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Article

Establishment of induced pluripotent stem cells from aged mice using bone marrow-derived myeloid cells

Zhao Cheng[1], Sachiko Ito[1], Naomi Nishio[1], Hengyi Xiao[2], Rong Zhang[1], Haruhiko Suzuki[1], Yayoi Okawa[3], Toyoaki Murohara[4], and Ken-ichi Isobe[1,*]

1 Department of Immunology, Nagoya University Graduate School of Medicine, 65 Turumai-cho, Showa-ku, Nagoya, Aichi 466-8520, Japan
2 Laboratory for Geriatrics, State Key Laboratory of Biotherapy, Sichuan University, Chengdu 610041, China
3 Department of Functioning Activation, National Institute for Longevity Sciences, National Center for Geriatrics Gerontology, Aichi 463-0003, Japan
4 Department of Cardiology, Nagoya University Graduate School of Medicine, 65 Turumai-cho, Showa-ku, Nagoya, Aichi 466-8520, Japan
* Correspondence to: Ken-ichi Isobe, Tel: +81-52-744-2134; E-mail: kisobe@med.nagoya-u.ac.jp

If induced pluripotent stem (iPS) cells are to be used to treat damaged tissues or repair organs in elderly patients, it will be necessary to establish iPS cells from their tissues. To determine the feasibility of using this technology with elderly patients, we asked if it was indeed possible to establish iPS cells from the bone marrow (BM) of aged mice. BM cells from aged C57BL/6 mice carrying the green fluorescence protein (GFP) gene were cultured with granulocyte macrophage-colony stimulating factor (GM-CSF) for 4 days. Four factors (Oct3/4, Sox2, Klf4 and c-Myc) were introduced into the BM-derived myeloid (BM-M) cells. The efficiency of generating iPS cells from aged BM cultured in GM-CSF was low. However, we succeeded in obtaining BM-M-iPS cells from aged C57BL/6 mice, which carried GFP. Our BM-M-iPS cells expressed SSEA-1 and Pou5f1 and were positive for alkaline phosphatase staining. The iPS cells did make teratoma with three germ layers following injection into syngeneic C57BL/6 mice, and can be differentiated to three germ layers *in vitro*. By co-culturing with OP9, the BM-M-iPS cells can be differentiated to the myeloid lineage. The differentiated BM-M-iPS cells proliferated well in the presence of GM-CSF, and lost expression of Nanog and Pou5f1, at least in part, due to methylation of their promoters. On the contrary, Tnf and Il1b gene expression was upregulated and their promoters were hypomethylated.

Keywords: induced pluripotent stem cells, aged mouse, bone marrow myeloid cells, gene expression, methylation, ChIP assay

Introduction

The generation of induced pluripotent stem (iPS) cells from murine and human somatic cells through forced expression of defined transcription factors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Jaenisch and Young, 2008) constitutes a major breakthrough in regenerative biology (Hanna et al., 2007). This technology opens the possibility of personalized cell therapies for treating human disease and/or repairing the damaged tissues of elderly patients. To use iPS cells to treat damaged tissues or repair aged organs, it will be necessary to establish iPS cells from elderly patients. Since bone marrow (BM) cells are relatively easy to obtain by BM aspiration, we asked if it was possible to establish iPS cells from BM cells of aged mice. Murine iPS cells have been established from mouse embryonic fibroblast (MEF) and adult fibroblast cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007). Although the efficiency of producing iPS cells from MEF is low, the efficiency of producing iPS cells from other cells is even lower. For example, T cells are difficult to convert to iPS cells without p53 deficiency (Hong et al., 2009). Here, we established iPS cells from aged BM-derived myeloid (BM-M) cells. We present data that show the efficiency of reprogramming aged BM-M cells and the pluripotency of the BM-M-iPS cells in generating teratoma with three germ layers. Further, we differentiated BM-M-iPS and MEF-iPS back to myeloid cells and compared the histological morphology, pluripotent gene expression, myeloid gene expression and myeloid cell surface marker expression of these cells. Finally, we compared the state of gene promoter methylation of undifferentiated BM-M-iPS and differentiated cells.

Results

Establishment of iPS cells from BM-M cells from aged mice

We attempted to establish iPS cells from aged mice, using BM cells from aged EGFP-C57BL/6 mice. Efforts to establish iPS cells by direct BM transduction with four retroviral factors were unsuccessful. Because it takes some time to establish iPS cells, BM cells without stimulation may die before the establishment of iPS cells. Thus, we used BM-M cells in granulocyte macrophage-colony stimulating factor (GM-CSF), which were actively proliferating. To ascertain the efficiency of iPS

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generation, we used MEF cells from C57BL/6, BM-M cells from 2-month-old C57BL/6 and 23-month-old C57BL/6. With MEF cells and 2-month-old BM-M cells, colonies appeared approximately 15 days after transduction. However, the number of colonies from MEF cells increased more rapidly than those from 2-month-old BM-M cells. As for 23-month-old BM-M cells, colonies appeared 30 days after transduction. The colony number was less than those obtained from MEF cells or 2-month-old BM-M cells (Figure 1B). One MEF-derived iPS (MEF-iPS) colony was expanded and was used in this report.

Then we tried to establish iPS clones by using BM of 21-month-old EGFP-C57BL/6 mice, which had been cultured for 4 days in GM-CSF. Since the efficiency of establishing iPS cells from aged mice was low, we treated the BM-M cells twice with four retroviral factors. After 1 month of culture in iPS medium, a few colonies emerged. We picked up two clones (1 and 2) and expanded them on mitomycin-C-treated SNL76/6 cells. The morphology of the cells was similar to that seen with established iPS cells (Figure 1C and Supplemental Figure S2). For further experiments we mainly used BM-M-iPS 1.

Pluripotent makers SSEA-1 and Pou5f1 were detected by immunocytochemical analysis in BM-M-iPS 1 cells and MEF-iPS cells. BM-M-iPS 1 cells expressed green fluorescence proteins (GFPs) (Figure 1D, upper, left) and expressed SSEA-1 and Pou5f1 (Figure 1D, upper, right). MEF-iPS cells also expressed SSEA-1 and Pou5f1 (Figure 1D, lower, right). We analyzed alkaline phosphatase (AP) activity of both BM-M-iPS 1 and MEF-iPS to examine the undifferentiated status of each iPS clone. Strong AP activity was observed in both BM-M-iPS 1 and MEF-iPS cells (Figure 1E). BM-M-iPS 1 had normal karyotypes (Supplemental Figure S3).

