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Differentiation of Mouse Embryonic Stem Cells into Blood

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Abstract

Embryonic stem (ES) cells can be maintained as pluripotent stem cells or induced to differentiate into many different somatic cell types. As ES-derived somatic cells can potentially be used for cell transplantation or cell-based therapy, ES cells have gained much scientific and general public attention. Successful derivation of blood from ES cells for tissue engineering will require a comprehensive understanding of inductive signals and downstream effectors involved in blood lineage development. Ideally, directed differentiation of ES cells into blood and isolation of pure hematopoietic progenitors will enhance our ability to utilize ES-derived blood cells for future clinical applications. The protocols provided in this unit describe methods of maintaining and differentiating mouse ES cells as well as identifying and isolating hematopoietic progenitors by utilizing flow cytometry and progenitor assays.

Keywords

embryonic stem (ES) cells; in vitro differentiation; hematopoietic progenitors

INTRODUCTION

The study of early events in hematopoietic development and the genes active during this process has been of great interest to investigators since the early 1900s. The modern tools of molecular biology and genetics, notably the development of genetic null animals through targeted gene deletion, have been utilized with great success to determine the individual genes and gene products required for discrete stages of hematopoietic development. Complicating this notion is the underlying importance of an intact hematopoietic system for mammalian development. Specifically, abrogation of a number of hematopoietic genes including *Gata1*, *Scl*, *Gata2*, and *Runx1* lead to embryonic lethality and preclude further analysis of animal tissues (Pevny et al., 1991; Tsai et al., 1994; Shivdasani et al., 1995; Okuda et al., 1996; Wang et al., 1996). Importantly, the use of embryonic stem (ES) cell-derived blood cells has allowed researchers to both circumvent early lethality as well as generate large numbers of specific types of cells for further genetic and biochemical analyses. To this end, it has become highly desirable for researchers to master the technique of generating blood cells of different lineages in vitro from ES cells carrying targeted mutations. Additionally, recent work using transgenic ES cells and a further understanding of the signaling and transcriptional requirements for hematopoiesis has led to the ability to

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augment gene dosage during specific temporal periods and to understand the role of specific soluble factors in the development of cell types of interest.

This unit contains protocols for in vitro differentiation of mouse ES cells to blood lineages in serum-containing (Basic Protocol 1) and serum-free (Alternate Protocol) conditions, assessing the differentiated cells by FACS (Basic Protocol 2), and maintaining ES cells (Support Protocol).

BASIC PROTOCOL 1

IN VITRO DIFFERENTIATION OF MOUSE ES CELLS TO BLOOD LINEAGES IN THE PRESENCE OF SERUM

Many different methods of in vitro differentiation of ES cells can efficiently be used to generate the progeny of all three primary germ layers—endoderm, ectoderm, and mesoderm. The most typical method is to differentiate ES cells in a stromal cell-independent manner to give rise to three-dimensional, differentiated cell masses called embryoid bodies (EBs; Wiles and Keller, 1991; Park et al., 2004). The following protocols are methods of differentiating ES cells to three-dimensional EBs in serum and serum-free medium (Alternate Protocol).

Materials—Mouse ES cells, 3rd passage or more after thawing

25-cm² tissue culture flasks (Techno Plastic Product AG, cat. no. 90026), gelatinized

14-ml polypropylene round-bottom tube (Becton Dickinson, cat. no. 352059)

IMDM (see recipe)

ES-IMDM (see recipe)

FCS for differentiation (see recipe)

Serum differentiation medium (see recipe)

Trypsin/EDTA (see recipe)

Bacterial petri-dishes (Valmark;; cat. no. 900) (Do not use tissue culture dishes)

0.1 % gelatin (see recipe)

37°C incubator with CO₂ supply system

Centrifuge (e.g., a SORVALL RT7-RTH250 model)

Set up cultures—1. Two days prior to setting up differentiation, split ES cells, seeding 4×10^5 ES cells per gelatinized 25-cm² flask into ?? ml ES-IMDM medium, without feeder cells (Support Protocol, step 8).

2. Change medium the next day.

Set up differentiation—3. Aspirate the medium from the flask.

4. Add 1 ml of trypsin/EDTA, swirl, and remove quickly.

5. Add 1 ml of trypsin/EDTA and wait until cells start to come off.

It usually takes about 10-30 sec. Do not over-trypsinize cells.

6. Stop the reaction by adding 1 ml of FCS (the same lot as to be used for differentiation) and 4ml of IMDM and pipet up and down to make a single-cell suspensions. Transfer to a 14-ml snap cap tube.

It is important not to have cell clumps.

7. Centrifuge for 5-10 min at $170 \times g$, room temperature. Discard supernatant.

8. Wash the cell pellet in 10 ml of IMDM without FCS. Centrifuge at $170 \times g$ for 5-10 min, room temperature. Discard the supernatant.

9. Re-suspend the cell pellet in 6 ml of IMDM with 10% FCS and count viable ES cells in an aliquot using a 2% eosin solution in PBS.

Make sure to count live ES cells only. For cell counting, eosin will stain the dead cells red. Do not count red cells.

