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14. ABSTRACT Mesenchymal stem cells (MSCs) differentiated from Induced pluripotent stem cells (iPSCs) have a potential application in clinic to treat osteoporosis and other skeletal diseases. Further engineering MSCs with homing factors like CXCR4 and osteogenic factors like shNoggin or FGF2 may increase the therapeutic effects. Toward these goals, we have generated iPSCs using lentiviral vectors from blood cells and integration-free iPSCs using episomal vectors. The generated iPSCs are pluripotent, as evident by expression of pluripotency markers and formation of teratoma. After differentiation of iPSCs into MSCs, the cells express MSC markers and can form bone nodule in in vitro culture. To improve the safety of this therapy, we developed an alternative strategy for generating MSCs: direct conversion of blood cells into MSCs rather than reprogramming blood cells into iPSCs followed by re-differentiation.					
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INTRODUCTION

Developing strategies to treat osteoporosis and related bone disease is of significant relevance to the health care needs of the Armed Forces. The commonly used maintenance therapy has serious adverse effects after long-term use. MSC-based therapy is considered most promising for systemic augmentation of bone strength¹. However, this approach has several limitations: 1) The available number of MSCs from one harvest is limited; 2) the osteogenic potential of MSCs from adult and aging patients is substantially lower than fetal MSCs²; 3) transplantation of stem cells that are engineered using retrovirus has a serious safety concern of insertional mutagenesis-associated tumorigenesis. A recent breakthrough of generating iPSCs from patient's own cells will provide a solution to all these potential problems³. iPSCs, unlike MSCs, can be expanded ex vivo to unlimited cell number, enabling us to use the prescreened best clone for therapy. Of note, generation of iPSC can rejuvenate the source cells from which iPSCs are derived, as evidenced by increased telomere⁴. Thus, we hypothesized that iPSCs-derived MSCs resemble more similarity to fetal MSCs in multi-potential differentiation abilities.

After 2 years of support by this grant, we have successfully generated mouse iPSCs using a novel episomal vector. Integration-free iPSCs generated without the use of lentivirus are more clinically relevant and potentially safer than lenti-iPSCs. We also developed an approach for differentiation of iPSCs into functional MSCs that are capable of trilineage in vitro differentiation. At the beginning of Year 2, the SOW was approved to make important changes. After extensive research, we developed an exciting technology: directly turning blood into MSCs. This novel approach is significantly better than what we proposed some 3 years ago in the original application. This breakthrough makes the therapy safer and more economical. We are looking for more grants to further develop this technology into a clinical therapy for treating severe bone diseases.

BODY

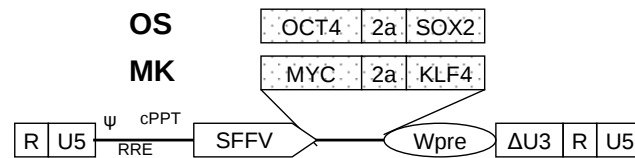
1. Generation of iPSCs

1-A) Generation of mouse iPS cells using lentiviral vectors.

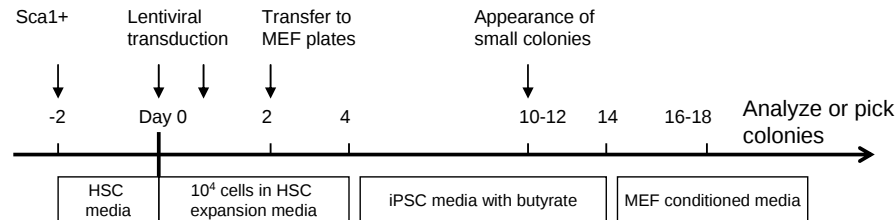
iPSCs are a type of pluripotent stem cell derived from non-pluripotent cells, typically adult somatic cells, by inducing forced expression of specific genes. In 2006, Yamanaka's group first converted fibroblasts into iPS cells by overexpressing 4 transcription factors—Oct4, Sox2, Myc and Klf4⁵. These factors were subsequently used in the successful generation of hPSCs in 2007⁶. This technical breakthrough has significant implications for regenerative medicine. The iPS cell technology lifts the big hurdle (i.e. immune rejection) in cell replacement therapy by providing MHC-identical autologous cells, which can be differentiated from patient-specific hPSCs. In addition, the "safe" clone that is engineered with therapeutic genes can be differentiated into HSCs for stem cell gene therapy.

To generate mouse iPSCs, we purified Sca1⁺ cells from bone marrow and transduced them with two lentiviral vectors that express OCT4 and SOX2, MYC and KLF4 (**Figure 1a**). Three days after transduction, cells were harvested for iPSC generation culture on the inactivated murine embryonic fibroblast feeder in the iPSC culture condition. Two weeks later, we observed the appearance of some mouse iPSC colonies (**Figure 1c**). We picked several colonies for further culture.

a. Lentiviral vector constructs



b. Experimental procedure



c. Mouse iPSC colonies

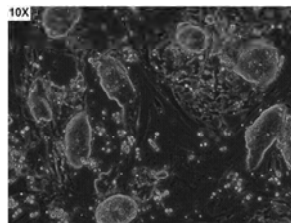
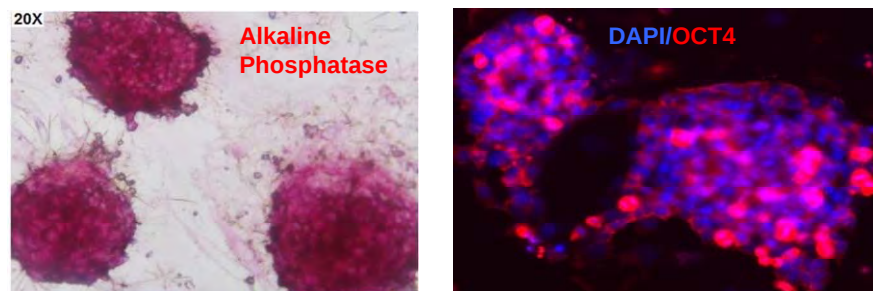


Figure 1. Generation of mouse induced pluripotent stem cells (iPSCs). **A.** Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of the reprogramming factors OCT4, SOX2, MYC and KLF4. Δ indicates the SIN design with partially deleted U3 of the 3' long terminal repeat. cPPT, central polypurine tract; Wpre, posttranscriptional regulatory element; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; ψ, packaging signal; 2a, a self-cleavage site derived from equine rhinitis A virus. **B.** Experimental strategy for reprogramming mouse Sca1⁺ cells using lentiviral vectors. **C.** Shown are iPSC colonies.

1-B) Characterization of mouse iPS cells

To test whether the generated iPS cells are bona fide pluripotent stem cells, we performed immunostaining and teratoma assay. As shown in **Figure 2**, the iPS cell colonies expressed pluripotency markers like alkaline phosphatase, Oct4, Sox2, and Nanog. The formation of teratoma in mice is the gold standard of pluripotency of pluripotent stem cells. We subcutaneously injected 1×10^6 iPS cells into each mouse. Four weeks after injection, teratomas were developed in all the mice (**Figure 2**). These data suggest that the generated iPS cells are pluripotent.



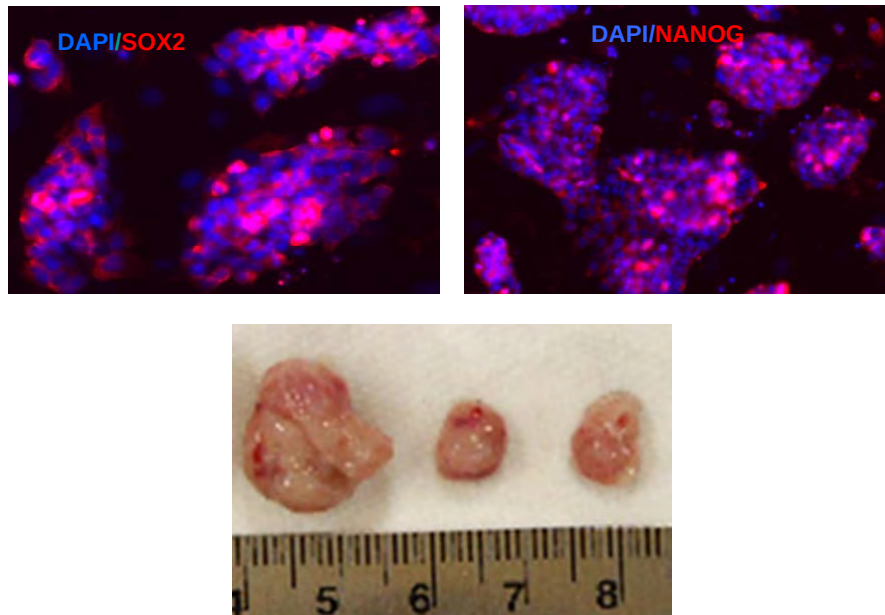


Figure 2. Characterization of mouse iPS cells. Immunostaining of iPS cells with stem cell markers alkaline phosphatase, Oct4, Sox2, and Nanog. **Bottom:** Teratomas from mice that were injected with iPS cells.

1-C) Generation of integration-free mouse iPS cells using episomal vectors.

For clinical applications, integration-free or footprint-free iPSCs need to be used to prevent potential adverse effects due to retroviral or lentiviral integration or due to the interference of residual expression of reprogramming factors on the differentiation of iPSCs into progenies of clinical interest.⁷⁻⁸ Toward this goal, several approaches have been used for obtaining integration or transgene-free iPSCs, including the use of plasmids,⁹ the Cre/loxP system,¹⁰⁻¹¹ adenoviruses,¹²⁻¹³ piggyBac transposon,¹⁴⁻¹⁵ minicircle DNA,¹⁶ protein transduction,¹⁷⁻¹⁸ sendai virus,¹⁹ and miRNA.²⁰ However, these methods suffer from low efficiency, require repetitive induction or selection, or require virus production. Synthetic modified mRNA might solve the problem,²¹ but it requires the daily addition of mRNA by lipofection.

Several investigators have used the EBNA1-based episomal vector due to its unique features: 1) only one transfection of vector DNA by nucleofection is needed for efficient reprogramming, and 2) the vector is lost in 5% or more cells after each cell division, leading to depletion of the episomal vector from cells after long-term passage. Recently, several groups have successfully used the pCEP4 episomal vector to generate footprint-free iPSCs.²²⁻²⁴ However, in those studies, five to seven factors, including strong oncogenes like MYC and/or simian virus 40 large T antigen (SV40LT) were used, which raises safety concerns for clinical use of iPSCs.

We improved the vector design by using a strong promoter SFFV and including wpre in the vector, which substantially increased expression of reprogramming factors (**Figure 3**). After multiple tests, we have successfully generated mouse iPSCs. We electroporated mouse cells with EV vectors that express OCT4, SOX2, MYC and KLF4 followed by culturing in mouse iPSC medium supplemented with small molecules like forskolin, CHIR99021, OAC-1 and PD0325901. At 2-3 weeks after culturing mouse cells on feeder cells, we observed 5-10 colonies. These colonies were picked for further culture in mouse iPSC medium.

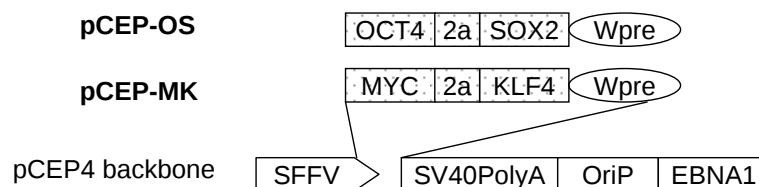
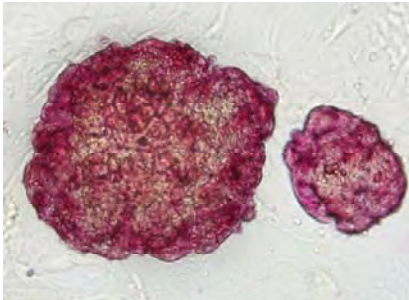


Figure 3. Schematic of episomal vectors used for generating integration-free iPSCs. Reprogramming factors were cloned into the pCEP4 backbone; their expression is driven by SFFV (Spleen focus-forming virus U3 promoter). 2a is a self-cleavage site derived from equine rhinitis A virus. Wpre, posttranscriptional regulatory element; SV40PolyA, polyadenylation signal from SV40 virus; OriP, EBV origin of replication; EBNA1, Epstein-Barr nuclear antigen 1, which plays essential roles in replication and persistence of episomal plasmid in infected cells.

We characterized these cells by ALP (alkaline phosphatase) staining, flow cytometry and teratoma assay. ALP staining showed that the generated mouse iPSC cells express pluripotency marker ALP at high levels (**Figure 4A**). Flow cytometry analysis showed that integration-free iPSCs have typical mouse iPSC phenotype that is SSEA1⁺ and SSEA3⁻ (**Figure 4B**). One month after injection of iPSCs into mice, teratomas were formed from injected cells. HE staining of the tumors demonstrated mouse iPSCs can form tumors composed of endoderm, ectoderm and mesoderm tissues. Taken together, we have generated integration-free mouse iPSCs with episomal vectors and these cells are truly pluripotent.

A. Mouse iPSC colony (ALP⁺)



B

SSEA1

SSEA3

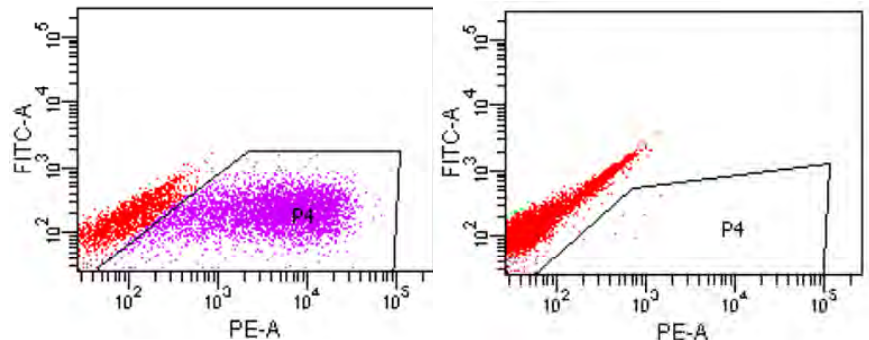


Figure 4. Generation of integration-free iPSCs from adult PBMCs with episomal vectors. (A) ALP staining of mouse iPSCs. **(B)** Flow cytometry analysis showed that mouse iPSCs express typical pluripotency markers like SSEA1, but do not express human iPSC marker SSEA3.

2. iPSC engineering, differentiation and MSC generation

2-A) Differentiation of mouse iPSCs into MSCs

After several tests, we have established a protocol that can efficiently differentiate mouse iPSCs into MSCs. iPSCs were harvested from MEF feeders and cultured in fibronectin-treated non-TC well plates with MEM10% FBS. Five days later, the formed embryoid body (EB) were dissociated into single cells or small clumps with collagenase and cultured in gelatin-treated well plates in MesenCult® MSC Basal Medium (Stemcell Technologies). After several passages, we analyzed iPSC-MSCs by immunohistochemical staining. **Figure 5** shows that iPSC-derived MSCs express markers of mouse MSCs.

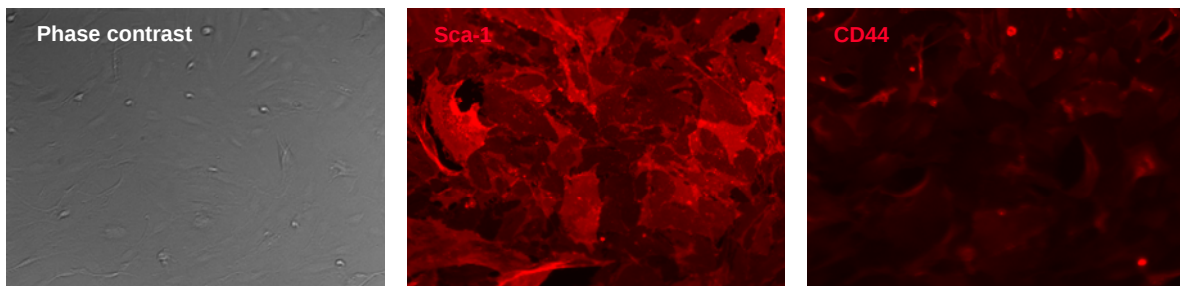


Figure 5. iPSC-derived MSCs manifest typical mesenchymal stem cell morphology and show high-level expression of typical mouse MSC markers Sca-1 and CD44.

We also tested the in vitro functionality of iPSC-derived MSCs using standard method. We found that these cells can be differentiated into adipocytes, osteogenic cells, and chondrocytes (**Figure 6**). These data suggest that our iPSC-MSCs are functional



Figure 6. Multilineage differentiation capacity of iPSC-derived MSCs. iPSC-derived MSCs were culture in differentiation media for 3 weeks and then stained with Alizarin Red S staining for osteogenic differentiation, Alcian Blue staining for chondrocytic differentiation, and Oil Red O staining for adipocytic differentiation.

2-B) Derivation of mouse BM-MSCs

BM MSCs were isolated and cultured using standard protocols. Bone marrow cells from C57BL/6 mice were collected by flushing the femurs and tibias from 6–8-week-old mice with MEM medium supplemented with 5% heat-inactivated fetal calf serum (FCS) (Invitrogen, CA, USA). Erythrocytes-depleted bone marrow cells were plated in MEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Culture medium was changed at day 2 to remove nonadherent cells. Whole medium was subsequently replaced weekly. The cells were grown for 2–3 weeks until almost confluent. Adherent cells were then detached by Accutase treatment and replated using a 1:4 dilution. We can culture these BM MSCs for up to 2 months.

2-C) Transduction of MSCs with lentivirus

In preparation for in vivo transplantation, we cloned several lentiviral vectors for animal studies (**Figure 6**). In all the vectors, GFP is also expressed to facilitate examination of transplanted MSCs by flow cytometry. CXCR4 is a commonly used factor to promote stem cell homing to the marrow niche. shNoggin may promote osteoblastic differentiation. Recently, we found that systemic FGF2 can strongly increase bone formation ²⁵.

We have generated lentivirus for these constructs. Co-culture of lentivirus with BM MSCs or iPSC-MSCs at an MOI of 1 led to a high transduction efficiency of ~95%. These results lay a foundation for our future studies.

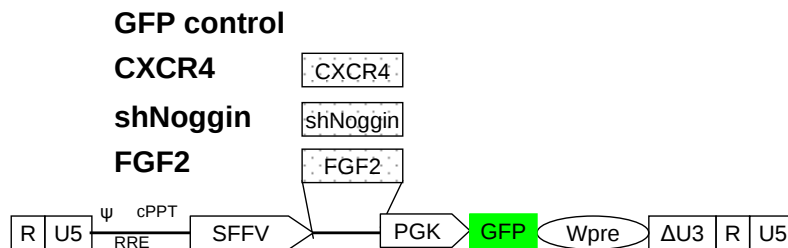


Figure 7. Lentiviral vectors. Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of homing factor CXCR4 and osteogenic factors shNoggin and FGF2. Δ indicates the SIN design with partially deleted U3 of the 3' long terminal repeat. cPPT, central polypurine tract; Wpre, posttranscriptional regulatory element; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; ψ, packaging signal. GFP expression is driven by PGK promoter.

3. Direct reprogramming peripheral blood cells into MSCs

An alternative strategy for blood stem cell-based skeletal gene therapy is to convert blood CD34⁺ cells directly into skeletal stem cells or mesenchymal stem cells. Last year, we found that induced mesenchymal stem cells (iMSCs) can be generated from cord blood CD34⁺ cells.²⁶ This objective is to generate MSCs from peripheral blood (PB) CD34⁺ cells. After transduction of PB CD34⁺ cells with Lenti SFFV-OCT4, iMSC colonies were formed in 1 week. The reprogramming of PB and CB CD34⁺ cells was equally efficient.

To optimize the reprogramming protocol, we tested whether small molecules capable of enhancing reprogramming of somatic cells into iPSCs, such as GSK3 inhibitor CHIR99021 (CHIR), MEK inhibitor, and ALK5 inhibitor, can also increase the efficiency of direct reprogramming of CB CD34⁺ cells into iMSCs. After a series of experiments, the only factor we found to substantially increase reprogramming efficiency was CHIR, which increased reprogramming efficiency from 3% to 15% (**Figure 8AB**). In addition, PB iMSCs were morphologically identical to CB iMSCs (**Figure 8C vs. 8D**). These data suggest that OCT4 can reprogram both fetal and adult hematopoietic cells at the same efficiency.

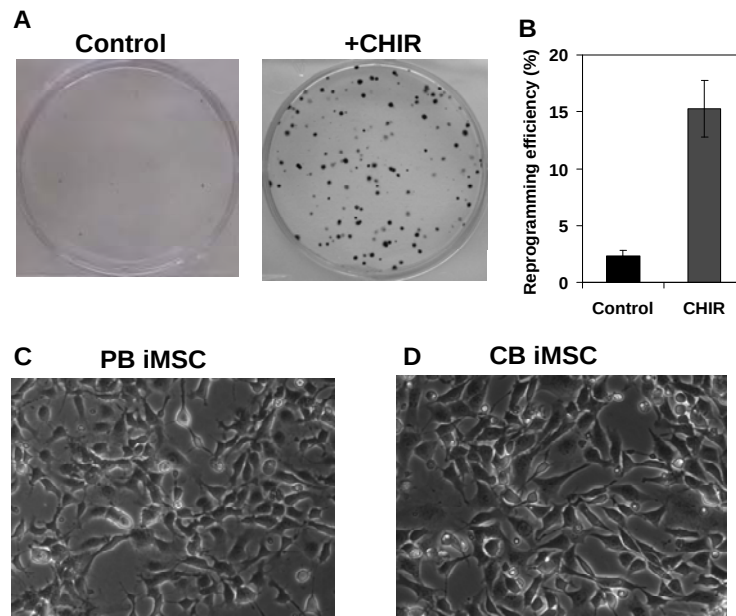


Figure 8. Efficient reprogramming of adult PB CD34⁺ cells into iMSCs. (A) MSC-like colonies formed from 1000 OCT4-transduced PB CD34⁺ cells in the absence (Control) or presence of CHIR99021 (+CHIR) after 9 days of culture in the MSC medium. (B) CHIR substantially increases reprogramming efficiency of adult PB cells ($n = 3$; $P < 0.01$). Error bar: mean $\pm$ SEM. Typical morphology of PB iMSCs (C) and CB iMSCs at 1 month after OCT4 transduction.

To test if iMSCs manifest typical markers of MSCs, we conducted FACS analysis. **Figure 9** shows that these cells express typical MSC markers like CD73, CD105, CD29, CD44, CD90 and CD166. In addition, PB iMSCs do not express hematopoietic and endothelial markers CD14, CD31, and CD34. These data suggest that we are able to generate MSCs from peripheral blood CD34⁺ cells.

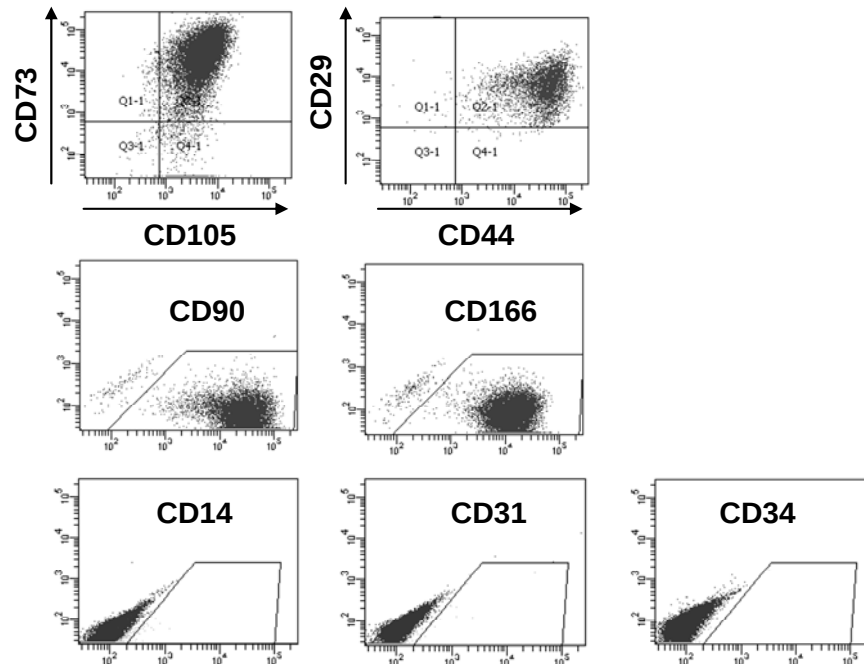


Figure 9: Phenotyping analysis of PB iMSC. Flow cytometry plots show that PB iMSCs express the MSC surface markers CD73, CD105, CD29, CD44, CD90, and CD166, while they do not express hematopoietic and endothelial markers CD14, CD31, and CD34.

