J Assist Reprod Genet (2014) 31:1013–1028
DOI 10.1007/s10815-014-0252-x

FERTILITY PRESERVATION

Alginate encapsulation supports the growth and differentiation of human primordial follicles within ovarian cortical tissue

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Received: 4 March 2014 / Accepted: 7 May 2014 / Published online: 21 May 2014

Abstract
Purpose In vitro follicle growth (IVFG) is an investigational fertility preservation technique in which immature follicles are grown in culture to produce mature eggs that can ultimately be fertilized. Although progress has been made in growing primate primary and secondary follicles in vitro, it has been a relatively greater challenge to isolate and culture primordial follicles. The purpose of this study was to develop methods to grow human primordial follicles in vitro using alginate hydrogels.
Methods We obtained human ovarian tissue for research purposes through the National Physicians Cooperative from nationwide sites and used it to test two methods for culturing primordial follicles. First, primordial follicles were isolated from the ovarian cortex and encapsulated in alginate hydrogels. Second, 1 mm × 1 mm pieces of 500 μm-thick human ovarian cortex containing primordial follicles were encapsulated in alginate hydrogels, and survival and follicle development within the tissue was assessed for up to 6 weeks.
Results We found that human ovarian tissue could be kept at 4 °C for up to 24 h while still maintaining follicle viability. Primordial follicles isolated from ovarian tissue did not survive culture. However, encapsulation and culture of ovarian cortical pieces supported the survival, differentiation, and growth of primordial and primary follicles. Within several weeks of culture, many of the ovarian tissue pieces had formed a defined surface epithelium and contained growing preantral and antral follicles.
Conclusions The early stages of in vitro human follicle development require the support of the native ovarian cortex.

Keywords Human · Primordial follicle · Fertility preservation · Ovary · alginate · In vitro follicle growth · Culture

Capsule A novel compound heterozygous mutation involving exon 1 and 10 resulting in LH/hCG resistance, infertility and female phenotype in XY and XX siblings.

Electronic supplementary material The online version of this article (doi:10.1007/s10815-014-0252-x) contains supplementary material, which is available to authorized users.

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Introduction

Cytotoxic treatments, including chemotherapy and radiation therapy, are common first-line cancer treatments. While these treatments are often effective and life-preserving, they can have a simultaneous and profound negative impact on women's reproductive health [1, 2]. Chemotherapy and radiation therapy can irreversibly damage ovarian follicles, ultimately contributing to conditions such as premature ovarian failure (POF), an increase in miscarriage rate, and perpetual health risks and psychological conditions associated with premature menopause [1, 3–7]. The field of oncofertility aims to explore and expand the reproductive options for both cancer patients and those with diseases that threaten reproductive function [8]. This has been accomplished through a combined approach of communication, education, and clinical and basic research [9, 10]. The oncofertility discipline has expanded female fertility preservation options and awareness of these options. Many experimental fertility options require trained physicians and scientists and specialized equipment. The ability to transport tissue where it

could be properly handled at designated sites, where trained scientists and specialized equipment are available, would alleviate these access limitations.

Standard methods for fertility preservation, endorsed by the American Society for Reproductive Medicine, now include cryopreservation of both eggs and embryos derived through assisted reproductive technologies (ART) [11]. However, these ART procedures require hormonal stimulation, which can both delay treatment of the disease and create a risk for some patients with hormone-responsive diseases [12]. Furthermore, these methods are generally not applicable to pre-pubertal girls [12, 13]. For these populations, ovarian tissue transplantation is a promising investigational technique. Ovarian tissue removal, cryopreservation, and later transplantation back into an individual has successfully restored both fertility and endocrine function [14–16]. In fact, autologous transplantations of thawed ovarian cortical tissue have resulted in 17 reported live births [17–30]. However, the data surrounding ovarian transplantation is controversial because typically only positive outcomes are reported, thus obscuring the success rate. Moreover, this technique has the underlying risk of reseeding cancerous cells in cases of blood borne malignancies and ovarian cancers [19, 31–34].

To overcome the limitations of ovarian tissue transplantation, an alternative fertility preservation approach, in vitro follicle growth (IVFG), is actively being developed [35, 36]. In this method, ovarian tissue or follicles isolated from ovarian tissue can be grown in vitro to produce mature gametes that could ultimately be used in standard ART procedures. Several techniques support the successful growth and survival of isolated primary and secondary mammalian follicles, including encapsulated in vitro follicle growth (eIVFG) systems. In eIVFG, isolated follicles are encapsulated in biomaterials, such as alginate hydrogels, which maintain follicular architecture throughout growth. Significantly, eIVFG has been used successfully in the growth and development of preantral follicles from several mammalian species including mouse, dog, non-human primate, and human [37–41].

A challenge of eIVFG in large mammalian species, however, has been the ability to recapitulate the entire continuum of folliculogenesis beginning at the primordial follicle stage. Although primordial follicle isolation and culture has been accomplished in species including the mouse and lamb, attempts to do the same in primates have been less successful [41–43]. Primordial follicles are difficult to isolate and culture because they are small, the connections between the granulosa cells and oocytes are minimal, and the ovarian environment surrounding them is comprised of dense extracellular matrix components [44, 45]. Nevertheless, the ability to grow primordial follicles in vitro has significant implications for oncofertility because primordial follicles are the most abundant follicle class, are present in females of all ages, and can withstand cytotoxic therapies and cryopreservation better than growing follicles [31]. Moreover, primordial follicles reside in the ovarian cortex which can be surgically removed following a minimally invasive laproscopic procedure that does not require a lengthy postponement of treatment or exposure to supraphysiologic hormone doses [46].

To address this gap in fertility preservation research, we recently developed a robust method to isolate primordial follicles from non-human primate ovaries and found that these follicles required encapsulation in high hydrogel concentrations to survive and grow during short-term culture [40]. This rigid biomaterial mimicked the dense cortex characteristic of the in vivo ovarian environment where primordial follicles are enriched. The purpose of this study was to extend these methods from non-human primate ovarian tissue to human. To perform this research, we first demonstrated that human ovarian tissue could be transported from nationwide sites at 4 °C for up to 24 h while still maintaining follicle health. Using this ovarian tissue we found that the primordial follicle isolation methods developed in the non-human primate could not be translated directly to human because of broad tissue heterogeneity likely due to variables such as age, disease diagnosis, and previous treatment history. Therefore, we cultured primordial follicles in situ within encapsulated cortical ovarian tissue pieces. We found that this method of eIVFG supported the survival, growth, and development of human primordial, transitional, and primary follicles in vitro for weeks, with evidence of antral follicle formation by 6 weeks. These results describe a hydrogel-based system to differentiate human primordial follicles in vitro within the context of the ovarian cortex, thereby providing the groundwork for future clinical fertility preservation options.

Materials and methods

Human ovarian tissue acquisition and transport

Human ovarian tissue was obtained from participants following informed consent under Institutional Review Board (IRB)-approved protocols. These participants were undergoing ovarian tissue removal and cryopreservation for fertility preservation at National Physicians Cooperative (NPC) sites that are part of the Oncofertility Consortium (oncofertility.northwestern.edu). As part of this investigational protocol, 80 % of participant ovarian tissue was cryopreserved for their future clinical use and up to 20 % of the tissue was designated for research to develop methods to grow follicles in vitro. We also obtained research tissue from another IRB-approved protocol under which women who were having their ovaries removed for other medical conditions could elect to have 100 % of their tissue donated to research. All participants were enrolled between 2010 and 2013 and ranged in age from 2 to 41 years old (median age: 20.5 years). All participants had a cancer diagnosis and 11 out of 32 had a previous history of therapy (radiation,

chemotherapy, immunosuppression) prior to ovarian tissue removal (Tables 1 and 2). For local cases, the research portion of the ovarian tissue was brought to the laboratory within 2–4 h of surgery (Table 2). For all other cases, the research tissue was transported to the laboratory in SAGE OTC Holding Media (Copper Surgical, Trumball, CT) at 4 °C for 14–24 h (Table 1). For some cases, a portion of the tissue was fixed in 10 % neutral buffered formalin both pre- and post-transport and processed for histological analysis as described below. The remaining tissue was allowed to equilibrate to room temperature upon arrival and then processed for encapsulation and culture as described below.

