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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Isolation of mouse ovarian follicles for single-cell RNA-seq and in vitro culture

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SUMMARY

Here, we provide a protocol combing single-cell genomics and functional experiments to study stepwise folliculogenesis. This protocol isolates different developmental stages of mouse ovarian follicles to perform single-cell RNA-seq and in vitro culture, allowing to dissect and validate key molecular events in guiding folliculogenesis, thus bringing non-growing oocytes into maturity. For complete details on the use and execution of this protocol, please refer to Gu et al. (2019) and Long et al. (2022).

BEFORE YOU BEGIN

Note: Animal work in this protocol was conducted under approval of the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences, China.

Introduction

Ovarian follicles are morphological and functional units of the ovary. In contrast to the broader knowledge of discrete signaling in regulating oogenesis, the holistic signatures of ovarian somatic cells and hierarchical instructions that coordinate folliculogenesis remain to be investigated. Folliculogenesis commences with breakdown of germ cell nests, in which a single oocyte is surrounded by a layer of granulosa cells. As follicles continue to grow, the recruitment of an envelope of theca cells signifies completion of follicle assembly. Genetic and molecular studies have identified fundamental pathways reliant on locally produced molecules in controlling mammalian oogenesis, under the direction of pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone (Edson et al., 2009). The stepwise folliculogenesis is highly instructed. Our recent single-cell studies further provided an insight into epigenetic hierarchy governing development transition of oocytes (Gu et al., 2019; Yan et al., 2021).

It is well known that oocytes are vulnerable to several maternal environmental exposures (Lane et al., 2014), and much progresses have also been made in understanding how the dysfunctional oocyte contributes to offspring disease predisposition (Saben et al., 2016; Risal et al., 2019). However, the mechanisms by which maternal inputs are transmitted to the oocyte remain largely unknown. Within ovarian follicle, reciprocal dialog between somatic cells and oocyte is a prerequisite for oocyte development. In obese mouse models, we recently discovered that deregulated signaling...
in follicular somatic cells could compromise oocyte quality, explaining impacts of maternal obesity on offspring health (Long et al., 2022).

When dissociating single cells from a whole ovary, stroma predominates. This protocol adopts a hand-pick method to capture oocytes and follicular somatic cells for single-cell RNA-seq for two reasons. Firstly, the number of oocytes is relatively small compared to somatic cells. To focus on how direct crosstalk between theca cells and granulosa cells could interfere manifestation of oocyte phenotype, this protocol starts with isolation and enrichment of mouse ovarian follicles at a succession of developmental stages, followed by manual picking to enrich oocytes and follicular somatic cells. Secondly, diameters of growing and mature oocytes (~30–100 μm) are larger than usual cell type, which is difficult to be captured by 10× chromium, as cell size suggested by 10× chromium is ~30 μm.

This protocol provides an experiment framework and detailed methods combing single-cell RNA-seq and in vitro culture to test function of candidate factors identified by single-cell analyses. For further complete information, please refer to our recent work (Gu et al., 2019; Long et al., 2022).

**Specification of mice age**

Follicles across growing stages predominate in ovaries of mice when puberty begins, and antral follicles can be collected from ovaries of young adulthood (Zheng et al., 2014; Qian and Guo, 2022). Oocytes and ovarian somatic cells contributed by female mice at different ages is a key to capture transcriptome dynamics across folliculogenesis. In this protocol, growing follicles are collected from ovaries of 3- to 4-week-old mice; antral follicles and ovulatory follicles are collected from ovaries of 7- to 8-week-old mice. It is worth noting that although mature oocytes can also be retrieved from mice until 8-month-old (in our experience), in this protocol, 7- to 8-week-old mice are recommended to be used as young adulthood to evade any potential effects arisen by aging.

**Note:** Experiments using mice must be performed in compliance with all related institutional and governmental rules.

**Specification of gonadotropin regimes**

In this protocol, oocytes and surrounding somatic cells are retrieved from either ovaries or oviducts. According to the tissue used, different regimes are used for administration of gonadotropins. Ovulatory follicles are retrieved from oviducts to collect metaphase II oocytes and cumulus cells (granulosa cells surrounding the ovulated oocytes), and mice used to collect ovulatory follicles need to be injected with sequential PMSG (pregnant mare serum gonadotropin) and hCG (human chorionic gonadotropin). Growing and antral follicles are dissociated from ovaries to collect growing oocytes and follicular somatic cells, and mice used to collect follicles from ovaries only need to be injected with PMSG.

**Dosages of gonadotropin administrated**

In this protocol, we prepared PMSG or hCG solution of 50 IU/mL (see in materials and equipment section). According to this, 0.1 mL (5 IU in total) of PMSG and 0.1 mL (5 IU in total) of hCG are used for 3- to 4-week-old mice; 0.2 mL (10 IU in total) of PMSG and 0.2 mL (10 IU in total) of hCG are used for 7- to 8-week-old mice.

**Note:** Dosages of gonadotropins are based on bodyweight of the mice, and whether interstrain variability in response to gonadotropins exists should to be considered, in addition to the possible difference in bodyweight among stocks. We used C57BL/6J females in this protocol. The average body weight for C57BL/6J females is 10–15 g at the age of 3- to 4-week, accordingly, 5 IU of gonadotropins are injected to mice at this age; the average body weight is 20–25 g at the age of 7- to 8-week, accordingly, 10 IU of gonadotropins are injected to mice at this age. Whether and to what extent the efficacy of gonadotropins would
be compromised in mice older than 6-month remains undetermined. Possibly dosages need to be increased for aged mice, however, care should be taken to avoid potential overdose. Furthermore, in our hands, this correlation between dosages of gonadotropin and body-weight of mice also works well in CD-1(ICR) mice.

