Akt is an important regulator of cell survival, growth, and glucose metabolism in many cell types, but the role of this signaling molecule in hematopoietic stem cells is poorly defined. Side population (SP) cells are enriched for hematopoietic stem cell activity and are defined by their ability to efficiently efflux Hoechst 33342. Bone marrow from Akt1-null mice exhibited a reduced SP fraction. However, bone marrow cellularity, growth factor-responsive progenitor cultures, and engraftable stem cells were normal in these mice. Treatment of bone marrow with LY294002, an inhibitor of the Akt effector protein phosphatidylinositol 3-kinase, led to a reversible loss of the SP fraction. Bcrp1, which encodes the Hoechst dye transporter, was translocated from the membrane to the intracellular compartment under conditions that promote the SP-depleted state. Lentivirus-mediated overexpression of Akt1 in bone marrow markedly increased the SP fraction, whereas there was no effect on bone marrow from *Bcrp1*−/− mice. These data suggest that Akt signaling modulates the SP cell phenotype by regulating the expression of Bcrp1.

The serine/threonine kinase Akt, also known as protein kinase B (PKB),1 functions downstream from phosphatidylinositol 3-kinase (PI3K) as an important mediator of insulin-like growth factor and insulin action, and it has been implicated in the control of cellular growth, survival, and metabolism (1, 2). In *Caenorhabditis elegans* and *Drosophila melanogaster*, modulation of PI3K/Akt signaling alters development, growth, and aging (3–5). Mammals express three isoforms of this kinase, Akt1/PKBα (6, 7), Akt2/PKBβ (8, 9), and Akt3/PKBγ (10, 11). Although these isoforms are encoded by separate genes, they share a high degree of sequence homology. Akt1 is the most ubiquitously expressed family member, whereas Akt2 is predominantly expressed in insulin-responsive tissues (12–14). Although initial analyses of the three gene products reveal similar biochemical characteristics (15), disruption of the individual genes produce different phenotypes. Akt1-null mice are viable but runted when compared with wild-type littermates (16, 17). In addition, Akt1-null mice have a greater sensitivity to apoptosis induced by genotoxic stress and other agents (17). In contrast, Akt2-null mice display a diabetic phenotype but they grow normally (18).

PI3K/Akt signaling is reported to regulate the differentiation of a variety of cell types including hematopoietic cells (19–23). Recently, it was shown that hydroxymethylglutaryl-CoA reductase inhibitors can function as pharmacological activators of Akt and mobilize bone marrow-derived endothelial progenitor cells through stimulation of the Akt signaling pathway (24–26). Furthermore, thymocytes from Akt1-null mice are reported to be more susceptible to spontaneous and stress-induced apoptosis (17). However, the properties of hematopoietic stem cells (HSC) have never been studied in mouse models that are deficient for Akt signaling.

Side population (SP) cells represent a rare fraction of bone marrow that is detected by dual-wavelength flow cytometry on the basis of their ability to efflux fluorescent Hoechst 33342 dye (27). These cells are of interest because they are highly enriched for HSC activity. SP cells have been identified in hematopoietic compartments of mice, humans, monkeys, and swine (28–30) and in non-hematopoietic tissues including skeletal muscle, brain, and lung (31–36). Hoechst dye exclusion is also a property displayed by embryonic stem cells (37). Stem cells express at least two different ATP-binding cassette (ABC) transporters that can mediate dye efflux. One is a breast cancer-resistant protein 1 (*Bcrp1*), also called ABCG2, and the other is a multidrug-resistant protein 1 (*Mdr1*), which is also known as P-glycoprotein (38–41). Recently, targeted gene ablation studies in mice have revealed that the hematopoietic SP cell phenotype is determined by *Bcrp1* but not by *Mdr1* (28, 42). *Bcrp1* expression is sharply down-regulated upon hematopoietic cell maturation, and it is not detected in mature blood cells (43). *Bcrp1*-null mice exhibit normal steady-state hematopoiesis and HSC number. These data indicate that Bcrp1 is a marker of HSC, but it is not necessary for stem cell self-renewal or differentiation.

