# Role of p53 in Hematopoietic Recovery After Cytotoxic Treatment

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Prompt reconstitution of hematopoiesis after cytoreductive therapy is essential for patient recovery and may have a positive impact on long-term prognosis. We examined the role of the p53 tumor suppressor gene in hematopoietic recovery in vivo after treatment with the cytotoxic drug 5-fluorouracil (5-FU). We used p53 knock-out (p53−/−) and wild-type (p53+/+) mice injected with 5-FU as the experimental model. Analysis of the repopulation ability and clonogenic activity of hematopoietic stem cells (HSCs) and their lineage-committed descendants showed a greater number of HSCs responsible for reconstitution of lethally irradiated recipients in p53−/− bone marrow cells (BMCs) recovering after 5-FU treatment than in the corresponding p53+/+ BMCs. In post–5-FU recovering BMCs, the percentage of HSC-enriched Lin− Sca-1+ c-Kit+ cells was about threefold higher in p53−/− than in p53+/+ cells. Although the percentage of the most primitive HSCs (Lin− Sca-1+ c-Kit+ CD34$^{low/−}$) did not depend on p53, the percentage of multipotential HSCs and committed progenitors (Lin− Sca-1+ c-Kit+ CD34$^{high/+}$) was almost fourfold higher in post–5-FU recovering p53−/− BMCs than in their p53+/+ counterparts. The pool of HSCs from 5-FU–treated p53−/− BMCs was exhausted more slowly than that from the p53+/+ population as shown in vivo using pre–spleen colony-forming unit (CFU-S) assay and in vitro using long-term culture-initiating cells (LTC-ICs) and methylcellulose replating assays. Clonogenic activity of various lineage-specific descendants was significantly higher in post–5-FU regenerating p53−/− BMCs than in p53+/+ BMCs, probably because of their increased sensitivity to growth factors. Despite all these changes and the dramatic difference in sensitivity of p53−/− and p53+/+ BMCs to 5-FU–induced apoptosis, lineage commitment and differentiation of hematopoietic progenitors appeared to be independent of p53 status. These studies suggest that suppression of p53 function facilitates hematopoietic reconstitution after cytoreductive therapy by: (1) delaying the exhaustion of the most primitive HSC pool, (2) stimulating the production of multipotential HSCs, (3) increasing the sensitivity of hematopoietic cells to growth factors, and (4) decreasing the sensitivity to apoptosis.
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**H**EMATOPOIESIS IS regulated by numerous growth factors, which act in concert to regulate proliferation (including self-renewal), differentiation, and apoptosis, thus generating a relatively constant pool of functionally mature blood cells. However, normal hematopoiesis can be perturbed by a variety of factors such as infection, reduced oxygen concentration, irradiation, and drugs. Cytostatics, which are routinely used as antineoplastic drugs, are toxic to bone marrow cells. In light of the importance of prompt bone marrow repopulation for patient outcome, analyses of the mechanisms regulating this process hold promise in leading to novel therapies that facilitate hematopoietic repopulation after cytostatic treatment or bone marrow transplantation.

The p53 tumor suppressor gene may be one of the key genes involved in regulating hematopoietic repopulation. Although hematopoiesis in p53 knock-out (p53−/−) mice appears to proceed normally,[1] numerous in vitro studies indicate that p53 is involved in proliferation, differentiation, and apoptosis of the hematopoietic cells.[2-7] Moreover, p53 deletions and mutations have been found at high frequency in acute leukemias and in chronic myelogenous leukemia in blast crisis.[8] The p53 phosphoprotein acts not only as a transcriptional activator of genes containing p53 binding sites,[9] but also as a potent inhibitor of transcription from many genes containing TATA boxes and lacking p53-binding sites.[10] p53 plays a critical role in cell proliferation by modulating the expression of genes such as WAF-1, which are required for progression through the cell cycle.[11] Furthermore, p53 is involved in the induction of apoptosis after DNA damage,[12] possibly by transactivating bax, a proapoptotic member of the bcl-2 family.[13]

In the present study, we examined the role of p53 in the in vivo recovery of hematopoiesis after treatment with cytostatic agents. Bone marrow cells obtained from p53 knock-out (p53−/−) and p53 wild-type (p53+/+) mice treated with 5-fluorouracil (5-FU) were tested for their in vivo repopulation ability and the clonogenic activity of hematopoietic stem cells (HSCs) and lineage-committed progenitors.

## MATERIALS AND METHODS

*Mice.* p53 knock-out inbred mice (C$_{57}$ BL/6TacfBR-[KO]p53N4) and p53 wild-type mice were purchased from Taconic Farms (Germantown, NY). Mice were 6 to 10 weeks old in all experiments.

*Isolation of bone marrow cells (BMCs).* Mice were injected intraperitoneally (IP) with 150 mg/kg 5-FU (SoloPak Laboratories Inc, Elk Grove Village, IL) or physiological saline on day 0 and sacrificed by cervical dislocation 2, 4, and 6 days later. BMCs were obtained from one femur, two tibias, and two brachial bones and suspended in Iscove's modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin (complete IMDM). Red blood cells were removed by lysis in hypotonic solution (0.85% NH$_4$Cl, 17 mmol/L Tris-HCl, pH 7.4) for 5 minutes on ice. The number of cells obtained from femur was multiplied by two because the contralateral femur was used for microscopy after fixation. Bone marrow stromal cells were removed by 2-hour incubation in complete IMDM at a concentration of 10$^6$ cells/mL

in a petri dish. Nonadherent cells were collected after gentle agitation and used for experiments.

*Bone marrow transplantation.* p53+/+ mice underwent total body irradiation (TBI) with a lethal dose of 875 cGy from a ^{137}Cs source and 48 hours later were injected intravenously (IV) with bone marrow cells as specified for each assay.

