Single OCT4 gene introduced into human bone-marrow derived mesenchymal stromal cells can generate putative iPS cells with the potential to differentiate into CD34+ hematopoietic progenitor cells ex vivo

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Research

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Title Page

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Single OCT4 gene introduced into human bone-marrow derived mesenchymal stromal cells can generate putative iPS cells with the potential to differentiate into CD34+ hematopoietic progenitor cells ex vivo

Abstract

Background: The ex vivo production of CD34+ hematopoietic progenitor cells from human bone-marrow mesenchymal stromal cells derived induced pluipotent stem cells (iPSCs) could serve as a feasible way to study patient-specific hematological disease from the perspective of hematopoietic differentiation. Different studies using virus-based or virus-free methods to reprogramming somatic cells into iPSCs by using fewer than four transcription factors, of which have the potential to differentiation in CD34+ hematopoietic progenitor cells. In this study, we demonstrate the generation of putative iPS cells from BMSCs with single OCT4 by plasmid transfection, which can differentiate into hematopoietic progenitor cells in defined culture system.

Objective: To generate induced pluripotent stem cells (iPSCs) from bone marrow stromal cells (BMSCs) using a plasmid pcDNA3.1 constructed with a single transcription factor gene OCT4 (pcDNA3.1-OCT4) and to evaluate the hematopoietic differentiation potential of the putative BMSCs-iPSCs.

Methods: BMSCs with ectopic high expression of OCT4 (BMSCs-OCT4) previously established by our group were cultured in traditional human ESC medium. Colonies with characteristic embryonic stem (ES) cell morphologies were selected and expanded in vitro. The undifferentiated status of putative BMSCs-iPSCs was confirmed by alkaline phosphatase (ALP) staining, telomerase activity assay, pluripotent marker expression and differentiation in vitro to form embryonic bodies（EBs） and in vivo teratoma formation. The expression of pluripotent markers

and ES markers were verified by RT-PCR, flow cytometry (FCM) and cellular immunofluorescence assay (CIFA). The hematopoietic differentiation potential into CD34+ progenitor cells by exposure to a defined culture system supplemented with a cocktail of hematopoietic growth factors was evaluated, of which the expression was confirmed by RT-PCR and FCM.

Results: BMSCs were successfully reprogrammed into pluripotent stem cells resembling ESCs by introduction single transcription factor OCT4 gene constructed into the eukaryogenic plasmid pcDNA3.1. The putative BMSC-iPSCs were positive for ALP and telomerase activity, as well as the pluripotent stem cell markers including TRA-1-60, SSEA4, TRA-1-81, SOX2 and NANOG as detected by FCM and CIFA. Moreover, the above MSCs-OCT4 could form EBs ex vivo and express ectoderm (TUBB3+, WNT1+), mesoderm (Brachyury+, TBX20+), and endoderm (SPARC+) genes. By treatment with a cocktail containing BMP4 (50ng/ml), IL-3 (10ng/ml), IL-6 (10ng/ml), Flt-3 Ligand (300ng/ml), SCF (300ng/ml) and G-CSF (50ng/ml), the proportion of CD34+ progenitor cells increased from 0.93±0.46% in untransfected parental BMSCs and 1.58±1.29% in undifferentiated BMSC-iPS cells to 16.16±1.27% and 25.40±3.08% in day 14 and 21 differentiated BMSC-iPS cells, respectively. Moreover, the proportion of CD34+ progenitor cells were higher in the group with diverse concentration of growth factor cocktail induction, the proportion of CD34+ cells reached 31.39±3.60% and 73.68±6.63% in day 14 and 21 differentiated BMSC-iPS cells, respectively.

Conclusion: In this study, we have clearly demonstrated the generation of putative iPS cells (or partly reprogrammed iPSCs from BMSCs with ectopic high expression of OCT4 by plasmid transfection). The BMSCs-derived iPSCs display the typical morphology and growth pattern as

iPS cells when they are maintained in undifferentiated pluripotent state. Moreover, the putative BMSCs-derived iPSCs can differentiate into hematopoietic progenitor cells in defined culture system containing a cocktail of six or seven growth factors. Our findings provide a feasible way to generate hematopoietic progenitor cells using patient-specific iPSCs generated by plasmid transfection for hematological disease modeling.

Key words: Induced pluripotent stem cells (iPSCs), Mesenchymal stromal cells (MSCs), Transcription factor, OCT4, hematopoietic differentiation, Reprogram, CD34+ progenitor cells

Background

From 2006 to 2007, groundbreaking researches demonstrated that induced pluripotent stem cells (iPSCs) can be established from mouse and human fibroblasts by transduction of four transcription factors including OCT4, SOX2, KLF4 and C-MYC (now called Yamanaka factors) (1, 2). The generated iPS cells exhibit characteristics similar to human embryonic stem cells (hESCs) in terms of morphology, ES marker expression and the ability to differentiate into three germ layers in vitro and in vivo. Since then, somatic cell reprogramming has opened up a new era for the generation of patient-specific or disease-specific iPS cells, which are useful for understanding the mechanism of pathogenesis and developing novel disease modeling(3-5). Human iPSCs have been successfully generated from different somatic cell types such as skin fibroblasts, peripheral blood cells, hepatocytes and adipose stem cells and so on, of which fibroblasts are the most commonly used target cells.

