Derivation of male germ cells from induced pluripotent stem cells *in vitro* and in reconstituted seminiferous tubules

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Abstract

Objectives: Previous studies have demonstrated that mouse- and human-induced pluripotent stem (iPS) cells can differentiate into primordial germ cells *in vitro*. However, up to now it is not known whether iPS cells would be able to differentiate into male germ cells *in vivo*. The aim of this study was to explore differentiation potential of iPS cells to male germ cells *in vitro* and *in vivo*.

Materials and methods: In this study, approaches using *in vitro* retinoic acid induction and *in vivo* ectopic transplantation were combined to induce iPS cells to become male germ cells.

Results: RT-PCR showed that expression of pre-meiotic and meiotic germ cell-specific genes was enhanced in iPS cell-derived embryoid bodies (EBs) compared to mRNA transcripts of iPS cells. Immunofluorescence analysis revealed that iPS cell-derived EBs positively expressed germ-cell markers VASA, c-Kit and SCP3. Furthermore, iPS cell-derived cells dissociated from EBs were injected with testicular cells into dorsal skin of mice. Histological examination showed that iPS cell-derived cells could reconstitute seminiferous tubules, and meanwhile, iPS cell-derived germ cells could settle at basement membranes of reconstituted tubules.

Conclusion: Our results suggest that iPS cells are able to differentiate into male germ cells *in vitro*

and that reconstituted seminiferous tubules may provide a functional niche for exogenous iPS cell-derived male germ cells. Derivation of male germ cells from iPS cells has potential application for treating male infertility and provides an ideal platform for elucidating molecular mechanisms of male germ-cell development.

Introduction

Low number and poor quality of human male germ cells are the main causes of infertility in married couples (1). Previous studies have shown that pluripotent stem cells such as embryonic stem (ES) cells are able to differentiate into germ cells *in vitro* (2–4); however, there are ethical problems concerned with the use of human ES cells and sources of human ES cells are limited. Thus, human ES cells are not an ideal cell source for producing germ cells. One of the major breakthroughs in stem-cell biology is establishment of induced pluripotent stem (iPS) cells from somatic cells by retroviral transduction of one or several pluripotent genes, including *Oct4*, *Sox2*, *c-Myc* and *Klf4* (5,6). iPS cells are very similar to ES cells in many aspects (7–9), and notably, iPS cells have some advantages over human ES cells: (i) there are fewer ethical issues using human iPS cells; (ii) sources for human iPS cells are abundant; (iii) mature cells derived from patient iPS cells can be used for patient-specific cell therapy without immunorejection; and (iv) it may be feasible to obtain male germ cells from iPS cells derived from a patient with azoospermia to treat male infertility. Recent studies have reported that iPS cells can generate germ cells by transmitting the germ line to the next generation (10,11) while Park *et al.* and Imamura *et al.* have both demonstrated that iPS cells can generate germ cells *in vitro* (12,13). Furthermore, Eguizabal *et al.* have induced differentiation

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of human iPS cells to post-meiotic cells and consistently obtained haploid cells from human iPS cells (14). However, to our knowledge, there are few studies on derivation of iPS cells into male germ cells both *in vitro* and *in vivo*.

Mammalian spermatogenesis mainly takes place in seminiferous tubules, which provide a functional micro-environment (the niche) for male germ cells (15). Thus, it is essential to establish a stem-cell niche in which to differentiate iPS cells into male germ cells. More recently, several studies have reported that ectopic grafting of dissociated testicular cells from neonatal donors could rearrange and form testis-like grafts under the dorsal skin of mice (15–17). Moreover, mouse germline stem cells co-engrafted with testicular cells might integrate into reconstituted seminiferous tubules and differentiate beyond meiosis and into spermatids (15). Immature testicular cells may reorganize and reconstitute seminiferous tubules to provide a supportive microenvironment for spermatogenesis.

In this study, we have hypothesized that iPS cells can differentiate into male germ cells in reconstituted seminiferous tubules. To study the potential of iPS differentiation to male germ cells *in vitro* and *in vivo*, iPS cells were induced using retinoic acid (RA) to generate male germ cells, and then iPS cell-derived germ cells and immature testicular cells were transplanted into nude mice by subcutaneous injection.

