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siRNA-mediated depletion of stable proteins in mouse oocytesAzusa Inoue^{1-4*}, Kazuko Sunaga⁶, Fugaku Aoki⁶ and Yi Zhang¹⁻⁵

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INTRODUCTION

Maternal proteins stored in mammalian oocytes play various roles in meiotic maturation, fertilization, and preimplantation development. To understand biological functions and molecular mechanisms underlying the dynamic developmental processes, it is necessary to deplete maternal proteins in oocytes. One way to achieve this is by generating a knockout (KO) mouse of a target gene; analyses of oocytes harvested from KO mice are helpful for understanding the gene function. Nevertheless, when the target gene is essential for embryonic development or germ cell development, it becomes impossible to collect oocytes from the KO mice. Although this issue could be overcome by generation of conditional KO mice using a Cre recombinase that is expressed by germ-line specific promoters such as TNAP, GDF9, and Zp3 (Andreu-Vieyra et al., 2010; Gu et al., 2011), generation of mutant mice can prove time consuming, thereby making this strategy challenging.

An alternative way to deplete maternal proteins is RNA interference (RNAi)-mediated knockdown of interested genes. This can be achieved by microinjection of short interfering RNA (siRNA) into oocytes. Many studies have indeed tried to deplete maternal proteins by injecting siRNA into metaphase II (MII)-stage oocytes (Okada et al., 2010; Wen et al., 2014; Wossidlo et al., 2011) or fully grown GV-stage oocytes (Inoue et al., 2012). However, oftentimes siRNA-injection at these stages cannot significantly deplete maternal proteins that have already been stored in oocytes. One of the reasons for this issue is that the loss of interested proteins after siRNA-injection solely depends on the protein half-life, which is different from the case of culture cells in which both the protein half-life and dilution through cell proliferation cooperatively contribute to the protein depletion. Although a prolonged culture of MII or GV oocytes following siRNA-injection may allow the targeted proteins to be further degraded, it impairs the quality of oocytes, and the oocytes are eventually fragmented (or MII oocytes are spontaneously activated) after a certain incubation period. Thus, the culture time following siRNA injection is limited when using MII or GV oocytes (for up to 8 or 24 h, respectively), which makes it difficult to deplete stable maternal proteins by this system. Another reason is that oocytes accumulate a large amount of mRNAs and proteins during oocyte growth to support the subsequent events including meiotic maturation and early development. Therefore, due to protein accumulation derived from growing oocyte, fully-grown GV- and MII-stages are sometimes not appropriate for protein depletion by siRNA injection.

To overcome these issues, we developed a novel knockdown system that allows depleting even stable proteins in mouse oocytes (Inoue and Aoki, 2010; Inoue and Zhang, 2014). In this system, follicles are collected from ovaries of 12 day-old mice, oocytes within the follicles are injected with siRNA, and then the injected follicles are cultured *in vitro* for 12 days until the oocytes reach the fully-grown GV-stage (**Figure 1**). This system provides a large enough time window following siRNA injection to degrade the target proteins via their half-life. *In vitro* maturation (IVM) followed by the *in vitro* growth (IVG) allows obtaining MII oocytes, and subsequent *in vitro* fertilization (IVF) provides zygotes and preimplantation embryos with depleted target maternal proteins (**Figure 1**). Application of this method will greatly help reveal the mechanism and function of molecular events occurring in mouse oocytes and preimplantation embryos.

