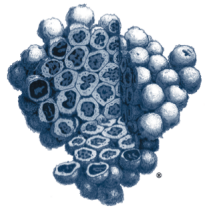



# The Ly-6A (Sca-1) GFP Transgene is Expressed in all Adult Mouse Hematopoietic Stem Cells

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# The *Ly-6A* (Sca-1) *GFP* Transgene is Expressed in all Adult Mouse Hematopoietic Stem Cells

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**Key Words.** *Hematopoietic stem cells · Sca-1 ·* Ly-6A GFP · *Transgenic mouse*

## ABSTRACT

**The Sca-1 cell surface glycoprotein is used routinely as a marker of adult hematopoietic stem cells (HSCs), allowing a >100-fold enrichment of these rare cells from the bone marrow of the adult mouse. The Sca-1 protein is encoded by the *Ly-6A/E* gene, a small 4-exon gene that is tightly controlled in its expression in HSCs and several hematopoietic cell types. For the ability to sort and localize HSCs directly from the mouse, we initiated a transgenic approach in which we created *Ly-6A* (Sca-1) green fluorescent protein (*GFP*) transgenic mice. We show here that a 14-kb *Ly-6A* expression cassette directs the transcription of the *GFP* marker gene in all functional repopulating HSCs in the adult bone marrow. A >100-fold enrichment of HSCs occurred by sorting for the *GFP*-expressing cells. Furthermore, as shown by fluorescence-activated cell sorting and histologic analysis of several hematopoietic tissues, the *GFP* transgene expression pattern generally corresponded to that of Sca-1. Thus, the *Ly-6A GFP* transgene facilitates the enrichment of HSCs and presents the likelihood of identifying HSCs in situ. *Stem Cells* *2002;20:514-521***

## INTRODUCTION

During adult stages, the hematopoietic system is constantly renewed from rare hematopoietic stem cells (HSCs) harbored in the bone marrow. HSCs can be retrospectively identified based on their functional repopulation properties observed only by transplantation into hematopoietic-depleted adult recipients. The properties that define HSCs are long-term, high-level repopulation of all hematopoietic lineages and the ability to self-renew [1, 2].

Using the transplantation assay to identify stem cells, HSCs have been enriched and characterized by flow cytometric sorting using a wide range of antibodies detecting cell-surface markers. The established surface marker profile of adult HSCs is high expression of c-kit and Sca-1, low expression of Thy-1, and an absence (or very low expression) of mature lineage markers, including CD3, CD4, and CD8 for T lymphocytes, B220 for B lymphocytes, Mac-1 for macrophages, and Gr-1 for granulocytes [3-5]. This sorting procedure has been verified by many laboratories and has been used to sort HSCs to relative homogeneity [6]. However, it is still unclear whether the use of antibody-mediated cell sorting has any activating effect on HSCs, since, for example, in lymphocytes, antibody-mediated cross-linking of some cell-surface proteins results in cell proliferation and/or differentiation.

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Sca-1, a glycoprotein-I-linked cell-surface glycoprotein, was one of the first epitopes used for antibody-mediated enrichment of murine HSCs. *Spangrude et al*. [3] used the Sca-1-specific monoclonal E13-161.7 antibody [7] for sorting HSCs from adult murine bone marrow to yield about a 100-fold enrichment of these cells [3, 4]. Since then, others have used Sca-1 as a marker for enrichment of embryonic day 14 (E14) fetal liver and E11 yolk sac HSCs [8]. While the Sca-1 antibody is useful for enrichment of viable HSCs, immunostaining of tissue sections, particularly from embryos, has been difficult. Hence, to more specifically localize Sca-1$^+$ cells in embryonic tissues and adult bone marrow, a transgenic approach with the gene encoding Sca-1 has been taken.

The Sca-1 epitope is encoded by the strain-specific *Ly-6E/A* allelic gene [9]. Examination of the specific expression pattern of *Ly-6E/A* has been difficult [9-11], since the *Ly-6* gene family consists of at least 18 highly homologous cross-hybridizing genes [12, 13]. However, it was found that the Ly-6A and Ly-6E proteins differ in their expression pattern on marrow-repopulating cells, with *Ly-6A* strains of mice expressing Sca-1 on 99% and *Ly-6E* strains expressing Sca-1 on 25% of such cells [11]. Nonetheless, the Sca-1 marker has proven useful in identifying HSCs, and the small size of the *Ly-6A/E* locus and flanking transcriptional regulatory elements make it useful in directing marker gene expression in HSCs in transgenic mice.