Follicle isolation, encapsulation, and culture

Ovarian tissue was processed into 500 μm–thick sections of cortex and medulla using a Thomas Stadie-Riggs Tissue Slicer. Primordial follicles were isolated as described previously for rhesus monkey [40]. Briefly, the 500 μm-thick sections of ovarian cortex were further processed into 1 mm × 1 mm pieces using a McIlwain Tissue Chopper or manually with scalpels. The tissue was then digested enzymatically in Minimum Essential Medium (αMEM-Glutamax, Invitrogen) supplemented with 1 % human serum albumin (HSA, Irvine Scientific), 0.08 mg/ml Liberase Blendzyme 3 (Roche Diagnostics), and 0.2 mg/ml DNase (Worthington Biochemical) for 30 min on a shaker at 37 °C and 5 % CO_2 in air. The tissue pieces were rinsed in SAGE OTC Holding Media to stop the enzymatic digestion and then mechanically agitated to release the follicles from the stroma into the media. The samples were passed through a double filtration apparatus fitted with a 70 μm cell strainer (BD Biosciences) and a 20 μm pore nylon net filter (Millipore). The filter was rinsed with 1 ml SAGE OTC Holding Media directly into a petri dish to recover the follicles.

Isolated primordial follicles were encapsulated in either 0.5 % or 2 % alginate (Sigma-Aldrich) in phosphate-buffered saline (PBS) as previously described [36]. Briefly, groups of follicles were transferred to alginate, and beads were formed by pipetting drops of alginate into the cross-linking solution (50 mM $CaCl_2$ and 140 mM NaCl). For short-term culture, beads were maintained in α-MEM-Glutamax supplemented with 1 % HSA. For long-term culture, the beads were transferred to individual wells of an Ultra Low Adhesion 96-well plate (Corning) and grown in Waymouth's media (Invitrogen) supplemented with 3 mg/ml HSA, 0.5 mg/ml bovine fetuin (Sigma-Aldrich), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (Sigma-Aldrich), 64 μg/ml ascorbic acid (2-P, Sigma) and 10 mIU/ml FSH (gift from Organon, Roseland, NJ) for up to 3 days.

For secondary follicle isolation, 500 μm-thick sections of the medulla were processed into 1 mm × 1 mm pieces and digested enzymatically as described previously [36]. Only immature follicles (Class 1 and 2) [47] that contained a clear, visible, centrally-located oocyte, healthy granulosa cells, and no signs of antrum formation were encapsulated and cultured. Selected follicles were embedded into diluted Growth Factor Reduced Matrigel (BD Cat 354230) matrix as previously described [36]. Briefly, Matrigel (BD Cat 354234) was thawed on ice, and diluted 1:3 with cold αMEM. The Millicell insert (PICM01250, Millipore) was filled with 100 μl diluted Matrigel and permitted to pre-gel for 30 min in a 37 °C, 5 % CO_2 incubator. After adding another 100 μl diluted Matrigel on the top of the first layer of Matrigel, single follicles were transferred into the middle of the second layer of Matrigel. The insert was then transferred to one well of a 24-well plate containing 400 μl of αMEM supplemented with 3 mg/ml HSA, 0.5 mg/ml bovine fetuin (Sigma-Aldrich), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (Sigma-Aldrich), and 10 mIU/ml FSH (gift from Organon, Roseland, NJ) [48]. Follicles were cultured at 37 °C with 5 % CO_2 for up to 21 days. Every 2 days, half of the culture media was exchanged. Fresh culture media was prepared weekly.

Fixation and staining of follicles

Follicles were fixed in 4 % paraformaldehyde (PFA) for 2 h at room temperature and then overnight at 4ºC in preparation for immunohistochemistry. Follicles for confocal microscopy studies were fixed in 4 % PFA at 37ºC for 1 h, followed by 1 h in wash buffer [49]. They were then stained overnight at 4ºC on a shaker with Rhodamine-Phalloidin (1:50 Molecular Probes, Invitrogen) that labels F-actin. The MSY2 antisera were a gift from R. Schultz and S. Medvedev (University of Pennsylvania). The primary antibodies were followed by incubation with 1:800 Goat anti-Rabbit Alexa 488 (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 Neoflaur 40x oil objective. Overlapping 1–3 μm sections were taken throughout each follicle imaged.

In situ cortical tissue preparation, encapsulation, and culture

For in situ culture, 500 μm-thick sections of cortical tissue were processed into 1 mm × 1 mm pieces, and pieces were identified as containing or not containing primordial follicles using transmitted light microscopy. Tissue pieces obviously containing secondary or larger follicles were excluded in an attempt to only culture tissue pieces with primordial follicles. Groups of 3–8 pieces were encapsulated in 0.25, 0.5, 1 or 2 % alginate and cultured in α-MEM-GlutaMAX supplemented with 1 % HSA, 1 mg/ml bovine fetuin (Sigma-Aldrich), 5 ng/ml insulin, 5 ng/ml transferrin, 5 ng/ml sodium selenite (Sigma-Aldrich) and 10 mIU recombinant human FSH (NV Organon) at 37 °C and 5 % CO_2

Table 1 Characteristics of participants enrolled in ovarian tissue cryopreservation research protocols through the National Physician's Cooperative of the Oncofertility Consortium whose tissue was transported to our site

Participant	Age at surgery	Diagnosis	Prior treatment
A	20	Cervical Clear Cell Carcinoma	None
B	29	Acute Lymphocytic Leukemia	Chemotherapy (cytarabine, methotrexate, daunorubicin, vincristine)
C	35	Cervical Mucinous Adenocarcinoma	None
D	22	Medullary Ovarian Cancer	None
E	17	Nasopharyngeal Rhabdomyoma	Radiation
F	15	Idiopathic Aplastic Anemia	Immunosuppressant (cyclosporine)
G	41	Uterine Adenocarcinoma	No information provided
H	2	Neuroblastoma	Chemotherapy
I	14	Acute Myeloid Leukemia	ANHL01P1 pilot group C
J	15	Medulloblastoma	None
K	35	Endometrial Cancer, history of melanoma	No information provided
L	39	Endometrial Cancer	None
M	17	Lymphoma	None
N	21	Invasive Endometrioid Adenocarcinoma	None
O	17	Ewing's Sarcoma	None
P	12	Acute Lymphoblastic Leukemia	Chemotherapy (cytarabine, etoposide, cytoxan, methotrexate)
Q	18	Anaplastic Large Cell Lymphoma	Chemotherapy (ALK inhibitor)
R	18	Hodgkins' Disease	Chemotherapy (ABVE-PC, DECA)
S	17	Hodgkins' Disease	None
T	8	Beta Thalassemia	None
U	41	Breast Cancer, triple negative	Chemotherapy (ACT)
V	36	Breast Cancer, BRCA+	None
W	18	Pineal Blastoma	None
X	13	Aplastic Anemia	None

in air. Encapsulated ovarian tissue was cultured between 1 and 6 weeks (Table 3). Alginate lysase was used to remove ovarian tissue from alginate at the end of culture. Recovered tissue was fixed in Modified Davidson's fixative (Electron Microscopy Sciences) at 4 °C for up to 24 h and processed for histological analysis as described below.