MATERIALS

Reagents

- M2 medium (Millipore #MR015-D)
- α -MEM with GlutaMax (Life technologies, #32571-036)
- α -MEM (Life technologies, #12571-063)
- KSOM (Millipore #MR-020P)
- Insulin solution (x800 stock)
 - Insulin, human recombinant, zinc solution, 4 mg/ml (Life technologies, #12585-014)
 - 20 μ l aliquot/tube, store at -20C.
- Transferrin solution (x1000 stock)
 - Transferin human (Sigma-Aldrich #T8158)
 - 5 mg/ml water, 20 μ l aliquot/tube, store at -20C.
- Sodium selenite solution (x1000 stock)
 - Sodium Selenite (Sigma-Aldrich, #S5261)
 - 5 μ g/ml water, 20 μ l aliquot/tube, store at -20C.
- PMSG solution (x1000 stock)
 - Gonadotropin, Pregnant Mare Serum (Millipore/Calbiochem, #367222)
 - 100 IU/ml water, 10 μ l aliquot/tube, store at -20C.
 - This PMSG stock solution should be made freshly every 3 months.
- Fetal Bovine Serum (FBS) (Sigma-Aldrich, #F0926)
 - 700 μ l aliquot/tube, store at -20C.
- EGF solution (x1000 stock)
 - Epidermal Growth Factor from murine submaxillary gland (Sigma-Aldrich, #E4127)
 - 10 μ g/ml water, 20 μ l aliquot/tube, store at -20C.
- Penicillin-Streptomycin (Life technologies #15140-122)
- Polyvinylpyrrolidone (PVP, Molecular Weight 360,000, Sigma-Aldrich #81440)
- Mineral oil (Sigma-Aldrich #M8410)
- siRNA
 - Purchase siRNAs from any of the following companies.
 - Silencer Select Pre-Designed & Validated siRNA (Life technologies;
<http://www.lifetechnologies.com/us/en/home/life-science/rnai/synthetic-rnai-analysis/ambion-silencer-select-sirnas/silencer-select-sirna.html.html>)

- ON-TARGETplus siRNA (GE Healthcare/Dharmacon;
<http://dharmacon.gelifsciences.com/sirna/on-targetplus-sirna-reagents-mouse/>)
- Custom Stealth siRNA (Life technologies;
<http://www.lifetechnologies.com/order/catalog/en/US/adirect/lt?cmd=ConfigureRNASingleTube&stealth=true>)

Dissolve siRNAs to 50 μM with nuclease-free water and store at -80C (siRNA stock). For the preparation of siRNA working solution, dilute the stock to 2-20 μM with nuclease-free water, aliquot (3 μl /tube), and store at -80C . The concentration of siRNA is dependent on the knockdown efficiency. To minimize a potential side effect, determine the minimum siRNA concentration (range: 2-20 μM) that can deplete the target proteins.

Media

(i) M2 medium (Millipore #MR015-D)

5 ml aliquot/tube and store at -20C . After thaw, store at 4C for up to 2 weeks.

(ii) *in vitro* growth (IVG) medium without PVP (Eppig and O'Brien, 1996; Hartshorne, 1997; Lenie et al., 2004; Smits and Cortvrindt, 2002)

Take 10 ml of α -MEM with GlutaMax (Life technologies, #32571-036) that is opened within 3 months. Add 12.5 μl insulin (f. 5 $\mu\text{g}/\text{ml}$), 10 μl transferrin (f. 5 $\mu\text{g}/\text{ml}$), 10 μl sodium selenite (f. 5 ng/ml), and 10 μl Penicillin & Streptomycin. After filtration, add 500 μl FBS (f. 5%) and 10 μl PMSG (f. 100 IU/L). Store at 4C for up to 1 month.

(iii) IVG medium with PVP (Hirao, 2012)

Take 40 ml of α -MEM with GlutaMax (Life technologies, #32571-036) and add 800 mg PVP (f. 2%). Gently mix for > 1 h at 4C until the PVP is completely dissolved. Filtrate and separate into 4 tubes (10 ml each). Store at 4C until use.

Add 12.5 μl insulin (f. 5 $\mu\text{g}/\text{ml}$), 10 μl transferrin (f. 5 $\mu\text{g}/\text{ml}$), 10 μl sodium selenite (f. 5 ng/ml), and 40 μl Penicillin & Streptomycin (Note: We use 1/10 concentration of manufacturer's recommendation) to the 10 ml medium. After filtration, add 500 μl FBS (f. 5%). Store at 4C for up to 2 weeks.