The transcriptional regulatory elements and 5′ and 3′ DNaseI hypersensitivity patterns of *Ly-6E* and *Ly-6A* have been examined and appear to be similar [14-17]. Deletional studies show that the 3′ flanking regions contain two hypersensitive sites, which are most likely responsible for high-level, γ-interferon-induced expression in vitro [18, 19]. This distal flanking element is also responsible for high-level, tissue-specific expression of a *lacZ* marker gene in transgenic mice [20, 21]. HSCs can be enriched from adult bone marrow based on *lacZ* transgene expression [20, 21]. However, not all HSCs are located within the lacZ-expressing population. Whether this is a result of the *lacZ* marker gene, the *Ly-6A/E* expression cassette, or the fluorescein di-β-D-galactopyranoside (FDG) substrate staining method [22] is uncertain.

Thus, to further examine, identify, and localize HSCs in the adult mouse, we created transgenic mice with a green fluorescent protein (*GFP*) gene within the context of the *Ly-6A* expression cassette. We report here that all bone marrow HSCs were positive for expression of GFP from the *Ly-6A* transgene. *Ly-6A GFP* was also expressed in other Sca-1$^+$ hematopoietic cells, and histological analyses revealed an interesting pattern of expression of GFP within the thymus, bone marrow, and spleen cells, suggestive of hematopoietic and endothelial cell expression. Thus, the *Ly-6A GFP* transgene marker facilitated the enrichment of functional adult repopulating HSCs and their identification in situ.

## Materials and Methods

### Transgenic Mice

The 14-kb Ly-6A cassette (pLy-6A14) was constructed as described previously [18, 21]. The enhanced GFP (EGFP) gene AgeI/AflII fragment containing simian virus 40 polyA in pEGFP-N1 (Clontech; Alphen aan den Rijn, The Netherlands; http://www.clontech.com/index.shtml) was modified, converting 3′ and 5′ AgeI/AflII sites to Nar1 sites using oligonucleotide adaptors. The 970-bp EGFP Nar1 fragment was cloned into pLy-6A14 to generate pLAG.

Fertilized (C57BL/10 × CBA)$F_1$ oocytes were microinjected with a 15-kb BamH1 fragment containing the *Ly-6A GFP* gene from pLAG (Fig. 1A). This fragment was gel purified for removal of all vector sequences. Positive founder animals were bred with (C57BL/10 × CBA)$F_1$ mice, and lines were maintained as heterozygotes. Polymerase chain reaction (PCR) analysis of tail DNA was used to identify founder transgenic mice. Animals were housed according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals.

### DNA and RNA Analysis

Genomic DNA (5-10 μg) for Southern blot analysis [20] was digested with BamH1 and electrophoresed through 1% agarose/Tris, acetate, EDTA gels prior to transfer to Hybond-N membranes. Transgene copy number controls were generated by addition of appropriate amounts of pLAG to nontransgenic genomic DNA. Filters were probed with *GFP* and *Thy-1* gene fragments. Normalization for DNA content of each lane was performed after phosphorimaging analysis of *Thy-1* signal. Copy number was determined subsequently by comparing the *GFP* signal obtained from the transgenic mice with that of the plasmid controls on the linear portion of the standard curve.

Total cellular RNA for Northern blot analysis was prepared using the lithium chloride/urea method, and 5-15 μg was fractionated on 1% agarose/formaldehyde gels [23] prior to transfer to Hybond-N membranes. Filters were probed with *GFP* and *Ly-6E* cDNA fragments. Probes used for hybridization to Southern or Northern filters were labeled by a random oligonucleotide priming procedure, incorporating $^{32}$P ATP. The fragments used were as follows:

