CHAPTER SIX

In Vitro Differentiation of Mouse Embryonic Stem Cells Into Primitive Blood Vessels

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Contents

Abstract

Mouse embryonic stem (ES) cells, derived from the inner cell mass of blastocyst stage embryos, undergo programmed differentiation *in vitro* to form a primitive vasculature. This programmed differentiation proceeds through similar processes of vasculogenesis and angiogenesis found during early vascular development *in vivo*. Partially differentiated ES cell clumps or embryoid bodies (EBs) first form blood islands that are subsequently transformed into a network of primitive blood vessels that contain lumens. Therefore, vascular differentiation of ES cells is an ideal model to study and manipulate early vascular development. Here we provide protocols for the routine maintenance of mouse ES cells

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and *in vitro* differentiation. We also include protocols for establishing transgenic ES cell lines and visualization of blood vessels by use of endothelial specific molecular markers.

1. Introduction

Mouse embryonic stem (ES) cells isolated from the inner cell mass of the blastocyst-stage embryo can be propagated *in vitro* to preserve their pluripotency or manipulated to give rise to multiple cell lineages. This unique property of ES cells has been extensively explored in the past decade to study early stages of mammalian embryonic development. In particular, differentiated ES cells have been instrumental in the understanding of early vascular development and hematopoiesis (Bautch, 2002; Doetschman *et al.*, 1985; Keller, 2005; Risau *et al.*, 1988; Wang *et al.*, 1992).

Differentiation of mouse ES cells *in vitro* can be achieved by removal of differentiation inhibitory factors from the media. In this scenario, ES cells undergo programmed differentiation, which results in the formation of predetermined cell types. These cell types include endoderm and several mesoderm derivatives, such as vascular endothelial cells and some hematopoietic cells (erythrocytes and macrophages). This process is very similar to differentiation in the mouse yolk sac, the first site of vascular and hematopoietic development in the embryo (Keller *et al.*, 1993; 1995; Vittet *et al.*, 1996; Wang *et al.*, 1992; Wiles and Keller, 1991). In addition, differentiated ES cells give rise to cardiac mesoderm that is not present in the yolk sac. We have established a method of differentiation of aggregated mouse ES cells into embryoid bodies (EBs), which are partially differentiated in suspension, then attached to a substratum. This method reproducibly gives rise to endothelial cells (15 to 20% of total cells) that are organized into a highly branched primitive vascular network (Fig. 6.1) (Bautch, 2002; Kearney and Bautch, 2003; Wang *et al.*, 1992).

Vascular development in differentiated ES cell cultures proceeds through the same processes of vasculogenesis and angiogenesis found *in vivo* (Risau, 1997). In the course of vasculogenesis, mesodermal precursor cells called angioblasts differentiate into early endothelial cells that then coalesce into a primitive vascular plexus. Hemangioblasts, common progenitors of both hematopoietic and endothelial cells, also contribute to vascular development (Choi *et al.*, 1998). Endothelial cells of the primitive plexus then proliferate and migrate, giving rise to a highly branched vascular network that can contain primitive erythrocytes.

We have previously shown that differentiating cultures of attached EBs initially form blood islands, reminiscent of those found in the yolk sac of developing embryos (Bautch *et al.*, 1996). Electron microscopy confirmed

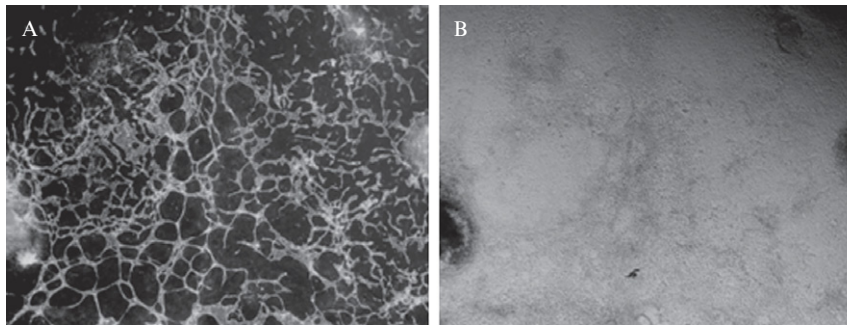

Figure 6.1 Programmed differentiation of ES cells *in vitro* results in the formation of primitive blood vessels. Attached ES cell cultures were differentiated until day 8, then fixed and stained with antibodies against PECAM to visualize blood vessels. (A) Immunofluorescence staining of differentiated ES cultures at day 8 with anti-PECAM antibodies; (B) phase-contrast image of corresponding area shows that other nonendothelial cell types are formed during ES cell differentiation.