*Competitive long-term reconstitution assay.* TBI-treated p53+/+ mice were injected with a mixture of $5 \times 10^4$ p53+/+ and $5 \times 10^4$ p53−/− BMCs. After 16 weeks, mice were sacrificed and genomic DNA was isolated from BMCs. The ratio of p53+/+ to p53−/− cells repopulating the host bone marrow was evaluated by quantitative polymerase chain reaction (PCR). Two sets of primers, 1-2 and 1-3, were used simultaneously to detect p53 wild-type and p53 knock-out alleles, respectively[14]: primer 1 (5′): GGGACAGCCAAGTCTGTTAT-GTGC, located upstream from the deleted region of the p53 gene and therefore common for both mutated and wild-type alleles; primer 2 (3′): CTGTCTTCCAGATACTCGGGATAC, located in the fragment deleted in the knock-out p53 allele and specific for the wild-type allele; and primer 3 (3′): TTTACGGAGCCCTGGCGCTCGATGT, located in the *Pol*II promoter region of the NEO cassette and present only in the knock-out allele. PCR was performed using 2 µg of genomic DNA and the products were separated by electrophoresis, transferred to a Zetabind membrane (Cuno Inc, Meriden, CT), and hybridized with an internal probe (5′ TTCCTCTTCAGCCTGTAGACTGTG 3′) specific for intron 1 in the p53 gene, thus recognizing PCR products from both the wild-type and the knock-out alleles. The ratio of these PCR products was compared with that in the calibration blot prepared using a mixture of p53+/+ and p53−/− cells in predetermined proportions.

*Flow cytometry.* The following antibodies were used: fluorescein isothiocyanate (FITC)–Sca-1, FITC–c-Kit, FITC–Gr-1, FITC–Mac-1, FITC–CD3, phycoerythrin (PE)–Ter-119, PE-B220, CD34 (all from Pharmingen, San Diego, CA), PE-CD4, PE-CD8 (both from Boehringer-Mannheim, Indianapolis, IN), and biotin-F4/80 (Serotec Ltd, Oxford, UK). Cells ($10^5$ per 100 µL of phosphate-buffered saline [PBS] + 2% FBS + 0.1% $NaN_3$) were incubated with Fc block (Pharmingen) for 10 minutes at room temperature followed by 45-minute incubation with the indicated antibodies at 4°C and, when appropriate, incubated with secondary antibody coupled to FITC or with streptavidin linked to PE. Flow cytometry was performed with EPICS Profile analyzer (Coulter Corp, Hialeah, FL).

*Cell sorting.* Lineage-positive (Lin+) cells were removed with a magnet (MPC-1; Dynal Inc, Oslo, Norway) after staining with a mixture of biotinylated antibodies (Gr-1, Mac-1, B220, CD4, CD8, and Ter-119) and incubation with Dynabeads (Dynal Inc, Great Neck, NY), according to the manufacturer's protocol. Cells in suspension were then incubated with streptavidin-Red 670 (SV-R670; GIBCO-BRL, Grand Island, NY) and sorted on Coulter cell sorter to remove remaining Lin+ cells. Lin− cells were subsequently incubated with the cocktail of PE-Sca-1, FITC–c-Kit, and biotin-CD34 antibodies, washed extensively, and further incubated with SV-R670, washed again and sorted using 3-color fluorescence-activated cell sorting (Epics Elite; Coulter Corp). Lin− Sca-1+ c-Kit+ CD34[high/+] and Lin− Sca-1+ c-Kit+ CD34^{low/−} populations were selected according to Osawa et al[15] and used for further experiments.

*Colony formation assay in methylcellulose.* Cells ($5 \times 10^4$) were resuspended in methylcellulose semisolid medium (HCC-4320, StemCell Technologies Inc, Vancouver, Canada) and plated in 35-mm wells in the presence of the following recombinant murine growth factors: 10 U/mL of interleukin-3 (IL-3; Genetics Institute Inc, Cambridge, MA), 50 U/mL of IL-2 (Genetics Institute Inc), 30 U/mL of IL-7 (Genetics Institute Inc), 10 ng/mL of kit ligand (KL; R&D System Inc, Minneapolis, MN), and 10 U/mL of erythropoietin (Epo; Amgen Inc, Thousand Oaks, CA). After 7 to 10 days, colonies and clusters were counted under an inverted microscope as described.[16]

To determine long-term clonogenic efficiency, cells were plated in methylcellulose in the presence of KL, IL-3, and Epo; the colonies were counted; and the cells were harvested 10 days later, washed in medium, counted, and replated ($10^4$ cells/plate) in the presence of the indicated growth factors.

*Megakaryocytic colony-forming unit (CFU-Meg) assay.* 4A5 hybrid-oma cells producing antimouse megakaryocyte antibodies[17] were obtained from Dr Paul Friese (University of Oklahoma Health Sciences Center, Oklahoma City, OK). Ascites were harvested from pristane-pretreated SCID mice injected IP with 4A5 cells. The IgG fraction was purified on a protein A affinity column (Oncogene Science, Uniondale, NY) and used for staining. Megakaryocytic colonies were grown in plasma cloth in the presence of recombinant IL-6, IL-3, and Epo as described.[18] CFU-Meg colonies were detected by staining the plasma cloths with antimouse megakaryocyte antibody (IgG) followed by FITC-conjugated antirat IgG. Colonies were counted under a fluorescence microscope.

*Fibroblast-like colony-forming unit (CFU-F) assay.* BMCs ($10^4$ from each mouse) were plated into 35-mm Petri dish in complete IMDM. After 2-hour incubation, floating cells were removed and adherent cells were cultured in Dulbecco's modified Eagle's medium + 10% FBS for 5 to 7 days. Colonies of fibroblasts were fixed in methanol, washed in PBS, stained with Giemsa and counted under a light microscope.

*Assay for LTC-ICs.* BMCs isolated from six p53+/+ and six p53−/− mice were evaluated for LTC-IC content in two sets of independent experiments as described.[19] Briefly, $5 \times 10^4$ BMCs recovered from 5-FU–treated mice were plated on irradiated (1,500 cGy) syngeneic murine stromal monolayers in 24-well plates (Corning, Cambridge, MA) containing 1 mL of IMDM (GIBCO BRL) supplemented with 12.5% calf serum (Hyclone, Logan, UT) and 12.5% horse serum (Hyclone). Cells isolated from every mouse were cultured in 16 independent wells. Half of the population of floating cells was collected every 2 weeks and fresh medium was added to the remaining cells. Collected cells derived from the same animal were pooled, washed, and analyzed for colony formation in methylcellulose containing 10 ng/mL recombinant murine KL as described.[16]

*CFU-S and pre–CFU-S assay.* BMCs ($10^5$) were injected IV into TBI-treated p53+/+ mice and after 12 days, spleens were removed and fixed in Teleyesnizky solution (70% ethanol, 5% acetic acid, and 2% formaldehyde), and CFU-S were counted. At the same time, $10^5$ BMCs were isolated and injected into secondary TBI-treated recipients. After 12 days, recipient mice were sacrificed, spleens were fixed in Teleyes-nizky solution, and pre–CFU-S were counted.