Histological analysis

All tissue processing, hematoxylin and eosin (H&E) staining, and Masson's Trichrome staining was performed by the Northwestern University Center for Reproductive Sciences Histology Core. Fixed tissue was processed using an automated

Table 2 Characteristics of participants enrolled in ovarian tissue cryopreservation research protocols through the National Physician's Cooperative of the Oncofertility Consortium whose tissue was obtained on site

Participant	Age at surgery	Diagnosis	Prior treatment
Y	40	Metastatic Breast Cancer	Hormone agonists (tamoxifen, goserelin)
Z	31	Cervical Cancer	None
AA	34	Breast Cancer	Chemotherapy (ACT), Hormone agonist (tamoxifen)
BB	38	Endometrial Cancer	None
CC	22	Wilms' tumor	Chemotherapy (doxorubicin, vincristine)
DD	16	Ewing's Sarcoma in the Breast	None
EE	37	Endometrial cancer	None
FF	27	Endometrial cancer	None

Table 3 Outcome of primordial follicle isolation protocol from human ovarian cortical tissue

Participant	Primordial follicles visible by light microscopy	Amount of research tissue	Isolated primordial follicles
A	Not Determined	3 large pieces	30
B	Yes	20 % of 1 ovary	0
C	No	20 % of 2 ovaries	5
D	Yes	20 % of 1 ovary	0
E	No	20 % of 1 ovary	8
F	Yes	20 % of 1 ovary	>100
H	Yes	20 % of 1 ovary	>200
I	Yes	20 % of biopsy	200
J	Yes	20 % of biopsy	0
L	Yes	20 % of 2 ovaries	300
CC	Yes	20 % of 1 ovary	>100
FF	Yes	20 % of 2 ovaries	200

tissue processor (Leica) and embedded in paraffin. Serial sections were cut 5 μm thick and stained with H&E using a Leica Autostainer XL (Leica Microsystems). Masson's Trichrome staining was performed based on manufacturer's instructions (Polysciences). All slides used for Masson's Trichrome staining were processed at the same time to minimize variations in staining intensity. Sections were imaged and analyzed in 100 μm increments in order to thoroughly examine each piece of inevitably variable size [50]. Only follicles with a visible oocyte nucleus within the section were counted. The section area was calculated using ImageJ software to determine the number of follicles within a defined area.

TUNEL staining

TdT-mediated dUTP Nick-End Labeling (TUNEL) staining was performed based on manufacturer's instructions (The DeadEnd™ Fluorometric TUNEL System, Promega). Paraffin-embedded sections from 9 participants with both fresh and transport tissue were analyzed in 2 experimental replicates. DAPI mounting medium (Vector) was used to counterstain the cell nuclear material. ImageJ software was used to analyze images and count fluorescein-positive and DAPI-positive cells. Positive and negative control slides from each experiment were used to determine the intensity threshold parameters for the fluorescein-positive signal. At least 4 sections from each tissue sample were screened and 3–5 representative frames spanning the section were imaged and counted. The percent of TUNEL-positive cells was calculated by dividing the number of fluorescein-positive cells by the number of DAPI-positive cells for each frame.

Statistical analysis

Plotting of results and statistical analysis was done using Microsoft Excel or GraphPad Prism. TUNEL-positive cell counts, follicle numbers, growth data and oocyte diameters were each analyzed using an unpaired *t*-test. A P value of <0.05 was considered significant.

Results

Human ovarian tissue transported at 4 °C for up to 24 h maintains preantral follicle function

There are currently 78 total NPC sites, with 36 that have active IRB-approved protocols to perform ovarian tissue removal and cryopreservation. These sites are located throughout the United States, so for many of the cases, there is a need to transport the ovarian tissue that is designated for research purposes. Although there has been a reported human live birth from ovarian tissue that was transported for approximately 20 h in a cooled container, this is a single case study [28]. Therefore, to more systematically determine the impact of ovarian tissue transport on gross tissue quality, we compared morphology and apoptotic index in tissues from the same participants both pre- and post-transport (Fig. 1). This subset of 9 participants ranged in age from 13 to 39 years old, and their ovarian tissue was maintained at 4 °C between 15.8 and 21.9 h total. We found that transported tissue from 5 participants remained histologically similar to the freshly fixed tissue (Fig. 1a, c, e). As a marker of the general health of the tissue, we quantified the number of apoptotic cells per defined tissue area. In these 5 cases, there was a similar percentage of TUNEL-positive cells between fresh and transported tissue from the same participant. However, transported tissue from 4 participants contained significantly more TUNEL-positive cells than the corresponding fresh tissue (Fig. 1a, b, d). This increase in TUNEL-positive cells was primarily limited to the edge of the post-transport tissue and was consistent with the

histology, which showed strong evidence of pyknotic nuclei (Fig. 1d). The observed inconsistency of stromal viability among this cohort of participants could be because the piece of ovarian tissue that was fixed pre-transport was distinct from the one that was fixed post-transport. Moreover, patient-specific variables such as participant age, diagnosis, or treatment history may impact overall ovarian tissue quality.

Because of the variability in stromal health observed in the fresh versus post-transport tissue, additional parameters to determine how transport affects ovarian tissue function were examined. We quantified the number of preantral follicles that could be isolated from either fresh or transported ovarian tissue using established methods [36]. Due to logistical constraints, pre- and post-transport tissues were from different participants for this analysis (Table 1 and 2). In an age-matched cohort we were able to isolate similar numbers of secondary follicles (8.0±3.5 and 11.9±11.8) from fresh and transported ovarian tissue, respectively (Fig. 2a). We examined the function of isolated secondary follicles by monitoring their ability to grow and differentiate in vitro. We found that secondary follicles, with an approximate starting diameter of 176.46±7.20, were able to grow to terminal diameters of 896.38±66.49 within 19–40 days of culture, irrespective of whether or not they were isolated from fresh or transported ovarian tissue (Fig. 2b). Moreover, equivalent percentages of follicles from fresh and transported ovarian tissue were able to differentiate and form antral cavities midway through culture (Fig. 2c and d). These IVFG parameters for human secondary follicles are consistent with our previously published work [36]. The oocytes within these follicles also expressed hallmarks of normal morphology, including cortical F-actin and cytoplasmic Germ cell-specific Y-box-binding protein (MSY2), one of the most abundant proteins in the oocyte (Fig. 2e). Taken together, these results confirm that human ovarian tissue can be transported for up to 24 h without compromising secondary follicle isolation and preantral follicle formation in vitro. Thus, we were able to use transported ovarian tissue to expand our source of research material and optimize methods to grow human follicles in vitro.

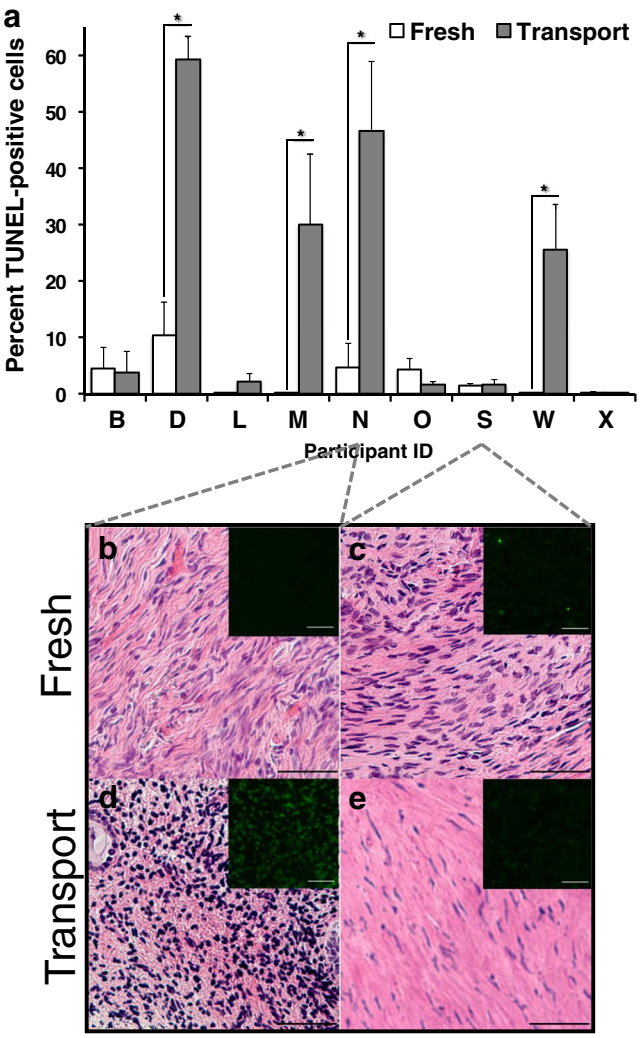

Fig. 1 The effects of ovarian transport at 4 °C for up to 24 h on tissue and follicle health. **a**) The percent of TUNEL-positive cells in fresh and transported tissue is plotted for each participant. **b–e**) H&E images of ovarian tissue, from participants N and S, that were fixed immediately (fresh) or fixed post-transport (transport). Representative TUNEL-staining for each sample is shown in the insets (TUNEL-positive cells are green, scale bar, 50 μm)

