Video Article

Use of Hematopoietic Stem Cell Transplantation to Assess the Origin of Myelodysplastic Syndrome

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Abstract

Myelodysplastic syndromes (MDS) are a diverse group of hematopoietic stem cell disorders that are defined by ineffective hematopoiesis, peripheral blood cytopenias, dysplasia, and a propensity for transformation to acute leukemia. NUP98-HOXD13 (NHD13) transgenic mice recapitulate human MDS in terms of peripheral blood cytopenias, dysplasia, and transformation to acute leukemia. We previously demonstrated that MDS could be transferred from a genetically engineered mouse with MDS to wild-type recipients by transplanting MDS bone marrow nucleated cells (BMNC). To more clearly understand the MDS cell of origin, we have developed approaches to transplant specific, immunophenotypically defined hematopoietic subsets. In this article, we describe the process of isolating and transplanting specific populations of hematopoietic stem and progenitor cells. Following transplantation, we describe approaches to assess the efficiency of transplantation and persistence of the donor MDS cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/58140/

Introduction

Myelodysplastic syndromes (MDS) represent a diverse set of clonal blood disorders characterized by ineffective hematopoiesis, morphologic evidence of dysplasia, and a propensity for transformation to acute myeloid leukemia (AML)[1,2,3,4]. Ineffective hematopoiesis is recognized as a maturation arrest in bone marrow, and results in peripheral blood cytopenias despite a hypercellular bone marrow[1,3]. The incidence of MDS has been variously estimated as 2-12 cases per 100,000 persons annually in the United States, and the incidence of MDS increases with age, making this an important condition to understand given the aging U.S. population[3,5]. Although most cases of MDS have no clear etiology, some cases of MDS are thought to be due to exposure to known genotoxic agents, including solvents such as benzene, and cancer chemotherapy[6].

MDS patients typically have acquired mutations in the MDS cells[7]. Although relatively uncommon, a number of MDS patients have acquired balanced chromosomal translocations involving genes such as NUP98, EVI1, RUNX1, and MLL (http://cgap.nci.nih.gov/Chromosomes/Mitelman). Our laboratory has a long-standing interest in chromosome translocations, which involve the NUP98 gene[8]. Transgenic mice that express a NUP98-HOXD13 (NHD13) transgene regulated by the Vav1 promoter and enhancer elements display all of the key features of MDS, including peripheral blood cytopenias, morphologic evidence of dysplasia, and transformation to AML[9].

Although MDS have been recognized for over 60 years[10], and are considered to be a clonal stem cell disorder, efforts to engraft human MDS cell in immunodeficient mice have been largely unsuccessful, because the MDS cells engraft poorly[11,12,13,14] and the mice do not develop clinical disease. In an effort to identify which hematopoietic cells can transmit MDS, we turned to the NHD13 model, and showed that we could engraft MDS as a disease entity that showed all of the cardinal features of human MDS, including peripheral blood cytopenias, dysplasia, and transformation to AML[15]. In this report, we present the technical details of these experiments, as well as approaches to further fractionate hematopoietic stem and precursor cells (HSPC), in an effort to identify MDS-initiating cells.

Protocol

The animal procedures described in this article were approved by the National Cancer Institute at Bethesda Animal Care and Use Committee, and conform to the policies contained within The Public Health Service Policy on Humane Care and Use of Laboratory Animals, The Animal Welfare Act, and the Guide for the Care and Use of Laboratory Animals.

