13, Part C

Directed differentiation of human embryonic stem cells into osteogenic cells

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Introduction

Human embryonic stem (ES) cells can be used as an *in vitro* system for studying bone formation and provide a cell source for production of bone (Sottile *et al*., 2003; Bielby *et al*., 2004; Cao *et al*., 2005; Karp *et al*., 2006; Ahn *et al*., 2006; Barberi *et al*., 2005; Olivier *et al*., 2006). Given the high incidences of bone trauma, cancer, and congenital and acquired disease, which are associated with over 500 000 bone graft procedures each year in North America, it is not surprising that bone is one of the most common transplanted tissue, second only to blood (Langer and Vacanti, 1993). Thus, one of the great challenges facing bone surgeons is to increase bone stock, or the amount of bone available for grafting. Current therapies for regeneration are fraught with many shortcomings including donor site morbidity, lack of suitable graft material, and osteogenic cell sources. The emerging field of bone engineering attempts to augment or replace the current approaches by utilizing the combination of liquid, gel, or solid carriers with a source of osteogenic cells. The scaffold or cell carrier, although temporary, can be engineered to support migration, proliferation and differentiation of osteoprogenitor cells and to aid in the organization of these cells in three dimensions. In one application, progenitor cell numbers are expanded *in vitro*, placed onto biodegradable matrices in combination with factors that stimulate osteogenic differentiation, followed by implantation into a bone defect site (Langer and

Vacanti, 1993; Alsberg *et al*., 2001). The ideal construct would eventually be replaced with host bone tissue. In addition to therapeutic applications, osteogenic cells and tissue engineered bone constructs derived from human ES cells can be used for screening drug candidates, for fabricating physiological systems to study bone biology, and for developing *in vitro* toxicity assays.

In choosing an appropriate cell source for bone engineering strategies, one must consider the capacity of the chosen cells to regenerate bone tissue and the ability to obtain a therapeutically relevant number of cells. The ratio of mesenchymal stem cells to total bone marrow cells within human bone marrow decreases from ∼1:10 000 in newborns to ∼1:1 200 000 at the age of 80 (Caplan, 1991). Therefore approaches which make use of bone marrow cells have to account for the scarcity of mesenchymal stem cells within the bone marrow, as well as the need to perform marrow biopsy to obtain the cells. Embryonic stem (ES) cells offer benefits including ease of isolation, ability to rapidly propagate without differentiation, and a greater capacity to give rise to different cell types (Bhatia, 2005; Thomson *et al*., 1998; Perlingeiro *et al*., 2001).

Although only few studies have reported osteogenic differentiation of human ES cells (Cao *et al*., 2005; Ahn *et al*., 2006), numerous culture strategies have been employed. In general, after expanding the human ES cells in an undifferentiated state, the cells may be (A) differentiated through an EB stage, (B) differentiated by plating them as embryoid bodies (EBs), or (C) differentiated by plating single cells into a 2D cell culture dish, as described in **Figure 1**. Plating of EBs may be useful to achieve cell adhesion (as human ES cell-derived aggregates tend to adhere more efficiently compared to single cells) or to isolate certain populations of cells based on their migration from the aggregate.

Application for human ES cell based bone engineering strategies face numerous challenges, for example directed differentiation towards the osteogenic lineage. Currently, human ES cell-based bone research is centered on elucidating soluble and immobilized cues and respective signaling mechanisms that direct osteogenic differentiation, on characterization and isolation of differentiated progeny cells, and on establishing protocols to improve the expansion and homogeneity of osteogenic cells (Sottile *et al*., 2003; Bielby *et al*., 2004; Cao *et al*., 2005; Karp *et al*., 2006; Ahn *et al*., 2006). This chapter focuses on fundamental concepts in osteogenic differentiation and key protocols and techniques for inducing the differentiation of human ES cells into functional osteoblasts. It must be emphasized that osteogenic differentiation of human ES cells is a relatively young field and much work remains to be done. For example, the process of human ES cell differentiation into osteoprogenitors is relatively inefficient and no more than 1:250–1000 adherent human ES cells gives rise to an osteoprogenitor cell (Karp *et al*., 2006). Given the assumption that clonal populations of human ES cells have the capacity to differentiate into multiple cells types, presumably individual clones could be forced to differentiate into a pure population of a desired cell type under specific media conditions. Therefore, in addition to the methods described herein, increasing the efficiency of osteogenic cells from human ES cells will likely require exquisite control over the cell microenvironment. Increasing the frequency of osteogenic cells derived from stem cells requires the presence of specific cues supplied by physical stimulation, soluble factors such as bone morphogenetic proteins, contact with other cell types, or

