

Isolated primate primordial follicles require a rigid physical environment to survive and grow *in vitro*

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BACKGROUND: *In vitro* follicle growth is a promising fertility preservation strategy in which ovarian follicles are cultured to produce mature and fertilization-competent oocytes. However, in primates, there has been limited success with *in vitro* follicle growth starting from primordial and primary follicles because adequate isolation methods and culture strategies have not been established. Understanding how to use primordial follicles for fertility preservation has significant implications because these follicles are the most abundant in the ovary, are found in all females and are fairly resistant to cryopreservation and chemotherapeutics.

METHODS: In the primate ovary, primordial follicles are concentrated near the collagen-rich ovarian cortex. To obtain these follicles, we separated the ovarian cortex prior to enzymatic digestion and enriched the primordial follicle concentration by using a novel double filtration system. To test the hypothesis that a rigid physical environment, as found *in vivo*, is optimal for survival, primordial follicles were cultured in different concentrations of alginate for up to 6 days. Follicle survival and morphology were monitored throughout the culture.

RESULTS: We found that primate ovarian tissue can be maintained for up to 24 h at 4°C without compromising tissue or follicle health. Hundreds of intact and viable primordial follicles were isolated from each ovary independent of animal age. Follicle survival and morphology were more optimal when follicles were cultured in 2% alginate compared with 0.5% alginate.

CONCLUSIONS: By mimicking the rigid ovarian environment through the use of biomaterials, we have established conditions that support primordial follicle culture. These results lay the foundations for studying the basic biology of primordial follicles in a controlled environment and for using primordial follicles for fertility preservation methods.

Key words: primordial follicle / follicle culture / primate

Introduction

In primate species, the primordial follicle pool is established shortly before birth, and it is widely accepted to be finite and non-renewable (Gougeon, 1996; Bristol-Gould *et al.*, 2006). Females are born with ~1 million primordial follicles, and this number continuously declines with age, as dormant primordial follicles are either lost to death or activated to proceed through folliculogenesis (Adhikari and Liu, 2009; Faddy *et al.*, 1992). Once the primordial follicle pool drops below one thousand, menopause ensues. The primordial follicle pool dictates a female's reproductive lifespan; so any diseases or iatrogenic treatments, such as cancer therapies, that result in primordial follicle depletion can lead to premature menopause, subfertility, infertility and associated symptoms (Jeruss and Woodruff, 2009; Schmidt *et al.*, 2010). Despite the importance of these follicles however, the

growth and regulation of primordial follicles both *in vivo* and *in vitro* are poorly understood.

Primordial follicles are a key population for fertility preservation as they are the most abundant follicle class, are present in all age females, can resist various chemotherapies, and can withstand cryopreservation and thawing (Newton *et al.*, 1996; Jin *et al.*, 2010a). Currently in the clinical setting, the fertility potential of the primordial follicle pool is harnessed through ovarian tissue cryopreservation followed by transplantation (Donnez *et al.*, 2011). This technique has resulted in 13 live births worldwide (Donnez *et al.*, 2011). However, ovarian tissue transplantation is technically demanding and only a few centres have reported success; additionally, it is not conclusively known whether the restored ovarian function is due to residual or transplanted tissue. Moreover, transplanting ovarian tissue back into cancer survivors has the risk of reintroducing cancer cells; so it is

contraindicated in cases of ovarian disease and blood-borne illnesses (Shaw et al., 1996; Meiorow, 1999).

Growing primordial follicles *in vitro* to obtain healthy, mature gametes that can be cryopreserved or fertilized is an alternative fertility preservation option to ovarian transplantation. In fact, several methods have been developed to grow follicles *in vitro*, including culturing follicles within the tissue (*in situ*) or culturing follicles isolated from the tissue. In the mouse, fertilization-competent oocytes have been obtained using a two-step culture system that begins with the culture of newborn ovaries (Eppig and O'Brien, 1996). Application of this *in situ* culture method, however, to large mammalian species has been more challenging. *In situ* culture of follicles in strips of human ovarian cortex for 4 days followed by isolation and culture of the resulting secondary follicles for an additional 6 days resulted in growth from primordial to antral stage follicles (Telfer et al., 2008). However, the timing of follicle growth in this system is highly irregular, as this process *in vivo* takes greater than 90 days, and the finding has yet to be repeated. *In situ* growth of primordial follicles to the antral stage has also been reported following xenotransplantation of human ovarian cortical strips, treated with a phosphatase and tensin homolog deleted on chromosome 10 (PTEN) inhibitor, into immune-deficient mice (Li et al., 2010). Although this is an important finding in terms of primordial follicle activation, this culture system is not translatable to the clinic. The limitations of *in situ* culture include a difficulty in assessing the starting material and the inability to monitor follicle progression during culture. Additionally, the ovarian stroma does not survive the freeze/thaw procedure well, and culturing follicles in dead or dying tissue is not optimal.

Growing isolated follicles *in vitro* has been successful in several species, resulting in live births in rodents, preimplantation embryos in rhesus macaque, metaphase II oocytes in baboon and Stage IV oocytes in human (Cortvriendt et al., 1996; Xu et al., 2006, 2009a,b, 2010, 2011a). These studies, however, have only been performed using secondary and larger stage follicles. To date, mature gametes have not been obtained from the culture of isolated primordial follicles, likely to be due to the challenge of manipulating these follicles. Primordial follicles are small, the physical connections between the oocyte and surrounding squamous granulosa cells are not well established and the conditions that support their activation and growth are poorly understood. Several studies have shown that primordial and primary follicles rapidly degenerate when cultured in a variety of ways (Hovatta et al., 1999; Abir et al., 2001; O'Brien et al., 2003). For example, primordial follicles isolated from human ovarian tissue using collagenase digestion and followed by culture in collagen gels resulted in degeneration of the follicles within 24 h (Abir et al., 1999, 2001).