*Western blotting.* BMCs ($10^6$) were lysed in RIPA buffer (PBS supplemented with 1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with proteinase inhibitors (1 mmol/L phenyl-methyl sulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mmol/L sodium orthovanadate, and 0.5 mmol/L EDTA). Lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting with antibodies against: PCNA WAF-1, bak, bcl-2, p53, actin (all from Oncogene Science, Cambridge, MA), bcl-x$_{LS}$, p16, and bax (all from Biotechnology Inc, Santa Cruz, CA). Secondary antibodies were from Amersham Life Science Inc (Arlington Heights, IL). Bands were detected with ECL kit (Amersham).

*Apoptosis assay.* Femurs were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. Sections were immobilized on slides and rehydrated. Apoptotic cells were detected using the TACS 2 TdT in situ apoptosis detection kit (Trevigen Inc, Gaithersburg, MD) according to the manufacturer's protocol.

*Histological and cytological analysis.* Bone marrow tissue sections were fixed in phosphate-buffered formalin and embedded in paraffin. Slides were stained with hematoxylin/eosin. Cytospin preparations were stained with Wright-Giemsa.

## RESULTS

*Role of p53 in 5-FU–induced toxicity to BMCs.* The number of BMCs in p53+/+ mice decreased dramatically after

injection of 5-FU, whereas the drug was markedly less potent in p53−/− mice (Fig 1). On day 6 after 5-FU administration, p53−/− mice contained fivefold to eightfold more BMCs than their wild-type counterparts. This phenomenon probably reflects the diminished apoptosis in p53−/− cells, because injection of 5-FU into the p53+/+ mice resulted in massive apoptosis of BMCs on days 2 and 4, whereas BMCs from p53−/− mice became apoptotic only sporadically (Fig 2).

Analysis of growth factor requirements of colony-forming units indicated that post–5-FU repopulating p53−/− BMCs, as compared with their p53+/+ counterparts, formed more colonies in threshold (0.1 U/mL), suboptimal (1 U/mL) and saturating (10 U/mL) concentrations of recombinant murine IL-3 (Fig 3). Thus, the absence of p53 in regenerating cells underlies their increased sensitivity to stimulation by growth factors, consistent with previous studies showing the importance of the p53 status in the response of hematopoietic cells to growth factor stimulation.[4]

The differences between p53+/+ and p53−/− BMCs in proliferation potential and sensitivity to apoptosis were confirmed by Western blotting analysis of proteins involved in cell cycle activity (PCNA, WAF-1, and p16$^{INK4a}$) and in apoptosis (bcl-2, bax, bcl-x$_L$, and bak) on days 0, 2, 4, and 6 after 5-FU injection (Fig 4). p53 expression was not detectable on day 0, but increased significantly on days 2, 4, and 6 in p53+/+ samples, in accord with previous findings.[20] As expected, p53 was not detectable in p53−/− samples. High levels of PCNA were detected in each sample of p53−/− BMCs after 5-FU treatment, whereas in p53+/+ BMCs, PCNA expression was decreased on day 2, undetectable on day 4, but again detectable on day 6 after injection of the drug. p16$^{INK4a}$ levels decreased slightly in p53+/+ BMCs on days 2, 4, and 6. In contrast, p16$^{INK4a}$ expression was low on days 0, 2, and 4, but markedly increased on day 6 in p53−/− BMCs. WAF-1 levels increased
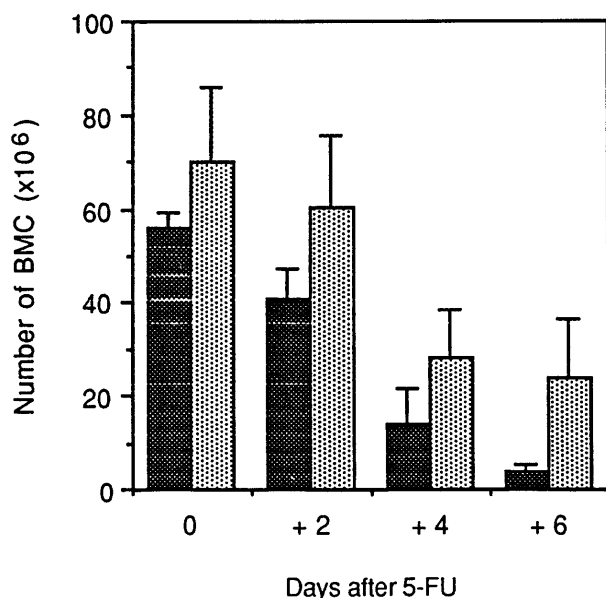
in both p53+/+ and p53−/− BMCs after 5-FU treatment, but the increase was detectable earlier and was more pronounced in the p53+/+ population. Together, these data confirmed that p53−/− BMCs regenerating after 5-FU had higher proliferative potential than their p53+/+ counterparts. Analysis of apoptosis-related proteins showed markedly enhanced bcl-2 levels in p53−/− BMCs on days 2 and 4, but low level expression at all other time points as in p53+/+ samples. Expression of bcl-x$_L$ was similar in both p53+/+ and p53−/− populations. Both bax and bak proteins were highly expressed on days 2 and 4 in p53+/+ BMCs, but undetectable in other samples including those from p53−/− cells. Thus, expression of proapoptotic effectors (bax, bak) relative to antiapoptotic (bcl-2, bcl-x$_L$) effectors was high in p53+/+ cells on days 2 and 4 after 5-FU treatment, whereas the reverse was observed in p53−/− cells.

