International Immunology, Vol. 23, No. 1, pp. 65–74
doi:10.1093/intimm/dxq458
Advance Access publication 6 December 2010

Successful differentiation to T cells, but unsuccessful B-cell generation, from B-cell-derived induced pluripotent stem cells

Haruka Wada[1], Satoshi Kojo[1], Chie Kusama[1,2], Naoki Okamoto[3], Yorino Sato[3], Bunpei Ishizuka[3] and **Ken-ichiro Seino[1,2,4]**

[1]Division of Bioregulation Research, Institute of Medical Science, St Marianna University School of Medicine, Kanagawa 216-8512, Japan
[2]Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Tokyo 102-0075, Japan
[3]Department of Gynaecology and Obstetrics, St Marianna University School of Medicine, Kanagawa 216-8512, Japan
[4]Division of Immunobiology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan

Correspondence to: K. Seino; E-mail: seino@igm.hokudai.ac.jp

Received 20 April 2010, *accepted* 1 November 2010

Abstract

Forced expression of certain transcription factors in somatic cells results in generation of induced pluripotent stem (iPS) cells, which differentiate into various cell types. We investigated T-cell and B-cell lineage differentiation from iPS cells *in vitro*. To evaluate the impact of iPS cell source, murine splenic B-cell-derived iPS (B-iPS) cells were generated after retroviral transduction of four transcription factors (Oct4, Sox2, Klf4 and c-Myc). B-iPS cells were identical to embryonic stem (ES) cells and mouse embryonic fibroblast (MEF)-derived iPS cells in morphology, ES cell marker expression as well as teratoma and chimera mouse formation. Both B-iPS and MEF-derived iPS cells differentiated into lymphocytes in OP9 co-culture systems. Both efficiently differentiated into T-cell lineage that produced IFN-γ on T-cell receptor stimulation. However, iPS cells including B-iPS cells were relatively resistant to B-cell lineage differentiation. One of the reasons of the failure of B-cell lineage differentiation seemed due to a defect of *Pax5* expression in the differentiated cells. Therefore, current *in vitro* differentiation systems using iPS cells are sufficient for inducing T-cell but not B-cell lineage.

Keywords: B cell, development, differentiation, ES cells, haematopoietic cells, iPS cells, OP9, reprogramming, T cell

Introduction

Pluripotent stem cells are being used extensively in biomedical research as they are proving invaluable for an array of potential applications. Pluripotency can be induced in mouse and human somatic cells by forced expression of *OCT4* and *SOX2* with a combination of either *KLF4* and *MYC* or *NANOG* and *LIN28* (1–4), resulting in the formation of induced pluripotent stem (iPS) cells. Differentiation of iPS cells into various cell types belonging to the three germ layers has been demonstrated by the analysis of teratomas generated from mouse and human iPS cells. In addition, the pluripotency of iPS cells is further evidenced by the contribution of iPS cell-derived cells to development of various organs of chimeric mice developed from iPS cell-introduced blastocysts (5). Regarding *in vitro* generation of cells of mesodermal lineage from iPS cells, differentiation into cardiac myocytes and endothelial cells from mouse iPS cells has been recently reported (6–8). However, it remains to be determined whether fully differentiated and functional haematopoietic cells can be generated from iPS cells by direct differentiation *in vitro*. Senju *et al.* (9) recently reported that mouse iPS cells can differentiate into macrophages and dendritic cells. Lei *et al.* (10) recently reported that mouse iPS cells can differentiate into T cells. However, information regarding differentiation into B cells is limited.

Differentiation of iPS cells into haematopoietic cells including lymphoid lineage is being considered for establishing new therapeutic tools for treating some haematological or immunological disorders. To assess the lymphoid lineage differentiation from iPS cells, we used the *in vitro* OP9 co-culture system. It has previously been demonstrated that haematopoietic stem and embryonic stem (ES) cells can differentiate into both T and B cells in this system (11). We were able to induce differentiation of mouse embryonic fibroblast-derived iPS (MEF-iPS) cells and found that they could easily differentiate into T-cell lineage, but not B-cell lineage, using this system (described below). We hypothesized that

this disparity was dependent on the source of iPS cells. Therefore, we attempted to generate iPS cells from B cells instead, using the 'classical' retroviral transduction of four Yamanaka transcription factors (Oct4, Sox2, Klf4 and c-Myc) (3) and successfully generated iPS cells from murine splenic B-cell-derived iPS (B-iPS) cells. Here, we report the generation of B-iPS cells and their efficient and reproducible differentiation into T-cell lineage *in vitro*. In contrast, the results indicated that iPS cells are relatively resistant to differentiate into B-cell lineage *in vitro*.

Materials and methods

Mice and cell lines

Imprinting control region (ICR) mice and non-obese diabetic (NOD)-SCID mice were purchased from Japan CLEA, Inc. (Tokyo, Japan). C57BL/6-Ly5.1 mice were purchased from RIKEN Bioresource Centre (Ibaraki, Japan). All animal procedures were approved by the St Marianna University Animal Care Committee. The mouse iPS-MEF-Ng-38C-2 cell line (MEF-iPS), which was generated from MEFs by retroviral transduction of Oct4, Sox2, Klf4 and c-Myc (5), was kindly provided by Dr Yamanaka (Kyoto University). MEF-iPS cells were maintained in DMEM medium supplemented with 15% FCS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 10 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (all from Invitrogen) containing 100× of recombinant human leukaemia inhibitory factor (LIF) supernatant (Wako, Tokyo, Japan) on feeder layers of irradiated MEF in 6-cm culture dishes. OP9 and OP9-DL1 cell lines were generous gifts from Dr Hiroshi Kawamoto [Research Center for Allergy and Immunology (RCAI), RIKEN, Yokohama, Japan] and were cultured as monolayers in OP9 media (α-MEM supplemented with 20% FCS, 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 U ml^{-1} penicillin, 100 μg ml^{-1} of streptomycin and 2.2 g l^{-1} sodium bicarbonate). The ES cell line (B6 Ly5.1 ES) was a generous gift from Dr Haruhiko Koseki (RCAI, RIKEN). ES cells were maintained in ES cell media DMEM high glucose supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 μM beta-mercaptoethanol, 15% FCS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} of streptomycin and recombinant human LIF supernatant on irradiated MEFs. MEFs were generated from embryos on day 14 as described previously (3).

Generation of iPS cells from peripheral B cells

pMXs vectors encoding Oct4, Sox2, Klf4 or c-Myc were established as described previously (3). Retroviruses were prepared as described previously (3, 5), and 8 μg ml^{-1} of polybrene (Sigma–Aldrich, St Louis, MO, USA) was added to the virus-containing supernatant. Murine splenic B cells were isolated with MACS beads (Miltenyi Biotech) as determined by $CD19^+$ expression (purity > 98%). The isolated $CD19^+$ cells were incubated in RPMI1640 medium supplemented with 10% FCS, 10 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol in the presence of 10 ng ml^{-1} IL-4 (Peprotech) and 1 μg ml^{-1} LPS (Sigma–Aldrich). After 24 h, the four reprogramming factors or Green fluorescent protein was introduced by retroviral transduction with centrifugation (780 × *g* for 60 min) and then incubated for 4 h in a 32°C, 5% CO_2 incubator and then incubated at 37°C. Viral transduction was performed twice over two consecutive days. Four days after the first transduction, RPMI medium was replaced with iPS cells medium. Twelve days after the first transduction, cells were plated onto irradiated MEF supernatant in iPS cells medium in 100-mm dishes. Seventeen days after the first transduction, iPS cell colonies were isolated in iPS cell lines.

