Slug, a highly conserved zinc finger transcriptional repressor, protects hematopoietic progenitor cells from radiation-induced apoptosis in vivo

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Summary

We show here that a zinc finger transcriptional repressor, Slug, which is aberrantly upregulated by the E2A-HLF oncoprotein in pro-B cell acute leukemia, functions as an antiapoptotic factor in normal hematopoietic progenitor cells. *Slug*$^{-/-}$ mice were much more radiosensitive than wild-type mice, dying earlier and showing accentuated decreases in peripheral blood cell counts, as well as abundant microhemorrhages and widely disseminated bacterial microabscesses throughout the body. *Slug* expression was detected in diverse subsets of hematopoietic progenitors, but not in more differentiated B and T lymphoid cells, and there was a significant increase in apoptotic (TUNEL-positive) bone marrow progenitor cells in irradiated *Slug*$^{-/-}$ mice compared to wild-type controls. These results implicate Slug in a novel survival pathway that protects hematopoietic progenitors from apoptosis after DNA damage.

Introduction

Slug, a zinc finger transcriptional repressor in the highly conserved Slug/Snail family of transcription factors (Hemavathy et al., 2001), was initially identified as a protein that regulates cell migration during early development in the chicken (Nieto et al., 1994). In *Drosophila,* Snail and several related proteins, including Escargot and Scratch, which show high (57%–79%) amino acid sequence similarity to Slug in the zinc finger region, have been implicated in embryonic patterning events during gastrulation and mesoderm formation, imaginal wing cell development, and neuronal cell fate determination (Grau et al., 1984; Fuse et al., 1996; Hayashi et al., 1993; Ashraf et al., 1999; Roark et al., 1995). In vertebrates, members of this transcription factor family are required for the epithelial-mesenchymal transition of mesodermal cells, migration of neural crest cells, and formation of neural tubes (Nieto et al., 1994; Sefton et al., 1998; Savagner et al., 1997; Carl et al., 1999). Snail-related proteins are also necessary for determination of left-right asymmetry (Isaac et al., 1997), are involved in apoptosis (Grimes et al., 1996; Metzstein and Horvitz, 1999; Inukai et al., 1999), and regulate the expression of E-cadherins (Oda et al., 1998; Batlle et al., 2000; Cano et al., 2000). Finally, many recent reports point to a role of Slug/Snail proteins in cancer (for a review, see Hemavathy et al., 2000).

The human and murine *Slug* genes encode highly conserved 267 aa proteins that contain five zinc fingers near their carboxyl termini, which mediate binding to tandem E box motifs (ACAGGTG; Inukai et al., 1999), and an amino-terminal transrepressor domain (Sefton et al., 1998; Zweidler-McKay et al.,

SIGNIFICANCE

Human cancer cells employ a wide variety of strategies to escape apoptosis. Insights into the existence of an evolutionarily conserved antiapoptotic pathway downstream of the Slug transcriptional repressor came initially from studies of human leukemia cells expressing the E2A-HLF chimeric oncogene. Our current studies indicate that Slug normally functions to promote the survival of bone marrow progenitors that have undergone DNA damage. Further study of this antiapoptotic transcription factor and the downstream genes it regulates may suggest methods to preserve apoptotic defense mechanisms in hematopoietic progenitor cells, a maneuver that could be used to ameliorate the side effects of cancer therapy on normal bone marrow.

1996). Jiang and coworkers (1998) recently reported that mice lacking *Slug* are fertile but show postnatal growth delays and eyelid abnormalities; their mesoderm and neural crest development appeared normal.

We identified human *SLUG* as a target gene of the chimeric E2A-HLF oncoprotein (Inukai et al., 1999), whose antiapoptotic activity transforms pro-B lymphocytes bearing the translocation t(17;19) (Inaba et al., 1992). The bZIP (basic region-leucine zipper) DNA binding and dimerization domain of *HLF* in the *E2A-HLF* oncogene is closely related to *ces-2*, a cell death specification gene in *Caenorhabditis elegans* (Metzstein et al., 1996; Inaba et al., 1996). Importantly, SLUG is a mammalian ortholog of CES-1, which acts downstream of *ces-2* in *C. elegans* (Metzstein and Horvitz, 1999; Inukai et al., 1999). *ces-1* is a member of the Snail family of transcriptional repressors that promotes cell survival, and must be inhibited by CES-2 before a developmental programmed cell death can occur in sister cells of neurosecretory motor neurons during development in *C. elegans* (Metzstein et al., 1996, 1998; Metzstein and Horvitz, 1999). The apparent convergence of cell death pathways, including CES-2/CES-1 in the worm and E2A-HLF/SLUG in human pro-B leukemia (Inaba et al., 1996; Inukai et al., 1999), suggests that SLUG may have an important regulatory role in the survival of lymphoid and perhaps other types of hematopoietic cells.

Here we report increased numbers of clonogenic hematopoietic progenitors in the bone marrow and spleen of *Slug*$^{-/-}$ knockout mice with normal peripheral blood cell counts. *Slug*-deficient mice also showed enhanced apoptosis of lineage-negative hematopoietic progenitor cells in response to DNA damage induced by total-body γ irradiation. These results implicate Slug as a survival factor in the protection of normal hematopoietic progenitor cells from genotoxic insults, and substantiate its proposed antiapoptotic role in leukemic B cell progenitors expressing the E2A-HLF oncoprotein.

Results

Generation and phenotype of Slug knockout mice

We generated different lines of *Slug* knockout mice by homologous recombination in embryonic stem cells, replacing expressed sequences with a *neo*^{R} cassette (Figure 1A). Three lines of heterozygous and homozygous animals carrying the targeted allele were successfully obtained from blastocyst injection and breeding of chimeric mice (Figure 1B). Analysis of mouse embryo fibroblasts established from E13.5 embryos verified a lack of *Slug* mRNA in the homozygous null animals (Figure 1C). *Slug*$^{-/-}$ mice were viable and fertile but demonstrated growth retardation after birth as well as eyelid malformations, a phenotype similar to that reported for an independently derived strain of *Slug*$^{-/-}$ mice (Jiang et al., 1998).

