

## ***Drosophila* Germline Stem Cells for In Vitro Analyses of PIWI-Mediated RNAi**

**Yuzo Niki, Takuya Sato, Takafumi Yamaguchi, Ayaka Saisho, Hiroshi Uetake, and Hidenori Watanabe**

### **Abstract**

The *Drosophila piwi* gene has multiple functions in soma and germ cells. An in vitro system provides a powerful tool for elucidating PIWI function in each cell type using stable cell lines originating from germline stem cells (GSCs) and ovarian soma of adult ovaries. We have described methods for the maintenance and expansion of GSCs in an established cell line (fGS/OSS) and an in situ hybridization method for analyzing *piwi*.

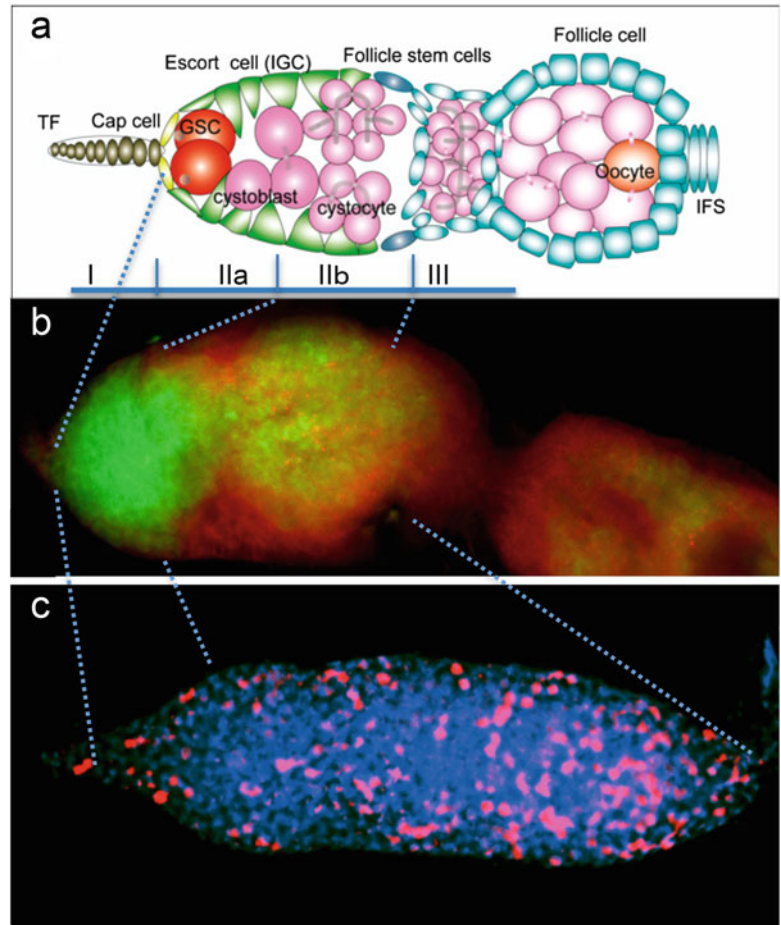
**Key words** *Drosophila* germline stem cells, Ovarian cells, fGS/OSS

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### **1 Introduction**

*Drosophila* P-element induced wimpy testis (*piwi*) was first described as a gene required for the maintenance and normal development of germline stem cells (GSCs) in the testes and ovaries [1–3]. The *Drosophila* GSC niche is a suitable experimental model for RNAi study because it is accessible to various molecular and genetic tools [4]. Piwi is localized in the nuclei of somatic and germinal ovarian cells. The renewal of GSCs is maintained by *piwi* expression in the ovarian somatic niche [2, 3, 5]. An additional role of Piwi in germline development is the formation of maternally inherited polar plasma [6]. This gene also plays a role in Piwi-interacting RNA silencing, e.g., the suppression of transposable element expression [7–11].