In recent years, mesenchymal stromal/stem cells (MSCs) are becoming a promising candidate for reprogramming due to their highly proliferative capacity and multipotency (6, 7) . Moreover, MSCs can be isolated from various sources and expanded in vitro for a long time. They provide an

environment favoring hematopoiesis and have been widely used in clinics for several years. Recently, MSCs derived from bone marrow or umbilical cord blood (UCB) or dental tissue have been successfully reprogrammed into iPSCs using the virus-mediated transduction (8, 9). However, virus-based approach for reprogramming is undesirable as a result of genomic integration and background expression of reprogramming factors (4, 10) . Thus virus-free methods have been developed to reprogramming somatic cells without viral genome integration. Plasmid-mediated reprogramming especially the episome plasmid containing EBNA/OriP is currently the most reliable and reproducible approach for integration-free reprogramming with less complication than viral protocols despite of its low reprogramming efficiency. It has been reported that utilizing the above plasmid system to successfully reprogram the unfractionated blood or umbilical cord blood (UCB) mononuclear cells (MNCs) into iPSCs (8, 11). It is worth of noting that the episomal vectors are transiently expressing foreign genes as well as regular plasmids.

Derivation of iPS cells from adherent cells such as bone marrow derived mesenchymal stromal cells (BMSCs) using the regular plasmid has rarely been reported due to its extremely low reprogramming efficiency (12). However, the multipotency cells may facilitate the generation of iPS cells with higher efficiency as reported by Kim JB et al (13, 14). In addition, certain studies have indicated that iPSCs can be generated by using fewer than four transcription factors (one or two Yamanaka factors) from cells expressing some of the Yamanaka factors endogenously (6, 8, 14, 15). Our previously study showed that ectopic high expression of transcription factor OCT4 in BM-MSCs may drive them to grow as ESC-like cells with “stemness” characteristics, which are capable of forming EBs ex vivo and up-regulate the expression of pluripotent transcription factors such as SOX2 and NANOG(16). Our results indicated that ectopic high expression of OCT4 in

BMSCs could convert them into a state of pluripotency. In our present study, we demonstrated the generation of putative iPS cells from BMSCs using plasmid by single transcription factor gene *OCT4*. The generated putative BMSCs-iPS cells resemble the ESCs in many aspects, including morphology, pluripotency marker expression and in vitro differentiation. Moreover, the aforementioned cells have the potential to differentiate into CD34+ progenitor cells by exposure to a defined culture system supplemented with hematopoietic growth factors. Our findings demonstrate a feasible induction system to generate cell model for the study of the mechanism of hematopoiesis and related diseases in the future.

Experimental Procedures

Cell culture

The bone marrow cells were obtained by aspiration after the informed consents were obtained from the parents or guardians and the ethics approval was required according to the guidelines from the Children's Hospital of Zhejiang University School of Medicine Committee on the Use of Human Subjects in Research (approval number: 2013-GJ-003; approval date: February 25, 2013). This study was approved by the Eithic Committee at Children's Hospital of Zhejiang University School of Medicine. Bone marrow-derived MSCs (BMSCs) were established by the whole bone marrow adherent cell culture method from acute lymphoblastic leukemia (ALL) patients as previously described in our study and saved in our laboratory. The characteristics of these cells have been identified (16). BMSCs (P4) were cultured at a density of 10^5 cells per 10cm2 in Dulbecco's Modified Eagle Medium (DMEM (Invitrogen, Carlsbad, CA)) (low glucose) supplemented with 20% fetal bovine serum (FBS), 100 U/ml of penicillin and 100μg/ml of streptomycin and were grown at 37°C in a humidified atmosphere of 5 % CO2 and the medium

was changed every two days. BMSCs were detached with 0.25% trypsin-EDTA (Haotian, Hangzhou, China) when cells reached 90% confluence and passaged at a 1:4 dilution under the same culture conditions. The optimal generation cells (P4) were transfected with a eukaryogenic plasmid pcDNA3.1-OCT4 for the OCT4 over-expression experiment to establish the cell lines (BMSCs-OCT4).

The putative iPS cells were generated and maintained in human ES cell medium supplemented with DMEM/F12 containing 20% Knockout Serum Replacement (KSR; Invitrogen), 0.1 mM Non-Essential Amino Acids (NEAA; Invitrogen), 0.1 mM 2-Mercaptoethanol (Invitrogen), 100 U/ml of penicillin and 100μg/ml of streptomycin and 4ng/ml recombinant human basic fibroblast growth factor (bFGF; Invitrogen).

Establishment of OCT4 over-expression BMSCs and Generation of putative iPS cells

The recombinant plasmid pcDNA3.1-OCT4 was prepared as previously described (16) and transferred into 2×10^5 BMSCs by liposome transfection according to the protocol of the manufacturer's instructions. The reprogramming process is initiated by continuous transfection by plasmid pcDNA 3.1 containing OCT4 gene. Consecutive transfections were performed to improve efficiency. Cultures were maintained at 37℃, 5% of CO2 with daily medium change. The cells with stable OCT4 expression were selected with continuous G418 (500 ng/ml) (G418 Sulfate, Sangon, Shanghai) resistance screening and consecutive single-cell subcloning, then the cells were transferred to 6-well culture plate for expansion culture, where the expression of OCT4 in the cells was verified by FCM, cellular immunofluorescence assay (CIFA), and RT-PCR. The selected colonies with stable expression of OCT4 were then cultured with ES medium 2 (DMEM/F12 supplemented with 2mM L-glutamine, 0.1mM β-mercaptoethanol, 2mM non-essential amino

acids, 10% FBS, 10% knockout serum replacement (KSR), 100 U/ml penicillin and 100 μg/ml streptomycin) for a week, followed by further culture with ES medium 1 (DMEM/F12 consisting of 20% KSR(Invitrogen), 2mM L-glutamine(Invitrogen), 0.1mM β-mercaptoethanol, 2mM non-essential amino acids, 4ng/ml basic fibroblast growth factor (bFGF), 100 U/ml penicillin and 100 μg/ml streptomycin) in 0.1% gelatin coated 6-well culture plate until putative iPS colonies formation. During the reprogramming, colonies were observed daily with a reversed microscope (Olympus) and picked out for expansion culture based on cell morphology. The medium was changed every other day. Colonies expanded up to 6 passages were used for determination of iPSC characteristics. Pluripotency marker analysis was performed as previously described (2, 7, 9). Alkaline phosphatase (AP) staining was performed with an alkaline phosphatase detection kit (Millipore) according to the manufacturer's instructions. Differentiation potential in vitro and in vivo analysis was carried out as described (2, 7, 9).

