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Generation of mesenchymal stromal cells from a HOXB4-expressing human embryonic stem cells colony

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Abstract

Background—HOXB4 transcription factor plays an important role in embryonic and adult hematopoiesis. Over-expression of HOXB4 in murine and human embryonic stem cells (ESCs) has been used to generate hematopoietic stem cells (HSCs) via embryoid body formation method.

Methods—We used FuGENE-6 based transfection of YPL2-HOXB4 vector to generate HOXB4-expressing colonies from human ESC line H9 and investigated the differentiation potential of these cells into primitive CD34+ hematopoietic cells, via co-culture methodology with OP9 murine bone marrow stromal cells. Expression of HOXB4 in transfected human ESC colonies and their derivatives was verified using immunocytochemistry and RT-PCR.

Results—Utilizing OP9 stromal cell co-culture methodology, we generated CD34+ cells from HOXB4-expressing H9 human ESCs at a frequency similar to, and not higher than, non-transfected human ESCs. However, we observed that some colonies of HOXB4-expressing human ESCs, not co-cultured on OP9 cells, differentiated into mesenchymal stromal/stem cells (MSCs) while preserving their HOXB4 expression. These HOXB4-expressing MSCs expressed CD29, CD73, CD44, CD90, CD105, and HLA-class I; were negative for the expression of CD34, CD45, CD54, CD71, CD106 and HLA DR; and could be differentiated into adipocytes and osteocytes.

Discussion—In our specific experimental system we observed that over-expression of HOXB4 in human ESCs did not improve the generation of CD34+ hematopoietic cells via OP9 co-culture methodology. Furthermore, we could generate MSCs from human ESCs over-expressing HOXB4.

Keywords

Human embryonic stem cells; Mesenchymal stromal/stem cells; HOXB4

INTRODUCTION

Human embryonic stem cells (ESCs) have the potential to revolutionize medicine by providing cellular therapeutic options for a wide variety of human diseases such as Parkinson's and Alzheimer's disease, diabetes, and hematological disorders [1]. Generation of both hematopoietic stem cells (HSCs) and more lineage restricted progenitor cells from ESCs has been studied extensively from both mouse and human ESCs. ESCs grown in a suspension culture without feeder cells spontaneously form complex cell aggregates called embryoid bodies (EBs), which contain components of all three germ layers [2]. Doetschman et al. were the first to note that EBs which develop from murine ESCs contain hematopoietic cells [3].

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This method has been widely used to generate primitive hematopoietic cells from murine [4–6] or human ESCs [7,8]. Alternatively, ESCs can undergo hematopoietic differentiation if they are grown in the presence of some type of hematopoietic supportive stromal cells [9]. This method has also been investigated extensively for the generation of hematopoietic cells from murine [10] or human ESCs [11–13], utilizing a variety of supportive stromal cells.

Nevertheless, until recently it was difficult to demonstrate robust *in vivo* hematopoiesis after the transplantation of primitive hematopoietic cells derived from ESCs *in vitro* using either the EB or stromal cell co-culture methodology. However, when Kyba et al. transduced day 6 murine EB-derived cells with the HOXB4 gene and then co-cultured them with OP9 murine bone marrow stromal cells, primitive hematopoietic cells expressing cell surface markers such as c-kit were generated [14]. Importantly, transplantation of these cells into mice resulted in hematopoietic reconstitution in both primary and secondary recipients indicating their long-term engraftment potential and thus their identity as HSCs. Since this seminal report the potential role of over-expression of HOXB4 in promoting generation of HSCs form murine or human ESCs have been extensively investigated, using a wide variety of methodologies and experimental conditions. Most of these studies involve a component of EB formation step to generate hematopoietic cells [15–19]. We initiated our current study to investigate the differentiation potential of human ESCs stably expressing HOXB4 utilizing the co-culture methodology with OP murine stromal cells instead of EB methodology, as this specific approach has not been reported yet.

METHODS

Human ESC culture

The human ESC line H9 was obtained from WiCell (Madison, WI). The cells were originally maintained in the undifferentiated state by culturing on irradiated murine embryonic fibroblasts (MEF). Prior to transfection cells were maintained in MEF- conditioned media (CM) on matrigel coated plates (BD Biosciences, San Jose, CA), for few passages to make sure no MEF cells left in culture, as described previously [20].

Construction of plasmids

HOXB4 gene was amplified from pTAT-HA-HOXB4 [21], kindly provided by Dr. Guy Sauvageau [22], with EcoR I and Xba I sites, and its sequence was confirmed. HOXB4 cDNA was then inserted into YPL2 vector [20], with EcoRI and XbaI, and designated YPL2-HOXB4 (Figure-1A). In this vector, there are two EF1-alpha promoters, one drives HOXB4 and the other drives Neomycin resistance gene expression (Figure-1-A). For the control transfection we used YPL2-GFP.