Human primordial follicles do not survive isolation and short-term culture

Human primordial follicle isolation and culture techniques have proven difficult because of the minimal established connections between the oocyte and the squamous granulosa cells at this developmental stage [51]. We recently developed robust methods to isolate and culture non-human primate primordial follicles using gentle enzymatic treatment, filtration, and encapsulation in rigid hydrogels [40]. Here we sought to translate these techniques to isolate primordial follicles from human ovarian cortical tissue. However, unlike our nonhuman primate studies in which the ovarian cortex was relatively uniform across different animals, we noticed wide heterogeneity in the human tissue likely due to participant-specific variables (Tables 1 and 2). For example, within the ovarian cortex where primordial follicles are enriched, there were noticeable differences in collagen deposition (Fig. 3a–d). We attempted to take this variability into account in our isolation procedure by adjusting e enzymatic treatment on a case-by-case basis. However, our success in routinely isolating intact primordial follicles was inconsistent despite these protocol modifications (Table 3). We attempted primordial follicle isolation using tissue from 12 participants and were able to collect a range of 0 to 300 follicles per participant (Fig. 3e and Table 3).

Of the follicles we recovered, many had lost integrity - with oocytes completely denuded from the surrounding somatic

a

Group	N	Age (yrs)	No. of Viable 2º Follicle Isolated
Fresh	8	30.8 ± 8.3	8.0 ± 3.5
Transport	10	30.9 ± 9.9	11.9 ± 11.8

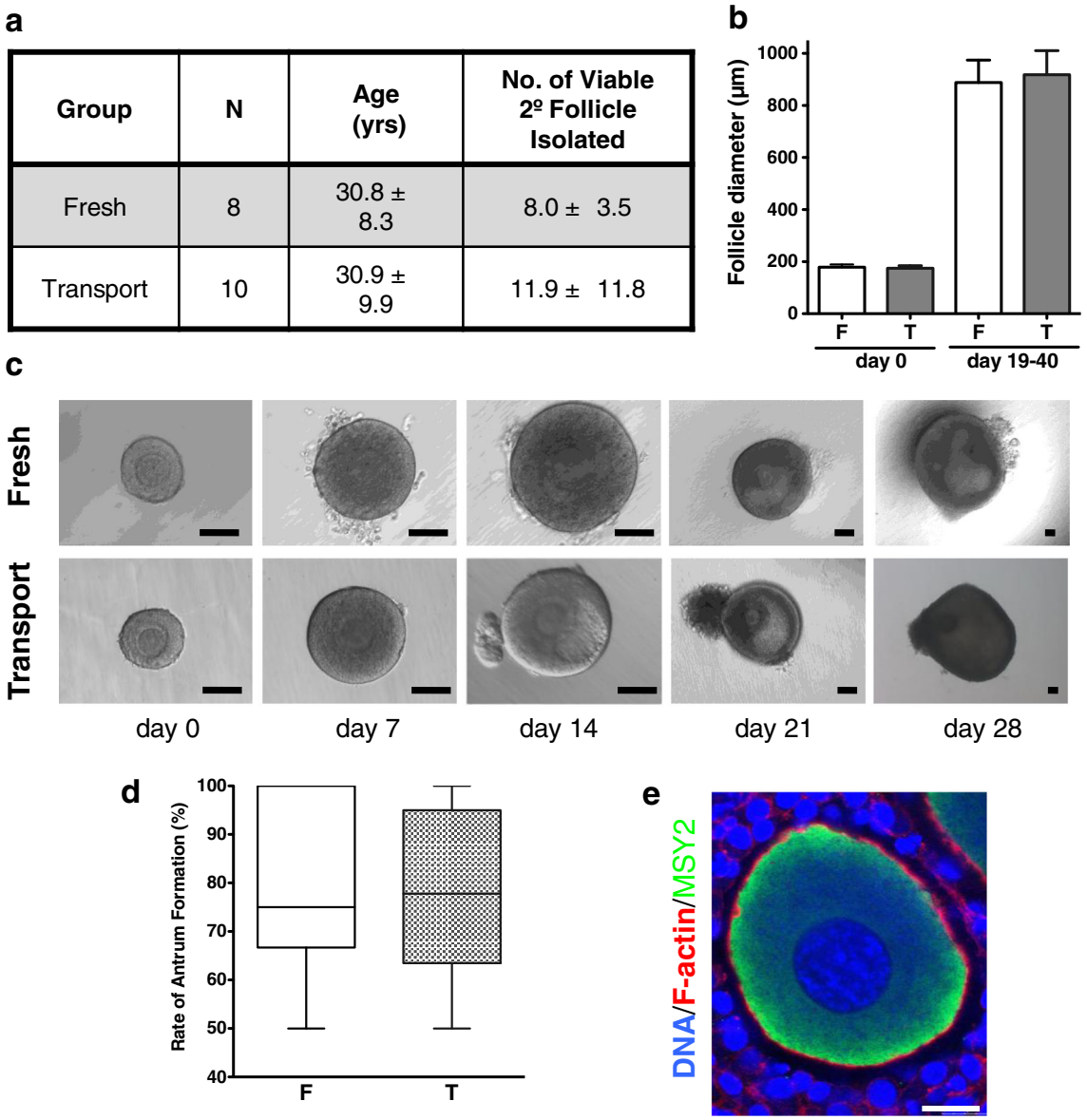

Fig. 2 Preantral follicles remained viable and functional when transported at 4 °C for up to 24 h. **a**) The number of secondary follicles are presented for the age-matched cohort of participants for fresh and transport tissue analysis. **b**) The follicle diameters upon collection (day 0) and upon termination (day 19–40) of culture are presented for fresh (F) and transported (T) tissue. **c**) Representative images of secondary follicles isolated (day 0) and tracked on day 7, 14, 21 and 28 of culture appear to grow similarly whether isolated from fresh (participant BB) or transported (participant V) tissue (scale bar, 100 µm). **b**) Follicular function is determined by the percentage of follicles that formed antrums in culture following isolation from fresh (F) or transport (T) tissue is shown. **e**) Normal follicular structure and cytoskeleton morphology is demonstrated within a cultured follicle isolated from transported tissue through staining with F-actin (red) and Germ cell-specific Y-box-binding protein (MSY2; green; scale bar, 10 µm)

cells (Fig. 3e arrows). To determine if those follicles that remained intact following isolation could survive in culture, we encapsulated them in 2 % alginate. This concentration of alginate was shown previously to support the survival and growth of isolated non-human primate primordial follicles [40]. However, after only 3 days of culture, none of the human primordial follicles survived as evidenced by complete dissociation of the oocytes and their companion granulosa cells (Fig. 3f inset). These results suggest that a single standard protocol for primordial follicle isolation from human ovarian cortex may not be feasible given the combined fragile nature of this follicle class and the heterogeneity of human samples.

Encapsulated in situ culture of ovarian tissue supports the long-term survival of human primordial follicles

An alternative approach to support primordial follicles in culture is to grow them within the context of the native tissue.