Teratoma formation by 21-month-old BM-M-iPS cells

In order to evaluate the pluripotency of iPS cells derived from aged BM-M cells, we transplanted the BM-M-iPS 1 cells to the dorsal flank of syngeneic C57BL/6 mice. We found that aged BM-M-iPS 1 cells produced teratoma 21 days after transplantation (Figure 2A). The teratoma contained various tissues (artery, stratum corneum, smooth muscle, gut-like epithelium,

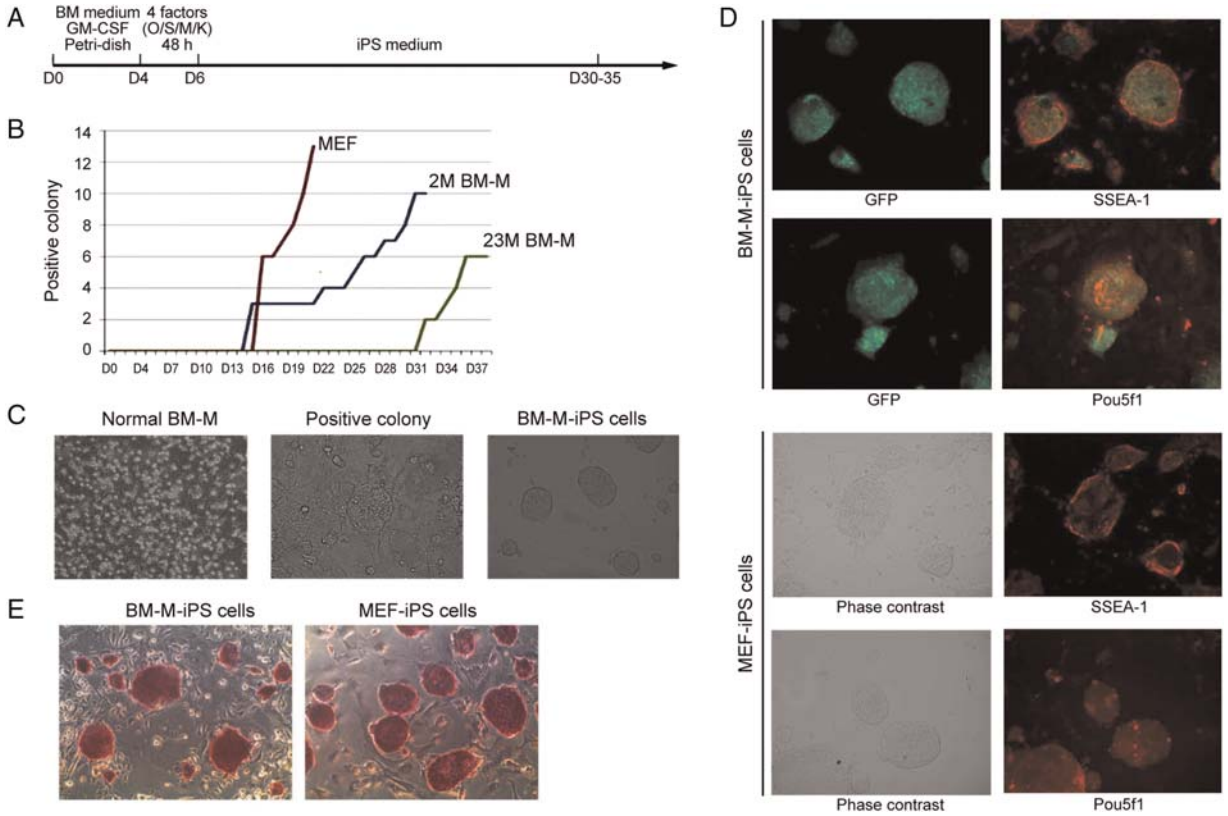

Figure 1 Establishment of iPS cells from aged BM-M cells and efficiency of the induction of MEF and BM-M cells. BM cells from 21-month-old EGFP-C57BL/6 mice were cultured in GM-CSF for 4 days. They were collected and seeded on MMC-treated SNL76/7 feeders then treated with four factors. Several colonies emerged, and each was trypsinized and transferred onto a 24-well plate. Two colonies were expanded and stocked in liquid nitrogen. One clone was used for further experiments. (**A**) Schema of production of iPS cells from BM-M cells. (**B**) The graph shows the different reprogramming efficiencies of MEF and BM-M cells from 2-month and 23-month-old C57BL/6 mice. Positive colonies first emerged at D15 from MEF (red), D15 from 2-month-old BM-M (blue) and D30 from 23-month-old BM-M cells (green). (**C**) Morphological changes (×100). Left: BM-M cells which were derived from 4 days culture of BM cells in the presence GM-CSF. Middle: newly appeared iPS colony. Right: established BM-M-iPS clone. (**D**) Immunostaining of aged BM-M-iPS cells (upper) and MEF-iPS (lower) (×100). The iPS cells were cultured on cover slide. They were observed by immunofluorescent microscope. Upper left: GFP protein was observed by 472.5 nm emission of fluorescence. Upper and lower right: cells were stained with murine anti-SSEA-1 and anti-Pou5f1. Then they were stained with secondary antibody PE-labeled anti-mouse IgG and observed by immunofluorescent microscope. Lower left: cell were observed by phase contrast channel. (**E**) BM-M-iPS cells and MEF-iPS cells were AP stained (×100). They were observed by Olympus microscope.

bronchial epithelium, adipose tissues, nerve and vein) belonging to three germ layers (endoderm, mesoderm and ectoderm) (Figure 2B). All the teratoma tissues expressed GFP protein (Figure 2C, upper). Tissues of teratoma were positively stained with alpha-smooth muscle actin (mesoderm), α-fetoprotein (endoderm) and neurofilament H (ectoderm), respectively (lower) (Figure 2C).

In vitro differentiation of 21-month-old BM-M-iPS cells

Then we try to differentiate BM-M-iPS 1 cells to three germ layers *in vitro*. We cultured BM-M-iPS cells by the hanging drop methods for 8 days to get embryoid body (EB). Then EBs were transferred to 24-well plates coated with 0.1% gelatin (Figure 3A). After 10 days culturing, they were stained with tissue-specific antibodies. Differentiated BM-M-iPS 1 cells expressed GFP (Figure 3B, left) and alpha-smooth muscle actin, α-fetoprotein and neurofilament H (Figure 3B, middle). We also differentiated MEF-iPS by the same way (data not shown). The proportions of the cells that express the differentiation marker were counted. The proportions of the cells, which express alpha-smooth muscle actin, were 50% in BM-M-iPS and 61.2% in MEF-iPS. Those of α-fetoprotein were 84.1% in BM-M-iPS and 51.7% in MEF-iPS, and those of neurofilament H were 68% in BM-M-iPS and 73.1% in MEF-iPS. These results indicated that differentiation capacity to three germ layers was not so different between BM-M-iPS and MEF-iPS.