Transfection

Plasmid transfection was performed as described before [20] with some modification. Briefly, 10 μg/well of YPL2-HOXB4 plasmid and 15 μl/well of FuGENE 6 (Roche Applied Science, Indianapolis, IN) were mixed in 100 μl OptiMEM medium, and incubated at room temperature for 30 minutes. Following incubation, the plasmid/FuGENE 6 mixture was added to human ESCs cultured in 6-well plates containing CM. Twenty-four hours after transfection, the media was changed to 2.5 mL of fresh CM containing 100 μg/mL of G418 (Invitrogen, Carlsbad, CA). After two weeks of G418 selection, the remaining colonies, undifferentiated or partially differentiated, were individually transferred to 4-well plates, each well containing one colony. One week later, cells from each well were distributed to 4 wells of a new 4 well-plate. From each of these secondary 4-well plates one well was immunostained with HOXB4 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) to identify HOXB4 positive colonies under microscope. Colonies that remained undifferentiated were used for co-culture

experiments with OP9 cells but for colonies with mesenchymal looking cells further culturing was done using MSC media (α-MEM containing 20% FBS).

Co-culture with OP9 cell

HOXB4+ human ESCs were co-cultured with OP9 stromal cells by plating undifferentiated HOXB4-expressing human ESC colonies at a density of 1×10^5 cells per mL onto six-well plates containing a confluent monolayer of OP9 cells, previously irradiated with 80 Gy, and differentiation medium containing α-modified minimum essential medium (α-MEM) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 0.1 nonessential amino acids, 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Co-cultured cells were then incubated at 37° centigrade (C)/5% CO_2 with half medium changes on days 4, 6, 8, 10, 12 and 14.

Immunostaining

All of the immunocytochemistry analyses were performed after fixation in 4% paraformaldehyde for 30 minutes, rinsing twice with phosphate-buffered saline (PBS), and incubation with 20% goat blocking serum in 4% Triton/PBS for 2 hours at room temperature. All primary antibodies, at 4 μg/mL in 4% Triton/PBS, were applied overnight at 4°C. The primary antibodies used for immunostaining included: anti-Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-HOXB4 (Developmental Studies Hybridoma Bank). For negative controls, we used the same concentration of the primary antibodies of mouse IgG isotype (Caltag, Burlingame, CA). After washing the cells three times with PBS, they were incubated with fluorescence-conjugated secondary goat anti-mouse antibody (Santa Cruz Biotechnology) at a 1:50 dilution.

Flow cytometry

The cells were dissociated with trypsin (0.25%) plus EDTA (1 mM), washed twice in PBS containing 0.1% bovine serum albumin (BSA), and suspended at a concentration of 6×10^5 cells/mL. Five μL of Phycoerythrin (PE) or Allophycocyanin (APC) conjugated antibodies were added to each 0.1 mL volume of the cells, incubated for 30 minutes at 4 °C in dark, washed twice in PBS containing 0.1% BSA, and then immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, Ca) with FlowJo acquisition and analysis software (Tree Star, Inc., San Carlos, CA). The antibodies used for detection of cell surface markers by FACS included: BD Biosciences antibodies: CD29 PE (#557332), CD34 PE (# 550619), CD44 PE (# 550989), CD73 PE (# 550257), CD90 APC (#559869), CD54 PE (#555511), CD106 PE (#555647), HLA-DR PE (#347363); eBiosciences (San Diego, Ca) antibodies: CD45 PE (clone HI30) and CD105 APC (Clone SN6); Santa Cruz antibody: CD71 PE (#SC7327); and Sigma antibody: HLA Class-I FITC (Clone W6/32). In each experiment control staining with the appropriate isotype monoclonal antibodies was included.

Reverse transcribed-polymerase chain reaction (RT-PCR)

The total RNA was extracted from 1 million cells with TRIZOL reagent using the manufacturer's recommendations (Invitrogen). One μg of total RNA was reverse transcribed using M-MuLV Reverse Transcriptase (Roche, Indianapolis, IN). PCR mixtures were prepared as described (Promega protocol for *Taq* polymerase, Madison, WI), denatured at 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds. A final extension at 72 °C for 10 minutes was performed after cycling. Primer sequences used included: HOXB4 sense: GAATTCAATGGCTATGAGTTCT, HOXB4 antisense TCTAGACTAGAGCGCGCGGGG; bone specific alkaline Phosphatase (ALP) sense TGGAGCTTCAGAAGCTCAACACAA, ALP antisense ATCTCGTTGTCTGAGTACCAGTCC; Bone Sialoprotein (BSP) sense AATGAAAACGAAGAAAGCGAAG, BSP antisense ATCATAGCCATCGTAGCCTTGT;

Peroxisome Proliferator Activated Receptor-γ (PPAR-γ) sense CTCCTATTGACCCAGAAAGC, PPAR-γ antisense GTAGAGCTGAGTTCTTCTCAG; Cyclophilin-A sense: CCGAGGAAAACCGGTACTAT, Cyclophilin-A antisense AGATTCTAGGATACTGCGAGCA.

Adipogenic Differentiation

Human ESC-derived MSCs were induced into adipocyte differentiation by exposure to 0.25 μM dexamethasone, 10 μg/mL insulin, and 0.5 mM isobutylxanthine (Sigma) in high glucose DMEM medium containing 10% FBS for 2–4 weeks [23]. Adipocyte-specific granules were detected via Red Oil O staining (Cambrex Bio Science, Walkersville, MD) according to the manufactory protocol. Cells were viewed with a phase contrast microscope; lipids appeared red and nuclei appeared blue. Differentiation of adipocytes was further confirmed using RT-PCR to detect expression of PPAR-γ.

