Video Article

Mouse Fetal Liver Culture System to Dissect Target Gene Functions at the Early and Late Stages of Terminal Erythropoiesis

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Abstract

Erythropoiesis involves a dynamic process that begins with committed erythroid burst forming units (BFU-Es) followed by rapidly dividing erythroid colony forming units (CFU-Es). After CFU-Es, cells are morphologically recognizable and generally termed terminal erythroblasts. One of the challenges for the study of terminal erythropoiesis is the lack of experimental approaches to dissect gene functions in a chronological manner. In this protocol, we describe a unique strategy to determine gene functions in the early and late stages of terminal erythropoiesis. In this system, mouse fetal liver TER119 (mature erythroid cell marker) negative erythroblasts were purified and transduced with exogenous expression of cDNAs or small hairpin RNAs (shRNAs) for the genes of interest. The cells were subsequently cultured in medium containing growth factors other than erythropoietin (Epo) to maintain their progenitor stage for 12 hr while allowing the exogenous cDNAs or shRNAs to express. The cells were changed to Epo medium after 12 hr to induce cell differentiation and proliferation while the exogenous genetic materials were already expressed. This protocol facilitates analysis of gene functions in the early stage of terminal erythropoiesis. To study late stage terminal erythropoiesis, cells were immediately cultured in Epo medium after transduction. In this way, the cells were already differentiated to the late stage of terminal erythropoiesis when the transduced genetic materials were expressed. We recommend a general application of this strategy that would help understand detailed gene functions in different stages of terminal erythropoiesis.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51894/>

Introduction

Erythropoiesis is the process of differentiation of multipotent hematopoietic stem cells to mature erythrocytes. This stepwise process includes the formation of committed erythroid burst forming units (BFU-Es), the rapidly dividing erythroid colony forming units (CFU-Es), and morphologically recognizable erythroblasts^{1,2}. Terminal erythropoiesis from CFU-E progenitor cells involves sequential erythropoietin-dependent and independent stages^{2,3}. In the early stage of terminal erythropoiesis, Erythropoietin (Epo) binds to its receptor on the cell surface and induces a series of downstream signaling pathways that prevent cell apoptosis and promote rapid cell divisions and gene expression^{1,4}. In the late stage of terminal erythropoiesis, erythroblasts undergo terminal cell cycle exit, chromatin and nucleus condensation, and extrusion of the highly condensed nuclei⁵.

Our understanding of terminal erythropoiesis has greatly improved in the last few decades, which is largely due to the successful use of several *in vitro* and *in vivo* mouse models⁶⁻⁹. Among these models, *in vitro* culture of mouse fetal liver erythroblasts provides many advantages including the ease of cell purification, fast proliferation and differentiation, and a closer mimic to human erythropoiesis^{10,11}. In this system, large numbers of erythroid progenitor cells from mouse fetal livers can be easily isolated by the single step purification of TER119 (a marker for the mature erythroid cells) negative erythroblasts. During the two-day culture of the erythroblasts, the differentiation of these cells can be monitored by a flow cytometric analysis based on surface expression of the transferrin receptor (CD71) and the TER119 antigen¹². In addition, enucleation of the terminally differentiation erythroblasts can be detected by a DNA maker (Hoechst 33342)¹³. Furthermore, the purified progenitors can be genetically modified by exogenous expression of cDNAs or small hairpin RNAs (shRNAs) for the genes of interest, which facilitates the mechanistic studies of the functions of gene expression on erythropoiesis^{11,13,14}.

On the other hand, the fast cell growth rate can be a double-edged sword since it is difficult to characterize gene functions in different stages of terminal erythropoiesis. In most cases, it is difficult to determine whether a specific gene functions in the early stage of terminal erythropoiesis since by the time the cDNAs or shRNAs expressed, the cells already passed the early stage. To solve this problem, we developed a unique system to dissect the early and late stages of terminal erythropoiesis. For the early stage of terminal erythropoiesis, genetically modified TER119 negative erythroblasts were cultured in Epo-free medium but containing stem cell factor (SCF), IL-6 and FLT3 ligand to maintain their progenitor status and allow the transduced cDNAs or shRNA to expression¹³. The cells were changed to Epo containing medium after 12 hr to induce cell proliferation and differentiation. In this way, when the cells started to differentiate, the transduced cDNAs or shRNAs were already expressed. For the late stage of terminal erythropoiesis, TER119 negative erythroblasts were cultured in Epo containing medium immediately after transduction.

Therefore, one can analyze the functions of the genes of interest in the late stage of terminal erythropoiesis. In summary, a broad application of this system would help dissect gene functions in different stages of terminal erythropoiesis.