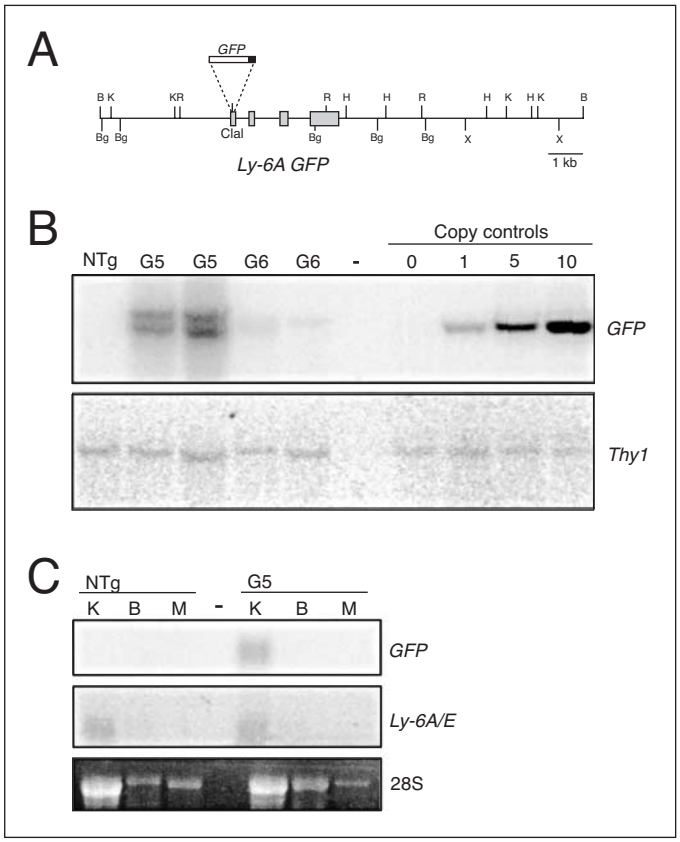


***Figure 1.*** **Ly-6A GFP** ***transgene construct and transgenic mouse characterization.*** *A) A schematic drawing of the 14-kb BamHI fragment of the* Ly-6A *gene and the insertion of the* EGFP *gene into the Cla1 cloning site. Restriction sites are indicated. B = BamH1, BG = BglII, K = Kpn1, R = EcoR1, H = HindIII, and X = Xba1. B) Southern blot analysis of DNA from two* Ly-6A GFP *transgenic lines, G5 and G6, and a nontransgenic control (NTg). Copy controls are nontransgenic DNA with* Ly-6A GFP *plasmid DNA added at 1, 5, and 10 copy equivalents. After hybridization with* GFP *and* Thy-1 *gene probes, the intensity of signal was determined by phosphorimaging. C) Northern blot analysis of total RNA from kidney (K), bone marrow (B), and muscle (M) of an adult G5 transgenic and a nontransgenic (NTg) littermate. Hybridization was performed with a* GFP *gene and* Ly-6E *cDNA probes, and the 28S RNA signal was used for RNA quantitation.*

(970 bp PCR EGFP products from pLAG); 1.2 kb Xba1-Nru1 *Thy-1* gene fragment from pD7 [24]; 761 bp EcoR1 *Ly-6E* cDNA fragment from pLy6.1-2R [13]. After hybridization, filters were washed to a stringency of 0.2 × standard saline citrate/0.1% SDS and exposed to a phosphorimager screen for quantitation using Imagequant software.

Genomic DNA (200 ng) from the peripheral blood of transplant recipients was analyzed by PCR using oligonucleotide primers for GAPDH-specific sequences, (GAPDH1) 5′CTTCACCACCATGGAGAAGG 3′ and (GAPDH2) 5′CC ACCCTGTTGCTGTAGCC 3′, and for *GFP*-specific sequences, (Ly6GFP) 5′GACAGAACTTGCCACTGTGC 3′ and (GFP) 5′AAGAAGATGGTGCGCTCCTG 3′.

DNA was subjected to an initial 5 minute denaturation at 94°C followed by 30 cycles of denaturation (1 minute at 94°C), annealing (2 minutes at 55°C), and elongation (2 minutes at 72°C). Serial dilutions of blood DNA from a transgenic animal were used as a control to evaluate the levels of donor cell reconstitution in transplanted mice.

### Analysis of Long-Term Multilineage Repopulating Activity

Bone marrow cells were isolated from the femurs and tibias of adult transgenic mice and sorted for GFP expression on a FACSVantage (Becton Dickinson; San Jose, CA; http://www.bd.com), and sorted adult bone marrow cells (in limiting dilution) were assayed for the presence of HSCs by intravenous transfer into irradiated adult recipients, as described [25, 26]. Briefly, (C57BL/10 × CBA)$F_1$ male recipients were exposed to a split dose of 900 rad of gamma-irradiation from a $^{137}$Cs source. Recipient mice were bled at 1 and >4 months after transfer and analyzed for percentage donor contribution by donor marker-specific PCR on DNA isolated from peripheral blood [20, 25, 26]. Reconstitution was evaluated by ethidium bromide staining of agarose gels, and in some cases, by Southern blot hybridization, as described previously [25-27]. To test for long-term multilineage hematopoietic repopulation, genomic DNA was isolated from peripheral blood, thymus, lymph node, sorted splenic B and T cells, and bone marrow myeloid and lymphoid cells. Percentage donor-cell contribution was analyzed by PCR, and in some cases, by Southern blot hybridization and phosporimaging.