Osteogenic Differentiation

Human ESC-derived MSCs were plated at low density ($1–2.5 \times 10^3$ cells/cm^2) on tissue-culture treated dishes in the presence of 10 mM β-glycerol phosphate (Sigma), 0.1 μM dexamethasone, and 200 μM ascorbic acid (Sigma) in alpha MEM medium containing 10% FBS for 3–4 weeks [23]. Osteogenesis was demonstrated by Von Kossa staining using silver stain and counterstaining with nuclear fast red, and then viewed on a phase contrast microscope. Osteogenesis was further confirmed by RT-PCR to detect bone specific ALP and BSP gene expression.

RESULTS

Generation of different colonies of HOXB4-expressing human ESCs

We used FuGENE 6-based transfection and our YPL2 vector to generate human ESC lines over-expressing human HOXB4 cDNA. Two different types of colonies appeared after YPL2-HOXB4 transfection and during G418 selection period. Colonies like colony#1 (Figure-1-C) showed differentiation towards mesenchymal looking cells, at the periphery of the colonies, similar to what we reported previously for non-transfected human ESC cultures [24, 25]. Colonies like Colony#2 maintained morphology of undifferentiated human ESC colonies (Figure-1-F). Both colonies expressed HOXB4 in RNA level as shown by RT-PCR (Figure-1-B) and protein level as shown by immunocytochemistry (Figure 1-D and 1-G). After the two-week selection period colonies like colony#1 were singly transferred into new 4-well plates and upon further passaging differentiation process continued, without any specific intervention, until all cells in the colony appeared to have mesenchymal looking morphology. All cells differentiated from this type of colony remained positive for HOXB4 as shown by immunocytochemistry (Figure-2-C). We did not find evidence of HOXB4 expression in BM-derived MSCs by immunocytochemistry (Figure-2-D). On the other hand, undifferentiated human ESCs (Figure-3-C) continue to express Oct-4, a marker of undifferentiated human ESCs [26]; however, in contrast, MSCs derived from HOXB4 expressing human ESCs were no longer expressing Oct-4 (Figure-3-D). These data provide evidence that MSCs derived from HOXB4-expressing human ESCs continue to express high levels of HOXB4 transcription factor but have lost the expression of markers of undifferentiated ESCs.

Generation of $CD34^+$ cell by co-culturing undifferentiated HOXB4-expressing human ESCs with OP9 cells

We co-cultured undifferentiated HOXB4-expressing human ESC colonies, similar to colony#2, or non-transfected H9 ESCs with OP9 cells for 2 weeks. Single-cell suspensions from day 4, 6, 8, 10, 12, and 14 of co-cultures were incubated with PE-CD34 antibodies and

percentages of CD34+ were analyzed with flow cytometry. In three sets of experiments, simultaneously comparing HOXB4-expessing human ESCs and non-transfected human ESCs, we could not detect any statistically significant difference in the percentage of CD34+ cells generated at any time point during the co-culture period (Figure-4).

Cells differentiated from HOXB4-expressing human ESCs without OP co-culture express cell surface markers of MSCs

We used CD73+, a well known marker for MSCs [27], as a screening marker to follow the phenotype of mesenchymal looking cells in our differentiation cultures. Two weeks after initiation of transfection of human ESCs, and when they were maintained in CM with 100 μg/mL G418, 36% of the total cells expressed CD73 on their cell surfaces as detected by flow cytometry. Upon three further passages and culture of the differentiating colonies in MSC media (α-MEM containing 20% FBS) all cells assumed a homogenous morphology. At this time the cells were positive for CD29, CD44, CD73, CD90, and CD105 cell surface markers, consistent with a MSC phenotype, but did not express hematopoietic markers such as CD34, CD45, or CD71 (Figure-5). Furthermore, these cells were positive for HLA class I, but negative for HLA-DR. Interestingly, our cells did not express CD106 marker. As we and others have already reported [24,25,28,29], our human ESC-derived MSCs showed slower proliferation after 12–15 passages; however, at these later passages they were still positive for MSC markers such as CD73 and CD105 (data not shown)

MSCs generated from HOXB4-expressing human ESCs differentiate into adipocytes and osteoblasts

We then tested the differentiation potential of MSCs generated from HOXB4-expressing human ESCs into adipocytes when they were cultured in the adipogenic media for 4 weeks. The characteristic fat globules were first observed at 2 weeks after incubation, as visualized with Oil Red O staining, and after 4 weeks of induction about 30% of the cells were positive for the fat globules (Figure-6-A). In addition, the induced cells were positive for adipocyte marker PPAR-γ by RT-PCR (Figure-6-B). When MSCs generated from HOXB4-expressing human ESCs were cultured in osteogenic media for 4 weeks, at day 28, the majority of cells showed calcium crystal (Brown-black) deposits (Figure-7-A). These cells were also shown to be positive for ALP and BSP, osteogenic specific, genes by RT-PCR (Figure-7-B).

DISCUSSION

HOX genes are a highly conserved family of 39 genes, organized in 4 genomic clusters (A, B, C, and D), encoding transcription factor proteins that play an essential role in cell fate determination in lower and higher organisms [30]. Gene over-expression experiments have demonstrated the particular role of HOXB4 in enhancing the self-renewal of adult HSCs [31, 32], and its key role in embryonic hematopoiesis as well [33]. However, despite the prominent role of HOXB4 gene in hematopoiesis, generation of different types of blood cells is a very complex process involving many other HOX or non-HOX genes [34–36]. Brun et al. showed that a mouse model lacking the entire HOXB4 gene exhibits a significantly only a mild reduction in the numbers of primitive progenitors and stem cells in adult BM and fetal liver [37]. Furthermore, they showed that HOXB4 is not required for the generation of HSCs or maintenance of steady state hematopoiesis. More recently, Bijl et al. demonstrated HOXB4 (−/−) and HOXB1-B9 (−/−) fetal liver cells have full competitive repopulation potential and can regenerate all myeloid and lymphoid lineages [38].

