In Vitro Oocyte Maturation and Preantral Follicle Culture from The Luteal Phase Baboon Ovary Produce Mature Oocytes

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ABSTRACT
Female cancer patients who seek fertility preservation but cannot undergo ovarian stimulation and embryo preservation may consider: 1) retrieval of immature oocytes followed by in vitro maturation (IVM); 2) ovarian tissue cryopreservation followed by transplantation or in vitro follicle culture (IVFC). Conventional IVM is carried out during the follicular phase of menstrual cycle. There is limited evidence demonstrating that immature oocyte retrieved during the luteal phase can mature in vitro and be fertilized to produce viable embryos. While in vitro follicle culture is successful in rodents, its application in nonhuman primates has made limited progress. The objective of this study was to investigate the competence of immature luteal-phase oocytes from baboon and to determine the effect of FSH on baboon preantral follicle culture and oocyte maturation in vitro. Oocytes from small antral follicle COCs with multiple cumulus layers (42%) were more likely to resume meiosis and progress to MII than oocytes with a single layer of cumulus cells, or less (23% vs. 3%, respectively). Twenty-four percent of mature oocytes were successfully fertilized by ICSI, and 25% of these developed to morula stage embryos. Preantral follicles were encapsulated in fibrin-alginate-matrigel (FAM) matrices, and cultured to small antral stage in a FSH-independent manner. FSH negatively impacted follicle health by disrupting the integrity of oocyte and cumulus cells contact. Follicles grown in the absence of FSH produced MII oocytes with normal spindle structure. In conclusions, baboon luteal-phase COCs and oocytes from cultured preantral follicles can be matured in vitro. Oocyte meiotic competence correlated positively with the number of cumulus cell layers. This study clarifies the parameters of the follicle culture system in nonhuman primates and provides foundational data for future clinical development as a fertility preservation option for women with cancer.

Introduction

Hormone stimulation followed by oocyte aspiration, *in vitro* fertilization (IVF) and embryo cryopreservation, is the most common approach for preserving fertility in female cancer patients prior to chemotherapy or radiation [1-5]. However, hormone stimulation requires a delay in cancer treatment, may be contraindicated in patients with hormone-sensitive malignancies, and is not an option for prepubertal girls. As the number of young cancer survivors continues to increase [6, 7], there is a need for fertility preservation options that do not require hormonal stimulation. Retrieval of immature oocytes followed by in vitro maturation (IVM) is suggested as an additional strategy for fertility preservation [8, 9]. Oocyte retrieval for IVM is commonly performed during the follicular phase of the menstrual cycle. Little data indicates whether primate immature oocytes retrieved during the luteal phase can undergo IVM, fertilization, and produce viable embryos. Only a few studies describe luteal phase-like oocyte retrieval performed during cesarean section; these studies reported that immature oocytes recovered during gynecologic surgery could be matured *in vitro* and fertilized [10-12]. Although a high concentration of circulating progesterone is present during pregnancy, this environment does not recapitulate the ovarian hormonal environment during the luteal phase.

Another potential alternative strategy for cancer patients involves ovarian tissue cryopreservation. Thawed tissue can be orthotopically transplanted [13-16], or immature follicles are retrieved for *in vitro* follicle growth, *in vitro* oocyte maturation (IVM) and fertilization [8, 9]. While ovarian tissue transplantation has been successful, it carries a risk of reintroducing malignant cells to the patient [17-19]. As an alternative, *in vitro* follicle culture techniques in rodent species are successful and progress is being made in optimizing the system for nonhuman primates and humans.

The development of 3-dimensional (3D) matrices for *in vitro* ovarian follicle culture is central to the success of these techniques thus far [20-22]. It is believed that 3D culture matrices may be able to maintain intercellular communication better within the follicle through the structural support of follicle architecture, specifically the transzonal projections (TZP) between the oocyte and the surrounding cumulus cells, which are necessary for oocyte growth *in vitro* [23, 24]. The impact of TZP maintenance on the success of IVM has been reported for mouse [25], rat [26], bovine[27], and human [12] , but not in baboon. Cumulus cells also play an essential role in promoting oocyte cytoplasmic maturation that is critical for subsequent embryo development. The culture methods that maintain the cumulus-oocyte interaction may support oocyte health.

In this study, we sought to optimize the *in vitro* follicle culture system and IVM in nonhuman primates by using preantral follicles and cumulus-oocyte complexes (COCs) from small antral follicles isolated from luteal-phase baboon ovaries. We aimed to answer the following questions: (1) Can oocytes from luteal-phase small follicles undergo meiosis and produce viable embryos? (2) Is COC morphology an indicator of oocyte meiotic competence? and (3) What criteria should *in vitro* follicle culture fulfill in order to produce meiotically competent nonhuman primate oocytes?

Materials and Methods
Ovary collection
Ovaries were obtained from 6 adult cycling baboons during the luteal phase, days 7-10 post-ovulation (DPO) (Table 1). Ovulation was detected by measuring peripheral serum levels of estradiol, beginning 7 days after the first day of menses. The day of the estradiol surge was designated Day -1, with Day 0 as the day of the ovulatory LH surge and Day 1 as the day of ovulation [28]. Luteal-phase ovaries were confirmed by presence of a corpus luteum (CL). Ovaries were transported to the laboratory at room temperature within 1 hour of retrieval. Experiments with animals and tissues were approved by the Animal Care and Use Committees of the University of Illinois at Chicago and Northwestern University, respectively.

