RAPID COMMUNICATION

Selection of Retrovirally Transduced Hematopoietic Cells Using CD24 as a Marker of Gene Transfer

By Robert Pawliuk, Robert Kay, Peter Lansdorp, and R. Keith Humphries

We have investigated the use of a cell surface antigen as a dominant selectable marker to facilitate the detection and selection of retrovirally infected target cells. The small coding region of the human cell surface antigen CD24 (~240 bp) was introduced into a myeloproliferative sarcoma virus (MPSV)-based retroviral vector, which was then used to infect day 4 5-fluorouracil (5-FU)-treated murine bone marrow cells. Within 48 hours of termination of the infection procedure CD24-expressing cells were selected by fluorescent-activated cell sorting (FACS) with an antibody directed against the CD24 antigen. Functional analysis of these cells showed that they included not only in vitro clonogenic progenitors and day 12 colony-forming unit-spleen but also cells capable of competitive long-term hematopoietic repopulation. Double-antibody labeling studies performed on recipients of retrovirally transduced marrow cells showed that some granulocytes, macrophages, erythrocytes, and, to a lesser extant, B and T lymphocytes still expressed the transduced CD24 gene at high levels 4 months later. No gross abnormalities in hematopoiesis were detected in mice repopulated with CD24-expressing cells. Our results show that the use of the CD24 cell surface antigen as a retrovirally encoded marker enables the rapid, efficient, and nontoxic selection in vitro of infected primary cells, facilitates tracking and phenotyping of their progeny, and should provide a unique tool to identify elements that regulate the expression of transduced genes in the most primitive hematopoietic cells.

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RECOMBINANT RETROVIRUSES provide an attractive vehicle for gene transfer based on their capacity for highly efficient infection and nontoxic integration into the genome of a wide range of cell types. Although the potential for retrovirus-mediated gene transfer efficiency approaches 100%, this may not be realized because of low viral titers or the failure to stimulate cells to divide, which is required for successful integration. Moreover, expression of the transferred gene(s) may not reach desired levels or be sustained, necessitating extensive analysis to identify critical regulatory sequences. Such problems are evident in current efforts to apply retroviral gene transfer to the most primitive hematopoietic cells that are rare and mostly non-cycling.

To aid in the identification, enrichment, and tracking of transduced target cells, a variety of selectable markers have been incorporated into retroviral vectors. The most widely used of these have been intracellular components that confer resistance to toxic compounds such as neomycin, hygromycin, chloramphenicol, methotrexate, mycophenolic acid, or various chemotherapeutic agents. However, use of these markers in selection protocols carry disadvantages that include nonspecific drug toxicity and difficulties in quantitating expression levels. The bacterial β-galactosidase gene (lacZ) and the human placental alkaline phosphatase gene have also been used to select transduced cells in vitro and as reporter molecules both in vitro and in vivo. However, the presence of an endogenous mammalian lysosomal β-galactosidase and problems in achieving adequate levels of expression of the exogenous β-gal gene have limited its effective use.

Genes encoding cell surface antigens have also been used as selectable markers of gene transfer to fibroblasts and more recently to human peripheral blood lymphocytes. The use of such genes offers several potentially significant advantages including the rapid and quantitative detection of transferred gene expression in the desired target cell population by flow cytometry; the efficient and nontoxic selection of transduced target cells by fluorescence-activated cell sorter (FACS) or other immuno-based selection techniques; and the tracking of transduced cells and their progeny both in vitro and in vivo. However, the applicability of this approach to primitive hematopoietic cells has not, as yet, been demonstrated. Moreover, the genes used to date as selectable markers are relatively large, leaving limited space in the retroviral vector for other genes of interest.

