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In vitro and *in vivo* differentiation of induced pluripotent stem cells into male germ cells

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ABSTRACT

The introduction of induced pluripotent stem cell (iPSC) lines has been a breakthrough in the field of stem cell research. However, the extent of pluripotency among those cell lines tends to be variable due to their different epigenetic signatures. Mouse iPS cell line 4.1 has been established via retroviral transfer of human transcription factors Oct4, Sox2, Klf4, and c-Myc; the germline competence of this line has not been determined. In the present study, we induced the differentiation of miPS-4.1 cells into male germ cells, *in vivo* and *in vitro*. In the *in vitro* model, the behavior of miPS-4.1 cells was identical to that of differentiating mouse embryonic stem cells (ESCs). We obtained primordial germ cell-like cells (PGC-LC) that were positive for alkaline phosphatase (AP) activity. In continuous culture, these cells expressed pluripotent marker Oct4 and male germline markers C-kit and MVH. For our *in vivo* model, miPS-4.1 cells were co-transplanted with neonatal testicular cell suspension. We observed ectopically reconstituted seminiferous tubule structures, in which the miPS-4.1 cells were homing and developing. In conclusion, we successfully induced the differentiation of miPS-4.1 cells into male germ cells, albeit their epigenetic characteristics. Our study provides a system to examine the mechanisms of male germ cell development and might help to supply an effective treatment for male infertility in the future.

1. Introduction

Germ cell lineage transmits the genetic and epigenetic information across the generations, making possible the conservation of species. However, our knowledge of the germline specification is still limited; *in vitro* recreation of germ cell development has been one of the biggest challenges in modern biology. The *ex vivo* gametogenesis can be divided into two phases: the first is the differentiation from pluripotent cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), into the primordial germ cell-like cells (PGC-LCs); and the second, controlled initiation and completion of meiosis. These two phases are fundamentally independent, and different in males and females.

Much progress has been achieved in the derivation of male germ cells from ESCs. In mice, male gametes derived from these cells have been shown to produce viable offspring [1,2]. In humans, some successful methods for deriving male germ cells from ESCs have been also reported [3–5] although the ethical arguments have been inevitable. However, the induction of germ cell differentiation from iPSCs seems to be more difficult. In several studies, PGC-LCs have been generated from either mouse or human iPSCs [6–8]. Even post-meiotic male haploid cells have been obtained [6]; however, it remains to be shown whether these haploid cells are reproductively competent.

Unlike the studies of differentiation from ESCs, similar work based on iPSCs is likely to be affected by the original properties of iPS cell lines. The residual epigenetic memories carried by iPSCs might promote or impede the differentiating process. Mouse iPS cell lines miPSC-4.1 and miPSC-11.1 have been derived from the male mouse neural progenitor cells via induced reprogramming using retroviral transfer of human transcriptional factors Oct4, Sox2, Klf4, and c-Myc. However, miPSC-11.1 cells have been successfully induced to form contractile cardiomyocytes, while iPS cells from line 4.1 have failed to do so under the same conditions. On the other hand, miPSC- 4.1 cells differentiated into endoderm lineages more easily than miPSC-11.1 cells [9]. It has been proven that miPSC-11.1 cells can contribute to the germline in chimera mice, and some recent related studies have reported the differentiation of miPSC-11.1 cells into male germ cells [10,11]. By contrast, the germline competence of miPSC-4.1 cells has not been determined so far.

Abbreviations: iPSC, induced pluripotent stem cell; ESC, embryonic stem cell; EB, embryoid body; PGC, primordial germ cell; GSC, germ stem cell.

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In the present study, we tried to induce miPSC-4.1 differentiation into male germ cells *in vitro* and *in vivo*. Using an *in vitro* protocol, we induced the iPSC-4.1cells into a PGC-like status. The resultant germ stem cell clones were characterized. At the same time, we developed a model of reconstituted seminiferous tubules, in which iPSC-4.1cells could properly differentiate into germ cells. In conclusion, we proved that the miPS-4.1 cell line has the male germline potential.

2. Materials and methods

2.1. Animals

Neonatal male ICR mice and adult male BALB/c-nu/nu mice were purchased from the Centre for Experimental Animals, Chinese Academy of Sciences, Shanghai, China. The animal procedures were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals [SYXK (Hu) 2008–0050)].

