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Derivation of Murine Induced Pluripotent Stem Cells (iPS) and Assessment of Their Differentiation Toward Osteogenic Lineage

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ABSTRACT

Induced pluripotent stem cells (iPSCs) have generated hope and excitement because of the potential they possess for generating patient-specific embryonic-like stem cells (ESCs). Although many hurdles remain to be solved before the cells can be applied clinically; studies directed toward understanding factors that control differentiation of the cells toward various cell lineages are prerequisites for their future application. In the present study, we generated murine iPSC and assessed their differentiation toward osteogenic lineage. Murine tail tip fibroblasts were reprogrammed into embryonic-like state by transduction with defined factors (Oct3/4, Sox2, c-Myc, and klf4) carried in a retroviral vector. The reprogrammed cells expressed ESC markers, gave rise to three germ layers as demonstrated by teratoma formation and immunofluorescence staining. These data confirmed that the reprogrammed cells exhibited ESC-like state. Treatment of iPSCs-derived embryoid bodies (EBs) with transforming growth factor beta 1 (TGF-β1) in the presence of retinoic acid enhanced generation of MSC-like cells. The MSCs-like cells expressed putative makers associated with MSCs; the cells deposited calcium in vitro when cultured in osteogenic medium. Interestingly MSCs-like cells generated from iPSC directed EBs by treatment with retinoic acid and TGF-β1 deposited more calcium in vitro than cells derived without TGF-β1 treatment. Taken together, the data demonstrate that iPSC give rise to MSCs-like state and that the cells have potential to differentiate toward osteoblasts. In addition, brief treatment of iPSC-derived EBs with TGF-β1 may be an approach for directing iPSC toward MSC-like state. J. Cell. Biochem. 109: 643–652, 2010.

KEY WORDS: iPSC; OSTEOBLAST DIFFERENTIATION; MINERALIZATION; EMBRYONIC STEM CELLS

Two types of stem cells are currently recognized. Adult-derived stem cells are present in most tissues and organs and are thought to play a role in maintaining the tissue integrity in cases of trauma or injury [Raff, 2003]. Adult-derived stem cells that have generated great excitement and interest are cells harvested from various prenatal sources and variously called multipotent mesenchymal stromal cells or mesenchymal stem cells [Horwitz et al., 2005; Chamberlain et al., 2007; Crisan et al., 2008; Uccelli et al., 2008; Caplan, 2009; Niyibizi and Li, 2009]. The biology of these cells is still poorly understood primarily because of the lack of markers that can unequivocally identify them. The cells are isolated by adherence to tissue culture plates and on functional assays in vitro and in vivo. The cells give rise to various cell types including those of bone and cartilage [Liu et al., 2009]. These cells have generated great interest because of their potential application in regenerative medicine. Ease of harvest in tissues like bone marrow and adipose tissue makes the cells attractive for various applications in regenerative medicine. The cells, however, have limitations in their application because they cannot be propagated indefinitely in culture; number of these cells also decrease with aging and there is evidence that the cells may exhibit reduced proliferation and differentiation with aging [Quarto et al., 1995; Izadpanah et al., 2006, 2008; Sharpless and DePinho, 2007; Mimeault and Batra, 2009; Wagner et al., 2009].

Embryonic stem cells (ESCs) derived from inner cell mass are pluripotent, thus they can give rise to any cell type of the body. In contrast to adult-derived stem cells, ESC can be cultured indefinitely

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while maintaining their stemness [Odorico et al., 2001; Keller, 2005]. Because of ethical concerns associated with generation of ESC, there is paucity of information on these cells regarding their potential application particularly in regenerating musculoskeletal tissues. Recently, it has been demonstrated that mouse and human fibroblasts can be reprogrammed into an ESC-like state by introducing combinations of four transcription factors; Oct-3/4, Sox2, c-Myc, and Klf4 [Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Park et al., 2008]. The reprogrammed cells are referred to as induced pluripotent stem cells (iPSCs). The cells have been shown to exhibit ESC-like state in terms of self-renewal, expression of ESC markers, and differentiation [Park et al., 2008; Kang et al., 2009]. The attractive feature of iPSC is that there is potential for generating patient-specific ESC for therapeutic purposes, drug screening, or for investigating the biology of stem cells in general. In the present report, we generated mouse-specific iPSC and assessed them for MSCs and osteoblasts differentiation in vitro so as to understand their potential future application in musculoskeletal tissue repair and regeneration.

MATERIALS AND METHODS

ISOLATION OF THE TAIL TIP FIBROBLASTS

To establish tail tip fibroblasts (TTFs), the tails from the 4-day old mice were peeled, minced into 1 mm pieces, placed into culture dishes, and incubated in DMEM containing 15% FBS and 1× non-essential amino acids (NEAA) (Invitrogen Corp., Carlsbad, CA) for 7 days. Cells that migrated out of the tail tip pieces were transferred to new plates and maintained in DMEM containing 15% FBS and 1% penicillin/streptomycin (P/S, v/v).

RETROVIRUS PRODUCTION

The plasmids containing the four factors; Oct-4, Sox2, c-Myc (58A mutant), and Klf4 were obtained from Addgene, Inc. (Cambridge, MA). The pMX-based retroviral vectors containing cDNAs for Oct-4, Sox2, c-Myc, and Klf4 were transfected into Phoenix packaging cell line using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's recommendations. Briefly, 36 μl of Lipofectamine 2000 transfection reagent were diluted in 1.5 ml OPTI-MEM and incubated for 5 min at room temperature. Plasmid DNA (9 μg) diluted in 1.5 ml OPTI-MEM was added to the mixture and incubated further for 30 min at room temperature. After incubation, the DNA/Lipofectamine 2000 mixture was added drop-wise onto Phoenix cells. The cells were then incubated overnight at 32°C in 5% CO_2. Twenty-four hours later, the medium was replaced with DMEM containing 10% FBS and 1% P/S (v/v). The supernatants containing the virus were collected at 48 h posttransfection, filtered through a 0.45 μm cellulose acetate, and used to infect target TTF generated above.