We have recently cloned cDNAs encoding the human hematopoietic cell surface antigen CD24, and its murine homologue the heat stable antigen (HSA). The function of these molecules is not yet resolved although CD24 has been associated with activation and differentiation events in B cells as well as the oxidative burst response in granulocytes, and roles for HSA in T-cell development and activation have been suggested. Both antigens are glycoproteins attached to the outer surface of the plasma membrane by a glycosyl phosphatidylinositol lipid anchor and are expressed on multiple lineages of hematopoietic cells. The mature peptides are only 30 to 35 amino acids in size with the entire coding region being encompassed within an approximate 240-bp DNA fragment. In addition, the mature CD24 and HSA proteins share only limited sequence homology (57%) with one another and antibodies to CD24 and HSA
are not cross-reactive. These features of small coding size, potential for cell surface expression on multiple hematopoietic lineages, and limited homology suggested to us that HSA and CD24 would be possible candidates are not cross-reactive. These features of small coding size, potential for cell surface expression on multiple hematopoietic lineages, and limited homology suggested to us that HSA and CD24 would be possible candidates.

In this study we show the feasibility of using CD24 for the identification and selection of retrovirally transduced primary cells of the murine hematopoietic system.

**MATERIALS AND METHODS**

**Animals.** Animals used in these experiments were 8- to 12-week-old (C57Bl/6J × C3H/HeJ)F1 (B6C3F1) male and female mice bred and maintained in the animal facility of the British Columbia Cancer Research Centre from parental strain breeders originally obtained from the Jackson Laboratories (Bar Harbor, ME). Mice used as bone marrow (BM) donors in competitive repopulation experiments were 10- to 14-week-old male or female (C57Bl/6J-Lyn-Pep3b × C3H/HeJ)F1 (PepC3F1) mice. B6C3F1 and PepC3F1 mice are phenotypically distinguishable on the basis of allelic differences at the Ly5 locus; B6C3F1 mice are homozygous Ly5.2 and PepC3F1 mice are Ly5.1/Ly5.2 heterozygotes.

**Retroviral vectors.** All experiments used a retrovirus derived from the JZen retroviral backbone provided by Dr S. Cory (Walter and Eliza Hall Institute, Melbourne, Australia). The 3' long terminal repeat (LTR) of JZen is derived from the myeloproliferative sarcoma virus (MPSV). To construct JZenCD24tkneo, a 310-bp Sal I fragment containing bp 1 to 303 of the published CD24 cDNA sequence and encompassing the entire 240-bp coding region was removed from PAX114 and inserted into the Xho I site of JZen-tkneo using standard procedures. JZen-tkneo was constructed by inserting into the Hpal-HindIII sites of JZen a 1,092-bp Smal-HindIII fragment from pT719tkneo that contains the neo gene linked to a mutant polyoma virus enhancer tandem repeat and herpes simplex virus thymidine kinase gene promoter isolated from pMC1neo.

**Cell lines.** The ecotropic packaging cell line, GP+AM12, and the amphotropic cell line, GP+E−86, were used to generate helper-free recombinant retrovirus. The cell lines were maintained in HX medium composed of Dulbecco’s modified Eagle’s medium (DMEM; StemCell Technologies, Vancouver, BC, Canada), 10% heat-inactivated (35°C for 30 minutes) newborn calf serum (GIBCO/ BRL Canada, Burlington, Ontario), hypoxanthine (15 μg/mL; Sigma Chemical Co, St Louis MO), xanthine (250 μg/mL, Sigma), and mycophenolic acid (25 μg/mL; Sigma). Viral packaging cells were maintained in HX medium supplemented with 1 mg/mL (approximately 0.6 mg/mL active compound) of the neomycin analog G418 (GIBCO/BRL). The interleukin-3 (IL-3)-dependent murine hemopoietic cell line Ba/F3 (from Dr G. Spangrude (Rocky Mountain Laboratory, Hamilton, MT)) and analysis by flow cytometry. Peripheral blood samples were depleted of erythrocytes by incubating them for 10 minutes on ice in the presence of 4 vol of sterile 1 mol/L NH4Cl solution. In some experiments photometric analysis of Ly5.1 donor-derived peripheral blood leukocytes was achieved through double-antibody labeling with Ly5.1-FITC in combination with one of Gr-1-PE (from hybridoma RB6-8C5 provided by Dr G. Spangrude) to identify granulocytes, Mac-1-PE (from hybridoma M1/70; American Type Culture Collection (ATCC), Rockville, MD) to identify macrophages, B220-PE (from hybridoma RA3-6B2; Dr Spangrude) to identify B lymphocytes, or Ly1-PE (from hybridoma TIB104; ATCC) to identify T lymphocytes as described below. Levels of expression of the transferred CD24 gene in cells from repopulated mice were assessed by staining peripheral blood samples with anti-CD24 tetrameric antibody complexes and R-phycocerythrin (R-PE) as described above. CD24 expression among peripheral blood leukocytes was analyzed by staining peripheral blood samples with anti-CD24/R-PE tetramers in combination with FITC-labeled Gr-1 to identify granulocytes.
cytes, Mac-1 for macrophages, Ly1 for T cells, and B220 for B cells.

