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Development of Feeder-Free Culture Systems for Generation of ckit+sca1+ Progenitors from Mouse iPS Cells

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Abstract

Patient-specific therapeutic cells derived from induced pluripotent stem (iPS) cells may bypass the ethical issues associated with embryonic stem (ES) cells and avoid potential immunological reactions associated with allogenic transplantation. It is critical, for the ultimate clinical applicability of iPS cell-derived therapies, to establish feeder-free cultures that ensure efficient differentiation of iPS cells into therapeutic progenitors. It is also necessary to understand if iPS cell-derived progenitors differ from those derived from ES cells. In this study, we compared the efficiency of three different feeder-free cultures for differentiating mouse iPS cells into ckit+sca1+ hematopoietic progenitor cells (HPCs) and compared how differentiation and functionality varies between ES and iPS cells. Our results indicated that both iPS and ES cells can be efficiently differentiated into HPCs in suspension cultures supplemented with secretion factors from mouse bone marrow stromal cells (OP9-DL1 conditioned medium). The functionality of these cells was demonstrated by differentiation into CD11c+ dendritic cells (DCs). Both ES and iPS-derived DCs expressed activation molecules (CD86, CD80) in response to LPS stimulation and stimulated T cell proliferation in a mixed lymphocyte reaction (MLR). Extensive quantitative RT-PCR studies were used to study the differences in gene expression profiles of ckit+sca1+ cells generated from the various culture systems as well as differences between ES-derived and iPS-derived cells. We conclude that a feeder-free system using stromal conditioned medium can efficiently generate HPCs as well as functional DCs from iPS cells and the generated cells have similar gene expression profile as those from ES cells.

Keywords

Induced pluripotent stem cells; iPS; ES cells; Feeder-free; Hematopoiesis; ckit; sca1; Gene expression

Introduction

ES cells are pluripotent cells that can self-renew indefinitely and be differentiated into virtually all types of cells in the three germ layers. With new advances in tissue engineering, therapies using ES cells are becoming clinical realities for treating several diseases, including certain cancers (e.g. leukemia, lymphoma, and myeloma) and immunodeficiency [1–3]. Recently, considerable efforts have been made to differentiate ES cells in vitro into hematopoietic stem cells [4] and further into blood cells including dendritic cells (DCs).

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Administration of murine ES-derived DCs in vivo demonstrated induction of anti-cancer immunity in mice [5–7]. These successes indicate that ES cells have the potential to become a scalable source of cells for DC-mediated immunotherapy [8]. Nevertheless, the unavailability of patient-specific human ES cells and the ethical concerns related to the use of human ES cells hinder the future clinical application of ES-derived DCs. Recent invention of induced pluripotent stem (iPS) cells may bypass this clinical obstacle [9].

iPS cells have been generated from a variety of human somatic and adult mice cells by ectopic expression of a small number of defined transcription factors [10–12]. These cells were demonstrated to be indistinguishable from ES cells in terms of DNA methylation, global gene expression, and more importantly, the development of viable chimaeras after being introduced into mouse blastocysts [10]. All these studies imply that iPS cells can be used as pluripotent starting material to substitute ES cells and generate lineage specific, therapeutic cell types [9]. In addition, the use of genetically-identical, patient-specific iPS cells derived from patients' own somatic cells can overcome immunological concerns associated with allogeneic or xenogeneic donor cells in clinical applications.

