Video Article

Derivation of Hematopoietic Stem Cells from Murine Embryonic Stem Cells

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Abstract

A stem cell is defined as a cell with the capacity to both self-renew and generate multiple differentiated progeny. Embryonic stem cells (ESC) are derived from the blastocyst of the early embryo and are pluripotent in differentiative ability. Their vast differentiative potential has made them the focus of much research centered on deducing how to coax them to generate clinically useful cell types. The successful derivation of hematopoietic stem cells (HSC) from mouse ESC has recently been accomplished and can be visualized in this video protocol. HSC, arguably the most clinically exploited cell population, are used to treat a myriad of hematopoietic malignancies and disorders. However, many patients that might benefit from HSC therapy lack access to suitable donors. ESC could provide an alternative source of HSC for these patients. The following protocol establishes a baseline from which ESC-HSC can be studied and inform efforts to isolate HSC from human ESC. In this protocol, ESC are differentiated as embryoid bodies (EBs) for 6 days in commercially available serum pre-screened for optimal hematopoietic differentiation. EBs are then dissociated and infected with retroviral HoxB4. Infected EB-derived cells are plated on OP9 stroma, a bone marrow stromal cell line derived from the calvaria of M-CSF^{-/-} mice, and co-cultured in the presence of hematopoiesis promoting cytokines for ten days. During this co-culture, the infected cells expand greatly, resulting in the generation a heterogeneous pool of 100s of millions of cells. These cells can then be used to rescue and reconstitute lethally irradiated mice.

Protocol

Differentiation of Embryonic Stem Cells

1. Collect a near confluent flask of embryonic stem cells (ESC) via treatment with ESC trypsin.
2. Resuspend cells in 5 mL of Differentiation media and transfer to a non-gelatin coated T25. Incubate at 37°C for 45 minutes. Retrieve supernatant and collect cells via centrifugation.
Note: Mouse embryonic fibroblasts (MEFs) from the ESC cultures attach readily to the non-gelatin coated T25 and thus are depleted from the culture during this plating step. If you are not culturing your ESC on MEFs, you can skip the pre-plate.
3. Resuspend MEF-depleted ESC in Differentiation media at 333,333 cells/50 mL. This concentration results in 100 ESC/15 mL.
4. Using multi-channel pipettor, plate ESC at 100 cells/15 mL drop on 15 cm² petri plates. With an 8-channel pipettor, you should be able to fit about 18-22 rows of drops per plate (about 5ml per plate).
Note: For this protocol, 2-5 15 cm² dishes of drops will be more than sufficient and should yield 2-5 x 10⁶ day six EB-derived cells.
5. Gently flip plates to invert drops. Incubate at 37°C 5% CO₂ for 48 hours.
6. Pool drops by gently swirling plates and transfer to 15 mL conical tube. Rinse plates with 4-6 mL of PBS and add to same 15 mL conical.
Note: You may pool up to five hanging drop plates. The EBs will grow as they continue to differentiate and if they are too concentrated they tend to form large clumps.
7. Allow pooled EBs to settle by gravity (about 10 minutes). Aspirate supernatant without disturbing EBs. Gently resuspend in 10 mL Differentiation media and transfer to 10 cm non-adherent petri plate.
8. Place petri plate on plate shaker at 50 rpm and incubate at 37°C 5% CO₂.
9. 24 hours later (day four of differentiation), swirl plates to concentrate EBs in center of petri dish. Carefully exchange 50% of media (5 mL) with 5 mL fresh Differentiation Media. Return plate to incubator for two more days.

EB dissociation and infection with MSCV-HoxB4-IRES-GFP

1. On day six of differentiation, transfer EBs to 15 mL conical tube. Allow to settle by gravity.
2. Aspirate media and resuspend in 10 mL PBS. Allow EBs to resettle by gravity. Aspirate PBS.
3. Add 250 mL dissociation enzyme mix and 1 mL PBS. Incubate in 37°C water bath for 20 minutes, occasionally swirling tube to mix enzymes and EBs.
4. Add 8 mL enzyme-free dissociation buffer.
5. Triturate mixture using 5 mL pipette until EBs are fully dissociated (about 10 times; excessive pipetting will increase death within the EB-derived cell preparation).
 1. Mixture will become cloudy with cells as EBs dissociate.
 2. Placing pipette tip against bottom of conical tube during trituration will create a shear force that will greatly facilitate dissociation.
6. Collect cells via centrifugation.
7. Resuspend EB-derived cells in 5 mL of 10% IMDM and count using trypan blue exclusion
Note: Between day four and day six of differentiation, EBs cavitate which results in a large amount of apoptosis and cell death. Thus, at day six, upwards of 30% of EB-derived cells may stain with trypan blue.
8. Resuspend EB-derived cells at 100,000 cells/2 mL of 10% IMDM+viral supernatant such that an MOI of 5-10 is achieved. Add protamine sulfate to a final concentration of 8 mg/mL.
Note: Prepare enough EB/viral supernatant mix to plate four 6-well plates (assuming 2 mL/well).
9. Plate 2 mL EB/viral supernatant mix per well of four 6 well plates pre-plated with OP9 stroma cells (see below).
10. Centrifuge plates at 2500 rpm at room temperature for 90 minutes. Transfer plates to incubator at 37°C 5% CO₂.
11. 4-6 hours later, harvest supernatant from plates and collect any potential cells remaining in suspension via centrifugation.