To elucidate the intracellular signaling mechanisms that control stem cell biology, we analyzed the bone marrow SP cells...
Bcrp1 Expression in SP Cells

EXPERIMENTAL PROCEDURES

Mice—Akt1-deficient mice on a C57BL/6J background were kindly provided by Dr. Morris J. Birnbaum (University of Pennsylvania). Akt2-deficient mice on a C57BL/6J background were kindly provided by Dr. Joseph Testa (Fox Chase Cancer Center). Bcrp1-null mice were on a mixed background (129/Ola and C57BL/6J) were kindly provided by Dr. Brian Sorrentino (St. Jude Children's Research Hospital). C57BL/6J mice and FVB mice were purchased from Charles River Laboratories (Wilmington, MA), and B6.SJL mice were from The Jackson Laboratories (Bar Harbor, ME). All mouse experiments were approved by the Institutional Animal Care and Use Committee of Boston University.

Hoechst and Antibody Staining for Flow Cytometry Analysis—Murine SP cell analysis was performed as described previously (30). Typically, bone marrow cells were isolated from six crushed bones (tibias, femurs, and iliacs) and stained with 5 μg/ml Hoechst 33342 (Sigma) in 2 ml of Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum and 10 mM HEPES at 37 °C for 90 min. After the incubation, cells were kept on ice immediately and washed twice with Hank’s balanced salt solution containing 2% fetal bovine serum and 10 mM HEPES. After these steps, cells were resuspended with Hank’s balanced salt solution medium and analyzed in a triple laser instrument (MoFlo®, DakoCytomation Inc., Fort Collins, CO) following propidium iodide staining (2 μg/ml) for excluding dead cells. SP gates were defined using non-treated wild-type bone marrow cells stained with 5 μg/ml Hoechst in each analysis. For analyzing the effect of inhibitors, LY294002 (10 μM) and PD98059 (10 μM) (Calbiochem), and verapamil (150 μM) (Sigma) were added to total bone marrow cells before 30 min of Hoechst staining. For KSL-antibody staining, bone marrow cells were incubated with phycoerythrin-conjugated Scf antibody (E13–161.7), fluorocyanin isothiocyanate (FITC)-conjugated c-Kit antibody (2B8) (BD Biosciences), and biotinylated Mouse Lineage Mixture antibodies consisting of CDS, CD11b, CD45R, Gr1, 7–4, and TER119 (2 μl each) (StemCell Technologies Inc., Vancouver, British Columbia, Canada). After washing twice, cells were resuspended into 100 μl of Hank’s balanced salt solution and incubated with streptavidin-RPE670-conjugated antibody (Invitrogen) for 30 min on ice. Samples were analyzed in a FACScan flow cytometer (BD Biosciences) using Cellquest software. For CD34-antibody staining, FITC-conjugated CD34 antibody (Caltag, Burlingame, CA) was used. Isotype control antibodies were also used for establishing positive-cell gating in KSL and CD34 staining.

Progenitor Colony Formation Assays—Fresh Akt1-deficient and wild-type whole marrow cells were assayed for in vitro colony-forming unit culture (CFU-c) and high proliferative potential colony-forming cell (HPP-CFC) assay using a double layer soft agar nutrient medium technique in 35-mm plastic culture dishes (Falcon) (44–48). The bottom layer consisted of the following: 1.0-ml underlay of 0.5% agar (Difco) technique in 35-mm plastic culture dishes (Falcon) (44–48). The bottom (HPP-CFC) assay using a double layer soft agar nutrient medium unit culture (CFU-c) and high proliferative potential colony-forming cell using non-treated wild-type bone marrow cells stained with 5 μg/ml Hoechst 33342 (Sigma) in 2 ml of Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum and 10 mM HEPES at 37 °C for 90 min. After the incubation, cells were kept on ice immediately and washed twice with Hank’s balanced salt solution containing 2% fetal bovine serum and 10 mM HEPES. After these steps, cells were resuspended with Hank’s balanced salt solution medium and analyzed in a triple laser instrument (MoFlo®, DakoCytomation Inc., Fort Collins, CO) following propidium iodide staining (2 μg/ml) for excluding dead cells. SP gates were defined using non-treated wild-type bone marrow cells stained with 5 μg/ml Hoechst in each analysis. For analyzing the effect of inhibitors, LY294002 (10 μM) and PD98059 (10 μM) (Calbiochem), and verapamil (150 μM) (Sigma) were added to total bone marrow cells before 30 min of Hoechst staining. For KSL-antibody staining, bone marrow cells were incubated with phycoerythrin-conjugated Scf antibody (E13–161.7), fluorocyanin isothiocyanate (FITC)-conjugated c-Kit antibody (2B8) (BD Biosciences), and biotinylated Mouse Lineage Mixture antibodies consisting of CDS, CD11b, CD45R, Gr1, 7–4, and TER119 (2 μl each) (StemCell Technologies Inc., Vancouver, British Columbia, Canada). After washing twice, cells were resuspended into 100 μl of Hank’s balanced salt solution and incubated with streptavidin-RPE670-conjugated antibody (Invitrogen) for 30 min on ice. Samples were analyzed in a FACScan flow cytometer (BD Biosciences) using Cellquest software. For CD34-antibody staining, FITC-conjugated CD34 antibody (Caltag, Burlingame, CA) was used. Isotype control antibodies were also used for establishing positive-cell gating in KSL and CD34 staining.

