Original Article

Efficient Generation of Induced Pluripotent Stem Cells from Human Bone Marrow Mesenchymal Stem Cells

(iPSCs / efficiency / p53 siRNA / VPA / Vc / mechanism)

X. YULIN, L. LIZHEN, Z. LIFEI, F. SHAN, L. RU, H. KAIMIN, H. HUANG

Bone Marrow Transplantation Centre, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, China

Abstract. Ectopic expression of defined sets of genetic factors can reprogramme somatic cells to induced pluripotent stem cells (iPSCs) that closely resemble embryonic stem cells. However, the low reprogramming efficiency is a significant handicap for mechanistic studies and potential clinical application. In this study, we used human bone marrow-derived mesenchymal stem cells (hBMMSCs) as target cells for reprogramming and investigated efficient iPSC generation from hBMMSCs using the compounds of p53 siRNA, valproic acid (VPA) and vitamin C (Vc) with four transcription factors *OCT4*, *SOX2*, *KLF4*, and c-*MYC* (compound induction system). The synergetic mechanism of the compounds was studied. Our results showed that the compound induction system could efficiently reprogramme hBMMSCs to iPSCs. hBMMSC-derived iPSC populations expressed pluripotent markers and had multi-potential to differentiate into three germ layer-derived cells. p53 siRNA, VPA and Vc had a synergetic effect on cell reprogramming and the combinatorial use of these substances greatly improved the efficiency of iPSC generation by suppressing the expression of p53, decreasing cell apoptosis, up-regulating the expression of the pluripotent gene *OCT4* and modifying the cell cycle. Therefore, our study highlights a straightforward method for improving the speed and efficiency of iPSC generation and provides versatile tools for investigating early developmental processes such as haemopoiesis and relevant diseases. In addition, this study provides a paradigm for the combinatorial use of genetic factors and molecules to improve the efficiency of iPSC generation.

Received May 1, 2012. Accepted August 27, 2012.

Research Project No. 20100471736 supported by research funding from National Post Doctor Science Fund Scheme and No. 2009C14011 supported by Major Program of Technology Research and International co-operative research of Zhejiang Province.

Corresponding author: He Huang, Bone Marrow Transplantation Centre, First Affiliated Hospital, Zhejiang University School of Medicine, No. 79 Qingchun Road, Hangzhou, Zhejiang Province 310003, China. Phone: +86 571 87236706; Fax: +86 571 87236562; e-mail: hehuang.zju@gmail.com

Abbreviations: AP – alkaline phosphatase, bFGF – basic fibroblast growth factor, EBs – embryoid bodies, ESCs – embryonic stem cells, FBS – foetal bovine serum, HDAC – histone deacetylase, hBMMSCs – human bone marrow-derived mesenchymal stem cells, iPSCs – induced pluripotent stem cells, MEFs – mouse embryonic fibroblasts, NOD/SCID – non-obese diabetic/severe combined immunodeficient, TBS – Tris-buffered saline, Vc – vitamin C, VPA – valproic acid.

Introduction

Induced pluripotent stem cells (iPSCs) can be generated from somatic cells by introducing four reprogramming factors (*OCT4*, *SOX2*, and *KLF4*/*c-MYC* or *NANOG*/*LIN28*) in mouse and human (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Okita et al., 2007; Yu et al., 2007). iPSCs closely resemble embryonic stem cells (ESCs) in morphology, gene expression, epigenetic signature and functional pluripotency. The genetic reprogramming approach has opened up tremendous opportunities to study the mechanism of development and to generate patient-specific cells for disease modelling as well as potential therapeutic applications without the controversies associated with conventional ESCs. However, iPSC reprogramming is an inefficient and slow process. The low efficiency of iPSC generation is a significant handicap for mechanistic studies and potential clinical transplantation (Scheper and Copray, 2009; Hanna et al., 2010; Okita and Yamanaka, 2011; Unternaehrer and Daley, 2011).

Recently, histone deacetylase (HDAC) inhibitor valproic acid (VPA) (Huangfu et al., 2008a), p53 short-interfering RNA (siRNA) (Zhao et al., 2008; Hong et al., 2009) and vitamin C (Vc) have been in succession reported to promote iPSC generation (Esteban et al., 2010). VPA regulates chromatin epigenetic modification by inhibiting HDACs and improves reprogramming efficiency (Huangfu et al., 2008b). Functional analysis of p53-regulated genes demonstrates that the p53-p21 pathway serves as a barrier in iPSC generation and p53 siRNA suppresses it to avoid cell apoptosis (Zhao et al.,

2008; Hong et al., 2009). Vc improves iPSC generation by alleviating cell senescence, accelerating gene expression changes, and promoting the transition of pre-iPSC colonies to a fully reprogrammed state (Esteban et al., 2010). It is known that several critical networks of signalling pathways exert functions harmoniously in cell reprogramming, making cells acquire immortalization and multi-potential, although the mechanism is unclear. Therefore, we hypothesized that p53 siRNA, VPA and Vc, combined to influence cell immortalization, chromatin modification, cell senescence and gene expression, might exert a synergetic effect on cell reprogramming and might have greater efficiency for iPSC generation than they did respectively by modulating several key signalling pathways during the cell reprogramming.

Beside that, human bone marrow-derived mesenchymal stem cells (hBMMSCs) have been delivered to treat diseases since 1956 (Thomas et al., 1959) and to date, hBMMSC-mediated cell therapies have rescued thousands of patients (Parekkadan and Milwid, 2010). The cell therapies have been proved to be a safe and effective treatment for various degenerative diseases and tissue injuries and their exploitation becomes a fast-growing field (Parekkadan and Milwid, 2010; Jung et al., 2012). Although being easily expanded *in vitro* and holding multipotency to differentiate into several lineages such as osteoblasts, adipocytes and chondrocytes (Pittenger et al., 1999), hBMMSCs are impeded by limited lifespan and limited differentiation potential for their further clinical application where the cells are cultured *in vitro* (Jung et al., 2012). However, iPSCs generated from hBMMSCs (hBMMSC-iPSCs) may have greater application potential for their pluripotency of forming three germ layers, indefinite passages and immunological compatibility, and may also have more virtues than other types of cell-derived iPSCs in disease treatment and regenerative medicine applications (Jung et al., 2012).

In this study, we used hBMMSCs as target cells for reprogramming and investigated whether the efficiency of iPSC generation could be greatly improved by compounds of p53 siRNA, VPA and Vc, together with the mechanism relevant for such improvement.