Recent efforts have focused on adapting ES cell-differentiation methods to iPS cells demonstrating that iPS cells can be differentiated into cells of the hematopoietic lineage [13]. Satoru et.al. reported generation of DCs in vitro from mouse iPS cells [14]. These iPS-derived DCs were functional and comparable to bone marrow-derived DCs in terms of T cell stimulation, antigen-processing and presentation, as well as cytokine production. In these studies iPS cells were differentiated into the hematopoietic lineage by co-culturing with a bone marrow stromal cell line (OP9) or its derivatives (e.g. notch-ligand transfected OP9 cells, OP9-DL1), which has been well established to support ES cell hematopoiesis and to generate lymphoid lineages [15, 16]. Despite significant advances, ultimate clinical application requires therapeutic cells to be free of stromal cell contamination, which is difficult to achieve through cell sorting techniques. Stromal cell-free differentiation cultures could provide a simple, scalable and clinically relevant process for stem cell-derived cells to be used in transplantation. Others have reported stromal cell-free suspension cultures to differentiate ES cells into various hematopoietic lineages [17, 18]. When culture conditions that maintain ES cells in the undifferentiated state are discontinued, the cells typically undergo spontaneous differentiation, with the formation of embryoid bodies (EBs). Under appropriate culture conditions, EBs can further differentiate to form primitive blood islands and can ultimately be stimulated to form each of the definitive hematopoietic lineages. In addition, recent reports have shown that ES cells do not have to be in close contact with stromal cells to give rise to lymphoid progenitors and hematopoietic precursors [19, 20]. Therefore, we hypothesized that hematopoietic stem or progenitor cells can be generated from iPS cells, by simply using OP9-DL1 conditioned medium which would prevent potential contamination from stromal cells while providing the necessary paracrine factors for iPS cell hematopoiesis.

In this study we compared the efficiency of three different feeder-free suspension culture systems for differentiating mouse iPS and ES cells into ckit+sca1+ hematopoietic progenitors (HPCs). The functionality of these HPCs was further investigated by differentiating them into the myeloid lineage, specifically to functional DCs. In addition, we evaluated the gene expression profile of HPCs generated from both iPS and ES cells under the three culture conditions by quantitative RT-PCR arrays. Our results indicate that both iPS and ES cells can be efficiently differentiated into HPCs in suspension cultures supplemented with secreted factors from mouse bone marrow stromal cells (OP9-DL1 conditioned medium). Functional CD11c+ DCs were obtained from both iPS and ES cells in all three culture conditions. Extensive gene expression analysis identified a number of

differentially expressed genes in HPCs generated from the feeder-free cultures but confirmed that ES and iPS-derived cells have similar gene expression profiles.

Materials and Methods

Cell Lines and Antibodies

R1 mouse ES cells [21] with 129/Sv $\times$ 129/Sv-CP back-ground were obtained from Dr. A. Nagy (Mount Sinai Hospital, Ontario, Canada). This cell line can also be purchased from American Type Culture Collection (ATCC; Manassas, VA). Mouse iPS cells were a gift from Dr. Kazutoshi Takahashi [22] in Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan. Murine embryonic fibroblasts (MEFs) were purchased from ATCC. Mytomicin C was purchased from Sigma Aldrich (St Louis, MO). Mouse bone marrow OP9 cells stably transfected with the Notch ligand DLL1 (OP9-DL1) [15] was a gift from Dr. J.C. Zuniga-Pflucker (Toronto, Canada). All antibodies for cell surface staining were purchased from BD Pharmingen (San Jose, CA). Granulocyte macrophage colony stimulating factor (GM-CSF) and recombinant mouse interleukin (IL)-3 for DC differentiation were purchased from Peprotech (London, U.K.). Lypopolysaccharide (LPS) from *Escherichia Coli* was purchased from Sigma-Aldrich (St. Louis, MO).

ES and iPS Cell Maintenance

R1 mouse ES cells and mouse iPS cells were maintained in an undifferentiated state by culturing on mitomycin C-inactivated MEF cells. ES and iPS cells were expanded in Knockout DMEM (Invitrogen, Carlsbad, CA) medium containing 15% ES cell-screened fetal bovine serum (defined FBS, Hyclone, Thermo Fisher Scientific Inc., Waltham, MA), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 100 U/mL penicillin G with 10 mg/mL streptomycin (Invitrogen), and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich). In addition, Leukemia Inhibitory Factor (ESGRO® LIF, Millipore, Temecula, CA) was included in the expansion medium at 1000 U/ml to maintain the undifferentiated status of ES and iPS cells. One passage prior to the differentiation culture, ES and iPS cells were seeded in gelatinized flasks without feeder cells and cultured using a pre-differentiation medium of IMDM (Invitrogen) containing 15% FBS (defined, Hyclone), 5×10^{-5} M 2-mercaptoethanol, 1000 U/ml LIF, 100 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids, 100 U/mL penicillin G, and 10 mg/mL streptomycin.

Cell Culture Media

IMDM complete medium (IMDM): IMDM (Invitrogen) with 15% FBS (ES-Cult for hematopoietic differentiation, StemCell Technologies, Vancouver, BC), 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich), 0.1 mM non-essential amino acids, 100 U/mL penicillin G, and 10 mg/mL streptomycin (all from Invitrogen).