Cumulus-oocyte-complex (COC) isolation and classification
Ovaries were quartered with a scalpel, and the medulla was separated from the cortex using curved scissors in MOPS-HTF medium (Cooper-Surgical, Trumbull, CT). Small antral follicles (Fig. 1) located on the border between the cortex and medulla were punctured with a 25-gauge needle and gently squeezed to release the COCs. Using a dissecting stereomicroscope, COCs were classified to 3 groups: 0L-COC, incomplete layer of cumulus cells; 1L-COC, 1-2 complete layers of cumulus cells; and ML-COC, at least 3 complete layers of compact cumulus cells. COC classification was performed by a single observer to ensure uniformity of COC types.

Preantral follicle isolation
The preantral follicles were isolated as previously described with minor modifications [23]. The ovarian cortex was cut into small pieces (approximately 1-2 mm^3) and tissue was enzymatically digested in αMEM (Invitrogen, Carlsbad, CA) containing 1% HSA (Irvine Scientific, Santa Ana, CA), 0.08 mg/ml Liberase Blendzyme 3 (Roche Diagnostics, Indianapolis, IN), and 0.2 mg/ml DNase (Worthington Biochemical, Lakewood, NJ) for 1 hour on a shaker inside incubator at 37 °C and 5% CO_2. After rinsing the cortex twice with MOPS-HTF medium, follicles were mechanically isolated using a 25-gauge needle into MOPS-HTF medium. The follicles were transferred to maintenance medium (αMEM, supplemented with 1% HSA, 100 IU/ml penicillin and 100 μg/ml streptomycin) and placed in an incubator at 37 °C and 5% CO_2. Only preantral follicles (class 1 and 2) [29] that contained a clear, visible, centrally-located oocyte, healthy granulosa cells, and no signs of antrum formation were encapsulated and cultured (see Fig. 7A).

Matrix preparation
Sodium alginate (55-65% guluronic acid) was provided by FMC BioPolymers (Philadelphia, PA); Tisseel was kindly provided by Baxter Healthcare (BioScience Division, Westlake Village, CA); and Growth Factor Reduced BD Matrigel^{TM} was purchased from BD Bioscience (BD Cat 354234, Bedford, MA). All biomaterials in this study were prepared as described previously [21, 23, 30]. Briefly, sterile alginate aliquots were reconstituted to 0.5% (w/v) in 1×PBS. Fibrinogen was reconstituted to 50 mg/ml in aprotinin (3000 KIU/mL) solution, and thrombin reconstituted to 50 IU/ml in 50 mM $CaCl_2$/140 mM NaCl, according to kit instructions (Baxter Healthcare). GFR-Matrigel was thawed on ice before use.

Follicle encapsulation and culture
In order to maximally deactivate the activity of the enzymes used for follicle isolation, preantral follicles were first embedded in 25% GFR-Matrigel for 1 hour as follows [23]. GFR-Matrigel was diluted 1:3 with cold αMEM and added to a V-bottom 96-well plate. After 10 minutes at room temperature, individual follicles were transferred into each well and the plate was incubated for 50 minutes at 37 ºC and 5% CO_2. Follicles were then retrieved from the Matrigel using blunt tip forceps.

The FAM matrix was prepared by mixing 25 μl fibrinogen (50 mg/ml), 25 μl alginate solution (0.25%), 40 μl 1×PBS and 10 μl GFR-Matrigel. Five to ten follicles were transferred immediately into the FAM mixture with a minimal amount of maintenance medium. Using a 10 μl pipette tip, individual follicles in 5 μl of FAM mixture were pipetted into the 50 IU/ml thrombin solution for crosslinking and gelatinization for 5 minutes. Fresh FAM mixture was prepared every 30 minutes until the encapsulation was completed. The crosslinked and gelled FAM beads, each containing a single follicle, were rinsed in maintenance medium and plated one per well in 96-well plates in 100 μl of basal culture medium: αMEM, 3 mg/ml HSA, 1 mg/ml bovine fetuin (Sigma-Aldrich, St. Louis, MO), 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium. Throughout isolation, encapsulation, and plating, follicles were maintained at 37 °C and pH 7. Encapsulated follicles were cultured at 37 °C in 5% CO_2 up to 14 days. Every other day, half of the medium (50 μl) was exchanged and stored at -80 °C for use in steroid assays.

Follicle culture in FSH-containing medium
In the first experimental culture phase, follicles from 4 baboons were randomly separated into 3 groups and grown for 10 days in culture medium supplemented with 0, 10, or 100 mIU/ml recombinant human FSH (NV Organon, Oss, The Netherlands). Follicles were then recovered from matrix and oocytes underwent IVM. In the second phase, follicles from 2 baboons were grown for 14 days in the absence of FSH, identified in phase 1 to produce cumulus-surrounded oocytes. Follicles were then recovered and the oocytes underwent IVM.