10. Add 6,000-10,000 ES cells per ml of differentiation medium to obtain day 2.75-3 EBs. Add 4000-5000 cells per ml to obtain day 4-5 EBs. Add ~ 2000 cells per ml and ~500 cells per ml to obtain day 6 and 10 EBs, respectively.

The cell number added for different EB stages is obtained empirically. We find that the culture becomes confluent and the EB differentiation becomes inefficient when more cells are seeded than recommended. Different hematopoietic progenitors can be identified at different time points. Blast colony forming cells (BL-CFCs) are measured between days 2.75-3, primitive erythroid progenitors between days 4-5 and definitive hematopoietic progenitors between days 6-10 EBs.

Primary differentiation is set up based on the cell number required for subsequent experiments. Total EB cell numbers typically obtained are as follows: Day 2.75, $\sim 0.5-1 \times 10^6$ EB cells/10 ml of differentiation medium; day 4, $\sim 2-3 \times 10^6$ EB cells/10 ml of differentiation medium; day 6, $\sim 3-5 \times 10^6$ EB cells/10 ml of differentiation medium.

We recommend differentiating ES cells in methylcellulose medium for obtaining late EBs (>days 8). The methylcellulose medium contains the same reagents as liquid differentiation medium, except that methylcellulose is added to 1% of the final volume.

For >day 6 EBs, also add Kit-Ligand (KL, final 1%) and IL-3 (final 1%). Feed 4-5 ml of differentiation medium per 100-mm petri-dish on day 6 for >day 8 EBs. Feeding medium contains 0.5 % methylcellulose instead of 1 %.

11. Grow the cells to the desired day and harvest for analysis or experimentation.

ALTERNATE PROTOCOL

IN VITRO DIFFERENTIATION OF MOUSE ES CELLS TO BLOOD LINEAGES IN SERUM-FREE MEDIUM

Mouse ES cells can also be differentiated in vitro to blood lineages in the absence of serum.

Additional Materials—(also see *Basic Protocol 1*)

Serum replacement (SR, Gibco/BRL 10828-028)

Serum-free differentiation medium (see recipe)

PFHM-II (Protein-Free Hybridoma Medium; Gibco 12040-077)

Set up cultures—1. Two days prior to setting up differentiation, split ES cells seeding 4×10^5 ES cells per gelatinized 25-cm² flask into ?? ml ES-IMDM medium without feeder cells (Support Protocol, step 8).

2. Change medium the next day.

Set up differentiation—3. Aspirate the medium from the flask.

4. Add 1 ml of trypsin/EDTA, swirl, and remove quickly.

5. Add 1 ml of trypsin/EDTA and wait until cells start to come off.

It usually takes about 10-30 sec. Do not over-trypsinize cells.

6. Stop the reaction by adding 1 ml of FCS (the same lot as to be used for differentiation) and 4ml of IMDM and pipet up and down to make a single-cell suspensions. Transfer to a 14-ml snap cap tube.

It is important not to have cell clumps.

7. Centrifuge for 5-10 min at $170 \times g$, room temperature.

8. Wash the cell pellet in 10 ml of IMDM without FCS. Centrifuge at $170 \times g$ for 5-10 min, room temperature. Discard the supernatant.

9. Re-suspend the cell pellet in 6 ml of IMDM with 10% FCS and count viable ES cells in an aliquot using a 2% eosin solution in PBS.

Make sure to count live ES cells only. For cell counting, eosin will stain the dead cells red. Do not count red cells.

10. Add 8,000-10,000 ES cells per ml of serum-free differentiation medium to obtain day 2.75-3 EBs. Add 6,000-7,000 cells per ml to obtain day 4-5 EBs. Add ~6,000 cells per ml and ~2,000 cells per ml to obtain day 6 and 10 EBs, respectively.

11. Grow the cells to the desired day and harvest for analysis or experimentation.

SUPPORT PROTOCOL

MOUSE ES CELL MAINTENANCE

Mouse ES cells grow rapidly with an average division time of ~8 hr. We normally split ES cells every 2 days and do not keep ES cells in culture for a long time after the cells are thawed. Typically, a new vial of cells is thawed after the previous batch of cells have undergone 5-6 passages. We found that ES cells maintained on feeder cells give consistent in vitro differentiation behavior. The following protocol describes how to maintain ES cells.

Materials—25-cm² tissue culture flasks (Techno Plastic Product AG 90026)

0.1 % gelatin (see recipe)

MEF Media (see recipe)

ES-DMEM (see recipe)

ES-IMDM (see recipe)

Trypsin/EDTA (see recipe)

ES cell freezing media (see recipe)

Mitotically arrested MEFs (PMEF; Specialty Media)

ES cells, frozen, passage 12 to 18

14-ml polypropylene round-bottom tube (Becton Dickinson; cat. no. 352059)

37°C incubator with CO₂ supply system

Centrifuge (we use a SORVALL RT7-RTH250 model)

Day 1—1. Gelatinize a 25-cm² flask by adding 3 ml of 0.1% gelatin. Swirl to cover the entire surface. Let it sit at room temperature for 10-20 min.

2. Thaw a vial of γ -irradiated (5000 rads) MEF cells in a 37°C water-bath and transfer cells to a 14-ml snap cap tube.

Specialty Media sells frozen aliquats of MEFs.