We investigated that if it was possible to differentiate aged BM-M-iPS cells back to myeloid cells. The BM-M-iPS 1 cells or MEF-iPS cells were co-cultured with OP9 cells for 5 days in OP9 medium, and then transferred to fresh OP9 cells in OP9 medium supplemented with GM-CSF for another 5 days (Figure 3C). Then, cells were transferred to 6-well plates, which had not been treated to promote cell adherence. We found myeloid-like cells at this point from both BM-M-iPS and MEF-iPS (Figure 3D). Cells were then expanded in petri dishes.

Gene expression of BM-M cells, BM-M-iPS cells, MEF-iPS cells and iPS cells after differentiation

We examined the expression of genes of BM-M-iPS cells to determine whether the expression pattern had changed from that

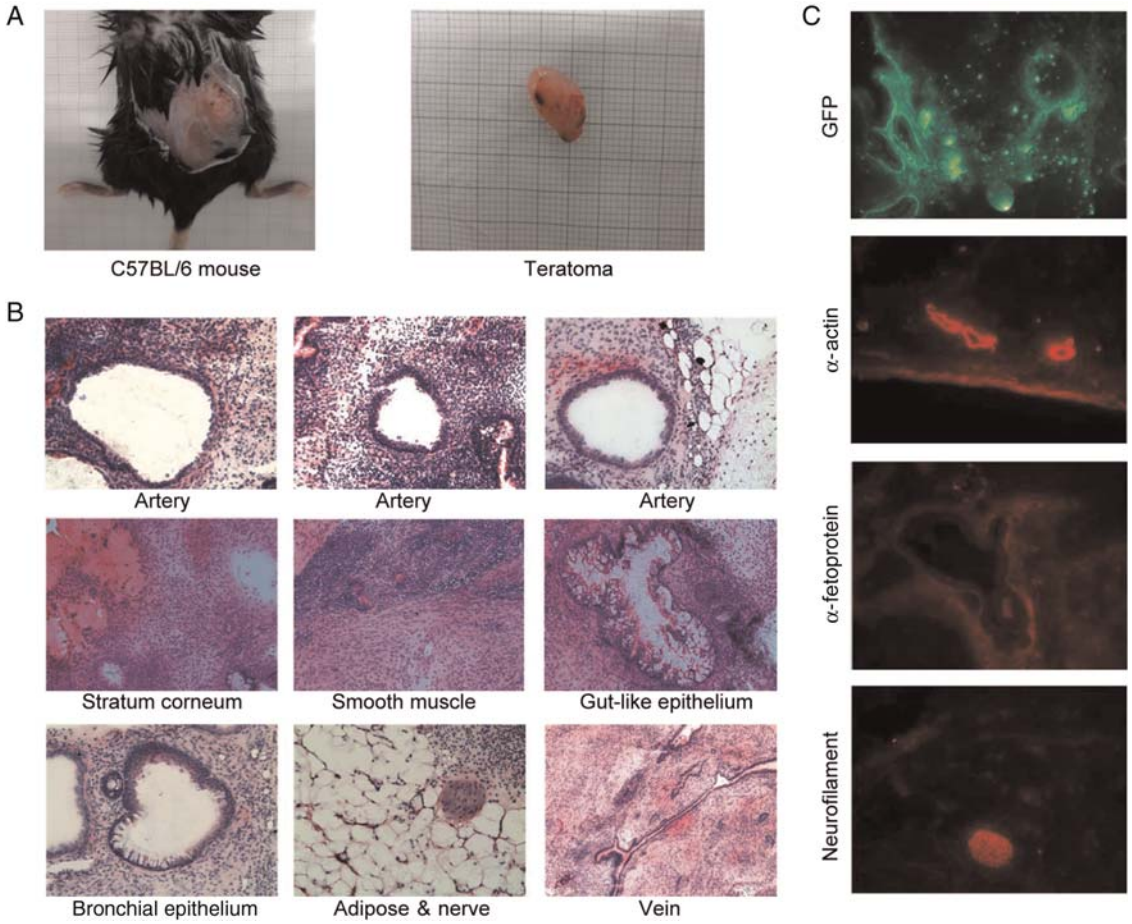

Figure 2 Teratoma formation of BM-M-iPS cells derived from 21-month-old C57BL/6 mice. (**A**) BM-M-iPS cells (1×10^7) were injected into the dorsal flank of C57BL/6 mice. After 21 days, the teratoma were collected and fixed in OCT compound, transferred to liquid nitrogen, then cut with a freezing microtome to 5 μm slices. (**B**) Slides were stained with hematoxylin and eosin. Upper panel shows artery like structures (×100). Middle panel shows stratum corneum, smooth muscle and gut-like structures (×100). Lower panel shows bronchial epithelium (×100), adipose and nerve (×100), and vein (×40) like structures. (**C**) Slides were stained with: rabbit anti-GFP, mouse anti-neurofilament H, mouse anti-α-fetoprotein or mouse anti-alpha-smooth muscle actin antibodies. Then stained with secondary antibody: anti-mouse IgG or anti-rabbit IgG, respectively (×100).

