Human embryonic stem cell culture for Tissue Engineering

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1 Embryonic stem cells

Embryonic stem (ES) cells are typically derived from the inner cell mass of a blastocyst[1, 2]. From the information gathered from the murine and other systems, human embryonic stem (hES) cells were successfully derived and characterized in 1998 [3, 4].

ES cells have three unique characteristics. The first is that they can maintain an undifferentiated phenotype [1, 2, 3, 4]. The second is that these cells are able to renew themselves continuously through many passages, leading to the claim that they are "immortal" [1, 2, 3, 4]. 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 [1, 2]. 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 Protocol for maintaining and expanding 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 fibroblasts feeder cells. Embryonic fibroblasts could be derived from murine [3, 4] or human[5] sources to sustain the undifferentiated phenotype. Thus far, most analysis on the use of co-cultures to prevent hES cell differentiation has been performed on murine embryonic fibroblasts (MEFs). Typically prior to use, MEFs are mitotically inactivated, either through irradiation or application of mitomycin, to prevent overgrowth of the feeder cells in the co-cultures. The factors that the MEF cells add to the medium are unknown, but they are crucial for maintaining hES cells in an undifferentiated state[4, 6, 7].

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[4, 6], allowing for free exchange of MEF secreted factors with the ES cells. In the second format, hES cells are grown on a layer of diluted Matrigel, which simulates the extracellular matrix that the MEFs provide[8]. The ES medium which is fed to these cells is derived from the medium that is cultured with mitotically inactivated MEFs in a separate plate for approximately 24 hours, and then transferred to the plate containing the hES on Matrigel. Thus, the unknown factors that MEF provide are available to the hES cells without their direct contact[8]. This second scenario is useful for applications when MEF contamination in subsequent steps is undesirable.

Two other factors which may play a role in the self-renewal of hES cells is basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF). LIF is of particular interest since its presence has been shown to be sufficient for self-renewal of mouse ES cells[9-11]. However, while 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[12].

To ensure that hES cells are pluripotent, their ability to differentiate into all germ layers must be analyzed *in vivo*. Currently, the most common technique to test for the pluripotency of hES cells is through injection of ES cells in an SCID mouse[8]. Due to the lack of immune system in these mice, the injected ES cells are not rejected and grow into tumors that can then be analyzed to ensure that the cells can differentiate into all germ layers. An *in vitro* alternative to analyze ES cell potency is to perform morphological and immuno-chemical assays in culture. The first 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 roughly spherical, 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 is to perform immunohistochemical or

immunofluorescent assays to test of the expression of ES cell specific markers[3, 5, 8, 13, 14] such as stage-specific embryonic antigens 3 and 4 (SSEA-3 and 4), Tra-1-60, Oct-4 and alkaline phosphatase (Fig 1).

2.1 Preparation of reagents and media

2.1.1 Stock solutions

1. Gelatin (1 % w/v) in sterile water. The solution should be autoclaved prior to use to ensure sterility.
2. bFGF, 10 μg bFGF in 1 mL of 0.1 % BSA in PBS. 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 ^{o}C.
3. LIF, 10^6 Units / mL. (Optional; see above discussion.)
4. Mitomycin, 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 resuspended in the bottle, use the syringe to draw the DMEM and mitomycin out of the bottle, and then dilute it to final concentration. Pass the diluted mitomycin/DMEM solution through a 0.22 μm low protein binding filter to ensure sterility. Aliquot and freeze at -20 ^{o}C until needed.
5. Collagenase type 4, 200 units collagenase per mL of DMEM, filtered through a 0.22 μm filter. Solution can be used for up to 2 weeks.

2.1.2 Murine embryonic fibroblast (MEF) media

Fetal bovine serum (FBS) 10% ,

Dulbecco's Modified Eagle medium (DMEM) 90%,

Filter the solution using a 0.22 μm filter bottle.

