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*Stem Cells* 2006;24;835-843; originally published online Oct 27, 2005;  
DOI: 10.1634/stemcells.2005-0383

**This information is current as of May 6, 2007**

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 **AlphaMed Press**

## Cultivation of Human Embryonic Stem Cells Without the Embryoid Body Step Enhances Osteogenesis In Vitro

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**Key Words.** Human embryonic stem cells • Bone tissue engineering • Embryoid bodies • Osteogenic cell frequency  
Matrix characterization

### ABSTRACT

Osteogenic cultures of embryonic stem cells (ESCs) are predominately derived from three-dimensional cell spheroids called embryoid bodies (EBs). An alternative method that has been attempted and merits further attention avoids EBs through the immediate separation of ESC colonies into single cells. However, this method has not been well characterized and the effect of omitting the EB step is unknown. Herein, we report that culturing human embryonic stem cells (hESCs) without the EB stage leads to a sevenfold greater number of osteogenic cells and to spontaneous bone nodule formation after 10–12 days. In contrast, when hESCs

were differentiated as EBs for 5 days followed by plating of single cells, bone nodules formed after 4 weeks only in the presence of dexamethasone. Furthermore, regardless of the inclusion of EBs, bone matrix formed, including cement line matrix and mineralized collagen, which displayed apatitic mineral (PO<sub>4</sub>) with calcium-to-phosphorous ratios similar to those of hydroxyapatite and human bone. Together these results demonstrate that culturing hESCs without an EB step can be used to derive large quantities of functional osteogenic cells for bone tissue engineering. *STEM CELLS* 2006;24:835–843

### INTRODUCTION

Current therapies for bone defects, including autograft and allograft transplantations, have inherent limitations, such as donor site morbidity and host immune rejection. Alternative therapies being considered involve the combination of liquid, gel, or solid carriers with a source of osteogenic cells. In one modality, progenitor cell numbers are expanded in vitro and cells are placed onto biodegradable scaffolds in combination with factors that stimulate osteogenic differentiation, followed by implantation into a bony defect site [1, 2]. Ideally bone will grow, the scaffold will degrade, and the patient will be left with functional bone tissue. In choosing an appropriate cell source for these types of bone engineering strategies, one must consider the capacity of the chosen cells to differentiate into cells that can produce bone.

Embryonic stem cells (ESCs) offer a potentially unlimited supply of cells that may be driven down specific lineages to give rise to all cell types in the body [3–5]. Recently, there has been great interest in examining the osteogenic potential of ESCs

derived from both mice [6–10] and humans [11–13]. Although cells derived from the mouse can be used as a tool to better understand the process of differentiation, there are considerable differences between murine and human embryonic stem cells (hESCs) [14]. Therefore, understanding cues that specifically induce osteogenic differentiation of hESC is important for tissue engineering and regenerative medicine.

Two methods have been examined in an attempt to stimulate the differentiation of hESCs into osteogenic cells. In one method, osteogenic cells are derived from three-dimensional cell spheroids called embryoid bodies (EBs) [11, 12]. EBs can be formed from either single-cell suspensions of ESCs or from aggregates of cells. EBs mimic the structure of the developing embryo and recapitulate many of the stages involved during its differentiation [15], and clonally derived EBs can be used to locate and isolate tissue-specific progenitors. EBs initiate many developmental processes and create suitable conditions for differentiation of cells into all three germ layers [16] and are generally formed through suspension [15] or hanging drop [17]

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methods. An alternative system that has been tested, but is not well characterized, avoids EBs through the immediate separation of ESC colonies into single cells, which are then plated directly into a cell adhesive culture dish [9, 11]. Given that each ESC has the capacity to differentiate into multiple cell types and that the formation of EBs leads to the formation of numerous cell types, we hypothesized that by omitting the EB step one could presumably exhibit a more uniform cell microenvironment and thus direct the differentiation of the ESCs more homogeneously.

To date, the generation of osteogenic cells from hESCs has required the presence of exogenous factors such as dexamethasone (DEX), L-ascorbic acid (AA) and sodium- $\beta$ -glycerophosphate ( $\beta$ gP) [11, 12]. DEX has been demonstrated to stimulate osteogenic differentiation for progenitor cells derived from multiple tissues [18, 19]. Under these media conditions, a fraction of the cells display osteogenic genes, including *Cbfa1*, an osteogenic transcription factor [20]. In addition, hESCs cultured in osteogenic media containing DEX, AA, and  $\beta$ gP express osteocalcin (OCN), as assessed by immunocytochemistry, and form mineralized nodules that stain positively for calcium and phosphorous as demonstrated using Alizarin Red and von Kossa stains, respectively [12]. However, when these classic stains were used to identify mineralized extracellular matrix (ECM) produced by hESCs derived both with or without an EB step, these stains did not localize to the same regions [11], which may be indicative of dystrophic mineralization. Furthermore, these stains were recently shown to provide erroneous results in the identification of a mineralized bone matrix without using other techniques, such as electron microscopy and Fourier transform infrared (FTIR) analysis, for verification [21]. Although there is evidence that osteogenic cells can be derived from hESCs, the matrix produced by these cells has not been thoroughly characterized. Thus, the positive identification of functional osteogenic cells derived from hESCs that are capable of producing *in vitro* bone has yet to be verified.

The goal of this study was to examine and compare the differentiation process within osteogenic cultures of hESCs with and without the formation of EBs. In addition, the ECM produced from these cultures was examined using various techniques and compared with human bone. Herein, we demonstrate that osteogenic cells derived from hESCs can produce many of the morphological hallmarks of *de novo* bone formation. In addition, we found major differences in the temporal development and sequence of *in vitro* bone formation that are dependent on the cell culture protocol.

