Video Article

Retroviral Infection of Murine Embryonic Stem Cell Derived Embryoid Body Cells for Analysis of Hematopoietic Differentiation

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Abstract

Embryonic stem cells (ESCs) are an outstanding model for elucidating the molecular mechanisms of cellular differentiation. They are especially useful for investigating the development of early hematopoietic progenitor cells (HPCs). Gene expression in ESCs can be manipulated by several techniques that allow the role for individual molecules in development to be determined. One difficulty is that expression of specific genes often has different phenotypic effects dependent on their temporal expression. This problem can be circumvented by the generation of ESCs that inducibly express a gene of interest using technology such as the doxycycline-inducible transgene system. However, generation of these inducible cell lines is costly and time consuming. Described here is a method for disaggregating ESC-derived embryoid bodies (EBs) into single cell suspensions, retrovirally infecting the cell suspensions, and then reforming the EBs by hanging drop. Downstream differentiation is then evaluated by flow cytometry. Using this protocol, it was demonstrated that exogenous expression of a microRNA gene at the beginning of ESC differentiation blocks HPC generation. However, when expressed in EB derived cells after nascent mesoderm is produced, the microRNA gene enhances hematopoietic differentiation. This method is useful for investigating the role of genes after specific germ layer tissue is derived.

Video Link

The video component of this article can be found at http://www.jove.com/video/52022/

Introduction

Murine embryonic stem cells (ESCs) are pluripotent, remaining undifferentiated and self-renewing in the presence of the cytokine Leukemia Inhibitory Factor (LIF)[1]. Upon withdrawal of LIF they will spontaneously differentiate into 3-dimensional (3D) structures called embryoid bodies (EBs)[2]. The 3D architecture allows for the development of the three germ layers ectoderm, endoderm, and mesoderm, which then later give rise to mature tissue types[3]. ESCs are an exceptional model for elucidating the molecular mechanisms of cellular differentiation, particularly the investigation of the development of early hematopoietic progenitor cells (HPCs)[4].

Gene expression in ESCs can be manipulated by several techniques that allow for the determination of a role for individual molecules in development. One of the most common techniques is to use homologous recombination to generate ESC lines, which lack a gene of interest[5,6]. There are also a number of techniques that have been used to overexpress genes. The first technique used to modify gene expression in ESCs was to infect them with recombinant retroviruses[7,8]. The gene of interest however is often silenced as the ESCs differentiate into progenitor and mature cell types. Use of lentiviruses has been successful in limiting the silencing of virally expressed genes[9]. Other viral vectors used for overexpression include adenovirus and adeno-associated virus[10]. In addition standard transfection techniques to stably introduce expression plasmids are widely used for ESC transgene expression[11]. One difficulty with these systems is that often expression of a specific gene has different effects depending on its temporal expression. For example, the Smad1 protein affects the development of hematopoietic cells differently during different stages of EB development[12,13].

This problem can be circumvented by the generation of ESCs that inducibly express a gene of interest. The most common system for inducibly expressing transgenes in ESCs uses the tetracycline resistance operon from *E. Coli (Escherichia coli)*. Several different tetracycline systems have been developed. One of the more popular strategies was developed by Kyba and colleagues[14]. They generated an ESC line (Ainv15), which has the reverse tetracycline transactivator gene inserted into the constitutively active ROSA26 locus. A tetracycline response element (TRE), a downstream LoxP site (locus of X-over P1 from bacteriophage P1), and a promoterless neomycin cassette were introduced into the HPRT (Hypoxanthine-guanine phosphoribosyltransferase) locus on the X chromosome. Using a CRE recombination approach a gene of interest along with a eukaryotic promoter to drive the expression of the neomycin resistance gene can be inserted into the LoxP site. Correctly targeted ESCs are isolated by G418 selection. These targeted clones then must be tested for doxycycline (or tetracycline) inducible expression of the transgene. This approach has successfully generated ESC lines that inducibly express HoxB4, Stat5, SCL, and Smad7[14-17]. However, generation of these inducible cell lines is time consuming. Described here is a method to disaggregate ESC-derived embryoid bodies (EBs) into single cell

suspensions, retrovirally infect the cell suspensions at different days of development, and then reform the EBs by hanging drop. Downstream differentiation is later evaluated by flow cytometry. In this article an example of how miRNA expression in EB-derived cells effects hematopoietic differentiation is shown. This method is useful for investigating the role of genes after specific germ layer tissue is derived.