On the day prior to use, take an arbitrary volume of the medium and add PMSG (f. 100 IU/L). After adding PMSG, store at 4C and use the medium within 2 days.

(iv) *in vitro* maturation (IVM) medium

Take 10 ml of α -MEM (Life technologies, #12571-063) that was opened within 3 months. Add 500 μ l FBS (f. 5%) and 10 μ l EGF (f. 10 ng/ml). Store at 4C for up to 2 weeks.

(v) *in vitro* fertilization (IVF) medium

HTF medium should be made by hand. Do not use a commercial HTF that is not effective for IVF following IVM. Components of the HTF are shown in **Table 1**.

(vi) Embryo culture medium

A commercial KSOM (Millipore #MR-020P) can be used. However, we recommend preparing it by hand because the developmental ratio of embryos in a homemade KSOM is higher than with commercial medium. Components of KSOM are shown in **Table 2**.

* Preincubate the media covered by mineral oil overnight in a CO₂ incubator, except for M2 medium.

Equipments

- CO₂ incubator (a humidified atmosphere of 5% CO₂/95% air at 37.8°C)
- FemtoJet (Eppendorf)
- Puller (Sutter instrument, Model P-97)
- Microforge (Narishige, MF-900)
- Borosilicate glass (Sutter instrument, #B100-75-10)
- Inverted microscopy for micromanipulation
- Nunc Cell Culture/Petri Dishes (Thermo Scientific, #150318)
- Femtotips Capillary Pipet Tips (Eppendorf, #930001007)

PROCEDURE

Collection of follicles

1. Collect follicles from 12 day-old B6D2F1 females (5.5-7.8 g).

Harvest ovaries into 300 μ l of M2 medium (i). Puncture them using a 30-gauge needle to release follicles.

Note: Softly (not to injure follicles), but thoroughly, puncture the ovaries.

2. Select follicles suitable for IVG.

Although >100 follicles can be collected from a female, most of them are not suitable for IVG. Follicles meeting all the morphological criteria described below have to be selected.

The criteria include the proper size of follicles (100-125 μm), the proper size of oocyte (50-65 μm), the central position of oocyte within the follicle, the round shape of both follicle and oocyte, and the high density of granulosa cells. Further information on selection criteria can also be found elsewhere (Hartshorne, 1997; Lenie et al., 2004; Pesty et al., 2006). Usually, 10-20 follicles per female can be collected.

Note: Strictly apply all the criteria described above. Discard follicles that do not meet any one of the criteria (see examples in **Figure 2A**) because they will fail to grow *in vitro*.

3. Transfer the follicles to the IVG medium without PVP (ii) and incubate for 1 h.

The 1 h incubation before microinjection softens the follicles, making them easy for microinjection.

4. Select follicles that meet all the criteria described above again.

The shape of follicles and oocytes change slightly during the 1 h incubation. Apply the strict criteria again.

5. Place ~15 follicles into a M2 droplet prepared in a microinjection chamber (see details in Ittner and Gotz, 2007).

Microinjection into oocytes within follicles

- 6-1. Prepare an injection capillary following the protocol for pronuclear injection (Ittner and Gotz, 2007). The Puller protocol setting we use is: P=300, Heat=635, Pull=75, Vel=75, Del=80. Fill siRNA solution with FemtoTips.

- 6-2. Prepare a holding pipette.

The Puller protocol setting we use is: P=500, Heat=800, Pull=80, Vel=120, Del=100. The inner diameter of holding pipettes should be 50-55 μm to hold a follicle very firmly (although the best diameter may depend on the instrument used, we recommend to make it larger than the one used for regular manipulation).

