Chapter 12

Manipulating the Avian Epiblast and Epiblast-Derived Stem Cells

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Abstract

Compared to eutherian mammals, birds retain a primitive form of epiblast development. Molecular studies of the avian epiblast can provide valuable insight for mammalian epiblast research. Here, we introduce several basic techniques in handling epiblast-stage embryos of the chick, the major model organism for avian developmental biology studies. We describe how to collect embryos for RNA extraction and gene expression analysis, to set up ex ovo New culture for overexpression, bead graft and small molecule-based inhibitor studies, and to carry out whole-mount RNA in situ hybridization analysis. We introduce a novel and simple method for molecular perturbation of the epiblast differentiation in ovo. We also describe how to perform primary chicken epiblast cell culture, to establish stable epiblast stem cell (Epi-SC) lines, and to assay for pluripotency in primary epiblast cells and Epi-SCs.

Key words Avian, Chicken, Epiblast, Stem cells, Pluripotency, New culture, Bead graft, In situ hybridization, Chicken ES cells, Epiblast stem cells

1 Introduction

Epiblast is a term used to describe the primitive ectoderm in amniotes (mammals, birds, and reptiles). In birds, it includes cells in both the area pellucida (equivalent to the mammalian epiblast) and the area opaca (similar to mammalian mural trophectoderm). Mammalian polar trophectoderm does not have an avian counterpart. Here, we will use the chick as the avian model and only concern ourselves with the area pellucida epiblast. We will refer to this part as the epiblast. It should be kept in mind that in addition to the three germ layers in the embryo proper, the epiblast in both the mammals and birds contributes to the extraembryonic mesoderm in its entirety and to the extraembryonic ectoderm partially.

The first day of chicken development takes place intrauterally. A fertilized and freshly laid hen's egg contains approximately

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40,000 cells and has reached the Eyal-Giladi and Kochav (EGK) stage X [1]. Intrauteral development (EGK I-X) and post-laying pre-streak development (EGK XI-XIV) are traditionally staged according to the EGK criteria [1] and correspond collectively to Stage 1 of the Hamburger and Hamilton (HH) system [2]. After the appearance of the primitive streak at HH2 (about 6–8 h of incubation post-laying), chicken development is staged according to the HH criteria [2]. Although the later half of the intrauteral development is relevant to issues concerning epiblast formation and pluripotency, technical limitations in obtaining these embryos preclude their routine use in labs without access to a poultry farm. In this chapter, we mainly discuss about how to handle chicken embryos during the first 18 h of its post-laying development (from stage EGK-X up to stage HH5). Most pluripotency genes are markedly down-regulated in the epiblast by HH5 [3, 4]. This period of chicken development corresponds roughly to E5.5–E7.5 of mouse development.

2 Materials

2.1 Manipulation and Analysis of Epiblast-Stage Chicken Embryos

1. Pannett-Compton Solution: H_2O, 40 ml of solution-A, 60 ml of solution-B in 1 l final volume. Pannett-Compton Solution-A: H_2O, 121 g NaCl, 15.5 g KCl, 10.42 g $CaCl_2$–$2H_2O$, and 12.7 g $MgCl_2$–$6H_2O$ in 1 l final volume. Autoclave. Pannett-Compton Solution-B: H_2O, 1.88 g Na_2HPO_4, and 0.188 g NaH_2PO_4–$2H_2O$ in 1 l final volume. Autoclave.
2. 20× PBS: H_2O, 175.3 g NaCl, 22.7 g Na_2HPO_4, and 4.7 g NaH_2PO_4–H_2O in 1 l final volume. Autoclave. For 1× PBT: 1× PBS with 0.1 % Tween-20.
3. 4 % PFA: Add 4 % w/v of paraformaldehyde powder with continuous stirring to preheated (65 °C) 1× PBS. Adjust pH to 7.5 with 1 N NaOH. Cool to RT and add EGTA to final concentration of 2 mM. Alternatively, premade 4 % Paraformaldehyde Phosphate Buffer Solution can be purchased from commercial sources.
4. Postfix: 4 % formaldehyde and 0.1 % glutaraldehyde in 1× PBT.
5. Pre-hybridization solution: H_2O, 50 % formamide, 1.3× SSC, 5 mM EDTA, 50 μg/ml yeast RNA, 0.002 % Tween-20, 0.005 % CHAPS, and 100 μg/ml Heparin. All glassware and tools in making this solution should be baked. Aliquot into 50 ml tubes. Store at −20 °C.
6. 20× SSC: H_2O, 175.3 g NaCl, 88.2 g Na_3Citrate–$2H_2O$ in 1 l final volume. Adjust pH to 5.3 with citric acid.
7. 10× TBST: H_2O, 8 g NaCl, 0.2 g KCl, 25 ml of 1 M Tris–HCl pH 7.5, and 11 g Tween-20 in 100 ml final volume.

8. Color reaction solution: NTMT (1 ml) + NBT (2.3 μl) + BCIP (2.3 μl). Scale up accordingly. NBT: 4-nitro-blue tetrazolium chloride solution (Roche). BCIP: 5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt solution (Roche). NTMT: H_2O, 1 ml of 5 M NaCl, 2.5 ml of 2 M Tris–HCl (pH 9.5), 1.25 ml of 2 M $MgCl_2$, 5 ml of 10 % Tween-20 in 50 ml final volume. Make fresh each time.
9. Affigel beads: Affi-Gel Blue beads (BioRad). AG1 ion exchange beads: AG1-X2 ion exchange beads (BioRad). Heparin-coated beads: Heparin immobilized on acrylic beads (Sigma).
10. Antibody blocking solution: 5 % heat-inactivated goat serum (30 min at 55 °C) and 1 mg/ml BSA in TBST.
11. pGEM-T Vector: pGEM-T Easy Vector System I (Promega).
12. Glass ring: Outer diameter 26 mm and inner diameter 23 mm. Custom made by local company (URIN, Kyoto, Japan).

2.2 Manipulation and Analysis of Epiblast Cells in Culture

1. Epiblast cell culture medium (ECC medium): KnockOut-DMEM (Invitrogen) supplemented with 20 % knockout serum replacement (KSR, Invitrogen), 2 % chicken serum, 1 mM sodium pyruvate, 1× nonessential amino acids, 2 mM L-glutamine, 1× nucleoside solution, 0.1 mM β-mercaptoethanol, 20 ng/ml recombinant chicken leukemia inhibitory factor.
2. 0.1 % gelatin solution: H_2O, 1 g gelatin in 1,000 ml final volume, followed by sterile filtration.
3. Ham-F12: F12 Nutrient Mixture.
4. Lipofectamine 2000 reagent: Lipofectamine™ 2000 Transfection Reagent (Invitrogen).
5. Mitomycin C solution: 1× PBS with 2 mg/ml mitomycin C.
6. 100× nucleoside solution: H_2O, 80 mg adenosine, 73 mg cytidine, 85 mg guanosine, 24 mg thymidine, and 73 mg uridine in 100 ml final volume, sterilized by passing through a 0.22 μm disposable filter.
7. Stem cell passaging tool: StemPro EZPassage (Invitrogen).
8. SuperScript™ III: SuperScript™ III First-strand Synthesis System (Invitrogen).
9. TOP10F *E. coli* strain: One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen).
10. Zeocin: Zeocin™ Selection Reagent (Invitrogen).
11. ProBond Resin: ProBond™ Purification System with Antibody (Invitrogen).
12. pSecTag2A Vector: pSecTag2 Expression and Purification kit (Invitrogen).
13. Anti-chicken Nanog polyclonal rabbit antibody: supplied by Hiroshima University [5].

3 Methods

3.1 Obtaining Epiblast-Stage Chicken Embryos

Basic embryological precautions should be taken in staging and handing early-stage chicken embryos (*see* **Note 1**).