Material and Methods

Cell culture

ICR mice (purchased from SLAC LABORATORY ANIMAL, Shanghai, China) were used for feeder cell preparation. Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (purchased from SLAC LABORATORY ANIMAL) were used for teratoma formation. All animal investigations were approved by the institutional animal care committee of Zhejiang University. hBMMSCs were used as target cells for reprogramming. hBMMSCs were obtained from bone marrow of healthy adult donors after informed consent. Samples were separated by Lymphocyte Separation Medium (human) (Haoyang Biological Manufacture Co. Ltd, Tianjin, China) and the mononuclear cells were collected and cultured in a flask at a density of 10^7 cells per 75 cm^2 in DMEM (Invitrogen, Shanghai, China) supplemented with 10 % foetal bovine serum (FBS) (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen) at 37 °C in 5% CO_2 atmosphere. hBMMSCs were trypsinized (0.25% trypsin-EDTA; Invitrogen) at 90% confluence and the passages 2–4 were used for iPSC generation. hBMMSCs were identified before iPSC induction.

Retroviral production

Retroviral supernatants were prepared as described previously (Takahashi et al., 2007) with some modifications. Briefly, each pMXs-based retroviral vector containing the human complementary DNAs (cDNAs) of *OCT3/4*, *SOX2*, *c-MYC* and *KLF4* (Addgene: http://www.addgene.org) was transduced into 293T cells with a mixture of two helper plasmids: pCMV-G and pCMV-GP (a gift from Associate Professor Weiyan Zheng of Zhejiang University) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Viral supernatants of 24–72 h after transfection were harvested, centrifuged at 400 *g* for 20 min and filtered through a 0.45 μm filter. hBMMSCs were transduced with an *OCT3/4* : *SOX2* : *c-MYC* : *KLF4* = 1 : 1 : 1 : 1 mixture of viral supernatants.

Generation and maintenance of induced pluripotent stem cells

hBMMSCs at passage 2–4 were seeded at 0.1 million cells per well in 6-well culture plates. Retroviruses with cDNAs encoding the four transcription factors were used to infect hBMMSCs for iPSC generation. To improve the reprogramming efficiency, p53 siRNA (Segment, Shanghai, China) was transduced to hBMMSCs together with the four transcription factors according to the manufacturer's instructions. The viral supernatant was changed to DMEM supplemented with 10% FBS, 2 μM VPA (Sigma, Shanghai, China) and 25 μg/ml Vc (Sigma) after the transduction for 6 h. Five days later (d6), cells were seeded onto the feeder layers of mitomycin C-treated ICR mouse embryonic fibroblasts (MEFs) in human ESC culture medium consisting of DMEM/F12 (Invitrogen), 20% KnockOut Serum Replacement (Invitrogen), 2 mM GlutaMAX (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 1% nonessential amino acids (NEAA, Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin, and 4 ng/ml basic fibroblast growth factor (bFGF, Invitrogen). VPA and Vc were still supplemented into the medium. Cultures were maintained at 37 °C, 5% CO_2 with daily medium changes. VPA was added for 7 days and Vc was supplemented until colonies were carried out for detection. The modified protocol of iPSC generation is shown in Fig. 1A. During the reprogramming, colonies were observed

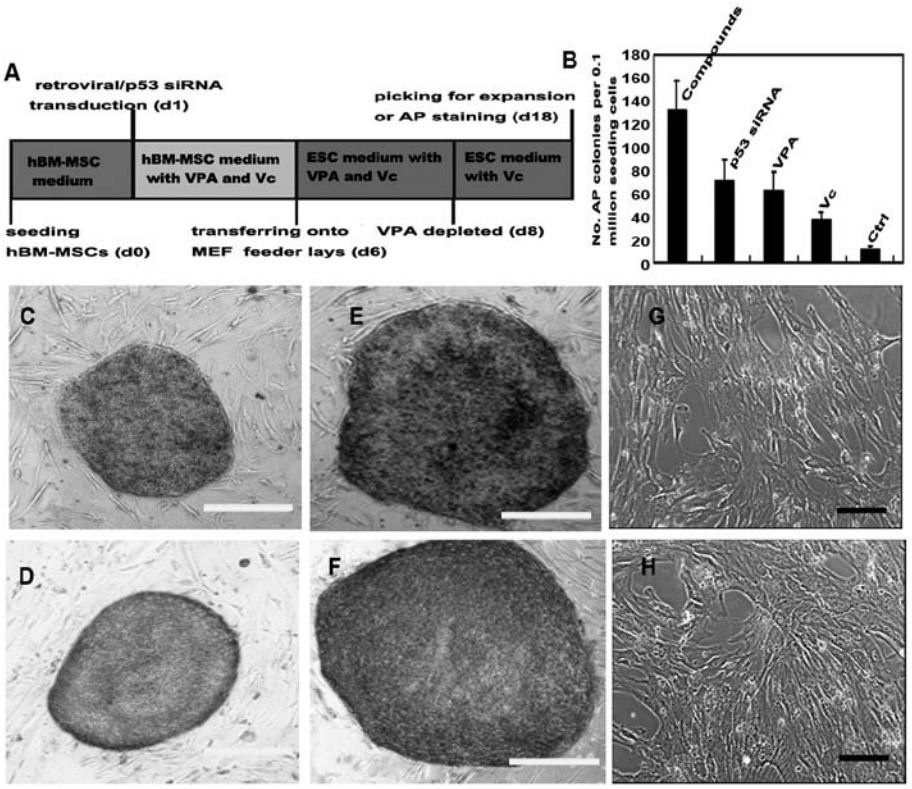


Fig. 1. Compound induction system improving iPSC generation from hBMMSCs. (**A**) Protocol for iPSC induction with the compounds of p53 siRNA, VPA and Vc along with the retroviral transduction encoding the four genes. d, day. ESC, embryonic stem cell. (**B**) The reprogramming efficiency was calculated by comparison of AP-positive colonies in different treatments. Representative hBMMSC-iPSC colonies (**C**) and AP staining (**D**) shown on the morphology and AP staining of human ESCs (**E** and **F**). No ESC-like and AP-positive colonies were observed in the control cultures of hBMMSCs (**G** and **H**).

with an inverted microscope (Olympus, Shanghai, China) every day. About three weeks after the initial transduction, colonies with human ESC-like morphology were picked up for alkaline phosphatase (AP) staining to examine the efficiency of the induction system according to manufacturer's recommendations or for expansion culture. For maintenance of undifferentiated cells, hBMMSC-derived iPSCs and human ESCs (H1, obtained from WiCell research institute, Madison, WI) were cultured in human ESC medium on mitomycin C-treated ICR MEFs at 37 °C, in 5% CO_2. Medium was changed every day.