Taken together, we have been able to generate MSCs from blood cells efficiently. The ability to generate large quantities of MSCs rapidly and efficiently from blood cells has important implications for regenerative medicine in general and in particular bone regeneration and bone fracture healing. An enticing future possibility is in vivo reprogramming of blood stem cells into mesenchymal stem cells for bone regeneration.

KEY RESEARCH ACCOMPLISHMENTS

- Generated mouse iPSCs from hematopoietic cells with lentiviral vectors.
- Developed a novel episomal vector and generated integration-free iPSCs.
- Differentiated mouse iPSCs into functional MSCs.
- Reprogrammed blood cells into MSCs.

REPORTABLE OUTCOMES

- Developed a novel episomal vector for generating integration-free iPSCs
- Developed a mouse integration-free iPSC cell line.
- Developed a novel technology for direct conversion of blood cells into mesenchymal stem cells
- This project contributed to the publication of 3 papers.
- This grant supported a technician who is now a PhD student.

CONCLUSION

We have generated mouse iPSCs from hematopoietic cells and differentiated iPSCs into functional MSCs. We also developed a novel episomal vector, which will have important applications in generating integration-free human iPSCs for clinical therapy. New findings in the past 2 years may have made our original proposal obsolete. As such, we changed the SOW and made a novel discovery in year 2: direct reprogramming blood cells into MSCs without iPSC generation. The impact of this discovery to whole field of regenerative medicine has yet to be unfolded in the coming years.

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APPENDICES

Publications

1. Meng X, Neises A, Su RJ, Payne KJ, Ritter L, Gridley DS, Wang J, Sheng M, Lau KH, Baylink DJ, **Zhang XB**: Efficient reprogramming of human cord blood CD34+ cells into induced pluripotent stem cells with OCT4 and SOX2 alone. Molecular Therapy. 2012 Feb;20(2):408-16. doi: 10.1038/mt.2011.258. Epub 2011 Nov 22.
2. Meng X*, Su RJ*, Baylink DJ, Neises A, Kiroyan JB, Lee W, Payne KJ, Gridley DS, Wang J, Lau KHW, Li G, **Zhang XB**: Rapid and efficient reprogramming of human fetal and adult blood CD34+ cells into mesenchymal stem cells with a single factor. Cell Research. 2013, May;23(5):658-72. doi: 10.1038/cr.2013.40.
3. Su RJ, Baylink DJ, Neises A, Meng X, Kiroyan JB, Payne KJ, Tschudy-Seney B, Duan Y, Appleby N, Kearns-Jonker M, Gridley DS, Wang J, Lau KHW, **Zhang XB**: Efficient generation of integration-free iPS cells from human adult peripheral blood using BCL-XL together with Yamanaka factors. PLOS ONE. 2013, 8(5): e64496. doi:10.1371/journal.pone.0064496.

Efficient Reprogramming of Human Cord Blood CD34⁺ Cells Into Induced Pluripotent Stem Cells With OCT4 and SOX2 Alone

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The reprogramming of cord blood (CB) cells into induced pluripotent stem cells (iPSCs) has potential applications in regenerative medicine by converting CB banks into iPSC banks for allogeneic cell replacement therapy. Therefore, further investigation into novel approaches for efficient reprogramming is necessary. Here, we show that the lentiviral expression of OCT4 together with SOX2 (OS) driven by a strong spleen focus-forming virus (SFFV) promoter in a single vector can convert 2% of CB CD34⁺ cells into iPSCs without additional reprogramming factors. Reprogramming efficiency was found to be critically dependent upon expression levels of OS. To generate transgene-free iPSCs, we developed an improved episomal vector with a woodchuck post-transcriptional regulatory element (Wpre) that increases transgene expression by 50%. With this vector, we successfully generated transgene-free iPSCs using OS alone. In conclusion, high-level expression of OS alone is sufficient for efficient reprogramming of CB CD34⁺ cells into iPSCs. This report is the first to describe the generation of transgene-free iPSCs with the use of OCT4 and SOX2 alone. These findings have important implications for the clinical applications of iPSCs.

Received 6 September 2011; accepted 31 October 2011; published online 22 November 2011. doi:10.1038/mt.2011.258

INTRODUCTION

The ability to generate induced pluripotent stem cells (iPSCs) from somatic cells has opened up a new avenue for regenerative medicine. Earlier studies used fibroblasts, such as those derived from a skin biopsy, to generate iPSCs by overexpression of Yamanaka factors (OCT4, SOX2, MYC and KLF4, or OSMK) or Thomson/Yu factors (OCT4, SOX2, NANOG, and LIN28).^{1,2} However, it takes several weeks to prepare cells from a skin biopsy for reprogramming.^{1,3} Later, hematopoietic stem/progenitor cells or CD34⁺ cells from mobilized peripheral blood, bone marrow, or cord blood (CB) captured much attention because blood cells

can be used immediately for reprogramming.^{4–6} However, isolation of mobilized peripheral blood and bone marrow is invasive, time consuming and has potential risks for the donor, while harvesting CB cells has none of these limitations. In addition, >400,000 fully characterized and HLA-typed CB units are stored in public banks and are readily available for clinical therapy.⁷ Moreover, CB has the youngest somatic cells and is expected to carry minimal genetic mutations induced by UV radiation.^{8,9} Due to its unique advantages as donor cells for the production of clinical-grade human iPSCs, CB is believed to be one of the best sources for reprogramming. An additional advantage is the potential of converting CB banks into iPSC banks for allogeneic cell-based therapy.¹⁰

For clinical applications, transgene-free or footprint-free iPSCs need to be used to prevent potential adverse effects due to retroviral or lentiviral integration or due to the interference of residual expression of reprogramming factors on the differentiation of iPSCs into progenies of clinical interest.^{11,12} Toward this goal, several approaches have been used for obtaining integration or transgene-free iPSCs, including the use of plasmids,¹³ the Cre/loxP system,^{14,15} adenoviruses,^{16,17} piggyBac transposon,^{18,19} minicircle DNA,²⁰ protein transduction,^{21,22} Sendai virus,²³ and miRNA.²⁴ However, these methods suffer from low efficiency, require repetitive induction or selection, or require virus production. Synthetic modified mRNA might solve the problem,²⁵ but it requires the daily addition of mRNA by lipofection and CB CD34⁺ cells are among the most difficult to transfect by lipofection.

Several investigators have used the EBNA1-based episomal vector due to its unique features: (i) only one transfection of vector DNA by nucleofection is needed for efficient reprogramming, and (ii) the vector is lost in 5% or more cells after each cell division, leading to depletion of the episomal vector from cells after long-term passage. Recently, several groups have successfully used the pCEP4 episomal vector to generate footprint-free iPSCs.^{26–28} However, in those studies, five to seven factors, including strong oncogenes like MYC and/or simian virus 40 large T antigen (SV40LT) were used, which raises safety concerns for the clinical use of iPSCs.

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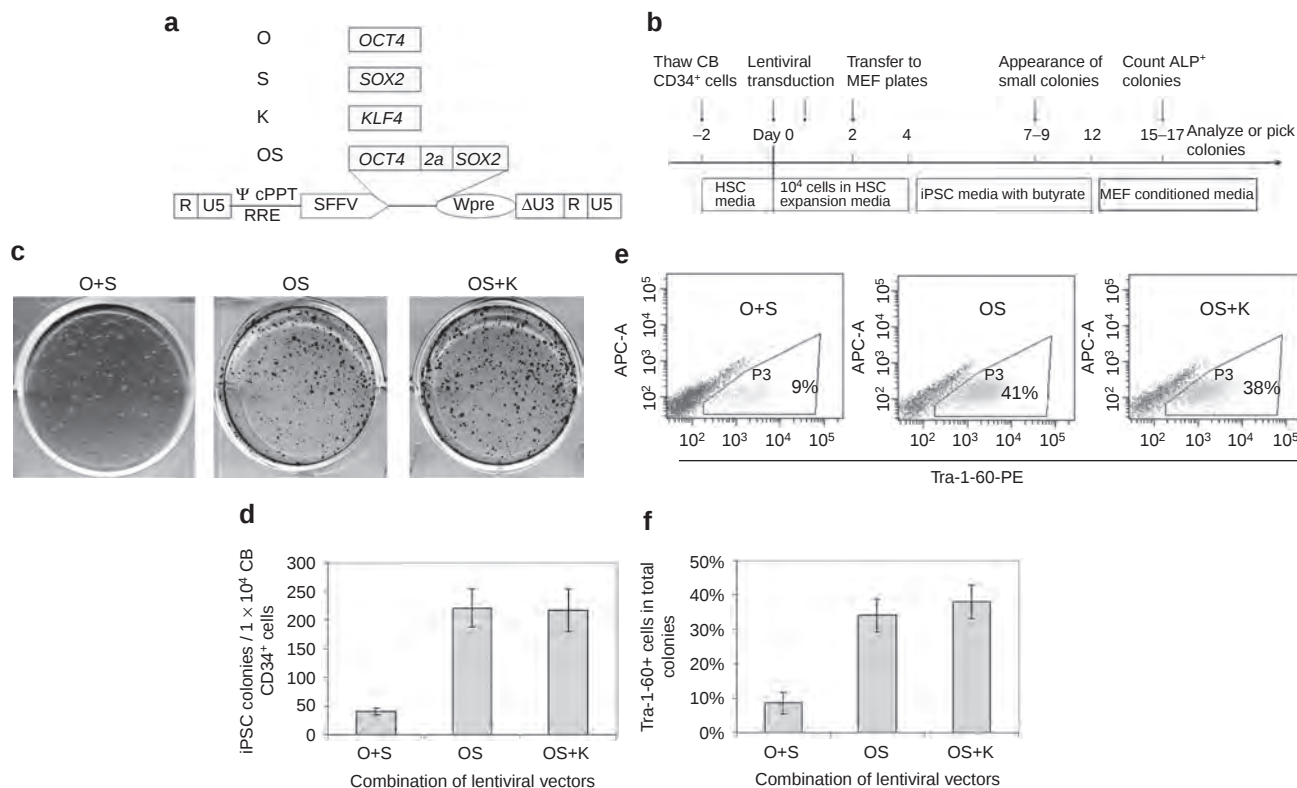


Figure 1 Lentiviral vector-mediated expression of OCT4 and SOX2 efficiently reprogram cord blood (CB) CD34⁺ cells into induced pluripotent stem cells (iPSCs). **(a)** Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of the human reprogramming factor OCT4, SOX2, KLF4. Δ indicates the SIN design with partially deleted U3 of the 3' long-terminal repeat. cPPT, central polypurine tract; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; Wpre, woodchuck post-transcriptional regulatory element; ψ, packaging signal; 2a, a self-cleavage site derived from equine rhinitis A virus. **(b)** Experimental strategy for reprogramming human CB CD34⁺ cells using lentiviral vectors. **(c)** Representative alkaline phosphatase (ALP) staining of iPSC colonies 16 days after lentiviral transduction of 1 × 10⁴ CB CD34⁺ cells. O, OCT4; S, SOX2; K, KLF4. **(d)** Numbers of induced pluripotent stem cells (iPSCs) generated from 1 × 10⁴ CB CD34⁺ cells. *n* = 3. O+S vs. OS: *P* < 0.05. OS vs. OS+K: no significant difference. Data shown are presented as mean ± SEM. **(e)** Representative fluorescence-activated cell sorting (FACS) diagram of TRA-1-60 expression in cells undergoing reprogramming. Cells at day 16 after transduction were harvested and analyzed. **(f)** Percentages of TRA-1-60 positive cells in reprogramming cultures. O+S vs. OS: *P* < 0.05; OS vs. OS+K: no significant difference. Data shown are presented as mean ± s.e.m. (*n* = 3).

Earlier studies showed that OCT4 and SOX2 alone can reprogram CB cells into iPSCs, but at a very low efficiency.⁹ We hypothesized that reprogramming efficiency might depend on expression levels of reprogramming factors, which largely relies on the promoters used. It is well known that the strength of promoters is contextual; several studies have shown that the spleen focus-forming virus (SFFV) promoter is stronger in primary hematopoietic cells or hematopoietic cell lines than many commonly used promoters like human elongation factor 1α (EF1α), cytomegalovirus, and A2UCOE (ubiquitous chromatin opening element).^{29–32} Thus, we set out to determine whether iPSCs can be efficiently generated from CB CD34⁺ cells with the SFFV promoter being used to drive expression of OCT4 and SOX2.

RESULTS

Balanced expression of OCT4 and SOX2 by a lentiviral vector efficiently reprograms CB CD34⁺ cells into iPSCs

It has been reported that overexpression of OCT4 together with SOX2 (O+S) using a retroviral vector in 2 individual constructs can reprogram CB CD133⁺ cells into iPSCs.⁹ However, the efficiency is as low as 0.002–0.005%, making this approach impractical for

many applications. We hypothesized that the low efficiency might be due to low-level expression of the reprogramming factors O+S mediated by retroviral vectors. To test this assumption, we cloned reprogramming factors into a lentiviral vector driven by a strong promoter SFFV (Figure 1a).

As detailed in Figure 1b and the Materials and Methods section, CB CD34⁺ cells were transduced with lentiviral vectors that express reprogramming factors followed by iPSC generation by culturing transduced cells on mouse embryonic fibroblasts (MEFs). Of interest, in the O+S condition, dozens of small colonies were observed in each well as early as 4–5 days after seeding transduced CB cells onto MEF layers, however, morphologically iPSC-like cells did not appear until a week later (data not shown). Analysis of these non-iPSCs by flow cytometry indicated that many cells expressed mesenchymal markers (data not shown). We also tested the combination of OCT4 and SOX2 (abbreviated as OS for clarity) in a single vector with the use of self-cleavage peptide sequence 2a. In this condition, no colonies were observed in the first week, and the first iPSC-like colonies appeared at 8–10 days after CB transduction. These data suggest that balanced expression of OCT4 and SOX2 may inhibit the outgrowth of non-iPSCs.

In the O+S condition, we routinely observed 300–600 total colonies from 10,000 transduced CB CD34⁺ cells 2 weeks after transduction. However, the majority of colonies were morphologically non-iPSCs and alkaline phosphatase (ALP) staining showed that ~20% of the colonies were iPSC-like (Figure 1c). In the OS condition, we observed 200–250 colonies in each well, with ~80% of the colonies being morphologically iPSCs, which was further confirmed by ALP staining (Figure 1c,d). In agreement with these results, fluorescence-activated cell sorting (FACS) analysis of the cells in the reprogramming cultures showed that only 9% of the cells in the O+S condition expressed the iPSC marker TRA-1-60, whereas ~40% of the cells in the OS condition were TRA-1-60 positive (Figure 1e,f).

Together, our findings demonstrate that OCT4 and SOX2 alone can efficiently reprogram CB cells into iPSCs and that balanced expression of the two factors that are linked with a 2a self-cleavage peptide sequence can increase reprogramming efficiency and inhibit growth of non-iPSC colonies.

KLF4 does not increase efficiency of lenti SFFV-OS-mediated reprogramming

Because the use of additional factors has been shown to boost reprogramming efficiency, we tested the effects of including other factors like KLF4 in reprogramming. In sharp contrast to expectations, we found that the addition of KLF4 (K) to OS did not increase the reprogramming efficiency. This surprising finding is unlikely to be explained by differential expression levels of reprogramming factors because the same OS vector was used in both conditions, and the expression of KLF4 was confirmed in preliminary studies. In OS conditions with and without K, 2% of transduced CB cells were successfully converted into iPSCs and ~40% of cells in the reprogramming culture expressed the iPSC marker TRA-1-60 (Figure 1c–f). This data suggests that the expression of OS, driven by the SFFV promoter, is sufficient to reprogram CB CD34⁺ cells at high efficiency and addition of other factors like KLF4 does not significantly increase the reprogramming efficiency.

Efficiency of OS-mediated reprogramming depends on OS expression levels

Having observed up to a 1,000-fold higher efficiency in converting CB cells into iPSCs by OS compared to the previous report,⁹ we speculated that differences in the expression levels of OS might explain the large difference in reprogramming efficiency. Transgene expression levels are largely determined by the strength of promoters; we thus cloned lentiviral vectors in which green fluorescent protein (GFP) expression is driven by the PGK, EF1, or the SFFV promoter to determine the strength of these promoters in CD34⁺ cells (Figure 2a). FACS analysis showed that GFP expression driven by the PGK or the EF1 promoters is ~85% or ~60% lower than expression driven by the SFFV promoter in CB CD34⁺ cells (Figure 2b,c). We reasoned that GFP is more stable than transcription factors; the GFP intensity may not reflect OCT4 or SOX2 expression levels. To address this issue, we cloned OCT4GFP fusion gene-expressing vectors driven by the three promoters. In this system, GFP is fused to the protein of interest. Thus the GFP expression, as measured by fluorescence intensity,

can reflect the expression level of its fusion partner.³³ Similarly, we observed that the SFFV promoter drove highest level expression of OCT4GFP in CB CD34⁺ cells, followed by the EF1 and the PGK promoters (Figure 2d). Of note, GFP intensity was decreased by ~20-fold in OCT4GFP-transduced cells, as compared to GFP-transduced cells, and the differences in expression of OCT4GFP were less pronounced than that of GFP, which reflect the rapid turnover of OCT4 in CB CD34⁺ cells. Together, these data suggest that the SFFV promoter drives significantly higher levels of transgene expression in CB CD34⁺ cells than the PGK or EF1 promoters.

To investigate the effects of low OS expression on reprogramming efficiency, we used the weaker PGK and EF1 promoters to drive OS expression. In more than five independent experiments, no iPSC colonies could be generated from 1×10^4 CB CD34⁺ cells that were transduced with lenti PGK-OS or lenti EF1-OS vectors (Figure 2e). Given that expression of OCT4 is decreased by ~50% when driven by EF1 as compared to the SFFV promoter (Figure 2d), this observation suggests that a 50% decrease in OS expression could lead to reprogramming failure. In hopes of increasing OS expression and thereby reprogramming efficiency, we synthesized an OS gene (*synOS*) that was codon optimized by DNA 2.0 (Menlo Park, CA). In contrast to our expectation, expression of OS at the protein level by *synOS* was ~20% lower than the wild-type human OS. Of note, this small decrease in OS expression translated into a fourfold decrease in reprogramming efficiency (data not shown). This observation further supports our conclusion that OS-mediated high-efficiency reprogramming critically depends on OS expression levels, and a slight decrease in OS expression leads to a substantial drop in reprogramming efficiency, whereas a 50% decrease results in reprogramming failure.

MYC and KLF4 facilitate reprogramming when OS expression levels are low

Having found that low-level OS expression is insufficient to induce CB reprogramming, we further asked whether this can be rescued by MYC and KLF4. As anticipated, in CB CD34⁺ cells that were transduced with EF1-OS or SFFV-MK alone, no iPSCs were generated. In contrast, after transduction of CB CD34⁺ cells with both EF1-OS and SFFV-MK, 0.1% cells were converted into iPSCs (Figure 2f). ALP staining and FACS analysis of iPSCs did not show any obvious differences in the expression of pluripotency markers when compared with iPSCs generated with SFFV-OS (data not shown). Of interest, when *MYC* and *KLF4* expression was driven by the EF1 promoter, which leads to lower expression levels, no iPSCs could be generated (data not shown). Together, these findings suggest that high-level expression of OS alone is sufficient for CB reprogramming, whereas reprogramming under low-level OS expression requires other reprogramming factors.

Generation of footprint-free iPSCs using an episomal vector

The successful generation of iPSCs with a lentiviral vector that expresses OCT4 and SOX2 alone prompted us to ask whether this approach would also work in a nonviral system. To test this, we shuttle cloned SFFV-OS from the lentiviral vector construct

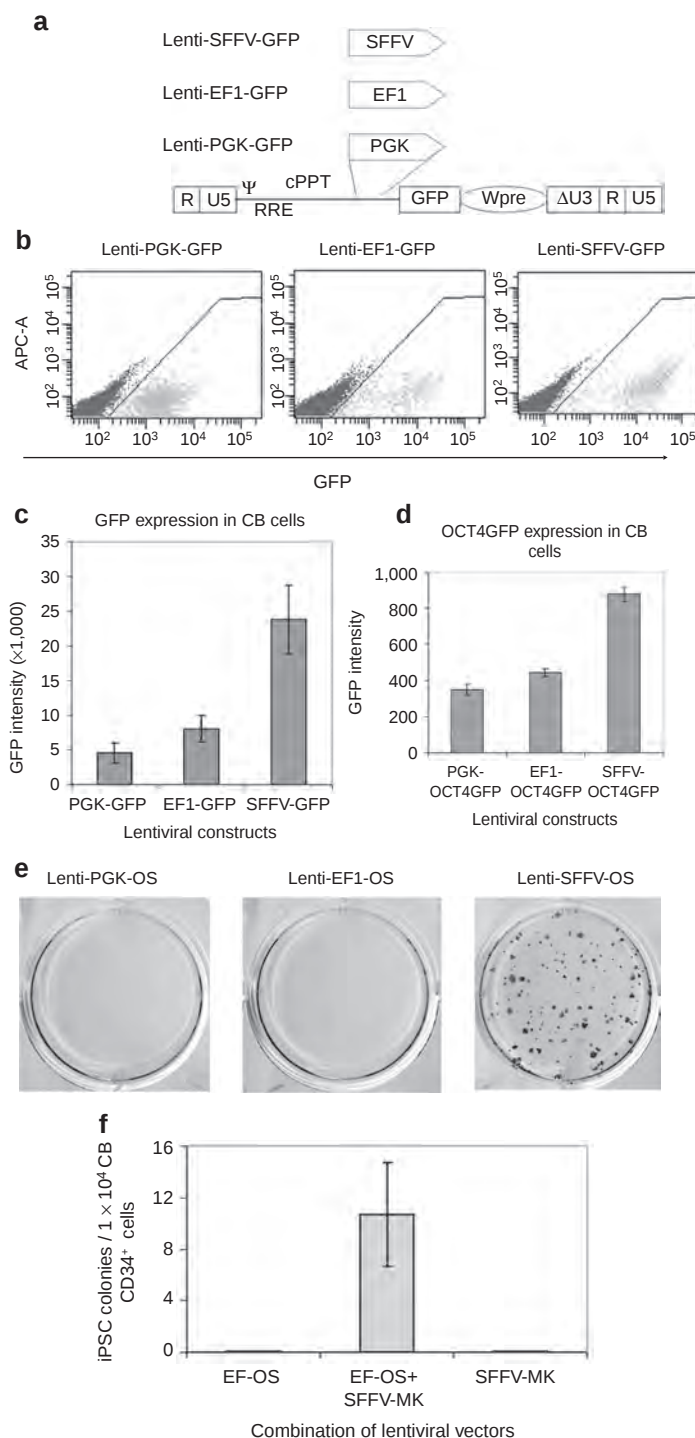


Figure 2 Efficiency of OCT4 and SOX2-mediated reprogramming depends on gene expression levels. **(a)** Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of green fluorescent protein (GFP). Δ indicates the SIN design with partially deleted U3 of the 3' long-terminal repeat. cPPT, central polypurine tract; EF1, elongation factor-1 α promoter; PGK, phosphoglycerokinase promoter; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; Wpre, post-transcriptional regulatory element; Ψ , packaging signal. **(b)** Representative levels of GFP expression driven by three different promoters in cord blood (CB) CD34⁺ cells. Fluorescence-activated cell sorting (FACS) analysis was conducted at 3 days post-transduction. **(c)** Distinct GFP expression levels driven by three different promoters in CB CD34⁺ cells. $n = 3$. PGK-GFP vs. EF-GFP: $P = 0.05$; EF-GFP vs. SFFV-GFP: $P < 0.05$. **(d)** Increased expression of OCT4GFP fusion gene driven by SFFV promoter compared to PGK and EF1 in CB CD34⁺ cells. FACS analysis was conducted at 3 days post-transduction. $n = 3$. PGK-OCT4GFP vs. EF1-OCT4GFP: $P = 0.06$; EF1-OCT4GFP vs. SFFV-OCT4GFP: $P < 0.01$. **(e)** Alkaline phosphatase (ALP) staining for iPSC cultures from CB cells transduced with PGK-OS, EF1-OS, and SFFV-OS. Note that no colonies were generated in PGK-OS, EF1-OS conditions. **(f)** Expression of MYC and KLF4 rescues failure of low level OS expression driven by EF1 promoter in generating induced pluripotent stem cells (iPSCs) from CB CD34⁺ cells. Graphed data are presented as mean $\pm$ SEM ($n = 3$).