Cellular immunofluorescence analysis (CIFA)

For cellular immunofluorescence analysis (CIFA), cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes and permeabilized with 0.2% Triton X-100 in PBS for 8 min and blocked with 4% normal goat serum in PBS (Boshide, Wuhan, China) for 30 min at room temperature. For detection of pluripotency markers by immunofluorescence, cells grown in 12-well plates were incubated with primary antibodies of rat anti-human OCT4 (1:100 dilution, R&D), SSEA-4 (1:100, Millipore), TRA-1-60 (1:100, Millipore), and TRA-1-81 (1:100, Millipore) for 30 min individually. After washing three times with PBST (0.1% Tween-20 in PBS), cells were incubated with the secondary antibodies of FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse IgM (KPL, 1:200 dilution) for 2 hours. Then 4',

6’-Diamidino-2-phenyl indole (DAPI, Kaiji, China) was used for nuclear staining. Fluorescence staining was observed under a fluorescence microscope (Olympus) and photographed.

RT-PCR analysis

The primer sets for reverse transcription PCR analysis of endogenous pluripotent genes and the three germ layer (endoderm, ectoderm, and mesoderm) marker genes were designed as shown in Table 1 using Primer Premier 5 software. Total RNA was prepared using Trizol (Invitrogen) according to the manufacturer’s protocol, and then used for cDNA synthesis. GAPDH was used as an internal control. Amplification was performed in 50 μl volume, and PCR reaction conditions were set as follows: 35 cycles of 94 °C for 30 s denature, 62 °C (OCT4-1143bp) or 60 °C (GAPDH, CMYC, KLF4, LIN28, NANOG, SOX2, OCT4-247bp, Brachyury, TUBB3, WNT1, SPARC, and TBX20) for 50 s annealing, and 72 °C for 90 s (OCT4-1143bp) or 30 s (the above eleven genes other than SOX2-966bp (72°C for 60 s)) extension, after an initial denature step of 94 °C for 5 min. Final extension at 72 °C for 10 min was performed. All reactions were repeated twice in triplicate using independently prepared cDNAs.

Flow cytometry analysis

Cell clumps were washed and dissociated with 0.25% trypsin-EDTA to prepare single cell suspension. For detecting ESCs surface markers including SSEA-4, TRA-1-60, TRA-1-81 expression (all the above antibodies were purchased from Millipore), cells were stained at 4℃ for 30 minutes with the above primary antibodies and then washed and stained with FITC-conjugated goat-anti-mouse IgG and IgM (GAM-FITC, purchased from Becton Dickinson). For detecting nuclear proteins (intracellular staining) of OCT4, SOX2 and NANOG expression, cells were fixed with PBS containing 4% paraformaldehyde and permeated with PBS (phosphate-buffered saline)

containing 0.2% Triton X-100 (Sigma) at 4℃ for 30 minutes. After washing, cells were stained with SOX2-PE (Becton-Dickinson), NANOG-PE (R&D Systems) and rat anti-human OCT3/4 primary antibody (McAb, R&D), respectively. Then, cells were washed and stained with FITC-conjugated goat anti-rat IgG (KPL, USA) at 4℃ for 30 minutes with light protection. For detecting hematopoietic surface marker expression, the following antibodies were used: CD34-PerCP, CD56-PE and CD45-FITC as detecting reagents and Mouse IgG1-PE, Mouse IgG1-PerCP and Mouse IgG1-FITC (all were purchased from Becton Dickinson, San Jose, CA, USA) were served as isotype control to determine the autofluorescence background. Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and Cell Quest software (Becton Dickinson, USA) was used for data acquisition and analysis. Independent experiments for flow cytometry assays were performed at least twice. For flow cytometric analysis, at least 5 000 events were collected and analyzed.

EB formation of putative BMSCs-iPS Cells

For embryoid body formation, BMSCs-iPS cells were dissociated into single-cell suspensions using 0.25% trypsin-EDTA, then plated on low-cluster 12-well plate (Nest) in the presence of DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS), 0.2mM L-glutamine, 1 x NEAA and 0.1mM β-mercaptoethanol (all from Invitrogen) for at least one week floating culture. The medium was changed every other day. In-vitro spontaneous differentiation of BMSC-iPS cells was performed following the protocol reported by Yamanaka S et al. with some modifications 2 and the primer sequences for ectoderm (TUBB3+ and WNT1+), endoderm (SPARC+ and AFP+) and mesoderm (Brachyury+ and TBX20+) were showed in Table 1. Then the cells were harvested and the expression levels of lineage representative markers for the three

embryonic germinal layers were analyzed.

Differentiation into CD34+ progenitor cells from BMSCs-iPS Cells

The hematopoietic differentiation potential of putative BMSCs-iPS Cells was examined by a method of EB formation and hematopoietic differentiation under a feeder-free condition as previously described (7, 17). For CD34+ progenitor cell differentiation, BMSCs-iPS cells were dissociated into single-cell suspensions using 0.25% trypsin-EDTA, then the floating cells were transferred to a 12-well low-cluster plate and cultured in MSCs medium with 20% FBS for 7 days. Then the cells were divided into two groups: the first group were transferred to culture medium supplemented with the following cocktail growth factors including BMP4 (50 ng/ml), SCF (300 ng/mL), Flt-3 Ligand (300 ng/mL), IL-3 (10 ng/mL), IL-6 (10 ng/mL) and G-CSF (50 ng/mL); while the second group cells were transferred to culture medium containing SCF (50 ng/ml) and Flt-3 Ligand (50 ng/ml) for 7-10 days, then transferred to the differentiation medium supplemented with the above cocktail growth factors (BMP4 (50 ng/ml), SCF (100 ng/mL), Flt-3 Ligand (100 ng/mL), IL-3 (10 ng/mL), IL-6 (10 ng/mL), GM-CSF (50 ng/ml) and G-CSF (50 ng/mL)) for additional 2-3 weeks to induce CD34+ progenitor cell formation. The cells were then prepared for further characterization for CD34 and CD45 expression by flow cytometry analysis and RT-PCR.