In vitro maturation (IVM)
Oocyte maturation was carried out using an IVM kit (Cooper-Surgical) at 37 °C in 5% CO_2 for 46-48 hours. The vendor-supplied IVM medium was supplemented with 100 mIU/ml FSH (NV Organon), 100 mIU/ml LH (Ares Serono, Randolph, MA), 1 IU/ml human chorionic gonadotropin (hCG) (Sigma), 10 ng/mL epidermal growth factor (EGF) (Sigma), and 5% (v/v) heat inactivated fetal bovine serum (FBS) (Invitrogen).

Ten to fifteen of each class of COCs from small antral follicles underwent IVM in an Organ Tissue Culture Dish (60 x 40 mm; Falcon/BD Biosciences, San Jose, CA) containing 1 ml maturation medium covered with embryo-grade mineral oil.

In vitro cultured preantral follicles were first recovered from the FAM matrix beads by incubation in a 10 IU/mL solution of alginate lyase in prewarmed MOPS-HTF medium for 10 minutes. The COCs were carefully separated from the surrounding follicle using 28-gauge insulin needles and individual COCs were transferred into a 15 μl droplet of IVM medium covered with embryo-grade mineral oil. After 46-48 hours, oocytes were denuded of cumulus cells with 0.3% hyaluronidase and gentle pipetting. Oocyte state was assessed using light microscopy and characterized as follows: germinal vesicle breakdown (GVBD), if the germinal vesicle was not present; GV, if there was an intact germinal vesicle; metaphase II (MII), if a polar body was present in the perivitelline space; and degenerated (DG), if the oocyte was fragmented or shrunken.

Intracytoplasmic sperm injection (ICSI) and embryo culture
Mature oocytes (MII) were inseminated by ICSI with frozen-thawed baboon sperm obtained by electro-ejaculation from males of proven fertility housed in the colony [31]. Fertilization was evaluated 16-18 hours after injection and considered normal if two pronuclei were observed. Embryos were individually cultured for 5 days in a 20 μl drop of embryo culture medium provided in the IVM kit (Cooper-Surgical) under mineral oil at 37 °C in 5% CO_2.

Follicle and oocyte measurement
During the follicle culture period, photographs of each follicle were captured using a Leica DM IL light microscope (Leica, Wetzlar, Germany) equipped with phase objectives, a heated stage, a Spot Insight 2 Megapixel Color Mosaic camera, and Spot software (Spot Diagnostic Instruments, Sterling Heights, MI). Follicle diameters were later measured using ImageJ software (National Institutes of Health, USA) as previously described [32, 33]. Oocyte diameters, minus the zona pellucida, were measured on day 0, when the oocyte was enclosed in the follicle, and on day 10 and 14, when the COC had been separated from the surrounding follicle.

Tissue sectioning and staining
Ovarian tissues and *in vitro* cultured follicles were fixed in 4% paraformaldehyde in 1× PBS overnight at 4 °C. Follicles were dehydrated in ascending concentrations of ethanol (70%-100%), and embedded in paraffin using an automated tissue processor (Leica, Manheim, Germany). 4-μm sections were cut and stained with Gomori's trichrome [34] for ovarian tissues or hematoxylin and eosin for *in vitro* cultured follicles.

Immunostaining and confocal imaging
Oocytes were fixed and extracted in a microtubule-stabilizing buffer with 4% formaldehyde at 37 °C for at least 30 minutes [35]. To visualize spindles and chromosomes, oocytes were incubated with mouse monoclonal anti-α-tubulin (1:200, Sigma) overnight at 4 °C, followed by Alexa Fluor 488-conjugated rabbit-anti-mouse IgG (1:400, Molecular Probes, Eugene, Oregon) for 1 hour at room temperature, and then were mounted in VectaShield with 1 μg/ml propidium iodide (Vector Laboratories, Burlingame, CA). Images were obtained using a laser scanning confocal microscope (Leica TCS SP5X, Manheim, Germany) under a × 63 oil immersion objective. For each spindle, a complete Z-axis scan was collected at 0.5 μm intervals, and 3D projection was analyzed on Leica SP5 software.

Statistics
Maturation data were analyzed using one-way ANOVA, followed by a paired t-test. Spindle data analysis was carried out with pooled data using chi-squared analysis. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using the software GraphPad Prism version 4.0.

Results
In vitro maturation of COCs from in vivo small antral follicle
Meiotic resumption
To investigate the correlation between COC morphology and oocyte meiotic competence, we collected 371 COCs from 1-2 mm small antral follicles located on the border between the ovarian cortex and medulla (Fig. 1). COCs were grouped according to the number of cumulus cell layers: 0L, 1L, or ML (Fig. 2A, D, G). After 48 hours IVM, the percentage of oocytes from each group that were in GV, MI, or MII stages was determined (Table 2). Most of oocytes in the 0L-COC group remained in the GV stage (Fig. 2A-C). Cumulus cell expansion was observed after 24 hours of IVM in the 1L-COC and ML-COC groups (Fig. 2E, H), and mature oocytes (MII) were seen after 48 hours (Fig. 2F, I). Significantly more oocytes from the ML-COC group resumed meiosis and progressed to the MII stage (42%) compared with oocytes from the 0L-COC and 1L-COC groups (3% and 23%, respectively; Table 2).