Because of the apparent cell survival advantage conferred by overexpression of *SLUG* in leukemias transformed by the chimeric E2A-HLF transcription factor (Inukai et al., 1999), we studied the lymphohematopoietic system of *Slug*$^{-/-}$ mice for histopathologic defects. The numbers of B and T lymphocytes (determined by flow cytometric analysis of surface markers), the mitogenic responses of splenic and thymic lymphocytes, and the serum levels of each class of immunoglobulin were essentially the same as those in wild-type littermate controls (data not shown). Circulating blood cell counts were normal in

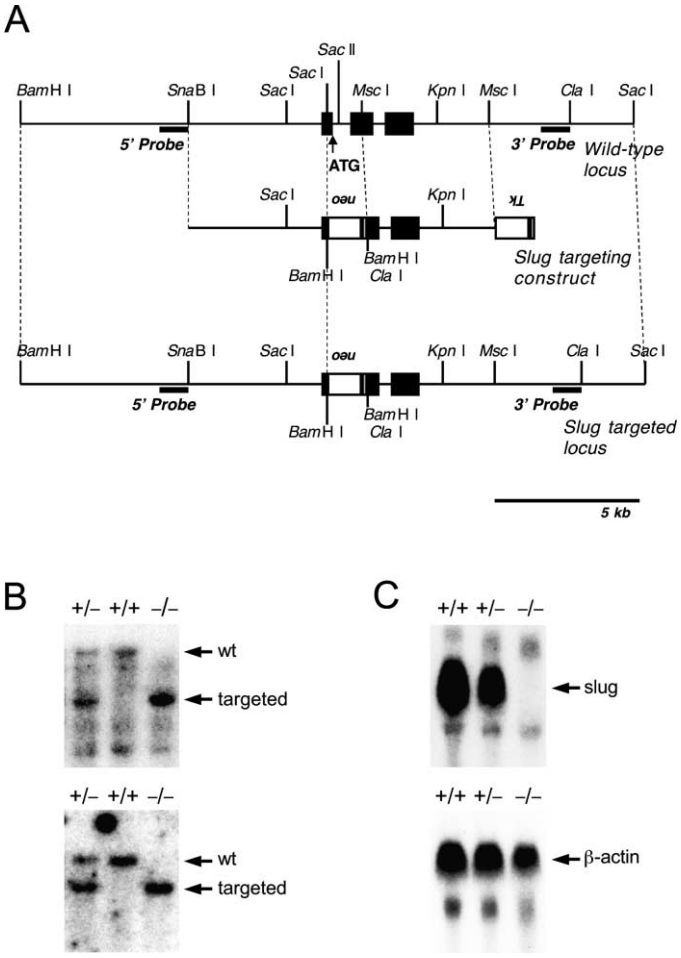

Figure 1. *Slug* gene targeting

A: *Slug* genomic locus and targeting: solid boxes, exons; bars, 5′ and 3′ flanking probes; *neo*^{R}, neomycin resistance gene; *Tk*, thymidine kinase.
B: Representative Southern analysis used to identify the targeted allele. BamHI-digested DNA from offspring at the F2 generation was hybridized with a 5′ (upper) or 3′ (lower) flanking probe as indicated in **A**. A >20 kb wild-type allele and an 8 kb targeted allele were detected with the 5′ probe, while the 3′ probe identified a >20 kb wild-type allele and a 16 kb targeted allele. **C:** Northern blot analysis of total RNA (30 μg) from mouse embryo fibroblasts. The *Slug* cDNA probe encompassed the entire coding sequence; β-actin served as an RNA loading control.

Slug$^{-/-}$ mice (Table 1), but the numbers of hematopoietic colony-forming progenitors (BFU-E, CFU-E, CFU-GM, and CFU-Meg) were increased relative to control values (Figure 2). In the bone marrow, levels of granulocyte-macrophage, erythroid, and megakaryocytic colony-forming units were approximately 2-fold higher per million cells in *Slug*$^{-/-}$ mice, while in the spleen they were about 4-fold higher, although bone marrow cellularity assessed by histology and spleen size was not increased.

These findings suggest that in the absence of *Slug* expression, hematopoietic progenitor pools must expand to maintain normal levels of differentiated blood cells in the circulation.

Slug$^{-/-}$ mice show increased sensitivity to γ irradiation

We next sought to perturb the mice in a way that would reveal defects in their cellular responses to DNA damage. Thus, *Slug*$^{-/-}$, *Slug*$^{+/-}$, and wild-type mice were exposed to 8 Gy of

Table 1. Peripheral blood cell counts in wild-type and *Slug*$^{-/-}$ mice*

	Genotype	
	Slug$^{+/+}$	Slug$^{-/-}$*
Number	12	9
Age	7.17 ± 0.94	7.11 ± 0.93
WBC, ×10^3/μl	6.14 ± 2.01	7.26 ± 1.59
RBC, ×10^6/μl	9.11 ± 0.45	9.05 ± 0.50
HGB, g/dl	14.45 ± 0.93	14.33 ± 0.81
HCT, %	50.63 ± 4.03	49.50 ± 3.28
MCV, fL	55.55 ± 2.45	54.71 ± 2.97
MCH, pg	15.83 ± 0.84	15.89 ± 0.60
MCHC, g/dl	28.75 ± 1.22	28.78 ± 1.30
PLT, ×10^3/μl	1166 ± 220	1149 ± 179

*Slug$^{+/+}$ and Slug$^{-/-}$ mice were littermates. Peripheral blood cells obtained from femoral arteries of the age and sex-matched subjects were tested using an automated complete blood cell counter. All values given as mean ± standard deviation. There are no significant differences between Slug$^{-/-}$ mice and wild-type (WT) littermate controls (unpaired t test). Each number is shown as mean ± S.D.
Abbreviations: RBC, red blood cells; WBC, white blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean red cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; PLT, platelet.

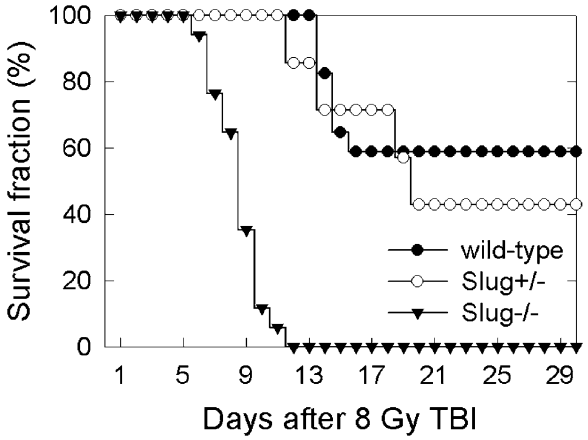

Figure 3. Sensitivity of *Slug*$^{-/-}$ and wild-type mice to total-body γ irradiation

Four-week-old littermates of three genotypes (*Slug*$^{+/+}$, n = 17; *Slug*$^{+/-}$, n = 7; *Slug*$^{-/-}$, n = 17) were given TBI at a dose of 8 Gy from a ^{137}Cs source. Their survival was monitored for 30 days.

total body γ irradiation (TBI), a dose lethal to approximately 50% of the wild-type animals (LD_{50}; Figure 3) (Hall, 1994). After this treatment, 7 of 17 wild-type mice died at a median time of 14.5 days, while all 17 of the *Slug*$^{-/-}$ mice died at a median of 8.5 days. Heterozygous animals (n = 7) were not hypersensitive to TBI, with responses paralleling those of littermate controls.