In this study, we used the characteristic architecture of the rhesus macaque ovary, which is similar to the human ovary in terms of morphology and physiology, to devise robust isolation and culture methods for primate primordial follicles. Primordial follicles are confined to the ovarian cortex, which is a rigid physical environment compared with the medulla where growing follicles are found. We therefore developed a novel double filtration method to isolate large numbers of primordial follicles with minimal manipulation specifically from thin layers of cortical ovarian tissue. These isolated follicles were then encapsulated in alginate hydrogels of varying concentrations and grown *in vitro*. We found that high alginate concentrations, which

mimic the rigid physical environment of the ovarian cortex, are essential to support the survival and growth of primordial follicles *in vitro*. These findings provide both the research and clinical fields with novel tools for the study and use of these follicles.

Materials and Methods

Ovarian tissue acquisition

The general care and housing of the monkeys were provided by the Panther Tracks Learning Center (Immokalee, FL, USA). Ovarian tissues obtained following necropsy of three female rhesus macaques (ages 6.5, 9.5 and 12.0 years old) were used. Immediately post-surgery, ovaries were cut into quarters. One quarter was fixed in neutral buffered formalin (NBF; Azer Scientific, Morgantown, PA, USA), and the remaining tissue was placed in a tube containing SAGE OTC Holding Media (Cooper Surgical, Trumbull, CT, USA). Tubes were placed in a styrofoam shipping container containing ice packs to maintain the temperature at 4°C. The ovarian tissue was shipped from Immokalee, FL to Chicago, IL. The time between surgery and tissue processing for experimental purposes was a total of ~18–24 h. Upon arrival in Chicago, a piece of tissue was fixed in NBF. The remaining tissue was allowed to equilibrate to room temperature and then processed.

Follicle isolation

Ovary quarters were sliced into 500 µm sections using a Thomas Stadie-Riggs Tissue Slicer (Thomas Scientific, Swedesboro, NJ, USA) to separate the cortex from the medulla. Cortical strips were then further cut into ~1 × 1 mm squares using scalpels. The tissue was enzymatically digested in α minimum essential medium (MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 1% human serum albumin (HSA, Irvine Scientific, Santa Ana, CA, USA), 0.08 mg/ml Liberase Blendzyme 3 (Roche Diagnostics, Indianapolis, IN, USA) and 0.2 mg/ml DNase (Worthington Biochemical, Lakewood, NJ, USA) for 30 min on a shaker inside a 37°C, 5% CO₂ incubator. To remove the enzymes and stop the digestion, the tissue pieces were rinsed twice in SAGE OTC Holding Media. The tissue was then mechanically agitated using manual pipetting to release the follicles from the stroma into the media. The sample was passed through a double filtration apparatus containing a 70 µm cell strainer (BD, Franklin Lakes, NJ, USA) and a glass microanalysis filter fitted with 20 µm pore nylon net filter (Millipore, Billerica, MA, USA). [Supplementary data, Fig. S1](#) illustrates the workflow and the filtration set-up. The nylon net filter was rinsed with 1 ml SAGE OTC Holding Media directly into a petri dish to recover the follicles. Follicles were either fixed for post-isolation analysis or processed for encapsulation and culture.

Follicle encapsulation and culture

Morphologically intact follicles of the correct stage were selected for encapsulation from the pool of isolated follicles. Alginate was prepared, and follicles were encapsulated in either 0.5% alginate or 2% alginate in phosphate-buffered saline (PBS), as previously described (Xu et al., 2009a). Briefly, groups of follicles were transferred to alginate, and beads were formed by pipetting drops of alginate into the cross-linking solution (50 mM CaCl₂ and 140 mM NaCl). After 2 min, the alginate beads were removed from the cross-linking solution and held in αMEM (Invitrogen) containing 1% HSA (Irvine Scientific) until all encapsulations were completed. The beads were then transferred to individual wells of an Ultra Low Adhesion 96-well plate (Corning, Lowell, MA, USA) containing 100 µl of αMEM culture media supplemented with 0.3% Serum Protein Substitute (Cooper Surgical), 1 mg/ml bovine fetuin (Sigma-Aldrich, St. Louis, MO, USA), 5 ng/ml insulin, 5 ng/ml transferrin, 5 ng/ml

sodium selenite (Sigma-Aldrich) and 10 mIU recombinant human FSH (NV Organon, Oss, The Netherlands). A subset of isolated follicles was transferred to separate wells of the culture plate in groups of ~50 without encapsulation. Throughout isolation, encapsulation and plating, follicles were maintained at 37°C and at pH 7.2–7.4. Follicles were cultured at 37°C in 5% CO₂ for up to 6 days. Half of the culture media (50 µl) was exchanged with fresh media every other day. Beads were imaged using a Leica DM IRB inverted microscope using 4× or 10× or 20× objectives (Leica Microsystems, Heidelberg, Germany). At the end of culture, follicles were fixed in the alginate beads with Modified Davidson's Fixative (Electron Microscopy Sciences, Hatfield, PA, USA) for 2 h at room temperature. For experiments involving follicles cultured loose on the bottom of low-adherence plates, the material was encapsulated in alginate immediately prior to fixation to facilitate processing.

Tissue sectioning and staining

Fixed tissue (ovarian tissue and beads) was dehydrated with increasing concentrations of ethanol (50–100%) and embedded in paraffin using an automated tissue processor (Leica, Manheim, Germany). Sections of 5 µm were cut with a microtome and mounted on Superfrost-Plus slides (Vector Laboratories Inc., Burlingame, CA, USA). Haematoxylin and eosin staining was performed using a Leica Autostainer XL (Leica Microsystems, Wetzlar, Germany). All tissue processing was performed by the Northwestern University Center for Reproductive Sciences Histology Core. Masson's trichrome staining was performed based on manufacturer's instructions (Polysciences, Inc., Warrington, PA, USA). Briefly, tissue sections were deparaffinized and rehydrated, followed by mordant in Bouin's solution for 15 min at 60°C. Slides were washed for 5 min in running tap water and then placed in Weigart's haematoxylin for 10 min. Slides were again washed for 5 min in running water, followed by staining in Biebrich Scarlet-Acid Fuchsin solution for 5 min. After brief rinsing in diH₂O, slides were transferred to phosphotungstic/phosphomolybdic acid for 10 min before staining in aniline blue for 5 min. Slides were rinsed in diH₂O and then fixed in 1% acetic acid for 1 min. Slides were dehydrated, cleared and dried before mounting.