Analysis of spindle apparatus of mature oocytes
Spindle morphology and chromosome alignment of *in vitro* matured oocytes from small antral follicle COCs was assessed using a previously described classification system [36]. Figure 3A-D shows representative images from the 1L-COC and ML-COC for each of the 4 classifications of nuclear status: (A) Bipolar spindle/aligned chromosomes show bipolar organization with pointed or flattened poles, microtubules converging at both poles, and all chromosomes present and evenly aligned at the equatorial plate (Fig. 3A); (B) Bipolar spindle/nonaligned chromosomes show bipolar organization with pointed or flattened poles, microtubules meeting at both poles, and chromosomes with varying degrees of misalignment (Fig. 3B); (C) Disarranged spindle/aligned chromosomes show clusters of disorganized microtubules, multipolar spindles, or spindles with microtubules not converging at one or both poles, and containing closely aligned chromosomes associated with microtubules (Fig. 3C); and (D) Severely disarranged or absent spindle/dispersed or absent chromosomes (Fig. 3D). No statistically significant differences were found between the 1L-COC and ML-COC groups with regard to the percentage of oocytes in each of the 4 categories (Table 3). All of oocytes that did not resume meiosis had surrounded nucleolus (SN), in which the nucleolus is surrounded by a rim of PI positive chromatin (Fig. 3E). MII oocyte was represented as Fig. 3F. There were insufficient numbers of MII oocytes in 0L-COC group to permit evaluation of nuclear status.

Fertilization and embryo development in vitro
Of the 33 mature oocytes (MII) from ML-COC group fertilized by ICSI, 8 demonstrated normal fertilization with two pronuclei. Four 2-cell embryos were obtained on day 2, and of these, 2 developed to the morula stage by day 4 (Fig. 4).

In vitro growth and maturation of preantral follicles

In the first experiment, 46 preantral follicles were encapsulated in FAM and cultured for 10 days in medium supplemented with 0, 10, or 100 mIU/ml FSH (Table 4). The overall survival rates of follicles among the 3 groups were comparable. Follicles exhibited FSH dose-dependent increases in follicle size. However, exogenous FSH negatively impacted follicle health (Fig. 5); in the presence of 100 mIU/ml FSH, granulosa cells became hypotrophic and the oocyte lost intercellular connection with the surrounding cumulus cells (Fig. 5B). After 10 days of culture COCs were recovered from follicles. All oocytes from the 10 and 100 mIU/ml FSH culture groups were already denuded (Fig. 5D), whereas oocytes cultured in the absence of FSH had at least one layer of cumulus cells (Fig. 5C). In addition, oocyte size was negatively correlated with FSH dose in culture (Table 4). Although IVM was performed on all of the GV-intact oocytes recovered, none of the oocytes resumed meiosis after 48 hours of IVM (data not shown).

Based on these results, 31 preantral follicles were encapsulated for the second experiment (Fig. 7A) and cultured for 14 days without FSH. With the additional 4 days of culture, follicles grew to diameters equal to those of follicles that were cultured in the presence of FSH for 10 days (Fig. 6A), and more than 50% of the follicles (17 out of 31) developed to the antral stage (Fig. 7B). In culture, oocytes grew to sizes comparable to those within small antral follicles *in vivo* (Fig. 6B). IVM was then carried out on 16 of the COCs, which have at least 1 complete layer of cumulus cells (Fig. 7C). Cumulus cells expanded within 24 hours (Fig. 7D). 13 of the 16 oocytes exhibited GVBD within 48 hours; two MII oocytes were obtained (Fig. 7E) and confirmed as having normal spindle structure and chromosome alignment by confocal microscopy (Fig. 7F).

Discussion

In this study, we first demonstrated that baboon oocytes from small antral follicles collected during the luteal phase could be matured *in vitro* and produce viable embryos. We also found that the meiotic competence of these oocytes was correlated to the number of surrounding cumulus cell layers. These studies provided preliminary data for the optimization of *in vitro* follicle culture system for nonhuman primate follicles. We subsequently demonstrated that luteal-phase baboon preantral follicles were able to reach the small antral stage after 14 days in culture, with oocytes reaching sizes similar to those from small antral follicles *in vivo*. The absence of FSH in the culture medium was critical to maintain a compact COC structure. For the first time in nonhuman primates, we also demonstrated that oocytes recovered from cultured follicles are able to undergo IVM and resume meiosis resulting in normal spindle structure.

Previous work in our lab and others revealed that the conditions required to support oocyte maturation *in vitro* are species-dependent. IVM is successful in rodents [37], and immature bovine oocytes from small antral follicles (2-8 mm in diameter) are matured *in vitro* to produce live offspring [38]. However, the application of IVM to nonhuman primate oocytes is not currently as successful. One study showed that only 29% of baboon immature oocytes, retrieved at the time of cesarean delivery, could progress to the MII stage *in vitro* [39]. Based on our findings, we speculate that the low IVM rate may be the result of pooling COCs, regardless of the number of cumulus cell layers. We achieved the highest percentage of MII oocytes (41.6%) from multilayer COCs, demonstrating a strong correlation between COC morphology and oocyte meiotic competence. These data support the idea that maintaining the cumulus-oocyte connection is essential for oocyte cytoplasmic maturation. Furthermore, the relative smaller oocyte diameter in 0L-COC group (~99.7μm) indicated that these oocytes might originate from less developed follicles [40, 41]; and the lack of surrounding cumulus suggested they might be from earlier atretic follicles [42]. Compared to the ML-COC group, there were fewer oocytes in the 1L-COC group that progressed to MII. Given that the 1L-COC oocytes had similar size and the same proportion of normal spindle structure and chromosome alignment as the ML-COC ones, those that did mature should have a