TRANSDUCTION OF TTFs AND PRODUCTION OF iPS CELLS

Twenty-four hours before transduction, TTFs were seeded at 8×10^5 cells per 100 mm dish. The virus-containing supernatants were supplemented with 4 μg/ml of polybrene. Equal amounts of viral supernatants containing each of the four retroviral vectors carrying each of the factors were mixed, added to the fibroblast dish, and incubated overnight. The titer of viral supernatants for each of the factors was not determined. This approach was used for most of the reports on somatic cell reprogramming [Takahashi and Yamanaka, 2006]. The cells were treated with the viral supernatants two more times and were then maintained in culture for 6 days. Six days after transduction, transduced fibroblasts were harvested by trypsinization and replated at 5×10^4 cells per 100 mm dish on murine embryonic fibroblasts (MEFs) feeder layers. Twenty-four hours later, the medium was replaced with Primate ES cell medium supplemented with 1,000 U/ml of leukemia inhibitory factor (LIF). The medium was replaced every other day. Twenty-one days after transduction, colonies were picked and transferred into 0.2 ml of ES cell medium (DMEM supplemented with 15% FBS, NEAA, L-glutamine, 1% P/S, 55 μm β-mercaptoethanol, and 1,000 U/mL of LIF). The colonies were mechanically dissociated to small clamps by pipetting up and down and were then transferred onto MEFs feeder in 24-well plates.

CULTURE OF iPSC

The iPSC generated from TTF were cultured on irradiated MEFs feeders in standard ES medium, DMEM supplemented with 15% FBS, NEAA, L-glutamine, 1% P/S, 55 μm β-mercaptoethanol, and 1,000 U/mL of LIF. As stated above the clones were picked at day 21 after initial transfer to MEF. After passage 1, the clones expanded rapidly and were trypsinized every 3–4 days. The reprogrammed fibroblasts were easily recognized by their morphological appearance [Blelloch et al., 2007; Meissner et al., 2007].

CHARACTERIZATION OF iPS CELLS

To confirm that the reprogrammed TTFs exhibited ESC-like state the following assays were performed.

Alkaline phosphatase activity (ALP) staining. Undifferentiated ESCs express ALP activity constitutively; the ALP activity was determined by cytochemical staining using standard protocols. Briefly, a staining solution containing 1-naphthyl phosphate and Fast red were prepared in phosphate-buffered saline (PBS) pH 9.2. The iPSC clones were fixed in methanol acetone 3:1 for 5 min and washed in PBS. The ALP staining solution was added onto the iPSC clones in 24-well plates and incubated at room temperature for 5–20 min. The staining solution was removed and the cells were washed in distilled water. The cells were examined under a light microscope for ALP activity and images were acquired.

Analysis of ESC markers expression by iPSC. Total RNA was extracted from 1×10^6 iPSC and differentiated iPSC-derived cells. Total RNA was extracted using RNeasy (Qiagen, Valencia, CA) as per the manufacturer's instructions. The mRNA was reverse transcribed to cDNA using SuperScript First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen Corp.) per manufacturer's instructions. cDNA was amplified using an Eppendorf Thermal Cycler at 94°C for 45 s, 58–65°C for 30–60 s, and 72°C for 60 s for 30–35 cycles, after initial denaturation at 94°C for 5 min. For genes associated with osteogenic differentiation; all primer sequences that were used were described previously [Li et al., 2007; Liao et al., 2008]; for expression of endogenous embryonic genes, all the primer sequences that were used were

described by Takahashi and Yamanaka [2006]. Triplicate PCRs were amplified using primers designed for β-actin as a control for assessing PCR efficiency.

For semi-quantitative RT-PCR, total RNAs were isolated. Expression of genes associated with osteoblasts differentiation at different time points was normalized against β-actin; each sample was analyzed in triplicate. The primers used were reported previously [Li et al., 2007]. Electrophoretic images were taken by BioSpectrum® Imaging System (Upland, CA) and were analyzed by Quantity One software (Bio-Rad, Hercules, CA).

Immunocytochemistry. The iPSCs were assessed for SSEA-1 (stage-specific embryonic antigen), Nanog, Oct-3/4, the markers of ESC- and iPSCs-derived embryoid bodies (EBs) for proteins representing each of the germ layer. The proteins examined were alpha fetal protein (AFP), a microtubule-associated protein 2 (MAP2), Troponin I, and α-SMA proteins which are associated with each of the germ layers. The cells were fixed in 4% paraformaldehyde for 10 min at room temperature. After washing in PBS, the cells were treated with normal 10% goat serum in PBS (Sigma, St. Louis, MO), 1% bovine serum albumin (BSA), and 0.1% Triton X-100 for 45 min at room temperature. Respective primary antibodies specific for each of the representative protein markers were added and incubated for 1 h. For visualization, secondary antibodies conjugated to fluorescent tags Cy3 or FITC were employed. The staining was observed under a fluorescent microscope and photographed. The nuclei were stained with DAPI.

Western blotting. Murine embryonic stem cells (mES) and iPSCs were lysed in 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 50 mM sodium fluoride, 2 mM sodium vanadate, 0.1% BSA, and complete protease inhibitors) (Pierce, Rockford, IL) on ice for 15–20 min. The lysate was centrifuged, the supernatant was collected, and analyzed by gel electrophoresis. The proteins were electroblotted onto PVDF membranes (polyvinylidene difluoride) using a mini-PROTEAN II system (Bio-Rad). The ES cell markers were detected using Nanog and Oct4 primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and were visualized by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce).

Teratoma formation. iPSCs were suspended in DMEM at 1×10^7 cells/ml. One hundred microliter aliquot of the cells was injected into the rear leg muscle of 4-week-old female SCID mice. Four to six weeks after injection, tumors were surgically dissected from the mice. The tumors were weighed, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. Tissue sections (10 μm) were cut and stained with hematoxylin and eosin.