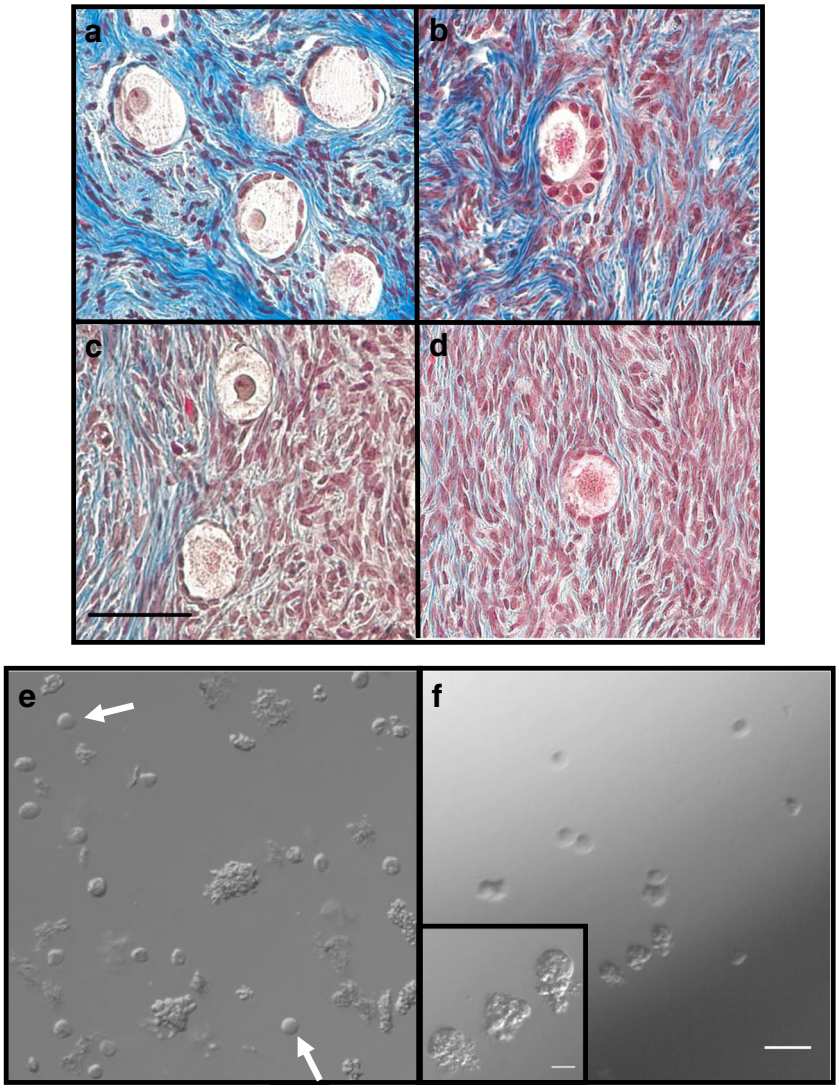

Fig. 3 Heterogeneous human ovarian cortex contributed to inconsistent primordial follicle isolation and culture protocols. **a–d**) Masson's Trichrome stain of ovarian cortical tissue highlights the variable collagen density (blue) surrounding primordial follicles between individual participants (A, participant H; B, participant FF; C, participant F; D, participant C; scale bar, 50 μm). **e**) Survival of primordial follicles following isolation protocols was poor as indicated by loss of integrity between the somatic cells and oocytes. Arrows highlight denuded oocytes. **f**) Follicles that remained intact following isolation were encapsulated in 0.5-2 % alginate (scale bar, 100 μm), but the granulosa cells dissociated from the oocytes by 3 days of culture (inset; scale bar, 25 μm)

This in situ approach has been applied with some success for human ovarian tissue [52–55] including encapsulating in a PEG-fibrinogen hydrogel [56]. We hypothesized that encapsulating small pieces of human ovarian cortex within alginate would provide additional physical support needed to maintain primordial follicles in culture. Acquiring accurate knowledge of the starting material composition is a challenge when performing in situ culture because follicles are distributed unevenly throughout the ovary and can be difficult to identify by non-invasive strategies [57]. To ensure that primordial follicles were present within the cultured ovarian cortex, we first validated our ability to accurately identify primordial follicles within the tissue. We were able to obtain 500 μm-thick sections of ovarian cortex, using a Thomas-Stadie Riggs Tissue slicer, and further processed these sections into 1 mm × 1 mm pieces. With these thin pieces, we were able to distinguish follicles within the tissue by simple light microscopy. Follicles were evidenced as areas of clearing within the dense tissue (Fig. 4a). To quantify our ability to detect follicles within the tissue, we analyzed the tissue by histology (Fig. 4b). We confirmed that we were able to predict which pieces of cortical ovarian tissue contained follicles 100 % of the time using light microscopy. Follicles were observed at a frequency ranging between 2.96 and 59.15 follicles per mm^2 (median=18.89 per mm^2, Fig. 4c bar). The majority of the tissue contained only quiescent or early growing follicles (65.22 % primordial, 23.91 % transitional and 7.61 % primary follicles; N=184 follicles). However, one participant contained secondary follicles at day 0 while all other participants contained 0 secondary follicles (3.26 % of total follicles counted). Additionally, the majority of these small follicles (>80 %) were observed in clusters within the tissue. These findings are consistent with previous observations that growing follicles are typically found within close proximity to each other and rely on paracrine signaling to support their growth [41, 57].

To determine whether encapsulating primordial follicles within the context of native ovarian tissue supports their

in vitro survival and growth, we cultured cortical pieces in 0.25, 0.5, 1 and 2 % alginate for 1 to 6 weeks (participants M-T, Table 4). In our previous study with isolated non-human primate primordial follicles, we found that 2 % alginate was required to provide the rigid physical environment small follicles require for survival [40]. However, here we found that different alginate concentrations did not obviously affect the extracellular matrix, the stroma, or follicle morphology during in situ culture as assessed by histology (representative images in Supplemental Fig. 1). Thus, subsequent data were pooled from all alginate conditions and analyzed according to the number of weeks in culture. We evaluated tissue survival within the cortical strip pieces using gross histology of H&E stained sections for each week of culture (Fig. 5a–c). Although individual cortical pieces were not tracked over time, the data represents an overall percentage of survival typical of such cultures. Cortical strip pieces containing live and healthy stroma were characterized by organized cells and elongated nuclei (Fig. 5a). In contrast, dead tissue lacked normal hematoxylin-stained nuclei and either appeared as mostly eosin-stained extracellular matrix material (Fig. 5b) or dark pyknotic nuclei characteristic of dead or dying cells as shown in Fig. 1d. We found that tissue survival in culture was 100 %, 85.4 %, 74.1 %, 52.4 %, and 46.67 % following 1, 2, 3, 4, and 6 weeks, respectively (Fig. 5c). Survival of more than 50 % of the tissue following 4 weeks of culture was significant given the basal composition of the media.

In addition to histological morphology, the health of the tissue was also evident by its ability to undergo ovarian surface epithelium remodeling characteristic of wound healing [58]. At the onset of culture, the cortical tissue pieces had rough edges due to processing with the tissue slicer and chopper (Fig. 6a). However, after 1 week of culture, most tissue pieces that exhibited healthy stroma had also differentiated from a square piece of tissue to a spherical body with a defined multilayered, smooth epithelial edge (Fig. 6b). This clearly defined surface epithelium was maintained for up to 4 weeks of culture but appeared to disintegrate in most samples by 6 weeks of culture (Fig. 7). This deterioration of the epithelial layer occurred concomitantly with an overall decline in the health of the stroma (Fig. 7g-h). Moreover, many of the follicles that remained in the tissue at 6 weeks were disorganized and lacked defined basement membranes (Fig. 7g arrowhead).