2.1.3 ES cell medium

Knockout (KO) serum, 20 %,

KO DMEM, 78.3 %,

Non essential amino acid solution, 1 %,

2-mercapto ethanol, 0.2 % (55 mM in DPBS),

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

LIF (10^3 Units / mL),

BFGF from a stock of 5 ng / mL.

Filter the solution using a 0.22 μm filter bottle.

2.2 Preparation of MEF cells

2.2.1 Seeding MEF cells

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

(ii) Leave the cryogenic vial in a 37 ^{o}C water bath until the contents begin to melt.

(iii) Transfer to a sterile hood.

(iv) Put the cells in a 15 mL Falcon tube.

(v) In a drop by drop manner add 5 mL of MEF medium to the tube.

(vi) Centrifuge the tube at 1000 rpm/min for 5 minutes.

(vii) Add 15 mL of MEF medium to a T75 tissue culture treated flask (or similar).

(viii) Remove the medium from the tube, and resuspend the cells in 2 mL of medium.

(ix) Seed the cells in the flask and place them in the incubator.

(x) Replace the medium with fresh medium the next day.

(xi) The MEFs can be maintained in culture for a few passages before losing their proliferative potential.

2.2.2 Seeding mitotically inactivated MEF for hES culture

(i) Remove the media that is in the flask of confluent MEF.

(ii) Add 7 mL of the mitomycin solution.

(iii) Leave the flask in the incubator for 2 hours.

(iv) While the MEF are incubating with the mitomycin, put 3-4 mL of 1% gelatin in the bottom of four 10 cm tissue culture dishes. Spread the gelatin to cover the entire surface and incubate at 37 ^{o}C until needed.

(v) After 2 hours, aspirate the mitomycin.

(vi) Wash 4 times with PBS.

(vii) Remove the last PBS washing.

(viii) Add 2 mL of trypsin solution.

(ix) Put the flask in the incubator until the cells are free-floating.

(x) Take the flask from the incubator and add 5 to 6 mL of MEF medium to stop the trypsin.

(xi) Pipette up and down ~10 times to break cell clumps and then move the contents to a 15 mL falcon tube.

(xii) Spin down at 1400 rpm for 5 minutes.

(xiii) During that time, take the gelatin coated plates from the incubator, remove the gelatin and add 10 mL of MEF medium to each plate.

(xiv) Resuspend the cells and divide the cells into the pre-treated flasks: usually 1 flask into 4 x 10cm plates.

2.3 ES cell expansion and passaging

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

2.3.1 Passaging ES with collagenase

(i) Aspirate the medium from the plates.

(ii) Add 4 mL of 200 unit / mL collagenase solution to each plate.

(iii) Leave the plate in the incubator for 30 to 45 minutes.

(iv) Add 5 mL of ES medium.

(v) Wash the plate gently to remove the ES colonies without removing MEFs from the bottom of the dishes.

(vi) Move the ES colonies to a 15mL falcon tube.

(vii) Wash the plate a second time with 3 mL ES medium to collect any ES colonies which were not taken the first time. Add to the 5 mL medium along with 4 mL collagenase solution in the 15 mL tube

(viii) Spin down at 800 rpm for about 3 minutes.

(ix) During that time, take plates prepared with mitomycin inactivated MEF from the incubator, remove the MEF medium and add 10mL of ES medium to each plate.

(x) Resuspend the ES cell pellet and pipette strongly to break the colonies into smaller pieces.

(xi) Spin down at 800 rpm for 3 minutes.

(xii) Aspirate the medium, add new medium, and pipette up and down to resuspend the colonies.

(xiii) Add the resuspended colonies to the mitomycin treated MEF plates. Split anywhere in the range from 1:4 to 1:10.

(xiv) Put the plates in the incubator. Change the medium daily.

2.3.2 Passage ES with trypsin

(i) Aspirate the media from the plates.

(ii) Wash one time with PBS.

(iii) Add 3mL of trypsin (0.25 or 0.1 units).

(iv) Leave the flask in the incubator 5 minutes.