### Fluorescent Antibody Surface Staining and Flow Cytometry

All antibodies used in flow cytometric sorting and analysis were obtained from PharMingen (Alphen aan den Rijn, The Netherlands; http://www.bdbiosciences.com/pharmingen). The monoclonal antibodies used were directly conjugated with either phycoerythrin (PE) or biotin and included: PE-anti-c-kit, PE-anti-Sca-1, PE-anti-CD4, PE-anti-CD8, PE-anti-B220, PE-anti-Mac, and biotinylated CD31 and CD34. Single-cell suspensions were prepared as described [21]. After incubation with specific antibodies for 30 minutes on ice, cells were washed twice and incubated with PE-conjugated streptavidin (Caltag Laboratories; Burlingame, CA; http://www.caltag.com) when required. Labeled cells were finally washed twice and filtered through a nylon mesh screen prior to sorting. One $\mu$g/ml Hoechst 33258 or 2 $\mu$g/ml 7 amino-actinomycin D were added to identify dead cells. To determine the background levels, cells were stained with fluorochrome-conjugated immunoglobulin isotype controls from Pharmingen. During the entire staining procedure, phosphate-buffered saline (PBS) containing 10% fetal calf serum and penicillin/

streptomycin was used. Cells were sorted using a FACSVantage SE (Becton-Dickinson); the purity of the sorted cells ranged from 89%-98%. Analyses were performed on a FACScan.

### Cryosectioning and Histology

Tissues (kidney, bone marrow, spleen, lymph nodes, thymus, and brain) from adult *Ly-6A GFP* mice were isolated and fixed for 1 hour at room temperature in 2% paraformaldehyde/PBS. Tissues were equilibrated in 20% sucrose/PBS overnight at 4°C, quick frozen in tissue tek, and stored in liquid nitrogen until cryosectioning. 10-$\mu$m thick sections were placed on glass slides, air-dried and mounted with vectashield (Vector Laboratories; Burlingame, CA; http://www.vectorlabs.com).

## Results

### Generation of *Ly-6A GFP* Transgenic Mice

To provide a means by which HSCs could be enriched without the use of antibodies or FDG (β-galactosidase substrate) and to facilitate in vivo HSC localization, we generated transgenic mice expressing the *GFP* reporter gene under the control of the *Ly-6A* gene regulatory sequences. Since our previous results with *Ly-6A* transgenic embryos showed incomplete expression of the inserted *lacZ* marker in bone marrow HSCs [20, 21], we sought to alleviate a possible experimentation problem (due to cell permeabilization necessary for FDG substrate entry into these cells) by using the *GFP* reporter gene. The enhanced *GFP* gene was inserted into the Cla1 cloning site of the first untranslated exon of *Ly-6A* (Fig. 1A) and was flanked by 4 kb of upstream and 10 kb of downstream genomic sequences containing regulatory elements necessary for high-level specific expression in adult bone marrow HSCs. Transgenic mice were generated, and mice from line G5, containing eight copies of transgene (hemizygous), were used in all the studies described here (Fig. 1B). As expected from other published studies [20, 21], Northern blot analysis of adult tissues revealed high-level transgene expression in the kidney (Fig. 1C). Low-level transgene expression was found in the bone marrow, and limited or no expression was found in muscle tissue. Thus, the *Ly-6A GFP* transgene produced a restricted expression pattern similar to that found previously in *Ly-6A lacZ* and *Ly-6E lacZ* transgenic mice [20, 21].

### *Ly-6A GFP* Expression in Tissues and Subsets of Hematopoietic Cells of Adult Transgenic Mice

Previously, it was shown, by immunostaining of sectioned adult hematopoietic tissues, that Sca-1 is expressed on some cells of the thymus, spleen, and lymph nodes [28]. Sca-1 is also expressed in the brain vasculature and the cortical tubules of the kidney [9, 29]. Hence, histologic sections from the hematopoietic tissues of *Ly-6A GFP* adult mice were examined for transgene expression under a fluorescence microscope. As shown in Figure 2, GFP signal was detected in some of the cells of the kidney (D), bone marrow (E), spleen (F), lymph nodes (G), thymus (H), and brain (I). Within the kidney,