2.2. Mouse embryonic stem cell (mESC) and mouse induced pluripotent stem cell (miPSC) culture

The mESC-H1.2 (40, XY) and Tg-EGFP-miPSC-4.1 (40, XY) lines have been originally established by Dr. JIN's team (Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences/Shanghai Jiao Tong University). The mESC-H1.2 line has been generated from a male C57BL/6J mouse, while the miPSC-4.1line is derived from the neural progenitor cells of the EGFP-transgenic C57BL/6J male mouse. Cells were cultured as described previously [9].

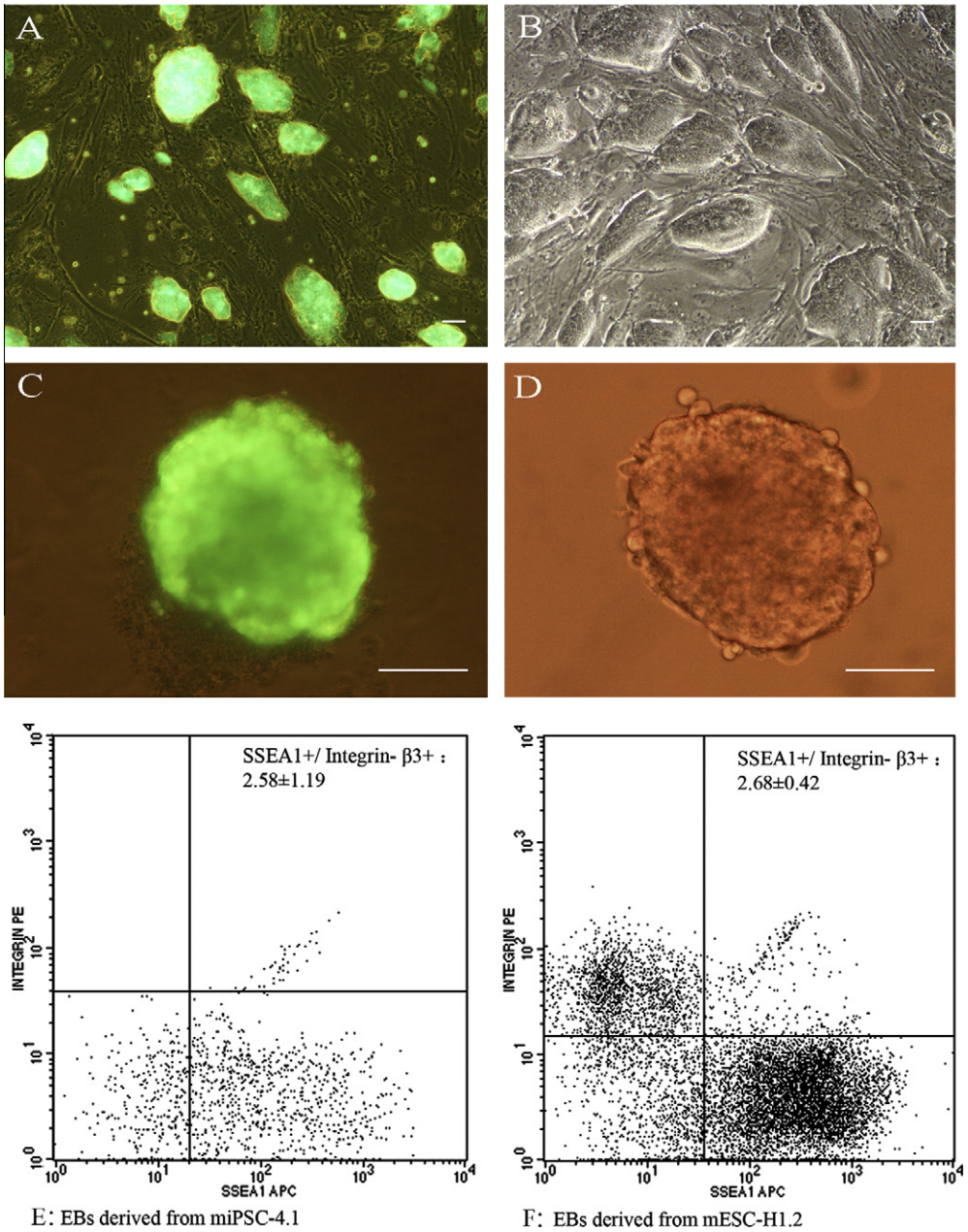

Fig. 1. Analyses of EBs derived from Tg-EGFP-miPSC-4.1 and mESC-H1.2 cell clones. (A–D) Morphology. (A) Tg-EGFP-miPSC-4.1 clones. (B) mESC-H1.2 clones. (C) 7-day EBs derived from miPSC-4.1. (D) 7-day EBs derived from mESC-H1.2. Green: (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) EGFP expression. Bars = 50 