-estradiol and 0.03 ng/ml for progesterone. Inhibin A, inhibin B and anti-Müllerian hormone (AMH) were measured using ELISA kits (DSL-10-28100, DSL-10-84100 and DSL-10-14400, Diagnostic Systems Laboratories, Webster, TX, USA) following manufacturer instructions. The intra-assay variations were 8.7% for inhibin A, 3.2% for inhibin B and 3.8% for AMH. The limits of sensitivity for inhibin A, inhibin B and AMH were 10, 10 and 20 pg/ml, respectively.

Results

Isolation and growth of human secondary follicles

In order to assess the ability of human secondary follicles to grow in 3D matrices, we isolated follicles from ovarian tissues donated from 14 different Oncofertility patients (Table I). The number of secondary follicles isolated, from each patient sample ranged from 0 to 9, which varied based on tissue amount, patient age, cancer type and whether the patient received prior chemo/radiation therapy.

Follicles were cultured for up to 30 days by embedding in matrigel ($n = 18$) or encapsulation in alginate ($n = 22$). The average size of

un-cultured isolated follicle was similar between culture groups, matrigel = 170.8 µm (± 51.1) and alginate = 178.4 µm (± 69.2). Matrigel embedded secondary follicles (Fig. 1A) or alginate encapsulated follicles (Fig. 1A inset) displayed at least two cell layers of granulosa cells. After 5 days of culture both groups of follicles displayed theca-like cells surrounding the follicle basement membrane (Fig. 1B). By day 15 of culture, follicles observed by brightfield microscopy contained a fluid filled antrum and distinct mural granulosa and cumulus granulosa cell types (Fig. 1C). The follicle also displayed a thickening of the theca-like cell layer. By day 30 of culture, the cumulus-oocyte complex was barely detectable by light microscopy due to the size of the follicle (Fig. 1D).

Because both matrigel and alginate environments supported follicle growth without significant differences (data not shown), the growth data were combined (Fig. 2). From day 0 to day 30 follicles linearly grew in size from 175 ± 10 to 715 ± 68 µm. Moreover, the oocyte size also increased in culture as the follicle grew three-dimensionally. After day 15 of culture, the oocyte could no longer be observed for measurement until the follicle was fixed for analysis. All secondary follicles that were grown for 30 days survived through the culture, and 75% of follicles developed visible antrum that commenced on approximately day 12 (± 3.9) of culture in follicles with an average diameter of 393.6 µm (± 93.6).

In vitro grown follicles secrete steroid and peptide hormones

To determine whether IVFG was similar to *in vivo* follicles in their capacity to secrete hormones, we analyzed growth media from individual follicles every other day of culture. Steroid hormones were not detected in the condition media before day 5. Increasing levels of steroid hormones, 17β-estradiol and progesterone, were measured

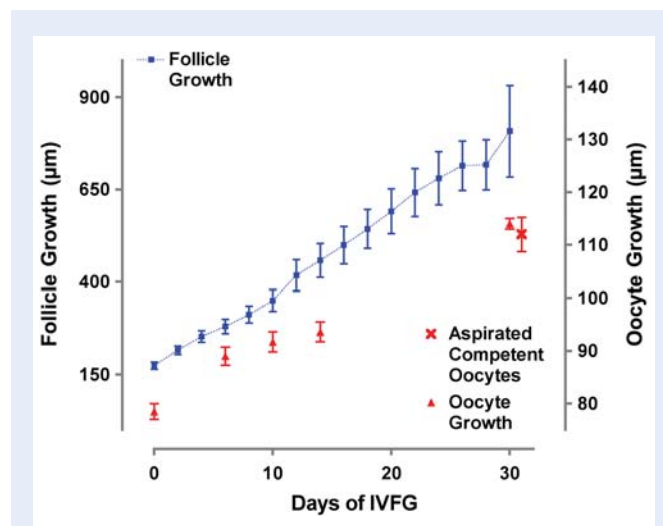


Figure 2 *In vitro* follicle and oocyte growth.

