Oocyte production from mouse pluripotent stem cells in culture

Katsuhiko Hayashi (hayashik@hgs.med.kyushu-u.ac.jp)
Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Orie Hikabe
Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Nobuhiko Hamazaki
Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Go Nagamatsu
Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

So Shimamoto
Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Norio Hamada
Department of Obstetrics and Gynecology, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Method Article

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Abstract

Generation of functional eggs from pluripotent stem cells in culture would have impacts on reproductive biology and regenerative medicine. We have recently established a novel culture system in which mouse pluripotent stem cells differentiate to mature oocytes in a manner similar to oogenesis in vivo. Importantly, the in vitro-generated oocytes are capable of full-term development. This protocol consists of a culture method that can be mainly divided into four periods: primordial germ cell-like cell \(PGCLC) derivation, primary oocyte differentiation \(in vitro differentiation: IVDi), oocyte growth to germinal vesicle \(GV) oocytes \(in vitro growth: IVG) and oocyte maturation \(in vitro maturation: IVM). In each process, a robust number of mouse oocytes can be generated in a dish.

Introduction

Oogenesis incorporates a unique sequence of differentiation processes, during which biologically significant events such as meiosis and oocyte growth are orchestrated with communication to surrounding somatic cells. Reconstitution of this process using pluripotent stem cells would provide an efficient tool for uncovering the molecular mechanism underlying oogenesis, as well as to more practically establish an alternative source of gametes for reproduction. Indeed, efforts have been made to reconstitute in vitro oogenesis,[1-3] though none of them succeeded in the production of functional oocytes from pluripotent stem cells. We previously established a culture system that produces primordial germ cell-like cells \(PGCLCs) from pluripotent stem cells. The PGCLCs were almost identical to primordial germ cells \(PGCs), the precursor of eggs and sperm, in terms of gene expression, epigenetic status and functionality.[4] Of note, PGCLCs gave rise to sperm and oocytes by transplantation into neonatal testes and adult ovaries, respectively,[4,5] which means that PGCLCs have the potential to give rise to functional gametes. It is also worth noting, however, that the PGCLC culture system reproduces only 4-6 days of embryogenesis, and thus an additional 4-5 weeks of oogenesis, which seem to be more complicated than early PGC differentiation, have remained to be reconstituted. Here, we established a culture system in which the entire process of oogenesis is reconstituted in a dish, yielding in vitro-generated oocytes that are capable of full-term development to healthy pups. Based on previous reports,[5-7] the culture system is composed of 4 periods; PGCLC derivation, IVDi, IVG and IVM, which produce PGCLCs, primary oocytes, fully grown GV oocytes and metaphase II \(MII) oocytes, respectively. The following is our step-by-step protocol for producing functional oocytes from pluripotent stem cells.

Reagents

1. Mice \(pregnant female ICR mice at 12.5 days post coitum \(dpc) 2. Female embryonic stem cells \(we use Blimp1-mVenus and stella-ECFP \(BVSC) -reporter lines, but these reporters are not essential for differentiation). 3. iPSCs from female embryonic fibroblast or tail tip fibroblast \(we use iPSCs harboring BVSC-reporter, but these reporters are not essential for differentiation). 4. BSA fraction V, 7.5% \(w/vol) solution \(Invitrogen, cat no. 15260-37) 5. Glasgow Minimal Essential Medium \(GMEM) \(Invitrogen, cat.