μm. (E–F) FCM analysis of derived EBs for SSEA-1 and integrin-β3 expression. (E) 7-day EBs derived from miPSC-4.1. (F) 7-day EBs derived from mESC-H1.2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

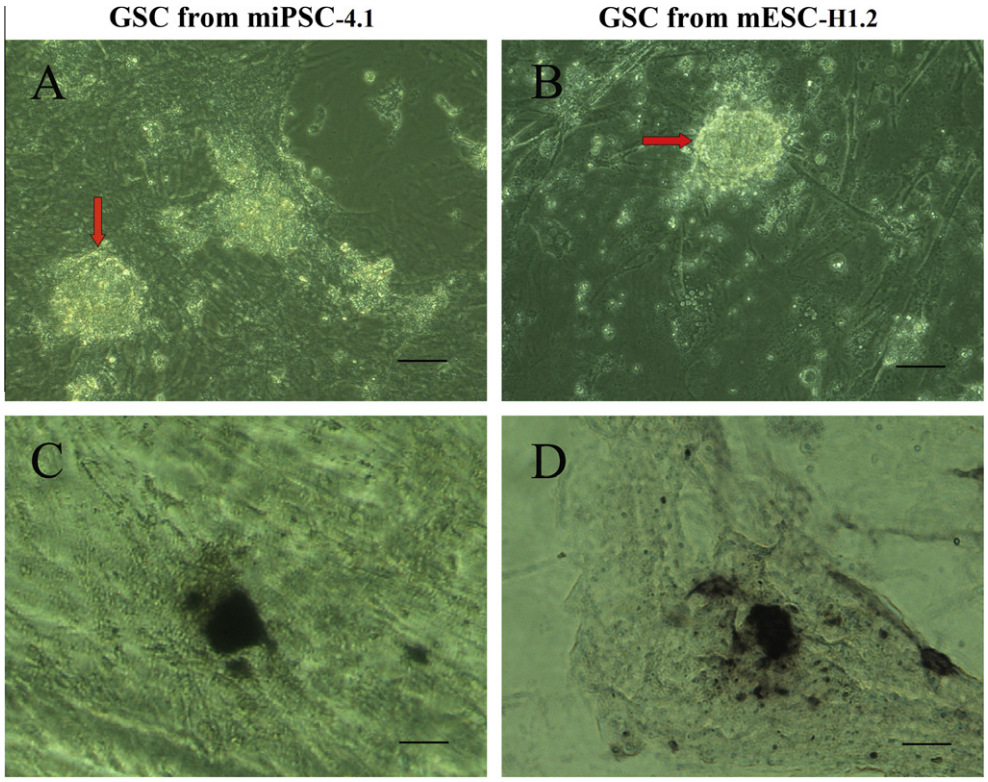

Fig. 2. Morphology and alkaline phosphatase staining of derived GSC clones. Bars = 20 μm.

2.3. Embryoid body (EB) formation and flow cytometry analysis

EBs were created using hanging drop method [9] and cultured in mESC/miPSC medium without LIF.

The EBs obtained using this method were digested using collagenase IV and hyaluronidase (Sigma) treatment at day 5, 7, and 10 of culture. The cells were then incubated with 1 μg/ml of Alexa Fluor 647-conjugated anti-mouse SSEA1 (Santa Cruz) and 1 μg/ml phycoerythrin-conjugated anti-mouse integrin-β3 (Abcam) at 4 °C for 30 min. The labeled cells were analyzed using FACS Calibur system (BD).