3. Add 9 ml of fresh MEF medium and centrifuge the cells for 5 min at 170 $\times$ g, room temperature. Aspirate the supernatant.

Be extremely careful not to disturb the cell pellet.

4. Resuspend the cells in 6 ml of MEF medium.

5. Aspirate gelatin solution from the flask and transfer MEF cells to the flask. Place the flask in a 37°C incubator with 5% CO₂.

All subsequent culture will be in the 37°C incubator with 5% CO₂.

We have also had great success with STO cells mitotically arrested with Mitomycin C instead of γ -irradiation. Treat STO cells that were seeded the previous day at 50,000/cm² with mitomycin C (Sigma, M0503, 10 μ g/ml in MEF medium) for 2-3 hr, wash and feed with 3 ml fresh MEF medium. Add ES cells the next day.

We typically thaw one vial of MEF cells into four 175-cm² flasks. When confluent, passage the cells once to sixteen 175-cm² flasks. Once confluent, trypsinize, collect cells in 50 ml of medium into a 50-ml tube and γ -irradiate (5000 rads) them. After irradiation, spin the cells at 170 $\times$ g for 5 min, room temperature. Aspirate the medium and resuspend the cell pellet in freezing media (90% FCS/10% DMSO) at a density of 1-1.25 $\times$ 10⁶ cells per ml per cryovial. Store cells at -80°C overnight before transferring them to liquid nitrogen (<-150°C).

Day 2—6. Thaw a vial of mouse ES cells in a 37°C water-bath and transfer cells to a 14-ml snap cap tube.

We typically use passages between 12 and 18 for starting the ES culture.

7. Add 9 ml of fresh ES-DMEM medium and centrifuge the cells for 5 minutes at $170 \times g$, room temperature. Aspirate the supernatant.

Be extremely careful not to disturb the cell pellet.

8. Resuspend ES cells in 6 ml of fresh ES-DMEM medium. Remove MEF medium from the 25-cm² flask containing feeder cells and transfer ES cells to the flask.

Day 3—9. Feed cells with 6 ml ES-DMEM medium. Prepare a new gelatin-coated 25-cm² flask and thaw out MEFs as in step 1.

Day 4 or 5—10. Split ES cells and passage onto MEFs. Aspirate medium and wash briefly with 1 ml of trypsin/EDTA. After trypsin/EDTA has been removed, add fresh 1 ml of trypsin/EDTA and place the flask in a 37°C incubator for 10-30 sec, enough time for the cells to lift off the flask.

Depending on the number of viable ES cells recovered from a vial of freshly-thawed cells, you may need additional one or two days in culture before the growth of the newly-thawed cells is sufficient. Ideally, a given flask will contain a large number of smaller colonies rather than a very small number of large colonies.

Passaging at the appropriate time will prevent the latter scenario.

11. Add 5 ml of ES-DMEM and pipet up and down to break up the cell clumps. Transfer to a 14-ml snap-cap tube and centrifuge for 5 min at $170 \times g$, room temperature.

12. Aspirate the supernatant and resuspend the cell pellet in fresh 6 ml of ES-DMEM medium.

13. Count cells, being careful to distinguish between ES cells and feeder cells.

ES cells are smaller, translucent and uniform in cell size, while mitotically inhibited MEFs are much bigger and granular.

14. Plate 8×10^5 ES cells in a new ??-cm² flask with MEFs in ?? ml ?? medium.

Second and subsequent passages—15. Day after the 1st passage: feed ES cells with ?? ml fresh ES-DMEM and prepare a new flask of MEFs.

16. Two days after the 1st passage: passage cells again as in steps 10 to 14.

We don't prepare cells to generate EBs after the first passage, as the ES cells don't differentiate well. We passage cells a minimum of twice after thawing before preparing cells to differentiate into EBs.

17. Day after the 2nd passage: feed cells with ?? ml fresh ES-DMEM and prepare new MEFs.

18. Two days after the 2nd passage: passage again; both onto MEFs to maintain the ES line and onto a gelatinized flask with ES-IMDM to prepare ES cells for in vitro differentiation. Prepare a flask as you would a flask for MEFs with 0.1% gelatin. After incubation, remove gelatin and add 6 mls of ES-IMDM. Plate 4×10^5 ES cells in this flask. Place 8×10^5 ES cells onto MEFs as well.

This makes a 3rd passage.

We normally prepare one 25-cm² flask of ES cells for in vitro differentiation. One can prepare more than one 25-cm² flask of cells depending on the scale of in vitro differentiation.

19. Day after the 3rd passage: Feed cells on MEFs and on a gelatinized flask with 6 ml of ES-DMEM or ES-IMDM, respectively. Prepare a new flask of MEFs.

20. Two days after the 3rd passage: passage cells on MEFs as on the 3rd passage: onto MEFs and onto a gelatin-coated flask. Use cells on a gelatinized flask from the 3rd passage to differentiate into EBs (Basic Protocol 1 or Alternate Protocol).

21. Repeat steps 15 and 16 until cells have been passaged 5-6 times and discard cells.

We find that ES cells do not differentiate well in culture after 5-6 passages.