that the blood islands are three-dimensional sacs lined by the endothelial cells, with evident cell–cell junctions. These sacs have lumens that are filled with hematopoietic cells. These blood islands later transform into a network of primitive blood vessels. Importantly, programmed differentiation of vascular endothelial cells in our model occurs in the same cellular microenvironment found during vascular development *in vivo*.

This vascular differentiation model has several important characteristics. First, the differentiated ES cell–derived primitive vessels do not experience blood flow. This precludes the study of remodeling, but it does allow us to identify flow-independent events during early vascular development. Second, even though vascular differentiation in our model occurs on reattachment of EBs, detailed analysis of vessel morphology by use of electron and confocal microscopy confirms that forming blood vessels have lumens (Fig. 6.2), allowing for three-dimensional imaging of vascular development *in vitro*. In summary, *in vitro* vascular differentiation of mouse ES cells closely recapitulates early vascular development *in vivo*, thus providing an ideal tool to study this process by use of molecular, cellular, and pharmacologic tools.

Another feature of this vascular differentiation model is the ability to easily analyze genetic manipulations. ES cells lacking the VEGF receptors Flt-1 (VEGFR-1), Flk-1 (VEGFR-2), or vascular endothelial growth factor A (VEGF) have severe defects in vascular development *in vitro*, mimicking the phenotypes *in vivo* (Bautch *et al.*, 2000; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Fong *et al.*, 1995; Kearney *et al.*, 2004; Schuh *et al.*, 1999; Shalaby *et al.*, 1995; 1997). We successfully used this model to analyze the vascular phenotype of ES cell lines with a deletion of *flt-1*, and we have

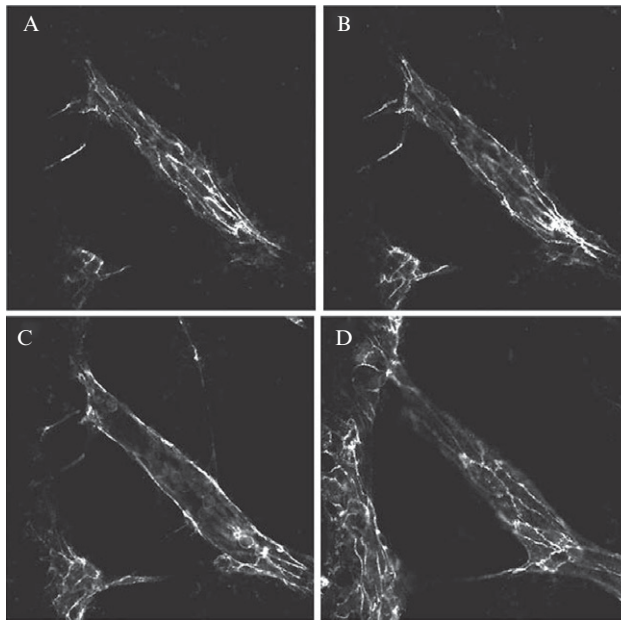

Figure 6.2 Blood vessels formed during programmed differentiation of ES cells contain lumens. Attached ES cell cultures were differentiated until day 8, and blood vessels were visualized with VE-cadherin antibodies. (A to D) Representative images in a z-stack series obtained by use of confocal microscopy. These images indicate that there is a lumen between the upper (A) and the lower (D) layer of endothelial cells in the blood vessel.

recently selectively rescued mutant phenotypes with transgenes (Kappas *et al.*, 2008). These studies provide additional verification of the model.

A number of studies describe blood vessel-specific marker expression in ES cell–derived blood vessels (Bautch *et al.*, 2000; Redick and Bautch, 1999; Vittet *et al.*, 1996). We and others have shown that vascular endothelial cells formed during *in vitro* differentiation of mouse ES cells express many of the same markers found *in vivo*. We routinely use CD31 (PECAM-1) and ICAM-2 antibodies to visualize blood vessels in differentiated ES cultures. Other markers include VE-cadherin, Flk-1, and Flt-1.