**Administration of gonadotropins**

- **Timing:** ~42–48 h

1. Hold the mouse, pierce through skin and muscle layer at the caudal left of the abdomen to inject PMSG into intraperitoneal cavity by using a disposable 1 mL-sterile syringe equipped with a 27-gauge needle.
2. To collect growing and antral follicles, ovaries are dissected directly 42–44 h after injection of PMSG.
3. To collect ovulatory follicles, 46–48 h after injection of PMSG, hCG is further injected, also through intraperitoneal administration. Oviducts are dissected 15–16 h after injection of hCG.

⚠️ **CRITICAL:** Make sure the entry point of the needle is at the right angle/ below skin and muscle layers. Appearance of a bleb at injection site indicates that the hormone solution was administrated subcutaneously rather than intraperitoneally, and this would compromise oocyte yield.

⚠️ **CRITICAL:** Avoid bladder during intraperitoneal administrations.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Cnmd, Rabbit Polyclonal (1:100) | Thermo Fisher Scientific | Cat# PAS-76974 |
| Anti-Rabbit-Alexa 555, Goat Polyclonal (1:1000) | Thermo Fisher Scientific | Cat# A-21429 |
| **Experimental models: Organisms/strains** |        |            |
| Mice: C57BL/6J (wild-type, female, 14-day-old) | Charles River | N/A |
| Mice: C57BL/6J (wild-type, female, 3- to 4-week-old) | Charles River | N/A |
| Mice: C57BL/6J (wild-type, female, 7- to 8-week-old) | Charles River | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| PMSG | ProSpec | Cat# HOR-272 |
| hCG | ProSpec | Cat# HOR-250 |
| Sodium chloride (NaCl) | Sigma-Aldrich | Cat# 59888 |
| Triton X-100 | Sigma-Aldrich | Cat# T9284 |
| M2 Medium | Sigma-Aldrich | Cat# M7167 |
| Type IV-S Hyaluronidase | Sigma-Aldrich | Cat# H4272 |
| Tyrode’s Solution, Acidic | Sigma-Aldrich | Cat# T1788 |
| Albumin, Acetylated from bovine serum | Sigma-Aldrich | Cat# B2518 |
| ITS Liquid Media Supplement (100 x) | Sigma-Aldrich | Cat# B3146 |
| EmbryoMax® Nucleosides (100 x) | Sigma-Aldrich | Cat# ES-008-D |
| Betaine | Sigma-Aldrich | Cat# 61962-50G |
| DPBS, no calcium, no magnesium | Gibco | Cat# 14190-144 |
| Leibovitz’s L-15 Medium | Gibco | Cat# 11415-064 |
| α-MEM Medium | Gibco | Cat# 12561056 |
| Penicillin-Streptomycin (100 x) | Gibco | Cat# 15140122 |
| Collagenase, Type IV | Gibco | Cat# 17104019 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| TrypLE Express Enzyme (1 x) | Gibco | Cat# 12604-021 |
| ProLong Gold Antifade Mountant with DAPI | Thermo Fisher Scientific | Cat# P36935 |
| Nuclease-Free Water | Thermo Fisher Scientific | Cat# AM9932 |
| Dynabeads "MyOne" Streptavidin C1 | Thermo Fisher Scientific | Cat# 65002 |
| UltraPure 1 M Tris-HCl, pH 7.5 | Thermo Fisher Scientific | Cat# 15567-027 |
| Superscript II reverse transcriptase | Thermo Fisher Scientific | Cat# 18064071 |
| dNTP mix | Thermo Fisher Scientific | Cat# R0192 |
| Recombinant RNase Inhibitor (40 U/mL) | Takara Bio | Cat# 2313B |
| Ampure XP Beads | Beckman Coulter | Cat# A63882 |
| Magnesium chloride (MgCl2) | VMR | Cat# J364-100G |
| 2 x KAPA HiFi HS ReadyMix | Roche | Cat# 7958935001 |
| Fetal Bovine Serum, New Zealand | VISTECH | Cat# SE200-ES |
| Tissue-Tek/O.C.T. Compound | Sakura | Cat# 4583 |
| Adhesion Microscope Slides | CITOTEST | Cat# 80312-3161 |
| recombinant human FSH (300 IU/0.5 mL) | Merck Serono | Cat# Gonal-f |
| Ethanol, absolute | In house | N/A |

Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| DNA Clean & Concentrator-5 (Capped) | Zymo Research | Cat# D4014 |
| Qubit dsDNA High-Sensitivity Kit | Invitrogen | Cat# Q32851 |
| NEBNext Ultra II DNA Library Prep Kit | New England Biolabs | Cat# E7645L |
| NEBNext Multiplex Oligos for Illumina | New England Biolabs | Cat# E73355 |

Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| TSO primer: AAGCAGTGGTATCAAC GCAGAGTACATrGrG+G | Integrated DNA Technologies | N/A |
| Oligo-dT primers: TCAGACGTGCTCTTTTCCGATC | Integrated DNA Technologies | N/A |
| ISPCR primer: AAGCAGTGGTATCAACGCAGAGT | Integrated DNA Technologies | N/A |
| 3’P2 primer: GTGACGTGAGTTC AGACGTTGCTCTCCTCCGATC | Integrated DNA Technologies | N/A |
| QP2 primer: CAAGCAGAAGACGGCATACGA | Integrated DNA Technologies | N/A |
| Short Universal primer: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC | Integrated DNA Technologies | N/A |

Other

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Abrasive stone | In house | N/A |
| Centrifuge, refrigerated | Eppendorf | S425R |
| Confocal Microscope | Carl Zeiss | LSM880 |
| Micro Scissors | JinZhong, Shanghai | Cat# W10110 |
| Micro Forceps | JinZhong, Shanghai | Cat# WA3090 |
| Fine Forceps | JinZhong, Shanghai | Cat# J3C040 |
| Needles, 27-gauge | BD Microlance | Cat# BD300635 |
| Rubber tubing | Sigma-Aldrich | Cat# A5177 |
| QSP 200, Filtered Tips | Thermo Fisher Scientific | TF140-200-Q |
| ThermoMixer F1.5 | Thermo Fisher Scientific | EP5384000020 |
| DNA LoBind Tubes, 1.5 mL | Eppendorf | Cat# 0030108051 |
| DNA LoBind Tubes, 2.0 mL | Eppendorf | Cat# 0030108078 |
| Qubit Assay Tubes, 0.5 mL | Axygen | Cat# PCR-05-C |
| 0.2-mL Thin Wall PCR Tubes with Flat Cap | Axygen | Cat# PCR-02-C |
| 15-mL High Clarity PP Centrifuge Tube | Corning | Cat# 352096 |
| 50-mL High Clarity PP Centrifuge Tube | Corning | Cat# 352070 |
| Focused-ultrasonicator | Covaris | Cat# M220 |
| Fragment analyzer | AATI | N/A |
| microTUBE Snap-Cap | Covaris | Cat# 520045 |
MATERIALS AND EQUIPMENT

Preparation of gonadotropins

Timing: ~1 h

**PMSG (pregnant mare serum gonadotropin) work solution**

| Reagent                        | Final concentration | Amount  |
|--------------------------------|---------------------|---------|
| PMSG                           | 50 IU/mL            | 1,000 IU|
| 0.9% sodium chloride           | 1 x                 | 20 mL   |

Store at −20°C for 6 months.

Gonadotropins are supplied as lyophilized powder. Reconstitute 1 vial (1,000 IU) of PMSG with 20 mL 0.9% sodium chloride to obtain a work solution of 50 IU/mL, filter through a 0.2 µm filter to sterilize, aliquot, and store at −20°C. Thaw PMSG work solution at room temperature 0.5 h before use. Avoid refreezing.

**hCG (human chorionic gonadotropin) work solution**

| Reagent                        | Final concentration | Amount  |
|--------------------------------|---------------------|---------|
| hCG                            | 50 IU/mL            | 12,500 IU|
| 0.9% sodium chloride           | 1 x                 | 250 mL  |

Store at −20°C for 6 months.

Reconstitute 1 vial (12,500 IU) of hCG with 250 mL 0.9% sodium chloride to obtain a work solution of 50 IU/mL, filter through a 0.2 µm filter to sterilize, aliquot, and store at −20°C. Thaw hCG work solution at room temperature 0.5 h before use. Avoid refreezing.

Preparation of digestive enzymes

Timing: ~1 h

**Hyaluronidase stock solution**

| Reagent                        | Final concentration | Amount  |
|--------------------------------|---------------------|---------|
| Hyaluronidase (lyophilized powder) | 10 mg/mL           | 30 mg   |
| M2 medium                      | 1 x                 | 3 mL    |
| Total                          | N/A                 | 3 mL    |

Store at −20°C for 6 months.

Reconstitute 1 vial (30 mg) of hyaluronidase with 3 mL M2 medium to first obtain a stock solution at 10 mg/mL, filter through a 0.2 µm filter to sterilize, aliquot. Avoid refreezing.
### Hyaluronidase work solution

| Reagent                        | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| Hyaluronidase (10 mg/mL)      | 0.3 mg/mL           | 0.3 mL |
| M2 medium                     | 1 x                 | 9.7 mL |
| **Total**                     | **N/A**             | **10 mL** |

Store at 4°C for 1 week.
Dilute to 0.3 mg/mL with M2 medium as work solution. Prewarm at 37°C for 5 min right before use.

### Collagenase stock solution

| Reagent                        | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| Collagenase, Type IV (lyophilized powder) | 10 mg/mL           | 100 mg |
| M2 medium                     | 1 x                 | 10 mL  |
| **Total**                     | **N/A**             | **10 mL** |

Store at −20°C for 6 months.
Reconstitute 100 mg Type IV Collagenase with 10 mL M2 medium to first obtain a stock solution at 10 mg/mL, filter through a 0.2 μm filter to sterilize, aliquot. Avoid refreezing.

### Collagenase work solution

| Reagent                        | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| Collagenase, Type IV (10 mg/mL) | 1 mg/mL            | 1 mL   |
| M2 medium                     | 1 x                 | 9 mL   |
| **Total**                     | **N/A**             | **10 mL** |

Store at 4°C for 2 weeks.
Dilute to 1 mg/mL with M2 medium as work solution. Prewarm at 37°C for 5 min right before use.