similar developmental capacity as ML-COC oocytes [41, 43]. Unfortunately, the low number of MII-stage oocytes prevented the direct characterization of fertilization and embryo development of 1L-COC oocytes. Oocytes that did not resume meiosis had a surrounded nucleolus (SN), which suggested that they had reached the nuclear meiotic competence. The failure of GVBD may be due to the cytoplasmic incompetence. While a few ML-COC oocytes could be matured in vitro, fertilized and developed to the morula stage, the overall rate was low, which could also be attributed to insufficient cytoplasmic maturation [35][44].

In monovular species, it is generally accepted that once the dominant follicle is selected, remaining small antral follicles in the same cohort become atretic. This concept forms the basis for the conventional method of IVM, with oocyte retrievals performed during the follicular phase [41, 45]. However, this dogma is challenged by several recent studies. Two early studies reported that immature oocytes obtained from women undergoing cesarean section could be matured and fertilized in vitro [10, 12]. Because progesterone concentration is high during pregnancy, the hormonal milieu prior to delivery resembles the luteal phase of the menstrual cycle. Other investigators subsequently proved that human immature oocytes retrieved during the luteal phase could be matured in vitro [8, 10, 46], with two 4-cell embryos obtained in one study [46]. In the current study, baboon oocytes collected from small antral follicles during the luteal phase could mature in vitro and produced embryos that underwent normal early development. This approach is distinct from those previously described in which COCs were retrieved from large antral follicles (<10 mm) aspirated from the ovarian surface. The ability of luteal-phase baboon small antral follicles to undergo IVM and the impact of the cumulus cells on oocyte development established two benchmarks for producing meiotically competent nonhuman primate oocytes in vitro.

Follicle culture systems are extremely successful in mice [21, 47-50], but have not yet been adapted successfully to human and nonhuman primates [51-58]. Recently, we demonstrated that rhesus monkey [22] and human follicles [23] could grow from the secondary to antral stage in an alginate-based 3D culture system. The cultured follicles contained healthy, growing oocytes that maintained TZPs with the surrounding granulosa cells [23].

Here, we took these studies an additional step forward to demonstrate the capacity of oocytes grown in vitro to reach the MII stage. We chose a semi-degradable FAM matrix containing fibrin, alginate, and Matrigel for this study because it provides a dynamic mechanical environment that facilitates follicle outward expansion while maintaining follicle architecture to enhance oocyte quality [30]. Interestingly, exogenous FSH had a negative impact on baboon follicle health and morphology; this was particularly surprising, as our previous studies showed that FSH was necessary for mouse, human and rhesus preantral follicle development in vitro [22, 23, 59]. In contrast, baboon follicles exposed to FSH in culture lost connections between oocytes and their surrounding cumulus cells, and produced significantly smaller oocytes than those from follicles cultured without FSH. Baboon granulosa cell FSH receptors may be more sensitive to human recombinant FSH [60]; it is unknown whether baboon FSH receptors are more like human or rhesus monkey receptors in this respect [61]. We suspect that the premature dispersion of cumulus cells relates to FSH-induced retraction of TZP, which has been reported in the mouse model [62]. Mammalian oocyte development only occurs when tight coordination and interaction between oocyte and cumulus cells is maintained [24, 63, 64]. Thus, while FSH-stimulated follicle growth appeared normal, the retraction of TZP decreased oocyte growth, development, and produced fewer oocytes with meiotic competence.

Taken together, we found that the production of meiotically competent baboon oocytes from in vitro follicle cultures could be achieved when (1) the follicle reached at least 0.5 mm, (2) the oocyte grew to 105 μm, and (3) functional cumulus-oocyte connections were maintained. These three morphological criteria could be used as a noninvasive method to determine the point at which nonhuman primate oocytes should be retrieved from the culture matrix for IVM. That said, and perhaps not unexpected, oocytes from

in vitro cultured follicles progress to MII oocytes at a lower frequency than oocytes from *in vivo* small antral follicles. Nonetheless, to the best of our knowledge, this is the first report of primate preantral follicle culture in which MII oocytes with normal spindle structure has been derived. Future work will optimize the culture system and determine whether cultured oocytes can be fertilized and support normal embryo development.

In conclusion, our studies revealed that luteal-phase baboon follicles can be cultured *in vitro* to produce meiotically competent oocytes. This research represents the next step in the ongoing effort to apply *in vitro* follicle culture systems to the primate. In time this technology may be applied to women with cancer who wish to preserve their fertility but who are not candidates for traditional IVF procedures.