In this chapter, we provide basic protocols for maintenance and *in vitro* differentiation of mouse ES cells. In addition, we have included protocols for visualization of blood vessels in differentiated ES cell cultures and for establishing transgenic ES cell lines. We have divided the chapter into sections, each containing a general description followed by protocols and troubleshooting notes.

2. Maintenance of ES Cells

ES cells are supplemented with serum and specific factors that prevent differentiation for propagation. The main factor used for this purpose is LIF (leukemia inhibitory factor), which can be obtained from two sources: (1) recombinant LIF, which is commercially available (Sigma, Stem Cell Technologies); and (2) medium conditioned by the 5637 human bladder cancer cell line (ATCC#HTB9), which can be added to ES cell growth medium. In our hands, conditioned medium works better than recombinant LIF and is more economical for routine ES cell maintenance.

Traditionally, ES cells were cultured on top of a feeder layer of mouse embryonic fibroblasts that provide necessary factors to prevent differentiation. However, for *in vitro* differentiation, ES cells must be cultured for several passages off feeder layers. Thus, we routinely culture ES cells without feeder cells and supplement with 5637 medium. ES cells grown on plastic surfaces form colonies that look flat and have some differentiated cells along the periphery compared with ES cell colonies grown on a feeder layer. Nevertheless, they provide consistent and reproducible results for vascular differentiation when used between passages 2 and 50.

2.1. Protocol #1: Passage of ES cells

ES cells should be passed frequently, at least every 2 to 4 days to prevent differentiation (see Note 1). All volumes are given for 6-cm dishes.

1. Aspirate off medium. Wash two times with 5 ml 1× PBS at room temperature.
2. Add 1 ml 0.25× Trypsin-EDTA solution to dish. Place dish in 37 °C incubator until most ES cell clumps dissociate on gentle agitation (3 to 5 min).
3. Stop the reaction by adding 4 ml of trypsin stop solution (20 to 40% FBS in PBS) to the dish. Break up the cell clumps by gentle pipetting up and down.
4. Coat dishes with 0.1 % gelatin solution in PBS for 1 h at 37 °C. Aspirate off gelatin solution, and add 5 ml prewarmed ES medium (contains 65.9% of 5637 conditioned media (Kappas and Bautch, 2007), 17.13% lot selected FBS, 82.5 μM (final concentration) of monothioglycerol (MTG), 15.18% DMEM-H, gentamicin (0.5 μg/ml) (see Note 2).
5. Add 2 to 3 drops of the cell suspension into the dish. Observe the size of the ES cell clumps under a microscope. ES cells should have no more than 4 to 6 cells/clump. If cell clumps are significantly larger, pipette the solution to further break apart cell clumps.

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

NOTES:

1. ES cells maintained without feeder cells normally look somewhat differentiated, especially around the edges. In our experience, passage of ES cultures in this state does not compromise *in vitro* differentiation. However, some ES cell lines, which look particularly differentiated, might benefit from enrichment with ES cells. To achieve this, we plate dissociated ES cells in a dish and let them attach for 30 min at 37 °C. During that time only differentiated cells should attach, whereas ES cells should remain in suspension. We then remove remaining unattached ES cells and plates them into a new dish.
2. We use FBS that has been screened for optimal ES cell maintenance (for protocol, please refer to Kappas and Bautch, 2007). In general, 50 to 75% of FBS lots approved for general tissue culture use are suitable for ES cell propagation.

2.2. *In vitro* differentiation of mouse ES cells

2.2.1. Generation of embryoid bodies (EBs)

We discuss protocols that allow ES cells to undergo a programmed differentiation that leads to formation of a primitive, lumenized vasculature (Bautch, 2002; Kearney and Bautch, 2003). For successful *in vitro* differentiation, ES cells are first aggregated into clumps that differentiate in suspension to form EBs. Subsequently, EBs are attached to tissue culture–treated plastic and spread while continuing a programmed differentiation (Fig. 6.3). Embryoid bodies can be generated by two different methods. One is treatment of ES cells with the enzyme Dispase, which results in detachment of ES cell colonies from the plastic and normally can provide a large amount of material for *in vitro* differentiation. Another alternative is the hanging drop method. It requires dissociation of ES cell colonies into a single cell suspension, followed by setting up small drops containing a specified