2.4. Induced differentiation of miPSCs and mESCs in culture

The 7-day EBs derived from miPSCs and mESCs were dissociated into single cells, seeded and cultured on the inactivated MEFs. For induction, germ cell selection medium was renewed daily. The medium was composed of miPSC/mESC culture medium, 15 ng/ml of basic fibroblast growth factor (Invitrogen), 30 ng/ml of stem cell factor (Invitrogen) and 2 μM all-trans retinoic acid (Sigma).

2.5. Alkaline phosphatase staining

The miPSCs, mESCs, and derived GSCs were fixed in 4% paraformaldehyde (PFA) for 15 min. Alkaline phosphatase activity was tested using AP Staining Kit (Beyotime).

2.6. Immunofluorescence

Samples were fixed in 4% PFA for 30 min. After washing with PBS, the cells were incubated with 5% BSA for 60 min. For Oct4 and MVH detection, cells were permeabilized by incubation with 0.5% Triton X-100 for 30 min. Then, the cells were incubated with appropriate primary antibodies (Abcam) at 4 °C overnight. The cells were washed with PBS, incubated with CY3-conjugated secondary antibody (Invitrogen) for 60 min, and then counterstained with DAPI. The results were recorded using Zeiss-LSM510 confocal microscope.

2.7. Transplantation of miPSCs and testicular cell suspension

All the operations were carried out under sterile conditions. Testis from 1–2 dpp ICR mice were homogenized in DMEM/F12 medium on ice. The supernatant was collected by centrifugation at 12,000 rpm for 10 min. This testis tissue extract was used in the transplantation.

Testicular cell suspension from 5–7 dpp mice was prepared by a two-step enzymatic digestion method [12].The miPSC suspension was prepared and mixed with the testicular cells resuspended in the testis tissue extract medium. The ratio of miPSC cells to testicular cells was 1:5–1:10. An equal volume of growth factor-reduced Matrigel Matrix (MGM, BD) was added to the cell suspension on ice. The final cell concentration was 2×10^7–5×10^7 cells/ml.

The recipient male BALB/c-nu/nu mice were anesthetized, and 50 μl of the prepared cell suspension was injected into the dorsal region of the mice. Each mouse was injected at 4–6 sites. The recipients were then castrated.

2.8. Live Imaging

To determine whether there were miPSC-derived $EGFP^+$ cells in the xenografts, the recipient mice were examined using IVIS-Spectrum System (Xenogen) at 2, 4, 6, and 8 weeks after transplantation.

2.9. Histological analysis of xenografts

The xenograft samples were collected at 2, 4, 6, 8, and 10 weeks after transplantation, subjected to serial paraffin sectioning and HE

staining. Otherwise, frozen sections were prepared for immunofluorescence examination as described above.

2.10. Statistical analysis

The results of flow cytometry were analyzed using student's *t*-test. *P* value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Primordial germ cell-like subpopulation in developing EBs

In our *in vitro* induction protocol, mESC-H1.2 cell line was used as a positive control. Stage-specific embryonic antigen 1 (SSEA-1) is a cell-surface marker expressed in mESCs, miPSCs, and early germ cells. Most recently, Integrin-β3 has been reported as a reliable surface marker of mouse PGC-LCs with spermatogenic capacity [13]. Using flow cytometry analysis, we analyzed mESC-H1.2- and miPSC-4.1-derived EBs at day 5, 7, and 10.We found a subpopulation of SSEA-1$^+$/Integrin-β3$^+$ cells, which represented the PGC-LCs. The 7-day EBs contained the highest percentage of SSEA-1$^+$/ Integrin-β3$^+$ cells; it was 2.58 ± 1.19% in miPSC-derived 7-day EBs and 2.68 ± 0.42% in mESC-derived counterparts (Fig. 1E and F). No significant differences were observed between these two groups ($p > 0.05$). Thus, we collected the 7-day EBs to isolate the PGC-like germ stem cells.

3.2. Germ stem cell formation after retinoic acid induction

The 7-day EBs derived from miPSC-4.1 and mESC-H1.2 were dissociated into single cells and cultured on inactivated MEF feeders in germ cell selection media. Retinoic acid (RA) has been shown to stimulate both the PGCs proliferation and the meiosis initiation through the CYP26/Stra8 signaling [14,15]; therefore, 2 μM RA was added as an inducer of germline specification.