Materials and methods

iPS cells and EB culture

Mouse iPS cells (Tg-EGFP-miPS11.1) were a gift from Prof. Yin Jin of Shanghai Institute of Health Sciences. As previously described, the iPS cells were generated from neural progenitor cells of EGFP transgenic C57BL/6J mice by retroviral transfer of human transcriptional factors Oct4/Sox2/Klf4/C-Myc; notably, this cell line has been demonstrated to be germline-competent (18). iPS cells were cultured on mouse embryonic fibroblast (MEFs) feeder layers inactivated by mitomycin C (Sigma, St. Louis, MO, USA). They were cultured in DMEM (Hyclone, Logan, UT, USA) containing 15% foetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 mg/ml leukaemia inhibiting factor (LIF; ESGRO; Millipore, Billerica, MA, USA), 100 U/ml penicillin plus 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA), 2 mmol/l L-glutamine (Gibco), 0.1 mmol/l β -mercaptoethanol (Gibco) and 0.1 mmol/l non-essential amino acids (NEAA; Gibco). Cells were passaged every 2–3 days and re-plated on fresh feeders. Cell culture was maintained at 37 °C in a humidified incubator with 5% CO₂.

EBs were formed by hanging drop and suspension culture as follows: iPS cells were aggregated in a hanging drop for 2 days and then transferred to suspension cultures for 3 days. During this 5-day culture process, LIF was removed from the medium. Thereafter, EBs were exposed to 2 μ mol/l RA (Sigma), or no treatment for 5 days.

RT-PCR analysis

Upon termination of EB culture, total RNA was extracted from EBs using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After DNase treatment to remove potential contamination with genomic DNA, two micrograms of total RNA was transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). RT-PCR was performed using primers shown in Table 1. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed.

Flow cytometric analysis

iPS cell-derived EBs were digested using 0.25% trypsin-EDTA (Gibco) to obtain single iPS cell suspensions. Cells were stained with PE anti-mouse SSEA1 (Biolegend, San Diego, CA, USA) for 30 min at 4 °C, then

Table 1. List of gene primers for RT-PCR

Genes	Sequences of gene primers (5'-to-3')	References
Stella	F: GACGCTTTGGATGATACAGACG R: GGTCTTTTCAGCACCCGACAACA	(24)
Vasa	F: ATTCTTGGAAGTCAGAAGCAGAAG R: CTGGTTGACCAATTCTCGAGT	(25)
Dazl	F: AATGTTTCAGTTCATGATGCTGCTC R: TGTATGCTTCGGTCCACAGAC	(26)
Stra8	F: CAACCTGCAAGATGGGAATC R: CTTCAAGCATCTGGTCCAACA	(28)
c-kit	F: CCCGACGCAACTTCCTTA R: CGCTTCTGCCTGCTCTTC	(27)
Scp3	F: TGCAGAAAGCTGAGGAACAA R: TGCTGCTGAGTTTCCATCAT	(28)
Haprin	F: CCAGAACATGAGACAGAGAG R: AGCAACTTCCTGAGCATACC	(28)
Tnp1	F: CAGCCGCAAGCTAAAGAC R: AAGACCACCAGGGCAGAG	(27)
Prrm1	F: AGCAAAAGCAGGAGCAGATG R: CTTGCTATTCTGTGCATCTAG	(28)
Zp1	F: GAGTGACTGTGTGCCATAG R: GCCACACTGGTCTCACTACG	(4)
Zp2	F: GCTACACACATGACTCTCAC R: GGTGACTCACAGTGGCACTC	(4)
β -actin	F: ACCAACTGGGACGATATGGAGAAG R: CTCTTTGATGTACGCACGATTTC	(28)

cells were washed twice in phosphate-buffered saline (PBS) and analysed using the FACSCalibur system (Becton Dickinson, San Jose, CA, USA). For each sample analysed, an aliquot of cells was labelled with mouse IgG conjugated to PE as isotype control.

Animals

ICR nude mice (BALB/c-nu/nu) were purchased from Shanghai Laboratory Animal Center of Chinese Academy. All experiments with animals were performed according to guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1996).

Transplantation of iPS cell-derived cells and testicular cells

Donor cells for transplantation were prepared from testes of mice in neonatal periods (1–2 days old). To harvest single cells from the tubules, they were digested using two-step enzymatic treatment (19). Briefly, testicular tissue was first treated with 1.5 mg/ml collagenase type II (Sigma) in DMEM at 37 °C for 20 min, then with digestion solution containing type II collagenase (1.5 mg/ml), hyaluronidase (1.5 mg/ml) and 0.25% trypsin-EDTA (Gibco) in DMEM at 37 °C for 20 min.

