Phenotypic Characterization, Osteoblastic Differentiation, and Bone Regeneration Capacity of Human Embryonic Stem Cell–Derived Mesenchymal Stem Cells

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To enhance the understanding of differentiation patterns and bone formation capacity of hESCs, we determined (1) the temporal pattern of osteoblastic differentiation of human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs), (2) the influence of a three-dimensional matrix on the osteogenic differentiation of hESC-MSCs in long-term culture, and (3) the bone-forming capacity of osteoblast-like cells derived from hESC-MSCs in calvarial defects. Incubation of hESC-MSCs in osteogenic medium induced osteoblastic differentiation of hESC-MSCs into mature osteoblasts in a similar chronological pattern to human bone marrow stromal cells and primary osteoblasts. Osteogenic differentiation was enhanced by culturing the cells on three-dimensional collagen scaffolds. Fluorescent-activated cell sorting of alkaline phosphatase expressing cells was used to obtain an enriched osteogenic cell population for in vivo transplantation. The identification of green fluorescence protein and expression of human-specific nuclear antigen in osteocytes in newly formed bone verified the role of transplanted human cells in the bone regeneration process. The current cell culture model and osteogenic cell enrichment method could provide large numbers of osteoprogenitor cells for analysis of differentiation patterns and cell transplantation to regenerate skeletal defects.

Introduction

THE TRANSPLANTATION OF STEM or progenitor cells may soon be an optional treatment for the repair of skeletal defects, particularly in wound beds with low numbers of osteoprogenitor cells or poor vascularization such as in irradiated or scared tissue sites. Such transplantation procedures would require adequate numbers of cells with a well-defined differentiation pattern and ease of procurement for bone regeneration [1,2]. Some of these challenges of cell transplantation may be met by the use of human embryonic stem cells (hESCs) that have the potential to differentiate into multiple cell types with relative ease of accessibility. By controlling cell culture conditions, differentiation of hESCs may be directed and restricted to desired cell lineages in potentially unlimited numbers [3,4].

Any potential use of hESCs for skeletal regeneration would require a reproducible method to ensure osteoblast differentiation and function. To reach this goal, a number of cell culture conditions have been used to induce differentiation of hESCs through the osteoblastic lineage with and without embryonic body formation [5–12]. Osteoblastic differentiation of hESCs has been achieved by introducing hESCs or hESC-derived mesenchymal stem cells (hESC-MSCs) in osteogenic medium supplemented with dexamethasone and ascorbic acid [5–12] or co-cultured with human primary bone-derived cells without the use of exogenous factors [13].

Differentiating hESCs into MSCs before undergoing lineage-specific differentiation provides the advantage of producing a large source of multipotent progenitor cells that can be expanded and differentiated into specified lineages such as bone, cartilage, or fat [4,14]. To date, the differentiation conditions for deriving MSCs from hESCs have required long culture periods [5], were dependent on a feeder layer, and demonstrated low yields of MSCs [10,15]. Generating MSCs in serum-free conditions supplemented with PDGF AB and FGF2 has also been reported [16]. To satisfy the likely demand for high numbers of progenitor cells to regenerate skeletal defects via a tissue engineering approach, cell culture conditions must be improved to assure appropriate and consistent differentiation of hESCs into MSCs on a large scale.

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The present study describes a cell culture method and osteogenic cell enrichment strategy that could provide large numbers of osteoprogenitor cells for analysis and cell transplantation. The progressive patterns of osteogenic differentiation and effects of three-dimensional matrix on osteogenic differentiation of hESC-MSCs in long-term culture suggest that the hESC-derived MSCs may be a good source of cells for skeletal regeneration.

Materials and Methods

Human embryonic stem cell culture

Human embryonic stem cells (hESCs) (BG01; Bresagen, Inc., Atlanta, GA) were cultured on irradiated mouse embryonic fibroblast (MEF) feeder layers following the protocol of the University of Michigan Stem Cell Core. Human ESCs were maintained in serum-free growth medium comprised of 80% DMEM-F12 supplemented with 20% (v/v) knockout serum replacement (KOSR), 200 mM L-glutamine, 10 mM nonessential amino acids (all from Gibco/Invitrogen, Carlbad, CA), 14.3 M β-mercaptoethanol (Sigma, St. Louis, MO), and 4 ng/mL β-FGF (Invitrogen). Cell cultures were incubated at 37°C in 5% CO_2 at 95% humidity and manually passaged every 7 days. Culture medium was changed every day.

Induction of mesenchymal stem cell differentiation

Aggregates of undifferentiated hESCs were cultured in mesenchymal stem cell culture medium (MSC medium) consisting of 80% α-minimum essential medium (α-MEM), 10% heat-inactivated fetal bovine serum (FBS), 200 mM L-glutamine, and 10 mM nonessential amino acids (all from Gibco) to induce mesenchymal differentiation of the undifferentiated cells [5]. Culture medium was changed every 3 days. Undifferentiated hESCs were manually harvested as cell aggregates and were seeded on fibronectin (Gibco)-coated plates (Corning Life Sciences, Lowell, MA) (20 µg/mL, 2 mL/60 mm plate) in a ratio of one to one. Cell adhesion and proliferation on the matrix were monitored under an inverted light microscope. When cells reached confluence (culture-day 14), cells were subcultured at a ratio of 1:2 on polystyrene-surfaced culture flasks (BD Falcon, Bedford, MA) using trypsin and ethylenediaminetetraacetic acid (0.25% trypsin/EDTA) (Gibco). Cells were then regularly passaged at confluence (7–10 days) at a ratio of 1:3. Differentiated cells derived from these culture conditions at passages 6–7 were designated hESC-MSCs and used in the analysis for MSC phenotypic characterization and differentiation potential. A karyotype analysis was performed by cytogenetic analysis on 20 G-banded metaphase cells of hESC-MSCs at passage 8 (Cell Line Genetics, Madison, WI).

Human bone marrow stromal cell culture

Human bone marrow was collected from patients undergoing iliac bone graft procedures with University of Michigan IRB approval and informed patient consent, and bone marrow stromal cells (BMSCs) were isolated and cultured as previously described [17]. Human BMSCs at passage 4 served as controls and were subjected to cell surface analysis for expressions of MSC surface antigens and induced to differentiate in osteogenic medium.

Mesenchymal stem cell phenotypic characterization

Characterization and comparison of the MSC phenotypes of hESC-MSCs and human bone marrow stromal cells (hBMSCs) in MSC medium were performed by examining expression of MSC cell surface antigens and performing functional differentiation analyses. Expression of MSC surface markers was analyzed by flow cytometric methods. Immunohistochemical staining was performed to identify expression of cell surface markers in mesodermal, ectodermal, and endodermal lineages. For functional differentiation, hESC-MSCs at passages 6–7 were induced to differentiate into adipogenic, chondrogenic, and osteogenic lineages in cell-specific culture medium.

Flow cytometric analysis of cell surface antigens

Expression of the cell surface antigen profile of MSCs was characterized using fluorescent-activated cell sorting (FACS). Human ES-MSCs were harvested using 0.25% collagenase in DPBS and trypsin 0.25% EDTA. After neutralization, single cell suspensions were washed in cold BSA (0.5% w:v) (Sigma) in DPBS and incubated at a concentration of 1×10^6 cells/mL in 1 µg/mL unconjugated goat anti-human IgG (Caltag/Invitrogen) on ice for 15 min to block nonspecific binding. Samples (2.5×10^5 cells) were then incubated on ice with the optimal dilution of conjugated monoclonal antibody (mAb) in 1 µg/mL unconjugate goat anti-human IgG in the dark. All mAbs were of the immunoglobulin G1 (IgG1) isotype. The following conjugated antibodies were used in the analyses: allophycocyanin (APC)-conjugated antibodies against CD44 (BD Pharmingen™, San Jose, CA) and alkaline phosphatase (R&D system, Minneapolis, MN). Fluorescein isothiocyanate (FITC)-conjugated mAbs against CD29, CD90, and CD45 (all from BD). Phycoerythrin (PE)-conjugated mAbs against CD49a, CD49e, CD73, and CD166 (all from BD), CD105 (eBioscience, San Diego, CA), and STRO-1 (Santa Cruz Biotechnology, Santa Cruz, CA). After 30 min incubation, cells were washed twice with ice cold 0.5% BSA in DPBS. Nonspecific fluorescence was determined by incubating cells with conjugated mAbs raised against anti-human IgG1 (all from BD).

At least 10,000 events were acquired for each sample using a fluorescent-activated cell sorting instrument (FACSCalibur; Becton Dickinson, San Jose, CA) and cell flow cytometry data were analyzed using CELLQUEST software (Becton Dickinson). The fluorescence histogram for each mAb was displayed alongside the control antibody. Percentages of positive cells were subtracted from the isotype control antibody of each conjugate. Single cell suspensions from two hESC-MSCs differentiated cell strains and one hBMSCs donor were analyzed.