Embryoid body formation and in vitro differentiation. iPSC colonies were trypsinized and transferred to ultralow attachment culture dishes (Corning, Corning, NY) to generate EBs. The EBs were maintained in DMEM supplemented with 15% FBS, 1 mM NEAA, 0.1 mM 2-mercaptoethanol, 1 mM L-glutamine, and 50 U/ml P/S for 3 days. After 3 days of suspension culture, EBs were transferred to 0.1% gelatin-coated plates and cultured in the same medium for another 3 days. The cells were then stained for the markers of the three germ layers. In other experiments, EBs were cultured further for 2 days in the presence of retinoic acid.

Generation of MSCs-like cells by treatment of iPSC-directed EBs with TGF-β1 or retinoic acid. After 3 days of EBs culture in suspension, some EBs were treated with retinoic acid (0.5 mM) and cultured further for 2 days. EBs were transferred to 0.1% gelatin-coated plates and cultured in the medium described above either in the presence or absence of transforming growth factor beta 1 (TGF-β1, 10 ng/ml) for 2 days. Following 2 days of TGF-β1 treatment, TGF-β1 treated and untreated cells were maintained in DMEM supplemented with 10% FBS and ascorbic acid. When the cells were near confluent, they were trypsinized and replated on 0.1% gelatin-coated tissue culture dishes and maintained in the same medium. After two passages, the cells were used for FACS analysis and osteogenic differentiation.

FACS analysis. The iPSC-derived EBs differentiated by retinoic acid or TGF-β1 treatment were harvested by trypsinization with 0.25% trypsin/EDTA and washed with cold PBS. Cell aliquots (1×10^6 cells) were incubated in a buffer containing antibodies to target surface antigens. Antibodies used for FACS analysis were phycoerythrin (PE) conjugated anti-CD13, anti-CD34, anti-CD44, anti-CD45, anti-CD73, anti-CD90, anti-CD117, and unconjugated antibodies against CD105 (all from BD Biosciences, San Diego, CA). Each sample was tested three times and two different samples were tested.

Osteogenic differentiation. The iPSC-derived EBs differentiated by retinoic acid or TGF-β1 treatment as described above were trypsinized, plated in six-well plates, and cultured in an osteogenic medium. The osteogenic medium consisted of α-MEM medium supplemented with 10% FBS, 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate, 10^{-7} M dexamethasone, and 1% P/S. The cells were maintained in culture with medium changes every 3 days for 28 days. After 28 days, the media were removed, and the cells were rinsed in PBS, fixed in 10% formalin. For Alizarin red S staining, the plates were treated with the Alizarin red solution and incubated for 5 min at room temperature. After 5 min, the plates were rinsed in distilled water and were then examined under a light microscope and scanned. For von Kossa staining, the plates were treated with 5% aqueous silver nitrate, and then exposed to UV lamp for 20 min. After washing in distilled water, the plates were treated with 2% sodium thiosulfate for 2 min and were examined under a light microscope and scanned.

Statistical analysis. Statistical analysis was carried out using SPSS® software (SPSS, Chicago, IL). One-way ANOVA was used to evaluate for differences in TGF-β1 treated and untreated samples for osteogenic differentiation and marker expression. Significance was set at $P < 0.05$.

RESULTS

iPSC GENERATION

Twenty-one days after transfection of the TTFs, clones of the reprogrammed cells (Fig. 1A, one clone shown) were picked based on morphological appearance and plated on MEFs. Upon replating, colonies were evident (Fig. 1B). The cells expanded rapidly in culture and were trypsinized and propagated every 2–3 days.

To determine if the reprogrammed cells exhibited ESC-like state, the cells were stained for alkaline phophatase activity. The results