12. Resuspend pellet (may be small) in 48 mL 10% IMDM+cytokines. Dispense 2 mL/well of resuspension to original four 6-well plates in which infection was performed and supernatant was collected.
Note: Always prepare 10% IMDM+cytokines fresh at time of use.
13. Incubate plates at 37°C 5% CO₂ for seven days. Colonies should be apparent by day four post infection and large and well-formed by day seven. A robust expansion should yield 40-60 colonies/well on day seven.
14. On day seven post-infection, collect and pool all cells (including OP9 stroma) from all wells by treatment with trypsin.
Note: DO NOT discard supernatant or PBS washes employed during trypsin treatment. At this point in the culture, some colonies may be only loosely adherent and there are many cells floating in suspension. Collect and pool all washes and supernatant to ensure that no potentially valuable cells are discarded.
15. Resuspend cells in 8 mL fresh 10% IMDM+cytokines. Distribute 2 mLs/flask into four T75 flasks. Add an additional 13 mLs 10% IMDM+cytokines to each flask. Incubate at 37°C 5% CO₂ for three days (a total of ten days post infection).

Culture and plating of OP9 stroma

1. Maintain OP9 stroma according to standard protocols in 20% a-MEM. OP9 cells will change properties when grown to confluency. Always split at 80% confluency at no more than 1:3 split.
2. 24 hours prior to infection of day six EB-derived cells with MSCV-HoxB4-IRES-GFP, plate 25,000 OP9 cells/well of four 6-well plates in 20% a-MEM.

Collection, fractionation and transplantation

1. Collect cells expanded on OP9 stroma for ten days via treatment with trypsin. Count cells.
Note: DO NOT discard supernatant or PBS washes employed during trypsin treatment. At this point in the culture, some colonies may be only loosely adherent and there are many cells floating in suspension. Collect and pool all washes and supernatant to ensure that no potentially valuable cells are discarded.
Note: A good expansion should yield between 40-50 x 10⁶ cells/original 6-well plate infected, for a total of 160-200 x 10⁶ cells.
2. Cells may be fractionated via magnetic bead selection or FACS according to standard techniques at this point in the protocol.
3. For transplantation, deliver cells via retro-orbital or tail vein injection to Rag-2/gc deficient recipients (weighing between 15-22 grams) subjected to a split dose of 9.25 Gy of irradiation 2.5 hrs. apart.
Note: It is CRITICAL that the weight of the animals fall between 15-22 grams at time of irradiation. If they are larger than 22 grams, 9.25 Gy will not be a sufficient dose of irradiation to ensure the appropriate ablation and cells may not mediate rescue from lethal irradiation and/or engraft the hematopoietic compartment. If transplanting larger animals, the appropriate irradiation dose must be determined experimentally.
4. A minimum dose of 2-5 x 10⁶ cells/animal is required to guarantee rescue from lethal irradiation. If fractionating cells, inject 2-5 x 10⁶ cell equivalents (e.g. if population of interest represents 10% of unfractionated pool of cells, inject 2-5 x 10⁵ cells/animal).
Note: If injecting >2 x 10⁶ cells, ALWAYS inject via tail vein to avoid teratoma formation in orbit, which may result when high numbers of cells are delivered retro-orbitally.
5. Maintain Rag-2^{-/-}/gc^{-/-} deficient recipients in autoclaved cages. Depending on the "cleanliness" of the animal facility, post-transplant animals may require administration of acidified water or Trimethoprim-Sulfasoxazole (Septra) for the 5 weeks following lethal radiation.
6. Expect >90% GFP+ ES-derived leukocytes in peripheral blood at 3 weeks post-transplant. Engraftment should be multi-lineage, although lymphocytes are known to silence retroviral HoxB4-IRES-GFP.

References