Cell colonies were emerging after 2 days of RA induction. These clones were flatter and less compact than miPSC/mESC clones, with positive AP activity (Fig. 2).

Immunofluorescence tests revealed that the pluripotent marker Oct4, as well as the male germline markers C-kit and MVH were expressed in these colonies (Fig. 3). These cell clones could be continuously cultured and passaged for 3 months. We designated them as PGC-like germ stem cells (GSC). Our *in vitro* results

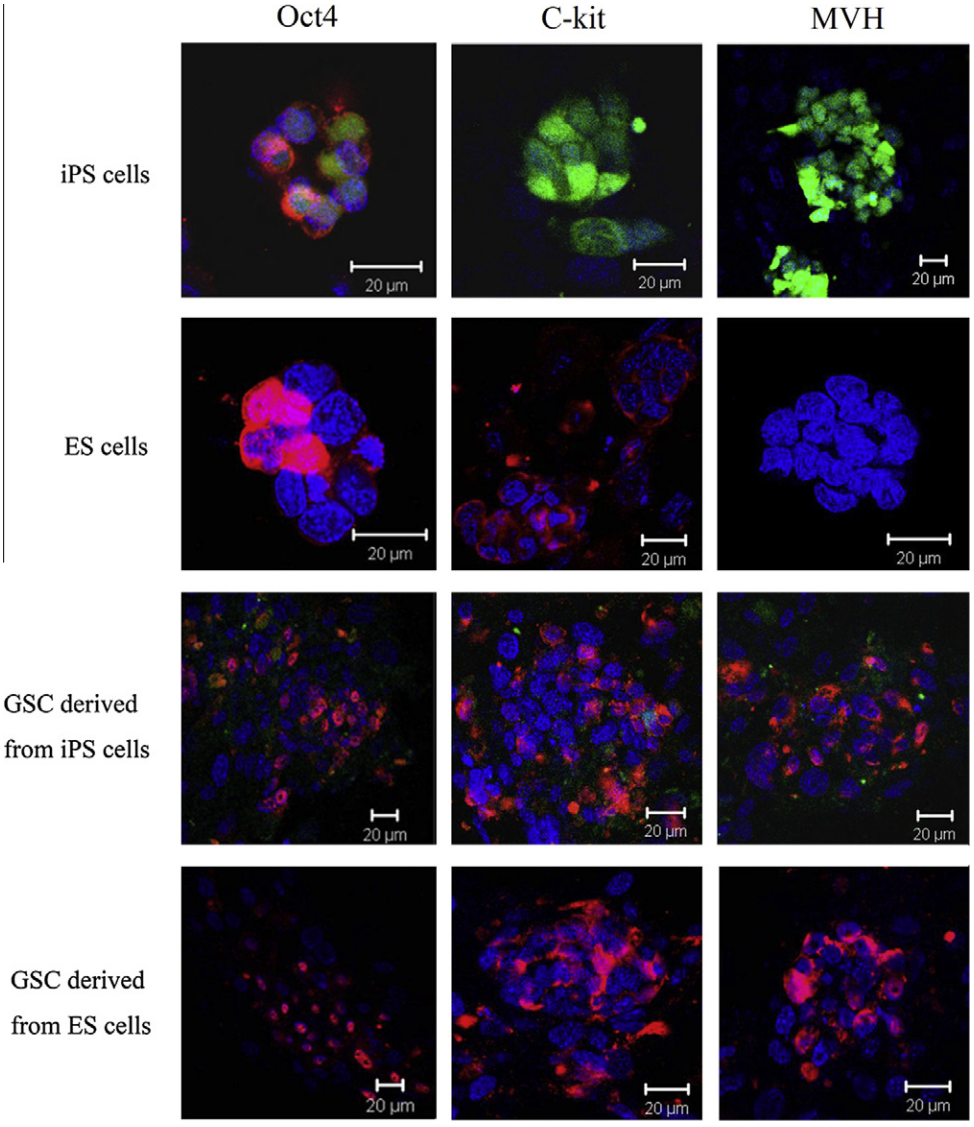

Fig. 3. Expression of Oct4, C-kit and MVH in miPSC-4.1, mESC-H1.2 and derived GSC cells Green: EGFP. Red: Oct4/C-kit/MVH. Blue: DAPI. Bars = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