**FACS sorting.** Cells were sorted on a FACStar® (Beckton Dickinson) equipped with a 5-W argon and a 30-mW helium neon laser. Cells were collected in sterile Eppendorf vials in medium with 50% FCS.

**In vitro clonogenic progenitor assays.** Sorted and unsorted BM cells were plated in 35-mm Petri dishes (Greiner, Stuttgart, Germany) in 1.1 mL culture mixtures containing 0.8% methylcellulose in α medium supplemented with 30% FCS, 1% bovine serum albumin (BSA), 10^{-6} M β-mercaptoethanol, 3 U/mL human urinary erythropoietin (Epo), 2% spleen cell conditioned medium (SCCM), and 10% agar-stimulated human leukocyte conditioned medium, all of which were obtained from StemCell Technologies. Cells were plated in the presence or absence of 1.5 mg/mL (~0.9 mg/mL, active compound) of G418 (GIBCO/BRL Canada) and incubated at 37°C in 5% CO₂. Large single and multilineage colonies were scored after 8 to 14 days of incubation according to standard criteria.²⁷

**CFU-S assay.** Lethally irradiated B6C3F1 mice (910-950 cGy, 110 cGy/min, ³⁷Cs γ-rays) were injected intravenously with 1 × 10⁷ to 1 × 10⁶ cells from the indicated cell fraction. Twelve days later, animals were killed and well-isolated macroscopic spleen colonies individually dissected and suspended for flow cytometric and DNA analysis.

**Competitive repopulating unit (CRU) assay.** Limiting numbers of sorted or unsorted BM cells derived from PepC3F1 (Ly5.1/Ly5.2) donors were injected into lethally irradiated B6C3F1 (Ly5.2) recipients together with a lifesparsing dose of Ly5.2 competitor BM cells; either 1 × 10⁷ marrow cells from a normal mouse or 2 × 10⁷ marrow cells from a compromised animal.⁴⁴ The function of these latter cells is to ensure the short-term survival of the recipient after the irradiation procedure. The level of reconstitution of recipients with donor (Ly5.1) cells was assessed at 5 or 16 weeks posttransplantation by flow cytometric analysis of peripheral blood samples (50 to 100 mL) obtained by tail-vein puncture.

**Southern blot analysis.** DNA was purified from NaDodSO₄/proteinase K-digested cells by phenol/chloroform extraction.⁴⁰ DNA was dialyzed for 16 hours against 1 × TE (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0) buffer and digested with Xbal or EcoRI (BRL) at 37°C for 12 to 16 hours. After ethanol precipitation, DNA was dissolved in 20 μL of 1 × TE buffer, separated on a 0.8% agarose gel, and transferred to a nylon membrane (Zeta-Probe; Bio-Rad, Richmond, CA) by blotting. Membranes were subsequently probed using a fragment of the neomycin resistance (neo⁸) gene or the CD24 cDNA ³²P labeled by random priming.

