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# Human Embryonic Stem Cell Culture for Tissue Engineering

Shulamit Levenberg[1], Ali Khademhosseini[2], Mara Macdonald[1], Jason Fuller[2], and Robert Langer[1,2]

*Department of Chemical Engineering[1], and Division of Biological Engineering[2], Massachusetts Institute of Technology, 77 Massachusetts Avenue, E25-342, Cambridge, Massachusetts 02139*

*Corresponding author: rlanger@mit.edu*



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## 1. EMBRYONIC STEM CELLS

Embryonic stem (ES) cells are typically derived from the inner cell mass of a blastocyst [Czyz and Wobus, 2001; Evans and Kaufman, 1981]. From the information gathered from murine and other systems, human embryonic stem (hES) cells were successfully derived and characterized in 1998 [Shamblott et al., 1998; Thomson et al., 1998].

ES cells have three unique characteristics. The first is that they can maintain an undifferentiated phenotype [Czyz and Wobus, 2001; Evans and Kaufman, 1981]. The second is that these cells are able to renew themselves continuously through many passages, leading to the claim that they are immortal [Czyz and Wobus, 2001;

Evans and Kaufman, 1981]. The third characteristic is that these cells are pluripotent, meaning that they are able to create all three germ layers (the endoderm, ectoderm, and mesoderm) of the developing embryo and thus can be manipulated to differentiate to form every cell type of an adult organism [Czyz and Wobus, 2001; Evans and Kaufman, 1981]. This third characteristic is what makes ES cells such a powerful tool in regenerative medicine. ES cells may potentially provide an unlimited supply of any of the hundreds of highly specialized cells that can be afflicted with disease in the human body, creating raw material for cell therapy and tissue engineering applications.

## 2. MAINTENANCE AND EXPANSION OF HUMAN EMBRYONIC STEM (HES) CELLS

To generate a therapeutically valuable tissue mass, it is crucial to maintain and expand hES cells in an undifferentiated state. Human ES cells require two to three environmental factors to prevent spontaneous differentiation. The first of these requirements are the factors derived from embryonic fibroblast feeder cells. Embryonic fibroblasts can be derived from murine [Shamblott et al., 1998; Thomson et al., 1998] or human [Richards et al., 2002] sources to sustain the undifferentiated phenotype. Thus far, most analysis on the use of cocultures to prevent hES cell differentiation has been performed on murine embryonic fibroblasts (MEFs). Before use, MEFs are mitotically inactivated, through either irradiation or application of mitomycin C, to prevent overgrowth of the feeder cells in the cocultures. The factors that the MEF cells add to the medium are unknown, but they are crucial for maintaining hES cells in an undifferentiated state [Itskovitz-Eldor et al., 2000; Reubinoff et al., 2000; Thomson et al., 1998].

There are two methods for using MEFs to prevent the differentiation of hES. In the first method, hES cells are cultured directly on a monolayer of mitotically inactivated MEFs [Itskovitz-Eldor et al., 2000; Thomson et al., 1998], allowing for free exchange of MEF-secreted factors with the ES cells. In the second method, hES cells are grown on a layer of diluted Matrigel, which simulates the extracellular matrix that the MEFs provide [Xu et al., 2001]. The ES medium that is fed to these cells is derived from medium conditioned by mitotically inactivated MEFs for approximately 24 hours. Thus the unknown factors that MEFs provide are available to the hES cells without their direct contact [Xu et al., 2001]. This second scenario is useful for applications when MEF contamination in subsequent steps is undesirable.

Two other factors that may play a role in the self-renewal of hES cells are basic fibroblast growth factor (bFGF; FGF-2) and leukemia inhibitory factor (LIF). LIF is of particular interest because its presence has been shown to be sufficient for self-renewal of mouse ES cells [Boeuf et al., 1997; Pease et al., 1990; Williams et al., 1988]. However, although the mouse and human models are similar in many ways, LIF alone does not prevent the differentiation of hES cells and its effect on the human system is not yet clear [Schuldiner et al., 2000].

To ensure that hES cells are pluripotent, their ability to differentiate into all germ layers must be confirmed in vivo. Currently, the most common technique to test for the pluripotency of hES cells is through injection of ES cells in a SCID mouse [Xu et al., 2001]. Because of the lack of an immune system in these mice, the injected ES cells are not rejected and grow into tumors that can then be examined to ensure that the cells can differentiate into all germ layers. Alternatively, morphological and immunochemical assays can be performed in vitro to confirm ES cell potency. The first step is to check the culture to ensure that the cells are morphologically similar to undifferentiated cells (i.e., tight colonies with high ratio of nucleus to cytoplasm). Human ES cells grow in compact colonies that, when undifferentiated, have a bright, even border. The colonies are rounded, with no jagged points or invaginations. The cells inside the colony should be homologous; no structures or variation should be noticeable. Colonies should be checked under a microscope before every passage. The second step is to perform immunohistochemical or immunofluorescent assays to test the expression of ES cell-specific markers [Ling and Neben, 1997; Richards et al., 2002; Shamblott et al., 1998; Xu et al., 2001; Zandstra et al., 2000] such as stage-specific embryonic antigens 3 and 4 (SSEA-3 and -4), Tra-1-60, Oct-4. and alkaline phosphatase (Fig. 3.1, See Color Plate 2).