*Role of p53 in repopulation of HSCs after 5-FU treatment in vivo.* To compare the ability of p53+/+ and p53−/− BMCs obtained from 5-FU–treated mice to rescue animals exposed to a lethal dose of TBI, decreasing numbers of p53+/+ and p53−/− BMCs were injected into recipient mice, and long-term survival was scored at 16 weeks postinjection. Lower numbers of p53−/− BMCs than p53+/+ BMCs were required to rescue TBI-treated recipient mice (Table 1), suggesting that post–5-FU repopulating p53−/− BMCs contain more HSCs capable of reconstituting hematopoiesis in vivo. BMCs from the p53+/+ and p53−/− mice not treated with 5-FU showed no difference in their ability to repopulate in TBI-treated recipients (data not shown). PCR analysis to assess the presence of the p53 knock-out and wild-type alleles in the mononuclear fraction of BMCs obtained from mice transplanted with p53−/− BMCs showed only the knock-out allele (not shown). Thus, long-term hematopoiesis in the transplanted hosts was caused by p53−/− marrow cells and not by normal marrow cells that may have escaped lethal irradiation. Analysis of blood smears and bone marrow sections showed normal hematopoiesis in mice transplanted with either p53+/+ or p53−/− BMCs (data not shown). Quantitative PCR followed by Southern blotting to assess the proportion of p53−/− to p53+/+ BMCs in TBI-treated recipient mice injected IV with a 1:1 mixture of both indicated a ratio of 10:1 to 100:1 in bone marrow obtained 16 weeks after transplantation (Fig 5). Mice were free of leukemia at the time of BMC collection as confirmed by histological analysis of bone marrow and other organs (blood, spleen, and thymus). These results suggest that the absence of p53 gene expression has a positive impact on the repopulation ability of BMCs after treatment with 5-FU.

*Role of p53 in regulation of HSCs after 5-FU treatment.* To determine whether the more vigorous repopulation ability of p53−/− BMCs reflected an increased number of HSCs, immunostaining followed by flow cytometry was used to quantitate HSCs. p53−/− BMCs that repopulated after 5-FU treatment contained about threefold more HSCs with the phenotype Lin− Sca-1+ c-Kit+ [21] than their p53+/+ counterparts (Table 2). Staining with anti-CD34 antibody discriminated two HSC subpopulations: Lin− Sca-1+ c-Kit+ CD34$^{low/−}$ (CD34$^{low/−}$, primitive HSCs) and Lin− Sca-1+ c-Kit+ CD34$^{high/+}$ (CD34$^{high/+}$, multipotential HSCs).[15] There was no difference in the percentage of CD34$^{low/−}$ cells between p53−/− and p53+/+ BMCs regenerating after 5-FU. However, the percentage of



Fig 1. Number of BMCs in p53+/+ (black bars) and p53−/− (shadowed bars) mice after injection of 5-FU. Results represent mean (standard deviation) from five mice/groups.
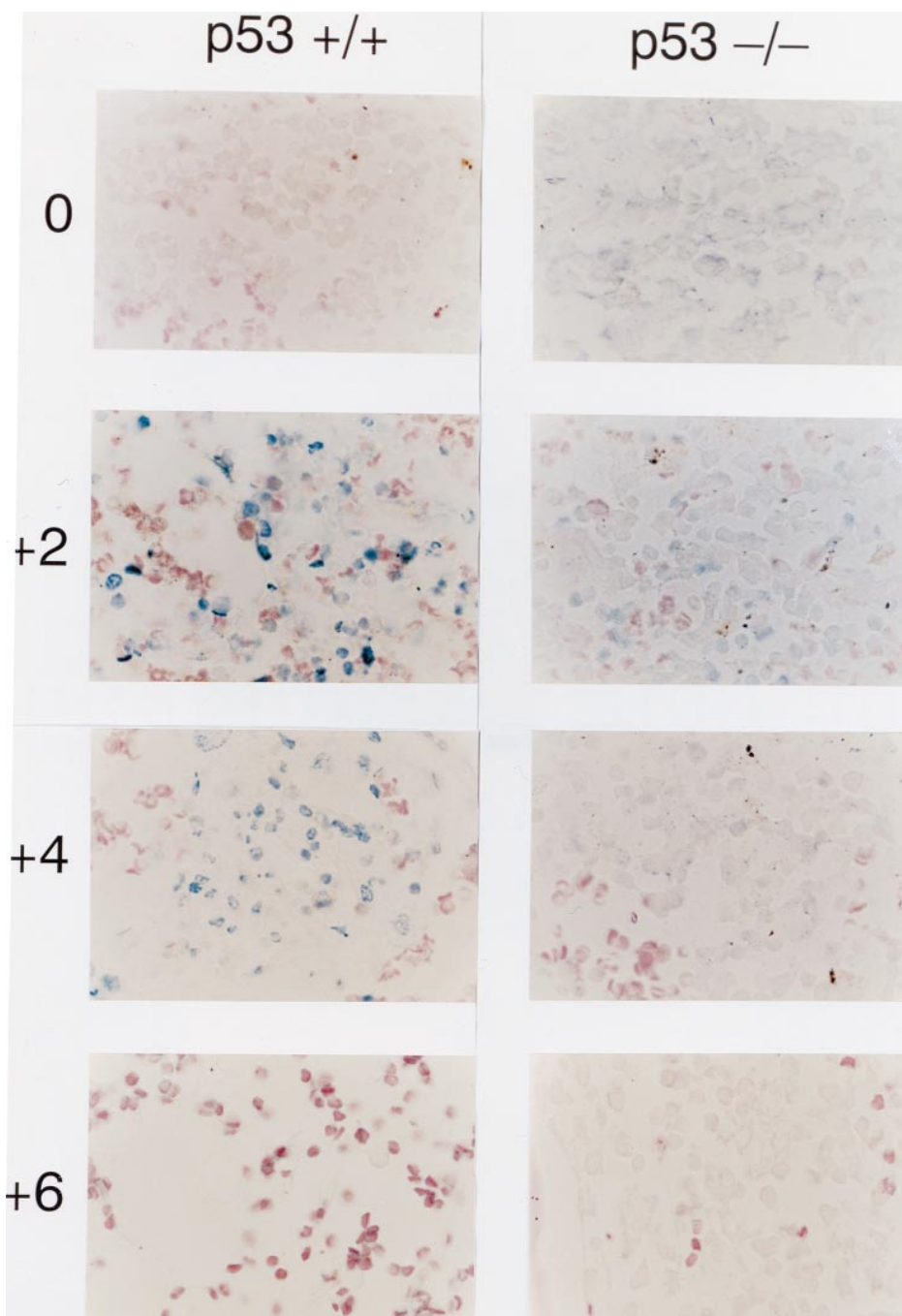
**Fig 2. Induction of apoptosis by p53 after 5-FU treatment.** Apoptotic cells were detected by TACS apoptosis assay in femoral bone marrows of p53+/+ (left panel) and p53−/− (right panel) mice on days 0, 2, 4, and 6 after 5-FU injection. Results are representative of three independent experiments.

