

# ***TLX1 (HOX11)* Immortalization of Embryonic Stem Cell–Derived and Primary Murine Hematopoietic Progenitors**

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## **ABSTRACT**

The ability to generate genetically engineered cell lines is of great experimental value. They provide a renewable source of material that may be suitable for biochemical analyses, chromatin immunoprecipitation assays, structure-function studies, gene function assignment, and transcription factor target gene identification. This unit describes protocols for *TLX1 (HOX11)*-mediated immortalization of murine hematopoietic progenitors derived from in vitro differentiated murine embryonic stem cells, or from primary mouse fetal liver or bone marrow. A wide variety of hematopoietic cell types have been immortalized using these procedures including erythroid, megakaryocytic, monocytic, myelocytic, and multipotential cell types. These lines are typically cytokine dependent for their survival and growth. *Curr. Protoc. Stem Cell Biol.* 7:1F.7.1-1F.7.19. © 2008 by John Wiley & Sons, Inc.

Keywords: murine cell immortalization • *TLX1* • *HOX11* • hematopoietic in vitro ES cell differentiation • hematopoietic progenitors

## **INTRODUCTION**

The ability to disrupt and/or manipulate selected genes in embryonic stem (ES) cells and whole animals has revolutionized the study of molecular and developmental biology. However, primary tissue from gene-targeted mice has limited applications because of the relatively small amount of material available, heterogeneity of cell types present, variability due to harvests from multiple animals, and cumbersome nature of accessing material for repeated experiments. Generation of immortalized cell lines from in vitro differentiated gene-targeted ES cells or primary cells from gene-targeted animals allows for a uniform, self-sustaining source of material for repeated studies.

Several different approaches have been reported for the immortalization of primary murine hematopoietic cells that include retroviral expression of *Myc* (Green et al., 1989), *Myb* (Gonda et al., 1989), *Hoxb8 (Hox-2.4)*; Perkins and Cory, 1993), *TLX1* (formerly called *HOX11*; Hawley et al., 1994a), *E2A-Pbx1* (Kamps and Wright, 1994), *MLL* (Lavau et al., 1997), *Lhx2* (Pinto do et al., 2002), *RARA* (Du et al., 1999), *Hoxa9* (Calvo et al., 2000; Schnabel et al., 2000), *Notch1* (Varnum-Finney et al., 2000), *v-raf/v-myc* (Coghill et al., 2001), *MYST3-NCOA2* (Deguchi et al., 2003), *Evi1* (Du et al., 2005), *HOXB6* (Fischbach et al., 2005), *HOXB4* (Zhang et al., 2007), *β-catenin* (Templin et al., 2008), and *Id1* (Suh et al., 2008). A subset of the genes utilized in these studies—most notably, *TLX1*—have also been demonstrated to efficiently immortalize ES cell–derived hematopoietic cells (Keller et al., 1998; Cantor et al., 2002; Shaw et al., 2006; Riz et al., 2007).

The protocols in this unit can be used to provide progenitor cells that are rare, transient, and otherwise difficult to purify, for further characterization and study. The following protocols were developed for retroviral *TLX1* immortalization of hematopoietic progenitors derived from in vitro differentiated murine ES cells (Basic Protocol) as well as from primary murine hematopoietic tissues (Alternate Protocol). Support Protocol 1 addresses the maintenance of *TLX1* retroviral producer cell lines. Support Protocols 2, 3, and 4 (respectively) address cloning, freezing, and characterizing of the immortalized cells.

**CAUTION:** *TLX1* is a well characterized human oncogene. The protocols described in this unit involve retroviral transduction of *TLX1* into murine cells. The retroviral particles are designed to be replication-defective and are generated in murine ecotropic retroviral producer cell lines. Nonetheless, caution should be taken when handling the retroviral supernatants. Standard microbiologic safety procedures (see <http://www.absa.org/restraining.html>) should be followed including the use of gloves, lab coats, and safety goggles when handling the retroviral supernatants. All materials and surfaces coming in contact with the retroviral supernatants should be decontaminated following standard procedures. The protocols described below should not be altered.

**NOTE:** The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.

**NOTE:** All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used throughout.

**NOTE:** All incubations are performed in a humidified 37°C/5% CO<sub>2</sub> tissue culture incubator unless stated otherwise.

## **BASIC PROTOCOL**

### **TLX1 IMMORTALIZATION OF ES CELL IN VITRO DIFFERENTIATED HEMATOPOIETIC PROGENITORS**

This protocol involves in vitro differentiation of murine ES cells to embryoid bodies (EBs) under conditions that promote hematopoietic development. The EBs (containing hematopoietic progenitor cells) are then disaggregated and the released cells transduced with *TLX1*-expressing retroviruses by coculture with *TLX1* retroviral producer cells to generate immortalized hematopoietic cells. Single cell clones are then isolated to produce cell lines.

#### **Materials**

Murine ES cells, gel-adapted (grown on gelatinized plates, not on feeder cells) and of low passage number (see UNIT 1C.4)

IMDM-ES-15 with MTG, LIF, and pen/strep (see recipe)

0.05% (w/v) and 0.25% (w/v) trypsin/EDTA (Invitrogen, cat. no. 25200 and 25300, respectively)

IMDM-ES-5 with MTG and pen/strep (see recipe)

Primary ES cell differentiation medium (see recipe)

*TLX1*-retroviral producer cell line (see Support Protocol):

GP+E-86/MSCV-HOX11 for cotransduction of the neomycin phosphotransferase (*neo*) gene conferring resistance to the neomycin analog Geneticin in mammalian cells *or*

GP+E-86/MSCVhyg-HOX11 for cotransduction of the hygromycin B phosphotransferase (*hyg*) drug resistance gene

Isocove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 12440)

IMDM-ES-15: IMDM supplemented with 15% (v/v) ES-FBS

Coculture medium (see recipe)

IMDM-ES-15 with glutamine and pen/strep (see recipe)  
Immortalized cell medium (see recipe)  
50 mg/ml Geneticin (Invitrogen cat. no. 10131-035) *or* 50 mg/ml hygromycin B (Mediatech) stock  
Gelatinized 25-cm<sup>2</sup> tissue culture flasks (see recipe)  
15-ml and 50-ml conical centrifuge tubes (sterile)  
Tissue culture centrifuge (refrigerated, benchtop centrifuge with swinging bucket rotor)  
100-mm petri dishes  
100-mm and 60-mm tissue culture dishes  
20-G needle and 3-ml syringe  
Hemocytometer  
Cell irradiator (optional)  
Additional reagents and equipment for counting viable cells (*UNIT 1C.3*)