Alkaline phosphatase staining and telomerase activity detection

Alkaline phosphatase staining was performed using the Alkaline Phosphatase Kit according to the manufactures' instructions. Telomerase activity was verified with a TRAPeze Telomerase Detection Kit according to the manufacturer's instructions (both kits were purchased from Millipore, USA). Each sample was separated by TBE-based 10% polyacrylamide gel

electrophoresis and the gel was stained with ethidium bromide.

Teratoma formation assay

For teratoma induction, putative BMSCs-iPS cells were passaged into a 6-well culture dish. After 3 days of culture, cells were harvested, resuspended in 200μl of DMEM/F12 medium, and injected in the subcutaneous space of naked mouse. Teratoma was harvested at 6-8 weeks. These experiments were approved by the Zhejiang University's Institutional Animal Care and Utilization Committee (IACUC). A total of 1 million iPS cells were harvested by Collagenase (Accuse) digestion, washed with PBS (phosphate-buffer saline), and resuspended in 200ul ES medium. Tumors were excised 6-8 weeks after injection and histologic processing was performed as previously described (7, 11). Tumors were collected and fixed with 4% paraformaldehyde/PBS. The paraffin-embedded tumors were sectioned and stained with hematoxylin and eosin.

Data analysis

The data presented in this study were statistically analyzed by SAS 9.2 (SAS Institute Inc., Raleigh, NC, USA), and the figures were processed by Microsoft Excel. The data including the expression levels of surface markers (CD34, CD45 and CD56) and pluripotent transcript factors (TRA--1-60, OCT4, SOX2 and NANOG) were described as mean ± standard deviation (SD). Analysis of variance was performed for the comparison of the parameters between the two groups at different passages. P value < 0.05 was considered to be statistically significant.

Results

Single OCT4 gene introduced into human bone-marrow derived mesenchymal stromal cells (hBMSCs) by plasmid transfection can generate putative iPS cells

We have previously reported that culture-expanded MSCs from leukemia bone marrow displayed

high proliferation and possessed the ESC-like characteristics by ectopic overexpression of OCT4 using plasmid transfection(16). To explore whether BMSCs with ectopic high expression of OCT4 could be reprogrammed into a state of pluripotency, we maintained the BMSCs-OCT4 in hESCs medium for several weeks to validate the ESC characteristics.

For the production of putative iPS cells, BMSC-OCT4 were cultured in hESCs medium supplemented with 80% DMEM/F12, 10% KSR, 10% FBS, 2mM L-glutamine, 2mM NEAA and 0.1mM β-mercaptoethanol for 7 days to expand, then were cultured in hESCs serum-free medium supplemented with 80% DMEM/F12, 20% KSR, 2mM L-glutamine, 2mM NEAA, 0.1mM β-mercaptoethanol and 4ng/ml bFGF to expand the cell clones. Cells underwent a series of changes, including morphologic transformation from fibroblast-like to round shape, with eventual formation of colonies with typical ESC morphology at approximately day 14-21 of culture (Fig.1A-b). Colonies resembling human ES or iPS cells were selected and picked out for expanding for several passages then performed for the subsequent experiments.

In order to confirm that the cells from the putative colonies were iPS cells, we examined the hES cell markers by cellular immunofluorescence assay, RT-PCR, FCM, ALP activity and telomerase activity assay. Cellular immunofluorescence assay (CIFA) showed that the putative iPS cells expressed hES cell-specific surface antigens such as SSEA-4 (stage-specific embryonic antigen 4), TRA-1-60 (tumor-related antigen) and TRA-1-81 (Fig.1B). These cells also expressed the ES cell-specific transcription factors such as OCT4, NANOG and SOX2, which was confirmed by RT-PCR (Fig.1E) and FCM (Fig.1D). We also examined the telomerase activity and the ALP staining, the results showed high telomerase activity in BMSC-iPS cells whereas negative in the untransfected parental cells and the heat-treated cells (Fig.1C). Moreover, the putative iPS cells

were positive for ALP staining (Fig.1A-c-d).

Embryoid body formation and differentiation in vitro and in vivo

Pluripotency of these putative BMSC-iPS cells was demonstrated by differentiation assays including EB formation, in vitro differentiation and in vivo teratoma formation. To ensure the differentiation potential of iPS cells, we performed the suspension culture to demonstrate EBs formation, showing the sphere structures of EBs (Fig.2A-b) after a week of culture. These cells were transferred onto the serum-free medium for at least one week to induce spontaneous differentiation. RT-PCR analysis demonstrated that the EBs-derived cells expressed 3 germ layer genes including ectoderm (*TUBB3*+, *WNT1*+), mesoderm (*Brachyury*+, *TBX20*+), and endoderm (*SPARC*+) genes (Fig.2B). The expression of 3 germ layer genes at various differentiation time including day 14 and day 26 was also compared (Fig.2B). The results showed that the mesoderm gene *Brachyury* appeared a little bit later in the second cocktail growth factor culture than the first combination. These data indicated that the putative iPS cells derived from the culture-expanded BMSCs could differentiate into cell types of three embryonic germ layers.