Alkaline phosphatase and immunofluorescence staining

Reprogramming efficiency was calculated as the number of iPSC colonies formed per number of infected cells seeded. iPSC colonies were identified based on ESC-like morphology and AP staining. AP staining was carried out with an alkaline phosphatase detection kit (Millipore, Beijing, China) according to the manufacturer's instructions. For immunoflurescence analysis, colonies were fixed with 4% paraformaldehyde at room temperature for 15 min. When nuclear proteins were detected, colonies were permeabilized with 0.1% Triton X-100 in PBS and then blocked with normal goat serum for 1 h at room temperature. Staining was performed using the following antibodies (Santa Cruz, Shanghai, China): anti-Oct4 (1 : 100), anti-Nanog (1 : 100), anti-SSEA-4 (1 : 100) and anti-Tra-1-81 (1 : 100) overnight at 4 °C. Secondary antibodies conjugated to FITC or Cy3 (Boster, Wuhan, China) were used for fluorescence detection followed by counterstaining with DAPI (Boster) to reveal the nuclei. Images were obtained using a confocal microscope (LSM 510 Meta, Carl Zeiss, Hangzhou, China).

RT-PCR analysis

The primer sets for reverse transcription RT-PCR analysis of expression of the endogenous pluripotent genes were designed using Primer Premier 5 software as shown in Table 1. Total RNA was prepared using Trizol (Invitrogen) according to the manufacturer's protocol. Total RNA (1 μg) was used for cDNA synthesis, using a ReverTra Ace-α kit (Toyobo Bio-Technology, CO., LTD, Shanghai, China) and oligo(dT) 20 primers. *GAPDH* was used as an endogenous control. PCR was performed in a 20 μl mixture containing 1× PCR buffer, 0.5 U of Taq DNA polymerase, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 μM of each primer and 2 μl of each RT product as a template. The following programme was carried out: initial denaturation for 4 min at 94 °C, 35 cycles of 94 °C for 15 sec, 51 °C for c-*myc*, *Nanog*,

Table 1. Primer sets for RT-PCR of the pluripotent genes and bisulphite-sequencing PCR for promoter assay of Oct4 and Nanog

Genes	Size (bp)	Sequence (5′ to 3′) F	Sequence (5′ to 3′) R
Primer sets for pluripotent gene RT-PCR			
Oct4	484 bp (55°)	TTCAGCCAAACGACCATC	GGAAAGGGACCGAGGAGTA
Sox2	190 bp (68°)	AAACAGCCCGGACCGCGTCAA	TCGCAGCCGCTTAGCCTCGT
Klf4	402 bp (55°)	CCACCGTGTCCTCGTCA	CAGGGCTGCCTTTGCT
c-*myc*	357 bp (51°)	GCTGCCAAGAGGGTCA	CGCACAAGAGTTCCGTAG
Nanog	168 bp (47°)	CCTATGCCTGTGATTTG	AGAAGTGGGTTGTTTGC
Rex1	492 bp (48°)	GGCAAAGACAAGACACC	GCAAATTCTGCGAGCT
hTERT	200 bp (59°)	CAGAGGTCAGGCAGCATCG	CGCCCGCTCGTAGTTGA
GAPDH	220 bp (53°)	AAGGTCGGAGTCAACGG	GGAAGATGGTGATGGGATT
For bisulphite-sequencing PCR			
Oct4	221 bp	*Oct4*-mF3	ATTTGTTTTTTGGGTAGTTAAAGGT
		Oct4-mR3	CCAACTATCTTCATCTTAATAACATCC
Nanog	164 bp	*Nanog*-mF3	TTAATTTATTGGGATTATAGGGGTG
		Nanog-mR3	AAACCTAAAAACAAACCCAACAAC

Rex1, 53 °C for *GAPDH*, 55 °C for *Oct4*, *Klf4*, 58 °C for *hTERT*, 68 °C for *Sox2* for 35 sec, and 72 °C for 1 min; and followed by 72 °C for 10 min. cDNA from hBMMSCs was used as negative control, while that from ESCs served as positive control.

DNA methylation analysis

For analysis of the methylation status of CpG sites on the promoters of *OCT4* and *NANOG*, genomic DNA of hBMMSC-iPSCs, ESCs and hBMMSCs was respectively isolated using a QIAamp DNA Mini Kit (QIAGEN, Shanghai, China), and then processed for conversion of unmethylated cytosines to uracil with EZ DNA Methylation-Gold Kit™ (ZYMO, Beijing, China) according to the protocol of the manufacturer. Converted DNA (50 ng) was amplified by PCR with *OCT4*-specific primers and *NANOG*-specific primers (Table 1). The conditions for PCR were 35 cycles, consisting of denaturing at 95 °C for 1 min, annealing and extension at 60 °C for 1 min, 72 °C for 1 min and followed by 72 °C for 10 min after the initial denaturing at 95 °C for 1 min. PCR products were collected with gel electrophoresis and purified with Gel Extraction kit (QIAGEN), then subcloned into bacteria using the TOPO TA Cloning Kit (Invitrogen) to be sequenced. The correlation between hBMMSC-iPSCs, ESCs and hBMMSCs was examined by Pearson correlation coefficients.

Differentiation potential in vitro and in vivo

To assess the *in vitro* differentiation potential, hBMMSC-iPSCs were cultured without the feeder layers and bFGF in DMEM supplemented with 10% FBS until embryoid bodies (EBs) appeared. Cells in EBs were cultured on 0.1% gelatin-coated culture dishes for differentiation *in vitro*. For teratoma formation, hBMMSC-iPSCs were collected and injected subcutaneously into the limbs of 5-week-old NOD/SCID mice (1×10^7 cells per injection site). After 8–10 weeks, teratomas were formed. For histological assay, teratomas were harvested and fixed with 4% paraformaldehyde, and then dehydrated, embedded in paraffin and serially sectioned at 6–8 μm thickness. After being processed with haematoxylin-eosin staining, their histology was studied to investigate the development of cells and tissues of the three germ layers.

