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Murine Retroviral Bone Marrow Transplantation Models for the Study of Human Myeloproliferative Disorders

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ABSTRACT

Human myeloproliferative diseases are common hematologic disorders characterized by clonal overproduction of maturing myeloid or erythroid cells, often caused by expression of a mutant, dysregulated tyrosine kinase (TK). These diseases can be accurately modeled in laboratory mice by the retroviral transfer of a mutant TK gene into murine hematopoietic stem and progenitor cells, followed by transplantation of these cells into irradiated recipient mice. This yields a model system for analyzing the molecular pathophysiology of these conditions and provides a platform for testing therapies, particularly molecularly targeted new chemical entities (NCEs). The Basic Protocol in this unit describes the preparation of mouse bone marrow cells to express the relevant human oncogene before transplanting them into irradiated recipient mice. An alternate protocol describes a similar technique that allows specific induction of lymphoproliferative disease by some TKs. Support protocols for generating and titering retroviral stocks are also included. *Curr. Protoc. Pharmacol.* 43:14.10.1-14.10.28. © 2008 by John Wiley & Sons, Inc.

Keywords: BCR-ABL • chronic myeloid leukemia • JAK2 • lymphoma • mouse model • polycythemia vera

INTRODUCTION

The classical myeloproliferative diseases (MPDs), which were first defined by Dameshek (1951), include chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF; also known as chronic idiopathic myelofibrosis). The MPDs are clonal disorders of hematopoiesis characterized by overproduction of mature myeloid or erythroid cells, abnormalities of hemostasis and thrombosis, and a tendency to progress to acute leukemia (Van Etten and Shannon, 2004). In the WHO classification, chronic eosinophilic leukemia (CEL) is included among the MPDs, while a closely related group of conditions with mixed myelodysplastic/myeloproliferative features is also recognized (Vardiman et al., 2002). Over the last several years there has been a revolution in the understanding of the pathogenesis of these disorders, with the recognition that somatic activating mutations in tyrosine kinases (TKs) occur in a subset of patients from each disease category (Krause and Van Etten, 2005). Biochemical studies in vitro, in cell lines, and in primary patient cells confirm these TKs are dysregulated and provide insight into the signaling pathways that are activated. However, a role of a TK in disease pathogenesis can only be established by its expression in the hematopoietic system in vivo.

Laboratory mice can be used as model systems for studying the genetics and pathophysiology of human leukemia using, as a strategy, the expression in mouse bone marrow (BM) of genes that are mutated or dysregulated in human leukemia cells. A primary motivation for this effort is to determine whether and how a particular genetic abnormality identified in a leukemic cell contributes to the malignant phenotype. If the recapitulation

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of some or all of the leukemia phenotype in mice is successful, the system can be used for studying the molecular pathophysiology of that disease. The goal in this case is a description, in biochemical terms, of the specific cellular abnormalities responsible for the development and clinical course of the malignancy and its response to treatment. Moreover, an accurate mouse model of leukemia can serve as a platform for testing potential new therapies, particularly those directed at specific molecular targets.

The power of mouse models derives from their ability to accurately recapitulate the malignant phenotype in primary cells in vivo. However, this comes at the price of significant complexity, with careful pathological and molecular analyses required to correctly interpret the findings (Van Etten, 2002). Thus, with proper precautions, mouse models can be used to answer important questions regarding the pathophysiology of human leukemia that are difficult, if not impossible, to address through studies of human primary cells and cell lines.

Described in this unit are methods to create a retroviral model system to study myeloproliferative disorders associated with disregulated TKs. In this system, retroviruses are used as the gene transfer vectors to stably express oncogenes (genes that are mutated in cancer cells) in hematopoietic cells. The Basic Protocol details the transduction of the mouse bone marrow cells with retroviral vectors containing disregulated TK oncogenes, followed by transplantation into irradiated recipient mice to induce a myeloproliferative disease. An additional protocol (Alternate Protocol) describes a similar technique to induce lymphoproliferative disease in mice by the BCR-ABL fusion of TK. Additional support protocols for generating (Support Protocol 1) and titering (Support Protocols 2 and 3) retroviral stocks are also included**.**

NOTE: All solutions and equipment coming into contact with living cells must be sterile, requiring the use of aseptic technique.

NOTE: All tissue culture incubations should be conducted in a humidified 37°C, 10% CO_2 incubator to maintain pH 7.4.

NOTE: All protocols using live animals must be reviewed and approved by the local Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL

BONE MARROW TRANSPLANTATION FOR INDUCTION OF CHRONIC MYELOPROLIFERATIVE SYNDROMES IN MICE

Clonality studies in human MPD indicate that the cell of origin is a pluripotent hematopoietic stem cell (HSC; Fialkow et al., 1977). When expressed in mouse bone marrow by retroviral transduction, the BCR-ABL TK induces CML-like myeloproliferative disease with overproduction of maturing neutrophils (Pear et al., 1998; Zhang and Ren, 1998; Li et al., 1999). Myeloid cells from these mice carry the retroviral provirus, with analysis of the proviral integration pattern by Southern blotting demonstrating that the CML-like disease is polyclonal, with the same spectrum of proviral clones present in neutrophils, macrophages, erythroid progenitors, B lymphocytes and, in some cases, T lymphocytes (Li et al., 1999; Million et al., 2002). Other studies confirm that the cells initiating the CML-like leukemia have the phenotypic properties of hematopoietic stem cells (Neering et al., 2007). These results indicate that successful induction of MPD in mice in the retroviral model requires efficient transduction of HSC. To accomplish this, donor mice are generally pretreated with 5-fluorouracil (5-FU), which kills dividing cells and recruits HSC into the cell cycle (Randall and Weissman, 1997). While 5-FU treatment increases the efficiency of HSC transduction and subsequent induction of MPD, its use is not absolutely necessary (Li et al., 1999). The bone marrow cells are first prestimulated in

culture for 24 hr with a cytokine cocktail, then subjected to two rounds of transduction with the oncogene-containing retrovirus on successive days. They are then transplanted into irradiated-recipient mice on the second day following harvest. Hematopoietic engraftment occurs in ∼12 to 14 days, and myeloproliferative disease develops within 2 to 8 weeks after transplant, depending on the oncogene employed.

Materials

Donor and recipient mice (see Critical Parameters)
  Donors: 6- to 10-week-old male BALB/c mice (from Jackson Laboratory or Taconic Farms)
  Recipients: 6- to 8–week-old female BALB/c mice (from Jackson Laboratory or Taconic Farms)
5 to 10 mg/ml 5-fluorouracil (5-FU) stock solution (Sigma, cat. no. 6627), prepared fresh in phosphate-buffered saline (PBS; 5-FU is poorly soluble, and agitating the solution at 37°C helps to dissolve it)
Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS; Cellgro, cat. no. 21-040-CV)
Ice
Bone marrow flush medium (see recipe)
70% ethanol
Erythrocyte lysis solution (see recipe or can be purchased from Sigma, cat. no. R7757)
Trypan blue solution
Ficoll-Paque (Stem Cell Technologies, cat. no. 7907)
Bone marrow prestimulation medium (see recipe)
Cytokines (see Critical Parameters)
  Recombinant murine IL-3 (1000× = 6 μg/ml; PeproTech, cat. no. 213-13)
  Recombinant murine IL-6 (500× stock = 5 μg/ml; PeproTech, cat. no. 216-16)
  Recombinant murine SCF (1000× = 50 μg/ml; PeproTech, cat. no. 250-0)
Virus spinfection solution (see recipe)
Oncogene-expressing retroviral stocks (see Support Protocol 1)
800 μg/ml polybrene (hexadimethrine bromide; Sigma, H-9268) stock in PBS store at −20°C
1 M HEPES, pH 7.4 (Invitrogen, cat. no. 15630-080)
Hanks' Balanced Salt Solution without Ca^{2+}/Mg^{2+} (HBSS, sterile; Cellgro Cat, cat. no. 21-023-CV)
Acidified water (pH 1.3 to 2.0, or between 2 to 10 ml conc. 38% HCl per liter of water)
Autoclaved chow

Tissue culture dishes (6-cm and 10-cm)
Bucket
10-ml sterile syringes, luer-lock
Single-use 27-G, 1/2-in. needles
Dissection tools (from Roboz) including:
  Curved dissection scissors
  Toothed forceps
  Bone rongeurs
Styrofoam dissecting board
21-G needles
Gloves
50-ml conical tissue culture tubes
Sterile tissue culture pipets (2-ml, 5-ml, 10-ml, and 25-ml)
Sorvall RT-6000 centrifuge or equivalent, with a swinging-bucket plate holder and HB1000B rotor, or equivalent

6-well tissue culture plates
Parafilm
Radiation source
Wide-mouth pipet
U-100 insulin syringes, 27-G needle, 1.0-ml volume
Microisolator cages
Rodent hematology analyzer (e.g., HemaVet, CDC Coporation)

Additional reagents and equipment for parenteral injections (Donovan and Brown, 2006a), rodent euthanasia (Donovan and Brown, 2006b), performing a viable cell count using a hemacytometer and trypan blue staining (Phelan, 2006), and blood collection from rodents (Donovan and Brown, 2006c)

CAUTION: 5-FU, a chemotherapeutic agent, is a mutagen and is classified as a hazardous chemical. Prepare only the minimal volume of stock solution required. Insulin U-100 syringes are recommended for injection as they have no residual volume and can be discarded into a sharps box. Discard left-over stock as a hazardous chemical according to institutional guidelines. Wear Nitro gloves at all times. Pregnant women should not handle 5-FU.

NOTE: Gamma irradiators are typically used, with cesium 137 as the source. Manufacturers include J.L. Shepherd and Atomic Energy of Canada. It is also possible to use a commercial X-ray source to condition the recipient mice, but anecdotal reports suggest that titration of the dose may be more difficult with X-ray machines.

Prime the donors

1. Weigh several members of a cohort of young mice to verify the weight (typically 18 to 25 g). Prime donor male BALB/c mice with 125 to 200 mg/kg 5-FU IV by tail vein injection (Donovan and Brown, 2006a).

   If necessary, 5-FU can also be administered intraperitoneally.

2. Four days after priming, sacrifice the mice by CO_2 asphyxiation followed by cervical dislocation (Donovan and Brown, 2006b).

   In the original publication (Daley et al., 1990) marrow was harvested 6 days post-5-FU injection. While marrow is harvested at day 4 post-5-FU for most gene transfer experiments, recent studies suggest that the beneficial effects of 5-FU on cell cycle status of long-term repopulating cells are seen within 2 days (Randall and Weissman, 1997). We usually harvest marrow on day 4 or day 5 after 5-FU injection.