1. Prepare spoon, scissors, and coarse and fine forceps (Fig. 1a). Regular wash (soap, tap water, distilled water, and 70 % ethanol) of these tools is sufficient for most experiments.
2. Eggs are flipped upside down from their storage/incubation position. This way the yolk is positioned lower inside the egg. An incision is made with the tip of coarse forceps (Fig. 1b, c).
3. Top part of the egg shell is opened and removed (Fig. 1c, d) and most of the albumen removed (or collected in a small beaker if needed) (Fig. 1d, e).
4. Position the embryo at the center by gently moving the yolk with a spoon or the back of forceps. Do not touch the embryo directly. Use scissors to make 3–4 quick cuts around the embryo (Fig. 1e, f).
5. Scoop up the embryo, together with the vitelline membrane above and some yolk below, with a spoon. Dip the embryo into PBS or Pannett-Compton solution (Fig. 1g).
6. Remove the vitelline membrane (with embryo) from the yolk with fine forceps (Fig. 1h). Move slowly over the embryo area.
7. In most cases after more than a few hours of incubation, the embryo stays attached to the vitelline membrane (Fig. 1h). In cases when the embryo stays attached to the yolk, use the blunt end of a Pasteur pipette to suck up and transfer the embryo (Fig. 1i).
8. Use coarse forceps to hold down the vitelline membrane and fine forceps (with tips closed) to gently dislodge the embryo from the vitelline membrane by scraping the surface of the vitelline membrane towards to the embryo (Fig. 1j). This way the edges of the embryo are dislodged from the vitelline membrane, while most regions of the embryo are not touched by the forceps (Fig. 1k).
9. Once freed from the vitelline membrane (Fig. 1l), the embryo can be transported using the blunt end of a Pasteur pipette from dish to dish with minimal damage, so long as it does not come into the liquid/air interface. Excessive yolk can be removed with forceps or gentle blows using a Pasteur or Gilson pipette.
10. If needed (e.g., for RT-PCR or large-scale transcriptomic analysis), the epiblast region can be cut out from the embryo (Fig. 1m). In most cases, embryos will be fixed for later use. Fix them flat. This is very important. The epiblast is slightly

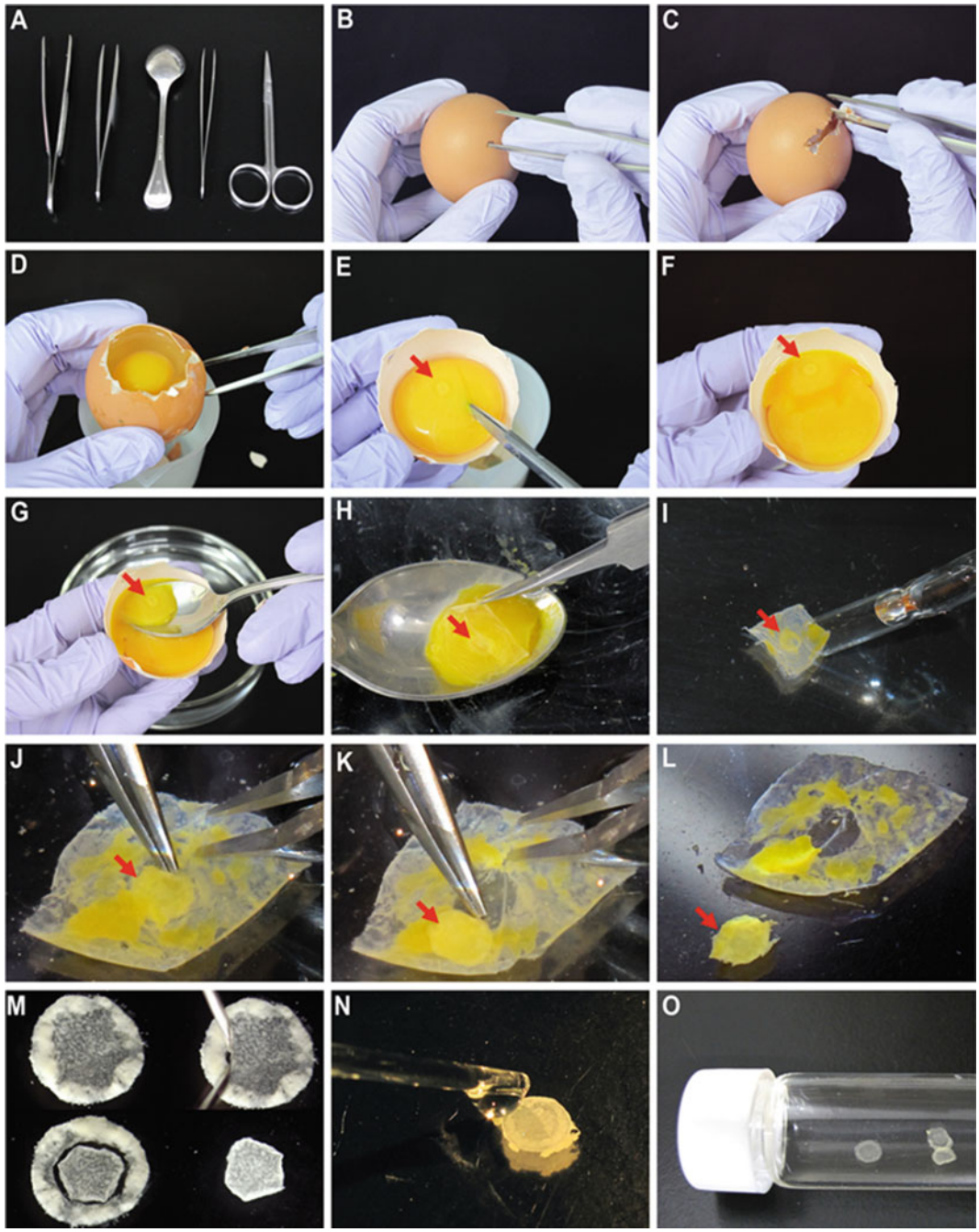

Fig. 1 Obtaining epiblast stage chick embryos. Unincubated eggs are opened (**a**–**d**) and embryos (*red arrow*) cut out and separated from the yolk as shown (**e-h**). Embryos are carefully detached from the vitelline membrane (**i–l**). The epiblast region can be cut out from freshly isolated embryos (**m**) or embryos can be fixed and used for subsequent analysis (**n**, **o**)

stretched in ovo, so it shrinks a bit in solution and can also fold up very easily. Flatten each embryo (it does not matter which side is facing up) with fine forceps, and further stretch it flat by removing all Pannett-Compton (or PBS) liquid around the embryo. Using a Pasteur pipette, gently add 1–2 drops of fixative (4 % PFA) on top (Fig. 1n). After 1–2 min, add a few drops. After 5–10 min of fixation, embryos can be handled quite roughly. With the blunt end of a Pasteur pipette, transfer embryos to a glass vial, fix for a couple of hours at RT or overnight at 4 °C (Fig. 1o).

3.2 Setting Up the New Culture

The New culture technique takes some practice to master. But it is the best ex ovo culture technique available for epiblast-stage chicken embryos and its versatility (*see* **Note 2**) justifies the initial steep learning curve.

1. Prepare glass tray, glass rings, watch glasses, forceps, and scissors (Fig. 2a). Regular wash of these tools (as in Subheading 3.1) is sufficient, but avoid using too much detergent and rinse with tap water thoroughly.
2. Pour 1–1.5 l of Pannett-Compton solution into a glass tray. Open eggs, collect thin albumen, remove as much thick albumen as possible without damaging the yolk. Pour intact yolk gently into the solution. Prepare a few yolks this way (up to 20 if you are experienced) and put several watch glasses inside the tray (Fig. 2b).
3. Use the back end of forceps to rotate the yolk so that the embryo is positioned on the top. Starting from one side of the yolk equator (Fig. 2c, e), cut all around the yolk with scissors (Fig. 2d, f).
4. Peel off the vitelline membrane with forceps gently and steadily (Fig. 2g). If the egg has been incubated for several hours, the embryo should stay attached to the vitelline membrane (Fig. 2f). If unincubated, the embryo tends to be detached from the vitelline membrane. In such cases, collect the embryo from the yolk using the blunt end of a Pasteur pipette, wash excessive yolk off the embryo and put the embryo back on to the vitelline membrane after the latter has been assembled. In the meantime, continue with the assembly of the vitelline membrane.
5. Move the vitelline membrane over a watch glass (yolky side up) (Fig. 2h) and position a glass ring on the membrane (Fig. 2i).
6. Use forceps to grab the membrane gently around the ring so that there is membrane margin all around the ring (Fig. 2j). Move the watch glass (with the embryo and the ring) out of the tray and on to the bench.