Western blot analysis

To examine the influence of combination treatment on the expression of P53, we performed Western blot to monitor P53 protein changes during hBMMSC reprogramming. For the effects on cell senescence, we selected samples treated with p53 siRNA transduction or VPA or Vc supplement as controls to assess the compounds of p53 siRNA,VPA and Vc on the expression of P53. The procedure was carried out as previously described (Hong et al., 2009) with a little modification. Briefly, cells were lysed in M-PER/M-PER protein extraction solution (Pierce, Hangzhou, China) to isolate total proteins after the substance treatments for three days. Proteins were quantified using a BCA kit (Pierce) and separated in 10% SDS-PAGE and then transferred to a PVDF membrane. After being blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST; 20 mM Tris-HCl, pH 7.2–7.5, 150 mM NaCl, and 0.1% Tween-20), the membranes were incubated with primary antibodies anti-P53 (1 : 200) (Santa Cruz) and anti-β-actin (1 : 200) (Santa Cruz) at 4 °C overnight. Following several washes in TBST, membranes were subsequently incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Boster) diluted 1 : 1,000 in blocking buffer. The membrane was washed three times with TBST. The signals were detected by the enhanced chemiluminescence reagents (ECL+, Pierce) and exposed to X-ray film. Relative expression of P53 was analysed using Image J software (National Institutes of Health) and β-actin values were used for control. Samples were analysed in triplicate.

Flow cytometry

hBMMSCs were transduced with virus containing the four transcription factors with or without the combined

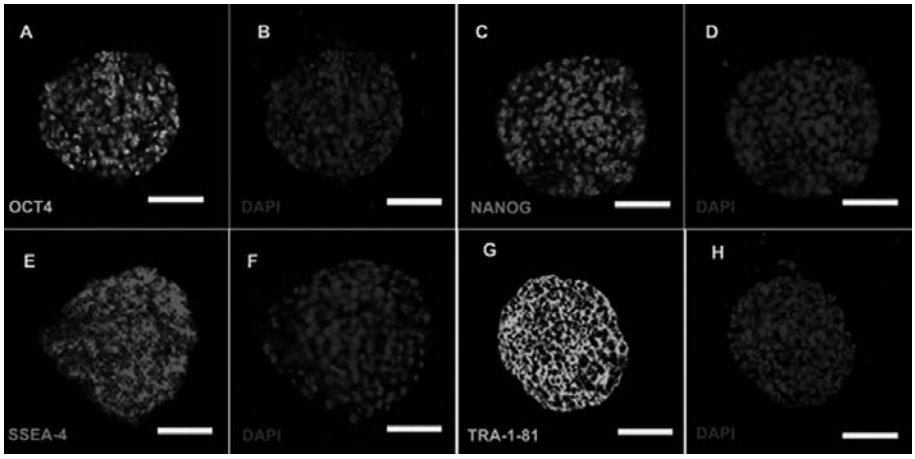


Fig. 2. Analysis of the marker expression of hBMMSC-iPSCs. Immunoassay of hBMMSC-iPSC colonies expressing ESC-specific markers OCT4 (**A** and **B**), NANOG (**C** and **D**), SSEA-4 (**E** and **F**) and TRA-1-81 (**G** and **H**). 4, 6-Diamidino-2-phenylindole (DAPI) staining was used to reveal the nuclei (**B, D, F** and **H**). Scale bar, 100 μm.

treatment of p53 siRNA, VPA and Vc as described in the above section on the generation and maintenance of induced pluripotent stem cells. After culture for 7 days, the samples were collected for flow cytometry analysis. Cells (x10^6) were fixed in 70% ice-cold ethanol overnight and treated with 100 μg/ml RNase A (Sigma) for 15 min and then incubated with 50 μg/ml propidium iodide (PI) in the dark. Flow cytometric analysis was performed using a BD FACSCalibur flow cytometer (Becton Dickinson, Hangzhou, China).

Statistical analysis

All data were presented as mean ± S.D. Differences between group means were assessed by analysis of variance using SPSS *t*-test (SPSS 16.0). Values of $P < 0.05$ were considered significant.

Results

Compound induction system improving iPSC generation from hBMMSCs

We cultured hBMMSCs to passage 2–4 before induction of reprogramming. hBMMSCs were positive for CD29, CD44, CD105, and CD166 and negative for CD34, CD45, CD14, HLA-DR. Multipotency of hBMMSCs into osteoblasts, adipocytes and chondrocytes was also demonstrated (data not shown). The protocol for hBMMSC reprogramming with the compound induction system was devised as shown in Fig. 1A according to the relevant reports (Takahashi et al., 2007; Huangfu et al., *2*008a; Esteban et al., 2010).

We retrovirally transduced hBMMSCs with cDNAs encoding four transcription factors (4TFs) together with p53 siRNA transduction according to the manufacturer's protocol. VPA and Vc were supplemented to the culture. In the study, we used p53 siRNA or VPA or Vc treatment alone together with 4TFs to investigate the efficiency of the compounds of p53 siRNA, VPA and Vc on the iPSC generation. On day 18 after induction, we performed AP staining to assess the induction efficiency of the different systems or disaggregated ESC-like colonies into parts of cell aggregates and reseeded them onto the feeder layers of mitomycin C-treated MEFs for expansion. In the compound induction system, some ESC-like colonies were observed 12–14 days after the transduction, while in the other induction systems, about 15– 22 days were needed to obtain ESC-like colonies. As for AP staining analysis, 132 ± 8 AP-positive colonies were observed in the compound induction system when we started with 1×10^5 hBMMSCs and its efficiency was higher than for the other treatments (Fig. 1B), while only 12 ± 3 AP-positive colonies were observed in the group induced with 4TFs only. Some ESC-like colonies exhibited AP activity (Figs. 1C and 1D) comparable in strength to ESCs (Figs. 1E and 1F), whereas hBMMSCs displayed weak AP activity, showing no ESC-like colony (Figs. 1G and 1H). When colonies were cultured for expansion, we observed many secondary colonies which were similar to ESC colonies in morphology. ESC-like colonies were expanded in subculture every 5 to 7 days using the standard ESC culture protocol.