1. Cell Preparation

1. Harvesting bone marrow nucleated cell (BMNC)
    1. Use only sterile materials. Sterilize re-usable instruments using a steam autoclave. Purchase needles, syringes, and plastic ware in single use sterile containers. Perform all animal and cell manipulations in a Class II, Type A2 Biological Safety Cabinet.
    2. Prepare BMNCs in a 14 mL round bottom tube containing 3 mL of HF2 (Hank's balanced salt solution supplemented with 2% fetal bovine serum).
    3. Euthanize C57Bl6 donor mice (positive for CD45 allele CD45.2), age typically 2-6 months, using a CO_2 chamber.
    4. Sterilize the mouse carcass by spraying 70% ethanol over the entire body with a spray bottle. Peel the skin from the legs, from the pelvis down to the ankle.
    5. Remove the femora and tibiae from the carcass using sterile scissors (straight, sharp/sharp, 11.5 cm) and forceps (Graefe, serrated, 0.8-mm tip width). Trim attached muscles clearly.
    6. Cut the proximal and distal ends of the tibia with the scissors. Holding the tibia with forceps, insert a 27-gauge 3 mL syringe containing 2.5 mL of HF2 into the tibial marrow cavity at the distal end of the tibia (ankle). Flush the bone marrow cells from the tibia using gentle pressure into the 14 mL round bottom tube.
        1. Repeat for the other tibia.
    7. Cut the proximal and distal ends of the femur with scissors. Flush the femur marrow cavity by inserting a 20-gauge needle attached to a 3 mL syringe containing 2.5 mL of HF2 into the distal end of the femur marrow cavity and applying gentle pressure to the syringe.
        1. Repeat for the other femur, collecting all bone marrow from both tibiae and femora in the same 14 mL round bottom tube.
    8. Disperse the marrow mass by aspirating up and down several times with the same 20-gauge needle attached to the 3 mL syringe.
2. Antibody staining of BMNC
    1. Count BMNC. Add 20 µL of a 3% acetic acid solution to 20 µL of the BMNC suspension generated in step 1.1.8. Then, count using a hemocytometer and an inverted microscope.
    2. Pellet the BMNC by gentle centrifugation at 450 x g for 5 min at 4 °C. Resuspend the pellet with HF2 using 90 µL per 10^7 cells and add 10 µL of biotinylated anti-lineage antibody cocktail to the cell suspension.
    3. After incubating for 20 min at 4 °C, wash the stained cells with 3 mL of phosphate-buffered saline (PBS).
    4. Resuspend the BMNC with 100 µL of HF2 per 10^7 cells. Add 2 µL of allophycocyanin (APC) conjugated anti-biotin antibody to the cell suspension, followed by incubation at 4 °C for 20 min.
    5. Rinse the stained BMNC with 3 mL of PBS and resuspend the BMNC with HF2 supplemented with 0.2 µg/mL of propidium iodide (PI) for flow cytometry cell sorting.
3. Flow cytometry cell sorting of BMNC
    1. Calibrate flow cytometry cell sorter. Optimize all photomultiplier tubes (PMTs), scatter, and fluorescence parameters using unstained cells and set within the linear range of each detector.
    2. Prepare unstained, PI-stained, and APC labeled lineage cocktail cells in parallel from step 1.2. Analyze for spectral compensation.
    3. Discriminate doublet cells by sequential gating of FSC-H *vs.* SSC-H, 640-SSC-A *vs.* 640-SSC-H, and 640-SSC-S *vs.* 640-SSC-W.
    4. Exclude nonviable cells using PI excited at 561 nm and emission collected with a 614/20 band pass filter. Excite lineage APC labeled cells at 640 nm and collect emission with a 671/30 band pass filter.
    5. Record fluorescence values as voltage height and collect at least 100,000 events in list mode data files to visualize the populations to be sorted.
    6. Calculate spectral compensation after acquiring three samples of 10,000 cells for the following: unlabeled cells, PI labeled cells, and anti-lineage antibody cocktail conjugated with APC labeled cells.
    7. Set three regions to sort Lineage negative (LN), Lineage positive (LP) with dim fluorescence intensity (LP1), and LP population with bright fluorescence (LP2) into 5 mL tubes containing 0.5 mL of 10% FBS media.
    8. Sort cells using a one drop envelop and purify abort mode.
    9. Perform post sort analysis with 1,000 total cells on each sorted sample to confirm the purity and viability of the sorted cells.
    10. Collect sorted cells by centrifugation at 450 x g for 5 min at 4 °C and resuspend cell pellet with 0.5 mL of HF2.
    11. Confirm sorted cell number with hemocytometer and Trypan blue. Adjust cell number with HF2 for transplantation.
    NOTE: A wide spectrum of BMNC populations can be isolated for transplantation using additional combinations of fluorochrome-conjugated antibodies in step 1.2.2 above.