(v) Add 6 mL of TNS (Trypsin natutralization solution).

(vi) Pipette the contents many times to remove all the cells.

(vii) Put them in a 15mL falcon tube. Pipette strongly**.**

(viii) Take a Pasteur pipette and remove the gelatin.

(ix) Spin down at 700 rpm for about 3 minutes.

(x) During that time, take new plates from the incubator, remove the gelatin and add 10mL of ES Medium.

(xi) Resuspend the cells and add to the dishes that contain MEF cells (usually split 1 to 4).

(xii) Put the plates in the incubator.

3 Protocols for differentiating ES cells

Human ES cells can be differentiated in culture through a number of different techniques. These techniques involve the removal of the chemical signals and molecular cues that induce stem cell self-renewal (the factors mentioned above), while at the same time providing molecular signals that induce differentiation[6, 12, 15-23]. Typically stem cells are differentiated 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 2). Another method which we have developed is the differentiation and organization of the cells on three dimensional polymer scaffolds (Levenberg et al, PNAS in press).

3.1 Embryoid body formation

EBs can be formed by a number of methods. The techniques include suspending cells in gels that restrict the migration of the cells, placing cells within non-adhesive dishes, or seeding cells within hanging drops that induce aggregate formation of the cells.

3.1.1 Embryoid body (EB) cell media

Knockout (KO) serum, 20 %,
KO DMEM medium, 78.3 %,
Non essential amino acid solution, 1 %,
2-mercapto ethanol, 0.2 % (55 mM in DPBS),
L-glutamine, 0.5 % (200 mM in 0.85% NaCl).
Filter the solution using a 0.22 micrometers filter bottle.
Serum and stock solutions should be stored at -20 ^{o}C.

3.1.2 Methycellulose formation

(i) Trypsinize hES cells as previously described.

(ii) Mechanically agitate the cells into single cells suspensions.

(iii) Suspend the ES cells in a medium of methylcellulose that is diluted with EB medium. Typical cell densities of 1x 10^5 cells / mL are desirable.

(iv) Immediately after the addition of the cells to a tube of methylcellulose, mix the contents vigorously.

(v) The methylcellulose solution is viscous and thus vortexing will form bubbles within the gel so allow 5-10 minutes for the bubbles to rise to the top.

(vi) Dispense the media and into 6 mm Petri dishes and using a 3 mL disposable syringe attached to a 16 gauge blunt end needle.

(vii) Put the plates in the incubator.

3.1.3 Nonadhesive dishes

(i) Collagenize the ES cells as previously described.

(ii) Resuspend the cells in EB medium and add to non treated polystyrene dishes.

(iii) Typically the cells are seeded so that one 10 cm ES plate is split into three 10 cm EB plates (or similar).

(iv) Put the plates in the incubator.

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

3.1.4 Hanging drop cultures

(i) Trypsinize ES cells as previously described.

(ii) To initiate the cultures, place a drop of medium containing the cells on the bottom side of the cap of a Petri dish. It is possible to place more than one drop on the cap of the Petri dish if there is sufficient space so that the drops will not touch each other.

(iii) Slowly place the cap 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.

(iv) Put the plates in the incubator.

3.2 2D confluence cultures

ES cells can also be differentiated within 2D cultures upon removal of the factors that induce their self-renewal. Thus for mES 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 using a collagenase protocol (similar to the protocol used to passage ES cells). The suspended ES cells can then be seeded directly on to tissue culture dishes that have been coated with gelatin.

3.3 3D cultures on polymer scaffolds

Recently we have demonstrated that hES cells can also be differentiated within biodegradable polymer scaffolds (Levenberg et al, PNAS, in press). Polymer scaffolds[24-26] 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[27]. In addition, directed degradation of scaffolds can be used as a tool for localized and controlled growth factor supplementation[28]. Ultimately, *in vitro*-differentiated constructs can potentially be used for transplantation.