CD34$^{high/+}$ cells was threefold to fourfold higher in the p53−/− population. No difference in the content of CD34$^{high/+}$ and CD34$^{low/−}$ populations was observed in BMCs obtained from p53−/− and p53+/+ mice not treated with 5-FU (data not shown).

To examine the role of p53 in the exhaustion of the HSC pool, $10^4$ CD34$^{low/−}$ and CD34$^{high/+}$ cells with the p53+/+ or p53−/− genotype were plated in methylcellulose in the presence of KL, IL-3, and Epo. Colonies were counted every 10 days and cells were collected and replated. Colony-forming ability of both CD34$^{low/−}$ and CD34$^{high/+}$ populations isolated

from BMCs of 5-FU–treated p53−/− mice persisted after more replatings as compared with that from p53+/+ BMCs (Fig 6A). p53 did not have any influence on lineage commitment and differentiation status of these cells, as indicated by morphological analysis of Wright-Giemsa–stained cytospin preparations of cells used for each replating (data not shown). All types of colonies showed predominantly cells of myeloid origin. Whereas the early 10-day cultures showed mainly myeloid precursors, the later 20- and 30-day cultures showed a preponderance of more mature myeloid cells, including neutrophils and macrophages, regardless of the origin and phenotype of the cells used
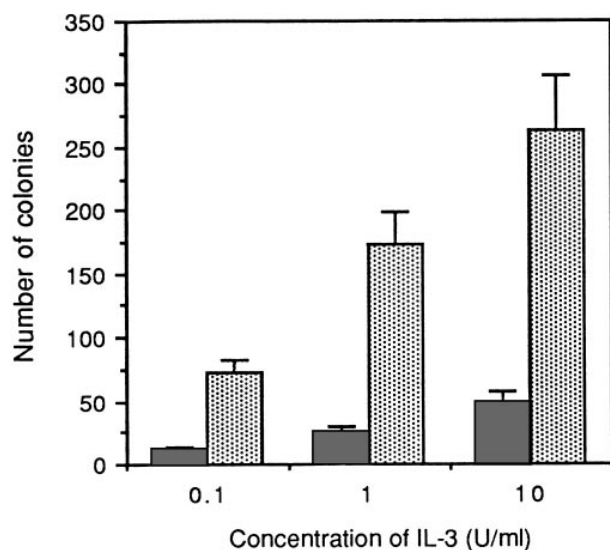
**Fig 3. Increased sensitivity of p53−/− BMCs to stimulation by IL-3.** p53+/+ (black bars) and p53−/− (shadowed bars) cells collected on day 6 after 5-FU administration were incubated in methylcellulose in the presence of the indicated concentrations of IL-3. Colonies and clusters were scored 10 days later. Results are mean (standard deviation) of three experiments.

responsible for the increased number of HSCs and the decreased rate of exhaustion of the HSC pool after treatment with 5-FU.

*Role of p53 in the recovery of lineage-specific progenitors after 5-FU treatment in vivo.* Morphological analysis of p53+/+ and p53−/− BMCs recovering after 5-FU treatment showed the presence of all major lineage-specific precursors for myelopoiesis, erythropoiesis, megakaryopoiesis, and lymphopoiesis (data not shown). Consistent with those findings, immunophenotyping analysis of these cell populations showed no statistically significant differences in the percentage of the following single-stained cell populations: B220+ and Ig+ cells (B lymphocytes and their precursors), CD3+ cells (T cells), TER-119+ cells (erythroid precursors), Mac-1 (myeloblasts and monocytes), F4/80+ (monocytes and macrophages), and Gr-1+ (granulocytes; data not shown). These data suggest that the p53 status does not affect the differentiation of post–5-FU regenerating marrow cells.

To analyze the effect of p53 on the proliferative potential of various lineage-specific precursors in BMCs regenerating after 5-FU treatment, in vitro clonogenic assays were performed in the presence of several growth factors. The colony-forming ability of myeloid (CFU-GM with IL-3), erythroid (BFU-E with KL + Epo), megakaryocytic (CFU-Meg with IL-6 + Epo), B-lymphocytic (B-CFU with IL-7) and T-lymphocytic (T-CFU with IL-2) precursors, as well as of stromal cells (CFU-F) was
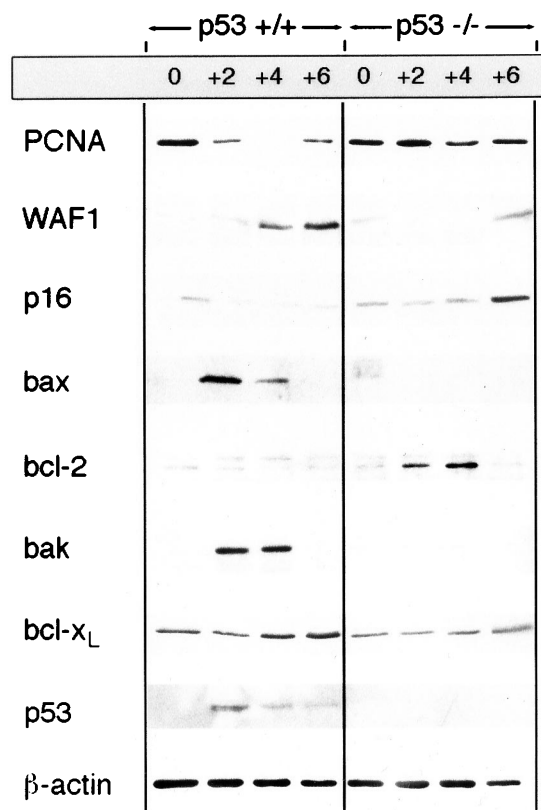
to establish the cultures. Consistent with the methylcellulose replating experiments, LTC-IC assay showed that 5-FU–treated p53−/− BMCs retained the ability to generate hematopoietic colonies for a longer time than the p53+/+ counterparts (Fig 6B).