Follicular viability assessment

Follicle viability was assessed by double fluorescent labelling with calcein AM and ethidium homodimer stains (Live/Dead Viability Assay, Molecular Probes, Inc., Eugene, OR, USA). Briefly, isolated follicles were incubated in 1 ml of α MEM (Invitrogen) containing 1% HSA (Irvine Scientific), 1 µm calcein AM and 2 µm ethidium homodimer for 40 min in an incubator at 37°C and 5% CO₂. Following staining, follicles were washed through α MEM containing 1% HSA and placed in a glass bottom dish (Bioprotech, Butler, PA, USA) in a drop of LI5 media (Invitrogen) containing 3 mg/ml polyvinylpyrrolidone (Sigma-Aldrich). Images were taken using a Leica SP5 inverted laser-scanning confocal microscope with a 40× or 63× oil immersion objective (Leica Microsystems). Images were processed using LAS AF software (Leica Microsystems).

Detection and quantification of apoptotic cells in ovarian tissues

Detection of apoptotic cells was done using the DeadEnd™ Fluorometric terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) System (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The positive control was treated with 10 units/ml DNase I for 10 min prior to labelling. Images were taken using a Leica SP5 inverted laser-scanning confocal microscope with a 10× objective (Leica Microsystems). To quantify the number of apoptotic cells, TUNEL positive cells were counted in three images each from three

separate, non-adjacent sections of each treatment group. Two independent, blinded counters were used, and totals were averaged for each treatment. The counts were then averaged over the total area counted in mm².

Whole mount follicle immunocytochemistry

Isolated follicles were fixed in 3.8% paraformaldehyde (Electron Microscopy Sciences) containing 0.1% Triton X-100 (Sigma-Aldrich) for 1 h at room temperature. After fixation, follicles were processed for fluorescent immunocytochemistry as described previously (Duncan *et al.*, 2009). Briefly, follicles were blocked in PBS containing 0.3% bovine serum albumin and 0.01% Tween-20 (blocking solution) and then incubated in a 1:2000 dilution of anti-MSY2 (gift from R. Schultz, University of Pennsylvania) for 16–18 h at 4°C. The primary antibody was detected using Alexa Fluor 488-conjugated Donkey anti-rabbit secondary antibody (Invitrogen). Follicles were simultaneously stained for F-actin with a 1:50 dilution of Rhodamine-phalloidin (Invitrogen). Follicles were washed in blocking solution and mounted in Vectashield with 4(6-diamidino-2-phenylindole) (DAPI) (Vector Laboratories). Images were captured with the confocal microscope as described earlier.

Results

Acquisition of ovarian tissue

Ovarian tissue was obtained from rhesus macaques following necropsy from a primate facility more than a thousand miles away. To determine whether primate ovarian tissue and the follicles contained within it could be maintained during transport for an extended time at 4°C, tissue was fixed before (fresh) and after transportation (transported, up to 24 h), and histological examination was performed to compare the ovarian stromal cells, follicles and oocytes. The morphology of the transported tissue was indistinguishable from that of the fresh tissue (Fig. 1A and B). Specifically, ovarian stromal cells in transported tissue were the same size and shape as the equivalent cell type in the fresh tissue. By haematoxylin and eosin staining, the cytoplasm and the nuclei of the stromal cells in both samples stained pink and blue, respectively, suggesting that they were healthy. There was not a significant amount of pyknotic nuclei that could be identified either in the stromal cells or in the follicle cells. Additionally, follicles of every size could be identified in both fresh and transported tissue, and each follicle type contained the appropriate number of granulosa cell layers (Fig. 1A and B, insets). The oocytes within the follicles also appeared morphologically normal with a zona pellucida and an intact germinal vesicle. The connections between the oocyte and granulosa cells also appeared intact by histology, as there were no visible gaps between these cell types.

To quantify the level of apoptosis that may have occurred between the time of ovary removal from the animal and the time of tissue processing, apoptotic cells were identified by TUNEL staining (Fig. 1C and D). Levels of TUNEL positive cells per mm² of ovarian tissue were comparable in fresh and transported tissue: 37.0 versus 40.7 TUNEL positive cells per mm² ($P = 0.8850$, Fig. 1E). In both fresh and transported tissue, TUNEL positive cells were predominantly found in the mural granulosa cells of antral follicles. The presence of apoptotic cells in larger follicles is consistent with what has been previously reported in multiple species, including primate (Yuan and Giudice, 1997). No TUNEL positive cells were detected in primordial, primary or secondary follicles or oocytes from either the fresh or