Teratoma formation, histological examination and blastocyst injections

iPS cells (3×10^5) suspended in PBS containing 10% FCS were inoculated into testes of NOD-SCID mice. Four weeks after the injection, tumours were surgically dissected from the mice and fixed in 4% formaldehyde. The samples were embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin. For blastocyst injections, a controlled number of B-iPS cells was micro-injected into ICR blastocysts and transferred to pseudopregnant female mice.

OP9 co-cultures

Differentiation of iPS cells was induced with a withdrawal of LIF from the culture in a non-treated plastic dish. By day 5 of culture, embryonic body-like round-shaped spheres were formed. The spheres were disrupted with 0.25% trypsin (Gibco-BRL). The resultant single-cell suspensions were replated at a density of 6×10^5 cells per 100-mm non-treated dish containing fresh OP9 cells with the addition of Flt3 ligand (20 ng ml^{-1} for B-cell lineage or 5 ng ml^{-1} for T-cell lineage differentiation; R&D Systems). On day 8 of culture, loosely adherent haematopoietic cells were harvested by gentle pipetting. Every 6 days thereafter, non-adherent iPS cell-derived haematopoietic cells were collected by vigorous pipetting, filtered through a 70-μm nylon mesh and transferred onto fresh OP9 (for B-cell lineage culture) or OP9/DL1 (for T-cell lineage culture) monolayers in OP9 media. On day 8 of culture, another Flt3 ligand and exogenous IL-7 (5 ng ml^{-1}; R&D Systems) were added. Both cytokines were included at all subsequent passages.

Flow cytometry and antibodies

Flow cytometry was performed with an FACScalibur® or FC500® instrument and analyzed by CellQuestPro® or FlowJo® software. FITC-conjugated anti-CD3 (clone 145-2C11) and -CD45 (clone 30-F11); PE-conjugated anti-CD8 (clone 53-6.7), -CD19 (clone 1D3), -CD25 (clone 7D4), IFN-γ (clone XMG1.2), TCRγδ (clone GL3), Flk-1 (clone 89B3A5); allophycocyanin-conjugated anti-CD4 (clone GK1.5), CD11b (clone M1/70), CD44 (clone IM7), TCRβ (clone H57-597), biotin-conjugated anti-CD3 (clone 145-2C11) and CD19 (clone 1D3) mAbs were purchased from Biolegend. A PE-conjugated anti-FoxP3 (clone FJK-16s) was purchased from eBioscience. An FITC-conjugated TCR panel was purchased from BD Biosciences. For analysis, live cells were gated based on forward and side scatter as well as lack of propidium iodide uptake.

T-cell stimulation assay

Non-adherent cells from iPS-cells/OP9-DL1 co-cultures at day 21 were collected by vigorous pipetting and filtered through a 70-μm nylon mesh. Collected cells were pre-plated into a fresh dish and cultured for 1 h at 37°C in 5% CO_2 incubator to avoid contamination of MEFs. Then the collected cells were stimulated for 2 days with plate-bound anti-CD3 (1 μg ml^{-1}; clone 145-2C11) mAb in differentiation medium in the presence of IL-2 (1 ng ml^{-1}) and anti-CD28 (1 μg ml^{-1}; clone 37.51). Cells were subsequently cultured for 6 h in the presence of phorbol myristate acetate/ionomycin. Intracellular staining for IFN-γ was performed with Cytofix/Cytoperm® (BD Bioscience) according to the manufacturer's instructions. Cells were analyzed by flow cytometry.

Bisulphite genomic sequencing analysis

Genomic DNA was prepared by using QIAGEN DNA extraction kit (QIAGEN). Whole genomic DNA was bisulphited by MethylEasy™ Xceed (Human Genetic Signatures); follow the manufacture's protocol. The resultant DNA was amplified using AmpliTaq® DNA polymerase (Applied Biosystems) and primers specific for the Pax5 promoter region 5′-TGGTTGATAATTGTGTTAGTATAGGG-3′ and 5′-AAACCCAAAAAACAACAATACC-3′ under the following conditions: 95°C for 1 min, 35 cycles of 95°C for 30 s, 53°C for 90 s, 72°C for 60 s and 72°C for 10 min. PCR product was cloned into pCR-TOPO vector using TOPO® TA cloning kit (Invitrogen). At least five individual bacterial clones were analyzed. DNA sequencing was performed using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

Gene rearrangement analysis

Genomic DNA was prepared by using QIAGEN DNA extraction kit (QIAGEN). PCR primers used for *Bcr* V-DJ and *Tcr* rearrangement analyses have been described previously (12, 13).

Reverse transcription–PCR

cDNA was generated with oligo dT primers and Superscript III (Invitrogen) from total RNA samples. Reverse transcription (RT)–PCR was performed with Amplitaq® (Applied Biosystems) for ES markers and lymphocyte differentiation markers. The primers used for ES cell markers analysis were described previously (3, 11). We used the following primers for development and differentiation analysis: 5′-ATGGAAGGGTTTTCCCTCACCGCC-3′ and 5′-GTCCACGCTCTGCAGCTCTGTGAA-3′ for *Pu.1*, 5′-TGCAGACATTCTAGCACTCTGG-3′ and 5′-ACATCTGCCTTCACGTCGAT-3′ for *Rag-1*, 5′-CCTGCCTCTCCTCCTCTTCT-3′ and 5′-CCCCTGGAGATGTCCTCATA-3′ for *Igα*, 5′-GATGCGGTGGAACACTTTCT-3′ and 5′-TAGTCTGGGTTGGGAACAGG-3′ for Cd3ε, 5′-CGCACTGACCACGAGCTTCAC-3′ and 5′-TCCAGGGACAGCACCTCATCTG-3′ for *E47*, 5′-AGCAACTGGACGCATGTATC-3′ and 5′-TCACCATCTCTGTAGTCAGG-3′ for *Il-7rα*, 5′-CAGAGCCTCCTCCCCCAACAG-3′ and 5′-GCTCAGAGGGGTGGGTAAGAT-3′ for *pTα*, 5′-TCCTCGGACCATCAGGACAG-3′ and 5′-CCTGTTGATGGAGCTGACGC-3′ for *Pax5*, 5′-ACTACCTCTGGAGCACAGCAGAA-3′ and 5′-ATAGGGCATGTCTGACAGGCACT-3′ for *Ikaros* and 5′-CCCTCCAACTGCAGTAGCTC-3′ and 5′-GCAAGGTCGGTGATTTTGTT-3′ for *Ebf1*. For *Oct4* detection, we used the following primers: 5′-CTGAGGGCCAGGCAGGAGCACGAG-3′ and 5′-CTGTAGGGAGGGCTTCGGGCACTT-3′ for total expression, 5′-TCCCTAGGTGAGCCGTCTTT-3′ and 5′-TTCATGTCCTGGGACTCCTC-3′ for internal expression, 5′-GTACAAAAAAGCAGGCTCCACC-3′ and 5′-GGTTCTCAATGCTAGTTCGCT-3′ for transcripts from *Oct4* vector. We also used the following primers: 5′-ACTTTTGTCCGAGACCGAGA-3′ and 5′-ATGTAGGTCTGCGAGCTGGT-3′ for *Sox2*, 5′-CAGCTTCATCCTCGTCTTCC-3′ and 5′-CGGGACTCAGTGTAGGGGTA-3′ for *Klf4*, 5′-GCCCAGTGAGGATATCTGGA-3′ and 5′-GAATCGGACGAGGTACAGGA-3′ for *c-Myc*. PCR products were separated by agarose gel electrophoresis and were visualized by ethidium bromide staining. All PCR products shown correspond to expected molecular sizes.