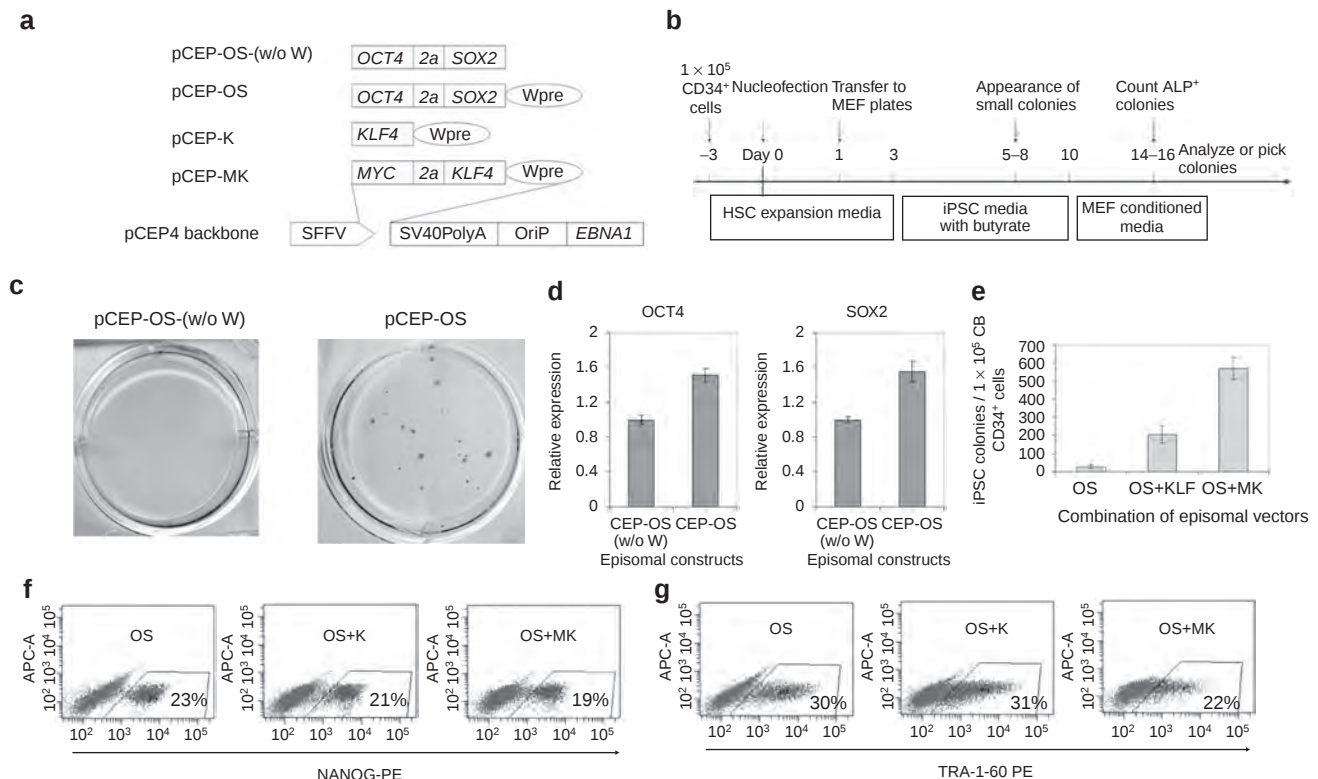


Figure 3 OCT4 and SOX2-mediated reprogramming using episomal vectors. (a) Schematic of episomal vectors used in this study for conversion of cord blood (CB) CD34⁺ cells into induced pluripotent stem cells (iPSCs). Reprogramming factors were cloned into the pCEP4 backbone; their expression is driven by spleen focus-forming virus U3 promoter (SFFV). 2a is a self-cleavage site derived from equine rhinitis A virus. Wpre, post-transcriptional regulatory element; SV40PolyA, polyadenylation signal from SV40 virus; OriP, EBV origin of replication; EBNA1, Epstein-Barr nuclear antigen 1, which plays essential roles in replication and persistence of episomal plasmid in infected cells. (b) Experimental strategy for reprogramming human CB CD34⁺ cells using EBNA1-based episomal vectors. (c) Representative alkaline phosphatase (ALP) staining shows that inclusion of the Wpre element in the episomal vector pCEP-OS (w/o W) results in successful reprogramming. $n = 3$. Colonies are from 1×10^5 CB CD34⁺ cells. (d) Inclusion of Wpre element in the CEP episomal vector increases gene expression. 293T cells were infected with same amount of plasmids. 3 days after transfection, OCT4 and SOX2 expression was examined by intracellular staining and fluorescence-activated cell sorting (FACS) analysis. $n = 3$. pCEP-OS (w/o W) vs. pCEP-OS: $P < 0.05$. (e) Numbers of ALP positive iPSC colonies at 16 days post-transfection of 1×10^5 CB CD34⁺ cells with pCEP-OS (OS) and pCEP-K (K) or pCEP-MK (MK). $n = 3$. OS vs. OS+K: $P < 0.05$; OS+K vs. OS+MK: $P < 0.05$. Expression of the iPSC markers (f) NANOG and (g) TRA-1-60 in cultures reprogrammed using three different combinations of episomal vectors. Cells were harvested for FACS analysis 20 days after nucleofection.

into a pCEP4 EBNA1/OriP-based episomal vector (Figure 3a). To generate iPSCs, 1×10^5 CB CD34⁺ cells were cultured in Iscove's modified Dulbecco's medium/10% fetal bovine serum with cytokines SCF, FL, and TPO. After 3 days of culture, the total cell number increased by approximately fivefold and all the cells were harvested for nucleofection with the pCEP-OS (w/o W) plasmid (Figure 3b). In three independent experiments, we failed to generate any iPSCs (left panel of Figure 3c). We reasoned that this failure might be due to the low-level expression of OS mediated by this vector. We then cloned woodchuck post-transcriptional regulatory element (Wpre), a post-transcriptional regulatory element that is commonly used in lentiviral systems to enhance gene expression levels, into the pCEP-OS (w/o W) plasmid (Figure 3a). As expected, the inclusion of Wpre in the episomal vector led to a 50% increase in OCT4 expression and a 55% increase in SOX2 expression (Figure 3d). Using pCEP-OS, we successfully generated ~20 iPSC colonies from the progeny of 1×10^5 freshly thawed CB CD34⁺ cells (Figure 3c,e).

To better compare our improved vector with published results, we evaluated the effects of KLF4 or MK (MYC and KLF4)

together with OS on the efficiency of CB reprogramming. With the addition of KLF4, the reprogramming efficiency increased by eightfold, and further inclusion of MYC led to an additional threefold increase (Figure 3e). Of interest, the appearance of the first iPSC-like colonies was observed at 9–10, 6–7, and 4–5 days after cells were transfected with episomal OS, OS+K, and OS+MK plasmids, respectively. This data suggests that addition of KLF4 and/or MYC accelerates the reprogramming process. Of note, using two episomal vectors that express four factors, we generated up to 600 iPSC colonies from 1×10^5 CB CD34⁺ cells, compared to 80 colonies from the same amount of CB CD34⁺ cells even with five factors (OSMK + LIN28).²⁶ These data suggest that our improved episomal vector is substantially more efficient in reprogramming CB cells into iPSCs than previously reported.

We conducted further tests to examine the differences in the expression of pluripotency markers between iPSCs generated with the three different combinations of episomal vectors. Immunostaining and FACS analysis showed that 20–30% of cells expressed the iPSC markers NANOG and TRA-1-60 in all the

three combinations, whereas including MYC appeared to decrease the portion of Tra-1-60 positive iPSCs in reprogramming culture (Figure 3f,g).

Taken together, these data demonstrate that we have developed an episomal vector in which increased expression of reprogramming factors leads to efficient reprogramming of CB cells

into iPSCs. We show for the first time, that iPSCs can be generated with the episomal vector that expresses only OCT4 and SOX2.

Characterization of iPSC colonies generated with the pCEP-OS plasmid

To characterize iPSCs, we randomly picked 10 colonies from the pCEP-OS reprogrammed cultures and passaged iPSCs for >3 months. Real-time PCR analysis with two pairs of primers showed that at passage 0, ~0.5 copy of the pCEP-OS plasmid per cell could be detected. After eight passages, the average copy number of residual CEP plasmid decreased to 0.001–0.007/genome and in 2 out of 10 clones, the presence of CEP plasmid was undetectable (Figure 4a). After 12 passages, residual episomal plasmid was disappeared in the majority of clones (data not shown). This finding is consistent with previous reports showing that the presence of episomal vector is undetectable in most iPSC colonies after 10–14 passages.²⁶

To extensively characterize pCEP-OS generated iPSCs, we selected several clones for a series of tests. Immunostaining of iPSC colonies showed that they expressed typical human iPSC-specific transcription factors OCT4, SOX2, NANOG, and surface markers SSEA-3, SSEA-4, and Tra-1-60 (Figure 4b). Karyotype analysis indicated a normal human karyotype for all the clones tested; one representative is shown in Figure 4c. Sulphite sequencing showed that both the *OCT4* and *NANOG* promoters were demethylated in three randomly picked iPSC clones (Figure 4d). When injected into immunodeficient NSG mice, iPSCs formed teratomas consisting of derivatives of all three embryonic germ layers, demonstrating the pluripotency of these iPSCs (Figure 4e). Together, these data suggest that *bona fide* transgene-free iPSCs can be generated from human CB CD34⁺ cells by nucleofection of a pCEP episomal plasmid that expresses OCT4 and SOX2 alone.

DISCUSSION

Here, we report that iPSCs can be generated from human CB CD34⁺ cells in 2–3 weeks with the use of OCT4 and SOX2 alone. We found that lentiviral vector-mediated transduction of OS is sufficient to reprogram 2% of transduced CB CD34⁺ cells into iPSCs. This efficiency is up to 1,000-fold higher than previously reported,⁹ which is attributed to the SFFV promoter-mediated high-level expression of OS. Furthermore, with the use of an

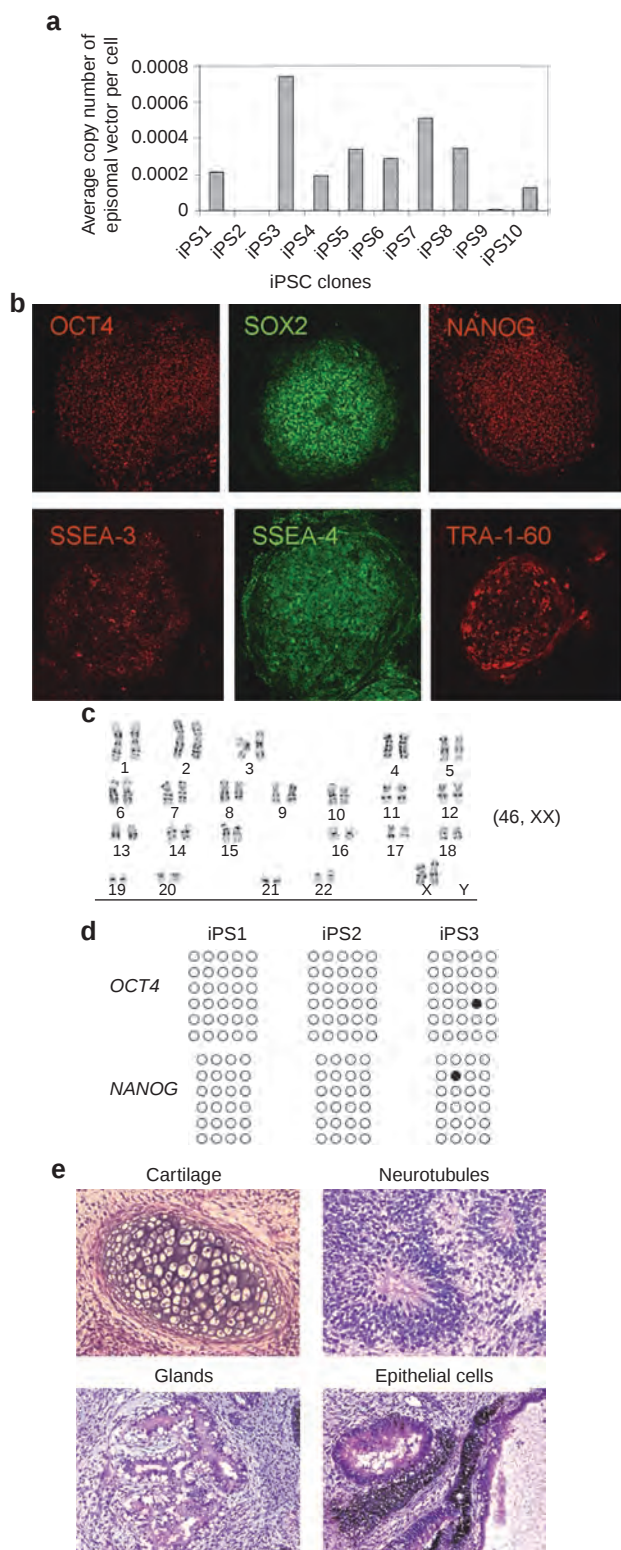


Figure 4 Characterization of induced pluripotent stem cells (iPSCs) generated with pCEP-OS. **(a)** Copies of residual episomal vectors after eight passages as indicated by real-time PCR. Data shown are from one pair of primers. Similar results were obtained with second pair of primers. **(b)** Immunohistochemistry analysis of a representative iPSC line showing expression of indicated pluripotency markers. Images were captured using the Zeiss LSM 710 confocal microscope with a $\times 10$ objective. **(c)** A representative karyogram of an iPSC clone. All analyzed iPSC clones showed a normal karyotype. **(d)** Bisulphite genomic sequencing of the *OCT4* and *NANOG* promoters indicates demethylation in three independent clones. Each horizontal row of circles represents an individual sequencing reaction of a given amplicon. Open and filled circles represent unmethylated and methylated CpG dinucleotides, respectively. **(e)** Hematoxylin and eosin (H&E) staining of representative teratoma from pCEP-OS cord blood (CB) iPSCs shows derivatives of three embryonic germ layers. Cartilage (mesoderm); neurotubules with rosettes (ectoderm); glands (endoderm); retina epithelial cells with pigments (ectoderm). Images were acquired using the Olympus microscope with a $\times 20$ objective.

improved OS-expressing episomal vector in which the inclusion of Wpre increases transgene expression by 50%, 20 footprint-free iPSCs can be generated from 1×10^5 CB CD34⁺ cells, an amount that can be purified from ~1 ml of CB. To the best of our knowledge, this is the first report that footprint-free iPSCs can be generated with only two factors.

Striking progress in iPSC reprogramming has been made over the past several years. iPSCs can be generated from almost any kind of mammalian cells. However, recent reports that describe exceedingly high rates of genetic point mutations and gene copy number variations have shifted the research focus from reprogramming efficiency to reprogramming safety.^{34,35} Two parameters are likely to be the key to the generation of safe iPSCs for clinical use: cell source and reprogramming method. It is widely accepted that CB is one of the best cell sources for reprogramming. However, one of the four transcription factors originally used by Yamanaka and Takahashi for cell reprogramming, MYC, is oncogenic. Overexpression of MYC has been shown to induce malignant transformation.³⁶ Another commonly used reprogramming booster SV40LT is also oncogenic. SV40LT functions by inhibition of the p53 and Rb-family of tumor suppressors and ectopic expression of SV40LT induces *in vitro* cellular transformation and *in vivo* tumorigenesis.³⁷ Although expression of reprogramming factors is only required for ~2 weeks, this short-term exposure to MYC may elicit adverse effects on genomic stability.³⁸ Therefore, we propose that an ideal combination of reprogramming factors should be devoid of factors whose overexpression has been demonstrated to induce cellular transformation and *in vivo* tumorigenesis.

With safety considerations in mind, we initiated experiments to optimize reprogramming conditions using only OS expressed by a lentiviral vector. We found that high-level expression of OS, driven by a strong promoter SFFV, led to the conversion of 2% of transduced cells into iPSCs. This efficiency is up to 1,000-fold higher than previously reported for these factors.⁹ An ~20% decrease in OS expression levels led to a fourfold decrease in efficiency. Moreover, when OS expression was decreased by 50% or more with the use of promoters like EF1 and PGK, no iPSCs could be generated from CB CD34⁺ cells. These findings establish that reprogramming of CB cells with OS critically depends on the expression levels of these genes. It is tempting to speculate that high-level expression of OCT4 and SOX2 alone could also reprogram other cells like fibroblasts. However, SFFV is not necessarily a strong promoter in cell types other than hematopoietic cells. For instance, the EF1 promoter drives higher-level expression of transgenes in fibroblasts than the SFFV promoter (data not shown).

To generate footprint-free iPSCs, we used an episomal vector. In the absence of the Wpre element, the OS-expressing pCEP episomal vector was insufficient to reprogram CB cells into iPSCs. However, an improved episomal vector design that included Wpre at the 3' end of the transgene and in front of the PolyA signal, led to the successful generation of iPSCs. Of note, sodium butyrate was used for ~10 days in our reprogramming culture. Omitting sodium butyrate led to a considerable decrease in reprogramming efficiency (data not shown). This data suggests that sodium butyrate is also crucial for episomal vector-mediated cellular

reprogramming. Characterization of iPSC colonies showed no differences in iPSC quality between different combinations of reprogramming factors, as evidenced by a series of *in vitro* and *in vivo* tests. Moreover, after 12 passages, no integration or residual episomal plasmid can be identified in most clones by sensitive real-time PCR analysis. However, a caveat is that this does not necessarily mean there is no integration of small fragments in these iPSC clones. Such fragments can only be detected by whole genome sequencing. While the reprogramming efficiency mediated by pCEP-OS is relatively low, this system is capable of generating sufficient numbers (20 iPSCs/ml of CB) of iPSCs for allogeneic cell therapy.

The generation of transgene-free iPSCs from CB cells has recently been reported by several groups. Yu and colleagues found that the use of episomal vectors expressing seven factors can highly efficiently reprogram CB cells; however no iPSCs could be generated in the absence of SV40LT expression.²⁷ Using a 5-in-1 vector (OSMK and LIN28), Cheng and colleagues were able to generate 80 iPSCs from 1×10^5 CB CD34⁺ cells.²⁶ From the same amount of cells, we can generate ~20 iPSCs with OS alone, and up to 600 iPSCs with OSMK. Considering that the addition of LIN28 increases reprogramming efficiency by three to fivefold,³⁹ our improved vector is at least 20-fold more efficient in reprogramming CB cells than plasmids used in previous studies. Our success is attributed to the inclusion of two features in the vector design: (i) the SFFV promoter, which drives higher levels of transgene expression in hematopoietic cells than PGK, EF1 or other promoters; and (ii) the Wpre element, which increases transgene expression by 50%. Wpre is commonly used in lentiviral vectors to improve transgene expression;³⁰ our findings suggest that Wpre is also functional in episomal plasmids and possibly other DNA vectors such as adenoviral vectors.

In summary, we are the first to report the successful generation of transgene-free human iPSCs with the use of OCT4 and SOX2 alone. All OS-reprogrammed iPSCs examined in our studies showed normal karyotypes. Future studies that compare genetic instability and mutation rates in iPSCs generated with OS alone versus combinations that include oncogenic factors like MYC will be an important next step on the path to clinical application of iPSCs.

MATERIALS AND METHODS

Cord blood. The use of CB was approved by the institutional review board of Loma Linda University (LLU) and written informed consent was obtained from all participants. CD34⁺ cells were purified with a CD34⁺ Microbead Kit (Miltenyi Biotec, Auburn, CA).

Construction of lentiviral and episomal vectors. Human OCT4, SOX2, MYC, and KLF4 cDNAs were purchased from Open Biosystems, Huntsville, AL and cloned into the pRRLSin.cPPT.PGK-GFP.WPRE lentiviral vector that was kindly provided by Luigi Naldini via Addgene, Cambridge, MA (Plasmid 12252).⁴⁰ Open reading frames of these reprogramming factors and PGK, EF1, or SFFV promoters were inserted into this vector by PCR cloning. For cloning OS or MK vectors, a 2A sequence was used to link OCT4 and SOX2, or MYC and KLF4.⁴¹ The EBNA1/OriP-based pCEP4 episomal vector was purchased from Invitrogen (Carlsbad, CA). For cloning pCEP-OS (w/o W), pCEP-OS, pCEP-K, or pCEP-MK vectors, the hygromycin resistance gene element and cytomegalovirus promoter were removed from the pCEP4 vector by digestion with endonucleases *NruI* and

BamHI, and inserts were cut from the counterparts of lentiviral vectors. All the constructs were verified by sequencing. For lentivirus production, a standard calcium phosphate precipitation protocol was used. Titters of $5\text{--}10 \times 10^7/\text{ml}$ were routinely achieved in our lab after a 100-fold concentration by centrifugation at $6,000g$ for 24 hours at 4°C .^{42,43}

Generation of iPSCs using lentiviral vector. Thawed CB CD34⁺ cells were cultured in hematopoietic stem cell culture condition: Iscove's modified Dulbecco's medium/10% fetal bovine serum supplemented with TPO, SCF, FL, and G-CSF each at 100 ng/ml, and IL-3 at 10 ng/ml.⁴⁴ Cytokines were purchased from ProSpec (East Brunswick, NJ). After 2 days prestimulation, 1×10^4 cells/well were seeded into non-TC treated 24-well plates that were precoated with RetroNectin (CH-296; Takara Bio, Shiga, Japan) for lentiviral transduction for 4–5 hours. A second transduction was conducted 24 hours later. One day after transduction, cells were harvested and transferred to 6-well plates, which were preseeded with a mitomycin C-inactivated CF-1 MEF feeder layer (Applied Stemcell, Menlo Park, CA). Passage five MEFs were used in our experiments. Cells were maintained in the hematopoietic stem cell culture condition for 2 more days before being replaced with iPSC media. The iPSC media used in our study is composed of knockout DMEM/F12 medium (Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 1 mmol/l GlutaMAX (Invitrogen), 2 mmol/l nonessential amino acids (Invitrogen), $1 \times$ penicillin/streptomycin (Invitrogen), 0.1 mmol/l β -mercaptoethanol (Sigma-Aldrich, St Louis, MO), 20 ng/ml FGF2 (ProSpec). To increase reprogramming efficiency, sodium butyrate⁴⁵ was added at 0.25 mmol/l from day 2–12, and cells were cultured under hypoxia⁴⁶ by placing culture plates in a Hypoxia Chamber (Stemcell Technologies, Vancouver, British Columbia, Canada) that was flushed with mixed air composed of 92%N₂/3%O₂/5%CO₂. Starting from day 10, MEF-conditioned medium was used. At day 14–16, ALP staining was conducted to quantitate iPSC colonies. Alternatively, all the colonies were harvested by Accutase (Innovative Cell Technologies, San Diego, CA) treatment for FACS analysis.

Immunostaining and flow cytometry. Staining for ALP was carried out using an ALP-staining kit (Stemgent, San Diego, CA) to quantitate iPSC colonies. For intracellular staining, cells were fixed for 30 minutes at room temperature in fixation buffer and permeabilization buffer (eBiosciences, San Diego, CA). After washing, cells were stained at room temperature for 2 hours with NANOG-PE (BD Pharmingen, San Diego, CA), followed by washing twice with permeabilization buffer. For staining of cell surface marker TRA-1-60-PE (Stemgent), cells were incubated with the antibody for 30 minutes at room temperature. Flow cytometric analysis was performed using FACS Aria II (BD Biosciences, San Jose, CA) with a 488-nm laser. Thirty thousand events were collected for each sample.

Episomal vector and nucleofection. Fresh or thawed 1×10^5 CB CD34⁺ cells were cultured in Iscove's modified Dulbecco's medium/10% fetal bovine serum supplemented with TPO, SCF, and FL at 100 ng/ml. Three days later, cells were harvested for nucleofection with a total of 12 μg CEP plasmid DNAs. Human CD34 Cell Nucleofector Kit (Lonza, Walkersville, MD) was used. Nucleofection was performed with Amaxa Nucleofector II using program U-008. Immediately after nucleofection, cells were cultured in a CH-296 pretreated well plate to facilitate the CB cell recovery. The next day, half of the cells were transferred to each well of MEF-coated 6-well plates. Cells were cultured the same way as for reprogramming with lentiviral vector. The total number of iPSC colonies was counted on day 16 post-transfection after ALP staining. At day 14–17, colonies were picked for further culture or harvested for FACS analysis.