## 2.1. Preparation of Media and Reagents

### 2.1.1. Stock Solutions

1. Gelatin (1% w/v) in sterile water. The solution should be autoclaved before use to ensure sterility.
2. bFGF: 10 μg of bFGF in 1 ml of 0.1% bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline lacking $Ca^{2+}$ and $Mg^{2+}$ (PBSA). 1 ml of the BSA solution should be used to resuspend the lyophilized bFGF. Immediately after resuspension, 250-μl aliquots should be stored at $-20\,^{\circ}C$.
3. LIF: $10^6$ units/ml. (Optional; See above discussion.)

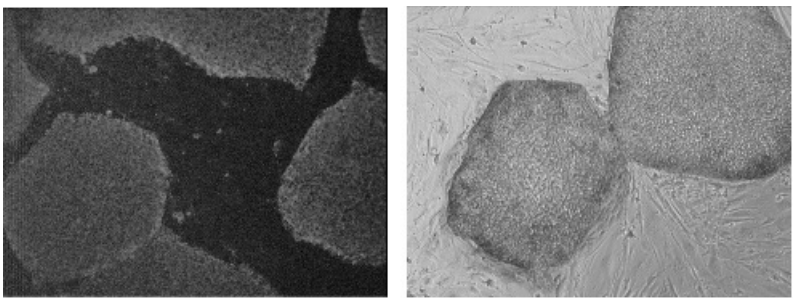

**Figure 3.1.** hES cell colonies grown on inactivated mouse embryonic fibroblasts. Colonies are stained with undifferentiated cell markers (See Color Plate 2): SSEA-4 (in red, left) and alkaline phosphatase (in blue, right).

4. Mitomycin C: 8 μg/ml in DMEM. Care should be taken when handling the powdered form of mitomycin. Use a syringe to add 5 ml of DMEM to the closed bottle of mitomycin by puncturing the top. Once mitomycin is resuspended in the bottle, use the syringe to draw the DMEM and mitomycin out of the bottle, and then dilute it to 8 μg/ml in DMEM and sterilize with a 0.22-μm low-protein-binding filter. Aliquot and freeze at −20 °C until needed.
5. Collagenase type IV: 200 units of collagenase per ml of DMEM, filtered through a 0.22-μm filter. Solution can be used for up to 2 weeks.
6. Trypsin, 0.5% in PBSA.

### 2.1.2. Murine Embryonic Fibroblast (MEF) Medium

(i) Fetal bovine serum (FBS) 10%

(ii) Dulbecco's modified Eagle medium (DMEM) 90%

(iii) Filter the solution, using a 0.22-μm filter bottle.

### 2.1.3. ES Cell Medium

(i) Knockout (KO) serum, 20%

(ii) KO DMEM, 78.3%

(iii) Nonessential amino acid solution, 1%

(iv) 2-Mercaptoethanol, 0.2% (55 mM in PBSA)

(v) L-Glutamine, 0.5% (200 mM in 0.85% NaCl)

(vi) LIF ($10^3$ units/ml)

(vii) bFGF from a stock of 5 ng/ml

(viii) Filter the solution, using a 0.22-μm filter bottle.

### 2.1.4. Embryoid Body (EB) Cell Media

(i) Knockout (KO) serum, 20%

(ii) KO DMEM medium, 78.3%

(iii) Nonessential amino acid solution, 1%

(iv) 2-Mercaptoethanol, 0.2% of a 55 mM stock in PBSA

(v) L-Glutamine, 0.5% of a 200 mM stock in 0.85% NaCl

(vi) Filter the solution, using a 0.22-μm filter bottle.

Serum and stock solutions should be stored at −20 °C.

## Protocol 3.1. Seeding Cryopreserved Murine Embryonic Fibroblast (MEF) Cells

***Reagents and Materials***

*Sterile*

- ❑ MEF cells, frozen stock, $1 \times 10^6$ cells/ampoule
- ❑ MEF medium
- ❑ Conical centrifuge tubes, 15 ml
- ❑ Culture flasks, 75 $cm^2$ (or similar)

***Protocol***

(a) Take the cells from storage in a liquid nitrogen tank.

△ *Safety notes.* Gloves must be worn to protect from frostbite. Great care must be taken with ampoules that have been immersed in liquid nitrogen as they may explode on warming if they have leaked and inspired liquid nitrogen. Wear a face mask, and thaw in a covered bath.

(b) Leave the cryogenic vial in a 37 °C water bath until the contents begin to melt.
(c) Transfer to a sterile hood.
(d) Put the cells in a 15-ml centrifuge tube.
(e) In a drop-by-drop manner add 5 ml MEF medium to the tube.
(f) Centrifuge the tube at 1000 rpm (125 *g*) for 5 min.
(g) Add 15 ml MEF medium to a 75-$cm^2$ tissue culture flask.
(h) Remove the medium from the tube, and resuspend the cells in 2 ml medium.
(i) Seed the cells in the flask, and place them in the $CO_2$ incubator.
(j) Replace the medium with fresh MEF medium the next day.
(k) The MEFs can be maintained in culture for a few passages (4–5 passages) before losing their proliferative potential.