After expansion up to passage 10, ESC-like colonies were characterized by standard procedures. Immunocytochemistry revealed that ESC-like colonies expressed pluripotency markers including OCT4 (Figs. 2A and 2B), NANOG (Figs. 2C and 2D), SSEA-4 (Figs. 2E and 2F), Tand RA-1-81 (Figs. 2G and 2H). Using RT-PCR we verified the expression of the pluripotent genes such as *Nanog, Oct4, Sox2, hTERT, Rex1* and we compared it with the levels in ESCs and hBMMSCs. The results showed that ESC-like colonies expressed typical pluripotent ESC genes at consistent levels with ESC, whereas hBMMSCs did not express these genes or expressed them at lower levels (Fig. 3A). There was no significant difference in the expression levels of *Oct4*, *Nanog* and *hTERT* between ESC-like colonies and ESCs. Next, we performed bisulphite genomic sequencing to analyse the methylation levels in the promoter regions of *Oct4* and

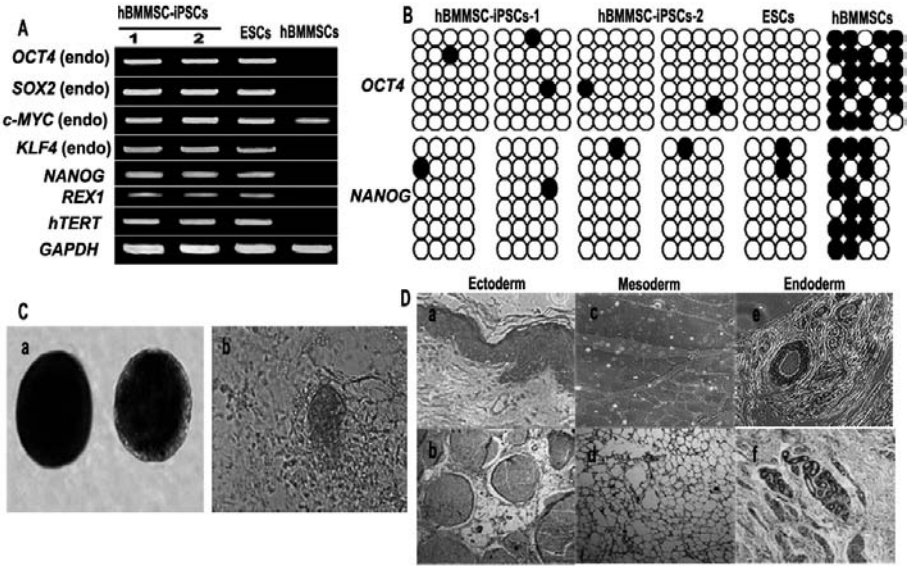


Fig. 3. Analysis of the gene expression and multi-potential of hBMMSC-iPSCs. (**A**) RT-PCR analysis of endogenous pluripotent gene mRNA expression in hBMMSC-iPSCs. Total RNA was isolated from two hBMMSC-iPSC lines, ESCs and hBMMSCs. (**B**) Analysis of the methylation status of the *OCT4* and *NANOG* promoters in hBMMSC-iPSCs subclones using bisulphite sequencing. Open and filled circles mean unmethylated and methylated CpG sites, respectively. (**C-D**) Pluripotency assay of hBMMSC-iPSCs. EBs were formed (**C a**) and spontaneously differentiated into many types of cells during the *in vitro* potential assay (**C b**). (**D**) Haematoxylin and eosin staining of teratoma sections of hBMMSC--iPSCs (10X). The amount of 1 x 10^7 of hBMMSC-iPSCs was injected subcutaneously into the limbs of NOD/SCID mice. Teratomas were obtained after 8–10 weeks. Ectoderm, epithelial-like and neural-like tissue (**D a, b**). Mesoderm, muscle--like and adipose-like tissue (**D c, d**). Endoderm, intestinal-like epithelium (**D e, f**).

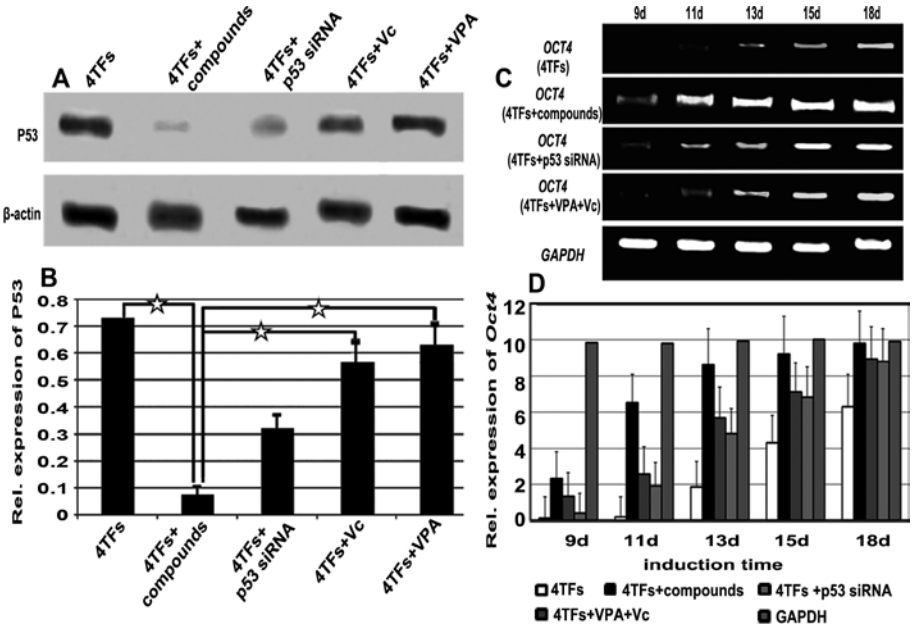


Fig. 4. Compounds of p53 siRNA, VPA and Vc synergized to improve the efficiency of the iPSC generation from hBMMSCs. (**A-B**) P53 protein levels were determined in the different induction systems. (A) Western blot analysis demonstrated that the expression of P53 was suppressed in the induction systems treated with the compounds or p53 siRNA or Vc alone, and the compound induction system was more efficient than the other systems. The induction system treated with VPA alone had no obvious effect on the expression of P53. (B) Relative expression of P53 in different treatments was analysed using gray-scale value assay and mean values ± standard deviation (S.D.) were shown for three independent experiments. (**C-D**) Detection of the pluripotent gene *OCT4* by RT-PCR and fluorescence intensity assays in the different induction systems. (**C**) RT-PCR analysis demonstrated that the expression of *OCT4* was activated in the different induction systems, and the compound induction system was more efficient than the other systems. (**B**) Relative expression of pluripotent gene *OCT4* in the different treatments was analysed using fluorescence intensity assay and mean values ± S.D. were shown for three independent experiments.

