

## Research Article

# Isolation of Female Germline Stem Cells from Mouse and Human Ovaries by Differential Adhesion

Maryam Saber , Pouya Tavakol, and Fereshteh Esfandiari 

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Correspondence should be addressed to Fereshteh Esfandiari; [fereshtehesfandiari@royaninstitute.org](mailto:fereshtehesfandiari@royaninstitute.org)

Received 14 May 2022; Accepted 5 August 2022; Published 7 September 2022

Academic Editor: Prem Yadav

Copyright © 2022 Maryam Saber et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Spermatogonial stem cell (SSC) counterparts known as female germline stem cells (fGSCs) were found in the mammalian ovary in 2004. Although the existence of fGSCs in the mammalian postnatal ovary is still under controversy, fGSC discovery encourages investigators to better understand the various aspects of these cells. However, their existence is not accepted by all scientists in the field because isolation of fGSCs by fluorescent activated cell sorting (FACS) has not been reproducible. In this study, we used differential adhesion to isolate and enrich fGSCs from mouse and human ovaries and subsequently cultured them *in vitro*. fGSCs were able to proliferate *in vitro* and expressed germ cell-specific markers Vasa, Dazl, Blimp1, Fragilis, Stella, and Oct4, at the protein level. Moreover, mouse and human fGSCs were, respectively, cultured for more than four months and one month in culture. Both mouse and human fGSCs maintained the expression of germ cell-specific markers over these times. *In vitro* cultured fGSCs spontaneously produced oocyte-like cells (OLCs) which expressed oocyte-relevant markers. Our results demonstrated that differential adhesion allows reproducible isolation of fGSCs that are able to proliferate *in vitro* over time. This source of fGSCs can serve as a suitable material for studying mechanisms underlying female germ cell development and function.

## 1. Introduction

Long-standing dogma that has claimed a fixed number of follicles existing in the mammalian ovary has been challenged recently as neo-oogenesis has been observed in the adult mouse ovary [1]. Subsequent isolation and culture of female germline stem cells (fGSCs) from mouse and human ovaries by fluorescent activated cell sorting (FACS) and magnetic activated cell sorting (MACs) reinforced the post-natal oogenesis [2, 3]. In these studies, fGSCs were sorted using the germ cell marker DDX4. However, sorting live fGSCs by application of this antibody is controversial due to its cytoplasmic domain [4]. The FACS-based isolation method yields a few number of mitotically active germline cells (<0.1% cells per ovary) [5]. While the existence of

fGSCs is the subject of extensive debate [4], their male counterparts (spermatogonial stem cells; SSCs) have been well characterized for many years [6]. SSCs are isolated by a simple and common method based on the differential attachment potential of germ cells and somatic cells [6]. This method enriches the SSC population simply by preparation of a gelatin-coated plate for trapping somatic cells and removes them from culture [7, 8]. This feasible approach was recently used for the enrichment of mouse fGSCs [9]. However, it remains to be answered whether differential adhesion allows the isolation of fGSCs from human ovaries. In this study, we intended to enrich fGSCs from human and mouse adult ovaries by the differential adhesion method. Easy culture of fGSCs could provide an *in vitro* source of material for studying mechanisms underlying female germ

cell development and function. Moreover, it may serve as a suitable source for female infertility treatments in future reproductive medicine.

## 2. Materials and Methods

**2.1. Animal.** In this study, we used female NMRI mice (6-8 weeks old, from Pasteur Institute, Tehran, Iran). The mice were housed under a standard animal facility (controlled atmosphere with 12:12 h light/dark cycles and temperature of 20–25°C). They had free access to water and food. All animal care studies and procedures were approved by the Royan Institutional Review Board and Institutional Ethical Committee of Royan Institute (No.: Ec/92/1026).

**2.2. Mouse Ovarian Cell Isolation.** For the preparation of cells, ovaries from four mice were pooled and isolated using a two-step enzymatic digestion method involving a 15 min incubation in the enzymatic solution containing collagenase type IV (800 U/ml prepared in HBSS) and DNase-I (1 µg/ml, Sigma-Aldrich) and a 10 min incubation with 0.05% trypsin-EDTA. When most of the cells were dispersed, the ovarian cells were washed 2 to 4 times in HBSS and centrifuged at 300 g for 5 min. Next, the supernatant was carefully removed from the pellet, the pellet was resuspended, and clumps of cells were removed by passing the suspension through a 70 µm nylon. Finally, the supernatant was removed, and the cells were resuspended in culture medium. Isolated cells were plated onto gelatin-coated culture plates ( $3 \times 10^5$  cells per 3 cm<sup>2</sup> plate, Falcon) in the culture medium containing α-MEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone), 1 mM sodium pyruvate (Invitrogen), 1 mM nonessential amino acids (Invitrogen), 1x penicillin-streptomycin-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1x concentrated N-2 supplement (R&D), 10<sup>3</sup> units/ml leukemia inhibitory factor (LIF, Royan Institute), 10 ng/ml recombinant human epidermal growth factor (rhEGF, Royan BioTech), 1 ng/ml basic fibroblast growth factor (bFGF, Royan BioTech), and 40 ng/ml glial cell-derived neurotrophic factor (GDNF, Royan BioTech). After 30 to 60 min of culture at 37°C, the supernatant that contained cells unattached to the plate was collected and transferred to a new 24-well plate coated with feeder cells (mitomycin C-treated MEFs). The medium was changed every 2–3 days, and when the cells reached confluence, they were digested using 0.05% trypsin (Invitrogen) followed by neutralization by adding 10% FBS and replated on fresh MEF at a 1:2 split ratio. The cells were cultured for four months.