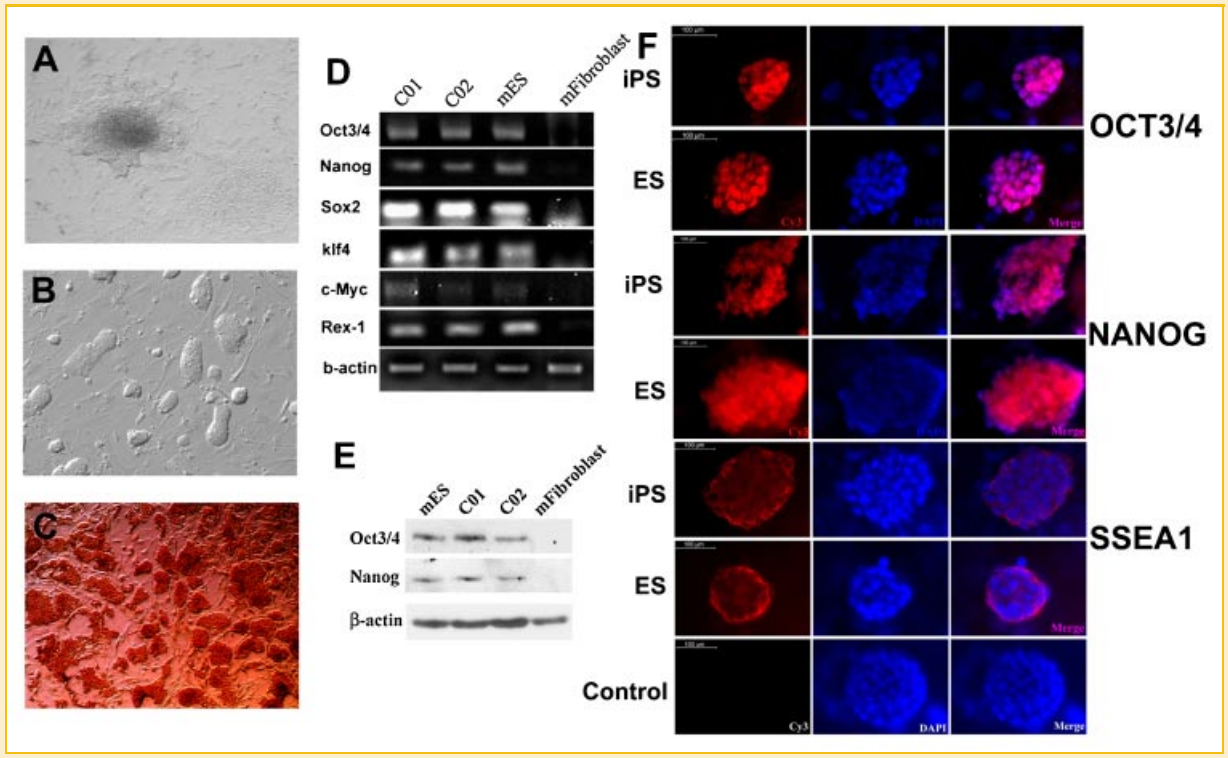

Fig. 1. iPSC generated from reprogrammed tail tip fibroblasts (TTFs) exhibit ESC-like state. A: Morphological appearance of iPSC clone 21 days after transfection of tail tip fibroblasts with defined factors and transfer on murine embryonic fibroblasts (MEFs). B: iPSC-derived colonies growing on murine embryonic fibroblasts. The cells exhibit ESC growth characteristics. Many cell colonies are evident suggesting self-renewal of iPSC. C: ALP activity of iPSC-derived colonies shown in B, all colonies are ALP positive, a characteristic of ESC indicating that the iPSC exhibit ESC-like state. D: Expression of ESC-specific genes by the iPSC demonstrating their ESC-like state. E: Western blot demonstrating synthesis of Nanog and Oct3/4 proteins by iPSC a characteristic of ESC. F: Immunofluorescence staining for ESC markers expressed by iPSC demonstrating expression of genes responsible for pluripotency in ESC. The iPSCs demonstrated staining for Oct/34 and Nanog in the nucleus as expected while SSEA-1, a stage-specific antigen is on the cell surface as expected. The murine ESC (ES) used as positive control shows similar staining. The data demonstrate that reprogrammed tail tip fibroblasts (TTF) exhibit ESC-like state. Staining was revealed by Cy3-conjugated secondary antibody, nuclei staining is revealed by DAPI (blue). Cy3 staining images are overlaid with DAPI staining to show location of each of the antigen analyzed. Original magnification: A, B 40× and F 400×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

showed that the cell colonies were positive for ALP activity as expected for ESC-like cells (Fig. 1C). The cells were also examined for the expression of the ESC markers by PCR and protein synthesis by Western blotting. Figure 1D shows that the fibroblasts we reprogrammed expressed genes characteristics of ESC (Oct3/4, Nanog, Sox2, klf4, c-Myc, and Rex-1). These data confirmed that the cells we generated exhibited ESC-like state. These data were further confirmed by Western blotting which showed that the ESC-associated factors were being expressed on protein level by the reprogrammed cells (iPSCs) (Fig. 1E). Next, we examined the ESC-associated proteins by immunocytochemistry to determine their location within the cells. Oct3/4 and Nanog are transcription factors that are critical for ESC pluripotency, thus they are located in the nucleus. Immunofluorescence staining indeed demonstrated that the factors were located within the nuclei of the reprogrammed TTFs (Fig. 1F). SSEA-1 was present on the cell surface as expected (Fig. 1F). Comparison of iPSC staining with murine ESC (ES) showed similar staining pattern. These data further demonstrated that the cells reprogrammed from the TTFs exhibited ESC-like state.

To further confirm that the reprogrammed cells exhibited characteristics of ESC, we assessed the cells for the ability to give rise to the three germ layers, ectoderm, endoderm, and mesoderm. Representative proteins of each layer were examined by immunofluorescence after differentiation of iPSC-directed EBs. EBs were made from iPSC and were then allowed to differentiate (Fig. 2A). The proteins examined were AFP which is expressed by the liver and is a representative of endoderm differentiation (Fig. 2B); Troponin I and α-SMA (Fig. 2C,F) proteins are associated with muscle and represent mesoderm differentiation; ectodermal lineage differentiation is represented by the expression of the MAP2 (Fig. 2D), a neural-specific protein that stabilizes microtubules which are critical for neuralite outgrowth. The immunofluorescence staining results clearly further confirm that the reprogrammed TTFs exhibit ESC-like state.

Finally, to further confirm the identity of the reprogrammed fibroblasts, we examined the ability of the cells to give rise to the three germ layers by assessing teratoma formation. The cells injected into the thigh muscles of SCID mice gave rise to tumor-like masses which upon histological examination demonstrated presence of tissues representing each of the germ layers (Fig. 3). The xenografts of mouse iPSC generated well-differentiated teratoma-like masses containing all three embryonic germ layers. Taken together all