Conditioned medium (CM): To prepare the conditioned medium, OP9-DL1 cells were cultured using minimum essential medium alpha (α -MEM) supplemented with 20% FBS (ES-Cult for hematopoietic differentiation, StemCell Technologies, Vancouver, BC), 2.2 g of sodium bicarbonate and 10 mg/mL streptomycin (Invitrogen). OP9-DL1 cells were grown until confluency in T150 tissue culture flasks and 24 ml of fresh medium was added every day (for 3 days). The conditioned medium was collected after 18–20 h and filtered using a 0.2 μ m filter.

IMDM+CM: The OP9-DL1 conditioned medium (CM) was concentrated 10 times by ultrafiltration using an Amicon centrifugal device (3 kDa; Millipore) and the retentate (1.0 ml) was diluted back to the initial volume with 9.0 ml of IMDM complete medium (IMDM).

ES and iPS Cell Differentiation under Various Suspension Culture Conditions

Upon reaching confluence, undifferentiated ES and iPS cells were harvested and cultured without LIF to initiate EB formation and differentiation. 5×10^5 cells were seeded as a suspension in a 100 mm low-attachment dish (Ultra Low Attachment dish, Corning Incorporated, Corning, NY) using 10 mL of the three different culture media described above: IMDM, CM and IMDM+CM. On day 7 of differentiation, ES and iPS cell growth (number of EBs formed) and hematopoietic differentiation (percentage of ckit+Sca-1+ cells by flow cytometry) were evaluated. The size and amount of EBs in each sample was determined by removing 200 μ l of differentiation culture volume for light microscopic imaging (EVOS microscope, Westover Scientific's Advanced Microscopy Group, Mill Creek, WA). EBs larger than 100 μ m diameter were counted.

ES and iPS Cell-Derived Dendritic Cells (DCs)

To direct the differentiation of ES and iPS cells into DCs, we followed previously reported DC generation protocol [23] with some modifications. In summary, after ES and iPS cells were differentiated for 14 days in suspension cultures, EBs were harvested from all the different culture conditions allowing them to settle in a 50-ml conical tube. Approximately 20–30 EBs were plated into 90-mm tissue culture dishes in 15 ml of differentiation medium (IMDM differentiation medium previously described supplemented with 25 ng/ml of rm-IL-3 and 25 ng/ml of rmGM-CSF). The dishes were incubated at 37°C, 5% CO₂ for 6 days. The medium was replaced at day 4 with fresh cytokines.

On day 6, lightly adherent DCs were harvested from the dishes and re-suspended in PBS+2 mM EDTA. These cells were transferred to a 24-well plate to induce activation in the presence of LPS at a final concentration of 1 μ g/ml in the same differentiation medium with GM-CSF and IL-3. After 2 days cells were collected and stained with different DC phenotype and activation markers (CD11c, CD86 and CD80) for flow cytometry. DC functionality was also evaluated by measuring T cell proliferation in a mixed lymphocyte reaction (MLR) assay as described below.

Generation of DCs from Mouse Bone Marrow Cells

As a control, DCs from bone marrow (BM-DCs) were generated following a previously described protocol [24]. Briefly, bone marrow cells were isolated from C57BL/6 mice and cultured in 24-well plates (0.5×10^6 cells/well) in RPMI-1640 medium supplemented with 10% FCS in the presence of recombinant murine GM-CSF and IL-4 (both used at 20 ng/ml; Peprotech). Half of the culture medium was changed on day 3 and day 5. The floating cells were harvested by pipetting at day 6 for LPS stimulation as described above. After 2 days, cells were collected, stained and assayed by flow cytometry for CD11c, CD80 and CD86, similar to iPS- and ES-derived DCs.

Mixed Lymphocyte Reaction

To evaluate the capacity of conditioned medium-generated DCs to induce T cell proliferation in vitro, either 1×10^5 or 2×10^5 ES and iPS cell -generated DCs were incubated for 72 h with 2×10^5 CFSE-labeled CD4⁺ T cells (1:1, 1:2 DC: T cell). CD4⁺ T cells were isolated from Balb/c splenocytes using CD4 magnetic beads (Miltenyi Biotech). The resultant CD4⁺ population was re-suspended in PBS and stained with Vybrant CFDA SE Tracer kit (Molecular Probes). Cultures were performed in triplicate in 96-well-flat bottom plates in a total volume of 200 μ l IMDM complete medium. Cell division was monitored on the basis of CFSE dilution by flow cytometry.

