Maintenance and In Vitro Differentiation of Mouse Embryonic Stem Cells to Form Blood Vessels

Embryonic stem (ES) cells are pluripotent cells that have been derived from the inner cell mass of blastocyst-stage embryos. Early studies of the ES differentiation model demonstrated that vasculogenesis and hematopoiesis were among the earliest developmental processes to occur (Doetschman et al., 1988; Schmitt et al., 1991; Wiles and Keller, 1991; Wang et al., 1992). Furthermore, the endothelial cells that differentiate during this model coalesce to form primitive blood vessels that are analogous to the first vessels that form in the developing embryo and yolk sac (Wang et al., 1992). Vascular development during mouse ES cell differentiation in vitro occurs by two processes; vasculogenesis and angiogenesis, similar to embryonic vascular development in vivo (Poole and Coffin, 1989; Risau, 1997). In vasculogenesis, mesodermally derived precursor cells, known as angioblasts, differentiate and coalesce to form a primitive blood vessel. In angiogenesis, endothelial cells from preexisting vessels coordinate cell proliferation and sprouting migration as a basis for expansion of vessels. Primitive erythrocytes and embryonic macrophages mature alongside vascular endothelial cells during ES cell differentiation (Wiles and Keller, 1991; Inamdar et al., 1997; Kearney and Bautch, 2003). Thus this model is ideal for studying molecular and cellular aspects of early vascular and hematopoietic development. This unit describes protocols for maintaining and differentiating mouse embryonic stem cells and methods of analyzing the vasculature via immunohistochemistry and β-galactosidase staining.

A protocol for the formation of embryoid bodies (EBs) and subsequent in vitro differentiation of mouse ES cells is described (see Basic Protocol). In combination with the in vitro differentiation protocol, an explanation of how to test different lots of fetal bovine serum (FBS) for maintaining differentiated cultures has been described (see Support Protocol 8). Methods for analysis of differentiation are included: testing for optimum ES cell differentiation via benzidine staining (see Support Protocol 1) and PECAM/Mac-1 double immunostaining (see Support Protocol 2), antibody immunolocalization (see Support Protocol 3), and *lacZ* staining of in vitro differentiated ES cells (see Support Protocol 4). Other protocols in this unit are designed to provide a method for maintaining mouse ES cells (see Support Protocol 5). A protocol for harvesting 5637 cell–conditioned medium, which contains the LIF that prevents differentiation, is explained (see Support Protocol 6). In addition, a protocol used for testing FBS lots from different manufacturers for ES Medium for maintenance is described (see Support Protocol 7).

NOTE: The protocols in this unit should be conducted under aseptic conditions using sterile reagents and equipment. Experiments should be performed in a Class II Biological Flow Hood.

NOTE: All incubations should be performed in a humidified 37°C, 5% CO_2 incubator, unless otherwise indicated.

PROGRAMMED IN VITRO DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

BASIC PROTOCOL

Mouse embryonic stem cells have the distinctive ability to differentiate into numerous cell types. In vitro ES cell differentiation complements their ability to contribute many tissue types in vivo, thus providing a unique model system for aspects of early mammalian

Contributed by Nicholas C. Kappas and Victoria L. Bautch
Current Protocols in Cell Biology (2007) 23.3.1-23.3.20

development. The conditions that are used initiate a programmed differentiation that leads to the formation of many of the cell types typically found in the mouse embryo and yolk sac. The following utilizes an unmanipulated differentiation protocol that involves the removal of differentiation inhibitory factors, thus allowing the ES cells to undergo a programmed differentiation to form cells that will in turn provide developmental signals to each other. In this case, cultures are examined for vasculogenesis and angiogenesis.

Materials

5- to 6-day-old ES cell culture (Support Protocol 5)
1× CMF-PBS (Sigma)
2.4 U/ml dispase, grade II stock (Boehringer-Mannheim; see recipe)
Differentiation medium (see recipe)

50-ml centrifuge tubes (Sarstedt), sterile
10-cm bacteriological petri dishes (do not use TC-treated plates)
24-well tissue culture plates (Costar)
Medidroppers (Fisher Scientific), autoclaved

NOTE: All volumes are given assuming that one 6-cm dish of ES cell colonies is being used. Double all volumes if using 10-cm dishes.

Collect ES cells

1. Choose the dish that has the best ES cell clumps for differentiation.

   ES cell clumps should be round and differentiated on the very edge and tight, shiny, and undifferentiated in the middle (Figure 23.3.1). ES cell clumps are collected from dishes after incubation for 5 to 6 days at 37°C without feeding after normal passage.

2. Aspirate medium from the ES cell dish. Wash two times, each time with 5 ml of cold 1× CMF-PBS. Aspirate PBS.

3. Add 1 ml of cold 1.2 U/ml dispase (diluted 1:1 with cold CMF-PBS just before use), and let dish sit at room temperature for 1 to 2 min. Check to see if ES cell clumps have detached from dish bottom by shaking dish.

   If the majority of cells clumps have not detached, let the solution sit longer. Dispase produces small clumps of cells that are used to start the differentiation.

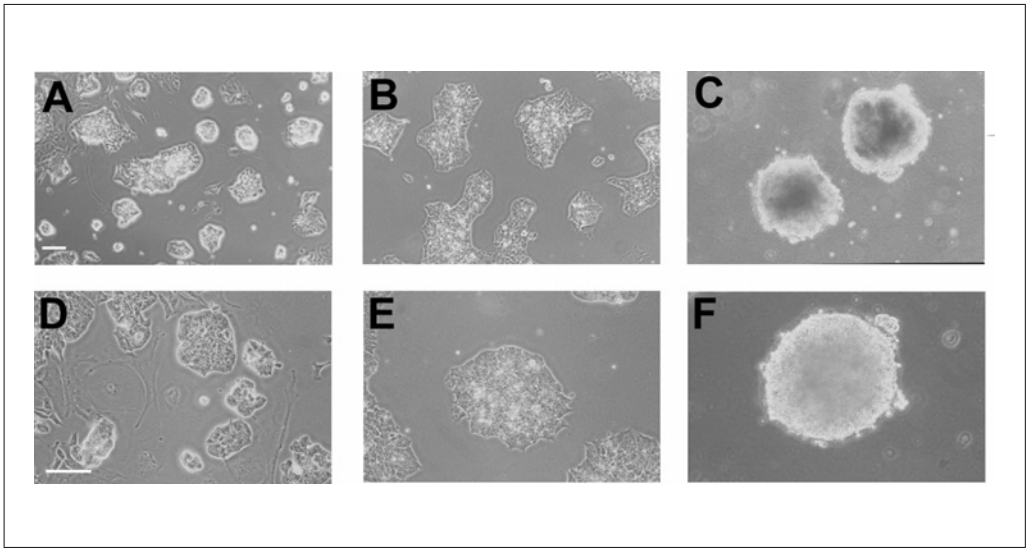

Figure 23.3.1 ES cells are typically processed for passage after 3 days of growth, when they are observed in tight, shiny clumps (**A, D**). ES cells undergo enzymatic disruption treatment for in vitro differentiation on day 5, when the ES clumps are large, but appear differentiated on the edges (**B, E**). Day 3 EBs (**C, F**) prior to plating (Bar = 100 μm).

4. When a majority of the cell clumps have detached from the dish, use a 5-ml pipet to gently transfer the cells into a 50-ml tube containing 35 ml of room temperature 1× CMF-PBS. Rinse the dish with 5 ml of 1× CMF-PBS and add the rinse to the 50-ml tube. Cap tube, and invert the tube once gently to mix.

