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Isolation and in vitro culture of primary cell populations derived from ovarian tissues of the rockfish, *Sebastes schlegeli*

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Abstract

This study was conducted to identify the general conditions for the isolation and in vitro culture of ovary-derived cells in rockfish (*Sebastes schlegeli*). The effects of three different enzymes on cell retrieval from ovarian tissues were evaluated first, and then the ovary-dissociated cells were cultured under various culture conditions, with varying basal media and culture temperatures, addition of growth factors, and/or culture types. We found that collagenase type I treatment was effective for cell isolation from ovarian tissues. From a total of 42 trials to evaluate the effects of basal media and culture temperatures on cell culture of ovary-dissociated cells, we observed that Leibovitz's L15 medium was more supportive than Dulbecco's modified Eagle's medium for culture, and the cells could grow at all three temperatures tested, 15, 20, and 25 °C, at least up to passage 2. However, growth factor addition did not improve cell growth. Introduction of suspension culture after monolayer culture expanded the culture period significantly more than did monolayer culture alone. Our results may provide a basis for developing an in vitro system for *S. schlegeli* germline cell culture, which will ultimately lead to improvement of the species.

Background

Establishment of an in vitro system can provide a useful tool for biotechnology applications as well as various research areas (Lakra et al., 2011; Smagghe et al., 2009; Smith, 2006; Stacey, 2012). Particularly, in vitro utilization of germline cells that possess greater developmental potential can offer a novel way of deriving superior animals through grafting this in vitro system onto existing genetic breeding and transgenic technology (Gong et al., 2008; Shin et al., 2008; Kim et al., 2009; Kim and Izpisua Belmonte, 2011). In fish, however, a limited number of studies has been conducted to establish in vitro systems using germline cells, and most of those have been restricted to small fish models (Hong et al., 2004; Kawasaki et al., 2012; Nóbrega et al., 2010; Wong and Collodi, 2013; Wong et al., 2013). Thus, there has been little research regarding farmed fish species (Lacerda et al., 2010).

Rockfish (*Sebastes schlegeli*) is a major farmed marine finfish, representing the second largest production of fish cultured in Korea (Jayasinghe et al., 2015). Thus, attempts to develop an in vitro system for culturing germline cells followed by improvement of the breed are important due to its commercial scale as a valuable food resource. In this study, as a first step towards the long-term goal of establishing a germline cell culture system in *S. schlegeli*, we first conducted primary cell cultures derived from *S. schlegeli* ovarian tissues to establish general guidelines for *S. schlegeli* cell culture by determining optimal culture conditions. We first compared the effects of three different enzymes on cell retrieval from ovarian tissues for efficient cell isolation and subsequently cultured the ovary-dissociated cells under various conditions to evaluate the effects of basal media, culture temperature, growth factors, and culture type on in vitro culture.

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Methods

Fish

Rockfish (*S. schlegeli*) were purchased from a local market from July to October. In total, 17 fish were used for this

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study, and the average weight and body length were 534.17 ± 64.46 g and 32.24 ± 1.71 cm, respectively. All procedures for animal management, euthanasia and surgery were complied with the guidelines of Institutional Animal Care and Use Committee (IACUC) of Pukyong National University and the ethical guidelines published by International Council for Laboratory Animal Science (ICLAS).