**2.3. Human Sample.** Ovarian biopsies were collected from women ( $n = 6$ ) with a mean age of 30 years (20-40 years), who underwent total abdominal hysterectomy for uterine fibroma or ovariectomy due to various gynecological pathologies other than ovarian pathology, infection, malignancy, or other pathologies. The study was performed after obtaining written informed consent from patients to use ovarian samples for research purposes. The tissue biopsies were collected during surgeries at Atieh Hospital and transferred in

α-MEM media containing antibiotics (penicillin 100 U/ml and streptomycin 100 mg/ml; Invitrogen) on ice, to Royan Institute as soon as possible. The Royan Institutional Review Board and Institutional Ethical Committee of Royan Institute (No.: Ec/92/1026) approved the use and preparation of the human ovarian samples in this study.

**2.4. Human Ovarian Cell Isolation.** Ovarian biopsies were first washed several times in HBSS that contained antibiotics and then cut into small pieces by using a scalpel and incubated in enzyme solution. Enzymatic digestion was performed based on the mouse model. The human fGSCs were cultured and maintained for more than one month. However, after passaging, cells did not develop or grow further.

**2.5. Viability Assessment.** After isolation of human and mouse tissue, at least 100,000 cells from the whole ovarian cells were harvested and aliquoted into FACS tubes. Then, the cells were washed two times with PBS and centrifuged at 300 g for 5 minutes. Then, the buffer was decanted from the pelleted cells, and the cells were resuspended in 100 µl of flow cytometry staining buffer; then, 5-10 µl of propidium iodide (PI) staining solution was added to each sample, mixed gently, and incubated for 1 minute in the dark. Finally, PI fluorescence was determined by a BD FACS Aria II instrument.

**2.6. Immunofluorescence Staining.** Cultured ovarian cells were washed with PBS and fixed in 4% PFA for 20 min. After permeabilization by 0.5% Triton X-100, the cells were incubated for 1 h in blocking buffer that consisted of PBS and 10% normal goat serum followed by an overnight incubation period with primary antibody (Table S1) in a humidified chamber at 4°C. The cells were subsequently incubated with an appropriate secondary antibody at room temperature for 45 min. For nucleus staining, 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used. Immunostaining without primary antibodies was used for negative control cells.

## 3. Results

**3.1. Isolation of Female Germline Stem Cells from Mouse Ovaries by Differential Adhesion.** The procedure for isolation of fGSCs is schematically presented in Figure 1(a). Cell suspension achieved from the mouse ovary using enzymatic digestion (Figure 1(b), A) showed >80% viability (Figure 1(c)). Five days after transferring the supernatant cells to the MEF-coated plates, we observed small round cells (about 5-10 µm) with little cytoplasm and a large ratio of nuclear plasma (Figure 1(b), B) similar to the FACS-sorted Ddx4-positive oogonial stem cells reported earlier [2]. The number of these cells increased during the culture, and they formed spherical or grape-like clusters without smooth borders (Figure 1(b), C and D).

**3.2. Characterization of Mouse Primary Female Germline Stem Cells.** In order to characterize fGSCs, we carried out immunofluorescence analysis. Immunostaining showed that