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Figure Legends

Figure 1: A collection of small antral follicles from a luteal-phase baboon ovary. (A) Small antral follicles observed under a stereomicroscope (arrowheads). (B) Gomori's trichrome-stained section of a luteal-phase baboon ovary with arrowheads indicating the location of small antral follicles on the border of the ovarian cortex and medulla. Bar = 1 mm.

Figure 2: Status of small antral follicle COCs status during IVM. (A-C) 0L-COC oocytes remained in the GV stage (black arrows) through 48 hours of IVM. (D-F) 1L-COC and (G-I) ML-COC oocytes displayed cumulus cell mucification within 24 hours and extruded the first polar body (F and I, arrows) by 48 hours. Bar = 50 μm.

Figure 3: Small antral follicle oocyte spindle morphology and chromosome alignment.
(A) Normal bipolar spindle/aligned chromosome; (B) Bipolar spindle/nonaligned chromosome; (C) Disarranged spindle/aligned chromosomes; (D) Severely disarranged or absent spindle/dispersed or absent chromosomes. (E) GV-stage oocytes with surrounded nuclei after 48 hours IVM. (F) A mature oocyte with a normal MII spindle and first polar body (FPB). DNA is stained with PI and appears red; α, β-tubulin was stained with Alexa Fluro 488 and appears green. Bar = 10 μm.

Figure 4: Characteristics of *in vitro* baboon embryo development. A total of 33 MII oocytes resulting from IVM of baboon small antral follicle COCs were fertilized by ICSI; by day 1, eight had visible pronuclei (2PN); by day 2, four embryos had reached the 2-cell stage; and by day 4, two embryos had reached the morula stage.

Figure 5: Characteristics of *in vitro* cultured preantral baboon follicles. (A) The follicle cultured in the

absence of FSH had a compact COC, whereas (B) the follicle cultured in 100 mIU/ml FSH showed separation of cumulus cells from the oocyte after 10 days. (C) A COC recovered after 10 days of culture in the absence of FSH had multiple cumulus layers, compared with (D) a denuded oocyte recovered from follicles cultured in the presence of FSH. Bar = 100 µm (A, B), 50 µm (C, D).

Figure 6: Follicle and oocyte size during *in vitro* preantral follicle culture. (A) Baboon preantral follicles grew continuously for 10 days in the presence of 10 or 100 mIU/ml FSH, or for 14 days in the absence of FSH. (B) After 14 days of culture without FSH, the average oocyte size increased from 95.0 ± 0.5 µm to 105.6 ± 2.1 µm, similar to the size of oocytes within small antral follicle COCs (*in vivo*; 104.6 ± 1.0 µm). Different letters indicate statistically significant differences ($P<0.05$).

Figure 7: Baboon preantral follicle growth in FAM matrix and IVM of recovered oocytes. (A) A preantral follicle (223 µm) isolated from the luteal-phase baboon ovarian cortex and encapsulated in FAM. (B) After 14-days in culture without FSH, the follicle developed to the small antral follicle stage (667 µm). (C) A compact COC was recovered from the FAM matrix for IVM. (D) Cumulus cells expanded after 24 hours of IVM. (E, F) The oocyte resumed meiosis, reached the MII stage within 48 hours, and showed normal spindle structure with the first polar body (FPB, black arrow). Bar = 100 µm (A-D), 50 µm (E), 10 µm (F).

Table 1. Baboon information and experimental design.

Baboon	Age (y)	DPO	Small antral COCs	Preantral follicles
1	16	13		
2	9	8	IVM + Confocal	IVFC in FSH-0, 10, 100 for 10 days
3	10	9		
4	11	7		
5	16	9	IVM + ICSI	IVFC in FSH-0 for 14 days
6	11	3		

DPO, day post ovulation; ICSI, intracytoplasmic sperm injection; IVM, *in vitro* maturation; IVFC, *in vitro* follicle culture.

Table 2. Oocyte status of small antral follicle at 0 and 48 hours of IVM.

Group	N	IVM-0 hour	IVM-48 hours			
		Diameter (μm)*	GV (%)	MI (%)	MII (%)	DG (%)
0L-COC	66	99.7 ± 0.8^a	93.3 ± 6.7^a	2.9 ± 2.9^a	2.9 ± 2.9^a	1.0 ± 0.9
1L-COC	112	102.6 ± 0.7^b	70.2 ± 7.9^a	7.0 ± 7.0^a	22.8 ± 7.4^b	0.0 ± 0.0
ML-COC	193	104.6 ± 1.0^b	50.8 ± 4.9^b	2.1 ± 1.4^a	41.6 ± 6.1^c	5.5 ± 4.8

Values are the average ± SEM from 6 independent experiments. Different letters within each column indicate statistically significant differences ($P<0.05$). N, total number of oocytes; GV, germinal vesicle; MI, metaphase I; MII, metaphase II; DG, degenerated.

Table 3. Spindle morphology and chromosome alignment in oocytes from small antral follicle COCs after 48 hours of IVM.

| | N | Spindle morphology and chromosome alignment | | | |
		A	B	C	D
0L-COC	3	Not Analyzed			
1L-COC	17	71.4%	14.3%	7.1%	7.1%
ML-COC	26	71.4%	9.5%	9.5%	9.5%

N, oocyte number. Note that only oocytes containing spindles with good 3D reconstruction were included in the analysis to confirm chromosome alignment. Spindle morphology and chromosome alignment classification system has been described previously (De Santis, et al. 2007).