Ovarian tissue collection and cell isolation

To collect ovarian tissues, healthy female rockfish were sterilized using 70 % ethanol (SK Chemicals, Sungnam, Korea) for 5 min. The ovarian tissues were removed from the bodies using sterilized surgical equipment and washed five times in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA). To evaluate the effects of different enzymes on cell isolation, 200.14 ± 0.11 mg ovarian tissue fragments were placed in 35-mm Petri dishes (SPL life Sciences, Pocheon, Korea) filled with different digestive solutions: DPBS supplemented with 0.05 % trypsin-EDTA (Gibco), 500 U/mL collagenase type I (Worthington Biochemical Corp., Lakewood Township, NJ, USA), and 500 U/mL collagenase type IV (Worthington Biochemical Corp.). Concentrations of each enzyme were determined on the basis of our previous study (Kim et al., 2014). Two collagenase solutions were prepared in Ca^{2+}/Mg^{2+}-containing DPBS, whereas the others were prepared in Ca^{2+}/Mg^{2+}-free DPBS. Then, the tissue fragments were chopped using a surgical blade and incubated for 30 min at 25 °C with periodic pipetting using wide-pore tips every 10 min. After digestion, tissue derivatives were filtered through a 40-μm cell strainer (BD Falcon, San Jose, CA, USA), and the cells were collected by centrifugation (400 g, 4 min). The cells were then treated with red blood cell (RBC) lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM $Na_2 \cdot EDTA$) for 5 min at 4 °C to remove RBCs and then collected by centrifugation (400 g, 4 min) after inactivation of the RBC lysis buffer by adding two volumes of Ca^{2+}/Mg^{2+}-free DPBS. Cell counting was conducted using a hemocytometer (Marienfeld, Lauda-Königshofen, Germany) after trypan blue (Gibco) staining. Cell viability was calculated as the number of live cells/number of total cells × 100. For cell culture, the cells were isolated from whole ovaries under the same protocol as above, but RBS lysis buffer was not used due to its detrimental effects on cell culture.

Culture media and supplements

Two basal culture media, Leibovitz's L15 medium (L15; Gibco) and Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 25 mM HEPES, were used in this study. For cell culture, both media were supplemented with 20 % (v/v) fetal bovine serum (Gibco) and a

1 % (v/v) mixed solution of penicillin and streptomycin (Gibco). According to the treatment groups, 10 ng/mL basic fibroblast growth factor (bFGF; Gibco), 25 ng/mL epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA), 1000 units/mL leukemia inhibitory factor (LIF; Millipore, Billerica, MA, USA), 1 % (v/v) fish serum from rainbow trout (FS; Caisson Laboratories, Smithfield, UT, USA), and/or 50 μg/mL medaka embryo extract (MEE) were added to the culture media. MEE was extracted as described previously (Lee et al., 2013).

Cell culture

The isolated cells were seeded in 12-well tissue culture plates (BD Falcon) coated with 0.1 % gelatin (Sigma-Aldrich). A high cell density, 1.28 ± 0.35 (mean ± standard deviation) × 10^6 cells/cm^2, was used for each culture, and the cells were cultured at 15, 20, or 25 °C in air. On day 3 after the initial seeding, cells were washed twice with Ca^{2+}/Mg^{2+}-free DPBS, and the culture medium was refreshed. Thereafter, half of the medium was replaced every 2 or 3 days. When the cells reached 80–90 % confluency, subculturing of the cells was conducted. The cells were washed twice with Ca^{2+}/Mg^{2+}-free DPBS and detached using 0.05 % trypsin-EDTA. After collecting the cells by centrifugation (400 g, 4 min), they were suspended in culture medium and seeded on 0.1 % gelatin-coated tissue culture plates by splitting at a 1:2 ratio. For suspension culture, the collected cells were suspended in L15 and cultured on 35 or 60 mm Petri dishes (SPL life Sciences) at a density of $3.5 × 10^4$ cells/cm^2. To exchange the medium in suspension cultures, cells and aggregates were collected by centrifugation (400 g, 4 min), and the aggregates were dissociated by treating with 0.05 % trypsin-EDTA for 3 min. After collecting the cells by centrifugation (400 g, 4 min), the cells were suspended in L15 and cultured on 35 or 60 mm Petri dishes. Medium exchange was conducted every 6 or 7 days. Primary cell attachment and cell morphology were examined visually under an inverted microscope (TS100-F, Nikon, Tokyo, Japan).
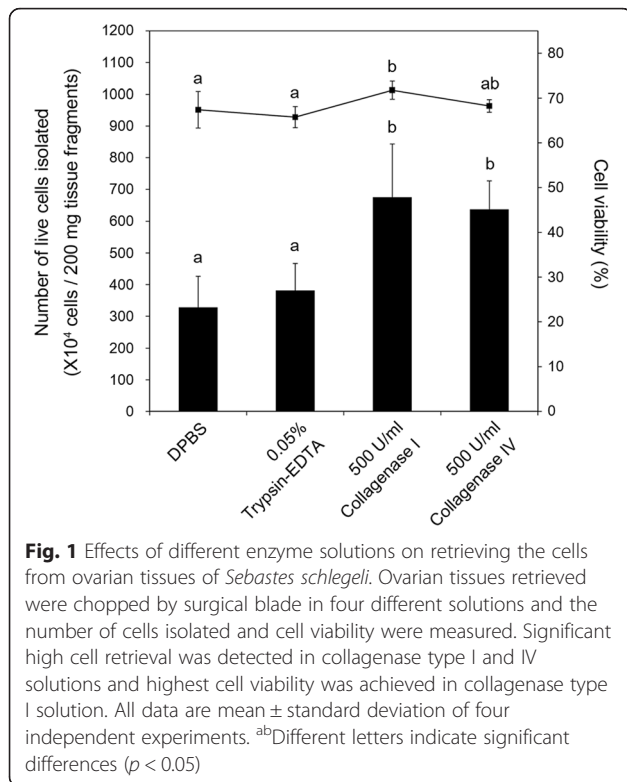
Statistical analysis