BASIC PROTOCOL 2

FACS ANALYSIS OF EB CELLS

Hematopoietic progenitors present within EBs can be assayed by FACS. A flow cytometer or fluorescence activated cell sorter (FACS) utilizes cells treated with monoclonal antibodies which are conjugated to different fluorochromes against cell surface proteins or intracellular markers to identify, analyze, and isolate specific EB cell population (Chung et al., 2002; Lugus et al., 2007). We typically use day 2-3 EBs to analyze mesoderm (FLK1⁺) by utilizing a FLK1 monoclonal antibody, and day 4-8 EBs to analyze hematopoietic (CD45⁺ and TER119⁺) and endothelial (CD31⁺ and VE-cadherin⁺) progenitors.

Materials—Mouse EB cells (Basic Protocol 1 and Alternate Protocol 1)

50-ml tube (Fisher, cat. no. 14-432-22)

7.5 mM EDTA (BioRad, cat. no. 161-0729)

Washing buffer: 4% (v/v) FCS in PBS

Staining buffer: washing buffer plus appropriated diluted antibody

V-shaped 96-well plate (Fisher, cat. no. 07-200-96)

20-gauge needle (Fisher, cat. no. 14826-5C)

5-ml polypropylene tube (VWR; cat. no. 60818-500)

CellQuest software (Becton-Dickinson) or FlowJo software (Tree Star, Inc.)

FACScan or FACS-Calibur Cytometers (BD Biosciences)

FCS for differentiation (see recipe)

Gene Mate Multichannel (12) pipettor (ISC BioExpress, model P-3912-200A)

1. Collect EBs in a 50-ml tube and centrifuge 1 min at 170 × g, room temperature, or by letting them settle at room temperature for 10-20 min.

2. Remove the supernatant and treat EBs with 1 ml of 7.5 mM EDTA /PBS (pH 7.4) for 1 minute in 37°C.

Trypsin/EDTA can be used to dissociate EB cells when FLK1 is the only antigen to be analyzed, as we find that FLK1 is resistant to trypsin/EDTA treatment.

3. Add 9 ml of IMDM to dilute EDTA. Vortex quickly and centrifuge the cells for 5 min at $170 \times g$, room temperature.

EDTA should be removed as soon as possible to minimize the exposure time to EDTA. Prolonged exposure to EDTA can lead to cell death.

4. Aspirate the supernatant and add 3 ml of washing buffer.

5. Pass through a 20-G needle 4-5 times to generate a single-cell suspension, and count the viable cell number with 2% eosin in PBS.

6. Centrifuge the cells for 5 min at $170 \times g$, room temperature. After centrifugation, aspirate the supernatant and re-suspend the cells at a density of 5×10^6 cells/ml in washing buffer.

7. Place cells into individual wells of a V-shaped 96-well plate at 5×10^5 cells/well. Centrifuge the plate at $170 \times g$, room temperature, for 5 min.

8. Aspirate the supernatant from the 96-well plate using a multi-channel pipettor. Add primary antibody at an appropriate dilution in 100 μ l of wash buffer. Incubate on ice (or 4°C) for 15 min.

If your primary antibody is directly conjugated to a fluorochrome, you can skip the secondary antibody staining and continue with step 13.

9. After incubation, add additional 100 μ l of wash buffer to the wells and centrifuge cells at $170 \times g$, room temperature, for 5 min. Remove the supernatant.

10. Wash cells in 150 μ l of washing buffer.

11. Centrifuge again. Repeat step 10 for a total of three washes.

12. After three washes, add 100 μ l of freshly diluted secondary antibody in staining buffer and incubate on ice (or 4°C) for 15 min in the dark.

The plate has to be kept in the dark if the secondary antibody is directly conjugated to fluorochrome.

13. After incubation, wash cells three times (steps 9-11), resuspend in 150 μ l of washing buffer, and transfer to a 5-ml polypropylene tube (VWR, #60818-500) for FACS analysis.

14. Acquire FACS data on a FACS Calibur (Becton-Dickinson) and analyze with CellQuest software (Becton-Dickinson) or FlowJo software (Tree Star, Inc.).

ALTERNATE PROTOCOL 2

CELL SORTING AND IN VITRO CULTURE OF SORTED CELL POPULATIONS

The staining for cell sorting is performed the same way as for FACS analysis. Prior to sorting, filter stained cells through a 40- μ m nylon-mesh cell strainer (BD Falcon 352340). Cells are sorted using FACS MoFlo (Becton-Dickinson) into a 14-ml tube (Fisher, #14-959-49B) containing 2 ml of FCS. Reanalyze the sorted cells on a FACS Caliber (Becton-Dickinson) to determine the sorting efficiency.

EB cells are notorious for their stickiness. For pure cell sorting with good yields, the sample must be as close to an absolute single-cell suspension as entirely possible.

BASIC PROTOCOL 3

HEMATOPOEITIC PROGENITOR ASSAYS

Hematopoietic progenitors present in EBs can also be assayed by directly replating EB cells. Day 2.75-3 EBs are typically used for blast colony assay (Kennedy et al., 1997; Choi et al., 1998), day 4 EBs for primitive erythroid colony assay, and day 6-10 EBs for definitive erythroid and myeloid progenitor analyses (Wiles and Keller, 1991; Keller et al., 1993).