Although we originally assumed over-expression HOXB4 in human ESCs would increase the efficiency of human ESC differentiation to CD34+ hematopoietic cells, we did not see increased numbers of CD34+ cells using the methodology of co-culturing with OP9 stromal

cells. The contrast between our studies and others which have shown improved generation of hematopoietic cells from HOXB4 over-expressing ESCs could have several explanations. Kyba et al. and Pilat et al. used murine, and not human, ESCs [14,15] to show generation of long term engrafting HSCs from HOXB4-transduced ESC cells. Bowles et al. showed that over-expression of HOXB4 in human ESCs using lipofection method augments their *in vitro* differentiation of them into hematopoietic cells via EB method [16]. Chan et al. also used EB methodology to generate engraftable primitive hematopoietic cells from human ESCs [17]. To our knowledge there is no report on the generation of HSCs from HOXB4+ human ESCs through OP9 co-culture methodology without going through EB formation step. Thus, our results could be explained by unknown mechanisms in which EB formation is necessary to direct the generation of hematopoietic cells from HOXB-expressing human ESCs. However, we can not exclude the possibility that other factors such as differences in the level of expression of HOXB4, as suggested by Unger et al. [39], or other variables specific to our experimental methodology could have contributed to this observation.

Interestingly, we observed some of our human ESC colonies over-expressing HOXB4 during our selection process and before their transfer onto OP9 cells started to differentiate into cells with MSC morphology; and upon further passaging and using MSC specific culture conditions all differentiated cells expressed cell surface markers typical of a MSC phenotype [27]. However, in contrast to bone marrow derived MSCs our HOXB4+ human ESC-derived MSCs did not express CD106. Nevertheless, MSCs derived from human adipose tissue [40], umbilical vein [41], and amnion [42] have also been reported to lack expression of CD106. Since MSCs differentiated from our HOXB4+ human ESCs continues to express HOXB4, both at the RNA and protein levels, we believe generation of MSCs was not due to silencing of HOXB4 transgene. However, since the majority of HOXB+ human ESC colonies did not differentiate into MSCs it is quite possible that expression of HOXB4 had no direct role in generation of MSCs and was just an “innocent bystander”. Nevertheless, a recent study by Karner et al. showed that forced high level expression of HOXB4 by a lentiviral vector promoted osteogenesis and caused reduction of the number of CD34+ cells, while low level of HOXB4 expression caused a higher number of CD34+ cells generated from human ESCs [43]. Since osteogenic cells are progenies of MSCs, this latter study corroborates our assumption that different levels of expression of HOXB4 under different conditions could activate different cellular differentiation pathways.

Finally, generation of fibroblast/mesenchymal looking cells at the periphery of human ESC colonies has been reported as a frequent phenomenon in ESC cultures. Original investigators showed that such cells were able to support growth of human ESCs and possessed some characteristics of mesenchymal cells [44–46]. Recently, many investigators, including our group, have been able to generate pure populations of fibroblast/mesenchymal looking cells from human ESCs with or without co-culturing with murine stromal cells; and show these cells have characteristics very similar to those reported for MSCs derived from bone marrow and other sources [24,25,28,29]. Thus, another working hypothesis of ours is that generation of mesenchymal cells could be the default pathway of differentiation of human ESCs. Such a differentiation process is reminiscent of the epithelial to mesenchymal transition, a ubiquitous process that plays a significant role, not only in the early stages of healthy embryonic development, but also in many disease processes including tissue reconstruction and carcinogenesis [47,48]. We propose it is possible that the inherent propensity of ESCs to differentiate into MSCs might have played a role in preserving this differentiation potential even in the presence of over-expression of HOXB4.