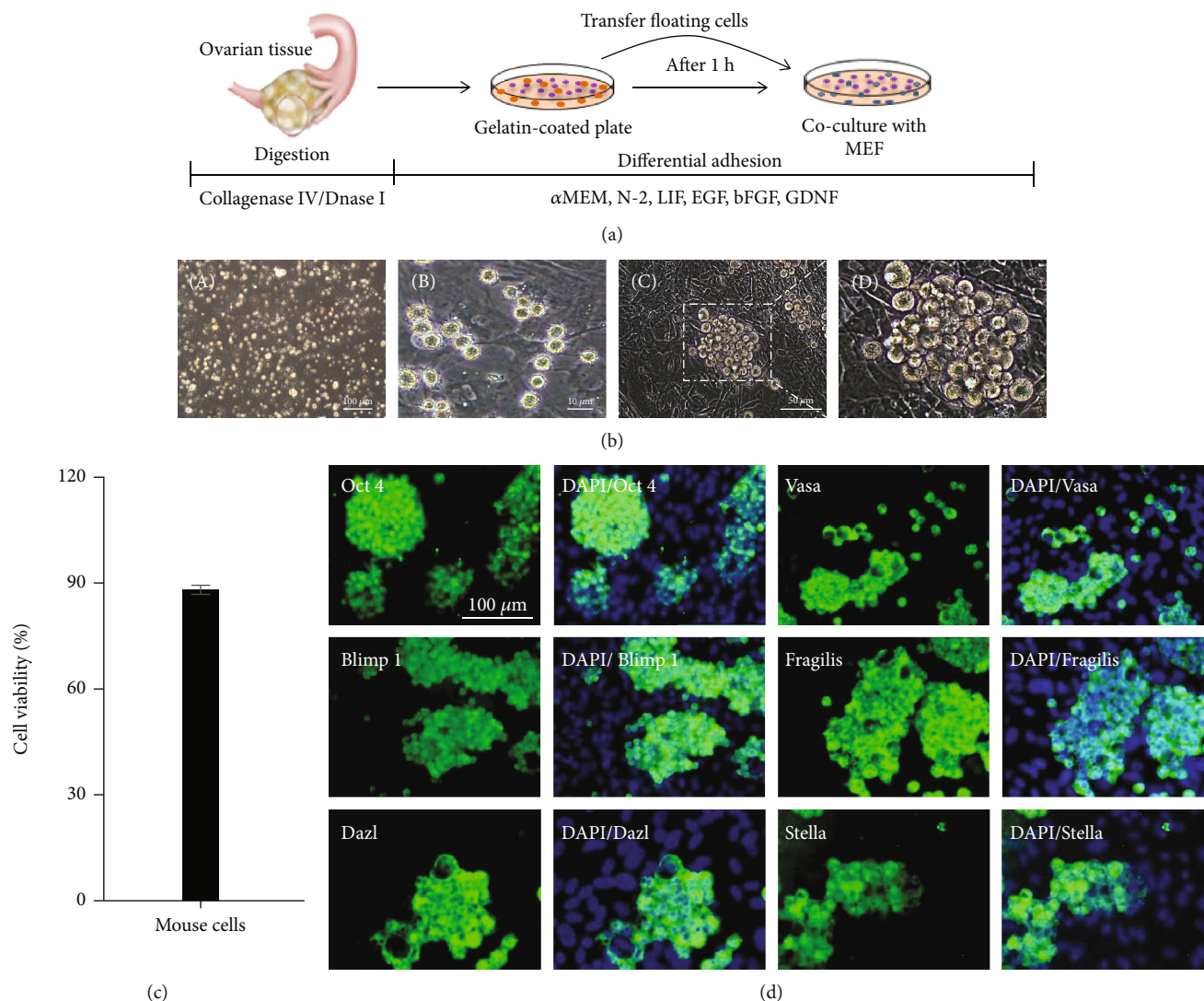


FIGURE 1: Isolation and culture of mouse female germline stem cells. (a) Schematic illustration of the culture system used for fGSC expansion. (b) Phase contrast images of mouse ovarian cell suspension after dissociation (A) and after 5 days of culture (B); spherical or grape-like clusters on day 30 in culture on MEF-coated plates (C, D; D at high magnification). (c) Viability percentage of mouse ovarian cells after ovarian tissue isolation by using PI staining analyzed by a BD FACS Aria II. (d) Characterization of established fGSCs. Expression of germ cell-specific markers in fGSCs detected by immunostaining analysis. The feeder cells were negative for these markers.

the cells were strongly positive for germline markers (Blimp1, Stella, Fragilis, Vasa, Dazl, and Oct-4 (Figure 1(d)) on day 20 of culture. These markers were not detected in mitotically inactive mouse embryonic fibroblasts (MEFs) used as a feeder (Figure 1(d)).

**3.3. Propagation of Mouse Female Germline Stem Cells in Culture by Differential Adhesion.** Mouse fGSCs proliferated during the culture and were passaged every 10-20 days. The cells were passaged for four months (Figure 2(a)). Immunostaining revealed expression of germ cell-specific markers in fGSCs after long-term culture ( $\geq 4$  months) (Figure 2(b)). Moreover, dual staining for Vasa and Ki67 (as a proliferative marker) demonstrated that fGSCs are actively proliferative in the culture (Figure 2(c)).

**3.4. In Vitro Differentiation of Mouse Female Germline Stem Cells to Oocyte-Like Cells.** During *in vitro* culture of mouse fGSCs, they spontaneously differentiated into large spherical cells (diameter up to  $48 \mu\text{m}$ ) and were morphologically similar to oocyte ((oocyte-like cells (OLCs)) (Figure 3(a)). We examined the expression of oocyte-specific markers in OLCs. Immunofluorescence analysis showed the expression of Gdf9, Vasa, and Zp1 (which are specific to the oocyte stage) at the protein level in OLCs (Figure 3(b)).