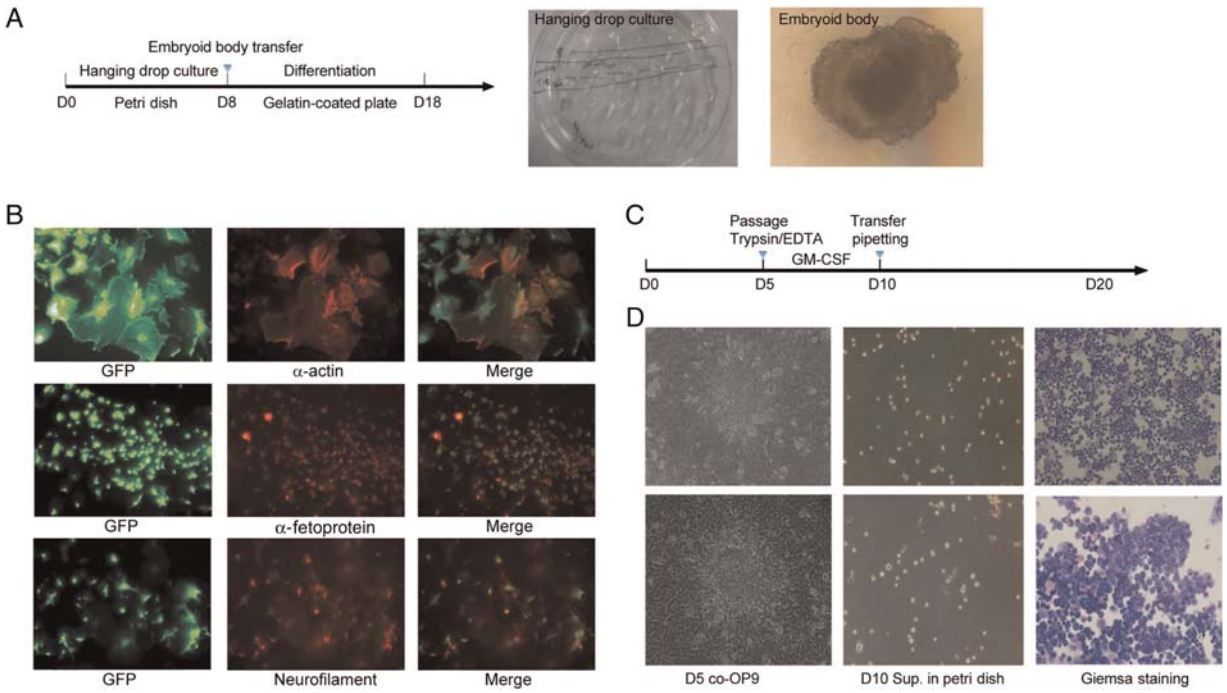

Figure 3 *In vitro* differentiation of 21-month-old BM-M-iPS cells. (**A**) Schema of EB formation and *in vitro* differentiation (×40). BM-M-iPS cells were hanging drop cultured for 8 days and transferred to 24-well plates. Figure shows hanging drop culture (left) and EB (right) taken by phase contrast microscope (Olympus). (**B**) Immunostaining of differentiated BM-M-iPS (×100). Upper panel shows the tissues belonging to mesoderm, which express α-actin. Middle panel shows the tissues belonging to endoderm, which express α-fetoprotein. Lower panel shows the tissues belonging to ectoderm, which express neurofilament H. (**C** and **D**) iPS cells were cultured on OP9, which were seeded on 0.1% gelatin-coated 6-well plates. After 5 days of culture, images were captured by phase contrast microscopy (×100). Upper, D5 MEF-iPS co-cultured with OP9. Lower, BM-M-iPS co-cultured with OP9. Phase contrast microscopy and cytospin May-Giemsa staining of differentiated iPS cells (×100). Upper, MEF-iPS differentiation. Lower, BM-M-iPS differentiation.

expected of myeloid cells to that of pluripotent stem cells. BM-M cells cultured 4 and 7 days in the presence of GM-CSF expressed myeloid cytokines (Tnf, Il1b), the chemokine CCL7 and transcription factors C/EBPα, and Pu-1. Expression of these factors in BM-M-iPS cells was reduced to the same level observed in MEF-iPS cells. As expected, BM-M-iPS cells expressed *Nanog*, *Pou5f1* and other pluripotent markers at the same level as MEF-iPS cells. After differentiation to myeloid lineage cells in GM-CSF, BM-M-iPS and MEF-iPS lost their expression of *Nanog*, and *Pou5f1*. However, they expressed myeloid cytokines (*Tnf*, *Il1b*), chemokine (*CCL7*) and transcription factors (*C/EBPα*, *Pu-1*) (Figure 4A).

Different patterns of gene promoter methylation of pluripotent stem cells and myeloid-differentiated cells

In order to characterize gene expression at the chromatin level, we performed chromatin immunoprecipitation (ChIP) assays. We found that the promoters of *Nanog* and *Pou5f1* were hypomethylated in BM-M-iPS. When differentiated to myeloid lineage cells, they were hypermethylated. Interestingly, the promoters of *Tnf* and *Il1b* were hypermethylated in BM-M-iPS. They were hypomethylated once they had differentiated to myeloid lineage cells (Figure 4B and C).

Expression of myeloid lineage cell surface makers in differentiated BM-M-iPS and MEF-iPS

Since BM-M-iPS expressed GFP protein, cell surface expression of myeloid lineage makers was analyzed with PE-labeled antibodies. Both myeloid-differentiated BM-M-iPS and MEF-iPS expressed CD11b and CD115, but expressed neither MHC class II nor CD11c. These results indicated that BM-M-iPS had been differentiated to myeloid lineage cells (Figure 4D).

Discussion

We successfully established iPS cells from aged mice using BM-derived myeloid cells. Our experiments demonstrated three important findings. First, iPS cells can be generated from BM-derived myeloid cells. Second, iPS cells can be derived from aged mice. Finally, the iPS cells derived from C57BL/6 mice expressed GFP, thus these iPS cells can be used for regenerative medicine experiments *in vivo*, allowing us to follow their differentiative fate.

For optimized clinical use of iPS cells for personalized cell therapies, it is important to select a relatively simple and safe way to obtain tissue sources (Amoh et al., 2009; VandenDriessche et al., 2009; Chen et al., 2010; Cho et al., 2010; Hwang et al., 2010; Seifinejad et al., 2010; Sun et al., 2010). Several sources are used to establish iPS cells. The most common sources are fetal fibroblasts and adult fibroblasts (Takahashi and Yamanaka, 2006; Wernig et al., 2007; Stadtfeld et al., 2008). Adult neural stem cells can be established from iPS cells with just two factors (Kim et al., 2008). Adult mouse hepatocytes and gastric epithelial cells were successfully used to generate iPS cells (Aoi et al., 2008). Immune cells, including B