Nanog. The results confirmed that the promoter regions were largely unmethylated in the colonies and ESCs, but methylated in hBMMSCs (Fig. 3B). To determine the differentiation potential of ESC-like colonies, we performed EB spontaneous differentiation *in vitro* and assessed teratoma formation *in vivo*. After suspension culture for 7 days, ESC-like colonies formed cell aggregates of typical EBs (Fig. 3C a). When EBs were seeded onto 0.1% gelatin-coated dishes for 7–14 days, spontaneous differentiation was observed (Fig. 3C b). When ESC-like colonies were injected subcutaneously into NOD/SCID mice, after 8–10 weeks teratomas were formed. Histological examination of the tumours revealed tissues representative of the three germ layers: epithelial- or neural-like tissue (ectoderm) (Figs. 3D a and b) (10X), muscle- or adipose-like tissue (mesoderm) (Figs. 3D c and d) (10X) and gut- or intestinal-like epithelium (endoderm) (Figs. 3D e and f) (10X). These findings indicated that ESC-like colonies had the multi-potential to differentiate into various cell types both *in vitro* and *in vivo*.

Collectively, ESC-like colonies generated in the compound induction system were bipotent and held the typical ESC properties, and therefore we made the conclusion that iPSCs derived from hBMMSCs (hBMMSC-iPSCs) were efficiently generated using the compound induction system.

Compound induction system down-regulating the expression of P53

Previous studies have shown that cell senescence is a main roadblock for reprogramming (Zhao et al., 2008; Hong et al., 2009) and we observed a similar phenomenon in the retrovirus transduction. In our study we employed the compounds of p53 siRNA, VPA and Vc to treat the target cells. p53 siRNA or VPA or Vc treatment alone served as controls. Western blot analysis was carried out for the expression of P53 in the different induction systems. The results showed that the expression of P53 was markedly suppressed in the compound induction system (Fig. 4A). The treatments with p53 siRNA or Vc alone also reduced the expression of P53 to some extent, and the treatment with VPA had no significant effect on the expression of P53 as shown in Fig. 4A. The relative expression of P53 was calculated with Imag J as shown in Fig. 4B compared with the expression of β-actin. The results demonstrated that the compound treatment was more efficient in suppressing the expression of P53 than p53 siRNA or Vc treatment alone. The treatment with VPA showed no obvious change in the expression of P53. Values of P were assessed by analysis of variance in the different induction systems using SPSS *t*-test (SPSS 16.0) and the result was shown in Fig. 4B.

Compound induction system up-regulating the expression of the pluripotent gene Oct4

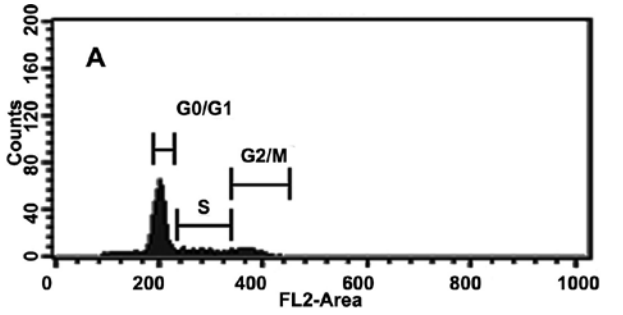
The critical step in the cell reprogramming was activation of the endogenous pluripotent genes by forced ectopic expression of the exogenous genes. Here, we also analysed the expression of endogenous gene *Oct4*, which had a critical function in cell reprogramming, by RT-PCR and fluorescence intensity assays. In this analysis, we tested the samples from the compound induction system, from the treatment with p53 siRNA and from the treatment with VPA and Vc for their effect on the cellular epigenetic modification. The results demonstrated that the gene was activated to expression on day 9 in the compound induction system but much less in the treatment with p53 siRNA and the combination treatment with VPA and Vc. The expression was undetectable in the 4TFs induction system. On day 11, the expression of *Oct4* reached a considerable level in the compound induction system, but was still lower in the other induction systems. During the process of reprogramming, the compound induction system clearly outshined the other treatments in the expression of endogenous *Oct4* (Figs. 4C and 4D). The treatment with p53 siRNA or the use in combination with VPA and Vc was also effective in promoting *Oct4* expression; however, the efficiency was lower than in the compound induction system. The relationship between the fluorescence intensity of *Oct4* expression and the time of induction was analysed as shown in Fig. 4D. The results confirmed that the gene expression gradually increased in the different induction systems as the reprogramming continued, and the efficiency of the compound induction system was better than the other treatments, even the combined use of VPA and Vc.

Compound induction system modifying cell cycle of hBMMSCs during reprogramming

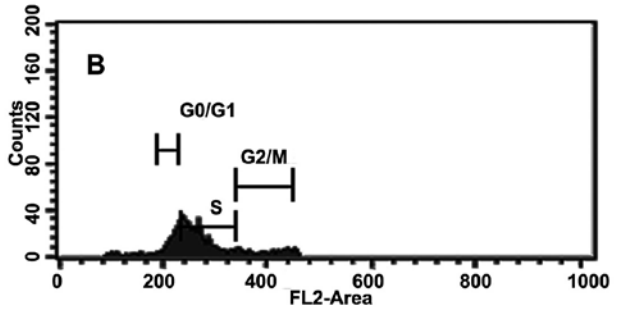
During the reprogramming process, we observed a relevant arrest in cellular proliferation. We inferred that cell senescence might be one of its reasons and we additionally analysed the cell cycle to investigate the cellular changes needed for obtaining the pluripotent state using flow cytometry. The samples were collected from the treatments of cDNA transduction containing the four transcription factors or together with the treatment of the compounds of p53 siRNA, VPA and Vc. The results showed that nearly 70 % of the cells were in G0/G1 phase in the compound induction system (Fig. 5A), while nearly 21 % of cells were in G0/G1 phase in the cDNA transduction system (Fig. 5B). The difference between the two groups was significant (68.45 % ± 4.36 % *vs.* 23.15 % ± 3.72 %) ($P < 0.05$).