**3.5. Isolation of Female Germline Stem Cells by Differential Adhesion from Human Ovaries.** Ovarian cells isolated from human ovarian biopsies showed  $>70\%$  viability. fGSCs were morphologically detectable among other cells as round cells with a large nucleus to cytoplasm ratio, and they were

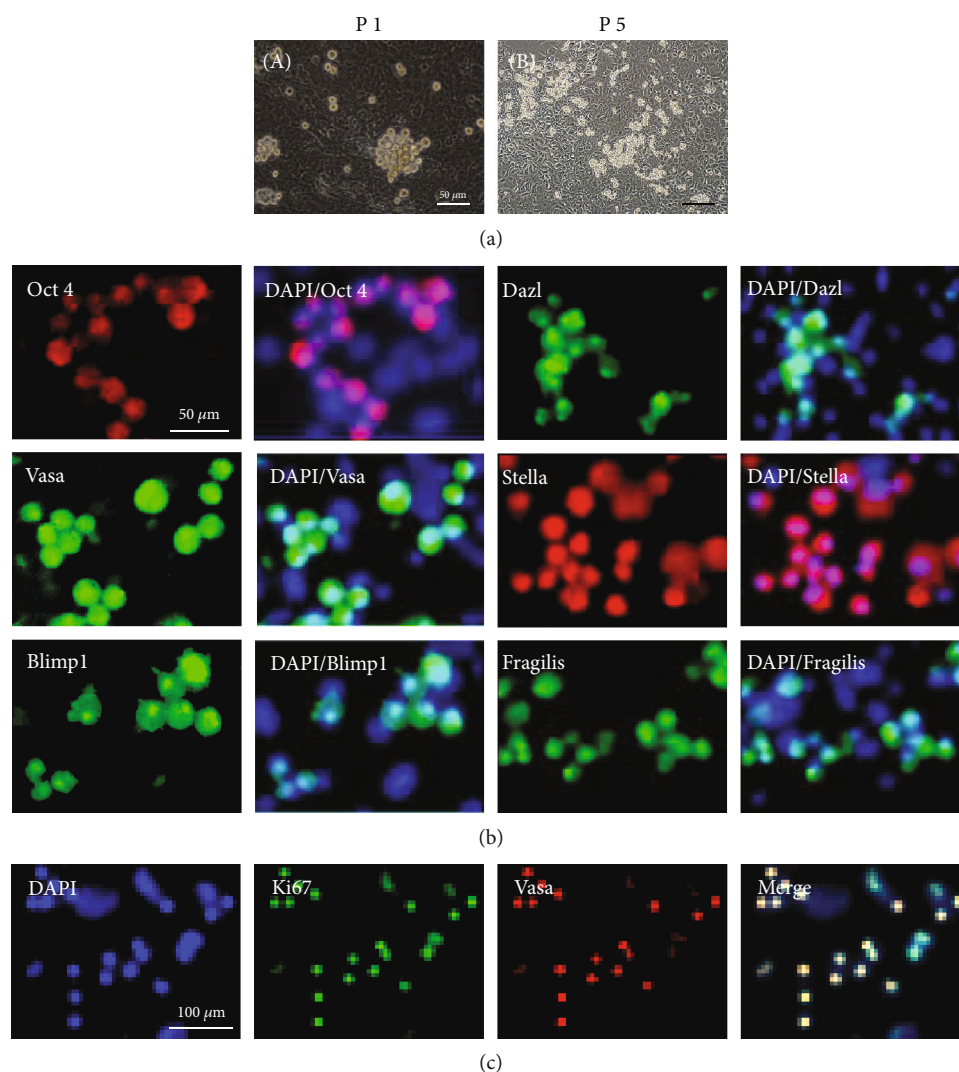


FIGURE 2: Characterization of established female mouse germline stem cells. (a) Phase contrast microscopy of cultured cells during passaging (day 30 at different passages (A: after passage 1; B: after passage 5) of fGSCs on MEF-coated plates. (b) Immunofluorescence staining for germline markers (Oct4, Vasa, Dazl, Blimp1, Stella, and Fragilis) in fGSCs (after 2 passages) in MEF-coated plates. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, blue) to visualize the nucleus. (c) Assessment of GSC proliferation by dual immunofluorescence for Vasa (red) expression and Ki67 incorporation (green).

undergoing mitosis in culture (Figure 4(a)). It was also possible to maintain the human fGSCs in culture for more than one month. We have been successful to isolate fGSCs from all 6 human samples. However, after passage, cells did not develop or grow further. Human fGSCs showed positive immunofluorescence staining for specific germline markers (BLIMP1, STELLA, FRAGILIS, VASA, DAZL, and OCT-4) on day 20 of culture. These markers were not detected in mitotically inactive mouse embryonic fibroblasts (MEFs) used as a feeder (Figure 4(b)). During the culture, the cells with a diameter of approximately  $60\ \mu\text{m}$  (oocyte-like cells) developed among proliferating small cells. These cells were positive for germ and oocyte markers including VASA, GDF9, and VASA in immunofluorescence staining (Figure 4(c)).