### Lysis buffer

| Reagent                        | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| RNase Inhibitor (40 U/μL)     | 1 U/μL              | 0.05 μL|
| 1% Triton X-100               | 0.475%              | 0.95 μL|
| Barcode oligo-dT primer (10 μM) | 0.3 μM             | 0.15 μL|
| Nuclease-free water           | N/A                 | 0.35 μL|
| dNTP (10 mM)                  | 1 mM each           | 0.5 μL |
| **Total**                     | **N/A**             | **2.0 μL** |

Prepare right before use.

### Elution buffer

| Reagent                        | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| Tris-HCl (1 M, pH 7.5)        | 10 mM               | 0.5 mL |
| Nuclease-free water           | N/A                 | 49.5 mL|
| **Total**                     | **N/A**             | **50 mL** |

Store at 4°C for 6 months.

### Biotinylated index primers

| Primer                        | Sequence                      |
|-------------------------------|-------------------------------|
| Index-primer-#1               | /Biotin/CAAGCAGAAGAGCAGGACGATCA/CAGAGCAGACGTGCTTCCGATC |

(Continued on next page)
### Culture medium setup

#### Follicle culture medium

| Reagent                        | Final concentration | Amount   |
|--------------------------------|---------------------|----------|
| α-MEM                          | 1 ×                 | 45.992 mL|
| Fetal Bovine Serum             | 5%                  | 2.5 mL   |
| Penicillin-Streptomycin (100 ×)| 1 ×                 | 0.5 mL   |

(Continued on next page)
**STEP-BY-STEP METHOD DETAILS**

This protocol provides a framework to decipher principles of folliculogenesis. To reach this goal, single-cell genomics followed by well-controlled *in vitro* cultures to relate comprehensive transcriptome data to causal mechanisms. This protocol involves 5 sections. **Section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles** and **section 2: preparation of single growing oocytes, fully grown oocytes and ovarian somatic cells from growing and antral follicles** are detailed methods to prepare follicular cell suspension for single-cell RNA-seq in **section 3: single-cell RNA-seq library construction**, **Section 4: isolating preantral follicles and culturing up to maturation** and **section 5: isolating immature granulosa cells for in vitro culture** offer detailed methods for follicle and follicular cell culture and depend on preference of researchers to follow this protocol.

**Section 1: Preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles.**

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| ITS Liquid Media Supplement (100 x) | 1 x                 | 0.5 mL |
| EmbryoMax® Nucleosides (100 x)   | 1 x                 | 0.5 mL |
| recombinant human FSH (300 IU/0.5 mL) | 100 mIU/mL         | 0.008 mL |
| **Total**                        | N/A                 | 50 mL  |

Store at 4°C for up to 2 weeks.

**Handling medium**

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| IT5 medium                       | 1 x                 | 44.5 mL |
| Fetal Bovine Serum               | 10%                 | 5 mL   |
| Penicillin-Streptomycin (100 x) | 1 x                 | 0.5 mL |
| **Total**                        | N/A                 | 50 mL  |

Store at 4°C for up to 2 weeks.

**Granulosa cells growth medium**

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| α-MEM                            | 1 x                 | 46 mL  |
| Fetal Bovine Serum               | 5%                  | 2.5 mL |
| Penicillin-Streptomycin (100 x)  | 1 x                 | 0.5 mL |
| ITS Liquid Media Supplement (100 x) | 1 x               | 0.5 mL |
| EmbryoMax® Nucleosides (100 x)   | 1 x                 | 0.5 mL |
| **Total**                        | N/A                 | 50 mL  |

Store at 4°C for up to 2 weeks.

**Granulosa cells starvation medium**

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| α-MEM                            | 1 x                 | 48.5 mL |
| Penicillin-Streptomycin (100 x)  | 1 x                 | 0.5 mL |
| ITS Liquid Media Supplement (100 x) | 1 x               | 0.5 mL |
| EmbryoMax® Nucleosides (100 x)   | 1 x                 | 0.5 mL |
| **Total**                        | N/A                 | 50 mL  |

Store at 4°C for up to 2 weeks.
Section 2: Preparation of single growing oocytes, fully grown oocytes and ovarian somatic cells from growing and antral follicles.

Section 3: Single-cell RNA-seq library construction.

Section 4: Isolating preantral follicles and culturing up to maturation.

Section 5: Isolating immature granulosa cells for in vitro culture.

△ CRITICAL: When preparing a hand-pulled micro capillary pipette for manipulating oocytes, the internal diameter of the opening should be slightly larger than an oocyte (~100–120 μm); and the opening tip need to be polished by swiftly touching a flame. This is important for removal of zona by brief Tyrode’s exposure without damaging oocytes. Handling these samples needs much practice.

Section 1: Preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles

△ Timing: ~0.5–1 h

1. Approximately 15–16 h after hCG injection (Figure 1A), humanely cull the mouse and place on an absorbent pad. Open abdominal cavity, cut peritoneum, and put back digestive system to expose reproductive organs, including two ovaries, two oviducts and two horns of uterus for each mouse.