5. Let the tube sit until the cell clumps have settled to the bottom of the tube.

Wash and plate the clumps

6. Aspirate all but 4 to 5 ml of CMF-PBS, carefully avoiding the ES cell clumps.

7. Add another 35 ml room temperature 1× CMF-PBS gently down the side of the 50-ml tube. Gently swirl the tube to redistribute the cell clumps. Cap tube, and invert gently to mix.

8. Let the tube sit until the cell clumps have settled to the bottom of the tube.

9. Aspirate the CMF-PBS (leaving 2 to 3 ml CMF-PBS/ES cell clump suspension at the bottom), and gently add 5 ml prewarmed (37°C) differentiation medium down the side of the tube.

10. Pipet 10 ml of prewarmed differentiation medium into a labeled 10-cm bacteriological petri dish. Using a 25-ml pipet, transfer the contents of the 50-ml tube (cell clumps/CMF-PBS/medium) to the 10-cm dish.

11. Check the density of cell clumps in each dish (~100 clumps). Incubate at 37°C in a humidified incubator with 5% CO_2.

    The authors attempt to achieve ~100 clumps/dish. Fewer clumps is not an efficient use of medium, while more clumps may allow for individual clumps to aggregate together.

Allow formation of embryoid bodies

12. Change the medium at least every other day after the dispase treatment (day 0). To feed the EBs, remove dish from incubator, place under hood, and gently swirl the dish in a circular manner so that the EBs migrate to the center of the dish.

13. Aspirate spent medium from the dish.

    This is easily done by aspirating along the inside edge of the dish (away from the EBs).

14. Add 10 ml of fresh prewarmed differentiation medium to the dish. Return dish to incubator.

Differentiate embryoid bodies

15. Set up reattachment cultures on day 3 after the dispase treatment (Fig. 23.3.1). Add 1.5 ml of prewarmed differentiation medium to each well of a 24-well tissue culture plate that is to be seeded with EBs.

16. Use a sterile medidropper to transfer EBs from the dish to the wells of a 24-well plate. Generally, dispense between 10 and 20 EBs per well.

    Holding the dish up and looking at it from underneath is helpful when determining the number of EBs in a well.

17. Ensure that the EBs are spread evenly in the well by gently shaking/moving the plate, or if necessary, use a pipettor with a sterile tip to gently pipet medium up and down in the well.

18. Place the plate in the incubator. Keep plate level when placed in incubator to ensure that the EBs do not settle to one side of the well. Incubate at 37°C in a humidified incubator with 5% CO_2.

    Attachment generally occurs within a few hours.

19. Feed the attached cultures every other day. To feed, aspirate medium, then slowly add 1.5 to 2 ml fresh prewarmed differentiation medium down the side wall of the well so as not to disturb the attached cultures.

20. Monitor the cultures for hematopoietic and vascular development (see Support Protocols 1 to 4).

    Using this protocol, the authors typically monitor vascular development in cultures that have been differentiated for 8 days (day of dispase treatment is day 0). Angioblast formation is generally observed at day 4 to 6, while vessel formation occurs at day 6 to 8.

    All volumes given are for staining cultures from a well of a 24-well plate. Gently add/remove all solutions from culture dishes, as harsh removal and addition of solutions may result in detachment of cultures.

SUPPORT PROTOCOL 1

BENZIDINE STAINING

Benzidine staining is performed on day-8 differentiated cultures to visualize the hemoglobin of red blood cells.

Materials

8-day differentiated ES cell cultures (Basic Protocol)
Benzidine solution (see recipe), 4°C
Differentiation medium (see recipe)
1× CMF-PBS (Sigma)

1. Remove 8-day differentiated cultures from incubator and add 0.2 ml of cold (4°C) benzidine solution per 2 ml (per well) of differentiation medium. Incubate at room temperature.

   Benzidine solution is added directly to the cultured cells while still in differentiation medium (i.e., do not rinse cells in CMF-PBS at this time).

   The benzidine reaction will occur immediately: upon addition of benzidine solution, the medium will turn a bluish/brown color, while positive cells will turn blue.

2. Observe/photograph cells within 1 hr, as the stain is not very stable.

   It is recommended that wells be rinsed once with 1× CMF-PBS before taking photographs of stained wells.

SUPPORT PROTOCOL 2

DOUBLE STAINING WITH PECAM AND Mac-1

PECAM/Mac-1 double staining is performed on day 10 differentiated cultures to visualize both blood vessels (PECAM) and embryonic macrophages (Mac-1).

Materials

Day-10 differentiated cultures (from Basic Protocol)
1× CMF-PBS (Sigma)
4% (w/v) paraformaldehyde fixative (see recipe)
1:500 trypsin solution (see recipe)
100% heat-inactivated fetal bovine serum
Staining medium (see recipe)
Primary and secondary antibodies (see recipe);
  Rat anti-mouse CD31 (PECAM-1; Mec 13.3; BD Pharmingen)
  FITC-AffinPure F(ab′)$_2$ fragment of donkey anti-rat IgG (Jackson Immunoresearch)
  Mac-1-BNHS (biotin coupled to Mac-1 antibody); clone M1/70; Rat anti-mouse Mac-1 (BD Pharmingen)
  Streptavidin-RPE (Southern Biotechnology Associates)
Rat serum staining medium (see recipe)

Treat cultures with trypsin

1. Remove day-10 differentiated cultures from incubator and bring plate to room temperature. Remove medium, wash cells twice with 1× CMF-PBS at room temperature, and fix cells in 4% paraformaldehyde (PFA). Use 1 ml of cold 4% PFA/well for 5 to 10 min at room temperature.
2. Aspirate PFA and add 2 ml room temperature 1× CMF-PBS/well. Allow dish to sit for 2 min before aspirating CMF-PBS. Repeat wash 1 more time. Either store at 4°C in CMF-PBS until ready to stain, or proceed with the staining protocol.
3. Add 0.2 ml room temperature 1:500 trypsin solution/well of a 24-well plate. Let 1:500 trypsin solution sit for no more than 60 to 75 sec at room temperature.

   Incubation times longer than 60 to 75 sec may result in complete detachment of differentiated cultures. This step slightly loosens cells and enhances Mac-1 staining.
4. Stop the trypsin reaction by adding 0.5 ml of room temperature 100% heat-inactivated FBS to the well. Immediately remove the solution from the well with gentle aspiration or pipetting.
5. Proceed with immunostaining or store in staining medium at 4°C.

Stain with PECAM

6. Gently add 1.5 to 2 ml (per well of a 24-well plate) of staining medium. Incubate for 1 hr at 37°C to block nonspecific sites.
7. Remove plate from incubator, aspirate staining medium, and add fresh staining medium containing Mec13.3 (PECAM antibody) at 1:1000 dilution. Incubate for 1 hr at 37°C.

   PECAM is expressed by mouse endothelial cells.

   Typically, 250 μl of staining medium/antibody is used per well of a 24-well plate.
8. Remove plate from incubator and aspirate PECAM antibody. Wash wells twice, each time with 2 ml room temperature staining medium.
9. Add fresh staining medium containing FITC-AffinPure F(ab')$_2$ donkey anti-rat antibody at 1:100 dilution. Incubate for 1 hr at 37°C in the dark.

   Typically, 250 μl of staining medium/secondary antibody is used per well of a 24-well plate.

Stain with Mac-1

10. Remove plate from incubator and aspirate FITC reagent. Wash wells twice, each time with 2 ml staining medium at room temperature.

    If desired, the plate can be stored at 4°C, in the dark, for later processing. Be sure to leave staining medium in wells.
11. Add 2 ml/well of rat serum staining medium. Incubate for 30 min at 37°C in the dark.

    If desired, the plate can be stored at 4°C, in the dark, for later processing. Be sure to leave rat serum staining medium in wells.
12. Remove rat serum staining medium. Add fresh rat serum staining medium containing Mac-1-BNHS at 1:800. Incubate for 1 hr at 37°C, in the dark.