## MATERIALS AND METHODS

All materials were used as received unless otherwise indicated. The following substrates were used: tissue culture polystyrene 75-cm [2] and 25-cm<sup>2</sup> flasks (BD Falcon; BD Biosciences, <http://www.bdbiosciences.com>) and BD Falcon 24-well plates. The  $\alpha$ -minimal essential medium ( $\alpha$ -MEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS; catalog no. 10437-028), 0.25% trypsin, nonenzymatic cell dissociation solution, and gentamicin were obtained from Invitrogen (Carlsbad, CA, <http://www.invitrogen.com>). The penicillin G, bovine serum albumin (BSA), amphotericin B (fungizone), AA, hexamethyldisilazane (HMDS),  $\beta$ gP, and DEX were obtained from Sigma-Aldrich (St. Louis, <http://www.sigmaaldrich.com>). An alkaline

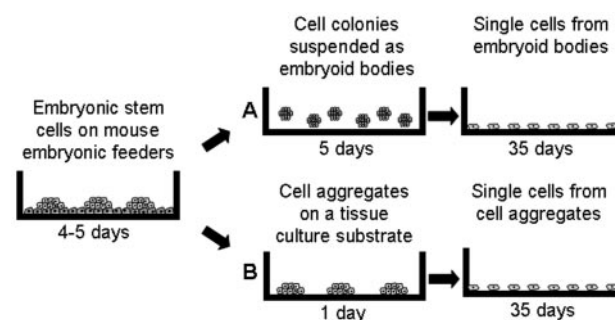
phosphatase (ALP) detection kit was obtained from JAS Diagnostics (Miami, FL, <http://www.jasdiagnostics.com>) and an OCN detection kit was obtained from Diagnostic Systems Laboratories Inc. (Webster, TX, <http://www.dslabs.com>). Mouse embryonic feeder cells were obtained from Cell Essentials (Boston, <http://www.cellessentials.com>).

## ES Cell Culture

hESCs (line H9, passages 25–45) were grown as cell aggregates on an inactivated mouse embryonic feeder layer, as previously described [22]. The hESCs were passaged every 4 days using 2 mg/ml type IV collagenase (Invitrogen). Undifferentiated hESC aggregates were removed from mouse feeders with 2 mg/ml collagenase for 2 hours. To obtain a single-cell suspension, cells were incubated at 37°C for 5 minutes in a solution with 1:2 (vol/vol) trypsin to cell dissociation solution with gentle pipetting. Cells were plated at a concentration of  $10^5$  cells per cm<sup>2</sup> in  $\alpha$ -MEM supplemented with 10% FBS and antibiotics consisting of 167 U/ml penicillin G, 50  $\mu$ g/ml gentamycin, and 0.3  $\mu$ g/ml amphotericin B. To examine the potential of the hESCs to produce mineralized ECM, two differentiation protocols were examined (Fig. 1). The first protocol (Fig. 1A) involved inducing the formation of EBs by transferring the hESC aggregates to non-tissue culture treated low attachment plates containing knockout Dulbecco's modified Eagle's medium supplemented with 20% knockout serum, 1 mM L-glutamine, 0.1 mM  $\beta$ -mercaptoethanol, and 1% nonessential amino acid stock (all from Invitrogen). EBs were cultured at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere, with a change of medium on the second day. After 4 days, EBs, which ranged in size from 300 to 2000  $\mu$ m were separated into single-cell suspensions and subsequently plated on tissue culture polystyrene. The second protocol (Fig. 1B) involved direct plating of the hESCs by skipping the EB step. Given that very few of the plated cells attached, undifferentiated hESC aggregates were initially plated on tissue culture dishes for 1 day prior to separating the cells into single-cell suspensions.

## Osteogenic Differentiation

To stimulate differentiation into osteogenic cells,  $\alpha$ -MEM containing and FBS was supplemented with  $10^{-8}$  M DEX, 50  $\mu$ g/ml AA, and 5 mM  $\beta$ gP [23, 24], together with antibiotics and



**Figure 1.** A comparison of culture protocols. Human embryonic stem cells were cultured on mouse embryonic fibroblast feeder layers for 4 days. The cell aggregates were removed from the mouse feeders with type IV collagenase and either suspended as embryoid bodies for 5 days and then plated as a single-cell suspension (A) or directly placed onto tissue culture Petri dishes for 1 day followed by plating as a single-cell suspension (B). Cultures from both protocols were grown for up to 35 days.

fungizone; 5 mM  $\beta$ gP has been used previously. Through interacting with specific glucocorticoid receptors, DEX has been demonstrated to stimulate osteogenic differentiation for progenitor cells derived from multiple tissues [18, 19]. AA is required for collagen assembly [25] and  $\beta$ gP is required for mineralization of the collagen [25]. The medium was changed every 2 to 3 days, and mineralized areas were observed by light microscopy and by electron microscopy as previously described [24]. Cultures were treated either with or without osteogenic supplements to assess directed or spontaneous differentiation into osteogenic cells, respectively. In some circumstances, individual components of the osteogenic media were employed to examine which components were responsible for the observed response. Unless otherwise stated, this medium is referred to as containing osteogenic supplements. All experimental groups were included within each experiment.

### Electron Microscopy

Prior to fixation, culture substrates were washed three times in PBS. Fixation was carried out for a minimum of 2 hours in Karnovsky's fixative at 4°C. After rinsing with cacodylate buffer three times, the dishes were dehydrated in graded alcohol (50, 70, 80, 90, 95, and 100%) and then air-dried in HMDS as previously reported [26]. Overlying cell layers and the collagenous matrix were partially removed with compressed air to facilitate examination of the elaborated ECM. The samples were then sputter-coated with carbon ( $\approx$ 250 Å) and examined on a JEOL JSM-5910 scanning electron microscope (JEOL-USA, Inc., Peabody, MA, <http://www.jeolusa.com>) equipped with a Rontec energy dispersive x-ray (EDX) detector for elemental analysis and mapping. Calcium-to-phosphate ratios (Ca/P) were obtained by integrating the area under the calcium and phosphate peaks.