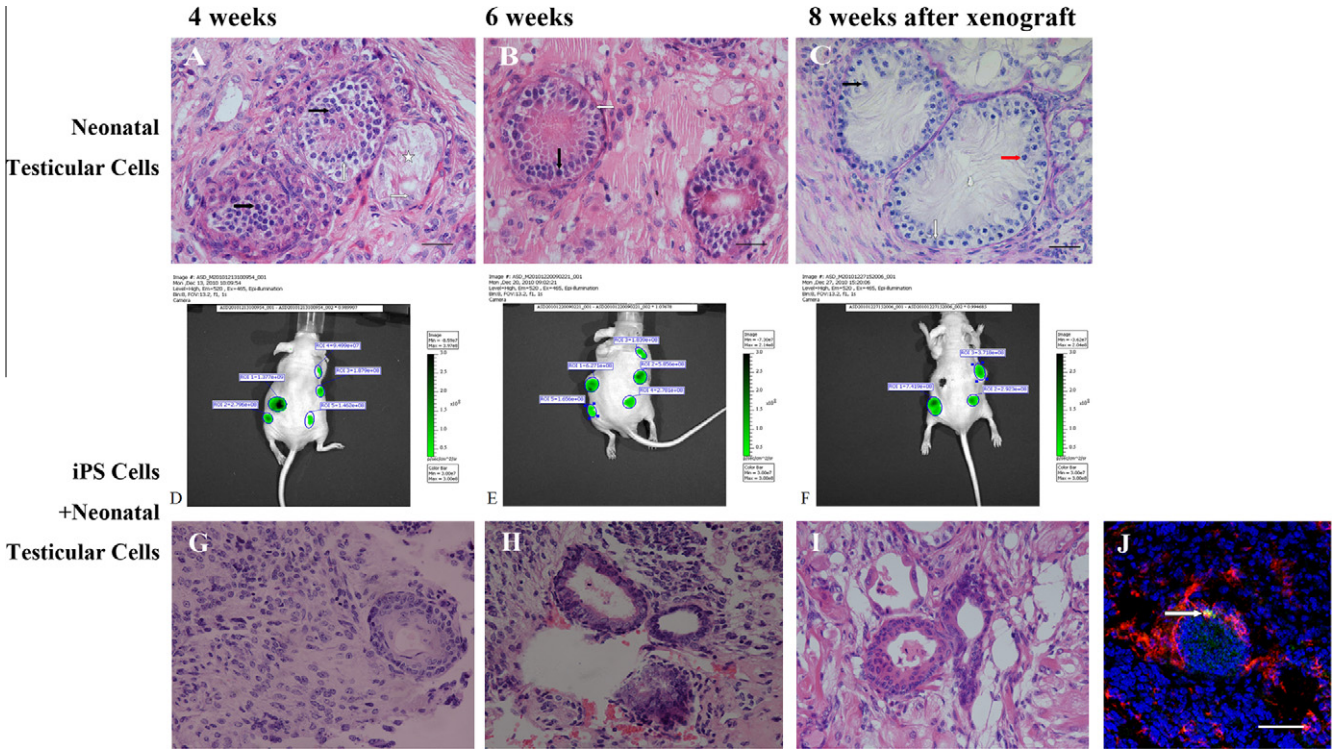

Fig. 4. Analyses of xenografts (A–C) HE staining of xenografts from neonatal testicular cell. White arrow: Sertoli cell. Black arrow: spermatogonia. Red arrow: pachytene spermatocyte. Bars = 20 μm. (D–F) Live-cell imaging of a recipient with xenografts generated by the co-transplantation of miPSC-4.1 and neonatal testicular cells. (G–I) HE staining of xenografts from co-transplantation. Bars = 20 μm. (J) MVH expression in 6-week xenograft generated by co-transplantation. Green: EGFP. Red: MVH. Blue: DAPI. Arrow indicates the $EGFP^+/MVH^+$ cells. Bars = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

revealed miPSC-4.1 cells with the potential of germline differentiation, indistinguishable from the mESC-H1.2 cell behavior.