*Drosophila* GSCs reside at the anterior tip of the germarium and are in direct contact with cap [12] and escort cells. One of the daughter cells of GSCs (called the cystoblast) leaves the niche and moves posteriorly with the help of escort cells [13, 14] (Fig. 1a). After four synchronous incomplete cycles of cytokinesis, the cystocyte (descendant from the cystoblast) is surrounded by



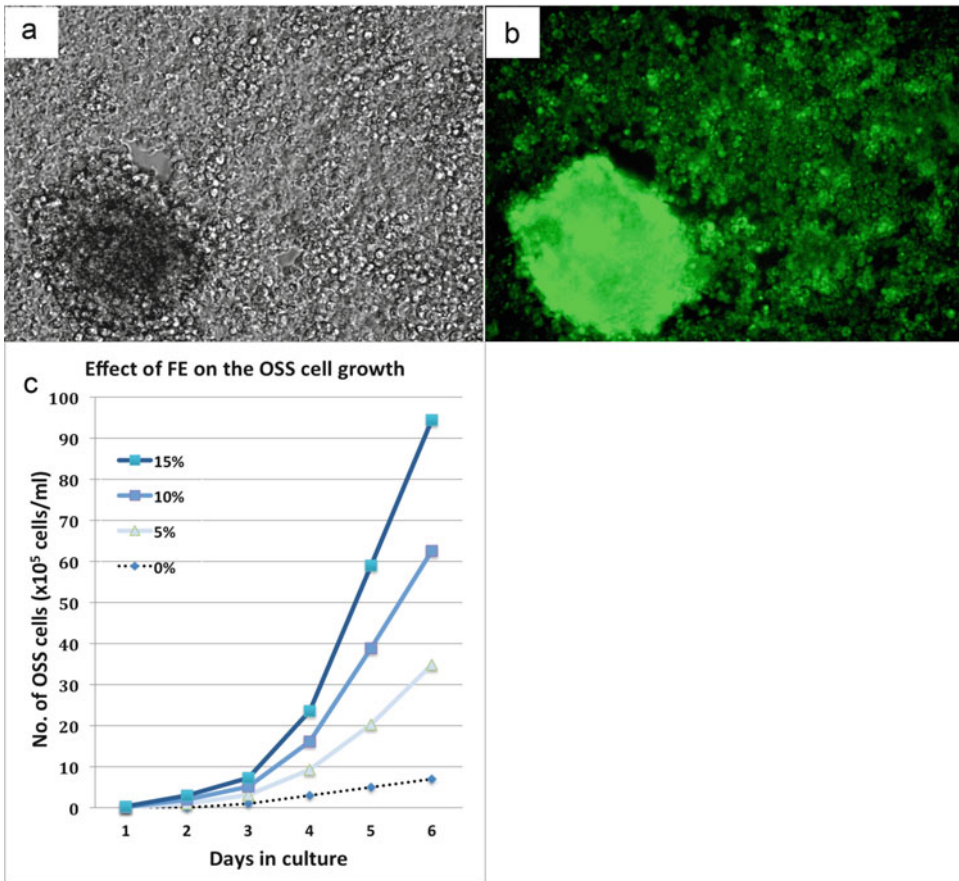
**Fig. 1** Diagram of wild-type germarium and immunostained *bam* germaria. (a) Diagram of wild-type germarium. GSCs associating with cap and escort cells at the anterior tip undergo self-renewal division. One of the daughter cells (cystoblast) moves posteriorly, dissociates from the cap cells, and undergoes four cycles of synchronous, incomplete cytokinesis to produce 16-cell cystocytes. The 16-cell cystocytes are then surrounded with prefollicular cells derived from follicular stem cells. The follicular cells undergo eight mitotic divisions resulting in more than 600 cells. (b) Immunostained *bam* germarium with anti-Vasa (green) and Fas3 (red) antibodies, a germ cell and follicular cell marker, respectively. The hypertrophy of the germarium begins from just posterior to the follicular stem cell location where an egg chamber would be formed during normal oogenesis. (c) A mitotic *bam* germarium stained with anti-BrdU antibody (red) after BrdU incorporation. Note that mitotic cells are prominent at the posterior region of the germarium

prefollicular cells originating from two follicle stem cells [15] (Fig. 1a). The follicular cells undergo approximately eight mitotic divisions and produce more than 600 cells that surround each maturing egg chamber [15, 16].