Induction of adipogenic differentiation

Culturing hESC-MSCs in adipogenic medium at 1×10^4 cells/cm² for 14 days induced adipogenic differentiation. Adipogenic medium was changed every 3 days and was comprised of 90% DMEM high glucose (Gibco), 10% heat-inactivated FBS (Gibco), 1 µM dexamethasone, 10 µg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.2 mM indomethacin (all from Sigma) [5]. Expression of adipogenic differentiation was determined by Oil Red O staining

on culture-day 14 using standard techniques as previously described [18].

Induction of chondrogenic differentiation

Chondrogenic differentiation was induced by culturing cell pellets in chondrogenic medium. For cell pellet cultures, 2×10^6 hESC-MSCs were suspended in 2 mL of chondrogenic medium in 15 mL centrifugation tubes and centrifuged at $600g$ for 5 min and cultured in chondrogenic medium for 21 days. Culture medium was changed every 3 days. Chondrogenic medium consisted of high glucose DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 mM sodium pyruvate, 40 µg/mL proline, 100 nM dexamethasone, 200 µM ascorbic acid (all from Sigma), and 10 ng/mL TGF-β3 (R&D systems). Determination of expression of chondrogenic differentiation was performed after 21 days of culture using safranin O staining and immunohistochemical identification of collagen type II following standard techniques [19].

Induction of osteogenic differentiation

Human ESC-MSCs were cultured in osteogenic medium at 5×10^3 cells/cm² to induce osteogenic differentiation. The differentiated cells were continually passaged every 4–5 days as cells approached about 80% confluence. Osteogenic medium was comprised of 90% α-MEM (Gibco), 10% heat-inactivated FBS (Gibco), 50 µg/mL ascorbic acid, 5 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma) [9]. Culture medium was changed every 3 days. Monitoring of osteogenic differentiation was performed when cells were first exposed to osteogenic medium (OS1) and when cells were passaged four times in osteogenic medium (OS4). The differentiated cells passaged in the osteogenic medium for the fourth time (hESC-MSCs-OS) were used in the analyses.

Osteogenic differentiation on two- and three-dimensional matrices

For two-dimensional cell cultures, hESC-MSCs that were serially passaged in osteogenic medium (hESC-MSCs-OS) were seeded in six-well plates (Corning Life Science) at 5×10^4 cells/well. Human ESCs-MSCs in standard culture medium with 10% FBS were monitored as negative controls. Osteogenic differentiation was characterized by quantifying alkaline phosphatase (ALP) activity, osteocalcin secretion into the culture medium, and levels of calcium content in the mineralized extracellular matrix (ECM). Detection of ALP activity was performed after 3 days and every 7 days thereafter for 28 days. Levels of osteocalcin in the culture medium and calcium content were monitored on culture-days 7, 14, and 28 ($n = 4$).

Three-dimensional cell culture

For three-dimensional cell cultures, 1×10^5 cells were seeded on 5×3 mm 3-D collagen composite scaffolds (BD Biosciences Discovery Labware, Bedford, MA). Scaffolds were neutralized in MSC culture medium over night at 37°C. Prior to cell seeding, excess fluid was removed from the scaffolds using filter paper. Hydrated scaffolds were placed individually in 60 mm bacterial cell culture plates (BD Falcon, Bedford, MA).

Differentiated hESC-MSCs (1×10^5 cells/30 µL) in osteogenic medium (OS4) were trypsinized and suspended in 20 mg/mL human fibrinogen (Sigma) in a 1.5 mL tube. Five microliters of human thrombin (100 unit/mL) (Sigma) was added to the fibrinogen-cell suspensions and then directly seeded on the collagen scaffold. Cell–fibrin gel and collagen scaffold constructs were maintained in culture medium (0.5 mL/60 mm plate) in a humidified incubator at 37°C with 5% CO_2 for 2 h and then 10 mL of osteogenic medium was added into each plate. The constructs were cultured in osteogenic medium for 28 days and culture medium was replaced every 3 days. Levels of ALP activity, osteocalcin secretion, and calcium content were monitored on culture-days 7, 14, and 28 ($n = 4$).

Characterization of osteoblastic phenotypes

A leukocyte alkaline phosphatase kit (Sigma-Aldrich Chemie, Steinheim, Germany) was used for ALP staining following the recommended protocol. Cells were counterstained with 0.05% neutral red (Sigma). For alizarin red staining, cells were fixed in Z-FIX aqueous buffered zinc formalin (Anatech, Battle Creek, MI) for 4 min and stained for 10 min with 1 mL of 40 mM Alizarin red (Sigma) following the standard protocol [18]. For von Kossa staining of mineralized matrix on the collagen scaffolds, after deparaffinization, slides were immersed in 2% silver nitrate (Sigma) with exposure to a 60-W lamp for 1 h, subsequently treated in 2% sodium thiosulfate (Sigma) for 5 min and counterstained with a 5% nuclear fast red solution following standard protocols [20].

Protein analysis, ALP activity, osteocalcin secretion, and calcium content were measured. For ALP activity and protein content analyses, cells were washed with DPBS and stored at −20°C prior to the analysis. For osteocalcin analysis, cells were washed with DPBS and incubated in serum-free culture medium (1 mL/one six-well plate) for 12 h, then the culture medium was collected and stored at −70°C for the analysis and cells were washed with DPBS and stored at −20°C for measurement of ALP activity and calcium and protein contents.

Cell lysis and cellular protein analysis

At each experimental end point, cells in six-well plates and the scaffolds were washed two times in DPBS (Gibco). Cells were lysed by incubation in 1% Triton X-100 (Sigma) in DPBS on ice for 1.5 h. Protein solutions were centrifuged at $2,000g$ for 15 min at 4°C. The supernatants were stored at −20°C for the analysis of ALP activity and protein content and cell pellets were stored for calcium content analysis.

The quantification of protein in cell lysates was performed according to the manufacturer's instruction (Bio-Rad Protein assay kit; BIO-RAD, Hercules, CA). The solutions were read at 650 nm (A650) in duplicate using a microplate reader (Biotek-µQuant; Bio-Tek Instruments Inc., Winooski, VT). Total cellular protein was determined on the same samples as the ALP activity and osteocalcin measurements [21].

Alkaline phosphatase assay

The cell protein solutions were assayed for ALP activity using Alkaline Phosphatase Yellow Liquid Substrate

for ELISA (Sigma) according to the manufacturer's instructions. One hundred microliters of protein solution was added to 1,000 μL of working solution and incubated for 1 h at 37°C and subsequently 250 μL of 3 M sodium hydroxide (Sigma) was added to each well to stop the reaction. Standards were prepared in concentrations ranging from 0 to 50 μM of *p*-nitrophenol (Sigma). The solutions were read at A405 nm in triplicate using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT). The concentration of *p*-nitrophenol in the assay was measured, correlated to standard concentrations, and normalized to the protein content in the cell lysate solutions (μM *p*-nitrophenol/mg protein). A total of five samples of each group were tested at each time point [21].

Osteocalcin assay

Quantification of osteocalcin in serum-free cell culture medium was performed according to the manufacturer's instruction using the Gla-type Osteocalcin EIA kit (TAKARA, Shiga, Japan). The solutions were read at A450 nm in duplicate using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT). A total cellular protein analysis was performed on the same samples as the ALP activity and calcium content measurements. The assay was performed on culture-days 7, 14, and 28. Levels of osteocalcin were reported as nanograms per milligram protein (ng/mg protein). A total of four samples of each group were tested at each time point [21].

Analysis of calcium content

The analysis of calcium deposition in the ECM of cells in six-well plates and on collagen scaffolds was modified from a previous report [22]. Briefly, the cell pellets and collagen scaffolds were washed two times in DPBS. Excess fluid was removed using absorbent filter paper and specimens were minced into small pieces with scissors and then incubated in 0.5 M HCl (Sigma) at 37°C for 12 h. The solutions were then centrifuged at 13,000*g* for 5 min and the supernatants were collected and maintained at −20°C for the analysis.

Calcium content in the cell lysate was quantified spectrophotometrically with cresolphthalein complexone, Quantichrom™ Calcium Assay Kit (DICA-500) (BioAssay Systems, Hayward, CA) according to the manufacturer's instruction. In a 96-well plate, 5 μL of sample and 200 μL of working solution were added to each well and incubated at RT for 5 min. The absorbance of the samples was read at A575 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT). The calcium concentration was calculated from a standard curve generated from a serial dilution of a calcium standard solution. Calcium content in the ECM was reported as milligrams of calcium per milligram protein (mg/mg protein). A total of four samples in each group were tested at each time point [21].