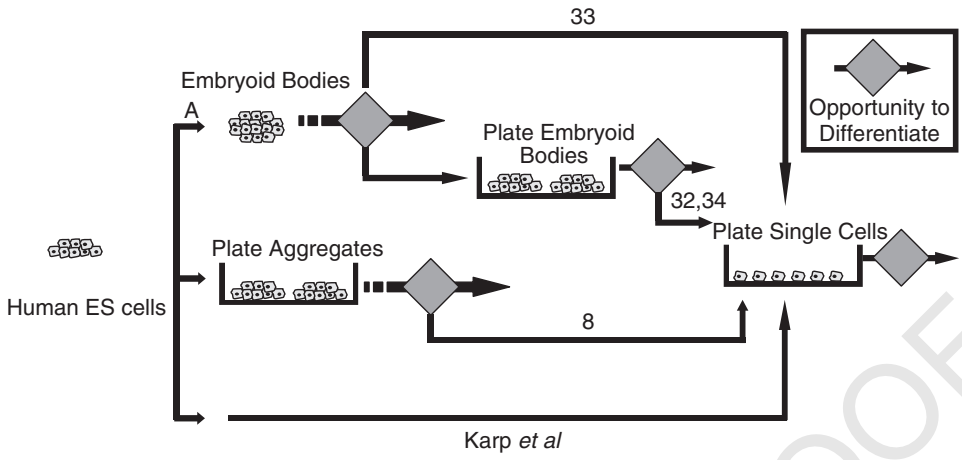

Figure 1 Flowchart of various methods for stimulating osteogenic differentiation of human ES cells. (**A**) Human ES cells are placed into suspension cultures as EB. After potential addition of differentiated media, the cells can either be plated as a single cell suspension, or plated directly onto a culture dish which may be followed by a single cell step. (**B**) Human ES cells may also be plated directly as cell aggregates. This may be useful to increase the number of adherent cells prior to separation as a single suspension. (**C**) Human ES cells may also be directly plated as a single cell suspension. Opportunities to differentiate may include addition of soluble cues and/or use of immobilized cues. Conditions which may be used to enhance osteogenic differentiation that are not included in this chart include: genetic manipulation, co-cultures, and placement of cells within bioreactors. There is also opportunity at various stages in the chart to purify populations of cells using flow cytometry or other means

by the substrate chemistry or morphology as recently reviewed (Heng *et al*., 2004). In addition, emerging microscale technologies for controlling the cellular microenvironment may also be useful for enhancing stem cell differentiation (Maniatopoulos *et al*., 1988)

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 classical stains to identify osteogenic cells, we believe that it is imperative to examine the matrix produced by the cells (Karp *et al*., 2006). Recently we demonstrated that human ES cells, regardless of being cultured with or without the EB step, can produce many of the hallmarks of *de novo* bone formation including an elaborate cement line matrix and overlying mineralized collagen (Karp *et al*., 2006) (**Figure 2**). In comparison to frequencies of osteoprogenitors derived form adult mesenchymal stem cells described above, by using standard osteogenic media with the inclusion of an EB step it maybe possible to achieve approximately one osteoprogenitor cell per ~1000–2000 adhered human ES cells. In comparison, when the EB step is omitted, we have demonstrated that the frequency can be increased to one osteoprogenitor cell per ~250 adhered cells.

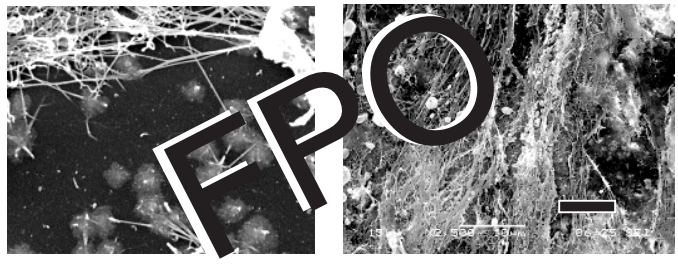

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Figure 2 Morphological characterization of bone produced from differentiated human ES cells. Scanning electron micrographs show (left) the deposition of mineralized globular accretions, which are reminiscent of the cement line formed by differentiating osteogenic cells. Collagen fibers can be see anchoring to the underling globular accretions. (right) Mineralized collagen is observed above the cement line matrix as verified by FTIR and EDX analysis. (Figure adapted from Karp *et al.*, 2006)

Overview of protocols

The methods presented here for differentiation of human ES cells to osteogenic cells, with or without the EB step, are complemented with a list of characterization techniques that allow for determination of success of differentiation and functional capacity of the resulting tissue.

Materials, reagents and equipment

OSTEOGENIC CELL CULTURE

Material/reagent	Vendor	Catalog number
α-MEM	Invitrogen	12571-089
Fetal Bovine Serum (FBS)	Invitrogen	10437-028
Dexamethasone	Sigma	D8893-1 MG
Ascorbic acid 2-phosphate	Sigma	A8960
Glycerol 2-phosphate disodium salt hydrate	Sigma	G9891
Penicillin G	Sigma	P3032
Gentamicin	Sigma	G1397-10 ML
Amphotericin B (Fungizone)	Sigma	A2942
Phosphate buffer saline (PBS)	VWR	72060-034

Material/reagent	Vendor	Catalog number
Trypan blue	Sigma	93595
Trypsin–EDTA solution	Sigma	T4049
Non-enzymatic cell dissociation solution	Sigma	C5914
70% ethanol solution	Sigma	E7148
ALKALINE PHOSPHATASE/VON KOSSA QUALITATIVE STAINING		
Formalin/formaldehyde	Sigma	11–0705
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma	7907
Sodium phosphate monobasic monohydrate ($NaH_2PO_4^{\cdot}H_2O$)	Sigma	S9638
N,*N*-Dimethylformamide (DMF)	Sigma	D4551
Silver nitrate (AgNO3)	Sigma	S8157
Sodium carbonate anhydrous (Na_2CO_3)	Sigma	S7795
Naphthol AS-MX phosphate disodium salt	Sigma	N5000
Tris-HCl (MW=157.6; pH 8.3; 0.2 M)	Sigma	88438
Red violet LB salt	Sigma	F1625
ALIZARIN RED STAINING		
Formalin/formaldehyde	Sigma	11–0705
Alizarin Red S	Sigma	A5533
TETRACYCLINE LABELING		
Tetracycline HCl ($C_{22}H_{24}N_2O_8 \cdot HCl$)	Sigma	T7660
QUANTITATIVE DETERMINATION OF ALKALINE PHOSPHATASE AND OSTEOCALCIN		
Osteocalcin immunostain 96-well kit	DSL	10–7600
BCA protein kit	Sigma	QPBCA-1KT
Alkaline phosphatase detection kit	Sigma	APF
96-well plates (UV transparent)	Corning	3370
IMMUNOHISTOCHEMISTRY		
Osteocalcin monoclonal antibody (human)	R&D	MAB1419
OCT-4 (human)	BD	611202
Alkaline phosphatase monoclonal antibody (human)	Sigma	A2064
Anti-Mouse IgG (whole molecule)-FITC antibody	Sigma	F9137-1 ML
DAPI nuclear stain	Mol. Probes	D21490
SCANNING ELECTRON MICROSCOPY PREPARATION		
Sodium cacodylate trihydrate	Sigma	C9722-50 MG
Calcium chloride	Sigma	222313
0.2N hydrochloric acid	Sigma	H9892
Paraformaldehyde	Sigma	P6148