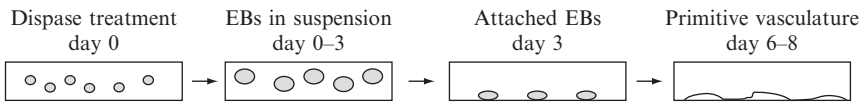

Figure 6.3 Schematic representation of programmed differentiation of ES cells *in vitro*. ES cell aggregates are detached at day 0 by Dispase treatment and allowed to differentiate into EBs in suspension. On day 3 EBs are attached to tissue culture dishes and differentiated until day 8. Formation of primitive blood vessels can be observed between days 6 and 8.

number of ES cells. The hanging drop method generates EBs of homogenous size and provides better synchronization of differentiation program; however, it is less suitable for scale-up. Here we describe the dissociation by Dispase method for EB generation, and for the hanging drop protocol see (Kearney and Bautch, 2003).

2.3. Protocol #2: Generation of EBs for *in vitro* differentiation with dissociation by dispase

All volumes are given for one 6-cm dish of ES cell colonies.

1. Choose the dish that has the best ES cell clumps for differentiation. ES cell clumps should be round and slightly differentiated on the very edge, and shiny and undifferentiated in the middle. ES cell clumps are collected from dishes after incubation at 37 °C for 5 to 6 days without feeding after normal passage.
2. Aspirate off media from ES cell dish. Wash two times with 5 ml of cold 1× PBS.
3. Add 1 ml of cold Dispase (Grade II stock, 2.4 U/ml, Boehringer-Mannheim) diluted just before use, and let dish sit at room temperature for 1 to 2 min.
4. When most of the cell clumps have detached from the dish, use a 5-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 1× 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 (approximately 10 min).
6. Aspirate all but 4 to 5 ml of PBS and add another 35 ml room temperature 1× PBS. Gently swirl the tube to redistribute the cell clumps. Cap tube, and invert gently to mix.
7. Let the tube sit until the cell clumps have settled to the bottom of the tube (10 min). Aspirate the PBS (leaving 2 to 3 ml PBS/ES cell clump solution at the bottom), and gently add 5 ml prewarmed (37 °C) differentiation medium down the side of the tube. Differentiation media contains DMEM-H supplemented with 20% lot selected FBS, 150 μM MTG (see Note 1).
8. Pipette 10 ml of prewarmed differentiation medium into a labeled Kord–Valmark bacteriologic 10-cm petri dish (see Note 2). By use of a 25-ml pipette (minimizes mechanical disruption of ES cell clumps), transfer the contents of the 50-ml tube (cell clumps/PBS/media) to the 10-cm dish.
9. Transfer the EBs to a new petri dish with fresh differentiation medium on day 2 by use of a Medidropper.

NOTES:

1. We use lot-selected FBS that ensures the best vascular differentiation of ES cells (for detail protocol see Kappas and Bautch [2007]).

2. It is crucial to use bacteriologic dishes at this point. They prevent attachment of ES cell clumps, allowing them to differentiate in suspension into EBs. Despite that, some ES clumps will still stick to the bottom of the dish, and they should be discarded after the medium is changed on day 2.

2.4. Differentiation of embryoid bodies (EBs)

On culturing ES cell clumps in suspension for 2 to 3 days, they round up and start a differentiation program to form EBs containing endoderm, mesoderm, and angioblast precursors. EBs can be reattached anytime between day 0 and day 4 after Dispase treatment, but in our hands, the best differentiation is achieved with EBs attached on day 3. Because dissociation by Dispase generates EBs of various sizes, selecting medium sized EBs that look "shiny" under the phase-contract microscope yields better results and more synchronized vascular differentiation. The density of EBs should be approximately 10 to 20 EBs/per well of 24-well dish. This density will promote optimal spreading of EBs, and they will cover most of the surface of the well by day 8 of differentiation. Vascular differentiation *in vitro* is normally assessed on day 8 after Dispase treatment. However, for certain applications, *in vitro* differentiation can be carried out until day 10 to 12. Care should be taken to provide fresh medium during the course of differentiation. In general, changing medium every 2 days is sufficient if EBs are seeded at the density described earlier.

2.5. Protocol #3: *In vitro* differentiation of EBs

The day of the Dispase treatment is day 0.