**RESULTS**

**The CD24 viral vector.** To explore the possible use of CD24 as a selectable cell surface marker, the retroviral vector depicted in Fig 1 was constructed. This vector contains the minimal 240-bp CD24 cDNA sequence encompassing the complete coding region under the control of the MPSV long-terminal repeat enhancer and promoter regulatory elements. For these initial feasibility studies neo⁸ was also included in the vector to aid in viral titering and to provide an independent means to assess gene transfer. A CD24 viral producer was generated using the ectotropic GP+ E⁻ 86 packaging line and had a titer of ~5 × 10⁴ CFU/mL as assessed by neo⁸ gene transfer to NIH-3T3 cells.

Transfer of the CD24 gene to hematopoietic cells was initially evaluated in IL-3-dependent murine Ba/F3 cells. Approximately 80% of Ba/F3 cells were found to express high levels of surface CD24 antigen after 2 days of cocultivation with CD24 viral producers and a further 5 days of growth in the absence of G418 selection (Fig 2). Similar levels of expression were detected as early as 12 hours post-infection (data not shown).

**FACS selection of CD24-transduced in vitro clonogenic progenitors and CFU-spleen (CFU-S).** Day 4–5 FU BM cells were cocultivated with CD24 viral producers for 24 hours and recovered nonadherent cells were cultured for a further 48 hours to allow expression of the transferred CD24 gene before flow cytometric analysis and cell sorting. As shown in a representative FACS profile for one experiment, approximately 1/3 of the BM cells recovered after cocultivation infection without prior growth factor prestimulation were positive for the CD24 cell surface antigen (see Fig 3, top panel). In three experiments 96% ± 3.6% of in vitro clonogenic progenitors recovered in the CD24⁺ fraction were G418-resistant compared with 35-69% in the unsorted marrow population (see Fig 3, bottom panel). Some G418-resistant progenitors (12% to 27%) were also detected in the CD24⁻ population, likely as a result of overlap between the CD24⁺ and CD24⁻ cell populations and the relatively low sorting threshold chosen.

CD24⁺ and CD24⁻ fractions were also assayed for day 12 CFU-S. A summary of findings from three experiments are presented in Table 1. All spleen colonies derived from the CD24⁺ fraction (37 of 37 analyzed) were positive both for proviral DNA sequences and significant levels of CD24

**Fig 1.** Schematic of the JZenCD24tkneo provirus. It incorporates a 240-bp portion of the CD24 cDNA encompassing the complete coding region; a thymidine kinase-neomycin resistance gene cassette (tkneo) from pMC1neo; and LTR sequences from the MPSV as described in Materials and Methods.
expression above background staining (range 5% to 79% of cells analyzed). The observation that not all cells within a colony expressed detectable levels of CD24 is likely a result of some admixture of contaminating inter-colony cells as well as differences in the absolute level of CD24 expression on cells within each colony. In contrast, only approximately 50% of spleen colonies derived from the unsorted marrow (13 of 30) showed evidence of gene transfer by Southern blot analysis. Furthermore, only 4 of 13 marked colonies were positive for expression of the transferred CD24 gene. Surprisingly, a significant proportion of spleen colonies derived from the CD24+ fraction (15 of 26) were also found to contain intact provirus, although only three expressed detectable levels of CD24. Thus, retrovirally transduced day 12 CFU-S can be successfully enriched based on their immediate expression of a transduced CD24 gene. In addition, the differentiating day 12 progeny of the CFU-S thus selected also show maintained expression of the transduced CD24 gene in vivo.

Selection by FACS of CD24-virus-infected CRU. Subsequent experiments were conducted to determine the feasibility of selecting CD24 transduced CRU by FACS. In an effort to facilitate gene transfer to repopulating cells, day 4 5-FU BM cells were prestimulated with growth factors for 48 hours before coculture with CD24 viral producer cells. The CD24 expression profiles of nonadherent cells recovered 48 hours after the coculture period for two experiments are shown in Fig 5. Greater than 50% of cells were CD24+ using this infection protocol compared to 33% with no prestimulation (see Fig 3, top panel). Unsorted and sorted CD24+ or CD24* cells (Fig 4, Expt 1) from Ly5.1 donor mice were injected into lethally irradiated Ly5.2 recipients under competitive repopulating conditions. In preliminary studies, injection of limiting numbers of BM cells recovered after the sorting procedure showed a competitive repopulating cell frequency of approximately 1/1 x 105 (data not shown). Therefore, recipients were transplanted with 104 or 4 x 105 unsorted or sorted cells in an effort to minimize the likelihood of CD24+ CRU contributing to the transplant due to contamination of the CD24+ fraction. Based on the proportion of uninfected control cells found within the CD24+ sorting window (0.7%, Fig 4, Expt 1), our calculations predict that for every 2 x 104 cells sorted less than 0.014 CD24+ (ie, contaminating) CRU would be found within the positive sort window because of occurrences such as nonspecific binding of the CD24/RPE tetrameric antibody complex.