Harvest bone marrow

3. Prepare 6-cm plates containing sterile CMF-PBS in a bucket with ice placed on the laboratory bench (Fig. 14.10.1A). In the tissue culture hood, load 10-ml syringes equipped with 27-G $^1/_2$-in. sterile needles with bone marrow flush medium (Fig. 14.10.2A).

4. On the bench, wash the donor mouse's abdomen with 70% ethanol.

5. Using scissors, snip the skin over the lower abdomen to make a short (0.5-cm) incision and pull the skin apart, dissecting the skin down over the lower extremities.

6. Anchor all four of the animal's feet to a Styrofoam dissecting board with 21-G needles. On one of the hind legs locate and sever the patellar tendon using scissors (Fig. 14.10.1B). Reflect the quadriceps muscle off the femur, and then sever the femur first at the knee and then at the hip joint just above the trichinae, using sharp scissors. If necessary, remove adherent muscle and tissue from the bone by blunt dissection. Place the bone in a dish of CMF-PBS on ice.

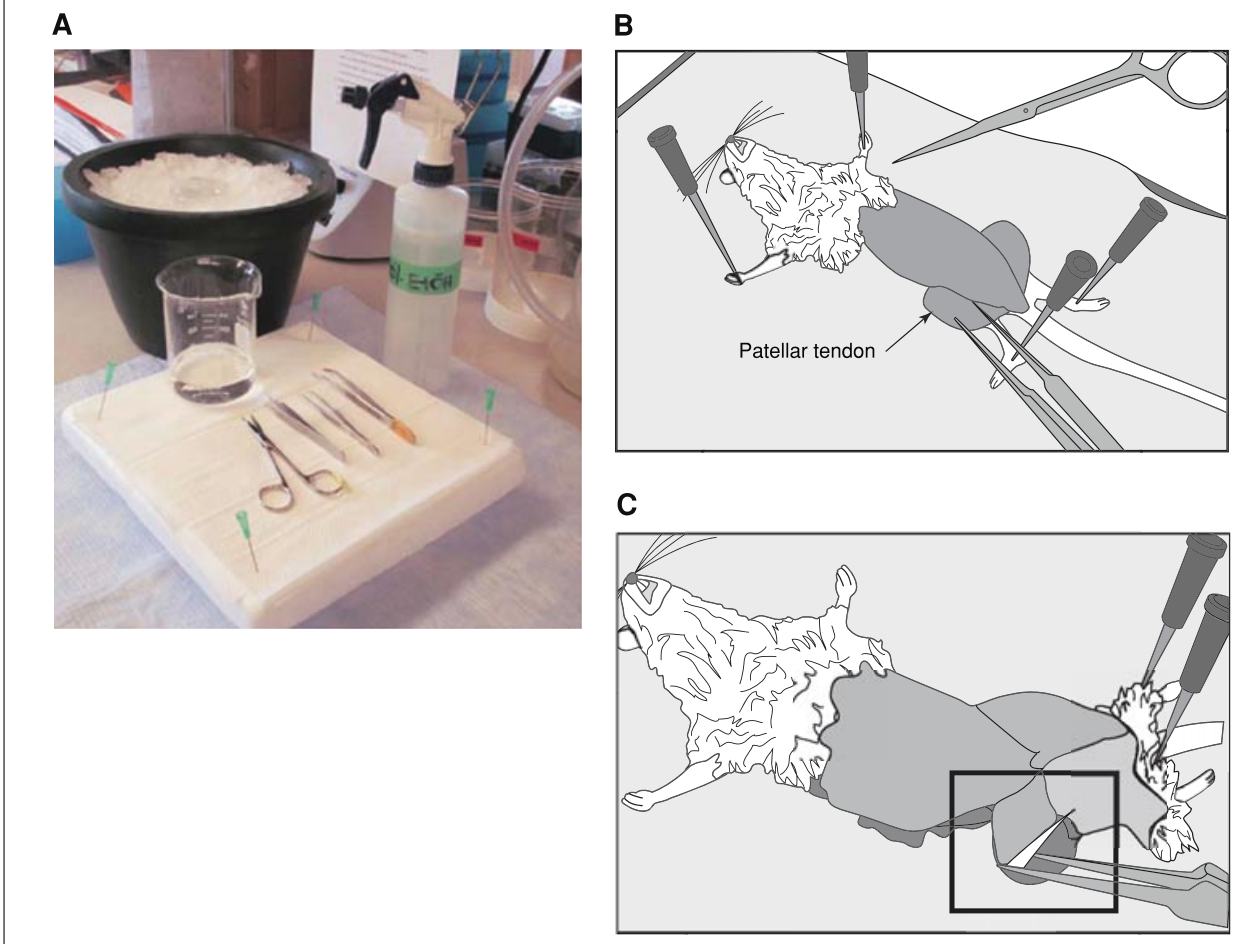

Figure 14.10.1 Dissection and removal of femur and tibia. (**A**) Dissection equipment setup. (**B**) Location of patellar tendon. (**C**) Position of tibia.

7. Remove the tibia by making a cut ~$^3/_4$ of the way across the distal tibia, then pulling the bone free of muscle and disarticulating at the knee (Fig. 14.10.1C). Place tibia in CMF-PBS on ice. Repeat femur and tibia removal for the other hind leg. Place all bones together in a dish of CMF-PBS on ice.

   It is usually convenient to harvest batches of two to four mice before flushing the marrow (total 8 to 16 bones), but long delays (>10 min) should be avoided since the blood will coagulate in the marrow, making flushing more difficult.

   The following steps are performed under sterile conditions (tissue culture hood).

8. Remove gloves and put on a new pair. Transfer bones in the dish to a tissue culture hood (Fig. 14.10.2A) and snip off the ends of long bones with scissors or bone rongeurs.

9. Using forceps, hold each bone over an open 50-ml conical tissue culture tube. Insert the needle of a 10-ml syringe containing bone marrow flush medium (prepared in step 3) into the marrow cavity, and flush with ~0.5 ml bone marrow flush medium (Fig. 14.10.2B). Repeat from other end of the bone. Repeat this flushing procedure for all bones, pooling all the marrow in the same 50-ml tube.

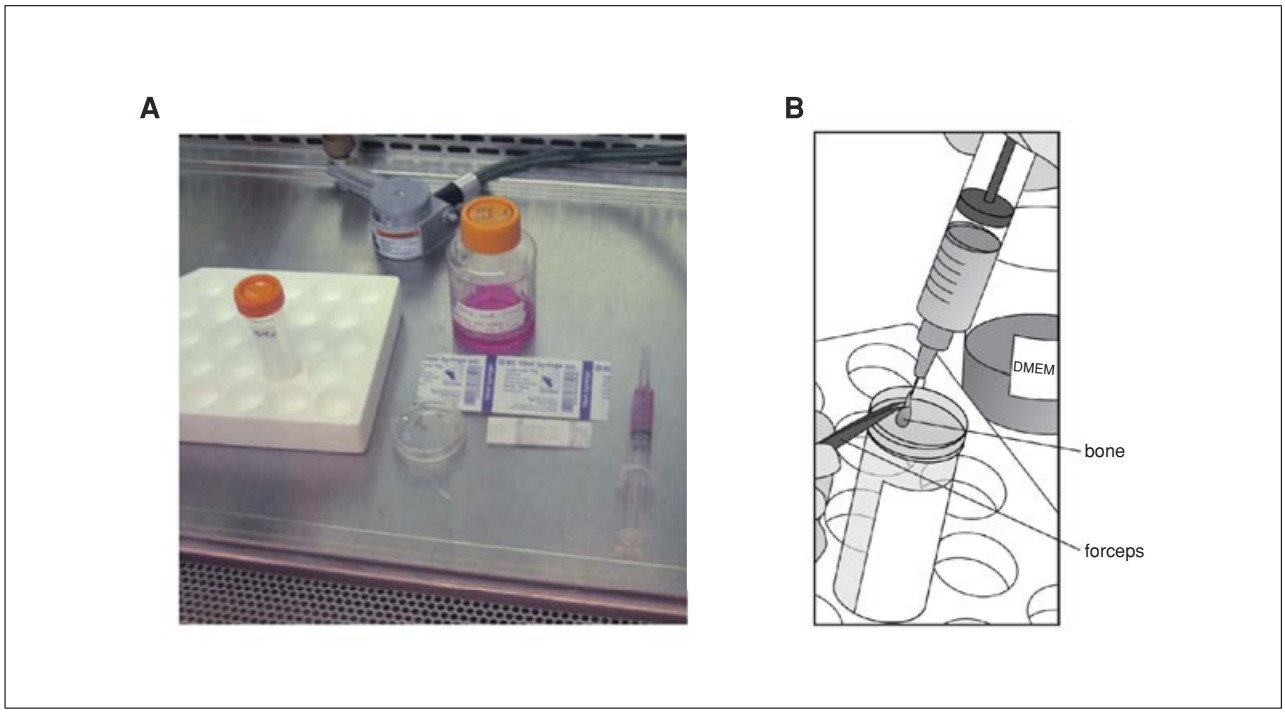

Figure 14.10.2 Bone marrow flushing procedure. (**A**) Equipment setup. (**B**) Flushing of marrow out of bone with a syringe of bone marrow flushing solution.

10. Pipet the marrow cells up and down with a 5- or 10-ml narrow-mouth tissue culture pipet to disperse any clumps. Allow particulate matter to settle briefly (1 min), and then transfer the supernatant to a fresh 50-ml tube.

    It is not necessary to strain the marrow through a nylon mesh filter.

11. After harvesting bone marrow from all donors, determine the cell yield by removing a 10-µl aliquot and diluting it into 40 µl of erythrocyte lysis buffer. Allow erythrocytes to lyse by placing the tube on ice for 5 min. Add 50 µl of Trypan blue solution and count viable cells on a hemacytometer (Phelan, 2006).

    Typical yield is 2 to 4 × 10^6 marrow cells per 5-FU-treated donor.

12. Centrifuge cells 15 min at 800 × *g*, room temperature in a Sorvall RT-6000 centrifuge or equivalent.

Remove erythrocytes from bone marrow preparations (optional)

Removal of erythrocytes from the marrow cells is not necessary, as they do not appear to affect either transduction or repopulation efficiency, although they make the cultures somewhat dense. Erythrocyte removal is recommended if purifying marrow cells pre- or post-transduction by flow cytometry or magnetic beads (e.g., using MACS MicroBeads, Miltenyi Biotec). There are two methods to accomplish this, NH_4Cl lysis and density centrifugation.

Removal of erythrocytes using NH_4Cl lysis

13a. Remove the supernatant after centrifugation in step 12, resuspend the cell pellet in ~3 ml erythrocyte lysis buffer, and incubate on ice 5 min.

14a. Dilute 5× with bone marrow flush medium and centrifuge (using the same conditions from step 12) to re-pellet the cells. Remove supernatant and wash pellet once with 5 ml bone marrow flush medium.

