

# Chapter 21

## Differentiation and Dynamic Analysis of Primitive Vessels from Embryonic Stem Cells

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### Abstract

Embryonic stem (ES) cells, which are derived from developing mouse blastocysts, have the ability to differentiate into various cell types *in vitro*. When placed in basal medium with added serum, mouse ES cells undergo a programmed differentiation favoring formation of cell types that are found in the embryonic yolk sac, including vascular endothelial cells. These *in vitro* differentiated endothelial cells form primitive blood vessels, analogous to the first vessels that form in the embryo and the yolk sac. This differentiation model is ideal for both genetic and pharmacological manipulation of early vascular development. We have made mouse ES cell lines that express endothelial-specific GFP or H2B-GFP and used these lines to study the processes of mammalian vessel development by real-time imaging. Here we describe protocols for making transgenic ES cells and imaging the processes of blood vessel development. We also provide methods for ES cell maintenance and differentiation, and methods for analysis of vascular marker expression.

**Key words:** Angiogenesis, *in vitro* differentiation, murine embryonic stem cell, Histone-2B, time-lapse imaging, cell division orientation, immunofluorescence, GFP.

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### 1. Introduction

Blood vessels in the embryo develop by two processes: vasculogenesis, the *de novo* formation of vessels from *in situ* differentiating endothelial cells, and angiogenesis, the generation of new blood vessels from endothelial cells of preexisting vessels (1, 2). Embryonic stem (ES) cells derived from developing mouse blastocysts are pluripotent, or have the ability to differentiate into various cell types *in vitro* and *in vivo*. *In vitro* differentiation of ES cells has been used to study vasculogenesis since the mid-1980s (3–5). When placed in basal medium with added serum, mouse ES cells undergo a programmed differentiation favoring formation of cell

types that are found in the embryonic yolk sac, including vascular endothelial cells (5–8). These *in vitro* differentiated endothelial cells form primitive blood vessels, analogous to the first vessels that form in the embryo and yolk sac. In this model, vessels develop in the context of other cell types that are normally associated with vascular development in the embryo. Thus, this model is ideal for studying cellular and molecular aspects of mammalian vessel development. Specifically, ES cell clumps are dissociated and differentiated into embryoid bodies (EBs). The EBs are then attached to a permissive surface, where they adhere and spread while continuing their differentiation program (8, 9). The EBs differentiate into lumenized primitive vessels in three dimensions and maintain a valid biological context, but the “pancake” feature of the flattened EBs allows for high-resolution imaging (10). This feature of the ES cell model, combined with fluorescent protein labeling and confocal microscopy, allows us to actually record the process of vessel development. Although some success in the culture and imaging of whole mouse embryos has been reported, this approach requires a more sophisticated image acquisition and data analysis system (11).

We have made mouse ES cell lines with vascular specific expression of GFP or H2B-GFP and used these cell lines to analyze the dynamic processes of vessel development by real-time imaging (12, 13). ES cells have the potential to be used for reconstituting vessels in a clinical setting, so studies using this model may eventually provide new insights into therapeutic vessel regeneration (14–16). Here we describe protocols for making transgenic ES cell lines and for time-lapse imaging of the primitive vessels. We also provide methods for maintaining and differentiating mouse embryonic stem cells and methods for analyzing the vasculature via immunofluorescence.

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## 2. Materials

### 2.1. Mouse ES Cell Maintenance

1. Collection of 5637 human bladder carcinoma cell line (ATCC #HTB9) conditioned media: 5637 cells are grown to confluence in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Bethesda, MD) (*see Note 1*) supplemented with 10% lot selected FBS (*see Note 2*). The cells are then incubated in collection media (DMEM + 5% FBS), and supernatant is collected every 48 h until the cells look suboptimal (usually after the fifth collection). Spin the supernatant for 10 min at 2,000 g, and filter through 0.22  $\mu\text{m}$  cellulose acetate filter units (Nalgene). Store filtered medium at 4°C or –20°C (if not used within 2 weeks). Combine collections and test the pooled conditioned medium prior to use.