Results

Generation of B-iPS cells

To generate B-iPS cells, we first isolated peripheral $CD19^+$ B cells from mouse spleen. The isolated $CD19^+$ cells were also determined to be $CD24^+$, CD45R $(B220)^+$ and IgM^+ (data not shown). $CD19^+$ cells were then activated with IL-4 and LPS and transduced with four retroviruses encoding Oct4, Sox2, Klf4 or c-Myc. Within 17 days of culture after the transduction, we obtained ~25 iPS colonies from 4×10^6 $CD19^+$ cells in our first experiment and ~30 colonies from 1×10^7 $CD19^+$ cells in our second experiment.

The obtained B-iPS colonies were expandable in culture and exhibited morphology similar to mouse ES and MEF-iPS cells (Fig. 1A). B-iPS cells expressed ES cell marker genes including *Nanog*, *Ecat* and *Gdf* similar to MEF-iPS and ES cells (Fig. 1B). In contrast, B-iPS cells did not express B-cell-specific transcription factor *Pax5* (Fig. 1B). We confirmed the rearrangement of B-cell receptors in B-iPS cells. Eight of 12 B-iPS cell colonies revealed a VDJ3 band, similar to splenic $CD19^+$ cells (Fig. 1C, upper), whereas the remaining four colonies revealed a VDJ2 band (Fig. 1C, lower). These data indicate that the source of B-iPS cells was *Bcr* gene-rearranged B cells, which was inherited by B-iPS cells.

We further examined teratoma formation. We injected three B-iPS cell lines into the testes of NOD-SCID mice. Four weeks after inoculation, macroscopic teratomas were observed in all injected mice. Histological examination showed that teratomas contained cell types representing all three embryonic germ layers (Fig. 1D). Blastocyst injection of B-iPS cells resulted in generation of chimeric offspring mice (Fig. 1E). These data clearly demonstrate that iPS cells can be derived from mouse peripheral B cells by forcing expression of the four Yamanaka factors (3) without any additional factors.

B-cell lineage differentiation of iPS cells and ES cells

Schmitt *et al.* (11) previously showed that B cells can be differentiated from embryonic or haematopoietic stem cells by day 20 in OP9 co-culture system in the presence of Flt3L and IL-7. Thus, we anticipated that iPS cells could also be differentiated into B-cell lineage using the OP9 co-culture system with slight modification. In the first 5-day culture

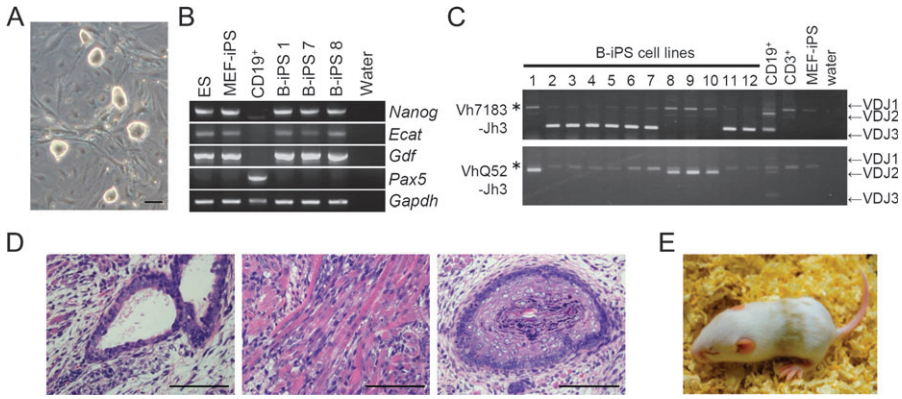

Fig. 1. Generation and characterization of B-iPS cells. (A) Morphology of iPS cells derived from mouse CD19$^+$ B cells. (B) ES cell marker expression of B-iPS cells. Transcripts from ES cells (R1), MEF-iPS cells, splenic CD19$^+$ B cells and three independently established B-iPS cell lines were analyzed by RT–PCR analysis. (C) Analysis of IgH V(D)J rearrangements in B-iPS cell lines. Genomic DNA was isolated from B-iPS cell line, splenic CD19$^+$ B cells, splenic CD3$^+$ T cells and MEF-iPS cells and analyzed by genomic PCR for the presence of IgH V(D)J gene rearrangements using primers as indicated. Asterisks indicate non-specific bands. (D) Histological analysis of teratoma from B-iPS cell line. Data of a B-iPS cell line 8 are shown. Thin-sectioned teratoma was stained by haematoxylin and eosin. Ciliated cell (endoderm, left), muscle fibre (mesoderm, centre) and dermal tissue (ectoderm, left) were shown. (E) Contribution of B-iPS cells to mouse embryonic development. B-iPS cells (line 8) were micro-injected in to blastocysts of ICR mice. Mice that have black- and white-coloured coat were born.

without LIF in a non-treated dish, embryonic body-like sphere formation was exhibited by B-iPS (Fig. 2A), MEF-iPS and ES cells (data not shown). These spheres contained mesoderm-like cells, which express Flk-1 (Fig. 2B). The spheres were then passed into OP9 feeder cells. By day 8 of the culture, cells similar to haematopoietic cells appeared (Fig. 2C). However, B-iPS and MEF-iPS cells only seldom showed B-cell lineage (CD19$^+$) differentiation (Fig. 3A), whereas ES cells and haematopoietic progenitor cells from foetal liver efficiently differentiated into a B-cell lineage (Fig. 3A and B). The MEF-derived iPS cell line (38C-2, established in Dr Yamanaka's laboratory, Kyoto University) occasionally expressed the B-cell marker CD19, but its frequency was very low compared with that of ES cells. We further tried the B-cell lineage differentiation from iPS cells by increasing the dose of cytokines, and by adding both vascular endothelial growth factor and bone morphogenetic protein-4, which were demonstrated to synergistically enhance lympho-haematopoietic cell generation (14), or LPS, which was shown to increase IgM$^+$-secreting B cells (15). However, these approaches did not enhance the differentiation of B-cell lineage in OP9 cells (data not shown). Therefore, even when B cells were used for the source of iPS cells, they are relatively resistant to differentiate into B-cell lineage with the current *in vitro* OP9 co-culture system. In addition, this co-culture did not induce differentiation of iPS cells into T-cell lineage that were traceable with the markers CD44, CD25, CD4 and CD8 (Fig. 3A).