For recipients transplanted with cells from the CD24+ fraction, 10 of 11 were found to be reconstituted with provirus-containing CRU 5 weeks posttransplantation (see Table 2). Expression of CD24 on peripheral blood leukocytes of these mice ranged from 7% to 24%. In three mice, evidence of the same proviral integration fragments in cells of both myeloid and lymphoid tissues suggested gene transfer to a totipotential repopulating cell. Retrovirally infected CRU were also found in the CD24- fraction, with 8 of 11 mice showing evidence of proviral marking in either BM and/or thymus DNA. However, none of these eight recipients showed detectable levels of CD24 expression on peripheral blood leukocytes. Of five recipients injected with unsorted BM cells,

### Table 1. Proviral Integration and CD24 Expression on Cells From Individual Spleen Colonies Derived From Sorted and Unsorted BM Cells After CD24 Virus Infection

<table>
<thead>
<tr>
<th>Cells Transplanted</th>
<th>Total No. Colonies Analyzed</th>
<th>No. Colonies* Positive for CD24 Expression</th>
<th>No. Colonies Positive for Proviral Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td>30</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Frac II (CD24+)</td>
<td>27</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Frac I (CD24-)</td>
<td>26</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>

Recipient mice were transplanted with 1 x 104 to 1 x 105 BM cells, which had been cocultured with CD24 viral producer cells, from one of each of the presort, CD24+ or CD24- marrow fractions as described in the legend to Fig 3. Well-isolated spleen colonies were dissected and analyzed for CD24 expression using flow cytometry and for proviral integration using Southern blot analysis. The table represents data accumulated over three separate experiments.

* A spleen colony was concluded to be positive for CD24 expression if >5% of the cells bound significant levels of the anti-CD24 tetrameric antibody complex.
Table 2. Proviral Integration and CD24 Expression on Cells From Competitively Repopulated Mice Assessed 5 Weeks Posttransplantation (Expt 1)

<table>
<thead>
<tr>
<th>Cells Transplanted</th>
<th>Reconstitution with retrovirally marked cells*</th>
<th>CD24 cell surface expression in PB cells (&gt;5%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td>5/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Sort Fraction I (CD24-)</td>
<td>8/11</td>
<td>0/11</td>
</tr>
<tr>
<td>Sort Fraction II (CD24+)</td>
<td>10/11</td>
<td>10/11</td>
</tr>
</tbody>
</table>

*Retroviral marking was assessed by Southern blot analysis of BM and thymus from each transplanted recipient. Blots were separately probed with 32P-labeled fragments of the neoR gene and CD24 cDNA with identical results.

†Expression of the CD24 antigen on peripheral blood leukocytes was assessed by staining peripheral blood samples depleted of erythrocytes with anti-CD24/R-PE tetrameric antibody complexes and analysis by flow cytometry.