The SAS (SAS Institute, Cary, NC, USA) software was used to analyze the effect of each treatment. If a significant main effect was detected by analysis of variance, treatments were analyzed subsequently by the least squares method or Duncan's method. Differences among treatments were considered significant at a P value < 0.05.

Results & discussion

Effects of different enzyme solutions on cell isolation

To develop an effective cell isolation method, we evaluated the effects of three different enzymes on cell retrieval and viability in the isolation procedure. As shown in Fig. 1, high levels of cell retrieval were achieved in the

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Fig. 1 Effects of different enzyme solutions on retrieving the cells from ovarian tissues of *Sebastes schlegeli*. Ovarian tissues retrieved were chopped by surgical blade in four different solutions and the number of cells isolated and cell viability were measured. Significant high cell retrieval was detected in collagenase type I and IV solutions and highest cell viability was achieved in collagenase type I solution. All data are mean ± standard deviation of four independent experiments. [ab]Different letters indicate significant differences ($p < 0.05$)

two groups treated with collagenase type I and collagenase type IV. The mean ± standard deviation numbers of live cells isolated from 200 mg tissue fragments were $328 \pm 98 \times 10^4$, $381 \pm 86 \times 10^4$, $675 \pm 168 \times 10^4$, and $637 \pm 90 \times 10^4$ in DPBS, 0.05 % trypsin-EDTA, 500 U/mL collagenase type I, and 500 U/mL collagenase type IV, respectively, from four independent experiments ($p = 0.0019$). In terms of cell viability, the cells isolated by collagenase type I treatment showed the highest value: 71.8 ± 2.0 % ($p = 0.0450$). Overall, we found that 500 U/mL collagenase type I treatment was an effective way to retrieve live cells from *S. schlegeli* ovarian tissues.

Cell isolation by tissue dissociation is a major step in cell culture, and many factors can affect tissue dissociation procedures. Of these factors, the choice of enzyme is critical to maximize the viable cell yield, and it can usually be determined by the type of tissue subjected to dissociation, because different tissues have different extracellular matrix compositions. However, due to the variability in the extracellular matrix according to the physiological status of the animal species and type of tissue, enzyme selection needs to be conducted empirically. Trypsin is a commonly used enzyme in tissue disaggregation, because it is effective on many cells and tissues. Similarly, the use of crude collagenase preparations has been found suitable for many mammalian cell cultures because of their effectiveness at breaking intercellular tissue matrices via collagenolytic and proteolytic activities (Freshney, 2010). Among the crude collagenase preparations evaluated, collagenase type I, which contains the original balance among collagenase, caseinase, clostripain, and tryptic activities (Can and Karahuseyinoglu, 2007), is widely used for the dissociation of many different kinds of tissues, including ovarian tissues in several mammalian species, such as the mouse (Gong et al., 2010), rat (Ando et al., 1999), and rabbit (Setrakian et al., 1993). Similar results were derived from our study, demonstrating suitability for *S. schlegeli* ovarian tissues as well. This provides fundamental information for cell culture of *S. schlegeli*, as well as other fish species, for which tissue dissociation procedures have not been well established. Nevertheless, additional conditions, such as the temperature, incubation time, and enzyme concentration for enzymatic dissociation, remain to be optimized in each case.