### ***Prepare ES cells***

1. Two days before inducing differentiation, split the murine gel-adapted ES cells at various dilutions (e.g., 1:10, 1:5, 1:3) into 10 ml of IMDM-ES-15 with MTG, LIF, and pen/strep and plate in gelatinized 25-cm<sup>2</sup> flasks. Refeed cells daily.
2. After two days, choose a flask with cells at ~25% to 50% confluency. Wash cells briefly with 2 ml of 0.05% trypsin/EDTA, and then add 2 ml of 0.25% trypsin/EDTA. Incubate until cells start to loosen from the flask (about 3 min).
3. Pipet up and down several times to dissociate cells and add the suspension to 10 ml of IMDM-ES-15 with MTG, LIF, and pen/strep in a 15-ml centrifuge tube.
4. Centrifuge 5 min at ~200 × g, 4°C in a tissue culture centrifuge.
5. Carefully remove the supernatant and resuspend the pellet in 5 ml of IMDM-ES-5 with MTG and pen/strep.
6. Count the number of viable cells in a hemacytometer (*UNIT 1C.3*).

### ***Differentiate ES cells to EBs***

7. Add 50,000 cells to each of three 100-mm **petri** dishes (do not use tissue culture dishes) containing 10 ml primary ES differentiation medium.

*The cells will probably need to be diluted 1:10, using IMDM-ES-5 with MTG and pen/strep. IMDM is used for dilutions here and in step 5 (instead of differentiation medium) in order to minimize waste of cytokines, which are expensive.*

*It is important to use petri plates (which are not coated) in this step because the ES cells will **not** form EBs on tissue culture dishes (which are coated with a material, usually poly-lysine, to promote cell attachment).*

8. Incubate for 6 or 7 days without changing the medium.

*The length of incubation will depend on stage of differentiation and cell type targeted for immortalization. We have noted lower immortalization efficiency from EBs differentiated for fewer than 6 days.*

9. During the EB differentiation, grow TLX1 retroviral producer cells in 100-mm tissue culture dishes, aiming to have cells at ~90% to 100% confluency on the day of coculture with the ES-derived hematopoietic progenitors (day 6 to 7 of EB differentiation).

*Typically, a confluent dish of producer cells split ~1:7 to 1:10 will be 90% to 100% confluent in ~3 days.*

### ***Dissociate EBs***

10. At the end of the EB differentiation period, combine the EBs and medium from the three dishes in a 50-ml conical tube. Centrifuge 5 min at  $\sim 200 \times g$ ,  $4^{\circ}\text{C}$ .
11. Carefully aspirate and discard supernatant. Resuspend the EBs in 5 ml of IMDM and then allow them to settle by gravity for  $\sim 5$  min.
12. Aspirate supernatant and add 3 ml of 0.25% trypsin/EDTA to the pellet. Incubate 3 min at room temperature.
13. Dissociate embryoid body cells by gentle passage through a 20-G needle six times.
14. Immediately add the suspension to 10 ml of IMDM-ES-15 to neutralize trypsin.
15. Measure the concentration of viable cells using a hemacytometer (*UNIT 1C.3*).
16. Centrifuge  $3 \times 10^6$  cells 5 min at  $\sim 200 \times g$ ,  $4^{\circ}\text{C}$ , and resuspend in 10 ml coculture medium.

### ***Prepare producer cells***

17. Irradiate the TLX1 retroviral producer monolayer with 3 Gy.

*The retroviral producer cell monolayer can be irradiated earlier in the day before dissociation of the EBs, and the irradiated cells left in the incubator until the time of the coculture.*

*Although irradiation of the producer cell line is recommended, it is not absolutely necessary because the immortalized hematopoietic cells grow in suspension and the producer cells are adherent. Therefore, any contaminating retroviral producer cells that get carried over after the coculture period can eventually be removed by serial passage of the populations on tissue culture plates. If gene-targeted cells containing a neo targeting construct are immortalized with the MSCV-HOX11 retrovirus, then any remaining producer cells can also be eliminated by selection in Geneticin.*

*If the producer cell line is not irradiated, the plating density should be slightly lower (e.g.,  $\sim 50\%$  to  $70\%$  confluent in a 100-mm tissue culture dish); otherwise, producer cells may become confluent and lift off the dish.*

18. Wash the irradiated cell monolayer once with 5 ml of IMDM-ES-15 with glutamine and pen/strep.

### ***Immortalize and passage EB cells***

19. Gently add the disaggregated EB cell suspension to the irradiated cell monolayer. Incubate the coculture for 3 days without changing the medium.
20. After 3 days, gently agitate the dishes to remove suspension cells loosely adherent to the monolayer and transfer the supernatant to a 15-ml conical tube.
21. Centrifuge 5 min at  $\sim 200 \times g$ ,  $4^{\circ}\text{C}$ .
22. Resuspend the cell pellet in 3 ml immortalized cell medium and incubate in 60-mm tissue culture dish.

*Over the next few days, clusters of proliferating cells in suspension should be visible.*

*In our experience, TLX1 immortalized cells are almost always IL-3 dependent for their growth and survival. Therefore, it is critical to include IL-3 in the medium after the coculture and in all subsequent steps. However, the IL-3-containing medium can be supplemented with other cytokines/growth factors, e.g., SCF, Epo (Keller et al., 1998; Yu et al., 2002; Riz et al., 2007).*

23. Select for TLX1-expressing cells (step 24) as early as 48 to 72 hr following the coculture, or passage cells in immortalized cell medium with the selective agent at 1:10 to 1:20 every 3 to 4 days, with gentle pipetting up and down to disrupt cell clusters.

**Select for TLX1-expressing immortalized cells**

24a. For cells transduced with the neo-carrying retrovirus cell line (GP+E-86/MSCV-HOX11): Add Geneticin to immortalized cell medium to a final concentration of 400  $\mu\text{g/ml}$ .