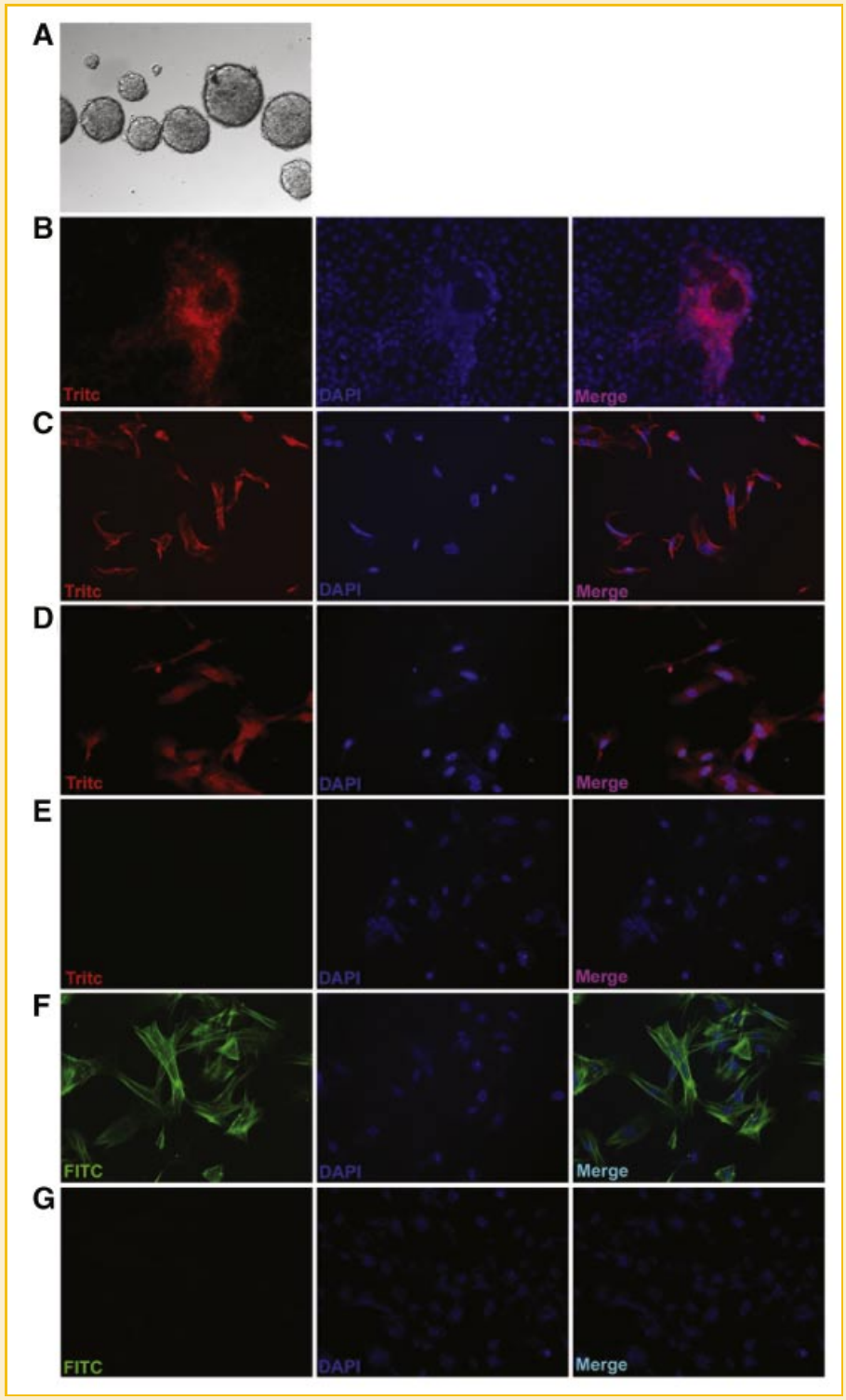

Fig. 2. Immunofluorescence staining for proteins representing each of the germ layer to demonstrate differentiation of iPSC to three germ layers. A: Embryoid bodies (EBs) made from (iPSCs) reprogrammed tail tip fibroblasts (TTFs). B: Alpha fetal protein (AFP) is expressed by the liver and is a representative of endoderm differentiation; the cells expressing the protein appear in aggregate. C,F: Troponin I and α-SMA proteins, respectively, are associated with muscle and they represent mesodermal differentiation. D: Differentiation into ectodermal lineage is represented by expression of microtubule-associated protein 2 (MAP2). E,G: No primary antibodies were added. DAPI staining is indicated in the middle column and overlaid images are shown in the third column on right. Embryoid bodies were generated from iPSC as described in the Materials and Methods Section and were then allowed to differentiate into the three germ layers. Differentiating EBs were used for immunofluorescence to assess expression of proteins that are representative of each of the germ layers. Original magnification: A 40$\times$ and B–G 200$\times$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

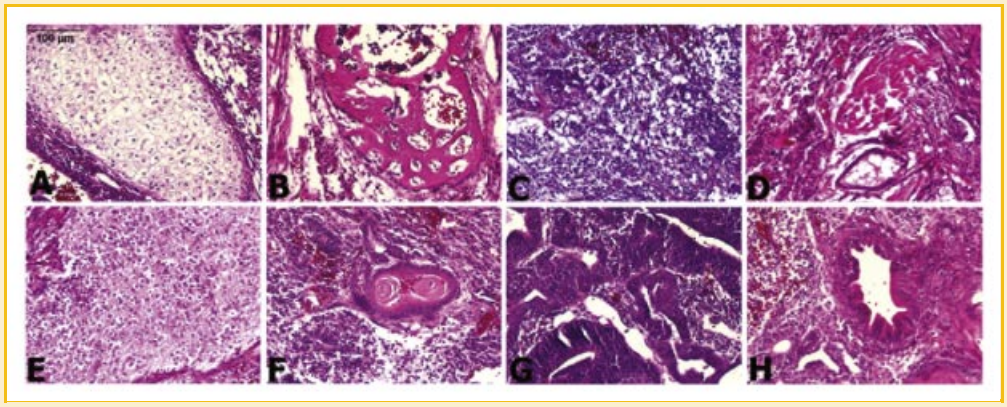

Fig. 3. Teratoma formation by the iPSC generated from the murine tail tip fibroblasts. The xenografts of mouse iPSC generated well-differentiated teratoma-like masses containing all three embryonic germ layers. Immunodeficient mouse recipients were injected with mouse iPSC intramuscularly. Resulting teratomas demonstrated the following features in ectoderm, mesoderm, and endoderm. Mesoderm: cartilage (A), bone (B), fat (C), muscle (D). Ectoderm: neural tissue (E), epidermis (F). Endoderm: glandular tissue (G), gut (H). All images were obtained from the same tumor. Tissue sections were stained with hematoxylin and eosin. Scale bar indicates100 μm and all micrographs are of the same size. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the analysis clearly established that iPSC generated here exhibit ESC-like state. The results are consistent with the established criteria for designation of reprogrammed somatic cells as iPSC [Takahashi and Yamanaka, 2006; Meissner et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Park et al., 2008].