- 6-3. Hold a follicle firmly (**Figure 2B**) and insert the injection capillary into the granulosa cell layers (do not penetrate the oocyte yet). With a single shot of the FemtoJet, release the siRNA solution within the granulosa cell layers, and estimate the amount of siRNA solution released from the capillary (**Figure 2C**). Adjust the injection pressure. **Troubleshooting.**

Note: It is important to adjust the injection pressure every time before penetrating the oocyte in order to inject a constant amount of siRNA into each oocyte. The amount of siRNA

solution released from the capillary can be estimated by the movement of granulosa cells.

6-4. Penetrate the capillary into the oocyte cytoplasm and inject siRNA. A successful injection is evident by movement of cytoplasmic granules within the oocyte.

6-5. After withdrawing the injection capillary, whip the capillary along the holding pipette several times to remove debris from the capillary tip and keep it clean.

Note: Because granulosa cells are sticky, the injection capillary frequently becomes partially clogged, making it difficult to inject a constant amount of siRNA into each oocyte. To avoid this problem, the capillary should be kept clean by whipping it along the holding pipette and flushing it with the maximum pressure of FemtoJet (see the manufacturer's instructions of FemtoJet) every time after withdrawal from a follicle.

6-6. Repeat steps 6-3 to 6-5.

6-7. Ten minutes after microinjection, transfer the injected follicles into the medium (ii) and incubate for 1 h.

***In vitro* growth (IVG)**

7. Discard follicles with lysed oocytes. Normally, up to 10% of oocytes die at this step.

8. Transfer the follicles into the IVG medium with 2% PVP (iii).

Up to 7 follicles can be cultured in a 50 μ l droplet. Four 50 μ l droplets can be prepared in a 35 mm dish.

Note: Do not place the follicles close to each other to avoid aggregation during IVG. When carrying the culture dish, be careful to minimize vibrations.

9. Change half of the culture medium (iii) every other day.

Prepare the IVG medium (iii) in which PMSG is freshly added, and then preincubate it in a CO₂ incubator overnight. Representative pictures of follicles cultured *in vitro* for 12 days can be found elsewhere (Inoue and Zhang, 2014; Lenie et al., 2004; Smits and Cortvrindt, 2002). After 12 days, 80-100% of oocytes normally reach the fully grown GV-stage.

Troubleshooting.

Note: Change the media very slowly to avoid detaching the follicles from the bottom of dish.

***In vitro* maturation (IVM)**

10. After 12 days of culture, harvest cumulus-oocyte complexes (COCs) using a glass pipette.
Do not completely denude the cumulus cells surrounding an oocyte (**Figure 3**).
11. Wash COCs 4-5 times and culture them in α -MEM (iv) for 15-18 h. Successful meiotic maturation is evident by cumulus expansion and the 1st polar body extrusion (**Figure 3**).
After IVM, 80-100% of oocytes can complete meiotic maturation.

***In vitro* fertilization (IVF)**

12. Harvest sperm from the caudal epididymides of a >8 week-old B6D2F1 male and incubate in 200 μ l HTF medium (v) for 1 h for capacitation.
13. Transfer the MII-stage oocytes with surrounding cumulus cells into a 100 μ l HTF medium.
Do not remove cumulus cells.
14. Inseminate 1.0-1.5 μ l of preincubated sperm ($>3 \times 10^4$ sperm cells).
15. Six to eight hours after insemination, transfer the fertilized oocytes into a 1:1 mixture droplet of HTF:KSOM and incubate for 30 min. Fifty-80% of oocytes become normally fertilized as determined by the presence of 2 pronuclei. ***Troubleshooting***.
16. Transfer the fertilized oocytes into KSOM (vi). Five days after fertilization, 20-40% of embryos can develop to the blastocyst stage. ***Troubleshooting***.