2. Grasp the upper end of uterus and gently pull away from body, make the first cut between uterus and utero-tubal junction (a narrow tube connects uterus and oviduct). Relocate the forceps near to utero-tubal junction, then a second cut is made between infundibulum of oviduct (the opening of oviduct) and ovary (Figure 1B). Repeat to cut off the other side of oviduct. Put oviducts into a drop of M2 medium in a 35-mm Petri dish.

Note: Prewarm M2 medium at 37°C for 5 min right before use.

△ CRITICAL: Pay attention not to break oviduct during dissection.

3. Use a pair of fine forceps to grasp isthmus and hold oviduct on the bottom of the Petri dish, immerse the oviduct in a drop of M2 medium. Use the second pair of fine forceps to tear ampulla (Figure 1C1) where cumulus-oocyte complexes are located, releasing the clutches of cumulus-oocyte complexes (Figure 1C2).

△ CRITICAL: In response to injection of gonadotropins, ampulla is usually swollen and transparent to be easily identified under a dissecting microscope.

4. Use a 20-μL tip to dispense 2 drops (20-μL) of hyaluronidase medium and 2 drops (20-μL) of M2 medium on the bottom of a 35-mm Petri dish (Figure 1D).

5. Transfer the collected cumulus-oocyte complexes by using forceps into the first drop of hyaluronidase. Under a dissecting microscope, incubate cumulus-oocyte complexes in hyaluronidase solution for 2–3 min (no more than 5 min) until the cumulus cells start to loosen. Oocytes surrounded by more sticky cumulus cells should be transferred by a finely pulled glass pipette into a second fresh drop of hyaluronidase, observe until cumulus cells fall off.

Note: For oocyte surrounded by cumulus cells that are easily shed, only the first drop of hyaluronidase is needed. For stickier cumulus cells, transfer of cumulus-oocyte complexes into the
second fresh drop of hyaluronidase is important to minimize exposure time in hyaluronidase. Pipette up and down with Pasteur pipette for a few times helps.

6. Use a hand pipette, pick up the dissociated individual oocytes and granulosa cells into a fresh drop of M2 medium and perform two washes in M2 medium droplets.
7. Use a 20-µL tip to dispense 2 drops (20-µL) of Tyrode’s medium and 2 drops (20-µL) of M2 medium on the bottom of a 35-mm Petri dish (Figure 1E).
8. Transfer a group of 10–12 oocytes into the first Tyrode’s drop, pipette up and down for 3–5 times, further transfer the oocytes into the second Tyrode’s drop, observe until zona pellucida gradually becomes thinning and disappears. Representative pictures of oocytes with zone before Tyrode’s treatment and without zona after Tyrode’s treatment are shown in Figures 1F1 and F2, respectively.

Note: Expunge the remaining medium in the hand Pasteur pipette into a blank Petri dish (for waste collection) before refill it with new Tyrode’s medium, as any trance of M2 carried would neutralize Tyrode’s effects.
9. Use a Pasteur pipette to pick up the zona-free oocytes into a drop of M2 medium to wash, then repeat once in another drop of M2.

10. Use a 20-μL tip to dispense several drops (20-μL) of 0.1% bovine serum albumin (BSA) (dissolved in 1× DPBS).

11. Use a Pasteur pipette to transfer granulosa cells (from step 6) and zona-free oocytes (from step 9) sequentially through two drops of 0.1% BSA (dissolved in 1× DPBS) to perform washes.

12. Pick single oocytes or granulosa cells into lysis buffer that is ready to prepare single-cell cDNA (complementary DNA).

**Note:** Promptly pick disaggregated single cells into lysis buffer and keep them immediately on ice.

**Section 2: Preparation of single growing oocytes, fully grown oocytes, and ovarian somatic cells from growing and antral follicles**

**Timing:** ~1–1.5 h

13. Approximately 42–44 h after injection of a single shot of PMSG, collect secondary follicles from ovaries of 3- to 4-week-old mice; collect secondary and antral follicles from ovaries of 7- to 8-week-old mice (Figure 2A).

14. Ovaries are dissected, carefully trimmed, and chopped into 0.5-mm³ cubes.

**Note:** The following digestive volume is recommended for 2 ovaries to ensure optimal performance.

15. Use a two-stage of digestion procedure to prepare single cell suspension. Digest the minced ovaries in 400μL 1 mg/mL Type IV collagenase at 37°C under 1,000 × rpm for 20 min in a ThermoMixer (Figure 2B).

16. Spin at 300 × g for 3 min at room temperature and discard the resulting supernatant.
17. Resuspend the pellet in 400 μL of prewarmed TrypLE Express at 37°C under 1,000 × rpm for 10–15 min (Figure 2B). Observe under a dissecting microscope to monitor status of cells during dissociation. It is important to gently homogenize by intermitting pipetting every 7–8 min during digestion.

△ CRITICAL: Visualize cell suspensions is important for evaluation of cell viability. If cell aggregates are observed, one should perform further gentle pipetting; if debris is observed, one should perform filtering. Once single-cell suspensions are obtained, promptly process to stop digestion. Avoid over incubation in digestive enzymes to avoid damaging cells or lysing.

18. Stop digestion by adding 400μL of prewarmed M2 medium, pipette the resulting cell suspension and transfer it into a 35-mm Petri dish, at this point single somatic cells or single oocytes could be obtained.