    Mac-1 is expressed by embryonic macrophages.

    Typically, 250 μl of rat serum staining medium/ antibody is used per well of a 24-well plate.

13. Remove Mac-1-BNHS and wash well(s) twice with 2 ml staining medium at room temperature. Add fresh staining medium containing streptavidin-R-PE at 1:800. Incubate for 1 hr at 37°C in the dark.

    Typically, 250 μl of staining medium/secondary antibody is used per well of a 24-well plate.

14. Remove streptavidin-RPE and wash well(s) once with 2 ml staining medium at room temperature, then aspirate. Next, wash well(s) twice with 2 ml 1× CMF-PBS. Add 2 ml CMF-PBS and store plate at 4°C in the dark.

15. Examine the plate using fluorescence microscopy with the appropriate filter sets.

SUPPORT PROTOCOL 3

IMMUNOLOCALIZATION

An important aspect of a model system is that it recapitulates the expression of molecular markers seen in vivo. Much of the vascular development that is observed in this in vitro ES differentiation system is analogous to blood island and vascular development that is observed in the mouse embryo and yolk sac. Fortunately, it is with general ease that one can observe primitive ES cell–derived mouse blood vessels using several markers and protocols. There are a number of identified molecular markers for the early mouse vasculature. Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD-31), VEGF (vascular endothelial growth factor) receptor 2 (Flk-1), VE-cadherin, and intercellular adhesion molecule 2 (ICAM-2) have proven particularly useful in visualizing primitive vessels. It has been shown that PECAM-1 and Flk-1 are expressed on mouse angioblasts and endothelial cells (Vittet et al., 1996; Redick and Bautch, 1999). Other vascular markers such as ICAM-2 and VE-cadherin are detectable later, when endothelial cells coalesce to form vessels. This is a protocol for visualizing expressed genes in the in vitro ES cell differentiation model system that utilizes antibodies specific for several vascular markers.

Materials

Differentiated ES cell culture (from Basic Protocol)
1× CMF-PBS (Sigma)
Methanol/actetone fixative (see recipe): 1:1 (v/v) methanol (Fisher Scientific)/acetone (Mallinckrodt) *or* 4% (w/v) paraformaldehyde (PFA; Polysciences; see recipe)
Staining medium (see recipe)
Primary and secondary antibodies (see recipe)
Fluorescent microscope, inverted (equipped with epifluorescence and camera)

NOTE: All volumes given are for staining cultures from a 24-well plate. Gently add and remove all solutions from culture plates, as harsh removal and addition of solutions may result in detachment of cultures.

Fix the cells

1. Remove differentiated cultures from incubator, and allow to slightly cool. Aspirate medium and wash twice with 2 ml/well of 1× CMF-PBS at room temperature.

2. Add 1 ml of cold methanol/acetone fixative/well and incubate for 5 min at room temperature.

    PECAM-1 and ICAM-2 antibodies work in both PFA and methanol/acetone fixatives. Both fixatives are listed here because some applications (not those described here) favor one fixative over another. The authors generally use methanol/acetone fixative because it is less harsh on the attached cultures.

3. Aspirate fixative and add 2 ml of room temperature 1× CMF-PBS/well. Allow plate to sit for 2 min before aspirating CMF-PBS. Repeat wash two more times. Either store at 4°C in CMF-PBS until ready to stain, or proceed with the staining protocol.

Stain the cells

4. If the plate has been stored at 4°C after fixation, allow the plate to come to room temperature.
5. Aspirate 1× CMF-PBS, and gently add 1.5 to 2 ml of staining medium. Incubate for 45 min to 1 hr at 37°C.
6. Remove plate from incubator, aspirate staining medium, and add fresh staining medium containing the properly diluted primary antibody. Incubate for 1 to 2 hr at 37°C.

   Typically, 250 μl of staining medium/primary antibody is used per well of a 24-well plate.

7. Remove plate from incubator, aspirate, and wash well(s) two times (2 to 3 min/wash) with 1.5 to 2 ml staining medium. Add fresh staining medium containing the properly diluted secondary antibody. Incubate for 1 hr at 37°C.

   Typically, 250 μl of staining medium/secondary antibody is used per well of a 24-well plate.

8. Remove plate from incubator, aspirate, and wash well(s) one time for 2 to 3 min with 1.5 to 2 ml staining medium. Aspirate wash, then wash one to two times (2 min/wash) using 1× CMF-PBS.
9. Aspirate last wash and add 1.5 to 2 ml of 1× CMF-PBS to each well, store in CMF-PBS at 4°C in the dark.
10. To visualize the vasculature, set up an inverted microscope equipped with epifluorescence and a camera.

β-GALACTOSIDASE STAINING

SUPPORT PROTOCOL 4

Another commonly used technique to visualize blood vessels in the in vitro ES cell differentiation model system relies on the β-galactosidase reporter system. The authors have mouse ES cells in which expression of the *lacZ* gene is under the control of a vascular promoter (Flk-1, Flt-1). The β-galactosidase protein is the gene product of *lacZ* and is easily assayed. β-galactosidase catalyzes the hydrolysis of the colorless substrate Xgal, to form a blue precipitate in β-galactosidase-expressing cells. This protocol describes the steps involved in β-galactosidase staining of ES cell–derived blood vessels.

Materials

Day-8 differentiated cultures in 24-well plate (Basic Protocol)
0.1 M phosphate buffer, pH 7.3 to 7.5 (see recipe)
Glutaraldehyde fixative solution (see recipe)
Xgal stain (see recipe)
Wash buffer (see recipe)

NOTE: All volumes given are for staining cultures from a 24-well plate. Gently add and remove all solutions from culture dishes, as harsh removal and addition of solutions may result in detachment of cultures.

1. Remove differentiated cultures from incubator, and allow to slightly cool. Aspirate medium and wash well(s) once with 1.5- to 2 ml 0.1 M phosphate buffer (pH 7.3 to 7.5) at room temperature.

2. Aspirate phosphate buffer and add 1 ml glutaraldehyde fixative solution to each well and fix for 5 min at room temperature.
3. Aspirate fixative. Wash cells three times (5 min/wash), each time with 1.5 to 2 ml 0.1 M phosphate buffer at room temperature.
4. Aspirate phosphate buffer. Add 200 µl Xgal stain to each well. Stain cells for 4 hr to overnight at 37°C depending on level of *lacZ* activity.
5. After staining, remove Xgal stain, add 1 ml wash buffer to each well, aspirate, then replace with 1 ml wash buffer to each well.
6. Examine plates for blue-staining structures indicating blood vessels (see Fig. 23.3.2C).