3.3. Ectopic reconstitution of seminiferous tubules by transplantation of miPSCs with neonatal testicular cells

To investigate further the potential of miPSC-4.1 to differentiate into germ cells, we used ectopic reconstitution approach. In the control group, testicular cells from 5–7 dpp mice were injected into the dorsal region of nude mice. Histological analysis demonstrated the formation of seminiferous-like tubules containing spermatogenic cells (Fig. 4A–C).

When Tg-EGFP-miPSC-4.1 cells were co-transplanted with neonatal testicular cells, the growth of xenografts was monitored periodically using a live-cell imaging system (Fig. 4D–F). In 94.4% (51/54) of the grafts, we detected seminiferous-like tubules surrounded by a basement membrane (Fig. 4G–I). $EGFP^+$ cells were observed in these xenografts, indicating the miPSC-4.1 origin. Importantly, immunofluorescence analysis demonstrated co-localization of EGFP and MVH in some reconstituted tubules, indicating that the miPSC-4.1 cells could differentiate into germ cells under appropriate conditions (Fig. 4J). To conclude, ectopic reconstitution provided an effective spermatogenic niche in which the differentiation of miPS-4.1 cells to germ cells was successfully induced.

4. Discussion

Personalized iPSC therapies could become the regenerative treatment of the future. For example, if the male gametes could be derived from iPS cell lines, the infertile patients could have their biological children.

Derivation of PGC-like cells from pluripotent cells *in vitro* has been the restrictive step in obtaining the gametes. Generally, PGCs are very few; they are sensitive to culture conditions and refractory to proliferation *in vitro* [16]. When human iPS cells are co-cultured with human fetal gonadal cells, the differentiation to PGC-LCs is enhanced, with specific feeder cells inducing and supporting this process [8].The effect of various factors during induction process, including bone morphogenetic proteins (BMPs), epidermal growth factor (EGF), and forskolin, needs to be further evaluated. Last but not least, the spontaneous initiation of meiosis should be carefully monitored, while the CYP26 inhibitor R115866 or retinoic acid might trigger the meiosis.

As discussed in the introduction, the second critical phase of gametogenesis is the meiosis. In mammalian spermatogenesis, this process is regulated by a highly specialized microenvironment mainly constructed by Sertoli cells, while the extracellular matrix and cytokines also play important roles. When spermatogenesis is recreated *ex vivo*, it is essential to mimic spermatogenic environment. It has been reported that, when injected subcutaneously into nude mice, testicular cells from neonatal donors reorganize into testis-like grafts. Moreover, when germline stem cells are co-transplanted with testicular cells, the stem cells can integrate into the newly formed seminiferous-like tubules and differentiate into spermatids [17]. Thus, ectopic reconstitution becomes a useful model to explore the regulation of spermatogenesis, especially the interactions between the niche and spermatogenic cells.

However, the safety and limitations of each iPS cell line should be prudently evaluated. In Hayashi's study [13], only one of the three iPS cell lines examined possessed the capacity for germline transmission. This capacity is determined by the intrinsic characteristics of iPSC lines. In our study, using both *in vivo* and *in vitro* methods, we confirmed the germline potential of miPSC-4.1 cell line. Our system could be used to elucidate the molecular mechanisms of male germ cell development and epigenetic regulation in iPSC differentiation.

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References

[1] N. Geijsen, M. Horoschak, K. Kim, J. Gribnau, K. Eggan, G.Q. Daley, Derivation of embryonic germ cells and male gametes from embryonic stem cells, Nature 427 (2004) 148–154.

[2] K. Nayernia, J. Nolte, H.W. Michelmann, J.H. Lee, K. Rathsack, N. Drusenheimer, A. Dev, G. Wulf, I.E. Ehrmann, D.J. Elliott, V. Okpanyi, U. Zechner, T. Haaf, A. Meinhardt, W. Engel, *In vitro*-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice, Dev. Cell 11 (2006) 125–132.