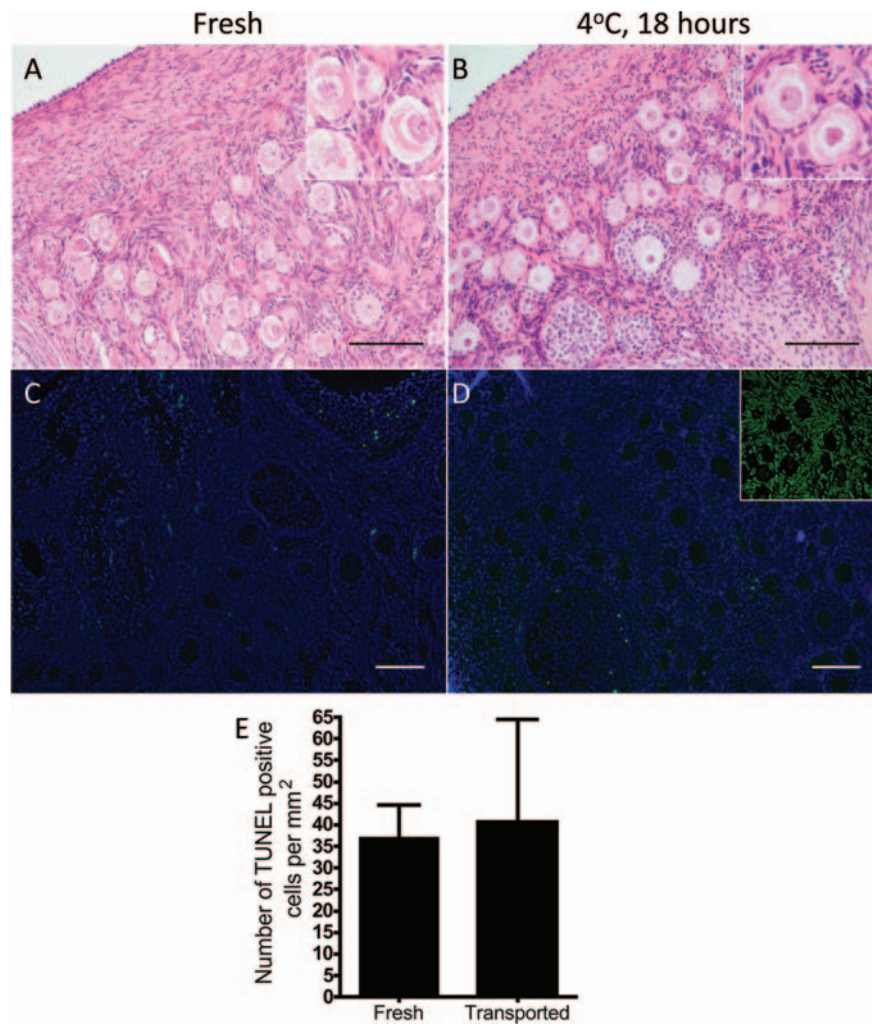


Figure 1 Morphology of ovarian tissue kept at 4°C for extended time is similar to that of fresh tissue. Representative histological sections of ovarian tissue stained with hematoxylin and eosin. Integrity of primordial follicles is similar (insets **A** and **B**). Apoptotic cells were detected in ovarian tissue by TUNEL labelling. TUNEL positive cells, green; DAPI, blue. Inset, Ovarian tissue treated with DNase to serve as a positive control for TUNEL labelling (**C** and **D**). Quantification of TUNEL positive cells per mm² (**E**). There were no statistically significant differences in the number of TUNEL positive cells between the two groups (*P*-value 0.8850). Scale bars: 100 μm.

transported tissue. Taken together, these data suggest that non-human primate ovarian tissue can be transported for extended periods without compromising the quality of the tissue.

To further verify the molecular and cellular integrity of the follicles in transported ovarian tissue, follicles were isolated from the tissue, fixed and analysed for several markers by immunocytochemistry and confocal microscopy. Filamentous actin staining was used to identify the status of the actin cytoskeleton. Because actin labelling demarcates the cell boundaries of the granulosa cells and the oocyte cortex, it was also used as an indirect measure of cellular structure. To identify the presence of an oocyte within the follicle, immunocytochemistry was also performed with an antibody against MSY2. MSY2, a cytoplasmic RNA-binding protein, is specifically expressed in the oocyte and comprises ~2% of the total protein in the oocyte (Yu et al., 2001). Finally, DNA staining was performed to assess the chromatin configuration of both the granulosa cells and the oocytes.

Primordial, primary and secondary follicles isolated from transported tissue appeared normal with respect to the markers analysed (Fig. 2). Primordial follicles had a single layer of squamous granulosa cells (Fig. 2A–D). Primary follicles had a single layer of cuboidal granulosa cells (Fig. 2E–H). Secondary follicles had multiple layers of granulosa cells, the exact number of which were dependent on follicle size (Fig. 2I–L). All of the follicle classes had an intact actin cytoskeleton (Fig. 2A, E and I) and an MSY2-positive oocyte (Fig. 2B, F and J). There was no evidence of pyknotic nuclei in either the granulosa cells or in the oocytes (Fig. 2C, G and K, insets). The chromatin of oocytes within primordial, primary and secondary follicles had a non-surrounded nucleus configuration (Fig. 2C, G and K, insets), representing a transcriptionally active state appropriate for these stages of oogenesis. These data suggest that follicles isolated from transported rhesus ovarian tissue are structurally intact and morphologically normal, indicating that the transport is not detrimental to individual ovarian follicles.