Undifferentiated GSCs accumulate in the anterior and posterior regions of the germarium in ovarian tumors of the *bam-of-marble* (*bam*) mutant. These correspond to regions IIb and III of the normal germarium (Fig. 1b). In addition to GSCs, prefollicular cells continue to overproduce in the germarium as the female ages. Observation of the *bam* ovary stained with an anti-BrdU antibody after BrdU incorporation reveals prominent mitotic GSCs and soma located posteriorly (Fig. 1c). More than 1,000 cells, consisting of similar number of GSCs and prefollicular cells, accumulate in each ovariole of a 20-day-old female (unpublished). It is possible that the posterior expansion of GSCs is sustained by Dpp and other growth factors produced by prefollicular cells.

We successfully cultured adult GSCs after dissecting adult tumorous ovaries of the genotype of *w<sup>1118</sup>; P[w hsp-70 bam<sup>+</sup>]<sup>11-d</sup> bam<sup>D86</sup>/bam<sup>D86</sup> P[ovo-LacZ] P[vasa-egfp]* for a long term [17]. In addition, we established a cell line consisting of germ and soma cells and named it fGS/OSS. We then selected subpopulations consisting of only somatic cells by checking for the absence of vas-Egfp expression and established more than 20 independent subpopulations of somatic cells. There were no conspicuous variations of cellular morphology among these subpopulations. The somatic cells were flat and less than 10  $\mu\text{m}$  in diameter. The uniformity of these subcultures indicates that the somatic cells were of one type and they originated from follicle stem cells and/or prefollicular cells. We named the somatic cell line OSS ([17, 18] and Fig. 2a, b). The OSS cells have a high mitotic activity that is similar to prefollicular cells in vivo. This cell line has been used for studying the biochemical nature of PIWI-related RNAi machinery in the ovarian soma [5, 19].

The maintenance and expansion of GSCs are sustained by growth factors supplied from OSS cells. GSCs and OSS cells directly associate with each other via DE-cadherin-mediated adherence junctions. In contrast to fGSCs, OSS cells can be maintained and expanded in culture under low nutrient conditions. Figure 2c shows an example of the growth curve of OSS cells with various concentrations of fly extract (FE) supplemented in the culture medium. Furthermore, the number of GSCs begins to decrease or is completely lost during rapid expansion of OSS cells. A possible explanation for this is the predominance of S- and M-phase OSS cells in the cell population. These cells would lose their functional ability to make contact with GSCs and produce sufficient quantities of growth factors. In addition GSC loss could be attributed to the formation of OSS cell clumps that surround GSCs as the former reach confluence (Fig. 2a, b). This would result in a lack of oxygen, nutrients, and space, all of which are necessary for survival and expansion of GSC. It is noteworthy that the contact inhibition observed in normal mammalian cell cultures does not occur in insect cultures. Thus, a key factor for the successful maintenance and expansion of GSCs in fGS/OSS culture is the suppression of their rapid expansion.



**Fig. 2** Characteristics of fGS/OSS and OSS cells. Phase-contrast (a) and fluorescent (b) images of confluent fGS/OSS cells. As cells become confluent, a characteristic cell clump is formed in which GSCs are surrounded with OSS cells. (c) Effect of FE on the expansion of OSS cells. Since OSS cells originate from prefollicular cells with high mitotic activity, they expand rapidly even when maintained in low concentrations of FE. They expand more than 30 and 90 times in culture media supplied with 5 and 10 % FE, respectively, within 5 days

## 2 Materials

Prepare all solutions using Millipore-filtered ultrapure water. The solutions are freshly prepared before each use. Use nuclease-free water in each experiment and endotoxin-free water in cell culture.

### 2.1 Cell Culture

1. Shields and Sang M3 insect medium (Sigma).
2. L-Glutamic acid potassium salt monohydrate (Sigma).
3. Potassium bicarbonate (Sigma).
4. Glutathione\* (Sigma).