### Histochemical Analysis and Quantification of Bone Nodules

**ALP, Von Kossa, and Tetracycline Staining.** Cell culture plates were fixed in 10% formalin-buffered saline for 20–30 minutes, washed once with ddH<sub>2</sub>O, and then left in ddH<sub>2</sub>O for 15 minutes. Plates were then stained for ALP by incubating for 40 minutes in a solution containing red violet 5-chloro-4-benzamido-2-methylbenzediazonium chloride hemi(zinc chloride) salt as previously reported [27]. Plates were then rinsed three to four times in ddH<sub>2</sub>O and stained with 2.5% silver nitrate for 30 minutes. After rinsing three to four times in ddH<sub>2</sub>O, plates were incubated in sodium carbonate formaldehyde for 1–2 minutes, rinsed, air-dried, and examined by light microscopy. For tetracycline staining, tetracycline was added to the culture medium 48 hours prior to terminating the cultures and visualized using a fluorescent light box equipped with a digital camera. Bone nodules were identified by the colocalization of ALP and von Kossa staining [27, 28]. Bone nodules within at least three entire wells of six-well plates were quantified under light microscopy for the experimental group. Cultures were also stained with safranin O/fast green for identification of glycosaminoglycans (cartilage). To compare the frequency of osteogenic cells derived from hESCs using the two culture methods, the numbers of mineralized regions that stained positively for both ALP and

von Kossa were manually quantified using light microscopic images. The values obtained were normalized to the number of adherent cells at the time of cell plating.

### FTIR

FTIR studies were conducted with a Nicolet Magna-IR 500 spectrophotometer (Thermo Electron Corporation, Waltham, MA, <http://www.thermo.com>). Dry samples were powdered, mixed with KBr, and pressed into pellets. The FTIR spectra were obtained by recording 128 scans between 4000 and 400 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup>. Plots were baseline corrected and analyzed over the range of 900 to 1725 as previously reported [9]. The mineral-to-matrix ratio was obtained by integrating the area under the curve between 900 and 1200 cm<sup>-1</sup> and dividing by the area under the curve between 1585 and 1725 cm<sup>-1</sup>. Spectra were also acquired from human humerus (Massachusetts General Hospital Bone Bank) and from hydroxyapatite (Clarkson Chromatography Ltd, South Williamsport, PA, <http://www.cccpcorp.com>).

### Immunocytochemistry and Flow Cytometry

Cell cultures were fixed in a 4% solution of paraformaldehyde in PBS for 20 minutes, washed in PBS, and incubated in 0.2% (vol/vol) Triton X-100 with 4',6-diamidino-2-phenylindole (DAPI) for 30 minutes to permeabilize cell membranes and stain the nuclei. After washing in PBS, cells were incubated in 1% (vol/vol) BSA for 20 minutes, rinsed, and then incubated for 1 to 4 hours with primary antibodies for OCN (R&D Systems Inc., Minneapolis, <http://www.rndsystems.com>) and OCT-4 (Bio-Vision Inc., Mountain View, CA, <http://www.biovision.com>) at a dilution of 1:100 contained within a 1% (vol/vol) solution of BSA. Cells were then rinsed and stained with the respective secondary antibodies in BSA for 2 hours. Any signal greater than that observed with the respective isotype (negative) controls was taken to be positive. As a negative control for immunocytochemistry, human umbilical vein endothelial cells (HUVECs) were used. These cells were cultured in EGM-2 medium (Clonetics; Cambrex Bio Science Baltimore, Inc., Baltimore, <http://www.cambrex.com>) supplemented with 10% FBS with medium changes every other day. For flow cytometry, hESCs derived without EBs after 20 and 30 days in culture ( $n = 1$ ) were stained with propidium iodide (PI) (2 mg/ml) and an ALP monoclonal antibody (B4-78 hybridoma; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at a dilution of 1:10 and subsequently analyzed under a fluorescence-activated cell sorting (FACS) scan flow cytometer (BD Biosciences). Live cells were analyzed using Cell Quest software (BD Biosciences).

### Statistical Analysis

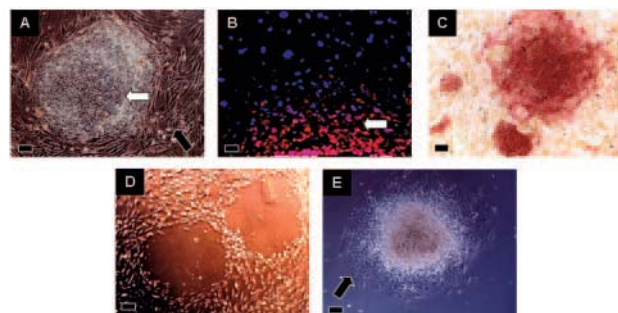
Unless otherwise stated, all experiments were performed in triplicate and the data presented are representative of three independent experiments. For single comparisons, an unpaired student's *t*-test was used. For multiple comparisons, analysis of variance was performed with the Tukey's honestly significant difference (HSD) test at a significance level of 95%. Error bars in bar graphs represent the standard deviation.

## RESULTS

hESCs grown on mouse feeders displayed a typical aggregate morphology (Fig. 2A) and stained positively for OCT-4 (Fig. 2B) and ALP (Fig. 2C), both markers for undifferentiated ESCs [29, 30]. hESC aggregates on mouse feeders did not stain positive for von Kossa (Fig. 2C) or OCN immunostaining (not shown), indicating the absence of mineralized regions. Type IV collagenase treatment removed most of the hESC aggregates, leaving the mouse feeders attached to the culture dish (Fig. 2D). For cells cultured with an EB step, placement of cell aggregates into low attachment plates provided a suitable environment for the formation of EBs, which were held in suspension for 5 days (not shown). When the EB step was omitted, very few of the day 4 hESCs obtained from the mouse feeders attached to the culture dish, irrespective of the substrate (fibronectin, gelatin, tissue culture). To circumvent this, after treatment with type IV collagenase, we plated entire cell aggregates on tissue culture dishes, to which cells quickly attached. After 24 hours, cells had migrated from the aggregate as observed using light microscopy (Fig. 2E). When hESCs were obtained from EBs,  $1.42 \pm 0.33\%$  of the seeded cells attached to the underlying culture dish, compared with  $2.1 \pm 0.60\%$  for the cells that were not obtained from EBs. The majority of the cells that did not attach were alive, as determined by toluidine blue exclusion, and gentle centrifugation of the culture dish (500g) did not improve the efficiency of cell attachment.