Mice exposed to an LD_{50} dose of TBI generally die from anemia, bleeding, and sepsis associated with bone marrow failure (Ellinger, 1945; Quastler, 1945). The accelerated death of *Slug*$^{-/-}$ mice after TBI suggested either accentuated damage of bone marrow progenitors or failure of an organ or organs other than bone marrow. To evaluate these alternatives, we sacrificed moribund *Atm*$^{-/-}$ and *Slug*$^{-/-}$ mice at 3–4 days and 8–9 days, respectively, after TBI. In contrast to *Atm*$^{-/-}$ mice, which showed the characteristic features of acute radioenteritis (Hall, 1994; Westphal et al., 1997; Kamijo et al., 1999) (Figures 4E and 4F), the mucosa of the entire intestinal tract of moribund irradiated *Slug*$^{-/-}$ mice appeared normal (Figures 4C and 4D) and was indistinguishable from that of wild-type mice (Figures 4A and 4B). Further examination of the *Slug*$^{-/-}$ mice revealed signs of sepsis and microabscesses in various tissues, including liver, kidney, brain, and small intestine (Figures 5A–5D). Peripheral blood cell counts were also lower in moribund *Slug*$^{-/-}$ mice than in wild-type controls (Supplemental Figure S1), except for platelet counts, which varied widely among individual *Slug*$^{-/-}$ mice. Taken together, these results implicate accentuated radiation-induced damage to bone marrow progenitors in the premature death of TBI-treated *Slug* null mice.

Rescue of hematopoiesis by Mpl-ligand in *Slug*$^{-/-}$ mice

Recovery from DNA damage-induced aplasia can be accelerated by the administration of hematopoietic growth factors and other cytokines (Lazarus and Rowe, 1994). In mice, a single dose of a truncated and pegylated form of thrombopoietin (rmMGDF, or recombinant murine megakaryocyte growth and development factor), given shortly after TBI, prevented pancytopenia by enhancing short-term multilineage repopulation of the spleen (Neelis et al., 1998). This effect can be attributed to the Peg-rmMGDF-mediated prevention of apoptosis in hematopoi-

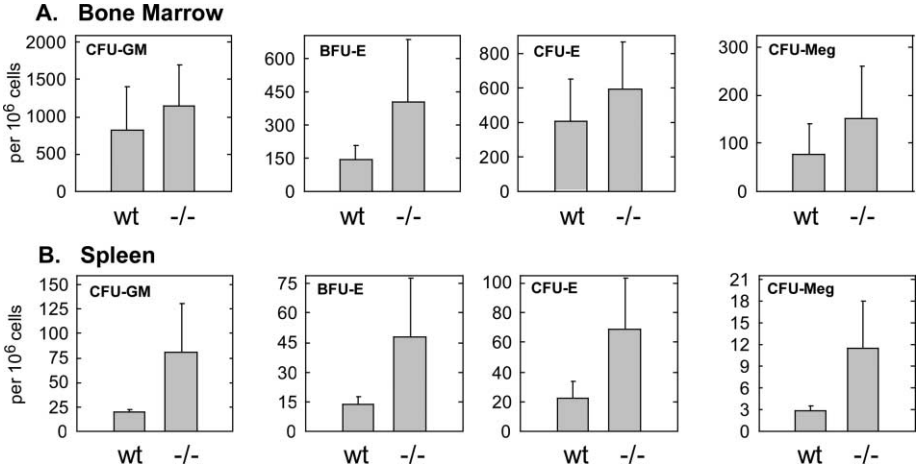

Figure 2. Colony-forming units in bone marrow and spleen of wild-type and *Slug*$^{-/-}$ mice

Numbers of colony-forming units per million viable cells plated from bone marrow (**A**) and spleen (**B**).
Cells from *Slug*$^{+/+}$ mice (n = 3) and *Slug*$^{-/-}$ littermates (n = 4) were propagated in methylcellulose-containing media, and colonies were counted and distinguished by morphology on day 3 (CFU-E) and day 7 (BFU-E, CFU-GM, CFU-Meg). Bars represent means ± standard deviation. Comparisons of colony-forming units between Slug$^{-/-}$ and wild-type littermates were not significantly different for bone marrow, but approach statistical significance for spleen cell values (p = 0.10 for CFU-GM, 0.11 for BFU-E, 0.07 for CFU-E, and 0.07 for CFU-Meg; two-tailed, unpaired t test).

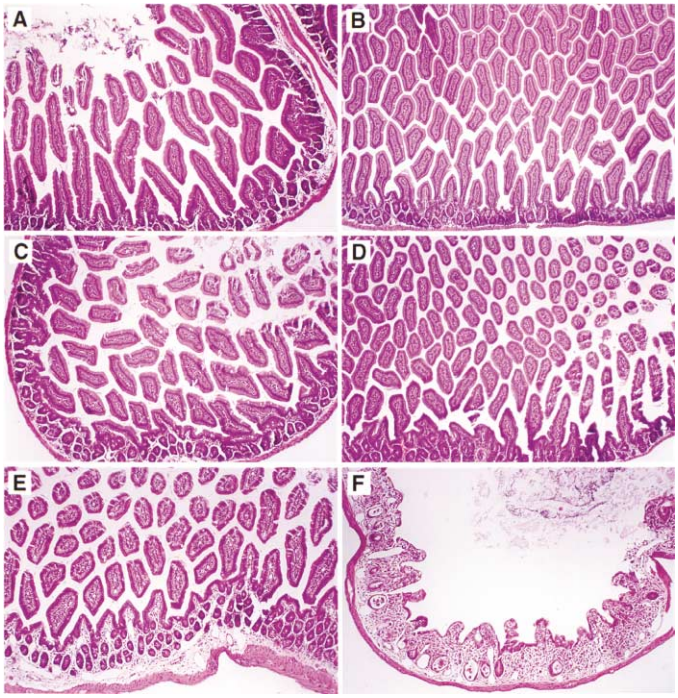

Figure 4. Absence of abnormalities in the gastrointestinal tract of moribund *Slug*$^{-/-}$ mice after 8 Gy of total-body γ irradiation

Hematoxylin and eosin staining of the small intestine of untreated or irradiated moribund wild-type mice (**A** and **B**), *Slug*$^{-/-}$ mice (**C** and **D**), and *Atm*$^{-/-}$ mice (**E** and **F**) at 70× magnification. The time at which mice of these three genotypes became moribund differed, as expected from previous experiments. Accordingly, *Atm*$^{-/-}$ mice were sacrificed on day 3, *Slug*$^{-/-}$ animals on day 8, and wild-type mice on day 13 after irradiation.

etic progenitor cells and their differentiated progeny after DNA damage; a similar mechanism has been reported for thrombopoietin rescue of multilineage (Lin$^-$ Sca-1$^+$) stem cells and human CD34$^+$ CD38$^-$ cells (Borge et al., 1997; Jacobsen et al., 1996).