5. Insulin\*\* (Sigma).
6. Fetal bovine serum (FBS; endotoxin-free).
7. 1 % NaOH.
8. 1 N HCl.
9. Fly extract (FE; *see* Subheading 3.3).
10. 96-Well culture plate (Sumilon: Sumitomo Bakelite).
11. Syringe filter (33-mm Millex filter unit: Millipore).
12. 10-mL Luer-lok™ syringe.
13. 1,000-mL Stericup® filter unit (Millipore).
14. 150-mL Stericup® filter unit (SCGVU01RE, Millipore).
15. 15-mL conical tube.

*\*Stock solution of 100× glutathione*

1. Dissolve 6.0 g of glutathione in 100 mL Milli-Q water.
2. Filter sterilize and store as 1.0-mL aliquots at –20 °C.

*\*\*Stock solution of 100× insulin*

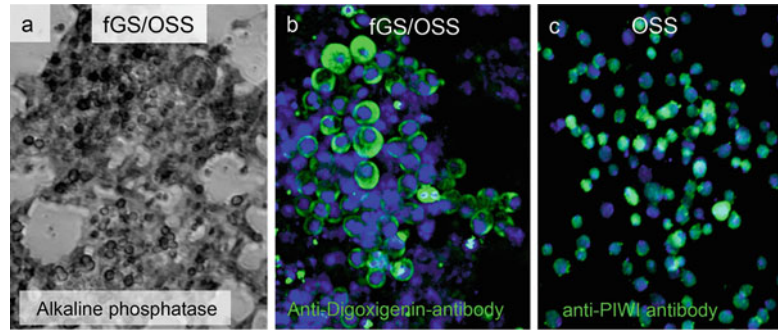
1. Dissolve 100 mg insulin in 100 mL Milli-Q water.
2. Add 100 µM HCl, filter sterilize, and store as 1.0-mL aliquots at –20 °C.

## **2.2 In Situ Hybridization**

1. ddH<sub>2</sub>O: Direct-Q®3 Ultrapure water system from Millipore.
2. *Drosophila* phosphate-buffered saline (PBS) (pH 7.4).
3. PBS plus Triton X-100 (PBT): 0.1 % Triton X-100 in 1× PBS.
4. Ethanol/PBT: 75 % ethanol in PBT, 50 % ethanol in PBT, and 25 % ethanol in PBT.
5. Fixative solution: 4 % (w/v) formaldehyde, 5 % (v/v) acetic acid, and 0.9 % (w/v) NaCl. Freshly prepared before use.
6. Hybridization buffer: 5× SSC, 50 % formamide, 100 µg/mL salmon sperm DNA, 50 µg/mL heparin, 0.1 % Triton X-100, and ddH<sub>2</sub>O.
7. PBT/hybridization buffer: 25 % PBT in hybridization buffer, 50 % PBT in hybridization buffer, and 75 % PBT in hybridization buffer.
8. 5 % bovine serum albumin (BSA): 5 % (w/v) BSA in PBT. Freshly prepared before use.

## **2.3 Colorimetric Detection (Alkaline Phosphatase, Fig. 3a)**

1. Anti-digoxigenin (DIG) antibody: Anti-DIG-AP Fab fragments from sheep (Roche).
2. NTMT: 100 mM NaCl, 100 mM Tris–HCl (pH 9.5), 50 mM MgCl<sub>2</sub>, and 0.1 % Triton X-100. Store at 4 °C.



**Fig. 3** *piwi* expression in fGS/OSS and OSS cells. Colorimetric detection of *piwi* mRNA with alkaline phosphatase (a) and fluorescence detection with anti-DIG–fluorescein antibody (b). (c) Immunostained OSS cells with anti-PIWI antibody. GSCs express higher levels of *piwi* mRNA than OSS cells. Weak signals in OSS cells stained with the anti-DIG–fluorescein antibody are masked by strong signals in GSCs. Accumulation of PIWI in the nuclei of OSS cells can be clearly observed when stained with the anti-PIWI antibody. Bar represents 10  $\mu$ m

3. NTMT–nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP): 4.5  $\mu$ L/mL NBT (75 mg/mL NBT in 70 % dimethylformamide) and 3.5  $\mu$ L/mL BCIP (50 mg/mL BCIP in dimethylformamide) in 1 mL NTMT. Solutions are freshly prepared before each use.