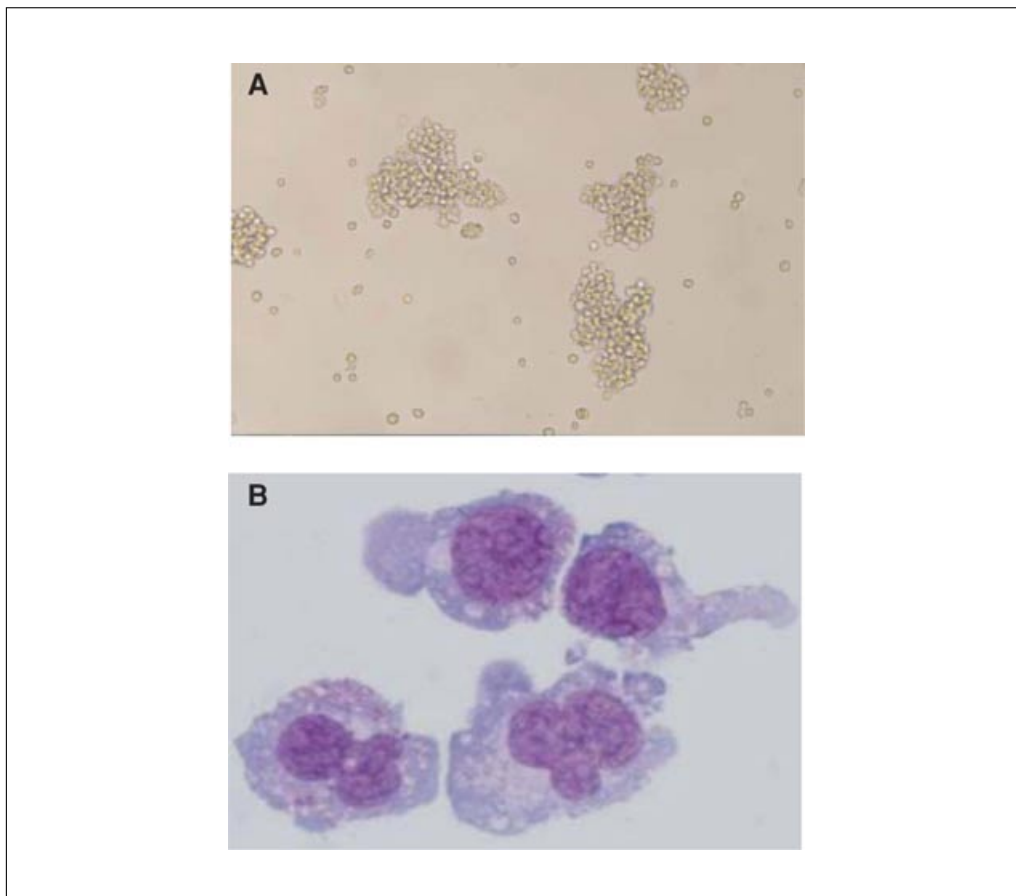
24b. For cells transduced with the hyg-carrying retrovirus cell line (GP+E-86/MSCVhyg-HOX11): Add hygromycin B to immortalized cell medium to a final concentration of 400  $\mu\text{g/ml}$ .

*While selection for retrovirally transduced cells with Geneticin or hygromycin B is recommended, it is not strictly required since TLX1-expressing cells will exhibit a growth advantage over nontransduced cells during in vitro passaging (Yu et al., 2002; Su et al., 2006) and nontransduced cells should eventually disappear from the cultures due to senescence. However, this may take as long as several months, especially for long-lived primary cells, e.g., mast cells.*

25. Clone Geneticin- or hygromycin B-resistant cells in methylcellulose (as described in Support Protocol 2) or by limiting dilution in liquid culture (see Fuller et al., 2001) using immortalized cell medium containing the appropriate selective antibiotic.

*See Figure 1F.7.1 for an example of a TLX1 immortalized cell line derived from in vitro differentiated ES cells.*

*The IL-3-containing methylcellulose and medium can be supplemented with other cytokines/growth factors, e.g., SCF, Epo (Keller et al., 1998; Yu et al., 2002; Riz et al., 2007).*



**Figure 1F.7.1** Photomicrographs of a TLX1 immortalized clonal hematopoietic cell line derived from in vitro differentiated FOG-1<sup>-/-</sup> ES cells. **(A)** Phase contrast photomicrograph of the suspension cell culture (original magnification 100 $\times$ ). **(B)** Bright field photomicrograph of May-Grunwald-Giemsa stained cytopun cells (original magnification 1000 $\times$ ). Note that the absence of FOG-1 contributes to the immature and uniform appearance of the cells.

26. Freeze clonal cell lines as described in Support Protocol 3.
27. Characterize the clonal lines, depending on the particular application, e.g., by identifying the hematopoietic cell lineages represented, and their relative state of maturation (Support Protocol 4).

**ALTERNATE  
PROTOCOL**

**TLX1 IMMORTALIZATION OF PRIMARY MURINE HEMATOPOIETIC  
CELLS**

In this protocol, primary hematopoietic cells are harvested from murine fetal liver (FL) or bone marrow (BM) and then cocultured with TLX1-expressing retroviral producer cell lines. After coculture, cells are selected for retroviral expression and cloned to generate immortalized cell lines. Procedures for immortalization of yolk sac–derived precursors cells have also been published (Yu et al., 2002).

***Additional Materials*** (also see *Basic Protocol*)

- Adult mouse
- Mouse dissection tools
- 70% ethanol spray solution
- 1.5-ml microcentrifuge tubes, sterile
- 1× PBS, sterile: diluted from 10× phosphate-buffered saline without calcium and magnesium (Sigma, cat. no. P7059) to 1× with water, and sterilized by passing through a 0.22- $\mu$ m filter
- 70- $\mu$ m cell strainer (BD Falcon; optional)
- 22-G needle and 1-ml syringe (for bone marrow progenitors)
- 23-G needle and 1-ml syringe (for fetal liver progenitors)
- 25-G needle and 1-ml syringe (for fetal liver progenitors)

**NOTE:** All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

***Prepare TLX1 producer cells***

1. Grow TLX1 retroviral producer cell to 90% to 100% confluence in a 100-mm tissue culture dish. Irradiate the cell monolayer with 3 Gy and return to the tissue culture incubator.

*Although irradiation of the producer cell line is recommended, it is not absolutely necessary since the immortalized hematopoietic cells grow in suspension and the producer cells are adherent. Therefore, any contaminating retroviral producer cells that get carried over after the coculture period can eventually be removed by serial passage of the populations on tissue culture plates. If gene-targeted cells containing a neo targeting construct are immortalized with the MSCV-HOX11 retrovirus, then any remaining producer cells can also be eliminated by selection in Geneticin.*

*If the producer cell line is not irradiated, the plating density should be slightly lower (e.g., ~50% to 70% confluent in a 100-mm tissue culture dish); otherwise, producer cells may become confluent and lift off the dish.*

***Prepare hematopoietic progenitor cells***

*For bone marrow–derived hematopoietic progenitors*

- 2a. Euthanize an adult mouse by carbon dioxide asphyxiation using compressed gas, or according to institutionally approved methods, and immediately spray the fur over the legs with 70% ethanol to disinfect. Dissect out both femurs and cut off ends.