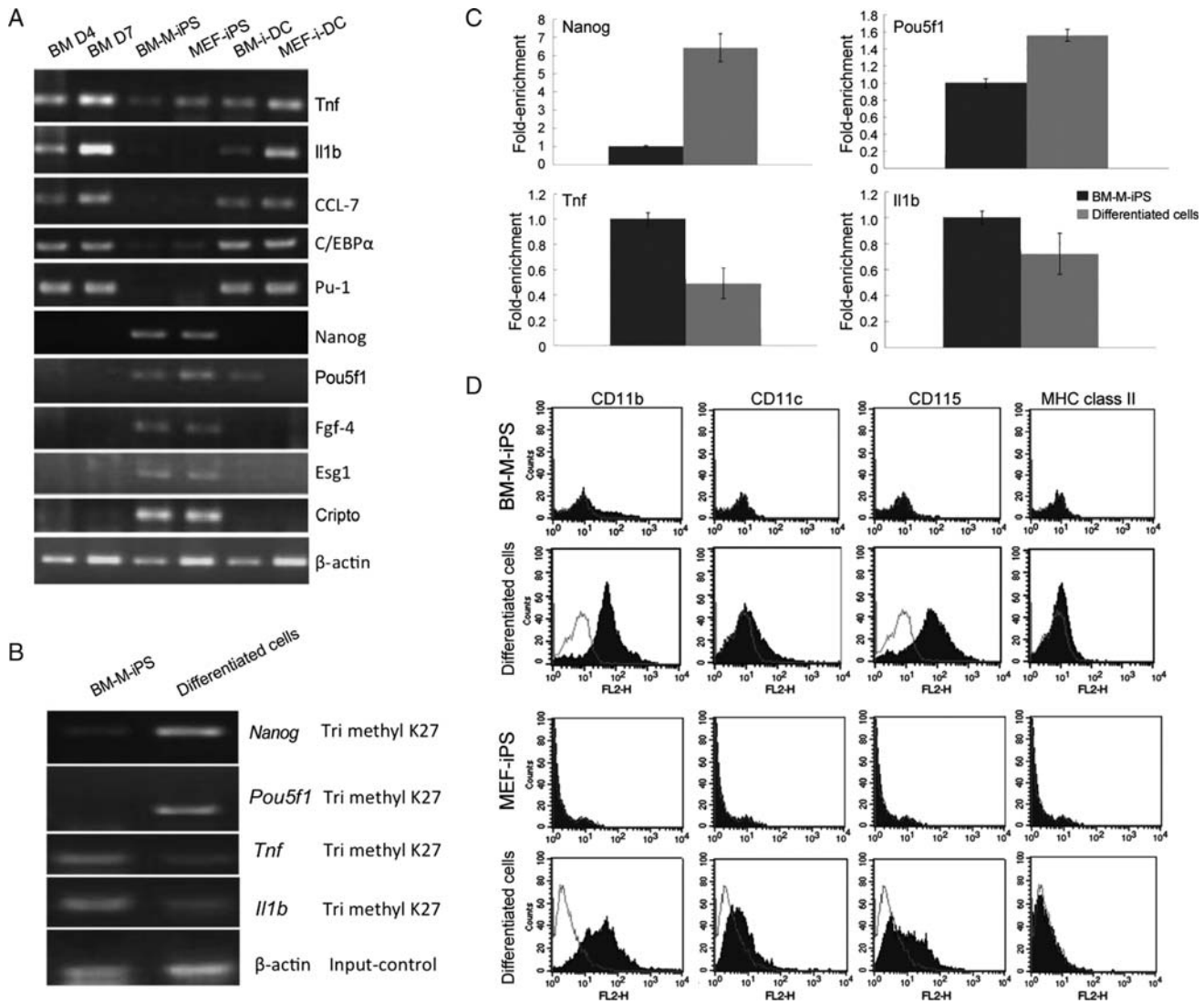

Figure 4 Gene expression, ChIP assays and cell surface markers of BM-M-iPS cells and differentiated BM-M-iPS cells. (**A**) Total RNA was extracted from 1×10^6 BM myeloid cells, which had been cultured for 4 days (BM D4) or for 7 days (BM D7), BM-M-iPS or MEF-iPS, differentiated BM-M-iPS (BM-i-DC) and differentiated MEF-iPS (MEF-i-DC). cDNA (500 ng) was used for RT-PCR analysis. Aged BM-M-iPS cells lost myeloid lineage gene expression, converting to a pattern consistent with pluripotent gene expression. (**B**) RT-PCR results of ChIP assay. Cells from undifferentiated BM-M-iPS or myeloid-differentiated BM-M-iPS cells were first fixed with formaldehyde and treated with nuclear lysis buffer to release protein–DNA complexes. The complexes were immunoprecipitated by anti-H3-K27 antibodies. Promoter regions of Pou5f1, Nanog, Tnf and Il1b DNA in immunoprecipitates were amplified by PCR using specific primers. Different methylation patterns of gene promoters in pluripotent stem cells and myeloid-differentiated cells were observed. (**C**) Real-time PCR of ChIP assays. (**D**) Cell surface markers of BM-M-iPS before and after differentiating to myeloid cells. BM-M-iPS or MEF-iPS cells were differentiated to myeloid cells in GM-CSF. Expanded cells were probed for cell surface markers for the myeloid lineage, PE-labeled anti-MHC class II, CD11b, CD11c, CD115.

cells (Hanna et al., 2008) and T cells (Hong et al., 2009), have been used to establish iPS cells. For regenerative medicine, it is not ideal to use B cells or T cells to establish iPS cells because of gene rearrangement. Okabe et al. (2009) used murine BM cells to establish iPS cells and showed no rearrangement. There is another report in which BM cells were used to establish iPS cells; Kunisato et al. (2010) reported the generation of iPS cells from adult murine BM cells. However, in those experiments, the sources of iPS cells from BM contained B cells in which genes had already been rearranged. In order to avoid gene rearrangement, we cultured BM cells for 4 days with GM-CSF, which induces myeloid lineage cells. Before transduction we stained the cells at this stage with several antibodies which react with myeloid cells. More than 98% were Gr-1/CD11b-positive cells and lacked B (IgM/B220) cell and T (CD4/CD8) cell makers. They have other myeloid cell makers (Supplemental Figure S1).

In the case of elderly patients, the use of iPS cells for personalized clinical cell therapy requires the establishment of iPS cells from relatively old autologous tissue. Here, we demonstrated that it was possible to establish iPS cells from 21-month-old C57BL/6 mice. It has been shown previously that fibroblasts from aged mice or highly differentiated hematopoietic cells have reduced capacities to produce iPS cells (Eminli et al., 2009; Li et al., 2009). Our experiments showed the efficiency of

iPS cell generation in aged mice is indeed lower than that of young mice (Figure 1B). Another problem is whether iPS cells from aged mice can be differentiated to somatic cells. It has been shown that human iPS cells established from adults are capable of differentiating into hemangioblast cells, but with dramatically decreased efficiency and early senescence (Feng et al., 2010). In our present experiments, aged BM-M-iPS cells could be differentiated into myeloid lineage cells and the differentiated cells proliferated well in the presence of GM-CSF. We did preliminary experiments to compare the differentiation capacity of BM-M-iPS (1 and 2) with that of MEF-iPS (iPS cells from our laboratory and iPS cells from Yamanaka's laboratory). Differentiation capacities of BM-M-iPS (1, 2) and MEF-iPS (iPS cells from our laboratory and iPS cells from Yamanaka's laboratory) were not greatly different (Supplemental Table S1). Further precise experiments are sure to be needed.