Removal of erythrocytes using Ficoll gradient purification

13b. Remove the supernatant after centrifugation in step 12 and resuspend the cell pellet in bone marrow flush medium to a final volume of ~3 ml per 10^7 cells. Using 15-ml conical tissue culture tubes, pour a 3 to 4 ml "foot" of Ficoll-Paque, then carefully layer 3 ml of cells (in medium) on top.

The maximum recommended cell density is ~2 × 10^7 cells per 15-ml tube. If desired, 50-ml tubes can be used instead.

14b. Centrifuge cells 30 min at 800 × *g*, room temperature in a Sorvall RT-6000 centrifuge. Following centrifugation, gently harvest medium and Ficoll to just above the cell pellet; although most mononuclear cells will collect in a band at the interface, with some viable progenitors scattered throughout the Ficoll phase. Dilute Ficoll at least 3× with bone marrow flush medium and pellet cells by centrifuging 30 min at 800× *g*, room temperature. Wash cells twice, each time with bone marrow flush medium and repeat the cell count (Phelan, 2006).

Prestimulate cells and transduce retrovirus

15. Resuspend the cells at 1 to 2 × 10^6 cells/ml in bone marrow prestimulation medium including cytokines. Place cells in the incubator in 10-cm tissue culture dishes, 10 ml/plate, for 24 hr.

Cytokines are added fresh for the required volume of medium.

Although this prestimulation step is optional, it is designed to increase the relative abundance of long-term repopulating cells and recruit them into the cell cycle, thereby improving their transduction by retroviruses.

16. The following day collect cells from the prestimulation plate by vigorous pipetting, rinsing the plates with 5 to 10 ml of bone marrow flush medium; there is normally a large adherent population of cells (fibroblasts, macrophages) that cannot be removed. Perform a viable cell count (Phelan, 2006) on a 10-µl aliquot as described in step 11. Pellet the cells by centrifuging (in the RT-6000 centrifuge) 10 min at 800 × *g*, room temperature.

17. While the cells are centrifuging, prepare the virus spinfection solution.

There are two basic methods of retroviral infection or transduction: cocultivation with a producer cell line (Daley et al., 1990), and direct supernatant infection with or without centrifugation, the method described here. Cocultivation is convenient and provides stromal support for hematopoietic stem cells, increasing their recovery. However, a producer cell line that yields a consistently high virus titer is required. Use of cell-free virus supernatant allows better matching of viral stocks for titer when several different virus stocks are being tested in an experiment (see Support Protocols 1, 2, and 3).

18. Remove supernatant from the cell pellet and resuspend the cells directly in freshly prepared spinfection solution (~10^6 cells/ml).

19. Distribute the spinfection cell suspension into a 6-well plate at a volume of 4 ml per well. Seal the plate lids with Parafilm and centrifuge in a Sorvall RT-6000 or equivalent, using an H1000B rotor and a swinging-plate holder, 90 min at 1500 × *g* (2500 rpm with this rotor), 32°C.

Centrifugation of the target cells increases the efficiency of retroviral infection several fold (Kotani et al., 1994). An alternative approach to increase transduction efficiency is to transduce cells on plates coated with Rectronectin (Panvera Laboratories), the CH-296 C-terminal fragment from fibronectin, which has been shown to increase efficiency of retroviral gene transfer, possibly by binding both cells and virus and inducing a colocalization effect (Hanenberg et al., 1996).

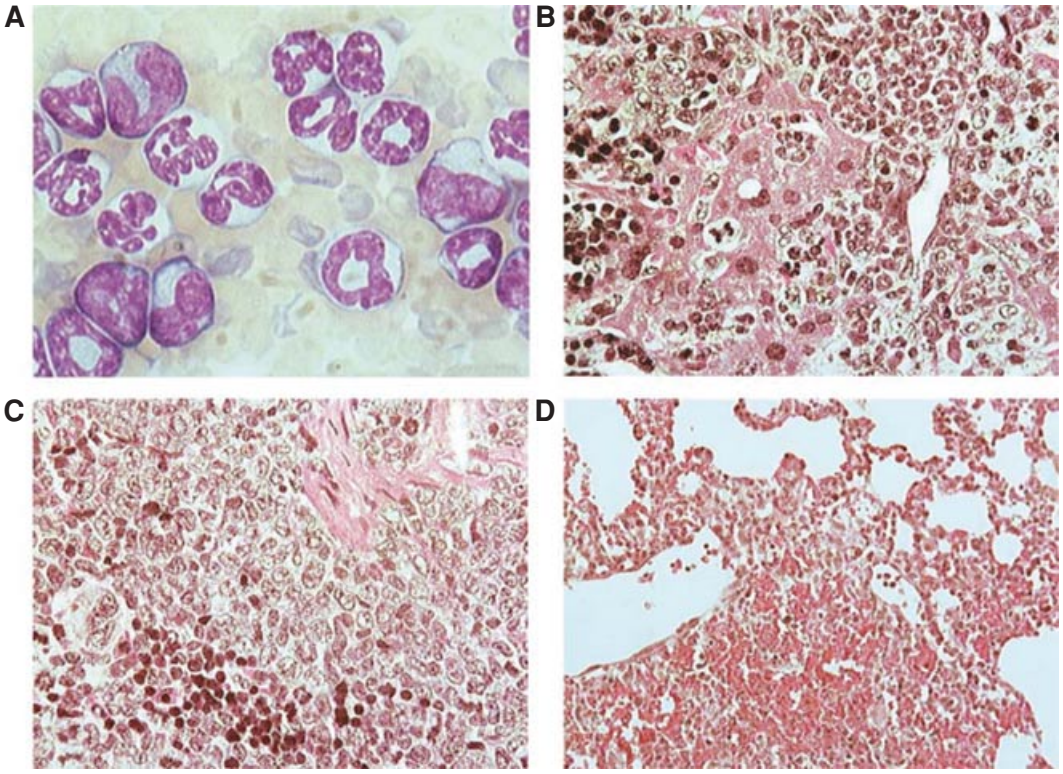

Figure 14.10.3 Histopathology of BCR-ABL-induced myeloproliferative disease in mice. (**A**) Peripheral blood smear (800×, Wright-Giemsa stain), demonstrating increased maturing myeloid cells with metamyelocytes, myelocytes, and occasional blast forms. (**B**) Liver (200×, Hematoxylin-eosin stain), demonstrating periportal infiltration with maturing myeloid and erythroid cells. (**C**) Spleen (200×, Hematoxylin-eosin stain), demonstrating disruption of follicular architecture with maturing myeloid cells. **(D**) Lung (75×, Hematoxylin-eosin stain), demonstrating alveolar hemorrhage and infiltration with myeloid cells. For color version of this figure see *http://www.currentprotocols.com*.

20. Gently resuspend the cells after centrifugation by pipeting the spinfection medium (but do not change medium), and place the plate in an incubator for 2 to 4 hr to allow virus adsorption.

21. Very gently, remove as much medium as possible from each well (~3 ml) and replace with fresh bone marrow prestimulation medium (i.e., no virus, polybrene, or HEPES). Incubate the cells overnight. If some cells are inadvertently removed when changing the medium, pellet the cells by centrifuging 5 min at 800 × *g*, room temperature. Remove supernatant, and add the cell pellet back to the well using a small volume of prestimulation medium.

22. The next day repeat the spinfection process (steps 17 to 21) by removing several milliliters of medium from each well (2 to 3 ml, equivalent to the volume of retroviral stock to be added) and adding an equal volume of freshly thawed retroviral stock containing the appropriate amounts of HEPES and polybrene for the total volume.

    It is not necessary to add additional cytokines to the medium at this point.

23. Centrifuge for 90 min at 1500 × *g* (2500 rpm), 37°C. After centrifugation, gently resuspend the cells in the same medium and return the plate to the incubator for 2 to 4 hr.

    Cells are now ready for collection and transplantation.

Transplant transfected cells

24. While the second spinfection is underway, irradiate female recipient mice with 900 cGy.

    Check the required exposure time for the particular radiation source. Irradiation is necessary for HSC engraftment to create space in the bone marrow niche (Bhattacharya et al., 2006). Depending on the experiment, lower radiation doses (200 to 450 cGy) can be used, but omitting radiation will result in very poor engraftment and disease induction. Note that the lethal irradiation dose differs between inbred mouse strains (i.e., 900 cGy for BALB/c, 1150 cGy for B6).

25. Collect bone marrow cells by vigorously rinsing the wells with a wide-mouth pipet. Wash the plate once with CMF-PBS to obtain the maximum number of cells. Pellet cells by centrifuging 10 min at 800 × *g*, room temperature.

26. Remove supernatant and resuspend cell pellet in a small volume (2 ml) of sterile HBSS and count viable cells with a hemacytometer (Phelan, 2006) after erythrocyte lysis.

27. Determine the quantity of cells to be transplanted per recipient mouse, and adjust the volume to administer the correct amount in a volume of 0.5 ml HBSS per recipient mouse.

    Typically, between 2 and 5 × 10^5 cells are transplanted per recipient. In the case of BCR-ABL mice, higher doses may yield animals with several distinct leukemias arising from different transduced progenitors (Li et al., 1999).

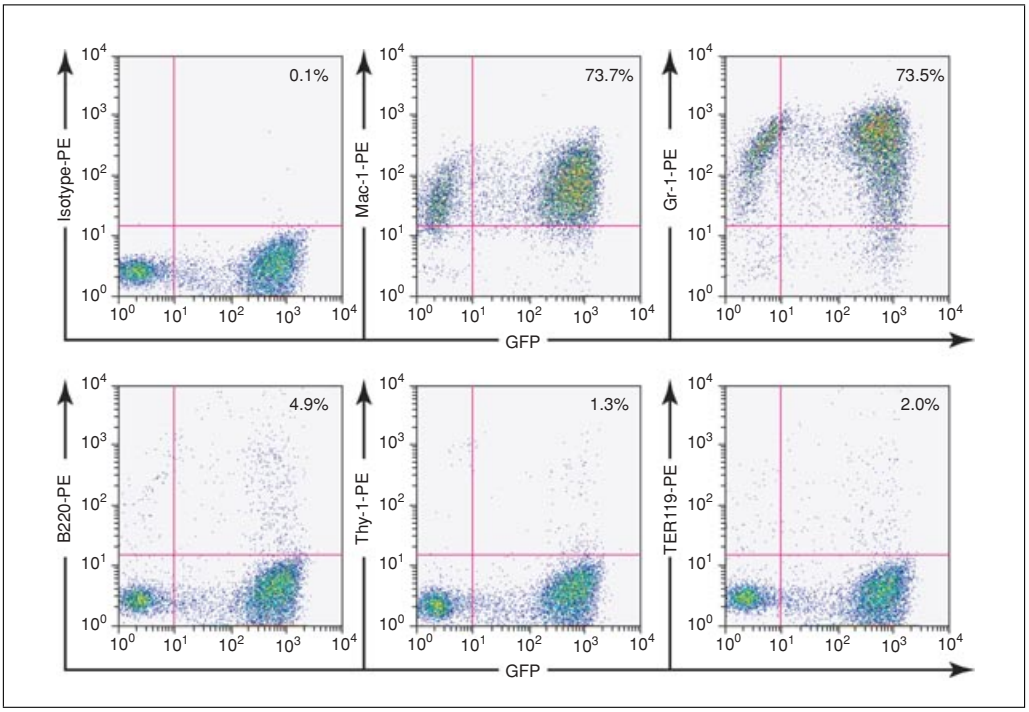

Figure 14.10.4 Flow cytometric dot-plot profiles of peripheral blood leukocytes from a mouse with CML-like disease induced by a retroviral vector co-expressing BCR-ABL and GFP. GFP fluorescence is on the *x* axis, while the *y* axis depicts intensity of PE-conjugated antibodies against different hematopoietic cell surface antigens. This mouse had leukocytosis with a peripheral blood leukocyte count of 150,000/μl, the majority of which were myeloid cells expressing Mac-1 (CD11b) and Gr-1. The fraction of cells in the upper/outer quadrant is displayed. Note the small population of GFP-negative myeloid cells, which probably represents malignant cells that have down-regulated or lost expression of GFP (see Li et al., 2001).