19. Use a 20-μL tip to dispense 2 drops (20-μL) of M2 medium on the bottom of a 35-mm Petri dish.

20. Use a hand glass pipette, pick up the dissociated individual oocytes and somatic cells into a fresh drop of M2 medium and perform two washes in M2 medium droplets.

21. Use a 20-μL tip to dispense 2 drops (20-μL) of Tyrode’s medium and 2 drops (20-μL) of M2 medium on the bottom of a 35-mm Petri dish (Figure 2C).

22. Remove zona pellucida encapsulating growing oocytes by Tyrode’s solution, and then wash zona-free oocytes immediately through 2 drops of M2 medium.

23. Wash somatic cells (from step 20) and zona-free oocytes (from step 22) in 0.1% BSA (dissolved in 1 x DPBS).

24. Pick individual zona-free oocytes or somatic cells into lysis buffer that is ready to prepare single-cell cDNA (Figure 2D).

Note: Promptly pick disaggregated single cells into lysis buffer and keep them immediately on ice.

Section 3: Single-cell RNA-seq library construction

◎ Timing: ~2 days

25. Individual single cells from step 12 or step 24 are manually picked into individual 0.2-mL PCR tubes by a hand glass pipette, vortex thoroughly for 30 s, incubate at 72°C for 3 min in a Thermal Cycler, then immediately place on ice.

26. Add 2.85 μL reverse transcription (RT) mix to each sample.

27. Vortex briefly and spin down at 1,000 × g for 1 min at 4°C, perform PCR in a Thermal Cycler as below:

| RT PCR cycling conditions | Temperature | Time  | Cycles |
|---------------------------|-------------|-------|--------|
| Steps                     | 1: 25°C     | 5 min | 1      |
|                           | 2: 42°C     | 60 min| 1      |
|                           | 3: 50°C     | 30 min| 1      |
|                           | 4: 70°C     | 10 min| 1      |
|                           | 5: 4°C      | Hold  | N/A    |

28. Add 7.5 μL PCR preamplification mix to each reaction.

29. Vortex briefly and spin down at 1,000 × g for 1 min at 4°C, perform PCR in a Thermal Cycler as below to amplify cDNA:
30. Pool 4 μL of each cDNA sample with different barcodes (up to 96 barcodes) together.
31. Purify once with Zymo DNA Clean and Concentrator-5 Kit according to manufacturer’s protocol, elute in 50 μL elution buffer. Purify twice with 0.8 × Ampure XP beads, elute in 21 μL elution buffer.
32. Check size distribution of cDNA samples by a Fragment Analyzer (AATI). The profile of a successful cDNA sample should have a peak at around 1.5 kb (see expected outcomes).
33. Use 30–40 ng cDNAs as template, add 29 μL index PCR mix, with Biotin index primer to perform a second amplification.
34. Perform PCR in a Thermal Cycler as below:

| Steps | Temperature | Time | Cycles |
|-------|-------------|------|--------|
| 1     | 95°C        | 3 min| 1      |
| 2     | 98°C        | 20 s | 4      |
| 3     | 65°C        | 30 s |        |
| 4     | 72°C        | 5 min|        |
| 5     | 98°C        | 20 s | 16     |
| 6     | 67°C        | 15 s |        |
| 7     | 72°C        | 5 min|        |
| 8     | 72°C        | 5 min| 1      |
| 9     | 4°C         | Hold | N/A    |

35. The biotinylated cDNAs are purified once with 0.8× Ampure XP beads, elute in 30 μL elution buffer.

**Pause point:** The cDNA samples can be stored at −20°C or −80°C for 8 weeks or longer.

36. Shear cDNAs into 300-bp fragments by an ultrasonicator (Covaris) according to manufacturer’s protocol.
37. Purify once with Zymo DNA Clean and Concentrator-5 Kit according to manufacturer’s protocol, elute in 50 μL elution buffer. Enriched by using Dynabeads® MyOne™ Streptavidin C1.
38. Construct RNA-seq libraries by using NEBNext Ultrall DNA Library Prep Kit for Illumina according to manufacturer’s instructions. Use QP2 primer and Short Universal Primer to conduct final library PCR amplification.
39. Pool the final RNA-seq libraries together, measure concentration by the Qubit Fluorometer, check size distribution and process to sequence. The expected average size of final libraries is around 200–800 bp, with a peak occurs at around 400 bp. The yield of the library is usually more than 100 ng.

**Pause point:** The RNA libraries can be stored at −20°C or −80°C for 8 weeks or longer.
Section 4: Isolating preantral follicles and culturing up to maturation

Timing: ~6.5 days

Note: How different systems should be chosen according to aim of the study is reviewed in (Simon et al., 2020). In this protocol, to trace growth of each individual follicle, the selected follicles are cultured singly into each well of a 96-well microplate; to avoid any perturbations to lyophilic steroids signalling, the follicles are cultured without mineral oil overlay.

To verify candidate factors instructing granulosa-theca interactions or stepwise transition across folliculogenesis, prepuberty mice at 14 days are used to isolate immature follicles for culture. At this age, early preantral follicles predominate, only a few follicles reach antral stage, no corpus lutea forms yet.

40. Prepare culture medium for follicle culture (see in materials and equipment section).
41. Sterilize surgery tools and pipettes by heating them in a sterilization oven in advance.
42. Dissect ovaries from 14-day-old female mice, trim and transfer them into L15 medium supplied with 10% FBS and 1 × penicillin-streptomycin.