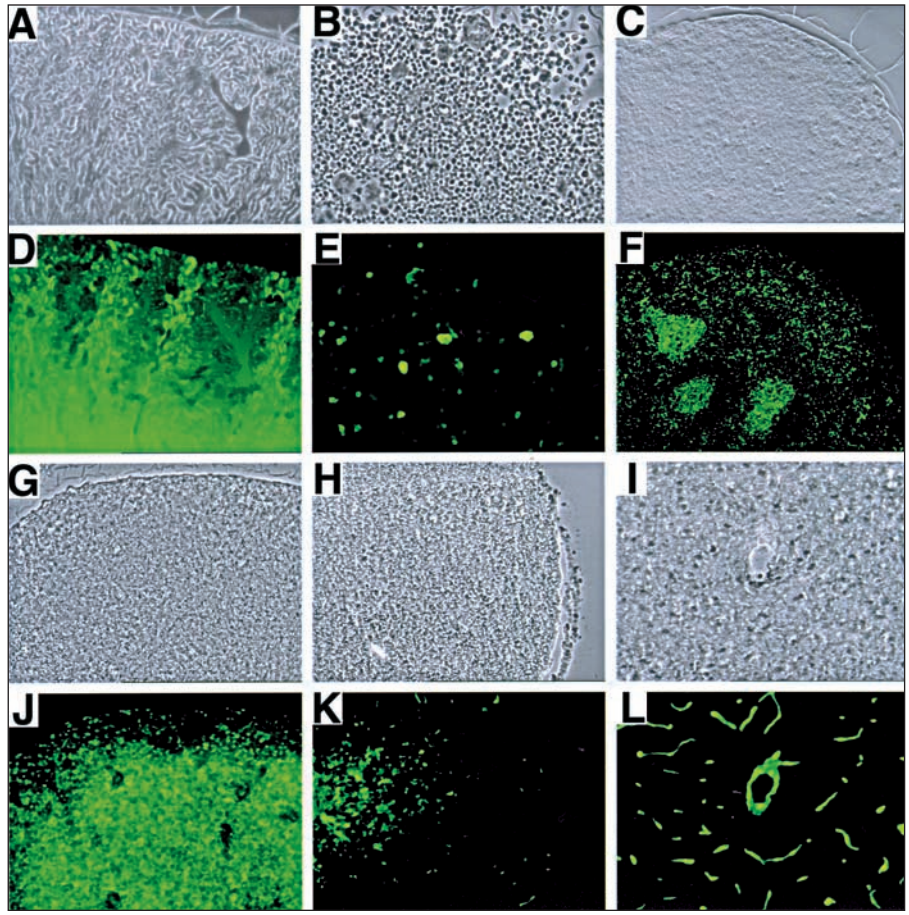


***Figure 2. Histologic sectioning and microscopy of* Ly-6A GFP *hematopoietic tissues.*** *Brightfield and fluorescent images, respectively, of kidney (A and D), bone marrow (B and E), spleen (C and F), lymph node (G and J), thymus (H and K), and brain (I and L). Tissues were cryosectioned at a thickness of 10 microns. Images B, E, H, K, I, and L were taken at 10× magnification. All other images were taken at 4× magnification.*

the cortical tubules were highly positive. The bone marrow showed highly GFP$^+$ endothelial cells in the vasculature and other scattered positive cells (presumably, HSCs, some progenitors, and stromal cells). In the peripheral hematopoietic tissues, the GFP expression pattern appeared to be strongest in the lymphoid areas of the spleen, in the lymph nodes, and in the medulla of the thymus (with a few subcapsular thymus cells also GFP$^+$). Finally, a vascular endothelial pattern of GFP expression was found in the brain. Thus, the general expression pattern of the *Ly-6A GFP* transgene was identical to the published Sca-1 immunostaining patterns [9, 28, 29].

To determine the percentage of GFP$^+$ cells in each of the tissues, flow cytometric analysis was performed. All hematopoietic tissues tested, i.e., bone marrow, thymus, lymph node, and spleen, contained GFP$^+$ cells (Table 1). The lymph node contained the highest percentage (80%) of cells expressing the GFP marker, while the bone marrow and thymus contained less than 20% of GFP$^+$ cells. Sca-1 antibody staining of cells from each of the hematopoietic tissues showed an overlap in the expression of GFP with the endogenous Sca-1 protein. Approximately 50% or more of Sca-1$^+$ cells in bone marrow, thymus, spleen, and lymph node were also GFP$^+$ (data not shown). In the lymph node, thymus, and spleen, 40% or greater of GFP$^+$ cells were Sca-1$^+$. However, in the bone marrow, a much lower percentage of GFP$^+$ cells were Sca-1$^+$, suggesting that GFP was expressed more highly or was more readily detectable than the Sca-1 antigen in the progenitors and stem cells of this tissue.

To determine the distribution of the GFP phenotype within hematopoietic lineages, antibodies directed against progenitor/stem cell markers and mature lineage markers were used in flow cytometric analyses (Table 1). In the bone marrow, cells of all mature lineages, T cell (CD4 and CD8), B cell (B220), and myeloid (Mac-1), and also progenitor/stem cells (c-kit, CD34, CD31) were GFP$^+$. Monocytes/granulocytes and their precursors (Mac-1$^+$) represented the largest percentage (72%) of bone marrow cells expressing GFP. In the thymus and lymph nodes, high percentages of T lymphoid cells, most notably CD4 cells, were found to be GFP$^+$. Cells of the myeloid lineage and the B lymphoid lineage in the spleen were also GFP$^+$. Thus, the *Ly-6A GFP* transgene was expressed to some degree in all hematopoietic lineages but appeared to be predominantly expressed in mature T cells in the lymphoid organs and in myeloid and immature hematopoietic progenitor/stem cells in the bone marrow.