Flow Cytometry

EBs were collected at day 7 of differentiation, and single cell suspension was attained by incubating them in Accumax (Innovative Cell Tech Inc., San Diego, CA) at 37°C for 1–2 h followed by filtering through a 40 µm nylon mesh. Cells were washed twice in FACS buffer (2% bovine serum albumin in HBSS) before staining and blocked for non-specific binding using anti-mouse CD16/CD32 Fc Block for 10 min at 4°C. Antibody stainings were performed in FACS buffer. Cell samples were analyzed using FACSCalibur (BD Biosciences, San Jose, CA). Live cells were distinguished from dead cells by gating using the forward and side scatter signals as well as by exclusion of 7-amino actinomycin D (eBioscience, USA) or propidium iodide (Invitrogen) stained cells. Non-stained cells or appropriate isotype control-stained cells were used to evaluate background fluorescence. All FACS data were analyzed with FlowJo (Version 7.2.5; Tree Star, Ashland, OR).

Real-Time PCR Array and Statistical Analyses

The RT² Profiler™ PCR Array System (SA Biosciences, Frederick, MD) with the mouse Stem Cell Array (PAMM-405) was used to assay global gene expression from EBs. Total RNA was isolated by using the RNeasy kit followed by on-column DNA digestion (Qiagen, Valencia, CA). Complementary DNA was synthesized from 1 µg of total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), and real-time PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using the SYBR green technology-based SuperArray RT² qPCR Master Mix (SA Biosciences). The $\Delta\Delta CT$ method of quantification was used to assess the fold change of individual genes (normalized to housekeeping genes) compared to undifferentiated iPS and ES cells. Fold change of gene expression was imported into ArrayStar™ (DNASTAR Inc., Madison, WI) and MeV microarray analysis software [25, 26]. Within MeV, one-way ANOVA was used to compare the different culture conditions for iPS and ES cells to produce a heat map with only those genes that showed statistically significant expression ($p < 0.01$). The changes in gene expression among the three different culture conditions for iPS and ES cells at day 7 and day 14 of differentiation were evaluated using the ArrayStar software ($p < 0.05$ was considered statistically significant). The data is represented as fold change of gene expression levels (log2) in each culture condition compared to control (undifferentiated cells) with error bars representing the standard error for each sample.

Results

Differentiation of iPS and ES Cells into ckit+sca1+ HPCs under Various Feeder-Free Conditions

As shown in Fig. 1a and b, after 7 days of differentiation, a significantly higher percentage of ckit+sca1+ cells was observed when either ES or iPS cells were cultured in OP9-DL1 conditioned medium (CM) as compared to IMDM-only medium. However, a significantly lower number of EBs were generated in CM than in IMDM (Fig. 1c) making the efficiency of ckit+sca1+ production (i.e. total number of ckit+sca1+ cells generated) in CM similar to that in IMDM-only medium. We suspected that OP9-DL1 CM favored hematopoietic differentiation, while IMDM medium stimulated cell expansion. Thus, we tested the combination of the differentiation and expansion effects from the two suspension cultures. We observed that the percentage of ckit+sca1+ cells generated from iPS/ES cells in IMDM medium supplemented with OP9-DL1 secretion factors (IMDM+CM) was lower than that in OP9-DL1 CM but higher than that in IMDM medium (Fig. 1a, b) indicating a balance between differentiation and proliferation. The number of EBs generated in IMDM+CM, nevertheless, was similar to that in IMDM alone (Fig. 1c). Therefore the total number of ckit+sca1+ cells generated from iPS/ES cells were significantly higher in IMDM+CM condition compared to that in IMDM or CM alone conditions (Fig. 1d).

