

Moran Foundation 2005-2006 Progress Report

Project Title: “Control of ELF4-gene expression”
Principal Investigator: H. Daniel Lacorazza, Ph.D.
Award Period: August 1st, 2005 – June 30th, 2006

Hypothesis and Specific Aims

Leukemic stem cells (LSC) are a small subset among leukemic cells believed to have properties similar to their progenitors: hematopoietic stem cells (HSC). The current chemotherapeutic approach is to target proliferating leukemic cells therefore sparing quiescent LSCs, which could potentially lead to relapses. The transcription factor ELF4 regulates the homeostatic proliferation of HSCs without perturbing significantly their proliferative response to stress.

The goal of this project is to study the regulation of ELF4 gene both in normal and leukemic cells and to identify factors that could regulate ELF4 expression. We propose to:

- 1- *Clone and characterize the ELF4 promoter*
- 2- *Develop a high-throughput cell-based assay to identify factors that could enhance the expression of ELF4 and the metabolic activity of hematopoietic cells.*

Results from this project will aid to define whether ELF4 expression is required to maintain leukemic stem cells and to identify small molecules able to modulate ELF4-expression.

Progress Report

Funds from this award supported in part the study of the behavior of HSCs in *Elf4*^{-/-} mice, work published last March in *Cancer Cell* as featured article on the cover (attached). Previous studies have implicated reduced levels of a transcription factor called ELF4 (a.k.a. MEF) with subtypes of leukemias, specifically acute myeloid leukemia (AML) of the subtype M2 and M3. We reported that ELF4, which is also targeted by the t(X:21)(q26;q22) in acute myelogenous leukemia, regulates the proliferation of primitive hematopoietic progenitor cells at steady state, controlling their quiescence. Hematopoietic stem cells are immature cells in the bone marrow that have the capacity to differentiate into all type of mature blood cells. *Elf4*-null HSC display increased residence in G₀-phase of the cell cycle with reduced *in vivo* incorporation of 5-bromodeoxyuridine and impaired cytokine-driven proliferation *in vitro*. Due to their increased HSC quiescence, *Elf4*-null mice are relatively resistant to the myelosuppressive effects of chemotherapy and radiation. Thus, ELF4 plays an important role in the decision of stem/primitive progenitor cells to divide or remain quiescent by regulating their entry to the cell cycle. A delicate

balance exists between self-renewal and differentiation of HSCs because the body must retain a sufficient population of HSCs while continually producing the multitude of new blood cells that are needed each day.

Bone marrow toxicity could be prevented by maintaining stem cells in a quiescence state during myeloablative treatments in cancer patients. However, another implication of this work is that *tumor stem cells* are more quiescent than more differentiated cancer cells and could use similar mechanisms to resist the effects of chemotherapy or radiation. Thus, we decided to study the regulation of ELF4 gene to then identify modulators of its expression. The advances made in each Aim are:

Aim 1- Clone and characterize the ELF4 promoter

ELF4 promoter is located within a 2-kb region upstream Exon 1 in a genomic sequence that contains a GC-rich region (1.3-kb CpG island). Several fragments within this region (Fig. 1) were amplified using primers that insert a KpnI and XhoI sites in the 5' and 3' ends respectively and human genomic DNA purified from KG-1 cells. Purified PCR fragments were ligated into the KpnI and XhoI sites of the pGL3-basic construct (promoter-less luciferase reporter gene) and confirmed by sequencing. This promoter region contains many regulatory sequences with putative binding sites for ETS-proteins, AML1, cEBP/ α , GATA3, a canonical T-box and RxR elements. We are in the process of testing these transcriptional regulators using ELF4-Luc plasmids. Preliminary, we found that ELF4, a member of the ETS-family of transcription factors, seems to regulate its own expression (Fig. 2). This needs to be further studied by site-directed mutagenesis of these sites. Noteworthy, a subset of patients with acute myeloid leukemia present low ELF4 levels, which could be associated to the presence of the oncoproteins AML1-ETO or PML-RAR α . In fact, AML1-ETO is able to block ELF4 self-regulation using same promoter assay (not shown) suggesting that deregulated ELF4 expression could be leukemogenic. One hypothesis is that AML1-ETO binds to DNA-bound ELF4 and recruit co-repressor molecules to ELF4 regulated genes.

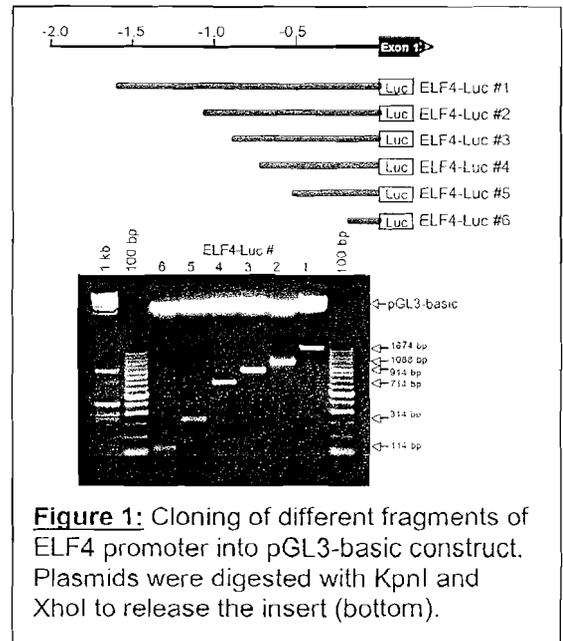


Figure 1: Cloning of different fragments of ELF4 promoter into pGL3-basic construct. Plasmids were digested with KpnI and XhoI to release the insert (bottom).

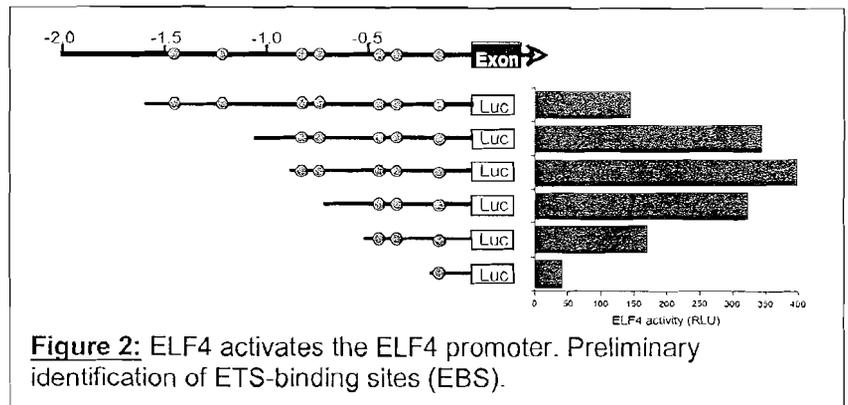


Figure 2: ELF4 activates the ELF4 promoter. Preliminary identification of ETS-binding sites (EBS).

Aim 2- Develop a high-throughput cell-based assay to identify factors that could enhance the expression of ELF4 and the metabolic activity of hematopoietic cells.

We are moving into this second aim in the near future. We will test first different hematopoietic cell lines to transfect with the ELF4-Luc plasmid by using the nucleofector system (Amaxa). Transfected cells will be then tested with different cytokines and growth factors (stem cell factor, IL-3, Flt3L, stroma derived factor 1, retinoic acid) and the activation of ELF4 promoter will be measured using a luminometer.

Significance

The normal balance between the self-renewal and differentiation of HSCs is perturbed in several leukemias. Quiescence is an alternative state for HSCs, and several genes that encode cell cycle regulatory proteins or transcriptional regulators have been shown to regulate this process. Treatments that decrease MEF/ELF4 expression could potentially improve hematopoietic recovery in cancer patients undergoing myeloablative treatments. However, quiescent leukemic stem cells could use similar mechanisms to escape from the cytotoxic effects of chemotherapy.

Passague and Wager highlighted our finding in a preview entitled “Regulating quiescence: new insights into hematopoietic stem cell biology” that appeared in *Developmental Cell* 2006 10: 415-417: *“using an impressive array of in vitro and in vivo assays, the authors show that loss of MEF expression in Mef-null mice slow HSC proliferation in response to cytokine stimulation and leads to an accumulation of mitotically quiescent HSC in vivo... the demonstration by Lacorazza and colleagues that HSC quiescence is an actively and dynamically controlled state that can be manipulated by particular transcriptional regulators suggests exciting possibilities for biomedical applications”*

Publications:

Lacorazza, H.D., Yamada, T., Liu, Y., Miyata, Y., Sivina, M., Nunes, J., and Nimer, S. The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. **Cancer Cell** (2006) 9, 175-187. (Article featured on the cover).

Lacorazza, H.D., and Nimer, S. Genetic Control of Stem Cell Quiescence. Review by invitation for **Cell Cycle**. *In preparation*.

Presentations:

2005- Department of Immunology, Retreat. “Role of the transcription factor ELF4 in Immunity and Hematopoiesis”.

2006- The Dan L. Duncan Cancer Center, Molecular Carcinogenesis Program, Baylor College of Medicine. “The transcription factor ELF4 regulates stem cell quiescence. Role in cancer”

Note: This project is still active

The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells

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Summary

The transcriptional circuitry that regulates the quiescence of hematopoietic stem cells is largely unknown. We report that the transcription factor known as MEF (or ELF4), which is targeted by the t(X;21)(q26;q22) in acute myelogenous leukemia, regulates the proliferation of primitive hematopoietic progenitor cells at steady state, controlling their quiescence. Mef null HSCs display increased residence in G₀ with reduced 5-bromodeoxyuridine incorporation in vivo and impaired cytokine-driven proliferation in vitro. Due to their increased HSC quiescence, Mef null mice are relatively resistant to the myelosuppressive effects of chemotherapy and radiation. Thus, MEF plays an important role in the decision of stem/primitive progenitor cells to divide or remain quiescent by regulating their entry to the cell cycle.