28. Transfer the cells into recipient mice by injecting 0.5 ml cell-solution into the animals i.v. in the lateral tail vein (Donovan and Brown, 2006a) using a U-100 syringe.

    It is also possible to inject the cells (in the same volume) via the retro-orbital venous plexus.

29. Following transplantation, place the recipient mice in microisolator cages, maximum of 5 mice per cage, with acidified water and autoclaved chow for two weeks post-transplant.

    The acidified water helps to protect against Pseudomonas infections arising from the gastrointestinal tract in the post-transplant period. Sterile environment and antibiotics are not required.

30. Monitor the development of MPD in the recipient cohort by serial analysis of peripheral blood collected by retro-orbital or submandibular venous plexus puncture (Donovan and Brown, 2006c). Complete blood counts can be determined manually or using an automated rodent hematology analyzer (such as the HemaVet, CDC Corporation).

    The hallmark of the CML-like disease induced by BCR-ABL is a dramatic increase in circulating myeloid cells, principally neutrophils and their precursors, with total leukocyte counts from 50 to 250 × 10^5 per μl, accompanied by infiltration of spleen, liver, and lungs (Fig. 14.10.3A). These malignant cells contain the retroviral provirus and express both BCR-ABL and GFP (if MIG-type vector is used), and display elevated levels of tyrosyl-phosphorylated proteins (Fig. 14.10.4). The syndrome is fatal due to respiratory

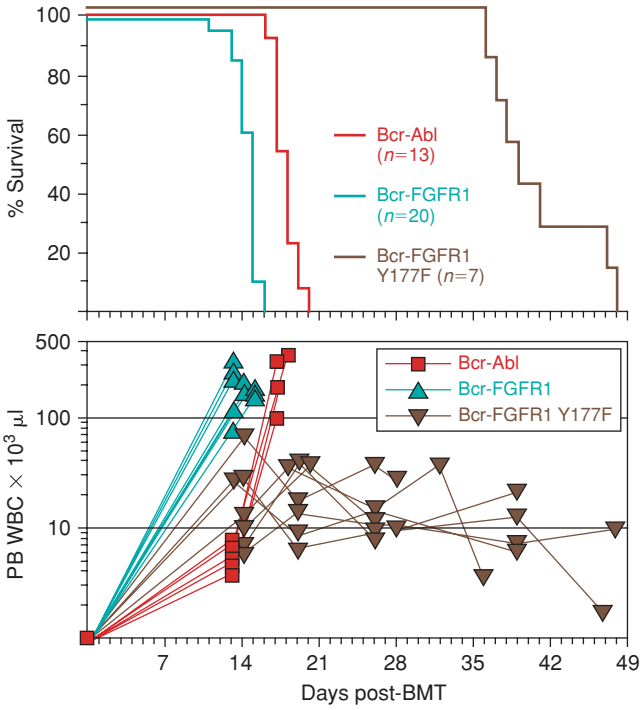

Figure 14.10.5 Top: Kaplan-Meier survival curve for bone marrow recipient mice with CML-like MPD induced by BCR-ABL (red curve) and the aggressive MPD induced by BCR-FGFR1 (green curve). Mutation of the Grb2 binding site at BCR Tyr177 greatly attenuates the latter disease (brown curve). Bottom: Scatter plot of peripheral blood leukocyte count (y axis, logarithmic scale) as a function of time after transplantation (x axis) for the recipients depicted in the top panel. Reproduced with permission from Roumiantsev et al. (2004). Abbreviations: PB WBC, peripheral blood white blood cells; BMT, bone marrow transplant. For color version of this figure see *http://www.currentprotocols.com*.

insufficiency caused by pulmonary myeloid cell infiltration. The median survival time for recipients depends on the disease clonality (Jiang et al., 2003), but is typically ∼3 to 5 weeks following transplantation (Fig. 14.10.5). Depending on the experimental goals, diseased mice can be analyzed by histopathology, analysis of cell signaling by flow cytometry and/or western blotting, and in vitro colony assays (Li et al., 1999; Van Etten, 2001b; Hu et al., 2004). Use of the model system for preclinical testing of new leukemia drugs is described in the Commentary.

DIRECT RETROVIRAL INFECTION AND BONE MARROW TRANSPLANTATION TO SELECTIVELY INDUCE B-LYMPHOID LEUKEMIA WITH BCR-ABL

ALTERNATE PROTOCOL

While the BCR-ABL oncogene is found in virtually every case of CML, it is also expressed in a subset of human B-lymphoid acute lymphoblastic leukemia (B-ALL) patients. How the same fusion TK could be involved in the pathogenesis of these two very different hematologic malignancies is one of the central questions in molecular oncology. Part of the explanation may be that the two conditions arise from expression of BCR-ABL in distinct bone marrow progenitors (Li et al., 1999). To demonstrate this, an efficient retroviral model of BCR-ABL-induced B-ALL has been developed, which is similar to the CML model except that the bone marrow donors are not treated with 5-FU and the transduction step is conducted without cytokines. When the transduced bone marrow is cultured on autologous stroma or a stromal cell line, BCR-ABL induces the outgrowth of immature B-lymphoid progenitors that are poorly leukemogenic in mice, providing a valuable in vitro assay for the initial steps of transformation (McLaughlin et al., 1987). This assay has been modified to include limiting dilution analysis, thereby allowing a quantitative assessment of initial BCR-ABL B-lymphoid transformation (Smith et al., 2003). If the transduced bone marrow is injected directly into irradiated recipients, all develop fatal acute B-lymphoid leukemia/lymphoma within 5 to 7 weeks. The leukemic blasts express B220 (CD45R), 6C3/BP1, CD24, and CD43 and have rearrangement of the immunoglobulin heavy chain gene locus with germline light chain loci, consistent with a late pro-B/early pre-B stage progenitor (Hardy et al., 1991). While the leukemias are oligo- to monoclonal and are efficiently transplanted, in contrast to the CML-like leukemia, provirus is not found in myeloid lineage cells or CFU-S_{12} from mice with B-ALL (Li et al., 1999). These results suggest that the cell initiating these leukemias is a committed lymphoid progenitor, rather than a multipotent stem/progenitor cell.

Described in the protocol below is the efficient transformation of primary marrow B-lymphoid progenitors and induction of B-ALL in recipient mice by BCR-ABL. For materials, see the Basic Protocol.

1. Following steps 3 to 12 from the Basic Protocol, harvest bone marrow cells from donor male BALB/c mice that have not been primed with 5-FU.

   Typical yield is 3 to 5 × 10^7 cells per donor.

   Lysis of erythrocytes is not necessary if the cells are to be transplanted directly following transduction. If a quantitative in vitro assay of transformation is planned, such as a Whittlock-Witte cultures (McLaughlin et al., 1989; Smith et al., 2003), or soft agar colony formation (Rosenberg and Baltimore, 1976) assay, then erythrocyte lysis is advisable (see steps 13a and 14a of the Basic Protocol).

2. Immediately prepare the spinfection solution with 50% (v/v) retroviral stock.

   As many as 10^7 cells can be transduced per 4 ml in each well of a 6-well plate.

   In the original paper (Roumiantsev et al., 2001), the transduction medium was supplemented with IL-7 (10 ng/ml), but no other cytokines. However, IL-7 is not required, and can be omitted.

3. Centrifuge the plates 90 min at 1500 × *g* (2500 rpm in RT-6000), 37°C (as described in step 19 of the Basic Protocol). After centrifugation, resuspend the cells in the same solution, and incubate 3 to 4 hr at 37°C in a tissue culture incubator to allow the virus to adsorb.

4. Irradiate the female recipient mice (450 to 900 cGy) during execution of the spin-fection and incubation steps.

5. After adsorption, collect the cells and centrifuge in a 50-ml conical tube (10 min at 800 × *g*, room temperature), wash once in 10 ml HBSS and perform a viable cell count (Phelan, 2006) after erythrocyte lysis, if applicable. Resuspend cells in HBSS at 2×10^6/ml and dispense 0.5 ml per U-100 syringe for tail vein injection (Donovan and Brown, 2006a).

6. Inject 10^6 cells/recipient mice via the lateral tail vein (Donovan and Brown, 2006a).

7. Provide the recipients with acidified water (pH 1.3 to 2.0, or between 2 to 10 ml conc. 38% HCl per liter of water) and autoclaved chow in microisolator cages during the post-transplant period (5 mice per cage).

   The acidified water helps to protect against Pseudomonas infections arising from the gastrointestinal tract in the post-transplant period. Sterile environment and antibiotics are not required.

8. Monitor disease.

   With p210 BCR–ABL retrovirus, all recipients die between 28 and 45 days post-transplant with B-ALL (Roumiantsev et al., 2001). The primary cause of morbidity or death in BALB/c mice as a consequence of this leukemia is a malignant pleural effusion accompanied by pleural hemorrhage. Malignant cells carry the retroviral provirus and express BCR-ABL and GFP (if a MIG-based vector is used). Depending on the experimental goals, diseased mice can be analyzed by histopathology, analysis of cell signaling by flow cytometry and/or western blotting, and in vitro colony assays, as described in several recent publications (see Anticipated Results and Li et al., 1999; Van Etten, 2001b; Hu et al., 2004). Use of the model system for preclinical testing of leukemia NCEs is described in the Commentary.