Note: Make sure the following steps to dissociate follicles for culture are performed in sterile conditions.

43. Transfer ovaries to a laminated-flow hood, immerse them in L15 medium supplied with 10% FBS and 1 × penicillin-streptomycin (15–20 μL medium for 2 ovaries), and mechanically chop into small pieces with a metal blade.

Note: To preserve follicular integrity and investigate intra-follicle cell-cell interactions, preantral follicles are released mainly by mechanical dissection with only brief collagenase treatment, rather than enzymic digestion.

44. Incubate minced ovary tissue in 1 mg/mL Type IV collagenase, at 37°C under 1000 × rpm for 10–15 min in a ThermoMixer, to dissociate connective tissues (Figure 3A). A representative picture showing chopped ovary tissue transferred into Type IV collagenase at the start of digestion (Figure 3B).
45. Spin at 300 × g for 3 min at room temperature, resuspend the resulting pellet in L15 medium and transfer them into a 35-mm Petri dish.
46. Released individual intact follicles are manually picked by using a hand pipette, based on two criteria: (1) with diameters around 120 μm; (2) oocyte located centrally within the follicle and is surrounded by 1–2 layers of granulosa cells, a basal membrane, and a layer of thecal cells (Figure 3C).
47. Under a dissecting microscope, pool the picked follicles and sequentially wash them through three 50-μL drops of L15 medium by a hand pipette, then randomly assign to different treatment groups.

Note: Perform washes <30 min if possible.

48. Use a 20-μL tip to dispense a 20-μL drop of follicle culture medium in each well of a 96-well plate. Culture the follicles singly in each well of a 96-well plate in an incubator at 37°C containing 5% CO₂, designating as day 1 of culture.
49. On day 2 of culture, further add 10 μL medium to each follicle droplet; thereafter refresh 10 μL of old medium with fresh medium in each follicle droplet every other day.
50. Perform morphological evaluation under a microscope every day until day 6 of culture.

Note: In normal conditions, follicles can grow and expand to form a well-organized structure, onset of antrum-like cavity formation. In this in vitro setting, phenotypes like initiation of granulosa-cell death under certain treatment could suggest a role of the treatment. We have previously used this system to study function of Notch signalling pathway in affecting folliculogenesis (Gu et al., 2019).

Alternatives: Morphological evaluation provides a convenient, time-sequential while relatively gross assessment of follicle growth. If needed, histological characteristics of sections of the cultured follicles could provide a detailed phenotype, for example, whether and when atresia and cell death of granulosa cells initiates (Cortvrindt et al., 1996).

Section 5: Isolating immature granulosa cells for in vitro culture

Timing: ~2.5 days

Granulosa cells isolated from immature follicles for in vitro culture are neat to circumvent its intrinsic and substantial heterogeneity during in vivo growth, thus provide a way to directly assess factors affecting granulosa differentiation.

Note: Prepuberty mice between postnatal days 12–14 are used to isolate immature granulosa cells for culture.

51. Prepare granulosa cells culture medium (see in materials and equipment section).
52. Collect preantral follicles as described in step 40 to step 46.
53. Incubate the collected preantral follicles in 400μL TrypLE Express at 37°C for 10–15 min, and gently pipette to liberate follicular cells (Figure 3D).
54. Stop digestion by adding an equal volume of M2 medium.
55. Spin at 300 × g for 5 min at room temperature and discard the resulting supernatant. Resuspend cells with M2 medium.
56. Filter cell suspension through a 30-μm cell strainer to obtain granulosa cells.
57. Centrifuge the flow-through at 300 × g for 5 min at room temperature and resuspend the pellet in granulosa cells growth medium.
58. Seed cells in a 24-well plate and the plating density is 1 × 10^6 cells/well. Culture cells in granulosa cells growth medium, allowing cells to adhere for 3 h in an incubator at 37°C containing 5% CO₂.
59. Granulosa cells are starved for 4 h in granulosa cells starvation medium prior to the beginning of experiment treatment. According to what treatment needed, add the substance in granulosa cells starvation medium for further culture (Figure 3D).

EXPECTED OUTCOMES

For the single-cell RNA-seq part, the profile of a successful cDNA sample should have a peak at around 1.5 kb (Figure 4A). The expected average size of final sequencing libraries is around 200–800 bp, with a peak occurs at around 400 bp (Figure 4B). The yield of the library is usually more than 100 ng. The UMAP (Uniform Manifold Approximation and Projection) validates that oocytes, granulosa cells and theca cells enrichment with our method in this protocol (Figure 5).

For the follicle culture part, on day 1 of culture, preantral follicle is composed of an oocyte surrounded by 1–2 layer(s) of granulosa cells, a basal membrane, and some adhering theca cells and/or interstitial cells (Figure 6). By days 6 of culture, in normal conditions, granulosa cells of follicles proliferate and break the basal membrane, the initial in vivo organization of follicle is lost while a diffuse or outgrowth pattern is observed (Figure 6).

For the granulosa cell culture part, the purity of isolated granulosa cells should be firstly examined by immunofluorescence analysis by using granulosa-specific markers, for example, Foxl2 (Forkhead box L2) and Cnmd (chondromodulin) (Figure 7). After a designed treatment, effects of the treatment can be evaluated with regard to proliferation, differentiation and steroidogenic capacity of granulosa cells.