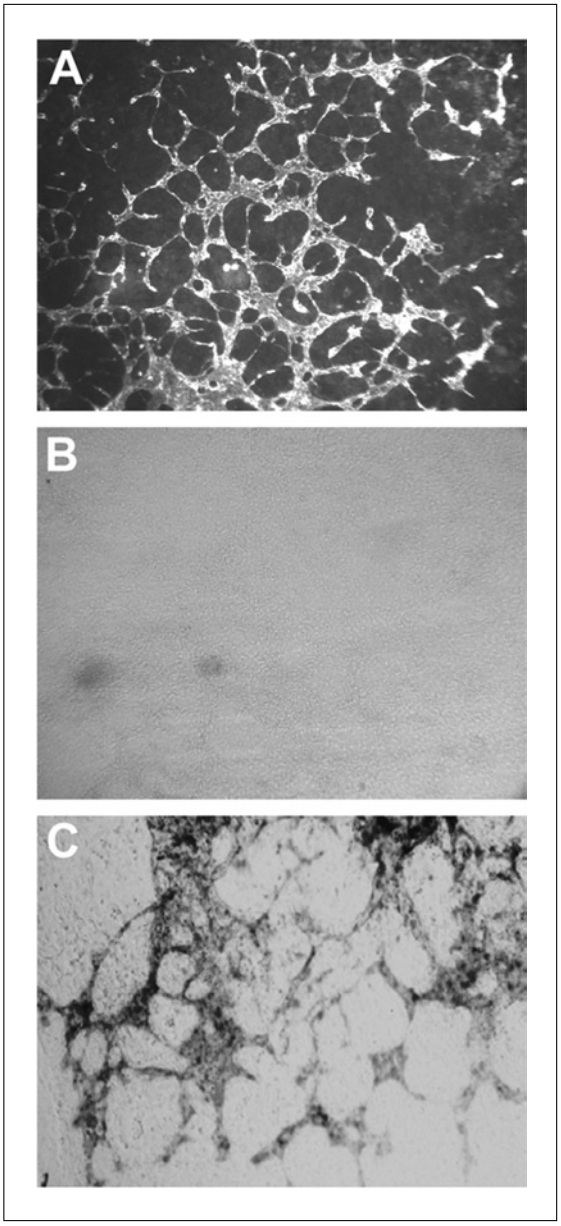

Figure 23.3.2 The vasculature of differentiated ES cell attached cultures can be visualized by both immunolocalization (PECAM stain) and enzymatic reaction (β-galactosidase stain). (**A, B**) ES cell culture differentiated to day 8, fixed and stained using the PECAM antibody, depicting a typical vascular pattern by immunofluorescence (**A**), and the corresponding phase image (**B**). (**C**) ES cell culture differentiated to day 8, fixed and stained for β-galactosidase, showing a vascular pattern (**C**) in phase.

CULTURING MOUSE EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER CELLS

There are numerous published protocols describing mouse embryonic stem cell (ES) maintenance (Bautch, 2002; Kearney and Bautch, 2003). Here the authors describe the maintenance of mouse ES cells for in vitro differentiation. Leukemia Inhibitory Factor (LIF) is a leading component that contributes to stem cell maintenance and prevention of cell differentiation. Traditionally, ES cells are maintained on a layer of feeder cells (usually mouse embryo fibroblasts or STO cells), which provide LIF to the surrounding ES cells. Here the authors describe the maintenance of ES cells using medium that has been conditioned by the 5637 human bladder cancer cell line. This cell line produces LIF that is subsequently used for ES cell maintenance. The authors prefer the 5637 cell–conditioned medium to commercial LIF because they feel that it best preserves the undifferentiated morphology of the ES cells grown off of feeder layers.

Materials

3- to 4-day-old ES cell dishes (*UNIT 23.2*), without feeder layer, passage 3 to 45
1× CMF-PBS (Sigma)
0.25× trypsin/EDTA (see recipe)
Trypsin stop solution (see recipe)
ES cell culture medium (see recipe)

Gelatin-coated dishes (see recipe)
6-cm tissue culture (TC)–treated culture dishes (Corning)

NOTE: All volumes are given assuming that one 6-cm dish of ES cell colonies is being used. Double all volumes if using 10-cm dishes. Prewarm all solutions to 37°C prior to use.

1. Remove 3- to 4-day-old ES cell dish(es) from 37°C incubator. Aspirate medium. Wash two times, each time with 5 ml 1× CMF-PBS at room temperature and then remove.

   ES cells must be passed frequently to prevent differentiation. The authors typically pass ES cells every 3 to 4 days. On the day of passage, ES colonies are typically found in tight, shiny clumps (Fig. 23.3.1). If the colonies are large and flat, the optimal time for passage has passed.

2. Add 1 ml 0.25× trypsin/EDTA solution to dish. Place the dish in 37°C incubator until a majority of ES cell clumps disassociate upon gentle agitation (1 to 3 min).

   This 1-ml volume should just cover the bottom of the dish.

3. Stop trypsinization reaction by adding 4 ml trypsin stop solution to dish. Gently draw the ES cells/trypsin stop solution up and down with a pipet a few times to break up the cell clumps.

4. Remove a gelatin-coated 6-cm dish from incubator. Aspirate gelatin solution, and add 5 ml pre-warmed ES cell culture medium. Add 2 to 3 drops of the cell suspension into the dish. Observe the sizes of ES cell clumps under a microscope.

   ES cells should be in a single-cell suspension, or in clumps of no more than 4 to 6 cells/clump. If cell clumps are significantly larger, pipet solution to further break apart cell clumps.

5. Place dish in 37°C incubator with 5% CO_2 and gently move dish in a back-and-forth motion in order to evenly disperse ES cells throughout the dish.

SUPPORT PROTOCOL 6

PREPARATION OF 5637 CELL–CONDITIONED MEDIUM (CM)

The conditioned medium of confluent 5637 cells contains LIF (leukemia inhibitory factor/differentiation inhibiting activity), which is used as a medium component for the maintenance of ES cell cultures.

Materials

5637 human bladder carcinoma cell line (ATCC #HTB9)
5637 cell growth medium (recipe)
Collection medium (see recipe)

6- and 15-cm tissue culture (TC)–treated culture dishes (Corning)
15- and 50-ml centrifuge tubes (Sarstedt)
200-ml centrifuge tubes, if desired (NUNC)
1-, 3-, 10, and 25-ml disposable pipets
0.22-μm cellulose acetate filter units with bottle (Corning) *or* Nalgene SFCA 0.2-μm bottle top filter with 33-mm neck to fit standard 500-ml glass bottles
500-ml bottles, autoclaved

NOTE: The following protocol describes the collection of 5637 medium from one 15-cm plate, however, more plates are typically processed for collection. Adjust volumes as needed if more plates are to be processed for 5637 collection.

1. Thaw and grow 5637 cells in 5637 cell growth medium to 80% to 90% confluence (usually 2 to 3 days after thaw) in 15-cm tissue-culture dishes. Grow cells in 37°C incubator.

   The authors typically use 25- to 30 ml 5637 cell growth medium/15-cm dish.

2. At cell confluence (2 to 3 days), remove dishes from incubator, aspirate 5637 cell growth medium and feed cells using collection medium. Use 25 to 30 ml collection medium/15-cm dish.

3. After 48 hr, remove the dishes from the incubator and carefully transfer the 5637–conditioned medium from the 15-cm dishes of cells to 50-ml or 200-ml centrifuge tubes using a 25-ml pipet. Avoid scraping the monolayer of cells. Add 25 to 30 ml of fresh collection medium and return plate to incubator.

   The addition of fresh medium to the dish allows for subsequent collection of additional 5637–conditioned medium.

4. Balance the volumes in the 50-ml tubes and centrifuge 10 min at 770 × *g*, 4°C.

5. In the hood, remove the supernatant from each tube and sterile filter through a 0.22-μm cellulose acetate filter unit.

   The supernatant will take a long while to filter.

6. After filtration is complete, place 3 ml of the filtered 5637–conditioned medium in a 6-cm dish into the incubator and check for contamination after 24 hr.

7. Collect conditioned medium every 2 to 3 days for 5 or 6 collections (30 ml/collection/plate). Combine/pool the filtered collected media. Store 5637 cell–conditioned medium up to 1 month at 4°C. Freeze medium at −20°C for use at a later time (up to 6 months). Use the conditioned medium in ES cell culture medium.

   Collect conditioned medium until the cells look sub-optimal (usually after 5th collection). Suboptimal cells will typically detach from the dish or have a disrupted cell monolayer.

ES cell culture medium containing individual pooled collections of conditioned medium is subsequently used for maintenance of ES cell cultures. It is important for 5637 cell– conditioned medium to maintain ES cells in an undifferentiated state. Overly differentiated ES cells, as evidenced by large, flat cells in colonies and no shiny center, are an indication of sub-optimal 5637 cell–conditioned medium.