To test the effects of Peg-rmMGDF in irradiated *Slug*$^{-/-}$ mice, we administered a single dose of the factor (50 μg/kg) immediately after 8 Gy of TBI. All 5 wild-type mice, but only 1 of 6 *Slug*$^{-/-}$ mice, survived after treatment with the growth factor. However, when the TBI dose was reduced to 6.25 Gy, 6 of 8 Peg-rmMGDF-treated *Slug*$^{-/-}$ animals survived, compared with none of 7 *Slug*$^{-/-}$ placebo controls (median time to death, 8.5 days; Figure 6). Platelet counts were significantly lower 8 days after 6.25 Gy of TBI in placebo- versus Peg-rmMGDF-treated mice (Figure 7B). Hemoglobin levels were higher in both groups of Peg-rmMGDF-treated mice than in carrier-treated animals (Figure 7A), while white blood cell counts recovered to the same levels with or without administration of growth factor (Figures 7C and 7D). Thus, Peg-rmMGDF appears to protect *Slug*$^{-/-}$ mice from ionizing radiation by improving the survival of hematopoietic progenitors and their progeny, suggesting that Mpl-ligand acts through pathways that bypass Slug-mediated defects in cell survival.

Lack of Slug does not sensitize thymocytes to apoptosis or upregulate p53 in irradiated bone marrow cells

Slug is widely expressed in various tissues and organs, except in brain and liver (Inukai et al., 1999). Within the hematopoietic compartment, Northern blot analysis revealed Slug expression in spleen and thymus, but not peripheral blood leukocytes (Inukai et al., 1999). Since thymocytes are highly sensitive to DNA damage-induced cell death mediated through p53-dependent pathways (Clarke et al., 1993; Lowe et al., 1993), they provide

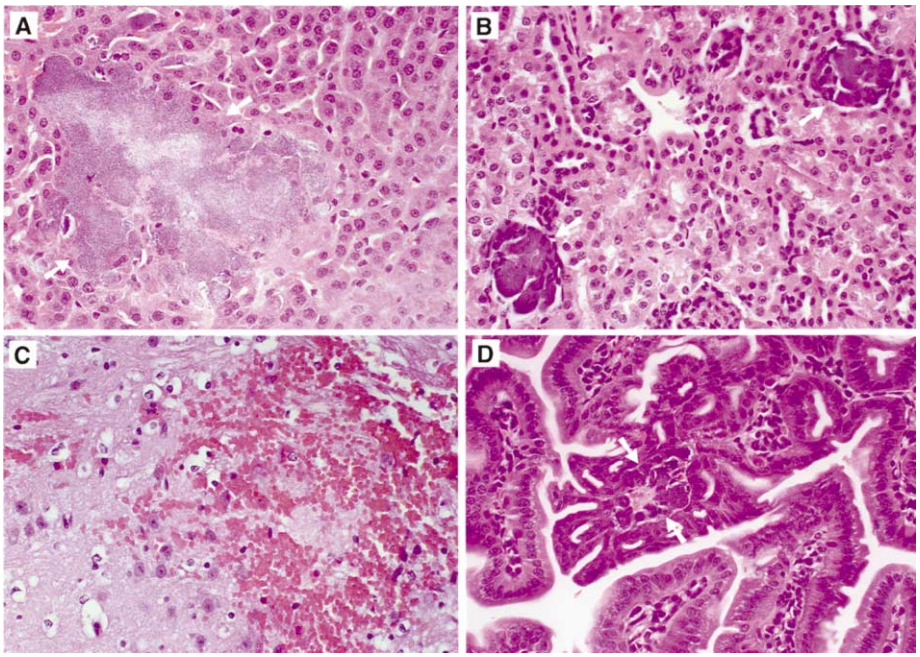

Figure 5. Microhemorrhages, signs of sepsis, and depressed blood cell counts in *Slug*$^{-/-}$ mice after total-body γ irradiation (8 Gy)

Hematoxylin and eosin staining of tissue sections showed bacterial foci in the parenchyma of the liver (**A**), within glomeruli of the kidney (**B**), and in the wall of the small intestine (**D**) of moribund *Slug*$^{-/-}$ mice. **C:** Bleeding in the brain was representative of bleeding in many other organs. Histologic sections are shown at either 170× (**A**) or 340× magnification (**B**, **C**, and **D**).

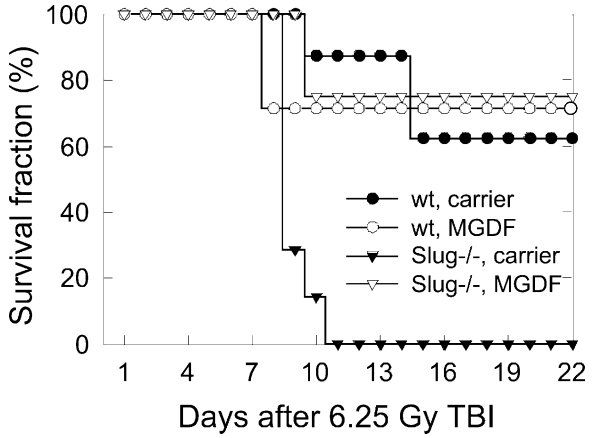

Figure 6. Mpl-ligand (Peg-rmMGDF) promotes survival of both wild-type and *Slug*$^{-/-}$ mice after TBI (6.25 Gy)

Mice were given normal mouse serum as placebo (n = 8, wild-type; n = 7, *Slug*$^{-/-}$ mice) or treated with 50 μg/kg Peg-rmMGDF (n = 7, wild-type; n = 8, *Slug*$^{-/-}$ mice) by intravenous tail vein injection immediately after TBI at 6.25 Gy. Survival was monitored for 22 days.

a means to test the interdependence of Slug and p53 regulatory pathways. In experiments shown in Supplemental Figure S2A, the extent of apoptosis in *Slug*$^{-/-}$ thymocytes was comparable to that in wild-type cells irradiated with 2 Gy and was only slightly higher than in controls when the radiation dose was increased to 4 Gy. These results suggest that Slug may promote hematopoietic cell survival through mechanisms that do not rely on interference with p53 activation. To test this prediction, we measured p53 protein levels in bone marrow cells of *Slug*$^{-/-}$ and wild-type mice at serial times after TBI. The controls, as well as *Slug*$^{-/-}$ mice, showed an increase of p53 at 2 to 4 hr, followed by a return to normal levels at 6 to 12 hr postirradiation (Supplemental Figure S2B), similar to a previous report (Maltzman and Czyzyk, 1984). Thus, *Slug*$^{-/-}$ hematopoietic cells appear to regulate p53 appropriately in response to DNA damage, suggesting that the putative Slug-dependent survival pathway either parallels or lies downstream of pathways responsible for regulation of p53.