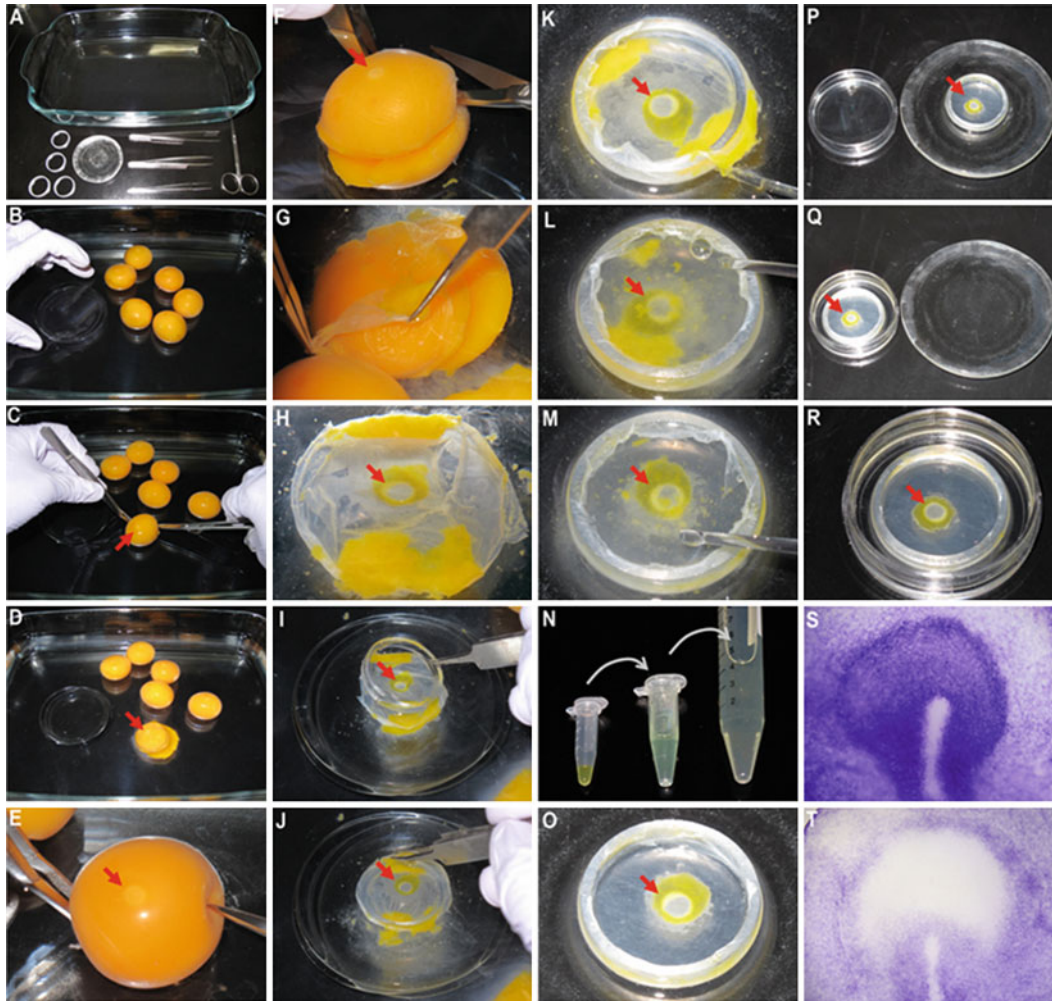

Fig. 2 Setting up New Culture. Egg yolks are collected in a glass tray filled with Pannett-Compton solution (**a–c**). Embryos attached to the vitelline membrane are obtained by generous circumferential cutting and careful dislodgement of the membrane plus embryos from the underlying yolk (**d–h**). The vitelline membrane (yolk side up) is carefully wrapped around and attached to a glass ring as shown (**i–m**). The assembled embryo is placed onto a 35 mm dish containing albumen (**o–r**). Albumen can be mixed with, e.g., small molecule-based inhibitors following successive dilution steps (**n**). Representative images of control and TGF-beta inhibitor (25 μM SB431542)-treated embryos reveal clear reduction in the expression of the pluripotency marker Nanog in the central epiblast upon New culture treatment in the presence of TGF-beta inhibition (**s**, **t**)

7. Reposition the ring as it may have shifted its position during the transfer. Use a Pasteur pipette to remove a bit of solution so that the top of the ring is exposed to the air. Use coarse forceps first, and then Pasteur pipettes (flame-polished to remove sharp edges) to grab the outer edges of the vitelline membrane over the ring (Fig. 2k). Because the top of the ring is exposed to the air, the membrane will (after 10–30 min) be attached to the ring after drying up. During this time window,

the membrane can be adjusted. An ideal outcome is that the vitelline membrane is taut under the ring and wraps around the top surface of the ring (Fig. 2l).

8. Cut off excessive vitelline membrane inside the ring (most lateral bits of the membrane) with scissors. Large pieces of yolk on the vitelline membrane inside the ring are removed with a Pasteur pipette (Fig. 2l, m). Always leave some liquid to cover the embryo. The assembly process may be stopped here for up to a couple of hours before moving on to the next step. Also at this step, the embryo can be removed for electroporation, detached embryos can be put back, and for bead graft, a few beads can be put onto the embryo.
9. Aliquot 2–3 ml of thin albumen in each 35 mm dish. For whole embryo treatment with small molecules, the chemical stock solution (or control solution), often dissolved in DMSO, is diluted in PBS first, followed by further dilution in the thin albumen (Fig. 2n). The volume of albumen in each dish can be reduced to 0.5–1 ml if cost of the chemical is a concern (e.g., SU5402).
10. Remove all Pannett-Compton solution from both inside the ring and on the watch glass outside the ring (Fig. 2o).
11. Move the ring (with the embryo) using coarse forceps from the watch glass to the 35 mm dish with albumen (Fig. 2p, q).
12. The vitelline membrane under the ring should bulge slightly upward. The embryo should be in the middle, at the highest point (Fig. 2r). The ring should naturally touch the bottom (remove some albumen if not) of the dish instead of floating on the albumen. Remove Pannett-Compton solution that has drained to the lowest point inside the ring. No albumen should leak inside the ring. Discard if so.
13. Put the lid on and put the petri dish inside a box moisturized with a wet towel. Incubate in a normal incubator (no need to have CO_2 or humidity control).
14. After a desired period of incubation, embryos are fixed and processed for further analysis. For example (Fig. 2s, t), embryos treated with a TGF-beta inhibitor from early-streak stage showed a reduction of Nanog expression in the central epiblast (Fig. 2t), whereas control-treated embryos exhibited normal expression (Fig. 2s).

3.3 Bead Graft and Subgerminal Cavity Injection

Epiblast-stage embryos can be experimentally manipulated using a number of techniques (*see* **Note 3**). Here we describe two of them, the bead graft technique and the subgerminal cavity injection technique.

3.3.1 Bead Graft

1. Embryos are prepared using the New culture method (Fig. 3a).
2. Prepare beads to be grafted. Beads come in different sizes. Pick the right bead size and pore size (for AG1 beads) suitable for your experiments. Bead preparation takes time, so carry out this step before or during New culture preparation. Aliquot 10–100 μl of beads to an Eppendorf tube. AG1 resins come as dry powdery beads, so aliquot an equivalent volume. These beads are relatively inexpensive and one bottle can last for