Troubleshooting

1. The injection capillary cannot be penetrated into follicles.
 - Hold follicles very firmly. Enlarge the inner diameter of a holding pipette. A representative image of holding pipette is found in **Figure 2B**.
 - The injection capillary should not be too thick.
 - Rotate the follicle and penetrate into the place where granulosa cell layers are relatively thinner.
2. The injection capillary becomes frequently clogged.
 - Whip the injection capillary along the holding pipette every time after injection.
 - Release siRNA solution with the maximum pressure every time after injection.
 - Increase the diameter of injection capillary tip.
 - Replace the capillary.
3. Oocytes do not grow. / Granulosa cells do not proliferate during IVG.

- Make sure to select high quality follicles only that meet all the criteria described above. Representative follicles with high quality are shown in **Figure 2B**.
 - Prepare PMSG stock solution freshly.
4. Fertilization ratio is low.
- Use homemade HTF instead of commercial formulation.
 - HTF should be freshly made at least every 2 months.
 - Increase sperm concentration for IVF.
5. Embryos do not develop to the blastocyst stage.
- Use homemade KSOM.
 - Check the quality of mineral oil, KSOM, and culture environments. The developmental ratio of haploid parthenogenetic embryos to the blastocyst stage (>30%) is a good indicator of optimal embryo culture condition.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Experimental scheme for siRNA-mediated knockdown of maternal proteins using the *in vitro* growth system. Follicles are isolated from 12 day-old mice, injected with siRNA, and cultured for 12 days *in vitro* until they reach the fully grown GV-stage. Following *in vitro* growth, oocytes are matured and fertilized *in vitro*.

Figure 2. Selection and microinjection of follicles. (A) Follicles unsuitable for IVG. Scale bar, 200 μm . (B) Image of microinjection into the follicles with high quality. Scale bar, 200 μm . (C) Estimation of the volume of released siRNA solution by a single shot within granulosa cell layers.

Figure 3. *In vitro* maturation following *in vitro* growth. Detailed explanations are described in the figure. Scale bars, 200 μm .