Encapsulated in situ culture of ovarian tissue supports the growth and differentiation of human primordial follicles

To determine whether this encapsulated in situ culture method supported folliculogenesis during the 6-week culture period, we quantified and classified the follicles within the cortical ovarian tissue pieces at each time point (Fig. 8a–d). Primordial follicles were those surrounded by squamous granulosa cells (Fig. 8a), while transitional follicles were classified as those

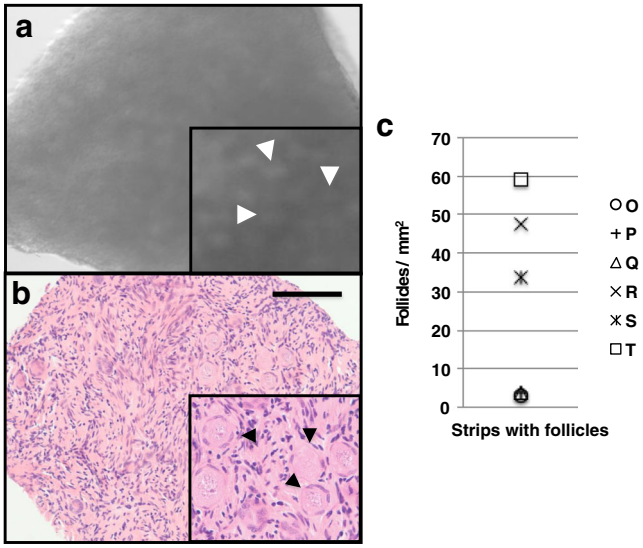

Fig. 4 Primordial follicles were reliably identified using only light microscopy within 500 μm sections of ovarian cortex. **a**) Transmitted light image of a thin section of ovarian cortex containing primordial follicles, which are visible as areas of clearing within the tissue (inset, arrowheads). **b**) H&E staining confirmed primordial follicles within the tissue (scale bar, 100 μm). Primordial follicles are characterized by an oocyte surrounded by an incomplete layer of squamous granulosa cells (inset, arrowheads). **c**) Follicles were counted within ovarian cortical tissue sections at 100 μm increments. There were between 2.96 and 59.15 follicles per mm^2. A line indicates the median follicle number (18.89 per mm^2)

with a mixture of squamous and cuboidal-shaped granulosa cells. Follicles with a single complete layer of cuboidal granulosa cells were identified as primary follicles, and those containing more layers were designated as secondary follicles (Fig. 8b). These different follicle classes were represented as a percentage of the total follicles counted at each culture time point (Fig. 8d).

At the onset of culture, the cortical ovarian tissue from each participant contained primordial and transitional follicles almost exclusively (Fig. 8d). However, within 2 weeks of culture, the follicle composition within the tissue shifted toward a higher percentage of growing follicles including both

Table 4 Experimental overview of encapsulated human ovarian cortex cultures

Participant	Culture conditions					
	1 wk	2 wk	3 wk	4 wk	6 wk	Alginate %
M	x	x				0.5
N	x	x	x			0.5
O	x	x	x			0.5
P				x		0.5
Q			x	x		0.5
R		x	x	x	x	0.25, 0.5, 1 & 2
S		x		x	x	0.25, 0.5, 1 & 2
T		x	x			0.5 & 2

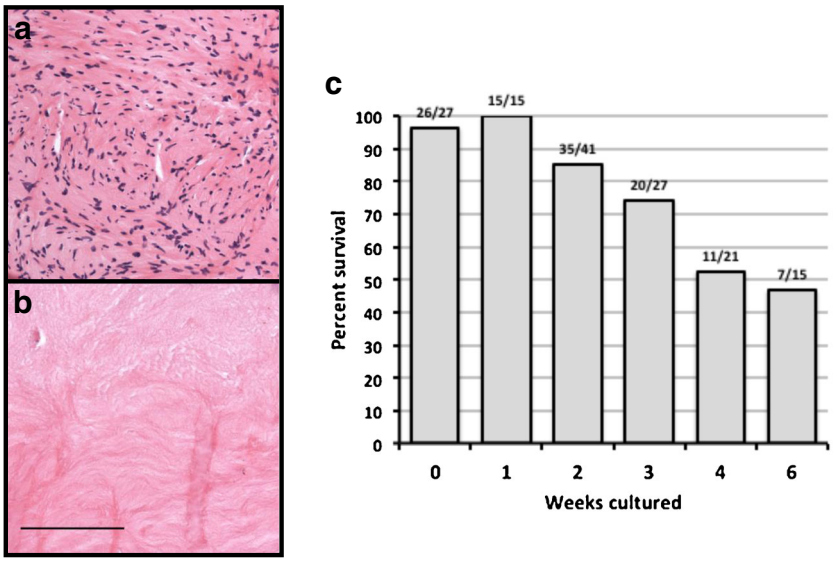

Fig. 5 The majority of ovarian cortical tissue survives long-term culture encapsulated in alginate hydrogels. Tissue survival was assessed by quantifying the number of pieces of ovarian cortex that contained **a**) live cells (hematoxylin-positive nuclear material) and **b**) dead cells (hematoxylin-negative). Representative images of stromal tissue are shown (scale bar, 100 μm). **c**) The percent survival of ovarian cortex was calculated as the number of tissue pieces that contained live cells over the total number of tissue pieces analyzed for the particular culture time point. Data from the entire 6-week culture period is plotted

primary and secondary follicles. Of note, we observed an antral follicle within cortical ovarian tissue from participant R at the terminal culture point (Fig. 8c). This follicle had a clear antral cavity and cellular differentiation into mural and cumulus granulosa cells had occurred. These findings suggest that encapsulated ovarian cortical tissue supports key morphological aspects of folliculogenesis.

Discussion

Primordial follicles dictate a female's reproductive lifespan. They are also the most abundant class of follicles, are present in females at any age and are therefore a critically important follicle class to harness for fertility preservation. Despite the importance of this follicle class, our ability to isolate and grow human primordial follicles in vitro remains limited [52, 53, 55, 59–64]. Here we describe a technique in which pieces of human ovarian cortex containing primordial follicles were identified, encapsulated within alginate hydrogels, and cultured for up to 6 weeks. More than 50 % of cortical tissue pieces maintained optimal tissue health and follicle morphology following 4 weeks of culture, and samples that initially contained mostly primordial follicles showed evidence of multi-layer follicle development following 3 to 6 weeks of culture. By 6 weeks of culture, appreciable tissue deterioration was observed under the culture conditions employed, suggesting that future studies are needed to

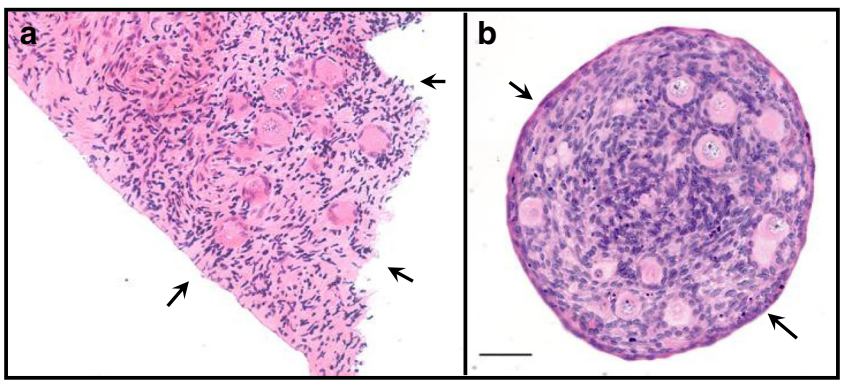

Fig. 6 Healthy ovarian tissue undergoes epithelial surface remodeling during culture. **a**) Processing of ovarian tissue into 1 mm × 1 mm pieces of 500 μm-thick cortex for encapsulation disrupts the edges of the tissue (arrows; participant O). **b**) This disruption likely stimulates a wound healing response, which causes the encapsulated tissue to differentiate to form an epithelial-like surface (arrows; participant R). Tissue pieces with clear epithelial differentiation typically contain healthy stroma and follicles (arrowheads). Representative images are shown (scale bar, 50 μm)