Material/reagent	Vendor	Catalog number
1N sodium hydroxide	Sigma	484024
Glutaraldehyde	Sigma	G5882
Ethanol	Sigma	E7148
Hexamethyldisilazane (HMDS)	Sigma	440191
FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)		
Potassium bromide (KBr)	Sigma	60090

Protocols

Osteogenic cell culture

For simplicity, we focus herein on the differentiation conditions for osteogenesis and not on the culture of human ES cells required prior to this stage; this was discussed in **Chapters 4, 5 and 6**. Typically, for differentiation experiments involving ES cells it is customary as a first attempt to apply culture conditions used for adult mesenchymal systems. Below we describe a widely accepted protocol that we have applied towards human ES cells as described in a recent publication (Karp *et al*., 2006). The differentiation medium is based on a protocol by Maniatopoulus *et al*. (1988) who derived progenitors from rodent bone marrow. In addition to describing the concentrations of various components, we provide details for preparation of stock solutions.

Standard medium for inducing osteogenic differentiation

Base:	85–90% (v/v) α-MEM
Serum:	10–15% (v/v) fetal bovine serum (FBS)
Dexamethasone:	10^{-7}–10^{-9}M
Ascorbic acid 2-phosphate:	50 μg/mL
Glycerol 2-phosphate disodium salt hydrate:	5 mM (Karp *et al*., 2003a)
Antibiotics including fungicide:	167 (U/mL) penicillin G
	50 (μg/mL) gentamicin
	0.3 (μg/mL) amphotericin B

Although we provide ranges for some of the components listed above, it is sometimes preferred to use 85% α-MEM, 15% FBS, and 10^{-8} M Dex. Typically we have found that increasing the FBS concentration from 10% to 15% can increase the number of bone nodules in our cultures. Although based on the lot of serum used (as discussed in the section on trouble shooting), it is likely best to test both 10% and 15%, as given the current price of serum, 10% would be more cost efficient.

Preparation of stock solutions

Dexamethasone

Is a synthetic member of the glucocorticoid class of hormones. Aside from being used clinically to treat many inflammatory and autoimmune conditions, it has been demonstrated to interact with specific glucocorticoid receptors leading to stimulation of osteogenic differentiation *in vitro* for progenitor cells derived from multiple tissues and animal sources (Karp *et al*., 2006; Aubin, 1999; Sarugaser *et al*., 2005; Qu *et al*., 1998; Lecoeur and Ouhayoun, 1997; Cornet *et al*. 2002; Karp *et al*, 2003b). However, *in vivo* dexamethasone administration results in decreased osteogenesis (Baron *et al*., 1992).

1. To make a 10^{-4} M (10000×) stock solution, add 25.5 mL of absolute ethanol to 1 mg of dexamethasone, mix and store at −20°C.
2. To prepare a 10^{-6} M supplement stock solution (100×), aseptically add 1 mL 10^{-4} M dexamethasone solution into 99 mL of culture media (containing α-MEM, FBS and antibiotics) and mix well.
3. Aliquot 2 mL per sterile tube and store at −20°C. This can be used at 1% [v/v] when making fresh media.
4. With all supplements, it is best not to refreeze thawed aliquots.

β-glycerophosphate (β-GP)

Is a source of organic phosphate added to the culture medium (Davies, 1996). Inorganic phosphate within α-MEM is in the soluble form typically in the physiological range and is readily available and quickly absorbed by cells. Organic phosphate refers to phosphate that is bound to organic matter such as proteins or glycerol. Organic phosphate must be broken down in order to become soluble phosphate. Osteogenic cells can facilitate this process (cell mediated) using alkaline phosphatase. To avoid non-specific precipitation of mineral, also referred to as ectopic or distrophic mineralization, generally the β-GP concentration should be 3.5–5.0 mM (Maniatopoulos *et al*., 1988; Aubin, 1999). Furthermore, in some cases, it may be necessary to add the β-GP after significant multi-layering has occurred as previously described (Baksh *et al*., 2003).

1. To make 100× supplement stock solution (500 mM), dissolve 10.8 g of glycerol 2-phosphate disodium salt hydrate in double distilled water at room temperature and make up to 100 mL.
2. Filter through 0.1 μm filter to sterilize.
3. Aliquot 2–3 mL per tube and store at −20°C. This can be used at 1% [v/v] when making fresh media.

Ascorbic acid (AA)

Ascorbic acid is an important cofactor for formation of hydroxyl praline which plays a key role in the stabilization of the collagen triple helix and thus collagen assembly. Given that collagen is the main organic component of bone, AA is included as a main supplement for osteogenic cultures (Maniatopolous *et al.*, 1988). Although most α-MEM formulations contain AA, the activity is completely lost after about 10 days at 4°C and much more quickly at 37°C (Feng *et al.*, 1977). Ideally, AA deficient α-MEM should be employed to ensure a known concentration. In addition, fresh AA (from frozen aliquots) should be added to media at each reefed, preferably every other day. If cultures are to be fed less frequently (i.e. within bioreactors), a long acting version of AA is preferred. L-Ascorbic acid 2-phosphate is a phosphate derivative of L-ascorbic acid and has more prolonged vitamin C activity in solution than does L-ascorbic acid.

1. To make 100× supplement stock solution (5 mg/mL) of short acting ascorbic acid (L-ascorbic acid, MW 176.12), add 0.5 g in 0.1 M PBS at room temperature and make up to 100 mL.
2. Filter through 0.1 μm filter to sterilize.
3. Aliquot 2–3 mL per tube and store at −20°C. These aliquots must be freshly thawed prior to use.