At the end of cell culture, EBs were digested using 0.25% trypsin-EDTA to obtain single-cell suspensions, then single-cell suspension was mixed with digested testicular cells. Cell number ratio of iPS cells to testicular cells was 1:3–5 and mixed cell suspension was added to the same volume of Matrigel Matrix (MGM; Becton Dickinson Labware, Bedford, MA, USA). In the region of $0.1\text{--}0.5 \times 10^6$ iPS cells and $0.5\text{--}1.5 \times 10^6$ testicular cells in MGM were injected into backs of anaesthetized nude mice and animals were then castrated, incisions being closed using Michel clips (7.5 mm; Miltex, York, PA, USA).

Histological and immunofluorescence analyses

Xenografts were dissected at 4, 6, 8 and 12 weeks, respectively. After fixation in Bouin's solution, xenografts were embedded in paraffin wax and cut at 4- to 8- μm thickness. Sections were stained with haematoxylin and eosin and observed by light microscopy for structure of seminiferous tubules. EB cells or frozen sections of xenografts were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.4% Triton/X-100 in PBS. Blocking was performed with 1% bovine serum albumin (BSA, Sigma) for 1 h prior to incubation with primary antibodies. Specimens were incubated with anti-VASA, c-Kit and SCP3 rabbit polyclonal antibodies (Abcam, Cambridge, UK) for 2 h at room temperature,

and goat anti-rabbit Alexa Fluor 594 (red)-labelled secondary antibody (A11072, Invitrogen) for 1 h. Replacement of primary antibodies with PBS was used as negative controls. DAPI was used to label cell nuclei and images were captured using confocal fluorescence microscopy (Leica, Mannheim, Germany).

Results

iPS cells differentiated into male germ cells in vitro by RA induction

EB formation is a common approach for differentiation of embryonic stem cells (20) and RA has been reported to promote differentiation of primordial germ cells (PGCs) and male gametes from embryonic stem cells (21,22). In this study, RA was used to treat EB for derivation of germ cells from iPS cells. EBs were formed by the hanging drop and suspension culture method. After 5 days' culture, EBs were formed, and then they were treated by 2 $\mu\text{mol/l}$ RA, or no treatment, for 5 days.

To detect germ cells derived from iPS cells, expression patterns of germ cell-specific genes in iPS cells, untreated EBs, and RA-treated EBs were compared by RT-PCR. As shown in Fig. 1, expression of Stella, a definitive marker of PGCs (23,24), was observed in both untreated EBs and RA-treated EBs. Expression of germ

Figure 1. Expression of germ cell-specific genes in undifferentiated iPS cells (iPS), untreated EBs (EB) and RA-treated EBs (EB+RA). Positive controls and negative controls used templates extracted from testis of 8-week-old ICR mice and mouse embryonic fibroblasts (MEF), respectively.

cell-specific genes *Vasa* (25) and *Dazl* (26) was enhanced in untreated EBs and RA-treated EBs compared to gene transcripts of iPS cells. Expression of c-kit, a hallmark of differentiating spermatogonia (27), was clearly upregulated in RA-treated EBs compared to gene transcripts of iPS cells and untreated EBs. Expression of *Scp3* (28,29) and *Haprin* (28,30), markers of meiotic germ cells and haploid germ cells, respectively, was also induced in EBs with RA treatment. Meanwhile, *Stra8*, an RA-responsive gene expressed in pre-meiotic mouse germ cells (28,31), was upregulated in RA-induced EBs. In contrast, there were no detectable expression of post-meiotic markers transition protein 1 (*Tnp1*) (27) and protamine 1 (*Prm1*) (21,28) in iPS cells, untreated EBs and RA-treated EBs. Interestingly, expression of female germ-cell markers *Zp1* and *Zp2* (4) were detected in untreated EBs, suggesting that iPS could differentiate into female germ cells spontaneously.

To further examine expression of germ-cell markers in iPS cell-derived cells, iPS cell-derived EBs with RA treatment were stained with anti- VASA, c-Kit and SCP3. Confocal images indicated VASA and c-Kit positive cells found in iPS cell-derived EBs (Figs 2,3a), suggesting that iPS cells could be induced into germ cells. SCP3, a marker expressed in spermatocytes from leptotene to late meiotic stages (29), was also observed

in RA-treated EBs, which indicated that iPS cells had differentiated into spermatocytes (Fig. 3b). For negative controls, sections were processed without primary antibody, and no signals were detected (data not shown).

To quantify germ cells derived from iPS cells, percentages of SSEA1⁺ cells of iPS, untreated EBs and RA-treated EBs were detected by flow cytometry. SSEA-1 is considered to be both a mouse pluripotency marker (4) and a putative PGC marker (32). As shown in Fig. 4, SSEA1 was highly expressed in iPS cells. However, percentages of SSEA1⁺ cells obviously decreased in differentiated EBs derived from iPS cells (Fig. 4B). Mean percentage of SSEA1⁺ cells of RA-treated EBs ($35.06 \pm 10.87\%$) was significantly higher than that of untreated EBs ($13.19 \pm 6.10\%$) ($P < 0.05$) (Fig. 4B), suggesting that RA could stimulate proliferation of PGCs.

