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The crucial role of mechanical heterogeneity in regulating follicle development and ovulation with engineered ovarian microtissue

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ABSTRACT

Contemporary systems for *in vitro* culture of ovarian follicles do not recapitulate the mechanical heterogeneity in mammalian ovary. Here we report microfluidic generation of biomimetic ovarian microtissue for miniaturized three-dimensional (3D) culture of early secondary preantral follicles by using alginate (harder) and collagen (softer) to fabricate the ovarian cortical and medullary tissues, respectively. This biomimetic configuration greatly facilitates follicle development to antral stage. Moreover, it enables *in vitro* ovulation of cumulus–oocyte complex (COC) from the antral follicles in the absence of luteinizing hormone (LH) and epidermal growth factor (EGF) that are well accepted to be responsible for ovulation in contemporary literature. These data reveal the crucial role of mechanical heterogeneity in the mammalian ovary in regulating follicle development and ovulation. The biomimetic ovarian microtissue and the microfluidic technology developed in this study are valuable for improving *in vitro* culture of follicles to preserve fertility and for understanding the mechanism of follicle development and ovulation to facilitate the search of cures to infertility due to ovarian disorders.

1. Introduction

The ovarian follicle consisting of a centrally located oocyte, an inner layer of granulosa cells, and an outer layer of theca cells is the fundamental functional tissue unit of mammalian ovary. Therefore, *in vitro* culture of ovarian follicles to obtain oocytes has been proposed as an attractive strategy for restoring infertility of women who are born with ovarian disorder and for preserving fertility of women who may want to delay child bearing or lose gonadal function due to aggressive medical treatment (e.g., chemotherapy) or exposure to environmental/occupational biohazards [1–3].

Both two and three-dimensional approaches have been developed for *in vitro* culture of ovarian follicles [4,5]. For two-dimensional (2D) culture on the surface inside culture dishes, both the theca and granulosa cells easily detach from the follicles, spread out, and attach on the surface of culture dishes, leading to a diffused morphology that is non-physiological [6]. Moreover, endogenous paracrine and autocrine factors produced by granulosa and theca cells are easily diluted into the bulk culture medium, which can negatively affect follicle development [7]. On the other hand, three-dimensional (3D) approaches involving the use of homogeneous hanging drop or hydrogel encapsulation (in millimeter sized alginate hydrogel) can better preserve the native 3D follicular architecture during *in vitro* culture [8–12]. Although these approaches have been successfully used for *in vitro* culture of follicles from inbred mouse model and contribute significantly to the understanding of follicle biology [8–12], they are far away from being used as an assisted reproductive technology (ART) to restore infertility or preserve fertility for humans. This is because most of the culture systems developed using inbred mice have been shown not to be directly applicable to even outbred mouse and primate models [6,13], not to mention humans. Moreover, both the 2D and homogeneous 3D (alginate hydrogel or liquid hanging drop) microenvironment used to culture follicles are non-physiological because it does not recapitulate the heterogeneous nature of the extracellular

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matrix (ECM) in the ovary with the ECM of medulla (inner region) being much softer than that in the cortex (outer region) [14–16]. The ECM is believed to not only provide a 3D network to support the ovarian tissue architecture but also regulate (together with soluble endocrine, paracrine, and autocrine factors) cell-ECM and cell–cell interactions that are important for follicle development [7,12,14,15].

To overcome these challenges, we employed outbred deer mice as the model to study the effect of culturing microenvironment on follicle development and ovulation with particular emphasis on mechanical heterogeneity that has been barely explored in the field of assisted reproduction. Deer mice are indigenous rodents in *North America* and believed to be more suitable than inbred mice for research aimed for medical applications due to their outbred nature as with humans [6,13]. Moreover, we engineered the *in vitro* culturing microenvironment of follicles by mimicking their native 3D milieu including the mechanical heterogeneity in ECM of medulla versus cortex and its 3D distribution. To create the mechanical heterogeneity native in ovary, we employed non-planar microfluidic flow-focusing devices to encapsulate early secondary preantral follicles in ~350 μm microcapsules composed of an alginate (harder) shell and collagen (softer) core. With the biomimetic ovarian microtissue, we investigated the role of mechanical heterogeneity in follicle development from the early secondary to antral stage. Because luteinizing hormone (LH) and epidermal growth factor (EGF) are conventionally believed to trigger and facilitate ovulation [17–22], we further investigated their effect on ovulation of the antral follicles obtained by *in vitro* culturing the biomimetic 3D ovarian microtissue.