Effects of media and temperature on the culture of ovary-dissociated cells

To determine the optimal culture conditions for *S. schlegeli* ovary-dissociated cells, we first investigated the effects of basal medium and culture temperature on primary cell attachment and continuous culture. Two different basal

Table 1 Culture outcome of *Sebastes schlegeli* ovary-dissociated cells according to temperature and basal media

Temperature (°C)	Basal media[a]	No. of cell populations tested	No. (%)[b] of cell populations initially attached	No. (%)[b] of cell populations subcultured to			
				Passage 1	Passage 2	Passage 3	Passage 4
15	L15	7	7 (100)	7 (100)	3 (43)	1 (14)	0 (0)
	DMEM	7	7 (100)	3 (43)	1 (14)	0 (0)	0 (0)
20	L15	7	7 (100)	6 (86)	3 (43)	1 (14)	0 (0)
	DMEM	7	7 (100)	3 (43)	0 (0)	0 (0)	0 (0)
25	L15	7	7 (100)	5 (71)	2 (29)	1 (14)	1 (14)
	DMEM	7	7 (100)	3 (43)	0 (0)	0 (0)	0 (0)
	Total	42	42 (100)	27 (64)	9 (21)	3 (7)	1 (2)

[a]For cell culture, basal media were supplemented with 20 % (v/v) fetal bovine serum and 1 % (v/v) mixed solution of penicillin and streptomycin
[b]Percentage of the number of cell populations tested

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Table 2 Effects of temperature and basal media on the culture of *Sebastes schlegeli* ovary-dissociated cells

Factors	Group	No. of cell populations tested	No. (%)[a] of cell populations initially attached	No. (%)[a] of cell populations subcultured to			
				Passage 1	Passage 2	Passage 3	Passage 4
Whole replicates	Merged	42	42 (100)	27 (64)	9 (21)	3 (7)	1 (2)
Basal media	L15	21	21 (100)	18 (86)	8 (38)	3 (14)	1 (5)
	DMEM	21	21 (100)	9 (43)	1 (5)	0 (0)	0 (0)
	P-value		-	0.003	0.0076	0.0754	0.3233
Temperature	15	14	14 (100)	10 (71)	4 (29)	1 (7)	0 (0)
(°C)	20	14	14 (100)	9 (64)	3 (21)	1 (7)	0 (0)
	25	14	14 (100)	8 (57)	2 (14)	1 (7)	1 (7)
	P-value		-	0.7475	0.6717	1.0000	0.3771

This table was derived from reallocation of data from Table 1
[a]Percentage of the number of cell populations tested

media, L15 and DMEM, were assessed in cell culture under three different temperatures of 15, 20, and 25 °C. As shown in Table 1, all 42 cell populations cultured showed primary cell attachment regardless of the experimental treatments. Of the cell populations, 64 % (27/42), 21 % (9/42), 7 % (3/42), and 2 % (1/42) grew and survived beyond the first, second, third, and fourth subcultures, respectively, but no cell population reached the fifth subculture. We found that L15 as a basal medium was better than DMEM for cell culture (Table 2). A higher number of cell populations grew to passage 2 when cultured in L15 than in DMEM (86 % vs. 43 % to passage 1, $p = 0.03$, and 38 % vs. 5 % to passage 2, $p = 0.0076$). However, no significant treatment effect on the initial cell culture was detected at the different culture temperatures, suggesting that the cultured cells can grow in the temperature range of 15 to 25 °C, at least up to passage 2. Significant differences in cell growth were observed between the cells cultured in L15 and DMEM. On the basis of many previous studies that reported successful fish cell culture in an air atmosphere (Abdul Majeed et al., 2013; Kim et al., 2014; Lee et al., 2015), we used an air atmosphere instead of CO_2 gas to culture *S. schlegeli* ovary-dissociated cells. L15 medium was originally designed for use in culture without CO_2, and DMEM was supplemented with HEPES to control the physiological pH (Will et al., 2011). Less support for DMEM for cell growth might be a result of phototoxicity caused by the production of hydrogen peroxide from light-exposed HEPES (Lepe-Zuniga et al., 1987; Zigler et al., 1985). More evidence may be derived from additional experiments using appropriate reactive oxygen species scavengers.