2. Recipient Mice Preparation and Hematopoietic Stem Cell Transplantation (HSCT)

1. Preparation of transplant recipients
    1. Maintain veterinary and husbandry care of congenic recipient mice (B6-LY5.1/Cr, CD45.1) in a specific pathogen free (SPF) animal facility.
    2. For prophylactic decontamination of the gastrointestinal track, provide sterile water containing 100 mg/L of ciprofloxacin to the recipients for 7 days prior to lethal dose (9 Gy) irradiation, and maintain for 14 days following irradiation.
    3. Provide a lethal dose (9 Gy) irradiation from a Cs source as follows. Use a trained, certified Cs operator. Set the Cs source instrument to deliver 70 cGy/min total body gamma irradiation. Place 10 mice in a custom-designed holder and insert the holder into the irradiator chamber. Deliver 9 Gy total body irradiation in 13 min and return the mice to their cages.
2. Hematopoietic stem cell transplantation
    1. Mix wild type (WT) BMNC from a congenic recipient mouse (B6-LY5.1/Cr, CD45.1) at a cell dose of 2 x 10^5 cells per mouse with 2 to 5 x 10^4 cells of the purified test BMNC prepared with flow cytometry sorting as described in 1.3 above. Mix cells in the same syringe.

NOTE: The WT BMNC provide a radiation sparing-effect to the recipient mouse and allow for the survival of the recipient mouse if the test cells do not support hematopoiesis. The WT BMNC used in this type of experiment express the CD45.1 allele, and thus can be distinguished from the test cells using antibodies which discriminate between CD45.1 and CD45.2. The WT BM cells are referred to as "helper cells", "radiation-sparing cells", or "competitor cells".

2. Adjust the cell mixture volume to 200 µL per recipient using sterile HF2 for facilitating the injection.
3. Transplant the cell mixtures to the lethally irradiated recipient mice *via* intravenous tail vein injection using a 1 mL syringe and 28-gauge needle, within 24 h of irradiation. Place the mouse tail under a heat lamp, as heat leads to tail vein dilation.
NOTE: Euthanize all the recipient mice at the end of the study and evaluate the disease progression by necropsy, histology, and flow cytometry.

3. Engraftment Assay using Flow Cytometry

1. To evaluate donor cell engraftment, collect peripheral blood (PB) from the recipient mice at 6th week, 12th week, and 16th week after transplantation.
2. Warm the recipients in the cage with heat lamp for about 1 min and place mouse into a restrainer.
3. Cut the tail vein with a scalpel (blade 10, #3 scalpel handle) and collect 100 µL of PB per recipient using a microcapillary tube containing EDTA as an anticoagulant.
4. Divide the collected PB into two equal aliquots by placing 50 µL in a sterile microcentrifuge tube. Use one aliquot for an automated complete blood count (CBC) (performed at the NCI Histopathology Core) and use the second aliquot for antibody staining and flow cytometry.
5. To detect donor engraftment by flow cytometry, lyse the red blood cells (RBC) by adding 1 mL of hypotonic RBC lysis buffer (8.29 g/L of NH_4Cl, 1 g/L of $KHCO_3$, 0.037 g/L of Na_2EDTA, pH 7.2) to 50 µL of the collected PB. Vortex the sample to mix and incubate 10 min at room temperature.
6. Centrifuge the lysed PB at 4,600 x g for 1.5 min. Carefully aspirate the supernatant and discard.
7. Partially disrupt the cell pellet by gently tapping or "flicking" the bottom of the tube. Add 1.3 mL of PBS into the pellet located in tube and centrifuge at 4,600 x g for 1.5 min. Carefully aspirate and discard the supernatant.
8. Disrupt the cell pellet by tapping or flicking, and add 200 µL of HF2 supplemented with 5% rat serum to the cell pellet.
9. Add anti-CD45.2 antibody (1 µL) conjugated with allophycocyanin (APC) to the cell suspension. Incubate at 4 °C for at least 30 min, and briefly vortex the mixture.
10. After the staining, wash the cells twice as described above, and resuspend with 400 µL of HF2 supplemented with 1 µg/mL of PI.
11. Detect engraftment using a 4-color flow cytometer. Obtain additional information on cell types within the peripheral blood by adding additional antibodies to detect specific cell types, such as B or T lymphocytes.
12. Record serial engraftment data using a spread sheet to allow for further analysis of data.

Representative Results

We show representative figures for results of several experiments. **Figure 1** shows a representative flow cytometry sorting experiment. During normal hematopoietic differentiation, as cells become committed to a specific hematopoietic lineage, they acquire lineage-defining cell surface markers and lose the potential for self-renewal. Therefore, in wild-type mice, stem cell self-renewal is confined to lineage-negative BMNC. In this experiment, we sorted BMNC from NHD13 bone marrow into lineage-negative, low lineage positive, and high lineage-positive cells. These sorted cells were then transplanted into WT recipients to determine self-renewal capacity.