Protocol

1. Embryoid Body (EB) Formation

1. Gelatin-adapt ESCs that have been maintained on mouse embryo fibroblasts (MEFs). Passage ESCs 3 times on 6 well tissue culture plate coated with 0.1% gelatin to remove MEFs.
    1. Grow cells in ESC maintenance media with LIF to keep the cells undifferentiated (**Table 1**). Make sure cells never exceed 80% confluence. Use low passage (10 or less passages after removing from MEFs) cells for differentiation.
2. On the day before ESCs are cultured for EB differentiation, passage the cells so they will be approximately 50-70% confluent the next day. Continue culturing cells in ESC maintenance medium to retain cell pluripotency.
3. On the day of the differentiation, aspirate ESC maintenance media and wash with 1 ml Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, and 11.9 mM phosphate buffer, pH 7.4) per well of a 6-well plate.
    1. Add 200 µl of 0.25% trypsin/EDTA to each well and incubate at 37 °C for 3 min.
    2. Inactivate the trypsin/EDTA with 800 µl of differentiation media. Pipet up and down in a 2 ml serological pipet with a p200 tip to break-up the cells into a single cell suspension. Ensure that the p200 tip fits snugly on the end so media does not go up between the pipet and tip interface.
    3. Pipet up and down approximately 5-10 times. Take care not to create bubbles during the pipetting.
4. Transfer cells to a 15 ml conical tube. Bring up the volume to 10 ml with PBS. Centrifuge at 315 x g for 5 min at room temperature.
5. Remove supernatant. Wash twice with 5 ml PBS to remove remaining media containing LIF. Centrifuge as in step 1.4 for each wash.
    1. Resuspend the final cell pellet in 2 ml of differentiation media (**Table 2**).
6. Count cells with a hemocytometer or cell counter and plate 6,000-10,000 cells/ml in a 10 cm Petri plate (non-tissue culture treated).
    1. Determine the exact cells/ml empirically for each ESC line. Use sterile Petri plates normally used for bacterial work or low adherence plates.
    NOTE: For best differentiation results, the EBs should form spheres that remain in suspension without attaching to the plate. Test several brands of plates to find ones with the least adherence.
    2. Incubate the differentiating ESCs in a 37 °C tissue culture incubator with 5% CO_2.

Reagent	Stock	Final Concentration	Volume	Company	Catalog Number
DMEM	1x		410 ml	Sigma/Aldrich	D5796
FBS (ES Screened)	100%	15%	75 ml	Hyclone	SH30070.03E
Non-essential Amino Acids	100x	1x	5 ml	Life Technologies	11140
L-Glutamine	100x	1x	5 ml	Life Technologies	35050
Penncillin/ Streptomycin	100x	1x	5 ml	Life Technologies	15070
β-mercaptoethanol	14.3 M	114 µM	4 µl	Sigma/Aldrich	M3148
Leukemia Inhibitory Factor (LIF)	10^7 units/ml	1,000 units/ml	50 µl	Millipore	ESG1107

Table 1: ESC Maintenance Media.

Reagent	Stock	Final Concentration	Volume	Company	Catalog Number
DMEM	1x		410 ml	Sigma/Aldrich	D5796
FBS (User Screened for optimal differentiation)	100%	15%	75 ml	Hyclone	SH30070.03E
Non-essential Amino Acids	100x	1x	5 ml	Life Technologies	11140
L-Glutamine	100x	1x	5 ml	Life Technologies	35050
Penncillin/ Streptomycin	100x	1x	5 ml	Life Technologies	15070
β-mercaptoethanol	14.3 M	114 µM	4 µl	Sigma/Aldrich	M3148

Table 2: Differentiation Media.