no. 11710-035) 6. Minimum Essential Medium Eagle, alpha modifications \(αMEM) \(Invitrogen cot. no. 12571-063 for IVDi, 32571-036 for IVG) 7. StemPro®-34 SFM \(1X), Liquid \(Gibco, cat. no. 10639-011) 8. PBS \(WAKO, cat. no. 045-29795) 9. Glutamax \(Invitrogen, cat. no. 35050-061) 10. MEM Non-essential amino acid \(NEAA) \(Invitrogen, cat. no. 11140-050) 11. Sodium pyruvate solution 100 mM \(Invitrogen, cat. no. 11360-070) 12. Sodium pyruvate \(Nacalai, cat. no. 29806-54), powder for IVM 13. Penicillin/Streptomycin \(Invitrogen, cat. no. 15070) 14. β-Mercaptoethanol, 55 mM \(Invitrogen, cat. no. 21985-023) 15. EDTA \(Nacalai, cat. no. 15111-45) 16. Knockout Serum Replacement \(Invitrogen, cat. no. 10828-028) 17. Dimethyl sulfoxide \(DMSO) \(Dojindo, cat no. 346-03615) 18. PD0325901 \(Stemgent, cat. no. 04-0006) 19. CHIR99021 \(Biovision, cat. no. 1667-5) 20. LIF \(Nacalai, cat. no. NU0012-2) 21. BMP4, recombinant human \(R&D Systems, cat. no. 314-BP-010) 22. EGF, recombinant mouse \(R&D Systems, cat. no. 2028-EG-010) 23. Retinoic acid \(Sigma, cat. no. R2625-50MG) 24. Trypsin-EDTA, 0.5% \(Invitrogen, cat. no. 15400-054) 25. Anti-SSEA1 Microbeads \(Miltenyi, cat. no. 130-094-530) 26. Anti-CD31 Microbeads \(Miltenyi, cat. no. 130-097-418) 27. Paraffin liquid \(Nacalai, cat. no. 26117-45) 28. Fetal Bovine Serum \(FBS) \(Invitrogen, cat. no. 10437-028 for IVDi, Euitech-Bio, cat. no. SBSU-0500 for IVG) 29. BMP15 \(R&D, cat. no. 5096-BM-005) 30. GDF9 \(R&D, cat. no. 739-G9-010) 31. Collagenase type IV \(MP Biomedicals, cat. no. 195110) 32. Polyvinylpyrrolidone \(PVP) \(Sigma, cat. no. PVP360-100G) 33. Follistim \(MSD) 34. Gonadotropin® \(ASKA) 35. ICI182780 \(Tocris, cat. no. 1047) 36. Ascorbic acid \(TCI, cat. no. G0394) 37. Hyaluronidase \(TCI, cat. no. H0164)

Equipment

1. Cell culture hood 2. CO_2 incubator \(37° C, 5%CO_2-95%air, 95% relative humidity) 3. Stereoscopic microscopy 4. Glass-calibrated pipettes 5. Pasteur pipette 6. Tirrill Burner 7. Glasscutter 8. Centrifuge 9. Aspirator 10. Hemocytometer 11. Micropipettes 12. Micropipettors for tips 13. Sterile plastic tips \(10µl, 200µl and 1000µl) 14. Pipettors for pipettes 15. Sterile plastic pipettes \(5-mL, 10-mL, 25mL and 50mL) 16. 15 and 50 ml conical centrifuge tubes 17. 4-well tissue culture plate 18. 6-well tissue culture plate 19. 96-well U-bottom low binding culture plate 20. Transwell-COL® 21. Sterile syringes \(1, 5, 20, 50 ml) for preparation of culture media 22. 0.22-µm-pore size syringe filter 23. Tungsten needle \(sharpened by electrical erosion) with a handle 24. 70µm cell strainers 25. MACS MS column 26. MiniMACS separator 27. Heat plate 28. FACSAriaII 29. Dissecting instruments \(surgical scissors, microforceps, surgical forceps)

Procedure

Solutions - GK15 medium: GMEM supplemented with 15% KSR, 1x non-essential amino acid, 1x sodium pyruvate, 1x Glutamine, 1x penicillin/streptomycin and 100µM β-mercaptoethanol - αMEM-based IVDi medium: αMEM supplemented with 2% FCS, 150 µM ascorbic acid, 1x Glutamax, 1x penicillin/streptomycin and 55 µM 2-mercaptoethanol - StemPro-34-based IVDi medium: StemPro-34 SFM supplemented with 10% FCS, 150 µM ascorbic acid, 1x Glutamax, 1x penicillin/streptomycin and 55 µM 2-mercaptoethanol - Wash medium: DMEM supplemented with 0.1% BSA fraction V - IVG-αMEM