Immunohistochemical staining

For staining of cell surface antigens, cells were seeded at 2.5×10^4 cells/cm^2 in Lab Tek Chamber slides (Nunc, Rochester, NY) and cultured in medium supplemented with FBS (Gibco) or osteogenic medium according to cell types. After 2 days in culture, cells were washed with DPBS and fixed in methanol at −20°C for 5 min and washed twice with DPBS. For paraffin-embedded sections, histological sections were deparaffinized and permeabilized with Triton (0.3% w:v) (Sigma) in DPBS for 30 min. For the detection of collagen type II expression in cell pellet cultures, following deparaffinization the sections were digested with 1 mg/mL pepsin from porcine gastric mucosa (Sigma) in Tris-HCL, pH 2.0 for 15 min at 37°C. In each washing step, cells on chamber slides and histological sections were washed in 0.5% BSA in DPBS.

To detect cell surface antigen expression on cultured cells, primary monoclonal and polyclonal antibodies were used in the following concentrations: rabbit polyclonal to CD105 1:200, mouse monoclonal to CD166 1:200, rabbit polyclonal to brachyury/Bry 1:50, mouse monoclonal to Runx2 1:200, and mouse monoclonal to alpha smooth muscle actin 1:200 (all from Abcam, Cambridge, MA), mouse monoclonal to vimentin 1:200, mouse monoclonal to collagen type I 1:200, goat polyclonal to α-fetoprotein 1:200, mouse monoclonal to VCAM-1 1:200, mouse monoclonal to estrogen receptor alpha (ERα) 1:200, and mouse monoclonal to β-catenin 1:200 (all from Santa Cruz Biotechnology). All immunohistochemical staining of cultured cells was performed at the same setting. To determine the expression of collagen type II in the ECM of cell pellets, mouse monoclonal antibody to collagen II, prediluted (Abcam) was applied.

To identify deposition of bone matrix on collagen scaffolds, mouse monoclonal antibodies to ALP 1:500 and rabbit monoclonal to osteonectin (Anti-Sprac) 1:200 (all from Sigma Life Science, St. Louis, MO), mouse monoclonal to osteopontin 1:200 (Santa Cruz Biotechnology) and mouse monoclonal to osteocalcin 1:100 (Abcam) were applied.

To identify the origin of transplanted cells, paraffin-embedded sections were stained with chicken polyclonal antibodies to green fluorescent protein (GFP) 1:200 (Abcam) and mouse monoclonal to human-specific nuclear antigen (HuNu) 1:50 (Chemicon/Millipore, Billerica, CA).

Negative controls were performed by omitting the primary monoclonal antibody. Throughout the study, the R.T.U. Vectastain Universal Elite ABC Kit and either Vector AEC, DAB or Vector® NovaRed™ (red) substrate kits (Vector laboratories, Burlingame, CA) were used. Except for the detection of chicken polyclonal to GFP, biotinylated anti-chicken IgG (H+L) (Vector laboratories) was used instead of prediluted biotinylated horse antibody anti-rabbit and mouse IgG (H+L) containing in the ABC kit. Immunohistochemical staining was performed according to manufacturer's instruction (Vector laboratories). Gill's formula hematoxylin (Vector laboratories) was used for counterstaining. Cells on chamber and histological slides detected with AEC and DAB substrates were covered with Crystal Mount Aqueous Mounting Medium (Sigma) and for cell pellet sections detected with NovaRed™ substrate, VectaMount™ permanent mounting medium was applied. Sections were then analyzed under a light microscope (Nikon Eclipse E600; Nikon, Shinakawa-ku, Japan).

Retrovirus-mediated infection of cells

Differentiated hESC-MSCs in osteogenic medium (OS4) were plated at a density of 5×10^3 cells/cm^2. After 24 h, cells were washed twice with DPBS and incubated with recombinant lentivirus vector (HIV-GFP) [23] (Vector Core, The

University of Michigan, MI) in 12 mL of α-MEM without FBS containing 2×10^6 transfection units/mL (2.4×10^7 transfection units in 12 mL) and 6 μg/mL polybrene (Sigma) in 225 cm² cell culture flask (BD Falcon) for 5 h with gentle manual shaking every 30 min. Subsequently, an equal volume of osteogenic medium was added and new osteogenic medium was replaced on the next day. Expression of GFP was monitored under a fluorescent microscope (Nikon Eclipse TE300; Nikon, Shinakawa-ku, Japan). On culture-day 3, cells were harvested for flow cytometric cell sorting of ALP expression for cell transplantation.

Flow cytometric cell sorting of ALP expressing cells for cell transplantation

GFP-transduced hESC-MSCs-OS were harvested using 0.25% collagenase in DPBS and 0.25% trypsin/EDTA 48 h after GFP-retroviral infection. Expression of ALP was detected using an APC-conjugated murine mAb against ALP (R&D system), according to the procedure stated earlier. Cell populations were sorted based on the levels of ALP expression. Sorted cells were seeded at a density of 1×10^4 cells/cm² in osteogenic medium. Culture medium was changed every 2 days. When the cells reached confluence (about day 5), cells were harvested for transplantation and 1×10^6 cells were analyzed for expression of pluripotent and osteoblastic genes using reverse transcriptase-polymerase chain reaction (RT-PCR).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed to compare expression of regulators of pluripotency (Oct4 and Nanog) and osteoblast-associated genes in undifferentiated hESCs, differentiated hESC-MSCs in osteogenic medium, and enriched ALP expressing osteoprogenitor cells for cell transplantation. BMSCs in osteogenic medium were tested as a positive control for expression of osteoblast-associated genes. Total RNA extraction was performed using Trizol (Invitrogen) and 1 μg of RNA was used for cDNA synthesis (ThermoScript™ RT-PCR System, Invitrogen) according to the manufacturer's instructions. Oct4 and Nanog primers used were for pluripotency markers, and the primers for Runx2, ALP, type I collagen (ColI), and bone sialoprotein (BSP) were used for markers of osteoblastic differentiation. β-actin was included as a housekeeping gene. Forward and reverse primer sequences are listed in Table 1. The RT-PCR was performed using Platinum

Taq DNA polymerase (Invitrogen) following the manufacturer's recommended cycling conditions. PCR products were analyzed on ethidium bromide-stained 1% agarose gel electrophoresis. A 100 base pair DNA ladder (Invitrogen) was applied to determine size of DNA bands.

Preparation of cell construct for transplantation

The expanded FCM-sorted cells were harvested using 0.25% collagenase in DPBS and 0.25% trypsin–0.53 mM EDTA. Cell pellets were resuspended in 20 mg/mL human plasma fibrinogen at a concentration of 3×10^6 cells per 50 μL in 1.5 mL tube containing 1.0 mg of Bio-Oss particles (particle size 0.25–1.0 mm) (Geistlich Pharma AG, Wolhusen, Switzerland). Cell suspensions were thoroughly mixed and placed in 1.5 mL tubes containing the cells-Bio-Oss suspension prior to transplantation.

Surgical procedure and cell transplantation in craniofacial defect model

All procedures were approved by the University of Michigan Committee on the Use and Care of Animals. Nine 5-week-old female immunocompromised mice (N:NIH-bg-nu-xid; Harlan Sprague Dawley, Inc., NC) were anesthetized with intraperitoneal injections of ketamine, (Ketaset, 75 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Ansed, 10 mg/kg; Lloyd laboratories, Bedford, OH). A semilunar scalp incision was made from right to left of the post-auricular areas, and a full-thickness flap was elevated. The periosteum overlying the calvarial bone was completely resected. A trephine was used to create a 5-mm craniotomy defect centered on the sagittal suture and the calvarial disk was removed.

Five microliters of human thrombin, 200 unit/mL (Sigma) was added in cell-Bio-Oss particle suspension and immediately placed over the entire area of the cranial defect. The defects were covered with a 6 mm diameter collagen membrane (Bio-Gide, Geistlich Pharma AG, Wolhusen, Switzerland) to ensure full coverage of the defect site and the incisions were closed with 4–0 chromic gut suture (Ethicon/Johonson&Jhonson, NJ). All mice were sacrificed 6 weeks after the implantation.

Radiographic and histologic analyses

Radiographic analysis was performed immediately after the calvariae were harvested (Faxitron X-ray Corporation,

TABLE 1.   REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION PRIMER SEQUENCES

Gene of interest	Forward primer	Reverse primer	cDNA (nucleotides)
Oct4	agtgagaggcaacctggaga	gccggttacagaaccacact	125
Nanog	ttccttcctccatggatctg	tctgctggaggctgaggtat	213
Runx2	ttacttacacccccgccagt c	tatggagtgctgctggtctg	139
ALP	ggacatgcagtacgagctga	ccaccaaatgtgaagacgtg	133
Collagen type I	ccccagccacaaagatcta	ctcgacttggccttcctctt	125
Bone sialoprotein	tgaatacgagggggagtacg	gatgcaaagccagaatggat	226
β-actin	atctggcaccacaccttctacaatgagctgcg	cgtcatactcctgcttgctgatccacatctgc	854

Lincolnshire, IL). The calvariae were subsequently fixed in aqueous buffered zinc formalin, Z-FIX (Anatech) and then were subsequently decalcified with a 10% EDTA solution for 2–3 weeks, dehydrated with a gradient of alcohols, and embedded in paraffin. Coronal sections 5 μm in thickness were cut and stained with hematoxylin and eosin and/or reacted with mAbs against human-specific nuclear antigen and GFP.