4 Protocols for isolating specific cells 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 ranges from using purely genetic approaches to approaches based on morphological and physical properties of the cells.

4.1 Immunostaining followed by cell sorting

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.

4.1.1 Fluorescence Activated Cell Sorting (FACS)

FACS is readily used to isolate distinct populations of cells at a rapid and reproducible rate[29]. In this technique, cell surface markers are labeled with antibodies bound to fluorescent signal molecules. Cells that have the surface marker become brightly labeled compared to cells without the marker. Using a cell sorting machine, the positive population can then be purified from a heterogeneous mixture of cells. The advantage with using FACS is the ability to use a combination of markers each with a distinctive fluorescent label. Thus, cells that co-express 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[30, 31]. In theory, if cell surface markers that define any cell population are known, that population can be isolated from developing EBs, which should have every cell type in the human body. This makes FACS a potentially powerful technique.

Despite this power there are several practical limitations with 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 which in process kills the cells, rendering them useless. 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 improve, FACS will only become more attractive as a method for isolating rare cell populations, both for study and for clinical applications.

4.1.2 Sample Immunostaining/ Sorting Protocol: Sorting out Endothelial Cells from EBs

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. Deviations for this have been noted below.

(i) Take EBs which have been cultured on non-adherent plates for 13-15 days (medium changes every 2^{nd}-3^{rd} day) from the incubator and place in the hood.

(ii) Remove the suspension culture of medium and cells and place in 15 mL conical tubes. Allow the EBs to settle out of the medium (5-10 minutes).

(iii) Aspirate the medium. Try to maximize the amount removed without disturbing the pellet.

(iv) Add 7 mL of trypsin to 3-4 15 mL tubes. Repeat as necessary for additional tubes. Cap tubes very tightly and put on an xyz shaker in an incubator for 5 minutes.

(v) 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 minutes

(vi) Add 7 mL of trypsin neutralizing solution (TNS) to each tube.

(vii) If desired, pour the cells through a cell filter to remove any clumps.

(viii) Centrifuge the cells for 3 min at 800 rpm.

(ix) Resuspend the cells in a small (1-2 mL) volume of 5% FBS in PBS sterile filtered solution. Count the cells.

(x) Reserve approximately 0.5 to 1 million cells for a negative control in a sterile Eppendorf tube. (If your antibody is not already conjugated to the florescent 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.

(xi) Spin the rest of the cells down and aspirate the medium.

(xii) Add an appropriate amount of the antibody label to 100-200 uL of 5% FBS in PBS. Resuspend the pellet in this minimal volume. Place on ice.

(xiii) Every 10 minutes, flick the tubes to make sure that mixing occurs

(xiv) After 1/2 hour incubation, dilute the 100 μL with 10 mL of 5% FBS/PBS. Spin down the cells.

(xv) Resuspend in 5 mL of 5% FBS/PBS and spin down (wash).

(xvi) Resuspend the cells in the volume of 5% FBS/PBS recommended by your sorting facility (1 mL or so) and place in a polyproplyene tube. Repeat for the control cells. Take to the cell sorting machine, along with collection tubes filled 3/4 with medium.

Magnetic sorting is an alternative approach which can be used to isolate the desired cells using positive or negative selection[32-34]. In this approach, instead of using a fluorescent label, a 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 using a biotin-streptavidin linkage. In this approach, all cells are then flown through a magnetic column. The cells that express the marker of interest are held within the column due to the magnetic attraction of the beads with 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 removing the magnetic potential of the column.

4.2 Selective markers (genetic engineering to select for resistant cells)

A technique that is currently under development to enrich for particular cell types is to engineer a cell's gene expression so that the desired progeny is enriched[35-37]. This process of enrichment can be induced either through the activation of suicide genes upon the expression of particular genes or the expression of genes that maintain the cells. For example, neomycin resistance can be engineered into ES cells. The expression of such genes can be regulated by the promoters that are activated for the desired cells. The use of

this technique and similar approaches promise 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 their adherent properties can been used to isolate for desired cell type. This has been used extensively in the isolation of mesenchymal stem cells from bone marrow populations[38]. 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 technique requires distinctive morphological properties of the desired cells. For example, beating cardyomyocytes can be easily detected 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 scale-up that is required for therapeutic applications.