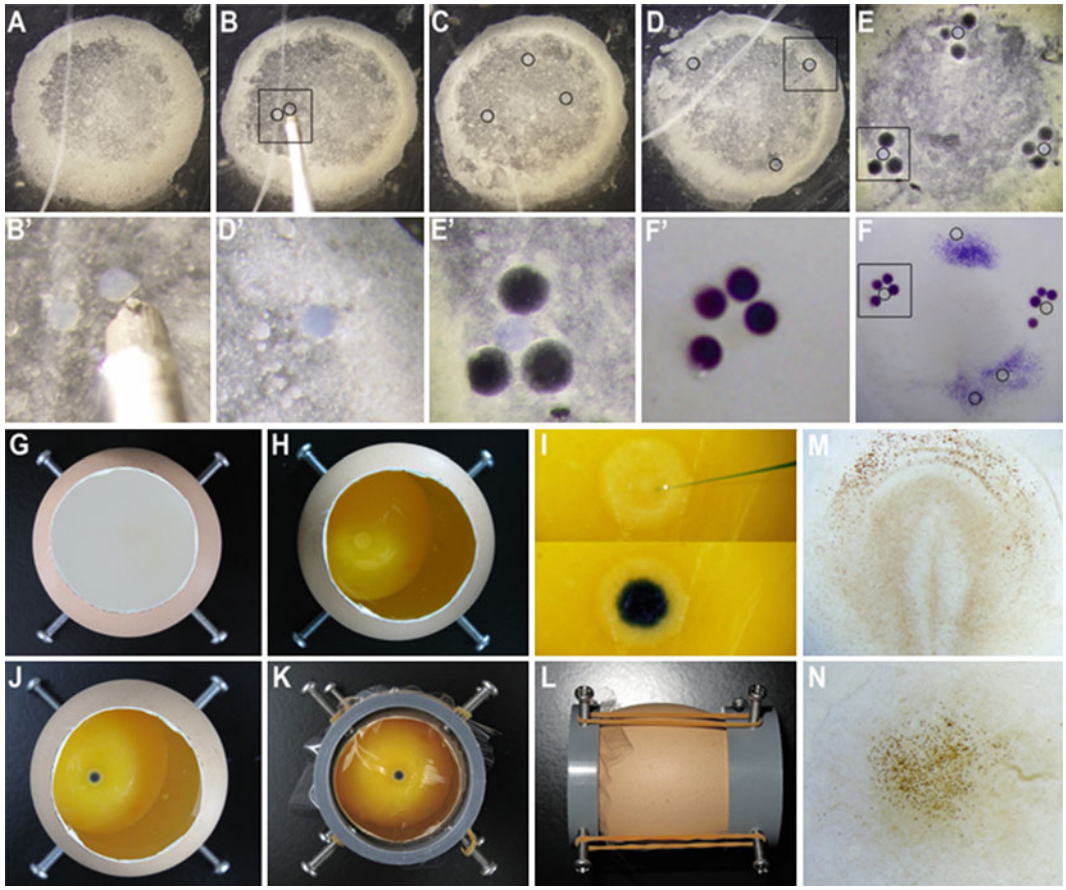

Fig. 3 Bead graft and subgerminal cavity injection. Embryos are prepared using the New culture setting. Growth factor-soaked beads are carefully placed on top of the embryo (**b, b′**) and positioned to the desired area using a sharp needle or metal tips (**c**, **d**, **d′**). Two types of beads can be combined (**e**, **e′**) with an example for such an experiment shown; induction of Brachyury by FGF can be inhibited when SU5402-soaked beads are placed in close proximity to FGF-loaded beads (**f**, **f′**). Unincubated eggs (embryo on top) are placed into prepared foster egg shells (**g**, **h**). Growth factor and/or small molecule solutions are injected into the subgerminal cavity using a fine pulled capillary and mouth pipetting (**i**, **j**). The injected egg is filled with albumen and sealed using plastic wrap, custom made rings, and rubber bands (**k**, **l**). Injected embryos are incubated horizontally and under rotation (90° every 90′). Examples of injected embryos are shown, with normal distribution of VASA positive PGCs in control-injected embryos (**m**), and abnormal number and location of PGCs after BMP injection (**n**)

many years. If the protein/small molecule to be loaded is dissolved in water-based solution as stock, wash the beads with PBS several times (each time a few minutes followed by short spins). If the stock is dissolved in DMSO, use DMSO to wash.

3. The stock solutions are normally kept as 5–10 μl aliquots, so only use equal or less volume of beads for loading. Mix beads and stock solution. Leave either on ice (for proteins) or RT (for small molecules) for at least a couple of hours.
4. Before use, wash beads 3× in PBS or Pannett-Compton solution. We wash by taking a couple of microliters out from the Eppendorf tube, and transferring successively into fresh wash solution three times.
5. Several (1–5) beads or bead combinations can be grafted on to each embryo. Beads can be added to the New culture after final assembly (Fig. 3b, b′) or at the step shown in Fig. 2m. Use fine forceps, a P20 pipette or any fine tips to add beads. Some beads may be sticky, add these beads as close to targeted locations as possible.
6. Use a 1 ml syringe needle or other fine metal tips to move beads to targeted areas (Fig. 3c, d, d′).
7. If a combination of beads is required, add a second type of beads (Fig. 3e, e′).
8. When all beads are in their targeted locations, remove excessive liquid from over the embryo and inside the ring using a capillary and mouth pipetting. Press beads down gently to make sure all beads are in contact with the embryo, and not floating on top of it.
9. Incubate. After intended period of incubation, make sure beads are still in place. If not, discard.
10. Fix the embryo and analyze the effect of the bead graft. An example is shown in Fig. 3f, in which three FGF-loaded beads induce Brachyury gene expression, and this induction is inhibited when both FGF- and SU5402-loaded beads are grafted.

3.3.2 Subgerminal Cavity Injection

1. Prepare foster egg shells (top part evenly cut with a circular saw) and large plastic rings as shown in Fig. 3g.
2. Transfer egg content from an unincubated egg to the foster egg shell (Fig. 3h).
3. Reposition the yolk with a spoon so that the embryo is on the top.
4. Use a fine pulled capillary and mouth pipetting, take up 5–10 μl of solution to be injected (growth factors or small molecules diluted in PBS or Pannett-Compton Solution, with a bit of fast green dye for visualization).

5. Inject 1–1.5 μl of solution per embryo into the subgerminal cavity (Fig. 3i, j). Do not inject above the epiblast or into the yolk. This is easy to achieve with only a little practice. The capillary opening should be large enough so that 1–1.5 μl can be injected in 2–3 s, but small enough so that no injected content leaks out afterwards.
6. Fill the injected egg with albumen (use a 10 ml syringe) to the very top. Cover the egg with a small piece of plastic wrap. Leave no trapped air bubbles. Cover the wrap with another plastic ring (Fig. 3k).
7. Tie together the top and bottom rings with rubber bands (Fig. 3k, l).
8. Incubate horizontally with 45–90° rotation every 90′.
9. After incubation, pour out the content, obtain the embryo as shown in Fig. 1 and analyze the effect of injected materials.
10. An example is shown in Fig. 3m, n. Embryo with control injection shows normal PGC specification (Vasa staining), whereas abnormal location and number of PGCs are seen in BMP-injected embryo.

3.4 RNA In Situ Hybridization

Whole-mount RNA in situ hybridization is the most basic technique in gene expression analysis. To achieve good results, follow basic precautions for RNA in situ analysis (*see* **Note 4**).

1. Make DIG or Fluorescein-labeled probes. This is an important step, but is the same for any type of RNA in situ analysis. So it is only briefly explained here. First, clone the region you intend to make the antisense probe of. We routinely use PCR method and pGEM-T-Easy as the host vector. Second, confirm the sequence and check the orientation by sequencing. Keep each construct as a stock. Third, either linearize the construct by digestion for antisense-directed transcription, or PCR the insert out using M13F/R, purify the DNA. Fourth, using the DIG or Fluorescein labeling kit, transcribe the antisense probe. Fifth, purify the probe. Keep it as a concentrated stock in a few milliliters of hybridization solution. Sixth, test each probe before experimental use.
2. Collect embryos as shown in Subheading 3.1. Fix them flat.
3. Remove the fixative completely and add 100 % methanol (with one rinse). Embryos can be stored this way for several weeks at −20 °C. Strong and clean probes may work with embryos kept this way for a couple of years.
4. Remove methanol, rehydrate in PBT by rinsing a couple of times and washing (nutating) for 30 min (Fig. 4a). Do not worry about yolk materials attached to the embryo. They will