Statistical analyses

Data were expressed as the mean value ± the standard error of the mean (mean ± SEM) and analyzed by one-way analysis of variance. Multiple comparisons with Dunnette T3 methods were used. The levels of statistical significance were set at $P < 0.05$.

Results

hESC-MSC cell culture

Aggregates of hESCs in MSC medium adhered and proliferated on fibronectin-coated plates. hESCs differentiated into cells with different morphologies in primary culture and reached confluence on day 7. Cells were allowed to mature to day 14 before they were passaged at a ratio of 1:3. The recovery rate and homogeneity of cell morphology after passaging increased with increasing passage number. In early passages, small groups of cells with a fibroblast-like morphology were observed and became more uniform in size and shape at passages 5–6 and higher (Fig. 1A–C). The cells could be passaged at least 12–15 times every 5–7 days. Because, as will be demonstrated with the results of our study, the cells exhibited a MSC-like phenotype, we termed the cells hESC-MSCs. The hESC-MSCs were karyotypically normal when tested at passage 8. All 20 G-banded metaphase cells demonstrated a normal male karyotype (Cell Line Genetics) (Fig. 1D).
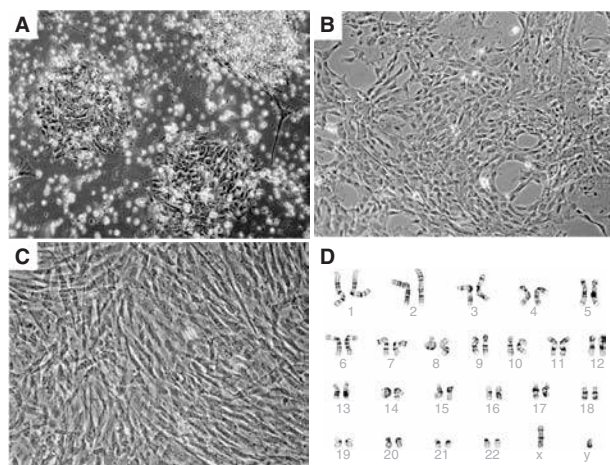

FIG. 1. Phase contrast images demonstrating morphological changes in aggregates of human embryonic stem cells (hESCs) cultured in medium supplemented with 10% FBS (20×): (**A**) 2 days after initial cell seeding; (**B**) after the first cell passage; (**C**) after the seventh cell passage; and (**D**) a normal karyotype for hESC mesenchymal stem cells eight passages after derivation.

Expression of MSC cell surface markers in differentiated hESC-MSCs

Flow cytometric analysis demonstrated that MSC cell surface markers were consistently and highly expressed in hESCs and hBMSCs in both MSC and osteogenic media (Fig. 2). The MSC surface markers CD29, CD44, CD49e, CD73, CD90, CD105, and CD166 were expressed to levels greater than 95% in both MSC and osteogenic media (Fig. 2A). Marked differences in levels of expression in osteogenic medium were noted for CD49a, ALP, and STRO-1. In osteogenic medium, levels of expression of CD49a and ALP were increased to levels higher than 90% and 40%, respectively, while levels of STRO-1 in hBMSCs decreased to less than 2%, but remained stable in hESC-MSCs (Fig. 2B). Expression of the hematopoietic markers, CD34 and CD45, were less than 0.5% in all cell culture methods (Fig. 2C).

Immunohistochemical determination of cell surface antigens

Expression of the MSC surface markers, CD105 and CD166, on hESC-MSCs and hBMSCs-MSCs was determined by immunohistochemical staining (Fig. 3A,B,M,N). Expression of the mesodermal marker, brachyury, and markers of cells in the mesenchymal lineage such as smooth muscle α-actin, collagen type I, and vimentin was also identified (Fig. 3C–F). Expression of VCAM-1 and the endodermal differentiation marker α-fetoprotein was not observed in any cultured cells (Fig. 3G,H). Intense staining of β-catenin and estrogen receptor was observed on both hESC-MSCs and hBMSCs-MSCs (Fig. 3I,J,O,P). High expression of Runx2, as demonstrated by nuclear staining, was observed in hESC-MSCs in osteogenic medium (OS4) (Fig. 3K). No nonspecific binding was found in the negative control (Fig. 3L).

hESC-MSCs differentiate into three different mesenchymal lineages

For adipogenic differentiation, Oil Red O staining demonstrated abundant deposition of lipids in the cytoplasm of differentiated hESC-MSCs (Fig. 4A). In chondrogenic medium, pellet cultures of hESC-MSCs demonstrated the morphology of chondroblast-like cells, consisting of cells with large nuclei in lacunae surrounded by ECM that stained positive for glycosaminoglycans with safranin O (Fig. 4B). Immunostaining of collagen type II confirmed the presence of a cartilaginous matrix in the cell pellet cultures (Fig. 4C). Human ESCs-MSCs exposed to osteogenic medium for the first time (OS1) on culture-day 14 were positive for ALP (Fig. 4D) and were able to mineralize the ECM (Fig. 4E), and the progression of osteogenic differentiation of hESC-MSCs in osteogenic medium was confirmed by increasing levels of ALP activity of hESC-MSCs in osteogenic medium (OS1) that reached the highest level on day 14 and was significantly higher than the levels of hESC-MSCs in MSC medium ($P < 0.01$) (Fig. 4G).

hESC-MSCs and enriched osteogenic cells cultured in osteogenic medium express osteoblastic genes but not pluripotent genetic markers

Undifferentiated hESCs differentiated into osteoblastic cells by losing their self-renewal capacity and expressing
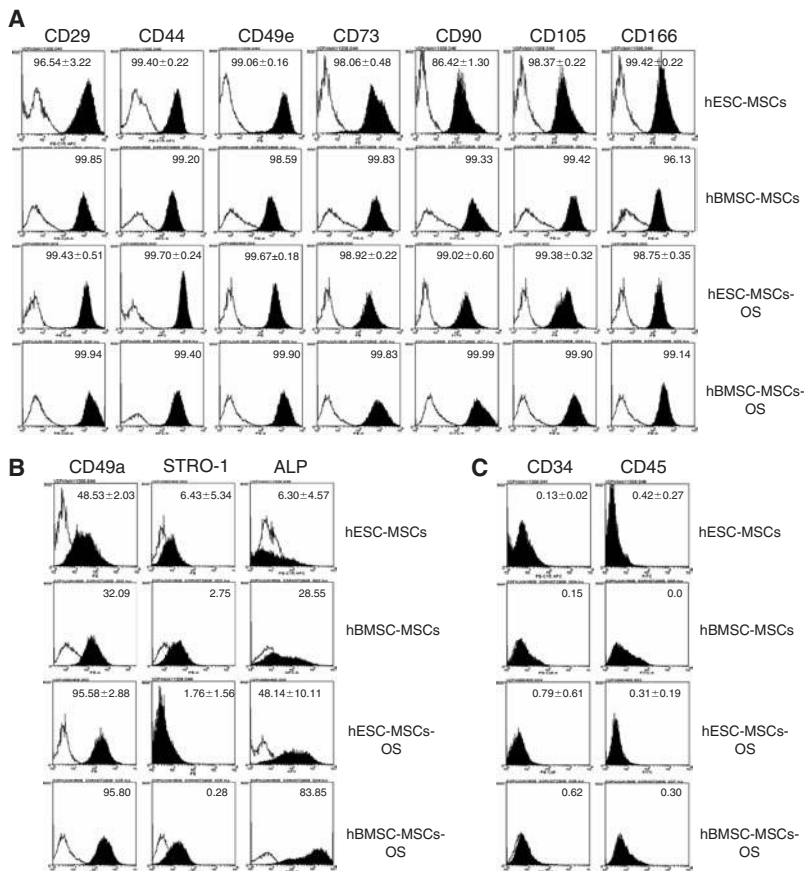
FIG. 2. Surface antigen profiling of human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) and human bone marrow stromal cells (hBMSCs) in MSC and osteogenic medium (OS). Cells were labeled and fluorescent-activated cell sorted (FACS) with: (**A**) antibodies against the MSC surface antigens that exhibited expression levels greater than 95%: CD29-FITC, CD44-APC, CD49e-PE, CD73-PE, CD90-PE-CY^{5TM}, CD105-PE, and CD166-PE; (**B**) antibodies against surface antigens that either increased or decreased markedly in osteogenic medium: CD49a-PE, STRO-1-PE, and ALP-APC; and (**C**) hematopoietic stem cell surface antigens: CD34 PE-CY^{5TM} and CD45-FITC. Data from hESC-MSCs and hESC-MSCs-OS were average values from two different cell strains at passage 6 and data from hBMSC and hBMSC-OS were derived from analysis of cells from one cell strain at passage 4. The white histogram represents isotype controls and the black histogram represents the conjugated antibody of each antigen.