## Protocol 3.2. Seeding Mitotically Inactivated Murine Embryonic Fibroblasts (MEF) for hES Culture

***Reagents and Materials***

*Sterile*

- ❑ Mitomycin C, 8 μg/ml (See Section 2.1.1. (iv))
- ❑ Gelatin, 1% (See Section 2.1.1. (i))
- ❑ PBSA
- ❑ Trypsin, 0.25% in PBSA (GIBCO-Invitrogen)
- ❑ Petri dishes, tissue culture grade, 10 cm
- ❑ Centrifuge tubes, 15 ml

***Protocol***

(a) Remove the medium that is in the flask of confluent MEF.
(b) Add 7 ml mitomycin C solution.
(c) Leave the flask in the $CO_2$ incubator for 2 h.
(d) While the MEF are incubating with the mitomycin, put 3–4 ml 1% gelatin in the bottom of four 10-cm Petri dishes. Spread the gelatin to cover the entire surface and incubate at 37 °C until needed.
(e) After 2 h, aspirate the mitomycin.
(f) Wash 4 times with PBSA.
(g) Remove the last PBSA wash.
(h) Add 2 ml trypsin solution.
(i) Put the flask in the $CO_2$ incubator until the cells are free-floating.
(j) Take the flask from the $CO_2$ incubator and add 5–6 ml MEF medium to stop the trypsin.
(k) Pipette up and down ~10 times to break cell clumps, and then move the contents to a 15-ml centrifuge tube.
(l) Spin down at 1400 rpm (250 *g*) for 5 min.
(m) While cells are centrifuging, take the gelatin-coated Petri dishes from the $CO_2$ incubator, remove the gelatin, and add 10 ml MEF medium to each dish.
(n) Resuspend the cells and divide equally among the pretreated dishes: usually 1 flask into 4 10-cm dishes.

### 2.3. ES Cell Expansion and Passaging

Collagenase is the preferred enzyme for passaging hES cells because it selectively removes the ES cell aggregates from the cocultures, without disturbing the MEF monolayer. Thus it is possible to enrich for hES cells during the passaging process.

## Protocol 3.3. Passaging Human Embryonic Stem Cells (hES) with Collagenase

***Reagents and Materials***

*Sterile*

- ❑ Collagenase, 200 U/ml (See Section 2.1.1 (v))
- ❑ ES medium (See Section 2.1.3)

***Protocol***

(a) Aspirate the medium from the dishes.
(b) Add 4 ml 200 U/ml collagenase solution to each dish.
(c) Leave the dish in the $CO_2$ incubator for 30–45 min.
(d) Add 5 ml ES medium.
(e) Wash the plate gently to remove the ES colonies without removing MEF from the bottom of the dishes.
(f) Move the ES colonies to a 15-ml centrifuge tube.

(g) Wash the plate a second time with 3 ml ES medium to collect any ES colonies that were not taken the first time. Add to the 9 ml of collagenase and medium already in the 15-ml tube.
(h) Spin down at 800 rpm (80 *g*) for about 3 min.
(i) During centrifugation, take plates prepared with mitomycin-inactivated MEF from the $CO_2$ incubator, remove the MEF medium, and add 10 ml ES medium to each plate.
(j) Resuspend the ES cell pellet, and pipette strongly to break the colonies into smaller pieces.
(k) Spin down at 800 rpm (80 *g*) for 3 min.
(l) Aspirate the medium, add new medium, and pipette up and down to resuspend the colonies.
(m) Add the resuspended colonies to the mitomycin-treated MEF plates. Split in the range from 1:4 to 1:10.
(n) Put the dishes in the $CO_2$ incubator.
(o) Change the medium daily.

## Protocol 3.4. Passaging Human Embryonic Stem (hES) Cells with Trypsin

*Sterile*

- ❑ PBSA
- ❑ Trypsin, 0.1%
- ❑ TNS: Trypsin neutralization solution
- ❑ ES medium
- ❑ MEF cells in gelatin-coated dishes (usually split 1 to 4; See Protocol 3.2, Step (n))
- ❑ Conical centrifuge tubes, 15 ml

***Protocol***

(a) Aspirate the medium from the dishes.
(b) Wash once with PBSA.
(c) Add 3 ml trypsin.
(d) Leave the flask in the $CO_2$ incubator 5 min.
(e) Add 6 ml TNS.
(f) Pipette the contents several times to remove all the cells.
(g) Put them in a 15-ml conical centrifuge tube and pipette vigorously.
(h) Spin down at 700 rpm (60 *g*) for about 3 min.
(i) During centrifugation, take new dishes from the $CO_2$ incubator, remove the gelatin, and add 10 ml ES medium.
(j) Resuspend the cells and add to the MEF cells in gelatin-coated dishes.
(k) Return the plates to the $CO_2$ incubator.