*Methods of euthanasia other than carbon dioxide asphyxiation may be used; however, we do not know if they would affect the cells.*

- 3a. Hold the cut femur with forceps over a sterile 15-ml conical centrifuge tube and use a 1-ml syringe fitted with a 22-G needle to flush the bone marrow space with 1 ml IMDMES-15 with glutamine and pen/strep (0.5 ml in each direction). Repeat to obtain a total of 2 ml of cell suspension, pooling the sample.
- 4a. Repeat step 3a, using the other femur, and combine the samples (total 4 ml of cell suspension).
- 5a. Cap and vortex the tube. Allow debris to settle by gravity at room temperature for 5 min.
- 6a. Transfer the supernatant to fresh tube and discard the pellet, which contains mostly bone fragments and fibrous debris.
 

*Alternatively, bone marrow fragments and debris can be removed from the flushed marrow by filtering through a sterile 70- $\mu$ m cell strainer (BD Falcon).*
- 7a. Measure the viable cell concentration using a hemacytometer (*UNIT 1C.3*).
- 8a. Centrifuge  $\sim 3 \times 10^6$  cells 5 min at  $\sim 200 \times g$ , 4°C, in a tabletop tissue culture centrifuge.
- 9a. Aspirate and discard the supernatant, and resuspend the cell pellet in 10 ml of coculture medium.

*For fetal liver–derived progenitors*

- 2b. Euthanize a pregnant female mouse at embryonic day 13.5 or 14.5 (E13.5 to E14.5) by carbon dioxide asphyxiation using compressed gas, or according to institutionally approved methods.
 

*Methods of euthanasia other than carbon dioxide asphyxiation may be used; however, we do not know if they would affect the cells.*
- 3b. Immediately spray the fur over the abdomen with 70% ethanol. Using forceps and sharp scissors (first soaked in 70% ethanol and wiped dry), incise the abdominal wall and extract the embryos. Place the embryos in a sterile 100-mm petri dish containing sterile  $1 \times$  PBS, and separate them by cutting with scissors.
- 4b. Open the yolk sacs and remove the fetuses, keeping them in the  $1 \times$  PBS. Using blunt dissection with sterile forceps and scissors, remove the fetal livers from the embryos' abdomens.
 

*The liver should come out easily at this stage of development.*
- 5b. Place one fetal liver per sterile 1.5-ml microcentrifuge tube containing 1 ml IMDM-ES-15 with glutamine and pen/strep.
- 6b. Prepare a single-cell suspension by drawing the tissue through a 23-G needle five times, followed by a 25-G needle five times.
- 7b. Pool cell suspensions from several fetal livers into a 15-ml conical centrifuge tube. Determine viable cell concentration using a hemacytometer (*UNIT 1C.3*).
- 8b. Centrifuge  $\sim 3 \times 10^6$  cells 5 min at  $\sim 200 \times g$ , 4°C, in a tabletop tissue culture centrifuge.
- 9b. Aspirate and discard the supernatant, and resuspend the cell pellet in 10 ml coculture medium.

*Specific progenitor cell populations from bone marrow or fetal liver can also be immunophenotypically isolated by fluorescence-activated cell sorting (FACS) and used for coculture with TLX1-expressing retroviral producer cell lines. For markers that can be*

used to select the progenitors at the various stages of differentiation, see Akashi et al. (2000) and Pronk et al. (2007).

### **Immortalize and passage the EB cells**

10. Wash the irradiated producer cell monolayer (from step 1) once with 5 ml IMDM-ES-15 with glutamine and pen/strep.
11. Gently add the bone marrow or fetal liver cell suspension ( $\sim 3 \times 10^5$  cells/ml) to the irradiated cell monolayer. Incubate to coculture for 2 to 3 days, without changing the medium.
12. At the end of the coculture period, gently agitate the dishes to remove suspension cells loosely adherent to the monolayer and transfer the supernatant to a 15-ml conical tube.
13. Centrifuge 5 min at  $\sim 200 \times g$ , 4°C.
14. Resuspend the cell pellet in 3 ml of immortalized cell medium and incubate in a 60-mm tissue culture dish.

*Over the next few days, clusters of proliferating cells in suspension should be visible.*

15. Select for TLX1-expressing cells (step 16) as early as 48 to 72 hr following the coculture, or passage cells 1:10 to 1:20 every 3 to 4 days, with gentle pipetting up and down to disrupt cell clusters.

*In our experience, TLX1 immortalized cells are almost always IL-3 dependent for their growth and survival. Therefore, it is critical to include IL-3 in the medium after the coculture and all subsequent steps. However, the IL-3-containing medium can be supplemented with other cytokines/growth factors, e.g., SCF, Epo (Keller et al., 1998; Yu et al., 2002; Riz et al., 2007).*

### **Select for TLX1-expressing immortalized cells**

- 16a. *For cells transduced with the neo-carrying retrovirus cell line (GP+E-86/MSCV-HOX11):* Add Geneticin to a final concentration of 400  $\mu\text{g/ml}$ .
- 16b. *For cells transduced with the hyg-carrying retrovirus cell line (GP+E-86/MSCVhyg-HOX11):* Add hygromycin B to a final concentration of 400  $\mu\text{g/ml}$ .

*While selection for retrovirally transduced cells with Geneticin or hygromycin B is recommended, it is not strictly required since TLX1-expressing cells will exhibit a growth advantage over nontransduced cells during in vitro passaging (Yu et al., 2002; Su et al., 2006) and nontransduced cells should eventually disappear from the cultures due to senescence. However, this may take as long as several months, especially for long-lived primary cells, e.g., mast cells.*

17. Clone Geneticin- or hygromycin B-resistant cells in methylcellulose as described in Support Protocol 2 or by limiting dilution in liquid culture (see Fuller et al., 2001) using immortalized cell medium containing the appropriate selective antibiotic.

*The IL-3 containing methylcellulose and medium can be supplemented with other cytokines/growth factors such as SCF, Epo, etc. (Keller et al., 1998; Yu et al., 2002; Riz et al., 2007).*

18. Freeze clonal cell lines as described in Support Protocol 3.
19. Characterize the clonal lines, depending on the particular application, e.g., by identifying the hematopoietic cell lineage(s) represented, and their relative state of maturation (Support Protocol 4).

## MAINTENANCE OF TLX1 (HOX11) RETROVIRAL PRODUCER CELL LINES

The GP+E-86/MSCV-HOX11 producer cell line is a murine fibroblast cell line that exports helper-free replication-defective recombinant retrovirus carrying the *TLX1* (*HOX11*) cDNA and the *neo* drug resistance gene (Hawley et al., 1994b). The GP+E-86/MSCVhyg-HOX11 producer cell line is a murine fibroblast cell line that exports helper-free replication-defective recombinant retrovirus carrying for the *TLX1* (*HOX11*) cDNA and the *hyg* drug resistance gene (Hawley et al., 1994b). The cell lines are available from the authors upon request.