### *Ly-6A GFP* Expression Marks All Functional Bone Marrow HSCs

To determine whether the *Ly-6A GFP* transgene was expressed in functional adult repopulating HSCs, we performed transplantation experiments with flow cytometric-sorted cells from adult bone marrow. A representative fluorescence profile of bone marrow cells sorted on the basis of endogenous Sca-1 and GFP expression is shown in Figure 3A. In this profile, at least five populations of cells are apparent; most predominant are the Sca-1$^-$GFP$^-$, Sca-1$^-$GFP$^{lo}$, and Sca-1$^-$GFP$^{hi}$, and less frequent are Sca-1$^+$GFP$^{lo+neg}$ and Sca-1$^+$GFP$^{hi}$. These five populations were sorted and injected into irradiated adult recipients at limiting dilution to test for long-term, high-level, multilineage repopulation. At greater than 4 months posttransplantation, peripheral blood DNA was tested for the presence of the *Ly-6A GFP* transgene marker. Only recipients showing greater than 10% donor cell repopulation using a semiquantitative PCR method for the *GFP* transgene were considered positive for HSC repopulation. The results of three independent sorting and transplantation experiments show that all HSC activity was found within the GFP$^{hi}$ fractions (Fig. 3B). These animals were high-level, multilineage engrafted as determined by donor marker PCR analysis of DNA from hematopoietic tissues and sorted populations of B, T, and myeloid cells (Fig. 3D). No HSC activity was found in the GFP$^{lo}$ or GFP$^-$ fractions. Although one animal receiving $5 \times 10^5$ Sca-1$^-$GFP$^-$ cells was positive, this recipient was only 10% engrafted by the donor cells, suggesting no enrichment for HSCs compared with the whole bone marrow control. As expected, most of the HSC activity was highly enriched in the Sca-1$^+$ fraction of the GFP$^{hi}$ cells. As few as 1,000 Sca-1$^+$ GFP$^{hi}$ cells resulted in reproducibly high-level multilineage repopulation (Fig. 3B). Limited HSC activity was also found in the Sca-1$^-$ GFP$^{hi}$ fraction, but only when $10^5$ cells were transplanted (Fig. 3C). These results demonstrate that GFP expression has no adverse effects on adult bone marrow HSCs and can be used to enrich for these cells by approximately 100-fold. Finally, these results, in combination with the expression

**Table 1.** Hematopoietic lineage marker expression on GFP$^+$ cells within adult tissues

| | Bone marrow | Thymus | Lymph node | Spleen |
|---|---|---|---|---|
| % GFP$^+$ | 15.4 | 10.8 | 80.7 | 26.3 |
| Marker | % marker$^+$ cells within GFP$^+$ population | | | |
| Sca-1 | 13.3 | 40.6 | 65.0 | 69.4 |
| c-kit | 9.7 | nd | nd | nd |
| CD34 | 8.5 | nd | nd | nd |
| CD31 | 35.0 | nd | nd | nd |
| CD4 | 8.9 | 83.1 | 62.1 | 41.8 |
| CD8 | 3.0 | 40.9 | 43.7 | 25.4 |
| B220 | 16.2 | nd | 22.8 | 48.5 |
| Mac-1 | 72.0 | nd | nd | 16.6 |

Abbreviation: nd = not done.