Preparation of differentiation medium

1. Add 375 mL of α-MEM to a sterile container.
2. Add 50 mL of fresh 10× antibiotics.
3. Add 75 mL of FBS (for 15% v/v).
4. Add 1% β-glycerophosphate stock (5 mM final concentration).
5. Add 1% dexamethasone stock (10^{-8}M final concentration).
6. Store at 4°C (for up to 2 weeks).
7. Upon each refeed, add 1% ascorbic acid freshly thawed stock (50 μg/mL final concentration).
8. Mix thoroughly.
9. Label with date of prepared antibiotic solution.

Alkaline phosphatase/von kossa qualitative staining

Reagents

10% Neutral formalin buffer (NFB) (store at room temperature)

Formalin/formaldehyde:	100 mL
Na_2HPO_4:	16 g
$NaH_2PO_4H_2O$:	4 g
Distilled water:	to 1L

2.5% Silver nitrate solution (store at room temp in the dark)

$AgNO_3$:	2.5 g
Distilled Water:	to 100 mL

Sodium carbonate formaldehyde (store at 4°C):

Formalin/formaldehyde:	25 mL
Na_2CO_3:	5 g
Distilled water :	to 100 mL

Other reagents

Naphthol AS MX-PO_4	0.005 g
N,*N*-Dimethylformamide (DMF)	200 µL
0.2 M Tris-HCL pH 8.3	25 mL
Red Violet LB salt	0.03 g
Distilled water	25 mL

Protocol

1. Remove media from dishes, rinse once in cold PBS.
2. Fix in 10% cold NFB for 30 min in chemical hood.
3. Remove buffer and rinse dishes in distilled water 3×.
4. Leave in distilled water for 15 min.
5. While waiting, prepare APase reagent using following protocol:

In an Eppendorf tube, dissolve 0.005 g of naphthol in 200 µl of DMF. Add to graduated cylinder with 25 mL of Tris-HCL and 25 mL Distilled water. Add 0.03 g of Red Violet salt to solution and filter with Whatman's No.1 filter paper immediately prior to adding to dishes to be stained.

6. Remove distilled water from cells and add APase reagent and incubate for 45 min at room temperature.
7. Rinse in distilled water 3–4 times.
8. Remove distilled water and stain with 2.5% silver nitrate for 30 min in the dark.
9. Remove silver nitrate and rinse with distilled water four times.
10. Prior to examination or drying dish, the color of the mineralized nodules can be deepened by adding sodium carbonate formaldehyde to the dish for 30 s to 2 min. One must observe closely as the black color may become too intense thus preventing analysis.
11. Remove sodium carbonate formaldehyde and rinse with slowly running tap water for 1 hr (do not let water fall directly onto dish, instead sink dishes within a large plastic tub).
12. Image/count nodules. Positive alkaline phosphatase staining appears bright red and positive von Kossa appears dark brown to black. The dish may be dried for indefinite storage.
13. A good negative control for the Von Kossa staining is to treat a test dish or well with 10% formic acid for 10 min prior to step 8. The formic acid should dissolve the mineral component of the matrix (i.e. the calcium phosphate) and thus the test dish should show a negative reaction.

Alizarin red staining

1. Fix cells in 10% NFB for 20–30 min.
2. Rinse cells 3× with distilled water.
3. Add 2% (w/v) solution of alizarin red in distilled water for 30 s to 5 min.
4. Rinse thoroughly with distilled water.

Calcium deposits should appear bright orange–red.

Tetracycline staining

Tetracycline staining

1. Prepare 900 μg/mL Tetracycline HCL solution in PBS and pass through a sterile filter (can store for 2 weeks at 4°C).

2. 24–48 hr prior to media change or termination of the culture, add 1% of stock tetracycline solution to media.
3. For analysis, rinse samples with PBS 3×
4. Fix in 100% cold ethanol (−20°C) for 2 hr.
5. Air dry samples and immediately image for green fluorescence.

Quantitative determination of alkaline phosphatase and osteocalcin

Given the availability of numerous kits available for quantifying alkaline phosphatase (a non specific early marker of osteogenesis and osteocalcin (a later marker of osteogenesis) expression, we have chosen to omit specific protocols here. Details for the protocol we used in a recent study with human ES cells can be found elsewhere (Karp *et al.*, 2006). For osteocalcin analysis, it is possible to analyze its release into the media, or osteocalcin that becomes entrapped within the produced matrix through a homogenization step. Similarly, alkaline phosphatase can be measured either within the media or within the cell membranes through a homogenization step.

Immunohistochemistry

1. Transfer• 300 μL of cell suspension to the wells of a chamber slide or slides of your choice. The choice of slide design is often dictated by the experiment. Some slides have four wells, some have eight, some are glass, and some are plastic. Glass is recommended because the slide becomes more versatile, and reduces the photo bleaching during the fluorescence or confocal microscopy analyses.
2. Allow cells to grow to confluence with the addition of fresh media.
3. Rinse cells 2× with PBS buffer.
4. Fix cells in freshly made 4% (v/v, in PBS) paraformaldehyde for 30 min.
5. Rinse cells 2× with PBS buffer. Do not let the cells dry at any step.
6. Incubate the cell preparations with 0.25–0.5% Triton X-100 in PBS for 10 min to permeabilize the membranes.
7. Rinse the cells 2× with PBS buffer.
8. Block the cells with 2–5% normal serum in PBS for 30 min (normal serum should be the same species as the secondary antibody is raised). This step is required to block non-specific binding of immunoglobulins. Alternatively, blocking with 1% (w/v, in PBS) BSA solution may be used.
9. Rinse cells 2× with PBS buffer.