Expression of Slug by subsets of hematopoietic progenitor cells

Since Slug plays a protective role in the bone marrow cells of mice that have been subjected to TBI, the first step in establishing its mechanism of action was to determine the subsets of hematopoietic progenitors expressing this factor. Thus, using multiparameter flow cytometry and cell sorting (FACS), we isolated cells with surface marker expression patterns that discriminate among long-term and short-term hematopoietic stem cells (LT- and ST-HSC), common lymphoid and myeloid progenitors (CLP and CMP), more restricted granulocyte-monocyte and megakaryocyte-erythroid progenitors (GMP and MEP), and pro-B and pro-T lymphoid progenitors, as described by Akashi and coworkers (Akashi et al., 2000). Using semiquantitative RT-PCR analysis of RNAs extracted from 2000 cells of each type, we determined that Slug is expressed by both long- and short-term hematopoietic stem cells, the common lymphoid and myeloid progenitors, and both MEP and GMP, but not by pro-B or pro-T cells (Figure 8). These results suggest a functional role of Slug in hematopoietic progenitors responsible for bone marrow repopulation after relatively high-dose but sublethal TBI.

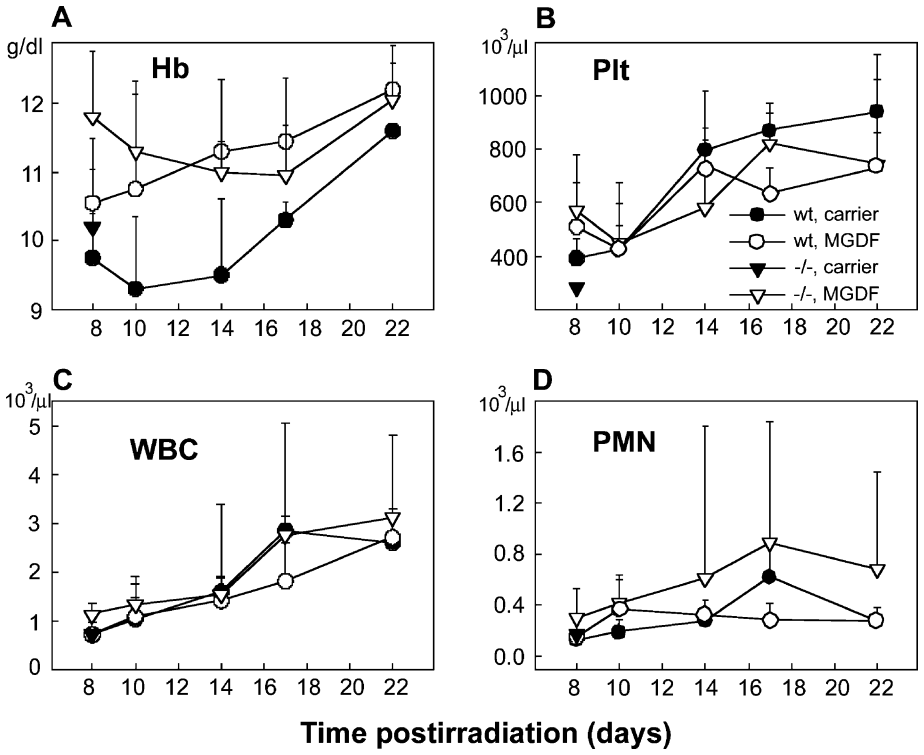

Figure 7. Blood cell recovery in *Slug*$^{-/-}$ versus wild-type mice when treated with MGDF after TBI (6.25 Gy)

Hemoglobin, Hb (**A**); platelets, Plt (**B**); white blood cells, WBC (**C**); and neutrophils, PMN (**D**), were monitored on days 8, 10, 14, 17, and 22 in mice given a single dose of 50 μg/kg Peg-rmMGDF or carrier immediately after TBI, as indicated. Only two of the seven carrier-treated *Slug*$^{-/-}$ mice were alive on day 8, and both died before day 10.

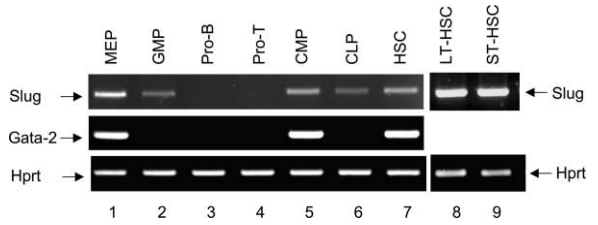

Figure 8. Patterns of Slug expression by myeloid and lymphoid progenitors in different stages of development

cDNA was synthesized from 2000 cells of each hematopoietic progenitor subset sorted from bone marrows by FACS. cDNAs were subjected to PCR with primers specific for the murine *Slug*, *Gata-2*, and *Hprt* genes. Abbreviations: HSC, hematopoietic stem cells; LT-HSC, long-term repopulating hematopoietic stem cell; ST-HSC, short-term repopulating hematopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; MEP, megakaryocyte-erythrocyte progenitor.

Increased radiation-induced apoptosis in hematopoietic progenitor cells of *Slug*$^{-/-}$ mice

Because expression of the Slug ortholog in *C. elegans*, CES-1, appears to be sufficient to protect NSM sister neurons from programmed cell death during development (Metzstein et al., 1998; Metzstein and Horvitz, 1999), we used TdT labeling of fragmented DNA ends (TUNEL) to compare levels of apoptosis in bone marrow cells from *Slug*$^{-/-}$ versus wild-type mice. We observed only a slight difference in the percent labeling of unselected marrow cells in slide assays conducted 6 hr after the mice received 6 Gy TBI (6.3% $\pm$ 0.7% for *Slug*$^{-/-}$ mice versus 3.3% $\pm$ 1.8% for wild-type mice; Figures 9A and 9B). This result was not surprising in view of the very low or absent expression of *Slug* by relatively differentiated bone marrow cells carrying lineage-specific differentiation markers.

We therefore repeated the TUNEL assay in whole bone marrow cells that had been separated into lineage-negative (Lin$^-$) and lineage-positive (Lin$^+$) fractions, using a cocktail of lineage-restricted antibodies conjugated to magnetic beads. The FACS analysis (Figure 9A) indicated that the lineage-negative cells, which are a mixture of stem and early progenitor cells, comprised about 12% of whole marrow cells in both *Slug*$^{-/-}$ and wild-type mice, and that enrichment for Lin$^-$ cells in a typical separation was 91%. By RT-PCR analysis, *Slug* expression was either very low or undetectable in whole bone marrow and Lin$^+$ cells, but was readily detectable in the Lin$^-$ cell fraction from unirradiated mice (Figure 9C). Similar percentages of TUNEL$^+$ cells were detected after TBI in the Lin$^+$ fractions from *Slug*$^{-/-}$ and wild-type mice (2.4% $\pm$ 1.2% versus 3.7% $\pm$ 2.2%, Figures 9A and 9B), while the Lin$^-$ stem and early progenitor cell fraction showed a significantly higher percentage of TUNEL$^+$ apoptotic cells (56.8% $\pm$ 9.7%) compared with wild-type Lin$^-$ cells (30.3% $\pm$ 12.5%; $P = 0.04$). In addition, *Slug* mRNA levels appeared to be increased in the Lin$^-$ bone marrow cells from wild-type mice following TBI (Figure 9C), suggesting that *Slug* expression is induced in hematopoietic progenitors following radiation-induced DNA damage.