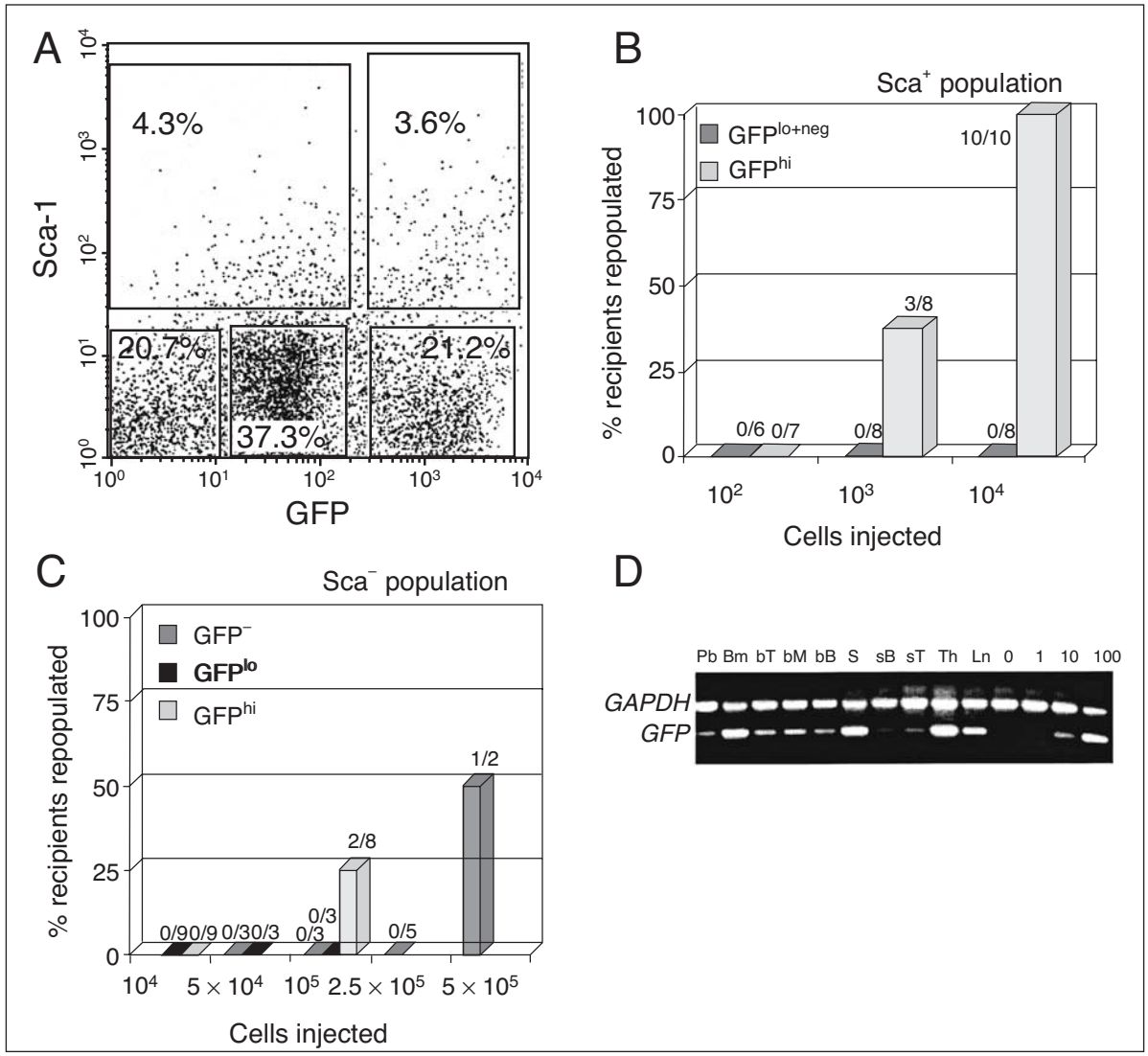


***Figure 3. A) Fluorescence profiles and gates used for sorting GFP⁺ and Sca-1 antibody stained* Ly-6A GFP *transgenic adult bone marrow are shown.*** *The percentages of bone marrow cells in each sorted population are indicated. B) Transplantation data from limiting dilution transplantation of Sca-1⁺ cells within the GFP$^{low+neg}$ and GFP$^{high}$ gates. The X axis shows the number of cells injected per recipient. The bars represent the percentage of recipients found positive for donor repopulation at greater than 4 months posttransplantation. The number of recipients repopulated/total number of recipients is indicated for each bar. C) Transplantation data from limiting dilution transplantation of Sca-1⁻ cells within the GFP⁻, GFP$^{low}$, and GFP$^{high}$ gates. The axis and bar indications are as described for (B). Note the higher cell numbers transplanted. D) Hematopoietic multilineage analysis of a recipient repopulated with Sca-1⁺GFP$^{high}$ cells. At greater than 4 months posttransplantation, DNA was isolated from peripheral blood (Pb), bone marrow (Bm), bone marrow T cells (bT), bone marrow myeloid cells (bM), bone marrow B cells (bB), spleen (S), spleen B cells (sB), spleen T cells (sT), thymus (Th), and lymph nodes (Ln). PCR analysis was performed using oligonucleotide primers for* GFP *(bottom band) and* GAPDH *(top band). 0, 1, 10, and 100 were the controls indicating percentage donor cell engraftment. Transplantation results were obtained from three independent experiments.*

observed in tissue sections, suggest that the GFP marker is more highly expressed by HSCs than is Sca-1.