## 3. INDUCTION OF DIFFERENTIATION IN ES CELLS

Human ES cells can be induced to differentiate in culture by a number of different techniques. These techniques involve the removal of the chemical signals and molecular cues that induce stem cell self-renewal (See Section 2), while at the same time providing molecular signals that induce differentiation [Assady et al., 2001; Itskovitz-Eldor et al., 2000; Kaufman et al., 2001; Kehat et al., 2001; Levenberg et al., 2002; Mummery et al., 2003; Reubinoff et al., 2001; Schuldiner et al., 2000, 2001; Xu et al., 2002; Zhang et al., 2001]. Typically, stem cells are induced to differentiate in two-dimensional cultures or within a suspension culture of cell aggregates or spheroids that can be derived clonally or from aggregation of many ES cells. These cell aggregates are called embryoid bodies (EBs) because they mimic and recapitulate many aspects of normal embryonic development (Fig. 3.2). Another method that we have developed is the induction of the differentiation and organization of the cells on three-dimensional polymer scaffolds [Levenberg et al., 2003].

### 3.1. Embryoid Body Formation

EBs can be formed by a number of methods including suspending cells in gels that restrict the migration of the cells, placing cells within nonadhesive dishes, and seeding cells within hanging drops that induce aggregate formation of the cells.

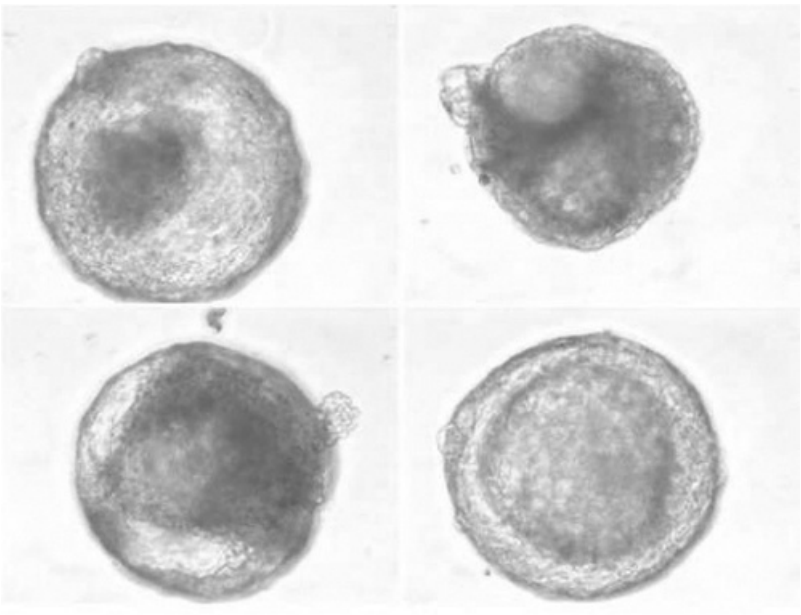

**Figure 3.2.** Human EBs (hEBs). hEBs grown in suspension in differentiation medium form spheres.

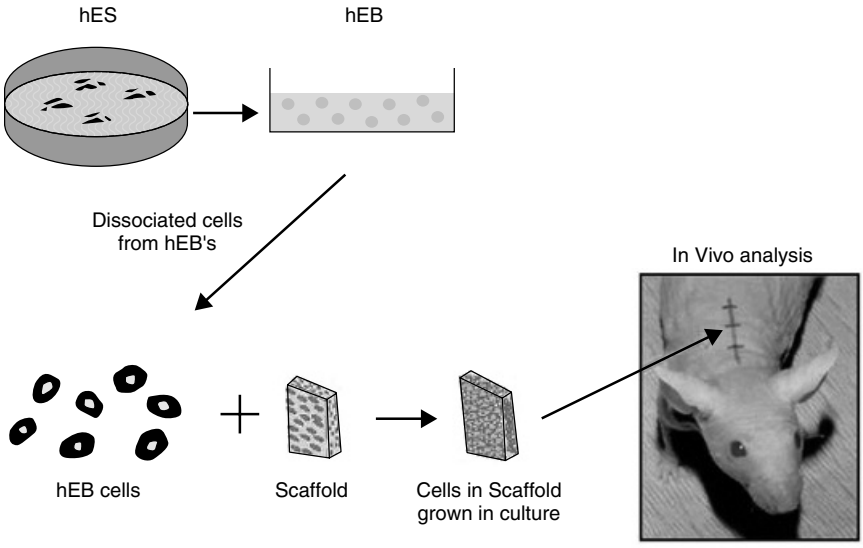


**Figure 3.3.** Differentiation of hES cells on 3D scaffolds. Cells are partially differentiated in EB. EB cells are dissociated, seeded into polymer scaffolds, and cultured in vitro. After culture in vitro and formation of tissue structure, the constructs are then implanted in vivo.

## Protocol 3.5. Formation of Embryoid Bodies in Methylcellulose

### *Reagents and Materials*

*Sterile*

- ❑ Methylcellulose in EB medium [Wiles and Keller, 1991] (can be bought from Stem Cell Technologies, Inc.)
- ❑ Trypsin, 0.1% in PBSA

### *Protocol*

(a) Trypsinize hES cells as previously described (See Protocol 3.4).
(b) Mechanically disperse the cells into a single-cell suspension.
(c) Suspend the ES cells in methylcellulose in EB medium at $1 \times 10^5$ cells/ml and immediately mix the contents vigorously by vortexing.
(d) The methylcellulose solution is viscous and vortexing will form bubbles within the gel, so allow 5–10 min for the bubbles to rise to the top.
(e) Dispense the medium and ES cells into 6-cm Petri dishes, using a 3-ml disposable syringe attached to a 16-gauge blunt-ended needle.
(f) Put the plates in the $CO_2$ incubator.