From day 0 to day 30, the average follicle size of the entire follicle population increased from 175 µm (± 10) to 715 µm (± 68), and the average oocyte size increased from 78.5 µm (± 1.5) to 114.0 µm (± 1.1). The terminal size of the oocytes grown *in vitro* was indistinguishable from oocytes aspirated from *in vivo* matured follicles during IVM. Data are represented as mean \pm SEM.

in conditioned media collected from individual follicle cultures on day 5 through day 30 (Fig. 3A and B). The ratio of 17 β -estradiol to progesterone decreased over the culture period (Fig. 3C). The peptide hormone inhibin A continually increased as follicles were cultured to 30 days whereas inhibin B increased from day 4 to day 16 but decreased from day 16 to day 30. AMH was also measured in cultured follicles and followed the secretion of inhibin B (Fig. 3). When comparing all hormones (Fig. 4A) over time in one single follicle (seen in Fig. 1), it was found that secondary follicles grown *in vitro* did not secrete steroid nor peptide hormones at detectable levels during the initial days of culture (days 0–4), however, all hormones were measurable by day 8. All hormones examined appeared to increase steadily until day 20 of culture after which levels of AMH began to drop slowly and inhibin B decreased rapidly. Both inhibin β -subunits (β A and β B) were localized to the mural granulosa cells of the same follicle at 30-days of culture (Fig. 4B and C).

In vitro follicle growth supports oocyte growth

Since the connection between somatic cells and the oocyte has never before been studied in human follicles, we used confocal reconstructions to observe the F-actin based connections (TZPs) in freshly isolated, early-antral follicles (Fig. 5A). We examined 14-day cultured follicles for the presence of TZPs and found that they maintain a dense network of TZPs emanating from the surrounding granulosa cells (Fig. 5B). We also reconstructed these cultured follicles and found them to be similar to the *in vivo* benchmark follicles (<http://oncofertility.northwestern.edu/research/human-follicle-maturation-in-vitro/shared-files>). This follicle also showed the formation of an early antrum and the differentiation of a mural granulosa and cumulus granulosa. Connexin 43, a gap junction protein, was also examined

in cultured follicles and was shown to be present at the cumulus granulosa cell junctions (Fig. 5C). Oocyte growth increased with time in culture from 75 μ m to a terminal size of 110 μ m (Fig. 2). This size was indistinguishable from competent oocytes aspirated from *in vivo* antral human follicles that went on to mature to MII stage in culture (Fig. 2). Healthy and intact GV's were seen during early stages of culture. Stage II GV's were observed in both fresh-isolated follicles (Fig. 5A, inset) as well as cultured follicles (14 days) (Fig. 5B inset) and progressed to stage IV by the end of culture (Fig. 5D).

Discussion

In vitro follicle growth provides the opportunity to expand research initiatives in follicle development, as well as to potentially provide expanded options to young cancer patients. Various groups (Abir *et al.*, 1997; Hovatta *et al.*, 1997; Wright *et al.*, 1999; Telfer *et al.*, 2008), including our own are working toward robust, reliable *in vitro* systems that will provide the next generation of research discoveries as well as 1 day aiding in fertility sparing plans for cancer patients. To achieve these two goals, we have adapted two 3D systems to human follicles. To the best of our knowledge, this is the first report in the humans showing the *in vitro* growth of large, antral follicles, which contains full size oocytes with stage IV GV's, from secondary stage *in vitro*. Importantly, the systems reported here can be further engineered to continue improving the *in vitro* environment in which these follicles grow. We suspect that the combination of engineered ECM (Kreeger *et al.*, 2006; Xu *et al.*, 2007), rigidity (Xu *et al.*, 2006a, b; West *et al.*, 2007) and the ability to temporally provide hormones (Kreeger *et al.*, 2005) to individual follicles will create the next generation of improvement in a system that has already demonstrated significant success in the murine model.