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Competitive Bone Marrow Transplantation—B6.SJL congenic mouse expressing CD45.1 and Akt1-deficient mice expressing CD45.2 antigen were used. Fresh male B6.SJL marrow cells (2 × 106) (CD45.1) were injected with an equal number of fresh male Akt1-deficient or wild-type whole marrow cells (2 × 106) (CD45.2) into lethally irradiated B6.SJL mice. These mice were exposed to 9 Gy in a single dose given at a dose rate of 0.85 Gy/min 3 h prior to cell infusion. Mice were sacrificed 16 weeks after cell infusion. Engraftment efficiency was analyzed by flow cytometry. Marrow was flushed from femurs, iliacs, and tibiae and then incubated with biotin-conjugated mouse anti-mouse CD45.1 mAb (BD Biosciences) and FITC-conjugated mouse anti-mouse CD45.2 mAb (Pharmingen) for 30 min in the dark on ice. Secondary biotin labeling with streptavidin allophycocyanin (Molecular Probes, Eugene, OR) was then performed (26, 27). At least 2 × 106 cells were stained, and a minimum of 20,000 events were analyzed per sample. The samples were kept on ice and in the dark prior to sorting. Propidium iodide (Sigma) was used immediately preceding flow cytometry to gate out dead cells (37). Samples were analyzed within 2 h of completion of antibody staining. Donor chimerism was calculated as CD45.2/(CD45.1 + CD45.2).

For lineage analysis, bone marrow was labeled with rat anti-mouse Ly6G/Gr1 (1B7 hybridoma), CD45B-220 (RA3 hybridoma), CD3 (T cells) (Pharmingen), CD4 (T-helper cells, MHC-II) (Pharmingen), or CD8 (T-suppressor cells, MHC-I) (Pharmingen) mAbs. Donor specificity was detected using a mouse anti-mouse biotin-CD45.2 mAb (Pharmingen). A FITC-polyclonal goat anti-rat mAb (Southern Biotechnology Associates, Birmingham, AL) and streptavidin allophycocyanin (Molec-
Table I
Parameter comparisons between Akt1+/−/ (wild-type) and Akt1+/−/ mice
Bone marrow cells (BMC) from 6 bones were assessed for total number, average cell size, cell cycle, and content of CD34⁺/ and c-Kit⁺/Scal⁺/Lin⁻ cells. Two to five individual mice were analyzed for each parameter.

|                | Body weight | BMC (million) | Cell size (FACS mean) | Cell cycle (G2/M %) | CD34⁺/ cells (%) | c-Kit⁺/Scal⁺/Lin⁻ cells |
|----------------|-------------|---------------|-----------------------|--------------------|------------------|------------------------|
| Akt1+/−/       | 17.8 ± 0.8  | 124 ± 37      | 564.2                 | 71.7/10.0/15.2     | 10.8 ± 2.7       | 1.03 ± 0.45             |
| Akt1−/−/       | 16.4 ± 0.5* | 120 ± 32      | 564.1                 | 69.4/9.8/18.5      | 10.4 ± 6.1       | 1.04 ± 0.45             |

*p < 0.05.