Reconstitution of seminiferous tubules of mice transplanted with iPS cell-derived male germ cells and testis cells

Testes harvested from neonatal mice were made into single-cell suspension and mixed with iPS cell-derived male germ cells and cell mixtures were then injected into backs of nude mice. Grafts were observed 3 weeks

Figure 2. Co-localization of EGFP and VASA in undifferentiated iPS and iPS cell-derived EBs with RA treatment (arrow). (a) iPS. (b) RA-treated EBs. (c) High magnification of (b). Scale bars = 75 μ m (a, b), 25 μ m (c).

Figure 3. Co-localization of EGFP and germ cell-specific markers in iPS cell-derived EBs with RA treatment (arrow). (a) c-Kit. (b) SCP3. Scale bars = 50 μ m (a), 25 μ m (b).

Figure 4. Flow cytometric analysis of SSEA1⁺ cells, iPS cells, untreated EBs and RA-treated EBs. (A) Example of flow cytometry of SSEA1⁺ cells. (B) Percentage SSEA1⁺ cells, iPS cells, untreated EBs and RA-treated EBs. ^a $P < 0.05$, compared to iPS cells; ^b $P < 0.05$, compared to untreated EBs.

after transplantation, and diameters reached to around 6–10 mm during the subsequent observation period (Fig. 5a). At different time points post-transplantation, xenografts were dissected from mice. Most xenografts grew into a testis-like tissue with rich blood supply (Fig. 5b). As shown in Fig. 6, grafts dissected at 4, 6, 8 and 12 weeks all have similar histological structure of

seminiferous tubules with germ cells arranged in an ordered sequence from the basement membrane to the lumen. Then reconstituted tubules were characterized by immunostaining with the germ cell-specific marker VASA and meiotic-specific marker SCP3. Immunohistochemical analysis confirmed that the reconstituted tubules of both 4-week and 12-week grafts had germ

Figure 5. Observation of xenografts 6 weeks after transplantation. (a) Nude mice with xenografts beneath the dorsal skin; (b) Testis-like grafted tissue dissected from mice showing blood vessels (arrow). Scale bar = 1 mm.

Figure 6. Histological observations of grafts at various time points post-grafting. (a) 4 weeks, (b) 6 weeks, (c) 8 weeks and (d) 12 weeks. Spermatocytes indicated by arrows. Scale bars = 40 μ m (a–c), 80 μ m (d).

cells associated (Fig. 7). However, spermatocytes were only observed in seminiferous tubules of 12-week grafts (Fig. 6d). Confocal images indicate that SCP3 positive cells were negative for EGFP (Fig. 8), which indicated that these spermatocytes in reconstituted tubules were derived from testicular cells of the neonatal mice. For negative controls, sections were processed without primary antibody; no staining was observed.

Co-localization of EGFP and male germ cell-specific markers in reconstituted tubules

To investigate whether iPS cell-derived germ cells would be able to integrate and continue differentiating

in reconstituted tubules, they were mixed with testicular cells and co-engrafted beneath dorsal skin of nude mice. Twelve weeks after transplantation, sections of xenografts were examined by immunostaining. Among three such sections stained with anti-VASA antibody, six EGFP-positive germ cells were observed in 3 of 21 reconstituted tubules with germ cells. As shown in Fig. 9, EGFP-positive cells were located near basement membranes of tubules, indicating that iPS cell-derived germ cells could integrate there. Confocal micrographs demonstrate the co-localization (Fig. 9), indicating that reconstituted tubules could provide a supportive niche for iPS cell-derived germ cells. Furthermore, all EGFP-positive germ cells had a single

Figure 7. Characterization of reconstituted tubules from grafts by immunostaining with VASA at various time points post-grafting. (a) 4 weeks and (b) 12 weeks post-grafting, by immunostaining with VASA. (c) Seminiferous tubules of 8-week-old mouse testis were used as positive control. Scale bars = 50 μ m (a, b), 75 μ m (c).

Figure 8. Characterization of reconstituted tubules from grafts at 12 weeks post-grafting, by immunostaining with SCP3. (a) SCP3 immunostaining in reconstituted tubules. (b) Higher magnification of (a). SCP3 positive cells indicated by arrows. Scale bars = 50 μ m (a), 10 μ m (b).

nucleus, excluding the possibility of EGFP-positive iPS cell fusion with germ cells from testicular cells. However, EGFP-positive germ cells were negative for SCP3 (data not shown), a meiosis-specific marker, indicating that iPS cell-derived germ cells did not differentiate into meiotic cells.