Introduction

The hematopoietic stem cells (HSCs) in the bone marrow continuously generate progeny that maintain a steady flow of peripheral blood cells. HSCs can remain quiescent, enter senescence, die, self-renew, or differentiate into multiple lineages. Adult HSCs are maintained quiescent in bone marrow niches where osteoblasts provide self-renewal signals, mainly BMP and Notch ligands (Calvi et al., 2003; Zhang et al., 2003). However, the downstream molecules involved in this process are largely unknown. At steady state, cytokines and chemokines drive a small percentage of HSCs to self-renew in order to maintain a constant number of stem cells. Following bone marrow ablation, by cytotoxic agents or radiation, stem cells are recruited from their quiescent niche to promote the rapid reconstitution of a depleted hematopoietic system. Stem cell quiescence is essential to protect the HSC pool from cell cycle-dependent injury (e.g., from toxins including chemotherapy) and from acquiring mutations during numerous rounds of replication. At the same time, the proper expansion and differentiation of HSCs is crucial for basal hematopoietic cell production and for reconstitution following myelotoxic injury.

A delicate balance between dormancy and cell cycle entry must take place to ensure maintenance of stem cell numbers

and adequate production of mature blood lineages. These events need to be tightly regulated, particularly during the transition of HSCs from G₀ to G₁ (from a quiescent to a cycling and potentially differentiating state). Most research has focused on mechanisms of self-renewal, and there is little information on the regulation of stem cell dormancy and its reentry into the cell cycle. The ability to expand HSCs would have clear clinical applications to stem cell transplantation and the correction of genetic disorders via gene transfer. Conversely, altering HSC dormancy could improve or impair the pace of hematopoietic recovery after bone marrow transplantation or chemotherapy. Furthermore, understanding how normal HSCs regulate quiescence and entry into the cell cycle will set the stage for defining these properties in leukemic stem cells.

MEF, also known as ELF4, is a member of the ETS family of winged helix-turn-helix transcription factors (Lacorazza and Nimer, 2003; Mao et al., 1999; Miyazaki et al., 1996, 2001). Recent studies have implicated MEF in tumorigenesis. The MEF gene is located on Xq26, within a region of LOH in advanced ovarian and breast carcinomas (Choi et al., 1997, 1998; Piao and Malkhosyan, 2002). While the MEF gene has tumor suppressor gene activity in lung carcinoma cell lines (Seki et al., 2002), MEF has also been identified as a site of integration in retroviral mutagenesis studies using the *Eμ-Myc Pim1^{-/-} Pim2^{-/-}*

SIGNIFICANCE

The normal balance between the self-renewal and differentiation of HSCs is perturbed in acute leukemia. Quiescence is an alternative state for HSCs, and several genes that encode cell cycle regulatory proteins or transcriptional regulators have been shown to regulate this process. We show that MEF, an ETS protein found rearranged by a rare chromosomal translocation in AML, regulates the quiescence of HSCs by facilitating cell cycle entry. MEF null hematopoietic cells demonstrate resistance to myelotoxic injury, conferring recipient mice with enhanced hematological recovery following chemotherapy or radiation exposure. Treatments that decrease MEF expression could potentially improve hematopoietic recovery in cancer patients undergoing myeloablative treatments. However, quiescent leukemic stem cells could use similar mechanisms to escape from the cytotoxic effects of chemotherapy.

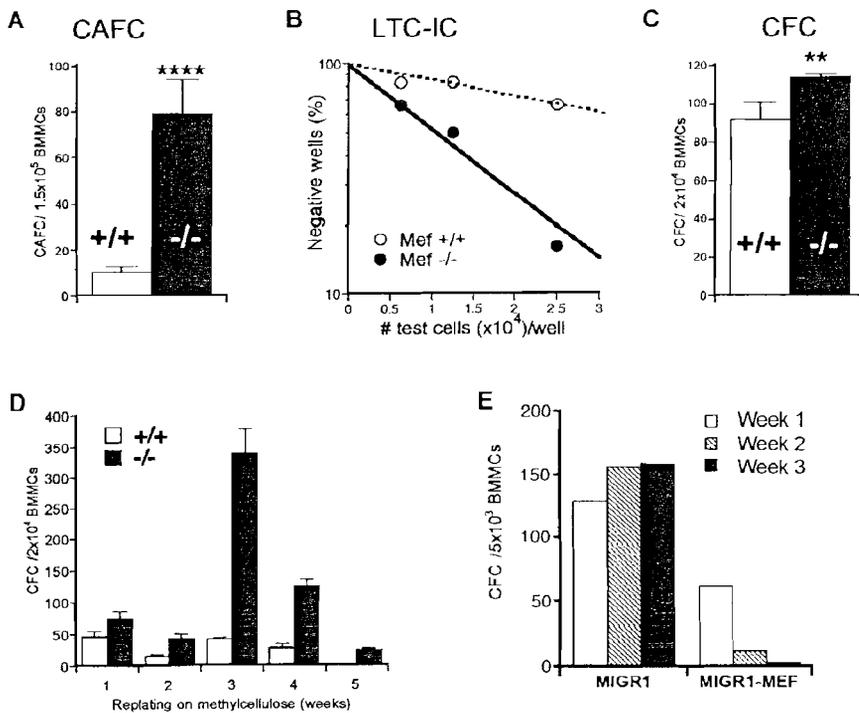


Figure 1. The number of CAFCs but not CFCs is increased in *Mef*^{-/-} bone marrow cells at steady state

A: The steady-state level of bone marrow primitive progenitor cells was evaluated using the cobblestone area-forming cell assay, scoring colonies at week 5 (*****p* < 0.0001).

B: LTC-IC was also used to enumerate primitive progenitor cells, using limiting dilutions of BMMCs that were cultured for 5 weeks on MS5 stroma and then cultured on methylcellulose for the readout.

C: Myeloid progenitors were quantified by methylcellulose culture using BMMCs from *Mef*^{+/+} or *Mef*^{-/-} mice (***p* < 0.01).

D: Methylcellulose cultures were serially replated weekly, for 5 weeks.

E: *Mef*^{-/-} BMMCs were transduced with retroviruses containing either the MEF cDNA or the empty vector; GFP-positive cells were purified and serially replated on methylcellulose culture for 3 weeks.

Data are shown as mean \pm standard deviation (*n* = 5).

and *cdkn2a*^{-/-} murine models (Akagi et al., 2004; Suzuki et al., 2002), and in MSCV-Sox4 virus-induced leukemia as well (Du et al., 2005). Three groups have reported that MEF expression is repressed by the AML1-ETO and PML-RAR α leukemia-associated fusion proteins, suggesting a role for dysregulation of MEF expression in the pathogenesis of acute myeloid leukemias (AML) (Alcalay et al., 2003; Muller-Tidow et al., 2004; Park et al., 2003). An analysis of MEF levels in AML patient samples showed that MEF RNA levels are particularly low in FAB M2 and M3 AML cells, which contain AML1-ETO and PML-RAR α , respectively, yet MEF RNA was detectably expressed in all samples (Fukushima et al., 2003). The recent identification of an ELF4-ERG fusion transcript generated by the t(X;21)(q26;q22) in a patient with AML definitively endorses the involvement of MEF (ELF4) in hematological malignancies (Moore et al., 2005).

Given the potential importance of MEF downregulation in human leukemic cells, we evaluated the behavior of hematopoietic stem and progenitor cells in *Mef*-deficient mice. We found that the bone marrow of *Mef*^{-/-} mice contains a higher fraction of HSCs (Lin⁻ Sca-1⁺ c-kit⁺ Flt3⁻ CD34⁻ cells), with more "side population" (SP) cells and more cobblestone area-forming cells (CAFCs). *Mef* null HSCs are more quiescent than normal at steady state, with lower 5-bromodeoxyuridine (BrdU) incorporation in vivo and minimal response to cytokine-driven proliferation in vitro. Nonetheless, *Mef* null HSCs can reconstitute an ablated host and compete with wild-type HSCs in secondary transplants. This quiescence confers *Mef*^{-/-} mice, and wild-type mice transplanted with *Mef*^{-/-} bone marrow cells, with clinically important protection from myelotoxic drugs (such as 5-FU and busulfan) and from radiation. The low levels of MEF expression that are seen in certain subtypes of AML may play an important role in AML pathogenesis by altering the growth properties of leukemic stem cells similarly to that observed in *Mef* null HSCs.

Results

Mef regulates the proliferation of hematopoietic progenitor cells

Mef RNA is expressed in lymphoid and myeloid cells including Lin⁻ Sca-1⁺ c-kit⁺ (LSK) cells, a population enriched in HSCs (Figure S1 in the Supplemental Data available with this article online). As this transcription factor is also involved in lymphoid cell development (Lacorazza et al., 2002) and is regulated during the cell cycle (Miyazaki et al., 2001), we examined whether MEF also plays a critical role in the behavior of HSCs in the bone marrow.

We first examined the number of primitive hematopoietic cells in the bone marrow of *Mef*^{-/-} compared to wild-type controls. We used the CAFC assay and the long-term culture-initiating cell (LTC-IC) assay, since they correlate with the in vivo repopulating potential of primitive hematopoietic progenitors (Ploemacher et al., 1991). *Mef*^{-/-} bone marrow cells contain at least 5-fold more CAFCs compared to wild-type bone marrow (Figure 1A). The increased frequency of primitive progenitors was also evident in the LTC-IC assay (Figure 1B). In contrast, methylcellulose colony-forming unit (CFU) assays, performed to quantify myeloid progenitor cells, showed no significant differences in the number or type of colonies formed (Figure 1C and data not shown), a result that is consistent with the normal blood counts observed in *Mef*^{-/-} mice (Table S1). Taken together, these data indicate the accumulation of primitive hematopoietic cells rather than committed progenitors in the absence of Mef.