medium: αMEM supplemented with 5% FCS, 2% PVP, 150 μM ascorbic acid, 1x Glutamax, 1x penicillin/streptomycin, 100 μM 2-mercaptoethanol, 55 μg/ml sodium pyruvate, 0.1IU/ml FSH \(Follistim), 15 ng/ml BMP15 and 15 ng/ml GDF9 - Collagenase wash medium: αMEM supplemented with 5% FCS - Collagenase solution: Collagenase wash medium with 0.1% Collagenase IV - IVM medium: αMEM supplemented with 5% FCS, 25 μg/ml sodium pyruvate, 1x penicillin/streptomycin, 0.1 IU/ml FSH, 4 ng/ml EGF, and 1.2 IU/ml hCG \(Gonadotropin®) - Hyaluronidase solution \(20x): 1% \(w/vol) dissolved in PBS **Making reconstituted Ovaries** \(rOvaries) Produce rOvaries according to the procedure reported previously[6] with slight modifications. 1. Sort BV-positive PGCLCs differentiated from ESCs or iPSCs by FACSAria II. 2. Centrifuge PGCLCs, removed supernatant and resuspend with appropriate volume of GK15 supplemented with 1μM retinoic acid. 3. Dissect female gonads from E12.5 embryos and dissociate them by 0.05% trypsin treatment.[6] 4. Purify E12.5 female gonadal somatic cells by incubation with SSEA1 and CD31 antibodies conjugated with magnetic beads, followed by magnetic separation through a MACS MS column, according to the manufacturer's instructions. Gonadal somatic cells should be in the flow through. 5. Centrifuge gonadal somatic cells, removed supernatants and resuspend with appropriate volume of GK15 supplemented with 1μM retinoic acid. 6. Aggregate 5,000 PGCLCs with 50,000 E12.5 gonadal somatic cells in a low-binding U-bottom 96-well plate in GK15 supplemented with 1 μM retinoic acid. 7. Placed in a CO_2 incubator and culture for 2 days. **IVDi culture** 8. Set a Transwell-COL on a 6 well plate and soak with αMEM-based IVDi medium. 9. Using glass pipette, transfer rOvaries from the 96-well plates on the Transwell-COL. 10. Place in a CO_2 incubator. 11. Change the medium at 2 days of culture. 12. At 4 days of culture, change a half volume of the medium to StemPro-34-based IVDi medium. 13. Change a half volume of the medium to fresh StemPro-34-based IVDi medium every other day. 14. Add 500 nM ICI182780 to the StemPro-34-based IVDi medium from 7 days to 10 days of culture. 15. At 21 days of culture, isolate individual follicles from the aggregates. Carefully remove interstitial cells between the follicles by a tungsten needle and then place the isolated follicles on the Transwell-COL. Leave a space between the follicles. During isolation, keep the plate warm on a heat plate. 16. After isolating all follicles, place the Transwell-COL on IVG medium in 6-well plate. **IVG culture** 17. After 2 days of culture, add 1ml collagenase solution on the follicles. 18. Leave for 10-20 min at 37° C. The time length depends on how much the interstitial cells stick to the follicle. 19. Wash 3 times with collagenase wash medium. 20. Place the Transwell-COL on IVG medium without BMP15 and GDF9 in 6-well plate, 21. Pour gently IVG medium without BMP15 and GDF9 to cover the follicles on the Transwell-COL. 22. Place the plate in a CO_2 incubator. 23. Change a half volume of IVG medium without BMP15 and GDF9 every other day. 24. After 11 days of culture, pick cumulus-oocyte complexes \(COCs) up by a fine glass capillary and transfer them into 500μl IVM medium covered with Paraffin liquid in a 4 well dish. **IVM culture** 25. Place the 4 well dish in a CO_2 incubator. 26. Culture for 16 hours. 27. Add 25μl hyaluronidase solution to the IVM medium, and removed cumulus cells from the oocytes. 28. Check first polar body extrusion.

Timing

It takes 10 days for PGCLC derivation, 3 weeks for IVDi, 11 days for IVG and 1 day for IVM.

Troubleshooting

1. In case of non-reporter ES/iPS cells, PGCLCs can be sorted by anti-SSEA1 and anti-CD61.[6] 2. For IVD, do not put the aggregates on the edge of Transwell-COL. 3. It is not absolutely required to isolate “single” follicles. A cluster of two to five follicles is still available. 4. Too long incubation with collagenase solution would result in denuded oocytes. On the other hand, too short incubation would compromise proliferation of granulosa cells.

Anticipated Results

In IVDi, 100-300 primary oocytes are obtained from one rOvary. In IVG and IVM, 50-150 COCs and 15-40 MII oocytes, respectively, are obtained. Resultant MII oocytes can be used for in vitro fertilization.

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