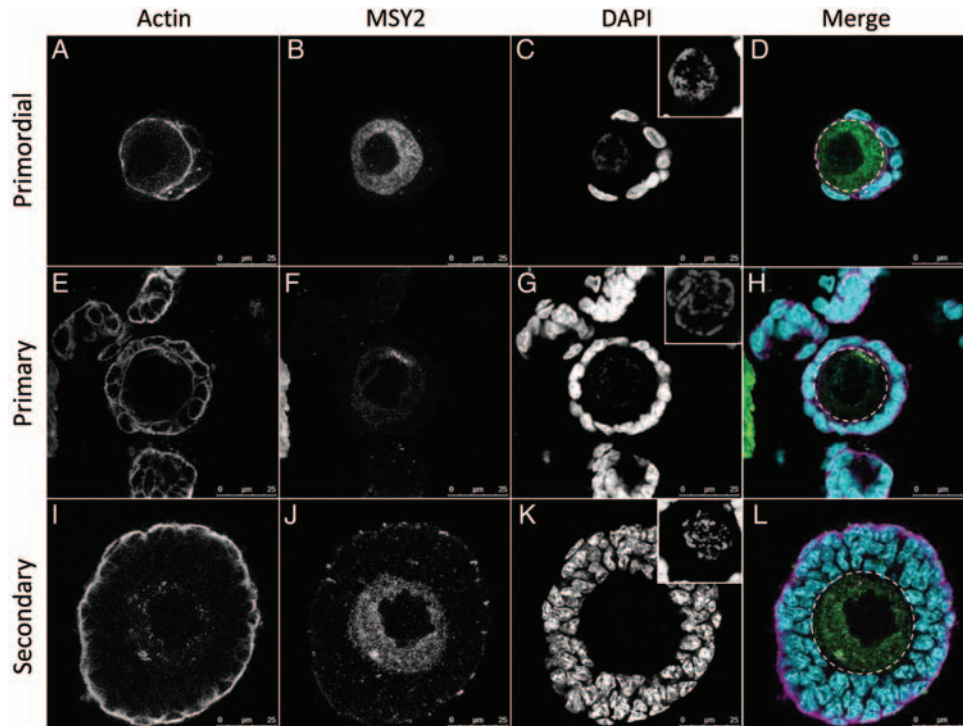


Figure 2 Follicles isolated from ovarian tissue held at 4°C for extended time express key structural and protein markers. Primordial (A–D), primary (E–H) and secondary (I–L) follicles were isolated from ovarian tissue held at 4°C for up to 18 h then fixed in 3.8% PFA + 0.1% Triton-X 100. Follicles were stained for Actin (A, E and I), MSY2 (B, F and J) and DAPI (C, G and K, insets highlight the oocyte nucleus). Oocytes are defined by dashed circles in merged images (D, H and L). Representative images are shown. Scale bars: 25 μm.

Isolation of primordial follicles and viability of isolated follicles

Primate ovaries are distinctly stratified with respect to organization of different stage follicles (Fig. 3A, Supplementary data, Fig. S1A). Primordial follicles are located in the cortical region, whereas antral and pre-ovulatory follicles are found most interior in the medullary region. Primary and secondary follicles are typically distributed in the tissue between these two distinct regions. We took advantage of this characteristic follicle localization to develop an isolation method that enriches for primordial follicles (Supplementary data, Fig. S1A–F). Using this method, an average of more than 500 follicles, ranging in size from 25.0 to 69.6 μm in diameter, were obtained from each pair of ovaries (Table 1, Fig. 3C). Based on this diameter range, this pool contained primordial, transitional and primary follicles. To assess the viability of isolated follicles following the isolation procedure, we performed a live-dead assay. All cells within intact follicles stained positive for calcein AM, indicating they were alive (Fig. 3D). The only cells that were positive for ethidium homodimer (i.e. dead cells) were cells not associated with a follicle, and were likely stromal cells or dissociated granulosa cells (data not shown).

Culture of isolated primordial follicles

Previous work has demonstrated that primordial follicles rapidly degenerate in culture, and one reason for this is likely because a culture environment that mimics the physical environment of the ovary has not been

established (O'Brien *et al.*, 2003; Hovatta *et al.*, 1999; Abir *et al.*, 2001). To characterize the physical environment in which primordial follicles reside, we stained histological cross sections of the rhesus ovary with a Masson's trichrome stain (Fig. 3A and B). Because this particular stain detects collagen fibres, it is used to distinguish cells from the surrounding connective tissue. We observed a distinct collagen gradient within the ovary, with the most concentrated regions existing at the cortex where primordial follicles are found (Fig. 3A and B). Thus, the collagen-rich cortical region of the ovary is likely to be a more rigid environment than the medullary region, which contains less collagen.

Based on these *in vivo* findings, we hypothesized that a more rigid physical environment is critical for primordial follicle survival and ultimately growth in culture. To test this hypothesis, groups of isolated primordial follicles were cultured for 6 days in conditions of differing rigidity, and their morphology and survival were tracked. Follicles were selected for culture from the filtered pool to include only those that appeared fully intact and that were between 20 and 40 μm in diameter, to enrich for primordial follicles. Follicles were either cultured loose in a low adherence plate or were encapsulated and cultured in 0.5 or 2% alginate (Supplementary data, Fig. S2 and Fig. 4A, D and G). These isolated follicles were cultured in groups (average of 66 ± 20 follicles/bead) to further mimic the ovarian environment, as primordial follicles are typically found in clusters within the ovary *in vivo* (Fig. 3A and B).

Morphological examination of follicles by light microscopy showed that the follicles that were not encapsulated had begun to dissociate

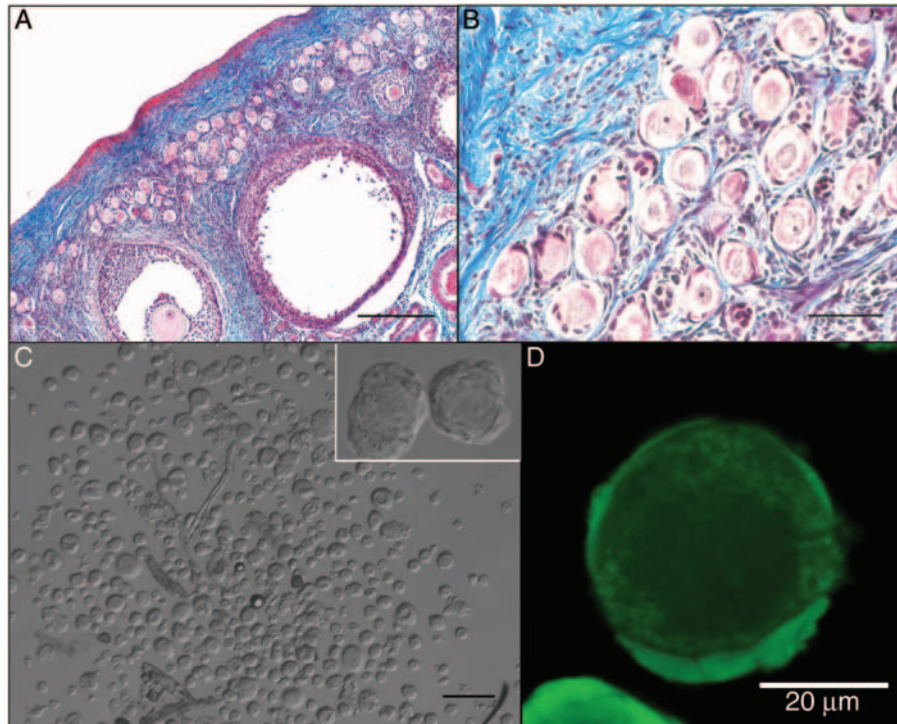


Figure 3 Separating the collagen-rich ovarian cortex containing primordial follicles from the rest of the ovary allows for efficient and robust isolation. Representative histological cross section of rhesus ovary stained with Masson's trichrome shows a gradient of collagen in the primate ovary (**A**, scale bar, 200 μm). Details of ovarian cortex stained with Masson's trichrome showing the pool of primordial follicles directly below the thick cortex (**B**, scale bar, 50 μm). Filtered pool of follicles obtained from a single animal following isolation and filtration; average diameter of follicles is 44.5 μm (**C**, scale bar, 200 μm). Viability assay showing that follicles are alive post-isolation and filtration; green fluorescence due to Calcein AM staining indicates a living cell (**D**, scale bar, 20 μm). Representative images are shown.