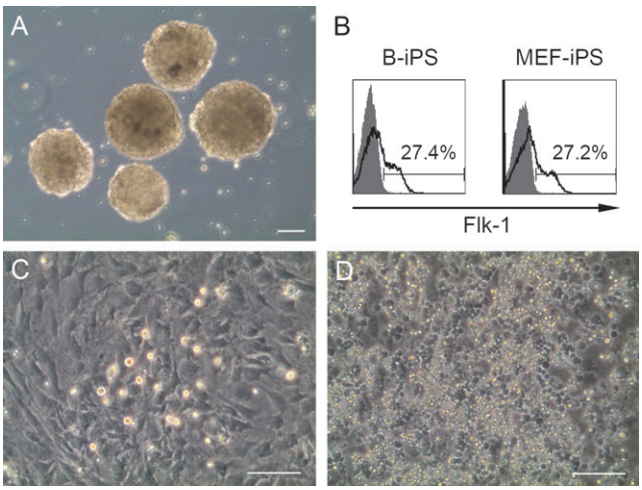

Fig. 2. Appearance of differentiating iPS cells. (A) Embryonic body-like spheres of B-iPS cells. B-iPS cells were cultured in differentiation media without LIF for 5 days. Scale bar: 100 μm. (B) Flow cytometry analysis of the embryonic body-like spheres. Single-cell suspension of the embryonic body-like sphere shown in (A) was examined for their expression of a mesoderm cell marker, Flk-1. (C) Hematopoietic cell formation. The single-cell suspension of embryonic body-like spheres was cultured on OP9 cells for another 3 days in the presence of Flt3 ligand. Scale bar: 100 μm. (D) Lymphocyte-like proliferation. At day 14, appearance of cells co-cultured with OP9-DL1 in the presence of Flt3 ligand and IL-7. Scale bar: 100 μm.

T-cell lineage differentiation of iPS cells

We further evaluated T-cell lineage differentiation of iPS cells *in vitro* using a co-culture system including OP9 cells expressing a Notch ligand, delta-like 1 (OP9-DL1), which has been shown to be essential for T-cell lineage differentiation (11). By way of embryonic body-like formation, B-iPS or MEF-iPS cells were co-cultured with OP9-DL1. By day 14, both iPS cell types turned into lymphocyte-like cells (Fig. 2D). On day 14, they gave rise to a population of cells expressing CD25 and/or CD44 and hence likely belonging to T-cell lineage resembling intrathymic differentiation (Fig. 4A). Rearrangement at the TCRβ locus (*Tcrβ*) is a hallmark of T-cell lineage commitment and is essential for progression of CD4/CD8 double-negative thymocytes to the double-positive stage, which occurs during normal αβT-cell development. To determine whether T cells derived from iPS cells co-cultured with OP9-DL1 cells undergo normal rearrangement

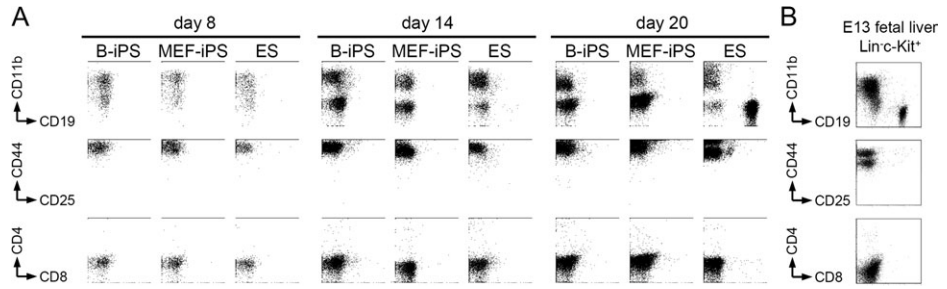

Fig. 3. Failure of B-cell lineage differentiation from iPS cells. (A) Flow cytometry analyses of B-iPS, MEF-iPS or ES cells differentiated with OP9 cells. On day 8, 14 and 20, expression of the indicated cell surface molecules on the differentiated cells were examined. (B) B-cell lineage differentiation of E13 fetal liver $Lin^{-}c\text{-}Kit^{+}$ cells on the OP9 monolayer. Flow cytometry was performed as in (A).

of the *Tcrβ* locus, we stained the differentiated cells collected on day 30 with various antibodies against TCRβ chain. The repertoire of TCRVβ in the generated T cells derived from B-iPS cells contained diversities as did those from MEF-iPS cells (Fig. 4B and data not shown). These results were confirmed by genomic PCR (Fig. 4C). Taken together, these data indicate that the iPS cell-derived T cells developed in the OP9-DL1 co-culture system have the potential to generate a diverse TCR repertoire.

During normal thymocyte development, T cells bearing both TCRαβ and TCRγδ develop in the thymus. To determine whether both populations of T cells also develop from iPS cells co-cultured with OP9-DL1 cells *in vitro*, we analyzed iPS cell-derived T cells for surface expression of TCRαβ and TCRγδ. These results showed that both populations of T cells developed from iPS cells in the co-culture model (data of B-iPS cell are shown; Fig. 4D).

iPS cell-derived T cells collected on day 20 and thereafter contained cells that were both CD4/CD8 double-positive and $CD8^{+}$ cells (Fig. 4A). To determine whether the TCRs expressed on these T cells were indeed functional, we stimulated the cells for 3 days in the plate-bound anti-CD3 antibody. Certain populations of the iPS cell-derived T cells produced IFN-γ in response to the TCR stimulation (Fig. 4E, gated on CD8). Furthermore, we added transforming growth factor-β to the culture of iPS cell-derived T cells in the presence of TCR stimulation. The addition of transforming growth factor-β enhanced the population of $FoxP3^{+}$ cells (Fig. 4F), which is the hallmark of regulatory T cells, as shown in naive T cells derived from normal adult lymphoid tissue (16). These data suggest that the iPS cell-derived T cells generated in this co-culture can respond to stimulation via TCR or cytokine receptors to a certain extent similar to naive T cells.

Analysis of gene expression in differentiating iPS cells

To elucidate the differentiation process of B-iPS cells at the molecular level, we assessed the expression of developmentally regulated genes by RT–PCR analysis. We analyzed the transcripts from whole cells of differentiating B-iPS, MEF-iPS and ES cells at day 20 of culture. A zinc finger transcription factor, *Ikaros*, and an Ets protein, *Pu.1*, both of which are known to be critical in regulation of haematopoiesis, were distinctly expressed in differentiating iPS cells co-cultured with either OP9 or OP9-DL1 (Fig. 5A and B, respectively). We also analyzed expression of the gene encoding the IL-7 receptor (*Il7r*), which is required for the survival and proliferation of lymphocyte progenitors. We found the *Il7r* transcripts in the differentiating cells from both conditions of T-and B-cell lineage differentiation (Fig. 5). Differentiating cells co-cultured with OP9-DL1 cells expressed substantial levels of Cd3, *Rag1* and *pTα*, which are essential for T-cell lineage development and were observed in normal thymocytes (Fig. 5B). These gene expression data are in agreement with the apparently normal development of T-cell lineage from iPS cells in OP9-DL1 co-culture (Fig. 4). In contrast, iPS cell-derived, but not bone marrow- or ES cell-derived, differentiating cells co-cultured with OP9 cells failed to express *Pax5*, which is critical for the development of B-cell lineage (Fig. 5A). Accordingly, transcripts encoding for Rag1, which is required for the B cell receptor (BCR) rearrangement, and for Igα, which is part of the pre-BCR complex, were not present in the cells differentiated from iPS cells co-cultured with OP9 cells (Fig. 5A). These data are in agreement with the fact that it was difficult to induce B-cell lineage differentiation using this co-culture system (Fig. 3). These data also suggest that the induction of T-cell, but not B-cell, lineage-specific gene expression in differentiating iPS cells was appropriately co-ordinated in these culture conditions. The immature regulated gene expression in iPS cells co-cultured with OP9 cells seemed to induce incomplete differentiation into B-cell lineage.