#### 4. Discussion

In the present study, we showed that differential adhesion allows efficient isolation and subsequent culture of fGSCs from both mouse and human ovaries. This approach allowed easy enrichment and culture of fGSCs that expressed germ cell markers in culture. Among different methods of cell isolation, the differential adhesion method is fast, easy, of low cost, and safe; this method causes the least negative effects on cell viability [10] [11] and is commonly used for *in vitro* cultures of male germ cells [12–14]. As long as 48 years ago, Steinberger and Steinberger [8] described that germ cells are enriched in the supernatant while somatic cells preferentially attach to the culture dish in single-cell suspensions of rats. A preferential expression of

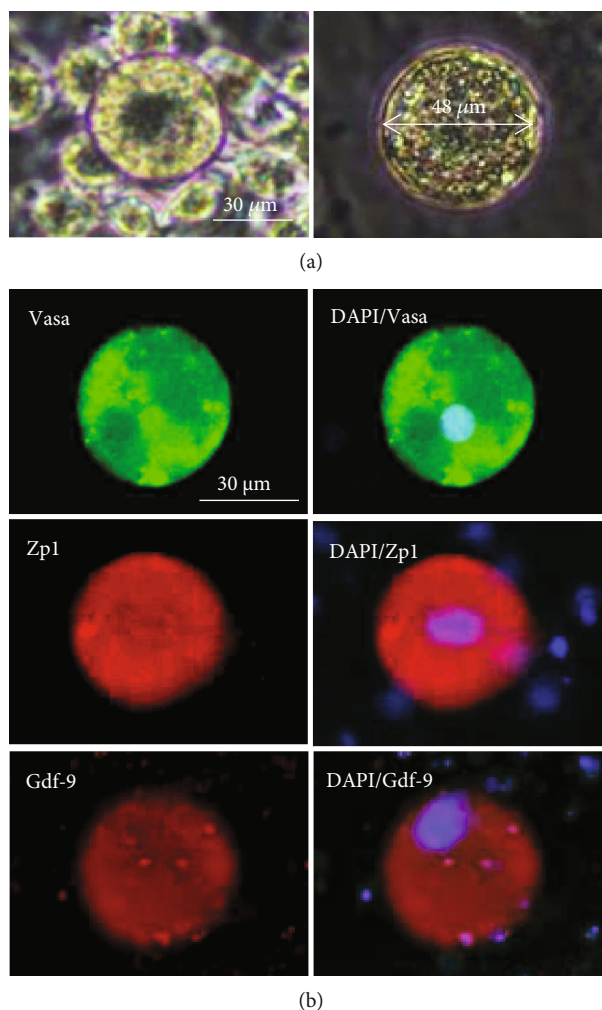


FIGURE 3: Characteristics of oocyte-like cells generated from female germline stem cells (fGSCs). (a) Phase contrast images of OLCs. Spontaneous generation of large spherical cells that morphologically resembled oocytes. (b) Immunofluorescence detection of germ cell and oocyte-specific markers in OLCs.

germ cell markers in the supernatant was also seen by Eildermann et al. [12] for marmoset testicular cell cultures and by Sadri-Ardekani et al. [14] and Kossack et al. [13] for human testicular cell cultures. It was proven that fibroblasts (MEFs) as the most commonly used feeder cells act as microniche by allowing secure attachment and proliferation of germ cells and secrete certain components into the media that support germ cell proliferation [15].

Compared to the FACS method for fGSC isolation, differential adhesion shortens the time of enrichment and provides the condition for accelerating the fGSC proliferation *in vitro*. In our experience, FACS-isolated fGSCs reached the confluency after 30 days of isolation (data not shown) while those isolated by differential adhesion reached the confluency 20 days after plating. It may be related to the few number of cells that are isolated by FACS.

We were able to culture the mouse fGSCs for three months in this system, and these cells maintained the expression of germ cell-specific markers (Vasa, Dazl, Blimp1, Stella, Fragilis, and Oct4) during this time. Moreover, fGSCs that were cultured by differential adhesion

increased in number and efficiently expressed a proliferation marker (Ki67) demonstrating active proliferation of the cells. We also found that during *in vitro* culture, fGSCs spontaneously produced OLCs with morphology and gene expression profile similar to native oocytes. The maximum size that OLCs grew was, respectively, 48 and 60  $\mu\text{m}$  in mouse and human, and they did not show strong expression of Sycp3 (data not shown) which is expressed during meiosis [16] that showed that they were at early stages of development. Improving culture conditions may promote OLC development *in vitro*. We used a culture medium which has been reported [3] previously to be suitable for the propagation of fGSCs. One of the most important factors in this media is LIF which promotes fGSC proliferation and inhibits differentiation [17, 18]. However, we could observe a differentiation leakage in this condition. The oocyte differentiation was very low in this condition, about 1% which is ignorable. It was shown that medium supplementation with follicle-stimulating hormone (FSH) and luteinizing hormone (LH) or the presence of cumulus cells in *in vitro* culture condition [19] leads to *in vitro* maturation