Discussion

Human iPSCs have been generated from various cell types even after their terminal differentiation, although the efficiencies and the factors used for regeneration are being verified (Nakagawa et al., 2008; Kim et al., 2009; Warren et al., 2010; Zhou and Ding, 2010; Chou et al., 2011). hBMMSCs are well-established adult stem cells which have the potential to self-renew and to differenti-



Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	1674	100.00	16.74
G0/G1	189, 230	1098	65.59	10.98
S	233, 343	213	12.72	2.13
G2/M	343, 452	161	9.62	1.61



Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	2148	100.00	21.48
G0/G1	189, 230	449	20.90	4.49
S	233, 343	1277	59.45	12.77
G2/M	343, 452	275	12.80	2.75

Fig. 5. Flow cytometry detection of the cell cycle of hBMMSCs during the reprogramming. The results showed that nearly 70 % of cells were in G0/G1 phase in the compound induction system (**A**), while nearly 21 % of cells were in G0/G1 phase in the 4TFs transduction system (**B**).

ate into multiple cell types, and have been employed in clinical cell therapy for many years (Pittenger et al., 1999; Parekkadan and Milwid, 2010; Jung et al., 2012). Thanks to the well-established features and the great clinical potential, hBMMSCs are targeted as a promising candidate for efficient generation of high-quality iPSCs (Jung et al., 2012). Oda et al. (2010) generated iPSCs from mesenchymal stromal cells derived from human third molars (wisdom teeth) by retroviral transduction. Niibe et al. (2011) generated iPSCs from PDGFRα$^+$ Sca-1$^+$ (PαS) adult mouse MSCs and PDGFRα$^-$ Sca-1$^-$ osteo-progenitors (OP cells). They found that mouse MSCs had a higher reprogramming efficiency compared with OP cells and fibroblasts. Ohnishi et al. (2011) generated iPSCs from frozen-stocked adipose tissue-derived MSCs and bone marrow-derived MSCs. In our study we used freshly isolated bone marrow-derived MSCs as target cells and obtained iPSCs successfully. The efficiency of iPSC production was greatly improved. The hBMMSC-derived iPSC populations showed typical properties of human ESCs such as the morphology, expression of pluripotent markers and the potential to differentiate into three germ layer-derived cells. Our data confirmed that bone marrow-derived MSCs are an ideal candidate for efficient generation of iPSCs.

Reprogramming somatic cells into a pluripotent state is an inefficient and slow kinetics process. Cell senescence is a main roadblock for reprogramming and the chemicals that alleviate cell senescence have been found to promote reprogramming (Banito et al., 2009; Kawamura et al., 2009; Utikal et al., 2009; Banito and Gil, 2010; Esteban et al., 2010; Li et al., 2010). A substantial reduction in efficiency may also result from incomplete reprogramming. In our study, the efficiency of iPSC generation was only about 0.01 % using the traditional procedure (Takahashi et al., 2007) with the retrovirus encoding the four transcription factors. By a combination treatment of p53 siRNA, VPA and Vc, the efficiency of iPSC generation from hBMMSCs increased more than 10-fold compared with the traditional procedure. Hong et al. (2009) showed that p53 and p21 suppressed iPSC generation. They found that up to 10 % of transduced MEFs lacking p53 became iPSCs, even without retroviral c-*myc*. p53 deletion also promoted induction of integration-free mouse iPSCs in plasmid transfection. Considering the important role of p53 in reprogramming, we investigated its expression during the iPSC induction. Our results showed that p53 siRNA and Vc both decreased the expression of P53, and the compounds of p53 siRNA, VPA and Vc had a synergetic effect on P53 suppression. These results indicated that p53 siRNA or Vc treatment alone reduced but did not abolish P53 expression, allowing the compounds of p53 siRNA, VPA and Vc to reduce it further.

OCT4 is the core regulatory factor of pluripotency (Kim et al., 2009), and so far it has been the only factor which cannot be replaced in reprogramming. DNA demethylation in the promoter regions of *OCT4* is a necessary step to achieve complete reprogramming of cells (Mali et al., 2010). Previous reports indicated that compounds that induce chromatin modifications by affecting either DNA methylation or histone acetylation greatly improve reprogramming efficiency of both mouse and human somatic cells (Huangfu et al., 2008a; Mali et al., 2010). In our induction system, we added the combination of HDAC inhibitor VPA with p53 siRNA and Vc to directly regulate *Oct4* and to ensure the acquirement and maintenance of pluripotency. Our data showed that the synergetic capacity of the combination to activate and up-regulate the pluripotent *OCT4* gene was higher than in the traditional procedure culture (Takahashi et al., 2007), and therefore prompted iPSC generation.

We further characterized the function of the compounds of p53 siRNA, VPA and Vc on the cell cycle during the reprogramming and the results showed that the compound induction system reduced hBMMSC proliferation by making them remain in the G0/G1 phase and preventing their entry in the S/M phase of the cell

cycle, and therefore promoted hBMMSC differentiation but not proliferation.

Molecules such as VPA have recently been reported to not only enhance reprogramming efficiencies, but also substitute for specific reprogramming factors. Melton and colleagues also demonstrated that addition of VPA enabled human fibroblasts to be reprogrammed with two factors (*OCT4* and *SOX2*) at an efficiency similar to three-factor (*OCT4*, *SOX2* and *KLF4*) reprogramming, thus negating the need for the oncogenes c-*MYC* or *KLF4* (Huangfu et al., 2008b). Whether the combination treatment of p53 siRNA, VPA and Vc can replace any of the reprogramming factors needs to be further elucidated.

During retrovirus transduction, we observed relevant cell senescence in the different induction systems. Following this observation, we performed an analysis of the effects of the compounds of p53 siRNA, VPA and Vc on reprogramming and we found that they, indeed, had a regulatory effect on the cell cycle. As demonstrated in this study, the number of cells in G0/G1 phase was significantly higher in the compound induction system than in the 4TFs transduction system ($P < 0.05$). Therefore, we suggest that compounds of p53 siRNA, VPA and Vc modulated the cell cycle and prevented cell entry into S/M phase, and therefore inhibited cellular proliferation. However, the mechanism should be further explored.

Taken together, our data suggest that the combination of p53 siRNA, VPA and Vc regulates hBMMSC reprogramming development at multiple levels, suppressing the expression of P53 and cell senescence, promoting expression of the pluripotent gene *OCT4*, preventing cells from proliferation and favouring their differentiation, and therefore improves the efficiency of iPSC generation. Our results highlight a straightforward method for improving the speed and efficiency of iPSC generation. In addition, the current study provides a paradigm for the combinatorial use of genetic factors and chemicals to improve the efficiency of iPSC generation.

Declaration of interest

The authors report no conflicts of interest and are responsible for the content and writing of the paper.

References

Banito, A., Rashid, S. T., Acosta, J. C., Li, S., Pereira, C. F., Geti, I., Pinho, S., Silva, J. C., Azuara, V., Walsh, M., Vallier, L., Gil, J. (2009) Senescence impairs successful reprogramming to pluripotent stem cells. *Genes Dev.* **23**, 2134-2139.