CpG methylation status of Pax5 promoter

Transcriptional silencing caused by DNA methylation of Pax5 promoter was observed in terminally differentiated B-cell lines (17). It was anticipated that such a CpG methylation in iPS cells caused the failure of *Pax5* expression. Thus, we analyzed the CpG sites in TATA-containing upstream promoter of *Pax5* in iPS cells. Bisulphite genomic sequencing analysis revealed that the *Pax5* promoter region in B-iPS and MEF-iPS cells were largely unmethylated as in splenic $CD19^{+}$ cells and ES cells (Fig. 6). It suggests that the failure of *Pax5* expression in iPS cells was not caused by the epigenetic modification of the *Pax5* gene.

Gene expression analysis in early stage of differentiation

To obtain insights about the failures of the B-cell lineage development from iPS cells, we further analyzed the B-lymphopoiesis in earlier stage of differentiation. On day 8 of

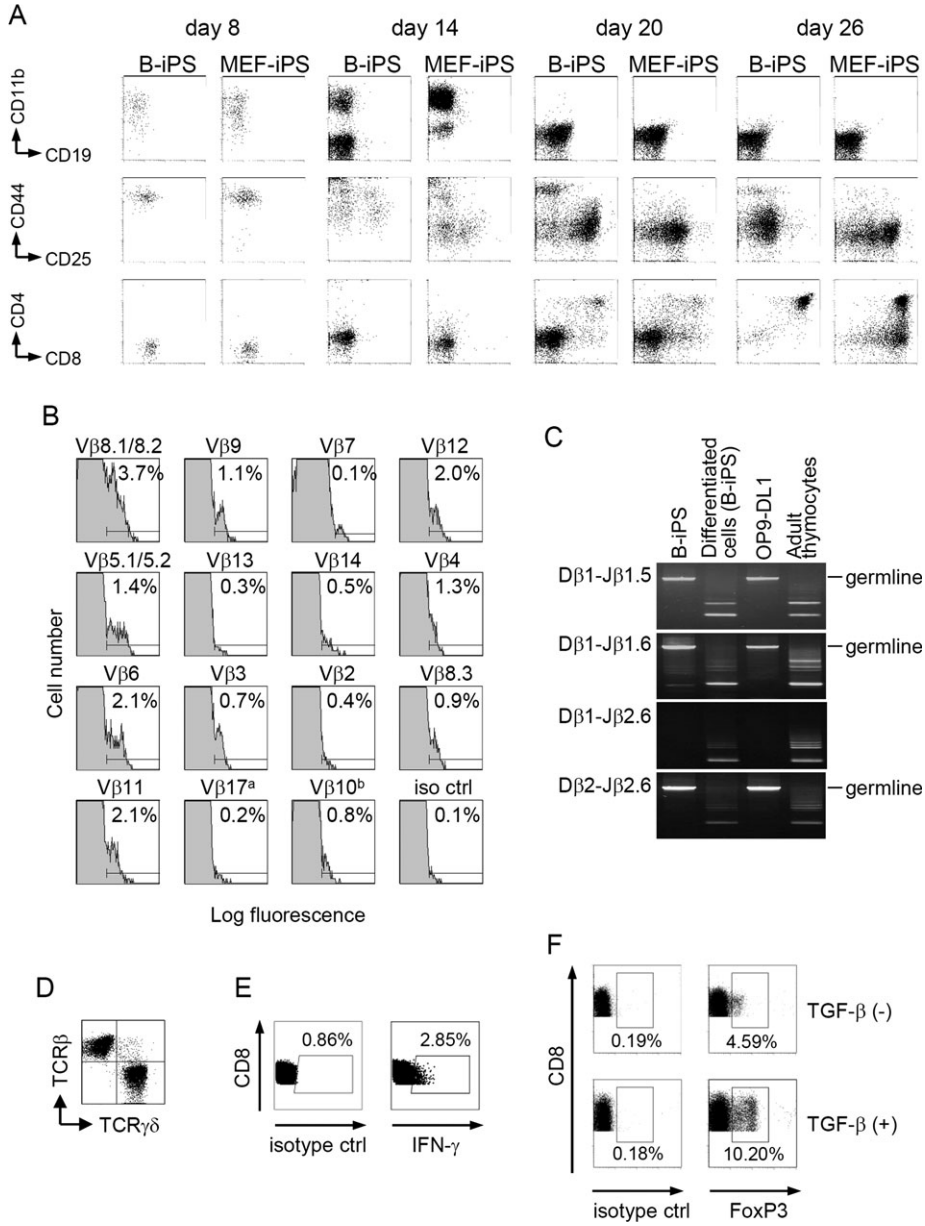

Fig. 4. Efficient T-cell lineage differentiation from iPS cells. (A) Flow cytometry analyses of iPS cells differentiated with OP9-DL1 cells. On day 8, 14, 20 and 26, expression of the indicated cell surface molecules on the differentiated cells were examined. (B) TCRVβ chain repertoire was analyzed with flow cytometry. The B-iPS cells differentiated with OP9-DL1 were examined at day 30 of culture. $CD3^+$ cells were gated. (C) Gene rearrangement analysis of *Tcr* locus. Genomic DNA was isolated from OP9-DL1, B-iPS cells (undifferentiated), B-iPS cells co-cultured on OP9-DL1 and mouse adult thymocytes as a control. *Tcr* gene rearrangement was analyzed by PCR. (D) αβT-cell and γδT-cell generation from B-iPS cells (day 30). $CD3^+$ cells were gated. (E) Intracellular analysis of IFN-γ secretion. Day 23 of the OP9-DL1 co-culture, generated cells from B-iPS cells were collected by vigorous pipetting through a 70-μm nylon mesh. Harvested cells were stimulated as described in Methods, and Golgi stop solution was put into the culture 6 h before the analysis. (F) Induction of $FoxP3^+$ regulatory T cells from B-iPS cell-derived T cells. Day 22 of the OP9-DL1 co-culture, differentiated cells were collected and put into the anti-CD3 mAb-coated well and cultured for another 2 days with 2 ng ml^{-1} of IL-2, with/without 5 ng ml^{-1} of transforming growth factor-β1. Intracellular FoxP3 expression was analyzed. $CD4^-CD8^+$ cells were shown.

the culture, losely attached cells were harvested and then c-kit^+ cells were magnetically sorted. Most of them expressed CD34 (data not shown), suggesting that these cells correspond to haematopoietic progenitor cells.