SUPPORT PROTOCOL 1

PRODUCTION OF RETROVIRUS STOCK BY TRANSIENT TRANSFECTION OF HEK 293T CELLS

Retrovirus is produced by transient transfection of 293T cells, a human renal epithelial cell line transformed with the adenovirus E1A gene product. The 293T cell line also expresses SV40 large T antigen, allowing for episomal replication of plasmids containing the SV40 origin and early promoter region. The 293 cells have the unusual property of being highly transfectable by calcium phosphate ($CaPO_4$) or lipofection transfection protocols, with up to 50% to 80% transfection efficiency being readily attainable (DuBridge et al., 1987). There are two distinct strategies for production of retroviruses using 293 cells. Bosc-23 cells are 293 cells engineered to stably express retroviral gag, pol, and env gene products (Pear et al., 1993), which can be directly transfected with a retroviral vector to produce retrovirus. The Phoenix system is a variation where hybrid Epstein-Barr virus (EBV)–retroviral vectors are maintained episomally in EBV nuclear antigen–expressing 293T cells, allowing stable retrovirus production over weeks to months (Swift et al., 1999). An alternative approach is to co-transfect 293 cells with a retroviral vector and plasmid(s) that express the gag-pol-env packaging functions, such as the single-genome packaging constructs kat (Finer et al., 1994) or pCL (Naviaux et al., 1996). The latter system is described below. For a video demonstration of retrovirus production from transfection of 293T cells, see *http://www.jove.com/index/details.stp?ID=550*.

The choice of the retroviral vector and host range of the packaging construct are important. For stable expression in murine hematopoietic stem cells, a vector based on the

myeloproliferative sarcoma virus is preferred (Stocking et al., 1985; Cherry et al., 2000). A common vector backbone is the MIG RI (Pear et al., 1998), containing an MPSV long terminal repeat sequence, a multiple cloning site for introduction of an oncogene, and a downstream internal ribosome entry site linked to the gene for enhanced green fluorescent protein (eGFP). For transduction of mouse HSC, a virus with an ecotropic host range is optimal, but such stocks are unstable at physiologic temperatures and cannot be concentrated by centrifugation. Amphotrophic packaging constructs express the envelope protein from the amphotrophic retrovirus 4070A and allow infection of a broad range of species, including human cells. However, in our experience amphotrophic virus is inferior to ecotropic virus for mouse HSC transduction. Pseudotyping the virus stock with the G-protein from vesicular stomatitis virus (VSV-G) is an alternative that allows transduction of cells from a broad range of species and the ability to concentrate the virus stock by ultracentrifugation (Ory et al., 1996).

Materials

293T cells (ATCC)
293T cell medium (see recipe)
0.05% Trypsin/EDTA (CellGro, cat. no. 25-051-CI)
25 mM chloroquine (1000× stock; Sigma, cat. no. C6628) in PBS (store up to 6 months at −20°C)
Retroviral oncogene-expressing vector [e.g., pMIG RI (Pear et al., 1998); see page 14.10.12]
TE (Tris-EDTA) buffer, pH 8.0 (*APPENDIX 2A*)
Packaging plasmid [e.g., pMCV-ecopac (Finer et al., 1994; available from the authors) or pCL (Naviaux et al., 1996; available from Dr. Inder Verma, Salk Institute)]
2 M $CaCl_2$ in dH_2O, sterile filtered (store up to 6 months at room temperature)
2× HBS, pH 7.05 (see recipe)

6-cm tissue culture plates
15-ml conical tube
P1000 pipettor
Suction pump
10-ml sterile syringes
Single-use 18-G needles
0.22-μm syringe filter
50-ml conical tubes
2- and 4-ml screw-top cryovials

1. The evening before transfection plate 293T cells at 4 to 5 × 10^6 cells/6-cm plate in 4 ml 293T cell medium. Place the cells in an incubator overnight.

   The 293 cells transfect best at higher densities. These cells must be passaged carefully and split 1:3 to 1:4 every three days with trypsin, otherwise they will tend to clump with replating and will not transfect as well. They are small cells, and optimal transfection density is ∼4.5 to 5.0 × 10^6 cells per 6-cm dish, plated the night before. This achieves ∼80% cell confluency on the day of the transfection. If expression of the TK gene from the retroviral vector might cause some cell toxicity or growth suppression, use the higher density for plating. As the cells are poorly adherent to glass and plastic, pipet solutions with care, from the side, using wide-mouth pipets. Optimal transfection results are obtained with 6-cm plates. Scaling up to 10-cm plates results in decreased transfection efficiency and lower titers.

2. The following morning gently remove medium and replace with 4 ml fresh 293T cell medium containing 25 μM chloroquine. Return cells to the incubator for at least 20 min.

Failure to change the medium will significantly decrease transfection efficiency. Addition of chloroquine improves transfection efficiency by stabilizing cell lysozomes and increasing the fraction of DNA that reaches the nucleus. This yields 2- to 4-fold higher virus titers. Sodium butyrate (another lysosome stabilizer) has also been used for this purpose. It is convenient to transfect the cells first thing in the morning.

3. Meanwhile, prepare the transfection mixture in a 15-ml conical tube. Recipe for one plate:

   10 μg retroviral oncogene-expressing vector DNA (in TE buffer)
   5 μg packaging plasmid DNA (in TE buffer)
   62 μl 2 M $CaCl_2$
   Bring total volume to 0.5 ml with sterile distilled water.

   The quality of DNA is very important. The preferred method of purification of both the vector and packaging plasmid DNAs is twice purified by CsCl-ethidium bromide buoyant density centrifugation. The concentration of the DNA should be at least 1 mg/ml, and the OD 260/280 ratio between 1.75 and 1.90. QIAGEN column-purified DNA will also work, although typically not as well. It is important that the combined volume of DNA solutions be small (<50 μl total per final milliliter) to avoid adverse effects of Tris and EDTA on the calcium phosphate precipitate.

   Scale up the mixture if multiple plates are to be transfected. Limit handling to a maximum of four plates at a time to minimize the delay in adding the transfection mixture to the cells.

4. At the time of transfection, add 0.5 ml of 2× HBS dropwise to the mixture using a P1000 pipettor while vigorously vortexing the tube. Immediately (within 1 min) add the final mixture to the plates, gently, drop by drop, scattering the drops uniformly over the surface of the medium. Return the plate to the incubator for 7 to 11 hr.

5. Gently remove the medium with a suction pump and very gently replace with 4 ml fresh 293T medium. Place in a 37°C humidified incubator until next day.

   Before changing the medium there should be a very fine, dust-like precipitate visible under the microscope. It is important to change the medium because chloroquine is toxic to the cells if incubated too long. If chloroquine is not used, changing the medium at this point is not required and the precipitate can be left overnight.

   Since at this point the cells are poorly adherent, pipet all solutions with care, from the side of the plate, using wide-mouth pipets.

6. The following day, around mid-day (18 to 24 hr before harvesting the virus), remove the medium, gently replacing it with 3 ml fresh 293T medium. Return the plate to the incubator.

   The reduction in volume helps to concentrate the virus stock.

7. The next morning (48 to 60 hr post-transfection), harvest the virus-containing supernatant by gently aspirating the medium from the plates with an 18-G needle and 10-ml syringe. Filter the supernatant through a 0.22-μm syringe filter. Pool all medium from multiple plates transfected with the same vector in a 50-ml tube.

8. Place 1- or 2-ml portions of virus supernatant in screw-top cryovials and store viral stocks for up to several months at −80°C until used.

   These frozen viral stocks can be thawed once with only a two-fold loss in titer.

   If desired, once harvested, the 293 cells can be used to make protein lysates for immunoblotting to check the expression of proteins encoded by the retroviral vector (e.g., the TK).

   Before using any virus, the titer needs to be determined. This is critical to match titers of different virus stocks in the same experiment, and to ensure efficient transduction of HSC (see Support Protocols 2 and 3).

CHECKING TRANSFECTION EFFICIENCY AND VIRUS PRODUCTION

To monitor transfection efficiency and virus production, transfect a control plate with pMFG-*lacZ*, a retroviral vector expressing the *E. coli* β-galactosidase gene together with the pMCV–Ecopac packaging construct (see Support Protocol 1). The virus supernatant is harvested as usual 48 to 60 hr post-transfection, and can be used for titering NIH 3T3 cells (Support Protocol 3). The plate of 293 cells is subsequently stained for *lacZ* expression using X-Gal.

Materials

293T cells transfected with pMFG-*lacZ* plasmid DNA (available from the authors; CsCl-purified, in TE buffer) together with the pMCV–Ecopac packaging construct (see Support Protocol 1)
Phosphate-buffered saline calcium- and magnesium-free (CMF-PBS)
25% glutaraldehyde (50× stock; store up to 2 years in the dark at −20°C)
1 M $MgCl_2$ (500× stock in dH_2O; Sigma, cat. no. M8266; store at room temperature)
0.7M $K_3Fe(CN)_6$ (20× stock potassium ferricyanide dissolved in dH_2O; Sigma, cat. no. P8131; store up to 6 months in the dark at room temperature)
0.7M $K_4Fe(CN)_6.3H_2O$ (20× stock potassium ferrocyanide dissolved in dH_2O; Sigma, cat. no. P9387; store up to 6 months in the dark at room temperature)
50 mg/ml X-Gal (50× stock in N,N–DMF; Sigma, cat. no. B9146; store up to 1 year in the dark at −20°C)
Additional reagents and equipment for harvesting the viral supernatant (Support Protocol 1)

NOTE: Volumes shown are for staining one 6-cm plate.

1. After harvesting the viral supernatant (Support Protocol 1), gently rinse the plate twice, each time with CMF-PBS.
2. Fix the cells with 0.5% glutaraldehyde for 10 min at room temperature.

   For example, use 40 μl of 50× glutaraldehyde stock in 2 ml of PBS

3. Gently rinse the plate three times, each time with PBS containing 2 mM $MgCl_2$, 5 min each time.

   For example, place 24 μl of 500× $MgCl_2$ in 6 ml PBS; use 2 ml at a time.

4. During this time, prepare 2 ml X-Gal staining solution in 1696 μl PBS, add:

   100 μl of 20× stock $K_3Fe(CN)_6$ (35 mM final concentration)
   100 μl of 20× stock $K_4Fe(CN)_6{\cdot}3H_2O$ (35 mM final concentration)
   100 μl of 50× stock X-Gal (1 mg/ml final concentration)
   4 μl of 500× $MgCl_2$ (2 mM final concentration)

5. Add the X-Gal solution to the plate, and incubate for 4 hr to overnight at 37°C in a humidified chamber.
6. Gently rinse twice, each time with distilled water, then let air-dry for permanent storage. Score plate for blue color under medium power without green filter or phase contrast.

   Typically, the transfection efficiency should be at least 50% to 80% blue cells, the number necessary to obtain good viral titers.