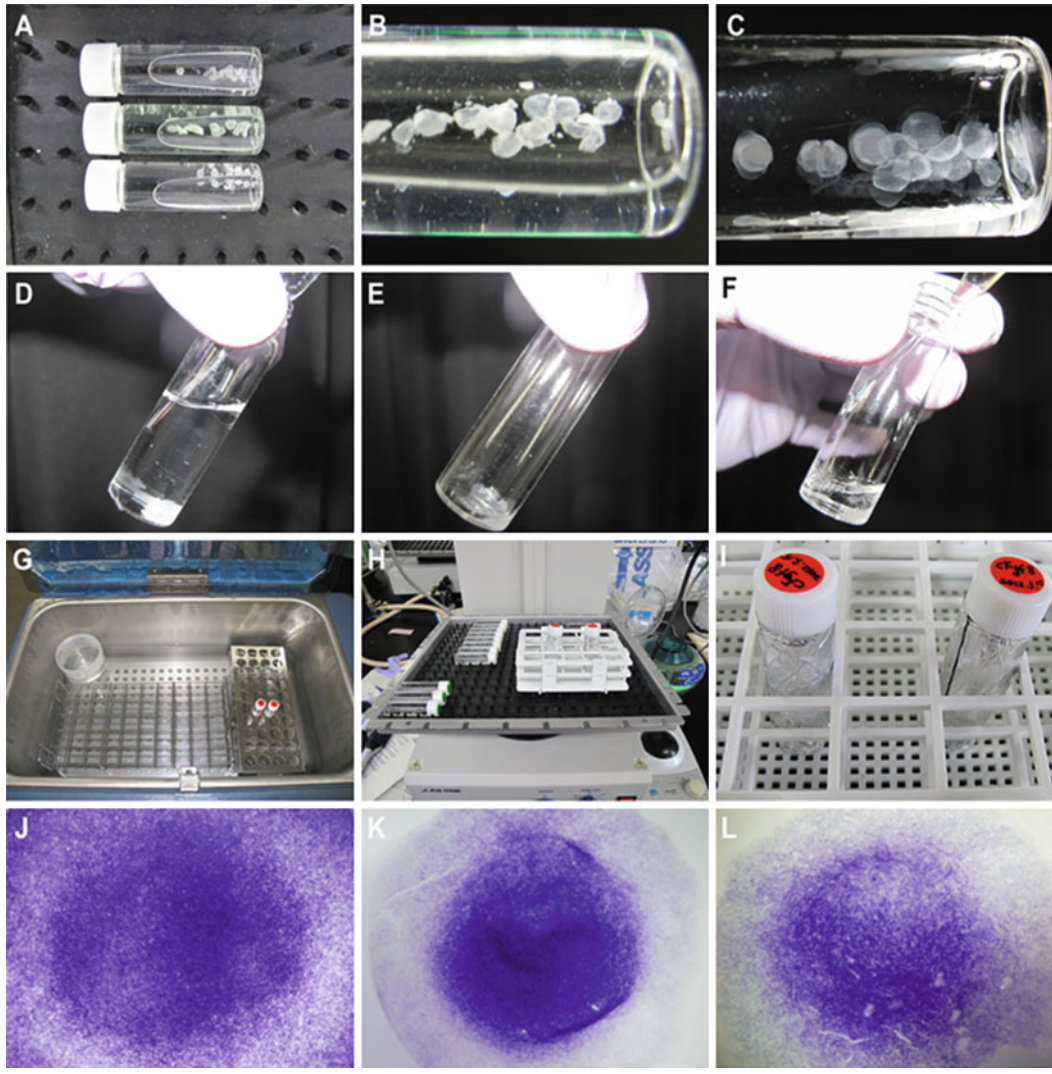

Fig. 4 RNA in situ hybridization. PFA and methanol fixed embryos (**a–c**) are pre-hybridized at 68 °C for several hours following Proteinase K treatment, post-fixation, and multiple PBT washes. Removal and addition of liquids should always be done from the side of the vial (**d–f**). Following pre-hybridization diluted DIG and/or Fluorescein-labeled probes are added to vials and incubated over night using a water bath at 68 °C (**g**). After multiple washing steps prior and post-incubation with an anti-DIG and/or anti-Fluorescein antibody solution, color development is initiated using a NTMT + BCIP + NBT solution (**h**, **i**). Following several hours up to several days of color development at RT or 4 °C, embryos are washed with TBST and used for further analysis. Representative in situ hybridization results of the pluripotency associated genes Nanog (**j**), Dnmt3B (**k**), and Lin28 (**l**)

come off during washes in subsequent steps (Fig. 4b) and embryos will eventually be free of yolk (Fig. 4c). When changing solutions in a vial, remove liquid from the side (do not touch or aspirate the embryos) and add fresh liquid to the side (do not drop directly onto the embryos) (Fig. 4d, e, f).

5. Digest the embryos with Proteinase K (Sigma) (10 μg/ml) for 15–30 min at RT. Leave the vials standing up, do not nutate. During and after this treatment, embryos are very fragile, but should not disintegrate (if so, adjust Proteinase K concentration or digestion time).
6. Remove Proteinase K very carefully, replace with postfix solution. Fix for 30 min to 1 h.
7. Remove postfix and wash with PBT (rinse 2× and wash 2× 10 min). Change into pre-hybridization solution.
8. Pre-hybridize in a water bath set at 68 °C (Fig. 4g) for at least 2 h. This step can be extended by several hours, or stopped by storing embryos in pre-hybridization solution at −20 °C for up to a couple of years.
9. Dilute labeled probes with pre-hybridization solution to desired dilutions (this should be tested out beforehand for each probe and diluted probes can be reused for many months). Diluted probe in pre-hybridization solution is called hybridization solution. Remove the pre-hybridization solution and add warmed-up hybridization solution. Hybridize overnight.
10. Remove the hybridization solution (and reuse) and wash with warmed-up pre-hybridization solution (3× rinses and 2× 30 min washes). Avoid contamination when working with multiple probes.
11. Change into 1:1 mix of pre-hybridization solution and TBST solution. Keep for another 30 min at 68 °C.
12. Wash in TBST (2× rinses and 3× 30 min washes) at RT.
13. Block in the blocking solution for 2 h at RT.
14. Incubate in the antibody solution (blocking solution + anti-DIG or anti-Fluorescein antibody (1:5,000 dilution)) overnight at 4 °C.
15. Wash in TBST (3× rinses and 3× 60 min washes) at RT.
16. Wash in NTMT for 10 min. Proceed to color development (NTMT + NBT + BCIP solution, protect from light) (Fig. 4h, i).
17. Monitor the color development in the first hour at RT. Decide on how long to continue at RT or move to 4 °C. If there is no signal in the first hour, the reaction can be left for several more hours at RT. If there are weak signals in the first hour, monitor closely every hour or so at RT or leave at 4 °C overnight. A good probe for a highly expressed gene will reach ideal signal/background ratio in 1–2 h. But for most moderately and specifically expressed genes, an ideal outcome is to have the best signal/background ratio within from several hours to overnight. Sometimes color reaction may take a few days. It is still good if there is no background or trapping signals.

18. To stop the color reaction, wash with TBST a few times (overnight if staining is strong). Change into fix solution and store at 4 °C for further analysis (photography, secondary staining, or sectioning).
19. Examples of in situ hybridization of pluripotency genes are shown in Fig. 4j (Nanog), Fig. 4k (Dnmt3B), and Fig. 4l (Lin28).

3.5 Primary Culture of Dissociated Epiblast Cells and Subculture of Epi-SCs

Refer to **Note 5** for a general description of avian epiblast-derived ES cells. Here we show how to make recombinant chicken LIF, prepare feeder layer, generate primary epiblast cell culture, and maintain Epi-SCs.

3.5.1 Production of Recombinant Chicken LIF

1. Total RNA is extracted from epiblast cells (*see* Subheading 3.1) with Trizol reagent and is used for reverse transcription PCR (RT-PCR) with the SuperScript™ III. Design and synthesize the appropriate oligonucleotide primers for amplification of chicken LIF. For cloning into pSecTag2A vector, modify the 5′-end sequence of the primers: forward primer 5′-GC*GCTAGC*CATGAGGCTCATCCCC-3′ (italics, *Nhe*I restriction site; underline, start codon); reverse primer 5′-GC*GTCGAC*CGCGGGGCTGAGGTGAGG-3′ (italics, *Sal*I restriction site).
2. Prepare the PCR mixture (50 μl final volume) in an amplification tube: 1.0 μl template DNA (approx. 100 ng), 5.0 μl 2.5 mM dNTPs, 5.0 μl 10× Ex Taq buffer, 1.0 μl forward primer (10 pmol/μl), 1.0 μl reverse primer (10 pmol/μl), 0.5 μl Takara Ex Taq DNA polymerase (5 U/μl), and 36.5 μl H_2O. Using a thermal cycler, heat the samples to 94 °C for 5 min and then run 30 amplification cycles in the linear range of 30 s at 94 °C (denaturation), 30 s at 60 °C (annealing), and 1 min at 72 °C (polymerization). Finally, hold for 10 min at 72 °C as an extension step and then store at 4 °C.
3. Digest both pSecTag2A and PCR products with *Nhe*I and *Sal*I, and purify the DNAs using a commercially available kit. The myc-epitope in pSecTag2A is excised from the vector using restriction enzymes. Ligate the PCR products into the pSecTag2A vector using a commercially available kit. Transform the recombinant vector to TOP10F *E. coli* strain and purify the recombinant vector using EndoFree Plasmid Maxi Kit.
4. Grow up CHO cells in Ham-F12 supplemented 10 % FBS or CHCC-OU2 cells in DMEM supplemented with 10 % FBS in a 6-well culture plate. The seeded cells are 90–95 % confluent at the time of transfection. Transfect 2.0 μg/ml of the recombinant vector using Lipofectamine 2000 reagent.
5. After incubation for 24 h, remove the medium including the recombinant vector and add fresh medium supplemented with

10 % FBS and 250 μg/ml of Zeocin. Feed the cells with the selective medium every 3 days until foci can be identified. Pick and expand 20–40 foci to obtain recombinant chicken LIF producing cells. Check the producing cells by immunoblotting analysis using the culture supernatant and anti-His HRP antibody (supplied with the kit) (Fig. 5a). Grow up the cells in DMEM supplemented with 10 % FBS and collect the culture supernatant of 500 ml.