Fig 4. Selection of CD24 virus-infected CRU by FACS. The CD24 expression profiles of day 4 5-FU uninfected control marrow cells (top panels) and day 4 5-FU BM cocultured with CD24 viral producer cells (bottom panels) 48 hours postinfection are shown for two independent experiments. Cells were stained with anti-CD24/R-PE tetrameric antibody complexes and analyzed by flow cytometry. In experiment 1, infected cells were sorted into CD24⁻ (I) and CD24⁺ (II) fractions and injected into lethally irradiated recipient mice under competitive repopulating conditions at 1 × 10⁵ to 4 × 10⁶ cells per mouse. Mice were analyzed 5 weeks posttransplantation for both CD24 cell surface expression on peripheral blood leukocytes and proviral integration in BM and thymus (results are shown in Table 2). For experiment 2, BM cells in the presort, CD24⁻, CD24⁺low, CD24⁺med, and CD24⁺high expressing fractions were injected into lethally irradiated recipient mice under competitively repopulating conditions at 1 × 10⁶ cells per mouse. Mice were analyzed 16 weeks posttransplantation for CD24 cell surface expression on their peripheral blood cells and proviral integration in BM and thymus (results are shown in Table 3 and Fig 5). Percentages of cells in the sort windows are indicated.
not detected although provirally marked cells were present in the BM and/or thymus of all of these animals. Additional analysis of BM and thymus DNA from these mice showed no gross rearrangements in proviral structure to account for the lack of expression in these recipients (data not shown). Lack of CD24 expression also did not appear to be a result of promoter interference because G418\(^\text{R}\) clonogenic progenitors were detected only in those mice showing CD24 expression on peripheral blood leukocytes (Table 3).

**Long-term multilineage expression of CD24.** To examine further the persistence and cell lineage pattern of CD24 expression after CD24 retroviral gene transfer, mice were transplanted under competitive repopulating conditions with relatively large numbers of unsorted BM immediately after infection by coculture with CD24 viral producer cells as above. Six mice received \(4 \times 10^8\) to \(2 \times 10^8\) cells estimated to contain 13 to 66 CRU based on a CRU frequency of 1/3,000 immediately post cocultivation. At 16 weeks postransplantation, analysis showed that Ly5.1\(^+\) (donor cell-derived) peripheral blood leukocytes were present in all mice (range 16% to 90%) and in five of the six mice peripheral blood leukocytes expressing CD24 (range 8% to 46%) were detectable. Double-antibody staining for CD24 and lineage-specific cell surface antigens showed that in all five of these recipients there were lymphoid, myeloid, and erythroid cells expressing the transduced CD24 gene. Representative flow cytometric profiles for one of these mice are shown in Fig 6. Further, high levels of CD24 expression were detected in all hematopoietic tissues tested in this mouse including BM, spleen, and thymus (see Fig 7).

**DISCUSSION**

In this study we have tested the utility of a cDNA encoding CD24, a human cell surface antigen, for the immediate post-

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**Table 3. Flow Cytometric Analysis of Peripheral Blood and BM Cells From Mice Competitively Repopulated With Sorted or Unsorted CD24 Virus-Infected Marrow Assessed 16 Weeks Posttransplantation (Expt 2)**

<table>
<thead>
<tr>
<th>Cells Transplanted</th>
<th>Mouse</th>
<th>Peripheral Blood</th>
<th>% G418(^\text{R}) BM Progenitors</th>
<th>Proviral Marking (BM and/or T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td>m1</td>
<td>10 (L)(^*)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m2</td>
<td>8 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m3</td>
<td>4 (L)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD24</td>
<td>m1</td>
<td>42 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m2</td>
<td>29 (L)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m3</td>
<td>7 (L)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m4</td>
<td>74 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD24(_{low})</td>
<td>m1</td>
<td>75 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD24(_{mod})</td>
<td>m1</td>
<td>13 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m2</td>
<td>8 (L/M)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>m3</td>
<td>70 (L/M)</td>
<td>17</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>m4</td>
<td>7 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD24(_{high})</td>
<td>m1</td>
<td>73 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m2</td>
<td>21 (L/M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>m3</td>
<td>7 (M)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Ly5.2 recipient mice were transplanted under competitive repopulating conditions with \(1 \times 10^8\) retrovirally infected Ly5.1 BM cells from the sort fractions indicated. Repopulation of recipients with Ly5.1 donor-derived cells was assessed by staining erythrocyte-depleted peripheral blood with FITC-conjugated Ly5.1 antibody and analysis by flow cytometry. Samples were also tested for expression of the transferred CD24 gene by staining cells with anti-CD24 tetrameric antibody complexes/R-PE and analysis by flow cytometry. The proportion of G418\(^\text{R}\) clonogenic progenitors in BM and proviral marking were determined as described in Materials and Methods.