SUPPORT PROTOCOL 3

TITERING VIRUS STOCKS BY FLOW CYTOMETRY, DRUG SELECTION, OR SOUTHERN BLOTTING

Typically NIH 3T3 cells are used for titering virus stocks as these cells are highly transducible with ecotropic retroviruses and easily manipulated. There are three complementary methods for titering retroviruses: flow cytometric detection of a fluorescence reporter protein (such as GFP), drug selection for an antibiotic resistance marker (such as neomycin resistance), and Southern blotting of genomic DNA. All three methods are described briefly below. In all cases, 3T3 cells are plated the day before titering, then transduced with serial dilutions of virus stock, and then harvested 48 to 72 hr later and subjected to FACS analysis, drug selection, or extraction of genomic DNA.

Materials

NIH 3T3 cells (ATCC)
3T3 cell medium (see recipe)
Retroviral stocks (e.g., MSCV-IRES/GFP or MSCV-IRES/Neo vectors; see Support Protocol 1), frozen
800 μg/ ml polybrene (100× stock; Sigma, cat. no. H9268); store at −20°C
Sterile phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS; CellGro, cat. no. 21-040-CV)
0.05% trypsin/EDTA (CellGro, cat. no. 25-051-CI)
FACS buffer (see recipe)
1 mg/ml neomycin in H_2O (G418; Sigma, cat. no. G1279); store at −20°C
Crystal Violet solution [1% (w/v) crystal violet in 20% methanol]
2× DNA lysis buffer (see recipe)
Proteinase K (solid; Roche, cat. no. 1245500)
25:24:1 (v/v/v) phenol:chloroform:isoamyl alcohol (25:24:1)
24:1 (v/v) chloroform:isoamyl alcohol
3M sodium acetate, pH 5.2
Isopropanol
70% ethanol
TE buffer, pH 8.0 (*APPENDIX 2A*)
Restriction enzyme (e.g., *Xba I*)
DNase-free RNase A (100 μg/ml stock)
DNA loading buffer (Voytas, 2000)
0.8% agarose/TBE slab gel (Voytas, 2000)
Radioactive probe

6-cm tissue culture dishes
37°C, 10% CO_2 incubator
1.5-ml microcentrifuge tubes
Fluorescence-activated cell analyzer (e.g., FACSCalibur; Becton Dickinson)
Benchtop microcentrifuge
37°C water bath
Pasteur pipet with end heat-sealed and melted into a small hook
Sorvall RT-6000 centrifuge
Nylon membrane
X-ray film *or* phosphor imager screen

Additional reagents and equipment for DNA extraction (*APPENDIX 3C*), determining DNA concentration by spectrophotometry (Gallagher and Desjardins, 2006), agarose gel electrophoresis (Voytas, 2000), Southern blotting (Southern, 1975), electroblotting (Li et al., 1999), and quantitation of radiolabeled DNA (Voytas and Ke, 1999)

1. Plate NIH 3T3 cells at 3×10^5 cells per 6-cm plate in 4 ml 3T3 medium. Place in incubator overnight (37°C, 10% CO_2). Prepare as many plates as there will be virus dilutions, plus one that will serve as a negative control (no virus).
2. The following morning thaw an aliquot of virus stock (from Support Protocol 1) and prepare a series of dilutions in 3T3 medium containing 8 μg/ml polybrene (10 μl/ml).

   For titering by FACS or Southern blotting, it is typical to use undiluted virus to prepare serial 1:3, 1:10, 1:30, and 1:100 dilutions. For titering by drug selection, recommended starting points for dilutions are 1:100, 1:1000, and 1:10,000.
3. Aspirate the medium from each plate and replace it with 1 ml viral dilutions. It is permissible to use the same pipet if starting with the most dilute solution and progressing sequentially to more concentrated solutions. Place plates back in the incubator for 3 to 5 hr, gently rocking the plate from time to time to increase absorption. Spinfection is not necessary.
4. At a time point 3 to 5 hr later, aspirate the virus and replace with 4 ml 3T3 medium. Place back in incubator.
5. At a time point 48 to 72 hr after transduction, aspirate the medium, rinse the plate with CMF-PBS and trypsinize the cells with several drops of 0.05% trypsin/EDTA. Five minutes later add 1.0 ml 3T3 medium to quench the trypsin and collect the cells.

   From this point, the protocol differs depending on the assay method employed.

To titer GFP-expressing virus by flow cytometry

6a. Pellet the cells in a 1.5-ml microcentrifuge tube by centrifuging 5 min at 800 × *g*, room temperature, and wash once with CMF-PBS. Resuspend cells in 1 ml FACS buffer, and analyze the cells on a fluorescence-activated cell analyzer (e.g., FACSCalibur; Fig. 14.10.6). Use nontransduced NIH 3T3 cells as negative controls.

   There should be >95% GFP–positive cells at lower virus dilutions (undiluted and 1:3 dilutions), which will gradually decrease at higher dilutions. A good virus stock will have close to 50% GFP+ cells at a 1:30 dilution.

7a. *Optional:* Calculate the proviral copy number. According to Poisson statistics, the average proviral copy per cell is given by the fraction of cells that failed to be transduced (i.e., GFP^- fraction), according to the following formula:

$$GFP^- \text{ fraction} = e^{-\lambda}, \text{ where } \lambda = \text{proviral copy number per cell.}$$

   Hence: $\lambda = -\log_e$ (GFP^- fraction). Therefore, a GFP^- fraction of ~0.37 (63% GFP^+) corresponds to about 1 copy per cell [$-\log_e(0.37) = 1.0$].

   Calculating proviral copy number is useful when matching the titers of viruses titered by FACS or by Southern blot.

To titer Neo-expressing viruses by drug selection

6b. Prepare 3T3 medium containing 1 mg/ml G418. For each dilution to be titered (typically, assay only the 1:1000 and 1:10,000 plates), prepare four 6-cm plates with 4 ml neomycin-containing medium

7b. Pipet the 1.0 ml of trypsinized 3T3 cells (from step 5) vigorously. Pipet either 50 or 100 μl of the cell suspension onto pairs of plates prepared with neomycin-containing medium (this gives an additional dilution factor of 1:10 or 1:20, respectively). Agitate the plates and place them in the incubator for 10 to 12 days.

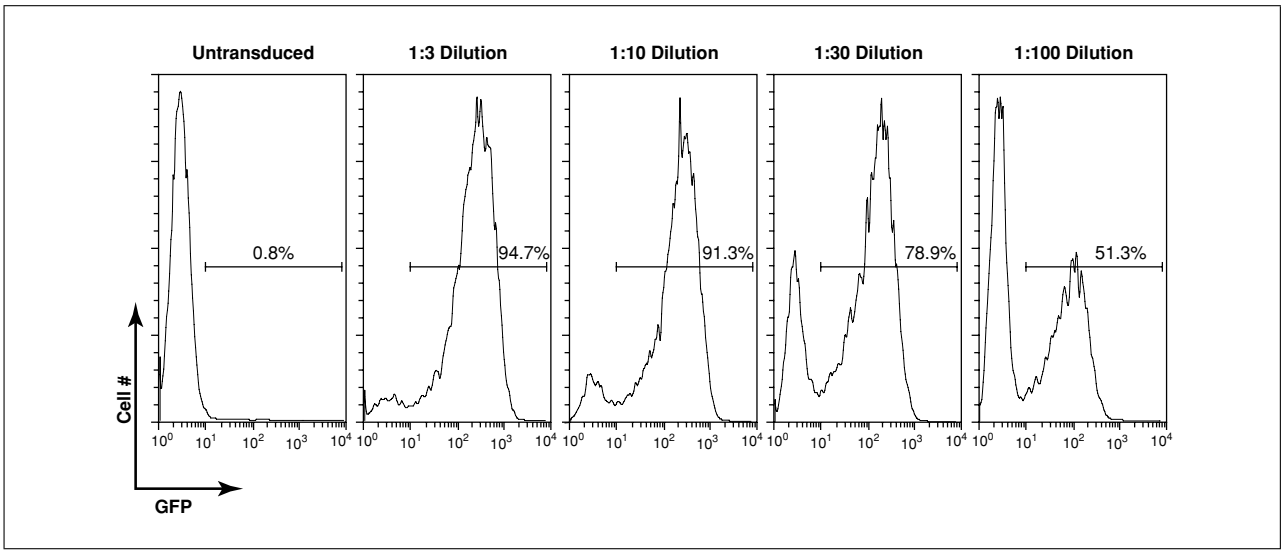

Figure 14.10.6 Titering GFP-expressing retrovirus stocks by flow cytometry. NIH 3T3 cells either untransduced or transduced with the indicated dilution of retrovirus stock were assessed 48 hour post-transduction for expression of GFP (*x* axis).

8b. After 10 to 12 days, aspirate medium off the plates, wash once with CMF-PBS, and fix/stain the plates for 5 min with Crystal Violet solution. After staining, rinse the plates under water and count the colonies visually.

9b. Determine viral stock titer as colony-forming units (CFU) per milliliter virus:

$$\text{Neo}^R \text{ titer (cfu/ml)} = (\#\text{ colonies}) \times (\text{cell dilution}) \times (\text{virus dilution})$$

For example, if 10 colonies are counted on a 1:20 dilution plate of the 1:10,000 virus stock dilution, this corresponds to a titer of 2×10^6 neo cfu/ml.

The neomycin titer is only a relative value to be used for comparing different virus stocks. The assay is highly nonlinear and cannot be used to calculate a multiplicity of infection, for instance. Titers of $\geq 2 \times 10^6$ neo cfu/ml are required for efficient transduction of murine HSC.

To titer viruses by Southern blotting

6c. Transfer trypsinized cells (from step 5) to a nonsterile 1.5-ml microcentrifuge tube. Pellet the cells by centrifuging in a benchtop microcentrifuge 5 min at $800 \times g$, room temperature.

7c. Resuspend the pellet in 0.25 ml of CMF-PBS, then add 0.25 ml of 2× DNA lysis buffer containing 0.25 mg/ml Proteinase K. Mix the tube well, as it will become viscous. Cap the tube and incubate overnight in a 37°C water bath.

8c. Extract DNA twice (*APPENDIX 3C*), each time with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), then extract the aqueous (upper) phase with an equal volume of 24:1 chloroform:isoamyl alcohol to remove traces of phenol.

9c. To the extracted aqueous phase add 1/10th vol of 3 M sodium acetate pH 5.2, mix, and then add 0.6 vol of room temperature isopropanol. Precipitate the DNA by inverting the tube. A visible clump of DNA should immediately appear. If no clump appears, place the tube overnight at −20°C to precipitate.

10c. Pellet DNA by centrifuging in a Sorvall RT-6000, 20 min at setting = 8, 4°C.