Fig. 2. In vitro progenitor colony comparison of Akt1-deficient and wild-type mice. 20,000 cells were plated in soft agar with a mixture of 7, 3, or 1 growth factors. After 14 days incubation in 5% CO₂, HPP-CFC and CFU-c colonies were counted according to size and density (see “Experimental Procedures”). Total number of colonies represents the combined values for HPP-CFC and CFU-c. The mean ± S.E. is shown. There was no significant difference found between Akt1-deficient and wild-type mice as calculated by Wilcoxon’s rank sum comparison.

Fig. 3. Competitive engraftment assay of Akt1-deficient versus wild-type mice. a, 2 million male B6.SJL marrow cells (CD45.1) were injected with an equal number of fresh male Akt1-deficient or wild-type C57BL/6J whole marrow cells (CD45.2) into lethally irradiated B6.SJL mice (9 Gy). The mean ± S.E. are presented. Sixteen-week engraftment assay showed equivalent multilineage engraftment for CD3, B220, and GR-1. Scatter-gated lineage analysis showed equivalent multilineage engraftment for CD3, CD4, and CD8. The mean ± S.E. are presented.

37 °C with or without LY294002 (10 μM). After 90 min of incubation, cells were fixed by ethanol and methanol solution (1:1 mixed) and permeabilized by 0.1% saponin prior to incubation with rabbit polyclonal anti-Bcrp1 antibody (Jackson ImmunoResearch, West Grove, PA). Cells were counterstained with DAPI. Images were viewed at ×60 magnification, and data were acquired with high resolution digital color camera and analyzed with deconvolution imaging effects using automated image analysis software (OpenLab, Improvision Inc., Lexington, MA).

Statistical Analysis—Data are shown as means ± S.E. For competitive engraftment and in vitro colony-forming assays, the Wilcoxon
Bcrp1 Expression in SP Cells

RESULTS

SP Cell Fraction in Akt-Null Mice—Nine-week old Akt1−/− and age-matched wild-type mice were used to assess SP cells in bone marrow. As shown in Fig. 1, a and b, the SP cell fraction is significantly reduced in Akt1−/− mice relative to wild-type mice. The SP fraction in Akt1−/− mice ranged from 0.02–0.06% compared with 0.07–0.12% in wild-type C57BL/6J mice (n = 5 each). To reduce experiment-to-experiment variability, SP fractions in age-matched Akt1−/− and wild-type mice were assessed on the same day and expressed as a ratio with the wild-type SP fraction designated as 1.0. Combined analyses from multiple experiments revealed that the SP cell fraction in Akt1−/− mice was less than half that seen in wild-type animals (Fig. 1c). In contrast, an analysis of Akt2−/− mice relative to wild-type mice (n = 5 each) revealed no detectable difference between the two strains (Fig. 1d).

Bone Marrow Cultures and Engraftment—The body weight of Akt1−/− mice was ~8% smaller than that of wild-type mice (Table I), whereas Akt2−/− mice had normal body size compared with wild-type mice (data not shown). These data are consistent with the findings of others (16–18). Despite this modest reduction in body size, Akt1-deficient mice had the same total number of bone marrow cells, and these cells were similar in size and cell cycle characteristics when compared with bone marrow from wild-type mice. Furthermore, no differences were apparent in the frequencies of CD34+ or c-Kit+ Sca1+Lin− (KSL) cell fractions between wild-type and Akt1-null mice (Table I). Finally, Akt1-null mice did not have any detectable differences in peripheral blood leukocyte count or hematocrit (data not shown).

Progenitor cell populations in Akt1-deficient mice were assessed with an in vitro assay (44–48). In vitro analysis comparing Akt1−/− with wild-type mice was performed by plating 20,000 fresh marrow cells in HPP-CFC and CFU-c assays for 14 days using a double layer soft agar nutrient medium technique. There were little or no differences in colony formation between Akt1−/− and wild-type marrow in response to 3, 3, or 1 growth factor mixtures (Fig. 2).