Banito, A., Gil, J. (2010) Induced pluripotent stem cells and senescence: learning the biology to improve the technology. *EMBO Rep*. **11**, 353-359.

Chou, B. K., Mali, P., Huang, X., Ye, Z., Dowey, S. N., Resar, L. M., Zou, C., Zhang, Y. A., Tong, J., Cheng, L. (2011) Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res.* **21**, 518-529.

Esteban, M. A., Wang, T., Qin, B., Yang, J., Qin, D., Cai, J., Li, W., Weng, Z., Chen, J., Ni, S., Chen, K., Li, Y., Liu, X., Xu, J., Zhang, S., Li, F., He, W., Labuda, K., Song, Y., Peterbauer, A., Wolbank, S., Redl, H., Zhong, M., Cai, D., Zeng, L., Pei, D. (2010) Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell Stem Cell* **6**, 71-79.

Hanna, J. H., Saha, K., Jaenisch, R. (2010) Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. *Cell* **143**, 508-525.

Hong, H., Takahashi, K., Ichisaka, T., Aoi, T., Kanagawa, O., Nakagawa, M., Okita, K., Yamanaka, S. (2009) Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* **460**,1132-1135.

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A. E., Melton, D. A. (2008a) Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat. Biotech.* **26**, 795-797.

Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W., Melton, D.A. (2008b) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat. Biotech.* **26**, 1269-1275.

Jung, Y., Bauer, G., Nolta, J. A. (2012) Induced pluripotent stem cell-derived mesenchymal stem cells: progress toward safe clinical products. *Stem Cells* **30**, 42-47.

Kawamura, T., Suzuki, J., Wang, Y. V., Menendez, S., Morera, L. B., Raya, A., Wahl, G. M., Izpisúa Belmonte, J. C. (2009) Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature* **460**, 1140-1144.

Kim, J. B., Greber, B., Araúzo-Bravo, M. J., Meyer, J., Park, K. I., Zaehres, H., Schöler, H. R. (2009) Direct reprogramming of human neural stem cells by OCT4. *Nature* **461**,649-643.

Li, F., He, Z., Shen, J., Huang, Q., Li, W., Liu, X., He, Y., Wolf, F., Li, C. Y. (2010) Apoptotic caspases regulate induction of iPSCs from human fibroblasts. *Cell Stem Cell* **7**, 508-520.

Mali P, Chou, B.-K., Yen, J., Ye, Z., Zou, J., Dowey, S., Brodsky, R. A., Ohm, J. E., Yu, W., Baylin, S. B., Yusa, K., Bradley, A., Meyers, D. J., Mukherjee, C., Cole, P. A., Cheng, L. (2010) Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes. *Stem Cells* **28**, 713-720.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., Yamanaka, S. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotech.* **26**,101-106.

Niibe, K., Kawamura, Y., Araki, D., Morikawa, S., Miura, K., Suzuki, S., Shimmura, S., Sunabori, T., Mabuchi, Y., Nagai, Y., Nakagawa, T., Okano, H., Matsuzaki, Y. (2011) Purified mesenchymal stem cells are an efficient source for iPS cell induction. *PLoS ONE* **6**, e17610.

Oda, Y., Yoshimura, Y., Ohnishi, H., Tadokoro, M., Katsube, Y., Sasao, M., Kubo, Y., Hattori, K., Saito, S., Horimoto, K., Yuba, S., Ohgushi, H. (2010) Induction of pluripotent

stem cells from human third molar mesenchymal stromal cells. *J. Biol. Chem*. **285**, 29270-29278.

Ohnishi, H., Oda, Y., Aoki, T., Tadokoro, M., Katsube, Y., Ohgushi, H., Hattori, K., Yuba, S. (2011) A comparative study of induced pluripotent stem cells generated from frozen, stocked bone marrow- and adipose tissue-derived mesenchymal stem cells. *J. Tissue Eng. Regen. Med.* **6**, 261-271.

Okita, K., Ichisaka, T., Yamanaka, S. (2007) Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313-317.

Okita, K., Yamanaka, S. (2011) Induced pluripotent stem cells: opportunities and challenges. *Phil. Trans. R. Soc. B* **366**, 2198-2207.

Parekkadan, B., Milwid, J. M. (2010) Mesenchymal stem cells as therapeutics. *Annu. Rev. Biomed. Eng.* **12**, 87-117.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A,, Simonetti, D. W., Craig, S., Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.

Scheper, W., Copray, S. (2009) The molecular mechanism of induced pluripotency: A two-stage switch. *Stem Cell Rev.* **5**, 204-223.

Takahashi, K., Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell* **126**, 663-676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872.

Thomas, E. D., Ashley, C. A., Lochte, H. L., Jaretzki, A., Sahler, O. D., Ferrebee, J. W. (1959) Homografts of bone marrow in dogs after lethal total-body radiation. *Blood* **14**, 720-736.

Unternaehrer, J. J., Daley, G. Q. (2011) Induced pluripotent stem cells for modelling human diseases. *Phil. Trans. R. Soc. B* **366**, 2274-2285.

Utikal, J., Polo, J. M., Stadtfeld, M., Maherali, N., Kulalert, W., Walsh, R. M., Khalil, A., Rheinwald, J. G., Hochedlinger, K. (2009) Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. *Nature* **460**, 1145-1148.

Warren, L., Manos, P. D., Ahfeldt, T., Loh, Y. H., Li, H., Lau, F., Ebina, W., Mandal, P. K., Smith, Z. D., Meissner, A., Daley, G. Q., Brack, A. S., Collins, J. J., Cowan, C., Schlaeger, T. M., Rossi, D. J. (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **7**, 618-630.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II., Thomson, J. A. (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920.

Zhao, Y., Yin, X., Qin, H., Zhu, F., Liu, H., Yang, W., Zhang, Q., Xiang, C., Hou, P., Song, Z., Liu, Y., Yong, J., Zhang, P., Cai, J., Liu, M., Li, H., Li, Y., Qu, X., Cui, K., Zhang, W., Xiang, T., Wu, Y., Zhao, Y., Liu, C., Yu, C., Yuan, K., Lou, J., Ding, M., Deng, H. (2008) Two supporting factors greatly improve the efficiency of human iPSC generation. *Cell Stem Cell* **3**, 475-479.

Zhou, H., Ding, S. (2010) Evolution of induced pluripotent stem cell technology. *Curr. Opin. Hematol.* **17**, 276-280.