2. Preparing EB Cells for Virus Infection

1. At the desired stage of development (between day 2 and day 3), transfer the EBs from the 10 cm Petri dish to 15 ml conical tube. Wash the plate with 5 ml PBS and add the wash to the conical tube.
2. Pellet the EBs at 315 x g at room temperature for 5 min. Wash the EB cells with 5 ml PBS.
3. Add 1 ml of thawed cell detachment solution (Containing proteolytic and collagenolytic enzymes) and place the tubes in a 37 °C water bath (or incubator) for 30 min with occasional agitation (from flicking or light vortexing).
4. Add 1 ml of differentiation media. Pipet up and down with a 2 ml pipet with a p200 pipet tip. Place the tube in 37 °C water bath again for 30-60 min.
5. Spin down the cells at 315 x g for 5 min and wash the cells with PBS.

3. Virus Infection

1. Resuspend the cells in 1.5 ml of differentiation media. Transfer the cell suspension to a 6-well non-tissue culture treated plate.
2. Prepare the retrovirus by standard techniques ahead of time. Add 5 to 100 µl of viral supernatant to cells depending on viral titer. Do not add polybrene to enhance infection as this inhibits the subsequent reformation of EBs.
3. Spinoculate the cells with virus by centrifuging at 2,120 x g for 90 min at room temperature.

4. Differentiating Viral Infected EB Cells in Hanging Drops

1. Add 2 ml of differentiation media to each well of infected EB cell suspension.
2. Pipet 3.5 ml of infected EB cell suspension into a sterile reagent reservoir for multipipettors. Use the multipipettor to pipet 15-20 rows of eight 20 µl drops onto the inverted lid of the 15 cm Petri plate.
3. Add 10 ml of sterile PBS or water to the bottom half of the Petri plate. Then invert the lid with the drops and place onto the Petri plate. Ensure that the drops are hanging upside down from lid with PBS or water below to keep the chamber humidified.
4. Place the dishes in the 37 °C tissue culture incubator with 5.0% CO_2.
5. After 2 days, collect EBs formed in the hanging drops by inverting lid and washing with 4.5 ml of differentiation media.
    1. Transfer media and EBs to a new 10 cm non-tissue culture Petri plate. Wash the lid once more with 3 ml of differentiation media and transfer to the 10 cm plate.
6. Harvest cells for analysis by flow cytometry after a total of 8 days culture starting from the initial transfer of cells to differentiation media (-LIF).

5. Preparing Cells for Flow Cytometer Analysis

1. Transfer cells to a 15 ml conical tube with a 10 ml pipet. Wash the plate with 5 ml PBS and transfer to the same conical tube.
2. Pellet cells at 315 x g. Wash pellets with 10 ml PBS.
3. Add 1 ml of thawed cell detachment solution and place the tubes in a 37 °C water bath for 30 min with occasional agitation. For detection of some cell surface antigens, trypsin may be used instead.
4. Add 1 ml differentiation media and pipet up and down with a 2 ml pipet. Use a p200 tip to the end of the pipet to help break-up digested EB into single cell suspension.
    1. Place the tube in a 37 °C water bath again for 60 min in order for the integral membrane proteins to recycle to the cell surface.
5. Pellet cells at 315 x g for 5 min.
6. Wash the cells with 0.1% Bovine Serum Albumin Fraction V (BSA) prepared in PBS (PBS/BSA) and incubate the cells with specific fluorescently tagged antibodies.