2. Experimental

2.1. Animals and materials

Peromyscus maniculatus bairdii (BW stock) deer mice were purchased from the *Peromyscus* Genetic Stock Center at the University of South Carolina, Columbia, SC and were maintained on a 16-8 h light–dark cycle. All procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and every effort was made to minimize animal suffering. A total of 15 animals were used in this study to ensure that follicles from at least 3 animals were used for each experimental condition except the 2D culture condition for which 2 animals were used. L-15 Leibovitz-glutamax and α-MEM-glutamax medium were purchased from Invitrogen. Fetal bovine serum (FBS) from Hyclone was purchased from Fisher Scientific. Sodium alginate was purchased from Sigma and purified by washing in chloroform with charcoal, dialyzing against deionized water, and freeze-drying. Unless specifically noted otherwise, all other chemicals were purchased from Sigma.

2.2. Isolation of early secondary preantral follicles

Early secondary preantral follicles (100–130 μm) were isolated from ovaries of female deer mice of 12–16-week old using a mechanical method reported by us previously [6]. Briefly, the ovaries were placed in 2 ml Leibovitz L-15 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin at 37 °C in 5% CO_2 air. Preantral follicles were obtained by using two 30 G needles to mechanically break the extracellular matrix between follicles in the ovarian tissue.

2.3. Fabrication of non-planar microfluidic device

To fabricate polydimethyl siloxane (PDMS, Dow Corning) microfluidic device, silicon master with patterned microfluidic channels was prepared using a multilayer (3-step UV exposure) SU-8 fabrication technique [23]. Briefly, photosensitive epoxy (SU-8 2025, MicroChem) was spun coated onto a 4-inch silicon wafer. The thickness of the first SU-8 coating was 100 μm. The wafers were then soft-baked at 95 °C for 20 min and exposed to UV light through the first shadow mask printed with the core channel. After a post-exposure baking at 90 °C for 10 min, an additional layer (50 μm) of SU-8 photoresist was spun coated, soft baked, exposed with a different shadow mask to pattern shell channel. The third layer for oil channel was similarly patterned. All three exposures were aligned using an EVG620 automated mask aligner. The SU-8 pattern on the substrate was developed in SU-8 developer (MicroChem) for 10 min, rinsed with isopropyl alcohol, and dried using nitrogen gas. PDMS pre-polymer was then poured on the silicon substrate and cured at 65 °C for 3 h to form PDMS slab. Thereafter, the PDMS slab embedded with microchannels (half-depth) was lifted off. Two PDMS slabs with the same channel design were then plasma-treated for 30 s using Harrick PDC-32 G plasma cleaner at 18 W and 27 Pa, wetted with methanol (to prevent instant bonding), and aligned and bonded together under microscope to produce the final microfluidic device. Assembled devices were kept on hotplate at 80 °C for ~ 10 min to evaporate residual methanol and further kept at 65 °C for 2 days to make it sufficiently hydrophobic for experiments.