Ebf1 is one of the principle determinants of the B-cell fate (19, 20), and its promoter is activated by one of the E2A splicing variants, E47 (21). *E47* was detected in the differentiated cells derived from B-iPS, MEF-iPS or ES cells as well as bone marrow $linage^-$ c-kit^+ cells (Fig. 7A). mRNAs for Id proteins, which are known to bind to the E-proteins and prevent them from binding to DNA, as well as *Ebf1*, were also detected in all the cell types tested. On the other hand, *Pax5* expression was not detected in iPS cell-derived haematopoietic progenitor cells (Fig. 7A). In this experiment, the Pax5 expression was not

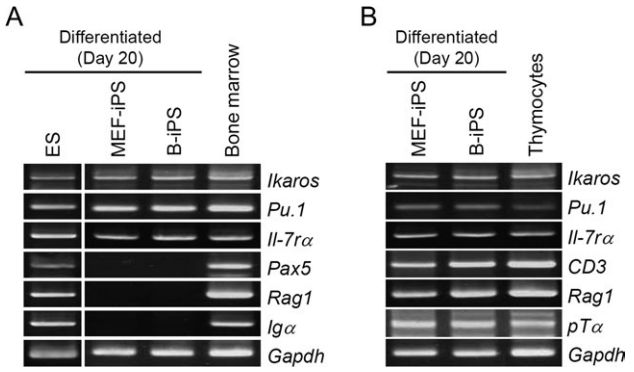

Fig. 5. Expression analysis of B- or T-cell lineage-correlated genes. B-iPS, MEF-iPS or ES cells were co-cultured on OP9 (A) or OP9-DL1 (B) for 20 days and transcripts analyzed from whole cells of differentiating B-iPS, MEF-iPS and ES cells. Mouse adult bone marrow (A) or thymocytes (B) were used as a control. The transcripts were analyzed by RT–PCR. Amount of transcripts between each samples were normalized by glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

detected in ES cell-derived cells either (Fig. 7A), but it clearly appeared in a later stage (see Fig. 5). Therefore, it seemed that, in the differentiated cells from iPS cells, the Pax5 expression was inhibited throughout the differentiation process.

Expression of the four Yamanaka factors in haematopoietic progenitors from iPS cells

We finally analyzed the expression of *Oct4*, *Sox2*, *Klf4* and *c-Myc* in the differentiated cells (Fig. 7B). *Klf4* and *c-Myc* expression were detected in the differentiated cells derived from B-iPS, MEF-iPS or ES cells as well as bone marrow linage$^-$ c-kit$^+$ cells. *Sox2* transcript was not detected in those cells. Importantly, *Oct4* transcripts were detected in B-iPS or MEF-iPS cell-derived haematopoietic progenitors and also slightly in the cells derived from ES cells. Detailed analysis using retrovirus vector-specific primers revealed that the *Oct4* transcripts in the cells derived from MEF-iPS or B-iPS cells were retrovirally transduced ones, but those in the cells derived from ES cells were endogenous (Fig. 7B). The ectopic *Oct4* expression seen in the cells differentiated from iPS cells may regulate the Pax5 expression and also B-cell lineage differentiation.

Discussion

In vitro culture methods are invaluable for defining specific cellular and genetic mechanisms that mediate lymphocyte development. It is widely known that ES cells can be differentiated into most blood cell lineages *in vitro* (22). The most commonly used method for inducing differentiation is the OP9 co-culture system. The OP9 cell line was established from calvariae of newborn *op/op* mice, which lack functional macrophage colony-stimulating factor (M-CSF) (23). OP9 cells enhance haematopoietic development by providing a supportive microenvironment for differentiation. The absence of M-CSF inhibits survival of monocytes and macrophages, which tend to outgrow other lineages in systems using wild-type stroma. Using this co-culture system, it has been well documented that ES cells can yield erythroid, myeloid and B-cell lineage cells (reviewed in ref. 22). However, it has been relatively difficult to generate T cells from unmanipulated ES cell-derived haematopoietic progenitor cells. Schmitt *et al.* (11) demonstrated an efficient induction of T cells from ES cells using OP9-DL1 cells that ectopically expressed the Notch ligand Delta-like 1. They showed that ES cells cultured with OP9-DL1 cells differentiated into haematopoietic cells, committed to T-cell lineage, underwent stage-specific proliferation and matured into CD4$^-$ and/or CD8$^+$ T cells *in vitro*. On the other hand, ES cells cultured on control OP9 cells differentiated into B-cell lineage as had been reported (11).

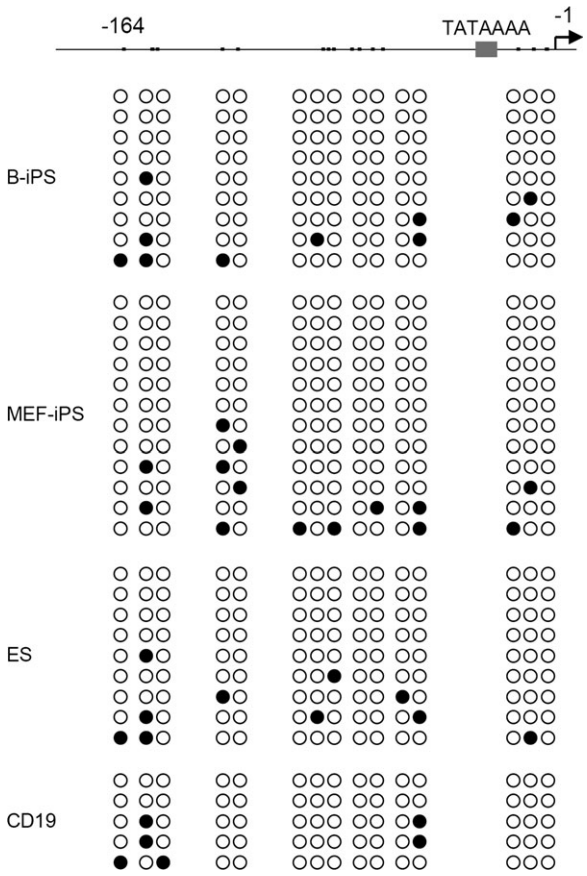

Fig. 6. Defect of *Pax5* expression was not due to CpG methylation of *Pax5* promoter. CpG methylation status of the *Pax5* promoter region containing 200 bp upstream from the transcription initiation site was analyzed by bisulphite genomic sequencing. Numbering at the top corresponds to the position relative to the published transcription initiation site (arrow) (18), and the filled small box indicates the position of the CpG site. Each row of circles represents a single cloned allele, and each circle represents a single CpG site (open circle, non-methylated cytosine; filled circle, methylated cytosine).

Using the above-mentioned OP9 co-culture system, in this study, we showed that iPS cells are competent to differentiate into T-cell lineage but are relatively resistant to differentiation into B-cell lineage *in vitro*. We used both MEF- and B-cell-derived iPS cells but failed to demonstrate reproducible B-cell lineage development *in vitro*. Analyses of gene expression data indicated that iPS cells are defective in expressing genes required for B cell, especially Pax5 throughout the differentiation process (Fig. 7), suggesting

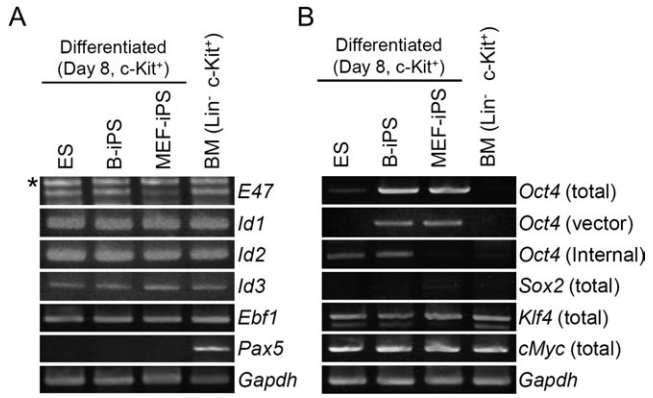

Fig. 7. Expression analysis in early stage of differentiation. B-iPS, MEF-iPS or ES cells were co-cultured on OP9 for 8 days, and c-kit$^+$ cells were harvested. Bone marrow lineage$^-$ c-kit$^+$ cells were used as a control. Transcripts for B-cell lineage development (A) and four Yamanaka factors (B) were analyzed by RT–PCR. Amount of transcripts between each samples was normalized by *Gapdh*. Asterisks indicate non-specific bands.