## Protocol 3.6. Formation of Embryoid Bodies in Nonadhesive Dishes

### *Reagents and Materials*

*Sterility*

- ❑ EB medium (See Section 2.1.4)

- ❑ Collagenase (See Section 2.1.1(v))
- ❑ Petri dishes, non-tissue culture grade, 10 cm

***Protocol***

(a) Detach the ES cells with collagenase as previously described (See Protocol 3.3).
(b) Resuspend the cells in EB medium and add to non-tissue culture-grade polystyrene dishes.
(c) Usually the cells are seeded so that one 10-cm ES dish is split into three 10-cm EB dishes (or similar).
(d) Put the dishes in the $CO_2$ incubator.

After one or two days, the cells typically form clusters that range in size from 50 to 1000 μm.

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## Protocol 3.7. Formation of Embryoid Bodies in Hanging Drop Cultures

***Reagents and Materials***

*Sterile*

- ❑ Trypsin, 0.1% in PBSA

***Protocol***

(a) Trypsinize ES cells as previously described (See Protocol 3.4).
(b) To initiate the cultures, place a drop of medium containing the cells on the inside of the lid of a Petri dish while the lid is turned upside down. Place more than one drop on the lid of the Petri dish if there is sufficient space that the drops will not touch each other. The lid is then quickly inverted right-side up while ensuring that the drops do not merge.
(c) Slowly place the lid back on the Petri dish so that the drop is suspended in the middle of the dish. It is suggested to examine the culture under phase-contract microscope to ensure that each drop contains cells.
(d) Carefully return the dishes to the $CO_2$ incubator.

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### 3.2. Two-Dimensional Confluent Cultures

ES cells can also be induced to differentiate within 2D cultures on removal of the factors that induce their self-renewal. Thus for mouse ES cells, LIF would be removed from the ES cells. However, in the case of hES cells care must be taken to remove the feeder cells from the cultures. This is typically done by dissociating the ES cells from the dishes by a collagenase protocol (similar to Protocol 3.3). The suspended ES cells can then be seeded directly onto tissue culture dishes that have been coated with gelatin.

### 3.3. Three-Dimensional Cultures on Polymer Scaffolds

Recently we demonstrated that hES cells can also be induced to differentiate within biodegradable polymer scaffolds [Levenberg et al., 2003]. Polymer scaffolds [Langer and Vacanti, 1999; Lavik et al., 2002; Niklason and Langer, 2001] represent a promising system for allowing formation of complex 3D tissues during differentiation. They provide physical cues for cell orientation and spreading, and pores provide space for remodeling of tissue structures [Vacanti and Langer, 1999]. In addition, directed degradation of scaffolds can be used as a tool for localized and controlled growth factor supplementation [Richardson et al., 2001]. Ultimately, in vitro-differentiated constructs can potentially be used for transplantation.

## 4. ISOLATION OF SPECIFIC CELL TYPES FROM CULTURES ORIGINATING FROM ES CELLS

So far no ES cell differentiation protocol has resulted in a pure population of cells. The heterogeneity in the ES cell-derived cultures necessitates the isolation of the desired cell types from a heterogeneous population of cells. There are a number of ways in which cells can be isolated for therapeutic or research applications. These methods range from purely genetic approaches to approaches based on morphological and physical properties of the cells.

### 4.1. Immunostaining Followed by Cell Fluorescence-Activated Cell Sorting (FACS)

Individual or combinations of various membrane-bound proteins can be used to distinguish different cell types from each other. Thus labeling cells with antibodies that are specific for particular surface proteins and then sorting the desired cells from the population is an approach that may be used for selecting desired cell types. FACS is readily used to isolate such distinct populations of cells at a rapid and reproducible rate [Eiges et al., 2001]. In this technique, cell surface markers are labeled with fluorescent antibodies. With a FACS flow sorter, the positive population can then be purified from a heterogeneous mixture of cells. The advantage of using FACS is the ability to use a combination of markers, each with a distinctive fluorescent label, for a multiparametric sort. Thus cells that coexpress three or four distinguishing proteins can be labeled and isolated, providing a robust method of isolating desired cells. This approach has been used clinically for characterizing and isolating bone marrow cells [Jurecic et al., 1993; Katayama et al., 1993]. In theory, if cell surface markers that define any cell population are known, that population can be isolated from developing EBs, making FACS a potentially powerful technique.

Despite this power there are several practical limitations of using FACS for cell isolation. For example, a distinctive set of cell surface markers may not be known, or even exist, for a desired cell type. In addition, internal markers such as proteins that reside within the cell cannot be used. Currently, cell permeabilization

is required to mark internal cell proteins, but this kills the cells. Furthermore, the fraction of cells in the desired population may be small (sometimes less than half a percent of the total number of cells), making subsequent expansion of the culture difficult. Finally, completely pure populations of cells are difficult to achieve. Thus if target cells that take a long time to go through population doublings are contaminated with even a few cells of a type that double quickly, within a few passages the culture will be overwhelmed with "weeds," or the undesired, quickly repopulating contaminant cells.