To test the pluripotency of the BMSC-iPS cells, in vivo teratoma formation was performed. The putative iPS cells were subcutaneously injected into dorsum of the naked mouse. 6-8 weeks after injection, half of the mouse (3/6) showed putative tumor formation (Fig.2C-a). However, histological examination of the tumors did not demonstrate the representative tissues of three embryonic germ layers but the primitive cells (Fig.2C-b-c). These findings indicated that a part of putative BMSC-iPS cells could not be fully reprogrammed. The down-regulation expression of pluripotency markers in cryopreservation cells confirmed the above phenomenon that the putative iPS cells could be converted into partly differentiated cells (Data not shown).

Generation of CD34+ hematopoietic progenitor cells and dynamic analysis during hematopoietic cell differentiation

To address whether putative BMSCs-iPS cells have the potential of differentiation toward the hematopoietic lineage, we carried out a defined culture condition protocol for CD34+ progenitor cell differentiation. Embryoid bodies (EBs) derived from BMSC-iPS cells were cultured in the medium supplemented with hematopoietic cytokines including IL-3, IL-6, Flt-3 Ligand, SCF, BMP4 and G-CSF. BMSC-iPS cells were divided into two groups: the first group was maintained in the above medium with aforementioned six factors, while the other group was maintained first in the culture with the presence of Flt-3 Ligand (50ng/ml) and SCF (50ng/ml) for 7-10 days, then cultured in medium with the cocktail containing IL-3, IL-6, Flt-3 Ligand (100ng/ml), SCF (100ng/ml), BMP4, GM-CSF (50ng/ml) and G-CSF for another 10-14 days. CD34+ progenitor cells were detected by flow cytometry. It demonstrated that the proportion of CD34+ progenitor cells increased from $0.93 \pm 0.46\%$ in untransfected parental BMSCs and $1.58 \pm 1.29\%$ in undifferentiated BMSC-iPS cells to $16.16 \pm 1.27\%$ and $25.40 \pm 3.08\%$ in day 14 and 21 differentiated BMSC-iPS cells, respectively (Fig.3A). Moreover, the proportion of CD34+ progenitor cells were higher in the group with diverse concentration of growth factor cocktails, in which the proportion of CD34+ cells reached $31.39 \pm 3.60\%$ and $73.68 \pm 6.63\%$ in day 14 and 21 differentiated BMSC-iPS cells, respectively (Fig.3B). Although further experiments are needed to address to what extent theses cells are able to differentiate into all hematopoietic lineages, cells positive for CD34 and CD45 (CD34+CD45+) could also be observed, indicating their hematopoietic differentiation after culture under the appropriate growth condition (Data no shown).

We also examined the expression of pluripotency markers including TRA-1-60, OCT4 and SOX2 in differentiated BMSC-iPS cells at different induction time by flow cytometry. The results showed decreasing expression of TRA-1-60 and OCT4 in differentiated BMSC-iPS cells, which suggested the differentiation into hematopoietic lineage of BMSC-iPS cells. Interestingly enough, in the second combination of growth factor culture system containing seven growth factors, the expression of SOX2 was gradually increased with CD56 expression detected by flow cytometry (Fig.3C). These results suggested that the hematopoiesis from putative BMSC-iPS cells was a dynamic process. During the EBs formation, RT-PCR results demonstrated that the mesoderm gene *Brachyury* was appeared 3 weeks later (Fig.2B), which to some extent could explain the kinetic process of CD34+ progenitor cells.

Discussion

Since the revolutionary discovery of induced pluripotent stem cells (iPSCs) by Yamanaka in 2006, reprogramming of somatic cells has opened up an entirely new perspective of obtaining patient-specific cell lines that can be further differentiated into the desired cell type. Virus-mediated gene delivery is the most common method for generation iPSCs despite of posing the risk of random genomic integration. Recent development has led to plasmid based protocols containing the Epstein-Barr virus (EBV)-derived oriP/EBNA1 becoming a promising system to avoid the above shortcomings. OriP/EBNA1 is a kind of transient transfection system but possessing the capacity to continuously express the reprogramming factors. Adult human fibroblasts have been successfully reprogrammed into induced neural stem cells using the plasmid based protocol (18) . Generation of iPSCs from unfractionated blood or umbilical cord blood (UCB) mononuclear cells (MNCs) utilizing the above plasmid system have also been reported (8,

11)8, 11. Moreover, the proliferation potential and the multipotency of the target cells are contributing to promote the generation of iPSCs (3, 4). In our previous study, we have established BMSCs with stable ectopic OCT4 expression with the ESCs-like characteristics by regular plasmid vector. Based on the above research status and our results, we tried to explore the possibility of iPS cell generation from BMSCs by plasmid transfection with single transcript factor OCT4. Our results showed that BMSCs with ectopic high expression of OCT4 changed their morphology after culture in ES medium for two weeks, and expressed the pluripotent markers and possessed the potential to EBs formation. However, the putative ESCs-like cells could not form teratoma in vivo, which indicated the above cells could be partly reprogrammed iPS cells. Further studies will be necessary to answer the question of the pluripotency or the partly reprogramming process presented in our study. However, a lot of studies have showed that iPSCs could be derived with one or two Yamanaka factors from somatic cells expressing high endogenous levels of one or two reprogramming factors, and the reprogramming efficiency could be greatly improved by certain defined small-molecule compounds(13-15) . The results from the published literatures confirmed the possibility of generation of iPSCs with single transcription factor OCT4 from mulitipoent cells such as BMSCs.