Figure 2 shows results from a separate experiment, in which lineage negative cells or unsorted cells from NHD13 donors with MDS were transplanted into lethally irradiated WT recipients. The NHD13 donor cells expressed the CD45.2 cell surface marker, whereas the WT competitor cells (see section 2.2.1 above) expressed the CD45.1 cell surface marker. **Figure 3** shows serial engraftment analysis of either unsorted NHD13 BMNC, or lineage-negative NHD13 BMNC. After approximately 16 weeks, the NHD13 cells outcompete the WT cells, as shown by the increasing percentage of CD45.2+ cells in the peripheral blood. **Figure 4** shows an example of leukemic transformation from a mouse transplanted with NHD13 MDS cells.

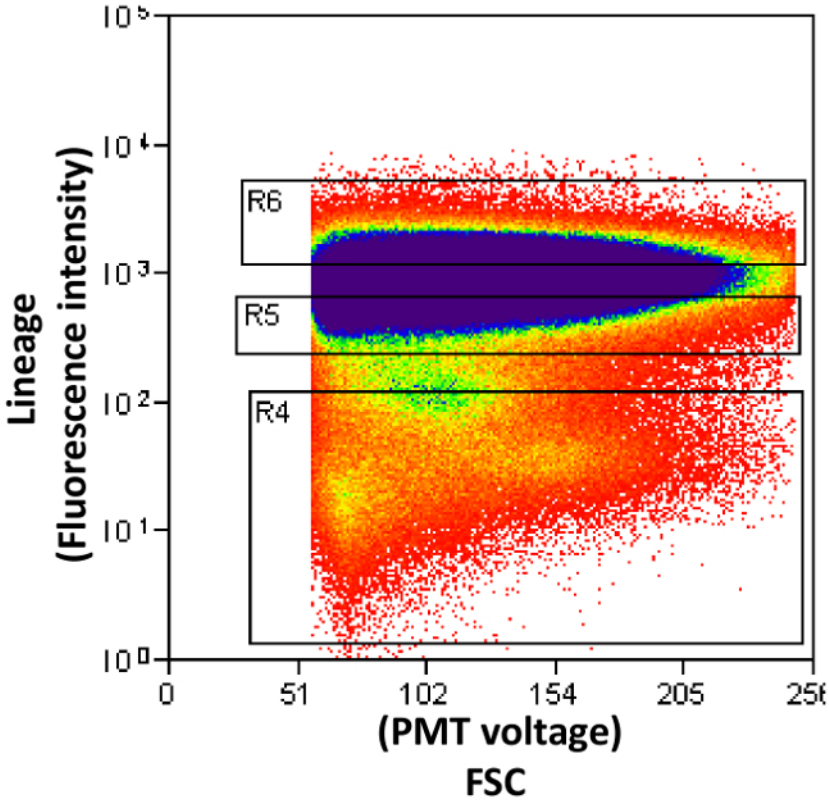

Figure 1: Flow cytometry sorting strategy of BMNC stained with lineage antibody cocktail. Each rectangular box on the dot plot represents the cell population sorted for HSCT to evaluate cell function. R4, lineage negative; R5, low lineage positive; R6, high lineage positive. Please click here to view a larger version of this figure.