that there is no sufficient reprogramming in the genetic region of iPS cells that is required for B-cell development. However, in the case of Pax5 promoter region, it seemed unlikely that an aberrant CpG methylation induced some epigenetic modifications, which are responsible for the loss of Pax5 expression (Fig. 6). Alternatively, some transcriptional or translational changes in iPS cells may affect gene expression required for B-cell development but not for T-cell development. In the absence of Pax5, B-cell development is arrested at the early pro-B-cell stage of development (24), and *Ebf1* expression appears important to activate the B-cell lineage programme (25). Therefore, we tried to rescue the B-cell lineage differentiation by enforced expression of Pax5 or Ebf1 in differentiated cells from iPS cells by using retroviral vectors encoding *Ebf1* or *PAX5* that have been reported to induce the B-lymphopoiesis (kindly provided by Dr Ikawa, ref. 12). However, these attempts did not induce the B-cell lineage differentiation from iPS cells (data not shown), suggesting an existence of some active inhibitor(s) for B-cell differentiation in the differentiated cells from iPS cells. In this context, the detection of *Oct4* (including transduced one) (Fig. 7) may explain the resistance of B-cell lineage differentiation as it has been reported that the amount of Oct4 affects the development and differentiation of ES cells (26).

Although no precise explanation has been offered to account for the difficulty of B-cell lineage differentiation from iPS cells, similar differences in lymphocyte development potential between human embryonic and umbilical cord blood-derived progenitor cells have been reported (27). Using a co-culture system similar to those we employed, the authors found that ES cells could be used to efficiently create functional NK cells, whereas T- and B-cell development was much more limited. In contrast, umbilical cord blood stem cells routinely generated NK, T cells and B cells in the co-culture system. Accordingly, the authors found that ES cell-derived, but not umbilical cord blood-derived, haematopoietic progenitor cells constitutively expressed some transcriptional factors, including the ID family genes (27), which promote NK cell development and repress both T-cell and B-cell development, by inhibiting the E-protein family of basic helix-loop-helix transcription factors such as E2A (28–30). In the B-cell lineage differentiation form iPS cells, similar transcriptional mechanisms may operate which repress gene expressions required for the B-cell development. In fact, the transcripts of ID family genes were detected in haematopoietic progenitor cells from iPS cells (Fig. 7A). Further studies are needed to clarify these issues.

In this study, we successfully reprogrammed peripheral B cells into iPS cells. Most published protocols are optimized to reprogramme adherent cells, such as fibroblasts and keratinocytes from skin or hair (31–35). However, using these sources requires time-consuming skin biopsies and expansion *in vitro* for several passages, which make the method relatively cumbersome, particularly when generating patient-specific iPS cells. In this sense, it is desirable to reprogram blood cells that are easily accessible and less exposed to environmental mutagens.

Recently, iPS cell lines were derived from bone marrow progenitor cells obtained from a mouse whose haematopoiesis was reconstituted from a single congenic haematopoietic stem cell, providing evidence that mouse haematopoietic cells can be reprogrammed to pluripotency (36). Derivation of iPS cells from post-natal human blood cells has also been reported. One study indicated that granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood CD34$^+$ cells from a healthy donor were reprogrammed to iPS cells (37). More recently, it was reported that CD34$^+$ cells from human cord blood and adult bone marrow from healthy donors could be reprogrammed to iPS cells without pre-treatment, like G-CSF mobilization (38). Moreover, in this report, several iPS cell lines were established from peripheral blood CD34$^+$ cells containing the JAK2-V617F mutation that is commonly found in haematopoietic progenitor cells of adult patients with myeloproliferative disorders (38). However, these reports all employed haematopoietic progenitor or stem cells as the source of iPS cells, which usually involve some complicated procedures.

Lymphocytes have been used for the source of iPS cells previously. The first report indicating a reprogramming of mouse B lymphocytes to pluripotency proved that terminally differentiated somatic cells are receptive to being reprogrammed to iPS cells (39). In this report, it was indicated that only pro- and pre-B-lymphocytes were reprogrammed with the four factors, whereas mature B lymphocytes were reprogrammed by the additional over-expression of C/EBPα or specific knockdown of the Pax5 transcription factor (39). Eminli *et al.* (40) reported that terminally differentiated B and T lymphocytes could be reprogrammed with over-expression of the four Yamanaka factors, but the efficiency was quite low, with levels up to 300 times lower than haematopoietic stem and progenitor cells. In these studies, 'secondary' iPS cells were derived from primary B or T lymphocytes of adult spleen, bone marrow, lymph nodes or embryonic liver of mice engineered to carry doxycycline-inducible *Oct4*, *Sox2*, *Klf4* and *Myc* retroviruses in every tissue (39, 40). Similarly, Hong *et al.* (41) recently reported that murine splenic T lymphocytes could be reprogrammed to iPS cells although only when *p53*-null, but not wild-type, mice were used as the

source of lymphocytes. Therefore, it has been concluded that mature B and T lymphocytes are resistant to reprogramming with the four Yamanaka factors when no additional modification is made. Although the efficacy of B-iPS cells generation was quite low in our study, the fact that we generated iPS cells from peripheral B cells using the 'classical' method (3) might expedite new studies in which human peripheral B cells are examined for use as a source for iPS cells generation, largely because they are much more accessible.

Gaining a better understanding of differentiation of T or B lymphocytes from pluripotent stem cells *in vitro* can potentially guide development of new therapeutic strategies against some immunodeficiency diseases, infectious diseases or cancers in the field of regenerative medicine. Our present study highlights an important issue that applies to the derivation of virtually all cell types from iPS cells and not just B lymphocytes with *in vitro* co-culture systems. *In vitro* culture and differentiation of iPS cells leads to complex conditions and results in a heterogeneous mixture of progenitor cells that are stimulated by stromal cells and multiple soluble proteins that affect iPS cells lineage differentiation and development. Additional cellular and molecular studies of iPS cells are necessary to reveal unknown experimental and clinical potentials of these cells, particularly in terms of their tendency in lineage commitment.

Funding

Ministry of Education, Culture, Sports, Science and Technology of Japan to H.W. and K.S; Mitsubishi Pharma Research Foundation; Kanagawa Nanbyo Foundation; Uehara Memorial Foundation to H.W.

Acknowledgements

We thank Dr Toshio Kitamura for Plat-E cells and pMX retroviral vectors. The MEF-iPS cell line was originally established by Dr Shinya Yamanaka (Kyoto University). We thank Drs Shiari Nozawa (St Marianna University) and Akinori Nozawa (Yokohama City University Medical Centre) for histological characterization of teratoma and Hajime Kamijuku and Mikako Koike for their technical assistance.