Maintain both cell lines in Dulbecco's modified Eagle medium with 4.5 g/liter glucose + 10% (v/v) heat-inactivated calf serum or fetal bovine serum (FBS). (It is acceptable to use calf serum for maintaining the producer cells to reduce costs, but FBS should be used in the Basic Protocol and Alternate Protocol.) Add Geneticin or hygromycin B to a final concentration of 400 µg/ml or 200 µg/ml, respectively, to maintain selection for the retroviral constructs. Passage cells by trypsinization with 0.05% trypsin/0.53 mM EDTA at room temperature. As soon as the cells loosen from the plate, neutralize the trypsin by adding serum to a final concentration of 10%. Split the cells at a dilution of 1:7 to 1:10 every 2 to 3 days, when they are typically 90% to 100% confluent.

The following patents have been issued that relate to the establishment of *TLX1* (*HOX11*)-immortalized ES cell-derived lines and use of the cells:

United States Patent No. 5,874,301

Title: Embryonic Cell Populations and Methods to Isolate Such Populations

Inventors: Keller, G.M., Hawley, R.G., and Choi, K.

Assignee: National Jewish Medical and Research Center, Denver, Colo.

Issued: February 23, 1999

United States Patent No. 6,110,739

Title: Method to Produce Novel Embryonic Cell Populations

Inventors: Keller, G.M., Hawley, R.G., and Choi, K.

Assignee: National Jewish Medical and Research Center, Denver, Colo.

Issued: August 29, 2000

United States Patent No. 6,555,318

Title: Method for Identification of Cell Growth or Differentiation Factors

Inventors: Keller, G.M., Hawley, R.G., and Choi, K.

Assignee: National Jewish Medical and Research Center, Denver, Colo.

Issued: April 29, 2003

United States Patent No. 6,576,433

Title: Method for Identification of Cell Growth or Differentiation Factors

Inventors: Keller, G.M., Hawley, R.G., and Choi, K.

Assignee: National Jewish Medical and Research Center, Denver, Colo.

Issued: June 10, 2003

United States Patent No. 7,374,934

Title: Cell Populations and Methods of Production Thereof

Inventors: Keller, G.M., Hawley, R.G., and Choi, K.

Assignee: National Jewish Medical and Research Center, Denver, Colo.

Issued: May 20, 2008.

## **CLONING OF IMMORTALIZED CELLS IN METHYLCELLULOSE**

This protocol describes a method for cloning Geneticin- or hygromycin B-resistant immortalized cells in methylcellulose-based medium. Alternatively, the cells can be cloned by limiting dilution (see Fuller et al., 2001) in immortalized cell medium containing the appropriate selective antibiotic, depending on which *TLX1* (*HOX11*) retroviral producer cell line was used for the immortalization (Geneticin for MSCV-HOX11 and hygromycin for MSCVhyg-HOX11).

### **Materials**

Suspension of immortalized cells (Basic Protocol or Alternate Protocol)

Methylcellulose medium (see recipe)

Immortalized cell medium (see recipe), containing the appropriate selection antibiotic

Tissue culture centrifuge (refrigerated, benchtop centrifuge with swinging bucket rotor)

15-ml centrifuge tubes (sterile)

5-ml syringe fitted with a 16-G needle

35-mm tissue culture dish (Falcon, cat. no. 35-1008)

150-mm bacterial dishes (Falcon, cat. no. 35-1058)

Inverted microscope

2- to 20- $\mu$ l micropipettor and sterile, disposable tips

96-well flat bottom tissue culture plate (Costar, cat. no. 3596)

24-well tissue culture plate (Falcon, cat. no. 35-3047)

Additional reagents and equipment for counting cells (*UNIT 1C.3*)

1. Pipet the suspension of immortalized cells up and down several times to break up clumps. Determine the cell concentration using a hemacytometer (*UNIT 1C.3*).
2. Centrifuge appropriate volumes of cell suspension containing 200, 2000, and 20,000 cells separately in 15-ml centrifuge tubes for 5 min at  $200 \times g$ ,  $4^{\circ}\text{C}$ .
3. Carefully aspirate off the supernatant, leaving a small amount ( $\sim 50 \mu\text{l}$ ) of medium behind so as not to disturb the cell pellet.
4. Add 3.5 ml methylcellulose mix containing the appropriate selection antibiotic (Geneticin or hygromycin) to each tube, using a 5-ml syringe fitted with a 16-G needle. Carefully mix the cell-methylcellulose mixture by drawing it up and down in the syringe several times. Try to avoid making air bubbles.
5. Using the syringe and needle, add  $\sim 1.5$  ml of each of the mixtures into separate 35-mm dishes. Place six dishes in a 150-mm bacterial dish, including an extra uncovered 35-mm dish filled with sterile water at the center (to prevent drying out of the methylcellulose). Cover the 150-mm bacterial dish, and place it in the tissue culture incubator.
6. Examine the cultures daily.  
*Typically, colonies are large enough to pick after  $\sim 1$  week.*
7. Choose dishes with well separated colonies. Under the inverted microscope, carefully aspirate selected colonies using a pipettor fitted with a disposable, sterile tip, and set at  $\sim 10 \mu\text{l}$ .
8. Immediately add the aspirated colony to a well of a 96-well tissue culture plate containing  $150 \mu\text{l}$  immortalized cell medium with the appropriate selection antibiotic. Wash out the tip several times by aspirating and expelling the liquid medium in the

well. Be careful not to cross-contaminate neighboring wells when passing the pipettor over the plate.

*Most of the colonies should grow nicely in the liquid culture.*

9. Examine the cultures daily, and expand them to 1-ml cultures in 24-well plates once they are growing well.
10. Expand to progressively larger culture volumes as necessary, and/or freeze aliquots as described in Support Protocol 3.

## **CRYOPRESERVATION OF IMMORTALIZED CELLS**

The following protocol describes a method we have found effective for cryopreservation of immortalized cells.

### ***Materials***

Suspension of immortalized cells (Basic Protocol, Alternate Protocol, or Support Protocol 2)

Immortalized cell medium (see recipe), ice cold

2× freezing medium (see recipe), ice cold

Tissue culture centrifuge (refrigerated, benchtop centrifuge with swinging bucket rotor)

15-ml centrifuge tubes (sterile)

1.8-ml cryovials (Nunc, cat. no. 377267)

−80°C freezer

Liquid nitrogen storage tank

1. Centrifuge  $1 \times 10^7$  immortalized cells in 15-ml centrifuge tubes 5 min at  $200 \times g$ , 4°C.
2. Aspirate and discard the supernatant, and resuspend the cells in 5 ml ice-cold immortalized cell medium.
3. Add 5 ml ice-cold 2× freezing medium. Pipet up and down to mix, and dispense 1-ml aliquots into each of 10 cryovials ( $\sim 1 \times 10^6$  cells/vial).
4. Cap vials and place on ice until they can be placed into a −80°C freezer.
5. Incubate vials in a −80°C freezer for 1 to 2 days, and then transfer to a liquid nitrogen tank for long-term storage.