### Culturing hESCs Without the EB Step Affects the Kinetics of ALP and OCN Expression

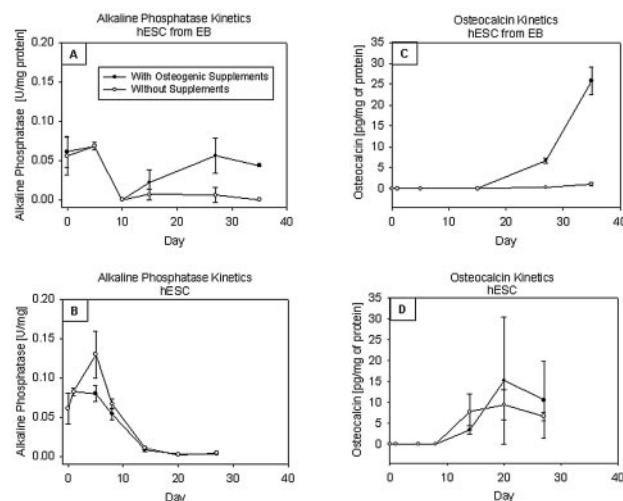
Undifferentiated hESCs exhibited a strong signal for ALP, which is an enzyme expressed by both hESCs [30] and osteoblasts [31], among other cell types. ALP expression remained relatively constant ( $p = .420$ ,  $n = 3$ ) after suspending cell



**Figure 2.** Examination of the cell colonies during various stages of culture. (A): Light microscopic images of human embryonic stem cell (hESC) colonies (white arrow) grown on mouse feeders (black arrow). (B): The primitive state of the hESC aggregates (white arrow) prior to differentiation experiments was verified by positive staining for the rhodamine-conjugated ESC marker OCT-4 (red). The mouse feeders (background) only stained for the blue 4',6-diamidino-2-phenylindole nuclear stain. (C): Day 5 hESC aggregates were positive for alkaline phosphatase, unlike surrounding mouse feeders. No evidence for von Kossa staining was observed, indicating that the aggregates did not contain any mineralized regions. (D): hESC aggregates were removed from the mouse feeders by treatment with type IV collagenase and then placed onto a tissue culture dish for 24 hours (E). During this time, cells migrated from the aggregate across the culture substrate and displayed a flattened morphology as observed with light microscopy (E). Scale bars = 100  $\mu\text{m}$  (A–D) and 200  $\mu\text{m}$  (E).

aggregates as EBs for 5 days (Fig. 3A). After plating a single-cell suspension from the EB, a rapid decrease in ALP was observed in cells treated with growth media, or media containing supplements (Fig. 3A). After 10 days, only the cells cultured in the presence of osteogenic supplements began to re-express significant levels of ALP. The expression of ALP reached a plateau after 20 days. At 27 days, the ALP signal was 10.1-fold ( $p = .023$ ,  $n = 3$ ) higher when osteogenic supplements were added to the medium. In contrast, for the cells cultured without the EB step (Fig. 3B), after an initial 58% greater ( $p = .052$ ,  $n = 3$ ) ALP expression level for the group without supplements, the ALP signal for both groups with and without supplements gradually decreased before reaching a plateau slightly above zero. This corresponded well with the flow cytometry data, which showed that approximately 10% of the hESCs cultured without the EB step in the presence of osteogenic supplements expressed ALP after both 20 and 30 days (not shown).

OCN, which is a late marker of osteogenesis that corresponds with induction of mineralization [31, 32], was first detected after 25 days in osteogenic cultures derived from EBs containing osteogenic supplements (Fig 3C) and displayed an upward trend when the cultures were terminated. After 35 days, cultures derived from EBs treated with osteogenic supplements displayed a 22.9-fold higher OCN signal ( $p < .001$ ,  $n = 3$ ) than



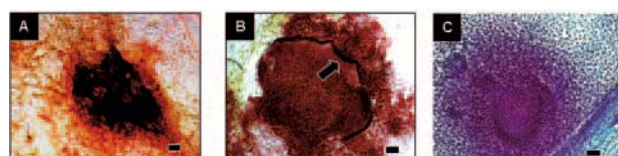
**Figure 3.** Kinetics of alkaline phosphatase (ALP) and osteocalcin (OCN) expression. (A): Undifferentiated hESCs initially displayed high levels of ALP that remained unchanged during the 5-day suspension as EBs. The ALP signal decreased sharply to undetectable levels after culturing single cells, derived from the EBs, in both media with and without osteogenic supplements. Following this, a sizeable increase in ALP signal was only observed with cells cultured in the presence of L-ascorbic acid, sodium- $\beta$ -glycerophosphate, and dexamethasone. When EBs were omitted from the culture protocol (B), a 35% higher level ( $p = .143$ ,  $n = 3$ ) of ALP was observed while culturing the cells for 24 hours as cell aggregates in the absence of mouse feeders. This was followed by a 58% increase ( $p = .052$ ,  $n = 3$ ) within cultures without supplements followed by a gradual decrease for both groups until reaching a plateau slightly above zero. For cells derived from EBs (C), OCN signal was only detected when osteogenic supplements were used and showed an upward trend when the cultures were terminated. (D): For cells derived without EBs, OCN was detected earlier and quickly reached a plateau regardless of the presence of osteogenic supplements. Abbreviations: EB, embryoid body; hESC, human embryonic stem cell.

those cultures without supplements. In contrast, OCN was first detected after 15 days in cells cultured without EBs (Fig. 3D) and quickly reached a plateau with no significant differences observed between the levels of OCN regardless of the supplementation regime.