Generation of Functional DCs from iPS and ES Cells under Various Culture Conditions

We examined the DC-differentiation potential of day14 EBs generated from mouse ES and iPS cells under different stroma-free culture conditions. Undifferentiated iPS and ES cells were differentiated for 14 days in three different suspension cultures (IMDM, IMDM+CM and CM) as previously described. Day 14 iPS- and ES cell-EBs from each culture condition were plated on tissue culture dishes and induced to differentiate into DCs using recombinant murine GM-CSF and IL-3. DC-like cell morphologies were visible at day 4 of culture for all three conditions, accumulating around the edge of the EBs (data not shown). On day 6 of differentiation, the floating and loosely attached cells with typical dendritic morphology were collected and dissociated into single cells. Subsequently, these cells were transferred into a new plate to induce activation in the presence of LPS. Flow cytometry analysis demonstrated the presence of CD11c+CD80+ and CD11c+ CD86+ populations in both ES-derived DCs and iPS-derived DCs (Fig. 2a, b). These cells were comparable in marker expression to BM-DCs (Fig. 2c).

To evaluate the functionality of iPS- and ES-derived DCs from feeder free cultures, we examined their capacity to stimulate T cells in a mixed lymphocyte reaction (MLR) assay. We used splenic CFSE-labeled CD4+ T cells, isolated from Balb/c mice, as allogenic responders. As shown in Fig. 2d, at 1:1 DCs to T cell ratio, iPS-derived DCs as well as ES-derived DCs are both capable of stimulating T cell proliferation, as measured by CFSE dilution. Similar results were obtained at 1:2 DC to T cell ratio (data not shown). In summary, we see a similar pattern of DC development from both iPS and ES cells differentiated in IMDM, IMDM+CM or CM. We conclude that feeder-free iPS-derived DCs are functionally similar to ES-derived DCs in stimulating T cell proliferation.

Gene Expression Profiles of ES- and iPS-Derived Cells

The differences between three different feeder-free culture conditions (IMDM+CM, IMDM and CM) for both iPS and ES cell differentiation were assessed using gene expression analysis. The global gene expression profile of EBs at day 7 and day 14 of differentiation from each culture was analyzed using the Mouse Stem Cell RT² Profiler™ PCR array. Of the 84 genes on the array, 34 genes for iPS cell differentiation and 30 genes for ES cell differentiation exhibited significant differences ($p < 0.01$) in expression levels between the various culture conditions at day 7 (Fig. 3a, b). As expected, the number of genes that showed significant differences ($p < 0.01$) across all culture conditions increased at day 14 indicating further progression to differentiation. (40 genes were significantly differed in iPS cell differentiation and 39 genes in ES cell differentiation) (Fig. 3c, d). Hierarchical clustering (HCL) of the data showed that biological replicates (-1,-2,-3) from each culture condition grouped together, indicating that experimental results were consistent across three independent experiments. We also observed that IMDM and IMDM+CM culture conditions grouped together and separated from CM, indicating that they are more similar to each other than to CM. This was consistently observed at day 7 and at day 14 for both iPS and ES cells. HCL displayed a clear grouping of statistically significant down and up-regulated genes (one-way ANOVA $p < 0.01$) across all three different culture conditions and both cell types at day 7 and at day 14 (Fig. 3). There was an increased expression of differentiation markers of all three germ layers across all culture conditions. For example, the expression of *Actc1*, a cardiac muscle alpha actin, Keratin 15 (*Krt15*), islet 1 (*Isl1*), the pancreatic and duodenal homeobox 1 (*Pdx1*) and the CD8 receptor alpha (*CD8a*) were significantly up-regulated at day 7 in all EB cultures (one-way ANOVA, $p < 0.01$). Overall, the expression of cytokines, growth factors and genes regulating cell-cell contact was increased at day 7 compared to undifferentiated cells and was further increased at day 14. Most of these genes were also differentially expressed across all three culture conditions. *Igf1*, *Gjb1* and *Fgf3* were up-regulated in all culture conditions, but their expression was higher in CM, compared to

IMDM and IMDM+CM, where it was similar. On the other hand, each of the day 7 and day 14EB groups exhibited a decrease in *Brachyury T* expression, a transiently expressed marker of mesoderm. Interestingly, this expression was lower in IMDM+CM and IMDM culture conditions compared to CM.