2. 7.5 mM monothioglycerol (Sigma) prepared with PBS, store at 4°C for up to 2 weeks.
3. ES cell culture media consists of 65.9%, 5637 conditioned medium, 17.13% lot selected FBS (*see Note 2*), 82.5 μM (final concentration) monothioglycerol (MTG), and 15.8% DMEM-H.
4. Gelatin (Type A porcine gelatin, Bloom Factor 200, Difco) is dissolved in tissue-culture water at 0.1%, filtered and stored in aliquots at 4°C. Tissue culture dishes are coated with 0.1% gelatin for at least 1 h and up to 1 week at 37°C.
5. Solution of trypsin (0.5 g/L) and EDTA (0.2 g/L) (Gibco/BRL, Cat. #25300) is aliquoted and stored at -20°C. Working solution (0.25×) is prepared by dilution in PBS.
6. Trypsin stop solution: 50% newborn bovine serum (NBS) in PBS.

## **2.2. *In vitro* Differentiation of Mouse ES Cells**

1. ES cells cultured for 5–7 days after passage in ES cell medium.
2. Differentiation medium: DMEM-H supplemented with 20% lot selected fetal bovine serum (FBS) (*see Note 3*) and 150 μM monothioglycerol (MTG).
3. Dispase Grade II stock (2.4 U/ml) (Boehringer–Mannheim) is aliquoted and stored at -20°C. Working solution is prepared freshly by dilution in PBS (1:1, V/V).
4. 10 cm Kord-Valmark bacteriological Petri dishes (Wilkem Scientific Co.)
5. Autoclaved medidroppers (Fisher Scientific, Cat. #13-711).

## **2.3. DNA Preparation and Electroporation**

1. Mouse ES cells cultured for 3–4 days in ES cell medium.
2. 10 mg/ml Protease K (Invitrogen, Cat. #25530-015).
3. T<sub>10</sub>E<sub>0.1</sub> solution: 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0).
4. 100 and 70% ethanol (EtOH).
5. 3 M NaAc (pH 5.2).
6. Cold and warm PBS.
7. 0.4 cm electroporation cuvettes (BioRad, Cat. #165-2088).
8. BioRad GenePulser II with capacitance extender (BioRad, Hercules, CA).
9. Geneticin (50 mg/ml) and hygromycin (50 mg/ml) from Roche.

## **2.4. Time-Lapse Imaging**

1. Slide flasks (Nunc).
2. Nikon TE300 inverted microscope (Melville, NY) with a Perkin Elmer spinning disk confocal head (Shelton, CT) and a heated stage.

**2.5. Platelet  
Endothelial Cell  
Adhesion Molecule  
(PECAM)  
Immunofluorescent  
Staining**

1. Fixatives: 4% paraformaldehyde (Polysciences Inc.), or freshly mixed cold acetone/methanol (MeOH) (1:1, V/V).
2. Staining media: PBS with 5% FBS (Hyclone) and 0.1% sodium azide (Fisher Scientific).
3. Primary antibody: Rat anti-mouse CD31 (PECAM-1) (Mec 13.3; BD Pharmingen).
4. Secondary antibody: FITC-AffinPure F(ab')<sub>2</sub> fragment of donkey anti-rat IgG (Jackson ImmunoResearch, Cat. #712-096-150).
5. Fluorescent microscope. We have an Olympus IX-50 inverted microscope (Melville, NY) outfitted with epifluorescence.