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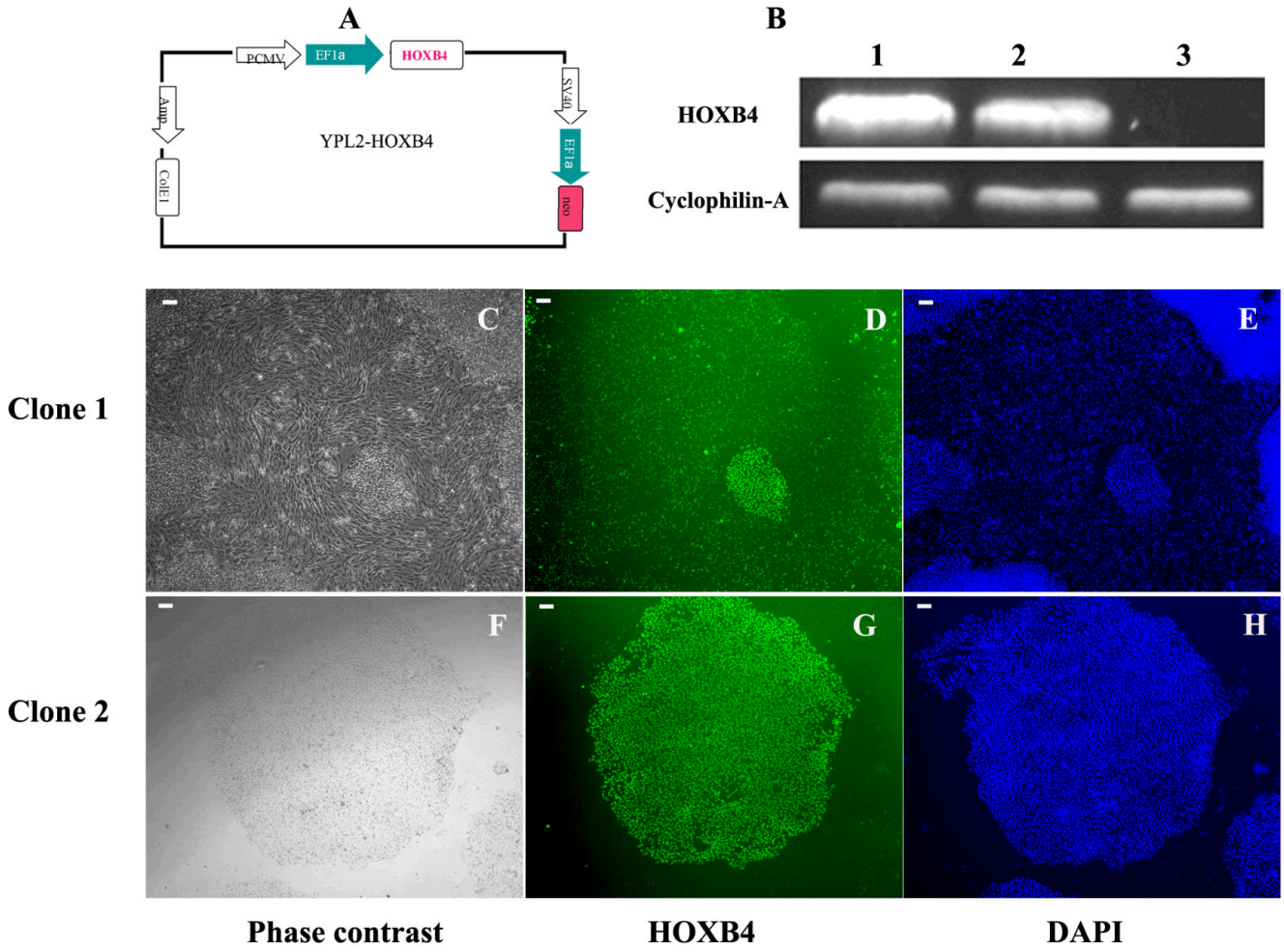

Figure 1.

A: HOXB4-expressing plasmid, B: RT-PCR using HOXB4 specific primers on RNA isolated from the 2 different colonies of HOXB4 transfected H9 ESC (column 1 from clone#1 and column 2 from clone#2), and from non-transfected H9 human ESCs (column 3). Cyclophilin-A was used as a house keeping control gene. Two colonies generated from HOXB4 plasmid transfection in H9 ESCs after 2 weeks of G-418 selection. Clone#1 showed mesenchymal cell morphology (C) but clone# 2 remained undifferentiated (F). Both clones showed HOXB4 expression in the cells (D, G). E and H are nuclei staining with DAPI. Scale bar = 100 μm.