Protocol

The experiments described in this protocol were performed in accordance with the guidelines and regulations set forth by Northwestern University Institutional Animal Care and Use Committee.

1. Preparation of Culture Medium

1. Prepare fibronectin solution. Add 1 ml of water to one vial of human fibronectin (1 mg). Leave solution in the tissue culture hood for 30 min without agitation. Transfer the total liquid to 50 ml of PBS to make a final concentration of 20 $\mu\text{g/ml}$ and gently mix well. Make the fibronectin solution fresh before coating the plate (see below).
2. Prepare 50 ml of Epo free medium (SCF medium). Combine the listed ingredients in **Table 1**. The medium can be stored at 4 °C for 1 month when made fresh.
3. Prepare 50 ml of Epo containing medium. Combine the listed ingredients in **Table 2**. The medium can be stored at 4 °C for 1 month when made fresh.

2. Fibronectin Coated Plate

1. Add 1 ml fibronectin solution to each well of a 6-well plate (or 0.5 ml to each well of a 12 well plate). Leave the plate in the tissue culture hood for 1 hr.
2. Rinse wells with sterile water twice and air dry the plate.
3. Wrap the plate and save in the cold room at 4 °C.

3. Purification of Fetal Liver Erythroblasts

NOTE: Use fetal livers from E13 to E15 timed pregnant mice (C57BL/6). E13.5 livers are preferred to maximize the yield of CFU-E progenitors. To obtain the timed pregnant mice, 6 to 8 weeks old male and female mice were placed in one cage (usually one male with one or two females). Female vaginal plugs were checked the next morning to determine if the mating was successful. The first day of gestation was the day after the plug was found.

1. Preparation of the Total Fetal Liver Cells
 1. Sacrifice the mouse by CO₂ inhalation and cervical dislocation. Spray the abdomen with 70% ethanol for disinfection. Open the abdomen with dissecting scissors to remove the uterus. Wash the uterus in 1x PBS.
 2. Dissect the fetuses under sterile conditions using a tissue culture hood and autoclaved tools and wash in 1x PBS.
 3. Dissect the fetal livers from the fetuses. Hold the body of the fetus with one forceps, gently pull the fetal liver (pink in color) away from the body with another forceps. Clean the fetal liver with the forceps to remove the associated fibrotic tissues. Place all fetal livers into fresh 1x PBS containing 10% fetal bovine serum.
 4. Mechanically disrupt fetal livers by pipetting up and down.
 5. Filter the cell suspension through a 40 μm cell strainer into a 50 ml conical tube. Pellet the cells at 800 x g for 5 min.
 6. Aspirate the supernatant and resuspend the cells in 1 ml PBS with 10% FBS (approximately 8 E13.5 fetal livers per ml).
2. Purification of TER119 Negative Erythroblasts
 1. Block the cells with rat IgG (0.2 mg/ml) on ice for 15 min.
 2. Without washing or centrifuging, add biotinylated anti-TER119 antibody to the cell suspension (1 $\mu\text{g/ml}$). Incubate for 15 min on ice.
 3. Wash in 1x PBS with 10% FBS (1:10) and centrifuge at 800 x g for 5 min. Resuspend the cells in 1 ml of PBS with 10% FBS.
 4. Add 75 μl streptavidin conjugated with magnetic particles and incubate for 10 mins on ice.
 5. Add the volume to 2.5 ml with PBS containing 10% FBS. Transfer the cell suspension into a 5 ml polypropylene round bottom tube.
 6. Insert the tube into a magnetic cell-sorting apparatus and incubate for 10 min. TER119 positive cells will attach to the side of the tube. The unattached TER119 negative erythroblasts will remain in the suspension.
 7. Pour the cell suspension into a new 5 ml polypropylene round bottom tube.
 8. Repeat steps 3.2.6 and 3.2.7 to remove the residual TER119 positive cells.
 9. Pellet the cells at 800 x g for 5 min. Aspirate off the supernatant. Resuspend the cells in 1 ml PBS containing 10% FBS and count live cells with Trypan Blue.

4. Transduction with Viruses Encoding cDNA or shRNA

1. Plate the purified TER119 negative fetal liver cells at 3-5 x 10⁵/well in a fibronectin coated 12-well plate (adjust the cell number if using a different plate).
2. Add polybrene to a final concentration of 10 $\mu\text{g/ml}$ to facilitate viral transduction. Spin infect the cells with lentiviruses or retroviruses, encoding cDNA or shRNA, at 800 x g for 1-1.5 hr at 37 °C.