LOT TESTING OF FBS FOR PASSAGE OF ES CELLS

SUPPORT PROTOCOL 7

A number of different lots of FBS (in parallel with the current lot) are tested in ES medium to ensure the best maintenance and growth of undifferentiated ES cells. To test different lots of FBS, ES cells are maintained in ES medium containing one lot of FBS (one lot of FBS/batch ES cell culture medium), for three passages. During these three passages, ES cells are analyzed for their proper morphology (shiny, tight clumps of cells) and growth. After this point, ES cells are differentiated to day 8, then analyzed for a healthy and normal vasculature pattern via PECAM antibody staining. In this way, ES cells maintained in medium containing different FBS lots during cell passage can be compared. Typically, three to four lots of FBS are tested independently in batches of ES cell culture medium.

Materials

ES cell culture medium (see recipe), prepared with different lots of FBS
3- to 4-day-old ES cell culture dish (Support Protocol 5)
6-cm tissue culture–treated culture dishes (Corning)

Additional reagents and equipment on in vitro differentiation of ES cells (Basic Protocol), passaging of ES cells (Support Protocol 6), and fixing and staining differentiated cultures (Support Protocol 3)

1. Prepare different batches of ES cell culture medium, with each batch containing a different lot of FBS including the currently used lot. To test FBS lots, use the same ES clone to test all lots.
2. Remove 3- to 4-day-old ES cell culture dish from 37°C incubator. Passage ES cells (Support Protocol 6) setting up one to two 6-cm plates of ES cells per lot of FBS to be tested.
3. Passage ES cells for three passages using the same ES cell culture medium and FBS lot to be tested to maintain the same group of ES cells for the duration of the three passages.

   One parameter used to assess whether an FBS lot is of good quality is if ES cells maintain an undifferentiated morphology of tight, shiny clumps for the duration of the three passages.

4. After completion of the third passage, in vitro differentiate the groups of ES cells (Basic Protocol).
5. On day 8 of in vitro differentiation, remove cells from incubator. Fix and stain differentiated cultures with anti-mouse PECAM antibody (Support Protocol 3).

   By maintaining/differentiating ES cells and assessing the vasculature (via PECAM antibody staining) of ES cells maintained in different lots of FBS, it is possible to evaluate the ability of each lot of FBS to properly maintain ES cells to form blood vessels.

SUPPORT PROTOCOL 8

LOT TESTING OF FETAL BOVINE SERUM FOR IN VITRO DIFFERENTIATION OF MOUSE ES CELLS

A number of different lots of FBS are tested in differentiation medium to ensure the best in vitro differentiation of ES cells into blood vessels. To test different lots of FBS, differentiated ES cells are maintained in differentiation medium containing a unique test lot of FBS (one lot of FBS/batch of differentiation medium), until day 8 of differentiation. Day 8 differentiated cultures are then fixed and stained for vasculature (PECAM stain; Support Protocol 2), red blood cell differentiation (benzidine stain; Support Protocol 1), and embryonic macrophages (Mac1 stain; Support Protocol 2). This way, differentiated cells maintained in medium containing different FBS lots can be compared by the expression of markers that are observed in differentiated ES cultures.

Typically, three to four lots of FBS, including the lot currently in use, are tested independently in batches of differentiation medium.

Materials

Differentiation medium (see recipe), prepared with different lots of FBS
5-day-old to 6-day-old ES cell culture (Support Protocol 5)

Additional reagents and equipment for in vitro differentiation of ES cells (Basic Protocol)

1. Make up different batches of differentiation medium, with each batch containing a different lot of FBS. To test FBS lots, use the same differentiated clone for all test media.
2. Remove 5-day-old ES cell dishes from incubator. Ensure that there is one 6-cm dish per batch of differentiation medium (containing a unique FBS lot) to be processed for in vitro differentiation.
3. In vitro differentiate ES cells (see Basic Protocol).
4. For each group of differentiated ES cells (corresponding to each FBS lot to be tested), set up attached cultures as described in Basic Protocol (steps 15 to 20). Attach each group of differentiated cells in four wells in each of two 24-well plates.

   The four wells of one 24-well plate will be used for benzidine staining, while the four wells of the other 24-well plate will be used for double immunostaining with PECAM and Mac-1.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antibodies, primary and secondary (and streptavidin-R-PE)

Dilute all antibodies in staining medium (see recipe).

Primary antibodies: Dilute rat anti-mouse PECAM-1 (CD31; Pharmingen) to 1:1000, rat anti-mouse ICAM-2 (3C4; Pharmingen) to 1:500, rat anti-mouse Mac-1-BNHS (Pharmingen, no. 01711D) to 1:800. Prepare just prior to use.

Secondary antibodies: Dilute reconstituted secondary antibody goat anti-rat Alexa Fluor 488 (Molecular Probes) to 1:250 (for detecting PECAM-1 and ICAM-2), FITC-AffinPure F(ab')$_2$ donkey anti-rat to 1:100 (Jackson Immunoresearch, no. 712-096.150), and streptavidin-R-PE (Southern Biotechnology, no. 7100-09) to 1:800. Store at 4°C after reconstitution (according to the manufacturer) for up to 6 months.

Do not freeze reconstituted antibodies.

Benzidine solution

Stock solution: Prepare a benzidine base stock solution that is 3% (w/v) benzidine base (Sigma) in 90% (v/v) acetic acid (Fisher Scientific)/10% water. Store up to 6 months at 4°C in the dark.

Working solution: To make benzidine solution, add 1 part benzidine base stock, 1 part 30% hydrogen peroxide, and 5 parts water. Prepare fresh for each use.

Cell growth medium, 5637

Heat inactivate FBS (lot tested for ES cell maintenance) at 55°C for 1 hr. Store up to 6 months at −20°C for future use.

Prepare growth medium:
DMEM-H containing:
10% (v/v) heat-inactivated FBS
50 μg/ml gentamicin (final concentration)
1× (75 μM) monothioglycerol (MTG; Sigma)
Freeze up to 6 months at −20°C. Avoid freeze thaws.
Once thawed, store at 4°C and use within 1 month.

Collection medium

Dulbecco's modified Eagle medium (DMEM-H; Invitrogen)
5% (v/v) heat-inactivated FBS
50 μg/ml gentamicin (final concentration)
1× (75 μM) monothioglycerol (MTG; Sigma)
Freeze up to 3 months at −20°C. Avoid freeze thaws.
Once thawed, store at 4°C and use within 1 month

Differentiation medium

Add gentamicin (Invitrogen) to a final concentration of 50 μg/ml per 500-ml bottle of DMEM-H (Invitrogen). Add lot-selected FBS (Support Protocol 8) to 20% (v/v) and MTG (Sigma) to 2× (150 μM final concentration). Store at 4°C. Use within 2 weeks.

Dispase

Dilute dispase, grade II stock (2.4 U/ml) 1:1 in 1× CMF-PBS (1.2 U/ml final). Use cold. Store at 4°C for up to 1 month.

ES cell culture medium

Stock solutions

DMEM-H/gentamicin: To a 500-ml bottle of DMEM-H add 0.5 ml of 50 mg/ml gentamicin (50 μg/ml final) and invert to mix.

100× MTG: Prepare a 7.5 mM solution of MTG in CMF-PBS. Store at 4°C.

FBS: Heat inactivate 100% lot-tested (Support Protocol 7) FBS at 55°C for 1 hr.

5637 cell–conditioned medium (Support Protocol 6)

Working solution:
100 ml 5637–conditioned medium (66% v/v final)
26 ml heat-inactivated, lot-tested FBS (17% v/v final)
1.5 ml 100× MTG [1× (75μm) final]
24 ml DMEM-H/gentamicin (16% v/v final)
Store at 4°C. Use within 2 weeks.