Materials—Mouse EBs (Basic Protocol 1 and Alternate Protocol 1) in ??-mm plate

Replating medium (see recipe)

Cellulase (see recipe)

Trypsin/EDTA (see recipe)

14-ml polypropylene round-bottom tube (Becton Dickinson; cat. no. 352059)

50-ml polypropylene conical tube (Becton Dickinson; cat. no. 352070)

Methylcellulose replating medium (see recipe)

35-mm and 150-mm bacterial dish (Becton Dickinson; cat. nos. 351008 and 351058)

3-ml syringe (Becton Dickinson; cat. no. 309585)

16- and 20-G needles (Becton Dickinson; cat. nos. 305198 and 305176)

Harvest EBs—1a. *For EBs in liquid*: Transfer medium containing EBs into a 50-ml tube. Wash the plate with IMDM and add to the 50-ml tube. Let it sit at room temperature for about 10-20 min.

EBs will settle down to the bottom of the tube.

1b. *For EBs in methylcellulose*: Add an equal volume of cellulase (2 U/ml, final 1 U/ml) to EBs growing in methylcellulose and incubate 20 min at 37°C. Collect EBs into a 50-ml tube. Wash the plate with ?? ml IMDM. Add the wash to the tube of cells and allow the cells to settle 10 to 20 min at room temperature..

2. Aspirate the medium, add 3 ml of trypsin/EDTA, and incubate for 3 min in a 37°C water bath.

Use collagenase for older EBs (>day 8, for example). When collagenase is used, incubate EBs for 1 hr at 37°C.

3. Vortex quickly and add 1 ml of FCS. Dissociate cells using a 20-G needle by passaging 4-5 times.

4. Transfer to a 14-ml snap cap tube and centrifuge for 5-10 min at $170 \times g$, room temperature. Discard the supernatant.

5. Re-suspend the cell pellet in 0.3-1 ml of IMDM with 10% FCS.
6. Count the viable cells in an aliquot with 2% eosin in PBS.

At this point, there should be no cell clumps.

7. Add cell suspension to a 14-ml snap-cap tube containing methylcellulose-replating medium (see recipe). Vortex thoroughly and let it sit at room temperature for 5-10 min.

Typically, the cells are used at $3-6 \times 10^4$ EB cells per 1 ml of methylcellulose replating medium.

8. Prepare 4 ml of methylcellulose replating medium for each group of three replica dishes for each sample. Using a syringe with a 16-G or 18-G needle, add 1 ml of methylcellulose mixture to each 35-mm bacterial dish. Spread the methylcellulose mixture by gently tapping.

The reason for making 4 ml of methylcellulose replating medium for each sample is that the methylcellulose medium is very viscous. The recipes for the blast colony, primitive erythroid colony, and definitive erythroid/myeloid progenitor assays are shown below.

9. Put several 35-mm bacterial dishes (up to 6 dishes) into a 150-mm bacterial dish with a 35-mm opened dish containing sterile water in the middle. Culture in a 37°C CO₂ incubator.

10. Count colonies under inverted microscope 4-7 days after replating.

Blast colonies develop from day 2.75-3 EBs contain cells with undifferentiated or blast morphology. Only blast colonies and secondary EBs form from days 2.75-3 EBs. Secondary EBs are compact and no individual cells can be identified, thus they can be easily distinguished from blast colonies.

Primitive erythroid colonies developing from day 4 EBs are small and compact.

Macrophage colonies developing from days 6-8 contain larger cells with granules. E-Mac colonies contain both erythroid and macrophages. Additional information on hematopoietic colonies is given in the original papers (Wiles and Keller, 1991; Keller et al., 1993; Kennedy et al., 1997; Choi et al., 1998).

REAGENTS AND SOLUTIONS

0.1 % gelatin—Dissolve gelatin (Sigma G-1890) at 0.1% (w/v) in PBS and autoclave. Store at 4°C.

DMEM—Dissolve 1 package of DMEM (Gibco; cat. no. 12100-046) powder in ~800 ml autoclaved distilled water. Add 3.024 g NaHCO₃ (Sigma S5761), 10 ml Penicillin/Streptomycin (10,000U Gibco/BRL; cat. no. 6005140PG), and 25 ml 1 M HEPES buffer (Gibco/BRL; cat. no. 380-5630 PG). Bring up to 1 liter with autoclaved distilled water, filter through 0.22-μm filter, and store at 4°C up to 1-2 months.

We normally use distilled water from Millipore Milli-Q purification system (QTUM000EX).

IMDM

Dissolve 1 package of IMDM (Gibco; cat. no. 12200-036) powder in ~800 ml autoclaved distilled water. Add 3.024 g NaHCO₃ (Sigma; cat. no. S5761) and 10 ml penicillin/streptomycin (10,000U Gibco/BRL; cat. no. 6005140PG). Bring up to 1 liter with autoclaved distilled water, filter through 0.22-μm filter and store at 4°C up to 1-2 months.

We normally use distilled water from Millipore Milli-Q purification system (QTUM000EX).