*This procedure can be scaled up to freeze more cells.*

*If there is poor viability after thawing, one can try substituting heat-inactivated fetal bovine serum (FBS) for the immortalized cell medium in step 2.*

## **IMMUNOPHENOTYPIC ANALYSIS OF IMMORTALIZED CELL LINES BY FACS**

Characterization of the clonal lines will depend on the particular application. However, identification of the hematopoietic cell lineages represented by the cell line, and their relative state of maturation, is common. A basic protocol for immunophenotypic analysis of hematopoietic lineage assignment by flow cytometry (FACS) and a list of suggested antibodies (Table 1F.7.1) are provided below. Additional procedures may include histochemical staining of the cells and/or reverse transcriptase-polymerase chain reaction (RT-PCR), northern blot analysis, or western blot analysis for lineage-specific and maturation specific gene expression. For a more detailed description and protocols pertaining to hematopoietic lineage analysis, see Baron (2005).

### **SUPPORT PROTOCOL 3**

### **SUPPORT PROTOCOL 4**

**Embryonic and  
Extraembryonic  
Stem Cells**

### **1F.7.11**

**Table 1F.7.1** Specific Cell-Surface Markers and Antibodies for Different Murine Hematopoietic Lineages

Lineage	Marker	Recommended supplier and antibody clone
Erythroid	CD71 (early)	BD Pharmingen clone C2
	Ter119 (late)	BD Pharmingen clone Ter-119
Megakaryocytic	CD41 <sup>a</sup> (early)	BD Pharmingen clone MSReg30
	CD61	BD Pharmingen clone 2C9.G2
	CD42b (late)	Emfret clone Xia.G5
Granulocytic	Gr-1 (Ly-6G)	BD Pharmingen clone RB6-8C5
Macrophage	Mac-1	BD Pharmingen clone M1/70
B lymphocyte	CD19	BD Pharmingen clone 1D3
	B220	BD Pharmingen clone RA3-6B2
T lymphocyte	CD3	BD Pharmingen clone 17A2

<sup>a</sup>CD41 is also present on earlier multipotential progenitor cells.

### Materials

Cell line suspensions (Basic Protocol or Alternate Protocol)

FACS wash buffer (see recipe)

Fluorochrome-conjugated antibodies and isotype matched control antibodies (see Table 1F.7.1); choice of fluorochromes depends on the flow cytometer being used and the combination of markers being analyzed

Tissue culture centrifuge (refrigerated, benchtop centrifuge with swinging bucket rotor)

15-ml conical centrifuge tube

12 × 75-mm FACS tubes (BD Falcon, cat. no. 35-2054)

Flow cytometer (FACS analyzer)

Additional reagents and equipment for counting cells (*UNIT 1C.3*)

1. Determine cell concentration of suspension using hemacytometer (*UNIT 1C.3*).
2. Centrifuge  $2 \times 10^6$  cells in a 15-ml conical centrifuge tube 5 min at  $200 \times g$ , 4°C.
3. Aspirate and discard the supernatant. Wash the cells two times with 1 ml FACS wash buffer, gently vortexing or pipetting up and down to mix. Centrifuge 5 min at  $200 \times g$ , 4°C, between washes.
4. After the last wash, resuspend the cells with 100  $\mu$ l FACS wash buffer.
5. Add 0.2  $\mu$ g fluorochrome-conjugated antibody (typically, 1  $\mu$ l of the recommended antibodies listed in Table 1F.7.1) or equivalent isotype-matched antibody to an identical aliquot of control cells. Incubate 30 min at 4°C, in the dark, with occasional agitation of the tube to mix the cells.

*The amount of antibody suggested is a starting point. Titrations may be needed to optimize cell staining.*

6. Centrifuge the cells 5 min at  $200 \times g$ , 4°C.
7. Aspirate and discard the supernatant. Wash the cells two times with 1 ml FACS wash buffer, centrifuging 5 min at  $200 \times g$ , 4°C, between washes.
8. Resuspend the final cell pellet with 1 ml of FACS wash buffer and transfer to 12 × 75-mm FACS tubes. Place samples on ice and keep in the dark.

*The stained cells can be stored for several hours at 4°C before analyzing.*

9. Analyze on a flow cytometer (FACS analyzer).

## REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see *SUPPLIERS APPENDIX*.

### *Ascorbic acid stock solution, 5 mg/ml*

Prepare a stock solution of 5 mg L-ascorbic acid (Sigma; A-4544)/ml autoclaved water and sterilize by passing through a 0.22- $\mu$ m filter. Prepare ascorbic acid stock solution fresh for each differentiation procedure.

### *Coculture medium*

Fetal bovine serum, ES grade (FBS-ES; Hyclone), heat-inactivated (15%, v/v, final)  
100 $\times$  penicillin/streptomycin solution (Sigma; P4333): 1 $\times$  (100 U penicillin/ml;  
100  $\mu$ g streptomycin/ml) final  
200 mM glutamine (2 mM final)  
Interleukin-3 (IL-3; R & D Systems; 10 ng/ml final)  
Interleukin-6 (IL-6; R & D Systems; 2 ng/ml final)  
Interleukin-11 (IL-11; R & D Systems; 5 ng/ml final)  
Stem cell factor (SCF; R & D Systems; 50 ng/ml final)  
Isocove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 12440) to  
make up the final volume  
Store up to 1 month at 4°C

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

### *FACS wash buffer*

10 $\times$  phosphate-buffered saline, without calcium and magnesium (CMF-PB; Sigma,  
cat. no. P7059) diluted to 1 $\times$  (final) with water  
Fetal bovine serum (FBS; Hyclone), heat-inactivated (1%, v/v, final)  
Sodium azide (0.1%, w/v, final)  
Store up to 6 months at 4°C

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

### *Freezing medium, 2 $\times$*

Fetal bovine serum (FBS; Hyclone), heat-inactivated (60%, v/v, final)  
Dimethyl sulfoxide (DMSO; Sigma, cat. no. D8418; 20%, v/v, final)  
Immortalized cell medium (see recipe; 20%, v/v, final)  
Store indefinitely at  $-20^{\circ}\text{C}$

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

*The addition of DMSO to the solution is an exothermic reaction and will produce heat. Cool the 2 $\times$  freezing medium on ice before adding to cells for cryopreservation.*

### *Gelatin 0.1% (w/v)*

Dilute 10 $\times$  phosphate-buffered saline without calcium and magnesium (CMF-PBS; Sigma, cat. no. P7059), to 1 $\times$  with water. Sterilize by passing through a 0.22- $\mu$ m filter. Add 0.5 g gelatin (Sigma, cat. no. G-1890) to 500 ml of the 1 $\times$  CMF-PBS and autoclave. Store up to 1 month at 4°C, in the absence of microbial contamination.