To further address the frequency of primitive progenitors in *Mef* null bone marrow, we performed serial replating studies using methylcellulose cultures (Figure 1D). Wild-type BMMCs cannot be replated more than three times, due to the progressive depletion of HSCs; in contrast, we saw a 8- to 10-fold higher frequency of primitive progenitors using *Mef*^{-/-} bone marrow cells

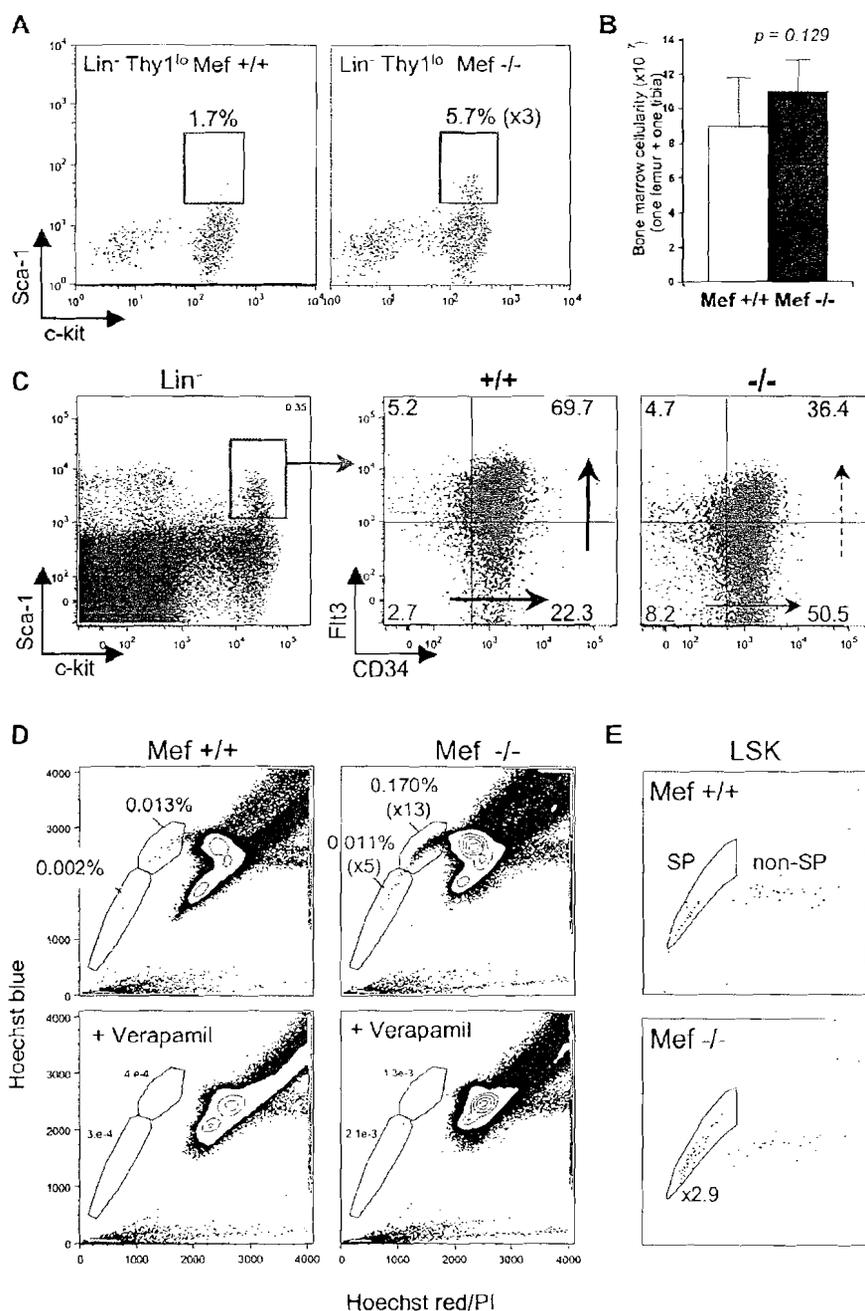


Figure 2. Increased primitive progenitor populations in the bone marrow of Mef-deficient mice

A: A greater percentage of Lin⁻ Thy1^{low} Sca-1⁺ c-kit⁺ cells are found in Mef^{-/-} bone marrow cells than in Mef^{+/+} bone marrow cells (representative dot plots are shown).

B: There is no difference in the bone marrow cellularity of wild-type and Mef-deficient mice (two femurs and two tibias were analyzed for six wild-type and six Mef^{-/-} mice; p = 0.130). Data are shown as mean ± standard deviation.

C: Expression of FIT3 and CD34 in LSK cells from wild-type and Mef^{-/-} mice. Mef null LSK cells show a significant increase of FIT3⁺CD34⁻ (3-fold) and FIT3⁻CD34⁺ (2.3-fold) LSK cells, which correspond to LT-HSC and ST-HSC. There is a decrease in the transition from ST-HSC to MPP (FIT3⁺CD34⁺ LSK cells).

D: Side population (SP) cells were identified by Hoechst 33342 staining and the use of blue and red filters (Goodell et al., 1996). The identification of SP cells was confirmed by their disappearance in the presence of Verapamil (50 μM). Two gates (SP-low and SP-intermediate) and the fold increase (Mef null/Mef wild-type) are shown.

E: SP cells contained within the Lin⁻ Sca-1⁺ c-kit⁺ population are also shown (Mef null cells show a 2.9-fold increase over wild-type).

at week 3. This was not sustained in later passages, showing that progenitors lacking Mef are not immortal. Rather, we believe that the additional time required for these cells to mature into committed progenitors indicates their primitiveness and ability to retain stemness in culture. This replating was seen using purified Mef^{-/-} Lin⁻ Sca-1⁺ c-kit⁺ cells and not Lin⁻ Sca-1⁻ c-kit⁺ cells (Figure S2), further demonstrating that primitive Mef null hematopoietic cells rather than committed progenitors have aberrant growth properties. To demonstrate that this effect is directly linked to the absence of Mef, we reintroduced Mef into Mef^{-/-} BMSCs using retroviruses that express MEF-IRES-GFP (MIGR1-MEF) or the empty IRES-GFP (MIGR1) and plated purified GFP-positive cells weekly in methylcellulose. Introduction of

Mef abrogated the “preservation” of early progenitor cells (Figure 1E), suggesting that restoring Mef expression restores the normal level of proliferation of Mef null hematopoietic progenitor cells, leading to their exhaustion in this in vitro assay.

These in vitro assays nicely correlate with the immunophenotypic characterization of various hematopoietic subsets within the bone marrow. Mef^{-/-} bone marrow contains three to four times more LSK Thy1^{low} cells than littermate controls (Figure 2A), based on their increased percentage in a normocellular bone marrow (Figure 2B). We detected more long-term HSC (LT-HSC) and short-term HSC (ST-HSC) based on Fit3 and CD34 expression (Fit3⁺CD34⁻ LSK cells = LT-HSCs and Fit3⁻CD34⁺ LSK cells = ST-HSCs [Yang et al., 2005]) in Mef null bone marrow

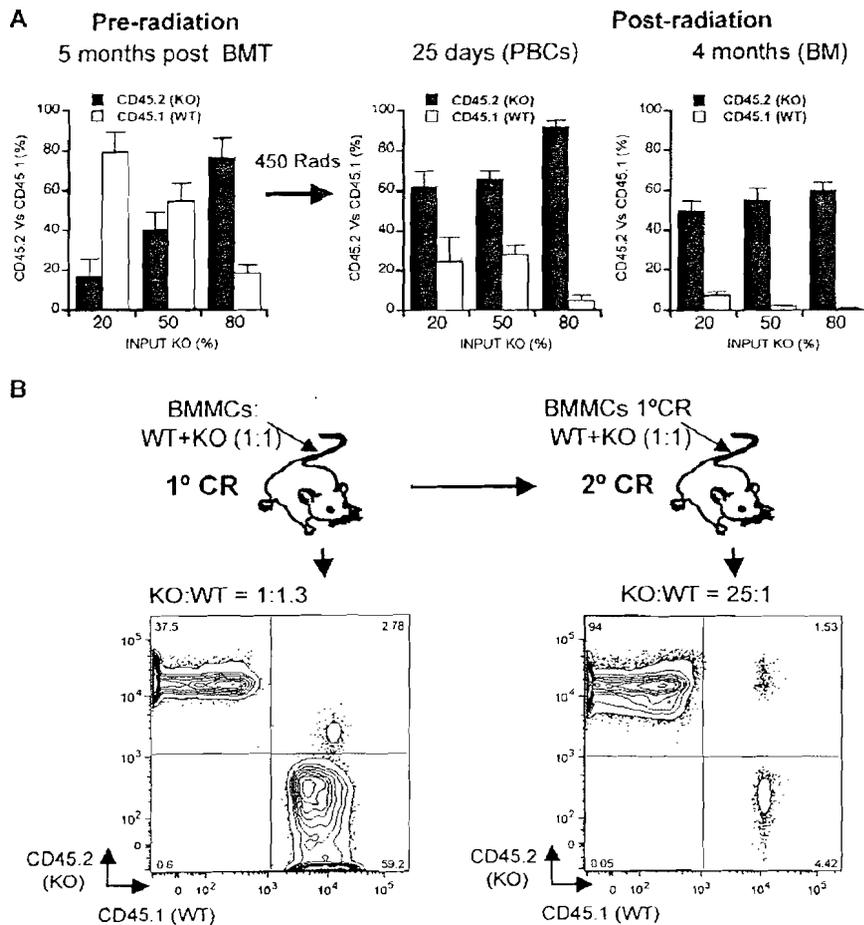


Figure 3. Transplantation studies to assess stem cell proliferation

A: The contribution of each donor (*Mef*^{+/+} versus *Mef*^{-/-}) to the peripheral blood cell populations 5 months after transplantation is shown for the competitive reconstitution study (far left). The same mice were then sublethally irradiated (450 rads), and the source of hematopoietic recovery was monitored in the peripheral blood 25 days after radiation. The contribution of donor cells was also evaluated in the bone marrow of recipient mice 4 months postirradiation (9 months posttransplant). Data are shown as mean \pm standard deviation ($n = 4$).