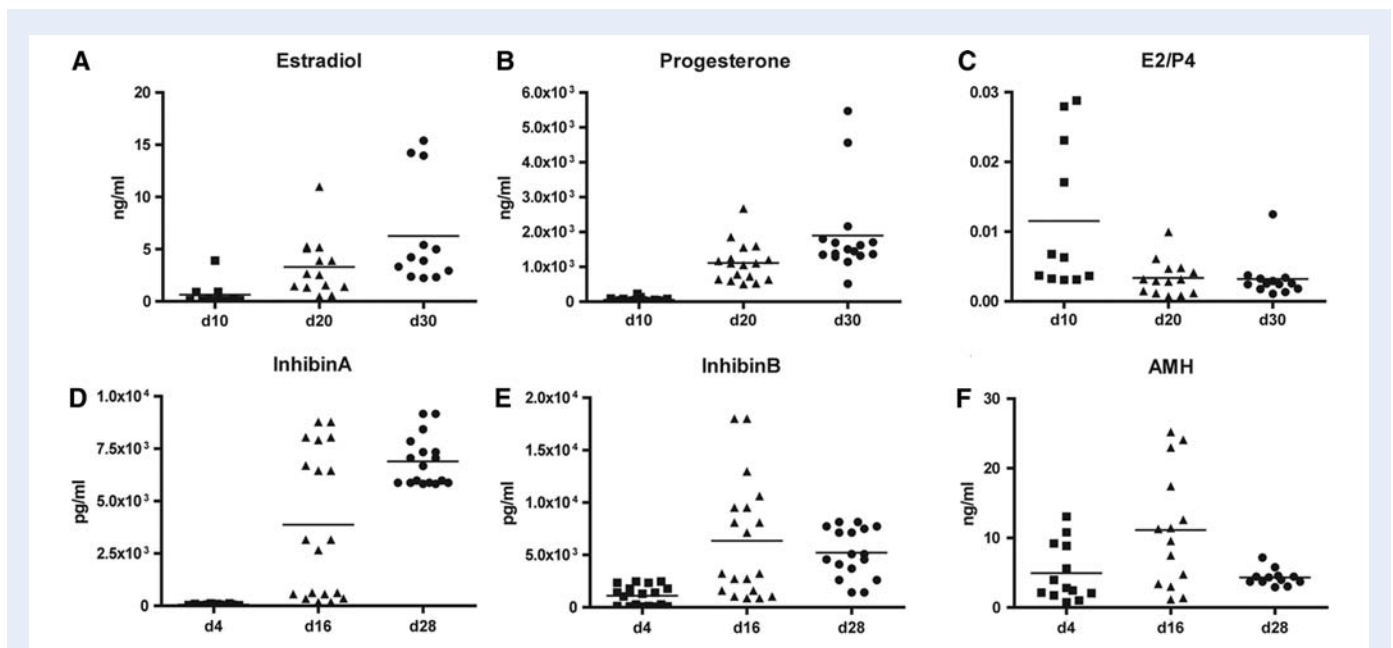
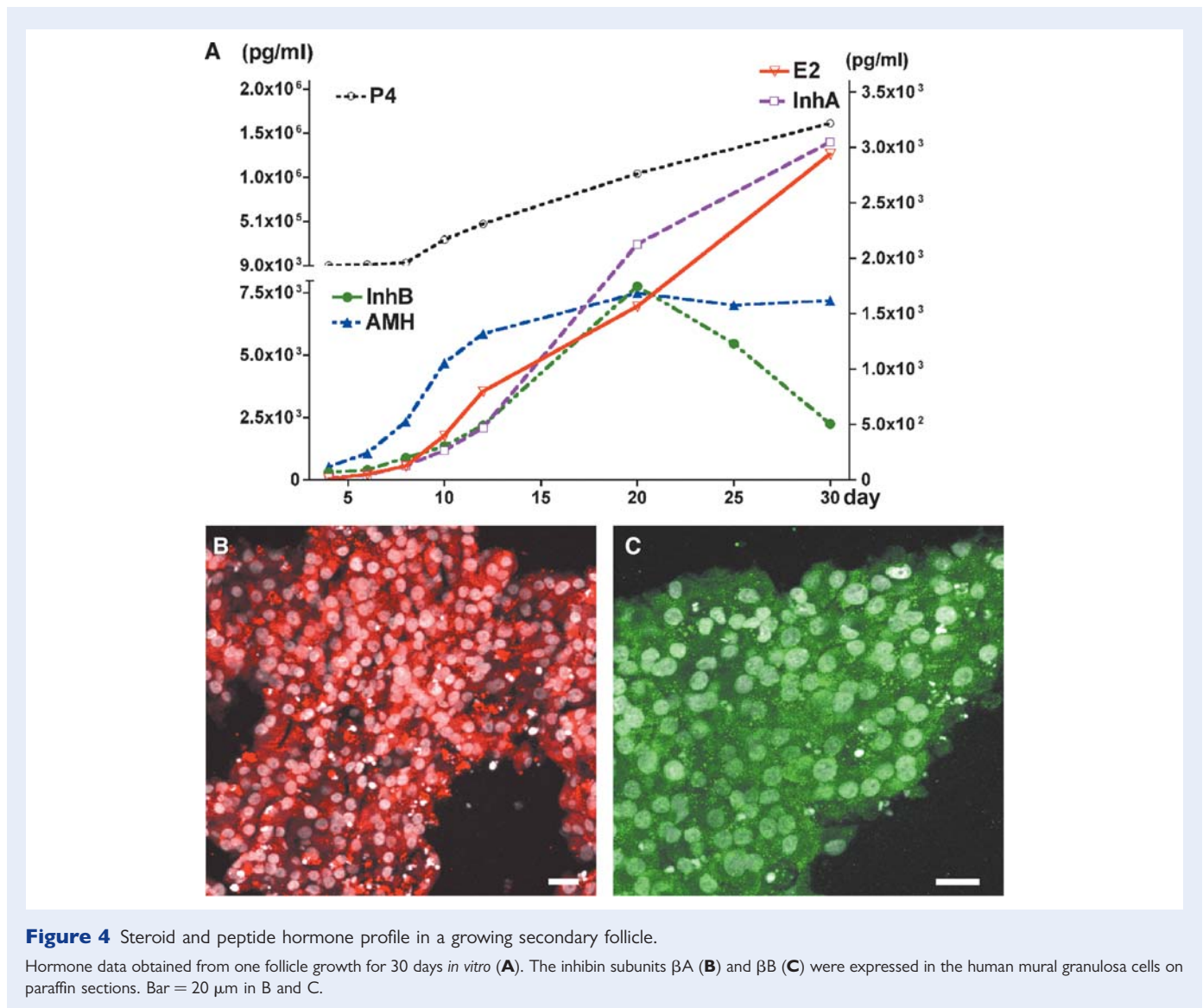