Authorship contributions and disclosure: H. W. designed and performed experiments, and collected and analyzed data. S. K. analyzed and interpreted data. C. K. performed experiments and collected and analyzed data. N. O., Y. S. and B. I. contributed to blastocyst injections. K. S. designed research, interpreted data and wrote the manuscript.

The authors declare no competing financial interest.

References

1 Park, I. H., Zhao, R., West, J. A. *et al.* 2008. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141.
2 Takahashi, K., Tanabe, K., Ohnuki, M. *et al.* 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861.
3 Takahashi, K. and Yamanaka, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663.
4 Yu, J., Hu, K., Smuga-Otto, K. *et al.* 2009. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 324:797.
5 Okita, K., Ichisaka, T. and Yamanaka, S. 2007. Generation of germline-competent induced pluripotent stem cells. *Nature* 448:313.
6 Mauritz, C., Schwanke, K., Reppel, M. *et al.* 2008. Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation* 118:507.
7 Narazaki, G., Uosaki, H., Teranishi, M. *et al.* 2008. Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation* 118:498.
8 Schenke-Layland, K., Rhodes, K. E., Angelis, E. *et al.* 2008. Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and haematopoietic lineages. *Stem Cells* 26:1537.
9 Senju, S., Haruta, M., Matsunaga, Y. *et al.* 2009. Characterisation of dendritic cells and macrophages generated by directed differentiation from mouse induced pluripotent stem cells. *Stem Cells* 27:1021.
10 Lei, F., Haque, R., Weiler, L., Vrana, K. E. and Song, J. 2009. T lineage differentiation from induced pluripotent stem cells. *Cell Immunol.* 260:1.
11 Schmitt, T. M., de Pooter, R. F., Gronski, M. A., Cho, S. K., Ohashi, P. S. and Zuniga-Pflucker, J. C. 2004. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated *in vitro*. *Nat. Immunol.* 5:410.
12 Ikawa, T., Kawamoto, H., Wright, L. Y. and Murre, C. 2004. Long-term cultured E2A-deficient haematopoietic progenitor cells are pluripotent. *Immunity* 20:349.
13 Kawamoto, H., Ohmura, K., Fujimoto, S., Lu, M., Ikawa, T. and Katsura, Y. 2003. Extensive proliferation of T cell lineage-restricted progenitors in the thymus: an essential process for clonal expression of diverse T cell receptor beta chains. *Eur. J. Immunol.* 33:606.
14 Nakayama, N., Lee, J. and Chiu, L. 2000. Vascular endothelial growth factor synergistically enhances bone morphogenetic protein-4-dependent lymphohematopoietic cell generation from embryonic stem cells *in vitro*. *Blood* 95:2275.
15 Cho, S. K., Webber, T. D., Carlyle, J. R., Nakano, T., Lewis, S. M. and Zuniga-Pflucker, J. C. 1999. Functional characterization of B lymphocytes generated *in vitro* from embryonic stem cells. *Proc. Natl Acad. Sci. USA.* 96:9797.
16 Chen, W., Jin, W., Hardegen, N. *et al.* 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 198:1875.
17 Danbara, M., Kameyama, K., Higashihara, M. and Takagaki, Y. 2002. DNA methylation dominates transcriptional silencing of Pax5 in terminally differentiated B cell lines. *Mol. Immunol.* 38: 1161.
18 Busslinger, M., Klix, N., Pfeffer, P., Graninger, P. G. and Kozmik, Z. 1996. Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. *Proc. Natl Acad. Sci. USA.* 93: 6129.
19 Nutt, S. L. and Kee, B. L. 2007. The transcriptional regulation of B cell lineage commitment. *Immunity* 26:715.
20 Lukin, K., Fields, S., Hartley, J. and Hagman, J. 2008. Early B cell factor: regulator of B lineage specification and commitment. *Semin. Immunol.* 20:221.
21 Smith, E. M., Gisler, R. and Sigvardsson, M. 2002. Cloning and characterization of a promoter flanking the early B cell factor (EBF) gene indicates roles for E-proteins and autoregulation in the control of EBF expression. *J. Immunol.* 169:261.
22 Olsen, A. L., Stachura, D. L. and Weiss, M. J. 2006. Designer blood: creating hematopoietic lineages from embryonic stem cells. *Blood* 107:1265.
23 Nakano, T., Kodama, H. and Honjo, T. 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 265:1098.
24 Nutt, S. L., Urbanek, P., Rolink, A. and Busslinger, M. 1997. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between foetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev.* 11:476.
25 Zhang, Z., Cotta, C. V., Stephan, R. P., deGuzman, C. G. and Klug, C. A. 2003. Enforced expression of EBF in hematopoietic stem cells restricts lymphopoiesis to the B cell lineage. *EMBO J.* 22:4759.

26 Niwa, H., Miyazaki, J. and Smith, A. G. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* 24:372.
27 Martin, C. H., Woll, P. S., Ni, Z., Zuniga-Pflucker, J. C. and Kaufman, D. S. 2008. Differences in lymphocyte developmental potential between human embryonic stem cell and umbilical cord blood-derived hematopoietic progenitor cells. *Blood* 112:2730.
28 Jaleco, A. C., Stegmann, A. P. and Heemskerk, M. H. 1999. Genetic modification of human B-cell development: B-cell development is inhibited by the dominant negative helix loop helix factor Id3. *Blood* 94:2637.
29 Heemskerk, M. H., Blom, B., Nolan, G. *et al.* 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J. Exp. Med.* 186: 1597.
30 Ikawa, T., Kawamoto, H., Goldrath, A. W. and Murre, C. 2006. E proteins and Notch signaling cooperate to promote T cell lineage specification and commitment. *J. Exp. Med.* 203:1329.
31 Aasen, T., Raya, A., Barrero, M. J. *et al.* 2008. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat. Biotechnol.* 26:1276.
32 Dimos, J. T., Rodolfa, K. T., Niakan, K. K. *et al.* 2008. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 321:1218.
33 Lowry, W. E., Richter, L., Yachechko, R. *et al.* 2008. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc. Natl Acad. Sci. USA* 105:2883.
34 Mali, P., Ye, Z., Hommond, H. H. *et al.* 2008. Improved efficiency and pace of generating induced pluripotent stem cells from human adult and foetal fibroblasts. *Stem Cells* 26:1998.
35 Park, I. H., Arora, N., Huo, H. *et al.* 2008. Disease-specific induced pluripotent stem cells. *Cell* 134:877.
36 Okabe, M., Otsu, M., Ahn, D. H. *et al.* 2009. Definitive proof for direct reprogramming of hematopoietic cells to pluripotency. *Blood* 114:1764.
37 Loh, Y. H., Agarwal, S., Park, I. H. *et al.* 2009. Generation of induced pluripotent stem cells from human blood. *Blood* 113:5476.
38 Ye, Z., Zhan, H., Mali, P. *et al.* 2009. Human induced pluripotent stem cells from blood cells of healthy donors and patients with acquired blood disorders. *Blood* 114:5473.
39 Hanna, J., Markoulaki, S., Schorderet, P. *et al.* 2008. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell* 133:250.
40 Eminli, S., Foudi, A., Stadtfeld, M. *et al.* 2009. Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. *Nat. Genet.* 41:968.
41 Hong, H., Takahashi, K., Ichisaka, T. *et al.* 2009. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* 460:1132.