LIF-conditioned medium—Chinese Hamster Ovary (CHO) cells transfected with the LIF (leukemia inhibitory factor) gene (Genetics Institute) are used as a source for LIF. Typically LIF is secreted from the cells into the medium at ~5 µg/ml.

MTG solution, 1.5×10^{-4} M—Prepare the solution by diluting MTG (Sigma; cat. no. M-6145) 1:10 in DMEM and adding 12.4 µl per 100 ml ES-medium. Prepare fresh.

Alternatively, β-mercaptoethanol (BME, Sigma; cat. no. M-7522) is used at 1×10^{-4} M. For a 100 × stock solution adding 72 µl of 14 M BME to 100 ml of 1 × PBS. To use, add 1 ml per 100 ml of ES-medium.

Make sure that MTG or BME is made fresh.

MTG solution, 4.5×10^{-4} M—Prepare the solution by diluting 26 µl of MTG into 2 ml of IMDM and adding 3 µl of diluted MTG per ml of differentiation medium. Prepare fresh

MEF medium—15% (v/v) FCS (pre-selected, same as for ES-DMEM)

1% (v/v) non-essential amino acids (Mediatech, Inc; cat. no. 25-025-CI)

1% (v/v) L-glutamine (Gibco; cat. no. 25030)

1.5×10^{-4} M MTG in DMEM (see recipe)

DMEM to 100%

Store at 4°C

ES-DMEM medium—15% (v/v) FCS (pre-selected)

2% (v/v) LIF (leukemia inhibitory factor) conditioned medium (see recipe)

1% (v/v) non-essential amino acids (Mediatech, Inc; cat. no. 25-025-CI)

1% (v/v) L-glutamine (Gibco; cat. no. 25030)

1.5×10^{-4} M MTG in DMEM (see recipe)

DMEM to 100%

Store at 4°C { *CE:AQ: How long? }

ES-IMDM medium—15% (v/v) FCS (pre-selected, same as for ES-DMEM)

2% (v/v) LIF (leukemia inhibitory factor) conditioned medium (see recipe)

1% (v/v) non-essential amino acids (Mediatech, Inc; cat. no. 25-025-CI)

1% (v/v) L-glutamine (Gibco; cat. no. 25030)

1.5×10^{-4} M MTG in IMDM (see recipe)

IMDM to 100%

Store at 4°C

FCS for ES culture—We normally prescreen FCS for ES culture. Typically, ES cells adapted to grow without feeder cells are used for testing serum. ES cells are maintained in test serum for 5-6 passages and scored for morphology; either differentiated or undifferentiated. A rapid, easy and quantifiable assessment of different FCS lots for use in ES cell propagation is to grow Oct4-GFP ES cells (Qi et al., 2004) in various lots of serum and perform FACS analyses to assess GFP-positivity.

A good lot of serum should maintain >95% of Oct4-GFP ES cells as GFP⁺ after 5-6 passages.

FCS for ES differentiation—We normally prescreen FCS for ES differentiation. Typically, ES cells are differentiated in test serum and analyzed by FACS for FLK1 staining or hematopoietic replating.

A good lot of serum should generate ~30%-50% of FLK1⁺ cells when day 3-4 EB cells are analyzed.

0.25% Trypsin/EDTA—Dissolve 2.5 g of trypsin (Sigma; cat. no. T-4799) in ?? ml of 1× PBS. Add 2.16 ml of 0.5 M EDTA and bring up to 1 liter with 1× PBS. Filter sterilize through 0.22-μm filter. Store aliquots at -20°C. Once thawed, store at 4°C for up to 1 month.

ES cell freezing medium—90% FCS with 10% DMSO (Sigma; cat. no. D2650). ES cells are frozen at a density of 2-3 × 10⁶ cells/ml of freezing media. Add 1 ml of cells to each freezing vial. Store cells at -80°C overnight before transferring them to liquid nitrogen (<-150°C).

Ascorbic acid—Prepare ascorbic acid (Sigma; cat. no. A-4544) solution fresh each time you set up differentiation. Dissolve ascorbic acid at 5 mg/ml in autoclaved water and filter sterilize (0.22-μm).

Methylcellulose, 2% (w/v)—Weigh a sterile 1-liter Erlenmeyer flask. Add ~450 ml of sterile water. Bring to boil on a hot plate and keep boiling for 3-4 min. Add 20 g of methylcellulose (Fluka; cat. no 64630), swirl quickly, and return the flask to the hot plate. Remove the flask quickly from the hot plate and swirl again when it starts to boil. Return the flask back to the hot plate. Repeat 3-4 times. Weigh the flask with the solution, subtract the weight of the flask and add sterile water (room temperature) to make up to 500 ml of methylcellulose mixture. Let it sit on bench to cool down to room temperature.

In a separate weighed flask, make 500 ml of 2× IMDM and filter sterilize (0.22-μm). Slowly add 2× IMDM to methylcellulose and mix vigorously. Put the mixture on ice until the right consistency is achieved. Make ~100 ml aliquots and store frozen at -20°C. When ready to use, thaw and use a syringe to disperse methylcellulose (do not use pipets).

ES differentiation media—See Table 1F.4.2 for the composition of various ES differentiation media.