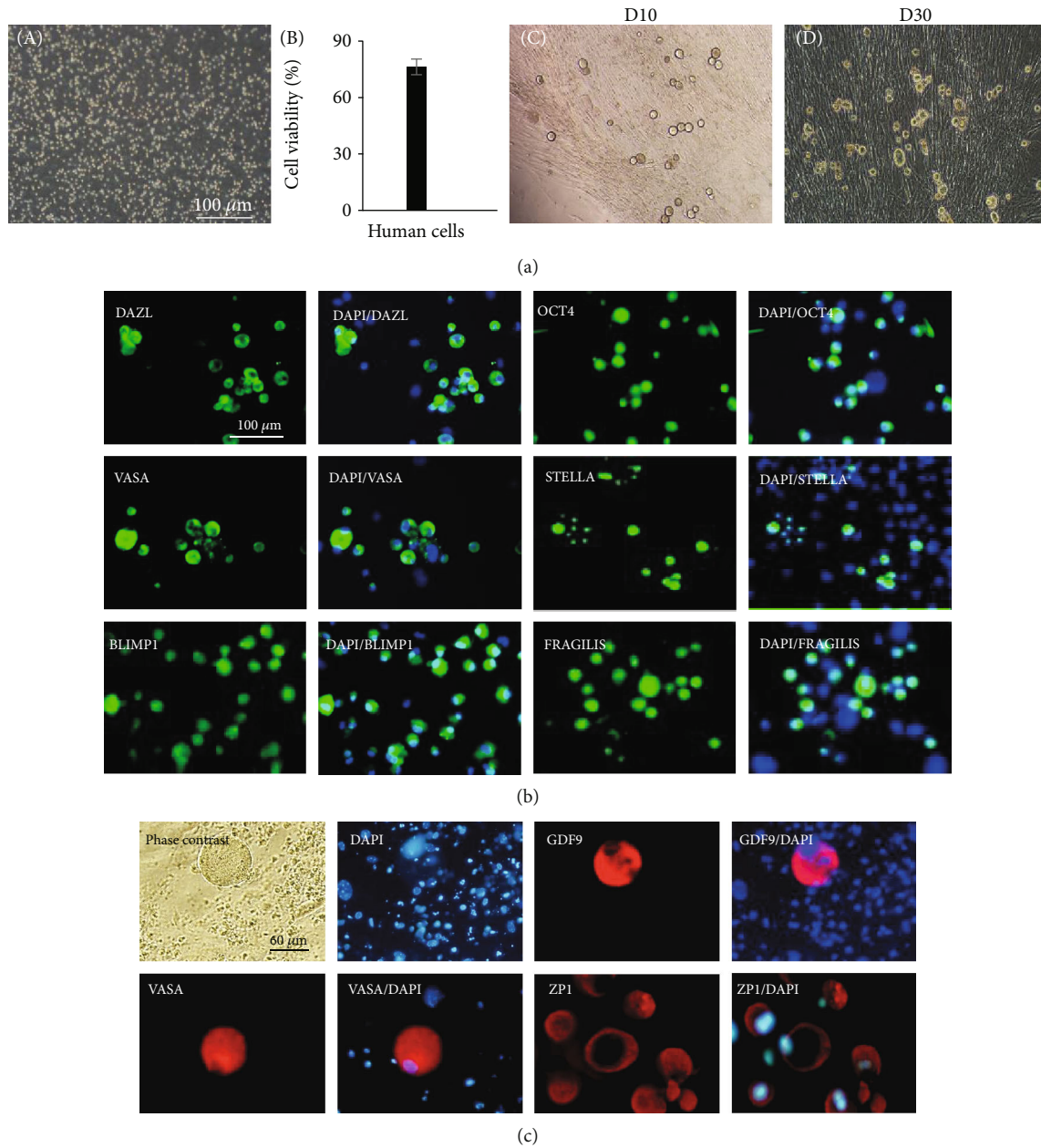


FIGURE 4: Characteristics of human female germline stem cells. (a) Phase contrast images of human ovarian cells after the isolation (A) and culture on different days (C and D); viability percentage of human ovarian cells after the isolation by using PI staining analyzed by BD FACS Aria II. (b) Immunofluorescence staining for germline markers in fGSCs on day 20 of culture in MEF-coated plates. (c) OLCs formed spontaneously from the fGSCs in the culture. Phase contrast and immunofluorescence staining images for oocyte markers (GDF9, ZP1, and VASA) of differentiated OLCs in the culture.