Confocal imaging. For immunostaining of iPSC colonies, iPSCs were cultured in chamber slides for 4–5 days. Cells were treated with fixation buffer and permeabilization buffer (eBiosciences) for 30 minutes before being stained overnight with PE or FITC-conjugated antibodies OCT4

(eBiosciences), SOX2 (BD Pharmingen), NANOG (BD Pharmingen), SSEA-3 (eBiosciences), SSEA-4 (eBiosciences), and TRA-1-60 (Stemgent). The samples were washed twice with permeabilization buffer, counterstained with 4',6-diamidino-2-phenylindole and coverslipped before being imaged. Imaging was performed using the Zeiss LSM 710 NLO laser scanning confocal microscope with a $\times 10$ objective at the LLU Advanced Imaging and Microscopy Core. High resolution monochrome image was captured using a Zeiss HRm CCD camera (Thornwood, NY).

Teratoma assay. The use of NOD/SCID/IL2RG^{−/−} (NSG) immunodeficient mice for the teratoma formation assay was approved by the Institutional Animal Care and Use Committee at LLU. NSG mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at the LLU animal facility. Approximately 1×10^6 iPSCs were harvested by Dispase (Invitrogen) digestion, washed with culture medium and resuspended in 200 μl DMEM/F12 diluted (1:1) Matrigel solution (BD, San Jose, CA). Cells were injected into the subcutaneous tissue above the rear haunch of NSG mice. At 6–8 weeks after injection of iPSCs, teratomas were dissected and fixed in 10% formalin. After sectioning, samples were embedded in paraffin and stained with hematoxylin and eosin and analyzed by a board certified pathologist.

Bisulphite sequencing. Bisulphite sequencing of genomic DNA from iPSC clones was used to assess methylation status of OCT4 and NANOG promoter. Genomic DNA was purified from human iPSCs by DNeasy Kit (Qiagen, Valencia, CA). The conversion of unmethylated cytosines to uracil was carried out using EZ DNA Methylation-Gold Kit (ZYMO Research, Irvine, CA). Approximately 1 μg genomic DNA was treated in each reaction, and 4 μl of elution was used for each PCR. PCR with primers OCT4-mF3/R3 and NANOG-mF3/R3, which were used by other investigators,⁴⁷ was carried out using Titanium Taq polymerase (Clontech Laboratories, Mountain View, CA): The cycling conditions were 95°C 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and finally 72°C for 7 minutes. The PCR products were cloned into a pJET1.2 vector (Fermentas, Glen Burnie, MD) and sequenced by MCLAB (San Francisco, CA).

Karyotyping and G-banding. GTG-banding chromosome analysis was carried out in the LLU Radiation Research Laboratories. Standard DNA spectral karyotyping procedures were followed and a HISKY Complete Cytogenetic System was used (Applied Spectral Imaging, Vista, CA). For each clone, 10 metaphases were analyzed and karyotyped. The data were interpreted by a certified cytogenetic technologist.

Real-time PCR. To determine the average copy numbers of residual or integrated CEP vector in iPSC clones, real-time PCR analysis was performed. Total DNA (genomic and episomal) was extracted from iPSCs using the DNeasy kit from Qiagen. Equal amounts of DNA (100 ng) isolated from naive cells (before nucleofection) were used as negative control, while a manual mixture of 1 copy pCEP-OS vector per genome was used as a positive control to calculate the average copy numbers of residual episomal vector in each iPSC after multiple passages. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on 7500 Fast Real-Time PCR System (Applied Biosystems). Two sets of primers were used to detect CEP plasmid DNA (in either episomal or integrated form): EBNA1-F: 5'-TTTAATACGATTGAGGGCGTCT-3', EBNA1-R: 5'-GGTTTTGAAGGATGCGATTAAG-3'; OSW-F: 5'-GGATTACAAGGATGACGACGA-3', OSW-R: 5'-AAGCCATACGGGAAGCAATA-3'. The amplification program consisted of 50°C for 2 minutes and 95°C for 10 minutes, and was followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Statistical analysis. Data are presented as mean $\pm$ s.e. of the mean (s.e.m.). Two-tailed Student *t*-test was performed. *P* value of <0.05 was considered statistically significant.

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Rapid and efficient reprogramming of human fetal and adult blood CD34⁺ cells into mesenchymal stem cells with a single factor

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The direct conversion of skin cells into somatic stem cells has opened new therapeutic possibilities in regenerative medicine. Here, we show that human induced mesenchymal stem cells (iMSCs) can be efficiently generated from cord blood (CB)- or adult peripheral blood (PB)-CD34⁺ cells by direct reprogramming with a single factor, OCT4. In the presence of a GSK3 inhibitor, 16% of the OCT4-transduced CD34⁺ cells are converted into iMSCs within 2 weeks. Efficient direct reprogramming is achieved with both episomal vector-mediated transient OCT4 expression and lentiviral vector-mediated OCT4 transduction. The iMSCs express MSC markers, resemble bone marrow (BM)-MSCs in morphology, and possess *in vitro* multilineage differentiation capacity, yet have a greater proliferative capacity compared with BM-MSCs. Similar to BM-MSCs, the implanted iMSCs form bone and connective tissues, and are non-tumorigenic in mice. However, BM-MSCs do not, whereas iMSCs do form muscle fibers, indicating a potential functional advantage of iMSCs. In addition, we observed that a high level of OCT4 expression is required for the initial reprogramming and the optimal iMSC self-renewal, while a reduction of OCT4 expression is required for multilineage differentiation. Our method will contribute to the generation of patient-specific iMSCs, which could have applications in regenerative medicine. This discovery may also facilitate the development of strategies for direct conversion of blood cells into other types of cells of clinical importance.

Keywords: mesenchymal stem cells; hematopoietic cells; direct reprogramming; CD34⁺ cells

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Introduction

Mesenchymal stem cells (MSCs) are somatic stem cells responsible for the regeneration of cartilage, tendon, ligament, muscle and bone, and thus hold great

promise for treating skeletal diseases [1–5]. Due to their immunomodulatory potential, MSCs have also been used in clinical and preclinical studies to treat multiple diseases including graft versus host disease (GVHD) [6], Crohn's disease [7], organ transplant rejection [8] and diabetes [9]. MSCs can be isolated from multiple tissues like bone marrow (BM) and adipose tissue [10]. However, the procedures are invasive and often yield limited numbers of MSCs for therapy. MSCs differentiated from human induced pluripotent stem cells (iPSCs) have been used for skeletal regeneration and tissue repair [11–13]. However, this approach is time-consuming, and the risk

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of teratoma formation due to trace amount of undifferentiated or contaminated iPSCs in iPSC-MSCs remains a concern. Thus, the direct reprogramming of somatic cells into MSCs presents an alternative to the iPSC approach and might decrease the risk of teratoma formation.

The direct reprogramming of one type of cells, most commonly fibroblasts, into another by ectopic expression of defined transcription factors has recently been achieved. Successful reprogramming of human or mouse fibroblasts into neural stem cells [14-15], cardiomyocytes [16-17] and hepatocytes [18-19] has been reported. The most readily available cell sources of adult human are skin and blood. The use of blood cells for reprogramming has unique advantages over dermal fibroblasts, as blood cells likely harbor fewer acquired genetic mutations induced by environmental insults, and can be easily obtained from patients with minimal invasiveness [20-23]. The first report of direct reprogramming of blood cells into somatic cells of clinical interest, such as neuronal cells, has recently been published [24]. Cord blood (CB)-CD133⁺ progenitor cells are reprogrammed to neuronal cells by ectopic expression of SOX2 and MYC at an efficiency of < 0.1%, requiring 3 weeks for the generation of neuronal cell-like colonies [24]. In addition, the use of the oncogene MYC poses a serious risk of tumorigenesis. Thus, we have been interested in identifying an approach that can generate MSCs safely, rapidly and efficiently from blood cells. Here, we present a novel approach for generating self-renewable, multipotent and non-tumorigenic induced mesenchymal stem cells (iMSCs) from human blood CD34⁺ cells by direct reprogramming with only one factor.

Results

Generation of iMSCs from CB-CD34⁺ cells

We have previously reported the efficient generation of iPSCs from human CB-CD34⁺ cells with a single vector that expresses OCT4 and SOX2 (Lenti SFFV-OCT4-2A-SOX2) [25]. When the two factors were expressed by two individual lentiviral vectors (Lenti SFFV-OCT4 and Lenti SFFV-SOX2), we observed that ~80% of the colonies morphologically resemble mesenchymal cells. This finding prompted us to explore whether OCT4 or SOX2 alone can reprogram CD34⁺ cells directly into MSC-like cells, which we term as induced MSCs (iMSCs) hereafter for brevity. We found that transduction of CB-CD34⁺ cells with Lenti SFFV-OCT4, but not Lenti SFFV-SOX2, induced iMSC formation (Supplementary information, Figure S1A), suggesting that OCT4 is responsible for the induced cell fate conversion. We also tested other reprogramming factors and found that MYC, NANOG, KLF4,

LIN28 or TBX3 alone completely failed to convert CB-CD34⁺ cells into iMSCs (Supplementary information, Figure S1B). Furthermore, the combination of these factors with OCT4 did not significantly increase the reprogramming efficiency. Thus, our subsequent studies focused on OCT4 only.

A successful iMSC conversion depends on OCT4 overexpression and MSC-conductive culture conditions. The transduction of CB-CD34⁺ cells with green fluorescent protein (GFP) control did not generate MSCs (Figure 1A and Supplementary information, Video S1), whereas OCT4-transduced cells formed colonies and were transformed morphologically into spindle-like cells within 3-4 days of culture in MSC-conductive conditions (Figure 1B and Supplementary information, Video S2). Some OCT4-transduced cells cultured in hematopoietic stem cell (HSC) medium manifested a spindle-like morphology, but died shortly after the formation of small colonies (Figure 1C and Supplementary information, Video S3). We also found that the use of human fibronectin-precoated non-tissue culture-treated (non-TC) culture plates that allow for efficient attachment of transduced CB-CD34⁺ cells is crucial for the rapid morphological transformation (1 week), whereas in the absence of fibronectin, the morphological transformation takes 2-3 weeks.

To optimize the reprogramming protocol, we tested whether small molecules capable of enhancing the reprogramming of somatic cells into iPSCs, such as GSK3 inhibitor CHIR99021 (CHIR), MEK inhibitor and ALK5 inhibitor, can also increase the efficiency of direct reprogramming of CB-CD34⁺ cells into iMSCs. After a series of experiments, we found that only CHIR can substantially enhance the reprogramming process; it increased the reprogramming efficiency from 3% to 16% (Figure 1D and 1E). Colonies were also substantially larger in size in the presence of CHIR (Figure 1D). Moreover, CHIR consistently increased the iMSC proliferation rate in long-term cultures (Figure 1F), and shortened the population doubling time from 35 h to 20 h. These data demonstrate that CHIR increases both the reprogramming efficiency and the *in vitro* proliferative rate of iMSCs.

The transition of CB-CD34⁺ cells to phenotypically mature iMSCs takes several weeks. During the first 2 weeks of the transdifferentiation, cells expressing the pan-hematopoietic marker, CD45, steadily decreased from > 99% to < 1%, whereas cells expressing the MSC marker, CD73, rapidly increased from 0% to > 70% (Figure 1G and Supplementary information, Figure S2A). MSC markers CD29 and CD44 were also expressed in HSCs, but at low levels. Of interest, we observed a 15-fold and 6-fold increase of the expression of CD29 and CD44, respectively, over 3-4 weeks (Supplementary

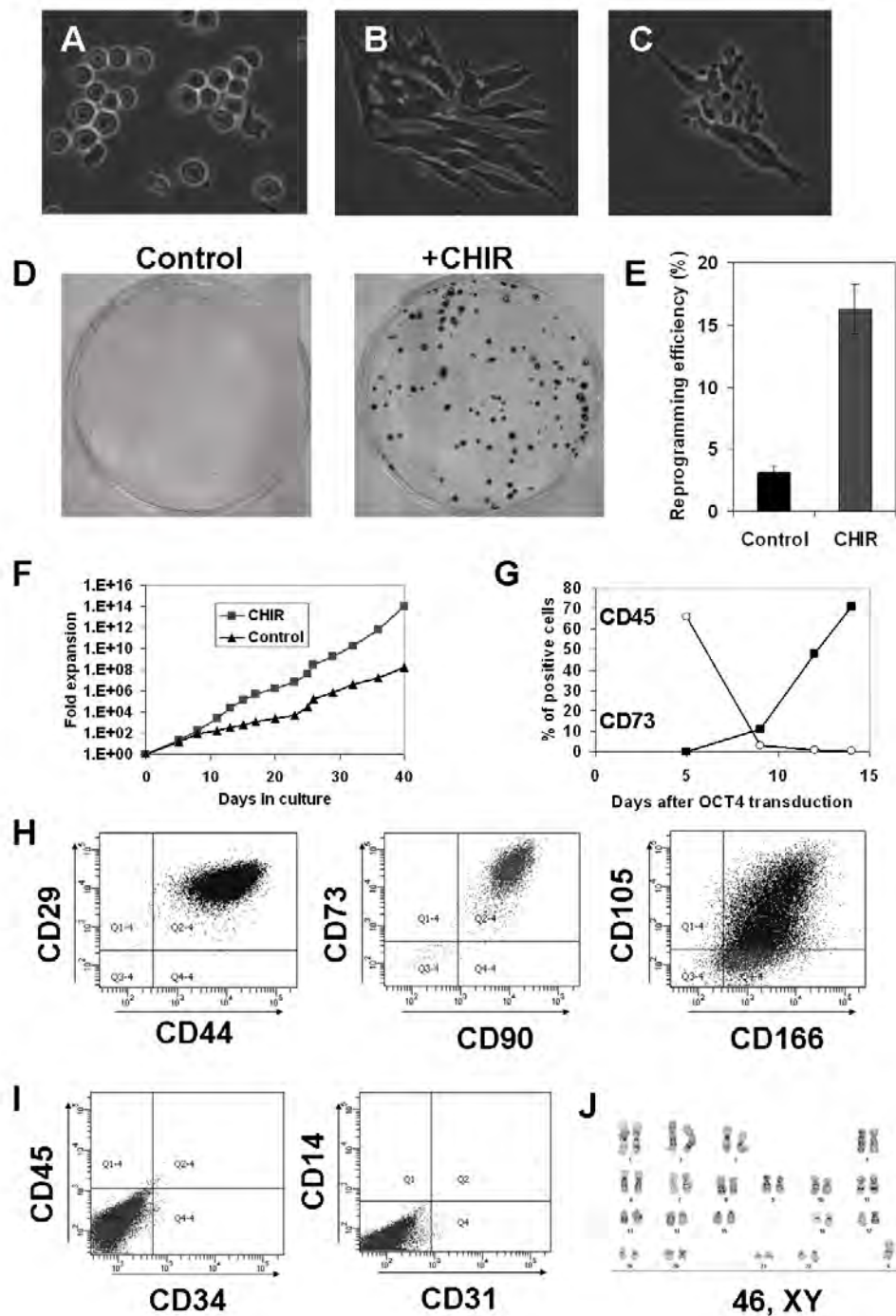


Figure 1 OCT4-transduced human CB-CD34⁺ cells give rise to MSC-like cells. **(A-C)** CB-CD34⁺ cells transduced with the GFP control vector retained the hematopoietic morphology at 5 days post-transduction **(A)**. OCT4-transduced CB-CD34⁺ cells developed a MSC-like morphology when cultured in MSC medium **(B)**, but the MSC-like cells did not survive when cultured in HSC medium **(C)**. **(D)** Colony formation at 9 days after seeding 1 000 OCT4-transduced CB-CD34⁺ cells in MSC culture conditions with or without CHIR. **(E)** Reprogramming efficiency with and without CHIR ($n = 3$, $P < 0.01$). Error bars indicate SEM. **(F)** iMSC fold expansion over time shows that CHIR promotes long-term *in vitro* proliferation of reprogrammed iMSCs. Data shown are a representative of 3 independent experiments with similar results. **(G)** Changes in the percentage of cells expressing the pan-hematopoietic marker CD45 and the MSC marker CD73, as measured by flow cytometry of OCT4-transduced cells over time. **(H)** Flow cytometry plots of typical MSC marker expression assessed at 1 month after OCT4 transduction. **(I)** Flow cytometry plots for expression of blood cell markers (CD45, CD14 and CD34) and endothelial cell marker CD31 assessed at 1 month after OCT4 transduction. **(J)** iMSCs show a normal karyotype after 3 months of culture.

information, Figure S2B-S2D). Four weeks after Lenti SFFV-OCT4 transduction, almost all the cells expressed typical MSC markers: CD29, CD44, CD73, CD90, CD105 and CD166 (Figure 1H), whereas the expression of the hematopoietic markers CD45, CD34 and CD14, or the endothelial marker CD31 was negligible (Figure 1I). These data provide evidence that fully reprogrammed iMSCs are phenotypically identical to MSCs from other sources.

OCT4-reprogrammed iMSCs are genetically stable and do not form tumors

We further examined the *in vitro* long-term proliferative capacity and potential risk of tumor formation of iMSCs. In five independent experiments, the iMSCs expanded robustly over the 4 months of culture, with a cell population doubling time of 20–22 h in the presence of CHIR (Supplementary information, Figure S3). Of note, the rate of cellular proliferation was virtually unchanged during the entire period of culture, with no signs of senescence or transformation based on the frequency of cell division. We asked whether OCT4 expression induces genomic instability after long-term *in vitro* culture. Karyotype analysis did not detect any major chromosomal abnormalities (Figure 1J). To further investigate the safety of these cells, we injected $1-2 \times 10^6$ iMSCs systemically or subcutaneously into 20 immunodeficient mice. Unlike iPSCs [25], iMSCs did not form tumors during 3 months of follow-up. In comparison, injection of iMSCs that were generated with OCT4 and MYC led to tumor formation at 1 month after systemic or subcutaneous inoculation (data not shown). These data suggest that the use of OCT4 for direct reprogramming of CB-CD34⁺ cells into iMSCs is a safe approach for rapid generation of large quantities of MSCs.

iMSCs are reprogrammed directly from hematopoietic progenitors, not from mature myeloid cells

As MSCs are present in the CB at frequencies ranging from 0 to 2.3 clones per 1×10^8 mononuclear cells or ~ 0.1 clones per ml [26], we asked whether OCT4 expands the contaminated MSCs in CB-CD34⁺ cells rather than directly reprogramming hematopoietic cells into iMSCs. The proliferation of the existing MSCs in the CB cannot explain iMSC generation, as no MSCs were generated from nontransduced CB-CD34⁺ cells after 2–3 weeks of culture under our MSC culture conditions. To unambiguously test this possibility, we cloned single CB-CD34⁺ cells in U-bottomed 96-well plates. After 10 days of culture in medium supplemented with cytokines TPO, SCF, FL, IL-3 and G-CSF, hematopoietic cell colonies were formed in $\sim 10\%$ of the wells (Supplementary

information, Figure S4). Random selection of 12 clones, followed by transduction with Lenti SFFV-OCT4 led to the formation of iMSCs in 33% cases (Supplementary information, Figure S4). These data demonstrate that iMSCs are directly reprogrammed from hematopoietic cells. Of interest, when CB-CD34⁺ cells were cultured in the condition that promotes myeloid differentiation for 20 days before OCT4-transduction, no iMSC colonies were generated from 12 randomly selected individual clones (data not shown). These data suggest that OCT4 likely reprograms hematopoietic progenitors into iMSCs, but cells in the terminal stages of myeloid differentiation cannot be reprogrammed by OCT4 overexpression.

Reprogramming to iMSCs requires a high level of OCT4 expression and lacks an iPSC stage

Next, we examined the potential mechanisms of OCT4-mediated direct reprogramming from CB-CD34⁺ cells into iMSCs. Consistent with our previous report [25], we found that the iMSC reprogramming critically depends on OCT4 expression levels. When we used a relatively weak promoter such as EF1 α or PGK that leads to 50%–60% lower expression of OCT4 compared to the SFFV promoter in hematopoietic cells [25], the reprogramming from CB-CD34⁺ cells into iMSCs completely failed. This result suggests that a high level of OCT4 expression is necessary for the successful reprogramming of hematopoietic cells.

We then asked whether a pluripotent state is induced during the transdifferentiation process. Apart from OCT4, whose expression level is similar to that in iPSCs (Figure 2A and Supplementary information, Figure S5), other pluripotency markers, such as SOX2, NANOG and TRA-1-60 were undetectable in iMSCs (Figure 2A). Bisulphite sequencing showed that OCT4 and NANOG promoters are hypermethylated (Figure 2B), suggesting that the endogenous OCT4 gene is not activated in iMSCs. We also found that OCT4 alone is insufficient for the reprogramming of CB-CD34⁺ cells into iPSCs and that the culture of iMSCs in iPSC-conductive conditions does not lead to the formation of iPSC colonies (Figure 2C and 2D). Taken together, our data suggest that reprogramming of CB-CD34⁺ cells into iMSCs does not go through an iPSC stage.

A high level of OCT4 expression inhibits iMSC differentiation

MSCs have the capacity to differentiate into adipocytes, osteoblasts and chondrocytes in differentiation-inducing conditions. However, iMSCs generated by transduction with the lentiviral vector SFFV-OCT4 (Lenti iMSCs) failed to differentiate into mature progenies of

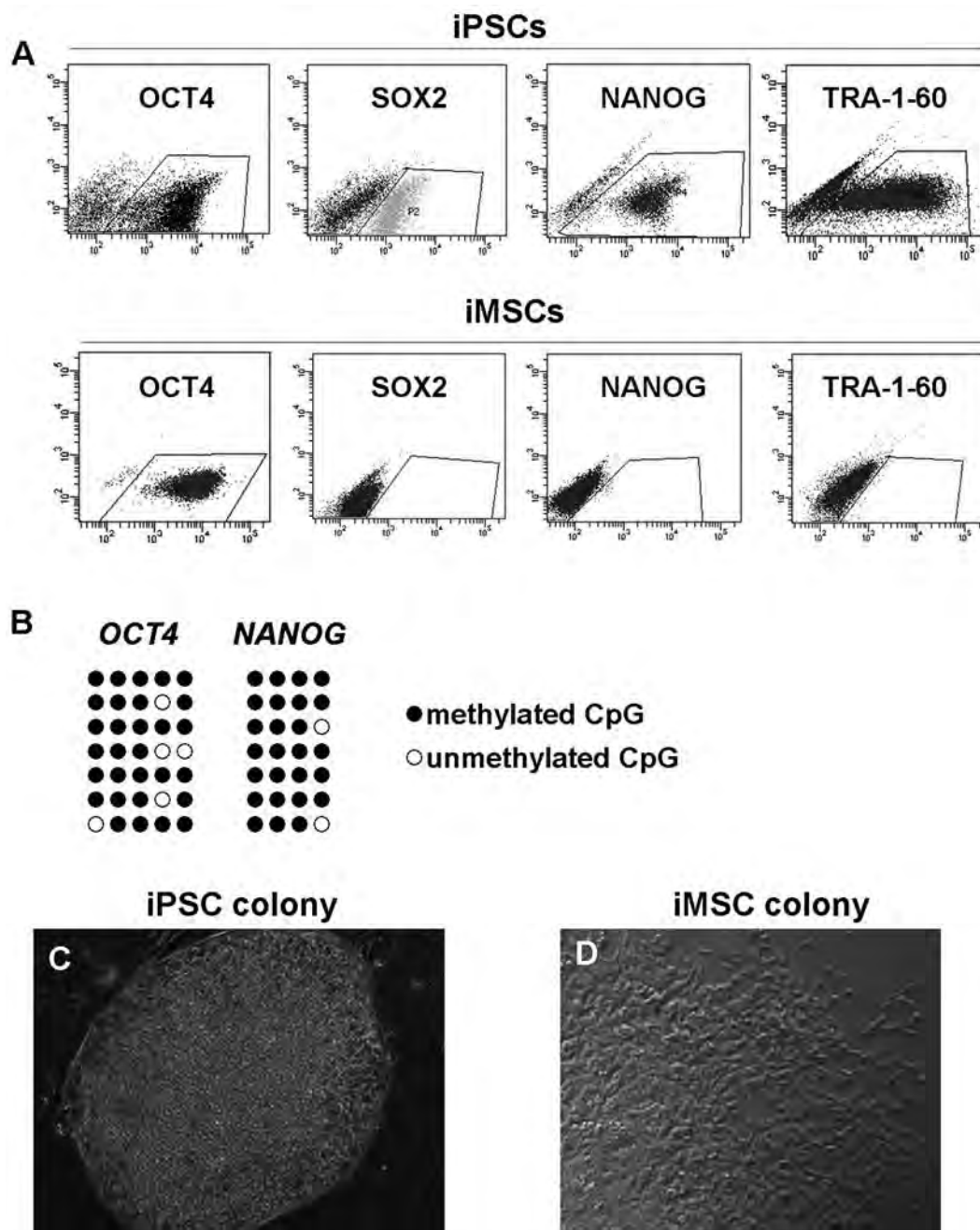


Figure 2 Reprogramming of CB-CD34⁺ cells into iMSCs bypasses the pluripotent state. **(A)** Flow cytometry plots show that OCT4, but not other iPSC markers such as SOX2, NANOG and TRA-1-60, is expressed in Lenti iMSCs at 1 month after transduction. As positive controls, iPSCs express OCT4, SOX2, NANOG and TRA-1-60. **(B)** Bisulfite sequencing shows that OCT4 and NANOG promoters remain hypermethylated in Lenti iMSCs after 2 months of culture. **(C)** A typical colony of iPSCs generated with OCT4 and SOX2. **(D)** A colony of iMSCs formed in iPSC culture conditions. OCT4-transduced CB-CD34⁺ cells did not form iPSC-like colonies even after 1 month of culture in iPSC medium.