Figure 3 Hormone levels in human follicle cultures.

17 β -Estradiol (A), progesterone (B), E2/P4 ratio (C), inhibin A (D), inhibin B (E) and AMH (F) were measured in individual follicles. Hormone levels on an individual follicle basis change throughout the culture period and reflect the overall maturation status of that individual follicle.



Follicle growth

Previous studies have shown human follicle development *in vitro* (Roy and Treacy, 1993; Abir et al., 1997; Telfer et al., 2008). Antrum formation was previously achieved using cultured secondary follicles (Roy and Treacy, 1993; Abir et al., 1997); this development was shown to be dependent on FSH (Abir et al., 1997). Theca-like cells were observed when unilaminar follicles developed to secondary follicles in a collagen gel culture system (Abir et al., 1999, 2001). More recently, primordial/primary follicles were shown to enter the growing pool of follicles and develop to early antral stage (~150 μ m) in 10 days using a two-step serum-free culture system in the presence of activin A (Telfer et al., 2008). In another study, primordial/primary follicles (34–51 μ m) isolated from cryopreserved tissue survived for 7 days and increased in size up to 44–70 μ m in alginate matrix culture system (Amorim et al., 2009). We used 3D culture systems to culture small secondary follicles (Class 1 and 2 as described by Gougeon, 1996). In our study, individual non-antral, secondary follicle grew substantially over 30 days, and reached an average size of

800 μ m. Our studies have also shown that 30 days of culture was enough time to grow follicles to 1 mm and produce oocytes with nuclear competence (stage IV GV). The growth of the oocyte correlated with an increase in follicle size; the fact that oocyte growth and development was supported in conjunction with follicle growth was a significant achievement. Though we have not yet explored whether these oocytes also have cytoplasmic competence after 30 days in culture, it has been shown that a follicle size of 1.6 mm can be aspirated for cumulus-oocyte complexes and matured *in vitro* (Chian et al., 2004), therefore 30 days of IVFG may be enough time for the secondary follicle to reach this step.