The expression of blood lineage markers during iPS and ES cell differentiation in three culture conditions (both days 7 and 14) are shown in Figs. 4 and 5. In general, across all conditions, we observed high expression of hematopoietic markers at day 7 which increased further on day 14 (Figs. 4a and 5a). At day 7, the expression of most hematopoietic markers was higher in IMDM+CM as compared to IMDM or to CM conditions. *CD8a* showed the highest expression consistently in all culture conditions. *CD8b1* expression in IMDM cultures differed between iPS and ES at day 7, showing a ~5-fold increase in iPS and an ~2-fold decrease in ES cells when compared to undifferentiated cells. *CD19* expression was only observed under IMDM and IMDM+CM conditions, increasing in both iPS and ES cells at day 14. The T-cell surface glycoprotein CD3 delta chain (*CD3d*) was expressed in all culture conditions for iPS and ES. However, its expression was higher in IMDM+CM and increased at day 14. *CD4* expression was very low or down-regulated in all conditions at day 7 and 14 of iPS differentiation. In contrast, during ES cell differentiation *CD4* expression was detectable at day 7 and increased at day 14.

The expression of mesenchymal and neuronal markers differed between iPS and ES cell differentiation. As shown in Figs. 4b, c and 5b, c, pro-collagens (*Col2a1* and *Col1a1*) were differentially expressed during iPS and ES cell differentiation as well as among the different culture conditions. *Col2a1* was highly expressed during both iPS and ES cell differentiation. However, its expression was significantly higher in IMDM and IMDM+CM than in CM cultures. On the other hand, *Col1a1* expression was low in iPS-derived cells, but high in ES-derived cells when compared to undifferentiated cells. This was consistently observed for all culture conditions. Similarly, *Pparg*, a peroxisome proliferator-activated receptor gamma, was down-regulated for iPS and up-regulated during ES cell differentiation. The other two mesenchymal markers shown in Figs. 4b and 5b (*Acan*, a proteoglycan important in cartilage formation, and *Bglap1*, a non-collagenous molecule important in bone-building) were highly down-regulated in iPS-derived cells at day 7, but their expression increased at day 14. A similar pattern was observed during ES cell differentiation, although the expression level of *Bglap1* was significantly higher in CM than in IMDM or IMDM+CM culture conditions. Among the neuronal markers examined, CD44 and S100b were differentially expressed during iPS and ES cell differentiation. CD44 was down-regulated in iPS and up-regulated in ES-derived cells across all culture conditions at day 7 and day 14. S100b was down-regulated during IMDM and IMDM+CM cultures (both iPS and ES cell cultures) but up-regulated in iPS CM cultures both at day 7 and day 14. Ncam1 was expressed in all culture conditions, but its expression was lower in iPS-derived than in ES-derived cell (Figs. 4c and 5c).

Lastly, we looked at the expression of the Notch-signaling pathway markers during iPS and ES cell differentiation. It is well recognized that the Notch cascade, consisting of Notch and Notch ligands, is a critical regulator of multiple differentiation processes during embryonic and adult life, including hematopoiesis. As shown in Figs. 4d and 5d, a similar gene expression pattern was observed for iPS and ES cell differentiation. The expression of two Notch-ligands, Dll1 and Dll3, was down-regulated in all culture conditions at day 7. Interestingly, Dll1 expression increased at day 14 only during iPS cell differentiation. However, Jagged 1, another Notch ligand, as well as the Notch receptors, Notch 1 and Notch2, were expressed in all the conditions for iPS, as well as ES cell differentiation.

Discussion

iPS cells have the clinical potential to achieve patient-specific cell therapies without the concern of ethics or immune rejection. We have developed a simple stromal cell-free culture condition for generating hematopoietic progenitors in vitro from iPS cells by supplementing OP9-secreted factors into IMDM medium. Such an approach is essential for removing potentially contaminating stromal cells that could compromise clinical translation of iPS cell therapy. A stroma-free approach could also enable large scale dynamic culture to cultivate large number of progenitor cells required for human applications [27].