osteoblast-associated genes, Runx2, ALP, collagen type I, and bone sialoprotein. Expressions of Oct4 and Nanog genes were found in undifferentiated hESCs at passage 58, but were not expressed in the cells differentiated in osteogenic medium (Fig. 5). hESC-MSCs exposed to osteogenic medium for the fourth time (OS4) cultivated on cell culture plates (hESC-MSCs-OS) and on collagen scaffolds (hESC-MSCs-OS-Scf) on culture-day 14 and the transplanted cells derived from FCM sorted-ALP expressing cells (ALP-FCM) expressed Runx2, ALP, and collagen type I similar to BMSCs in osteogenic medium (BMSCs-OS). Only hESC-MSCs-OS-Scf and BMSCs-OS expressed bone sialoprotein at culture-day 14 (Fig. 5).

Osteogenic differentiation of hESC-MSCs is promoted by a three-dimensional collagen matrix

During cell culture in osteogenic medium, all groups demonstrated similar patterns of ALP activity (Fig. 6). ALP activity gradually increased to reach the highest levels then decreased and remained stable during the later stages of culture. A spontaneous osteogenic differentiation of hESC-MSCs in MSC medium was suggested by the expression of ALP activity reaching the highest level on day 14 (24.95 ± 4.00, $P < 0.01$). ALP activity of hESC-MSCs in osteogenic medium on cell culture plate, hESC-MSCs-OS reached the highest levels on day 21 (219.01 ± 10.00, $P < 0.01$) and was stabilized by day 28. In a group of hESC-MSCs in osteogenic medium on collagen porous scaffolds, hESC-MSCs-OS-Scf, the activity was highest on day 14 (755.32 ± 83.65, $P < 0.01$) then decreased significantly at day 28 ($P < 0.05$). The activity

of hBMSCs in osteogenic medium on cell culture plate, hBMSCs-OS was relatively high on day 3, reaching the highest level on day 7 (680.54 ± 30.94, $P < 0.01$) and became stable afterward, expressing lower levels on days 14, 21, and 28 (Fig. 6).

The highest level of activity among groups was found on day 7 in a group of hBMSCs in osteogenic medium and on day 14 in hESC-MSCs in osteogenic medium on collagen porous scaffolds. At each experimental time point, levels of ALP activity from hESC-MSCs in osteogenic medium on cell culture plates were significantly higher than hESC-MSCs in MSC medium and lower than hESC-MSCs in osteogenic medium on collagen scaffolds and hBMSCs in osteogenic medium on cell culture plate ($P < 0.01$) (Fig. 6).

Osteocalcin secretion in culture medium

Levels of osteocalcin secretion from hESC-MSCs in osteogenic medium on collagen scaffolds was consistently high (41.96 ± 3.54) and significantly higher than hESC-MSCs in osteogenic medium on cell culture plates throughout the cell culture period ($P < 0.05$). Levels of osteocalcin secretion from hESC-MSCs in osteogenic medium on cell culture plates were significantly increased on day 28 (29.45 ± 1.19, $P < 0.01$) (Fig. 7).

Levels of calcium content in the ECM

Differentiation of hESC-MSCs in osteogenic medium into mature osteoblasts was further supported by mineralization of the ECM (Fig. 7). Levels of calcium deposition per
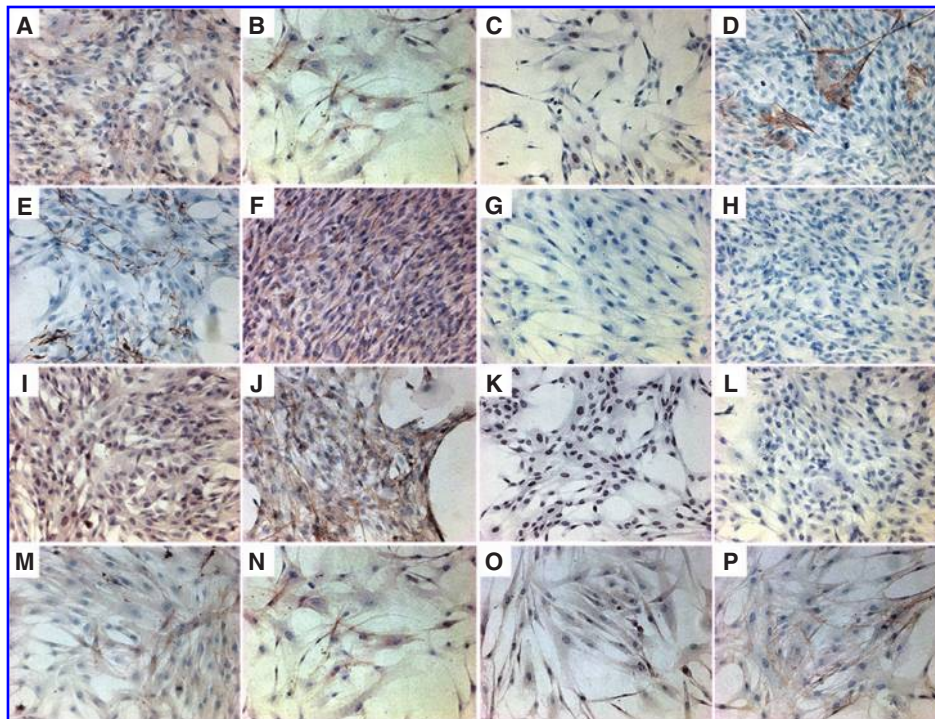
FIG. 3. Immunohistochemical staining of surface antigens on human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) cultured in MSC medium (**A–J**), hESC-MSCs in osteogenic medium (**K**), negative control (**L**), and human bone marrow stromal cells (hBMSCs) in MSC medium (**M–P**). Positive staining is indicated by red-brown cytoplasmic staining in all images except nuclear staining of brachyury (**C**) and Runx2 (**K**). (**A**) hESC-MSCs-CD105, (**B**) CD166, (**C**) brachyury, (**D**) smooth muscle α-actin, (**E**) collagen type I, (**F**) vimentin, (**G**) VCAM-1, (**H**) α-fetoprotein, (**I**) estrogen receptor α (ERα), and (**J**) β-catenin. (**K**) hESC-MSCs in osteogenic medium-Runx2. (**L**) Negative control omitting monoclonal antibody. (**M**) hBMSC-MSCs-CD105, (**N**) CD166, (**O**) ERα, and (**P**) β-catenin. (AEC substrate and hematoxylin counter stain, magnification 20×.)
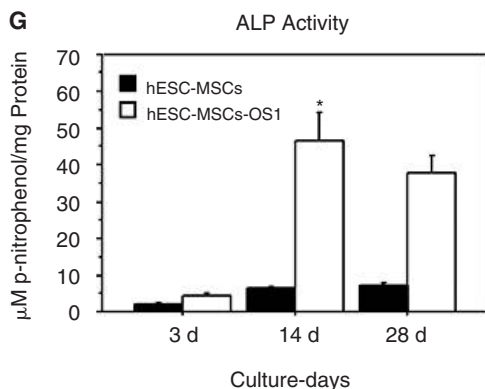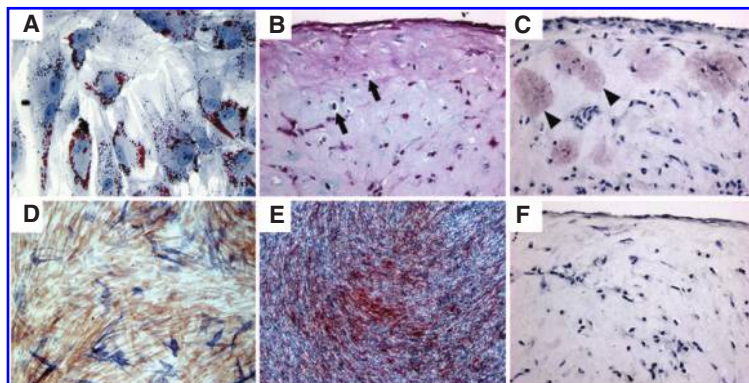