10. Incubate the fixed cells with anti-human monoclonal antibodies, for 1 hr (*Note*: antibody concentration should be determined by titration of the stock solution and testing on a known positive specimen. Usually, working concentrations are in the range of 10–20 µg/mL. However, depending on the source of antibodies, this concentration could vary significantly). It is advisable to run the appropriate negative controls. Negative controls establish background fluorescence and non-specific staining of the primary antibodies. It should be isotype-matched, not specific for cells of the species being studied and of the same concentration as the test antibody. The degree of autofluorescence or negative control reagent fluorescence will vary with the type of cells under study and the sensitivity of the instrument used.
11. Rinse cells 2× with PBS buffer.
12. Add fluorescence conjugated secondary antibody in the appropriate concentration, and recommended for the monoclonal antibody used previously, for 30 min.
13. Rinse cells 2× with PBS buffer.
14. Add DAPI nuclear staining (2 µg/mL, in PBS) for 10 min.
15. Remove the DAPI solution, add a drop of fluorescence mounting media and cover the slide with a coverslip.
16. Examine the cells under the microscope.

Scanning electron microscopy (SEM)

Karnofsky's Fixative (helps to preserve cell morphology for electron microscopy)

For 10 mL

2.5 mL 8% paraformaldehyde (final concentration of 2.0% (v/v) paraformaldehyde).

1 mL 25% glutaraldehyde (final concentration of 2.5% (v/v) glutaraldehyde).

5 mL 0.2 M cacodylate buffer.

Make up to 10 mL with distilled water, adjust pH to 7.2–7.4 with 1N NaOH or 1N HCl.

8% Paraformaldehyde (make up fresh on the day for better staining)

a. Dissolve 2 g of paraformaldehyde in 25 mL of water that is already at 60°C (do not let temperature go above 60°C).
b. Stir for 10 min.

c. Add 1–2 drops of 1N NaOH and wait 10 s (the solution should go from cloudy to clear — add more NaOH if required).

d. Let cool until the solution reaches room temperature.

e. Filter.

0.2 M Sodium cacodylate buffer

*All work with sodium cacodylate should be performed in a fume hood.

Sodium cacodylate tryhydrate	8.56 g
Calcium chloride	25.0 g
0.2N hydrochloric acid	2.5 mL

Dilute to 200 mL with distilled water, pH 7.4

SEM preparation

1. Prior to fixation, culture substrates should be washed 2–3 × with α-MEM and then with 0.1 M cacodylate buffer 2–3 × (dilute 0.2 M cacodylate buffer with distilled water).
2. Fixation is best carried out for a minimum of 2 hr in Karnovsky's fixative at 4°C.
3. Rinse with 0.2 M cacodylate buffer three times,
4. Dehydrate in graded alcohols (50%, 70%, 80% 90%, 95% and 100%) for 10 min each. The final step should include at least 2–3 wash steps in 100% anhydrous ethanol.
5. Replace ethanol with 100% HMDS (in a fume hood) and let stand for 30 min.
6. Repeat Step No. 5.
7. Remove HMDS and air dry in fume hood or desiccator.
8. Cut samples to appropriate size and shape for SEM.
9. Mount samples on aluminum stubs using carbon tape.
10. If observation of the matrix/culture surface is desired (i.e. cement line matrix), the overlying cell layers and the collagenous matrix can be partially removed by applying small blasts of compressed air.
11. To reduce charging, apply a small amount of colloidal silver or carbon paint and leave to dry. This paint is useful to bridge the culture substrate to the metal stub — this any charging that occurs can be quickly transferred away from the sample to the metal stub.

12. Sputter coat with gold, platinum/palladium, or carbon. Carbon is useful if energy dispersive X-ray analysis is to be performed to reduce chance of interfering peaks.

13. Store samples in a desiccator.

Energy dispersive X-ray analysis (EDX)

Typically instrumentation for this is attached to a scanning electron microscope or a transmission electron microscope and can be quite useful for elemental analysis and mapping. For example, the presence of calcium and phosphorous can be determined. Furthermore, semi-quantitative calcium to phosphate ratios (Ca:P) can be obtained by integrating the area under the Ca and P peaks. When performing such analysis, it is important to have a positive control consisting of crystalline hydroxyapatite which should have a ratio of 1.67:1.

Fourier transform infrared spectroscopy (FTIR)

*It is imperative for the fixing cells prior to FTIR that PBS is not used. The phosphate in the buffer can interfere with the phosphate signal from the sample.

1. Remove part of the cell culture substrate (approximately 0.5–1.0 mg), using a spatula, and place the sample into an agate mortar containing approximately 100 mg of KBr.

2. The sample and KBr must be ground to a fine powder until it sticks to the mortar.

3. Take the powder sample and place it into a FTIR die-set. Press the powder, using a hand or hydraulic presser, into a pellet with a thickness of about 1 mm. A good KBr pellet is transparent. Opaque pellets give poor spectra, because little of the infrared beam passes through them. White spots in a pellet indicate that the powder is not ground well enough, or is not dispersed properly in the pellets.

4. Before, placing the pellet in a FTIR sample holder, a reference background should be taken. In general, 128 scans between 4000 and 450 cm^{-1}, with a resolution of 2 cm^{-1}, are acquired.

5. Place the pellet in a sample holder and acquire the spectra.

Analysis

Positive identification of bone nodules

To ensure positive identification of bone nodules formed in culture, we recommend using a variety of assays (**Figure 3 I**). Prior to such studies, it is important to understand the hallmarks of *de novo* bone formation as illustrated in **Figure 3 II and Figure 3 III**)