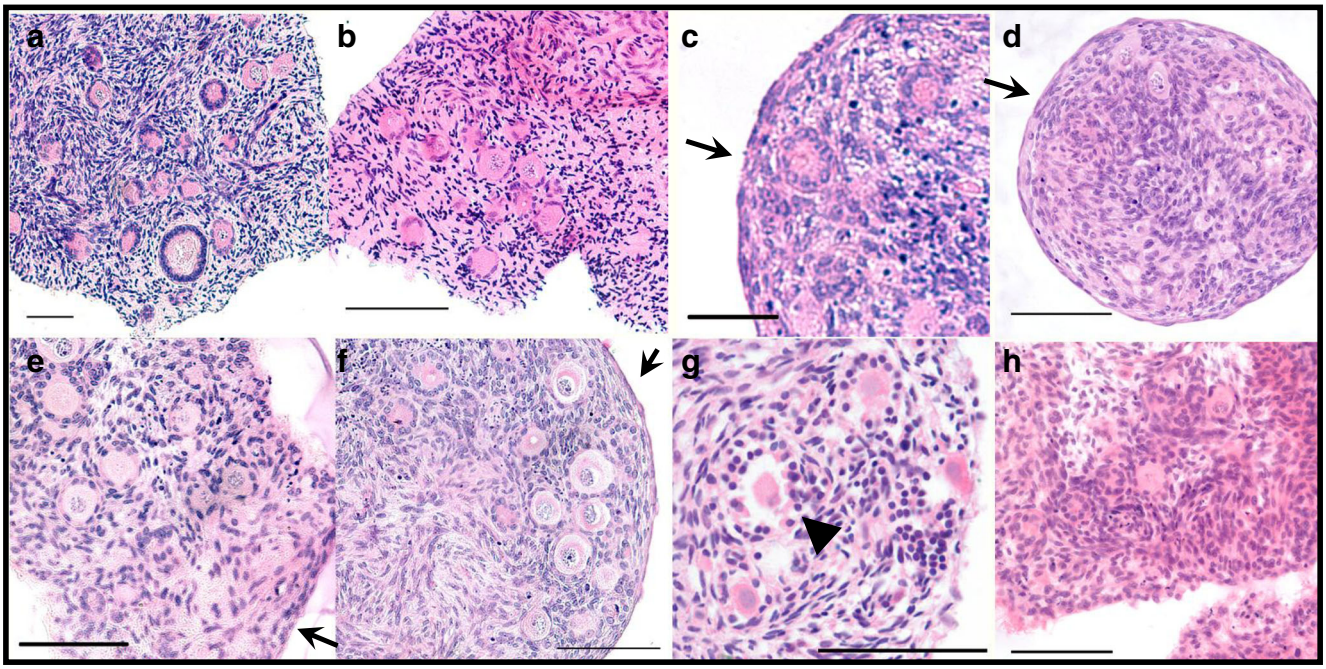

Fig. 7 Alginate-encapsulated culture of ovarian cortex maintains and supports follicles for at least 4 weeks. Representative cortical strip pieces fixed on day 0 (**a**, participant R; **b**, participant M) or following 2 weeks (**c**, participant S; **d**, participant, R), 3 weeks (**e**, participant R), 4 weeks (**f**, participant P) or 6 weeks (**g**, participant R; **h**, participant S) of culture are shown (scale bar, 100 μm). Arrows highlight the differentiated surface epithelium edge of the cortical tissue in 2, 3 and 4-week cultures. Evidence of general tissue degeneration was common at 6 weeks. The arrowhead points to a disorganized secondary follicle. This degeneration corresponded to the absence of the defined epithelial surface that was present earlier in culture

optimize long-term culture. Nevertheless, there was evidence of a morphologically normal antral follicle at this terminal culture point. The findings presented herein not only improve our understanding of human follicle development in vitro, but they provide key procedural insights into research with human ovarian tissue.

As oncofertility expands, there will be increased patient demand for both standard and investigational fertility preservation techniques. Many of these methods require highly trained individuals or specialized equipment to perform, which could limit access to individuals who are in close geographical proximity to qualified centers. Our findings, however, indicate that this limitation may be remedied because the majority of human ovarian tissue maintained at cold temperatures for up to 24 h retains viability and, more importantly follicle function. These findings are consistent with previously published data that demonstrates that both male and female primate reproductive tissues can be kept at cold temperatures [28, 65]. Of note, this transport only preserves preantral follicles as cumulus-oocyte-complexes within antral follicles deteriorate rapidly at cold temperatures ([66] and unpublished observations). In practice, ovarian tissue could be transported nationwide or globally to reputable sites that perform tissue processing and cryopreservation by slow freezing or vitrification. Tissue transport, therefore, has the potential to not only increase patient access to fertility preservation options, but as demonstrated here, can also facilitate the acquisition of rare human ovarian tissue for critical basic research.

Performing basic research with human tissue is essential but difficult due to limited availability and inherent heterogeneity among samples. Previously we used non-human primate ovarian tissue as a model to develop techniques to isolate and culture primordial follicles from large mammalian species [40]. Non-human primate ovaries are similar to human ovaries in terms of architecture, with primordial follicles enriched in the outer dense ovarian cortex and growing follicles found in the inner, less dense medulla [40]. Using non-human primate tissue, we were able to routinely isolate hundreds of intact primordial follicles per ovary from individual animals [40]. However, when we applied the same techniques to human ovaries, our results were inconsistent, and follicle integrity was greatly compromised as a consequence. We found that this inconsistency was likely attributable to wide differences in extracellular matrix composition surrounding the primordial follicles in thin sections of the ovarian cortex. The heterogeneity among participants is not unexpected as many participant-specific variables could impact the quality and composition of the acquired ovarian tissue. These variables include, but are not limited to, the participant's age, disease diagnosis, previous medical treatment history and/or environmental exposures. A recent publication also suggests that in vitro growth of both isolated follicles and follicles within cortical tissue may differ between prepubertal and