Nevertheless, as the body of knowledge of cell surface markers and techniques for sorting cells improves, FACS will only become more attractive as a method for isolating rare cell populations, both for study and for clinical applications.

## Protocol 3.8. Separating Endothelial Cells from EBs by Immunostaining and Flow Sorting

*Note:* The antibody that is utilized in this protocol has already been conjugated to a fluorescent marker. However, it is possible that under different circumstances, the cell surface marker would be bound by an antibody that would then be attached to a secondary antibody containing the fluorescent signal. Protocol variations for this have been noted below.

### *Reagents and Materials*

*Sterile*

- ❑ EBs, 13- to 15-day culture(with medium changed every 2nd–3rd day)
- ❑ Trypsin, 0.1% in PBSA
- ❑ TNS: trypsin neutralizing solution
- ❑ FBS, 5% in PBSA
- ❑ Conical centrifuge tubes, 15 ml

(a) Take EBs, which have been cultured on nonadherent dishes for 13–15 days, from the $CO_2$ incubator and place in the hood.
(b) Remove the suspension of cells in medium and place in 15-ml conical centrifuge tubes.
(c) Allow the EBs to settle out of the medium (5–10 min).
(d) Aspirate the medium. Try to maximize the amount removed without disturbing the pellet.
(e) Add 7 ml trypsin to 3 to 4 15-ml tubes. Repeat as necessary for additional tubes. Cap tubes very tightly and put on an xyz shaker in an $CO_2$ incubator for 5 min.
(f) Remove tubes from the incubator and pipette up and down strongly to dissociate EBs. If necessary, place back in the incubator for an additional 2 min.
(g) Add 7 ml TNS to each tube.
(h) If necessary, pour the cells through a cell filter to remove any clumps.

(i) Centrifuge the cells for 3 min at 800 rpm (80 *g*).
(j) Resuspend the cells in a small (1–2 ml) volume of 5% FBS in PBSA.
(k) Count the cells.
(l) Reserve approximately $0.5–1 \times 10^6$ cells for a negative control in a sterile Eppendorf tube. (If your antibody is not already conjugated to the fluorescent signal and you will have to apply a secondary antibody containing the fluorescent marker, also reserve a fraction for secondary antibody only). Place this tube on ice.
(m) Spin the rest of the cells down and aspirate the medium.
(n) Add an appropriate amount of the fluorescently labeled antibody to 100–200 μl 5% FBS in PBSA.
(o) Resuspend the pellet in this minimal volume and place on ice.
(p) Every 10 min, flick the tubes to make sure that mixing occurs.
(q) After 30-min incubation, dilute the 100 μl with 10 ml 5% FBS-PBSA.
(r) Spin down the cells.
(s) Resuspend in 5 ml 5% FBS-PBS and spin down (wash).
(t) Resuspend the cells in the volume of 5% FBS-PBS recommended by your sorting facility (1 ml or so) and place in a polypropylene tube. Repeat for the control cells.
(u) Take to the cell sorting machine, along with collection tubes $\frac{3}{4}$-filled with medium.

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Magnetic sorting is an alternative approach that can be used to isolate the desired cells by positive or negative selection [Luers et al., 1998; Wright et al., 1997; Zborowski et al., 1999]. In this approach, instead of using a fluorescent label, small magnetic beads that attach to the primary antibody can be used to label particular cells. The beads are typically attached to the primary antibody by a biotin-streptavidin linkage. In this approach, all cells are then passed through a electromagnetic column. The cells that express the marker of interest are held within the column because of the magnetic attraction of the beads to the column. Thus cells that do not express the desired antigen are washed through the column and collected. Subsequently, the cells that are retained within the column can be collected by switching off the electromagnet.

### 4.2. Genetically Engineered Selectable Markers

A technique that is currently under development to enrich particular cell types is to engineer a cell's gene expression so that the desired progeny is enriched [Friedrich and Soriano, 1991; Moritoh et al., 2003; Soria et al., 2000]. This process of enrichment can be induced either through the activation of suicide genes on the expression of particular genes or the expression of genes that maintain the cells. For example, neomycin resistance (*neo*, aminoglycoside phosphotransferase) can be engineered into ES cells in a construct with a lineage-specific promoter. The expression of neomycin resistance can then be regulated by the promoters that are

activated for the desired cells. The use of this technique and similar approaches promises to be a powerful tool for directed differentiation of ES cells and is an area of active research.

### 4.3. Preferential Detachment and Attachment

Different cell types express various levels of a number of cell adhesion molecules such as integrins. Thus the cell's adherence properties can be used to isolate a specific cell type. This has been used extensively in the isolation of mesenchymal stem cells from bone marrow populations [Pittenger et al., 1999]. However, its utility in ES cell culture has not been tested vigorously.

### 4.4. Hand Enrichment (Mechanical Isolation of Defined Structures)

Hand enrichment of desired cell types in the form of colonies or mechanical isolation of defined structures is another method of isolating the desired cells. The use of such a technique requires visibly distinct morphological properties of the desired cells. For example, beating cardiomyocytes can easily be dissected from a culture of heterogeneous cells. Thus it is possible to isolate the desired cells from a heterogeneous culture based on distinctive morphological properties. However, it is anticipated that such methods will not be efficient for the scale-up that is required for therapeutic applications.