5. Culture of the Transduced TER119 Negative Mouse Fetal Liver Erythroblasts

- To test gene functions in the early stage of terminal erythropoiesis (**Figure 1** dashed lines).
 - Culture the transduced cells in Epo free medium (SCF medium) for 12 hr to allow the expression of the transduced genes and maintenance of the progenitor state.
 - Centrifuge the cells at 800 x g for 5 min. Aspirate the supernatant. Resuspend the cells in 1 ml Epo containing medium in each well of a 12-well plate.
 - After culture for 24 or 48 hr, harvest the cells for further assays.
- Test gene functions in the late stage of terminal erythropoiesis (**Figure 1** solid lines).
 - Culture the transduced cells immediately in Epo containing medium.
 - After culture for 24 or 48 hr, harvest the cells for the further assays.

6. Staining of the Cultured Fetal Liver Cells for Flow Cytometric Analysis

- Wash and resuspend the cells in 1x PBS. Harvest 2×10^5 cultured cells for flow cytometric analysis.
- Incubate the cells with fluorophore-conjugated antibodies for 20 min at room temperature in the dark. To test differentiation, use APC conjugated anti-CD71 and PE conjugated anti-TER119. To test enucleation, use Hoechst 33342 stain in addition to the other antibodies.
- Wash cells with 1x PBS. Resuspend the cells in 0.5 ml PBS containing 1% FBS and propidium iodide (PI) (1 μ g/ml) to stain the dead cells and exclude them from analysis.
- Conduct flow cytometric analysis. Analyze single color stained and unstained control cells first for adjustment and compensation of the flow cytometer.

Representative Results

Figure 1 outlines the experimental strategies. The protocol consists of two independent conditions for targeting the functions of the signaling molecules in the early and late stages of terminal erythropoiesis. TER119 negative fetal liver erythroblasts were purified from E13.5 mouse fetus. Flow cytometric analysis of fetal liver erythroid cells before and after purification demonstrated that the purification was efficient (**Figures 2A and 2B**). For the early stage of terminal erythropoiesis (**Figure 1**, dashed lines), after viral transduction, the cells were cultured in Epo-free medium (SCF medium) for 12 hr. During this period, the cells showed minimal differentiation (**Figure 2C**) with no significant apoptosis observed in the transduced cells (GFP positive) (**Figure 3**). The cells started to differentiate after transferring to Epo-containing medium (**Figure 4**), which was similar to the cells cultured in Epo containing medium immediately after transduction (**Figure 1**, solid lines and **Figure 5**). During the 2 day culture in Epo containing medium in both methods, most of the erythroid progenitor cells ($CD71^{low} TER119^{low}$) exhibited induction of the transferrin receptor (CD71) and TER119 on day 2 ($CD71^{high} TER119^{high}$) (**Figure 4A and Figure 5A**). Enucleation occurred on day 2, as observed by $Hoechst^{high} TER119^{high}$ (erythroblasts) and $Hoechst^{low} TER119^{high}$ (reticulocytes) (**Figure 4B and Figure 5B**). It is notable that cells immediately culture in Epo (solid line) was relatively more efficient for differentiation and enucleation than cells cultured first in SCF medium (dashed line).

Figure 1. Schematic overview of the experimental strategy. Dashed and solid lines represent methods for studying signaling molecules in the early and late stages of terminal erythropoiesis, respectively.

Figure 2. Flow cytometric analysis of mouse fetal liver erythroid cells before and after purification of TER119 negative erythroblasts. (A) Total fetal liver cells stained with TER119 and CD71. (B) Purified TER119 negative cells stained with TER119 and CD71. (C) Purified TER119 negative cells after 12 hr culture in SCF medium. [Please click here to view a larger version of this figure.](#)

Figure 3. Flow cytometric analysis of apoptosis after culture in Epo-free medium (SCF medium) for 12 hr. (A-B) The TER119 negative erythroblasts were transduced with control lentiviruses (A) or lentiviruses encoding green fluorescent protein (GFP) (B). The percentage of the GFP positive transduced cells was presented after 12 hr of culture in SCF medium. (C-D) Analysis of apoptosis in GFP negative (C) and positive (D) cells from (B).

Figure 4. Flow cytometric analysis of differentiation and enucleation of the transduced erythroblasts cultured in SCF medium followed by Epo medium. (A) As in Figure 3, GFP negative and positive cells were gated for differentiation analysis using CD71 and TER119 after the cells were changed to Epo medium and cultured for an additional 48 hr. **(B)** Enucleation assay using Hoechst and TER119 of the indicated cells after 48 hr in Epo medium.

Figure 5. Flow cytometric analysis of differentiation and enucleation of the transduced erythroblasts directly cultured in Epo medium. (A) The TER119 negative cells were transduced with lentiviruses encoding GFP. The cells were immediately cultured in Epo medium after transduction. Cell differentiation analysis of GFP negative and positive cells was performed using CD71 and TER119 after 48 hr in culture. (B) Enucleation assay of the indicated cells using Hoechst and TER119 after 48 hr in culture.