Although all three culture conditions studied here favored iPS differentiation, the combination of conditioned medium (CM) and IMDM achieved maximal number of ckit+sca1+ cells. Previous studies have established that Stem Cell Factor (SCF) and Interleukin-7 (IL-7), both secreted by OP9 cells, are essential for hematopoiesis and HPC survival [20, 28, 29]. Therefore, the presence of components that favor both proliferation (IMDM) and differentiation (CM) likely increased the efficiency of hematopoiesis as compared to only proliferation stimulating factors or differentiation stimulating factors. However, it is imperative to take into consideration that the observed increase in differentiation in CM and IMDM+CM cultures could be due not only to OP9 secreted factors, but also to the alpha-MEM medium that is used to culture OP9 cells. As we addressed in Materials and Methods, to prepare the IMDM+CM condition we concentrated the OP9CM medium (alpha-MEM based) 10 times and diluted this concentrate back to its initial volume using IMDM medium. Therefore, there are three components, IMDM, OP9-secreted factors and alpha-MEM, which could be responsible for making IMDM+CM a better culture medium, in terms of HPC generation.

Several studies have concluded that iPS cells possess all features of ES cells and are indistinguishable from ES cells in their pluripotent potential [10, 22]. In our study, the hematopoietic differentiation capacity of iPS cells was compared directly to ES cells, since most hematopoietic differentiation methods have been established for ES cells. There was no significant difference between iPS and ES cells in their ability to generate c-kit+Sca-1+ HPCs. Further, we used the method described by Fairchild and his colleagues [23] to determine whether HPCs generated from stroma-free cultures of iPS and ES cells could be further differentiated into functional DCs. EBs formed in the three stroma-free culture conditions were harvested at day 14, and further differentiated in the presence of GM-CSF and IL-3. There were no significant differences in DC generation efficiency between the culture conditions. The in-vitro expanded cells from day14 EBs formed adherent cell clusters, resembling those observed in cultures of bone marrow-derived DCs. However, the ES- or iPS-derived cells showed higher attachment to the plate than the loosely attached cells from bone marrow cultures. This could be a reason why the number of DCs obtained from each culture was relatively low. It has been suggested by others [24] that the differentiation of these cells in bacterial-quality Petri dishes could help to reduce attachment which inhibits DC generation. Nevertheless, we were able to obtain enough DCs to demonstrate the functionality of the iPS- and ES-derived cells in all three culture conditions.

Flow cytometric analysis of the ES- and iPS-derived cultures revealed a population of CD11c+ cells that expressed CD80 and CD86 following LPS stimulation in a similar pattern but at a lower level than bone marrow derived-DCs. In addition, upon co-culture with allogeneic CD4+ T cells, these DCs stimulated T cell proliferation, indicating that they are functional. Previous studies have reported generation of functional DCs from mouse iPS and ES cells by using OP9 co-cultures [24]. The DCs generated from our protocol seems to be similar, based on the morphology, surface phenotype and function. The generation of DCs from human ES cells and more recently from iPS cells has also been reported [9, 30, 31]. In

a recent study, it was shown that human ES cells could be differentiated into immunostimulatory dendritic cells under feeder-free suspension cultures [30]. Similarly to our work, day 14 EBs were used to generate functional DCs. These human ES-cells derived DCs showed migratory cytokine secretion and allo-stimulatory properties comparable to monocyte-derived DCs.

One of the most informative results of this work is provided by the gene expression analysis. iPS and ES cells differed significantly in the expression profiles of several genes, both at day 7 and 14. These differences could be the result of differences in the kinetics of the differentiation process between iPS and ES cells [13]. It is also possible that the observed differences could be a result of epigenetic memory in iPS cells that favor their differentiation along specific lineages, especially those related to their tissue of origin [32, 33]. However, the iPS cell line used here was generated from mouse embryonic fibroblasts and recent studies show that embryonic tissues are more efficiently reprogrammed than adult tissues [32, 33].

Although there are genes that were significantly differentially expressed between iPS and ES cells, the overall gene expression patterns for both cell types were similar at days 7 and 14 of differentiation in each culture condition. The gene expression analysis also confirmed that iPS, as well as ES cells, can be differentiated into all three germ layers using the stroma-free suspension cultures. As expected, the expression of differentially expressed genes increased at day 14, indicating further differentiation into hematopoietic, neuronal or mesenchymal lineages. Consistent with our results on the number of generated c-kit+/Sca-1+ HPCs, we observed that the expression of several hematopoiesis-related markers were higher in IMDM+CM cultures as compared to IMDM and CM. As expected, there was a decrease in *Brachyury T* expression at days 7 and 14. This is a transiently expressed marker of mesoderm that has been reported to be maximal at day 4, but decreases at day 7 and later periods of ES cell differentiation [27, 34]. Furthermore, the Notch receptors Notch 1 and Notch 2, key components of the notch-signaling pathway were expressed under all conditions. The Notch signaling pathway plays an important role in hematopoiesis and allows maintenance of HPCs [20, 35]. However, the Notch ligands Dll1 and Dll3 were both down-regulated. It is possible that the strength of notch signaling is maintained by dynamically controlling the ligand-receptor ratio in these cultures and should be studied in further details.