6. Flow Cytometry Analysis for Hematopoietic Progenitors

1. Place 10^6 EB derived cells in a 5 ml 12 x 75 mm round bottom tube. For adjusting flow cytometer compensation settings, use compensation beads according to manufacturer instructions.
2. Pellet cells at 315 x g. Pour off supernatant and resuspend pellet in residual PBS/BSA solution in the tube.
3. For detecting ESC HPCs, incubate the cells with fluorescent labeled antibodies: anti-mouse CD41-PE (R-Phycoerytherin) and anti-mouse CD117 (cKit)-APC/CY7 (Allophycocyanin Cyanine 7).
    1. Dilute antibodies 1:100 in PBS/BSA. Add 100 µl of diluted antibody to each sample. Determine the correct dilution of antibody for each vendor, clone, and lot or antibody. Determine optimal dilution for labeling cells individually.
4. Incubate samples on ice in the dark for 30 min.
5. Add 2 ml PBS/ BSA and pellet cells at 315 x g for 5 min.
6. Aspirate supernatant and resuspend pellet in 300 µl of PBS or another isotonic buffer.
7. Filter resuspended cells through a cell strainer cap (0.35 µM) to remove large cell aggregates.
8. Analyze cells on a flow cytometer.

Representative Results

In these studies gelatinized RW4 (Derived from 129X1/SvJ mouse strain) ESCs were used. EBs were isolated at 3 days of differentiation. For best results the EBs should be spherical and non-adherent (**Figure 1A, B**). After spinoculation with virus co-expressing the fluorescent marker

GFP along with a gene of interest, EBs were reformed by the hanging drop method. EBs were successfully reformed from cell suspensions prepared from 2.0, 2.5, and 3.0 day EBs. However, EBs could not be reformed from cell suspensions prepared from day 4.0 EBs. The chimerism of the EBs between infected and non-infected cells was observed by fluorescence microscopy at day 8 of differentiation (**Figure 2A, B**). The density of the EBs often makes the EBs appear to be 100% GFP positive. However, focusing on different focal planes can reveal the contribution of GFP positive and negative cells to the EBs (**Figure 2B**).

For analysis of HPC development, chimeric EBs were dissociated into single cell suspensions after 8 days of differentiation. Antagonizing the activity of miRNA(s) of the *mirn23a* cluster (miRs-23a, 24-2, and -27) results in an inability of ESCs to differentiate into blood progenitors (Manuscript in preparation). To determine if overexpression of the *mirn23a* cluster has the opposite effect, ESCs were infected at the onset of differentiation. However, this results in generation of decreased HPCs. Since the *mirn23a* cluster may be involved in TGFß/BMP/Smad signaling[18-21], the cluster may have distinct effects when expressed at different stages of EB development similar to the BMP4 activated Smad1 protein[12,13]. Using this protocol, EB single cell suspensions were infected after 3 days of differentiation. EBs were reformed by hanging drop, and subsequently analyzed at day 8 for HPC generation by assaying CD41 and CD117 cell surface expression by flow cytometry. CD41+ single positive cells are a pool of primitive and definitive HPCs, whereas CD41+CD117+ cells contain only definitive HPCs[22,23]. We observed a significant increase in the overall population of CD41+ HPCs in the MSCV-mirn23a infected cells compared to the MSCV control virus infected cells (**Figure 3**). The figure also demonstrates that a high contribution of infected cells to the reformed EBs can be achieved. It is important to infect EBs with both a control virus expressing the fluorescent protein alone, as well as infecting cells with the experimental virus. Infected populations (GFP+) should then be compared for differences in phenotype. Examining differences between uninfected cells versus infected cells may give erroneous results. Comparing GFP- (uninfected), and GFP+ (infected) populations there is a difference in the CD41 populations in both the MSCV and MSCV-mirn23a cultures (**Figure 3**). This increase may be due to retroviruses infecting proliferative cells.