6. Recombinant chicken LIF is expressed as a fusion protein linked to a 6× Histidine tag and purified by affinity chromatography method using ProBond Resin. Add 10 ml of the washed resin to 500 ml of the culture supernatant, and aliquot the supernatant in 50 ml tubes. Use gentle agitation such as end-over-end rotation for 2 h at 4 °C. Pour the resin in a 2.5 cm × 10 cm column. Wash the column with 100 ml native wash buffer (supplied with the kit). Repeat washing three more times.
7. Elute the protein with native elution buffer (supplied with the kit). Collect 10 ml fractions and analyze with SDS-PAGE (Fig. 5b). Pool the major fractions containing the recombinant chicken LIF in a clean tube. Dialyze against cold PBS at 4 °C overnight. If necessary, concentrate to about 100 μg/ml using Amicon Ultra-15.
8. Aliquot the recombinant chicken LIF into 1.5 ml tubes, store at −80 °C until use. Using this method, 0.5–2 mg of purified recombinant chicken LIF can be obtained.

3.5.2 Preparation of Feeder Layer

1. Grow STO cells in DMEM supplemented with 10 % FBS until confluent.
2. Remove medium and add fresh medium supplemented with 10 % FBS and 10 μg/ml of mitomycin C.
3. After incubation for 2 h, remove the medium, and wash the culture dishes in PBS (4× rinses) at RT.
4. Add PBS with 0.025 % trypsin and 1 mM EGTA, and incubate for 5 min.
5. Resuspend the cells in a volume of DMEM supplemented with 10 % FBS and spin the cells in the DMEM for 5 min at 500 × *g* (3× rinses) at RT.
6. Plate the cells to gelatin-coated culture dishes at a concentration of 10,000–15,000 cells/cm^2 and culture at 37 °C with 5 % CO_2 for 24 h. The culture dishes are gelatinized with 0.1 % gelatin solution for a minimum of 30 min before use.
7. STO feeder layers should be prepared and used within 4 days.

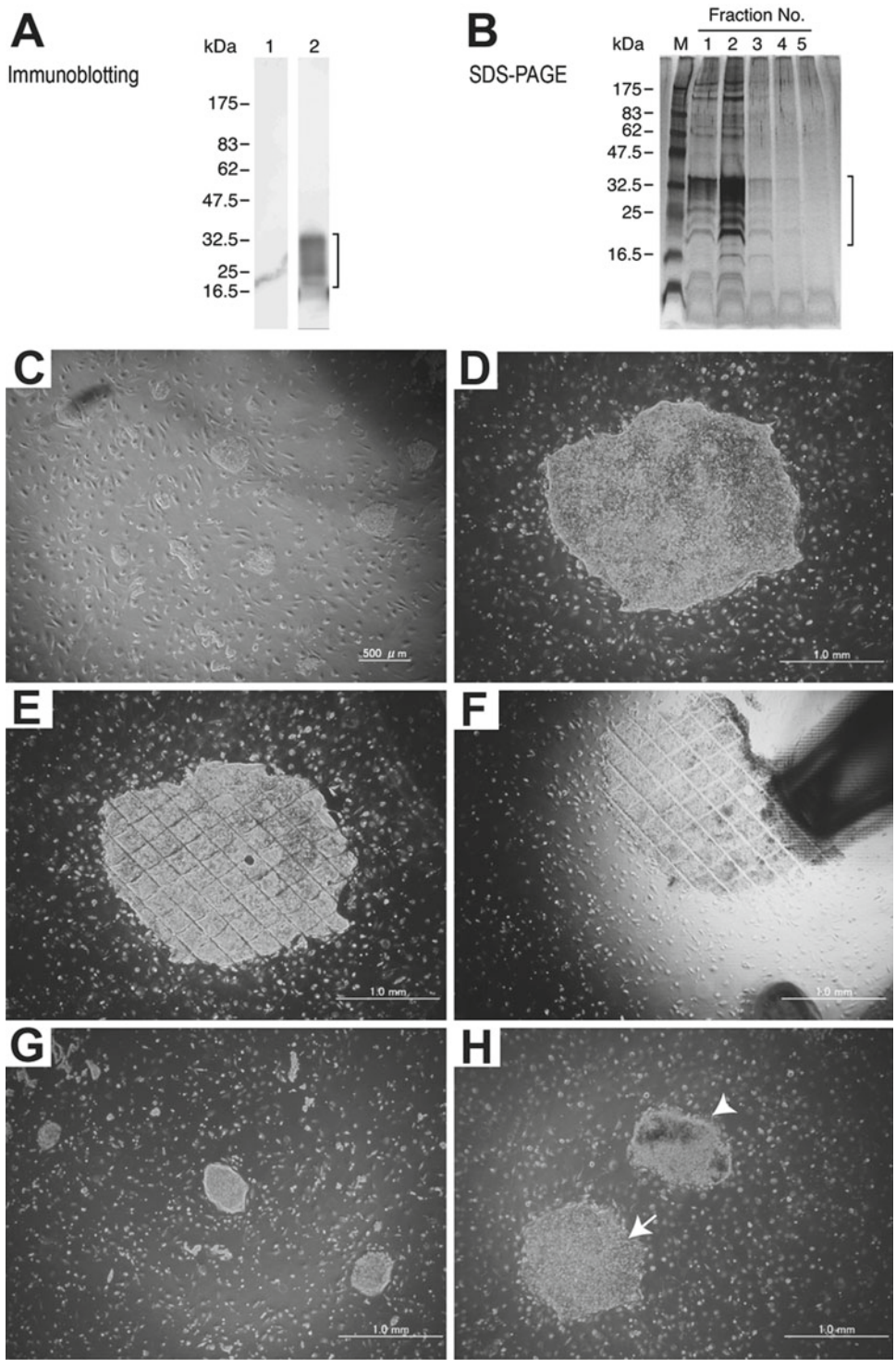

Fig. 5 Primary culture of dissociated epiblast cells and subculture of Epi-SCs. Recombinant chicken LIF in the supernatant was analyzed using 12.5 % SDS-PAGE, blotted to blotting membrane, and reacted with anti-His HRP antibody. *Lanes 1* and *2* were incubated with negative control antibody and anti-His HRP antibody, respectively (**a**). The fractions were analyzed using 15 % SDS-PAGE and the silver-staining method. Molecular

3.5.3 Primary Culture of Epiblast Cells

1. Warm ECC medium and KO-DMEM at 37 °C and keep them warm until ready for use.
2. Pick up dissected epiblast tissues (*see* Subheading 3.1 and Fig. 1m), and transfer them to a 1.5 ml siliconized tube.
3. Centrifuge at 120 × *g* for 3 min, and then remove the supernatant.
4. Add 0.5 ml of ECC medium and gently suspend with a micropipette.
5. Remove the culture medium from STO feeder layer plated on a 35 mm culture dish, wash the dish with KO-DMEM, and add 2 ml of ECC medium.
6. Add 0.5 ml of the cell suspension to the culture dish and culture at 37 °C with 5 % CO_2 and 3 % O_2.
7. After 2 days add 0.5 ml of ECC medium. The cultured epiblast cells form small and round colonies (Fig. 5c).
8. Change one-half of the total medium every 2 days. After 5–6 days, the colonies grow to the size of approximately 1 mm in diameter (Fig. 5d).