Table 4. Results of baboon preantral follicle culture in FAM matrices.

Group	N	Survival	Follicle Diameter (μm)*				Oocyte Diameter (μm)[†]	
			Day 0	Day 4	Day 8	Day 10	Day 0	Day 10
FSH-0	16	73.6	283.0±11.6	304.1±13.5^a	359.2±30.8^a	398.0±35.0^a	93.5±3.8	112.0±6.7^a
FSH-10	16	84.0	293.9±13.6	394.8±38.7^b	434.0±41.3^b	508.5±52.2^b	94.6±3.0	101.8±6.7^b
FSH-100	14	78.0	287.7±9.4	439.0±23.7^b	500.8±21.5^c	505.6±17.8^b	92.9±8.2	98.4±2.9^c

Different letters within each column indicate statistically significant differences ($P<0.05$). N, starting follicle number. *Values are the average ± SEM; [†]Values are the average ± SD.
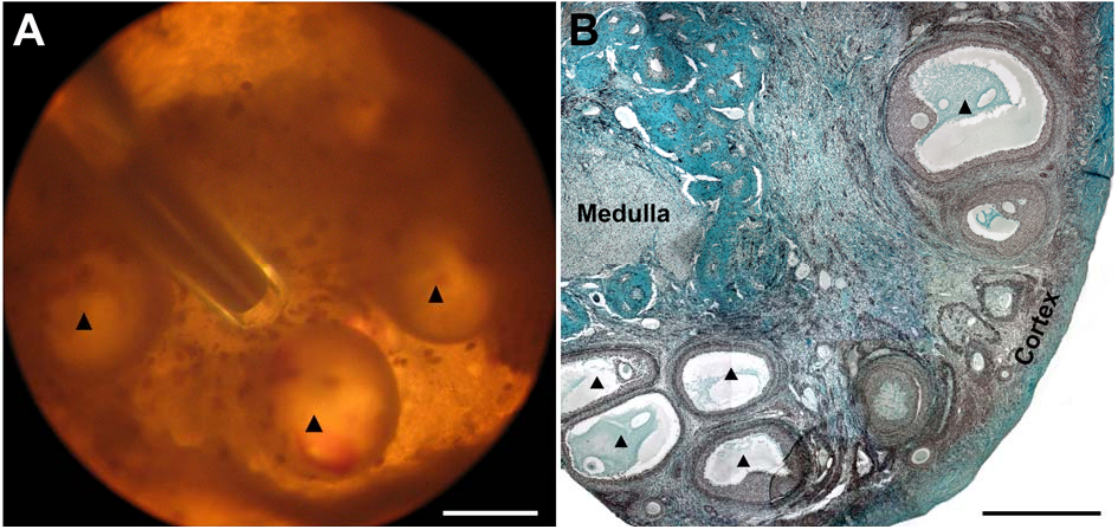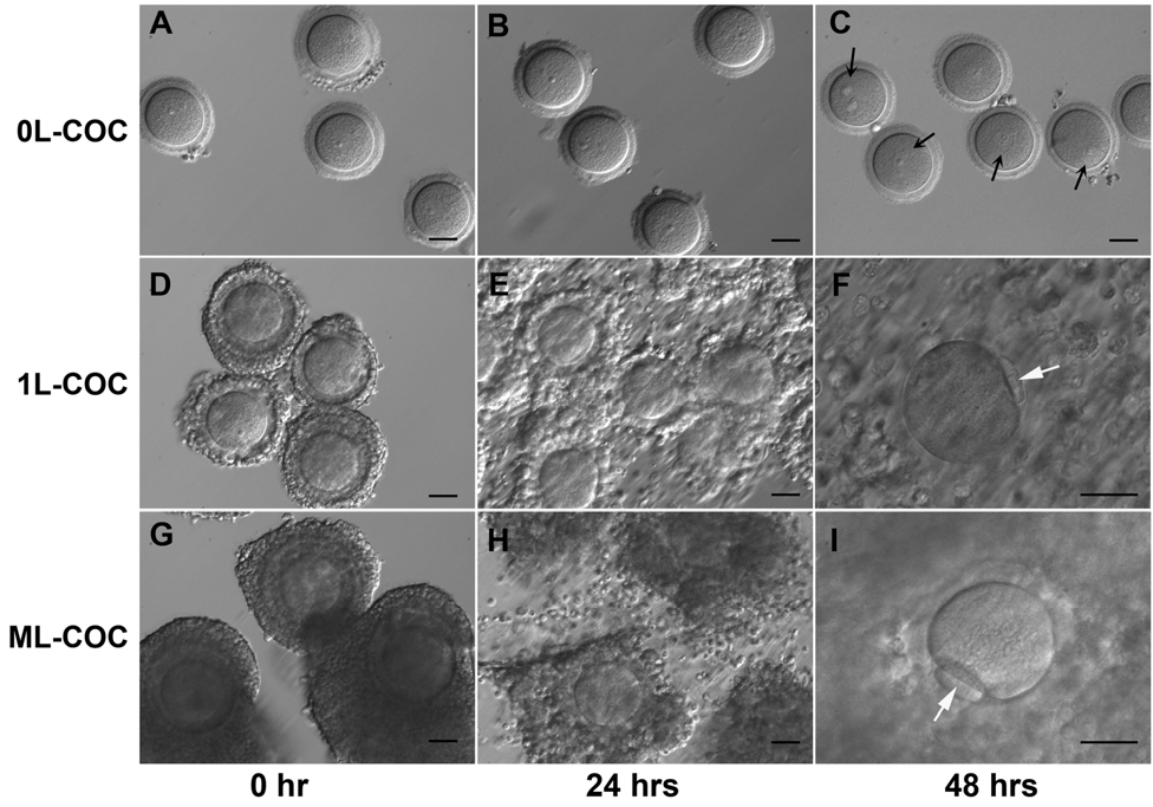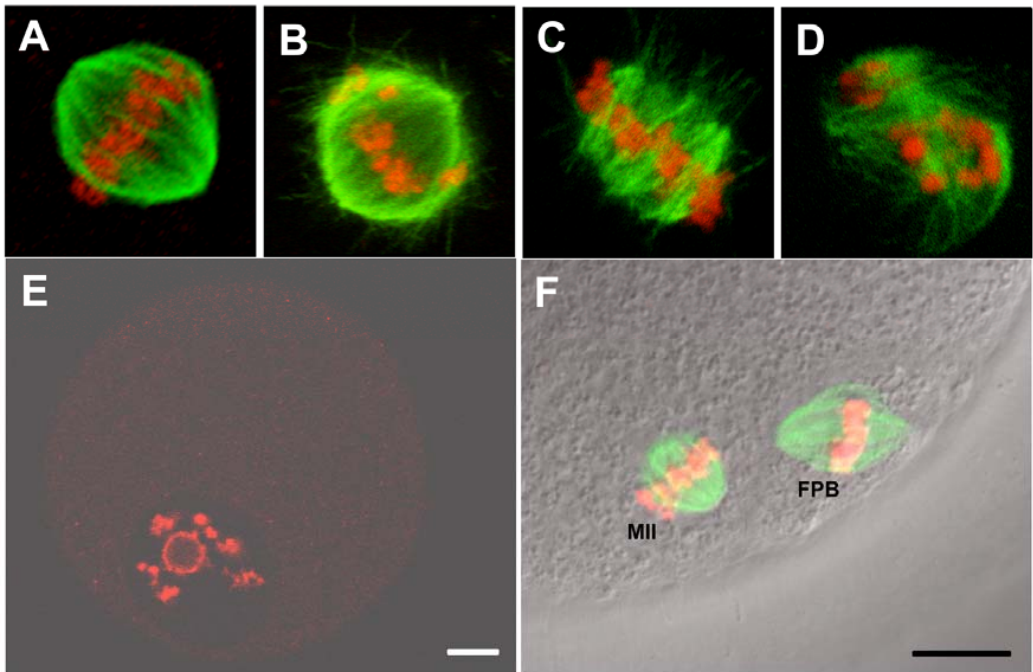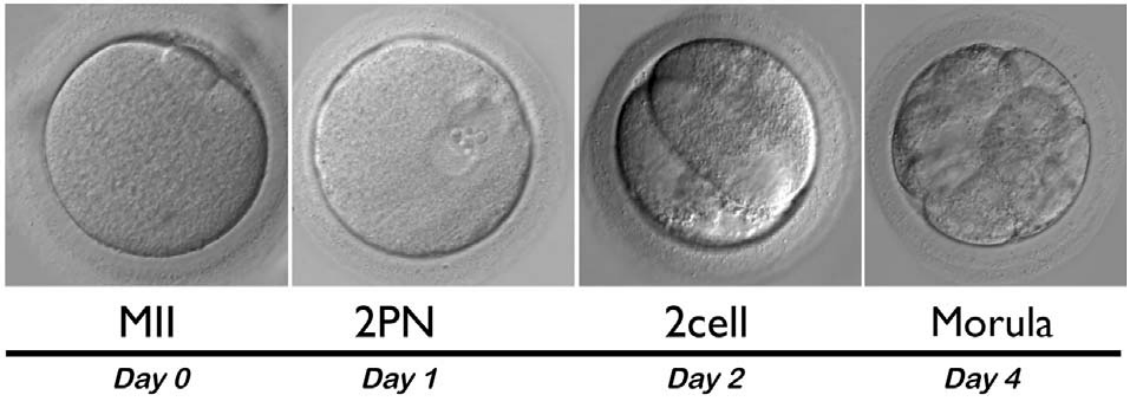
Figure 1

Figure 2

0L-COC

1L-COC

ML-COC

0 hr   24 hrs   48 hrs

Figure 3

Figure 4

| MII | 2PN | 2cell | Morula |
| Day 0 | Day 1 | Day 2 | Day 4 |

Figure 5

Figure 6

A — Follicle Growth

Legend: FSH-0, FSH-10, FSH-100

(y-axis: µm, 250–600; x-axis: Day 0, Day 4, Day 8, Day 10, Day 12, Day 14)

B — Oocyte Growth

(y-axis: µm, 85–110; x-axis: Day 0, Day 14, In Vivo)

Figure 7