Discussion

Here we show that Slug-deficient mice have a defect in hematopoiesis that is potentiated by TBI. Although peripheral blood

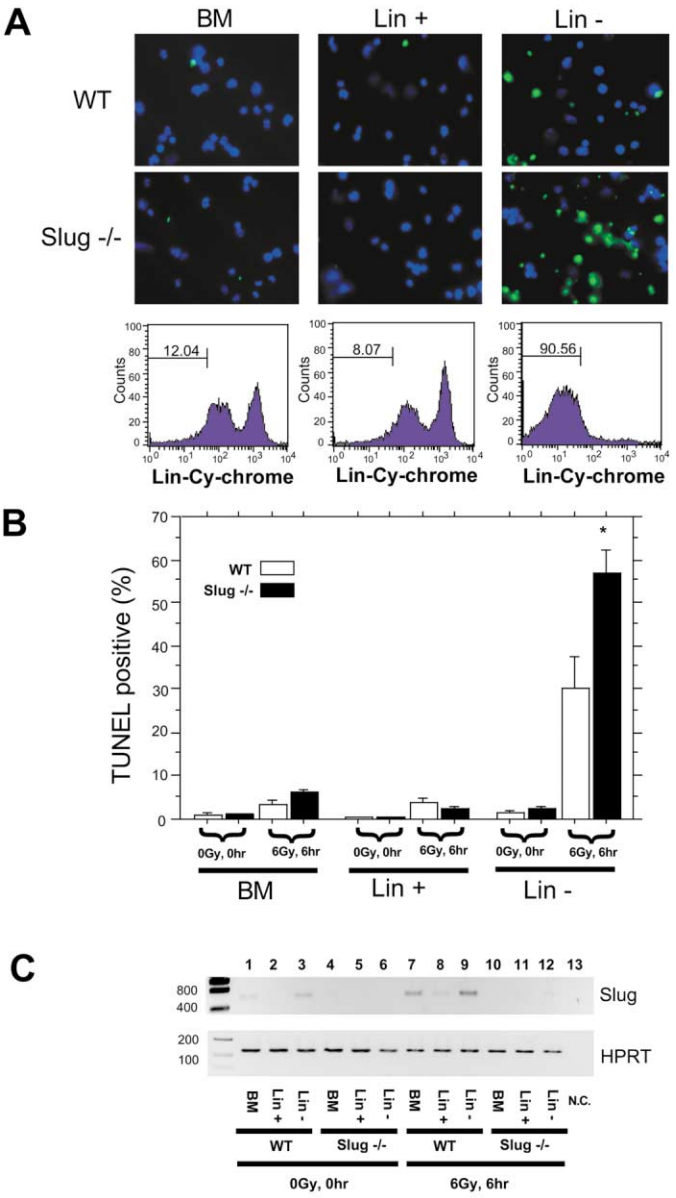

Figure 9. Increased apoptosis after radiation-induced DNA damage in hematopoietic progenitors of Slug$^{-/-}$ versus wild-type mice

A: Representative TUNEL staining of unseparated, lineage$^+$ (Lin$^+$) and lineage$^-$ (Lin$^-$) bone marrow cells from wild-type (WT; upper panel) and *Slug*$^{-/-}$ mice (middle panel), analyzed 6 h after the mice received 6 Gy total-body irradiation. Apoptotic cells are shown in green (TUNEL-positive), and nuclei in blue (DAPI DNA staining). The lower panel shows representative data obtained by FACS analysis of unseparated cells and cells from each fraction after staining with a lineage-restricted monoclonal antibody panel (see Experimental Procedures); the calculated percentages of Lin$^-$ cells are shown.

B: Percentages of TUNEL$^+$ cells calculated from three independent experiments for unseparated bone marrow cells (BM), and the Lin$^+$ and Lin$^-$ fractions from WT (open bars) and Slug$^{-/-}$ mice (solid bars). Results are shown before (0 Gy, 0 hr) and at 6 hr after 6 Gy total-body irradiation (6 G, 6 hr). The asterisk (*) indicates $P < 0.05$ (unpaired t test), and the error bars represent one standard error.

C: The expression of *Slug* (upper panel) and *Hprt* (lower panel, as a control) messenger RNA in bone marrow, Lin$^+$, and Lin$^-$ cells from WT and *Slug*$^{-/-}$ mice before (0 Gy, 0 hr) and 6 hours after 6 Gy total-body irradiation (6 Gy, 6 hr).

profiles were normal in untreated *Slug*$^{-/-}$ mice, the numbers of hematopoietic clonogenic progenitors in spleen and bone marrow were increased. After 8 Gy TBI (the LD_{50} for wild-type controls), each of the *Slug*$^{-/-}$ mice succumbed early to radiation effects, with normal histologic findings in all organ systems except the hematopoietic system. Administering Mpl-ligand after TBI largely reversed this increased radiosensitivity. DNA damage produced by γ irradiation normally induces apoptosis in subsets of hematopoietic progenitor cells. By performing TdT end-labeling (TUNEL) assays on the relatively small fraction ($<$10%) of bone marrow cells that lack surface markers of differentiated blood cells (the so-called Lin$^-$ fraction), we were able to directly demonstrate increased TBI-induced apoptosis in enriched populations of hematopoietic progenitors from *Slug*$^{-/-}$ compared to wild-type mice. Taken together, our results suggest that a lack of Slug accentuates the hematopoietic toxicity of γ irradiation-induced DNA damage in vivo, leading to increased apoptotic cell death among the hematopoietic progenitor cells normally required to repopulate the bone marrow and blood after sublethal gentoxic injury.