In our present study, we tried to evaluate the hematopoietic capacity of putative BMSCs-iPS cells (partly reprogrammed iPSCs). Interestingly, we found that the partly reprogrammed iPSCs derived from BMSCs could be induced to differentiate into the CD34+ progenitor cells in defined culture condition based on the spontaneous differentiation approach involving EB formation. Moreover, the proportion of CD34+ progenitor cells varied with the growth cytokine composition. Our results were not very consistent with other studies (6, 11). One possible explanation for this

discrepancy is that the induction efficacy and cellular function of CD34+ progenitor cells from iPSCs are distinct to some extent with the differentiation protocols, cell derivation methods and cytokine composition. We found higher expression level of CD34+ progenitor cells in the second growth factors supplement (supplied with Flt3-Ligand and SCF for 7-10 days followed by continuous culture in fully defined growth medium with 7 growth factors) than that in first combination. One of the possible explanations for this result is that early-acting hematopoietic signaling initiated is feasible for the CD34+ progenitor cell generation. Our findings are supported by another study reported in the literature (7). Moreover, the expression of surface marker CD56 was also increased besides CD34, along with the gradual expression of *SOX2*, one of the pluripotency marker gene. One possible explanation for this is that the CD34+/CD56+ could be a kind of stem cells as showed by the pluripotent marker expression in separated tumor cells cultured in vitro (data not shown) , which highly expressed the pluripotency marker *SOX2* gene. Maucksch et al. has showed that over-expressing SOX2 and PAX6 by plasmid transfection could reprogram fibroblasts into neural precursor-like cells (19), which supported our hypothesis. The potential mechanism behind the above phenomenon merits further study.

OCT4 is not only a very important transcript factor in regulating the pluripotent state of embryonic stem cells (ESCs) but also a pivotal and indispensable transcription factor in somatic cell reprogramming. It has been demonstrated that the other two transcription factors NANOG and SOX2, as well as OCT4, also played a similar important role in maintaining the pluripotency of ESCs(20, 21). In our study, we found that high expression of pluripotent markers SOX2 and NANOG in putative BMSCs-iPS cells (partly reprogrammed iPSCs), the expression levels of the above two transcript factors were down-regulated and decreased in the differentiation process. The

results indicated that OCT4, SOX2 and NANOG co-operated with each other to maintain the pluripotency. Our results have addressed the question to some extent that during reprogramming process transient expression of ectopic transcript factor OCT4 could initiate endogenous pluripotent marker expression and prompt the multipotent BMSCs transit into pluripotent state(18). The underlying mechanism related the pluripotency maintenance warrants further study.

The shortcomings of this study include the following: firstly, the underlying molecular mechanisms of partly reprogrammed iPSCs have not been clearly investigated and warrants further intensive study. Secondly, efficient commitment to functional CD34+ progenitor cells from putative BMSCs-iPSCs should be performed to confirm the hematopoietic differentiation potential. Lastly, but not least, the function of the proportion of CD34+/CD56+ cells remain to be answered and is worthy of in-depth study.

Conclusion

In this study, we demonstrate the generation of putative iPS cells (or partly reprogrammed iPSCs) from BMSCs with ectopic high expression of OCT4 by plasmid transfection, and the BMSCs-derived iPSCs display the typical morphology and growth pattern as iPS cells when they are maintained in undifferentiated pluripotent state. They express ESCs markers (including SSEA4, TRA-1-60 and TRA-1-81) and pluripotent factors (such as OCT4, NANOG and SOX2), capable of differentiation into three germ layers in vitro. Moreover, the putative BMSCs-derived iPSCs could differentiate into hematopoietic progenitor cells in defined culture system containing a cocktail with seven growth factors. Our findings provide a feasible way to generate hematopoietic progenitor cells using patient-specific iPSCs generated by plasmid transfection for hematological disease modeling.

Author Contributions

Xiaoping Guo conceived, designed and carried out the study, and drafted the manuscript. Sisi Li provided the technical assistance and performed some of FCM and participated in its data analysis. Wenwen Weng participated in BM-MSCs cultue and PDT analysis. Xiaojun Xu performed the statistical analysis and analyzed the data. Chan Liao, Diying Shen and Jingying Zhang contributed reagents and analysis tools. Yongmin Tang participated in the design of the study and helped to draft and edit the manuscript. All authors read and approved the final manuscript.

Abbreviations

iPSCs: induced pluripotent stem cells; BM-MSCs: bone marrow derived mesenchymal stromal cells; ALP: alkaline phosphatase; ESCs: embryonic stem cells; EBs: embryonic bodies; FCM: flow cytometry; CIFA: cellular immunofluorescence assay; PDT: population doubling time; TFs: transcription factors; UCB: umbilical cord blood; MNCs: mononuclear cells; FBS: fetal bovine serum; DMEM: Dulbecco's Modified Eagle Medium; KSR: knockout serum replacement; bFGF: basic fibroblast growth factor; PBS: phosphate-buffer saline NEAA: Non-Essential Amino Acids

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

Xiaoping Guo conceived, designed and carried out the study, and drafted the manuscript. Sisi Li provided the technical assistance and performed some of FCM and participated in its data analysis. Wenwen Weng participated in BM-MSCs cultue and PDT analysis. Xiaojun XU performed the statistical analysis and analyzed the data. Chan Liao, Diying Shen and Jingying Zhang contributed reagents and analysis tools. Yongmin Tang participated in the design of the study and helped to draft and edit the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The informed consents were obtained from the parents of guardians and the ethics approval was required according to the guidelines from the Children's Hospital of Zhejiang University School of Medicine Committee on the Use of Human Subjects in Research. All the procedures described in this report have been approved by the Children's Hospital of Zhejiang University IRB. (Approval number: 2013-GJ-003; Approval date: 2013-02-25). All animal procedures described in this report have been approved by Zhejiang University's Institutional Animal Care and Utilization Committee (IACUC).

Consent for publication

Not applicable.

Conflict of interests

No potential conflict of interests to declare.