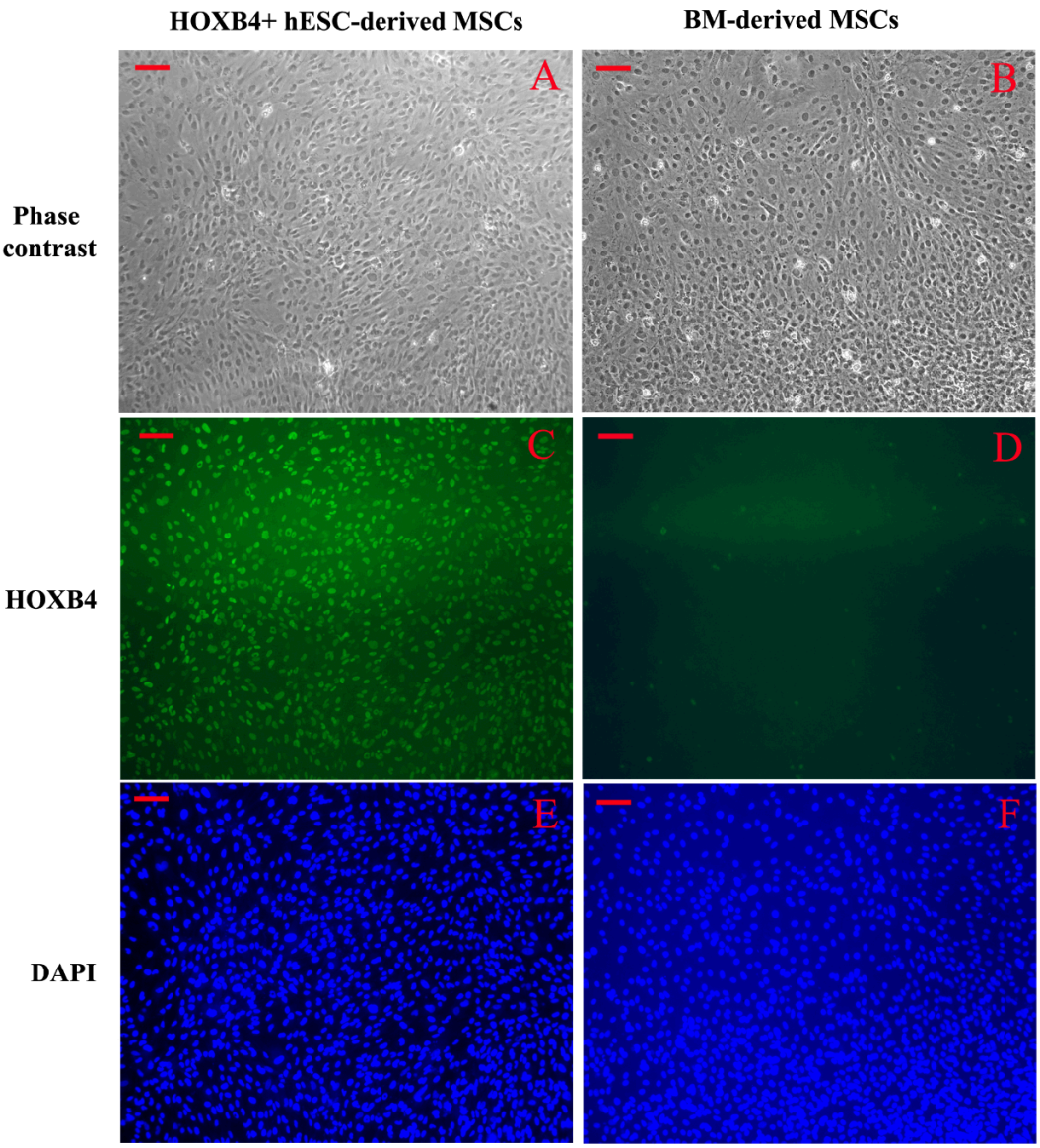

Figure 2.

Comparison of HOXB4 immunostaining in mesenchymal stromal cells (MSCs) differentiated from HOXB4+ human embryonic stem cells (ESCs) (A, C, E) and bone marrow (BM)-derived MSCs (B, D, F). BM-derived MSCs do not express HOXB4. A: Phase contrast view of human ESC-derived MSCs; B: Phase contrast view of BM-derived MSCs; C: HOXB4 antibody staining of MSCs derived from HOXB4+ human ESCs, and D: HOXB4 antibody staining of BM-derived MSCs; E and F: DAPI staining of cell nuclei. Scale bar = 100 μm.