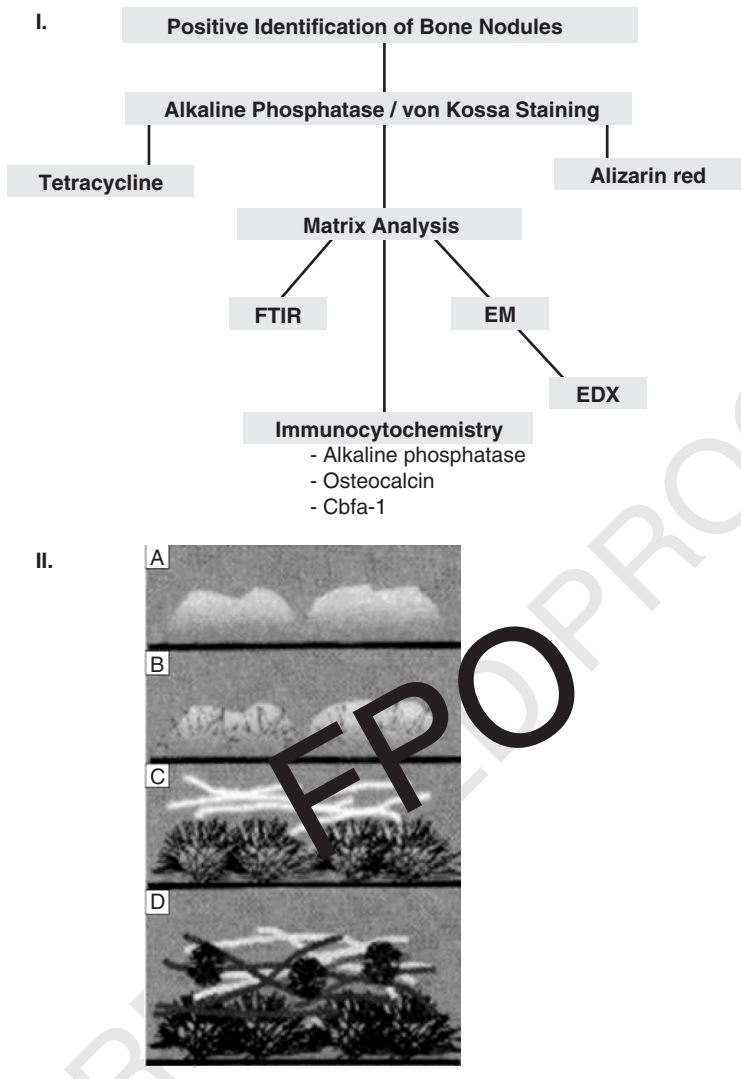

Figure 3 **I.** Experimental flow chart showing steps involved in the positive identification of bone nodules. After first confirming the presence of bone nodules with an alkaline phosphatase/von Kossa stain, the matrix should be examined with FTIR and/or with electron microscopy. Immunocytochemical analysis using a variety of osteogenic markets can be used to justify the results, but on their own are not sufficient to conclude the presence of bone nodules. **II.** Cascade of *de novo* bone formation on a solid surface. (**A**) Differentiating osteogenic cells initially secrete an organic matrix that is rich is non-collagenous proteins which mediates (**B**) the nucleation and formation of calcium phosphate crystals. (**C**) Collagen fibers assemble and anchor to this cement line matrix. (**D**) The overlying collagenous matrix is mineralized (Adapted from Davies, 1996). **III.** A cross-section through a bone nodule illustrates the various phases of *de novo* bone formation and the associated cellular phenotypes (adapted from)

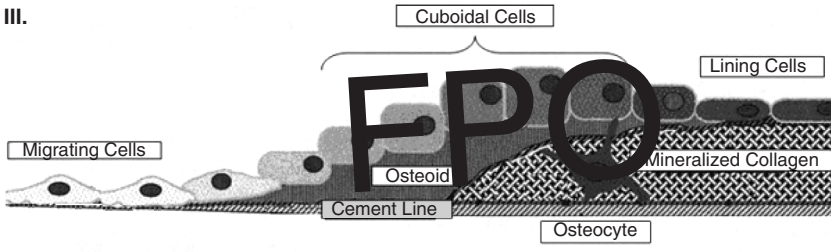

Figure 3 (*continued*)

and what information can be gained from each assay. Instead of relying on merely calcium stains or enzyme stains (i.e. for alkaline phosphatase) investigators are strongly encouraged to first use a dual stain for both alkaline phosphatase and von Kossa (Karp *et al*., 2006; Purpura *et al*., 2003) as described in detail above. Only based on this data can a retrospective analysis be used to correctly derive frequencies of recruited osteoprogenitors. Colonies that express APase but not von Kossa may be representative of a variety of cell types (including those of the osteogenic) but without further analysis, these cells cannot be considered as osteogenic, whereas those staining positive for both APase and von Kossa can be "loosely" considered as colony forming unit osteoblasts (CFU-O) (Baksh *et al*., 2003; Purpura *et al*., 2003). Although, further analysis is required to justify this (Karp *et al*., 2006). If calcium stains such as alizarin red are used to stain for mineralized bone nodules, the pattern of staining should mimic the von Kossa staining pattern, otherwise this could indicate dystrophic mineralization or the presence of other cations that may be identified with this stain. Following positive identification of CFU-O, it is useful to examine the produced matrix with FTIR to confirm the mineral is organized in an apatite form (Bonewald *et al*., 2003). (**Figure 4**). In addition to these methods of analysis, hallmarks of *de novo* bone formation can be examined through ultrastructural studies with electron microscopy.[63]• This is one of the only methods to date that can be used to detect the cement line matrix. Energy dispersive X-ray analysis is also useful for semi-quantitatively examining the calcium to phosphate ratio, which in hydroxyapatite is 1.67:1.

• Q6

Osteogenic markers

For a list of human ES cell markers refer to **Chapter 7**. Alkaline phosphatase is also a marker for osteogenic cells, it is important to use other markers to ensure complete differentiation of the human ES cells. In addition to alkaline phosphatase, classical osteogenic markers include:

Osteocalcin (bone Gla protein, OCN)

Is believed to be exclusively found in bone tissue and dentin. It accounts for 10–20% of the non-collagenous protein in bone and contains three residues of gamma carboxy