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### 3. Methods

**3.1. Culturing Mouse  
ES Cells in the  
Absence of Feeder  
Cells**

There are numerous published protocols describing mouse ES cell maintenance (5, 7–10, 17). ES cells are traditionally maintained on a feeder layer of mouse embryo fibroblasts or STO cells, which provide LIF (leukemia inhibitory factor) to prevent differentiation. Here we describe the maintenance of mouse ES cells in the absence of feeder cells. In this situation, LIF can be provided by several other sources: (1) commercially available LIF; (2) the medium of COS cells transiently transfected with LIF-expressing plasmids; (3) harvesting medium conditioned by the 5637 cell line. We prefer the 5637 cell conditioned medium because in our hands it preserves the undifferentiated morphology of the ES cells better than the other two options.

*All volumes are given assuming that one 6 cm dish of ES cell colonies is being used.*

1. Aspirate off medium. Wash two times with 5 ml pre-warmed PBS.
2. Add 1 mL of 0.25× trypsin–EDTA solution to dish (this volume should just cover the bottom of the dish). Place dish in 37°C incubator until a majority of ES cell clumps dissociate upon gentle agitation (1–3 min).
3. Stop trypsinization reaction by adding 4 ml of trypsin stop solution to dish. Gently pipette the ES cells/trypsin stop solution up and down a few times to break up the cell clumps.
4. Remove a gelatin-coated 6 cm dish (*see Section 2.1*) from incubator. Aspirate off gelatin solution, and add 5 ml pre-warmed ES medium. Add two to three drops of the cell suspension into the dish. ES cells should be in a single cell suspension, or in clumps of no more than four to six cells/

clump. If cell clumps are significantly larger, then pipette solution to further break apart cell clumps.

- Place dish in 37°C incubator with 5% CO<sub>2</sub> and gently move dish in a “back and forth” motion in order to evenly disperse ES cells throughout the dish.

### 3.2. *In vitro* Differentiation of Mouse Embryonic Stem Cells

The ES cells are cultured for 5–7 days in ES cell medium after normal passage, and then digested with dispase. The ES clumps generated by dispase treatment are resuspended in differentiation medium in bacteriological dishes for 3 days to form EBs that contain endoderm and mesoderm, as well as hemangioblasts, which are the precursors of both vascular and hematopoietic cells. The EBs are then reattached to tissue culture treated dishes to spread and continue the process of differentiation. The process of differentiation is shown in **Fig. 21.1**.

- 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. ES cell clumps are collected from dishes after incubation at 37°C in ES cell medium for 5–7 days without feeding after normal passage.
- Aspirate off media from ES cell dish(es). Wash two times with 5 ml of cold 1× PBS. Aspirate PBS.
- Add 1 ml of cold dispase (diluted 1:1 with cold PBS just before use), and let dish sit at room temperature for 1–2 min. Check to see if the ES cell clumps have detached from dish bottom by shaking the dish. If the majority of cells clumps have not detached, let the solution sit longer.
- When a majority of the cell clumps have detached from the dish, use a 10 ml pipette to gently transfer the cells into a 50 ml tube containing 35 ml of room temperature 1× PBS. Rinse the dish with 5 ml of 1× PBS and add the rinse to the 50 ml tube. Cap tube, and invert the tube once gently to mix.
- Let the tube sit until the cell clumps have settled to the bottom of the tube (around 10 min).
- Aspirate all but 4–5 ml of PBS, carefully avoiding the ES cell clumps.

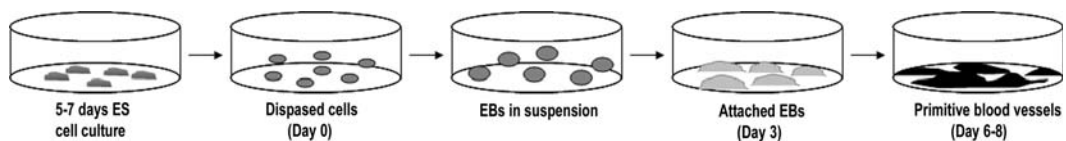


Fig. 21.1. Scheme for programmed differentiation of mouse embryonic stem cells. ES cells of 5–7 days old are dispersed in suspension by dispase digestion (day 0). They are allowed to grow in suspension for 3 days, and then allowed to attach to tissue culture plastic dishes. At days 6–8 primitive blood vessels are well established.