## 5. CHARACTERIZATION OF ISOLATED ENDOTHELIAL PRECURSOR CELLS

As described above (See Section 3), several techniques are available for inducing differentiation of ES cells in the absence of self-renewing agents, and the resulting mixture of cells can be enriched for a specific combination of surface receptor expression by one of the above isolation techniques. The cells derived by these means must be characterized to validate gene expression, phenotype, and in vivo functionality.

Recently, we established the successful isolation of endothelial cells from human ES cells [Levenberg et al., 2002]. The isolation procedure was as follows. The hES cells were grown on gelatin-coated dishes over mitomycin-treated MEF. The growth medium consisted of 80% KO DMEM and 20% KO serum-free formulation, with glutamine, and supplements of $\beta$-mercaptoethanol, bFGF, LIF, and nonessential amino acids [Schuldiner et al., 2000]. To form EB aggregates, the cells were dissociated with 1 mg/ml collagenase type IV and grown in Petri dishes. EBs at 13–15 days were dissociated with trypsin and incubated with fluorescently labeled CD31 antibody for 30 min before cell sorting with a FACStar flow sorter. The $CD31^+$ cells were replated and grown in vitro in endothelial cell growth medium.

### 5.1. Expression of Endothelial Markers

Through studies in animal models, and more recently in humans, a number of related markers, transcriptional factors, adhesion molecules, and growth factor

receptors for endothelial cells have been identified including endothelial cell adhesion molecules such as PECAM1/CD31, vascular endothelial cadherin and CD34; growth factor receptors such as vascular endothelial growth factor receptor-2 and Tie-2; and transcription factors GATA-2 and GATA-3. These molecules have been used to characterize endothelial cells by RNA/gene expression assays (RT-PCR, Northern blot, in situ hybridization) or by immunostaining for protein expression and localization in cell structures [Levenberg et al., 2002].

### 5.2. LDL Incorporation

Functional characterization of endothelial cells involves measuring the uptake of acetylated low-density lipoprotein (ac-LDL) using the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL). This assay apparently has no effect on endothelial cell growth rate at incubation conditions of 10 μg/ml Dil-Ac-LDL for 4 hours at 37 °C {Voyta, 1984}. We have shown that human embryonic-derived $CD31^+$ cells stained brightly for Dil-ac-LDL [Levenberg et al., 2002].

### 5.3. Analysis of In Vitro Tube Formation

Three-dimensional matrices such as collagen or Matrigel are often used to analyze endothelial cell differentiation, vascularization potential, and organization into tubelike structures in vitro. In this method, cells are seeded either on or in the gel (either by mixing the cells with the gel or seeding in between two layers of the gel) [Balconi et al., 2000; Hatzopoulos et al., 1998; Levenberg et al., 2002; Yamashita et al., 2000]. Capillary tube formation can be evaluated by phase-contrast microscopy after seeding the cells for several hours or up to a few days. The effect of growth factors on these processes can also be studied by the addition of growth factors to the culture medium. The structure of the capillary network, stability of the cords over time, and lumen formation (by electron microscopy of the tube cross sections) can be used to characterize the tube structure and lumen size [Grant et al., 1991; Vernon et al., 1995].

### 5.4. Analysis of In Vivo Vessel Formation

In vivo testing is useful for studying the therapeutic potential of ES cell-derived endothelial cells. Various methods have been used to analyze involvement of implanted endothelial cells in the host vasculogenesis and angiogenesis processes. One method involves injecting endothelial cells into chicken embryos to analyze the vasculogenesis potential of the cells and incorporation into vascular structure in the developing embryo [Hatzopoulos et al., 1998]. Endothelial precursors have also been injected into infarcted myocardium and ischemic hindlimb to analyze the effects of the cells on neovascularization and angiogenesis processes [Kocher et al., 2001]. Another method involves seeding endothelial cells into polymer scaffolds and then implanting the cell-scaffold construct in vivo to analyze vessel formation within the implant [Nor et al., 2001]. This technique has been used to

characterize the endothelial cells derived from hES cells. The cells were seeded on highly porous biodegradable polymer scaffolds fabricated from poly-L-lactic acid (PLLA) and poly(lactic acid-co-glycolic acid) (PLGA) that are commonly used as scaffolds for tissue engineering. Sponges seeded with embryonic-derived $CD31^+$ cells were implanted in the subcutaneous tissue of SCID mice and analyzed by immunostaining with human-specific endothelial markers after 1 week and 2 weeks of implantation. We have shown that the implanted cells formed blood vessels in vivo that appeared to anastomose with the mouse vasculature [Levenberg et al., 2002].

Briefly, $PECAM1^+$ cells ($1 \times 10^6$) were resuspended in 50 μl of a 1:1 mix of culture medium and Matrigel and allowed to absorb into the PLLA/PLGA polymer sponges. After a 30-min incubation at 37 °C to allow for gelation of the Matrigel, the cells plus scaffolds were implanted subcutaneously in the dorsal region of 4-week-old SCID mice. After transplantation (7 or 14 days), the implants were retrieved, fixed overnight in 10% (v/v) buffered formalin at 4 °C, embedded in paraffin, and sectioned for histological examination.