### Culturing hESCs Without the Formation of EBs Generates Spontaneous Bone Nodules and Results in Greater Osteogenic Cell Numbers

After 10–12 days, the cells and ECM produced by cultures grown without EBs stained for both ALP and von Kossa, respectively, in both the presence and absence of supplements (Fig. 4A). Three-dimensional (3D) hemispherical structures appeared (Fig. 4B) that displaced the mineralized regions to the margins of these structures (black arrow). The mineralized nodules did not stain red for safranin O (Fig. 4C), demonstrating that glycosaminoglycans were not present in this matrix. The emergence of 3D structures was evidenced by light microscopy (Fig. 5A) and corresponded with a decrease in ALP activity as observed by qualitative ALP staining. Visual observation of ALP staining corresponded well with biochemical and colorimetric analysis.

hESCs from EBs contained areas of intense ALP staining (Fig. 5A) only when cultured in the presence of DEX. These regions began to mineralize after 4 weeks and stained positively for von Kossa (Fig. 5A), OCN (Fig. 5A), and tetracycline (not shown). However, when cultured in the presence of only growth media or growth media containing AA and  $\beta$ gP, intense ALP staining and mineralized areas were not observed. In contrast, hESCs that were cultured without EBs formed 3D structures that emerged from the underlying monolayer of cells and stained positively for ALP (Fig. 5A), von Kossa (Fig. 5A), tetracycline (not shown), and OCN (Fig. 5A). The formation of these mineralized aggregates was independent of the presence of AA,  $\beta$ gP, and DEX. Von Kossa expression was localized to the margins of the 3D structures, and colocalization of tetracycline demonstrated that these regions were actively mineralizing upon termination of the cultures (not shown). To ensure that the data were not false positives, a number of controls was performed. Human umbilical vein endothelial cells that served as a negative control for immunocytochemistry did not stain positive for OCN (not shown). Furthermore, day 4 aggregates of hESCs grown on



**Figure 4.** Culturing human embryonic stem cells (hESCs) without the embryoid body (EB) step leads to the formation of three-dimensional structures with mineralized areas containing calcium phosphate. **(A):** After 10 to 12 days, the matrix produced by hESCs cultured without the EB step, in both the presence and absence of supplements, stained for both alkaline phosphatase (ALP) and von Kossa. **(B):** After 12 days, three-dimensional hemispherical structures emerged, displacing many of the mineralized regions to the margins of these structures (black arrow), which was followed by a substantial decrease in ALP signal (not shown). **(C):** The mineralized matrix did not stain for safranin O, indicating that these regions did not contain glycosaminoglycans, which are associated with cartilage. All scale bars represent 1  $\mu$ m.

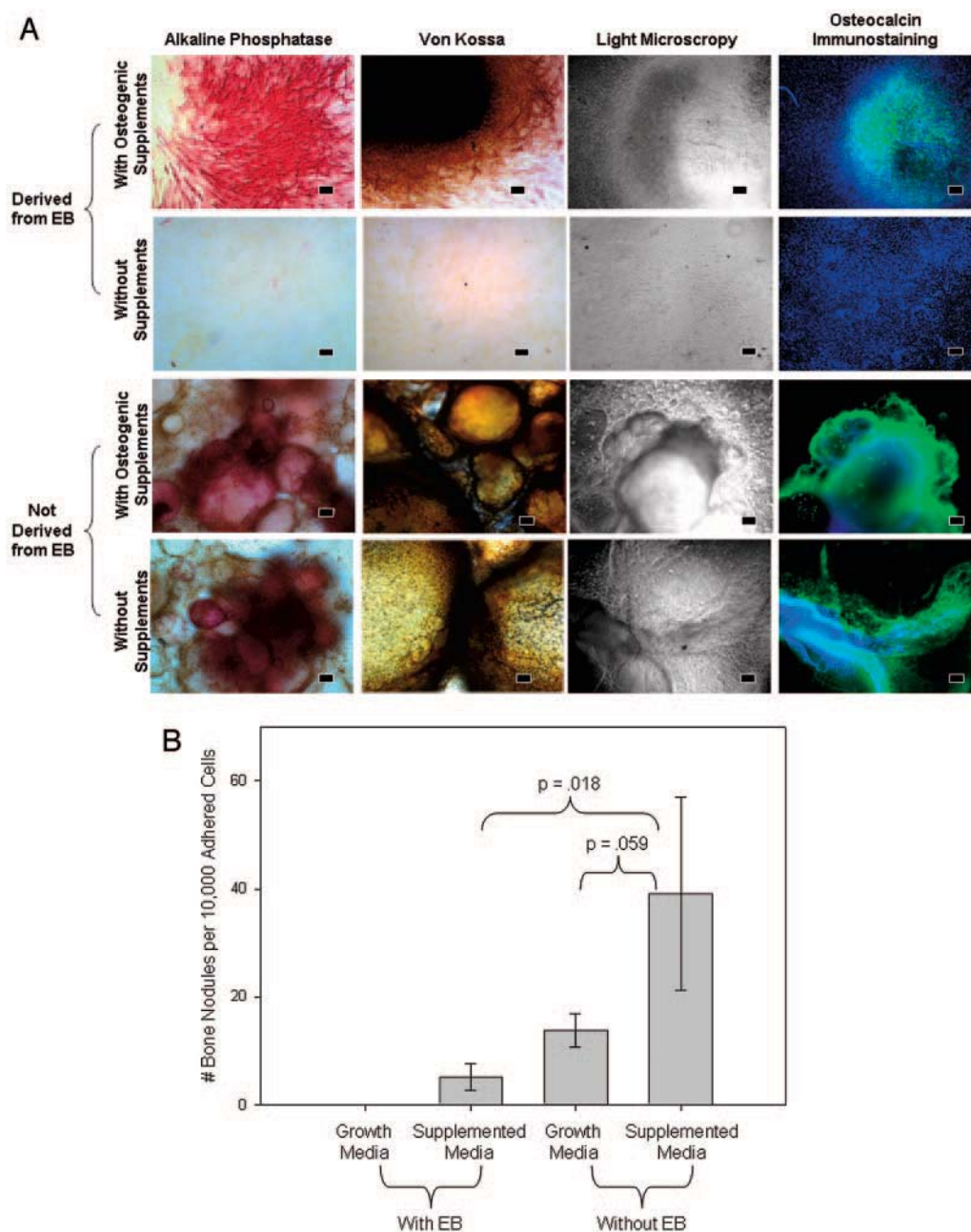
mouse feeders that served as a negative control did not stain positive for von Kossa, tetracycline, or OCN.