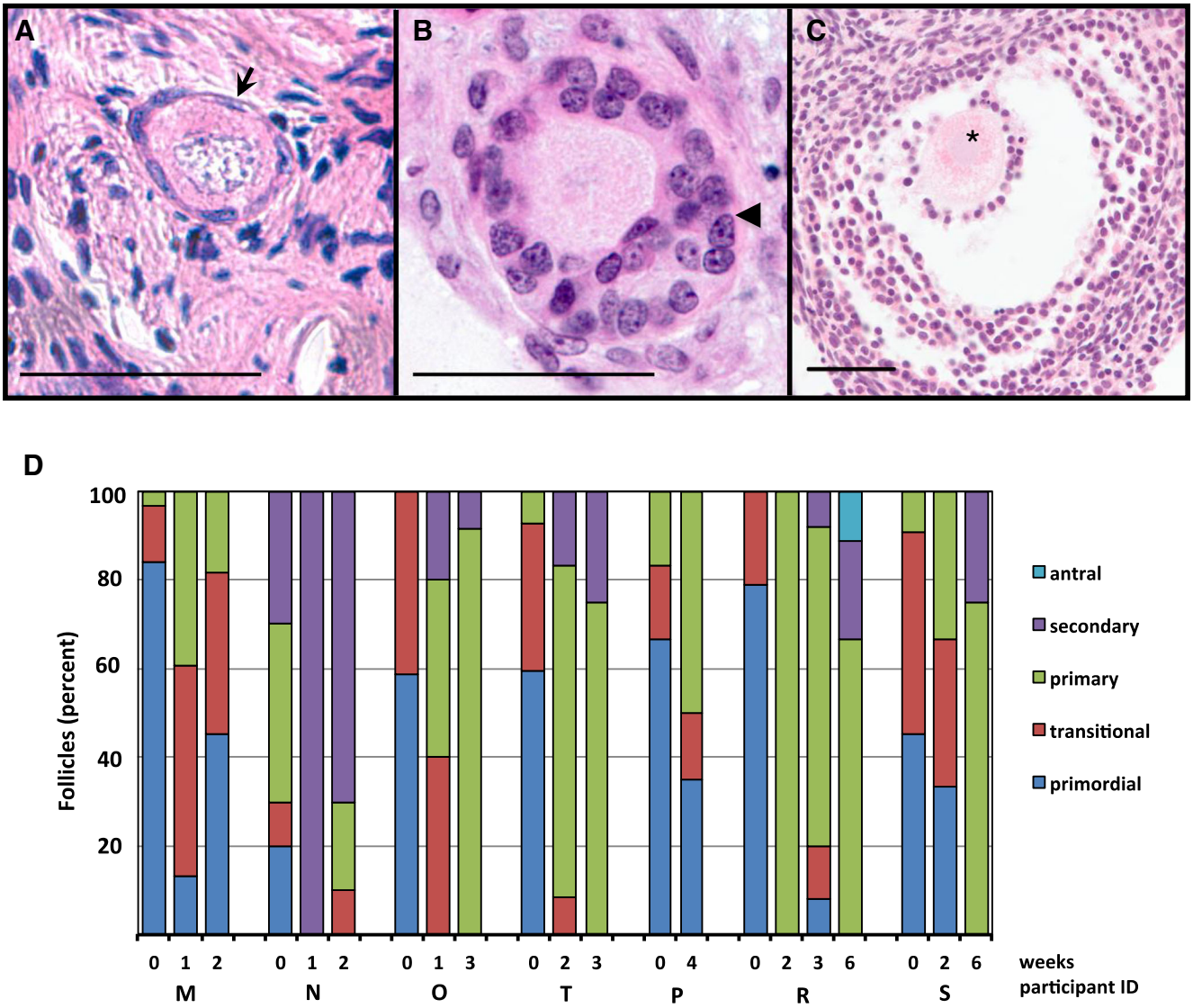

Fig. 8 Alginate encapsulation and culture of human ovarian cortex supports folliculogenesis. Representative images of a primordial (**a**, participant M, day 0) and a secondary follicle (**b**, participant T, 2 weeks) within the cultured ovarian tissue are shown. An arrow and arrowhead highlight the squamous granulosa cells and multiple granulosa layers, respectively. **c**) An antral follicle was identified within a piece of ovarian cortex that was cultured for 6 weeks (participant R). The cumulus-oocyte-complex is highlighted by an asterisks and the antral cavity is apparent as the white fluid-filled space (scale bars, 50 μm). **d**) The distribution of follicle classes at each culture time point is plotted for individual participants

postpubertal patients [67]. Given this heterogeneity, it is not surprising that it is quite challenging to develop and apply standard procedures to isolate and grow primordial follicles from human ovarian tissue. Interestingly, we have not noticed equivalent difficulties in isolating human secondary follicles probably because these larger follicles have a more pronounced and protective basement membrane, and the connections between the somatic and germ cells are well-established.

Additional significant challenges of performing tightly-controlled studies with human ovarian tissue are having firm knowledge of the starting material composition and being able to track tissue health non-invasively. Follicles are not distributed evenly within the human ovary, and so it is difficult to predict whether a specific piece of ovarian tissue contains follicles, especially in older individuals where total follicle numbers decline [57, 68, 69]. However, being able to reliably identify follicles is particularly important when trying to accurately evaluate outcomes of culture conditions. To this end, methods, including staining ovarian tissue with vital dyes, have been developed to identify follicles within intact tissue [70]. Here, we developed a method to visualize follicles in thin sections of human ovarian tissue using only light microscopy. We validated this approach by histology and demonstrated that we were able to identify tissue pieces that contained predominantly primordial, transitional, and primary follicles with 100 % accuracy. Thus, we could be confident that the

tissue we cultured actually contained follicles and that any appreciable follicle development occurred in vitro.

Moreover, we discovered a new non-invasive indicator of tissue health. We found that within the first week of culture, the outer cells of the cortical ovarian pieces differentiated into a smooth epithelial surface and became spherical. It is known that the mouse ovarian surface epithelium has the capacity to remodel via increased proliferation following wounding when encapsulated and cultured in alginate, and we suspect that a similar phenomenon occurred during our in situ human cultures [58]. This defined epithelial surface was maintained in most tissue pieces for 4 weeks of culture, but appeared to breakdown by 6 weeks. The ability of the tissue to remodel seemed to correlate with tissue survival and follicle quality, indicating that this morphology may be a potential non-invasive marker of tissue health.

Although we were not able to reliably isolate intact primordial follicles from human tissue, our ability to identify this follicle class within intact cortical tissue allowed us to perform in situ cultures following encapsulation in alginate. As noted by us and other groups, isolated human primordial follicles survive only approximately 24 h in culture, but a larger proportion of follicles survive when cultured in cortical strips [52–55]. The integrity of the whole follicle unit is essential for healthy oocyte development and must be maintained in native and artificial environments [37, 38, 71, 72]. We found previously that isolated non-human primate primordial follicles require a rigid physical environment composed of 2 % alginate to maintain integrity and survive in culture. In contrast, when primordial follicles were cultured in situ, the tissue health and follicle quality was independent of the alginate concentration used (0.25 %, 0.5 %, 1 %, and 2 %). This observation is likely because the native tissue provides sufficient physical support to maintain follicle integrity during the early stages of growth, and alginate encapsulation serves to keep the tissue pieces intact.

This method of ovarian cortex eIVFG resulted in secondary follicle development in five out of the seven cases. Of note, in four of the five cases in which we observed secondary follicles, the initial tissue samples contained primordial and transitional follicles almost exclusively. Thus, these secondary follicles most likely developed during culture. It has been estimated that primate secondary follicles require approximately 120 days to develop from primordial follicles in vivo [47]. However, evidence from multiple groups suggests that follicle development in culture is accelerated [60, 73]. One report identified preantral follicles in human cortical strips cultured for only 6 days following activin stimulation [74]. Other studies, using a wide variety of culture conditions, report a range of successes, from tissue degeneration to appreciable follicle growth within 2 weeks [55, 56, 75, 76]. Data described here support the premise that secondary follicles require approximately 3 weeks in culture to develop from primordial follicle-containing cortical strips, as this occurs in 4 out of 5 participant tissues. However, the heterogeneity amongst studies highlights the need for continued research in this area.

Although ovarian cortex eIVFG supports folliculogenesis, we observed appreciable tissue degeneration and follicle disorganization between 4 and 6 weeks of culture, suggesting the need for optimized stage-specific culture conditions. For example, Akt pathway stimulation using pharmacological PTEN inhibitors has been shown to drive primordial follicle activation of human ovarian cortical tissue that was xenotransplanted into immunodeficient mice [77]. Moreover, a similar follicle activation protocol resulted in a human live birth in a patient with primary ovarian insufficiency [78]. These protocols, which stimulate the global activation and growth of primordial follicles, could be combined with a multi-step culture protocol to increase the yield of mature gametes from an individual. For example, ovarian cortical tissue grown through eIVFG would support primordial follicles to secondary follicles, which then could be isolated and grown individually within alginate hydrogels as demonstrated previously [36]. A challenge of multi-step cultures that is often overlooked, however, is developing methods to isolate growing preantral follicles from tissue that has been cultured for weeks without damaging the follicle. If follicles can be isolated successfully, signaling molecules such as activin, insulin, and IGF-1 could be added into the media to support and stimulate survival and growth [79].

Developing methods to sustain primordial follicle activation and growth in vitro is a prime goal of fertility preservation researchers. This technology has the potential to benefit females of all ages but particularly pre-pubertal populations for whom ovarian tissue cryopreservation is the prime fertility preservation option. We have found that the number of pediatric participants who have had ovarian tissue cryopreserved through the Oncofertility Consortium has increased nearly 10-fold in the past 5 years (our unpublished data). Thus, there is a large clinical drive to ensure that the fertility needs of these patients are ultimately met when they are ready and able to start a family. Being able to grow primordial follicles in vitro is a necessary bridge to the translation of even more investigational technologies such as the derivation of gametes from stem cells. The results presented here provide a solid foundation for these future studies.

Acknowledgments The authors would like to acknowledge all participants who donated ovarian tissue for the research portion of this protocol and the personnel of the National Physicians Cooperative who facilitated these studies. We also thank Megan Romero and Keisha Barreto from the Ovarian Histology Core at Northwestern University for their technical expertise and R. Schultz and S. Medvedev from the University of Pennsylvania for the use of their MSY2 antisera. This research is funded in part by the National Institutes of Health, Eunice Kennedy Shriver National Institute of Child Health and Human Development through the U54 HD076188 grant. The Oncofertility Consortium® is funded through the NIH Roadmap for Medical Research, Grant UL1DE19587 and PL1CA133835.

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