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Figures

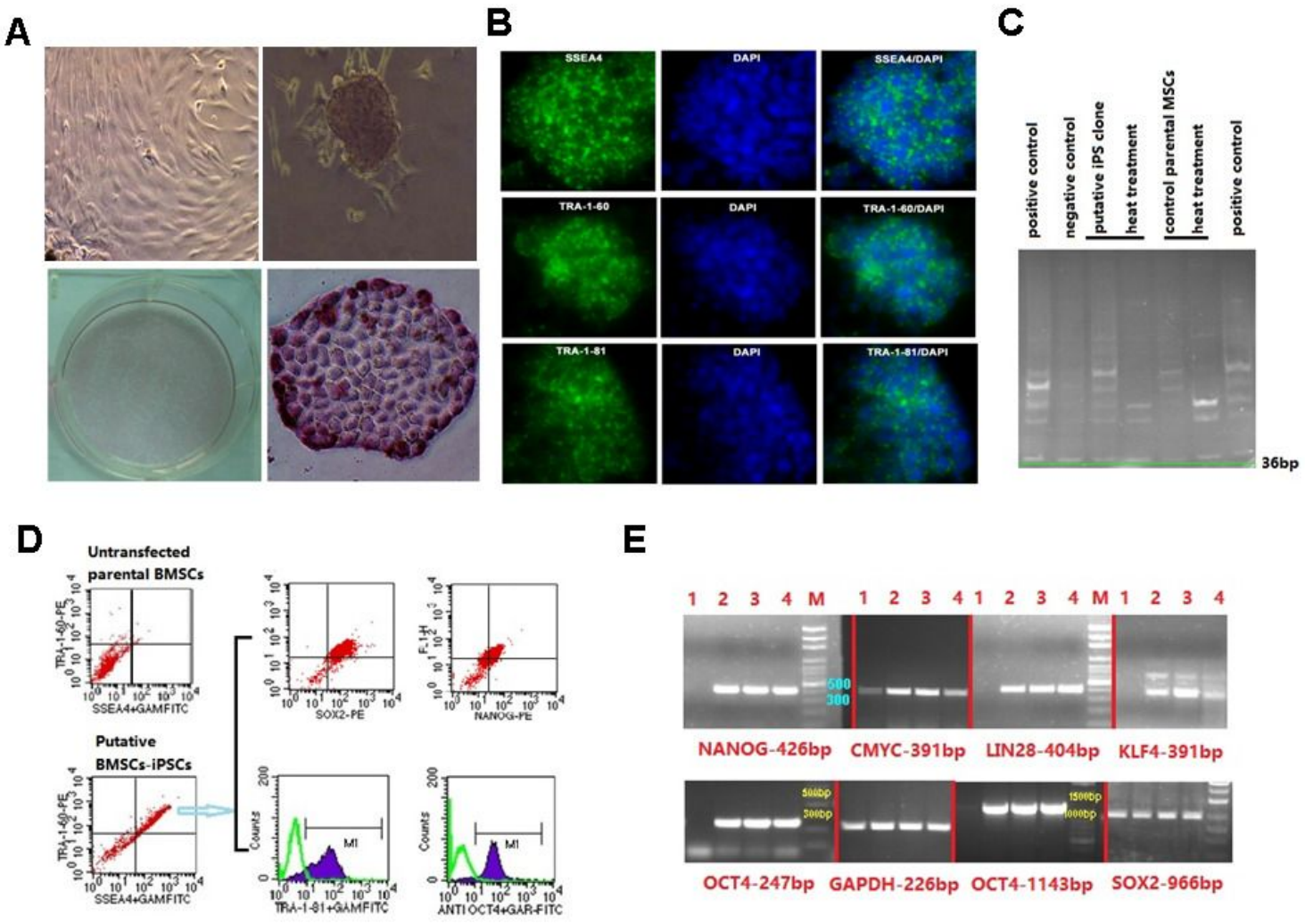

Figure 1

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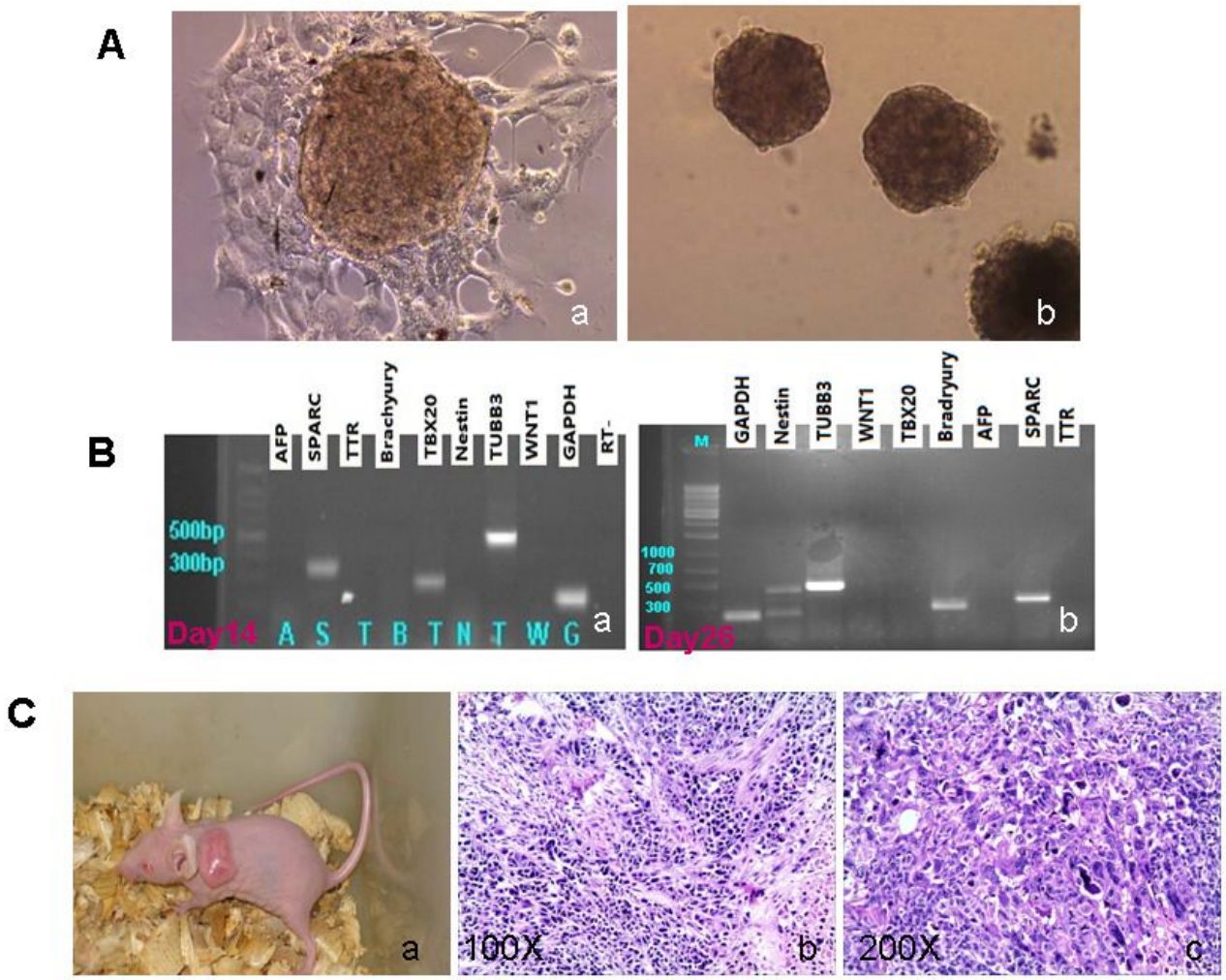