Role of Slug in normal hematopoiesis and leukemogenesis

Slug is widely expressed in murine and human tissues, including the hematopoietic system, and is ectopically expressed in leukemic pro-B cells expressing the E2A-HLF oncoprotein (Inukai et al., 1999). Human SLUG, like E2A-HLF, can inhibit apoptosis, suggesting that it participates in the transformation of leukemic blasts expressing E2A-HLF, most likely as a downstream target of the oncoprotein (Inukai et al., 1999). The lack of expression of Slug by normal pro-B cells (Figure 7) argues that E2A-HLF acts not by invoking a normal survival pathway in B lymphocytes, but rather by aberrantly activating a Slug-mediated survival pathway normally used by more primitive hematopoietic cell progenitors. Our results suggest that hematopoietic progenitor cells in *Slug*$^{-/-}$ mice are the targets of accentuated apoptosis because of a deficiency of this survival-promoting transcription factor. An increase in the colony-forming progenitor cell compartment with normalization of circulating differentiated blood cells is best explained by impaired survival of hematopoietic progenitor cells, resulting in a feedback loop that leads to an enlarged progenitor pool with a higher-than-normal "output" of cells needed to maintain normal levels of the affected lineages.

Absence of Slug accentuates the effects of γ irradiation-induced aplasia

After receiving what normally would be the LD_{50} dose of TBI, all of the *Slug*$^{-/-}$ mice died from sepsis, anemia, and bleeding at earlier times than wild-type mice. Results implicating accentuated loss of repopulating hematopoietic progenitor cells include lower peripheral blood cell counts in *Slug*$^{-/-}$ compared with *Slug*$^{+/+}$ mice at day 8 or 11 after irradiation. Moreover, a thrombopoietin analog that promotes multilineage hematopoietic cell survival after myelosuppression (Borge et al., 1996; Ulich et al., 1999) was sufficient to restore blood counts in *Slug*$^{-/-}$ mice to levels approximating those in *Slug*$^{+/+}$ mice, at a time when all placebo-treated *Slug*$^{-/-}$ animals had died from the consequences of pancytopenia.

Normal survival pathways and candidate downstream targets of Slug

The paucity of information on the timing and regulation of apoptosis in developing hematopoietic cells makes it difficult to predict components of the normal pathways regulated by Slug. However, provocative hints can be drawn from the *C. elegans* model. Slug is the closest mammalian ortholog of CES-1, which is negatively regulated by CES-2 in pathways controlling the fate of neuronal cells in the worm (Metzstein and Horvitz, 1999; Inukai et al., 1999). Repression of *ces-1* leads in turn to upregulation of *egl-1*, whose product interacts directly with the BCL-2-like protein CED-9, leading to activation of the CED-3 caspase (Conradt and Horvitz, 1998). Thus, it seems reasonable to look to EGL-1, the cell death-inducing effector antagonized by CES-1, for clues to the analogous mammalian pathway. EGL-1 contains a BH3 domain (BCL-2 homology domain 3) and is thought to function in a manner similar to that of mammalian proapoptotic BH3 proteins (del Peso et al., 2000; Conradt and Horvitz, 1998; Letai et al., 2002). We therefore suspect that Slug may function as a negative regulator of one or more BH3-containing proapoptotic proteins in differentiating hematopoietic cells. The growing list of candidate mammalian proteins of this class includes: BAX, which is mutationally inactivated in lymphoid neoplasms and which together with BAK mediates p53-independent suppression of tumorigenesis (Meijerink et al., 1998; Degenhardt et al., 2002); HRK, which is specifically induced in hematopoietic progenitor cells after growth-factor withdrawal (Sanz et al., 2000); Noxa, which induces apoptosis when overexpressed in vitro (Oda et al., 2000); and BIM, whose proapoptotic function was shown to be critical for homeostasis within the hematopoietic compartment (Bouillet et al., 1999). Other representatives of the BH3 family of proapoptotic proteins, such as BAD or BID, are primarily regulated at the post-transcriptional level by phosphorylation or cleavage by caspases and N-myristoylation (Harada et al., 1999; Gross et al., 1999; Zha et al., 2000), and therefore are less attractive candidates for Slug-mediated transcriptional regulation.

Further studies are needed to define the pathways through which Slug exerts its protective effect in hematopoietic progenitor cells that have received radiation-induced genotoxic damage. Expression of Slug by subsets of hematopoietic stem and progenitor cells and induction of RNA levels after irradiation in Lin$^-$ populations enriched for these cell types is certainly consistent with the interpretation that Slug acts cell autonomously within hematopoietic cell lineages. We are currently breeding Slug knockout mice into an isogenic C57Bl/6 background, so that transplant experiments can be performed to conclusively address this issue.

Antiapoptotic mechanisms hold considerable promise as targets for molecular therapy. As tumor cells evolve, they develop increasingly complex strategies to avoid programmed cell death (Green and Evan, 2002), suggesting that the correction of specific antiapoptotic lesions might be needed to ensure rapid and efficient destruction of malignant clones. Unfortunately, not enough is known about the repertoire of antiapoptotic pathways exploited by human cancers. Our data implicate the Slug transcriptional repressor as a major survival factor in human hematopoietic progenitor cells. As such, the pathways it regulates could contribute to the toxicity of chemotherapy and radiation to bone marrow progenitor cells, which is dose-limiting with many therapeutic regimens in current use. Slug may also

contribute to the marked resistance of transformed myeloid leukemia and other tumor cells to chemotherapy and radiation, especially in settings in which dosages of DNA-damaging agents are too low to eradicate the entire tumor cell population. Exploitation of SLUG in cancer therapy will require additional studies to delineate the pathway through which this survival factor exerts its protective effects. It will be especially important to establish whether Slug acts only in committed progenitors, or whether it also is able to promote the survival of hematopoietic stem cells (LT-HSC) that possess self-renewal and long-term repopulating capacity.

Experimental procedures

Generation of *Slug*$^{-/-}$ mice

A bacterial artificial chromosome (BAC) clone containing the murine *Slug* gene was isolated from a 129/SvjE mouse genomic library (Genome Systems, St. Louis, MO) by use of a 0.8 kb cDNA probe, which contains nucleotides 1 to 609. To construct the targeting vector, we deleted a 1.2 kb SacI/MscI fragment containing exon 1 and part of exon 2 and replaced it with a *neo*^{R} cassette.

Embryonic stem (ES) cells (RW4, Genome Systems) were electroporated with 10 μg of linearized targeting vector and selected with G418 antibiotic (Sigma Chemicals, St. Louis, MO) and 1-[2′-deoxy-2′-fluoro-β-D-arabinofuranosyl]-5-iodouracil (FIAU; Bristol-Myers Squibb, Princeton, NJ) (van Deursen et al., 1995). 380 ES clones doubly resistant to G418 and FIAU were analyzed for homologous recombination by BamHI digestion of the genomic DNA and hybridization with a 1.0 kb probe (5′ probe in Figure 1A). Three ES clones heterozygous for *Slug* were injected into C57Bl/6 blastocysts, which were then implanted into the uteri of pseudopregnant F1 B/CBA foster mothers and allowed to develop to term. Male chimeras from three clones selected by agouti coat color were mated to C57Bl/6 females. Germline transmission was obtained with all three clones. F1 animals were tested for the presence of the deleted *Slug* locus by Southern blotting of tail DNA, and heterozygous F1 males and females were interbred to generate F2 littermates taken for subsequent studies. *Atm* knockout mice were used as a reference because of their reported hypersensitivity to irradiation (Herzog et al., 1998).