7. Add another 35 ml room temperature 1× 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 PBS (leaving 2–3 ml PBS/ES cell clump solution at the bottom), and gently add 5 ml pre-warmed (37°C) Differentiation medium down the side of the tube.
10. Pipette 10 ml of pre-warmed differentiation medium into a Kord Scientific 10 cm Petri dish (*see Note 4*). Using a 25 ml pipette, transfer the contents of the 50 ml tube (cell clumps/PBS/media) to the 10 cm dish.
11. Check the density of cell clumps in each dish. We attempt to achieve approximately 100 clumps/dish. Incubate at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The day of the disperse treatment is day 0.
12. Transfer the EBs to a new Petri dish with fresh media on day 2. Gently swirl the old dish in a circular manner so that the EBs migrate to the center of the dish. Transfer the EBs to a new dish with a sterile medidropper.
13. Set up reattachment cultures on day 3 after the disperse treatment. Add 4 ml of pre-warmed differentiation media to each slide flask that is to be seeded with EBs.
14. Use a sterile medidropper to transfer EBs from the dish to slide flasks. Generally, dispense between 30 and 40 EBs per slide flask. Holding the dish up and looking at it from underneath is helpful when determining the number of EBs in a slide flask.
15. Keep plate level and gently move the slide flask when placed in incubator to ensure that the EBs spread evenly in the slide flask. Incubate at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Attachment generally occurs within a few hours.
16. Feed the attached cultures every other day until days 6–8 (*see Note 5*). To feed, aspirate off media, then slowly add 4 ml fresh pre-warmed differentiation medium down the top cover of the slide flask so as not to disturb the attached cultures.

### **3.3. DNA Preparation and Electroporation**

1. Linearizing DNA for electroporation: For a 20 kb construct use 20 µg DNA. Linearize plasmid according to standard procedure. Check plasmid for complete digestion on agarose gel.
2. Add SDS to 0.5% final concentration, add Proteinase K to 0.2 µg/µl final concentration, bring up to 500 µl volume with T<sub>10</sub>E<sub>0.1</sub>. Incubate at 37°C for 30 min.

3. Heat inactivate the restriction enzyme according to the demands of that specific enzyme (*see* **Note 6**).
4. Add 3 M NaAc to 0.3 M final concentration and two volumes of 100% EtOH. Precipitate on ice for 30 min or store overnight at  $-20^{\circ}\text{C}$ .
5. Centrifuge DNA at 12,000  $g$  for 15 min, wash with 1 ml of 70% EtOH, then centrifuge another 10 min. Under the tissue culture hood carefully remove supernatant and let dry for approximately 10 min, then resuspend in sterile PBS to 1  $\mu\text{g}/\mu\text{l}$ .
6. Trypsinize the cells as usual (*see* **Section 3.1**). Stop trypsin by adding an equal volume of FBS.
7. Spin down the cells in a clinical centrifuge on speed #4 for 2 min. At the same time count the cells using a hemocytometer.
8. Aspirate the supernatant, resuspend the cells in cold PBS to  $2 \times 10^7$  cells/0.5 ml, and mix with the DNA. We usually split the DNA into two different fractions (such as 17.5 and 2.5  $\mu\text{g}$ ) and do two electroporations for each construct.
9. Transfer cell/DNA mixture to chilled cuvettes. Make a control sample of cells without added DNA.
10. Chill cuvettes on ice for 20 min.
11. Electroporate at the following settings: 300  $\mu\text{F}$ , 250 V on high capacitance. Pulse once (*see* **Note 7**).
12. Incubate cuvettes on ice for 20 min.
13. Transfer each sample to two 10 cm dishes (contain 1/4 or 3/4 cells in 10 ml ES cell medium). Bring the volume up to 10 ml for each dish with ES medium.
14. After 24 h, change to ES medium with the selective drug. We use Geneticin (G418) or Hygromycin at 200  $\mu\text{g}/\text{ml}$ .
15. Feed culture every other day with ES medium containing the appropriate drug. Clones should form and expand over the next 12–14 days. There should be no clones on the “no DNA” control plate.
16. When the clones reach approximately 1 mm in diameter, they can be picked and moved to a new dish. Aspirate the media, add 10 ml PBS, aspirate, then add fresh PBS so it only covers the cells (5 ml for one 10 cm plate).
17. Add trypsin–EDTA (1:4 in PBS) to a 48-well plate (20  $\mu\text{l}$ /well), keeping it as a droplet.
18. Pick colonies from the original plate with plugged pipet tips (new tip for each colony) and put one colony in each trypsin droplet. We usually pick up 20–30 colonies.