B: Primary recipients were transplanted with a mixture of wild-type and *Mef*^{-/-} BMMCs (1×10^5 BMMCs each). After reconstitution, PBCs showed a similar contribution of each donor. Wild-type (CD45.1⁺) and *Mef*^{-/-} (CD45.2⁺) cells were then purified from the bone marrow of these mice and then transplanted into secondary recipients in a ratio 1:1. After hematologic reconstitution, there is a predominance of *Mef* null-derived cells in peripheral blood.

cells than in wild-type cells (Figure 2C), with a concomitant decrease in Flt3⁺CD34⁺ LSK cells (multipotent progenitors [MPPs]). Although this suggests a developmental holdup in the maturation of HSCs to MPPs, stem cell function is not significantly affected, as *Mef* null LSK cells are able to reconstitute host hematopoiesis in a myeloablative transplant model (data not shown).

We used another assay to identify murine HSCs, namely dual-wavelength flow cytometric detection of cells stained with Hoechst 33342, which is based on the ability of HSCs to efflux this dye (Goodell et al., 1996). The Hoechst-stained SP identifies HSCs with long-term repopulating activity by a functional feature rather than by the expression of cell surface proteins. As expected, *Mef*-deficient BMMCs contain a 5-fold greater number of SP cells (Figure 2D), even gating on the highest dye efflux activity (SP^{low}) (0.002% versus 0.011% for +/+ and -/-, respectively). This population, SP^{low} cells, is known to be nearly homogenous for quiescent, long-term HSCs (Camargo et al., 2006). Since SP cells could include some committed progenitors, we also assessed SP cells within the LSK cell compartment and observed a consistent increase (at least 2.9-fold) in the number of SP-LSK cells (Figure 2E). To validate our SP cell detection, we also performed this assay in the presence of verapamil and saw disappearance of the SP cells (Figure 2D). Based on all these assays, we conclude that in the absence of MEF

hematopoietic stem/primitive progenitor cells accumulate in the bone marrow, primarily LT-HSCs and ST-HSCs.

Bone marrow transplant reveals a functional defect in the cell cycle

We used a competitive repopulating assay and the distinct congenic markers (CD45.1 and CD45.2) to define the long-term reconstituting ability of *Mef*^{-/-} BMMCs and to enumerate stem cell numbers. By injecting BMMCs from *Mef*^{-/-} (C57BL/6, CD45.2⁺) and *Mef*^{+/+} (B6.SJL, CD45.1⁺) into B6/SJL.F1 (which express CD45.1⁺ and CD45.2⁺) at different input ratios (containing 0%, 20%, 50%, 80%, or 100% *Mef*^{-/-} BMMCs) we can clearly examine the origin of blood cell production after reconstitution. We observed the same magnitude increase in LSK cells in the bone marrow of B6/SJL.F1 mice transplanted with 100% *Mef*^{-/-} BMMCs as in the *Mef* null mice (Figure S3), further demonstrating the cell-intrinsic nature of the primitive progenitor cell accumulation. The percentage of LSK cells in the bone marrow of these mice increases as the percent input of *Mef*^{-/-} BMMCs increases (Figure S3), yet we did not observe a greater contribution of *Mef*^{-/-} BMMCs to the steady-state circulating lymphoid cells (Figure S3) or myeloid cells (Figure 3) 5 months after hematologic reconstitution. This suggests that even though *Mef*^{-/-} BMMCs contain more primitive progenitors

they do not have a repopulating advantage over wild-type progenitors in a competitive situation.

The greater contribution of *Mef*^{-/-} HSCs following radiation could be due to either increased survival of *Mef*^{-/-} cells during the radiation or to enhanced proliferation in response to stress. We did not observe a competitive advantage over wild-type cells in primary competitive repopulation (CR) experiments, despite using different limiting doses (data not shown). We believe this illustrates a limitation of in vivo CR experiments, namely the use of this assay to determine the number of functionally abnormal stem cells (due to cell cycle defects). However, when we purified *Mef*^{+/+} (CD45.1+) and *Mef*^{-/-} (CD45.2+) cells, obtained from mice previously transplanted with a mixture of wild-type: knockout (1:1), and used these cells in a secondary CR assay, the vast majority of PBCs were derived from the *Mef*^{-/-} bone marrow cells, even though the donor mice had approximately 50:50 peripheral blood mixed chimerism at the time of the harvest (Figure 3B). *Mef*^{-/-} bone marrow cells clearly out-compete wild-type cells that are compromised from the primary transplant. Thus, although *Mef*^{-/-} BMMCs contain more HSCs with repopulating capacity, this can only be seen in a secondary CR, due to their cell cycle deregulation.

To recapitulate hematological recovery in mixed bone marrow chimera mice, animals transplanted for more than 5 months with both *Mef*^{+/+} and *Mef*^{-/-} cells were sublethally irradiated. The vast majority of the peripheral blood cells were at that time derived from the *Mef*^{-/-} progenitor cells (Figure 3A). Most importantly, 4 months following the radiation treatment (and 9 months after the initial transplant) nearly all the bone marrow cells of the transplanted mice were derived from *Mef* null stem cells (Figure 3A). This indicates that the proportion of *Mef*^{-/-} to wild-type HSCs in the bone marrow of transplanted mice was higher than the ratio of BMMCs initially transplanted and points to a proliferative defect at steady state that is overcome by the response to stress (i.e., radiation).

Mef regulates the quiescence of primitive hematopoietic progenitors

The accumulation of primitive progenitor cells in *Mef*^{-/-} mice could be cell intrinsic and due to enhanced self-renewal, quiescence, or survival. Alternatively, faulty environmental cues could lead to alteration of their behavior, reflecting cell extrinsic effects of *Mef* loss. CFSE studies showed normal homing ability; we also observed nearly normal G-CSF induced PBPC migration (data not shown). We next performed serial bone marrow transplants and saw no improvement in the survival of mice that received bone marrow cells serially transplanted four times (Figure S4). This implies that enhanced stem cell self-renewal may not be the primary cause of the stem cell accumulation that occurs in *Mef*^{-/-} mice, even though we did find greater CAFC and CFC activity in mice serially transplanted twice with purified *Mef* null LSK cells, indicating preservation of stem cell properties (Figure S4). The absence of a survival advantage in the serial transplantation assay, despite a larger pool of primitive progenitors, suggests that *Mef*^{-/-} HSCs have impaired proliferative properties.

To assess the ability of *Mef* null HSC to enter the cell cycle, we measured DNA content in purified LSK cells incubated for 24 hr in the presence and absence of SCF, IL-3, and IL-6. The percent of *Mef*^{-/-} LSK cells in S phase increased by only 24%, whereas wild-type LSK cells showed at least a 52% increase (Figure 4A).

Furthermore, by monitoring the growth of individual LSK cells, placed one cell per well into 96-well plates, we showed that *Mef*-deficient cells grow more slowly than wild-type cells (ten representative clones per genotype are shown in Figure 4B), demonstrating impaired cytokine responsiveness at the single LSK-cell level.

The impaired proliferation at steady state suggests decreased movement of stem cells into the cell cycle, perhaps the transition from G₀ to G₁. We used multiparameter flow cytometry (Pyronin Y and Hoechst 33342 to monitor RNA and DNA content, respectively) to distinguish between G₀ and G₁, as quiescent primitive progenitors are defined as cells with low Pyronin Y staining within the G₀/G₁ population of cells (Figure 4C). The percentage of cells in G₀ within the Lin⁻ Sca-1⁺ cells was clearly higher in the *Mef* null cells than the *Mef*^{+/+} control cells (Figure 4D). We also measured Ki67 expression on purified Lin⁻ Pyronin-Y^{low} cells; we found more Ki67-negative cells in *Mef* null LSK Pyronin-Y^{low} cells compared to wild-type, which was also evident by measuring Ki67 expression on gated LSK cells (Figure S5). The increased quiescence observed in *Mef* null cells suggests that *Mef* regulates the entrance of primitive progenitor cells into the cell cycle at steady state.

To confirm the reduced proliferation of LSK cells using in vivo assays, we determined the proportion of LSK cells that incorporate BrdU over a 2 day period. Nuclear BrdU was measured in LSK cells using a DNA-labeling protocol. Only 20% of *Mef* null LSK cells proliferated during 48 hr BrdU exposure while the BrdU incorporation into wild-type LSK cells was 60% (Figure 4E). Thus, *Mef* null LSK cells proliferate less than normal under steady-state conditions.

Mef null BMMCs are protected from cell cycle-dependent toxicity

The presence of more quiescent HSCs in *Mef*^{-/-} mice could result in enhanced recovery from myelosuppressive treatments that target cycling hematopoietic cells. To measure the kinetics of hematopoietic recovery, we administered a single dose of a chemotherapeutic agent (i.e., 5-FU) and serially followed peripheral blood counts. 5-FU-treated *Mef*^{-/-} mice had less severe leukopenia than wild-type mice, with more rapid recovery (Figure 5A). The speedy restoration of myeloerythroid cell lineages improved the overall survival, as fewer *Mef* null mice succumbed to life-threatening infections due to severe neutropenia (100% survival compared to 33% in wild-type mice). The milder leukopenia seen after 5-FU treatment is likely due to the presence of a more quiescent and more abundant stem cell pool (defined as LSK CD34⁻ Flt3⁻ and LSK CD34⁺ Flt3⁻ cells) in the bone marrow. LSK CD34⁺ Flt3⁻ cells can efficiently protect myeloablated mice from life-threatening cytopenias (Yang et al., 2005). To address the possibility that *Mef* deficiency could alter the expression of enzymes involved in metabolizing 5-FU, we also tested busulfan, a different cytotoxic agent that also targets proliferating cells. As shown in Figure S6, WBC recovery post-busulfan is also faster in *Mef*-deficient mice, with milder leukopenia compared to wild-type mice. As irradiated *Mef*^{-/-} mice also display a more rapid recovery of WBC counts (Figure S6), this protection from myelosuppression is likely due to a combination of enhanced quiescence and an increased number of HSCs.