of oocyte [20–22]. So, in order to study the differentiation potential of fGSCs, we used a different culture media supplemented with 10% FBS, 10 ng/ml hEGF, 5  $\mu$ l/ml insulin/transferrin/selenium, 0.05 IU follicle-stimulating hormone (FSH), and 0.03 IU luteinizing hormone (LH). Moreover, we removed LIF from fGSCs which is another inducer for oocyte differentiation [23, 24]. When using this differentiation media, more oocytes were differentiated, and the size of oocytes which was differentiated in this condition was larger than those formed spontaneously during propagation (the data have not been reported). Further inves-

tigations are required to reach optimal conditions for achieving more developed OLCs.

In line with our study, the isolation of fGSCs from mouse ovaries was reported recently [9]. However, they did not apply this method to the isolation of fGSCs from human ovaries. Here, we showed efficient isolation of fGSCs from human ovaries by differential adhesion for the first time. Moreover, ovarian surface scarping has been used for the isolation of fGSCs in humans. This method was employed for the first time in 2004 by the Bukovsky group [25] and then by Virant-Klun et al. [26] and Parte et al. [27] in order

to isolate fGSCs from the ovarian surface epithelium (OSE) of the human ovaries. The initial number of cells that were obtained by using these methods is very low, and the scraped cells (putative fGSCs) underwent spontaneous differentiation not only into the OLCs but also into other cells such as neural-like cells [16].

## 5. Conclusion

In summary, we established the differential adhesion method for fGSC isolation from mouse and human ovaries. The fGSCs expressed germ cell-specific markers in culture and proliferated actively over time. Importantly, they were able to differentiate into OLCs *in vitro*. This culture system allows enrichment of fGSCs to serve as an *in vitro* model for studying basic aspects of reproductive biology and as a promising source to be used in future reproductive medicine.

## Abbreviations

SSCs: Spermatogonial stem cells  
 fGSCs: Female germline stem cells  
 FACS: Fluorescent activated cell sorting  
 MACs: Magnetic activated cell sorting  
 OLCs: Oocyte-like cells  
 MEFs: Mouse embryonic fibroblasts.

## Data Availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

## Ethical Approval

This study was approved by the Cell Science Research Center of Royan Institute. The Institutional Ethical Committee of Royan Institute approved all animal studies and procedures and preparation of the human ovarian samples for this study (No.: Ec/92/1026).

## Consent

Informed consent was obtained from all participants.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Authors' Contributions

M.S. and F.E. designed the experiments and drafted the manuscript. M.S. conducted the experiments and analyzed the experimental data. P.T. helped with animal handling and collection of ovarian tissues. F.E. supervised the research and took part in revising the manuscript. All the authors studied and approved the final manuscript.

## Acknowledgments

We thank all personnel in histology and animal labs at the Royan Institute. This work was supported by a grant from the Royan Institute (No.: 91000177).

## Supplementary Materials

The list of antibodies used for immunofluorescence staining has been illustrated as a table in the supplementary file. (*Supplementary Materials*)

## References

- [1] J. Johnson, J. Canning, T. Kaneko, J. K. Pru, and J. L. Tilly, "Germline stem cells and follicular renewal in the postnatal mammalian ovary," *Nature*, vol. 428, no. 6979, pp. 145–150, 2004.
- [2] Y. A. White, D. C. Woods, Y. Takai, O. Ishihara, H. Seki, and J. L. Tilly, "Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women," *Nature Medicine*, vol. 18, no. 3, pp. 413–421, 2012.
- [3] K. Zou, Z. Yuan, Z. Yang et al., "Production of offspring from a germline stem cell line derived from neonatal ovaries," *Nature Cell Biology*, vol. 11, no. 5, pp. 631–636, 2009.
- [4] H. Zhang, W. Zheng, Y. Shen, D. Adhikari, H. Ueno, and K. Liu, "Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 31, pp. 12580–12585, 2012.
- [5] L. Zarate-Garcia, S. I. Lane, J. A. Merriman, and K. T. Jones, "FACS-sorted putative oogonial stem cells from the ovary are neither DDX4-positive nor germ cells," *Scientific Reports*, vol. 6, no. 1, article 27991, 2016.
- [6] D. G. de Rooij and M. F. Kramer, "Spermatogonial stemcell renewal in rats and mice," *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, vol. 85, no. 2, pp. 206–209, 1968.
- [7] D. Langenstroth, N. Kossack, B. Westernstroer et al., "Separation of somatic and germ cells is required to establish primate spermatogonial cultures," *Human Reproduction*, vol. 29, no. 9, pp. 2018–2031, 2014.
- [8] A. Steinberger and E. Steinberger, "In vitro culture of rat testicular cells," *Experimental Cell Research*, vol. 44, no. 2-3, pp. 443–452, 1966.
- [9] M. Wu, J. Xiong, L. Ma et al., "Enrichment of female germline stem cells from mouse ovaries using the differential adhesion method," *Cellular Physiology and Biochemistry*, vol. 46, no. 5, pp. 2114–2126, 2018.
- [10] K. Guan, F. Wolf, A. Becker, W. Engel, K. Nayernia, and G. Hasenfuss, "Isolation and cultivation of stem cells from adult mouse testes," *Nature Protocols*, vol. 4, no. 2, pp. 143–154, 2009.
- [11] B. Q. Kreider, A. Messing, H. Doan, S. U. Kim, R. P. Lisak, and D. E. Pleasure, "Enrichment of Schwann cell cultures from neonatal rat sciatic nerve by differential adhesion," *Brain Research*, vol. 207, no. 2, pp. 433–444, 1981.
- [12] K. Eildermann, J. Gromoll, and R. Behr, "Misleading and reliable markers to differentiate between primate testis-derived multipotent stromal cells and spermatogonia in culture," *Human Reproduction*, vol. 27, no. 6, pp. 1754–1767, 2012.