#### 2.4 Fluorescence Detection (Fig. 3b)

1. Anti-DIG antibody: Anti-DIG–fluorescein Fab fragments from sheep (Roche). Store at 4 °C in the dark.

### 3 Methods

Perform all procedures at room temperature unless otherwise specified. Prepare and handle culture medium and cells at a clean bench or in a clean room.

#### 3.1 Preparation of M3 (BF) Medium

Prepare the M3 (BF) medium according to the manufacturer's protocol.

1. Dissolve M3 powder in 700–800 mL sterilized Milli-Q water.
2. Add 1.0 g L-glutamic acid potassium salt monohydrate and 0.5 g potassium bicarbonate.
3. Adjust the medium to pH 6.8 with 1 % NaOH.
4. Add Milli-Q water to a final volume of 1 L.
5. Filter sterilize the medium through a 1,000-mL Stericup filter unit and store at 4 °C.



### 3.2 Preparation of Culture Medium for fGS/OSS and OSS Cells

1. Mix 50–60 mL of the above M3 (BF) medium, 10 mL of heat-inactivated FBS (pre-incubated at 50 °C for 10 min), and 1.0 mL each of 100× glutathione and 100× insulin. Make up to a final volume of 100 mL by the adding M3 (BF) medium.
2. Sterilize the mixed medium by passage through a 150-mL Stericup filter unit and store as 10-mL aliquots in 15-mL conical tubes at 4 °C.

### 3.3 Preparation of FE

FE is prepared according to the method of Currie et al. [20].

1. Prepare young adult flies of wild-type Oregon-R, aged 1–2 days post emergence. Two hundred flies are required for a 1.5-mL of aliquot.
2. Sterilize the flies with 70 % ethanol for 10 min.
3. Wash the sterilized flies three times with sterilized PBS and once with M3 (BF) medium supplemented with 10 % FBS.
4. Homogenize 200 sterilized flies with 1.2 mL of M3 (BF) medium containing 10 % FBS (1,000 flies with 6.0 mL of the culture medium) on ice.
5. Centrifuge the homogenate at 1,500×*g* for 20 min at 4 °C.
6. Heat inactive the supernatant at 60 °C for 5–10 min.
7. Centrifuge the heat-inactivated supernatant at 6,000×*g* for 10 min at 4 °C.
8. Transfer the supernatant into a fresh 1.5-mL tube and repeat centrifugation at 10,000×*g* for 20 min.
9. Perform a second centrifugation of the supernatant at 15,000×*g* for 60 min at 4 °C.
10. Make up to a final volume of 1.5 mL by adding M3 (BF) medium + 10 % FBS.
11. Store the FE aliquot at –20 °C after filter sterilization.

### 3.4 Maintenance and Expansion of fGS/OSS Cells

The most critical factor for the survival of GSCs is the supply of an adequate concentration of FE. A second limiting factor is cellular density as previously mentioned. Select GSC-rich subpopulations after splitting into smaller cell populations. Less than 100 cells are sufficient for expansion of fGS/OSS cells.

1. Filter sterilize the culture medium and FE mixture (*see Note 1*).
2. Inoculate the small cell population of fGS/OSS or OSS cells into one well of a 96-well culture plate.
3. Add the above mixed culture medium to a final volume of 200 µL in each well.
4. Maintain the plate in a Tupperware box or small container at 25 °C (*see Note 2*).

5. Check for cell viability and cell expansion. Within 24 h a healthy culture will contain somatic cells spreading from the cell masses and round GSCs in association with the soma.
6. Perform daily checks on the growth of GSCs on the somatic sheet.
7. Feed the cells every 5–7 days by replacing half of the culture volume with fresh culture medium.
8. After reaching confluence, the cells are transferred to a fresh well on the culture plate by gently scraping and pipetting the cellular sheet with the tip of a 200- $\mu$ L micropipette (*see Note 3*).