As an important factor controlling the physicochemical culture properties, the optimal culture temperature is largely dependent on the body temperature of the animal from which the cells were

Table 3 Effects of various growth factors on the culture of *Sebastes schlegeli* ovary-dissociated cells

Composition of culture medium	No. of cell populations tested	No. (%)[a] of cell populations initially attached	No. (%)[a] of cell populations subcultured to	
			Passage 1	Passage 2
B	6	6 (100)	6 (100)	2 (33)
B + E	3	3 (100)	3 (100)	1 (33)
B + F	3	3 (100)	3 (100)	1 (33)
B + L	3	3 (100)	3 (100)	1 (33)
B + E + F	3	3 (100)	3 (100)	1 (33)
B + E + L	3	3 (100)	3 (100)	1 (33)
B + F + L	3	3 (100)	3 (100)	1 (33)
B + E + F + L	3	3 (100)	3 (100)	1 (33)
B + FS	3	3 (100)	3 (100)	2 (67)
B + MEE	3	3 (100)	3 (100)	2 (67)
B + FS + MEE	3	3 (100)	3 (100)	2 (67)

B, L15 medium containing 20 % fetal bovine serum and 1 % mixed solution of penicillin and streptomycin; E, 25 ng/ml epidermal growth factor; F, 10 ng/ml basic fibroblast growth factor; L, 1000 units/ml leukemia inhibitory factor; FS, 1 % fish serum; MEE, 50 μg/ml medaka embryo extract
[a]Percentage of the number of cell populations tested

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derived (Freshney, 2010). In the case of *S. schlegeli*, a wide range of temperatures was tested, because as a poikilotherm, it can tolerate a wide temperature range (Md Mizanur et al., 2014). Because all of the temperatures tested supported the growth of *S. schlegeli* ovary-dissociated cells to passage 2, we decided to fix the culture temperature for further study at 15 °C due to the tolerance of cultured cells to low relative to higher temperatures (Freshney, 2010). Further examinations targeting stable continuous cell lines may be required to determine the optimal culture temperature.

There may be several reasons why cell growth was limited to passage 1 or 2 in most cases. It may be the original senescence timing of *S. schlegeli* ovary-dissociated cells, or it may be caused by a lack of appropriate signaling molecules stimulating cell growth. Next, we evaluated the effects of various growth factors, including EGF, bFGF, LIF, FS, and MEE, on cell growth in L15 medium and 15 °C

culture temperature. We confirmed again that ovary-dissociated cells can grow and survive at least to passage 1 when cultured under these conditions regardless of added growth factors but observed that none of the growth factors tested induced further growth of the cultured cells (Table 3). These results may indicate that the limitation in cell growth was due to the senescence of cultured cells at passage 1 or 2, although additional signaling molecules that originate from *S. schlegeli* or allied species need to be tested.

Effects of culture types on in vitro maintenance of ovary-dissociated cells

Throughout the culture of *S. schlegeli* ovary-dissociated cells, we observed that all cultured cell populations formed primarily a monolayer in culture (Fig. 2a), but they detached spontaneously from the substratum as the culture progressed, resulting in the removal of a
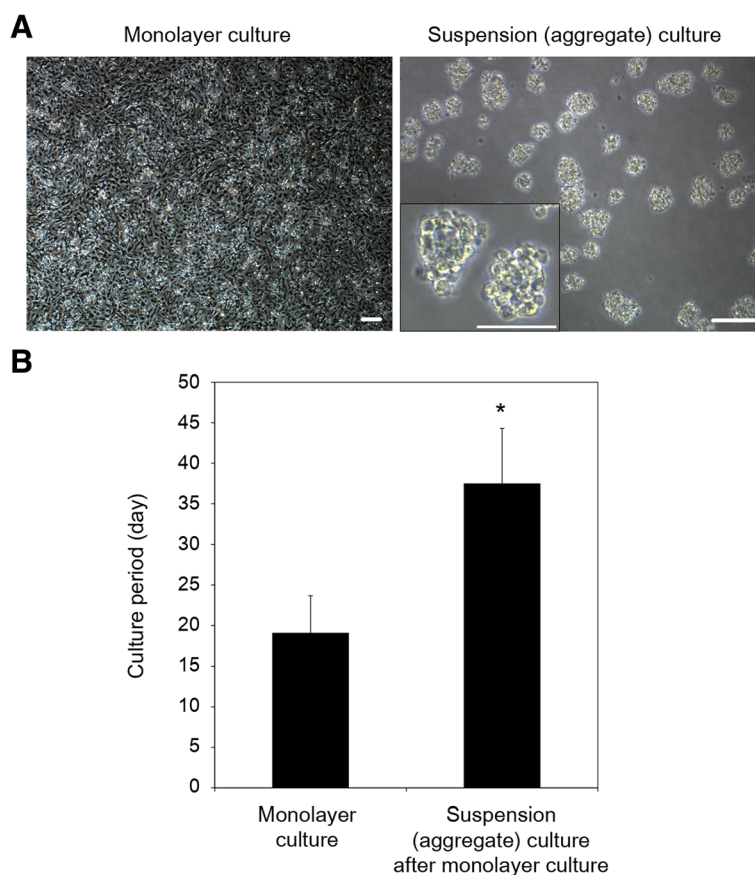