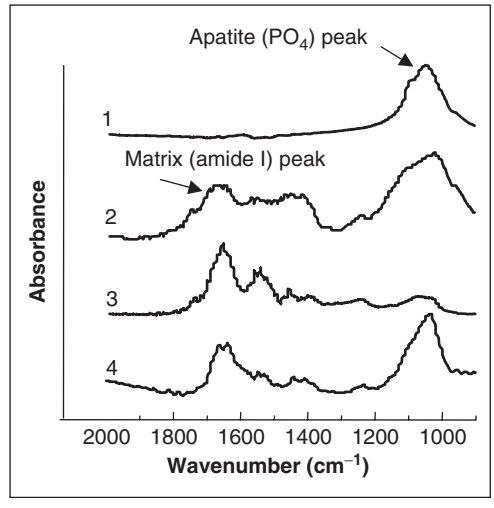

Figure 4 FTIR spectra of (curve 1) hydroxyapatite, (curve 2) human bone, (curve 3) human ES cells cultured under conditions without dexamethasone, (curve 4) human ES cells cultured under conditions with dexamethasone. The mineral and matrix peaks from human bone and hydroxyapatite are comparable to the extracellular matrix produced by the human ES cells

glutamic acid which allows it to bind strongly to bone tissue. This is a late marker of osteogenesis and its expression typically corresponds with mineralization.

Osteopontin (OPN)

Is a glycoprotein synthesized by a variety of cell types including osteoblasts, hypertrophic chondrocytes, macrophages, smooth muscle cells and endothelial cells. Osteopontin is one of the extracellular proteins that constitute the organic component of bone.

Bone sialoprotein (BSP)

Is a phosphorylated glycoprotein that is expressed almost exclusively in bone and other mineralized tissues. BSP is believed to be involved in the nucleation of hydroxyapatite at the mineralization front of bone.

Osteonectin (Secreted protein acidic and rich in cystine — SPARC)

Is a matrix associated glycoprotein that binds to hydroxyapatite and collagen.

Core binding factor alpha1 (Cbfa1 or RUNX2)

Is the osteogenic master gene. It is a transcriptional activator of osteoblast differentiation during embryonic development and is also expressed in differentiated osteoblasts.

Collagen 1

Is the main organic component of bone (**Figure 3 II**) and is also found in other tissues including skin and tendon.

Fourier transform infrared microscopy (FITR)

Through examination of spectral vibrations using FTIR, the nature of the mineral and matrix of bone matrix can be characterized within the same sample without the use of molecular markers. For FTIR analysis of mineralized matrix, there are two main methods which can be used. In one method, dry samples are powdered, mixed with KBr, and pressed into pellets. Alternatively, samples may be analyzed directly within the culture dish which facilitates additional analyses using alternate assays. Regardless of the instrument used, the region of interest spans from 840–1725 cm^{-1} as previously reported (Shimko *et al*., 2004). A single or double band between 900–1200 cm^{-1} (depending on the instrument resolution) is characteristic of the presence of a phosphorus based apatite phase which has the general formula $A_5(PO_4)_3$ where A are cations typically consisting of calcium. The PO_4 can be substituted to a limited extent by a carbonate anion or HPO_4^{-2}. Hydroxyapatite, which has the following composition: $Ca_{10}(PO_4)_6(OH)_2$, may be substituted with a variety of ions which may alter the width the bands. For example calcium may be substituted with lead, strontium, sodium, magnesium, potassium, lithium, iron, manganese, zinc and copper. The hydroxyl group may be substituted by fluorine, chlorine or carbonate. One important metric for examination of bone tissue includes the mineral to matrix ratio which can be obtained by integrating the area under the curve between 900–1200 cm^{-1} (which includes phosphate υ_1 and υ_3 absorption bands) and dividing by the area under the curve between 1585 and 1725 cm^{-1} (collagen amide I band). Although methods exist for quantifying the carbonate fraction (integrating 840 and 890 cm^{-1} (carbonate υ_2 absorption band) and dividing by the area under the phosphate absorption region) and the crystallinity (1020-to-1030 ratio), the ability to perform these types of analysis depend strongly on the spatial resolution and signal to noise ratio of the instrument and the purity of the sample.

Troubleshooting

How do I improve the frequency of osteogenic differentiation, can serum be the problem?

There can be significant differences between lots or suppliers of serum and certain lots of serum may not support osteogenic differentiation and production of bone

nodules. Therefore, it may be useful to assess the ability of the serum to produce bone nodules with a conventional model such as primary rat bone marrow cells where the frequency of bone nodules (or osteoprogenitors) should be approximately 1 in 500 adherent cells (Purpura *et al*., 2004). Using a typical rodent cell culture system, osteogenesis *in vitro* has been demonstrated to culminate in the formation of mineralized nodules which are discrete islands of bone that display histological, ultrastructural and immunohistochemical similarities to bone formed *in vivo* (Baksh *et al*., 2003; Purpura *et al*., 2004). Therefore, it is typically best to screen serum from a variety of lots or suppliers prior to moving ahead with human ES cells experiments. This normally involves determining frequencies of osteoprogenitor cells with a well defined osteogenic differentiation system — i.e. primary rodent cells. After determining the serum that produces the greatest frequency of osteoprogenitor cells (determined indirectly through counting APase and von Kossa stained nodules), it is advisable to purchase enough serum for all projected experiments. Typically it is best to test samples of serum from various suppliers prior to beginning experiments with human ES cells. After determining the serum that produces the greatest frequency of osteoprogenitor cells (determined indirectly through counting APase and von Kossa stained nodules), it is advisable to purchase an excess of serum required for all projected experiments.

Why do I see so much non-specific staining?

Although a number of publications report supplementation of with beta glycerophosphate (β-GP) at a concentration of 10 mM (Sottile *et al*., 2003; Bielby *et al*., 2004) (twice the concentration we suggest), this concentration has been associated with increased levels of dystrophic mineralization (Bonewald *et al*., 2003) and thus 5 mM is more advisable. Previous work has demonstrated that aberrant mineralization and cell death may occur when β-GP is greater than 6 mM (Gronowicz *et al*., 1989). We demonstrated that 5 mM is sufficient for development of bone nodules from differentiated human ES cells (Karp *et al*., 2006). In addition, specific mineralization of osteogenic cultures can be confirmed by demonstrating that mineral stains including tetracycline, alizarin red and von Kossa produce similar staining patterns.