LIMITATIONS

This protocol allows to enrich oocytes and capture rare follicular somatic cells to focus on soma-oocyte communications within a follicle. However, an unbiased whole ovary transcriptome cannot be profiled using this protocol, because individual follicles are first isolated to prepare single cell suspension. Further, this protocol is more laborious than whole ovary dissociation followed by high-throughput 10x scRNA-seq. Dissociation of follicles needs to be performed by an experienced experimenter to ensure cell integrity and viability. Pick follicles, single cells, and removal of zona pellucida are also skillful.

TROUBLESHOOTING

Problem 1
Just a few ovulated oocytes are found after superovulation (visible in the before you begin).

Potential solution
Low efficiency of superovulation could be due to several reasons.

Allow newly arrived mice to get acclimated for one week before use, as shipping process, new facility and environment would interfere with several physiological responses. For example, a changed dark-light cycle is known to disturb endogenous LH peak in response to PMSG administration during superovulation.
**Figure 4. AATI Fragment Analyzer electropherograms show size distribution of cDNA sample and RNA library**

(A) A representative example of cDNA size. Profile of a successful cDNA sample shows a peak at about 1–2 kb, and the amount of short fragments is low. DNA profile is measured in relative fluorescence units (RFU). LM, lower marker. UM, upper marker.

(B) A representative example of sequencing cDNA library. The average size of a successful cDNA library is 200 bp to 800 bp, and a broad peak is observed at about 400 bp. DNA profile is measured in relative fluorescence units (RFU). LM, lower marker. UM, upper marker.
Check that the gonadotropins are not expired. Each new batch of gonadotropins should be tested before the beginning of experiments, as efficacy of gonadotropins varies. This can be tested by performing superovulation for fertile females and checking the number of oocytes that can be recovered. In our experience, 15–25 oocytes can be recovered from 6- to 7-week-old C57BL/6J mice.

Dosages of gonadotropins are mostly determined by bodyweights of mice, usually 5 IU of PSMG or hCG is used for 4-week-old mice and 10 IU of PSMG or hCG for 8-week-old mice. Optimization of dosages is also recommended, as possibly there are also strain variations in response to gonadotropins.

**Problem 2**
Ampulla is not enlarged after superovulation (visible in the section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles, step 3).

**Potential solution**
Occasionally ampulla is not swollen 15 h post hCG injection, likely because the cumulus-oocyte complexes start to diffusing. Oocytes can be collected by flushing from infundibulum to utero-tubal junction by using a needle attached to a 1 mL-syringe. Pay attention to gently flush and avoid M2 medium splashing through the narrow utero-tubal junction, which would lose almost all the oocytes. About 0.1 mL prewarmed M2 is needed for each side of oviduct flushing.

**Problem 3**
Zona-free oocytes stick to a Petri dish (visible in the section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles, step 8).

**Potential solution**
Zona-free oocytes sticking to a plastic dish would be difficult to recover without damage. Thus, avoid leaving oocytes reaching bottom of Petri dish by gently pipette up and down, and transfer the
oocytes into M2 medium quickly after zona disappears. Prolonged exposure in Tyrode’s solution impairs oocytes quality and can be lethal in extremely prolonged exposures.

**Problem 4**
Incomplete zona removal (visible in the section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles, step 8).

**Potential solution**
After Tyrode’s incubation, oocytes should be checked under a 25 × magnification to determine whether a thin zona remains, as this would compromise efficiency of cell lysis. Incomplete zona removal is mostly caused by carryover of M2 medium that neutralizes Tyrode’s. If this is the case, prepare another fresh drop of Tyrode’s solution and repeat zona-removal step. Also check that the Tyrode’s solution is not expired.

**Problem 5**
Quality of cDNA sample is poor (visible in the section 3: single-cell RNA-seq library construction, step 32).

**Potential solution**
The key for RNA-seq is to ensure high RNA quality. A large amount of short fragments in cDNA size distribution profile indicates RNA degradation. To avoid RNA degradation, make sure to set up workbench, tubes, and pipette tips free from RNase and DNase. To ensure cell viability and achieve high-quality mRNA, minimize exposure time of cells in digestive enzymes and promptly pick individual cells into lysis buffer after dissociation. Cells in lysis buffer should be kept on ice until processed to RT.

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**Figure 6.** Representative pictures showing morphologies of an isolated preantral follicle at day 1 of culture, and a diffused follicle after 6 days of culture.
Black arrowhead indicates the oocyte, white arrowhead indicates onset of an antrum-like cavity. Scale bar, 50 μm.

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**Figure 7.** Characterization of in vitro cultured granulosa cells
Cultured primary granulosa cells are stained for the granulosa cell marker Cnmd, verifying the purity of isolated cells. Scale bar, 20 μm. Adapted with permission from (Long et al., 2022).
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fuo Guo (guofan@ioz.ac.cn).

Materials availability
This study did not generate new material or reagents.

Data and code availability
For detailed analysis and the datasets generated across folliculogenesis, please refer to (Gu et al., 2019; Long et al., 2022).

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AUTHOR CONTRIBUTIONS

F.G. conceived and designed the project. J.Q., R.Z., and R.Y. performed experiments. X.L. performed single-cell analysis. J.Q. and F.G. wrote the manuscript. All the authors approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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