Although it was recently reported that bone marrow cells undergo senescence following myelosuppressive therapies,

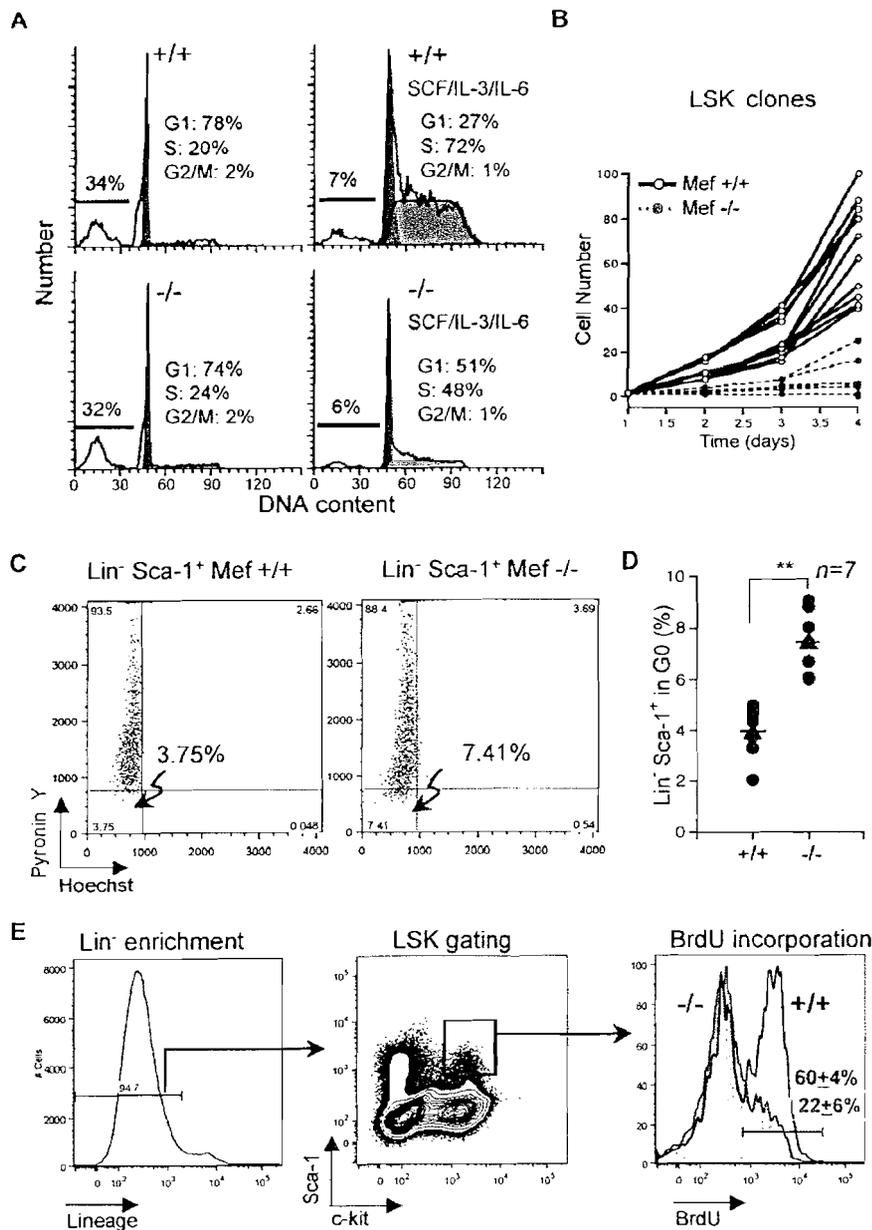


Figure 4. Loss of *Mef* leads to increased quiescence of hematopoietic stem cells

A: Defective entry to cell cycle of *Mef*^{-/-} LSK cells in response to a 24 hr incubation with SCF, IL-3, and IL-6. Cell cycle analysis was performed on nuclei stained with propidium iodide and analyzed using the ModFit software. LSK cells were purified from four mice per group.

B: Single LSK cells were purified by cell sorting into 96-well plates (1 cell/well), and then cell growth was evaluated microscopically for each different clone. Only ten representative clones for each group are shown.

C: Multiparameter flow cytometry was used to determine the percentage of hematopoietic progenitor cells in the G₀ phase of the cell cycle. Total bone marrow cells were stained with anti-Sca-1, with a lineage cocktail (Lin), and with Pylonin Y and Hoechst 33342. Lin⁻ Sca-1⁺ cells in G₀ are defined as cells with low Pylonin Y content that contain 2n DNA (G₀/G₁).

D: The percentage of Lin⁻ Sca-1⁺ cells in G₀ is plotted (n = 7; **p < 0.005, two-tailed distribution of two samples with equal variance).

E: The proliferation of LSK cells was measured by in vivo BrdU incorporation. Lower proliferation of *Mef* null LSK cells was seen (22% versus 60% for wild-type; n = 3).

evidenced by increased levels of p16 and p19 (Wang et al., 2006), *Mef*^{-/-} bone marrow cells isolated at different times post-5-FU treatment showed a similar pattern of p16 and p19 expression as the wild-type group (Figure S7), implying that loss of *Mef* does not affect chemotherapy-induced cell senescence.

To correlate the hematopoietic recovery observed in the peripheral blood with bone marrow morphologic features, we examined the location of hematopoietic cells within the femurs of *Mef*^{-/-} mice following 5-FU treatment. We saw no appreciable difference in the trabecular bone, the growth plate, or the epiphysis of untreated wild-type and *Mef*^{-/-} femurs stained with hematoxylin and eosin (Figure 5B). This suggests that *Mef*-deficient mice have no gross alterations in stem cell niches

(Calvi et al., 2003; Zhang et al., 2003). Examining femoral sections at various time points showed that although the initial bone marrow findings are similar in *Mef*^{-/-} and *Mef*^{+/+} femurs (Figure 5C), clusters of hematopoietic cells can be observed as early as day 4 in the osteoblastic zone of the femur of *Mef*^{-/-} mice. Six days post-5-FU, the bone marrow of *Mef*^{-/-} mice had retained over 50% of its cellularity in contrast to wild-type mice (10%); the expansion of megakaryocytes and myeloid cells is also increased in ablated *Mef* null mice (data not shown). The early appearance of polyploid megakaryocytes in the vascular niche, which is usually observed at much later times (Avecilla et al., 2004), accounts for the more rapid platelet recovery seen following 5-FU administration to *Mef*-deficient mice (data not shown).

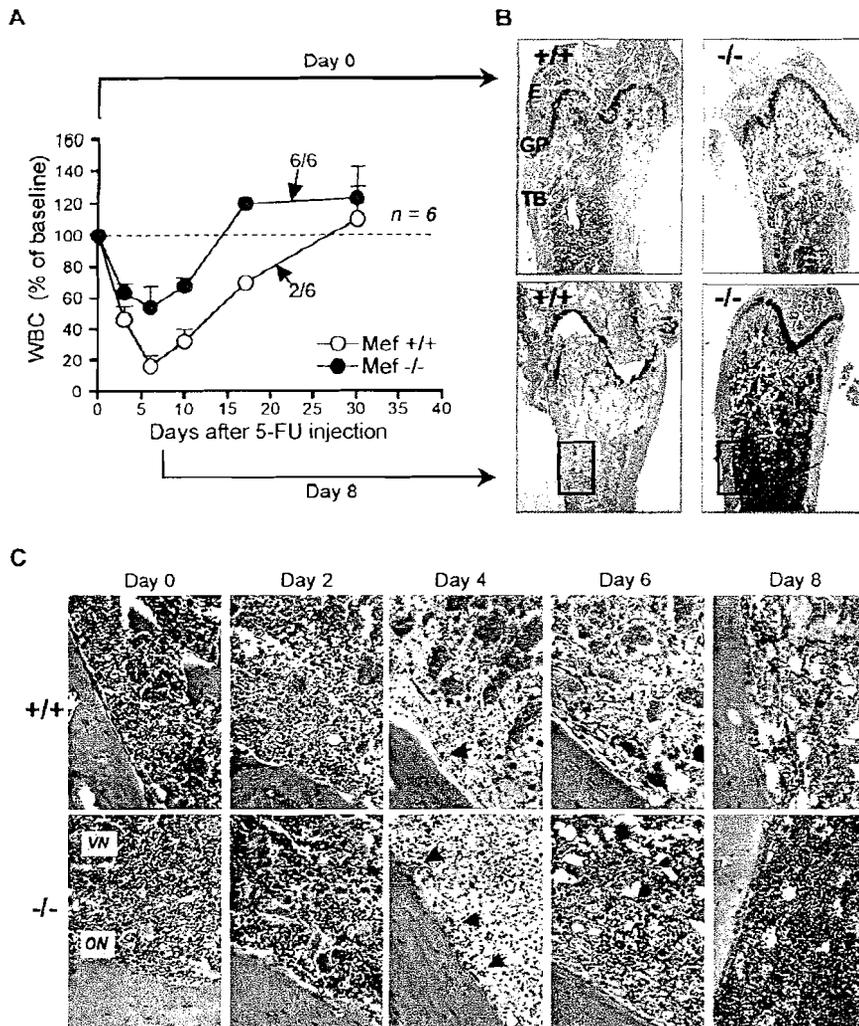


Figure 5. Hematopoietic recovery after treatment with myelotoxic agents

A: Hematopoietic reconstitution was followed by serial peripheral blood count monitoring of mice (*Mef*^{+/+} and *Mef*^{-/-}) injected with a single dose of 5-FU (200 mg/kg, i.p.). WBC counts are shown as a percentage of the initial values for each group of mice (mean \pm standard deviation; $n = 3$ for each time point). Overall survival is indicated for the mice treated with 5-FU.