1. Set up reattachment cultures on day 3 after the Dispase treatment (see Note 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.
2. 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 (see Note 2). Holding the dish up and looking at it from underneath is helpful when determining the number of EBs in a well.
3. Ensure that the EBs are spread evenly in the well by gently shaking/moving the plate, or if necessary, use a pipetter with a sterile tip to gently pipette the medium up and down in the well.
4. Incubate at 37 °C in a humidified incubator with 5% CO_2. Attachment generally occurs within a few hours.
5. Feed the attached cultures every other day. To feed, aspirate off medium, and then slowly add 1.5 to 2 ml fresh prewarmed differentiation medium down the sidewall of the well so as not to disturb the attached cultures (see Note 3, 4).

NOTES:

1. EBs can be attached at any point from right after Dispase treatment to day 4. By day 5, EBs usually start becoming cystic. We routinely set up attachment cultures at day 3, which reproducibly results in good vascular differentiation.
2. EBs can be plated into any size of dishes. We plate EBs into 24-well plates for antibody detection by immunofluorescence and use larger dishes for RNA or protein analysis. Adjust EB numbers for the surface area of the well or dish.
3. Providing fresh medium to differentiating EBs ensures optimal conditions for vascular differentiation. Changing the medium every 2 days is usually sufficient; however, if the medium color turns light orange or yellow, it should be changed more frequently.
4. With this protocol, we 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, whereas vessel formation occurs at day 6 to 8.

3. Generation of Transgenic ES Cell Lines

The boom in recombinant technology during the past decades resulted in the creation of different methods for generating loss-of-function or gain-of-function mutations for particular genes. ES cell lines with heterozygous or homozygous deletion of certain genes were made by means of homologous recombination (Fung-Leung and Mak, 1992). Other approaches include generation of transgenic ES cell lines that overexpress genes of interest. Moreover, tissue-specific expression of transgenes is achieved by use of tissue-specific promoters. We have successfully created several transgenic ES cell lines expressing reporters that are used for real-time imaging of vascular development in differentiated ES cultures (Kearney *et al.*, 2004; Zeng *et al.*, 2007). At least two approaches exist for production of transgenic ES cell lines, namely, random integration or targeted integration into a specific locus. For random integration, we designed constructs expressing cytoplasmic or nuclear (fused to Histone2B) GFP under the PECAM promoter-enhancer with a drug resistance gene (either neomycin or hygromycin) (Fig. 6.4). The constructs were linearized and electroporated into ES cells followed by selection of drug-resistant colonies.

For targeted integration we chose the ROSA26 locus. The mouse ROSA26 locus has become a preferred site for integration of transgenes, because it can be targeted with a high efficiency, it is expressed in most cell types, and it is not subject to gene silencing (Irion *et al.*, 2007; Soriano, 1999).

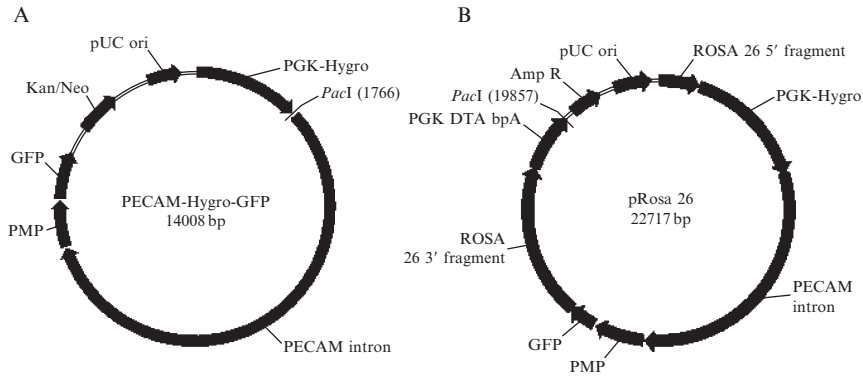

Figure 6.4 Constructs for random (A) and targeted (B) integration of transgenes into DNA. (A) PGK-Hygro-hygromycin resistance gene driven by PGK (phosphoglycerate kinase) promoter, for positive selection; PMP-PECAM minimal promoter; GFP–reporter gene. (B) pROSA26 5′ and 3′ fragments are homologous to the sequences in the ROSA26 locus and serve as sites of homologous recombination for targeted integration of the gene of interest; PGK-Hygro-hygromycin resistance gene driven by PGK promoter, for positive selection; PMP-PECAM minimal promoter; GFP-reporter gene; PGK DTA bpA-diphtheria toxin, driven by PGK promoter, serves for negative selection, because it is only expressed if homologous recombination did not occur.