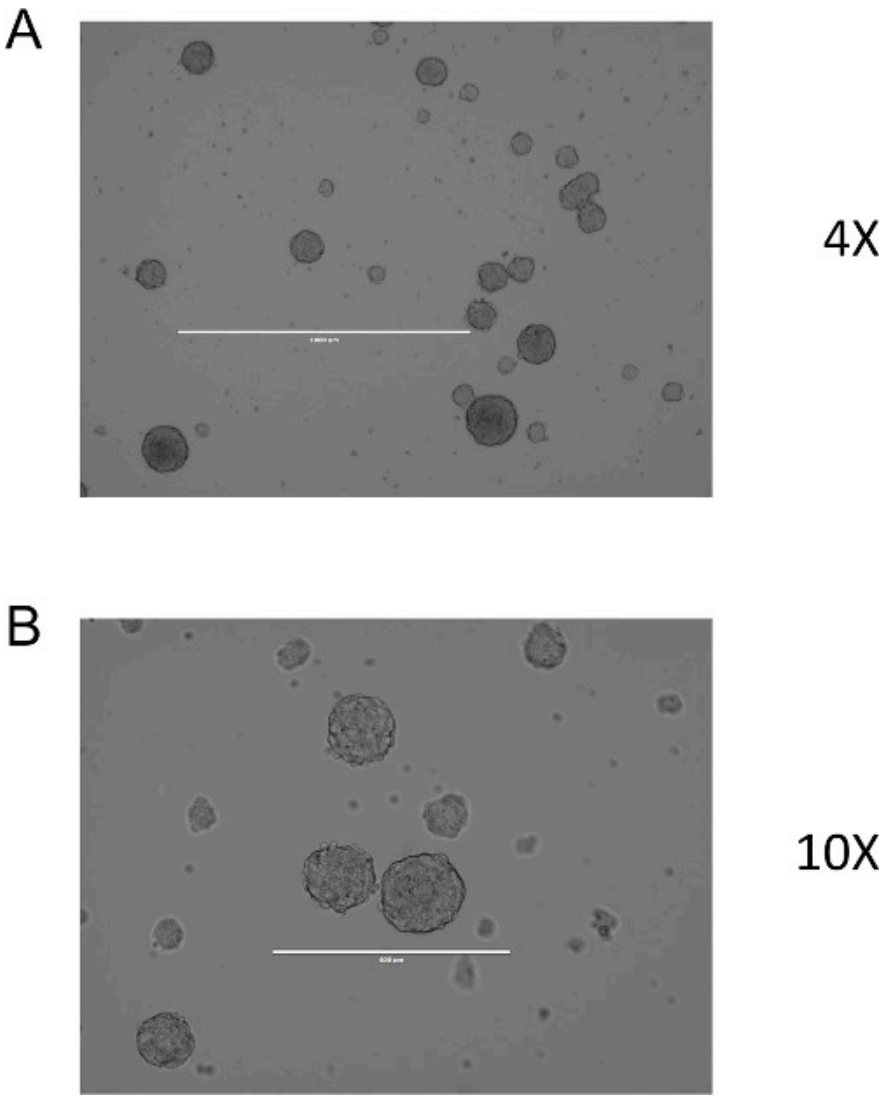

Figure 1: Representative Day 3 Embryoid Bodies. RW4 ESCs were switched from ESC maintenance media into differentiation media and cultured in 10 cm plates. **(A)** 4X and **(B)** 10X images are shown of embryoid bodies that developed after 3 days of culture. In the 4X image the bar represents 1,000 µm, whereas in the 10X image the bar represents 400 µm.