B: Gross morphology of femurs from untreated mice and mice 8 days after 5-FU injection is shown. Slides were stained with hematoxylin-eosin. The epiphysis (E), growth plate (GP), and trabecular bone (TB) regions of metaphysis are indicated.

C: Bone marrow morphology was examined following a myelosuppressive dose of 5-FU at higher magnification in the diaphysis of the bone (indicated by rectangles in **B**). Localization of the osteoblastic niche (ON) and vascular niche (VN) are indicated. Arrowheads show clusters of hematopoietic cells in the osteoblastic zone 4 days after 5-FU administration.

The rapid recovery from a myeloablative treatment in *Mef* null mice seems paradoxically opposed to the increased quiescence observed in homeostasis. However, the steady-state maintenance of HSCs and subsequent expansion of hematopoietic progenitors postablation is likely controlled by different mechanisms. We monitored stem cell expansion post-5-FU treatment using SP cell detection and found the *in vivo* proliferative response of *Mef* null cells to myeloablation similar to that seen in *Mef*^{+/+} mice (Figure 6A). We also observed similar BrdU incorporation in LSK cells and Lin⁻ Sca-1⁺ (LS) cells post-5-FU treatment (Figure 6B). Thus, although the absence of *Mef* enhances the quiescence of primitive progenitors, its absence does not prevent cell division in response to myeloablative treatment.

Resistance to chemotherapy is intrinsic to *Mef* null HSCs

The improved hematopoietic recovery from 5-FU treatment seen in *Mef* null mice is cell intrinsic and transplantable. Normal B6 mice transplanted with *Mef*^{-/-} bone marrow mononuclear cells showed the same rapid recovery of peripheral blood

WBC and platelet counts post-5-FU (Figure 7A) as *Mef* null mice (Figure 5A). Similarly, increased numbers of hematopoietic cells are found in the osteoblastic and vascular zones of the femurs of mice receiving *Mef* null BMSCs on day 3 (Figure 7A), when bone marrow ablation is usually reached (Figure 5). Remarkably, wild-type mice transplanted with *Mef* null BMSCs also exhibit an expansion of megakaryocyte (Figure 7A), which again manifests itself as more rapid platelet recovery post-5-FU.

Given the importance of stem cell quiescence in regulating hematopoiesis, we investigated whether the acute lowering of MEF expression in human hematopoietic cells could lead to the same perturbations in cell cycle that we observed in *Mef* null mice. We reduced MEF expression in human cord blood CD34⁺ cells by siRNA and observed reduced proliferation (percentage in S phase) and increased numbers of quiescent cells (percentage in G₀, defined as Ki67-negative cells within G₀/G₁ phase of cell cycle) even when MEF levels were only reduced by 30%–40% (Figure 7B). Thus, decreasing MEF levels in these human cells enhances quiescence, which could similarly provide *in vivo* protection from myelosuppression.

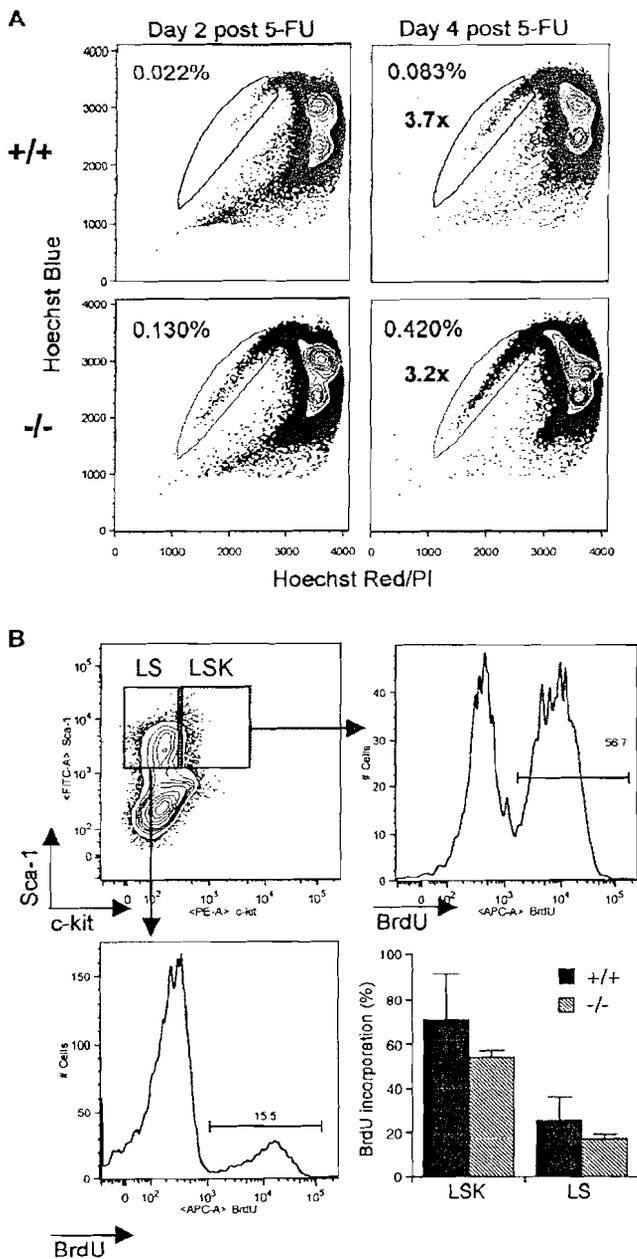


Figure 6. Normal expansion of *Mef*^{-/-} hematopoietic progenitor cells during bone marrow ablation

A: SP cells were identified by flow cytometric analysis in BMMCs obtained from mice 2 and 4 days after 5-FU administration. The number of SP cells that resisted this treatment is indicated as percentages.

B: In vivo proliferation of LSK and LS (*Lin*⁻ *Sca-1*⁺) cells following 5-FU treatment. Mice (*n* = 3) were injected with 5-FU 2 days before the BrdU injection and then left for 2 additional days with BrdU in their water. Statistical analysis is shown in a bar graph (mean \pm standard deviation).

Discussion

The preservation of HSCs throughout life is essential to ensure an adequate supply of mature blood cells under physiologic and stress situations. HSC numbers remain constant over

time, with approximately 8% of the HSCs randomly undergoing cell division each day to generate another HSC and/or to differentiate along specific lineages (Passegue et al., 2003). The fate of HSCs that commit to enter the cell cycle is self-renewal, differentiation, senescence, or apoptosis. The molecular determinants of stem cell maintenance have not been studied in detail, although the involvement of *Hox* proteins such as *HoxB4* (Antonchuk et al., 2002) and polycomb group proteins such as *Bmi-1* (Lessard and Sauvageau, 2003a, 2003b) in stem cell self-renewal has been unveiled. The regulation of stem cell dormancy is particularly relevant to bone marrow failure states, and for patients treated with myelotoxic chemotherapy or radiation. Furthermore, efficient engraftment of HSCs during bone marrow transplantation may depend on their cell cycle status, in fact their quiescence (Passegue et al., 2005).

We have shown that loss of the transcription factor *Mef* leads to increased stem cell quiescence, diminished in vitro cytokine-induced cell proliferation of primitive progenitors, and in vivo resistance to cell cycle-dependent myelotoxicity. These findings demonstrate that *Mef* facilitates the entrance of HSCs to the cell cycle, and bone marrow transplantation studies show that these properties are cell intrinsic and directly due to the absence of *Mef* expression. The absence of *Mef* results in an accumulation of primitive hematopoietic cells with repopulating capacity (both LT-HSCs and ST-HSCs), which is accompanied by enhanced steady-state quiescence, no change in cell survival, and minimal changes in self-renewal (not detectable in serial bone marrow transplantation [sBMT] assays). This accumulation is not due to the "retention" of HSCs in the bone marrow microenvironment, as we have seen nearly normal mobilization of stem cells from the bone marrow of *Mef*-deficient mice into the bloodstream in response to G-CSF (data not shown).

We have confirmed the increased proportion of primitive progenitors in *Mef*^{-/-} mice by a variety of in vitro and in vivo assays commonly used to identify stem cells, including CAFC, LTC-IC, immunophenotyping (*Lin*⁻ *Thy1*^{low} *Sca-1*⁺ *c-kit*⁺ cells, SP-LSK cells, and *CD34*⁻ *Flt3*⁻ LSK cells), CFU-S₁₂ (data not shown), and serial replating in methylcellulose. Multilineage rescue of ablated mice using *Mef*^{-/-} LSK cells confirm that they do indeed contain HSCs with long-term repopulating capacity when transplanted into both primary and secondary recipients. Interestingly, although *Mef* null primitive progenitor cells do not enter the cell cycle efficiently at steady state, their progeny do expand normally in vivo, following bone marrow transplantation or during recovery from chemotherapy. The role of *MEF* in cell cycle regulation in HSCs versus committed progenitors shows its cell stage-specific effects. As a result of *MEF* loss, LT-HSCs and ST-HSCs tend to accumulate, due to a lower proliferation capacity without a major impact on self-renewing divisions. Lymphoid and myeloid development proceeds normally in the progenitor populations, leading to nearly normal peripheral blood counts.