5 Characterization of isolated precursor cells (example of endothelial cells)

As described earlier (section 3), several techniques are available for 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 using one of the isolation techniques. The cells derived from this pathway must be characterized to validate gene-expression, phenotype, and *in vivo* functionality.

Recently, we have established the successful isolation of endothelial cells from human ES cells [17]. 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 supplements of glutamine, ß-mercaptoethanol, bFGF, LIF, and nonessential amino acids [39]. 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 cytometer. 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; 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[17].

5.2 LDL incorporation

To characterize endothelial cells, a functional method involves measuring the uptake of acetylated-low density lipoprotein (ac-LDL) using a fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL). This assay has seemingly no effect on endothelial cell growth rate at incubation conditions of 10 μg / mL Dil-Ac-LDL for 4 hours at 37 ˚C [40]. We have shown that the human embryonic-derived CD31+ cells stained brightly for Dil- ac-LDL [17].

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 tube-like 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 if the gel [17, 41-43]. Capillary tube formation can be evaluated by phase-contrast microscopy after seeding the cells for hours or days. The effect of growth factors on these processes can also be studied by the addition growth factor 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 [44, 45].

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 [41]. Endothelial precursors have also been injected into infracted myocardium and ischemic hind limb to analyze the effects of the cells on neovascularization and angiogenesis process[46]. 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 [47]. 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 following one week and two weeks of implantation. We have shown that the implanted cells formed blood vessels in vivo that appeared to anastomose with the mouse vasculature [17].

Briefly, PECAM1$^+$ cells (1×10^6) were resuspended in 50 µl of 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 matrigel, the cells plus scaffolds were implanted subcutaneous in the dorsal region of 4-week-old SCID mice. After transplantation (7 or 14 days), the implants were retrieved, fixed overnight in 10% (vol/vol) buffered formalin at 4°C, embedded in paraffin, and sectioned for histological examination.

6 Protocols for 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 weather it is best to expand these undifferentiated cells to large number, or if it is more beneficial to differentiate the cells and then expand them once differentiated. Regardless of the order a bio-reactor capable of overcoming the nutritional and metabolic limitations characteristic of large cell numbers will be required.

6.1 Expansion of cells in undifferentiated state

Although the fundamental transport processes governing cells growth are no different for differentiated or undifferentiated cells, undifferentiated cell do have some special requirements to maintain expansion in the undifferentiated state such as their requirement for feeder cells. This requirement complicates the use of a steady state chemostat reactor or other such reactors capable of inducing “ideal mixing”. However, promising results on the growth of hematopoietic[48], neural[49], and ES and progenitor cell numbers in stirred suspension bioreactors cultures have been obtained[50].

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, etc) that may influence the viability and turnover of specific stages and types of stem cells.

Stem cell properties are the result of their 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."

6.2 Expansion of differentiated cells

The expansion of differentiated cells derived from ES cells would be identical to the expansion of that adult tissue regardless of its source.

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 to direct the differentiation of these cells followed by the

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 co-culture 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 the differentiated cells onto scaffolds

Seeding differentiated cells into scaffolds will be identical to seeding any cell into the corresponding scaffold (examples are in this text 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 which induce the differentiation of ES cells. In addition, ES cells have been shown to fuse with somatic cells and repair or replace the adult cell. 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 [26] .

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 using the cells in the clinics. These modifications include growth conditions without feeder cells to have a clean population ES cells and scale up of the cell culture. In addition, protocols for specific differentiation of the cells to desire cell type are required including methods for isolating desired cell type and characterization of the isolated cells. Other challenges in the use of hES in tissue engineering include to ensure safety and efficacy of the cells *in vivo,* to ensure that the cells are immunologically compatible with the patient and will not form

tumors and to enhance current tissue engineering methods. We are getting close to a 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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Legends to Figures.