3.5.4 Subculture and Maintenance of Epi-SCs

1. After 5–6 days, the colonies of cultured epiblast cells are ready for mechanical passaging by using a razor or a stem cell passaging tool. Do not cut up the colonies too finely at this step.
2. Warm ECC medium and KO-DMEM at 37 °C and keep them warm until ready for use.
3. Aspirate the medium from the epiblast cell cultured dish. Wash once with 2 ml of ECC medium. Replace with 1 ml of ECC medium.
4. Cut the colonies using a razor or a stem cell passaging tool under the stereomicroscope (Fig. 5e). Collect the segmented colonies with a micropipette (Fig. 5f) and break up cell clumps by gentle pipetting.
5. Seed these cells to new 35 mm dish(es) plated with feeder layer cells (typically at a 1:1 or 1:2 passaging ratio).
6. After 1 day, small colonies start to form (Fig. 5g). After 3 days from passaging, both healthy (arrow) and unhealthy

Fig. 5 (continued) size markers: *lane M*. The molecular mass of recombinant chicken LIF is 20–35 kDa (**b**). When dissociated epiblast cells are cultured with ECC medium, they form small and round colonies after 2 days (**c**). After 6 days, the colonies grow to the size of over 1 mm in diameter (**d**). For subculture, colonies are cut with a stem cell passaging tool (**e**). The segmented colonies are collected with a micropipette (**f**). After 1 day from passaging, small colonies start to form (**g**). After 3 days from passaging, both healthy (*arrow*) and unhealthy (*arrowhead*) large colonies start to form (**h**)

(arrowhead) large colonies start to form (Fig. 5h). Remove the unhealthy colonies with a micropipette.

7. After 4–5 days from passaging, remove the unhealthy colonies and subculture only the healthy colonies. Change half of the total medium every 2 days.
8. Repeat **steps 3–7**. After about 3 weeks of primary culture, these cells can grow stably and can be considered to be chicken Epi-SCs.

3.6 Evaluation of Pluripotency in Epi-SCs

Currently, tools for evaluating the pluripotency of cultured chicken Epi-SCs are limited (*see* **Note 6**). This is due primarily to the lack of good antibodies for pluripotency markers and of suitable in vivo differentiation assays. Here we describe two basic evaluation protocols: RT-PCR analysis for Oct4 and Nanog, and immunostaining analysis for Nanog.

3.6.1 mRNA Expression Analysis of Oct4 and Nanog

1. Using a micropipette, collect the Epi-SC colonies from a 60 mm culture dish into a 1.5 ml Eppendorf tube.
2. Wash the cells in RNase-free PBS by mild centrifugation. Remove the supernatant carefully.
3. Total RNA is extracted from the Epi-SCs (Trizol method) and is used for reverse transcription PCR (RT-PCR) with SuperScript™ III. The primers for expression analysis of chicken Oct4, Nanog, and GAPDH (internal control) are shown in Fig. 6a.
4. Prepare the PCR mixture (50 μl final volume): 1.0 μl template DNA (approx. 100 ng), 5.0 μl 2.5 mM dNTPs, 5.0 μl 10× Ex Taq buffer, 1.0 μl forward primer (10 pmol/μl), 1.0 μl reverse primer (10 pmol/μl), 0.5 μl Takara Ex Taq DNA polymerase (5 U/μl), and 36.5 μl H_2O.
5. Using a thermal cycler, heat the samples to 94 °C for 5 min and then run optimal amplification cycles in the linear range of 30 s at 94 °C (denaturation), 30 s at optimal temperature (annealing, *see* Fig. 6a), and 1 min at 72 °C (polymerization). Finally, hold for 10 min at 72 °C as an extension step and then store at 4 °C.
6. Analyze the amplification products on a 1.5–2 % agarose gel (Fig. 6b).

3.6.2 Immunostaining of Nanog

1. Gelatinize chamber slides with 0.1 % gelatin solution for 1 day.
2. Plate mitomycin C-treated STO cells (*see* Subheading 3.5.2) to the slides at a concentration of 10,000–15,000 cells/cm^2 and culture at 37 °C with 5 % CO_2 for 24 h.
3. Aspirate the medium from the slides. Wash once with ECC medium and replace with ECC medium.

A Primers for exoression analysis using RT-PCR

Genns	Sequences	Annealing (C°)	Cycles
Nanog			
forward	TGACCACAGAGCAGAAAACG	58	24
reverse	CAGCCATGAACGGATACAGG		
Oct4			
forward	GGGGCTGGCTCTGGGCACGCTCTAT	60	22
reverse	CCTGGGGACTGGGCTTCACACATTT		
GAPDH			
forward	GCACGCCATCACTATCTTCCAG	58	22
reverse	CGGCAGGTCAGGTCAACAACAG		

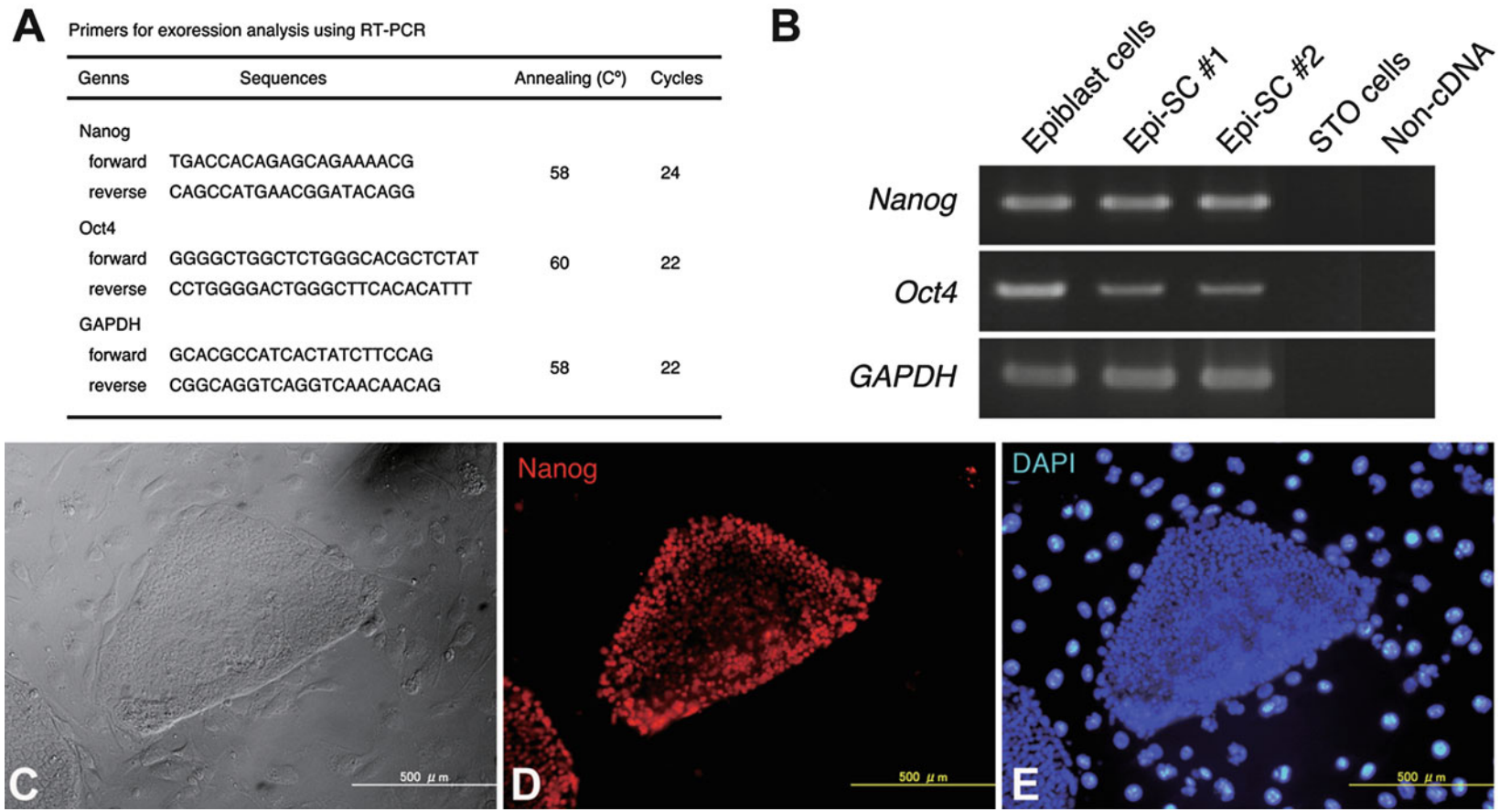

Fig. 6 Evaluation of pluripotency in Epi-SCs. Primer information for expression analysis of Nanog and Oct4 is shown in (**a**). RNAs are isolated from Epi-SCs successively subcultured for 30 days (#1 in **b**) and for 50 days (#2 in **b**). RNAs isolated from uncultured epiblast cells (positive control) and STO cells (negative control) are also examined with the same primers. Chicken GAPDH gene is used as an internal control. In immuno-analysis, Nanog is seen to be expressed in the nucleus of most cells of an Epi-SC colony (**c**–**e**)