We found that the gene expression pattern of the pluripotent markers of aged BM-M-iPS cells was similar to that of MEF-iPS cells (Figure 4A). BM-M-iPS cells can be differentiated back to myeloid cells by co-culturing with OP9 in the presence of GM-CSF (Senju et al., 2003; Vodyanik et al., 2005; Ji et al., 2008; Choi et al., 2009; Senju et al., 2009). The differentiated cells proliferated in the presence of GM-CSF and expressed myeloid cell surface markers CD11b and CD115, and Tnf, Il1b, CCL-7, C/EBPα, Pu-1 mRNAs (Figure 4A). Thus, differentiated cells have markers similar to those of monocytes or macrophages.

It has been shown that gene expression can be regulated by histone methylation. Histone methylation causes gene silencing and heterochromatin formation, which is an essential mechanism of epigenetic regulation (Sun and Allis, 2002; Tenney et al., 2006; Kondo et al., 2008; Vaissiere et al., 2008; Shukla et al., 2009). Expression of *Nanog* and *Pou5f1* is important for maintaining the pluripotency of both embryonic stem cells and iPS cells (Loh et al., 2006; Liang et al., 2008; Tay et al., 2008). Using chromatin immuno-precipitation assays, we investigated histone H3 methylation of both *Nanog* and *Pou5f1* in undifferentiated BM-M-iPS cells and differentiated BM-M-iPS cells. The results of ChIP assays indicated that both *Nanog* and *Pou5f1* were hypomethylated in undifferentiated BM-M-iPS cells and hypermethylated in differentiated BM-M-iPS cells, which is consistent with previous reports of the epigenetic regulation of *Nanog* and *Pou5f1* genes in embryonic stem cells (Hattori et al., 2007; Fouse et al., 2008). In contrast, both Tnf and Il1b were hypermethylated in undifferentiated BM-M-iPS cells and hypomethylated in differentiated cells, which explains the gene expression patterns obtained with RT-PCR (Figure 4B,C). The results of ChIP assays support the idea that changes in histone methylation may play an important role in both the reprogramming required to achieve pluripotency and the directed differentiation of pluripotent stem cells, which affects the expression of genes associated with pluripotency and those required for myeloid differentiation.

Materials and methods

Mice

C57BL/6 (C57BL/6J) mice were purchased from SLC Japan. EGFP-C57BL/6 mice (C14-Y01-FM131Osb) carrying pCAG-EGFP (CAGpromoter-EGFP) were purchased from Riken with the permission of Dr Okabe (Okabe et al., 1997). All mice were maintained in the Animal Research Facility at the Nagoya University Graduate School of Medicine under specific pathogen-free conditions and used according to institutional guidelines.

Cell culture

For MEF isolation, uteri isolated from 13.5 days pregnant mice were washed with phosphate-buffered saline (PBS). The head and visceral tissue were removed from each isolated embryo. The remainder of the bodies were washed in fresh PBS, minced using a pair of scissors, transferred into a 0.25-mM trypsin/1-mM EDTA solution (3 ml per embryo) and incubated at 37°C for 20 min. After incubation, an additional 3 ml per embryo of PBS was added, and the mixture was incubated at 37°C for 20 min. After trypsinization, an equal amount of medium (6 ml per embryo of DMEM containing 10% FBS) was added and aspirated with a pipette to promote tissue dissociation. Then, cells were filtered through a mesh, seeded at 1×10^6 cells per 10 cm dish and incubated at 37°C in 5% CO_2.

BM myeloid cells were generated according to the method of Inaba et al. (1992). Briefly, BM cells were cultured in RPMI 1640 medium (10% FBS), 300 g/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 mM 2-mercaptoethanol (Sigma Aldrich) containing 0.3% GM-CSF supernatant (from murine GM-CSF producing Chinese hamster ovary cells, a gift from Dr T. Sudo, Toray Silicon, Tokyo, Japan). Floating and loosely adherent clustering cells were collected on day 4 and used as BM myeloid cells.

Plat-E packaging cells, which were used to produce retroviruses, were kindly provided by Dr Kitamura (Morita et al., 2000), and maintained in DMEM containing 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 μg/ml puromycin (Sigma) and 100 μg/ml of blasticidin S (Funakoshi).

SNL76/7 feeder cells were clonally derived from the STO cell line transfected with a $G418^R$ cassette and an LIF expression construct (Williams et al., 1988).

OP9 cells were purchased from Riken. OP9 cells were maintained by culturing in α-MEM (Gibco 12571) supplemented with 1% L-glutamine, 1% non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin and 20% non-inactivated FBS at 37°C with 5% CO_2. iPS cells control were provided by the Riken cell bank (Wako, Japan), with the permit of Dr S. Yamanaka.

All iPS cells were maintained by culturing in KO-DMEM (Gibco) supplemented with 1% L-glutamine, 1% non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 5.5 mM 2-mercaptoethanol and 15% FBS (iPS medium) at 37°C with 5% CO_2.

Retroviral infection

One day before transduction, Plat-E cells were seeded at 5×10^5 cells/well in 6-well culture plates. pMXs-c-Myc, pMXs-Klf4, pMXs-Sox2 and pMXs-Oct3/4 were purchased from Addgene. The pMXs-based retroviral vectors were introduced into Plat-E cells using Fugene 6 transfection reagent (Roche) according to the manufacturer's recommendations. After incubation, the DNA/Fugene 6 mixture was added drop by drop onto Plat-E cells. Cells were then incubated overnight at 37°C with 5% CO_2.

Twenty-four hours after transduction, the medium was replaced. MEF or BM myeloid cells were seeded at 5×10^5 cells on 1×10^6 mitomycin C-treated SNL76/7 feeders in 6 cm dishes. After 24 h, virus-containing supernatants derived from these Plat-E cultures were filtered through 0.45 μm cellulose acetate filters (Schleicher and Schuell) and supplemented with 4 μg/ml polybrene (Nacalai Tesque). BM myeloid cells were incubated in the virus/polybrene-containing supernatants for 48 h and MEF for 24 h. After infection, medium was replaced with iPS medium. Clones were collected and expanded for further experiments.