To verify the absence of *Slug* messages in knockout mice, we explanted mouse embryo fibroblasts according to a standard method (Kamijo et al., 1997). Total RNA was prepared with TRIZOL reagent. After agarose-gel electrophoresis, RNA was transferred to a nylon filter membrane, which was then exposed to a radiolabeled probe comprising the whole coding sequence of mouse *Slug* cDNA.

Total-body irradiation and Mpl-ligand rescue

Three genotypes of mice (*Slug*$^{+/+}$, *Slug*$^{+/-}$, and *Slug*$^{-/-}$), 4 to 5 weeks of age, were treated with total-body irradiation at a dose of 8 Gy (^{137}Cs source) at a rate of 313 cGy/min. The mice were subsequently housed together, fed ad libitum, and checked frequently for any signs of radiation-induced morbidity. When *Slug*$^{-/-}$ mice became moribund, they were killed and their tissues were fixed with 4% paraformaldehyde for subsequent histopathologic examination.

To assess the myeloprotective effect of Mpl-ligand, we administered a single dose of Peg-rmMGDF (50 mg/kg; kindly supplied by Amgen Inc., Thousand Oaks, CA) or normal mouse serum (vehicle control) immediately after TBI. Blood cell counts were performed on days 8, 10, 14, 17, and 22 postirradiation.

Clonogenic progenitor assays

Mice were killed between 4 to 8 weeks of age to collect splenocytes and bone marrow cells from femurs and tibias. Colony-forming cells were identified by propagating 1×10^5–1×10^7 viable cells in methylcellulose medium containing rmIL-3 (10 ng/ml), rhIL-6 (10 ng/ml), rmSCF (50 ng/ml), and rhEPO (3 U/ml) (Stem Cell Technologies, Vancouver, BC). The colonies were then classified according to their morphologic features by transmission light microscopy. CFU-E were counted 3 days after the propagation, while BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-Meg were counted on day 7.

Thymocyte apoptosis assay

Thymocytes undergoing apoptosis were detected by a reported method (Lowe et al. 1993). Briefly, isolated thymocytes were either left untreated and propagated directly in DMEM medium (Mediatech Cellgro, Herndon, VA) supplemented with 5% fetal bovine serum (Bio Whittaker, Walkersville, MD) and 25 mM HEPES (pH 7.2), or were irradiated in vitro at doses from 2 to 4 Gy before propagation. Cells were cultured for 12 hr before TUNEL (TdT-dUTP nick end-labeling) assay and flow cytometric analysis.

Analysis of p53 protein levels in bone marrow cells

Immunoblotting of p53 protein was performed with pooled bone marrow cells representing two mice of each genotype at each timepoint. Cells were lysed by sonication, and lysates were resuspended in Laemmli's sample buffer (Biorad, Hercules, CA) supplemented with 1 M dithiothreitol (DTT; Sigma) and boiled for 10 min. Protein samples were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter membrane. p53 protein was detected with an antimouse p53 sheep antibody (Ab-7; Calbiochem, La Jolla, CA). Actin was detected as loading control (antibody C-11; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Analysis of gene expression by subsets of hematopoietic stem and progenitor cells

2×10^3 cells with surface marker expression patterns typical of hematopoietic stem cells (HSC) and various types of myeloid and lymphoid progenitors were sorted from mouse bone marrows by FACS, as previously described (Akashi et al., 2000). Total RNA was prepared with TRIZOL reagent and subjected to cDNA synthesis with the SuperScript first-strand cDNA synthesis system (GIBCO, Inc.). PCR was performed for 40 cycles of 94°C for 30 s; 55°C for 45 s; and 72°C for 45 s with the following primers: 5′-GCGAACTG GACACACACACAGTTAT and 5′-CCCCAGTGTGAGTTCTAATGTGTCC for *Slug*; 35 cycles and 5′-CTCTACCACAAGATGAATGGACAGAACC and 5′-GTAGGATGTGTCCAGAGTGACTAAAAGGTG for Gata-2; and 32 cycles and 5′-GTAATGATCAGTCAACGGGGGAC and 5′-CCAGCAAGCTTGCAACCT TAACCA for HPRT.

Bone marrow cell separation for TUNEL assays

Bone marrow cells were obtained from wild-type and *Slug*$^{-/-}$ mice. After removing the dead cells by Ficoll-Paque (Amersham Pharmacia Biotech, Sweden) density gradient centrifugation, we enriched for hematopoietic progenitor cells by using StemSep magnetic beads (StemCell Technologies, Vancouver, Canada). Cytospin preparations were prepared with 1×10^4 cells from each fraction for TUNEL staining, 5×10^4 cells were used for semiquantitative RT-PCR analysis of *Slug* expression, and 1×10^5 cells were used for FACS analysis with Lin$^+$ antibodies.

FACS analysis

1×10^5 cells of each cell fraction were stained for FACS analysis by using a cocktail of biotinylated mouse monoclonal antibodies (ly-1, B220, Mac-1, TER119, Gr-1, and 7-4 [StemCell Technologies, Vancouver, Canada], 10 min. at 4°C in a cold room). Washed cells were then stained with streptavidin-Cy-Chrom (BD Biosciences) for 10 min., also in a cold room. After washing, the cells were analyzed with a FACSCalibur (BD Biosciences) flow cytometer.

TUNEL and DAPI staining

Cells were centrifuged onto slides (Cytospin, 1000 rpm for 2 min), fixed in 4% paraformaldehyde for 1 hr at room temperature, and then permeabilized for 2 min. in 0.1% sodium citrate containing 0.1% Triton X-100 in a cold room. The TUNEL assay was performed with a commercial kit (In Site cell death detection kit; Roche, Germany). After TUNEL staining, cells were counterstained with DAPI (1 μg/ml; Sigma) for 12 min. at room temperature. Cells were washed in 1× PBS for 5 min. and examined by fluorescence microscopy (Optiphot-2; Nikon, Japan). The experiment was done in triplicate, with at least 300 cells analyzed in each assay. Percentages of TUNEL-positive cells were compared for statistical significance by use of an unpaired t test.

Acknowledgments

We thank Shuhua Qi, Madoka Inoue, and Anne Delahaye-Brown for their technical assistance, John Gilbert for editorial review and critical comments,

and Doris Dodson for manuscript preparation. This work was supported by grants (CA-59571, NS-39867, and NS-37956) from the National Institutes of Health. A.I. was supported by a Karnofsky Memorial Fellowship in Cancer Research, St. Jude Children's Research Hospital. M.G.S. was supported by the Max Kade Foundation Inc., N.Y.

Received: September 12, 2002
Revised: September 23, 2002

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