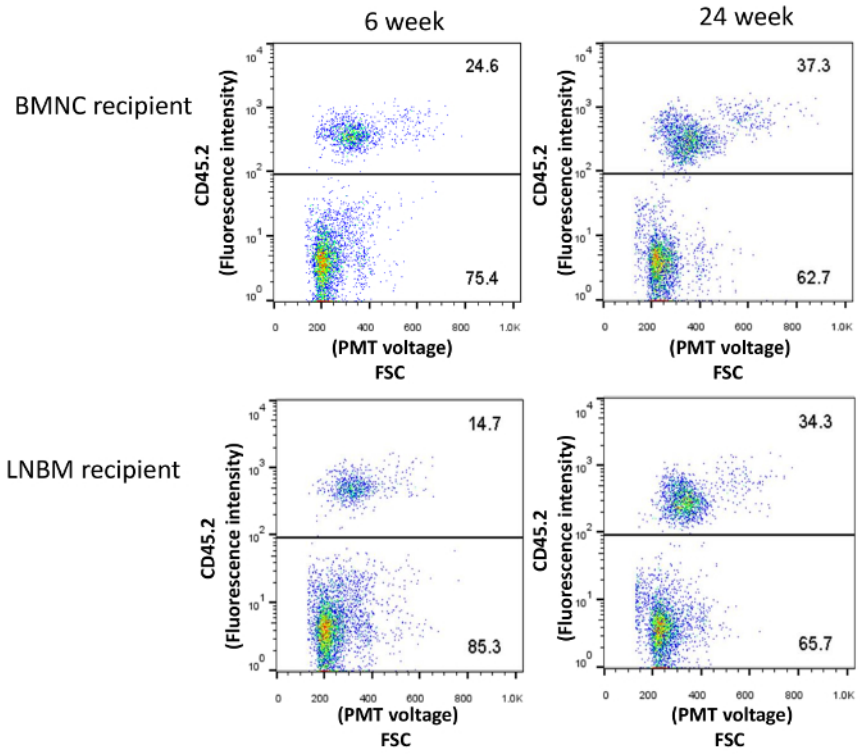

Figure 2: Representative FACS profiles for engraftment assay using donor specific anti-CD45.2 antibody. BMNC recipient was transplanted with 1 X 10^6 NHD13 BMNC (CD45.2+) and LNBM recipient with 5 X 10^4 NHD13 lineage negative BM cells (CD45.2+). In each case, 2 x 10^5 WT BMNC (CD45.1+) were used as competitor cells. Numbers in the upper and lower boxes represent CD45.2 positive (derived from NHD13 donor) and CD45.2 negative (derived from WT competitor cells), respectively, at post-transplantation 6 week and 24 week. Please click here to view a larger version of this figure.

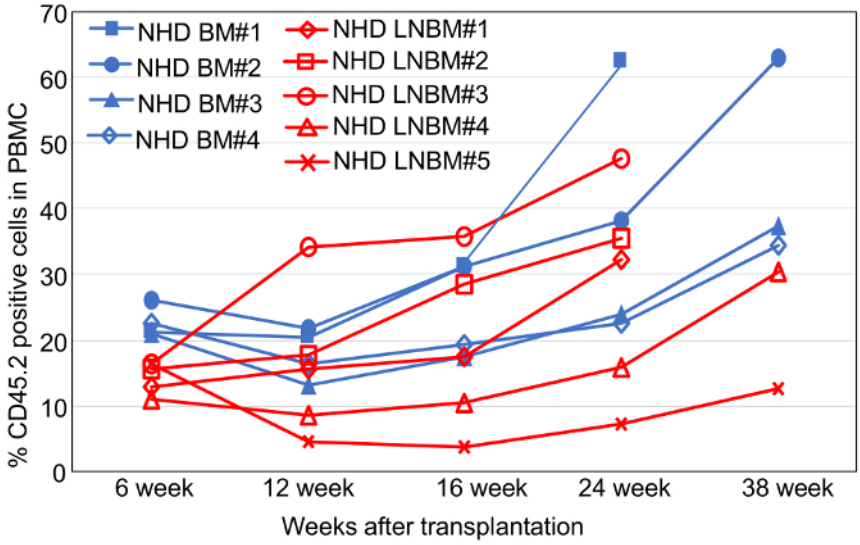

Figure 3: Engraftment kinetics of individual recipients after transplantation. Each line on the graph shows serial engraftment assays of an individual recipient mouse. BMNC recipients were transplanted with 1 X 10^6 cells of NHD13 whole BMNC, and LNBM recipients were transplanted with 5 X 10^4 of NHD13 lineage negative BM after sorting. NHD indicates NHD13 donor. BM, BMNC recipient mice; LNBM, lineage negative BM recipient; PBMC, peripheral blood mononucleated cell. In all cases, the NHD13 donor cells gradually outcompete the WT cells. Please click here to view a larger version of this figure.