FIG. 4. Phase contrast images of functional differentiation of human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) in (**A**) adipogenic medium with Oil Red O staining on Day 14 demonstrating red lipid droplets in cytoplasm (20×); (**B**) chondrogenic medium with safranin O staining of the extracellular matrix (ECM) and cells with large nuclei in lacunae (arrow) on Day 21 (40×); (**C**) immunohistochemical staining showing patchy red staining of collagen type II (arrowheads); (**D**) Alkaline phosphatase (ALP) staining (blue staining) in hESC-MSCs in osteogenic medium (OS1) with neutral red counter stain on Day 14; (**E**) alizarin red staining of mineralized ECM on Day 21 (20×); (**F**) negative control with omission of a primary antibody; (**G**) Progression of ALP activity of hESC-MSCs in MSC medium, hESC-MSCs, and hESC-MSCs in osteogenic medium, hESC-MSCs-OS1, for a period of 28 days. The symbol (*) represents the highest level of ALP activity in comparison within and between groups ($P < 0.01$) ($n = 5$).
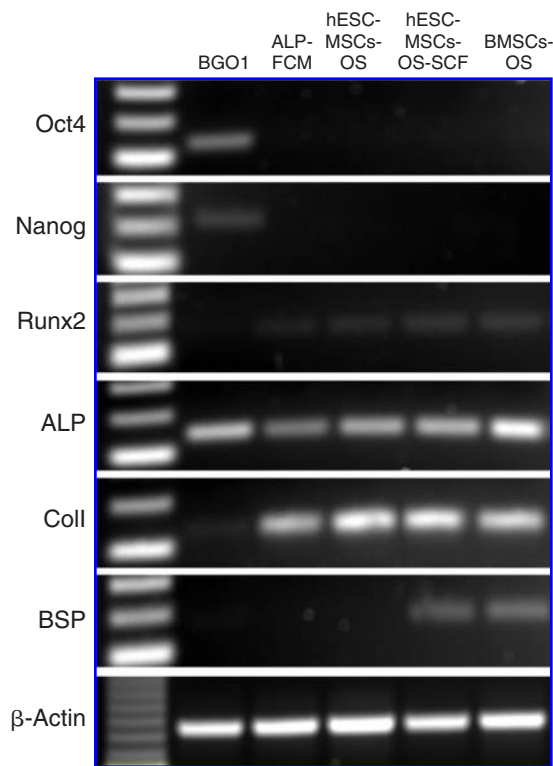
FIG. 5. Gene transcription analysis comparing expression of pluripotent regulator genes, Oct4 and Nanog and the osteoblast-associated genes, Runx2, alkaline phosphatase (ALP), collagen type I (ColI), and bone sialoprotein (BSP) in undifferentiated hESCs at passage 58 (lane 1, BGO1), FCM-sorted ALP expressing cells (lane 2, ALP-FCM), hESC-MSCs in osteogenic medium (lane 3, hESC-MSCs-OS), on three-dimensional collagen scaffolds (lane 4, hESC-MSCs-OS-Scf), and BMSCs in osteogenic medium (lane 5, BMSCs-OS) on culture-day 14. BMSCs-OS served as positive control and β-actin as a housekeeping gene. See Table 1 for primers and amplicon sizes.

milligram protein in a group of hESC-MSCs in osteogenic medium on cell culture plates were significantly increased from day 7 to day 14 and from day 14 to day 21 ($P < 0.01$) and stabilized on day 28 (131.98 ± 3.93) (Fig. 8).

Noncollagenous bone matrix formation

von Kossa and immunohistochemical staining of bone matrices of hESC-MSCs on porous collagen scaffolds in osteogenic medium demonstrated mineralization and deposition of noncollagenous matrix, ALP, osteonectin, osteopontin, and osteocalcin on the peripheral area of the scaffold, where cells grew densely and formed a multilayer cell lining. Accumulation of cells and matrixes on the outer area of the scaffolds indicated uneven distribution of cell growth within the structure of the scaffolds (Fig. 9).

hESC-MSCs are capable of regenerating bone in calvarial defects

The transplanted cells were derived from the FCM-sorted hESC-MSCs in osteogenic medium expressing ALP with and without GFP expression. The FCM analysis demonstrated high levels of expression of the MSC cell surface markers, CD49a (91.7%), CD90 (94.4%), and CD105 (96.2%), with low expression level of STRO-1 (<0.5%). Expression of ALP in this population was 48.0%, and 20% of ALP-positive cells expressed GFP (Fig. 10A, B). The sorted cells were subsequently expanded in osteogenic medium for 5 days after cell sorting before they were transplanted into the calvarial defects in nude/SCID mice.

Histologic analysis demonstrated the formation of multiple nodules of new bone in the area near to and on the margin of the defects. Bone marrow formation was found within the unorganized matrix of some bone nodules. The central area of defects was filled with loose fibrous tissue and vascular infiltration. Resorption of the Bio-Oss particles and remnants of the Bio-Gide membrane were not observed. No evidence of teratoma formation was found in any of the samples (Fig. 10C, E).
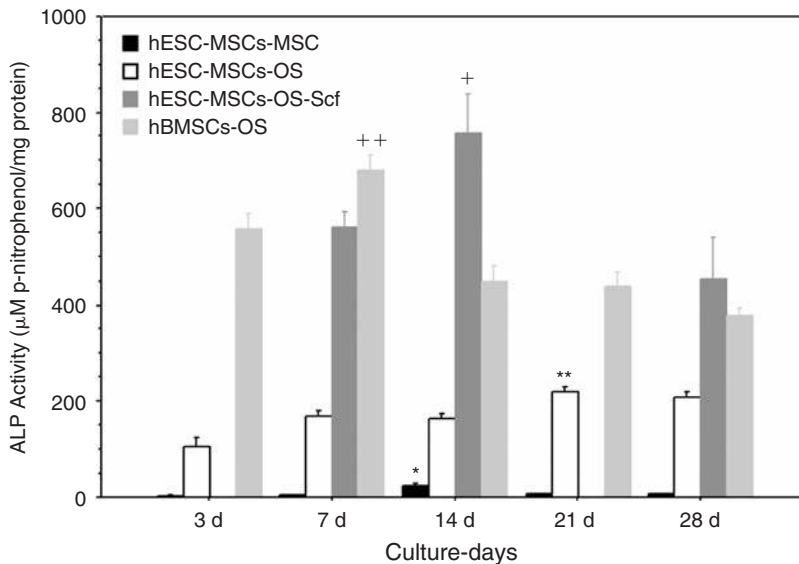

FIG. 6. Alkaline phosphatase (ALP) activity in cultured cells. Human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) in MSC medium (hESC-MSCs-MSC); hESC-MSCs in osteogenic medium (hESC-MSCs-OS); hESC-MSCs in osteogenic medium on collagen scaffolds (hESC-MSCs-OS-Scf); and human bone marrow stromal cells (hBMSCs) in osteogenic medium (hBMSCs-OS), for a period of 28 days. The symbol (*) represents significant changes in ALP activity of hESC-MSCs ($P < 0.01$), (**) hESC-MSCs-OS ($P < 0.01$), (+) hESC-MSCs-OS-Scf ($P < 0.05$), and (++) hBMSCs-OS ($P < 0.01$). $n = 5$, mean $\pm$ SEM.
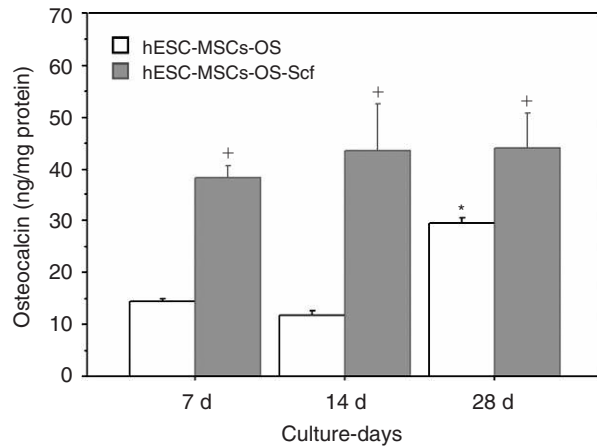
FIG. 7. Levels of osteocalcin secretion in cultured cells. Human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) in osteogenic medium (OS) on cell culture plates and on collagen scaffolds (Scf) for 28 days. The symbol (*) represents a significant increase in osteocalcin secretion from Days 7 and 14 to Day 28 ($P < 0.05$) and (+) significant higher levels of osteocalcin in hESC-MSCs-OS-Scf than hESC-MSCs-OS ($P < 0.05$) ($n = 4$, mean ± SEM).

FIG. 8. Calcium content in the extracellular matrix (ECM) of human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) in osteogenic medium on cell culture plates for a culture period for 28 days. The symbol (*) represents significant difference from Days 7 and 21 and 28 ($P < 0.01$) ($n = 4$, mean ± SEM).