To evaluate engraftable stem cells in Akt1−/− mice, an in vivo competitive assay was employed. This assay compared engraftable stem cells between B6.SJL mice (CD45.1) and Akt1−/− or wild-type mice (CD45.2) in lethally irradiated B6.SJL hosts (Fig. 3a). The results showed a slight advantage to the Akt1-deficient marrow compared with the wild-type controls (p = 0.041). Lineage analysis of the engrafted cells showed no significant differences (Fig. 3b and c).

Apoptosis in Bone Marrow and SP Cells—To test whether

Fig. 4. Apoptosis in bone marrow cells from wild-type and Akt1−/− mice. a, approximately 300,000 bone marrow cells from the indicated strains of mice were incubated with 5 µg of Hoechst 33342 per ml for 90 min. Propidium iodide (2 µg/ml) was added immediately prior to flow cytometry. The apoptotic, propidium iodide-positive fraction is indicated by the box in the density dot plot analyses. b, quantitation of apoptotic, propidium iodide-positive fraction with bone marrow cells from wild-type control (n = 10), Akt1−/− (n = 5), or Akt2−/− (n = 5) mice incubated with Hoechst 33342 for 90 min. Bone marrow cells from wild-type mice (n = 10) were also preincubated with LY294002 (10 µM) for 30 min prior to the addition of Hoechst 33342 for 90 min. c, Hoechst 33342 toxicity in KSL cells. KSL cells were isolated from wild-type or Akt1−/− mouse bone marrow and incubated with the indicated concentrations of Hoechst 33342. Propidium iodide was then added, and viability was assessed by microscopy (n = 3 per experimental condition). d and e, Hoechst 33342 toxicity in SP cells. SP cells were also preincubated with LY294002 (10 µM) for 30 min prior to the addition of Hoechst 33342 (10 µM) and LY294002 (10 µM) for 30 min prior to the addition of Hoechst 33342 for 90 min. Viability was assessed by the addition of propidium iodide (PI) followed by phase contrast (d) and fluorescent microscopy (e) to determine total and apoptotic cell numbers, respectively. f, quantitation of Hoechst 33342 toxicity in SP cells isolated from wild-type or Akt1−/− mice. Wild-type SP cells were also preincubated with LY294002 (10 µM) for 30 min prior to the addition of Hoechst 33342 for 90 min. Viability was assessed by the addition of propidium iodide (PI) followed by phase contrast (d) and fluorescent microscopy (e) to determine total and apoptotic cell numbers, respectively.
the diminished SP fraction in Akt1-null mice could be due to an increase in the frequency of apoptosis, bone marrow cells from wild-type and Akt1\(^{-/-}\) mice were assessed for their relative sensitivities to Hoechst 33342 toxicity. For these experiments, total bone marrow cells were exposed to 5 \(\mu\)g of Hoechst 33342 per ml for 90 min prior to the addition of propidium iodide and flow cytometry analysis (Fig. 4a). The fraction of apoptotic cells, assessed by their inability to exclude propidium iodide, was not different in the bone marrow from wild-type, Akt1\(^{-/-}\), or Akt2\(^{-/-}\) mice (Fig. 4b). Furthermore, brief incubation with the PI3K inhibitor LY294002 (10 \(\mu\)M) had no effect on the apoptotic cell fraction.

To examine Hoechst 33342 toxicity with a purified fraction of bone marrow, KSL cells were isolated from the bone marrow of wild-type and Akt1\(^{-/-}\) mice. A 16-h incubation with Hoechst 33342 led to a dose-dependent increase in apoptotic, propidium iodide-positive cells (Fig. 4c). Under the conditions of these assays, there was no detectable difference in toxicity between wild-type and Akt1\(^{-/-}\) KSL cells at any dose of Hoechst 33342 examined, nor was there a detectable difference in the frequency of viable wild-type and Akt1\(^{-/-}\) KSL cells in the absence of Hoechst treatment (Table I and data not shown).