MSCs (Figure 3A-3C). A previous study shows that ectopic expression of OCT4 blocks the differentiation of epithelial progenitors [27]. We thus hypothesized that a high level of OCT4 expression promotes self-renewal,

but inhibits differentiation of iMSCs. As predicted, after knockdown (KD) of OCT4 by 80% with an shOCT4 lentiviral vector (Supplementary information, Figure S5), iMSCs differentiated into mature adipocytes, osteoblasts

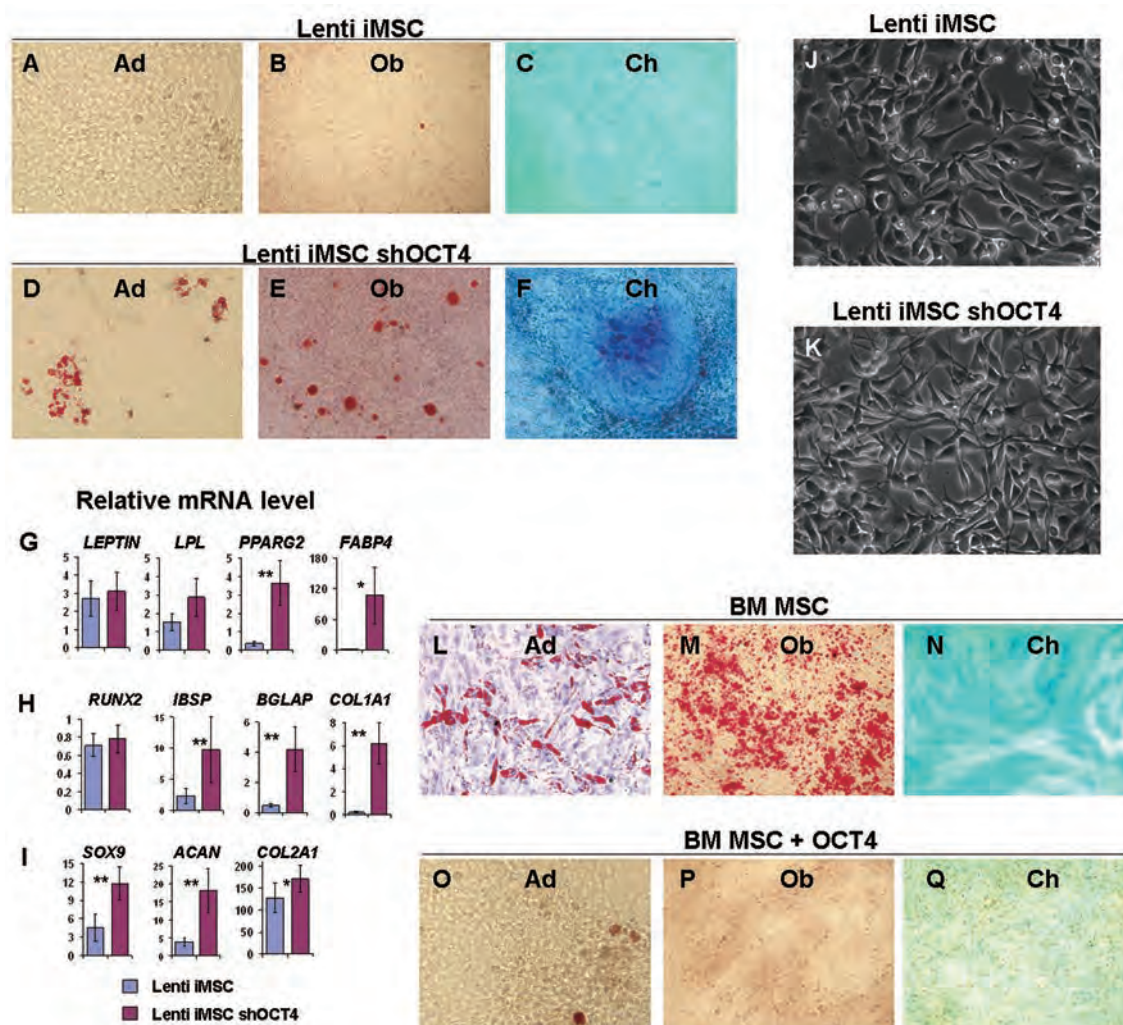


Figure 3 A high level of OCT4 expression in iMSCs inhibits the *in vitro* differentiation, whereas OCT4-KD allows for multilineage differentiation. (A–F) Lenti iMSCs do not differentiate into mature adipocytes (Ad), osteoblasts (Ob) or chondrocytes (Ch) under differentiation induction conditions for the indicated lineages (A–C), whereas shRNA-mediated OCT4-KD allows for the terminal differentiation of Lenti iMSCs (D–F). (G–I) RT-qPCR analyses of genes associated with adipogenic (G: *LEPTIN*, *PPARG2*, *LPL* and *FABP4*); osteoblastic (H: *RUNX2*, *IBSP*, osteocalcin (*BGLAP*) and *COL1A1*) and chondrogenic (I: *SOX9*, aggrecan (*ACAN*) and *COL2A1*) differentiation before or after OCT4-KD. mRNA expression levels were normalized to levels in the BM-MSC control. ** $P < 0.01$, * $P < 0.05$. $n = 10$ –20. Error bars indicate SEM. (J) Typical morphology of Lenti iMSCs at 1 month after OCT4 transduction. (K) Typical morphology of Lenti iMSCs at 2 weeks after OCT4-KD. (L–N) Images of *in vitro* differentiation of BM-MSC to Ad, Ob or Ch. (O–Q) Differentiation of BM-MSCs was blocked after Lenti-OCT4 transduction. Red stained are oil droplets in D and L, and bone nodules in E and M. Mucopolysaccharides secreted by chondrocytes are stained blue in F and N.

and chondrocytes (Figure 3D–3F). To quantitate the effects of OCT4-KD on MSC differentiation, we conducted real-time RT-PCR analyses. The results showed that OCT4-KD leads to significant upregulation of a majority of genes expressed specifically during adipocytic (Figure 3G), osteoblastic (Figure 3H) and chondrocytic (Figure 3I) differentiation. Of interest, OCT4-KD neither affected the long-term MSC proliferation potential, nor the expression of MSC markers (Supplementary informa-

tion, Figure S6). We also observed that OCT4-KD leads to appreciable changes in morphology: Lenti iMSCs-expressing shOCT4 became more spindle-like compared with regular Lenti iMSCs (Figure 3J and 3K). To further evaluate the roles of OCT4 in MSC differentiation, we transduced BM-MSCs with OCT4. As expected, OCT4 overexpression in BM-MSCs led to differentiation failure (Figure 3L–3Q). Taken together, these data demonstrate that a high level of OCT4 blocks MSC differentiation,

and a reduction of OCT4 expression allows for multilineage differentiation.

Generation of integration-free iMSCs with an episomal vector

As a high level of constitutively expressed OCT4 inhibits iMSC differentiation and the use of integrating viral vector may limit the potential clinical applications, we attempted to reprogram CB-CD34⁺ cells into iMSCs by a transient OCT4 overexpression with a non-integrating episomal vector (EV) [25, 28]. After nucleofection of CB-CD34⁺ cells with EV SFFV-OCT4 plasmid DNA, we successfully generated iMSCs in 2 weeks, which we hereafter term as EV iMSCs for brevity. After 1 month of culture, OCT4 expression in EV iMSC decreased to baseline levels as observed in BM-MSCs (Supplementary information, Figure S5). EV iMSCs and BM-MSCs were morphologically identical (Figure 4A and 4B). In addition, EV iMSCs did not differ from regular MSCs in the expression of typical MSC markers (Supplementary information, Figure S7).

We next tested the *in vitro* differentiation potential of EV iMSCs in comparison with BM-MSCs. EV iMSCs fully differentiated into adipocytes, osteoblasts and chondrocytes, with the similar differentiation potentials as BM-MSCs (Figures 3L-3N, 4C-4E and Supplementary information, Figure S8). Further, results from RT-qPCR analyses showed similar expression levels for a majority of lineage-associated genes in adipocytes, osteoblasts and chondrocytes differentiated from EV iMSCs as compared to those differentiated from BM-MSCs (Figure 4F-4H). Of interest, the expression of *SOX9*, *ACAN* and *COL2A1* appeared to be higher in chondrocyte cultures of EV iMSCs than those of BM-MSCs. These data suggest that EV iMSCs are similar to BM-MSCs in the *in vitro* multilineage differentiation potency.

In vivo functionality of iMSCs

To evaluate the potential for clinical application of iMSCs, we further investigated the *in vivo* functionality of EV iMSCs. Cells were transduced with GFP to facilitate the analyses of human cells in mice. HA-TCP bone graft substitute blocks loaded with iMSCs were subcutaneously implanted in nude mice. Analyses of the implants 2-3 months later showed that iMSCs differentiated into bone tissue, connective fibers and adipose tissue in all 10 animals, and into skeletal muscle fibers in the majority of the implants (Figure 5A-5D). Immunohistological staining of GFP confirmed that the differentiated tissues were derived from human EV iMSCs (Figure 5E). Of interest, the implanted BM-MSCs differentiated into bone tissue, connective fibers and adipose tissue but not skeletal mus-

cle fibers (Supplementary information, Figure S9). These data suggest that iMSCs may hold a functional advantage in promoting muscle regeneration.

Long-term proliferation and self-renewal of multipotent iMSCs depend on a low level of ectopic OCT4 expression

Primary adult human BM-MSCs display slowed proliferation after ~1 month in culture, and eventually stop growing and lose multilineage differentiation potential. We thus tested the *in vitro* proliferative potential of EV iMSCs. EV iMSCs showed an enhanced *in vitro* proliferative capacity compared with BM-MSCs — more than 100-fold more MSCs can be generated from the CB by transient OCT4 transfection than from the same volume of BM at 6 weeks after initiation of *in vitro* culture (Figure 6A). After ~15 passages in culture, EV iMSCs discontinued cell division and manifested the typical morphology of aged MSCs (Figure 6B).

Together with the observation of an unrestricted proliferative potential of Lenti iMSCs, we hypothesized that a low level of OCT4 expression may allow for long-term iMSC proliferation without blocking differentiation. To test this hypothesis, we selected a very weak promoter, the Sca1 promoter, which drives OCT4 expression at ~5% of the level in Lenti iMSCs or iPSCs (Supplementary information, Figure S5). After transducing EV iMSCs with Lenti Sca1-OCT4, these cells can be cultured for more than 30 passages, with a doubling time of ~40 h. In addition, Sca1-OCT4 iMSCs at passage 30 were still morphologically indistinguishable from BM-MSCs at earlier passages (Figure 6C). In addition, Sca1-OCT4 iMSCs readily differentiate into adipocytes, osteoblasts and chondrocytes in induction cultures (data not shown), suggesting that maintaining a low level of OCT4 expression in iMSCs allows for long-term proliferation, yet does not affect the *in vitro* multilineage differentiation potential of these cells.

Generation of iMSCs from adult human peripheral blood CD34⁺ cells

Our findings with fetal blood prompted us to test whether OCT4 can also reprogram adult human peripheral blood (PB) cells into iMSCs. After transduction of PB-CD34⁺ cells with Lenti SFFV-OCT4, iMSC colonies were formed within 1 week. The reprogramming of PB- and CB-CD34⁺ cells was equally efficient. CHIR also increased the reprogramming efficiency of PB-CD34⁺ cells from 3% to 15% (Figure 7A and 7B). In addition, PB-Lenti iMSCs were morphologically and phenotypically identical to CB-Lenti iMSCs (Figure 7C vs Figure 3J, and data not shown). These data suggest that OCT4 can reprogram both fetal and adult human hematopoietic

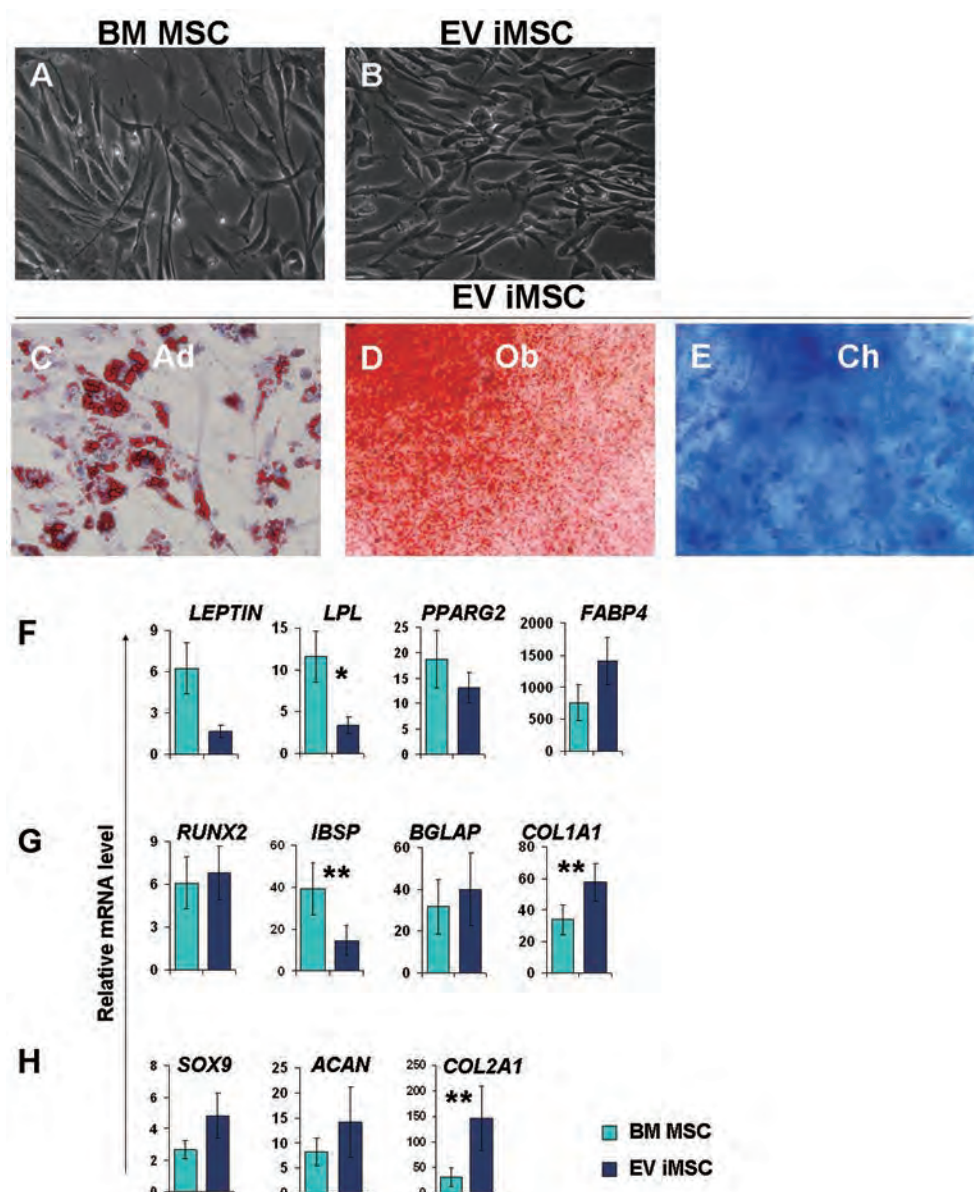


Figure 4 *In vitro* multilineage differentiation capacity of integration-free episomal iMSCs. **(A)** Typical morphology of BM-MSCs at passage 5. **(B)** Typical morphology of EV iMSCs at passage 5. BM-MSCs and EV iMSCs are morphologically indistinguishable. **(C-E)** iMSCs generated with episomal vector EV SFFV-OCT4 robustly differentiated into mature adipocytes (Ad) with oil droplets (red), osteoblasts (Ob) that form bone nodules (red) or chondrocytes (Ch) that secrete mucopolysaccharides (blue) after 3 weeks of induction culture. **(F)** RT-qPCR analyses of genes associated with adipogenic differentiation *LEPTIN*, *LPL*, *PPARG2* and *FABP4*. **(G)** RT-qPCR analyses of genes associated with osteoblastic differentiation *RUNX2*, *IBSP*, *BGLAP* and *COL1A1*. **(H)** RT-qPCR analyses of genes associated with chondrogenic differentiation *SOX9*, *ACAN* and *COL2A1*. The mRNA expression levels in **F-H** are normalized to the levels in BM-MSCs before the induction of differentiation. ** $P < 0.01$, * $P < 0.05$. $n = 10-20$. Error bars indicate SEM.

cells into iMSCs at a similar efficiency.

Discussion

Here, we report that a single transcription factor, OCT4, allows for rapid and efficient reprogramming of

human CD34⁺ cells from either CB or adult PB directly into iMSCs. The ability to reprogram blood cells into self-renewable iMSCs at an unprecedented efficiency has important implications for regenerative medicine. Although MSCs have been isolated from many sources [10], our approach for generating iMSCs from the pa-

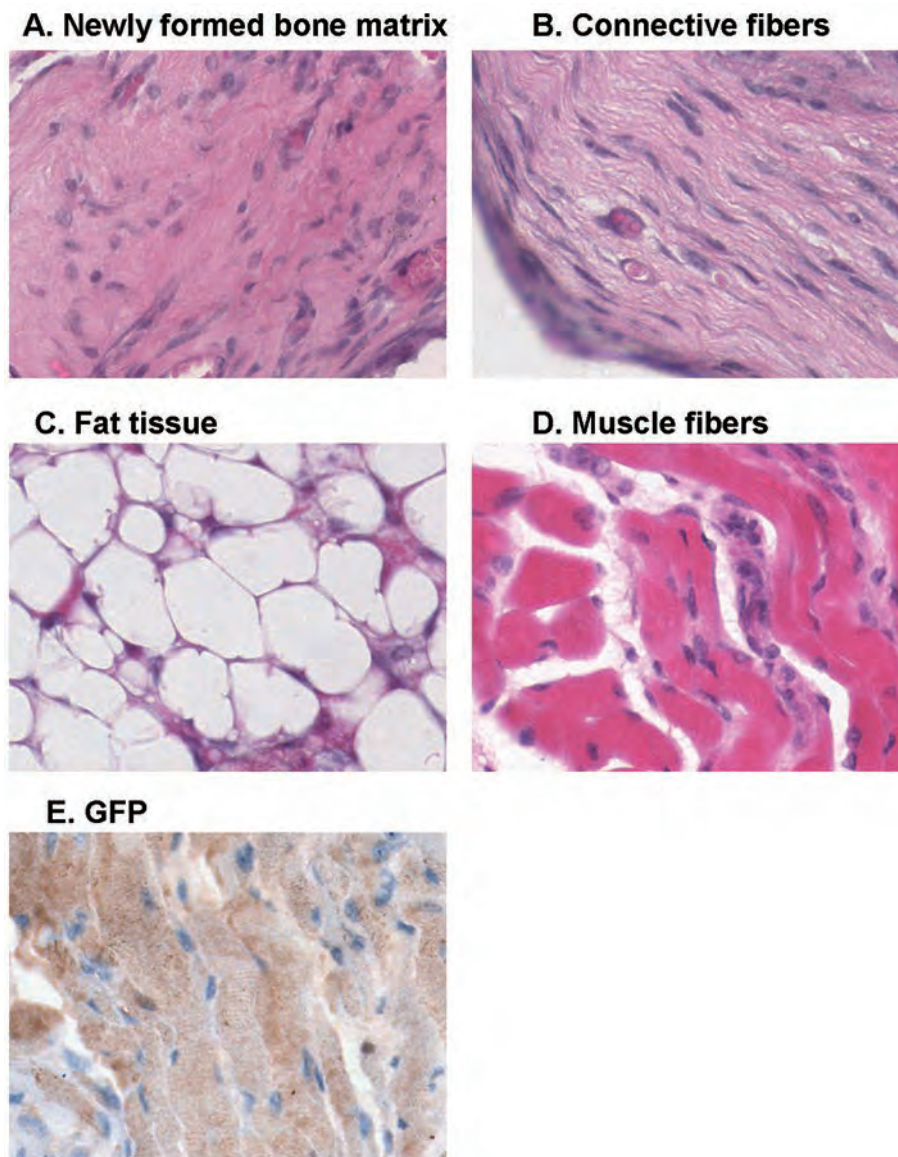


Figure 5 *In vivo* multilineage differentiation capacity of iMSCs. Two to three months after subcutaneous implantation of 1×10^6 EV iMSCs in HA-TCP blocks to nude mice, the implants were harvested for microsectioning and H&E staining analyses. **(A)** Newly formed bone matrix derived from the implanted iMSCs (400 $\times$). Bone matrix was formed in all of the implants ($n = 10$). **(B)** Connective tissues derived from the implanted iMSCs (400 $\times$). Connective tissue was formed in all the implants ($n = 10$). **(C)** Adipose tissue derived from implanted iMSCs (400 $\times$). Adipose tissue was formed in all the implants ($n = 10$). **(D)** Muscle fibers differentiated from the implanted iMSCs (400 $\times$). Muscle fibers were found in most of the implants ($n = 10$). **(E)** The expression of GFP demonstrates that cells are differentiated from human GFP-transduced EV iMSCs (400 $\times$). Brown colored tissues are stained GFP⁺ cells.

tient's own blood cells should find its applications in the treatment of diseases such as arthritis and muscular dystrophy. The iMSCs are multipotent, being able to differentiate into different types of MSC progenies both *in vitro* and *in vivo*, and are not tumorigenic. In addition, with the use of an episomal vector, integration-free iMSCs can be generated. We also demonstrate a dosage

effect of OCT4: a high level of OCT4 is required for efficient reprogramming, but blocks *in vitro* multilineage differentiation, whereas a low level of OCT4 promotes long-term self-renewal and proliferation but does not affect differentiation of iMSCs.