## Discussion

We have shown here that the *Ly-6A GFP* transgene faithfully marks all HSCs in adult bone marrow. This is in contrast to previous transgenic mice made with the *Ly-6A* and *Ly-6E* expression cassettes directing the transcription of the *lacZ* marker gene [20, 21]. In those mice, some HSCs did not express *lacZ*. It is possible that those transgenic HSCs were inefficiently labeled by FDG. Not all HSCs may be sufficiently permeabilized so as to allow entry of

FDG and, thus, appeared to be negative for *lacZ* expression. In addition, too much permeability may allow leakage of the substrate and again result in no or low FDG signal. Alternatively, it may be possible that expression of the bacterial *lacZ* is regulated in transgenic mice (for purposes such as viability) so that only low-level expression is allowed in HSCs. Despite the incomplete isolation of bone marrow HSCs from *Ly-6E lacZ* and *Ly-6A lacZ* transgenic mice, the *GFP* marker in the *Ly-6A* cassette was optimally expressed in all bone marrow HSCs and allowed the enrichment of all HSCs. Thus, the *Ly-6A* cassette appears to contain all the necessary transgene regulatory elements for HSC-specific expression. And when used together in a transgene context, the *Ly-6A* cassette and the *GFP* marker are highly advantageous in maintaining a viable, unmanipulated pool of HSCs in the absence of enzyme substrate addition or antibody staining.

Is Sca-1 surface glycoprotein limiting on the surface of some HSCs and hematopoietic cells? The expression profile of *Ly-6A GFP* in adult tissues and hematopoietic cells is reminiscent of the Sca-1 profile [9, 10, 28, 29], particularly in mature hematopoietic cells of T lymphoid, B lymphoid, and myeloid lineages. However, our fluorescence-activated cell sorting profiles suggest that not all $GFP^+$ cells were Sca-$1^+$. Indeed, the fluorescence signal produced by GFP was more intense. This is most likely due to the fact that, within the G5 transgenic line of mice, there are eight copies of the transgene, compared with the normal diploid copy number of the endogenous *Ly-6A/E* gene encoding Sca-1. Furthermore, unlike the cell-surface localization of Sca-1, which requires extra processing steps, such as transport through the plasma membrane, glycosylation, and GPI linkage, GFP is an easily expressed cytoplasmic protein. Thus, GFP may be a better marker than Sca-1 in low-expressing cells of the various hematopoietic tissues, particularly HSCs.

Although we have not directly compared limiting dilution transplantation of Sca-1-sorted HSCs with GFP-sorted HSCs, the general enrichment gained by *Ly-6A GFP* sorting is about 100-fold. This is similar to the Sca-1 enrichment levels established in the published literature [3, 10]. While differences do not appear to exist on the level of bone marrow HSC enrichment, we are examining whether differences exist between antibody-mediated sorting and GFP sorting in the intraembryonic tissue-generating HSCs at the earliest stages of mouse development. This earliest site, the aorta-gonads-mesonephros (AGM) region, generates the first adult repopulating HSCs at E10.5 [27]. Interestingly, in related studies, we reproducibly isolated only 50% of HSCs from the AGM region using the Sca-1 antibody, but could isolate 100% of AGM HSCs using *Ly-6A GFP* marker expression [30]. This supports the notion that the *Ly-6A GFP* marker is more efficiently expressed and identifies the Sca-$1^{low}$-expressing population of HSCs as they are being generated within the embryo. This may be particularly useful in future studies of precursor-progeny relationships and possible lineage relationships of HSCs with endothelial cells.

Further to this point, when we used a *lacZ* marker gene in the *Ly-6A* cassette, we found high-level β-galactosidase expression in the mesonephros of the E11 AGM but no expression in the dorsal aorta, the site where the first AGM HSCs are generated [21]. HSC sorting procedures based on the FDG substrate for β-galactosidase were unsuccessful. However, we have found GFP-expressing cells lining the walls of the dorsal aorta in *Ly-6A GFP* embryos, and these cells contained all the adult repopulating HSC activity [30]. While both *lacZ* and *GFP* marker gene expressions were found in the mesonephros, only the *GFP* reporter was optimally expressed in AGM HSCs. Thus, *GFP* expression from the *Ly-6A* cassette in the G5 line of transgenic mice serves as an excellent tool for HSC enrichment and for the localization of the HSCs in the adult as well as the embryo. Further transgenic studies will focus on the deletion mapping of HSC-specific transcriptional regulatory elements so as to express exogenous genes only in HSCs. Combined with inducible expression, the *Ly-6A* transgenesis will allow for a better understanding of HSC regulation and migration during each precise stage of development and adult hematopoietic maintenance.

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