\* Phenotypic analysis of Ly5.1 donor-derived peripheral blood leukocytes was achieved by double-antibody labeling with Ly5.1-FITC antibody in combination with one of Gr.-1-PE to identify granulocytes, Mac-1-PE to identify macrophages, Ly-1-PE to identify T lymphocytes and B220-PE to identify B lymphocytes. (L) represents lymphoid restricted repopulation, (M) myeloid restricted repopulation, and (L/M) stands for multilineage (lymphoid and myeloid) repopulation.

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**Fig 5. Hematopoietic reconstitution from CD24 retrovirus-infected competitive repopulating cells as assessed by Southern blot analysis of proviral integration in BM (B) and thymus (T) 16 weeks posttransplantation.** Recipients received \(1 \times 10^8\) cells of CD24 virus-infected marrow from presort, CD24\(_{low}\), CD24\(_{mod}\), or CD24\(_{high}\) fractions as shown for Expt 2 Fig 4. Individual recipient mice are labeled as m1-m4. DNA (20 \(\mu\)g) from each tissue sample was digested with EcoRI, an enzyme that cuts once within the CD24 provirus sequence. Shown are results of a blot probed with a \(^{32}\)P-labeled fragment of the neo\(^+\) gene; identical results were observed using the CD24 cDNA as a probe. The positive control represents DNA obtained from a retrovirally infected Ba/F3 clone harboring two copies of provirus.
numbers of selected CD24+ cells showed proviral integration in virtually all animals in which donor cells were detected. Because long-term repopulating cells are such rare cells and have been difficult to purify to homogeneity, it has been difficult to analyze the diversity in properties and behavior of individual cells of this type, particularly after their transplantation in vivo. The ability to obtain, before transplant, a population of cells that are 100% provirally marked should now greatly enhance the power of studies aimed at addressing such questions. In addition, this type of strategy should be useful as a preclinical model for the development of more effective gene transfer strategies to long-term human repopulating stem cells.

The recovery of competitive repopulating cells in the top 8% of CD24-expressing cells provides direct evidence that the MPSV LTR enhancer and promoter are able to drive high-level gene expression in the most primitive hematopoietic cells present in adult marrow tissue. The use of such a selectable marker should be useful for optimizing vectors to achieve high and sustained levels of transferred gene expression in very primitive hematopoietic cells and ultimately for studies aimed at the genetic manipulation of stem cell infection selection of hematopoietic cells transduced with a retrovirus containing this cDNA. FACS analysis in combination with functional studies showed that under the conditions used, Ba/F3 cells, a factor-dependent hematopoietic cell line, as well as primary marrow in vitro clonogenic progenitors, day 12 CFU-S and, most significantly, the earliest cells capable of competitive long-term hematopoietic repopulation all expressed CD24 within 48 hours of termination of the infection procedure. Southern blot analysis of BM and thymus cells from mice competitively repopulated with limiting B220-FITC

**Fig 6.** Expression of the transferred CD24 gene on cells of multiple hematopoietic lineages from a mouse that had been competitively repopulated with CD24 retrovirus-infected BM 4 months previously. Erythrocyte-depleted peripheral blood samples were subjected to double-antibody labeling with anti-CD24/R-PE tetrameric antibody complexes in combination with one of Gr-1-FITC to detect granulocytes, Mac-1-FITC to detect macrophages, Ly1-FITC to detect T lymphocytes, and B220-FITC to detect B lymphocytes and analyzed by flow cytometry. The control mouse shown here and in Fig 7 is a recipient that had been transplanted only with helper BM cells.