Due to the limited proliferative potential of MSCs derived from adult bone marrow or adipose tissue, many

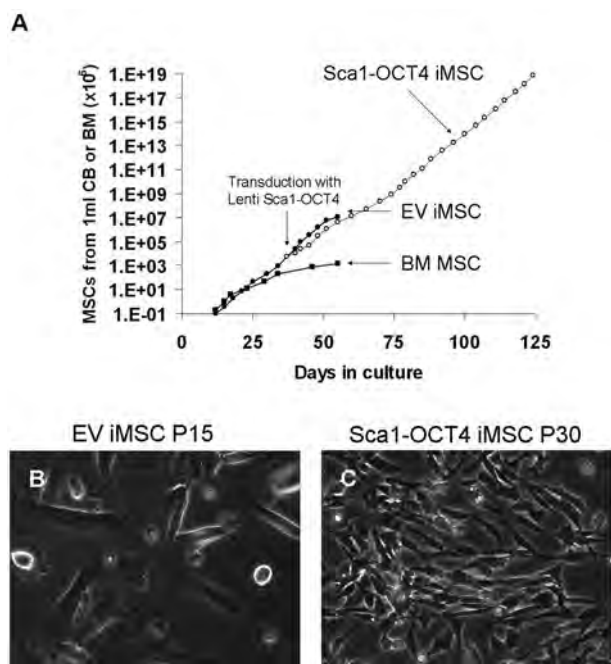


Figure 6 Integration-free EV iMSCs have greater proliferative potential than BM-MSCs and a low level of OCT4 expression allows for long-term expansion of iMSCs. **(A)** Numbers of MSCs generated from 1 ml BM by regular culture vs those from 1 ml CB by OCT4-mediated direct reprogramming. Integration-free EV iMSCs were generated by nucleofection of human CB-CD34⁺ with the EV SFFV-OCT4 plasmid. 1 ml whole BM and CB-CD34⁺ cells (1×10^5) from 1 ml CB were used in this experiment. Total MSCs were calculated by multiplying the numbers of MSCs with splitting factors of each passage. Sca1-OCT4 iMSCs were generated by transduction of EV iMSCs with Lenti Sca1-OCT4, in which the Sca1 promoter drives low-level OCT4 expression. Data shown are one representative result of 3 experiments with similar trends for *ex vivo* expansion of BM-MSCs, EV iMSCs and Sca1-OCT4 iMSCs. **(B)** Senescence morphology of EV iMSCs after 15 passages in culture. **(C)** Typical morphology of Sca1-OCT4 iMSCs after 30 passages in culture. After long-term *in vitro* culture, Sca1-OCT4 iMSCs still morphologically resemble BM-MSCs at low passages.

investigators have focused on the generation of MSCs from iPSCs. Earlier reports have shown that iPSC-MSCs are superior to adult MSCs in proliferative capacity and exhibit better or similar therapeutic effects in treating limb ischemia or allergic airway diseases, and promoting the ectopic formation of vascularized bone [11, 29–31]. The iMSC approach sidesteps the need for iPSC generation, and thus shortens the time required for generating large quantities of patient-specific MSCs from several months to several weeks, and lowers or even abrogates the risk of teratoma formation. We also demonstrate that iMSCs can differentiate to generate skeletal muscle,

pointing to their potential applications in treating muscular disorders.

OCT4 is a master transcription factor that helps to maintain the pluripotency of embryonic stem cells and plays a pivotal role in the generation of iPSCs [32–34]. However, whether OCT4 is expressed in somatic stem cells like MSCs is controversial. Many earlier reports of OCT4 expression in somatic stem cells are likely due to artifacts caused by background noises and the expression of *OCT4* pseudogenes [35–36]. In addition, conditional knockout studies in mice demonstrate that OCT4 is functionally dispensable in self-renewal and differentiation of adult BM-MSCs [37]. However, the observation that OCT4 is not required for *in vivo* self-renewal of MSCs does not necessarily argue against the expression of OCT4 in MSCs during *ex vivo* culture and its potential effects on the functionality of MSCs. Recent reports have provided evidence that OCT4 expression is higher in MSCs at early passages compared with MSCs at late passages [38–39]. They also found that OCT4-KD in MSCs decreases the proliferation potential and enhances spontaneous differentiation of MSCs, whereas OCT4 overexpression in MSCs increases proliferation and sup-

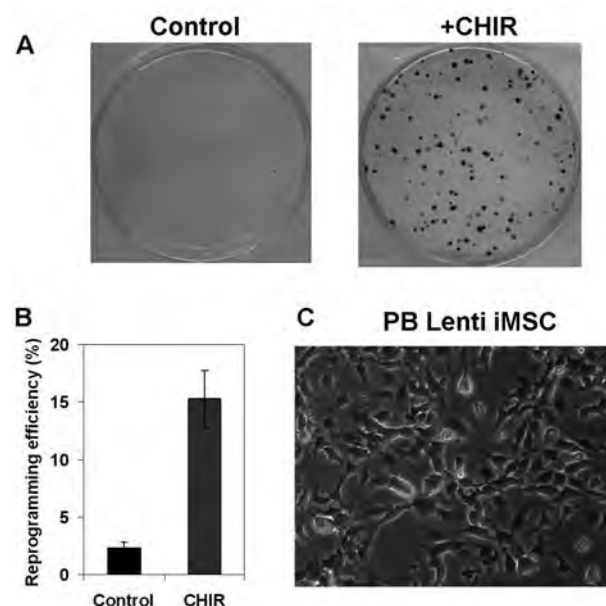


Figure 7 Efficient reprogramming of adult PB-CD34⁺ cells into iMSCs. **(A)** MSC-like colonies formed from 1 000 OCT4-transduced PB-CD34⁺ cells in the absence (control) or presence of CHIR99021 (+CHIR) after 9 days of culture in the MSC medium. **(B)** CHIR substantially increased the reprogramming efficiency from adult PB-CD34⁺ cells into iMSCs ($n = 3$, $P < 0.01$). Error bars indicate SEM. **(C)** Typical morphology of PB-Lenti iMSCs at 1 month after OCT4 transduction.

presses spontaneous differentiation [39]. These data suggest an important role of OCT4 in maintaining the self-renewal of MSCs. This conclusion is further supported by a transgenic mouse study showing that ectopic OCT4 expression blocks the differentiation of epithelial progenitors [27]. Consistent with these observations, we show that a high level of ectopic OCT4 expression promotes long-term proliferation but inhibits iMSC differentiation, and that when OCT4 expression drops to baseline levels, iMSCs can initiate differentiation and eventually undergo senescence.

The use of OCT4 for iMSC generation is likely a safe approach due to several reasons. First, OCT4 expression is detected in germline tumors, but rarely identified in somatic tumors [40-41]. Second, although the inducible expression of OCT4 induces dysplasia in epithelial tissues, withdrawal of the induction stimulus leads to a complete reversal of the tumor-like phenotype. In addition, no mesenchymal tumor was observed in the OCT4 transgenic mouse model [27]. Third, iMSCs cultured in iPSC conditions do not form iPSC colonies, thus it is unlikely that iMSCs will induce teratoma formation after transplantation. In support of this notion, injection of Lenti iMSCs that express a high level of OCT4 does not induce tumors in immunodeficient mice. In contrast, coexpression of MYC in iMSCs did induce tumor formation (data not shown). Fourth, OCT4-generated iMSCs manifest a normal karyotype even after long-term culture, suggesting that ectopic OCT4 expression does not induce genomic abnormalities. In comparison, when MYC together with OCT4 was used for iMSC generation, chromosomal aneuploidies were observed (data not shown). Fifth, decreasing OCT4 expression to 5%-10% of the level expressed in iPSCs does not affect long-term self-renewal and expansion of iMSCs. A low level of OCT4 expression is sufficient to maintain iMSC self-renewal, but does not affect its multilineage differentiation, thus is unlikely to pose any significant risk in clinical use.

We provide compelling evidence that MSCs can be directly reprogrammed from hematopoietic cells. Although vascular and other endothelial cells also express CD34, the source cells we used for reprogramming also express CD45 (Supplementary information, Figure S2A and data not shown), arguing against the possibility that iMSCs are derived from endothelial cells. The time-lapse video also demonstrates the morphological changes from hematopoietic cells to fibroblast-like cells (Supplementary information, Video S2). In addition, single-cell cloning experiment rules out the possibility that OCT4 overexpression expands contaminated MSCs in CD34⁺ cells (Supplementary information, Figure S4). Due to the

unprecedented high reprogramming efficiency of 16%, our findings cannot be explained by the proliferation of trace amount of endothelial cells, MSCs or other types of unknown cells that express CD34.

Many *in vitro* studies used integrating-viral vectors for direct cell reprogramming. Although silencing of the viral vector was observed in some studies [14], reactivation of the reprogramming factor may still pose a long-term risk after cell transplantation. Here, we show that OCT4 expression via a nonintegrating episomal vector is sufficient to induce direct reprogramming of CD34⁺ cells into iMSCs, making this approach one step closer to clinical therapy.

The capacity for long-term undifferentiated proliferation of iMSCs in the presence of low levels of OCT4 expression has important implications for MSC-based therapy. A low level of OCT4 expression in iMSCs does not affect iMSC terminal differentiation. This provides the possibility of a safe autologous MSC-based therapy, as one could select a clone with lentiviral vector integration at the “genomic safe harbors” for expansion and therapy. In this study, we used a weak promoter (Sca1) to drive the low-level OCT4 expression in EV iMSCs. Alternatively, identification of small molecules or microRNAs that can turn on OCT4 expression in MSCs may be useful for promoting the self-renewal of iMSCs and even adult MSCs.

Our study represents the first report that somatic stem cells can be efficiently reprogrammed from hematopoietic cells in adult human peripheral blood. Direct conversion of fibroblasts into cells of clinical interests has been reported by many groups. Due to the obvious advantages of using blood cells compared with fibroblasts derived from skin biopsy, our findings provide a foundation for further investigations into the possibility of direct conversion of blood cells into other cell types such as neural stem cells [14], cardiomyocytes [16-17] and hepatocytes [18-19]. Alternatively, due to the biological similarities between fibroblasts and MSCs [42-43], iMSCs may also constitute a cell source comparable to fibroblasts for generating cells of clinical interest.

Materials and Methods

CB and PB CD34⁺ cells

The use of human CB was approved by the Institutional Review Board of Loma Linda University and written informed consent was obtained from all participants. CD34⁺ cells were purified with a CD34 Microbead Kit (Miltenyi Biotec, Auburn, CA, USA). Mobilized PB CD34⁺ cells were purchased from AllCells LLC (Emeryville, CA, USA).

Lentiviral vectors and episomal vector

In conducting work involving the use of recombinant DNA, the investigators adhered to the current version of the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules. The lentiviral vectors used in this study have been described previously [25]. In these vectors, the SFFV, EF1, PGK or Sca1 promoters were used to drive OCT4 expression at different levels in CD34⁺ cells [25]. The details of lentiviral vector packaging and titering have been published elsewhere [44]. In brief, the calcium precipitation method was used for generating lentiviral vector. After 100-fold concentration by ultracentrifugation, the biological titers of vectors were determined by transducing HT1080 cells. For generating integration-free iMSCs, OCT4 was subcloned into our improved ENBA/oriP-based episomal vector, in which the SFFV promoter and Wpre element were included to drive high-level transgene expression in hematopoietic cells [25]. For OCT4-KD, the shOCT4 lentiviral vector (catalog no: RHS3979-9573664) was purchased from Thermo Scientific Open Biosystems.

BM-MSCs

Human BM samples were purchased from AllCells, LLC. To generate BM-MSCs, whole BM was cultured in human fibronectin (BD Biosciences, San Jose, CA, USA)-precoated culture plates with the Mesenchymal Stem Cell (MSC) Medium Kit from Applied Biological Materials Inc. (ABM; Richmond, BC, Canada). The cultures were kept at 37 °C with 5% CO₂ in a water-jacketed incubator. Cells were cultured under hypoxia by placing culture plates in Hypoxia Chambers (Stemcell Technologies, Inc., Vancouver, BC, Canada) that were flushed with mixed air composed of 92% N₂/3% O₂/5% CO₂. Culture medium was changed every 1-2 days. Approximately 10 days later, when MSC-like colonies appeared, cells were passaged every 3-5 days after treatment with accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA) for 5 min. BM-MSCs at passages 3-6 were used in this study as a positive control. Some BM-MSCs were purchased from ABM.

iMSC generation with lentiviral or episomal vectors

To generate Lenti iMSCs, thawed human CB-CD34⁺ cells or G-CSF-mobilized PB-CD34⁺ cells (AllCells) were cultured in HSC culture medium: Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Grand Island, NY, USA)/10% fetal bovine serum (FBS; ABM) supplemented with TPO, SCF and Flt3 ligand (FL) each at 100 ng/ml, and G-CSF and IL-3 at 10 ng/ml [45]. Cytokines were purchased from ProSpec (East Brunswick, NJ, USA). CD34⁺ cells were cultured for 2 days before transduction. For transduction, cells were inoculated in fibronectin fragment CH-296 (RetroNectin; Takara Bio Inc., Shiga, Japan)-pretreated non-TC well plates and transduced with Lenti SFFV-OCT4 at a multiplicity of infection (MOI) of 2-5 for 4-5 h. A second transduction was performed the next day. To determine transduction efficiency, one well of cells were transduced with Lenti SFFV-GFP. After 2 transductions, 80%-95% cells were GFP⁺. OCT4-transduced cells were cultured in MSC medium. 4 to 6 days later, when CD34⁺ cells were morphologically transforming into MSC-like colonies, cells were split at a ratio of 4-6:1 to human fibronectin-treated non-TC plates. In some cultures, GSK3 inhibitor CHIR99021 (ABM) was also added at a final concentration of 3 μM. For long-term culture, cells were passaged every 2-3 days and cultures were maintained in hypoxia.

To generate integration-free iMSCs, fresh or thawed 1×10^6

CB-CD34⁺ cells were cultured in IMDM/10% FBS supplemented with TPO, SCF and FL at 100 ng/ml. 1 to 2 days later, cells were harvested for nucleofection with a total of 10 μg EV SFFV-OCT4 plasmid DNA. Human CD34 Cell Nucleofector[®] Kit (Lonza, Köln, Germany) was used. Nucleofection was performed with Amaxa Nucleofector II using program U-008. Immediately after nucleofection, cells were suspended in HSC medium and gently transferred to a CH-296-pretreated non-TC well plate and spun down for 5 min at 400× *g* to improve cell survival. Starting the next day, MSC medium was added to the culture wells. At 1-2 weeks after nucleofection, when MSC-like colonies were formed, cells were passaged to human fibronectin-treated non-TC 6-well plates. In the first 2 weeks of culture, CHIR99021 (ABM) was also added at a final concentration of 3 μM. For long-term culture, episomal iMSCs were passaged every 2-4 days and cultures were maintained in hypoxia.

Single CD34⁺ cell cloning and iMSC generation

To rule out the possibility that some MSCs are carried along during CD34 cell enrichment, we conducted single cell cloning. Single CB-CD34⁺ cells were cultured in four U-bottomed 96-well plates in IMDM/10% FBS supplemented with TPO, SCF and FL at 100 ng/ml, and IL-3 and G-CSF at 10 ng/ml. Ten days later, colonies with more than 100 cells were formed in ~10% wells. Twelve clones were randomly selected and transferred to a CH-296-pretreated non-TC 12-well plate. After overnight transduction with Lenti SFFV-OCT4, cells were cultured in a mixture of HSC medium and MSC medium at a 20:80 ratio. At 5 and 8 days after transduction, cultures were refreshed with MSC medium. CHIR99021 (ABM) was added throughout the culture. At 10 days after transduction, the culture plate was stained with crystal violet stain.

Calculation of reprogramming efficiency

To calculate transdifferentiation efficiency, 1000 Lenti SFFV-OCT4-transduced CD34⁺ cells were seeded into CH-296-pretreated non-TC 6-well plates. HSC medium and MSC medium were added at a 20:80 ratio. In some wells, 3 μM CHIR99021 (ABM) was added to the medium. Five days later and every 2 days thereafter, the culture was refreshed with MSC medium. At 9 days after transdifferentiation of culture, the plate was stained with crystal violet stain and colonies were enumerated.

Cell imaging

Phase contrast pictures were taken using a Nikon converted microscope with a 10× objective. For time-lapse live cell imaging, CD34⁺ cells were transduced with Lenti SFFV-OCT4 or GFP control vectors. One day after transduction, 1×10^4 cells were seeded in each well of a CH-296-pretreated non-TC 6-well plate. Cells were cultured in HSC medium or MSC medium supplemented with 3 μM CHIR99021. At day 4, culture medium was changed. Phase contrast pictures of cells in culture were taken every 2 h for 6 days in a BioStation CT (Nikon Instruments Inc.) at UCR (University of California Riverside) Stem Cell Center Core Facility. The pictures were edited and videos composed with ImageJ.

Flow cytometry

To phenotype iMSCs, cells were stained with hematopoietic markers CD14, CD34 and CD45, endothelial marker CD31, MSC markers CD29, CD44, CD73, CD90, CD105 and CD166, and

iPSC markers OCT4, SOX2, NANOG and TRA-1-60. All antibodies except otherwise specified were purchased from eBioscience, Inc. (San Diego, CA, USA). For staining of cell surface markers, CD14-FITC, CD29-APC (BD Biosciences), CD31-PE, CD34-PE, CD44-PE, CD45-FITC, CD73-APC, CD90-PE, CD105-APC, TRA-1-60-PE, CD166-PE (BioLegend, Inc., San Diego, CA, USA) cells were incubated with each antibody for 30 min at room temperature in FACS buffer in the dark. For intracellular staining, cells were fixed for 20 min at room temperature in a fixation buffer supplemented with 10% permeabilization buffer (eBioscience). After 2 washes with FACS buffer, cells were incubated with OCT4-PE, SOX2-FITC or NANOG-PE (BD Biosciences) for 2 h at room temperature in the dark. Following incubation, the samples were washed twice with FACS buffer supplemented with 10% permeabilization buffer. Flow cytometric analysis was performed using the FACS Aria II with a 488 nm laser (BD Biosciences).

In vitro differentiation of MSCs

For adipocytic differentiation, 1×10^5 iMSCs or BM-MSCs were seeded in each well of TC-treated 6-well plates. Adipocytic differentiation was induced with 1 μ M dexamethasone (Sigma-Aldrich Corp, St Louis, MO, USA), 1 μ M troglitazone (Sigma), 10 μ g/ml insulin (Sigma), 0.5 mM isobutylxanthine (Sigma), 5 ng/ml of FGF2 (ABM) and 10% FBS in α -MEM (Life Technologies) with 1% penicillin/streptomycin (P/S). For osteoblastic differentiation, 2×10^5 cells were seeded in each well of TC-treated 6-well plates. Osteoblastic differentiation was induced with 0.1 μ M dexamethasone (Sigma), 200 μ M ascorbic acid (Sigma), 10 mM β -glycerol phosphate (Sigma), 10 ng/ml BMP2 (ABM), 10 ng/ml BMP4 (ABM) and 10% FBS in α -MEM. For chondrogenic differentiation, 4×10^5 cells were seeded in each well of TC-treated 6-well plates. Chondrogenic differentiation was induced in α -MEM/10% FBS/1% P/S supplemented with 0.1 μ M dexamethasone, 200 μ M ascorbic acid, 5.33 μ g/ml linoleic acid, 0.35 mM L-proline, 10 ng/ml TGF β 3 (Stemgent, San Diego, CA, USA), 10 ng/ml TGF β 1 (ABM) and 1% ITS (insulin-transferrin-selenium; Life Technologies).

All cultures were maintained with 5% CO₂ in a water-jacketed incubator at 37 °C, and culture media were changed every 2-3 days. Three to four weeks after differentiation culture, cells were fixed in 10% neutral buffered formalin for 15 min before staining. Adipocytes were stained with Oil Red O solution for 15 min. Oil Red O working solution was made by diluting Oil Red O stock solution with nanopure water at a 3:2 ratio. Oil Red O stock solution was made by dissolving 0.5 g of Oil Red O powder in 100 ml isopropanol with gentle heat and then filtered with a 5 μ m syringe filter. After 15 min of incubation with 1 ml of Oil Red O working solution, the stain was washed out with 60% isopropanol. Bone nodule formation was evaluated by Alizarin Red staining. After formalin fixation, 1 ml of Alizarin Red staining solution was added for 5 min and the cells were washed with nanopure water. Alizarin Red staining solution was made by dissolving 2 g of Alizarin Red in 100 ml of nanopure water. The pH was adjusted to 4.3 with 10% ammonium hydroxide and then filtered with a 5 μ m syringe filter. To stain mucopolysaccharides associated with chondrocytic differentiation, fixed cultures were stained with Alcian Blue staining solution for 30 min. The cultures were later washed in tap water for 2 min and then rinsed in nanopure water. Alcian Blue staining solution was prepared by dissolving 1 g of Alcian Blue in 100 ml of 3% acetic acid solution. The pH was adjusted to 2.5 using acetic acid.

RNA isolation and quantitative real-time RT-PCR

To induce *in vitro* multilineage differentiation of iMSCs and BM-MSC controls, cells were cultured in conditions detailed above. At 2 or 3 weeks after differentiation culture, cells were harvested using cell scrapers or by treating with trypsin (ABM). Total RNA was extracted with Trizol reagent and RNeasy kit (Qiagen, Inc., Valencia, CA, USA). Reverse transcription was performed using the EasyScript Plus cDNA Synthesis Kit (ABM), following the manufacturer's recommendations. Quantitative real-time RT-PCR (qPCR) was performed as previously described [44]. Expression of differentiation-associated genes at 2 or 3 weeks after induction of cultures were not significantly different, and thus were combined for analysis. Expression of *OCT4* and lineage-specific markers was normalized to the expression of *ACTB*. The sequences of primers for qPCR are as follows: *ACTB* forward, 5'-TCGTGCGTGACATTAAGGAG-3'; reverse, 5'-GGCAGCTCGTAGCTCTTCTC-3'; *LEPTIN* forward, 5'-GAAGACCACATCCACACACG-3'; reverse, 5'-AGCTCAGCCAGACCCATCTA-3'; *LPL* forward, 5'-ATTTGCCCTAAGGACCCC-3'; reverse, 5'-ATGACAGGTAGCCACGGAC-3'; *PPARG2* forward, 5'-AGAAGCCTGCATTCTGCAT-3'; reverse, 5'-TCAAAGGAGTGGGAGTGGTC-3'; *FABP4* forward, 5'-TACTGGGCCAGGAATTTGAC-3'; reverse, 5'-GTGGAAGTGACGCCTTTCAT-3'; *RUNX2* forward, 5'-TTTGCCTGGGT-CATGTGT-3'; reverse, 5'-TGGCTGCATTGAAAAGACTG-3'; *BGLAP* forward, 5'-TCACACTCCTCGCCCTATTG-3'; reverse, 5'-TCGCTGCCCTCCTGCTTG-3'; *IBSP* forward, 5'-GCAGTAGTGACTCATCCGAAGAA-3'; reverse, 5'-GCCTCAGAGTCTTCATCTTCATTC-3'; *COL1A1* forward, 5'-GC-CATCAAAGTCTTCTGC-3'; reverse, 5'-ATCCATCGGTCATGCTCT-3'; *ACAN* forward, 5'-CGCTACTCGCTGACCTTT-3'; reverse, 5'-GCTCATAGCCTGCTTCGT-3'; *SOX9* forward, 5'-GACTTCCGCGACGTGGAC-3'; reverse, 5'-GTTGGGCG-GCAGGTACTG-3'; *COL2A1* forward, 5'-TCCCAGAACAT-CACCTACC-3'; reverse, 5'-AACCTGCTATTGCCCTCT-3'; *OCT4* forward, 5'-CTTGCTGCAGAAGTGGGTGGAGGAA-3'; reverse, 5'-CTGCAGTGTGGGTTTCGGCA-3'.

Tumor formation assay

The use of NOD/SCID/IL2RG^{-/-} (NSG) immunodeficient mice for the tumor formation assay was approved by the Institutional Animal Care and Use Committee at Loma Linda University (LLU). In conducting research using animals, the investigators adhered to the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals, and the principles set forth in the current version of the Guide for Care and Use of Laboratory Animals, National Research Council. NSG mice were purchased from the Jackson Laboratory (Bar Harbor, MA, USA) and maintained at the LLU animal facility. To facilitate detection of human cells in mice, iMSCs were transduced with GFP. 1×10^6 Lenti iMSCs were harvested by Accutase digestion, washed with culture medium and re-suspended in 200 μ l MSC medium-diluted (1:1) Matrigel solution (BD Biosciences). Cells were then injected into the subcutaneous tissue of NSG mice. As a positive control, clumps of $\sim 1 \times 10^6$ iPSCs in Matrigel were injected subcutaneously. In some mice, 2×10^6 Lenti iMSCs were also injected via tail vein. Two to three months after transplantation, mice were euthanized to examine the presence of solid tumor in multiple organs. To detect the presence of leukemia, BM cells were harvested for FACS analysis.

Bisulphite sequencing

Bisulphite sequencing of genomic DNA from iMSCs was used to assess methylation status of *OCT4* and *NANOG* promoter. Genomic DNA was purified with a DNeasy Kit (Qiagen, Inc.). The conversion of unmethylated cytosines to uracil was carried out using EZ DNA Methylation-Gold Kit (ZYMO Research Corp., Irvine, CA, USA). The experimental procedure was detailed in a previous publication [25].