4. Seed the segmented colonies of Epi-SCs to the slides and culture at 37 °C with 5 % CO_2 and 3 % O_2. After 2 or 3 days, aspirate the medium from the slides and wash with cold PBS (3× rinses).
5. Add 4 % PFA and incubate for 30 min at RT. Wash in 10 mM glycine–PBS (3× rinses) and rewash in cold PBS (2× rinses).
6. Add 0.1 % Triton-X-100–PBS and incubate for 5 min at RT. Wash in cold PBS (3× rinses).
7. Block in 3 % BSA–PBS for 15 min at RT. Aspirate the blocking solution and add ×100 of anti-chicken Nanog polyclonal rabbit antibody diluted in 1 % BSA–PBS for 1 h at RT. Wash in 0.1 % BSA–PBS (6× rinses).
8. Add ×100 of Alexa Fluor 594-conjugated anti-rabbit IgG antibody and incubate for 1 h at RT. Wash in 0.1 % BSA–PBS (6× rinses).
9. Mount the slide with Vectashield mounting media. The sample is examined using fluorescence microscopy (Fig. 6c–e).

3.7 General Remarks

The chick model is an essential complement to the mouse/human models in epiblast biology. The mammals and reptiles (including birds) are two main branches of the amniotic vertebrates, and development of primitive mammals has a lot in common with that of the reptiles. Eutherian mammals (humans, mice, etc.) have many derived features in their early development which are difficult to understand without the knowledge of comparative amniote embryology. Studies using the chick, the main nonmammalian model organism, can therefore offer essential insights in understanding the formation, differentiation, and pluripotency maintenance of the epiblast and epiblast stem cells.

4 Notes

1. Fertilized and freshly laid eggs purchased from poultry farms can be stored at 16 °C for up to 1 week. Eggs are either warmed up briefly (30′) for the unincubated stage or incubated at 38.5 °C for up to 18 h to reach desired stages. Depending on the weather and flock conditions, developmental stage of embryos from unincubated eggs varies, and this needs to be taken into consideration when designing an experiment. Epiblast-stage embryos are fragile and easily damaged by metal tools or at the liquid/air interface. Keep embryos submerged in liquid and do not grab them directly with forceps. A basic understanding of how to stage and operate on early-streak stage embryos is necessary [6].

2. New culture was invented by Denis New [7] with several modifications made afterwards [8]. It has been the most powerful ex ovo culture method for pre-streak and early-streak stage chicken embryos. Using this culture method, young embryos can be experimented upon using a variety of techniques (tissue graft, bead graft, DNA electroporation, time-lapse imaging, and whole embryo treatment with small molecules). Although it is tedious to set up and takes practice to master, the New culture and its versatile applications are indispensible for avian epiblast-related studies.
3. To study a specific gene or pathway during early avian development, localized treatment is often preferred. The New culture method (Subheading 3.2) can be modified for such purpose. Localized effects can be achieved through bead graft or targeted DNA electroporation. For protein absorption, Heparin-coated agarose/acrylic beads or Affigel blue beads are commonly used. We have also successfully used hydrogel to embed and release proteins locally. For absorption of small molecules, AG1 ion exchange beads are used. A great variety of small molecules (e.g., agonists or antagonists of kinases, receptors, or membrane channels) are currently available. But proteins are limited to secreted growth factors. They are often expensive to acquire from commercial sources and in general their efficacy in such experimental settings has not been tested. This limitation is circumvented by DNA electroporation. DNA constructs expressing any gene of interest can be electroporated and their effect analyzed locally in cells expressing the gene of interest, either through a protein tag, 2A-peptide-mediated co-expression of a marker gene, or co-electroporation of a marker gene-encoding construct. The drawback of electroporation, especially for early-stage embryos, is that there is a minimum of 2 h (for GFP) needed to transcribe and translate introduced genes. Any effect from an electroporated gene may not be evident until at least 4–6 h culture after electroporation. In addition to the New culture-based methods, we have also tested a method for in ovo analysis of the epiblast-stage embryos. We call it the subgerminal cavity method. The subgerminal cavity is the space between the yolk cell membrane and the epiblast/hypoblast. Its lateral margin is the germ wall where the yolk cell and deep layer cells of the germ wall adhere to each other. In unincubated eggs, epiblast cells are directly exposed to the subgerminal cavity. Growth factors and/or small molecules can be injected into this space, and their influence on epiblast cell differentiation can be analyzed after a short period of incubation. The advantage of this method is that it is in ovo, easy to learn and quick to perform (compared to the New culture) and its effect

on epiblast or embryonic development is rapid (compared to the electroporation method).

4. There are a few protocols for RNA in situ hybridization of chicken embryos. The main differences are in the ingredients for the hybridization solution and the hybridization temperature. We follow the protocol of the Stern lab [9]. This protocol has worked well for us. We have also used the same protocol successfully for reptilian and other avian embryos. Pre-gastrulation and early gastrulation-stage embryos are fragile, so throughout the entire in situ process (solution changes, washing, etc.), care should be taken to avoid damage to the embryos. The "tissues" at these stages are only a few cell-layers thick, so there is minimal worry of tissue trapping or trapping-related background staining. Key to success is the quality of DIG- or Fluorescein-labeled probes and the washes after hybridization and antibody incubation. Routine precautions for RNA-related work (baking glassware and metal tools and using DEPC-treated water) should be taken when preparing stock solutions.
5. Chicken epiblast cells derived from a stage X (EGK) embryo have the capacity to contribute to all three germ layers and germ cells when transplanted into a recipient embryo of the same developmental stage [10, 11]. Thus, attempts have been made to establish chicken embryonic stem (ES) cells by in vitro culture of early epiblast cells [5, 12–14]. All of the established ES cells are of the epiblast origin, and we describe them as epiblast stem cells (Epi-SC). The pluripotency and growth of cultured epiblast cells can be maintained by the addition of chicken leukemia inhibitory factor (LIF) [15] or of conditioned medium from buffalo rat liver cells [13] to the culture medium. We follow the protocol of epiblast culture using chicken LIF. Recombinant chicken LIF protein [5, 16] is obtained from recombinant CHO-K1 cells (ATCC #CCL-61) or CHCC-OU2 cells (supplied by the United States Department of Agriculture [17]) transfected with the chicken LIF gene. The epiblast cells or Epi-SCs are plated onto a layer of mitotically inactivated Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) cell (ATCC #CRL-1503) feeders. Chicken epiblast stem cells in culture are similar in morphology to primate ES cells and consist of circular colonies with clear contour and each individual cell visible.
6. Mouse and human ES cells express a number of pluripotency markers (Oct4, Nanog, Sox2, Rex1, Utf, etc.). In chickens, however, only Oct4 and Nanog have been reported [3, 5]. These factors are required for the maintenance of pluripotency and self-renewal of chicken ES cells [16]. Our unpublished results indicate that several other pluripotency markers, such as

Dnmt3B and Lin28, can also serve as good markers for avian Epi-SCs. The list will undoubtedly increase as more whole-genome analyses are being performed for early-stage chicken embryos. Pluripotency of cultured chicken Epi-SCs can be evaluated by assaying the expression level of these genes using RT-PCR or immunostaining. For RT-PCR analysis, the GenBank accession numbers for chicken Oct4 and Nanog are DQ867024 and DQ867025, respectively. For immunostaining, unfortunately, specific antibodies against these proteins are not commercially available, and the commercially supplied antibodies against their mammalian orthologues do not show satisfactory cross-reactivity.

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