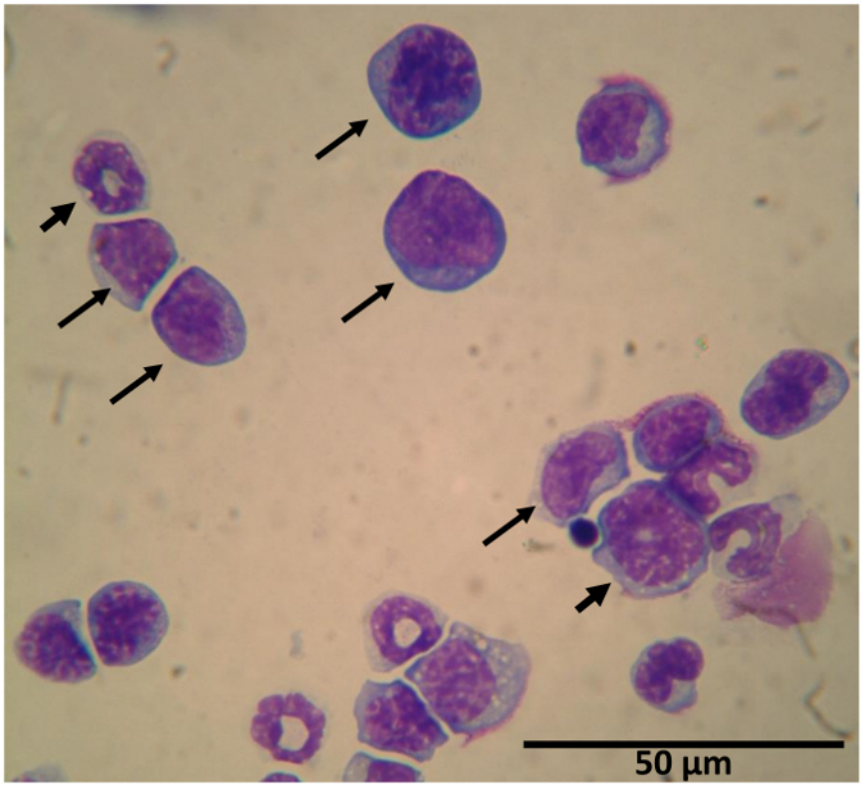

Figure 4: May-Grünwald Giemsa (MGG) staining of bone marrow (BM) from MDS recipient that progressed to AML. Note the presence of numerous blasts and immature forms. Arrows indicate blasts, and arrowheads indicate immature forms. Original 400X. Please click here to view a larger version of this figure.

Discussion

Although MDS are a clonal hematopoietic stem cell disorder, the MDS "stem", or initiating cells, have not yet been characterized. We previously demonstrated that MDS can be transplantable to WT mice using bone marrow from NHD13 mice by HSCT, characterized by macrocytic anemia, leukopenia, neutropenia, and morphologic evidence of dysplasia[15]. In addition, competitive repopulation assays identified a growth advantage of cells from the NHD13 MDS bone marrow. Taken together, these findings imply the existence of an MDS stem or initiating cell. Additional experiments are now in progress, aimed at refining the immunophenotypic characteristics of an MDS initiating cell[15] using more defined stem and progenitor cell markers (CD150, CD48, c-Kit and Sca-1)[16] combined with the lineage marker staining method described in this protocol.

Identifying MDS initiating cells using NHD13 mice involves extensive flow cytometry sorting procedure. The *ex vivo* manipulation of primary BMNCs places stress on the cells, potentially resulting in cell death or functional loss. Therefore, efforts should be made to reduce the time of *ex vivo* manipulation including the time of flow cytometry sorting. The maker and model of flow cytometry instruments and flow dynamics are additional variables that may affect cell viability after sorting. The HSCT procedure requires cell infusion through a blood vessel. Although tail vein injection may be a more challenging technique than retro-orbital injection, in our experience, tail vein injection can be performed more rapidly than retro-orbital injection as the recipient mice are not anesthetized for the tail vein injections. Limitation of injection volume (maximum 150 µL) is another issue in retro-orbital injection[17], as a higher cell concentration may lead to additional loss of cells in the course of cell preparation, such as shearing of cells on the wall of syringes and needles.