Figure 1

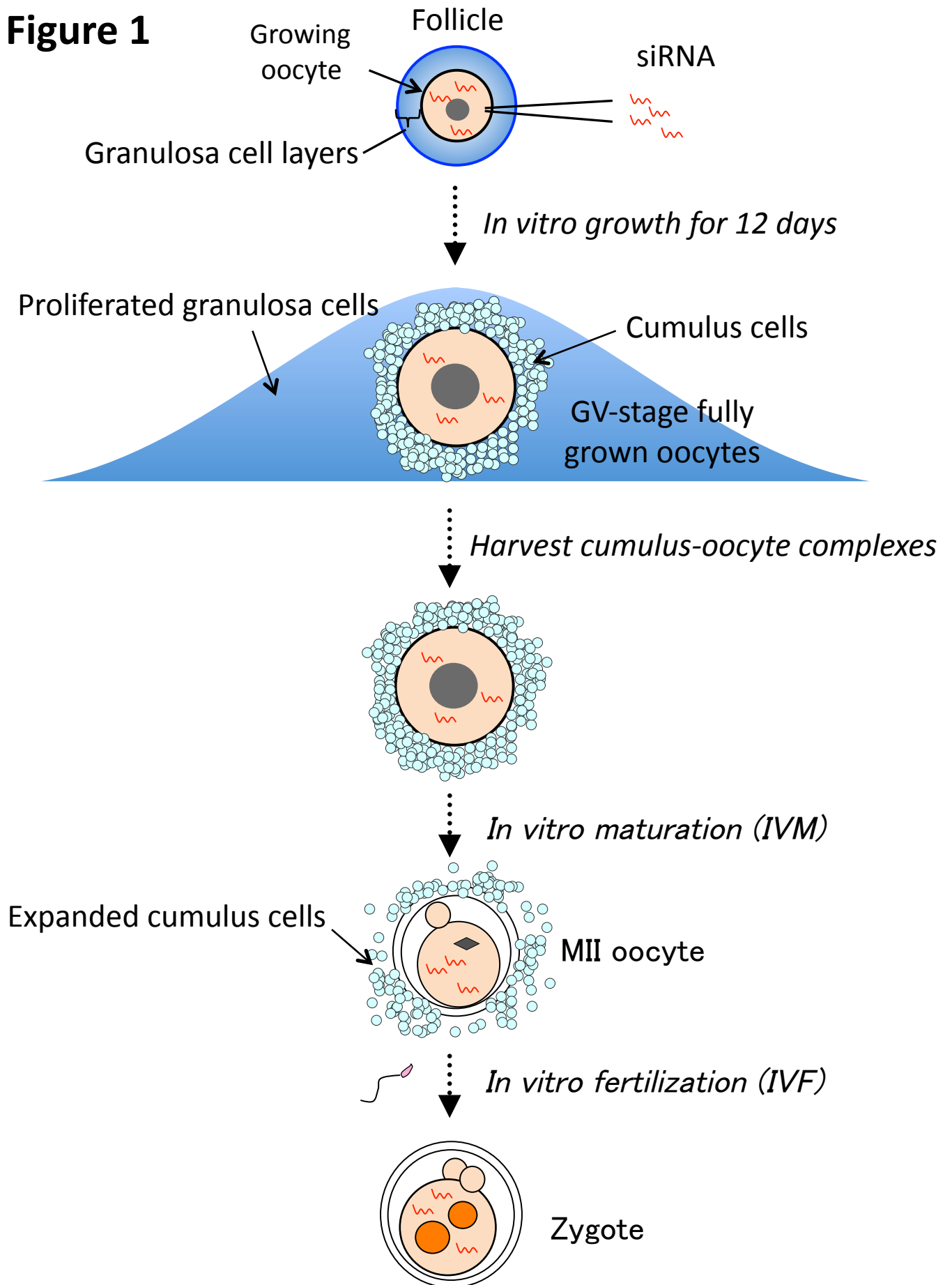
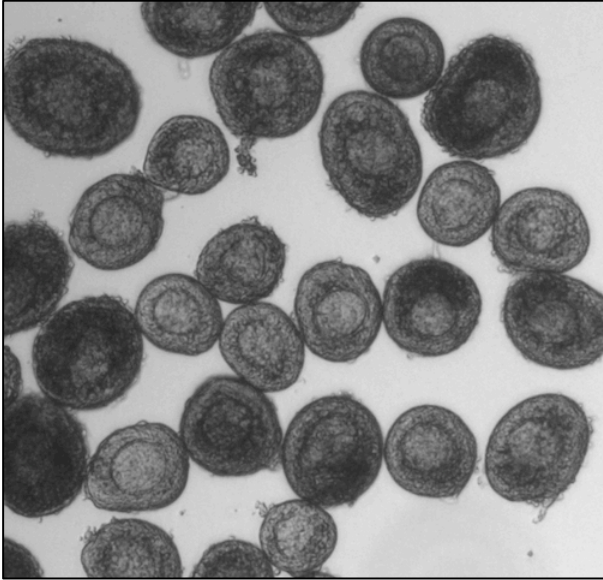
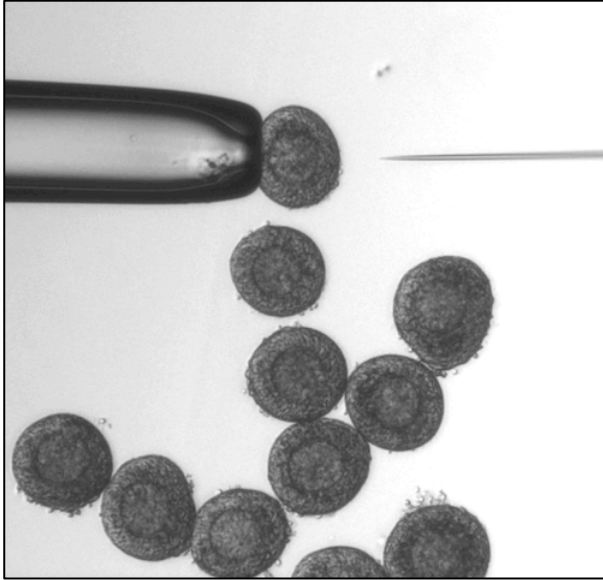