Methylcellulose mixes for progenitor assays—See Table 1F.4.3 for the composition of the methylcellulose mixes for the progenitor assays

PDS (Animal Technologies, Inc. TX)

Erythropoietin (EPO), Amgen Epogen NDC 55513-126-10

VEGF, R&D Systems 293-VE

IL-1 β , R&D Systems 401-ML

IL-6, R&D Systems 406-ML

IL-11, R&D Systems 418-ML

G-CSF, R&D Systems 414-CS

GM-CSF, R&D Systems 415-ML

M-CSF, R&D Systems 416-ML

PFHM-II (Protein Free Hybridoma Medium) (Gibco 12040-077)

Transferrin (Human) in IMDM (Boehringer-Mannheim/Roche 652202)

Cellulase, 2x—Dissolve cellulase (Sigma; cat. no. C-1794) in PBS at 2 U/ml. Filter sterilize through 0.45- μ m filter. Store at -20°C up to 1-2 months.

Collagenase—Dissolve 1 g of collagenase (Sigma; cat. no. C0310) in 320 ml 1 $\times$ PBS. After filter sterilization, add 80 ml of FCS. Make ?? ml aliquots and keep at -20°C up to 1-2 months.

D4T conditioned medium (CM): D4T endothelial cells (Kennedy et al., 1997; Choi et al., 1998) are cultured in 10% FCS in IMDM. Remove medium and change to 4% FCS in IMDM when the culture becomes ~80% confluent. Culture an additional 72-96 hr, and collect the supernatant. Centrifuge for 5 min at 170 $\times$ g, room temperature, to remove cell debris, and filter sterilize the supernatant utilizing a 0.45- μ m filter unit. Make 10-ml aliquots, and keep at -80°C. Once thawed, D4T CM is kept at 4°C for about 1 week.

Kit ligand: Kit ligand is from medium conditioned by CHO cells transfected with a KL expression vector (Genetics Institute, Cambridge, MA).

IL-3: IL-3 is from medium conditioned by X63 AG8-653 myeloma cells transfected with a vector expressing IL-3 (Karasuyama and Melchers, 1988).

COMMENTARY

Background Information—An alternate source of embryonic cells for the studies of early embryonic events is the in vitro differentiated progeny of ES cells. ES cells differentiate efficiently in vitro and give rise to three dimensional, differentiated cell masses called embryoid bodies (EBs, reviewed in Park et al., 2005). ES cells can also be differentiated on stromal cells or type IV collagen without intermediate formation of the EB structure (Nakano et al., 1994; Nishikawa et al., 1998). Many different lineages have been reported to develop within EBs, including neuronal, muscle, endothelial, and hematopoietic lineages (reviewed in Park et al., 2005). Of these, the hematopoietic lineage has been the most extensively characterized. Hematopoietic progenitors develop sequentially within EBs. The first to develop is the Blast Colony-Forming Cell (BL-CFC). BL-CFCs are transient and develop prior to the primitive erythroid population (Choi et al., 1998; Lugus et al., 2007). Definitive erythroid and myeloid progenitors develop shortly after primitive erythroid

progenitors. BL-CFCs form blast colonies in response to vascular endothelial growth factor (VEGF), a ligand for the receptor tyrosine kinase, FLK1. Gene expression analysis indicated that cells within blast colonies (blast cells) expressed a number of genes common to both hematopoietic and endothelial lineages, including *Scl*, CD34, and *Flk1* (Kennedy et al., 1997). In addition, blast cells are clonal and give rise to primitive, and definitive hematopoietic as well as endothelial cells when replated in media containing both hematopoietic and endothelial cell growth factors (Kennedy et al., 1997; Choi et al., 1998). The developmental kinetics of various hematopoietic lineage precursors within EBs and molecular and cellular studies of these cells have demonstrated that the sequence of events leading to the onset of hematopoiesis within EBs is similar to that found within the normal mouse embryo. In addition, EBs provide a large number of cells representing an early/primitive stage of development which is otherwise difficult to access in an embryo. Therefore, the in vitro differentiation model of ES cells is an ideal system for obtaining and studying primitive progenitors of all cell lineages.

Critical Parameters and Troubleshooting

For ES cell maintenance and differentiation: 1. We recommend that ES cells are healthy and fresh. Mouse ES cells grow rapidly with an average division time of about 8 hr. Therefore, ES cells require frequent splitting. We normally split ES cells every 2 days and do not keep ES cells in culture for a long time after the cells are thawed. Typically, a new vial of cells is thawed after the initial cultures have undergone 5-6 passages. We recommend that ES cells be passed at least one time after the thaw before setting up differentiation. We typically set up three independent differentiations from one thaw.

2. For ES cell differentiation, we add more cells for ES lines that differentiate poorly.

3. We find that liquid differentiation is good for obtaining early EBs (up to days 5-6) and methylcellulose differentiation for obtaining late EBs (days 6-14). The methylcellulose medium contains the same reagents as liquid differentiation medium, except that methylcellulose is added to 1% of the final volume.

4. Some maintain ES cells on gelatinized flasks without feeder cells. We find that ES cells maintained on feeder cells give more consistent in vitro differentiation results compared to those maintained on gelatinized flasks.