11c. Using a Pasteur pipet with end heat-sealed and melted into a small hook, grab the clump of DNA, dip briefly in a microcentrifuge tube containing 70% ethanol, then transfer the DNA to a small volume of TE buffer (0.2 to 0.3 ml), aiming for a final DNA concentration of 0.5 to 1.5 mg/ml.

12c. Determine DNA concentration by spectrophotometry (Gallagher and Desjardins, 2006).

An A_{260} of 1.0 indicates ~50 μg/ml double-stranded DNA.

13c. Digest the genomic DNA using a restriction enzyme that cuts twice within the provirus (for example, *Xba I*, which cuts within each LTR.) Perform the digest according to the enzyme manufacturer's instructions, in a small volume (35 to 50 μl) of appropriate buffer using 5 to 8 μg of genomic DNA. Unless the genomic DNAs have been previously treated with RNase, add 1.0 μl of DNase-free RNase A (100 μg/ml stock) to each digest. Digest the DNA for 3 to 5 hr at the appropriate temperature.

For controls, include genomic DNA samples from control cell lines containing a single integrated provirus (Li et al., 1999). This item is also available from the authors.

14c. Add 1/10 vol DNA loading buffer and load the samples on a 0.8% agarose/TBE slab gel (Voytas, 2000). Load serial dilutions of the same virus stock in adjacent lanes.

15c. Electrophorese (Voytas, 2000) the samples overnight at no more than 60 to 70 V constant voltage.

16c. Transfer the DNA to a nylon membrane using either the osmotic transfer method of Southern (Southern, 1975), or by electroblotting (Li et al., 1999).

Detailed protocols for Southern blotting can be found in Brown (1999).

17c. Probe the membrane with a radioactive probe for a proviral sequence that is located on the restriction fragment generated from the provirus by the enzyme chosen.

For Xba I digest of MSCV-IRES/GFP vectors, the GFP gene can be used for this.

18c. Wash the blot, and expose to X-ray film or a phosphor imager screen (Voytas and Ke, 1999).

19c. Quantify the intensity of the common proviral band detected by the probe and compare it to the intensity of the same band in the single-copy control samples.

This will allow a direct calculation of the proviral copy number in the sample.

PREPARING WEHI-3B CELL-CONDITIONED MEDIUM (WEHI-CM)

SUPPORT PROTOCOL 4

WEHI medium is included in the pre-stimulation and transduction medium as an additional source of IL-3 and perhaps other cytokines. WEHI-3B is a murine myelomonocytic cell line that constitutively produces IL-3 due to insertional activation of the IL-3 gene by an endogenous retrovirus (Lee et al., 1982). This protocol describes how to make the conditioned medium.

Materials

WEHI-3B cells (ATCC) in culture
Growth medium (DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin)

175-cm^2 flasks
50-ml sterile plastic tubes
0.45-μm filter system

1. Split WEHI cells a final time at ~1:5 into several 175-cm^2 flasks with 100 ml growth medium in each flask.
2. Allow cells to grow until they are confluent ($\geq 1 \times 10^6$ cells/ml) and stationary and the medium begins to turn orange from the pH change.
3. Collect the medium by pelleting the cells by centrifuging 10 min at 800 × *g*, room temperature, in 50-ml sterile plastic tubes, pooling supernatants and sterile-filtering the medium through a 0.45-μm filter system.
4. Apportion 25-ml aliquots into 50-ml conical tubes and store up to 1 year at −80°C until needed.
5. Test the IL-3 content of the WEHI CM by determining the dilution that supports maximal proliferation of an IL-3–dependent cell line, such as Ba/F3.

   Typically, a dilution of ~5% to 8% (v/v) of WEHI CM gives maximal proliferation of Ba/F3 or 32D cells.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Bone marrow flush medium

DMEM, high glucose (4500 mg/liter; Cellgro, cat. no. 10-017-CV)
5% (v/v) WEHI CM (see Support Protocol 4)
10% fetal bovine serum (FBS; heat-inactivated at 56°C for 30 min; Invitrogen, cat. no. 16000-044)
1% penicillin/streptomycin solution (from a 100× stock; HyClone, cat. no. SV30010)
Sterile filter through a 45-μm filter unit
Store up to 1 month at 4°C

Bone marrow prestimulation medium

DMEM, high glucose (4500 mg/liter; Cellgro, cat. no. 10-017-CV)
15% fetal bovine serum (FBS; heat-inactivated at 56°C for 30 min; Invitrogen, cat. no. 16000-044)
5% (v/v) WEHI CM (see Support Protocol 4)
1% of 200 mM L-Glutamine (from a 100× stock; Cellgro, cat. no. 25-005-CI)
1% penicillin/streptomycin solution (from a 100× stock; HyClone, cat. no. SV30010)
Sterile filter through a 45-μm filter unit
Store up to 1 month at 4°C
Add fresh for the quantity needed:
1 μg/ml of 1 mg/ml Ciprofloxicin (from 1000× stock in PBS; Miles Pharmaceuticals)
Cytokines (final concentrations):
  Recombinant murine IL-3 (1000× = 6 μg/ml; PeproTech, cat. no. 213-13)
  Recombinant murine IL-6 (500× stock = 5 μg/ml; PeproTech, cat. no. 216-16)
  Recombinant murine SCF (1000× = 50 μg/ml; PeproTech, cat. no. 250-0)

DNA lysis buffer, 2×

100 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
2% SDS
50 mM EDTA, disodium
Store up to several months at room temperature.

Before use, add 1 mg/ml proteinase K (fresh) to the required volume to be used.

Erythrocyte lysis solution

150 mM NH_4Cl
10 mM $KHCO_3$
0.1 mM EDTA, pH 7.4 Store up to 6 months at room temperature

This solution can be purchased from Sigma (cat. no. R7757).

FACS buffer

Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS; CellGro, cat. no. 21-040-CV)
0.05% (w/v) NaN_3
1% BSA or 2% FBS
Store up to 6 months at 4°C

HBS, 2×

8.0 g NaCl
0.37 g KCl
106.5 mg Na_2HPO_4 (anhydrous; 201.1 mg if 7 × H_2O)
1.0 g dextrose (D-glucose)
5.0 g HEPES (Invitrogen, cat. no. 15630-080)
Dissolve in 450 ml dH_2O (milli-Q)
Adjust pH to 7.05 with NaOH
Adjust volume to 500 ml with dH_2O
Sterile filter through a 0.45-μm filter unit
Store at room temperature (with the cap on tight) up to ~2 months

A batch of HBS should be used only for a maximum of ~2 months. After that time, the pH seems to drift and transfection efficiency drops. It does not help to freeze the solution for storage.

Spinfection solution (4 ml at 10^6 cells/ml)

Viral supernatant (see Support Protocol 1), 2 ml (final dilution 1:2)
300 μl FBS (15% of total nonvirus volume; heat-inactivated at 56°C for 30 min; Invitrogen, cat. no. 16000-044)
200 μl of 5% (v/v) WEHI-3B cell conditioned medium (see Support Protocol 4)
40 μl penicillin/streptomycin (1/100 volume; Hyclone, cat. no. SV30010)
40 μl of 200 mM L-glutamine (1/100 volume; Cellgro, cat. no. 25-005-CI)
40 μl of 1 M HEPES (1/100 volume; Invitrogen, cat. no. 15630-080)
4 μl Ciprofloxicin (1/1000 volume; Cipro, 1000× stock in PBS; Miles Pharmaceuticals)
4 μl IL-3 (1/1000 volume; Peprotech, cat. no. 213-13)
8 μl IL-6 (1/500 volume; Peprotech, cat. no. 216-16)
4 μl SCF (1/1000 volume; Peprotech, cat. no. 250-0)
40 μl Polybrene (1/100 volume; Sigma, cat. no. H-9268)
DMEM (Cellgro, cat. no. 10-017-CV) to 4 ml (contains 4 × 10^6 cells)

continued

In case the quantity of marrow cells requires several wells, multiply each quantity listed above by the total number of wells. The final total volume of cell solution is determined by the total number of cells. The viral supernatant can be as much as, but not more than, 50% of the final solution, and the optimum cell concentration should be 10^6 cells/ml. It is possible to increase the total number of cells/well up to about 1×10^7.

It is also possible to use a higher dilution of virus stock (1:3, 1:4, etc.) if necessary to match titers between different virus stocks. In this instance, increase the amount of DMEM added, keeping FBS at 15% (v/v) of nonvirus volume.

Polybrene is a polycationic compound that reduces electrostatic repulsion between virus and cell membrane and increases infection efficiency.

3T3 cell medium

DMEM (4500 mg/liter; Cellgro, cat. no. 10-017-CV) containing 4.5 g/liter glucose
10% fetal bovine serum (FBS; heat-inactivated at 56°C for 30 min; Invitrogen, cat. no. 16000-044)
1% penicillin/streptomycin (from a 100× stock; HyClone, cat. no. SV30010) Sterile filter through a 0.45-μm filter unit
Store up to 1 month at 4°C

293T cell medium

DMEM containing 4.5 g/liter glucose
10% FBS (heat-inactivated 30 min at 56°C; Invitrogen, cat. no. 16000-044)
1% penicillin/streptomycin (from a 100× stock from HyClone, cat. no. SV30010)
1% L-glutamine (from a 100× sock from Cellgro, cat. no. 25-005-CI)
1% non-essential amino acids (NEAA; from a 100 × stock from Cellgro, cat. no. 25-025-CI)
Sterile filter through 0.45-μm filter unit
Store up to 2 months at 4°C

COMMENTARY

Background Information

Over the last several years there has been a revolution in the understanding of the pathogenesis of MPDs, with the recognition that somatic activating mutations in tyrosine kinases (TKs) are found in a subset of patients with each disease category (Krause and Van Etten, 2005). For example, some cases of chronic myelomonocytic leukemia (CMML) are associated with activation of platelet-derived growth factor receptor β (PDGFRβ) through chromosome 5q translocations that fuse PDGFRβ with TEL (Golub et al., 1994) or many other partners (Jones and Cross, 2004). Fusion of PDGFRα to FIP1L1 through interstitial deletions on chromosome 4q are found in a subset of patients with CEL/hypereosinophilic syndrome (Cools et al., 2003; Griffin et al., 2003), while activating point mutations in c-KIT are found in some patients with systemic mast cell disease (Furitsu et al., 1993). Patients with 8p11 myeloproliferative syndrome (EMS) (Macdonald et al., 2002) have myeloproliferation frequently accompanied by non-Hodgkin's lymphoma. Both myeloid and lymphoma cells share translocations involving chromosome 8p, which lead to fusion of the receptor TK fibroblast growth factor receptor-1 (FGFR1) to multiple partners, including ZNF198 (Xiao et al., 1998). Recently, a somatic mutation (V617F) in JAK2, a nonreceptor tyrosine kinase required for erythropoietin receptor signaling (Parganas et al., 1998), was found in nearly every patient with PV, and in 30% to 60% of those with ET and CIMF (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Together, these observations indicate that dysregulated tyrosine kinases are found in the majority of the chronic myeloproliferative-like syndromes.