- [13] N. Kossack, N. Terwort, J. Wistuba et al., "A combined approach facilitates the reliable detection of human spermatogonia in vitro," *Human Reproduction*, vol. 28, no. 11, pp. 3012–3025, 2013.
- [14] H. Sadri-Ardekani, S. C. Mizrak, S. K. van Daalen et al., "Propagation of human spermatogonial stem cells in vitro," *JAMA*, vol. 302, no. 19, pp. 2127–2134, 2009.
- [15] N. Y. Choi, Y. S. Park, J. S. Ryu et al., "A novel feeder-free culture system for expansion of mouse spermatogonial stem cells," *Molecules and Cells*, vol. 37, no. 6, pp. 473–479, 2014.
- [16] J. L. Syrjanen, I. Heller, A. Candelli et al., "Single-molecule observation of DNA compaction by meiotic protein SYCP3," *eLife*, vol. 6, article e22582, 2017.
- [17] H.-T. Bui, N. Van Thuan, D.-N. Kwon et al., "Identification and characterization of putative stem cells in the adult pig ovary," *Development*, vol. 141, no. 11, pp. 2235–2244, 2014.
- [18] K. Onishi and P. W. Zandstra, "LIF signaling in stem cells and development," *Development*, vol. 142, no. 13, pp. 2230–2236, 2015.
- [19] S. Tanghe, A. Van Soom, H. Nauwynck, M. Coryn, and A. de Kruijff, "Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization," *Molecular Reproduction and Development*, vol. 61, no. 3, pp. 414–424, 2002.
- [20] C. E. Farin, K. F. Rodriguez, J. E. Alexander, J. E. Hockney, J. R. Herrick, and S. Kennedy-Stoskopf, "The role of transcription in EGF- and FSH-mediated oocyte maturation in vitro," *Animal Reproduction Science*, vol. 98, no. 1-2, pp. 97–112, 2007.
- [21] J. E. Fortune, "The early stages of follicular development: activation of primordial follicles and growth of preantral follicles," *Animal Reproduction Science*, vol. 78, no. 3-4, pp. 135–163, 2003.
- [22] K. Morohaku, Y. Hirao, and Y. Obata, "Developmental competence of oocytes grown in vitro: has it peaked already?," *The Journal of Reproduction and Development*, vol. 62, no. 1, pp. 1–5, 2016.
- [23] V. Cadoret, P. Jarrier-Gaillard, P. Papillier, D. Monniaux, F. Guérif, and R. Dalbès-Tran, "Leukaemia inhibitory factor modulates the differentiation of granulosa cells during sheep in vitro preantral to antral follicle development and improves oocyte meiotic competence," *Molecular Human Reproduction*, vol. 27, no. 9, article gaab051, 2021.
- [24] L. M. Salvador, C. P. Silva, I. Kostetskii, G. L. Radice, and J. F. Strauss III, "The promoter of the oocyte-specific gene, *Gdf9*, is active in population of cultured mouse embryonic stem cells with an oocyte-like phenotype," *Methods*, vol. 45, no. 2, pp. 172–181, 2008.
- [25] A. Bukovsky, M. R. Caudle, M. Svetlikova, and N. B. Upadhyaya, "Origin of germ cells and formation of new primary follicles in adult human ovaries," *Reproductive Biology and Endocrinology*, vol. 2, no. 1, p. 20, 2004.
- [26] I. Virant-Klun, N. Zech, P. Rozman et al., "Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes," *Differentiation*, vol. 76, no. 8, pp. 843–856, 2008.
- [27] S. Parte, D. Bhartiya, J. Telang et al., "Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary," *Stem Cells and Development*, vol. 20, no. 8, pp. 1451–1464, 2011.