The effect of p53 on 5-FU–induced exhaustion of primitive HSCs was also examined in spleen colony formation assays. Thus, 10⁵ BMCs obtained from p53−/− and p53+/+ mice 6 days after 5-FU treatment were injected into TBI recipients and 12 days later CFU-S were scored. A total of 10⁵ BMCs harvested from the mice used to examine CFU-S formation were retransplanted into secondary TBI-treated recipients and pre–CFU-S were counted 12 days later. p53 had only a moderate effect on the number of CFU-S formed by post–5-FU BMCs (Fig 6C). Morphological analysis of the colonies showed no major differences. Numerous large pre–CFU-S were formed by post–5-FU recovering p53−/− BMCs in secondary recipients (Fig 6C), whereas p53+/+ marrow cells did not form classical pre–CFU-S, although numerous microscopic nodules of hematopoiesis were noted. At low power, microscopy of p53−/− pre–CFU-S showed large cellular nodules obliterating normal splenic architecture. At high power, colonies composed of both erythroid and myeloid cells, with the latter showing partial inhibition of maturation, were evident. In contrast, evaluation of the p53+/+ microscopic nodules showed an overall, preserved architecture of the spleen with small, but numerous, cellular aggregates. High power view (×600) showed mixed hematopoietic cell populations with a preponderance of erythroid precursors and a smaller number of myeloid cells. No difference in CFU-S– and pre–CFU-S–forming ability was found in p53+/+ and p53−/− BMCs obtained from mice not treated with 5-FU (data not shown).

Together, the above results indicate that the absence of p53 is



**Fig 4. Western blot analysis of cell-cycle related and apoptosis-related proteins in p53+/+ and p53−/− BMCs repopulating after 5-FU treatment.**

**Table 1. Long-Term Repopulation Ability of Post–5-FU Recovering p53+/+ and p53−/− BMCs**

| No. of Cells Injected | No. of Survivors/Total | | |
|---|---|---|---|
| | Source of Cells: | p53 +/+ | p53 −/− |
| $10^5$ | | 4/10 | 8/10 |
| $10^4$ | | 0/10 | 2/10 |
| $10^3$ | | 0/10 | 0/10 |

TBI-treated recipient mice were injected IV with the indicated numbers of p53−/− or p53+/+ BMCs collected on day 6 after 5-FU treatment. Survival was scored 16 weeks later. Results represent two independent experiments.

higher in post–5-FU regenerating p53−/− than in p53+/+ BMCs (Fig 7).

## DISCUSSION

The regeneration of mature blood cells after cytotoxic treatment results from the proliferative activity of a small number of HSCs that have a high, but limited, capacity for self-renewal.[22,23] Despite much recent progress in identifying cytokines that regulate the proliferation of HSCs and lineage-specific descendants after cytoreductive therapy, the genetic mechanism responsible for the intrinsic control of self-renewal and differentiation of these cells remains largely undefined. Increasing evidence points to the importance of the p53 tumor suppressor gene in regulating hematopoiesis.[2-7] Using marrow cells from p53 knockout and wild-type mice, we found that p53 can have a profound effect on the proliferation and exhaustion of HSCs as well as on the proliferation of lineage-specific precursors in bone marrow regenerating after 5-FU treatment. However, p53 had no measurable effects on lineage determination and differentiation of these cells. Nevertheless, this 5-FU–induced dysregulation of cell growth does not appear to lead to leukemia and is not translated into any altered output of mature blood cells or altered commitment to any specific blood cell lineage, at least during our period of observation (5 months). Our data also suggest that other regulatory mechanisms involving p16[INK4a] and WAF-1 may compensate the loss of function of p53. The changes that occur in the stem cell population during the recovery from 5-FU treatment seem likely to reflect relevant physiological processes that are important during bone marrow transplantation and chemotherapy procedures.

Randall and Weissman[24] reported that after 5-FU treatment, the number of HSCs (Lin− Sca-1+ c-Kit+ Thy1.1[low]) in p53+/+ mice begins to increase on day 2, and approximately 50% of these cells are in S/$G_2$/M phase on day 6. Thus, BMCs for most of our studies were collected on day 6 after 5-FU

injection. The finding that fewer p53−/− BMCs regenerating after 5-FU treatment are required to rescue long-term hematopoiesis in TBI-treated recipient mice suggests that these cells contain more HSCs than their p53+/+ counterparts. Indeed, the percentage of Lin− Sca-1+ c-Kit+ cells, which are highly enriched with HSCs,[21] was almost threefold higher in p53−/− than in p53+/+ BMCs recovering after 5-FU treatment. No differences were observed in untreated mice. However, the pool of HSCs can be subdivided into long-term reconstituting primitive HSCs (p-HSCs), with extensive self-renewal capacity, and transiently repopulating HSCs (t-HSCs) that only self-renew in mice for 3 to 6 weeks.[25] Both populations are essential, at different times, for the rescue of hematopoiesis in TBI-treated animals. Recently, the immunophenotype of both populations have been determined[15] as Lin− Sca-1+ c-Kit+ CD34^{low/−} (p-HSCs) and Lin− Sca-1+ c-Kit+ CD34[high/+] (t-HSCs). We found that the percentage of p-HSCs in p53−/− and p53+/+ BMC populations is similar after 5-FU administration and also in untreated mice (data not shown). However, 5-FU was responsible for an almost fourfold increase in the percentage of t-HSCs in p53−/− BMCs as compared with p53+/+ BMCs. Thus, it seems likely that the long-term repopulating capacity of 5-FU–treated p53−/− BMCs results from the increased content of t-HSCs, which are essential for hematopoietic recovery during the critical initial weeks after transplantation until the p-HSC descendants become the major source of long-term hematopoiesis. Moreover, p53−/− mice contained five to eight times more BMCs than did p53+/+ mice, so that at 6 days after 5-FU treatment the number of p-HSCs and t-HSCs were five to eight times and 20 to 32 times higher, respectively, in the p53−/− mice. Bone marrows in long-term reconstituted animals transplanted with a 1:1 mixture of p53−/− and p53+/+ BMCs regenerating after 5-FU contained 90% to 99% of p53−/− cells. Thus, even if the percentage of p-HSCs is similar in 5-FU-treated p53−/− and p53+/+ mice, the long-term repopulating activity of the former cells is much higher, possibly because of the increased production of t-HSCs. Apparently, p53 plays an important role in BMC regeneration after 5-FU treatment by controlling not only the total number of p-HSCs in the organism, but also the production of their early descendants, t-HSCs, which represent the "functional" subpopulation responsible for the actual repopulation.