Gelatin-coated dishes

Add 2 ml of 0.1% (w/v) gelatin (Type A gelatin, porcine, Bloom Factor 200; Difco) in CMF-PBS (Sigma) per 6-cm dish or 4-ml per 10-cm dish. Ensure that bottom of dish is gelatin-coated by tilting dish back and forth. Incubate dish for at least 1 hr at 37°C.

Dishes can be prepared up to 1 week in advance, however, to ensure that there is no bacterial or fungal contamination, check the dish under a microscope.

Glutaraldehyde fixative solution

For 50 ml:
0.4 ml 25% (v/v) glutaraldehyde (Polysciences)
2.5 ml 100 mM EGTA, pH 7.3 (Sigma)
0.1 ml 1 M magnesium chloride (Mallinckrodt)
47 ml 0.1 M phosphate buffer (see recipe)
Prepare fresh for each use

Methanol/acetone fixative

Prepare a 1:1 (v/v) mixture of methanol/acetone fresh on the day of use.

Methanol and acetone can be stored separately at −20°C.

Monothioglycerol (MTG)

Prepare 100× MTG stock by adding 32.5 µl of monothioglycerol stock (Sigma no. M6145) to 50 ml CMF-PBS (7.5 mM final). Store at 4°C and use within 2 weeks.

Paraformaldehyde (PFA) fixative, 4% (w/v)

Add 2 g PFA (Polysciences) powder to 50 ml 1× CMF-PBS in a 50-ml conical centrifuge tube. Heat solution to 60°C, shaking occasionally until PFA is in solution. Cool to room temperature, then filter through a 0.45-µm filter fitted to a syringe. Divide into 3-ml aliquots and freeze up to 6 months at −20°C. Alternatively, store at 4°C in dark and use within 2 weeks.

Phosphate buffer, 0.1 M (pH 7.3 to 7.5)

Prepare 500 ml 0.1 M phosphate buffer solution by combining 115 ml 0.1 M sodium phosphate monobasic (Mallinckrodt) and 385 ml 0.1 M sodium phosphate dibasic (Mallinckrodt). The mixture should give a pH between 7.3 and 7.5. Store at room temperature and use until solution becomes cloudy.

Rat serum staining medium

5% (v/v) rat serum
3% (v/v) FBS, heat inactivated 1 hr at 55°C
0.1% (w/v) NaN_3 (Fisher Scientific)
1× CMF-PBS
Store up to 6 months at 4°C

Staining medium

3% (v/v) FBS, heat inactivated 1 hr at 55°C
0.1% (w/v) NaN_3 (Fisher Scientific)
1× CMF-PBS
Store up to 6 months at 4°C

Trypsin/EDTA, 0.25×

Add 1 ml 1× trypsin/EDTA stock solution (0.2% (w/v) trypsin/0.53 mM EDTA; Invitrogen) to 3 ml CMF-PBS. Store at 4°C. Use within 2 weeks.

Trypsin solution, 1:500

Dilute 2.5% (w/v) trypsin (no EDTA; Invitrogen) 1:500 using 1× CMF-PBS. Prepare fresh each time.

Trypsin stop solution

35% (v/v) FBS, heat inactivated 1 hr at 55°C
1× CMF-PBS
Store up to 1 month at 4°C

Wash buffer

For 200 ml:
0.4 ml 1 M magnesium chloride (Mallinckrodt)
2 ml 2% (v/v) Nonidet P-40 (Sigma)
197.6 ml 0.1 M phosphate buffer (see recipe)
Filter sterilize
Store at room temperature until solution becomes cloudy

Xgal stain

For 50 ml:
2 ml of 25 mg/ml Xgal stock (Bethesda Research Labs) in dimethyl formamide (Fisher Scientific)
0.106 g potassium ferrocyanide (Sigma)
0.082 g potassium ferricyanide (Sigma)
48 ml wash buffer (see recipe)
Store at 37°C for 2 weeks or until cloudy

COMMENTARY

Background Information

Embryonic stem (ES) cells are pluripotent cells that have been derived from the inner cell mass of developing blastocysts (Evans and Kaufman, 1981). ES cells maintained on embryonic fibroblasts in culture can repopulate all different cell lineages, including the germ line, after being injected into host blastocysts (Bradley et al., 1984). Furthermore, in vitro studies over the past twenty years have shown that ES cells can differentiate into a variety of cell lineages under appropriate conditions in culture (Keller, 2005). Many of the cell types that differentiate under theses conditions are those that are found in the developing embryo and yolk sac, such as hematopoietic cells, endoderm, and endothelial cells (Wiles and Keller, 1991; Schmitt et al., 1991; Wang et al., 1992; Keller et al., 1993). The vascular endothelial cells that arise during ES cell differentiation have the potential to form primitive blood vessels, comparable to the vessels that first form in the developing embryo and yolk sac.

An extensive discussion of nonmouse ES cell differentiation into endothelial cells and vascular structures is beyond the scope of this unit, but several excellent reviews have recently highlighted similarities and differences between mouse and human or nonhuman primate ES cells (reviews: Smith, 2001; Pera and Trounson, 2004; Hematti et al., 2005). Although human ES cells (hES) differ from mouse ES cells in requirements to maintain their undifferentiated status, hES can be induced to differentiate and form embryoid bodies similar to their mouse counterparts. Several reports have characterized endothelial progenitors, endothelial cells, and vascular structures derived from hES cells, although the development of hematopoietic cells and vascular structures takes longer than with mouse ES cell differentiation (Levenberg et al., 2002; Gerecht-Nir et al., 2003; Wang et al., 2004).

These recent findings suggest that the basic cell-cell interactions and differentiation programs are shared among the different species, but that the protocols must be modified when changing the species source of ES cells.

Mouse ES cells were initially cultured on embryonic fibroblast feeder cells (Evans and Kaufman, 1981; Martin, 1981), or with leukemia inhibitory factor (LIF) once it was identified as a factor to prevent differentiation (Smith et al., 1988; Williams et al., 1988). It was subsequently shown that LIF activates the transcription factor STAT3, and this activation is critical to ES cells (Matsuda et al., 1999). Sources of LIF include: recombinant LIF that is commercially available, transient transfection of COS cells with LIF-expressing plasmids, and harvesting medium conditioned by the 5637 human bladder cancer cell line (Kearney and Bautch, 2003). The authors prefer the 5637–conditioned medium because they feel it best preserves the undifferentiated ES cell morphology, perhaps because other factors in the medium co-operate with LIF to efficiently maintain undifferentiated ES cells. There are several methods used for initiating ES cell differentiation. ES cells are removed from medium containing LIF and cultured in differentiation medium in suspension where they aggregate to form a colony of partially differentiated cells known as an embryoid body (EB; Doetschman et al., 1985; Wang et al., 1992; review: Keller, 1995). Alternatively, ES cells are cultured directly onto stromal cells to provide an environment conducive to hematopoietic differentiation (Nakano et al., 1994). A commonly used stromal cell line for such differentiation is the OP9 cell line, isolated from CSF-1 deficient op/op mice (Yoshida et al., 1990). A third method utilizes the culture of ES cells in a monolayer on extracellular matrix proteins (Nishikawa et al., 1998). Also, EBs can be disaggregated into single ES cells and plated in methyl cellulose, and subsequent analysis of the colonies that form shows that EBs contain many of the hematopoietic progenitors found in bone marrow (Schmitt et al., 1991). More recent studies have shown that single cells disassociated from EBs and plated in methyl cellulose generate blast-cell colonies that indicate the existence of a common progenitor for hematopoietic and endothelial cells (Kennedy et al., 1997; Choi et al., 1998).