19. Incubate in 37°C for a few minutes. At the same time, add ES medium (we remove drug from the ES cell medium at this point) to a gelatin-coated 24-well plate.
20. Stop the trypsin–EDTA reaction by adding an equal volume of FBS, and transfer the cells to the wells of the 24-well plate.
21. Expand the colonies (clones) by passing them to six-well plates after 2–3 days, then to individual 6 cm dishes after another 2–3 days.
22. Screen individual clones by *in vitro* differentiation (*see Section 3.2*). Check GFP expressions if the cells are electroporated with a GFP construct. Maintain (*see Section 3.1*) and freeze (*see Note 8*) the colonies in the meantime. We freeze two to three vials (from one 6 cm dish) for each clone, then freeze more vials for the good clones after screening.

### 3.4. Time-Lapse Imaging of Blood Vessel Formation

1. Differentiate ES cells using the described protocol (*see Section 3.2*). Attach EBs to slide flasks on day 3 (*see Note 9*).
2. At days 7–8 of differentiation, take the slide flask out of the incubator and tighten the cap. Using a fluorescent microscope, look for areas with nice vasculature that express GFP or H2B-GFP. Circle the area(s) on the bottom side of the slide flask with a Sharpie marker.
3. Set up the slide flask on a heated stage under an inverted microscope with a spinning disk confocal head, and find the area you wish to image (*see Note 10*). The diagram of the system is shown in **Fig. 21.2**.

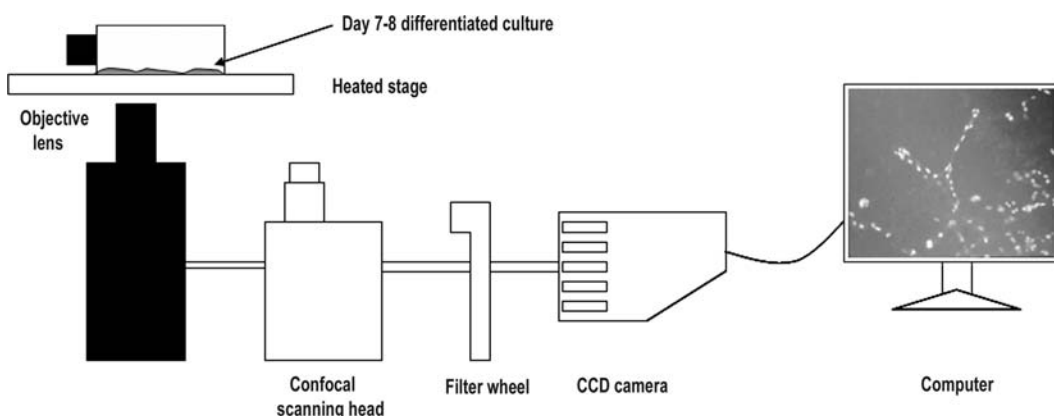


Fig. 21.2. Schematic illustration of a confocal time-lapse imaging system for *in vitro* differentiated ES cultures. A sealed slide flask containing the culture is placed on a heated stage. The laser light excites the GFP, and the emitted light goes to the objective lens, through the confocal scanning head, the filter wheel, and to the CCD camera. The image is projected onto the computer screen and can be saved as a TIFF file. The compilation of images is played as a time-lapse “movie” after collection.