Fig. 2 Effects of culture type on culturing *Sebastes schlegeli* ovary-dissociated cells. Four cell populations derived from monolayer culture were detached on substratum at first or second passage and subsequently cultured as a form of aggregates in suspension manner for each individual. Culture period was measured individually on all cell populations cultured. **a** Morphology of the cells and cell aggregates in monolayer and suspension culture, respectively. Box within the picture of suspension culture shows a magnified image of aggregates. Scale bar = 100 μm and 50 μm in pictures and box, respectively. **b** Comparison of culture period between monolayer culture and suspension (aggregate) culture after monolayer culture. Combination of monolayer and suspension culture induced a statistically significant long-term maintenance of the cell populations compared with the sole monolayer culture. The data are mean ± standard deviation of nine and four independent experiments in monolayer and suspension culture, respectively. Asterisk indicates significant difference, $p < 0.05$

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large proportion of the cells during media exchange. Eventually, it brought about a shortening of the culture period in vitro. To increase the culture period of the cells, we attempted a suspension culture after a monolayer culture and compared the total culture period between suspension culture after monolayer culture and monolayer culture alone. Four cell populations in the monolayer culture derived from different individuals were detached artificially from the substratum using 0.05 % trypsin-EDTA treatment at the first or second passage and subsequently cultured in a suspension manner individually. The cells in suspension culture formed cell aggregates spontaneously (Fig. 2a), which were maintained without significant morphological changes. A significant increase in culture period was detected in suspension culture after monolayer culture versus monolayer culture alone (Fig. 2b; 37.5 ± 6.8 vs. 19.1 ± 4.6 days, $p < 0.0001$). Short-term primary cultures have limited application because of a lack of reproducibility and cell homogeneity. Thus, long-term culture or development of continuous cell lines is required for certain biotechnological applications of cultured cells (Bols et al., 1994). Consequently, extension of the culture period by introducing suspension culture is important in that it provides more time to add additional treatments for establishing long-term cultivable cells, such as spontaneous induction of cell immortalization and artificial induction of cell transformation. Additionally, aggregate formation in suspension culture has potential advantages, such as ease of cell maintenance, culture at high cell densities (Kyung et al., 1992), and provision of a three-dimensional microenvironment (Welter et al., 2007). These suggest the potential for further development of this culture system. Additional studies to activate cell proliferation followed by establishment of continuous cell lines are needed.

Conclusions

We report the general conditions for in vitro culture of *S. schlegeli* ovary-dissociated cells. Tissue dissociation and cell isolation can be implemented effectively using collagenase type I, and the cells can be cultured in L15 medium under a range of temperatures. Additionally, a combination of monolayer and suspension culture can extend the culture period significantly. The results of this study provide a basis for establishing an in vitro system for *S. schlegeli* germline cell culture.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JHR carried out the experiments. SSB, CGJ, and HJK participated in experimental design and data analysis. SPG conceived and designed the study, analyzed the data, and wrote manuscript. All authors read and approved the final manuscript.

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