Figure 2

A



B



C

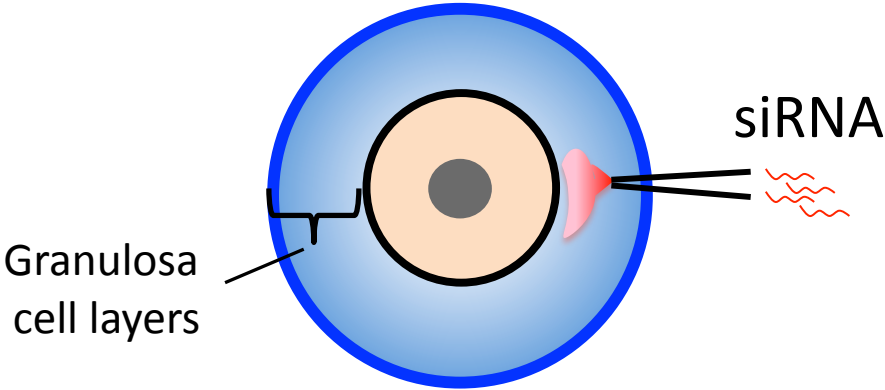
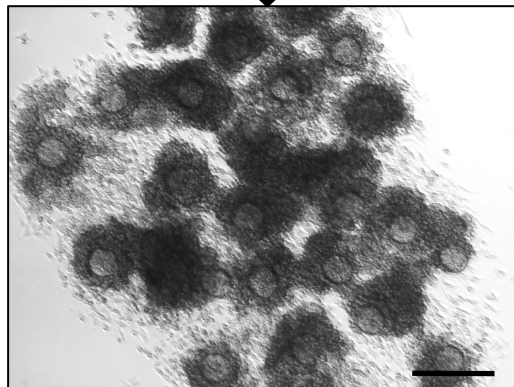
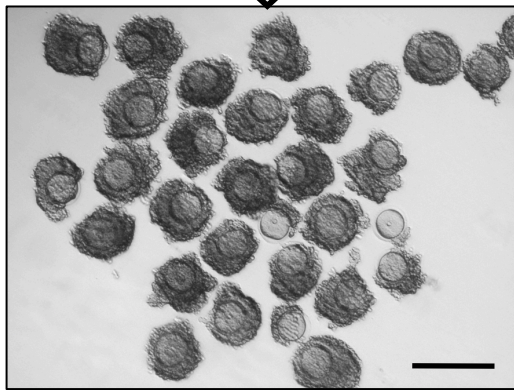
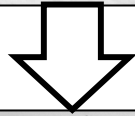
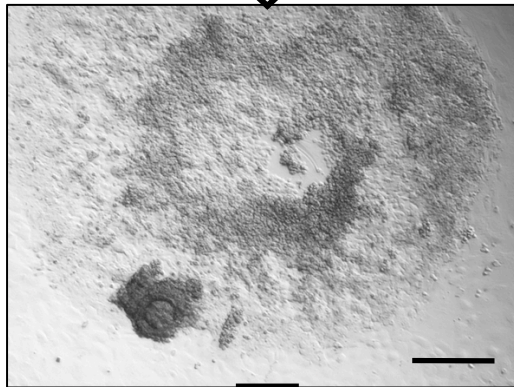
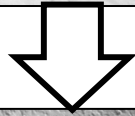
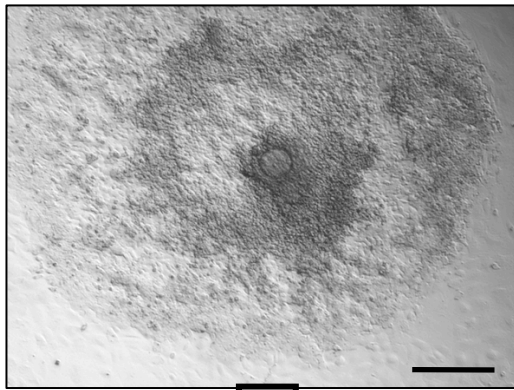


Figure 3



After *in vitro* growth, detach cumulus-oocyte complexes (COCs) from the proliferated granulosa cells by pipetting.