Immunostaining

For immunostaining, cells were fixed in 4% formaldehyde and tissues were fixed in pre-cooled acetone. Non-specific binding sites were blocked with 0.2% BSA and 1% goat serum in PBS. Cells were incubated with optimal dilutions of primary antibody and followed by fluorescence-labeled appropriate antibody. They were observed and photographed with a Keyence BZ-8000 microscope.

Detection of iPS cells by marker staining

ES-AP detection kit (Cat no. SCR004; Chemicon) according to the manufacturer's instructions. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde (EMD Chemicals) diluted in 1 × PBS (Mediatech) for 10 min at room temperature. After washing with 1 × TBST, the cells were blocked with 4% normal goat serum and 0.2% BSA (Sigma), in 0.1% Triton X-100 (Fisher Scientific) for 1 h at room temperature. Primary antibodies included mouse anti-SSEA-1 (1:50; Chemicon), mouse anti-Oct4 (1:50, Chemicon).

Teratoma formation and histological analysis

iPS cells were suspended at 1×10^7 cells/ml in PBS. C57BL/6 mice were anesthetized with diethyl ether. A total of 100 μl of the cell suspension (1×10^6 cells) were injected subcutaneously into the dorsal flank. Three weeks after the injection, the teratoma was surgically dissected from the mice. Samples were embedded in OCT compound. Sections were stained with hematoxylin and eosin.

EB formation and in vitro differentiation

In vitro differentiation by EB formation was analyzed following the protocol of Ohnuki et al. (2009) with some modifications. In brief, iPS were suspended at the concentration of 3×10^3 cells/20 μl iPS medium and hanging drop cultured in petri dish for 8 days to get EBs. EBs were transferred to 0.1% gelatin-coated plates to induce further differentiation for 10 days. Differentiated markers such as α-fetoprotein (R&D MAB1368) for endoderm, α-smooth muscle actin (Sigma A2547) for mesoderm and neurofilament H (Cell signaling no. 2836) for ectoderm were analyzed by immunocytochemistry.

RT-PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's recommended protocol. Residual genomic DNA was digested and removed using DNase I (Roche) treatment. First-strand cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen) for RT-PCR and oligo d(T)12–18 primers. The cDNA was diluted with DNase-free water at a concentration of 10 ng/μl. RT-PCR was performed using the Ex-Taq PCR kit (Takara) according to the manufacturer's instructions. The following primers were used: *β-actin* (f) 5′-AGTGTGACGTTGACATCCGT-3′, *β-actin* (r) 5′-GCAGCTCAGTAACAGTCCGC-3′; *Tnf* (f) 5′-GCCCATATACCTGGGAGGAG-3′, *Tnf* (r) 5′-CACCCATTCCCTTCACAGAG-3′; *Il1b* (f) 5′-GCCCATCCTCTGTGACTCAT-3′, *Il1b* (r) 5′-AAGGCCACAGGTATTTTGTCG-3′; *CCL-7* (f) 5′-GCATGGAAGTCTGTGCTGAA-3′, *CCL-7* (r) 5′-AGAAAGAACAGCGGTGAGGA-3′; *C/EBPα* (f) 5′-ATCCCAGAGGGACTGGAGTT-3′, *C/EBPα* (r) 5′-AGCATAGACGTGCACACTGC-3′; *Pu-1* (f) 5′-CTTCCAGTTCTCGTCCAAGC-3′, *Pu-1* (r) 5′-TTTCTTCACCTCGCCTGTCT-3′; *Nanog* (f) 5′-AAGTACCTCGACCTCCAGCA-3′, *Nanog* (r) 5′-CGTAAGGCTGCAGAAAGTCC-3′; *Pou5f1* (f) 5′-CCAATCAGCTTGGGCTAGAG-3′, *Pou5f1* (r) 5′-CTGGGAAAGGTGTCCCTGTA-3′; *Fgf-4* (f) 5′-CGTGGTGAGCATCTTCGGAAGTGG-3′, *Fgf-4* (r) 5′-CCTTCTTGGTCCGCCCGTTCTTA-3′; *Esg1* (f) 5′-GAAGTCTGGTTCCTTGGCAGGATG-3′, *Esg1* (r) 5′-ACTCGATACACTGGCCTAGC-3′; *Cripto* (f) 5′-ATGGACGCAACTGTGAACATGATGTTCGCA-3′, *Cripto* (r) 5′-CTTTGAGGTCCTGGTCCATCACGTGACCAT-3′.

Chromatin immunoprecipitation assays

ChIP assays were performed by using anti-H3-K27 antibodies (Abcam ab6002). Normal rabbit IgG (Sigma) was used as the negative control to verify immuno-precipitation specificity. BM-M-iPS cells (1×10^7) and BM-M-iPS differentiated cells were treated with 1% formaldehyde for 10 min at room temperature to form DNA–protein cross-links. Samples were then treated with cell lysis buffer, nucleus lysis buffer, then sonicated on ice until chromatin fragments reached 300–1000 bp in size. Samples were diluted in dilution buffer then pre-cleaned with protein A, followed by incubating with antibodies at 4°C overnight. On the next day, samples were treated with protein A and protein G at 4°C for 2 h, then washed with high-salt buffer three times, LiCl buffer and low-salt buffer once, followed by TE buffer twice. After washing, samples were resuspended in elution buffer, then treated with proteinase K at 65°C overnight. On the third day, samples were purified using QIAquick PCR purification kit. The PCR amplification was performed under the following conditions: 95°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, final extension 72°C for 5 min. Quantitative real-time PCR was performed as described previously.

Primers were designed as follows: *Nanog* (f) 5′-GGTTAGAGTGCTTTCACTCAC-3′, *Nanog* (r) 5′-GCTGGCTTCAGACTTACTGC-3′; *Pou5f1* (f) 5′-GTGAGGTGTCCGGTGACCCAAGGCAG-3′, *Pou5f1* (r) 5′-CGGCTCACCTAGGGACGGTTTCACC-3′; *Il1b* (f) 5′-CCGCACATCCTGACTTAAAATGTA-3′, *Il1b* (r) 5′-TTATTTCCCCCTGGACAATTGT-3′; *Tnf* (f) 5′-ATGCACACTTCCCAACTCTAAG-3′, *Tnf* (r) 5′-CTTCTGAAAGCTGGGTGCATAAG-3′; *β-actin* (f): 5′-GCTTCTTTGCAGCTCCTTCGTTG-3′, *β-actin* (r): 5′-TTTGCACATGCCGGAGCCGTTGT-3′.