### *Gelatinized flasks*

Prior to addition of ES cells, add enough 0.1% gelatin solution (see recipe) to coat the surface of the flask. Incubate at room temperature for 20 min. Remove excess gelatin solution before adding cells. Store up to 6 months at room temperature.

### ***IMDM-ES-5 with MTG and pen/strep***

Fetal bovine serum, ES grade (FBS-ES; Hyclone), heat-inactivated (5%, v/v, final)  
α-monothioglycerol (MTG; Sigma, cat. no. M-1753; 12.4 μl/liter final)  
100× penicillin/streptomycin solution (Sigma; P4333): 1× (100 U penicillin/ml;  
100 μg streptomycin/ml) final  
Isocove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 12440) to  
make up the final volume  
Store up to 1 month at 4°C

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

*Use MTG stock from a bottle that has been open for <6 months.*

### ***IMDM-ES-15 with glutamine and pen/strep***

Fetal bovine serum, ES grade (FBS-ES; Hyclone), heat-inactivated (15%, v/v, final)  
2 mM L-glutamine  
100× penicillin/streptomycin solution (Sigma; P4333): 1× (100 U penicillin/ml;  
100 μg streptomycin/ml) final  
Isocove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 12440) to  
make up the final volume  
Store up to 2 months at 4°C

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

### ***IMDM-ES-15 with MTG, LIF, and pen/strep***

Fetal bovine serum, ES grade (FBS-ES; Hyclone), heat-inactivated (15%, v/v, final)  
α-monothioglycerol (MTG; Sigma, cat. no. M-1753; 12.4 μl/liter final; first diluted  
from stock, in IMDM, if necessary)  
Leukemia inhibitory factor (LIF; R & D Systems; 1000 U/ml final)  
100× penicillin/streptomycin solution (Sigma; P4333): 1× (100 U penicillin/ml;  
100 μg streptomycin/ml) final  
Isocove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 12440) to  
make up the final volume  
Store up to 1 month at 4°C

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

*Use MTG stock from a bottle that has been open for <6 months.*

### ***Immortalized cell medium***

Fetal bovine serum (FBS; standard tissue culture grade; Hyclone; heat-inactivated;  
15%, v/v, final)  
100× penicillin/streptomycin solution (Sigma; P4333): 1× (100 U penicillin/ml;  
100 μg streptomycin/ml) final  
L-glutamine (2 mM final)  
Recombinant murine interleukin-3 (IL-3; R & D Systems, cat. no. 403-ML;  
10 ng/ml final) *or* X630-recombinant IL-3-conditioned medium (Karasuyama  
and Melchers, 1988; 10%, v/v, final)  
Isocove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 12440) to  
make up the final volume  
Store up to 2 months at 4°C

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

### ***Methylcellulose medium***

2.5× Methocult stock solution (StemCell Technologies, cat. no. 03234) diluted to  
1× (final) with immortalized cell medium (see recipe)  
L-glutamine (2 mM final)

*continued*

100× penicillin/streptomycin solution (Sigma; P4333): 1× (100 U penicillin/ml; 100 µg streptomycin/ml) final  
IL-3 (R & D systems, cat. no. 403-ML; 10 ng/ml final)  
Geneticin (Invitrogen, cat. no. 10131-035; 400 mg/ml final) *or* hygromycin B (400 mg/ml final) for selection (depending on the TLX1 retroviral producer cell line used for immortalization)  
Prepare fresh for each experiment

*Prewarming the methylcellulose at 37°C for a few minutes in a water bath makes it easier to work with.*

### **Primary ES differentiation medium**

1:1 fetal bovine serum (FBS; Hyclone), heat-inactivated/plasma-derived serum (PDS; Antech), 15% (v/v) final  
L-glutamine (2 mM final)  
α-monothioglycerol (MTG; 0.004%, v/v, final; first diluted from stock, in IMDM, if necessary)  
5 mg/ml ascorbic acid stock solution (see recipe; 50 µg/ml final)  
Protein-free hybridoma medium (PFHMII; Invitrogen, cat. no. 23600-026; 5%, v/v, final)  
100× penicillin/streptomycin solution (Sigma; P4333): 1× (100 U penicillin/ml; 100 µg streptomycin/ml) final  
IL-11 (5 ng/ml final)  
Recombinant murine or rat stem cell factor (rSCF; also called KL, kit-ligand; R & D Systems; 50 ng/ml final)  
Isocove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 12440) to make up the final volume  
Store up to 1 month at 4°C

*Heat inactivation of FBS is carried out for 30 min at 56°C.*

## **COMMENTARY**

### **Background Information**

The diverged homeobox-containing gene *TLX1* (formerly called *HOX11*) was first identified in T cell acute lymphoblastic leukemia carrying the t(10;14)(q24;q11) chromosomal translocation (Dube et al., 1991; Hatano et al., 1991; Kennedy et al., 1991; Lu et al., 1991). In this translocation, the T cell receptor δ chain gene regulatory sequences become juxtaposed to the 5' promoter region of the *TLX1* gene, leading to aberrant *TLX1* expression in the T cell lineage. A similar translocation, t(7;10)(q35;q24), places the T cell receptor β chain gene regulatory sequences into the *TLX1* gene 5' promoter region, causing a similar effect. Subsequent studies showed that retroviral expression of *TLX1* immortalizes murine hematopoietic cells derived from bone marrow cells, fetal liver, yolk sac, and in vitro differentiated ES cells at high frequency (Hawley et al., 1994a; Keller et al., 1998; Yu et al., 2002; Zhang et al., 2002; Yu et al., 2003; Owens et al., 2006). A wide variety of hematopoietic cell types have been immortalized, including

erythroid, megakaryocytic, myelocytic, and monocytic lineages. Interestingly, there are no reports of *TLX1* immortalization of T lymphocyte progenitors, even though its misexpression disrupts T lymphocyte differentiation and leads to T cell leukemia in humans (Owens et al., 2006). Essentially all of the reported *TLX1*-immortalized cell lines are IL-3 dependent for their growth and survival.