To compare the frequency of osteogenic cells derived from hESCs, the numbers of mineralized regions that stained positively for both ALP and von Kossa were quantified after 30 days and 14 days in cultures obtained with and without EBs, respectively. Cells grown from EBs cultured in the presence of osteogenic supplements produced  $5.1 \pm 2.4$  bone nodules per 10,000 cells, whereas the cells grown from EBs without supplements did not produce bone nodules (Fig. 5B). In comparison, cells that were grown without EBs in media with and without osteogenic supplements produced  $39.1 \pm 17.8$  and  $13.8 \pm 3.1$  bone nodules per 10,000 cells, respectively. Cells cultured without EBs in the presence of osteogenic supplements produced significantly more bone nodules than cells cultured with EBs in the presence of osteogenic supplements ( $p = .018$ ).

### Differentiated hESCs Produce Many of the Hallmarks of De Novo Bone Formation, Including a Mineralized Collagenous Matrix Containing Calcium Phosphate

When hESCs were cultured from 5-day EBs in the absence of supplements (Fig. 6A), or in the presence of AA and  $\beta$ gP but not DEX (not shown), the matrix produced was reminiscent of unmineralized collagen [24], as observed by scanning electron microscopy (SEM). However, regions that appeared to contain ectopically deposited calcium and phosphorus were observed and verified by a high calcium-to-phosphorus ratio (2.71:1) and standard deviation (0.64) as observed with energy dispersive X-ray analysis (EDX). When DEX was added in addition to the other supplements (Fig. 6B), distinct areas of mineralization were observed within the collagen fibers, which displayed a Ca/P of  $1.78:1 \pm 0.01$ . The ECM produced by the cells obtained from EB-free cultures without supplements appeared to contain small mineral deposits and had a Ca/P of  $2:00:1 \pm 0.10$  (Fig. 6C). When osteogenic supplements were added, regions containing thickened fibers and large mineral deposits were observed (Fig. 6D) and the Ca/P was only  $1.64:1 \pm 0.04$ . Hydroxyapatite samples served as a positive control and displayed a Ca/P of  $1.69:1 \pm 0.11$ , which was similar to its theoretical value of 1.67:1. Human humerus (Fig. 6E) contained thick mineralized collagen fibers with a Ca/P of  $1.52:1 \pm 0.09$ . The lower Ca/P ratios observed when osteogenic supplements were added, regardless of the inclusion of EBs, were primarily a result of a greater presence of phosphorus, as observed from the EDX spectra.

FTIR analysis was conducted to examine and compare the mineralized ECM with hydroxyapatite and human bone (Fig. 6F). The mineral peak from hydroxyapatite and the mineral and matrix peaks from human bone were comparable with those of the ECM produced by the hESCs irrespective of whether the cells were derived from EBs. However, human bone had a mineral-to-matrix ratio of 4.7:1 (Fig. 6F2), which was substantially higher than for the ECM produced by hESCs derived with EBs in the absence (0.5:1, Fig. 6F3) and presence (2.1:1, Fig. 6F4) of osteogenic supplements. In contrast to the ECM produced by hESCs derived with an EB step, the ECM produced by hESCs derived without EBs was similar regardless of whether the cells were cultured in the absence (1.5:1, Fig. 6F3) or presence (1.4:1, Fig. 6F4) of osteogenic supplements.

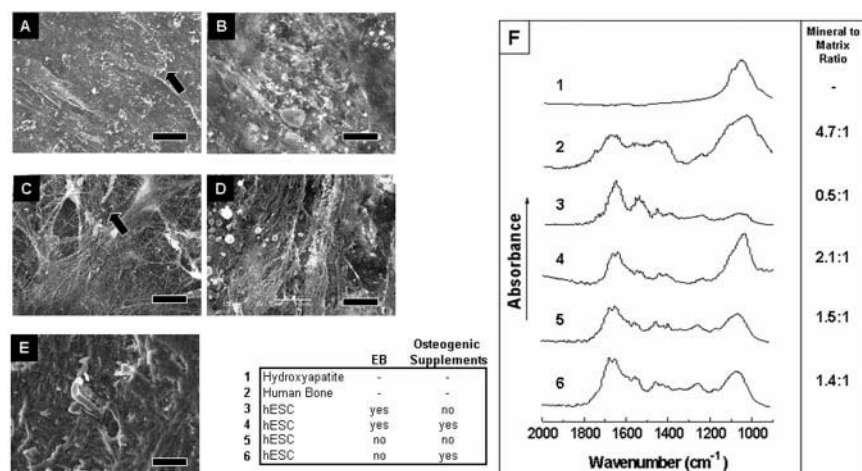


**Figure 5.** The human embryonic stem cell (hESC) culture protocol affects both the gross morphology and the quantity of bone nodules. **(A):** Alkaline phosphatase (ALP), von Kossa, and osteocalcin (OCN) immunocytochemical staining of hESCs after 35 days in culture. Cells derived from EBs only showed significant ALP, von Kossa, and OCN staining when cultured in the presence of osteogenic supplements, compared with the condition without supplements. In contrast, cells derived without the EB step stained for ALP, von Kossa, and OCN irrespective of the addition of osteogenic supplements. Regardless of the inclusion of the EB step, OCN and tetracycline (not shown) staining localized to similar regions that stained positive for von Kossa. Scale bars = 100  $\mu\text{m}$  for ALP- and von Kossa-stained images and 500  $\mu\text{m}$  for immunocytochemistry images. **(B):** Frequency of bone nodule formation per 10,000 adhered cells. Cells grown from EBs produced bone nodules ( $5.1 \pm 2.4$ ) only in the presence of osteogenic supplements. In comparison, cells grown without EBs cultured in growth media and media containing osteogenic supplements produced  $13.8 \pm 3.1$  and  $39.1 \pm 17.8$  bone nodules per 10,000 plated cells, respectively. Abbreviation: EB, embryoid body.