Table 1 Ovarian tissue characteristics.

Animal	Age	Amount of tissue used for isolation	Number of primordial follicles isolated
1	6.5 years	1.75 ovaries	460
2	9.5 years	1.5 ovaries	889
3	12.0 years	1.5 ovaries	632

into separate granulosa cells and oocytes by 3 days of culture (Fig. 4B). By 6 days of culture, the dissociated granulosa cells had clustered together to form a large mass (Fig. 4C, arrow). Cells that were not part of the mass were completely denuded oocytes, as immunocytochemistry analysis demonstrated that they stained positive for MSY2 and did not have surrounding granulosa cells (Fig. 4C, arrowheads and Supplementary data, Fig. S3). In contrast to the non-encapsulated follicles, those encapsulated in either 0.5 or 2% alginate retained their structure after 3 days of culture (Fig. 4B, E and H). In both conditions, the majority of follicles appeared to have an oocyte completely surrounded

by at least one layer of granulosa cells. However, by 6 days of culture, distinct differences were observed between follicles cultured in 0.5 and 2% alginate. The follicles encapsulated in 0.5% alginate had begun to dissociate, as many oocytes had begun to extrude from the granulosa cells (Fig. 4F, inset, representative image). In contrast, follicles encapsulated in 2% alginate still retained the proper follicle morphology with the oocyte completely surrounded by granulosa cells (Fig. 4I, inset, representative image).

To obtain a more detailed view of the follicle structures within the beads, alginate beads were fixed at different culture times and processed for histological examination by haematoxylin and eosin staining. Histological examination confirmed what was observed by light microscopy. At the start of culture, follicles were both primordial and primary, composed of only squamous granulosa cells or a single layer of cuboidal granulosa cells, respectively (Fig. 5A and B). These follicles averaged $31.3 \pm 7.5 \mu\text{m}$ in diameter (Supplementary data, Fig. S4). Only those structures that were an oocyte fully enclosed by granulosa cells were measured. By Day 6 of culture, the non-encapsulated follicles had lost their integrity; oocytes were devoid of any surrounding granulosa cells, and the somatic cells were found in large clusters (Fig. 5C and D). There were also no fully-intact, morphologically normal follicles present within 0.5% alginate beads at the end of culture (Fig. 5E and F). In contrast, follicles encapsulated in 2% alginate, however, not only maintained granulosa-

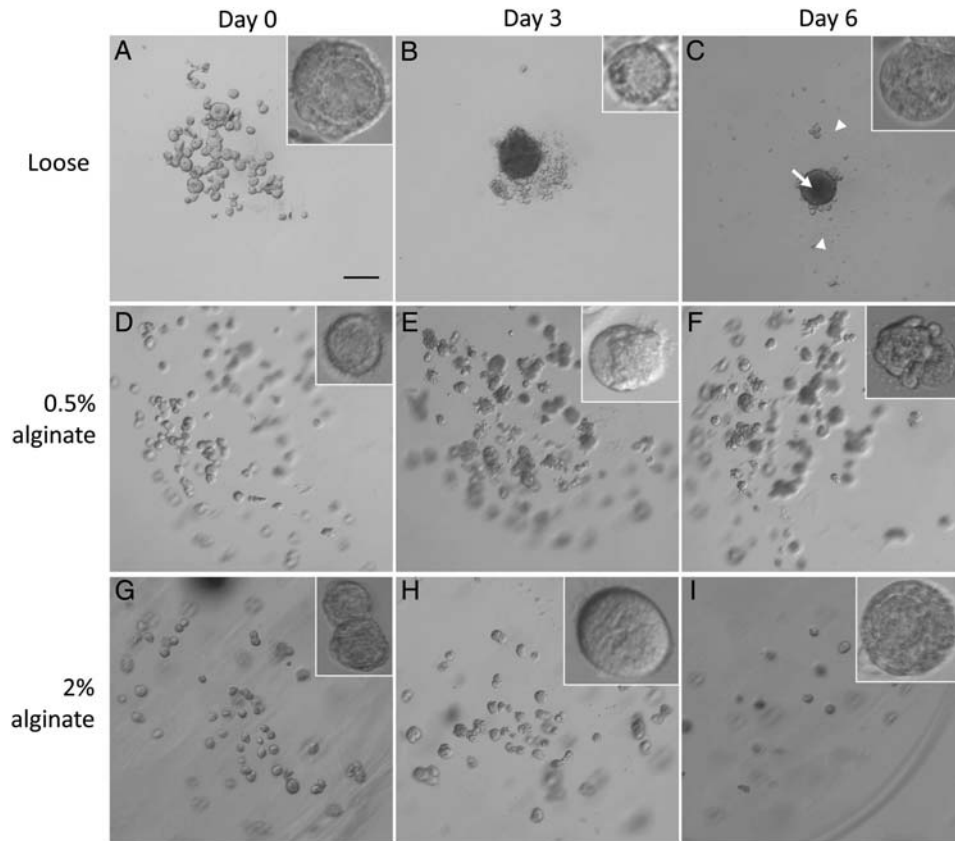


Figure 4 Primordial follicles survive and maintain follicular integrity when cultured in 2% alginate. Groups of approximately 60 isolated follicles (average follicle number per bead 66 ± 20) were not encapsulated (loose, **A–C**), or encapsulated in 0.5% alginate (**D–F**) or in 2% alginate (**G–I**) and cultured for up to 6 days. Follicles are distributed throughout the thickness of the bead and therefore, only a subset are in focus for images shown. Insets show representative follicles from each culture condition. Follicles cultured in the absence of biomaterial dissociate completely, resulting in completely denuded oocytes and a cluster of somatic cells (**C**, arrowheads and arrow, respectively). Scale bar: 200 μm .