Why do I get an insufficient number of bone nodules from embryoid bodies?

While EB are used as a model for recapitulating the simultaneous formation of multiple tissues during embryonic development, achieving high frequencies of osteoblasts in this system may present a challenge. This may be due to complex cell−cell and cell−matrix interactions in addition to gradients of biomolecules. This creates several microenvironments within each EB where gradients of biomolecules can present different stimuli to the cells. Thus a system devoid of EB may be useful to improve the derivation efficiency of osteogenic cells where one would anticipate more homogenous microenvironments (Karp *et al*., 2006). When using H1 cells it may be more difficult to differentiate these cells along the osteogenic lineage due to problems associated with EB formation with these cells as has been described in one study (Sottile *et al*., 2003). We found that H9 cells readily differentiated to osteogenic lineage with increased frequencies when the EB step is skipped. H9

cells are most commonly used for study of osteogenic differentiation of human ES cells to date.

How do I avoid contamination during differentiation experiments?

To avoid contamination, it is imperative that reconstituted antibiotics stored at 4°C are only kept for 2 weeks. Typically it is convenient to make fresh antibiotic and fully supplemented media solutions once per week. If in some cases penicillin/streptomycin is not enough to stop contamination and antifungal agents such as Fungizone (amphotericin) should be added.

Why do I see unusual cell morphology in SEM images?

If the morphology of cells and matrix within the electron micrographs is not of suitable quality, it may be of interest to replace the final preparation step using
• Q7 HMDS with critical point drying as previously described.[63]• Although the HMDS step significantly reduces processing time, critical point drying is the gold standard method for preparing samples for publication quality images.

How do I determine the level of spontaneous differentiation?

Cultures treated without osteogenic supplements may be used to assess spontaneous differentiation. It may be useful to include ascorbic acid and betaglycerophosphate without dexamethasone (or other differentiation stimulating factors). This condition will thus provide the supplements that osteogenic cells would be able to use to form mineralized collagenous tissue, yet these supplements should not be able to stimulate osteogenic differentiation on their own.

Why do I get variable results from alkaline phosphatase staining?

It is important to know that alkaline phosphatase is both a marker for human ES cells and for osteogenic cells. Given that conventional antibodies for APase cannot differentiate between these two forms, it may be useful to examine APase kinetics during differentiation. One should observe an initial high level of APase followed by a decrease to almost zero and then an increase. This corresponds to a high number of human ES cells that differentiate and lose their APase expression followed by differentiation into osteogenic cells indicated by the re-expression of APase.

How does alkaline phosphatase (APase) and von Kossa (VK) staining work?

The APase/VK protocol is used to stain mineralized nodules in culture (**Figure 5**). Undifferentiated human ES cells exhibit a strong signal for APase, which is a hydrolase enzyme expressed by both human ES cells (Draper *et al*., 2002)

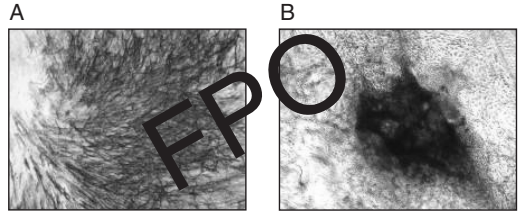

Figure 5 Alkaline phosphatase and von Kossa staining results. (**A**) Unmineralized regions containing colonies of fibroblast will stain red without any black staining. Alternatively, (**B**) mineralized bone nodules will exhibit positive dual staining typically consisting of a black core with red around the boarders

and osteoblasts (Aubin *et al*., 1995) amongst other cell types and is discussed in **Chapter 7.**• APase is present in all tissues throughout the body, and most concentrated in the liver, bile duct, kidney, bone and placenta and its chemical structure varies depending on where it is produced. It is possible that alkaline phosphatases produced by human ES cells and by osteoblasts likely represent different isoenzymes although this needs to be verified. Furthermore, most antibodies and assays for examining alkaline phosphatase cannot distinguish between these isoenzymes.

• Q8

• Q9 Von Kossa has been used to stain mineralized tissues since the •19th century[85]. In a standard reaction, calcium is displaced from phosphate ions and replaced by silver ions, generated from the addition of silver nitrate. Therefore, the Von Kossa technique is used to directly demonstrate the anion (e.g. phosphate) and indirectly the cation (e.g. calcium). The reaction is promoted through providing activation energy with ultraviolet light for 20 min (or a 100 W incandescent desk lamp for 1 hr) thereby reducing the silver, which replaced the calcium, to metallic silver which is visualized as black deposits. The deposits appear black just as small metal particles do when they are shaved from a normally shiny metallic surface. Unreacted silver is typically removed with 5% sodium thiosulfate for 5 min.

An alternative protocol that we prefer avoids exposure to light. Instead, the intensity of the black stain (reduction reaction) can be achieved through addition of sodium carbonate formaldehyde as described below. For those interested, von Kossa protocols using UV light induced reduction reactions are readily available on the internet.

Although immunocytochemistry is a useful technique to identify osteogenic cells and related extracellular proteins, we believe it should be a complement to other techniques describe above. It should be noted that cellular markers for osteogenic cells do not necessarily correlate with the ability of those cells to produce bone nodules (Gronowicz *et al*., 1989). For differentiation experiments from human ES cells, it is important to examine the percentage of human ES cells that remain during differentiation and determine the degree of differentiation by determining the percentage of Oct4+ cells.

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Queries in Chapter 13, Part C

Q1. What does asterisk after Robert Langer's name signify?
Q2. References have been converted to Harvard system. Please check all citations.
Q3. We have shortened the running head, as it exceeds the page size.
Q4. Kindly check quality of figures 2,3,5
Q5. Confirm μL meant here.
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Q7. What is meant by 63 here?
Q8. Reference to APase not found in Chap 7. Please clarify.
Q9. What is meant by 85 here?
Q10. Olivier *et al*. (2006): volume and page nos?