Karyotyping and G-banding

Giemsa (GTG)-banding chromosome analysis was carried out in the LLU Radiation Research Laboratories. Standard DNA spectral karyotyping procedures were followed and a HiSKY Complete Cytogenetic System was used (Applied Spectral Imaging, Inc. Vista, CA, USA). Ten metaphases were analyzed and karyotyped. The data were interpreted by a board-certified cytogenetic technologist.

In vivo MSC differentiation assay

Experimental procedures were approved by the Animal Care and Use Committee of the Chinese University of Hong Kong. 1×10^6 GFP-transduced EV iMSCs or BM-MSCs were absorbed into the porous Skelite[®] resorbable HA-TCP bone graft substitute blocks (size: $0.4 \times 0.4 \times 0.4$ cm) for 2 h to allow cell attachment. The cells-HA/TCP block was then implanted subcutaneously into the dorsal side of female nude mice. At 8-12 weeks after implantation, the implants were harvested and fixed with 10% neutral buffered formalin, decalcified with 9% formic acid for 2-3 weeks and embedded in paraffin for histological examination. The 5- μ m microsectioning slides were deparaffinized and rehydrated, and endogenous peroxidase was quenched by rinsing in 3% hydrogen peroxide in methanol for 20 min. Hematoxylin and Eosin (H&E) staining was performed using standard protocol. To identify progeny of human GFP-expressing iMSCs, we conducted immunohistological staining against GFP. After washing in PBS, antigen retrieval was carried out in 10 mM citrate buffer at 37 °C for 10 min, and followed by 3 washes in PBS. The samples were blocked with 10% goat serum in 1% BSA/PBS for 1 h at room temperature. Primary antibody against GFP (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added and the blocking solution was used as negative control. After overnight incubation at 4 °C, the samples were washed in PBS 3 times and incubated with HRP-conjugated anti-rabbit secondary antibody (1:200; Santa Cruz Biotechnology) for 1 h at room temperature. After PBS washing, peroxidase substrate DAB (Dako, Carpinteria, CA, USA) was used for color development (1 min for GFP). The samples were immersed in distilled water, dehydrated and cleared before being mounted with DPX.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Mann-Whitney *U* test was performed for RT-qPCR data and two-tailed Student's *t*-test was performed for other comparisons. *P* < 0.05 was considered statistically significant.

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(Supplementary information is linked to the online version of the paper on the *Cell Research* website.)

Efficient Generation of Integration-Free iPSCs from Human Adult Peripheral Blood Using BCL-XL Together with Yamanaka Factors

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Abstract

The ability to efficiently generate integration-free induced pluripotent stem cells (iPSCs) from the most readily available source—peripheral blood—has the potential to expedite the advances of iPSC-based therapies. We have successfully generated integration-free iPSCs from cord blood (CB) CD34⁺ cells with improved oriP/EBNA1-based episomal vectors (EV) using a strong spleen focus forming virus (SFFV) long terminal repeat (LTR) promoter. Here we show that Yamanaka factors (OCT4, SOX2, MYC, and KLF4)-expressing EV can also reprogram adult peripheral blood mononuclear cells (PBMCs) into pluripotency, yet at a very low efficiency. We found that inclusion of BCL-XL increases the reprogramming efficiency by approximately 10-fold. Furthermore, culture of CD3⁺/CD19[−] cells or T/B cell-depleted MNCs for 4–6 days led to the generation of 20–30 iPSC colonies from 1 ml PB, an efficiency that is substantially higher than previously reported. PB iPSCs express pluripotency markers, form teratomas, and can be induced to differentiate in vitro into mesenchymal stem cells, cardiomyocytes, and hepatocytes. Used together, our optimized factor combination and reprogramming strategy lead to efficient generation of integration-free iPSCs from adult PB. This discovery has potential applications in iPSC banking, disease modeling and regenerative medicine.

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Introduction

The successful generation of induced pluripotent stem cells (iPSCs) from human somatic cells has revolutionized our understanding of the development and regeneration of cells, tissues and organs, igniting new hope for replacement therapies.[1,2,3] This breakthrough has been recently recognized by the Nobel Committee for Physiology or Medicine.[4,5,6] The development of novel approaches for generating integration-free iPSCs has eliminated the concern of integrating virus-associated genotoxicity in clinical applications. Transposon [7,8] and excisable polycistronic lentiviral vectors [9,10,11,12,13,14] can be used to generate integration-free iPSCs, but a second step is necessary to remove the transgenes once reprogramming has been achieved. Many one-step approaches such as adenovirus vectors, [15,16] plasmids, [17,18,19] minicircle DNAs, [20,21] artificial chromosome vectors [22] and protein transduction [23,24] are very inefficient in generating integration-free iPSCs. Relatively

efficient approaches that have been readily reproduced in different labs include Sendai virus vector, [25,26,27,28,29,30] modified mRNA, [31,32,33] and oriP/EBNA1-based episomal vectors (EV). [34,35,36,37,38,39,40,41,42] The most cost effective approach is EV, because there is no need for packaging of viral vectors and one simple infection instead of daily or multiple additions of factors is sufficient for successful reprogramming. EV is a plasmid containing two elements from Epstein-Bar virus: oriP and EBNA1. Binding of the EBNA1 protein to the virus replicon region oriP maintains a relatively long-term episomal presence of plasmids in mammalian cells. The unique features of EV make it an ideal vector for generating integration-free iPSCs. EV yields expression of reprogramming factors at sufficiently high levels for several cell divisions, thus allowing for successful reprogramming after only one infection, while the gradual depletion of plasmids during each cell division leads to the generation of integration-free iPSCs after approximately 2 months of culture.

Although fibroblasts from skin biopsy or other sources were initially used in many studies for the generation of iPSCs, mononuclear cells (MNCs) from peripheral blood (PB) have been widely accepted as a more convenient and almost unlimited resource for cell reprogramming. [43,44,45,46] PB MNCs are a mixed population, containing lymphoid cells like T cells and B cells and non-lymphoid cells that include myeloid cells as well as 0.01–0.1% CD34⁺ hematopoietic stem/progenitor cells (HSCs). In earlier studies, mature T or B cells were efficiently converted to iPSCs with Sendai virus or EV plasmids. [25,42,47] However, iPSCs generated from T/B cells contain T cell receptor (TCR) or immunoglobulin (IG) gene rearrangements, restricting their broad applications in regenerative medicine. [45,46,48] Therefore, we and many other investigators have attempted to generate integration-free iPSCs from non-lymphoid cells. [35,39,40,41] However, only 1–5 integration-free iPSC colonies can be generated from 1 ml of PB in these reports. Thus, further improvements in reprogramming efficiency are necessary to make the EV-based approach for reprogramming PB widely applicable. Building on our previous finding that our improved EV vector design leads to efficient reprogramming of cord blood (CB) CD34⁺ cells, [40] here we further develop this approach for the generation of integration-free iPSCs from adult PBMNCs. More recent approaches generate up to 10 iPSC colonies from 1 ml of PB in non-T cell culture conditions with 7 factors including EBNA1 and shRNA against TP53 (also known as p53). [42] However, expression of EBNA1 and TP53 shRNA synergistically inhibits the genome guardian p53, which raises concerns about the genomic integrity of iPSCs generated using this approach. [49] Thus, we avoided the use of TP53 shRNA in this study.

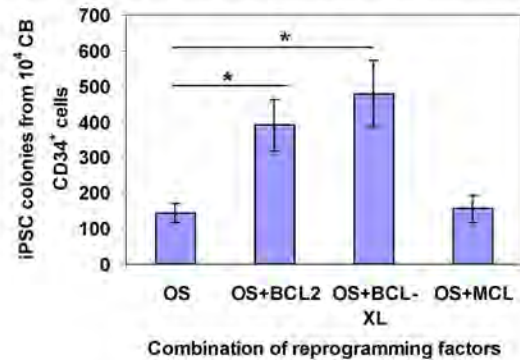
Results

BCL-XL enhances the efficiency of OS-mediated reprogramming of both CB CD34⁺ cells and PBMNCs

We have found that balanced expression of OCT4 and SOX2 (OS) driven by a single strong promoter efficiently induces CB CD34⁺ cells into pluripotency. [40] It was also reported that Bcl2 increases reprogramming efficiency of mouse fibroblasts by 2–3 fold. [50] We thus hypothesized that anti-apoptotic factors in the Bcl-2 family may increase OS-mediated human blood cell reprogramming. To test this hypothesis, we cloned anti-apoptotic factors BCL2, BCL-XL (isoform Bcl-X(L) of BCL2L1), and MCL1 into a lentiviral vector under the control of the spleen focus forming virus (SFFV) promoter. We chose this promoter because we and others found that the SFFV promoter drives higher-levels of transgene expression in primary hematopoietic cells or cell lines than commonly used promoters like human elongation factor 1alpha (EF1), human phosphoglycerate kinase (PGK), and cytomegalovirus (CMV). [40,51,52]

Consistent with our previous report, [40] 1–2% of OS-transduced CB CD34⁺ cells were converted to iPSCs (**Figure 1A**). Inclusion of BCL2 or BCL-XL increased reprogramming efficiency by ~3-fold ($P < 0.05$), while MCL1 had no obvious effect on enhancing OS-mediated reprogramming. We then conducted the same study using adult PB. MNC were isolated from several donors (age 22–43) by Ficoll-Hypaque density gradient centrifugation and cultured for 4–6 days. Of note, OS alone could also induce adult blood cells into pluripotency, although the efficiency was 100-fold lower than reprogramming of CB CD34⁺ cells (**Figure 1B**). Of interest, the effects of the three anti-apoptotic factors on OS-mediated reprogramming of PBMNCs were identical to that of CB CD34⁺ cells (**Figure 1B**).

A. Reprogramming of CB CD34⁺ cells with lentiviral vectors



B. Reprogramming of PB MNCs with lentiviral vectors

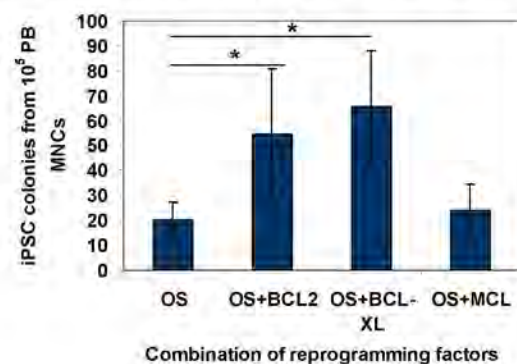


Figure 1. BCL-XL significantly enhances OS-mediated reprogramming of cord blood and peripheral blood cells with lentiviral vectors. (A) Differential effects of BCL2 family members on enhancing OS-mediated reprogramming of CB cells. CB CD34⁺ cells were cultured for 2 days before lentiviral transduction. CB iPSC colonies were enumerated at 2 weeks after transduction of reprogramming factors. Data shown are presented as mean $\pm$ SEM (n = 4). OS: OCT4 and SOX2. * indicates $P < 0.05$. (B) Differential effects of BCL2 family members on enhancing OS-mediated reprogramming of PBMNCs. PBMNCs were cultured for 4–6 days before lentiviral transduction. PB iPSC colonies were enumerated at 3 weeks after transduction of reprogramming factors. Data shown are presented as mean $\pm$ SEM (n = 4). OS: OCT4 and SOX2. PBMNCs, peripheral blood mononuclear cells. * indicates $P < 0.05$. BCL2 and BCL-XL significantly increased reprogramming of both CB CD34⁺ cells and PBMNCs. doi:10.1371/journal.pone.0064496.g001

BCL-XL appeared to be more potent than BCL2 in enhancing reprogramming, even though the difference did not reach statistical significance. Therefore, we elected BCL-XL in our integration-free iPSC studies.

We have shown that our lentiviral vector design enables high-level expression of reprogramming factors, leading to a 1000-fold increase in reprogramming of CB CD34⁺ cells relative to previously reported. [40,53] To investigate if this conclusion also holds for PB reprogramming, we compared our results with a similar study reported by Daley's lab. [46] They generated iPSCs from human PBMNCs at an efficiency of up to 0.001% with four factors (OS + MYC and KLF4 or MK) expressed by lentiviral vectors. The reprogramming efficiency with OS alone was 0.02% (**Figure 1B**); with the addition of MK, 0.2% PBMNCs can be reprogrammed into iPSCs. Addition of MK to OS increased PBMNC reprogramming efficiency by 10-fold, which is similar to our results with CB CD34⁺ cells. [54] These data suggest that our

lentiviral vector-mediated PBMNC reprogramming is at least 200-fold more efficient than other vectors. [46]

Generation of integration-free iPSCs from PBMNCs with episomal vectors

We have developed an improved oriP/EBNA-based episomal vector in which the SFFV promoter drives high-level transgene expression in hematopoietic cells and Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) stabilizes transcribed mRNA, thereby increasing expression of transgenes. [40] This EV vector also led to successful generation of integration-free iPSCs from CB, even with OS alone. Here we used the same EV backbone to express several factors for reprogramming of PBMNCs. Our intent was to generate integration-free iPSCs from non-lymphoid PBMNCs, thus we cultured cells in conditions that favor expansion of HSCs and myeloid cells. After nucleofection of cultured PBMNCs with EV plasmids, cells were transferred to 6-well plates, pre-coated with feeder cells, for 3–4 weeks of culture. In contrast to CB, OS-expressing EV plasmid alone failed to reprogram PBMNCs, while inclusion of BCL-XL led to successful reprogramming (**Figure 2A**). A similar enhancing effect of BCL-XL was observed in OSK or OSMK-mediated reprogramming: BCL-XL increased reprogramming efficiency by up to 10-fold (**Figure 2B**). With the use of three EV plasmids expressing OS, MK and B (BCL-XL), up to 10 iPSC colonies could be generated (**Figure 2B**). Of interest, in the absence of MYC, OS+K+B also induced PBMNCs to pluripotency at a similar efficiency (**Figure 2B**).

To test whether the generated iPSCs are from non-lymphoid cells in PBMNCs, we nucleofected fractionated cells with EV plasmids expressing OS+MK+B. iPSCs were generated from the population of cells expressing the myeloid lineage marker, CD33, but not from the CD33[−] cells (**Figure 2C**). To provide further evidence that iPSCs were indeed generated from non-lymphoid cells, we fractionated PBMNCs using antibodies that recognize T cells (CD3) and B cells (CD19). Consistent with our findings in the previous experiment, the T/B cell enriched population (containing CD3⁺ T and 19⁺ B lymphocytes, respectively) failed to generate any iPSCs, while the same number of T/B-depleted cells (CD3[−]/19[−] cells) generated dozens of iPSCs (**Figure 2C**). Taken together, these data demonstrate that the integration-free iPSCs generated with our approach are derived from non-lymphoid cells.

An incidental finding in the above experiments showed that T/B-depleted cells generated substantially more iPSC colonies (**Figure 2C**). We thus used PBMNCs from 4 donors to test whether T/B cell depletion can increase reprogramming efficiency. Whole PBMNCs or CD3[−]/19[−] cells were cultured in the same conditions for 4 days and then nucleofected with EV plasmids expressing OS+MK+B. Approximately 10-fold more iPSC colonies were generated from 1×10⁶ CD3[−]/19[−] cells than from 1×10⁶ whole MNCs. Since the amount of CD3[−]/19[−] cells was only ~30% that of MNCs after purification, this population generated iPSCs ~3-fold more efficiently than when they were plated as unfractionated PBMNCs with T and B cells present ($P < 0.05$) (**Figure 3A**). In our pilot studies, we found that freshly isolated PBMNCs often failed to be reprogrammed and, after 10 days of *ex vivo* culture, the yield of iPSCs also substantially decreased. We then used CD3[−]/19[−] cells to determine the optimal culture duration for efficient reprogramming. We found that if cells were cultured for less than 2 days, only a few colonies were obtained, whereas 4–6 days of culture led to the generation of more than 20 iPSC colonies from 1 ml of PB (**Figure 3B**). Culturing cells for more than 8 days led to a significant decrease in reprogramming efficiency (**Figure 3B**). Take together, T/B cell

depleted PBMNCs that are cultured for 4–6 days under conditions that favor nonlymphoid cell expansion can be efficiently reprogrammed to iPSCs with EV plasmids that express five factors (OS+MK+BCL-XL).

Characterization of integration-free iPSCs derived from adult PB

PB iPSCs, generated as described above, robustly proliferated under human iPSC culture conditions for more than 20 passages. PB iPSC colonies showed a tight morphology characteristic of human pluripotent stem cells (**Figure 4A**). Consistent with earlier reports, [35,40,55] qPCR analysis of iPSCs after 10 passages showed that the average copy number of residual EV plasmids decreased to less than 0.01 copy per cell in 6 out of 6 iPSC clones, suggesting that after long-term culture, EV plasmids are depleted from almost all cells. Karyotype analysis indicated a normal human karyotype for all of the clones tested; one representative karyotype is shown in **Figure 4B**. Immunostaining of iPSC colonies showed expression of pluripotency markers like OCT4, SOX2, NANOG and SSEA4 (**Figure 4C**). Two months after being subcutaneously injected into immunodeficient mice, iPSCs formed teratomas consisting of derivatives of all three embryonic germ layers (**Figure 4D**). Together, these data demonstrate that integration-free PB iPSCs are morphologically, phenotypically and functionally identical to pluripotent stem cells.

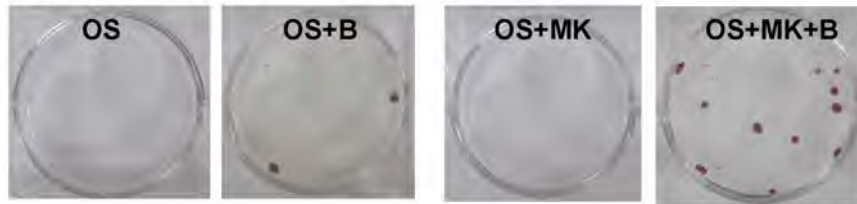
In vitro multilineage differentiation potential of integration-free PB iPSCs

We further investigated whether PB iPSCs can be induced to differentiate into cells of different lineages in culture. We found that PB iPSCs were readily differentiated into mesenchymal stem cells (MSCs) in MSC-conductive culture conditions (**Figure 5A**). More than 90% cells expressed typical markers of MSCs including CD73, CD105 and CD166. Furthermore, after 3 weeks of induction culture, PB iPSC-derived MSCs differentiated into adipocytes, osteoblasts and chondrocytes (**Figure 5A**). These data suggest that MSCs differentiated from integration-free PB iPSCs are morphologically and functionally indistinguishable to bone marrow-derived MSCs. [56] To differentiate PB iPSCs into hepatocytes, cells were initially induced to a relatively homogenous population of definitive endoderm cells, which were further differentiated into hepatocyte-like cells within 7 days. The differentiating cells underwent a series of morphological changes. After Day 7, the cells manifested a polygonal shape and round single or double nuclei with many cytoplasmic vesicles, characteristic features of mature hepatocytes (**Figure 5B**). Immunohistochemical analysis showed that ~90% cells iPSC-derived hepatocytes expressed liver-specific genes like alpha fetoprotein (AFP), albumin (ALB), and alpha 1-antitrypsin ($\alpha 1$ -AT) (**Figure 5B**), thus providing evidence of *bona fide* differentiation into hepatocytes. We also investigated the cardiac differentiation capacity of PB iPSCs. After 2 weeks of culture in cardiomyocyte differentiation medium, dozens of beating colonies were observed in each well of 6-well plates. Immunostaining of these cells showed the majority of cells expressed the Troponin I marker, confirming their identity as cardiomyocytes (**Figure 5C**). Taken together, these in vitro differentiation data demonstrate that integration-free PB iPSCs can be induced to differentiate into MSCs, hepatocytes and cardiomyocytes.

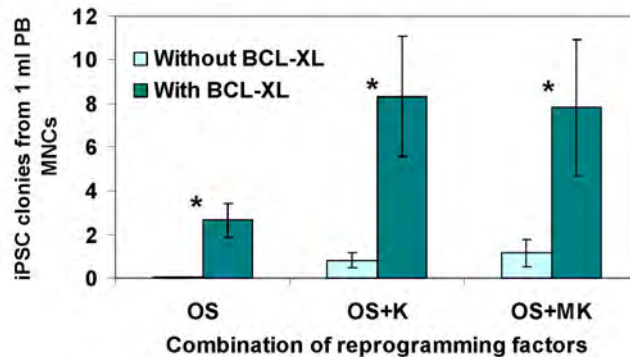
Discussion

Here we report that integration-free iPSCs can be efficiently generated from human adult PBMNCs in 3–4 weeks with EV

A. Episomal vector mediated reprogramming of PB MNCs



B. BCL-XL substantially enhances PB reprogramming



C. iPSC colonies generated from fractionated cells in PB MNCs

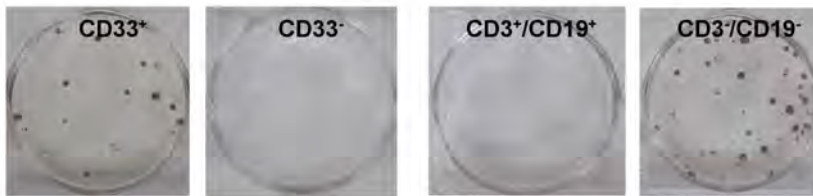


Figure 2. Generation of integration-free iPSCs from adult PB MNCs with episomal vectors. (A) ALP staining of iPSCs at 4 weeks after nucleofection of PB MNCs with reprogramming factor-expressing episomal vectors. OS, OCT4 and SOX2; MK, MYC and KLF4; B, BCL-XL. PB MNCs were cultured for 4–8 days before nucleofection. 1×10^6 PB MNCs were nucleofected and then seeded into each well. (B) Inclusion of BCL-XL increases PB reprogramming efficiency by up to 10-fold. PB MNCs were cultured for 4–8 days before nucleofection. ALP-positive iPSC colonies were enumerated at 3–4 weeks after nucleofection. Data are presented as mean $\pm$ SEM (n = 6). In all 3 conditions, BCL-XL significantly increased reprogramming efficiency. * indicates $P < 0.05$. (C) iPSC are generated from PB MNCs expressing the myeloid lineage marker, CD33, but not lymphoid cells (CD3⁺ and CD19⁺ cells) in PB MNCs. ALP staining of iPSCs at 4 weeks after nucleofection of fractionated PB MNCs with episomal vectors OS+MK+B. CD33, myeloid marker; CD3, T cell marker; CD19, B cell marker. 1×10^6 indicated cells were nucleofected and then seeded into each well. ALP staining was conducted at 4 weeks after nucleofection.

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plasmids that express five factors, OS+MK+BCL-XL. In previous studies we improved vector design to increase reprogramming efficiency of CB CD34⁺ cells by 10–20 fold. [40] Here we build on this improved vector design and show that inclusion of BCL-XL in the factor combination increases reprogramming efficiency by ~10-fold. When we also enrich PB MNCs for non-lymphoid cells (CD3[−]CD19[−]) and combine this with *ex vivo* culture for 4–6 days, we can generate 20–30 iPSC colonies from 1 ml of PB. Consistent with our previous report on CB reprogramming, [40] we found that lentiviral vector-mediated transduction with OS alone can also induce PB MNCs into pluripotency, while inclusion of MK increases the reprogramming efficiency to a level that is more than 200-fold higher than previously reported. [46] Thus, our improved vector design provided a primary tool that with the inclusion of BCL-XL allows us to achieve unprecedented efficiency in PB MNC reprogramming. The improved vector design and the identifica-

tion of BCL-XL as a powerful reprogramming-enhancing factor have important implications for cellular reprogramming of hematopoietic cells.