The increased stem cell pool in *Mef* null mice is likely due to their increased quiescence. The abundance of LT-HSCs and ST-HSCs in *Mef* null mice, as a result of increased dormancy, enables a faster hematologic recovery postmyelosuppression. Although the converse could also be true, that the increase in stem cell numbers triggers the increased quiescence, our transplantation experiments suggest that this is not the case, as consistent biological properties are seen independent of the infused cell dose. The increased quiescence in the absence of *MEF* was

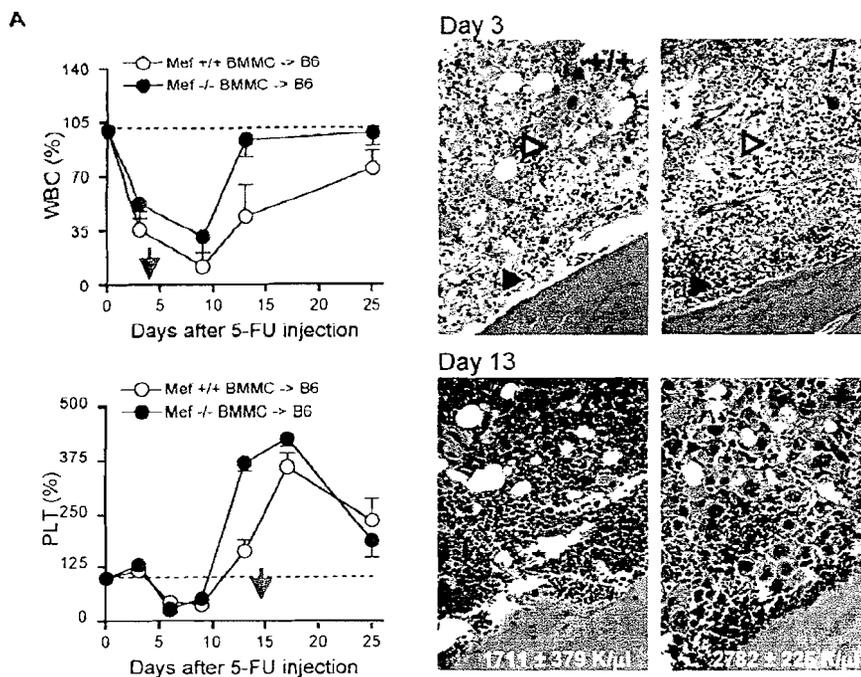
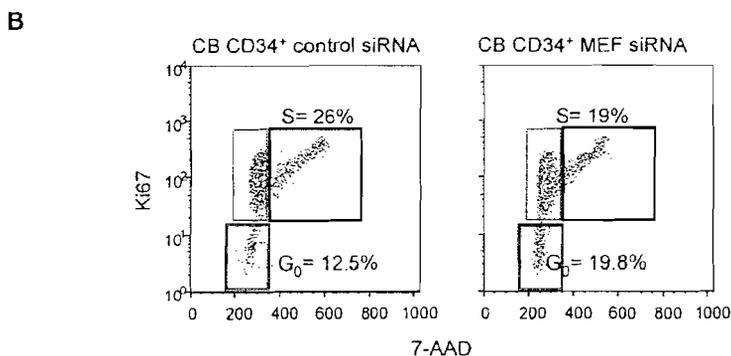


Figure 7. Enhanced hematopoietic recovery is due solely to hematopoietic progenitors and their resistance to 5-FU

A: Wild-type mice transplanted with Mef^{+/+} or Mef^{-/-} BMBCs were injected with 5-FU, and the hematopoietic recovery was measured in peripheral blood (WBC and PLT counts). Data are shown as mean \pm standard deviation; $n = 3$ for each time point. The histology of femurs 3 days posttreatment is shown on the right (filled arrowheads, osteoblastic zone; empty arrowheads, vascular niche). Megakaryocytic expansion in the femur is shown at day 13 post-treatment (multinuclear megakaryocytes are indicated with arrowheads). The number of platelets (PLT) in the peripheral blood at day 13 is also indicated.

B: Effect of deregulated MEF expression in human hematopoietic cells. Cord blood CD34-positive cells were transfected with control and MEF siRNA. Ki67 and DNA content were then analyzed by flow cytometry.



evidenced by the incorporation of BrdU *in vivo*, by *in vivo* resistance to cell cycle-dependent cytotoxicity, by measurements of Pyronin-Y^{low} cells in the G₀/G₁ phase, and by assessing Ki67 expression within LSK Pyronin-Y^{low} cells. Furthermore, acute knockdown of MEF *in vitro* using siRNA increased quiescence of human CD34⁺ cells.

Several cell cycle regulators have been shown to play key roles in hematopoiesis. The absence of the cdk inhibitor p27 (kip1) affects the proliferation of progenitor cells rather than HSCs (Cheng et al., 2000a). In contrast, mice deficient in the p21 cdk inhibitor have increased stem cell numbers with higher proliferative capacity but also lower quiescence than normal (Cheng et al., 2000b). This leads to stem cell exhaustion following serial 5-FU administration or sBMT. A recent study shows that the cdk inhibitor p18 INK4C acts earlier than p21 in G₁, and its absence increases the self-renewing divisions of HSCs (Yuan et al., 2004). Mef also regulates the size of the primitive progenitor cell pool, regulating the release of HSCs from quiescence. This suggests that p21 and Mef could regulate stem cell pool size via opposing effects.

We propose a model where MEF (ELF4) facilitates cell cycle entry of primitive progenitors from their quiescent state, acting earlier than p18 and antagonizing p21 (Figure 8). The enforced expression of GATA-2 has been shown to block the expansion of Lin⁻ Sca-1⁺ cells (Persons et al., 1999), whereas GATA-2 haploinsufficiency results in fewer CD34⁺ LSK cells with poor competitive repopulating ability. Increased quiescence is seen in GATA-2^{-/-} cells in the presence of normal self-renewal. Thus, MEF and GATA-2 could be the first transcription factors to be implicated in the maintenance of stem cell quiescence (Rodrigues et al., 2005) (Figure 8A). The zinc finger repressor Gfi-1 appears to restrict the proliferation of HSCs, likely in a p21-dependent fashion as Gfi-1 null HSCs show downregulation of p21 (Hock et al., 2004; Zeng et al., 2004). We have seen no major changes in the level of p21, p27, p57, cyclin D, or cyclin A protein levels in Mef null BMBCs (data not shown). The contrasting effects of Mef and Gfi-1 in terms of cell cycle regulation, CR, and engraftment capacity might be due to their different actions on gene expression, one being an activator (Mef) and the other a repressor (Gfi-1). Although Tie-2 expression has been recently

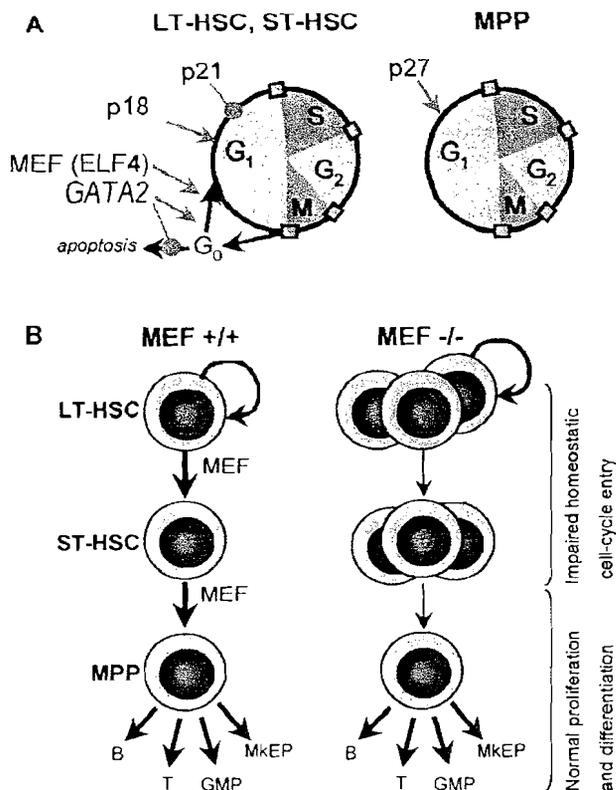


Figure 8. Model of MEF function on the proliferation of hematopoietic stem cells

A: Diagram depicting the cell cycle of stem cells (left) and progenitor cells (right) and the cell cycle regulators known to control their proliferation.

B: LT-HSCs were defined in this work as Lin⁻ Sca-1⁺ c-kit⁺ SP cells and as Lin⁻ Sca-1⁺ c-kit⁺ CD34⁻ Flt3⁻ cells. ST-HSCs were identified as Lin⁻ Sca-1⁺ c-kit⁺ CD34⁺ Flt3⁻ cells. In the absence of MEF, we found a higher fraction of primitive progenitors (LT-HSC and ST-HSC) in spite of normal differentiation into lymphoid and myeloid cells. The abundance of ST-HSCs further contributes to the enhanced hematologic recovery seen post-myeloid suppression, in addition to the increased primitive progenitor cell quiescence.

linked to quiescent HSCs (Arai et al., 2004), we have not detected significant alterations of Tie-2 expression in Mef null bone marrow cells (data not shown). A full molecular delineation of the Mef^{-/-} HSCs is planned; such characterization will help define the cell-intrinsic mechanisms that control stem cell maintenance.