### **3.5 In Situ Hybridization**

Several modifications were applied to the procedures reported in previous studies [21–25] and to the manufacturer's protocol [26] in order to optimize the protocol for use in a 96-well culture plate. Addition or removal of reagents by pipette must be performed with care throughout the experiment to avoid detachment of adherent cells from the well. Note that Triton X-100 can result in the production of unwanted bubbles.

#### *Cell Preparation*

1. Inoculate a 96-well culture plate with a small population of cells ( $1 \times 10^3$  to  $10^4$  cells/mL) and culture for 24–48 h (*see Note 4*).
2. Remove the medium (*see Note 5*).
3. Fix the cells for 30 min in the fixative solution.
4. Perform a second fixation in 100 % ethanol for 10 min after removal of the first fixative solution.
5. Rehydrate the cells by passage through an ethanol/PBT gradient (75, 50, and 25 %) with 10 min in each solution.
6. Perform two 10-min washes in PBT.
7. Prehybridize by adding hybridization buffer for 10 min and then incubate for 60 min at 55 °C. This step is optional.
8. Remove the hybridization buffer.

#### *Hybridization*

9. Dilute the DIG-labeled RNA probe (*see Note 6*) in 100  $\mu$ L of hybridization buffer (10–20 ng/well).
10. Denature the DIG-labeled RNA probe at 80 °C for 5 min and store the probe at 4 °C in a PCR machine until use.
11. Add 100  $\mu$ L of the prepared probe to the cells and hybridize at 60 °C for 18 h.
12. Remove the probe.
13. Perform two 30-min washes in prewarmed hybridization buffer at 60 °C.



14. Rinse for 20 min in prewarmed 25 % PBT/hybridization buffer at 60 °C.
15. Rinse for 20 min in prewarmed 50 % PBT/hybridization buffer at 60 °C.
16. Rinse for 20 min in 75 % PBT/hybridization buffer.
17. Perform two 20-min rinses in PBT.
18. Block the cells in 5 % BSA for 30 min.
19. Remove the BSA solution.

*Colorimetric Detection (Alkaline Phosphatase) (Fig. 3a)*

20. Stain the cells for 60 min with a 1:500 dilution of anti-DIG-AP in BSA.
21. Perform four 20-min washes in PBT.
22. Perform three 5-min rinses in NTMT.
23. Add NTMT-NBT/BCIP in the dark. Check the cells every 5 min under the microscope.
24. Remove NTMT-NBT/BCIP after the desired incubation period (*see Note 7*).
25. Perform three 5-min washes in PBT (*see Note 8*).

*Fluorescence Detection (Fig. 3b)*

26. Stain the cells overnight with a 1:500 dilution of anti-DIG-fluorescein antibody in BSA at 4 °C.
27. Perform four 20-min washes in PBT.

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## 4 Notes

1. The quality of FE may vary between laboratories. Thus, the optimal concentration of FE and FBS should be determined according to individual experimental design. For example, if the cells are intended for in situ hybridization and histochemistry, then the amount of FE and FBS required is less (<5 % each), whereas for biochemistry, 10–15 % FE and FBS would be required.
2. Add 200 µL sterilized Milli-Q water to the outermost wells of the plate to avoid desiccation.
3. Dissolve cell masses by treatment with 0.1 % trypsin + 0.02 % EDTA at 37 °C for 5 min when cell masses or cell clumps are tightly associated.
4. A confluence level of approximately one-third ( $4.0 \times 10^5$  cells/mL), allows easy capture of individual cell images at the end of the experiment.
5. Leave approximately 20 µL of the medium in the well. Removal of all the medium from the well is likely to lead to drying out and cell loss.

6. Prepare DIG-labeled RNA probes [26]. We use alkaline hydrolysis to produce 500-bp RNA probes.
7. Check under the microscope for NTMT-NBT/BCIP color changes in the samples.
8. Samples are washed at 4 °C in the dark because this helps the residual NTMT-NBT/BCIP to react with the probes more slowly.

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