To assess directly the effect of Hoechst 33342 on SP cell viability, SP cells were isolated from wild-type and Akt1\(^{-/-}\) mouse bone marrow by standard procedures and then incubated with 5 \(\mu\)g of Hoechst 33342 per ml. Following a 90-min...
incubation, propidium iodide was added to the cultures, and viability was determined by phase contrast and fluorescence microscopy (Fig. 4). The frequency of apoptotic, propidium iodide-positive, cells did not differ between the SP fractions isolated from wild-type or Akt1/H11002/H11002 mice. Furthermore, inclusion of LY294002 in the incubation mix with wild-type SP cells for 90 min did not lead to a detectable change in the frequency of apoptosis.

**Effects of LY294002 on SP and KSL Cell Fractions—** To assess further the role of PI3K signaling in SP cells, wild-type bone marrow cells were incubated with LY294002 for 30 min before Hoechst 33342 dye addition and flow cytometry. This brief treatment with LY294002 almost completely abrogated the SP cell population (Fig. 5). These results were confirmed with another strain of mice (Fig. 5b). The SP fraction could be recovered when LY294002-treated bone marrow cells were washed and treated with Hoechst dye in fresh media in the absence of the inhibitor. The effect of LY294002 was as effective as the calcium channel blocker verapamil at reducing the SP cell population (Fig. 5c). Treatment with the mitogen-activated protein kinase inhibitor PD98059 (10^{-6} M) had no effect on the SP cell fraction.

The effect of LY294002 on the KSL population was also examined (Fig. 6a). Total bone marrow cells were incubated with 10 μM LY294002 for 2 h prior to flow cytometry. This treatment had no detectable effect on the fraction of KSL cells in multiple experiments (Fig. 6b).

**Bcrp1 Expression in SP Cells—** Hoechst dye efflux by SP cells requires the action of the ABC half-transporter Bcrp1 (37, 40, 42). Therefore, SP cells were immunostained using rabbit polyclonal anti-mouse Bcrp1 antibody to assess directly the effect of LY294002 on transporter expression. Deconvoluted fluorescent microscopy revealed that Bcrp1 was predominantly expressed on the plasma membrane of non-treated cells (Fig. 7a). DAPI staining revealed a high nucleus to cytoplasm ratio that is typical of SP cells. Interestingly, treatment of these cells with LY294002 for 90 min led to the translocation of Bcrp1 from the plasma membrane to the cytoplasmic compartment (Fig. 7b). Total fluorescence intensity was similar in both groups, indicating that PI3K inhibition predominantly affected cellular localization rather than overall Bcrp1 protein level.

**Forced Expression of Akt Increases the SP Fraction—** An Akt1-lentivirus transduction system was developed for transfecting Akt genes into bone marrow cells (Fig. 8a). After bone marrow isolation, cells were transduced with lentivirus constructs expressing the myristoylated (constitutive active) Akt1 or β-galactosidase (LacZ) for 24 h prior to Hoechst staining. A relatively high transfection efficiency was reflected by anti-V5 tag FITC-conjugated antibody staining of bone marrow cells (Fig. 8b). As shown in Fig. 8c, enforced expression of Akt1 significantly increased the SP cell fraction compared with the lacZ transfection control. The bone marrow from Bcrp1^{-/-} mice displayed a marked reduction in the number of SP cells (Fig. 8d), consistent with data reported previously (42). Furthermore, enforced expression of Akt1 in Bcrp1^{-/-} bone marrow did not lead to an increase in the SP cell number, suggesting that Bcrp1 mediates the action of Akt1 signaling on Hoechst dye efflux.

**DISCUSSION**

In this study multiple lines of evidence are provided to suggest that the PI3K-Akt signaling axis is an important regulator of the bone marrow-derived SP cell phenotype. First, it was
shown that the SP fraction in bone marrow from Akt1-null mice was reduced compared with wild-type mice. Second, transduction of bone marrow with the constitutively active form of Akt1 led to an increase in the SP fraction. Third, treatment of bone marrow with the PI3K inhibitor LY294002 led to a rapid reduction in the observed SP fraction that was effective as verapamil, a calcium channel blocker that inhibits the efflux of the Hoechst dye. In contrast, the mitogen-activated protein kinase inhibitor PD98059 had no effect on the SP fraction. Moreover, LY294002 did not affect the bone marrow KSL fraction.