While this protocol discusses the formation of EBs by enzymatic disruption of ES cell clumps, an alternative method of creating EBs is by culturing ES cells in hanging drops for several days (Wobus et al., 1991; *UNIT 23.2*). Whether forming EBs by enzymatic disruption or by hanging drops, differentiation of the EBs can be performed in suspension rather than as attached cultures, as described here. EBs, which contain an inner and outer layer, can be expanded to form a large lumen and are called cystic embryoid bodies (CEBs; Wang et al., 1992). CEBs have hemoglobinized areas that are indicative of hematopoietic development, primitive blood vessels, and areas of beating that indicate differentiation of cardiac muscle (Doetschman, et al., 1985; Risau et al., 1988; Wang et al., 1992).

All methods of ES cell differentiation have both advantages and disadvantages. The formation of EBs (either enzymatic disruption or hanging drop) has the advantage of providing for a cellular structure that enhances cell-cell interactions. Cells within an EB provide critical developmental cues involved in differentiation of other cell types (review: Keller, 2005). However, EBs have the potential to generate unknown factors that can complicate analysis of developmental programs. The advantage of coculturing ES cells with stromal cells is that stromal cells can provide a beneficial growth environment for hematopoietic development. However, undefined factors produced by stromal cells may influence the differentiation of ES cells to one cell type over another (review: Keller, 2005). Separation of differentiated ES cells from the stromal cell layer has also been found to be a disadvantage of this method. The formation of EBs is sometimes preferred over the formation of CEBs, because reattachment of EBs lends itself to enhanced experimental manipulations such as in situ localization and image analysis (Kearney and Bautch, 2003).

The protocol of ES cell differentiation from EBs that is described here provides a tool for further understanding the process of vascular differentiation (vasculogenesis) and vascular expansion (angiogenesis) in an in vitro setting that is analogous to the in vivo setting. A number of markers for the early vasculature have been identified and are particularly useful, and protocols for immunolocalization and β-galactosidase staining described here are beneficial tools for analyzing vascular marker expression.

Critical Parameters

All tissue culture should be performed under sterile conditions in a class II biological flow hood. All reagents and media should be sterile. Pipet tips and medidroppers should be

sterilized by autoclaving. All bottles should be thoroughly washed and autoclaved before use. All incubations should be performed in a humidified 37°C, 5% CO_2 incubator, unless otherwise indicated.

ES cells should be passed on day 3 or 4 after the last passage. As the source of LIF is depleted over time, ES cells will become more differentiated, thus passage of ES cells on day 3 is preferred over day 4. Enzymatic dissociation of ES cells using trypsin should be carried out for no longer than 2 to 3 min as described. Longer incubations have the potential to cleave cell surface proteins important for cell viability. Once the trypsin reaction is stopped with trypsin stop solution, it is important to gently draw cells up and down with a pipet for further ES cell dissociation, as harsh handling of the ES cells at this step can result in decreased cell viability. The authors typically culture ES cells for 50 passages, after which, maintenance of cell viability declines and undifferentiated ES cells are not preserved.

For ES cell culture, 5637 cell–conditioned medium is a critical component for ensuring that ES cells are maintained in their undifferentiated state. Testing of pooled collections of 5637 cell–conditioned medium on ES cells for proper ES cell maintenance is essential. The lot of FBS that is utilized for proper maintenance of mouse ES cells is also a critical factor in ES cell maintenance. Thus, lot testing of FBS as described in Support Protocols 7 and 8 is essential. FBS and 5637 cell–conditioned medium aliquots should be stored at −20°C and repeated freeze-thaws should be avoided.

When choosing 5- to 6-day-old ES cells for in vitro differentiation, it is important to choose the dish containing ES clumps that are undifferentiated in the middle (tight and shiny appearance), but differentiated along the edges (Figure 23.3.1). This morphology indicates a partial differentiation that promotes further differentiation. After treating the ES cells with dispase to generate small clumps of cells, it is imperative to then transfer the cell clumps to bacteriological dishes to prevent them from sticking to the dish bottom. This will ensure that the cell clumps will form EBs in suspension culture. Some cell clumps will invariably attach to the dish, but are discarded upon EB transfer to attachment dishes. The authors have found that timely feeding of attached cultures with fresh differentiation medium is important for good differentiation. The pH of medium can be monitored with phenol red, and if the medium on the cultures is light orange or yellow after 24 hr, it should be changed every day. Daily feedings are sometimes necessary for densely seeded wells or for later days of a time course (past day 8) when there are a large number of cells in each well. The lot of FBS that is utilized for proper maintenance and differentiation of ES cells is also a critical parameter and it should be emphasized that often different lots are optimal for maintenance versus differentiation. Thus, lot testing of FBS as described in Support Protocol 8 is essential. When performing immunolocalization experiments, it is important to set up control conditions for the experiment. Set up attached cultures that do not receive primary antibody (staining medium alone), but do receive secondary antibody. This step should help determine if the secondary antibody exhibits nonspecific binding.

Troubleshooting

Maintaining undifferentiated ES cells during ES cell passage is a commonly encountered problem. There are a few potential causes for overly differentiated ES cells: (1) The 5637 cell–conditioned medium does not contain enough LIF. Thus, it is important to test the different pools of combined 5637 collections on ES cells to determine which pool is optimal at keeping ES cells undifferentiated. (2) The FBS lot is not efficient in maintaining ES cells. Testing lots of FBS from different manufacturers is critical for proper ES cell maintenance. (3) Cell passage number is too high. The authors typically stop maintaining ES cells once they reach passage 50.

If the ES cells begin to differentiate in culture after 1 or 2 days since passage, it is advisable to immediately feed cells with fresh ES medium. If ES cells do not adhere to the gelatin-coated dish, make sure that the gelatin-coated dishes are used within 1 week after preparation.

When feeding EBs on day 2 after enzymatic disruption of ES clumps for in vitro differentiation, many of the EBs stick to the dish bottom. If this occurs, it may be necessary to transfer the EBs to a fresh 10-cm bacteriological dish using a medidropper.

When setting up attached cultures for in vitro differentiation, it is best to select medium-sized EBs for attachment (Figure 23.3.1). Larger EBs do not adhere as well, and while smaller EBs do attach to the dish, they often do not spread out and differentiate as well. Another factor that can influence ES cell differentiation is the quality of FBS. Thus, testing lots of FBS from different manufacturers is critical for optimal ES cell differentiation.

A problem that is commonly encountered when fixing and staining differentiated ES cultures is detachment of cells from the dish. As mentioned in Support Protocol 4, the authors prefer to use methanol/acetone fixative over PFA when applicable, because methanol/acetone fixative is less harsh on the attached cultures. However, it is recommended that attention be given to treating the cultures as gently as possible during the fixing and staining procedures.

If background signal problems occur after β-galactosidase staining, try increasing the pH of the phosphate buffer. The β-galactosidase staining procedure works at a pH of 8.5.

Anticipated Results

Although maintenance and passage of mouse ES cells with 5637 cell–conditioned medium should result in cultures containing a population of only undifferentiated ES cells, the authors typically do not observe this. In general, they notice that ~80% of cultured cells appear to be undifferentiated ES cells on day 3. This percentage goes down significantly if the batch of 5637 cell–conditioned medium is sub-optimal.

Attached cultures of EBs are set up on day 3 following the dispase treatment. By day 4 of differentiation (1 day after attachment of EBs), most EBs have attached to the bottom of the plastic dishes, and the rest are removed by aspiration. This attachment is strengthened as the EBs spread out further on the dish bottom over time. Angioblasts are first observed between days 4 and 6 of differentiation. Vessels are routinely visualized between days 6 and 8, with some expansion of the vasculature after day 8. A typical time course to observe and analyze vascular development would begin on day 5 and conclude on day 8. While cultures are viable past day 8, they begin to deteriorate around day 10 of culture under differentiation conditions.