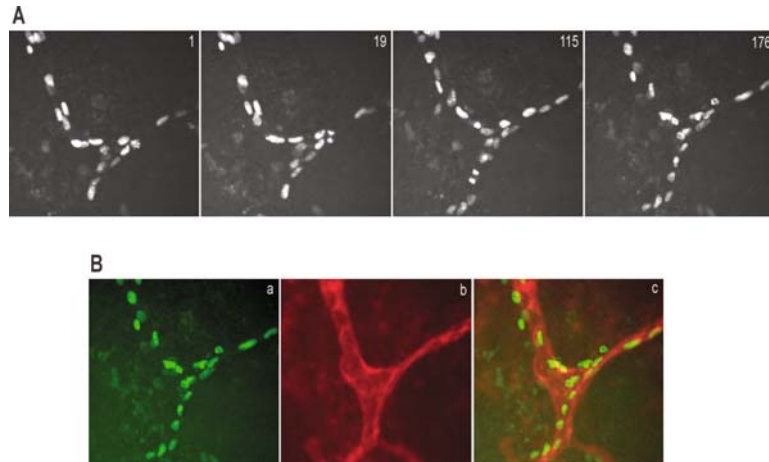


Fig. 21.3. Time-lapse imaging of ES cell differentiation cultures. An ES cell line expressing endothelial cell-specific H2B-GFP was differentiated to day 8. It was imaged on a Perkin Elmer spinning disk confocal microscope. Culture is fixed right after imaging and stained for PECAM-1. (A) Frames of a time-lapse movie. Time is in minutes at the top right corner of each panel. (B) Last frame of the movie in green (a), the same field with PECAM-1 stain in red (b), and the overlay of the two images (c). (See Color Plate 21)

4. Acquire time lapse images using acquisition software (*see Note 11*). We take images once every minute for 1 s, but other options are possible. We usually image for 2–10 h. Although the latter times sometimes yield some degradation of the sample, we rarely see sample degradation with total times of less than 8 h. Images from a time-lapse movie are shown in **Fig. 21.3(A)** and Color Plate 21.
5. Proceed immediately to fixation and PECAM staining (*see Section 3.5.*) after time lapse imaging is done.

### 3.5. ES Cell Culture Fixation and PECAM Staining

*These Procedures Assume the use of One Slide Flask.*

1. Aspirate media and wash twice with 4 ml of  $1 \times$  PBS at room temperature.
2. Add 2 ml of cold fixative and incubate for 5 min at room temperature (1:1 methanol/acetone fixative or 4% PFA fixative) (*see Note 12*). Aspirate fixative and wash twice with PBS, incubating 2 min at room temperature for each wash.
3. Gently add 2 ml of staining medium to the slide flask. Incubate for 45 min to 1 h at  $37^{\circ}\text{C}$ .
4. Remove slide flask from incubator, aspirate staining medium, and add 1 ml fresh staining medium containing the properly diluted primary antibody. Incubate for 1–2 h at  $37^{\circ}\text{C}$ .
5. Remove slide flask from incubator, remove primary antibody (*see Note 13*), and wash two times (2–3 min/wash) with 3 ml staining medium. Add 1 ml fresh staining medium containing the

- properly diluted secondary antibody. Incubate for 1 hr at 37°C, and protect the secondary antibody from light (foil wrapped).
6. Remove slide flask from incubator, remove secondary antibody (*see Note 13*), and wash once (2–3 min/wash) with 3 ml staining medium. Aspirate, then wash two times (2 min/wash) using 1× PBS.
  7. Aspirate last wash and add 2 ml of 1× PBS to the slide flask, store in PBS at 4°C in the dark.
  8. Obtain images of PECAM staining of the imaged area under an inverted microscope outfitted with epifluorescence and a camera. Overlay image with the last time lapse image acquired. Example of the images is shown in **Fig. 21.3 (B)**.