There are two different strategies to express dysregulated TKs in the mouse hematopoietic system: transgenic mice and retroviral gene transfer into bone marrow followed by transplantation. Both methods have their particular advantages and limitations (reviewed in Van Etten, 2001a). Transgenic mice allow production of a uniform cohort of diseased animals

by breeding, although multiple new transgenic lines must be generated for each new TK mutant to be studied and the transgene is present in all tissues in the embryo and adult. Since activated TKs can have deleterious effects during development, attempts to express certain oncogenic TKs via a traditional transgene or knock-in have been frustrated by toxicity and silencing (Heisterkamp et al., 1991; Jaiswal et al., 2003). These observations suggest that more sophisticated conditional transgenic approaches (Huettner et al., 2000) may be necessary to express dysregulated TKs in mice. By contrast, in the retroviral BM transduction/transplantation systems described herein, it is easy to test the leukemogenicity of new TKs and TK mutants by simply preparing new retroviral stocks. Furthermore, the effect of expression of a TK in distinct subsets of BM progenitors can be assessed. The major drawback is the labor-intensive and technically demanding nature of the experiments.

Critical Parameters and Troubleshooting

The major drawbacks of the retroviral model system are the complex nature of the assay and the many steps required. Significant care is necessary at every point in the protocol to ensure ultimate success. When problems are encountered with failure to efficiently induce MPD in recipient mice, the single most common source of difficulty is retroviral titers that are too low. It is absolutely essential to have virus stocks that are capable of single-copy transduction of 3T3 cells at 1:10 and, preferably, 1:30 dilutions, as discussed in Support Protocol 3. The second most common problem is excessive loss of viable cells during the two-day prestimulation and spinfection procedure. A decrease in the viable cell number of up to 80% is not unusual, but increased cell losses might be indicative of problems with the cytokines, the centrifugation step, or the 5-FU dose or mode of administration.

Donor and recipient mice

The source of mice can be any viral antibody-free (VAF)-certified vendor. Young males 6 to 10 weeks of age are preferred as donors as this allows use of a Y chromosome-specific probe (such as Zfx/Zfy or Sry) for determining the donor versus recipient status of cells after transplant (Miles et al., 1997). Although older donors (6 to 8 months of age) can also be used, the frequency and transducibility of HSC is decreased with these subjects. BALB/c mice are the preferred inbred strain for these studies. This strain appears to be uniquely sensitive to induction of MPD by retroviruses, as BCR-ABL will induce CML-like disease in C57Bl/6 mice, but less efficiently than in BALB/c (Li et al., 2001).

Cytokines

The number and concentrations of cytokines in the prestimulation and transduction medium have never been specifically tested. Maximal physiological concentrations of at least one stem cell–active cytokine (IL-3 or SCF) is necessary, to ensure hematopoietic stem cell survival during the 2-day culture period (Fletcher et al., 1991).

Anticipated Results

Properly applied, the protocols detailed in this unit should allow the reproducible generation of cohorts of mice with distinct myeloproliferative diseases, depending on the particular mutant TK employed. Some specific examples of the distinctive diseases induced by particular dysregulated TKs in this model system are provided below.

BCR-ABL: The murine CML-like disease, induced upon retroviral transduction of bone marrow, is a fatal myeloproliferative disease characterized by peripheral blood leukocytosis with greatly increased neutrophils and infiltration of spleen, liver, and lungs with maturing myeloid cells, leading to hepatosplenomegaly and ultimately death from respiratory failure. Myeloid cells from these mice express BCR-ABL protein, contain increased levels of tyrosine-phosphorylated proteins (Roumiantsev et al., 2001), and carry the retroviral provirus in chromosomal DNA. The disease is very similar to the human illness in terms of the cell of origin, transplantability, and disease progression. The CML-like leukemia is responsive to treatment with TK inhibitor drugs (Wolff and Ilaria, 2001) and to immunotherapy (Krause and Van Etten, 2004). However, the retroviral model system does have some drawbacks and limitations (reviewed in Van Etten, 2001a). The polyclonal nature and short latency of murine CML-like leukemia differ from human CML, which is monoclonal and more chronic. However, this is not an important pathophysiological difference, but merely reflects transplantation of multiple BCR-ABL-transduced progenitors into each recipient. When a lower titer virus is used, or limiting numbers of transduced cells are transplanted, oligo- to monoclonal disease with a longer latency is observed (Daley et al., 1990; Jiang et al., 2003). In addition, BCR-ABL is

expressed from the proviral LTR at several-fold higher levels than are typically found in chronic-phase CML cells (Li et al., 1999; Barnes et al., 2005), which may also affect disease latency. In addition, human CML cells are typically haploid for the *BCR* and *c-ABL* genes and can express the reciprocal ABL-BCR fusion product. However, the fact that BCR-ABL expression alone recapitulates the disease in mice argues these differences are not critical for leukemogenesis.

The 8p11 myeloproliferative syndrome (EMS) is a novel disorder characterized by MPD and non-Hodgkin's lymphoma, usually of immature lymphoblastic T cell phenotype. Both myeloid and lymphoma cells share balanced chromosomal translocations involving 8p11, demonstrating that the disease arises in HSC (Inhorn et al., 1995; Macdonald et al., 1995). The translocations fuse the cytoplasmic domain of fibroblast growth factor receptor-1 (FGFR1), encoded on 8p11, to several different N-terminal partner proteins, most commonly ZNF198. Interestingly, several patients who presented with typical CML rather than EMS had t(8;22) and fusion of BCR to FGFR1 (Demiroglu et al., 2001; Fioretos et al., 2001). Using the retroviral model system, it was demonstrated that ZNF198-FGFR1 induced EMS-like disease in mice, with MPD and lymphoblastic T lymphoma that arose from common multipotential stem/progenitor target cells (Roumiantsev et al., 2004). Mutation of FGFR1 Tyr766, a binding site for the SH2 domain of phospholipase C-γ1 (PLC-γ1), abolished activation of PLC-γ1 in the leukemic cells and attenuated both the MPD and lymphoma (Roumiantsev et al., 2004). BCR-FGFR1 bound to the adapter protein Grb2 via BCR Tyr177 and induced CML-like MPD in mice, whereas a BCR-FGFR1 Y177F mutant lacked Grb2 binding and caused EMS-like disease. These results established a mouse model of EMS, and implicated signaling pathways originating from both kinase and fusion partner in the pathogenesis of EMS and CML (Roumiantsev et al., 2004).

Mutant JAK2 in the non-CML MPDS: A somatic mutation (V617F) in the JH2 pseudokinase domain of the JAK2 tyrosine kinase is found in the majority of patients with polycythemia vera (PV), and in some with essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (CIMF; Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). The authors of this unit have expressed murine JAK2 wild-type (WT) or a JAK2 V617F mutant using retroviral bone marrow transduction and transplantation in two different strains of inbred mice, C57Bl/6 (B6) and BALB/c (Zaleskas et al., 2006). In both B6 and BALB/c mice, JAK2 V671F induced nonfatal polycythemia, characterized by increased hematocrit and hemoglobin, reticulocytosis, and splenomegaly that were manifest by 3 weeks post-transplantation and were sustained for at least several months. JAK2 V617F also induced significant leukocytosis and neutrophilia that was much more pronounced in BALB/c mice than in B6. Platelet counts were not significantly increased in either strain. By contrast, peripheral blood counts in recipients of bone marrow transduced with JAK2 WT were normal and not significantly different from recipients of vector-transduced bone marrow. These results suggest that the principal effect of expression of JAK2 V617F in hematopoietic stem/progenitor cells is expansion of the erythroid lineage. The strain-dependent leukocytosis suggests that other genetic loci may modulate the hematopoietic phenotype of this activated JAK2 mutation. The lack of thrombocytosis implies that additional events may be required for JAK2 V617F to cause essential thrombocythemia. Similar results have been reported by several other groups (Lacout et al., 2006; Wernig et al., 2006).

Preclinical therapeutic testing: Collectively, these results illustrate the rich phenotypic diversity of MPD-like syndromes induced using the mouse retroviral transduction model system, and emphasize the opportunities for exploiting the system for the study of disease pathogenesis and therapy. These retroviral model systems can be used to assess the therapeutic efficacy of drugs that inhibit the enzyme activity of the TK (tyrosine kinase inhibitors, TKIs) or target critical downstream signaling pathways. For example, the ABL tyrosine kinase inhibitor imatinib has therapeutic activity in the mouse retroviral model of CML-like leukemia induced by BCR-ABL. Imatinib, dosed once or twice a day by oral gavage, can decrease leukocyte counts and splenomegaly and prolong survival of mice with BCR-ABL-induced CML-like MPD (Wolff and Ilaria, 2001). Some second-generation ABL kinase inhibitors, including dastainib also inhibit the activity of SRC family TKs. Testing of these dual ABL/SRC inhibitors in retroviral models of BCR-ABL-induced CML-like MPD and B-ALL demonstrate that inhibition of the SRC kinase family has therapeutic benefits in BCR-ABL-induced B-ALL, but not in CML (Hu et al.,

2004, 2006). Candidate drugs can also be delivered parenterally to mice with MPD in these models, either by injection or by implantable osmotic minipumps. In mice with polycythemia induced by retroviral expression of JAK2 V617F, a 2 week treatment with AG490 using implanted minipumps (Alzet Corporation) caused significant decreases in hematocrit and reticulocyte counts, providing proof-of-concept that a JAK2 inhibitor may have therapeutic efficacy in the treatment of this condition (Zaleskas et al., 2006).

Time Considerations

Prior to beginning a bone marrow transplant experiment the investigator must have corresponding viral stocks and know their titers. If none are present, allow extra time for preparing and characterizing the viral stocks (Support Protocols 1, 2, and 3). Assuming the retroviral plasmid is cloned and healthy cells are growing well (293T and 3T3 NIH cell-lines), preparing a new viral stock will take 3 days from plating producer cells to collecting the virus-containing supernatant. Titering the new virus will require another 3 to 10 days, depending on the protocol selected. Actual hands-on time is limited to a few hours, depending on the number of plates and different viruses.

If 5-FU priming is used, count 4 days prior to bone marrow retrieval, then 2 days in culture before the actual transplant. Hands-on time is limited to a few hours, mostly the day of 5-FU injection and bone marrow retrieval, depending on the number of donor mice. Once the transplant is performed, engraftment and successful repopulation takes ∼15 days during which the mice have to be checked for survival. Stringent disease monitoring begins after 15 days.

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