Time Considerations

Passaging mouse ES cells

Approximately 15 min should be allowed to prepare all solutions. Maintaining mouse ES cells is fairly time efficient. After allowing solutions to warm to appropriate temperatures (5 to 15 min depending on volumes), a beginner can anticipate about 20 to 30 min to process 1 to 2 6-cm dishes of ES cells.

Preparation of 5637 cell–conditioned medium (CM)

After 5637 cells are thawed and passed, cells are typically grown for 48 to 72 hr (or until 80% to 90% confluency in a 15-cm plate. At this point growth medium is replaced with collection medium, and the cells are cultured for a further 48 hr. Approximately 45 min should be allowed to process one 15-cm plate for 5637 cell–conditioned medium. More time should be added if more than one 15-cm plate is processed (typically about 5 min more for every plate added).

Enzymatic disruption of ES cells (dispase treatment)

Approximately 15 min should be allowed to prepare all solutions, and 25 to 30 min should be allowed to process one 6-cm dish of ES cells. More time should be added if more than one 6-cm dish is processed (typically about 2 to 3 min for every added dish).

In vitro differentiation

Medium must be changed on day 2 after the dispase treatment. After warming differentiation medium to 37°C, ~3 to 4 min should be allowed to aspirate and feed one dish of EBs. The time required to set up attached cultures varies depending on the number of samples that are to be processed and the number of wells to be plated. It typically takes only a couple of minutes to plate one sample into four wells of a 24-well plate. Differentiated samples must be fed every other day after plating. Generally, it takes 5 min or less to feed the wells of a 24-well plate.

Benzidine staining

Approximately 10 to 15 min should be allowed to prepare all of the solutions for the benzidine staining protocol. Including incubation times, ~5 min to 1 hr should be allowed for completion of protocol (benzidine stain does not typically last longer than 1 hr).

Double immunostaining with PECAM and Mac-1

Approximately 20 to 30 min should be allowed to prepare all of the solutions for this protocol. Including incubation times, ~7 to 7.5 hr should be allowed to double immunostain 24 wells of a 24-well plate.

Immunolocalization

Approximately 10 to 15 min should be allowed to prepare all of the solutions for the

immunolocalization protocol. Including incubation times, ~4 to 4.5 hr should be allowed to stain all 24 wells of a 24-well plate with antibody.

β-galactosidase staining

Approximately 30 to 40 min should be allowed to prepare all of the solutions for β-galactosidase staining protocol, and ~30 to 40 min should be allowed to complete all protocol steps leading up to the addition of Xgal stain. Cells are incubated with Xgal stain for 4 hr to overnight depending on the level of β-galactosidase activity.

Literature Cited

Bautch, V.L. 2002. Embryonic stem cell differentiation and the vascular lineage. *In* Methods in Molecular Biology, Vol. 185: Embryonic Stem Cells: Methods and Protocols (K. Turksen, ed.) pp. 117-125. Humana Press. Totawa, N.J..

Bradley, A., Evans, M., Kaufman, M., and Robertson, E. 1984. Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* 309:255-256.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J., and Keller, G. 1998. A common precursor for hematopoietic and endothelial cells. *Development* 125:725-732.

Doetschman, T., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines; formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27-45.

Doetschman, T., Williams, P., and Maeda, N. 1988. Establishment of hamster blastocyst-derived embryoid stem (ES) cells. *Dev. Biol.* 127:224-227.

Evans, M. and Kaufman, M. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Gerecht-Nir, S., Ziskind, A., Cohen, S., and Itskovitz-Eldor, J. 2003. Human embryonic stem cells as an in vitro model for human vascular development and the induction of vascular differentiation. *Lab. Invest.* 83:1811-1820.

Hematti, P., Obrtlikova, P., and Kaufman, D. 2005. Nonhuman primate embryonic stem cells as a preclinical model for hemtopoietic and vascular repair. *Exp. Hematol.* 33:980-986.

Inamdar, M., Koch, T., Rapoport, R., Dixon, J.T., Probolus, J.A., Cram, E., and Bautch, V.L. 1997. Yolk-sac-derived murine macrophage cell line has a counterpart during ES cell differentiation. *Dev. Dyn.* 210:487-497.

Kearney, J. and Bautch, V.L. 2003. In vitro differentiation of mouse ES cells: Hematopoietic and vascular differentiation. *Methods Enzymol.* 365:83-98.

Keller, G. 1995. In vitro differentiation of embryonic stem cells. *Curr. Opin. Cell Biol.* 7:862-869.

Keller, G. 2005. Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. *Genes Dev.* 19:1129-1155.

Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M. 1993. Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell Biol.* 13:473-486.

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N., and Keller, G. 1997. A common precursor for primitive erythropoiesis and definitive hematopoiesis. *Nature* 386:488-493.

Levenberg, S., Golub, J., Amit, M., Itskovitz-Eldor, J, and Langer, R. 2002. Endothelial cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:4391-4396.

Martin, G. 1981. Isolation of a pluripotent cell line from the early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. 1999. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J.* 18:4261-4269.

Nakano, T., Kodama, H., and Honjo, T. 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 265:1098-1101.

Nishikawa, S.I, Nishikawa, S., Hirashima, M., Matsuyoshi, N., and Kodama, H. 1998. Progressive lineage analysis by cell sorting and culture identifies FLK+VE-cadherin cells at a diverging point of endothelial and hematopoietic lineages. *Development* 125:1747-1757.

Pera, M. and Trounson, A. 2004. Human embryonic stem cells: Prospects for development. *Development* 131:5515-5525.

Poole, T. and Coffin, J. 1989. Vasculogenesis and angiogenesis: Two distinct morphogenetic mechanisms establish embryonic vascular pattern. *J. Exp. Zool.* 251:224-231.

Redick, S. and Bautch, V.L. 1999. Developmental platelet endothelial cell adhesion molecule expression suggests multiple roles for a vascular adhesion molecule. *Am. J. Pathol.* 154:1137-1147.

Risau, W. 1997. Mechanisms of angiogenesis. *Nature* 386:671-674.

Risau, W., Sariola, H., Zerwes, H-G., Sasse, J., Ekblom, P., Kemler, R., and Doetschman, T. 1988. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development* 102:471-478.

Schmitt, R., Bruyns, E., and Snodgrass, H. 1991. Hematopoietic development of embryonic stem cells in vitro: Cytokine and receptor gene expression. *Genes Dev.* 5:728-740.

Smith, A. 2001. Embryo-derived stem cells: Of mice and men. *Annu. Rev. Cell Dev. Biol.* 17:435-462.

Smith, A., Heath, J., Donaldson, D., Wong, G., Moreau, J., Stahl, M., and Rogers, D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336:688-690.

Vittet, D., Prandini, M.-H., Berthier, R., Schweitzer, A., Martin-Sisteron, H., Uzan, G., and Dejana, E. 1996. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* 88:3424-3431.

Wang, L., Li, L., Shojaei, F., Lavec, K., Cerdan, C., Menendez, P., Martin, T., Rouleau, A., and Bhatia, M. 2004. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity* 21:31-41.

Wang, R., Clark, R., and Bautch, V.L. 1992. Embryonic stem cell-derived cystic embryoid bodies form vascular channels: An in vitro model of blood vessel development. *Development* 114:303-316.

Wiles, M. and Keller, G. 1991. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* 111:259-267.

Williams, R., Hilton, D., Pease, S., Willson, T., Stewart, C., Gearing, D., Wagner, E., Metcalf, D., Nicola, N., and Gough, N. 1988. Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336:684-687.

Wobus, A., Wallukat, G., and Hescheler, J. 1991. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. *Differentiation* 48:173-182.

Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., and Shultz, L.D. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 345:442-444.

Contributed by Nicholas C. Kappas and Victoria L. Bautch
The University of North Carolina at Chapel Hill
Chapel Hill, North Carolina