Culture the COCs for *in vitro* maturation (IVM).

Oocytes reach the MII-stage and cumulus cells are expanded after IVM.

Table 1. Components of HTF medium			
Reagent	Company	Catalog number	mg/200 ml DW
Water	Sigma-Aldrich	270733	200 ml
NaCl	Sigma-Aldrich	S7653	1187
KCl	Sigma-Aldrich	P9333	70
MgSO ₄ ·7H ₂ O	Sigma-Aldrich	M2773	9.8
KH ₂ PO ₄	Sigma-Aldrich	P5655	10.8
CaCl ₂ ·2H ₂ O	Sigma-Aldrich	C3306	151
NaHCO ₃	Sigma-Aldrich	S5761	420
Glucose	Sigma-Aldrich	G6152	100
Sodium lactate (ml)	Sigma-Aldrich	L1375	0.68 ml
Sodium Pyruvate	Sigma-Aldrich	P4562	7.4
Penicillin G	Sigma-Aldrich	P4875	15
Streptomycin	Sigma-Aldrich	S1277	10
Phenol Red (μl)	Sigma-Aldrich	P0290	20 μl
<p>* Store at 4C for up to 2 months. * Add 10 mg/ml BSA (Sigma-Aldrich, A4503), filtrate, and then store at 4C for up to 2 weeks.</p>			

Table 2. Components of KSOM			
Reagent for Stock	Company	Catalog number	mg/500 ml DW
DW	Sigma-Aldrich	W1503-500ML	200 ml
Polyvinyl alcohol (PVA)	Sigma-Aldrich	P8136	50
NaCl	Wako USA	191-17351	2774
KCl	Wako USA	164-13122	93.3
MgSO ₄ ·7H ₂ O	Wako USA	165-04242	24.7
KH ₂ PO ₄	Wako USA	132-09232	20.4
EDTA, 2Na	Wako USA	343-01861	1.9
Phenol Red (μl)	Sigma-Aldrich	P0290	50 μl
* Dissolve PVA in 80C for 3 h. * This stock solution can be stored at 4C for 3 months. * Take 100 ml and add the following reagents to make the working solution.			
Reagent for Working	Company	Catalog number	mg
Stock solution			100 ml
Glucose	Wako USA	047-31161	50
Sodium Pyruvate	MP Biomedicals	219965425	2.2
NaHCO ₃	Wako USA	197-01302	210
Sodium lactate (ml)	Sigma-Aldrich	L7900	185 μl
BSA	Millipore	12657-5GM	400
* After filtration, the working solution can be stored at 4C for 1 month. * Take 1 ml and add the following reagents before use.			
Amino Acid Mix	see below		15 μl/ml
Glutamine stock	see below		10 μl/ml
Calcium stock	see below		10 μl/ml
Reagent for Amino Acid Mix	Company	Catalog number	ml
x50 MEM Amino Acids Solution	Life technologies	11130-051	5 ml
100X MEM Non-Essential Amino Acids Solution	Life technologies	11140-050	10 ml
* Aliquot and store at -80C.			
Reagent for Glutamine Stock	Company	Catalog number	mg/10 ml DW
L-Glutamine	Sigma-Aldrich	G8540	146.1
* Filtrate, aliquot, and store at -80C.			
Reagent for Calcium Stock	Company	Catalog number	mg/10 ml DW
CaCl ₂ ·2H ₂ O	Wako USA	039-00431	249.9
* Filtrate and store at 4C.			