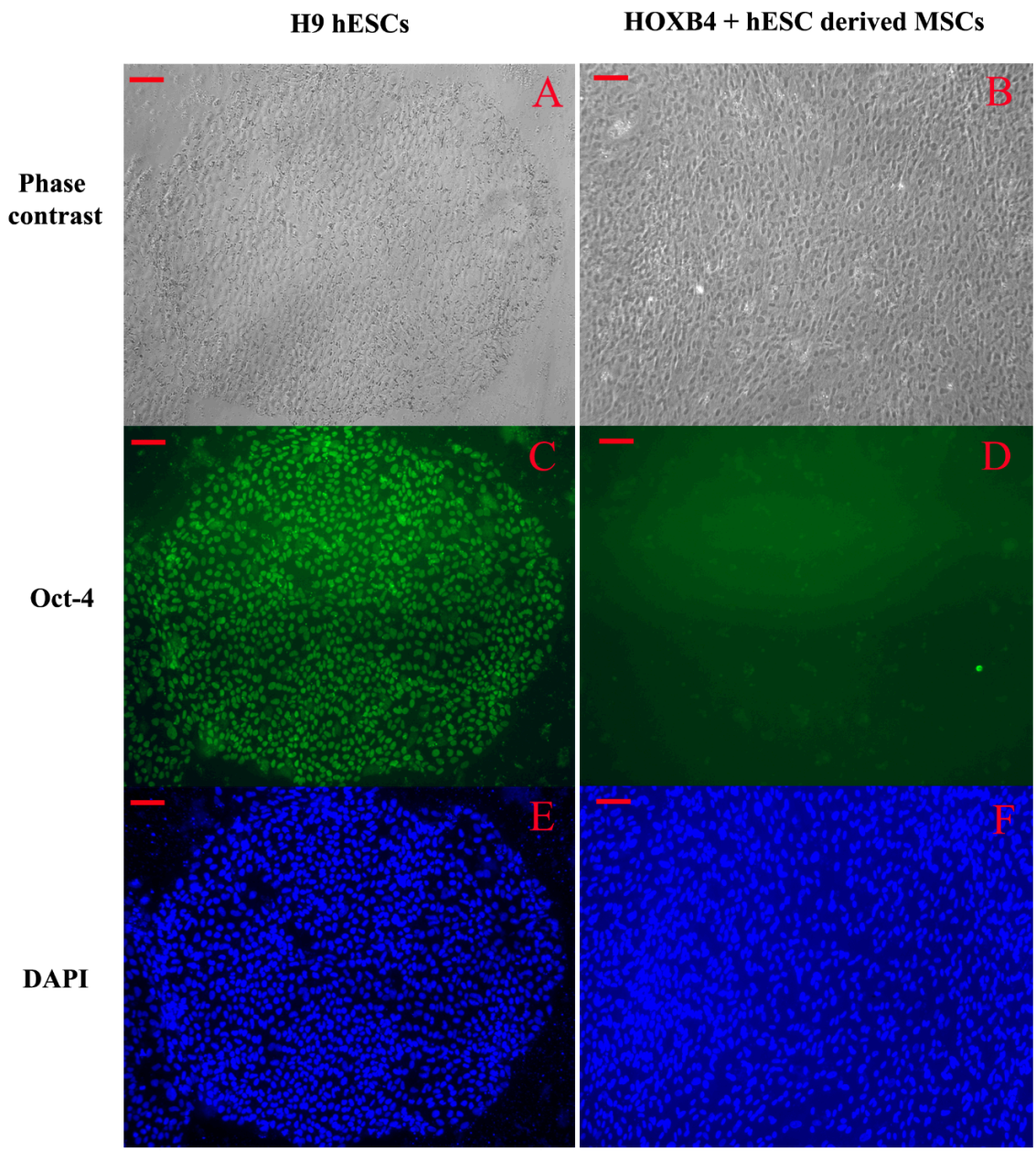

Figure 3.

A: Phase contrast view of a colony of H9 human embryonic stem cells (ESCs) and B: a colony of mesenchymal stromal cells differentiating from HOXB4+ human ESCs; C and D: OCT4-specific antibody staining of H9 human ESCs and MSCs from HOXB4+ human ESCs, respectively; E and F: DAPI staining of nuclei for the same field. Scale bar = 100 μm.

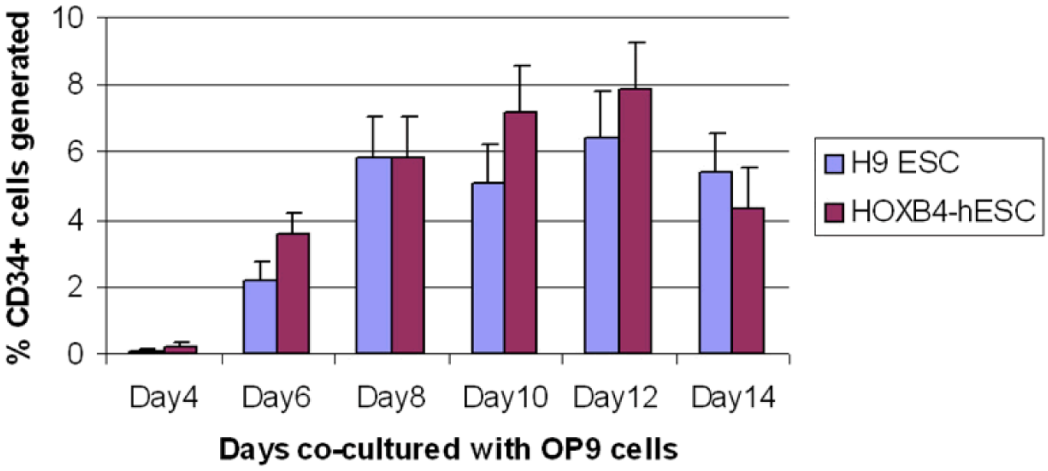

Figure 4.
Flow cytometric analysis of HOXB4+ human ESCs co-cultured with OP9 cells. Over-expression of HOXB4 in human ESCs did not cause any statistically significant differences in CD34+ cell generation.

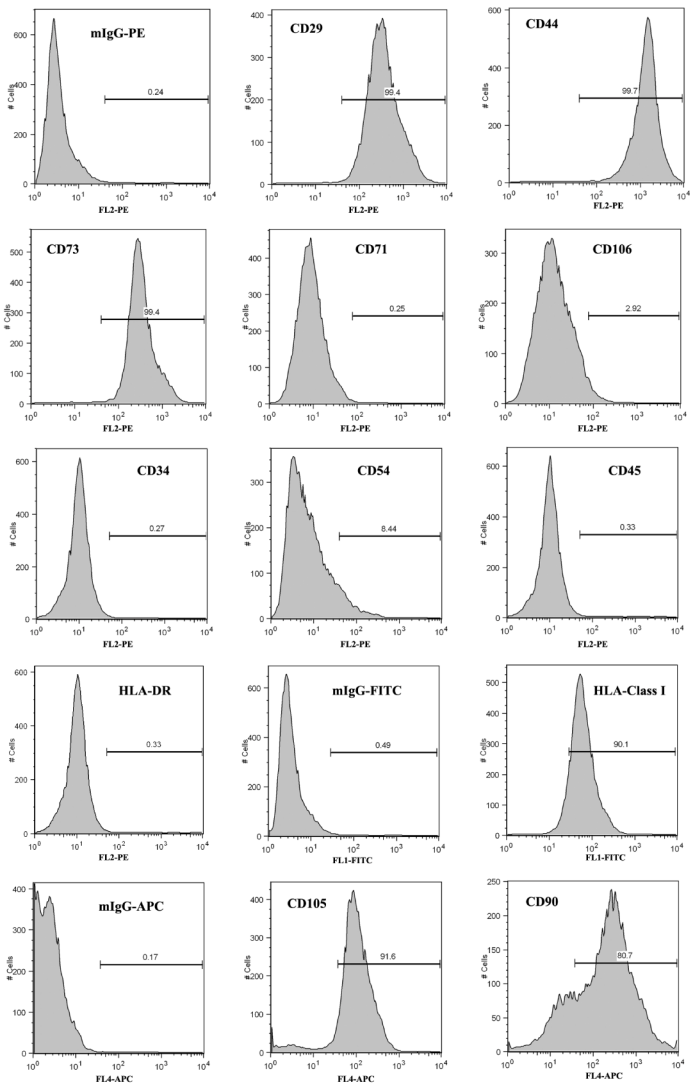

Figure 5.