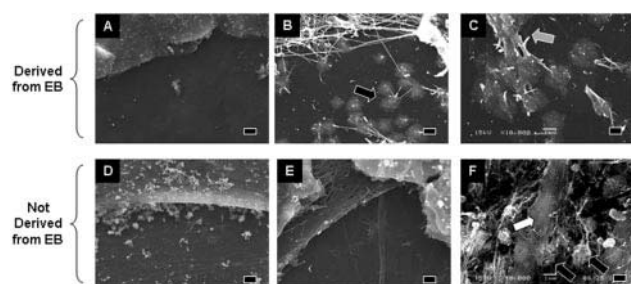
### The hESC Culture Protocol Affects the Sequence of De Novo Bone Formation

To examine if the osteogenic cells derived from hESCs could produce the cement line matrix, which is the first matrix produced by differentiating osteogenic cells and separates new bone from the old bone surface during bone remodeling

[25], electron microscopy was employed. Compressed air was used to remove the overlying cell and collagenous matrix to expose the underlying ECM/culture dish interface. The hESCs derived from EBs that were cultured in the absence of supplements (Fig. 7A) or in the presence of AA and  $\beta\text{gP}$  (not shown) did not produce the cement line matrix. However,



**Figure 6.** hESCs are capable of producing morphologically identifiable bone that contains calcium and phosphorus with an apatitic phase. Scanning electron microscopy was used to examine the morphology of the matrix produced by osteogenic cultures of hESCs. Regardless of whether cells were derived from embryoid bodies (EBs) (A, B) or not (C, D), cultures produced thin collagenous fibers that appeared to display traces of ectopic mineral, as observed by their grainy appearance (arrow) even in the absence of supplements (A, C). The collagenous matrix became mineralized (B, D) in the presence of L-ascorbic acid, sodium- $\beta$ -glycerophosphate, and dexamethasone. In comparison, the matrix within human bone contained thick, densely packed collagen fibers (E). (F): Fourier transform infrared spectra and mineral-to-matrix ratios (obtained by integrating the area under the curve between 900 and 1200  $\text{cm}^{-1}$  [phosphate bands] and dividing by the area under the curve between 1585 and 1725  $\text{cm}^{-1}$  [amide I band]). The mineral peak from hydroxyapatite and the mineral and matrix peaks from human bone were comparable with those of the extracellular matrix produced by the hESCs irrespective of whether the cells were derived from EBs. However, hESC cultures derived from EBs in the absence of supplements produced a substantially smaller mineral peak, which corresponded to a low calcium-to-phosphate ratio. Scale bars = 10  $\mu\text{m}$  (A–E). Abbreviation: hESC, human embryonic stem cell.



**Figure 7.** Examination of the bone matrix/tissue culture interface with scanning electron microscopy. (A–E): The matrix/culture dish interface was revealed through removal of cells and extracellular matrix. Shown are cultures in the absence of (A, D) and in the presence of (B, C, E, F) osteogenic supplements. Unlike cells grown in the absence of supplements (A), cultures derived from EBs grown in the presence of osteogenic supplements (B, C) displayed 1- $\mu\text{m}$ -sized mineralized globular accretions (black arrow) that were reminiscent of the cement line formed by differentiating osteogenic cells. These globules were produced by the cells through pseudopodia (grey arrow). In contrast, cement line globules were not observed at the matrix/culture dish interface when hESCs were obtained without the EB step, regardless of the supplementation regime (D, E). However, when these cells were cultured in the presence of osteogenic supplements, 1- $\mu\text{m}$ -sized globules (black arrows) were observed entrapped within the overlying collagenous matrix (white arrow) (F). All scale bars represent 1  $\mu\text{m}$ . Abbreviation: EB, embryoid body.

when DEX was added in addition to the other osteogenic supplements, a mineralized cement line matrix was revealed as evidenced by SEM (Fig. 7B, 7C) and by elemental mapping using EDX (not shown). In contrast, when hESCs were derived from an EB-free system, cement line matrix on the culture dish was not observed, regardless of the addition of

osteogenic supplements (Fig. 7D, 7E). However, globules that are reminiscent of the cement line matrix were observed suspended within the overlying collagenous matrix (Fig. 7F).

## DISCUSSION

In this study, we examined the capacity of hESCs to differentiate into osteogenic cells using cultures derived with and without an EB step. Osteogenic cells were identified based on the biomarkers ALP and OCN, bone nodule formation, and the formation of cement line matrix. Given that the frequency of osteoprogenitor cells can only be demonstrated retrospectively through examining the culture surface for de novo bone formation [32], we quantified the number of bone nodules as an indirect measure of the number of osteoprogenitors within our cultures. The number of bone nodules produced by hESCs derived from EBs in our study ( $5.1 \pm 2.4$  bone nodules per 10,000 attached cells) was similar to the number reported in previous experiments in which hESCs were derived using a similar protocol. Specifically, Bielby et al. [12] reported a maximum of 38 bone nodules from differentiated hESCs in a 35-mm dish (which has a surface area of 9.6  $\text{cm}^2$ ) at a seeding density of 5,200 cells/ $\text{cm}^2$ , which equates to 7.6 bone nodules per 10,000 cells. Because approximately 85% of their cells attached [J.M. Karp and R.C. Bielby, personal communication], this translates into 8.9 nodules per 10,000 attached cells. Although it is useful to compare results between studies, these comparisons must be interpreted with caution given that previous experiments with hESCs have used 10 mM  $\beta\text{gP}$  [11, 12] (twice the concentration used in this study), which has been associated with greater levels of dystrophic mineralization [21]. Because EBs are used as a model for recapitulating the simul-



taneous formation of multiple tissues during embryonic development, achieving much greater frequencies of osteoblasts in this system may present a challenge. Thus, a system devoid of EBs may be useful to improve the derivation efficiency of osteogenic cells. In this study, a 7.6-fold greater ( $39.1 \pm 17.8$ ) number of bone nodules was observed when the EB step was omitted from the culture protocol. In addition, the nodules produced from cultures without EBs formed after 10–12 days, compared with 4 weeks in cultures derived from EBs. The relatively rapid production of bone nodules in cultures derived without EBs is supported by the early detection of OCN as observed in Figure 3.