Our gene of interest was inserted together with the PECAM promoter/enhancer sequence, a drug-resistance cassette, and a diphtheria toxin expression cassette (for negative selection) into a ROSA targeting vector (see Fig. 6.4). On electroporation into ES cells, the gene of interest integrates into the ROSA locus through homologous recombination. Diphtheria toxin driven by the PGK promoter should be expressed only in cells with random integration of the targeted transgene, providing a negative selection.

In our hands, random integration yields ES clones with a range of gene expression, which can be advantageous when different levels of transgene expression are desired (Kappas *et al.*, unpublished results). On the other hand, expression of transgenes from the ROSA locus under the PECAM promoter/enhancer results in overall lower levels of expression, but with equivalent levels of transgene expression among different ES clones. This can be useful if the effects of different genes on vascular development are to be compared (Kappas *et al.*, 2008). However, in our hands, certain approaches have worked for some transgenes but not for others. For example, by use of random integration we created ES cells lines with vascular endothelial–specific expression of a GFP reporter either in the cytoplasm or in the nucleus (Kearney *et al.*, 2004; Zeng *et al.*, 2007). However, the same approach did not work for a dsRed reporter transgene. Subsequently, we achieved endothelial-specific expression of dsRed by adding insulator sequences in front of the PECAM promoter (G. Zeng and V. L. B., personal communication; West *et al.*, 2002).

3.1. Protocol #4: Electroporation of DNA constructs into ES cells

1. Digest DNA with a restriction enzyme to linearize the plasmid. Choose an enzyme that cuts once and outside of the promoter/transgene/drug resistance sequences. Run an aliquot of the digest on the gel to check for complete digestion. Use 20 μg of DNA for a 20-kb construct.
2. Add SDS to 0.5% final concentration, add proteinase K to 0.2 μg/μl final concentration, adjust volume up to 500 μl with 10 m*M* Tris/0.1 m*M* EDTA solution. Incubate at 37 °C for 30 min. to inactivate the restriction enzyme.
3. Add 1/10 volume of 3 *M* NaAc and 2 volumes of 100% ethanol. Precipitate on ice for 30 min or store overnight at −20 °C.
4. Centrifuge DNA at 12,000*g* for 15 min, wash with 70% ethanol, and centrifuge another 10 min. Under the tissue culture hood, remove the supernatant and let dry for approximately 10 min, then resuspend in sterile PBS to 1 μg/μl.
5. Dissociate ES cells with trypsin as described earlier (see Note 1). Stop the reaction by adding trypsin stop solution. Count the cells and then spin them down.
6. Remove the supernatant, and then resuspend the cells in cold PBS to a concentration of 2×10^6 cell/0.5 ml and mix with DNA.
7. Transfer cell/DNA mix into prechilled cuvettes and incubate on ice for 20 min. Make a control sample of cells without DNA.
8. Electroporate with following settings: 250 V, 10 msec on ECM 830 electroporator (BTX Harvard Apparatus). Pulse once and incubate on ice for 20 min (see Note 2).
9. Transfer the sample into a 50-ml tube with 10 ml of medium, then plate in two 10-cm dishes (contain 25% or 75% of cells). Bring the volume up to 10 ml with ES medium.
10. After 24 h, change to ES medium containing the drug for selection. We use Geneticin (G418) or hygromycin at 200 μg/ml.
11. Change selection medium every other day. Clones should form during next 12 to 14 days. There should be no clones in the control plate without DNA.
12. Pick the clones when they reach 1 mm in diameter, generally on day 12 to 14. Rinse the dish with the PBS, add fresh PBS so it only covers the surface (5 ml for a 10-cm dish). Pick 20 to 30 colonies with fresh sterile pipet tips and put them into a prepared 48-well plate containing 20 μl of trypsin-EDTA in each well. Incubate for 20 to 30 min at 37 °C.
13. Stop the reaction by adding an equal volume of FBS, and transfer cells to the wells of the 24-well plate coated with gelatin.
14. Expand the clones by passing them into a 6-well plate after 2 to 3 days and to 6-cm dishes after another 2 to 3 days.