Because the increased production of t-HSCs may reduce the pool of p-HSCs, we investigated the role of p53 in the exhaustion of the HSC pool after 5-FU treatment. Unfortunately, we could not use the in vivo BMC serial retransplantation assay, because most of the mice transplanted with p53−/− BMCs developed lymphomas after 5 to 6 months, consistent

**Fig 5. Competitive long-term reconstitution assay. Detection of the p53 knock-out and the wild-type allele in BMCs of TBI-treated mice injected 16 weeks earlier with a 1:1 mixture of p53+/+ and p53−/− BMCs by PCR followed by Southern blotting (left panel: 1, 2, 3, and 4 individual mice). Known mixtures of p53+/+ and p53−/− cells (percentages indicated on the top or bottom of the blot) were analyzed to generate a calibration blot (right panel).**

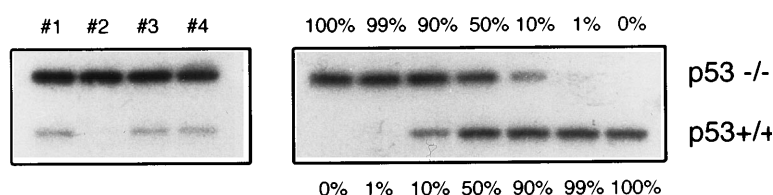Table 2. Frequency of HSCs in Post–5-FU Regenerating
p53+/+ and p53−/− BMCs

| Immunophenotype | Frequency of HSCs (%) | | |
|---|---|---|---|
| | Source of Cells: | p53+/+ | p53−/− |
| Lin⁻ Sca-1⁺ c-Kit⁺ CD34low/⁻ | | 0.022 ± 0.020 | 0.021 ± 0.016 |
| Lin⁻ Sca-1⁺ c-Kit⁺ CD34high/⁺ | | 0.037 ± 0.021 | 0.137 ± 0.021 |
| Lin⁻ Sca-1⁺ c-Kit⁺ | | 0.059 | 0.158 |

Cell subpopulations were obtained after immunosorting p53−/− or p53+/+ BMCs collected on day 6 after 5-FU treatment. Results are mean ± standard deviation from three independent experiments.

with previous observations.[1,26] Therefore, we used an in vivo pre–CFU-S assay and in vitro LTC-IC and methylcellulose replating assays. Although p53−/− and p53+/+ BMCs after 5-FU treatment developed similar numbers of CFU-S only p53−/− BMCs regenerated cells forming pre–CFU-S. Thus, p53 expression facilitates the exhaustion of cells forming pre–CFU-S, which are believed to belong to the p-HSC pool.[27] LTC-IC assay and methylcellulose replating assay confirmed the longer duration of colony-forming ability in p53−/− than in p53+/+ HSCs regenerating after 5-FU. In summary, both in vivo and in vitro assays showed that the absence of p53 in

BMCs regenerating after 5-FU treatment not only increases the pool of HSCs, but also delays their exhaustion. The increase of the total number of p-HSCs in p53−/− mice after 5-FU treatment may reflect the reduced sensitivity to apoptosis and increased proliferative capacity of p53−/− BMCs. The quiescent state of most p-HSC[28] may explain the absence of any difference in the percentage of these cells after 5-FU treatment. In contrast, most t-HSCs may represent cycling cells and, because p53−/− cells have a proliferative advantage, the total number as well as the percentage of those cells in BMCs regenerating after 5-FU treatment is increased in p53−/− mice as compared with p53+/+ mice. In accord with this hypothesis, p53−/− BMCs recovering after 5-FU treatment displayed a much higher multilineage clonogenic activity in the presence of growth factors. This effect appeared to rest in the proliferative advantage of p53−/− cells rather than the different number of precursor cells, because immunophenotypic analysis showed no significant differences in the content of lineage-specific precursors in p53−/− and p53+/+ BMCs. It is also possible that in the absence of p53, the production of t-HSCs from p-HSCs is increased by a different unknown mechanism.