2.4. Rheological characterization of hydrogel materials for making ovarian microtissues

Rheological measurements were carried out using a TA instrument AR-1000N rheometer. For alginate and collagen (type I, BD Biosciences) hydrogels, 40 mm parallel plate and plate-cone geometries were used, respectively. Stress sweeps at a constant frequency of 1 Hz were first performed to obtain the linear viscoelastic region for collecting subsequent data. Frequency sweeps were performed in the linear viscoelastic regime to determine values of the storage (G′) and loss (G″) modulus. The data at 1 Hz are reported in Fig. 1 for comparison. More rheology data showing the shearing rate (or frequency) dependence of the moduli of the various materials are given in Supplementary Fig. S1. The 2% and 0.5% alginate hydrogels were prepared and gelled on mold using calcium infused mineral oil for 30 min, followed by washing with mannitol solution and transferring onto the rheometer plate. Collagen gels were prepared directly on the rheometer, for which 0.5% collagen solution was placed on the rheometer plate at 4 °C and gelled by raising the rheometer temperature to 37 °C for 30 min. To determining the time dependent mechanical properties, the samples were prepared in the same way and further incubated in the basal culture medium of follicles at 37 °C in 5% CO_2 incubator till measurement at the desired times.

2.5. Encapsulation of early secondary preantral follicles to produce ovarian microtissues

The fluid entering the core microchannel via I-1 (Fig. 2) was 0.5% collagen or sodium alginate (non-oxidized by default) solution. The dispatching fluid was the same as the core fluid. The fluid entering the shell microchannel via I-2 was 2% sodium alginate or a mixture of 1% sodium alginate and 1% sodium alginate with oxidization using a protocol reported previously [24,25]. For convenience, the mixture is called oxidized alginate (O-alg) in this work. To increase the viscosity in the core solution, which was found to be necessary for formation of core–shell structure, 1% sodium carboxymethyl cellulose was included. For the extraction channel, 1% sodium carboxymethyl cellulose solution was used which was necessary for forming stable interface between oil emulsion and the aqueous phase. All the solutions were sterile and buffered with 10 mM HEPES to maintain pH at 7.2 before use. Further, osmolality of all the solutions were maintained at 300 mOsm by the addition of D-mannitol. To make mineral oil infused with calcium chloride for flowing in the oil channel, stable emulsion of mineral oil and 0.7 g/ml calcium chloride solution (volume ratio: 3 to 1 with the addition of 1.2% SPAN 80) was prepared by sonication for 1 min using a Branson 450 digital sonifier. Water in the emulsion was then removed by rotatory evaporation for ~2 min at 55 °C. All solutions (except collagen that was kept at 4 °C) were injected into the microfluidic device using syringe pump at room temperature to generate microcapsules in oil phase and then extract them into aqueous phase. Flow rates for core, dispatching, shell, oil, and aqueous extracting fluids were 50 μl/h, 30 μl/h, 120 μl/h, 2 ml/h, and 4 ml/h, respectively. Outlets of the device were connected to a 50 ml centrifuge tube containing M2 medium (Millipore) to collect microtissues at room temperature.

2.6. Preparation of ovarian cell-conditioned medium

To prepare ovarian cell-conditioned medium, ovarian cells were first isolated by following a protocol reported previously [6]. Briefly, the ovaries of 12–14 week-old of deer mice were collected and chopped after the removal of adherent tissues such as fat pad. The specimens were incubated initially for 30 min in a dissociation medium consisting of a 50:50 (v:v) mixture of 0.25% (v/w) trypsin-EDTA (ethylenediaminetetraacetic acid) and DMEM (Dulbecco's modified eagle's medium) supplemented with 750 units/ml type I collagenase and 0.03% (v/v) fetal bovine serum at 37 °C in 5% CO_2 air. The dissociated cells were filtered through a 40 μm filter and subsequently centrifuged at 390 × *g* for 4 min. The collected cells were further cultured onto a 60 mm culture dish in 5 ml DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. After 20 h of culture in the dish, the DMEM-based medium was removed and the cells were washed once using 1× PBS. A total of 5 ml (non-conditioned) α-MEM–glutamax medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin solution was then added into the dish. The cells were incubated with the non-conditioned medium at 37 °C in 5% CO_2 air for two days and the resultant conditioned medium (CM) was collected and the procedure was repeated once to eventually make a total of 10 ml conditioned medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 100 mIU/mL recombinant human follicle stimulating hormone (FSH) for further use.

2.7. In vitro culture of early secondary preantral follicles encapsulated in microtissues

For 2D culture, the early secondary preantral follicles were placed singly in 10 μl drops of ovarian cell conditioned culture medium overlaid with mineral oil in