Upon establishing that the reprogrammed cells exhibit ESC-like state, the cells were assessed for potential to differentiate toward osteogenic lineage. iPSCs were differentiated into EBs followed by treatment with retinoic acid and by culturing the cells on gelatin-coated plates in the presence or absence of TGF-β1 for 2 days. First, the resulting cells were expanded and assessed for proliferation and expression of the putative MSC markers at passage 2. The morphological appearances of the cells resulting from either treating iPSC-derived EBs with or without TGF-β1 are shown in Figure 4A. Proliferation analysis of both cell populations showed that with extended culturing, the cells generated by TGF-β1 treatment exhibited a higher rate of proliferation than untreated cells (Fig. 4B). Analysis of the expression of the putative MSCs cell surface markers revealed that the cells expressed markers associated with MSCs. Both cell subpopulations expressed CD73 and CD105 but a higher number of cells expressing these antigens were present in a population generated by TGF-β1 treatment (Fig. 4C). Interestingly there were higher numbers of cells expressing CD34 in a population of cells resulting from non-TGF-β1 differentiation protocol. These data suggest that brief treatment of iPSC-directed EBs with TGF-β1 in addition to retinoic acid generated high levels of cells expressing MSCs markers (Fig. 4D). Taken together the data suggested that TGF-β1 may modulate production or expansion of MSC-like cells from iPSC-derived EBs.

We next examined the ability of the cells to differentiate toward osteogenic lineage. We examined both cell populations as well as undifferentiated cells. iPSC-directed MSCs derived from TGF-β1 and retinoic acid treatment or retinoic acid treatment alone were assessed for osteogenic differentiation. The results showed that both cell populations exhibited potential to differentiate toward osteogenic lineage in the presence of osteogenic factors as indicated by Alizarin red and von Kossa staining (Fig. 5A,B). Quantitation of the amount of calcium deposited by the two cell populations showed that iPSC-directed MSCs generated by TGF-β1 treatment deposited more calcium than non-TGF-β1-derived MSCs (Fig. 5C). von Kossa staining revealed similar results (Fig. 5A). The cells that were not cultured in osteogenic medium demonstrated minimal mineral deposition (Fig. 5A–C).

Semi-quantitative PCR analysis of osteoblast gene expression by differentiating TGF-β1 or non-TGF-β1-derived MSCs is shown in Figure 6. Runx2 and osterix were expressed early at low levels within the EBs stage (Fig. 6A). There was a gradual increase in expression of these genes with culturing. Runx2 expression was constantly expressed at higher levels in non-TGF-β1-derived MSCs than the TGF-β1-derived MSCs (Fig. 6B). Osterix expression was also expressed in EBs at low levels prior to differentiation toward osteogenic lineage; this transcription factor, however, increased in expression by the cells with time in culture and was expressed at high levels in iPSC-directed MSCs generated by TGF-β1 treatment. Osteocalcin was expressed by the putative MSCs cultured in osteogenic medium and was detected at week 1 of culture. The expression of osteocalcin increased with culturing with maximal expression at week 3; interestingly putative MSCs derived by TGF-β1 treatment exhibited higher expression of osteocalcin when maintained in osteogenic medium (Fig. 6B). Undifferentiated iPSC clone (C01) expressed ESC markers as expected.

DISCUSSION

In this report, we have shown that by using PMX retrovirus carrying the reprogramming factors we were able to generate cells from TTFs with ESC-like state. iPSCs have generated excitement and hope because of the future potential they possess for generation of patient-specific stem cells. Although there are still obstacles before the cells can be used for clinical application, it is important to begin to understand future application of these cells for musculoskeletal tissue repair and regeneration. Recent reports have shown that iPS