cell–oocyte associations, but also a follicle-like structure throughout culture (Fig. 5G and H). These follicles had oocytes that were fully surrounded by granulosa cells. Some follicles had multiple layers of granulosa cells, indicating follicle growth. The average diameter of the follicles cultured in 2% alginate after 6 days of culture was $58.4 \pm 13.2 \mu\text{m}$ (Supplementary data, Fig. S4). Together, this data suggest that a higher percentage alginate, which mimics a more rigid environment, provides a suitable environment for the culture of isolated primate primordial and early primary follicles.

Discussion

In this study, we demonstrated that primate ovarian tissue can be transported at 4°C for up to 24 h without compromising follicle integrity, developed a robust technique for isolating hundreds of primordial follicles from the ovarian cortex irrespective of animal age and determined that a more rigid physical environment maintains follicular architecture that supports follicle growth during short-term culture.

The ability to perform research using ovarian tissue from large mammalian species has been dictated by proximity to specialized animal facilities. Here we show that ovarian tissue and the follicles

within it can be maintained in a healthy state when kept at 4°C in simple media for several hours, thus allowing the tissue to be transported for use nationwide. Histology of transported ovarian tissue showed healthy stromal cells, follicles and oocytes and TUNEL staining indicated levels of apoptotic cells comparable to those found in freshly fixed tissue. Additionally, follicles isolated from transported tissue had an intact actin cytoskeleton and the oocytes within them expressed MSY2 specifically in the cytoplasm. Although this study focuses on tissue from the rhesus macaque, we have found similar results with bovine and human ovarian tissue (data not shown). The finding that ovarian tissue from large mammalian species can be transported will not only increase tissue accessibility for research purposes, but it also has the potential to transform human clinical practice as well. For example, because several potential fertility preservation options, including ovarian tissue cryopreservation and *in vitro* follicle growth, are specialized techniques, there are limited centres worldwide that perform these procedures. Although previous work has shown that ovarian tissue can be transported up to 4 h (Schmidt *et al.*, 2003), this time may not be suitable for patients who live large distances from centres that offer these procedures, particularly in the USA. With the ability to ship ovarian tissue in a simple manner for

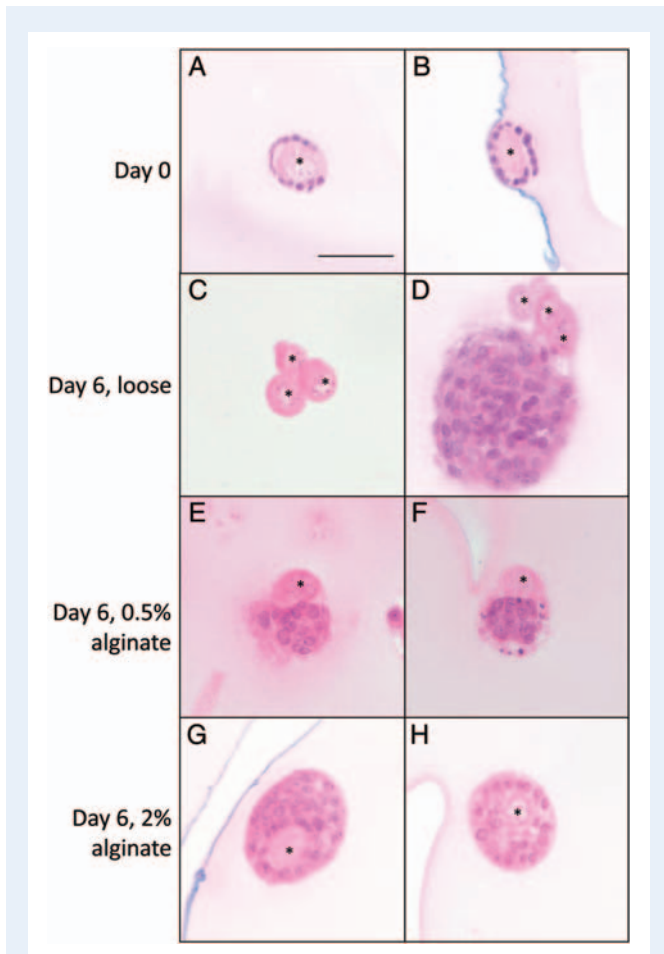


Figure 5 Histological analysis of isolated follicles cultured for 6 days in varying alginate concentrations shows differential morphology. Histological sections were stained with hematoxylin and eosin. Isolated follicles post-encapsulation at Day 0 (**A** and **B**). Cultured follicles at Day 6 without encapsulation (**C** and **D**), or encapsulated in 0.5% alginate (**E** and **F**), or 2% alginate (**G** and **H**). Representative images from each condition are shown (asterisks represent oocytes). Faint pink staining in the background is due to the alginate in which the follicles are embedded. Scale bar: 50 μm .

prolonged time, patients can have their surgery at a centre close to home, and the tissue can be transported to the appropriate location for processing.