The reprogramming into pluripotency of unmobilized adult PB MNCs cells that lack the DNA alterations present in T and B lymphocytes (non-lymphoid cells) has been a long-sought goal. Recent technological breakthroughs make it possible to generate integration-free iPSCs from non-lymphoid cells in PB cells, but at relatively low efficiencies. Studies showed that 6–7 factors are necessary to achieve successful reprogramming of PB MNCs. In one report, the use of 6 factors (OSMK+LIN28+SV40 Large T antigen or SV40LT) leads to generation of ~1 iPSC colony per ml of PB. [41] In others, the use of 7 factors (OSK+LIN28+NA-NOG+MYCL1+SV40LT or OSK+LIN28+MYCL1+TP53 shRNA+EBNA1) leads to the generation of 5–10 iPSC colonies from 1 ml of PB. [39,42] In comparison, with only 5 factors

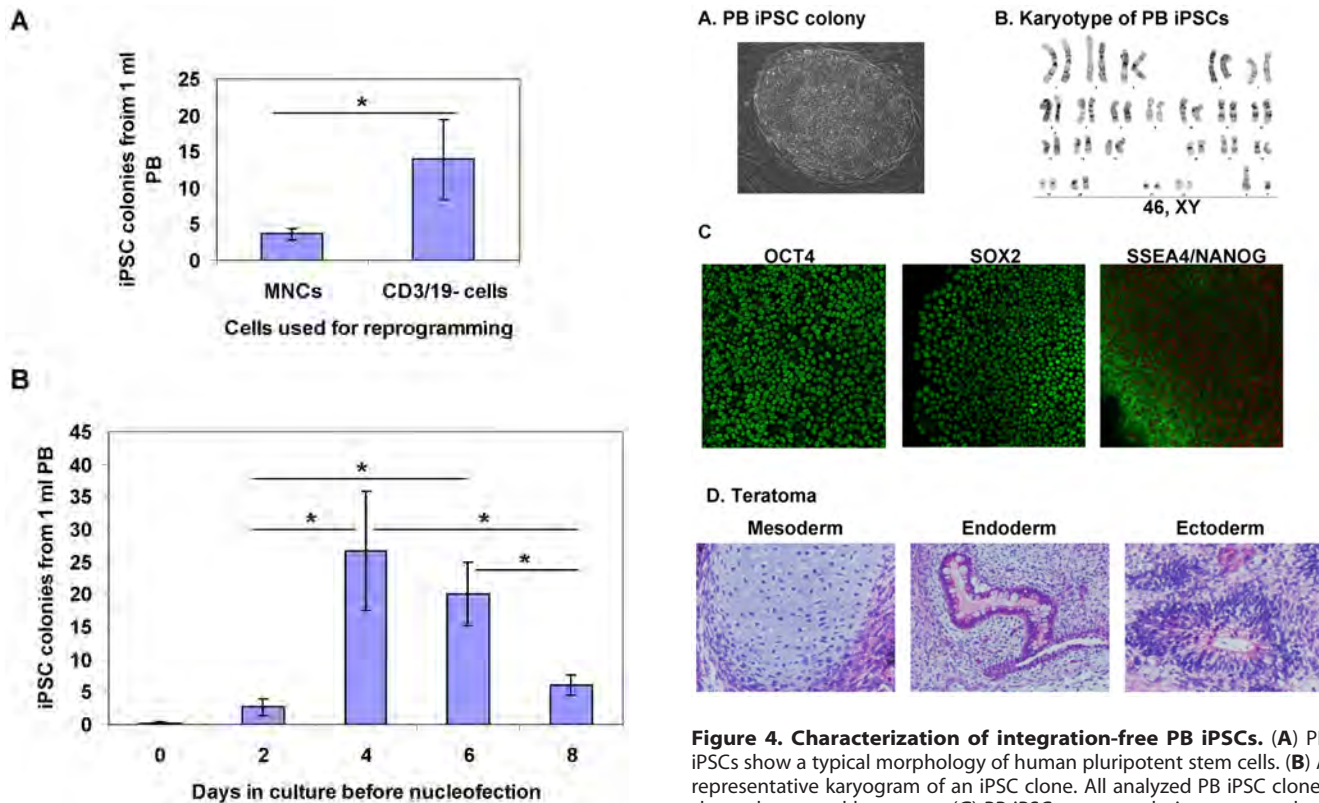


Figure 3. Optimization of the PBMNC reprogramming protocol. (A) Depletion of CD3⁺ and 19⁺ lymphoid cells increases reprogramming efficiency. Whole PBMNCs or CD3⁺/CD19⁺ cells (T/B cell-depleted PBMNCs) were cultured for 4 days before nucleofection with episomal vectors expressing OS (OCT4 and SOX2), MK (MYC and KLF4) and B (BCL-XL). 1×10^6 cells were used for nucleofection. The numbers of iPSC colonies were counted at 3–4 weeks after nucleofection and numbers of iPSC colonies per 1 ml of PB were calculated by normalization to the amount of starting peripheral blood. Data shown are presented as mean $\pm$ SEM (n=4). * indicates $P < 0.05$. (B) Culturing PB CD3/19⁺ cells for 4 days allows maximum reprogramming. CD3/19⁺ cells (T/B cells-depleted) PBMNCs were cultured for 0 to 8 days before nucleofection with episomal vectors expressing OS, MK and B. 1×10^6 cells were used for nucleofection. The numbers of iPSC colonies were counted at 3–4 weeks after nucleofection. Graphed data are presented as mean $\pm$ SEM (n=6). * indicates $P < 0.05$. doi:10.1371/journal.pone.0064496.g003

(OS+MK+BCL-XL), we have achieved significantly higher reprogramming efficiency: 20–30 iPSC colonies from 1 ml of PB. With the inclusion of additional factors such as LIN28, Mir-302, TP53 shRNA and/or EBNA1, it is possible that we might generate ~ 100 iPSC colonies from 1 ml of PB, an efficiency that is 10–100 fold higher than previously reported. [39,41,42] The success is largely due to the use of the SFFV promoter in our vectors, which drives higher levels of transgene expression in hematopoietic cells than promoters like EF1 and PGK. Of interest, when only 4 factors (OS+KLF4+BCL-XL) were used, no obvious change in reprogramming efficiency was observed compared to conditions with MYC (Figure 2B). In some applications, MYC may pose potential risks and thus MYCL1 has been proposed to replace MYC. [38,42,57] Here we show that neither MYC nor MYCL1 are necessary for reprogramming PBMNCs, potentially broadening the applications of our approach.

Our studies demonstrate that the addition of BCL-XL increases reprogramming efficiency by ~ 10 -fold in all the three factor

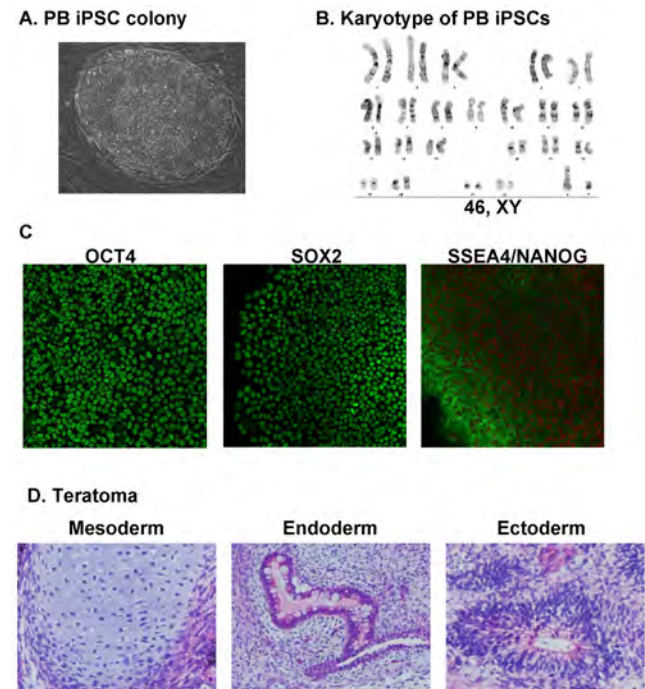


Figure 4. Characterization of integration-free PB iPSCs. (A) PB iPSCs show a typical morphology of human pluripotent stem cells. (B) A representative karyogram of an iPSC clone. All analyzed PB iPSC clones showed a normal karyotype. (C) PB iPSCs express pluripotency markers OCT4, SOX2, NANOG and SSE4. Shown are representative confocal images captured using the Zeiss LSM 710 confocal microscope with a 20 $\times$ objective. (D) PB iPSCs form teratoma in immunodeficient mice. H & E staining of representative teratoma from PB iPSCs shows derivatives of 3 embryonic germ layers. Cartilage (mesoderm); glands (endoderm) and neurotubules (ectoderm). Images were acquired using the Olympus microscope with a 20 $\times$ objective. doi:10.1371/journal.pone.0064496.g004

combinations we tested (Figure 2B); it is tempting to speculate that BCL-XL can also substantially enhance other factor combination-mediated reprogramming. This observation can be explained by three facts. First, the anti-apoptotic effect of BCL-XL may have led to better survival of cells that ectopically expressed reprogramming factors after transfection with EV plasmids. Second, a recent study shows that overexpression of BCL-XL in human embryonic stem cells (ESCs) not only attenuates apoptosis, but also upregulates the expression of adhesion molecules, which facilitate cell-matrix interactions. [58] The latter may be particularly important for the reprogramming of nonadherent PBMNCs, because tight adherence of PBMNCs to culture wells or matrix is the first step of successful reprogramming. Third, genomic analysis of human ESCs after long-term culture has identified recurrent amplifications at 20q11.21, a chromosome region that harbors the gene encoding BCL-XL. [59] This finding suggests that BCL-XL may also play a role in long-term self-renewal and proliferation of ESCs and iPSCs.

We found that integration-free PB iPSCs are reprogrammed from cells that express the CD33 myeloid marker, but not cells that express the T or B lymphocyte makers (CD3 or CD19, respectively). The culture conditions we used are favorable for the growth of HSCs and myeloid cells, but lack factors such as IL-2, IL-7 and anti-CD3 that support the growth and survival of lymphoid cells. In addition, nucleofection protocol we used is optimized for electroporation of nonlymphoid cells, in particular CD34⁺ cells. Thus, another benefit of our approach is that iPSCs

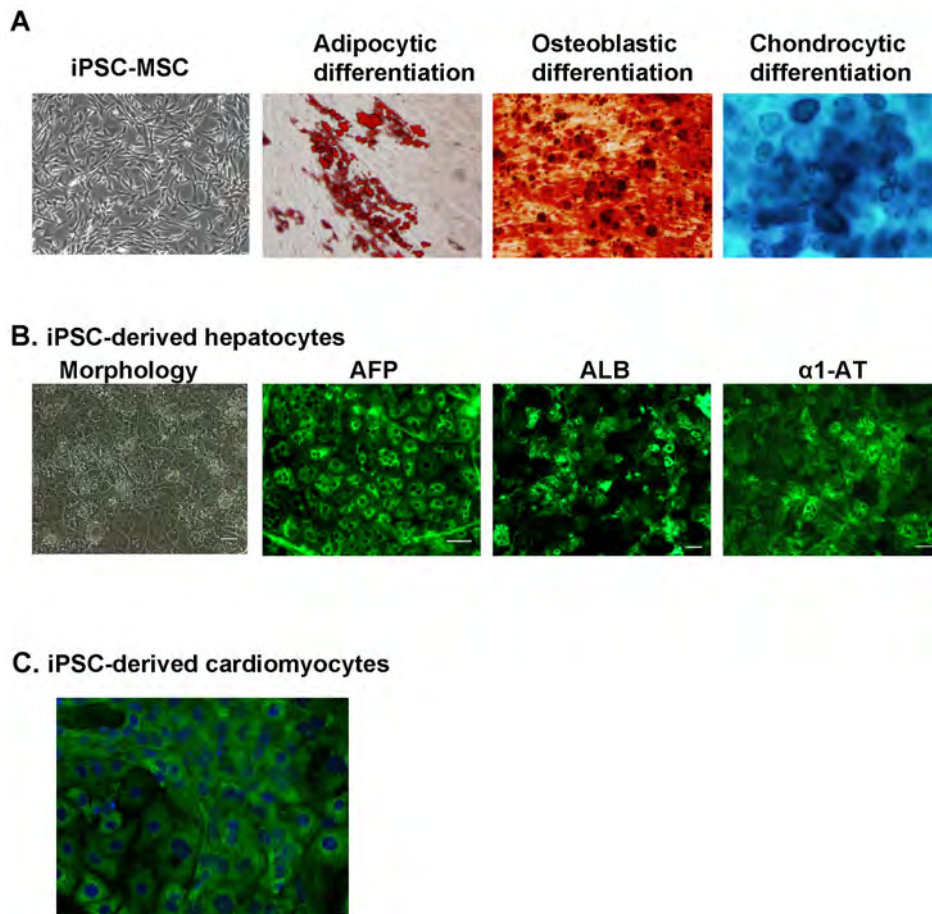


Figure 5. In vitro multilineage differentiation of integration-free PB iPSCs. (A) Differentiation of PB iPSCs into MSCs. iPSC-MSCs show a typical MSC morphology and are capable of differentiation into adipocytes, osteoblasts and chondrocytes. Oil Red O stains the oil droplets of adipocytes. Alizarin Red stains the bone nodules formed by osteoblasts. Alcian Blue stains acid mucopolysaccharides synthesized and secreted by chondrocytes. (B) PB iPSC-derived hepatocytes show a typical morphology of hepatocytes at 25 days after hepatocytic differentiation. These cells also express markers AFP, albumin (ALB), and alpha 1-antitrypsin (α 1-AT). The differentiated cells were stained with monoclonal antibody against AFP, goat anti-albumin, and goat anti-alpha 1-antitrypsin at 18 days after differentiation culture. (C) PB iPSC can be induced to differentiation into cardiomyocytes that express Troponin I marker. Cell nuclei were counterstained with DAPI.

doi:10.1371/journal.pone.0064496.g005

are generated from non-lymphoid cells, which eliminates the need for screening of PB iPSCs to assure that they do not harbor genetic rearrangements at the TCR or IG gene.

The use of our lentiviral vector to express the four Yamanaka factors leads to the generation of more than 200-fold more iPSC colonies compared to one early study that used another vector backbone to express the same factors. [46] This lentiviral vector is likely to have broad application in the direct reprogramming of CB or PBMNCs into cells of clinical importance. Indeed, we recently found that SFFV-mediated expression of OCT4 directly reprograms blood CD34⁺ cells into induced MSCs, whereas the use of EF1 or PGK promoter failed to do so. [56] This result highlights the importance of choosing appropriate promoters when using lentiviral vectors to express genes in question.

Generation of integration-free iPSCs without the use of integrating viral vectors represents one step closer to the clinical application of iPSC-based therapy. However, there are other safety concerns of iPSCs, including epigenetic memory of starting cells, genetic mutation during reprogramming and immunogenicity of iPSC-derived cells. Epigenetic memory and aberrant epigenomic reprogramming have been identified in iPSCs. [60,61] However, long-term passage of iPSCs has been found to

be able to diminish epigenetic signature inherited from the parent cells. [62] Furthermore, inclusion of ascorbic acid in the culture media and the induction of high-level expression of Oct4 and Klf4 are able to prevent aberrant epigenetic variation, allowing for the generation of “all-iPSC mice. [63,64] Early studies also showed high-level genetic aberrations in iPSCs, [65,66,67] but recent studies indicate that these identified mutations are largely due to fixation of preexistent rare mutations in the parental cells. [68,69] Our exome sequencing analysis showed only 1.3 coding mutations per CB iPSC, suggesting that *de novo* mutations during CB reprogramming are negligible. [54] The report of immunogenicity of iPSCs even after syngeneic transplantation revealed another “dark side” of iPSCs. [70] However, comprehensive investigations into this issue by two labs could not reproduce that finding and instead demonstrated negligible immunogenicity of differentiated cells from iPSCs. [71,72] Taken together, although early reports sparked pessimism over the therapeutic potential of iPSCs, several recent studies demonstrated their safety.

In summary, we are able to generate 20–30 integration-free iPSCs from the non-lymphoid cells in 1 ml of PB using EV plasmids expressing 4–5 factors including BCL-XL. To the best of our knowledge, this is the most efficient approach for generating

iPSCs from PB with non-viral vectors thus far. In addition, the lentiviral vectors and EV plasmids reported here are likely to have other applications like direct reprogramming of blood MNCs cells into other types of cells for clinical therapy.

Materials and Methods

Cord blood and peripheral blood

The use of CB and PB was approved by the Institutional Review Board of Loma Linda University and written informed consent was obtained from all participants. CB CD34⁺ cells were purified by MACS as described previously. [40] MNCs were obtained by standard density gradient centrifugation with Ficoll-Hypaque (1.077 g/ml) at room temperature. Some PBMNCs were directly purchased from AllCells (Emeryville, CA). The age of male and female PB donors ranged from 22 to 43 years old. In some studies, cells enriched for the myeloid lineage marker, CD33, or T/B depleted (CD3[−]/CD19[−]) cells were used. CD33⁺ cells were purified by staining with CD33-PE antibody (eBioscience; San Diego, CA) followed by staining with PE Microbead Kit (Miltenyi Biotec, Auburn, CA). CD3[−]/CD19[−] cells were collected from flow-through cells after being stained with CD3-PE and CD19-FITC (eBioscience) followed by isolation using anti-PE and FITC Microbead Kits (Miltenyi).

Lentiviral and episomal vectors

Human BCL2, BCL-XL, and MCL1 cDNAs were purchased from Thermo Scientific Open Biosystems (Huntsville, AL). Open reading frames (ORFs) of these genes were cloned into lentiviral vector Lenti SFFV-OS under the control of the SFFV promoter as detailed previously. [40,54] The backbone of this vector was modified from a lentiviral vector originally designed by Dr. Luigi Naldini. [73] The oriP/EBNA1-based episomal vectors EV SFFV-OS, EV SFFV-KLF4 and EV SFFV-MK have been described previously. [40] To drive expression of 2 genes, a self-cleavage peptide sequence from equine rhinitis A virus (E2A) was used to link the 2 genes. [9,40] EV SFFV-BCL-XL was cloned by inserting BCL-XL into the EV backbone. All the constructs were verified by DNA sequencing. Lentiviral vector packaging and titering have been detailed elsewhere. [74] Biological titers of 5–10×10⁷/ml were routinely achieved in our lab after a 100-fold concentration by centrifugation at 6000 g for 24 hr at 4°C. [40,74]

Generation of iPSCs using lentiviral vector

For generation of iPSCs from CB, we followed our previously published protocol. [40,54] To generate PB iPSCs, human PBMNCs were cultured in HSC culture conditions. [75,76] Iscove's modified Dulbecco's medium (IMDM)/10% FBS supplemented with TPO, SCF, FL and G-CSF each at 100 ng/ml, IL-3 at 10 ng/ml, and StemRegenin1 or SR1 (Cellagen Technology; San Diego, CA) [77] at 1 uM. Cytokines were purchased from ProSpec (East Brunswick, NJ). After 6–8 days of culture, 1×10⁵ cells per well were seeded into non-TC treated 24-well plates that were pre-coated with fibronectin fragment RetroNectin or CH-296 (Takara Bio, Inc., Shiga, Japan). [78] Lentiviral transduction was conducted for 5–6 hr with a multiplicity of infection (MOI) of 4. One day after transduction, cells were harvested and transferred to 6-well plates, which were pre-seeded with inactivated rat embryonic fibroblast (REF) feeder cells (Applied Biological Materials Inc. or ABM; Richmond, BC, Canada). Cells were maintained in the HSC culture condition for 2 more days before being gradually replaced with iPSC medium. The iPSC medium is composed of Knockout DMEM/F12 medium (Invitrogen) sup-

plemented with 20% Knockout Serum Replacement (KSR) (Invitrogen; Carlsbad, CA), 1 mM GlutaMAX (Invitrogen), 2 mM nonessential amino acids (ABM), 1× penicillin/streptomycin (ABM), 0.1 mM β-mercaptoethanol (Sigma-Aldrich Corp; St. Louis, MO), 20 ng/ml FGF2 (ABM), and 50 μg/ml ascorbic acid. [63,79] Culture medium was changed every 2 days. To increase reprogramming efficiency, an inhibitor of histone deacetylase sodium butyrate [80,81] was added at 0.25 mM every 2 days from day 2 to 10, and cells were cultured under hypoxia throughout the experiment by placing culture plates in a hypoxia chamber (Stemcell Technologies, inc., Vancouver, BC, Canada) that was flushed with mixed air composed of 92% N₂/3% O₂/5% CO₂. [40,82][54]. Starting from day 10, REF-conditioned medium was used.

Generation of integration-free iPSCs with episomal vectors

PBMNCs or fractionated cells (CD33⁺ or CD3[−]/CD19[−]) cells were cultured under HSC conditions for 2–8 days. To generate integration-free iPSCs, cells were nucleofected with 15–20 μg EV plasmid DNA using human CD34 Cell Nucleofector® Kit (Lonza). In each nucleofection, 10 μg EV SFFV-OS, 5 μg EV SFFV-BCL-XL, 5 μg EV SFFV-KLF4 or 5 μg EV SFFV-MK were used. 1×10⁶ cells were nucleofected with Amaxa Nucleofector II using program U-008. Immediately after nucleofection, cells were cultured in CH-296 pretreated well plates. The next day, cells were transferred to each well of 6-well plates that were preseeded with REF. Cells were cultured the exactly same way as for reprogramming with lentiviral vector expect that every 7–10 days, 2–3×10⁵ freshly thawed inactivated REF feeder cells were added into each well. The number of ALP-positive iPSC colonies was counted at 3–4 weeks after nucleofection. Some iPSCs colonies were picked for further culture. iPSCs were passaged every 5–7 days by treating with Dispase (Invitrogen). After 10–20 passages, iPSCs were further characterized with other assays.

ALP staining

To determine the number of iPSC colonies, culture plates were stained with an ALP-staining kit (Stemgent, Inc. San Diego, CA). ALP-positive iPSC colonies were enumerated at 2–3 weeks after transduction for lenti iPSCs or 3–4 weeks after nucleofection for episomal iPSCs.

Confocal imaging

For immunostaining of iPSC colonies, iPSCs were cultured in chamber slides for 4–5 days. Cells were treated with fixation buffer and permeabilization buffer (eBioscience) for 30 min before being stained overnight at 4°C with PE or FITC conjugated antibodies anti-OCT4 (eBioscience), anti-SOX2 (BD Pharmingen; San Diego, CA), anti-NANOG (BD Pharmingen), and anti-SSEA-4 (eBioscience). Confocal imaging was performed using the Zeiss LSM 710 NLO laser scanning confocal microscope with a 20× objective at the Loma Linda University Advanced Imaging and Microscopy Core. High resolution monochrome images were captured using a Zeiss HRm CCD camera.

Teratoma assay

The use of NOD/SCID/IL2RG^{−/−} (NSG) immunodeficient mice for the teratoma formation assay was approved by the Institutional Animal Care and Use Committee at Loma Linda University (LLU). NSG mice were purchased from the Jackson Laboratory (Sacramento, CA) and maintained at the LLU animal facility. Approximately 1×10⁶ iPSCs were suspended in 200 ul

DMEM/F12 diluted (1:1) Matrigel solution (BD) and injected into the subcutaneous tissue above the rear haunch of NSG mice. At 2 months after implantation, teratomas were dissected and fixed in 10% formalin. After microsectioning, samples were stained with hematoxylin and eosin (H & E) and analyzed by a board certified pathologist.

Karyotyping and G-banding

GTG-banding chromosome analysis was carried out in the LLU Radiation Research Laboratories. Standard DNA spectral karyotyping procedures were followed and a HiSKY Complete Cytogenetic System was used (Applied Spectral Imaging, Inc. Vista, CA). For each clone, 10 metaphases were analyzed and karyotyped.

In vitro differentiation of integration-free PB iPSCs

To differentiate PB iPSCs into MSCs, iPSCs were cultured with Mesenchymal Stem Cell (MSC) Medium Kit (ABM) for 4–5 days. Cells were then treated with Accutase (Innovative Cell Technologies, Inc., San Diego, CA) and further cultured in fibronectin (BD)-precoated non-tissue culture treated well plates. After 5 passages under MSC culture conditions, cells were induced to differentiate into adipocytes, osteoblasts, and chondrocytes as detailed previously. [56] After 3 weeks of culture in differentiation medium, Oil Red O, Alizarin Red, and Alcian Blue staining for adipocytes, osteoblasts, and chondrocytes, respectively, were conducted. [56]

To differentiate iPSCs into cardiomyocytes, small clusters of iPSCs were cultured in differentiation medium consisting of StemPro-34 (Invitrogen), supplemented with 2 mM GlutaMAX, 50 µg/ml ascorbic acid, and 4×10^{-4} M monothioglycerol (MTG) (Sigma). [83] Cytokine Activin A (R & D Systems; Minneapolis, MN) was used at 50 ng/ml for one day and 10 ng/ml BMP4 (R&D Systems) was added for four days. After 12 days of culture, beating colonies of cardiomyocytes were observed. The identity of cardiomyocytes was confirmed by immunostaining with cardiomyocyte-specific marker Troponin I (R&D Systems).

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Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Two-tailed Student *t* test was performed. *P* values of <0.05 were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: XBZ. Performed the experiments: RJS AN XBZ XM JBK BTS YD NA MKJ DSG. Analyzed the data: XBZ DJB JW KHWL. Contributed reagents/materials/analysis tools: KJP. Wrote the paper: XBZ.

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