**Fig 7.** Flow cytometric analysis of CD24 expression in the hematopoietic tissues of a mouse competitively repopulated with CD24 retrovirus-infected BM 4 months posttransplantation. Cells were stained with anti-CD24/R-PE tetrameric antibody complexes. Peripheral red blood cells either were or were not depleted before the staining procedure.
The most dramatic evidence of continued CD24 expression at the time of selection was also maintained in their progeny, expression observed in primitive retrovirally infected cells behavior. An interesting finding of this study was that CD24 expression did not decrease with time after transplantation. Compared to CD24 expression in peripheral blood leukocytes 5 to 6 weeks posttransplantation (experiment 1), whereas such cells were not observed in animals repopulated with CRU from the CD24+ fraction despite the presence of provirus in the marrow and/or thymus of a number of these. Sustained expression of CD24 in recipients of initially selected CD24+ CRU was also observed 4 months posttransplantation, although in a small proportion of mice despite the persistence of retrovirally marked cells in myeloid and/or lymphoid tissues in all. Neither gross rearrangement in proviral structure (as assessed by Southern blot analysis) or promoter interference between the CD24 and neo genes could account for the lack of CD24 expression observed in these mice. The loss of CD24 expression in some long-term repopulated mice transplanted with CD24+ selected cells is suggestive of a shutdown of exogenous promoter activity in vivo, a phenomenon that has been previously reported. Such shutdown of promoter activity may be related to the methylation status of the regulatory elements. However, others have reported the continued expression of transferred genes such as human CD85 and the human glucocerebrosidase gene for long periods posttransplantation. However, it is important to note that in our studies recipients were purposely transplanted with only one or few stem cells to enable the analysis of gene expression at the clonal level whereas in the studies mentioned above recipients were not transplanted at limiting dilution. Indeed, we observed that virtually all animals receiving an excess of 10 (unselected) CRU showed continued long-term expression of the transferred CD24 gene consistent with previous results of others. Our results would suggest that at least for long-term repeat-driven gene expression the phenomenon of promoter shutdown may be more widespread than previously appreciated. Moreover, it may be that mice repopulated with limiting numbers of CD24+ selected cells continue to express the transferred CD24 gene but at levels below that which can be detected with FACS. Because of the ease and sensitivity of methods for monitoring transduced CD24 expression in peripheral blood cells, this vector construct should be well suited for further studies of vector modifications that may abrogate promoter shutdown in primitive hematopoietic cells and their long-term progeny.

The CD24 cell surface antigen can be used not only as a marker for the selection of retrovirally infected target cells but also as a reporter molecule for the phenotyping of target and progeny cells. Double-staining procedures performed on the peripheral blood of irradiated recipients repopulated with CD24 virus-infected cells showed that all hematopoietic lineages were able to express the transferred CD24 gene including granulocytes, macrophages, erythrocytes and to a lesser extent, B and T lymphocytes. Depressed levels of expression in lymphoid cells may reflect cell lineage-specific differences in MPSV promoter activity. Therefore, these results would suggest that the use of CD24 might aid in the identification of regulatory elements that differentially optimize transferred gene expression in specific tissues or cell lineages. Of particular interest in this regard was the noted expression of transduced CD24 on mature red blood cells. This may provide unique advantages for its use as a reporter molecule to identify erythroid specific regulatory elements and should, at the very least, facilitate the design of vectors for use in gene therapy applications to red blood cell disorders. No gross abnormalities in hematopoiesis were observed in mice expressing high levels of CD24 after transplantation with CD24 virus-infected marrow compared with normal control animals. Further, sustained multilineage expression in recipient mice at least 4 months posttransplantation shows that the use of such a foreign antigen as a retroviral marker is compatible with long-term expression in hematopoietically reconstituted lethally irradiated recipients. In conclusion, this study has shown the feasibility of using the CD24 cell surface antigen as a selectable marker and a reporter molecule in primary hematopoietic cells including the most primitive elements of this system. This technique should prove useful as a method for rapidly testing various retroviral infection protocols as well as the identification of regulatory elements that optimize gene expression in primitive hematopoietic stem cells or other target cells of interest. Extension of this approach to the human setting is now being pursued using retroviral vectors containing the murine CD24 homologue, HSA.

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Selection of retrovirally transduced hematopoietic cells using CD24 as a marker of gene transfer

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