The final expansion of the follicle is largely attributed to the size of the antrum, which is associated with a prodigious increase in hormones (principally estrogen) necessary to drive an ovulatory signal (LH) from the pituitary gland. Moreover, the follicle must grow outward from the perimedullary zone to permit contact between the follicle surface and the ovarian surface in anticipation of ovulation. *In vitro* systems do not have to phenocopy the properties of the follicle

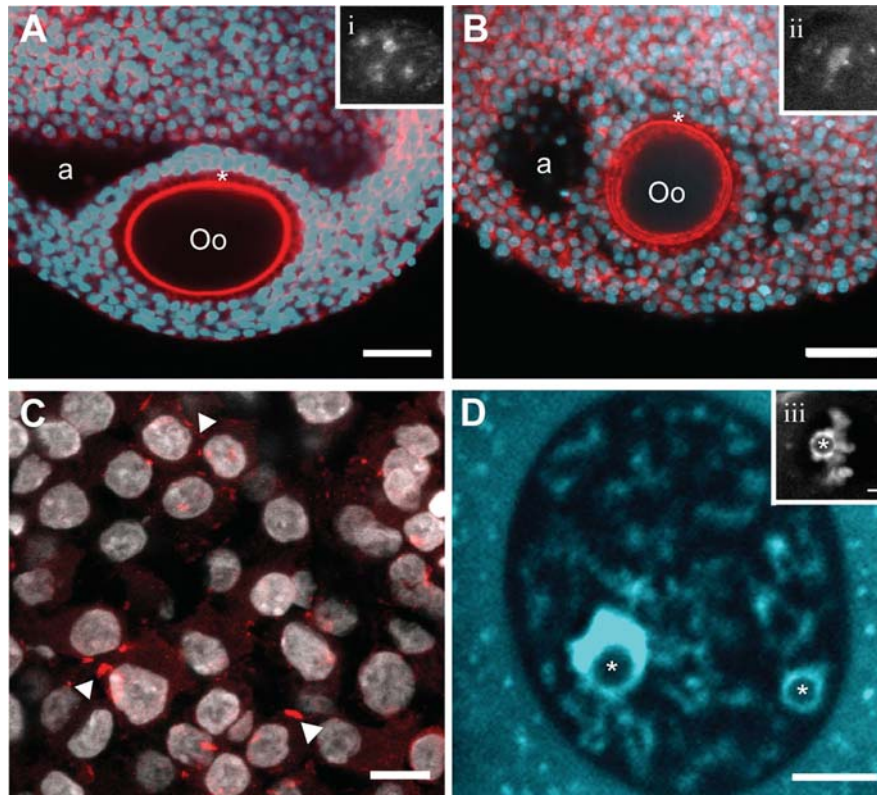


Figure 5 *In vitro* growth follicles resemble *in vivo* follicles.

(A) A fresh-fixed human follicle, and (B) an isolated human follicle grown in alginate for 14 days show similar characteristics including an antral cavity (a) as well as the development of the cumulus-oocyte complex (Oo = oocyte). TZPs (*) are maintained between the oocyte and surrounding granulosa cells (shown by F-actin in red). In both follicles, the oocytes are incompetent to resume meiosis as shown by the GV stage II (insets i and ii, chromatin/DNA). Scale represents 50 μm . (C) Localization of connexin-43 (arrowheads) to the gap junctions between granulosa cells in *in vitro* growth follicles show the cell–cell interactions that are important for bidirectional transport of small molecules between cells and that are essential for follicle growth and cell differentiation. Scale represents 20 μm . (D) An oocyte from an *in vitro* growth follicle contains a stage IV GV (chromatin/DNA-blue) represented by a chromatin-surrounded nucleolus (*). Scale represents 5 μm . This can be compared with a fresh-isolated human cumulus-oocyte complex that is competent to undergo meiotic resumption as depicted by the stage IV GV (inset iii, scale represents 5 μm) containing a chromatin surrounded nucleolus (*).

that relate to maintaining a menstrual cycle and extruding an oocyte—they simply have to accommodate the oocyte to the point where nuclear and cytoplasmic maturation can occur. Thus, our goal for the human follicle growth project is not to generate a 20 mm follicle, rather, to determine what length of culture and what antrum size is necessary to produce an oocyte competent to resume meiosis *in vitro*. Based on the data presented here, our culture conditions support follicle growth to an appropriate *in vitro* terminal size.