Flow cytometric analysis of MSCs differentiated from HOXB4+ human ESCs. MSCs differentiated from HOXB4+ human ESCs express CD29, CD44, CD73 CD90 and CD105, and are positive for HLA-Class I (ABC); they do not express CD34, CD45, CD71, and HLA-DR.

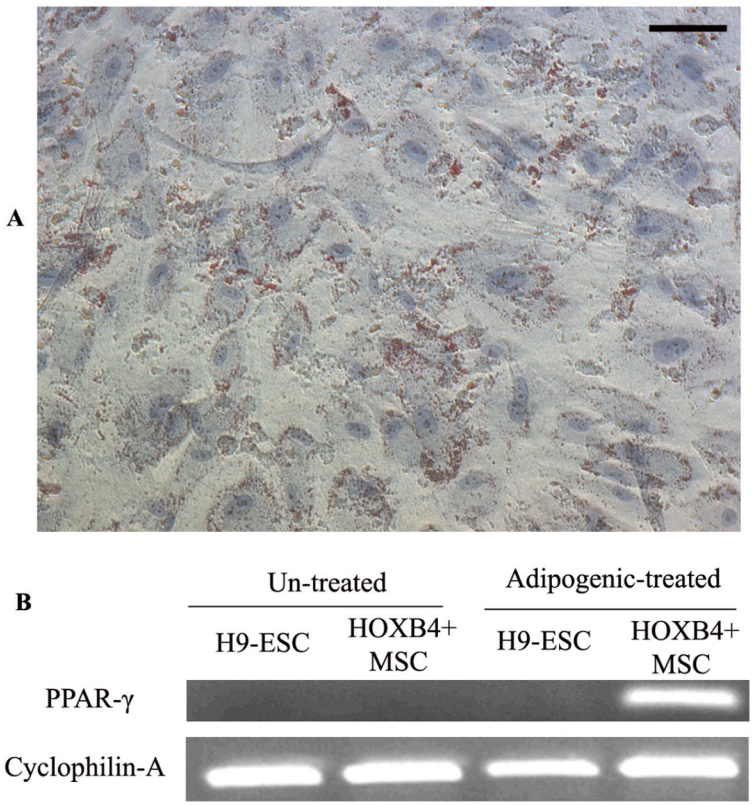

Figure 6.
Adipogenic differentiation of MSCs differentiated from HOXB4+ human ESCs when cultured in the adipogenic media. The adipocytes were characterized by accumulation of lipid (red) vacuoles inside the cells (Oil Red O staining) (A), and further verified by RT-PCR for Peroxisome Proliferator Activated Receptor-γ (PPAR-γ) gene (B).

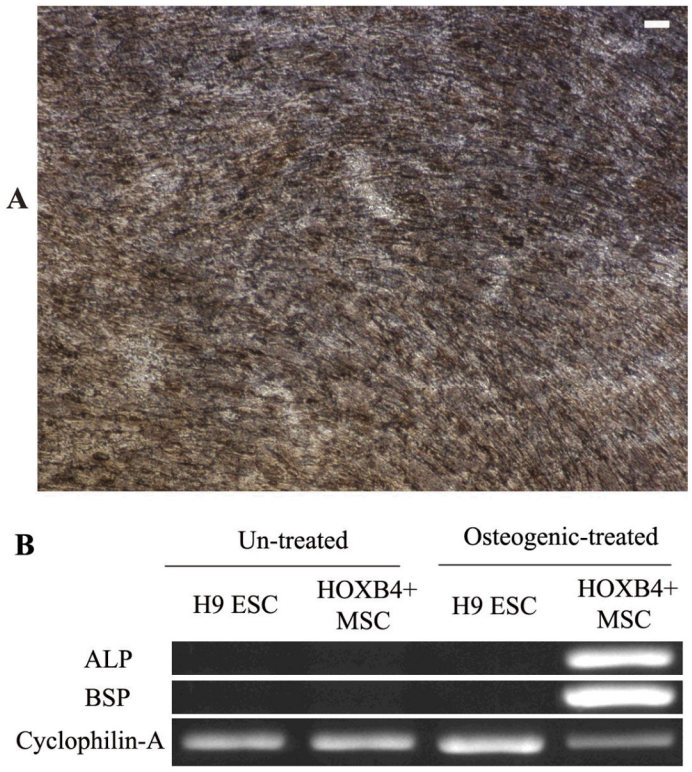

Figure 7.
Osteogenic differentiation of mesenchymal stromal cells derived from HOXB4+ human embryonic stem cells (ESCs) in the osteogenic media. Osteoblast generation is indicated by presence of Ca+ crystals shown as dark brown spots (von Kossa staining) (A), and verified by RT-PCR for expression of bone-specific alkaline Phosphatase (ALP) and bone sialoprotein (BSP).