Figure 1. HES cell colonies grown on inactivated mouse embryonic fibroblasts. Colonies are stained with undifferentiation markers: SSEA-4 (in red-right) and alkaline phosphatase (in blue- left).

Figure 2. Human EBs. hEB grown in suspension in differentiation medium form spheres.

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

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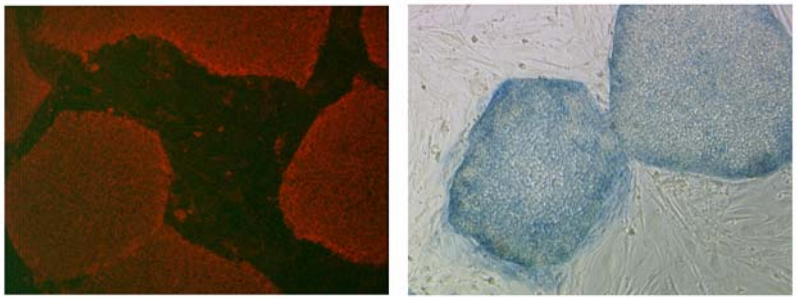
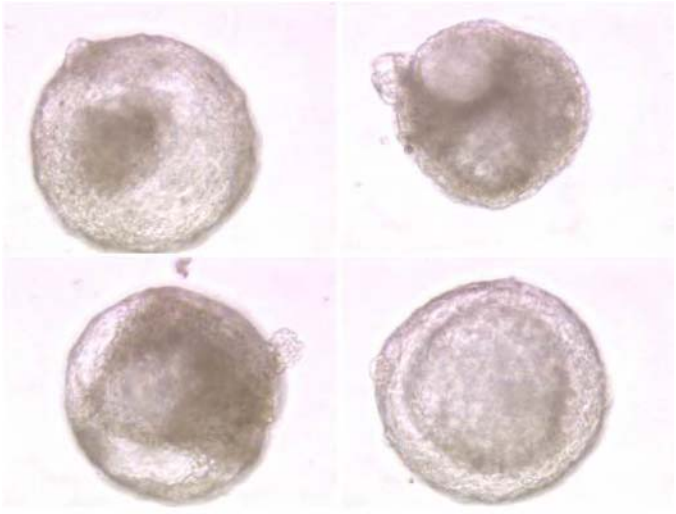


Figure 1.



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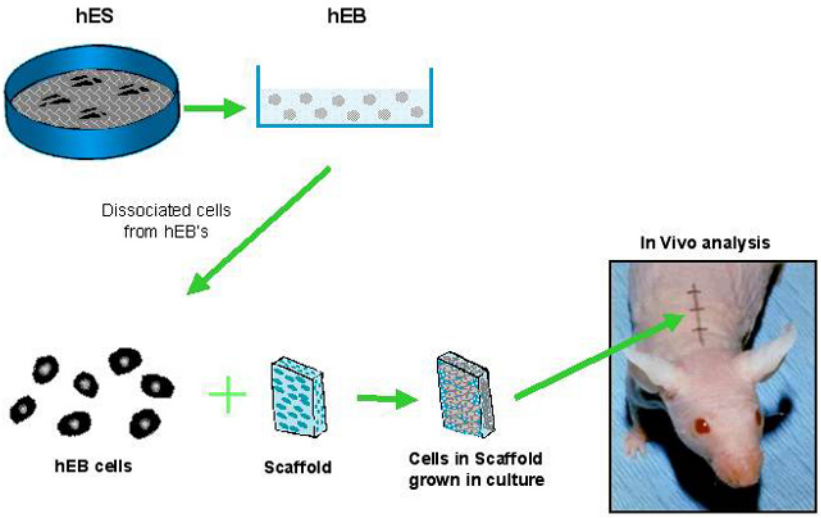

Figure 3