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#### 4. Notes



1. The DMEM is supplemented with Gentamicin (0.5 µg/ml final, GIBCO/BRL) before being used for medium preparation.
2. It is important to use serum that has been screened for ES cell maintenance. Prescreened serum is commercially available, but we have had good success screening serum lots in our own laboratory. In general, 50–75% of lots approved for general tissue culture use are suitable for ES cell maintenance.
3. Testing lots of FBS from different manufacturers is critical for optimal ES cell differentiation. We found serum that is not heat inactivated favors differentiation, although the serum for ES cell maintenance should be heat inactivated.
4. Do not use tissue culture treated dishes at this step because the EBs will stick to the plastic. The bacteriological dishes dramatically reduce the attachment of the ES cell clumps to the bottom of the dish during this period, which is crucial in obtaining a good differentiation.
5. Using this protocol, we typically monitor vascular development in cultures that have been differentiated for 7–8 days (day of dispass treatment is day 0). Angioblast formation is generally observed at days 4–6, while vessel formation occurs at days 6–8.
6. If the restriction enzyme cannot be heat inactivated, add an equal volume of tris equilibrated phenol/chloroform. Mix by inverting four to six times, centrifuge at 12,000 g for 10 min. Transfer upper layer to new tube, then continue to step 4 of **Section 3.3**.
7. Electroporation protocol with BioRad GenePulser II can be converted to a compatible one with other electroporator models. For example, we have used an ECM 830 (BTX

Harvard Apparatus), with settings at 250 V, 10 msec, 1 pulse. It worked as efficiently as the BioRad GenePulser II.

8. Generally, freeze two vials (1 ml/vial) of cells per 6 cm dish of ES cells. Prepare a 20% DMSO/80% FBS solution, chill on ice. Detach cells from tissue culture plates per usual. Spin cells down in a clinical centrifuge on speed #4 for 2 min. Resuspend cells in FBS at one-half the final volume. Cool cells on ice for 2–5 min. Dropwise, with gentle swirling of tube, slowly add an equivalent volume of cold 20% DMSO/80% FBS solution. Distribute 1 ml volume of the cells to the cryotube. Freeze cells overnight at  $-70^{\circ}\text{C}$  in a Nalgene freezer box for cryo-freezing. Move the cells to liquid nitrogen vessels the next day.
9. Slide flasks are required if you use a simple heated stage as we do, but if you have a more complex (and costly) plexiglass incubator that sits atop the stage for temperature and gas, you can then use whatever fits in the incubator for imaging.
10. We have a Nikon TE300 inverted microscope (Melville, NY) with a Perkin Elmer spinning disc confocal head (Shelton, CT), but there are now several nice options and combinations to choose from.
11. We use Metamorph (Version 6.1, PLACE) software for acquisition and data analysis, but other software packages are available.
12. PECAM-1 and ICAM-2 antibodies work in both PFA and MeOH/acetone fixatives. Both fixatives are listed here since some applications (not described here) favor one fixative over another. We prefer MeOH/acetone fixative since the cell layers stay attached to the dish more tightly, and it yields brighter staining. The PFA fixed cells tend to come up off the dish during the staining procedure. The H2BGFP is stable in both fixatives, while cytoplasmic GFP will diffuse in MeOH/acetone fixation.
13. Primary and secondary antibodies can be saved for subsequent experiments by storing at  $4^{\circ}\text{C}$  (the staining media contains 0.1% sodium azide). We reuse the antibodies up to three times over several months.

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