Discussion

iPS cells, which are generated by transduction of transcription factors of somatic cells (11), have the same pluripotential ability to differentiate into all cell lineages, as ES cells. Numerous previous studies have demonstrated

Figure 9. Co-localization of EGFP-positive cells with VASA in reconstituted tubules of xenografts (arrow). Scale bar = 50 µm.

that ES cells can differentiate into primordial germ cells and early gametes (32,33) and recently, it has been reported that mouse iPS cells could be induced into PGCs (13). Park *et al.* and Panula *et al.* have also reported that human iPS cells can differentiate into PGCs *in vitro* (12,34). However, many attempts have been made to induce iPS cells to become germ cells *in vitro*, but as yet it is not known whether they would be able to differentiate into germ cells *in vivo*. Moreover, our understanding so far of iPS cells' potential to generate germ cells *in vitro* and *in vivo* remains very limited.

Transplantation into seminiferous tubules of infertile recipients is widely applied to induce spermatogenesis from germ cells (35), however, this orthotopic grafting technique still has many disadvantages including low efficiency in most species except mice, and detrimental pre-treatment to the recipients (36). Thus, ectopic grafting, which rearranges reconstituted tubules in testis-like grafts in mice, has become a useful model to induce spermatogenesis.

In the present study, *in vitro* differentiation and ectopic grafting of iPS cell-derived germ cells with testicular cells were combined to induce derivation of male germ cells from iPS cells. First, expression of germ-cell genes of different stages in iPS cells, untreated EBs and RA-treated EBs were compared, to analyse germ-cell differentiation at the molecular level. Results of RT-PCR

demonstrated that both untreated and RA-treated EBs expressed early germ-cell marker gene *Stella* and germ cell-specific genes *Vasa*, *Dazl* and *Stra8*. However, expression of differentiating spermatogonia marker gene *c-kit*, meiotic gene *Scp3* and post-meiotic gene *Haprin* were clearly enhanced in RA-treated EBs compared to untreated EBs. Previous studies have reported that RA signalling controls entry of germ cells into meiosis in mice and humans (37,38). Our results have confirmed that RA can act as a meiosis-inducing factor in differentiation of iPS cells into male germ cells. Moreover, no expression of post-meiotic genes *Tnp1* nor *Prm1* was detected in either untreated or RA-treated EB. These results suggested that *in vitro* differentiation of RA-treated EBs remained in pre-meiotic and meiotic stages, consistent with previous investigation (13). In addition, expression of oocyte-specific markers *Zp1* and *Zp2* was observed in untreated EBs, which suggested that iPS could spontaneously differentiate into female germ cells.

Secondly, phenotypes of iPS cell-derived EBs were confirmed by immunostaining and flow cytometry. Immunofluorescence revealed that iPS cell-derived EB expressed VASA, c-Kit and SCP3, indicating that iPS cells could differentiate into meiotic germ cells during EB formation and RA induction. Flow cytometry showed that RA-treated EBs contained more SSEA1⁺ cells than untreated EBs, but less SSEA1⁺ cells than iPS

cells, which is consistent with previous work showing that RA can rapidly cause differentiation of ES cells while supporting proliferation of PGCs (4). Finally, to induce differentiation into germ cells *in vivo*, iPS cell-derived cells dissociated from EBs were injected into mice together with testicular cells. Histological examination confirmed that immature testicular cells could reconstitute seminiferous tubules and furthermore, iPS cell-derived germ cells settled in at basement membranes of reconstituted tubules. Results of the study are consistent with previous findings that reconstituted tubules can provide a functional niche for exogenous germ cells (15).

We also found that no further differentiation of iPS cell-derived germ cells was observed in reconstituted seminiferous tubules, indicating that the tubules did not provide a totally suitable microenvironment for *in vivo* differentiation. This may be due to initially deficient germ cells and/or impaired meiotic and post-meiotic differentiation (39), or immature testicular cells not containing appropriate proportions of the various cell types, or else lack of specific environmental input (40). Thus, it was necessary to improve ectopic grafting method to provide a stable niche to induce complete spermatogenesis.

In summary, we have demonstrated that male germ cells could be derived from iPS cells both *in vitro* and *in vivo*. Moreover, the system of using ectopic grafting can be applied for inducing exogenous germ-cell differentiation *in vivo*.

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