## 6. SCALE-UP OF ES CELLS IN TISSUE ENGINEERING

The widespread clinical use of ES cells as tissue engineering precursors will require optimization and standardization of large-scale production of these cells. Fortunately, cells can be expanded nearly indefinitely in the undifferentiated state, but some question remains as to whether it is best to expand these undifferentiated cells to large numbers, or if it is more beneficial to induce differentiation of the cells and then to expand them once differentiated. Regardless of the order, a bioreactor capable of overcoming the nutritional and metabolic limitations characteristic of large cell numbers will be required.

### 6.1. Expansion of Cells in Undifferentiated State

One major distinction between the handling of undifferentiated and differentiated cells is the requirement of undifferentiated cells for feeder cells. This requirement complicates the use of a steady-state chemostat reactor or other such stirred bioreactors. However, some promising results on the growth of hematopoietic [Zandstra et al., 1994], neural [Kallos and Behie, 1999], and ES and progenitor cell numbers in stirred suspension bioreactors cultures have been obtained [Zandstra and Nagy, 2001].

Steady-state stirred suspension reactors are easily scalable and relatively simple. Their relatively homogeneous nature makes them uniquely suited for investigations of different culture parameters (e.g., $O_2$ tension, cytokine concentration, serum components, medium exchange rates) that may influence the viability and turnover of specific stages and types of stem cells.

Stem cell properties are the result of the expression of a specific subset of genes, changes in the expression of which determine exit from the stem cell compartment

into functional cell lineages. Although there is still much to learn about the genes involved in such changes (as well as how they are regulated), it is clear that stem cells interact with many molecules in their extracellular milieu via transmembrane receptors (or receptor complexes) to maintain their viability, and to effect change in their cell cycle progression and differentiated state. A key feature of any stem cell culture system is the combination of cytokines it delivers to the microenvironment of the cells, and how the concentrations of these cytokines and their associated receptors are maintained over time.

Significant efforts have been made to define cytokine and growth factor supplementation strategies to control stem cell responses. The cytokine composition of the medium is particularly challenging to optimize in stem cell cultures because multiple cell types compete for several cytokines that each influence stem cell fate directly or indirectly.

## 7. PROTOCOLS FOR USING ES CELLS IN TISSUE ENGINEERING

One of the major goals of isolating hES cells is their future use as precursor cells for tissue engineering. One option is direction of the differentiation of these cells followed by isolation of the desired cell type. These differentiated cells are theoretically identical to their somatic cell counterparts and therefore can be seeded into scaffolds and implanted identically to any other somatic cells. However, it has been shown that coculture with adult cells directs the differentiation and integration of ES cells with their surrounding cells. This discovery leads to the interesting concept of seeding and implanting undifferentiated ES cells, allowing them to differentiate in vivo.

### 7.1. Seeding Differentiated Cells onto Scaffolds

Seeding differentiated cells into scaffolds will be identical to seeding any cell into the corresponding scaffold (See Section 5.4).

### 7.2. Seeding Undifferentiated ES Cells for In Vivo Differentiation

Adult cells are known to express and excrete some of the proteins and factors that induce the differentiation of ES cells. In addition, ES cells have been shown to fuse with somatic cells and to repair or replace the adult cells. Logically, if undifferentiated ES cells are seeded into scaffolds and transplanted into the site of tissue damage they may differentiate to regenerate the damaged tissue. This process has not yet been attempted in humans but has been successful in treating mice with spinal cord injuries [Langer and Vacanti, 1999; Lavik et al., 2002; Niklason and Langer, 2001].

## 8. CONCLUSION AND FUTURE PERSPECTIVES

ES cells have generated a great deal of interest as a source of cells for tissue engineering. Protocols for growing hES cells are established but will need to be

modified for use of the cells in the clinic. These modifications include establishing growth conditions without feeder cells, to generate a pure population of ES cells, and scale-up of the cell culture. In addition, protocols for the induction of specific differentiated phenotypes are required, including methods for isolating the desired cell types and their characterization. Other challenges in the use of hES in tissue engineering include ensuring the safety of the cells in vivo, that is, ensuring that the cells are immunologically compatible with the patient and will not form tumors, and enhancing efficacy by improving current tissue engineering methods. We are getting close to the day when ES cells can be manipulated in culture to produce fully differentiated cells that can be used to create and repair specific tissues and organs.

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## SOURCES OF MATERIALS

| Item | Catalog No. | Supplier |
|---|---|---|
| bFGF | 13256-029 | Invitrogen (GIBCO) |
| Cell filter | | BD Biosciences (Falcon) |
| Centrifuge tubes, 15 ml | | BD Biosciences (Falcon) |
| Collagenase IV | | |
| DMEM | 11965-118 | Invitrogen (GIBCO) |
| FBS | 16000-044 | Invitrogen (GIBCO) |
| Glutamine | | |
| Knockout DMEM | 10829-018 | Invitrogen (GIBCO) |
| Knockout serum | 10828 | Invitrogen (GIBCO) |
| LIF | ESG1106 | Chemicon |
| 2-Mercaptoethanol | | |
| Nonessential amino acid solution | 11140-035 | Invitrogen (GIBCO) |
| Trypsin Neutralizing Solution (TNS) | | Cambrex (Clonetics) |

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