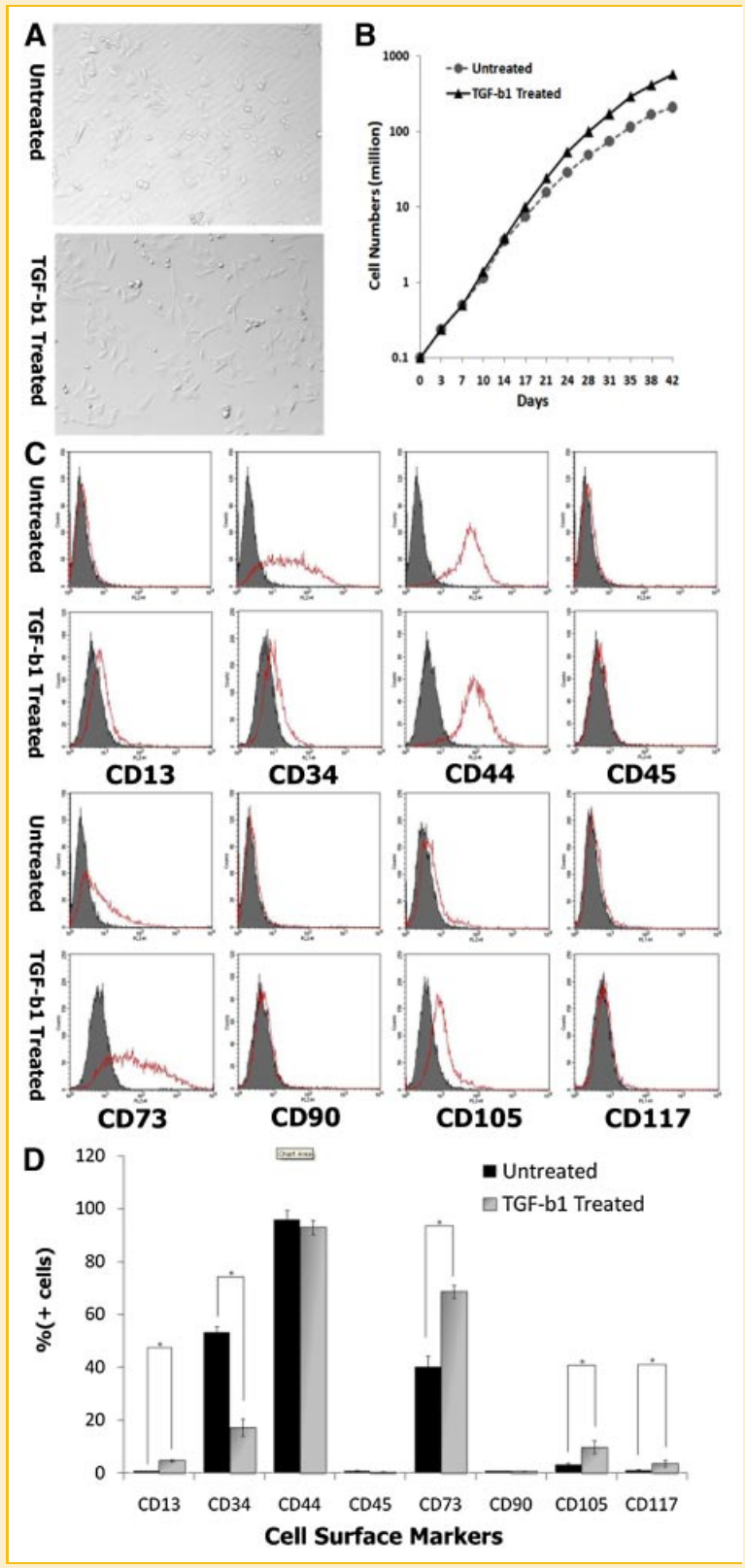

Fig. 4. Comparison of MSC-like cells from iPSC generated with or without TGF-β1 treatment. A: Morphological appearance of MSCs-like cells generated from iPSC-directed EBs by either treatment with or without TGF-β1. B: Proliferation of MSCs-like cells with extended culturing; MSCs-like cells generated by TGF-β1 treatment exhibited higher proliferation rate with extended culturing. C: Putative surface marker expression by MSC-like cells generated from iPSC-directed EBs by treatment with or without TGF-β1. D: Relative percent (mean ± SD) of cells expressing each marker within each cell preparation. TGF-β1-derived cells contain high levels of cells expressing CD73 and CD105 suggesting the presence of higher levels of MSC-like cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

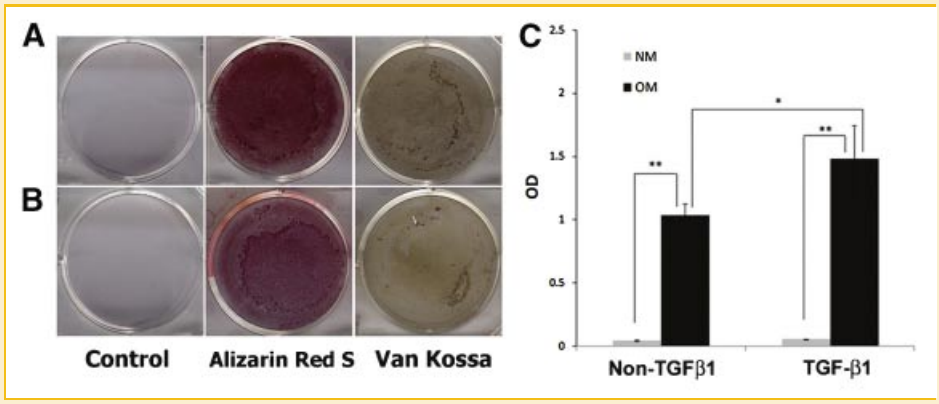

Fig. 5. Osteoblastic differentiation of MSCs-like cells generated from iPSC with or without TGF-β1 treatment. A: MSC-like cells derived by treatment of iPSC-directed EBs with TGF-β1, cultured in the presence or absence of osteogenic factors and stained with Alizarin red at 28 days. B: MSC-like cells derived from iPSC-directed EBs without TGF-β1 but exposed to retinoic acid treatment, cultured in the presence or absence of osteogenic factors and stained with Alizarin red at 28 days. C: Relative amount of calcium deposited by cells derived by treatment of iPSC with or without TGF-β1. MSCs-like cells derived by TGF-β1 treatment showed higher mineral deposition than non-TGF-β1-derived MSCs following culture in osteogenic medium (OM). Alizarin red deposits shown in (A) and (B) were extracted and their absorbance was determined. The absorbance levels represent calcium deposits. Each experiment was carried out in triplicates and repeated twice. Osteogenic medium (OM), normal medium (NM). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells can be generated without using viral vectors thus bringing iPSC one step closer to clinical application [Kaji et al., 2009; Zhou et al., 2009]. A proof of concept report demonstrated that iPSC can be used to treat sickle cell anemia [Hanna et al., 2007]. In the present report, we investigated the potential of the cells to give rise to MSC-like state and to differentiate toward osteogenic lineage. The results showed that TGF-β1 may play a role in directing iPSC toward MSC-like state or may play a role in the expansion of the cells. The cells derived by treatment of iPSC-derived EBs with TGF-β1 deposited more calcium in vitro than cells derived without TGF-β1 treatment. These findings are of interest because they suggest that MSCs-like cells can be generated from iPSC by brief exposure to TGF-β1 in the presence of retinoic acid. Previous studies have shown that brief exposure of hESC to BMP-4 induced their differentiation toward mesodermal lineage [Zhang et al., 2008]. In the present report, although TGF-β1 was added after EBs formation, presence of the growth factor may have enhanced mesodermal differentiation [Sanders and Prasad, 1991]. TGF-β family of proteins and downstream signaling smads play key roles in hematopoietic differentiation [Mishra et al., 2005]. TGF-β has been shown to inhibit proliferation of early multipotent hematopoietic stem cells [Mishra et al., 2005]. In the present report, we have shown that TGF-β1 treatment of EBs enhances production of MSC-like cells expressing putative MSCs markers with no evidence of hematopoietic stem markers, thus implying inhibition of hematopoietic stem cell development. In the present report, TGF-β1 was not used for osteogenic differentiation but for differentiation of EBs-derived cells toward MSCs. Differentiation of MSCs toward bone, cartilage, or adipose tissue is controlled by select members of TGF-β family. TGF-β is stimulatory for embryonic myoblasts but it inhibits progression of differentiation and maturation of myoblasts, osteoblasts, and adipocytes but stimulates chondrocytes differentiation. TGF-β1 may therefore be playing a role in expansion of MSCs generated from iPSC-directed EBs.