Although these experiments are focused on the NHD13 model for MDS, the HSCT sorting and transplant approaches described here can be used for any genetically engineered mouse. In addition to determining the characteristics of self-renewing hematopoietic cells, this transplant approach can be used for other purposes. For instance, if one desires to obtain a large cohort of mice with MDS bone marrow to assess the efficacy of treatment with a small molecule, a large cohort (30+ mice) can be generated by transplanting WT mice with MDS BM. Alternatively, the HSCT strategy can be reversed. In an attempt to cure mice of MDS, NHD13 mice with MDS can be transplanted with WT BM, and the success of the transplant can be monitored by assessing the proportion of hematopoietic cells that express CD45.1 (which marks WT BM) as opposed to CD45.2 (which marks MDS BM)[18].

Disclosures

We have nothing to disclose.

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References

1. Corey, S. J. *et al.* Myelodysplastic syndromes: the complexity of stem-cell diseases. *Nature Reviews Cancer.* **7** (2), 118-129 (2007).
2. Garcia-Manero, G. Myelodysplastic syndromes: 2015 Update on diagnosis, risk-stratification and management. *American Journal of Hematology.* **90** (9), 831-841 (2015).
3. Heaney, M. L., & Golde, D. W. Myelodysplasia. *The New England Journal of Medicine.* **340** (21), 1649-1660 (1999).
4. Nimer, S. D. Myelodysplastic syndromes. *Blood.* **111** (10), 4841-4851 (2008).
5. Aul, C., Giagounidis, A., & Germing, U. Epidemiological features of myelodysplastic syndromes: results from regional cancer surveys and hospital-based statistics. *International Journal of Hematology.* **73** (4), 405-410 (2001).
6. Pedersen-Bjergaard, J., Christiansen, D. H., Desta, F., & Andersen, M. K. Alternative genetic pathways and cooperating genetic abnormalities in the pathogenesis of therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia.* **20** (11), 1943-1949 (2006).
7. Uy, G. L. *et al.* Dynamic changes in the clonal structure of MDS and AML in response to epigenetic therapy. *Leukemia.* **31** (4), 872-881 (2017).
8. Gough, S. M., Slape, C. I., & Aplan, P. D. NUP98 gene fusions and hematopoietic malignancies: common themes and new biologic insights. *Blood.* **118** (24), 6247-6257 (2011).
9. Lin, Y. W., Slape, C., Zhang, Z., & Aplan, P. D. NUP98-HOXD13 transgenic mice develop a highly penetrant, severe myelodysplastic syndrome that progresses to acute leukemia. *Blood.* **106** (1), 287-295 (2005).
10. Block, M., Jacobson, L. O., & Bethard, W. F. Preleukemic acute human leukemia. *Journal of the American Medical Association.* **152** (11), 1018-1028 (1953).
11. Thanopoulou, E. *et al.* Engraftment of NOD/SCID-beta2 microglobulin null mice with multilineage neoplastic cells from patients with myelodysplastic syndrome. *Blood.* **103** (11), 4285-4293 (2004).
12. Kerbauy, D. M., Lesnikov, V., Torok-Storb, B., Bryant, E., & Deeg, H. J. Engraftment of distinct clonal MDS-derived hematopoietic precursors in NOD/SCID-beta2-microglobulin-deficient mice after intramedullary transplantation of hematopoietic and stromal cells. *Blood.* **104** (7), 2202-2203 (2004).
13. Benito, A. I. *et al.* NOD/SCID mice transplanted with marrow from patients with myelodysplastic syndrome (MDS) show long-term propagation of normal but not clonal human precursors. *Leukemia Research.* **27** (5), 425-436 (2003).
14. Medyouf, H. *et al.* Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. *Cell Stem Cell.* **14** (6), 824-837 (2014).
15. Chung, Y. J., Choi, C. W., Slape, C., Fry, T., & Aplan, P. D. Transplantation of a myelodysplastic syndrome by a long-term repopulating hematopoietic cell. *Proceedings of the National Academy of Sciences of the United States of America.* **105** (37), 14088-14093 (2008).
16. Pietras, E. M. *et al.* Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell Stem Cell.* **17** (1), 35-46 (2015).
17. Yardeni, T., Eckhaus, M., Morris, H. D., Huizing, M., & Hoogstraten-Miller, S. Retro-orbital injections in mice. *Lab Animal.* **40** (5), 155-160 (2011).
18. Chung, Y. J., Fry, T. J., & Aplan, P. D. Myeloablative hematopoietic stem cell transplantation improves survival but is not curative in a pre-clinical model of myelodysplastic syndrome. *PLoS One.* **12** (9), e0185219 (2017).