Figure 2

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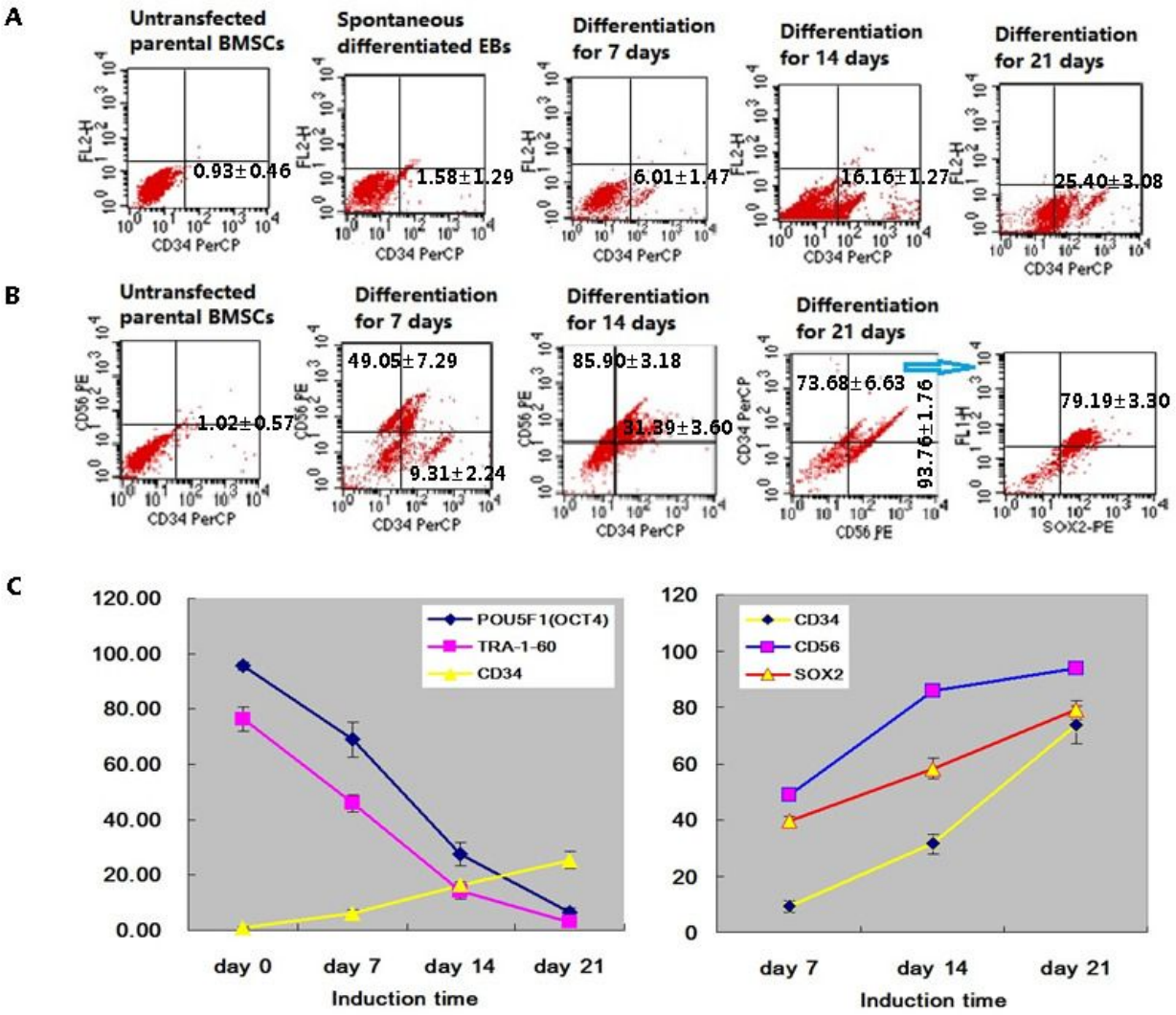

Figure 3

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