FACS analysis showed that differentiating iPSC-directed EBs gave rise to cells that exhibit MSCs characteristics including expression of the putative surface markers. Cells expressing CD73 and CD105 are predominant in MSCs harvested from postnatal tissues. Putative MSCs generated from iPSC-derived EBs comprised a high population of cells expressing CD73 antigen, about 75%, while CD105 expressing cells comprised about 15% of the cell population. Interestingly CD34 expressing cells decreased in the cell population derived from treatment of iPSC-derived EBs with TGF-β1. These data suggest that brief treatment of iPSC-derived EBs with TGF-β1 may favor differentiation of the cells toward MSCs-like state. There were no CD45 expressing cells in differentiating iPSCs. The majority of the TGF-β1-derived MSCs expressed CD73 and CD44 antigens. Previous studies showed that cells sorted for CD73 from differentiating hESC gave rise to cells that differentiated into osteoblasts, chondrocytes, adipocytes in vitro, and gave rise to engraftable myoblasts [Barberi et al., 2007]. The present findings on iPSCs differentiation toward putative MSCs are in agreement with these previous findings.

Several reports have shown that the number of stem cells decrease with aging and that the cells exhibit reduced proliferation and differentiation with aging [Quarto et al., 1995; Sharpless and DePinho, 2007; Mimeault and Batra, 2009; Wagner et al., 2009]. In contrast, the ESC exhibit unlimited proliferation capacity, thus they can be generated in high numbers and maintained in culture for many generations under appropriate conditions without losing their stem cell potential. In addition, the ESC can give rise to any cell type in the body, thus they are appropriate for transplantation and regeneration of various tissues and organs, making them superior candidates for application in regenerative medicine. The draw back in using ESC is that patient-specific cells cannot be generated thus presenting a high risk for rejection upon transplantation. iPSCs would obviate this concern because patient-specific ESC-like can be generated reducing potential of cell rejection upon transplantation. To compensate for the lower pool of stem cells in aged

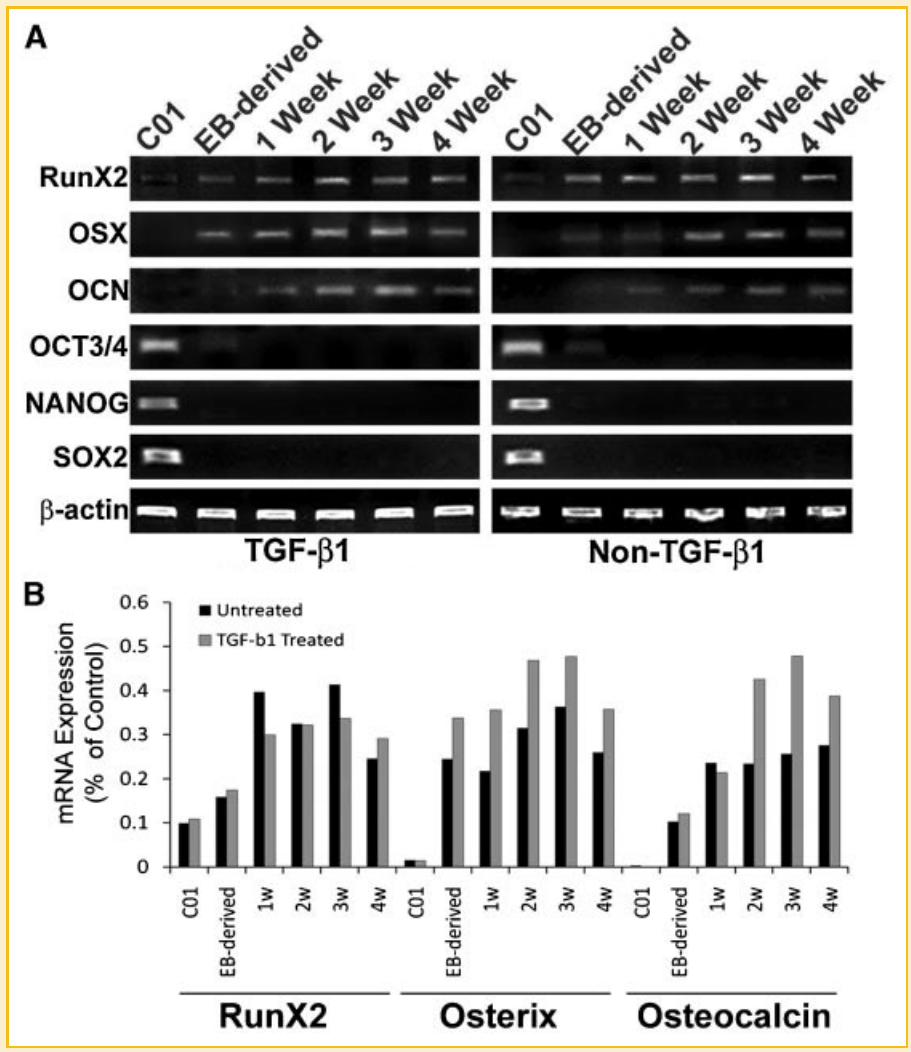

Fig. 6. Semi-quantitative PCR analysis of osteoblast-associated genes expressed by MSC-like cells generated from iPSCs with or without TGF-β1 treatment. A: Electrophoretic profile of genes analyzed at weekly intervals in MSC-like cells cultured in the presence of osteogenic factors. B: Relative mRNA levels of Runx2, Osx, and OCN analyzed at weekly intervals in MSCs maintained in osteogenic medium. Runx2 expression levels by MSCs-like cells generated by treatment with or without TGF-β1 and cultured in osteogenic medium were relatively similar. Osx and OCN expressions were higher in MSCs derived by TGF-β1 treatment. The data confirm that the TGF-β1-derived cells exhibited higher osteogenic potential than non-TGF-β1-derived cells. C01 = iPSC Clone 01, EB = Embryoid body. EBs and C01 were not cultured in osteogenic medium.

musculoskeletal tissues, fibroblasts generated from aged individuals can be reprogrammed to generate ESC-like cells which can be directed for differentiation toward desired cell lineages.

Taken together, the data presented in this report show that iPSCs have potential to give rise to MSCs-like state and the resulting cells can be induced to differentiate toward osteoblasts lineage at least in vitro. TGF-β1 appears to play a role in directing iPSC-derived EBs toward MSCs-like state.

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