5. When ES cells differentiate poorly, we check MTG and ascorbic acid. We typically open a new bottle of MTG every 1-2 months. Ascorbic acid needs to be made fresh every time a differentiation experiment is set up.

6. It is important to put only 4×10^5 ES cells per 25-cm² flask 2 days prior to differentiation. The ES cell confluency in ES-IMDM medium should not exceed 80%. ES cells differentiate poorly, if the cells are too confluent, but they also differentiate poorly if the culture is too sparse.

For replating: 1. D4T conditioned medium (CM) appears to be important to obtain healthy blast colonies. D4T is an endothelial cell line which was generated from day 4 EB cells by infecting retroviruses expressing polyoma middle T gene (Kennedy et al., 1997; Choi et al., 1998). We have not determined if other endothelial cell conditioned media will also support blast colony formation.

2. We typically use plasma-derived serum (PDS) for primitive erythroid and other myeloid colony replating. The red color of erythroid colonies appears to be more vivid in cultures containing PDS. Pre-made methylcellulose mixture (Methocult GF M3434, cat. no. 03434)

purchased from Stemcell Technologies Inc., can also be successfully used for replating day 4 and 9 EBs.

Anticipated Results—We typically analyze FLK1⁺ cells from day 3-5 EBs. For R1 ES cells, FLK1⁺ cells represent ~10% in day 3 EBs; ~30-50% in day 4 EBs; and ~20% in day 5 EBs. Blast colony forming cells (BL-CFCs) typically represent ~1-3% of day 2.75-3 EBs. Primitive erythroid progenitors represent ~10% of day 4 EBs. Definitive hematopoietic progenitors represent about 1% of day 6-7 EBs. About 4% to 7% and 2% to 4% of day 6 EBs express CD45 and TER119, respectively (Zhang et al., 2005). It is important to note, however, that the kinetics of FLK1 expression as well as hematopoietic progenitor development can be different among different ES lines. Individual lines need to be examined independently.

Time Considerations—We typically set up 2-3 consecutive differentiations once ES cells are thawed. It takes about 3-4 weeks to complete one round of 2-3 differentiations including hematopoietic replating and counting.

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Table 1F.4.1

Antibodies Used to Characterize Mesoderm, Endoderm and Hematopoietic Progenitors

Target antigen [alternate name(s)]	Primary antibody (dilution; supplier)	Secondary Antibody (dilution; supplier)
FLK1 (VEGF-R2, Ly-73)	PE-conjugated rat anti-mouse FLK1 (1:200; BD Pharmingen; cat. no. 555308)	-
CD45 (Ly-5)	FITC-conjugated anti-mouse CD45 (1:200; eBioscience; cat. no. 11-0451)	-
TER119(Ly-76)	FITC-conjugated anti-mouse TER119 (1:200; eBioscience; cat. no. 11-5921)	-
CD31 (PECAM-1)	PE-conjugated rat anti-mouse CD31 (1:200; BD Pharmingen; cat. no. 553373)	-
VE-Cadherin (CD144, Cadherin-5)	Purified rat Anti-mouse CD144 (1:500; BD Pharmingen; cat. no. 555289)	Alexa Fluor®488 goat anti-rat IgG(H+L) (1:1000; Invitrogen; cat. no. A11006)

Table 1F.4.2**ES Differentiation Media Composition**

	Serum differentiation (liquid)	Serum differentiation (Methylcellulose)	Serum Free differentiation (liquid)
2% Methylcellulose	-	55%	-
FCS (pre-selected)	15%	15%	-
Serum Replacement	-	-	15%
Ascorbic acid (5mg/ml)	50μ/ml	50μ/ml	50μ/ml
L-glutamine (200mM)	2mM	2mM	2mM
MTG (see recipe)	$4.5 \times 10^{-4}\text{M}$	$4.5 \times 10^{-4}\text{M}$	$4.5 \times 10^{-4}\text{M}$
PFHM-II	-	-	5%
IMDM	Up to 100%	Up to 100%	Up to 100%

Table 1F.4.3**Methycellulose Mixes for Progenitor Assays**

	Blast	Primitive erythroid	Definitive erythroid and myeloid
2% Methylcellulose	55%	55%	55%
FCS	10%	-	-
PDS	-	10%	10%
Ascorbic acid	12.5 µg/ml	12.5 µg/ml	12.5 µg/ml
L-glutamine	2 mM	2 mM	2 mM
Transferrin	200 µg/ml	200 µg/ml	200 µg/ml
MTG	4.5×10^{-4} M	4.5×10^{-4} M	4.5×10^{-4} M
D4T CM (see recipe)	20%	-	-
VEGF	5ng/ml	-	-
KL	1%	-	1%
EPO	-	2 units/ml	2 units/ml
PFHM-II	-	5%	5%
IL-1	-	-	5ng/ml
IL-3	-	-	1%
IL-6	-	-	5ng/ml
IL-11	-	-	5-25ng/ml
G-CSF	-	-	2-30ng/ml
GM-CSF	-	-	3-5ng/ml
M-CSF	-	-	2-5ng/ml
IMDM	Up to 100%	Up to 100%	Up to 100%