Our results suggest that p53 is a key regulator of the proliferation of hematopoietic progenitor cells responsible for long- and short-term repopulation, whereas it does not affect
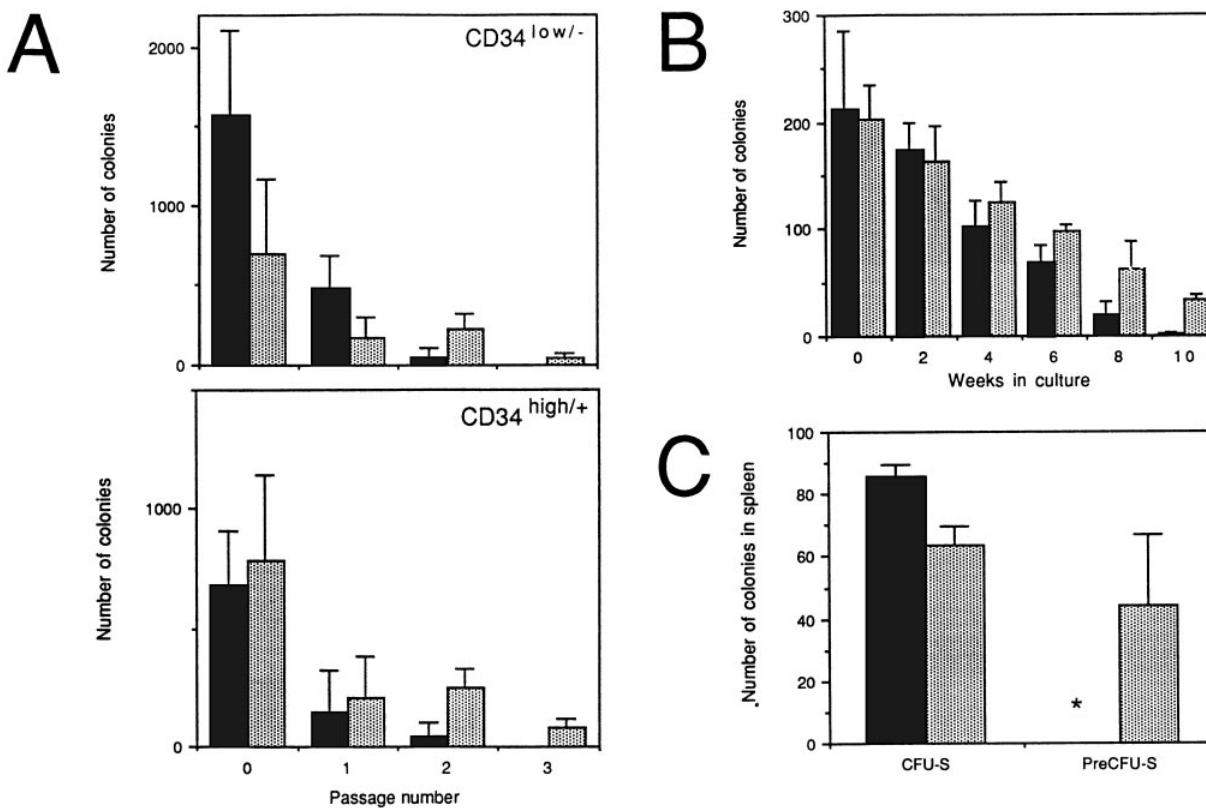


Fig 6. Role of p53 in HSC exhaustion. (A) Number of colonies arising from 10⁴ Lin⁻ Sca-1⁺ c-Kit⁺ CD34low/⁻ (upper panel) or Lin⁻ Sca-1⁺ c-Kit⁺ CD34high/⁺ (lower panel) cells passaged every 10 days in methylcellulose semisolid medium containing KL, IL-3, and Epo. Results are mean (standard deviation) from three experiments. (B) BMCs were cocultured on irradiated stromal layers and clonogenic activity was measured every 2 weeks in the presence of KL. Results are mean (standard deviation) from two independent experiments (3 mice per experiment). (C) CFU-S and pre–CFU-S were counted on day 12 after BMC transplantation. Mean (standard deviation) from six mice. * Only microscopic colonies were present. Black and shadowed bars represent results from p53+/+ and p53−/− cells, respectively.
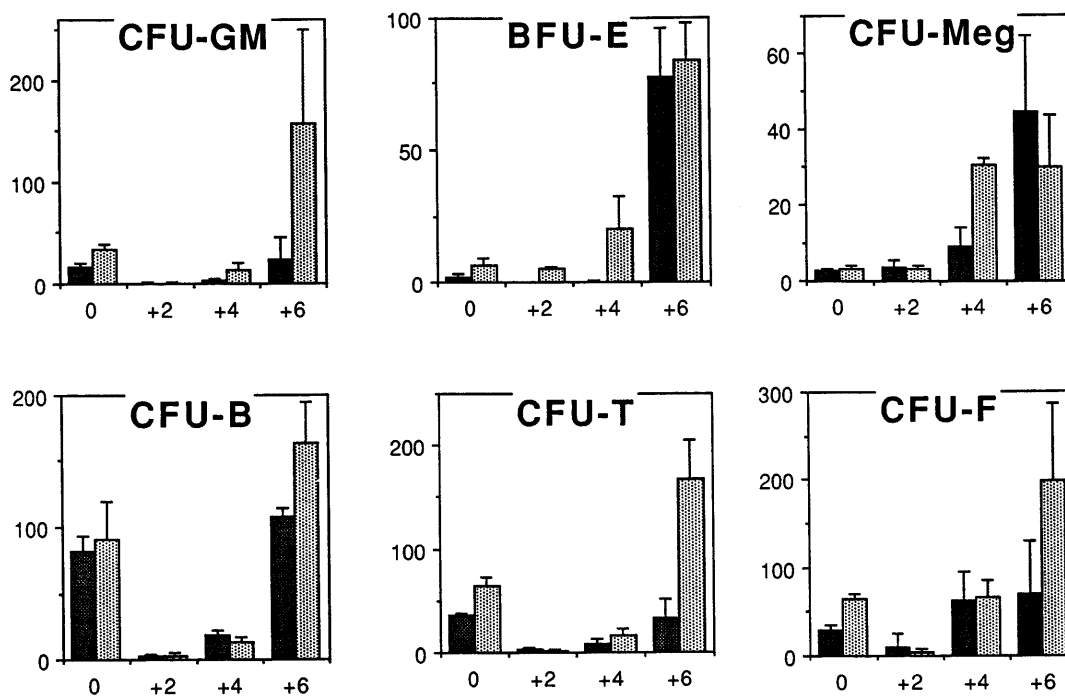
Fig 7. Clonogenic activity of various hematopoietic progenitors and stromal cells in bone marrows of p53+/+ (black bars) and p53−/− (shadowed bars) mice analyzed on days 0, 2, 4, and 6 after 5-FU treatment. Results are mean (standard deviation) from three independent experiments. Y axis, number of colonies; X axis, days after 5-FU treatment.

lineage determination and differentiation of committed progenitor/precursor cells. These findings raise the possibility that the manipulation of p53 expression might delay the decline of HSCs that occurs after chemotherapy and/or bone marrow transplantation.

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