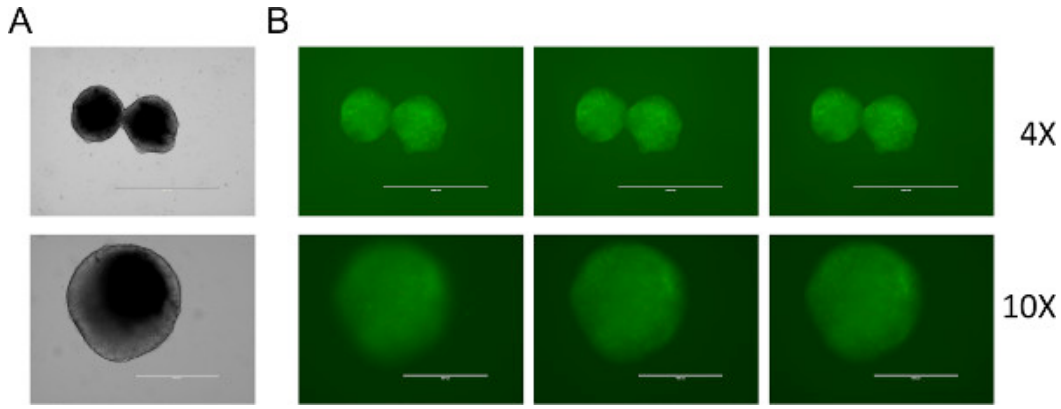

Figure 2: Eight day Embryoid Bodies (EB) reformed from retroviral infected day 3 EB cells. (A) Left hand side images are bright field. **(B)** The three fluorescent columns of fluorescent images to the right are the same EB taken in different focal planes. In the 4X images the bar represents 1,000 µm, whereas in the 10X images the bar represents 400 µm.

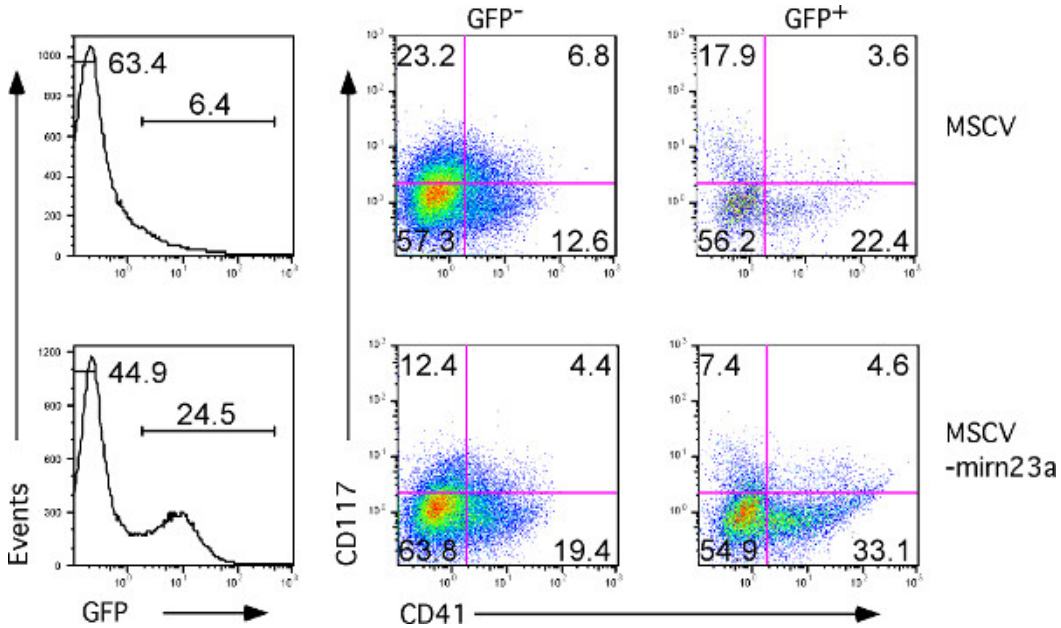

Figure 3: Flow cytometry analysis of hematopoietic progenitor cells produced in chimeric EBs. Single cells suspensions were prepared from d3 EBs, and infected with the indicated retroviruses. EBs were reformed by hanging drop, and cultured an additional 8 days. Single cell suspensions were generated and incubated with fluorescently labeled antibodies to CD41, and CD117. Left hand histogram plots show the GFP- (non-infected cells) and GFP+ (infected cells) gates. The right hand panels show color dot plot of cells positive for CD41, and CD117 expression in the GFP- and GFP+ gates. Primitive hematopoietic progenitors are found in the CD41+ fractions, and definitive progenitors are present in both the CD41+CD117-, and CD41+CD117+ fractions. Please click here to view a larger version of this figure.