A frontier in the field of oncofertility is to be able to use primordial follicles for fertility preservation purposes, as these follicles are the most abundant and are present in females of all ages. However, primordial follicles are difficult to study because of their small size and susceptibility to dissociation upon isolation. By taking advantage of the architecture of the ovary in the non-human primate, we developed an efficient and robust mechanism to isolate large numbers of primordial and small primary follicles, or resting follicles. Similar to human ovarian tissue, in the rhesus macaque ovary, the majority of the primordial follicles reside in the most cortical region of the ovary with the larger, growing follicles more interior in the medullary region. We used a tissue slicer to obtain thin cortical ovarian sections. Such tissue was suitable for brief and even enzymatic digestion, which is especially

critical when isolating primordial follicles which can dissociate rapidly (Abir et al., 1999, 2001). Because manual isolation from digested tissue results in follicle damage, we released the follicles from the tissue using vigorous agitation. To enrich for primordial follicles, we used a double filtration method to remove stromal cells, larger follicles and remaining tissue pieces. By brightfield microscopy, whole mount immunofluorescence analysis and histological analysis of the isolated follicles, we determined that the follicles in our pool were composed of primordial, transitioning and small primary follicles. In the primate, this group of follicles is extremely slow growing and is considered the resting pool (Gougeon, 1996). Although it would be optimal to have a pure population of primordial follicles to study, these follicles all exist within the ovary together and share many biological characteristics, such as oocyte size and growth rates.

Another significant challenge has been the ability to keep primordial follicles intact during culture. We have shown previously that the physical *in vitro* environment is critical in regulating follicle function. For example, secondary and larger follicles encapsulated and cultured in 0.25% alginate grow larger, produce more hormones and have better quality gametes compared with those grown in higher alginate concentrations of up to 3% (Xu et al., 2006; West et al., 2007). Interestingly, we found that unlike larger follicles, isolated primordial follicles require a higher alginate concentration to survive and grow in culture. In the absence of any encapsulation, isolated primordial follicles dissociated by 3 days of culture, resulting in denuded oocytes and clumps of somatic cells. In 0.5% alginate, follicles maintained their integrity for up to 3 days, but no intact follicles were observed after 6 days of culture. In contrast, in 2% alginate, not only the isolated primordial follicles maintained their integrity, but also the granulosa cell layer began to grow by 6 days of culture. These findings are also consistent with another study that reported that isolated human primordial follicles remain viable during short-term culture in 1% alginate (Amorim et al., 2009). Together, these *in vitro* studies correlate with the *in vivo* observation that primordial follicles are primarily found within the collagen-dense, rigid ovarian cortex. By mimicking the physical environment of the ovary, we can maintain follicle integrity and even promote growth during culture.

Although we observed follicle growth over the 6 days of culture in 2% alginate, we did not observe appreciable oocyte growth (data not shown). However, oocyte growth is not thought to occur in the primate until the granulosa cells begin the period of rapid growth associated with the secondary follicle stage (Gougeon, 1996). We have also observed, in histological sections from rhesus macaque ovaries, the presence of follicles that have at least a partial second layer of granulosa cells that do not appear to have a growing oocyte (unpublished observations). Thus, the follicle growth pattern we observed *in vitro* may mirror what occurs *in vivo* when the oocyte is on the cusp of its growth period. Culturing the isolated primordial follicles for longer times will demonstrate if this is indeed the case.

We were able to observe evidence of follicle growth, potentially due to spontaneous follicle activation *in vitro*, yet other factors will likely have to be added to the culture system to obtain global activation of primordial follicles. Although we enriched for primordial follicles, we cannot exclude the possibility that the growth we observed in culture is due to follicles that are already activated. Based on several gene knockout mouse models, it has been demonstrated that genes involved in the PI3K signaling pathway, including *Foxo3*

and *Pten*, are important for regulating primordial follicle activation (Castrillon *et al.*, 2003; John *et al.*, 2008; Reddy *et al.*, 2008). Exposure of ovarian tissue to bpV(pic), a small molecule inhibitor of PTEN, has been used to activate primordial follicles *in vitro* (Li *et al.*, 2010). Future studies will be performed to determine whether such compounds are sufficient to support global activation of isolated primordial follicles in the system we have established.

Once follicles are activated either *in vitro* or *in vivo*, their growth must be supported. The *in vivo* ovarian environment where a follicle resides is dynamic, with small follicles found in the rigid cortex and larger follicles located in the less rigid medulla. Long-term cultures starting with primordial follicles will, therefore, require a highly tunable physical environment. Given that alginate is a non-degradable hydrogel, it is unlikely to support the volumetric expansion that must occur during follicular development from the primordial to the antral stage. Next-generation biomaterials that are semi-degradable or completely degradable, including fibrin-alginate interpenetrating networks (FA-IPNs) and poly(ethylene glycol) hydrogels, respectively, provide follicles with a dynamic physical environment (Shikanov *et al.*, 2009; Shikanov *et al.*, 2011). For example, in the FA-IPN, alginate is blended with the biodegradable fibrin. As a follicle grows within the FA-IPN, the plasmin it produces degrades the fibrin, allowing it to ultimately grow in a less rigid environment. This system has shown great promise in the growth of secondary follicles from several species (Jin *et al.*, 2010b; Shikanov *et al.*, 2009; Xu *et al.*, 2011b). Future studies will be done to determine whether such dynamic systems support the growth of primordial follicles as well.

The methods described here will not only provide novel ways to study the basic biology of primordial follicles, but translation of this work to the human has significant implications for fertility preservation. First, human ovarian tissue could be transported to centres nationwide at 4°C without compromising follicle health, thus providing patients equal access to fertility preservation centres. Second, our methods provide the foundation for being able to obtain fertilization-competent oocytes from isolated primate follicles grown *in vitro* beginning from the primordial follicle stage instead of the secondary stage. For patients wishing to preserve their fertility, especially those for whom ovarian transplantation is contraindicated, primordial follicles could be isolated from fresh or transported tissue and grown *in vitro*, and the oocytes obtained from these methods could be cryopreserved for the patient's future use. This would eliminate the follicle loss associated with tissue cryopreservation and thawing, while maximizing the fertility potential for patients of nearly all ages.

Supplementary data

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

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Authors' roles

J.E.H. was involved in conception and design, data collection, analysis and interpretation and manuscript writing. F.E.D. was involved in conception and design, data collection, analysis and interpretation and manuscript writing. T.K.W. took part in conception and design, manuscript writing and final approval of manuscript.

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Conflict of interest

The authors have nothing to disclose.

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