Hormones

We next investigated whether the human ovarian follicles grown *in vitro* were hormonally active. The pattern of hormone secretion from the growing human follicles was similar to the pattern observed during the human menstrual cycle (Welt *et al.*, 1999; Klein *et al.*, 2004). The earliest detectable hormone released (above baseline) from the follicle was AMH. AMH secretion was followed by estradiol and the inhibins. Inhibin A and 17 β -estradiol were positively correlated throughout follicle development, although inhibin B levels fell as the follicles achieve ‘dominant’ status. Average AMH decreased

as the inhibin A increased and inhibin B fell (Fig. 3). AMH remains elevated in the individual follicle depicted in Fig. 4. Understanding the relationship between AMH and follicle/oocyte maturity is a future goal of this work. Baseline levels of progesterone from newly isolated follicles were unexpectedly higher than one might predict, however, progesterone levels have never been measured in a secondary human follicle. The effects of the progesterone on follicle development were unknown, but did not impact follicle growth or oocyte development. Like the other hormones, progesterone secretion was maintained at basal level for the first week of culture and did not increase until estrogen and the peptide hormones levels rose. Importantly, the ratio of estradiol to progesterone was consistent during the last 2 weeks culture after the antrum has formed, which indicated no significant luteinization has occurred in the cultured follicle. We can confirm the relative health of granulosa cells by observing the low cytoplasm to nucleus ratio in the confocal images of freshly isolated and *in vitro* cultured follicles. Moreover, the inhibin βB subunit was known to be expressed in healthy granulosa cells and not in luteinized human tissue (Groome *et al.*, 1996; Klein *et al.*, 2004). Thus, the ovarian follicles grown *in vitro* were capable of sustained hormone

production, which phenocopied the patterns of the hormones secreted by follicles *in vivo*.

Oocyte status

In addition to overall follicle size and growth patterns, the development and maturation of the oocytes described here mirrors that of *in vivo* matured follicles, or freshly isolated *in vitro* matured follicles. The key anatomical structures that provide communication routes between the somatic cells and the oocyte are the TZPs (Albertini and Barrett, 2003). Although these actin-based cytoplasmic processes are known to be present in mature human cumulus-oocyte complexes, their existence in immature human follicles, although expected, has not previously been established. We isolated fresh, immature human follicles that were fixed, and then imaged by confocal microscopy. Importantly, we found that cultured immature human follicles did indeed maintain a dense network of TZPs. Because we were able to maintain these structures with our 3D matrices, oocyte growth increased with time in culture from 75 μm to a terminal size of 110 μm . This size was indistinguishable from meiotically competent oocytes aspirated from small antral human follicles *in vivo* (Cavilla et al., 2008). During the early stages of culture, freshly isolated follicles and cultured follicles (14 days) had displayed healthy, appropriately staged, intact GV's (nucleoli stage II), which progressed to stage IV by the end of culture. It was documented that oocytes with GV stage IV developed nuclear competence and that, depending on cytoplasmic competence, these oocytes should be able to resume meiosis in preparation for fertilization (Combelles et al., 2002). Thus, the *in vitro* 3D culture system supported coordinated follicle and oocyte growth creating an environment in which the major steps of human follicle development were maintained.

Our studies revealed that early secondary follicles, once removed from the restricted environment of the ovary, had the capability of surviving and growing in culture. By using a 3D supportive matrix, follicles were able to maintain bidirectional communication between somatic cells and the germ cell creating an environment conducive for oocyte growth and proper steroid production. The next frontier will be maturation of *in vitro* grown oocytes to MII, fertilization and embryo development, and the adaptation of these techniques to primordial follicles and cryopreserved tissues.

Author's Role

M.X. design, acquisition of data, analysis, interpretation. S.L.B. acquisition of data, interpretation and critical review. E.W.-F., L.A.K. and S.E.K. acquisition of data, analysis. L.D.S. interpretation, critical review. T.K.W. design, interpretation, critical review, final approval.

Supplementary data

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

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