15. If the transgene was fused to GFP and expresses in ES cells, identify positive clones by use of fluorescent microscopy. In addition, screen the colonies by *in vitro* differentiation.
16. Freeze the positive clones. Generally, freeze 2 vials of cells per 6-cm dish of ES cells. Prepare 2× freezing medium (20% DMSO/80% FBS), chill on ice. Detach cells from tissue culture plates as described previously. Spin cells down in a clinical centrifuge on speed #3 for 5 min. Resuspend cells in FBS at half the final volume. Cool cells on ice for 2 to 5 min. Drop wise, with gentle swirling of tube, slowly add an equivalent volume of cold freezing medium. Distribute 1 ml volume of the cells to the cryotube. Freeze cells overnight at −70 °C in a Nalgene freezer box for cryofreezing. Move the cells to liquid nitrogen vessels the next day.

NOTES:

1. In general, three 10-cm dishes of ES cells provide enough cells for one electroporation.
2. Electroporation can be performed with other compatible electroporator models. For example, initially we used a BioRad Gene Pulser II, and it worked as efficiently as the BTX ECM 830 model.

3.2. Immunodetection of blood vessels

Primitive vascular networks formed in the course of programmed differentiation of attached EBs can be visualized by immunofluorescence detection with antibodies for endothelial specific markers. A number of specific molecular markers are used to detect endothelial cells. These include Flk-1 (Ly-73, B-D Pharmingen), VE-cadherin (11D4.1, B-D Pharmingen), CD31 or PECAM-1 (Mec13.3, B-D Pharmingen), and ICAM-2 (3C4, B-D Pharmingen). We and others have shown that Flk-1 and PECAM are expressed on mouse angioblasts and endothelial cells. In addition, PECAM is expressed in ES cells and some hematopoietic cells (Redick and Bautch, 1999). Endothelial ICAM-2 and VE-cadherin are only detectable later, when endothelial cells coalesce to form primitive vessels.

3.2.1. Protocol #5: Visualization of ES cell–derived blood vessels with antibodies against PECAM or ICAM-2

For immunostaining, EBs are plated at day 3 after Dispase treatment into 24-well plates. We normally stain 4 wells for each ES cell line. Differentiated ES cell cultures are processed for immunostaining on day 8 of differentiation.

1. Rinse plates with 1× PBS two times.
2. Aspirate PBS. Fix in an ice-cold mix of acetone/methanol (1:1) for 5 min at room temperature (RT) (see Note 1).

3. Wash two times with PBS, 5 min each (see Note 2).
4. Block in staining media (3% FBS in PBS/0.1% sodium azide) for 1 h at 37 °C.
5. Incubate with primary antibodies (Ab) diluted in staining medium for 1 h at 37 °C or overnight at +4 °C (0.25 ml volume is sufficient for one well of 24-well plate). Use rat anti-mouse PECAM Ab (Mec 13.3, BD Pharmingen) at 1:1000 dilution or rat anti-mouse ICAM-2 Ab (3C4, B-D Pharmingene) at 1:500 dilution.
6. Wash two times with PBS, 5 min each.
7. Incubate with secondary Abs diluted in staining medium for 1 h at 37 °C. For secondary Abs, we use goat anti-rat Ab conjugated to Alexa fluor 488 or 555 (Molecular Probes, Invitrogen).
8. Wash with PBS two times and observe under a fluorescent microscope (see Note 3).

Stained ES cultures can be stored at +4 °C for several weeks.

NOTES:

1. Alternately, differentiated ES cultures can be fixed in 4% fresh paraformaldehyde (PFA) for 6 min at RT. This fixation procedure normally yields weaker PECAM staining, and cells are more loosely attached to the surface of the well. However, some antibodies (i.e., VE-cadherin) require PFA for visualization.
2. Care should be taken during all washing steps. To avoid detachment of EBs from the plastic, add PBS slowly along the wall and aspirate it carefully without touching the bottom of the well.
3. For imaging with confocal microscopy, EBs can be attached and differentiated in slide flasks (NUNC) or chamber slides (Lab-Tek chamber slide, NUNC).

2. Concluding Remarks

Programmed differentiation of mouse ES cells results in the formation of primitive blood vessels in a process very similar to early vascular development *in vivo*. This approach provides a reliable model for analysis of both pharmacologic and genetic manipulations of early mammalian vascular development.

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