To engineer human bone tissue *in vitro*, naturally, it is relevant to thoroughly characterize the matrix produced by the cell source of interest and compare it with that of native bone. Although numerous staining techniques have been used to detect the presence of osteoblasts in culture, expression of osteogenic markers such as ALP does not directly correlate with production of bone nodules [28]. Conventional wisdom holds that cells capable of forming bone are more useful for engineering bone tissue than cells that express osteogenic markers yet do not produce bone. Therefore, in addition to using classic stains to identify osteogenic cells, it is imperative to examine the matrix produced by the cells. Using a typical rodent cell culture system, osteogenesis *in vitro* has been demonstrated to culminate in the formation of mineralized nodules that are discrete islands of bone that display histological, ultrastructural, and immunohistochemical similarities to bone formed *in vivo* [25, 28]. With respect to ESCs, to date, there is only one study that has characterized the mineralized matrix produced by a differentiated murine population [9]. They found that the mineralization process did not parallel conventional osteogenesis, and their spectroscopic analysis demonstrated that the Ca/P of the mineral phase was 1.26:1, compared with 1.67:1 for hydroxyapatite. Therefore, it is unclear whether the nodule-like structures described thus far in ESC cultures are indeed bone nodules that resemble bone formed *in situ* or are representative of dystrophic mineralization. Given that only von Kossa and/or alizarin red have been used for identification and quantification of *in vitro* bone nodules from osteogenic cultures of hESCs [11, 12], the positive identification of *in vitro* bone formed from hESCs has yet to be verified. In this study, we demonstrated that differentiated hESCs can produce a mineralized matrix that displays colocalized staining for ALP and von Kossa and displays many of the hallmarks of *de novo* bone formation, including a cement line matrix and mineralized collagen. We show that the matrix produced by the differentiated hESCs, irrespective of the culture protocol, contains an apatitic mineral phase with calcium and phosphorous in a ratio that is similar to that for hydroxyapatite and human bone. Although hypertrophic chondrocytes may express ALP and OCN, and produce a mineralized collagenous matrix that stains positively for von Kossa [33, 34], the absence of glycosaminoglycans and the presence of a cement line matrix demonstrates that that these cells are osteogenic.

The results reported previously for murine ESCs cultured without EBs are in contrast to the results we present here and likely represent innate differences between humans and mice. For example, murine ESCs cultured without the EB step fail to spontaneously differentiate into osteogenic cells [9], and ALP expression remains relatively constant, regardless of the pres-

ence of osteogenic supplements. Therefore, for clinical application of osteogenic cells derived from ESCs, it remains crucial to focus efforts on understanding the differentiation processes in the human system. Differences between murine and human ESCs have been described previously in detail elsewhere [14, 35].

Whereas omitting the EB step from the culture protocol improves the efficiency of osteogenic differentiation, the emergence of 3D structures impedes the development of *in vitro* bone. Therefore, purification of the osteogenic population for bone engineering applications would likely need to be performed prior to the emergence and dominance of these structures at day 10–12. This is when the hESCs have lost their stem cell properties, as evidenced by a decrease in ALP. Unlike cultures of hESCs that were derived from EBs, in which ALP expression re-emerged corresponding to the appearance of osteogenic cells, for the hESCs derived without EBs, the ALP signal continued to decrease before reaching a low, yet detectable, level. This low level of ALP and the relatively low OCN signal that quickly reached a plateau were likely produced from the osteogenic cells that were displaced to the margins of the 3D structures. Although speculative, we believe that the emergence of the 3D structures corresponded to the appearance and dominance of a nonosteogenic cell type. The presence of mineralized globules of cement line within the collagenous matrix suspended above the culture surface was likely a result of the forces exerted by these cells, which displaced the bone nodules to the margins of the 3D structures. In addition, the low mineral-to-matrix ratio observed for hESCs cultured without EBs, compared with the matrix produced by hESCs cultured with EBs, may be explained by the dominance of a nonosteogenic cell type that hindered the growth and maturation of the bone matrix. However, future work is required to more thoroughly examine this.

Although these findings are encouraging and will ultimately be useful for osteogenic cell-based therapies, the ability to simulate the differentiation of hESCs into osteogenic cells remains relatively inefficient. If indeed clonal populations of ESCs have the capacity to differentiate into multiple cell types, conventional wisdom holds that individual clones could be forced to differentiate into a pure population of a desired cell type under specific media conditions. Thus, increasing the efficiency of deriving osteogenic cells from hESCs would likely require better control over the cell microenvironment. Evidently, factors other than DEX,  $\beta$ gP, and AA are required to improve the efficiency of differentiation of hESCs into osteogenic cells. Increasing the frequency of osteogenic cells may require the presence of specific cues supplied by physical stimulation, soluble factors such as bone morphogenetic proteins, contact with other cell types, or the substrate [36]. In this early stage of exploring the potential of ESCs for bone therapies, it is not only crucial to characterize the behavior of these cells and their respective production of bone matrix, but also understand which cues are required to stimulate bone formation. Once these cues have been identified, only then can one begin to harness the potential of these cells for therapeutic use.

## CONCLUSION

In this study, we found that the kinetics of bone formation and the number of bone nodules derived from hESCs are significantly affected by the inclusion of the EB step in the culture protocol. By omitting an EB step, we demonstrated that

a significantly greater number of osteogenic cells can be more rapidly achieved. In addition, we found that osteogenic cultures of hESCs can produce many of the hallmarks of de novo bone formation.

#### ACKNOWLEDGMENTS

We acknowledge technical support from Roger Li, Hannah Seong, and George Eng, and useful conversations with Professor Bjorn Olsen (Harvard University, Oral and Developmental Biology). We also thank Professor Peter Zandstra (University of Toronto, Institute for Biomaterials and Biomedical Engineering,

Toronto, ON, Canada) for critically reviewing this manuscript. Human bone (humerus) was generously provided by the Massachusetts General Hospital Bone Bank (Boston), and hydroxyapatite was generously provided by Clarkson Chromatography Ltd. J.M.K. is supported by a Natural Sciences and Engineering Research Council of Canada fellowship, and L.F. is supported by a Fundação para a Ciência e Tecnologia (SFRH/BPD/14502/2003) postdoctoral fellowship. This work was supported by NIH grant RO1-DE16516-01.

#### DISCLOSURES

The authors indicate no potential conflicts of interest.

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**Cultivation of Human Embryonic Stem Cells Without the Embryoid Body Step  
Enhances Osteogenesis In Vitro**

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*Stem Cells* 2006;24;835-843; originally published online Oct 27, 2005;  
DOI: 10.1634/stemcells.2005-0383

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