The mechanism of *TLX1* cell immortalization remains incompletely understood. An intact homeodomain is required, suggesting direct transcriptional effects due to DNA binding activity (Owens et al., 2003). However, overexpression of *TLX1* deregulates genes involved in controlling G1/S cell cycle progression and disrupts a G2/M cell-cycle checkpoint, apparently by indirect mechanisms mediated in part via inhibition of PP1/PP2A phosphatase activity (Kawabe et al., 1997; Riz and Hawley, 2005). *TLX1* expression also alters the subcellular distribution of CREB-binding protein (CBP), a transcriptional coactivator with histone acetyltransferase activity

(Riz et al., 2007). It seems likely that multiple mechanisms contribute to *TLX1* immortalization of hematopoietic progenitor cells.

Examples of how *TLX1* immortalized cell lines have been utilized experimentally include: (1) structure-function study of the GATA transcriptional cofactor Friend of GATA-1 (FOG-1; Cantor et al., 2002); (2) investigation of the role of the SHP-2 tyrosine phosphatase in interleukin-3 signaling (Yu et al., 2003); (3) chromatin immunoprecipitation assays demonstrating FOG-1 facilitation of GATA-1 chromatin occupancy (Pal et al., 2004); (4) establishment of a link between the proapoptotic BCL-2 family member Bid and the DNA-damage response (Zinkel et al., 2005); (5) testing the function of mitoferrin, a novel mitochondrial iron transporter (Shaw et al., 2006); and (6) transcription factor target gene identification using Cre-mediated excision in *TLX1* immortalized cells generated from *SCL<sup>flox/flox</sup>* (H. Mikkola, pers. commun.) and *Runx-1<sup>flox/flox</sup>* mice (M. Yu and A.B. Cantor, unpub. observ.).

On a note of caution, the process of immortalization by *TLX1* results in perturbed or arrested differentiation potential (Hawley et al., 1994a; Keller et al., 1998; Dixon et al., 2007; Riz et al., 2007). This should be taken into consideration when designing cell fate experiments utilizing the *TLX1* immortalized cell lines.

### **Critical Parameters and Troubleshooting**

In our experience, the protocols for *TLX1* immortalization of hematopoietic progenitors described in this unit yield cell lines quite readily. When using in vitro differentiated ES cells as a source of target cells, the time of EB harvest is an important consideration. We typically use EBs harvested at 7 days of culture. We have generated cell lines from 6-day-old EBs, but the efficiency seems to be lower. The stage of EB differentiation used for the immortalization will also theoretically affect whether primitive or definitive hematopoietic progenitors are targeted. We reported *TLX1* immortalization of embryonic precursors with both primitive and definitive hematopoietic potential from day 7 EBs (Keller et al., 1998). Two of the lines had the capacity to generate cells with both primitive and definitive erythroid potential. A third line was limited to definitive erythroid potential, but also had myeloid potential. We have also found that the quality and reproducibility of the ES cell in vitro differentiation declines when using older stock solu-

tions of MTG. We therefore try to avoid using MTG stocks that are greater than 6 months old.

Most cells will continue to replicate after the coculture with *TLX1* retroviral producer cells regardless of whether they are transduced by the *TLX1* retroviruses. However, these non-transduced cells should eventually undergo senescence. Mast cells are particularly long-lived and may be retained in primary cultures with IL-3 for many weeks before naturally senescing. Although one can “wait out” the eventual senescence of these nonimmortalized cells by serial passaging, one can also select for the *TLX1*-immortalized cells earlier by including Geneticin or hygromycin B (depending on the *TLX1* retroviral vector used) in the medium beginning 48 hr after coculture with the retroviral producer cells. If not used up front, resistance to Geneticin or hygromycin B should be tested to ensure that the cells have been transduced with the retroviral vectors. In addition, immunoblot analysis of the final cell lines should be performed to ensure expression of *TLX1*. This migrates as an ~37-kDa protein and can be detected using affinity-purified anti-*TLX1* polyclonal antibody (HOX11 C18; Santa Cruz Biotechnology). Because immortalized hematopoietic cells typically grow in suspension, it is relatively easy to separate them from any residual retroviral producer cells, which are adherent; this is also easily accomplished by cloning in methylcellulose. However, the monolayer of producer cells can be irradiated with 3 Gy prior to adding the hematopoietic target cells to ensure that the producer cells do not contaminate future cell lines. Finally, MSCV-HOX11 retroviral producer cells can be eliminated by selection in Geneticin if the immortalized cell lines are derived from gene-targeted cells expressing the *neo* gene.

In our experience, *TLX1*-immortalized cells are strictly dependent on IL-3 for survival and growth. Therefore, it is critical to add IL-3 to the medium during the coculture with the retroviral producer cells, or at least soon after. We have used conditioned media from WEHI-3B cells (Lee et al., 1982) or X630-recombinant IL-3 cells (Karasuyama and Melchers, 1988) as a source of IL-3, but have found better cell growth and viability using recombinant murine IL-3 (10 ng/ml, R&D Systems).

### **Anticipated Results**

After the 3-day coculture period, proliferation of the transferred suspension cells should be apparent within a few days, with cells

growing as clusters in liquid medium. Even after cloning the cell lines, the wild-type cell cultures may remain heterogeneous with a mixture of self-renewing multipotent progenitor cells of various potentials, and differentiated progeny at various stages of maturation. Use of gene-targeted cells as the source of immortalized cells may lead to more homogenous cell populations if the disrupted gene is required for a specific stage of hematopoietic differentiation. For example, we generated a FOG-1<sup>-/-</sup> cell line from in vitro differentiated FOG-1<sup>-/-</sup> ES cells (Cantor et al., 2002; Fig. 1F.7.1). This cell line is blocked in erythroid and megakaryocytic maturation (as expected), and therefore has a more homogenous morphology. Although genetic complementation of the cells by retrovirally expressed FOG-1 rescues the terminal maturation block, only about 35% of the cells differentiate in this case, as measured by staining with *o*-dianisidine for hemoglobin production. A large proportion of cells remain undifferentiated, presumably reflecting the self-renewing population of immortalized progenitor cells.

### Time Considerations

Overall, generation of *TLX1*-immortalized cell lines can be accomplished within a couple of weeks. For the in vitro differentiation protocol, the ES cells are first differentiated for 7 days. The resultant EBs are then dissociated and directly cocultured on prepared *TLX1* retroviral producer cell monolayers for 3 days. At this point, the transduced cells can be cultured as a pool, or cloned immediately in the presence of Geneticin or hygromycin B (and IL-3). Colonies should be detectable within about 2 weeks and expanded accordingly. Cell lines should be maintained by splitting at a dilution of ~1:10-1:20 every 3 to 4 days. The doubling time of the cells may be in the range of ~36 to 48 hr.

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