Human cells were identified in the newly formed bone

To determine the role of transplanted human cells in the bone regeneration process, expression of human-specific nuclear antigen and GFP expressed by the transplanted cells was investigated. The localization of specific human nuclear antigen in osteocytes within the new bone demonstrated cells of human origin (Fig. 10D). Likewise, the red-brown staining of AEC substrate on GFP antigen–streptavidin complexes (Fig. 10F) on osteocytes in the newly formed bone provided evidence of human cells participating in the regeneration of bone. Host calvarial bone (Fig. 10G), mouse femur (Fig. 10H), and a negative control that consisted of the omission of primary antibodies (Fig. 10I, J) did not demonstrate positive staining.

Discussion

This study describes the derivation and characterization of MSCs from hESC aggregates by serial passaging the differentiated cells in culture medium supplemented with FBS to direct the differentiation along the mesenchymal lineage. We also further characterized and compared osteoblastic differentiation of hESC-MSCs to hBMSCs, studied the effects of a three-dimensional matrix on osteogenic differentiation, and investigated the bone regeneration capacity of the differentiated cells in a skeletal defect.

The derivation of MSCs from hESCs for tissue regeneration is a promising alternative to the use of adult stem cells. The potential for self-renewal and multilineage differentiation distinguish hESCs from adult stem cells. Human ESCs represent an unlimited source of genetically identical progenitor cells that creates the opportunity to manipulate the early stages of MSC differentiation [5,14,24]. In contrast, the numbers of adult stem cells in the bone marrow and adipose tissue are limited and decreased with age [25,26] and
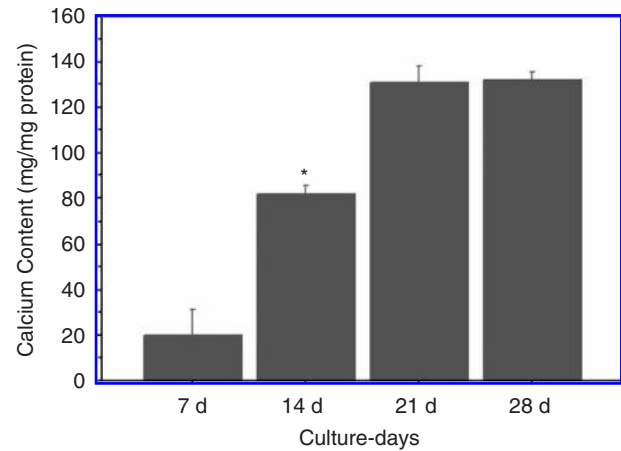
are present at different stages of differentiation at the time of harvest [27].

The current study demonstrated alternative cell culture conditions to a previous study [12] by reporting that the differentiation of hESCs aggregates into multipotential MSCs did not require formation of embryonic body intermediates. Here, we did not introduce the hESCs to osteogenic medium at the EB stage, rather, the implanted cells were enriched osteoprogenitor cells derived from serial expansion of aggregates of hESCs in culture medium supplemented with FBS [6–9,11]. We then further cultured the hESC-MSCs in osteogenic medium and transplanted a FAC-sorted population in vivo.

Serial passaging of hESC-MSCs and hESC-MSCs-OS in specialized culture medium every 4–5 days restricted differentiation of cells into specific lineages and generated large numbers of homogenous osteoprogenitor cells for cell stocks and transplantation [10,12], which are significant advantages over continuous cell culture. This is because serial passaging of cells allows the expansion of cells at an early stage of differentiation [10]. In addition, passaging the differentiated cells at a preconfluence stage helped to maintain cells in a proliferative stage for transplantation. When cells are allowed to differentiate for a period of time without subculture, the proliferating potential of cells is inhibited by intercellular contact and osteogenic differentiation of cells would advance into mature differentiation stage as demonstrated by expressions profile of ALP, osteocalcin, and calcium contents (Figs. 6–8). Furthermore, fully confluent cells are more difficult to dissociate for cell transplantation and mature cells have a diminished capacity to differentiate in vivo, leading to apoptosis of transplanted cells and a decrease of osteoprogenitor cells being able to sequentially differentiate into functional osteoblasts [28,29].

The current study supports previous studies that following an expansion period of five to six passages, hESC-derived MSCs exhibited the uniform fibroblast-like morphology, expressed high levels of MSC cell surface antigens, such as CD29, CD44, CD49a, CD49e, CD73, CD90, CD105, and
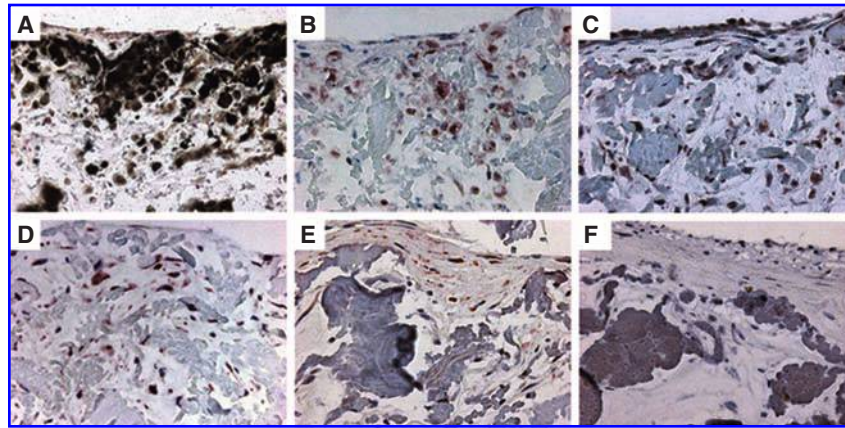
FIG. 9. von Kossa and immunohistochemical staining of paraffin-embedded sections of human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) in osteogenic medium on collagen scaffold for 28 days demonstrating mineralization of the extracellular matrix (**A**) and deposition of noncollagenous bone matrix on the scaffold (**B–E**) (20X). (**A**) von Kossa staining (black stain) and immunohistochemical staining of noncollagenous matrix proteins (**B**) ALP, (**C**) osteonectin, (**D**) osteopontin, and (**E**) osteocalcin secreted by cells on the scaffold. (**F**) Negative control with omission of primary antibodies. The antigen–antibody complexes were detected by AEC substrate yielding a red-brown stain (**B–E**).
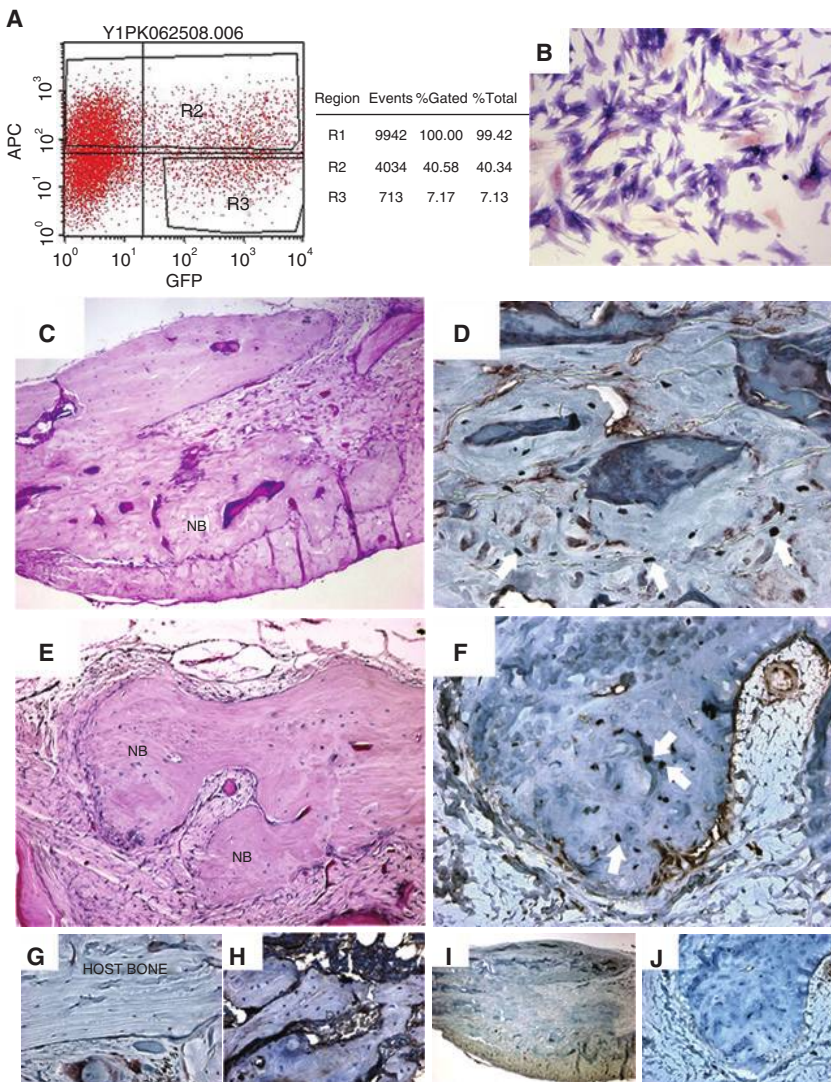

FIG. 10. Intramembranous bone formation in calvarial defects by the transplanted osteoblast-like cells derived from human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs) cultured in osteogenic medium. (**A**) FAC sorting of APC-conjugated alkaline phosphatase (ALP) and green fluorescent protein (GFP) of cells before transplantation (Gate R2). (**B**) ALP staining of the sorted ALP-positive cells (blue) counterstained with neutral red (red) (20×). (**C,E**) Histological images of newly formed bone (NB) near the margins of the defect (H&E, 40×). (**D**) Staining of human-specific nuclear antigen (HuNu) and (**F**) GFP staining of osteocytes in new bone matrix (arrow). (**G**) Negative staining of HuNu mAb on the mature host bone. (**H**) HuNu staining of mouse femur. (**I**) Negative control of HuNu and (**J**) GFP staining with omission of primary antibodies. Magnification of images **C–F** was 40× and **G–J** was 20×.