[3] N. Bucay, M. Yebra, V. Cirulli, I. Afrikanova, T. Kaido, A. Hayek, A.M. Montgomery, A novel approach for the derivation of putative primordial germ cells and sertoli cells from human embryonic stem cells, Stem Cells 27 (2009) 68–77.

[4] K. Tilgner, S.P. Atkinson, A. Golebiewska, M. Stojkovic, M. Lako, L. Armstrong, Isolation of primordial germ cells from differentiating human embryonic stem cells, Stem Cells 26 (2008) 3075–3085.

[5] W. Wei, T. Qing, X. Ye, H. Liu, D. Zhang, W. Yang, H. Deng, Primordial germ cell specification from embryonic stem cells, PLoS ONE 3 (2008) e4013.

[6] C. Eguizabal, N. Montserrat, R. Vassena, M. Barragan, E. Garreta, L. Garcia-Quevedo, F. Vidal, A. Giorgetti, A. Veiga, J.C. Izpisua Belmonte, Complete meiosis from human induced pluripotent stem cells, Stem Cells 29 (2011) 1186–1195.

[7] S. Panula, J.V. Medrano, K. Kee, R. Bergstrom, H.N. Nguyen, B. Byers, K.D. Wilson, J.C. Wu, C. Simon, O. Hovatta, R.A. Reijo Pera, Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells, Hum Mol Genet 20 (2011) 752–762.

[8] T.S. Park, Z. Galic, A.E. Conway, A. Lindgren, B.J. van Handel, M. Magnusson, L. Richter, M.A. Teitell, H.K. Mikkola, W.E. Lowry, K. Plath, A.T. Clark, Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells, Stem Cells 27 (2009) 783–795.

[9] C. Li, H. Yu, Y. Ma, G. Shi, J. Jiang, J. Gu, Y. Yang, S. Jin, Z. Wei, H. Jiang, J. Li, Y. Jin, Germline-competent mouse-induced pluripotent stem cell lines generated on human fibroblasts without exogenous leukemia inhibitory factor, PLoS ONE 4 (2009) e6724.

[10] S. Yang, J. Bo, H. Hu, X. Guo, R. Tian, C. Sun, Y. Zhu, P. Li, P. Liu, S. Zou, Y. Huang, Z. Li, Derivation of male germ cells from induced pluripotent stem cells in vitro and in reconstituted seminiferous tubules, Cell Prolif. 45 (2012) 91–100.

[11] Y. Zhu, H.L. Hu, P. Li, S. Yang, W. Zhang, H. Ding, R.H. Tian, Y. Ning, L.L. Zhang, X.Z. Guo, Z.P. Shi, Z. Li, Z. He, Generation of male germ cells from induced pluripotent stem cells (iPS cells): an *in vitro* and *in vivo* study, Asian J. Androl. 14 (2012) 574–579.

[12] K. van der Wee, M.C. Hofmann, An *in vitro* tubule assay identifies HGF as a morphogen for the formation of seminiferous tubules in the postnatal mouse testis, Exp. Cell Res. 252 (1999) 175–185.

[13] K. Hayashi, H. Ohta, K. Kurimoto, S. Aramaki, M. Saitou, Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells, Cell 146 (2011) 519–532.

[14] M. Pellegrini, D. Filipponi, M. Gori, F. Barrios, F. Lolicato, P. Grimaldi, P. Rossi, E.A. Jannini, R. Geremia, S. Dolci, ATRA and KL promote differentiation toward the meiotic program of male germ cells, Cell Cycle 7 (2008) 3878–3888.

[15] E. Trautmann, M.J. Guerquin, C. Duquenne, J.B. Lahaye, R. Habert, G. Livera, Retinoic acid prevents germ cell mitotic arrest in mouse fetal testes, Cell Cycle 7 (2008) 656–664.

[16] M. Saitou, M. Yamaji, Germ cell specification in mice. Signaling, transcription regulation, and epigenetic consequences, Reproduction 139 (2010) 931–942.

[17] K. Kita, T. Watanabe, K. Ohsaka, H. Hayashi, Y. Kubota, Y. Nagashima, I. Aoki, H. Taniguchi, T. Noce, K. Inoue, H. Miki, N. Ogonuki, H. Tanaka, A. Ogura, T. Ogawa, Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules, Biol. Reprod. 76 (2007) 211–217.