It could be possible, in the future, to generate large scale therapeutic cells that are genetically matched to a patient by using iPS cells de-differentiated from relatively small numbers of donor somatic cells. Stromal cells-free cultures could provide the means to produce therapeutic cells from iPS cells in large scale. Future work needs to define the secretory factors from OP9 cells and develop a serum-free medium with these factors.

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Fig. 1.

Hematopoietic differentiation of iPS and ES cells in various stroma-free cultures. iPS and ES cells were differentiated through EB formation in three different suspension culture conditions: IMDM medium alone (IMDM); OP9-DL1 conditioned medium contains secreted factors (CM); IMDM medium supplemented with CM from OP9-DL1 (IMDM+CM). All data shown are day 7 of iPS and ES cell differentiation culture. **a** Representative percentage of HPCs (ckit+sca1+ cells) in total live cells of EB culture. **b** Percentage of HPCs from 3 representative experiments. Statistical analysis was done by one-way ANOVA comparing three different culture conditions. * $p < 0.01$. **c** EB counts in each culture condition at day 7 of differentiation. Statistical analysis was done by student *T* test (IMDM vs CM; IMDM+CM vs CM). * $p < 0.05$ **d** Total number of ckit+sca1+ cells from various culture conditions. The numbers were calculated by multiplying the percentage of ckit+sca1+ cells to the total live cells recovered. Statistical analysis was done by one-way ANOVA. * $p < 0.01$. All error bars shown are standard deviations

Fig. 2. Dendritic cell generation from iPS and ES cells. Surface phenotype of iPS (**a**) and ES (**b**) cell-derived DCs in three different culture conditions (IMDM, IMDM+CM, CM). Percentages in double positive quadrants represent CD11c+cells expressing CD86 or CD80. Quadrants were established using isotype control antibodies. **c** Surface phenotype of mouse bone marrow (BM) derived DC (BM-DCs). **d** In vitro T-cell proliferation following activation with iPS or ES-derived DCs. The response of CFSE-labeled CD4+ T cells to allogenic stimulation from iPS or ES-derived DCs was measured by CFSE dilution. Results shown in the histogram are percentages of proliferating T cells (1:1 DC to T cell ratio). The filled-histogram represents un-stimulated CFSE-labeled T cells

Fig. 3.

Gene expression profiles of EBs from three different suspension cultures (CM, IMDM+CM, IMDM) at day 7 (**a**, **b**) and day 14 (**c**, **d**) of iPS and ES cells differentiation. Hierarchical clustering represents genes that are up-regulated (red) and down-regulated (green), compared to undifferentiated cells, in the various culture conditions (One-way ANOVA, $p < 0.01$) at day 7 (**a** (ES), **b** (iPS)) and day 14 (**c** (ES), **d** (iPS)) of differentiation. -1, -2 and -3 are biological replicates for each culture condition

Fig. 4. Expression profile of specific genes differentially regulated during iPS cell differentiation in three different culture conditions (IMDM, IMDM+CM, CM) at day 7 and day 14. Bar graphs represent fold change (log2) of gene expression level compared to undifferentiated iPS cells ($n=3$). **a** Hematopoietic, **b** Mesenchymal, **c** Neuronal and **d** Notch-signaling pathway markers. Error bars represent standard error for each sample ($n=3$)

Fig. 5. Expression profile of specific genes differentially regulated during ES cell differentiation in three different culture conditions (IMDM, IMDM+CM, CM) at day 7 and day 14. Bar graphs represent fold change (log2) of gene expression level compared to undifferentiated ES cells ($n=3$). **a** Hematopoietic, **b** Mesenchymal, **c** Neuronal and **d** Notch-signaling pathway markers. Error bars represent standard error for each sample ($n=3$)