CD166, and did not express the hematopoietic lineage markers, CD34 and CD45 (Fig. 2) [10,12,30]. These findings are consistent with the observed phenotype of MSCs from bone marrow (BMSCs) [31,32] and hESCs-derived MSCs [12,16,30] with slight differences in the degree of expression (Fig. 2).

Expression of the STRO-1 antigen, which is expressed on multipotential BMSC progenitor cells, was clearly detected in hESC-MSCs at levels comparable to BMSCs [33] (Fig. 2B). Furthermore, intense staining suggesting high expression levels of estrogen receptor-α (ER-α) and β-catenin on hESC-MSCs and hBMSCs found in the current study (Fig. 3I,J and O,P) strengthen the similarity of phenotypes between hESC-MSCs and BMSCs and suggests potential roles of estrogen and Wnt signaling in controlling osteogenic differentiation of hESC-MSCs [34–36]. Additionally, the homogenous fibroblast-like cell morphology (Fig. 1C) [12,30], expression of proteins associating with mesenchyme, such as α-smooth muscle actin, collagen type I, and vimentin [37], and a lack of α-fetoprotein expression (Fig. 3C–H) indicate that in the described culture conditions, hESCs differentiated specifically into cells of the mesenchymal lineage [3,32].

Increasing levels of ALP and low level of STRO-1 expressions of cells in osteogenic medium (Fig. 2B) indicated an early stage of osteoblastic differentiation [33]. The continual high expression of all MSCs surface markers on the differentiated cells in osteogenic medium (Fig. 2A) is consistent with the observations of MSC cell surface antigens on osteoblasts in various differentiation stages suggesting their association with proliferation and differentiation of cells [38–40]. The expression of CD49a (α1 integrin) may promote osteogenic differentiation of hESC-MSCs in osteogenic medium, as it was markedly increased in the differentiated cells (Fig. 2B) and found strongly expressed on osteoblasts [41]. Only hBMSCs expressing CD49a have been shown to form multipotential colony forming unit-fibroblasts (CFU-Fs) in vitro [42,43]. Taken together, the findings support a model in which cell surface antigens of MSCs are not specifically expressed on MSCs [32] and advocate their roles on regulating growth and osteogenic differentiation [38–40,44,45].

Expression of brachyury (Fig. 3C) in hESC-MSCs cultured in MSC medium and Runx2 in hESC-MSCs cultured in osteogenic medium (Fig. 3K) suggest that hESCs differentiated to MSCs and subsequently differentiated into osteoblastic cells [6,33,46]. The sequential pattern of differentiation of hESC-MSCs into mature osteoblasts (Figs. 6–8) was similar to the temporal pattern observed in hBMSCs and primary osteoblasts [33,47,48]. Human ESCs-MSCs-OS demonstrated the progression of osteoblastic differentiation into three temporal phases: proliferation, matrix maturation, and mineralization phases [38,49], as indicated by the temporal pattern of expressions of ALP activity, levels of osteocalcin and calcium content in ECM (Figs. 6–8).

The differentiated hESC-MSCs exhibited the phenotypes of mature osteoblasts by producing osteocalcin and depositing calcium within the ECM [47,49] (Figs. 6–8). The cells also secreted noncollagenous bone proteins such as osteonectin, osteopontin, and osteocalcin when grown on the porous collagen scaffolds (Fig. 9). Furthermore, the findings that only hESC-MSCs-OS on collagen scaffold expressed BSP (Fig. 5) and levels of ALP activity and osteocalcin secretion on the three-dimensional matrix were significantly higher than cells on two-dimensional matrix ($P < 0.01$) (Figs. 6 and 7) were consistent with previous reports that three-dimensional collagen scaffolds and fibrin gel support cell growth and promote osteoblastic differentiation of human MSCs and osteoblast-like cells in osteogenic medium [50,51]. This is likely due to the promotion of cell–cell and cell–ECM interactions in the three-dimensional matrix that are crucial for efficient growth and differentiation [52–54].

The current study also demonstrates the bone formation capacity of the enriched osteoprogenitor cell population derived from hES cells in the calvarial defect model (Fig. 10) and emphasizes the importance of delivering lineage-specific cells into such a skeletal defect to ensure formation of a predetermined tissue type and avoid spontaneous differentiation into undesired lineages [14]. The osteoprogenitor cell population in the differentiated hESC-MSCs in osteogenic medium was enriched by selective cell sorting using expression of ALP as a cell surface marker (Fig. 10A, B). The advantage of ALP selective cell sorting is to enrich the numbers of ALP-positive colonies [55] and to obtain an enriched population of preosteoblasts in the late proliferation stage (STRO-1−/ALP+) [33]. However, it is possible that ALP sorted cells may be contaminated with pluripotent cells, as ALP is expressed in undifferentiated hESCs [6], and is also expressed in cultured preosteoblastic and osteogenic cells [33,55]. To determine the presence of undifferentiated hESCs or pluripotent cells in the osteogenically differentiated cells, RT-PCR was performed to compare expression of key regulators of pluripotency, Oct4 and Nanog genes [56] and the osteoblast-related genes, Runx2, collagen type I, and bone sialoprotein [33,57] in undifferentiated hESCs, differentiated hESC-MSCs in osteogenic medium, and the enriched population of ALP expressing cells for transplantation (Fig. 5).

The gene expression profiles of Oct4 and Nanog, Runx2, collagen type I, and bone sialoprotein demonstrate that the cells in osteogenic medium (hESC-MSCs-Os) differentiated into osteoblasts and lost their self-renewal capability, as there was no expression of Oct4 and Nanog in the differentiated cells and the ALP-enriched population, but expression of osteoblasts-related genes was enhanced [33,57] (Fig. 5). This conclusion is also supported by the lack of teratoma formation in the transplanted site up to 6 weeks in vivo (Fig. 10). Taken together, the data demonstrate that there was no contamination of undifferentiated hESCs in the population of osteogenically differentiated hESC-MSCs.

The fate of transplanted cells in the bone formation process was verified by identifying the presence of human cells in the matrix of the newly formed bone. Immunohistochemical staining of a specific nuclear antigen and identification of transfected GFP protein ensured identification of human cells functioning in the defect site (Fig. 10D,F) [58]. These data support previous reports that hESCs exposed to an osteogenic induction environment in vitro were able to form bone in ectopic sites [8,9,20,28] and emphasize that the differentiated cells were lineage-specific and able to form bone in the calvarial defect (Fig. 10).

In the current study, transplanted cells did not regenerate enough bone to bridge the critical size cranial defect. Based on studies of bone formation capacity of hBMSCs, this limitation may be related to poor survival of cells after transplantation that was compromised by local environmental factors such as hypoxia and delayed angiogenesis [59] and ability of the cell delivery vehicle to support osteogenic differentiation [60,61]. Uneven distribution of Bio-Oss particles in the defect site and their small particle size (0.25–1.0 mm) might also

diminish bone formation capacity of the transplanted cells [62]. It should be noted that this model was not intended to demonstrate regeneration of a critical size bony defect, but rather to demonstrate bone formation by hES-derived MSCs in a skeletal defect. Optimal conditions for the complete regeneration of such defects by cells derived from hES cells have yet to be established.

In conclusion, the results demonstrate that aggregates of hESCs in the current cell culture model could be a valuable source of MSCs and osteoblast-like cells in various differentiation stages for analysis and transplantation and suggest that osteogenic differentiation of hESC-MSCs can be modulated through the modification of porous three-dimensional matrices. Furthermore, FAC sorting of ALP expressing cells serve as an example of a method to isolate lineage-specific cells derived from hESCs to form bone in skeletal defects in vivo.

Acknowledgments

Author Disclosure Statement

Authors declare no conflict of interest in connection with the article.

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