Determination of karyotypes

Karyotypes of iPS cells were identified by quinacrine-Hoechst staining at the International Council for Laboratory Animal Science (ICLAS) Monitoring Center (Kawasaki, Japan).

Supplementary data

Supplementary data for this article are available online at http://jmcb.oxfordjournals.org.

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References

Amoh, Y., Kanoh, M., Niiyama, S., et al. (2009). Human hair follicle pluripotent stem (hfPS) cells promote regeneration of peripheral-nerve injury: an advantageous alternative to ES and iPS cells. J. Cell. Biochem. *107*, 1016–1020.

Aoi, T., Yae, K., Nakagawa, M., et al. (2008). Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science *321*, 699–702.

Chen, S.J., Chang, C.M., Tsai, S.K., et al. (2010). Functional improvement of focal cerebral ischemia injury by subdural transplantation of induced pluripotent stem cells with fibrin glue. Stem Cells Dev. *19*, 1757–1767.

Cho, H.J., Lee, C.S., Kwon, Y.W., et al. (2010). Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. Blood *116*, 386–395.

Choi, K.D., Yu, J., Smuga-Otto, K., et al. (2009). Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. Stem Cells *27*, 559–567.

Eminli, S., Foudi, A., Stadtfeld, M., et al. (2009). Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. Nat. Genet. *41*, 968–976.

Feng, Q., Lu, S.J., Klimanskaya, I., et al. (2010). Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence. Stem Cells *28*, 704–712.

Fouse, S.D., Shen, Y., Pellegrini, M., et al. (2008). Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. Cell Stem Cell *2*, 160–169.

Hanna, J., Wernig, M., Markoulaki, S., et al. (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science *318*, 1920–1923.

Hanna, J., Markoulaki, S., Schorderet, P., et al. (2008). Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell *133*, 250–264.

Hattori, N., Imao, Y., Nishino, K., et al. (2007). Epigenetic regulation of Nanog gene in embryonic stem and trophoblast stem cells. Genes Cells *12*, 387–396.

Hong, H., Takahashi, K., Ichisaka, T., et al. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature *460*, 1132–1135.

Hwang, D.Y., Kim, D.S., and Kim, D.W. (2010). Human ES and iPS cells as cell sources for the treatment of Parkinson's disease: current state and problems. J. Cell. Biochem. *109*, 292–301.

Inaba, K., Inaba, M., Romani, N., et al. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. *176*, 1693–1702.

Jaenisch, R., and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell *132*, 567–582.

Ji, J., Vijayaragavan, K., Bosse, M., et al. (2008). OP9 stroma augments survival of hematopoietic precursors and progenitors during hematopoietic differentiation from human embryonic stem cells. Stem Cells *26*, 2485–2495.

Kim, J.B., Zaehres, H., Wu, G., et al. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature *454*, 646–650.

Kondo, Y., Shen, L., Cheng, A.S., et al. (2008). Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat. Genet. *40*, 741–750.

Kunisato, A., Wakatsuki, M., Kodama, Y., et al. (2010). Generation of induced pluripotent stem cells by efficient reprogramming of adult bone marrow cells. Stem Cells Dev. *19*, 229–238.

Li, H., Collado, M., Villasante, A., et al. (2009). The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature *460*, 1136–1139.

Liang, J., Wan, M., Zhang, Y., et al. (2008). Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. Nat. Cell Biol. *10*, 731–739.

Loh, Y.H., Wu, Q., Chew, J.L., et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat. Genet. *38*, 431–440.

Morita, S., Kojima, T., and Kitamura, T. (2000). Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. *7*, 1063–1066.

Ohnuki, M., Takahashi, K., and Yamanaka, S. (2009). Generation and characterization of human induced pluripotent stem cells. Curr. Protoc. Stem Cell Biol. *9*, 4A.2.1–4A.2.25.

Okabe, M., Ikawa, M., Kominami, K., et al. (1997). 'Green mice' as a source of ubiquitous green cells. FEBS Lett. *407*, 313–319.

Okabe, M., Otsu, M., Ahn, D.H., et al. (2009). Definitive proof for direct reprogramming of hematopoietic cells to pluripotency. Blood *114*, 1764–1767.

Seifinejad, A., Tabebordbar, M., Baharvand, H., et al. (2010). Progress and promise towards safe induced pluripotent stem cells for therapy. Stem Cell Rev. *6*, 297–306.

Senju, S., Hirata, S., Matsuyoshi, H., et al. (2003). Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. Blood *101*, 3501–3508.

Senju, S., Haruta, M., Matsunaga, Y., et al. (2009). Characterization of dendritic cells and macrophages generated by directed differentiation from mouse induced pluripotent stem cells. Stem Cells *27*, 1021–1031.

Shukla, A., Chaurasia, P., and Bhaumik, S.R. (2009). Histone methylation and ubiquitination with their cross-talk and roles in gene expression and stability. Cell Mol. Life Sci. *66*, 1419–1433.

Stadtfeld, M., Maherali, N., Breault, D.T., et al. (2008). Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell *2*, 230–240.

Sun, Z.W., and Allis, C.D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104–108.

Sun, N., Longaker, M.T., and Wu, J.C. (2010). Human iPS cell-based therapy: considerations before clinical applications. Cell Cycle *9*, 880–885.

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

Takahashi, K., Tanabe, K., Ohnuki, M., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

Tay, Y., Zhang, J., Thomson, A.M., et al. (2008). MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature *455*, 1124–1128.

Tenney, K., Gerber, M., Ilvarsonn, A., et al. (2006). *Drosophila* Rtf1 functions in histone methylation, gene expression, and notch signaling. Proc. Natl. Acad. Sci. USA *103*, 11970–11974.

Vaissiere, T., Sawan, C., and Herceg, Z. (2008). Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat. Res. *659*, 40–48.

VandenDriessche, T., Ivics, Z., Izsvak, Z., et al. (2009). Emerging potential of transposons for gene therapy and generation of induced pluripotent stem cells. Blood *114*, 1461–1468.

Vodyanik, M.A., Bork, J.A., Thomson, J.A., et al. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood *105*, 617–626.

Wernig, M., Meissner, A., Foreman, R., et al. (2007). *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature *448*, 318–324.

Williams, R.L., Hilton, D.J., Pease, S., et al. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature *336*, 684–687.