The SP stem cell phenotype is defined by the action of the ABC half-transporter Bcrp1 (42). The observation that transduction with constitutively active Akt increases the SP fraction in bone marrow from wild-type but not Bcrp1-null mice suggested that PI3K-Akt signaling modulates Bcrp1 expression. Deconvoluted fluorescent microscopy revealed Bcrp1 expression on the plasma membrane of isolated SP cells. Brief treatment with LY294002 led to Bcrp1 translocation to the cytoplasmic compartment of these cells. It is well known that PI3K-Akt signaling controls the cellular localization of a number of proteins including GLUT4, which is required for insulin-stimulated glucose uptake, by a process that involves the cycling of GLUT4 between the plasma membrane and specialized intracellular vesicles (50). It has also been shown that PI3K activity regulates Mdr1 and Mdr2 transporter activity in the canalicular membrane of liver (51). Therefore, it is reasonable to speculate that the PI3K-Akt-mediated regulation of the SP fraction observed here is mediated, at least in part, through a regulation of Bcrp1 localization. In this regard, it has been shown recently that lung SP cells express Bcrp1 on their cell surface, whereas smooth muscle cells express Bcrp1 intracellularly and are incapable of Hoechst 33342 efflux (34).

PI3K-Akt signaling is an important regulator of cellular survival, and a number of cell types in Akt1-deficient mice display an increase in spontaneous and stress-induced apoptosis (17). However, it is unlikely that the differences in SP cell fraction observed under conditions of reduced PI3K-Akt signaling are due to the differential effects of Hoechst dye on SP cell viability. Here it was shown that SP, bulk bone marrow, or KSL cells from wild-type and Akt1-deficient mice do not differ in their sensitivity to Hoechst dye-induced toxicity. Furthermore, brief incubation with LY294002 did not promote toxicity in SP or bulk bone marrow cell fractions. However, our data do not rule out the possibility that PI3K-Akt-mediated regulation of Bcrp1 expression is important for cellular survival in other contexts. For example, it has been shown that Bcrp1-deficient hematopoietic cells exhibit a greater sensitivity to mitoxantrone-induced toxicity (42). Therefore, given the general nature of PI3K-Akt signaling in controlling cellular survival (1), it is possible that PI3K-Akt-mediated regulation of Bcrp1 expression is a mechanism by which stem cells are protected from naturally occurring genotoxic xenobiotics.

Whereas Akt1 deficiency led to a reproducible reduction in the SP fraction of bone marrow, bone marrow from these mice did not show a deficiency in competitive engraftment in vivo or colony formation in vitro. Furthermore, bone marrow characteristics, including cell number, cell cycle status, and fractions of CD34+ and KSL cells did not differ between the two strains of mice. Therefore, the reduction in Akt signaling associated with Akt1 gene ablation results in a reduction in Hoechst dye efflux with little or no effect on stem cell function. Presumably, Akt1 is required for full Bcrp1 expression in SP cells, and in its absence these cells are maintained but appear outside of the SP region upon flow cytometry. This hypothesis is consistent with the observation that the elimination of the SP cell fraction by LY294002 could be reversed following the removal of this inhibitor.

Recently, the signaling and transcriptional regulatory mecha-
organisms that control stem cell biology have received considerable attention (52–54). Although our data show that the PI3K-Akt signaling axis is important for Bcrp1 expression and hence, the SP phenotype, questions still remain regarding the larger role of this signaling system in the biology of SP cells. Akt1, -2, and -3 genes share a high degree of sequence homology, suggesting a considerable degree of functional overlap between these three genes. The ablation of individual Akt genes results in mice with relatively subtle phenotypes (16–18), but the combined deletion of Akt1 and Akt2 genes results in perinatal lethality with multiple developmental defects (55). Thus, it is possible that further suppression of PI3K-Akt signaling could influence the stem cell activities of SP cells. In this regard, PI3K-Akt signaling has been implicated in the regulation of cell death-mediated regeneration of tissue including migration, survival, and differentiation (reviewed in Ref. 56). Therefore, dissection of the PI3K-Akt signaling axis in SP cells could provide further insights into the molecular regulation of stem cell behavior.

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Bcrp1 Expression in SP Cells

39075

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Bcrp1 Expression in SP Cells

39075