Discussion

As discussed above, ESC clones that inducibly express a gene of interest can be generated using doxycycline systems, however, generation of these lines is time-consuming and labor intensive. Described in this protocol is a method to express a gene of interest in single cell suspension prepared from ESC derived EBs. These infected cells are then reformed into EBs by hanging drop to examine subsequent differentiation. In the example (**Figure 3**), it is shown that expression of the *mirn23a* cluster enhances hematopoietic development when expressed at day 3 of differentiation. At this point the development of nascent germ layer tissue has occurred including early mesoderm that gives rise to blood progenitors. At day 2 and 3 of RW4 ESC differentiation, expression of genes representing ectoderm (Pax6), endoderm (FoxA2), and mesoderm (T, Twist, and Tbx6) are detected by quantitative reverse transcriptase PCR (Q-RT-PCR, Data not shown). For *mirn23a* to enhance HPC production, it may need to be expressed in early mesoderm. This technique allows for the ability to determine if a transgene has different effects upon its temporal expression without expending a lot of effort to generate novel reagents. Upon observing phenotypes associated with distinct temporal expression, an investigator may want to now spend the effort to generate inducible lines where the expression of the transgene can be tightly controlled.

Other applications for this technique include performing rescue experiments in ESCs where both alleles of a gene of interest have been deleted (Double Knockout ESCs, DKOs). The wildtype gene or mutated versions of the deleted gene could be reintroduced into the differentiating EB cells. Additionally rescue of a phenotype by genes downstream of the deleted gene can be be assayed. This method can be used to examine the cell intrinsic nature of the observed phenotypes. For example in hematopoiesis, defects have been observed in genes that affect either the

hematopoietic stem and progenitor cells themselves or sometimes the defect may affect cells in the microenvironment that are needed to support the development of stem and progenitor cells[16,24]. In this system, if the defect is intrinsic to hematopoietic progenitor cells, then reintroduction of the wildtype gene will only rescue hematopoietic development in the GFP+ (retroviral infected) cells. If the defect were non-cell autonomous, then retroviral infection would result in a rescue of hematopoietic development being observed in both GFP+ and GFP- populations.

In this protocol flow cytometry is used to identify hematopoietic progenitor cells by the cell surface expression of CD41 and CD117. Alternatively, after 8 days of differentiation infected GFP+ cells could be isolated by fluorescence activated cell sorting (FACs). The isolated cells could then be assayed for the presence of specific hematopoietic progenitors by hematopoietic colony assays in methylcellulose media as we have previously described[25,26]. Similarly, RNA can be extracted from sorted cells and analyzed for expression of lineage specific genes.

The advantage of the described technique is that it requires minimal effort to generate the needed reagents, and allows for temporal expression of a transgene in the developing EB. If a viral vector co-expressing the gene of interest with a fluorescent protein is already available the experiment can be performed immediately. There are a few limitations to be considered with choosing to use this protocol. The technique outlined here does require the ability to produce high-titer retrovirus to have a sufficient number of cells for analysis. Also note that use of the retrovirus can result in insertional mutagenesis that could yield a phenotype. However since this protocol is examining a pool of infected cells instead of a clonal population, it is less likely that a phenotype due to insertional mutagenesis will be observed over the short time of culture. The major limitation of the technique (compared to an inducible system such as at tet system) is that expression of the transgene cannot be silenced at a later time point, and there is no ability to control the levels of expression. ESCs that express a transgene under the control of doxycycline regulation is advantageous since it allows not only for turning on the gene in a temporal manner, but also allows for turning off the gene at a later timepoint. Varying the amount of an inducer such as doxycycline will also allow the user to fine-tune expression. Lastly the inducible systems allow for reproducible expression of the transgene in all cells, whereas in this retroviral protocol there is chimeric expression of the transgene. As discussed above though this chimeric expression could be valuable for some applications. The inducible systems clearly offer a lot of advantages. However, due to the ease and decreased expense of the technique described here, many investigators will find it useful for their studies of embryonic stem cell differentiation.

Disclosures

The authors have no competing financial interests to disclose.

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