Tumorigenesis is a multistep process where cells accumulate genetic alterations that result in their progressive transformation. MEF-deficient mice seem to have a normal lifespan, without spontaneous tumor formation. However, MEF^{-/-} HSCs might be prone to transformation, as a larger stem cell pool could increase the likelihood that these cells accumulate additional mutations. Leukemia cells could benefit from inactivation of MEF (via mutations or via transcriptional repression such as occurs with AML1-ETO, which can block MEF function [Mao et al., 1999]). Studies implicating the MEF (also known as ELF4) gene in malignant transformation include its downregulation by two human leukemia-associated fusion proteins (Alcalay et al., 2003; Muller-Tidow et al., 2004; Park et al., 2003), its potential role as a tumor suppressor gene in solid tumors (Seki et al., 2002), and also its upregulation by retroviral insertional

activation in murine cancer models (Akagi et al., 2004; Du et al., 2005; Suzuki et al., 2002). These features are in fact similar to those implicating the AML1 transcription factor in hematologic malignancies, namely the functional downregulation of AML-1 function by AML1-ETO (Frank et al., 1995), haploinsufficiency studies in a familial AML predisposition syndrome (FPD/AML) implicating AML1 as a tumor suppressor gene (Song et al., 1999), and the retroviral insertional activation of Runx1, Runx2, or Runx3 in murine cancer models (Stewart et al., 1997, 2002; Wotton et al., 2002). Although lack of AML1 function during development is embryonically lethal, the lack of MEF alters stem cell biology but does not block hematopoietic development.

In other studies, we have shown that deregulated MEF expression can be tumorigenic in solid tumors; overexpression of MEF in ovarian cancer cell lines increases proliferation and aggressiveness, and MEF overexpression transforms NIH3T3 cells leading to tumor growth in nude mice (J.-J. Yao, Y.L., H.D.L., R.A. Soslow, J.M. Scandura, S.D.N., and C.V. Hedvat, unpublished data). The recent discovery of t(X;21)(q26;q22) in a patient with AML that fuses exon 2 of the MEF gene with most of the coding region of the ERG gene, which encodes another ETS protein, strongly supports the role of MEF dysregulation in hematological malignancies (Moore et al., 2005). In this female patient, the t(X;21) generates at least haploinsufficiency for MEF if not MEF deficiency due to X inactivation. Furthermore, it deregulates ERG expression, which is now transcriptionally controlled by the MEF promoter.

Advances in our understanding of HSC biology will hasten the development of new therapeutic approaches to cancer. HSCs may make an active choice to neither self-renew nor differentiate, but rather to remain quiescent. MEF regulates the quiescence of primitive hematopoietic progenitors; therefore, treatments that block or diminish MEF expression could contribute to improve hematopoietic recovery postmyelosuppression. Hematopoiesis in Mef^{-/-} mice is far less affected by 5-FU administration than that in normal mice, leading to minimal cytopenias and fewer fatal infections. Our studies using siRNA to lower MEF levels in human CD34⁺ cells and enhance quiescence suggest that this could even be clinically relevant. Myelotoxicity induced by chemotherapy or radiotherapy could be prevented by maintaining stem cells in a quiescent state during their administration to cancer patients. However, another implication of our work is that tumor stem cells are more quiescent than more differentiated tumor cells and could use similar mechanisms to resist the effects of chemotherapy or radiation (Chevallier et al., 2004; Nakamura et al., 2002; Snowden et al., 2003).

Experimental procedures

Mice

The generation of Mef-deficient mice was described previously (Lacorazza et al., 2002). C57BL/6 (CD45.1⁺), B6.SJL (CD45.2⁺), and F1 B6/SJL mice were purchased from Jackson Laboratories. All mice were maintained in MSKCC Animal Facility and Baylor College of Medicine Animal Facility, according to IACUC-approved protocols, and kept in Thorensten units with filtered germ-free air.

Stem and progenitor cell assays

To evaluate the most primitive progenitors, we used the CAFC assay. Briefly, 1.5×10^5 bone marrow cells were seeded on MS5 stroma and cultured in α MEM containing 10% FBS, 10% horse serum, 1 μ M hydrocortisone, and 1 mM glutamine. Medium was semireplenished every week, and "cobblestone" colonies were scored at week 5 and expressed as number of CAFC

per 1.5×10^5 BMMCs. In LTC-IC assays, serial 2-fold dilutions were done using initially 50,000 BMMCs/well; 12 replicates of each bone marrow were performed. After 5 weeks of weekly semireplenishment of the media, cells were trypsinized and plated on methylcellulose media (MethoCult GF M3434, StemCell Technologies) and cultured for 10 days before the percentage of negative wells per dilution was scored. Frequencies were calculated using Poisson statistics (L-cal program from StemCell Technologies).

The content of primitive myeloid progenitors was determined by the day 12 CFU-S assay. Briefly, recipient mice were irradiated and injected with 1×10^5 bone marrow cells. Spleens were dissected 12 days posttransplant and fixed in Bouin's solution. Colonies per spleen were counted by visual inspection and expressed as number of CFU-S₁₂ colonies per 1×10^5 BMMCs.

Clonogenic progenitors were determined in methylcellulose medium (MethoCult GF M3434, StemCell Technologies) using 2×10^4 BMMCs per well (6-well plate). Colonies were scored after 10 days of incubation and expressed as number of CFUs per 2×10^4 BMMCs.

Bone marrow transplantation

Recipient mice were lethally irradiated with 10 Gy of whole-body irradiation. Two to three million BMMCs were injected intravenously into the lateral tail vein of recipient mice previously warmed under a heat lamp. Animals were daily monitored for signs of toxicity and sacrificed 2 months postinfusion. sBMT was used to test the self-renewal capacity of the *MeF^{-/-}* stem cells. We used Lin⁻ Sca-1⁺ c-kit⁺ cells (HSCs) purified by cell sorting (MoFlow cell sorter, Cytomation); cells were then injected (5000 LSKs per mouse) into lethally irradiated mice (B6.SJL). We also performed sBMT using donor-derived BMMCs. B6 (CD45.2) mice were used as donors, and B6.SJL (CD45.1) mice were used as recipients. For each transplant, donor-derived cells (CD45.2+) were purified by cell sorting and injected into lethally irradiated B6.SJL mice (approximately $1-2 \times 10^5$ BMMCs/mouse). In both cases, hematopoietic reconstitution was monitored in peripheral blood, as described below.

In the CR study, we injected different percentages of BMMCs from wild-type (B6.SJL) and *MeF^{-/-}* (B6) mice (0%, 20%, 50%, 80%, or 100%) into lethally irradiated B6.F1 mice (CD45.1/CD45.2). After 5 months of reconstitution, peripheral blood was obtained by retro-orbital eye bleeding. The RBCs were lysed, and the PBMCs were stained with anti-CD45.2 FITC and anti-CD45.1 PE and analyzed by flow cytometry. These mice were then irradiated with 4.5 Gy of whole-body radiation, and hematopoietic recovery in peripheral blood was analyzed at different time points, as described below.

Measurement of cell cycle parameters using Pyronin Y and Hoechst staining

Bone marrow cells were first stained for the Lin cell surface markers and for Sca-1. Then, cells were resuspended in a Hank's balanced salt solution, 20 mM HEPES, 1 g/l glucose, 10% FCS, 1.7 μ M Hoechst 33342 and incubated for 1 hr at 37°C. After a single wash, bone marrow cells were incubated in the same buffer containing Pyronin Y (1 μ g/ml) for an additional hour at 37°C. Finally, cells were analyzed using a MoFlow cytometer (Cytomation) and FlowJo program (Cytomation).

BrdU incorporation

Mice received an intraperitoneal injection of 3 mg BrdU (Becton Dickinson) and admixture of 1 mg/ml of BrdU (Sigma) to drinking water for 2 days. Bone marrow cells were isolated and lineage negative purified using the BD-Imag system (Becton Dickinson) and then stained with c-kit PE and Sca-1 FITC antibodies. Then, the analysis of BrdU incorporation was performed using the APC BrdU flow kit (Becton Dickinson).

Cell cycle analysis

LSK cells were purified by cell sorting pooling four mice per genotype (wild-type and knockout). Cells were incubated for 24 hr in Xvivo-15 supplemented (or not) with SCF (100 ng/ml), IL-3 (10 ng/ml), and IL-6 (6 ng/ml). Cells were centrifuged and resuspended in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100) containing 100 μ g/ml RNase and 50 μ g/ml propidium iodide. Samples were analyzed within 1 hr using the FACScanto flow cytometer (Becton Dickinson) and the Modfit LT software (Verity).

Detection of SP cells

We followed the SP detection procedure using Hoechst dye as previously described (Goodell et al., 1996). Briefly, BMMCs were resuspended at 1×10^6 cells/ml (DMEM + 10% FBS) and incubated with Hoechst 33342 (5 μ g/ml) for 90 min at 37°C. Cells were then washed and stained for cell surface markers (lineage, Sca-1, and c-kit). Analysis was performed on a MoFlo flow cytometer (Cytomation) equipped with a 351 nm laser for UV. Red and blue fluorescence derived from UV excitation was separated with a 610 dichromatic DRSP. Blue Hoechst fluorescence was collected with a 450/20 filter, and red Hoechst/PI fluorescence was collected via a 675 ES/LP filter. We collected 5×10^6 events to have enough cells in the SP-LSK gate.

Myelosuppressive treatments

We used 5-FU (200 mg/kg, i.p.), busulfan (15 mg/kg, s.c.), and 4.5 Gy radiation for myelosuppression, in order to follow hematopoietic recovery in the peripheral blood. PBCs were monitored once a week in each mouse but not more than three times overall. Therefore, each experimental group (wild-type and knockout) was formed by two sets of three to four mice per set that were bled alternatively. Retro-orbital peripheral blood (<100 μ l) was obtained from anesthetized mice and collected into EDTA-coated capillary tubes. Complete blood counts and differential counts were measured in the Genetically Engineered Mouse Phenotyping Core (MSCKK/Cornell/Rockefeller) using an automated blood cell counter.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, seven supplemental figures, and two supplemental tables and can be found with this article online at <http://www.cancerres.org/cgi/content/full/9/3/175/DC1/>.

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