Ingredient	Quantity
IMDM	41.385 ml
Fetal bovine serum (FBS, 15%)	7.5 ml
b-mercaptoethanol (10 ⁻⁴ M)	50 μ l (10 ⁻¹ M stock in IMDM)
Penicillin-Streptomycin (1%)	500 μ l
Glutamine (2 mM)	500 μ l
Stem Cell Factor (50 ng/ml)	25 μ l from 100 ng/ml stock
FLT3 Ligand (FLT-3L) (30 ng/ml)	30 μ l from 50 ng/ml stock
IL-6 (20 ng/ml)	10 μ l from 100 ng/ml stock

Table 1. Ingredient for SCF medium (Epo free medium).

Ingredient	Quantity
IMDM	36.3 ml
FBS (15%)	7.5 ml
Bovine Serum Albumin (BSA, 10%)	5 ml (10% stock in IMDM)
Insulin (10 mg/ml)	50 μ l from 4 °C stock

Holo-transferrin (200 mg/ml)	200 μ l (50 mg/ml in Water, -20 °C stock)
b-mercaptoethanol (10^{-4} M)	50 μ l (10^{-1} M stock in IMDM)
Penicillin-Streptomycin (1%)	500 μ l
2 mM Glutamine	500 μ l
Epo (2 U/ml)	33 μ l (3,000 U/ml stock)

Table 2. Ingredient for Epo containing medium.

Discussion

Here we present a unique system to chronologically analyze mouse fetal liver terminal erythropoiesis. Through the application of different culture conditions, we successfully dissected terminal erythropoiesis in early and late stages. This is particularly important to determine the mechanisms of genes with multiple functions. For example, Rac GTPases play important roles in different stages of terminal erythropoiesis. Inhibition of Rac GTPases in the early stage of terminal erythropoiesis influences cell differentiation and proliferation. On the other hand, inhibition the activity of Rac GTPases in the late stage of terminal erythropoiesis blocks enucleation without affecting cell differentiation, proliferation, or survival¹³.

Several important steps in this protocol should be kept in mind. First, E13.5 mouse fetal livers are preferred for the purification of TER119 negative cells. Older embryos can be used but the proportion of the progenitors out of the total fetal liver cells will be low. Second, it is important to use low endotoxin BSA in Epo medium since it is critical for enucleation, although the mechanism is not clear. Third, although fibronectin was reported to be important for erythropoiesis¹⁵, we found that the use of fibronectin coated plate was optional. It does not affect cell proliferation, differentiation, or enucleation in our system. However, fibronectin can be useful for the cells to attach to the plate that might be important for certain applications such as immunostaining. Fourth, a small percentage of reticulocytes or late stage erythroblasts may lose GFP signal that was carried with the transduced cells. Alternatively we use human CD4 as a marker to track the transduced cells. Finally, previous reports recommended removal of erythropoietin from the culture on day 1¹¹. We found that it was not necessary to do so since there was no significant difference on cell differentiation, proliferation, or enucleation with or without removal of erythropoietin. On the contrary, change of medium may cause cell loss.

Using the same strategy, we recently discovered more than 30 genes that play novel functions in different stages of terminal erythropoiesis (Zhao *et al.*, unpublished results). Like Rac GTPases, many of these genes play dual functions in both early and late stages of terminal erythropoiesis. This indicates that signaling pathways in terminal erythropoiesis are closely interconnected. Genes involved in actin cytoskeleton remodeling and nuclear condensation are those that tend to play dual functions due to the critical roles of these signaling pathways throughout terminal erythropoiesis. On the other hand, genes involved in pathways such as heme synthesis and generation of erythroid specific plasma membrane tend to be more restricted in the early stage of terminal erythropoiesis^{16,17}. Although there are certain limitations of this experimental strategy, such as relatively low infection efficiency compared to transduction of cell lines, and difficulty of knocking down proteins with long half-life, we recommend a routine practice to use our system given the importance of dissecting gene functions in different stages of terminal erythropoiesis. In addition, this experimental strategy can also be used for other applications. For example, using the SCF medium culture condition, genes that are involved in the maintenance of stem cell properties such as self-renewal or differentiation could be analyzed. Using the method for analyzing late stage erythropoiesis, one could also determine the function of genes that could be involved in autophagy for the removal of organelles.

Broadly, this experimental strategy can be applied to the functional investigation of other hematopoietic systems such as megakaryopoiesis and myelopoiesis. Successful application of this strategy would also reveal the hidden side of the molecular mechanisms of specific genes in different developmental stages of blood cells.

Disclosures

The authors declare that they have no competing financial interests.

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