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Targeted Disruption of the *flk2/flt3* Gene Leads to Deficiencies in Primitive Hematopoietic Progenitors

Katrin Mackarehtschian,* Jeff D. Hardin,†
Kateri A. Moore,* Sharon Boast,†
Stephen P. Goff,† and Ihor R. Lemischka*
*Department of Molecular Biology
Princeton University
Princeton, New Jersey 08544
†Department of Biochemistry and Molecular Biophysics
Columbia University
College of Physicians and Surgeons
New York, New York 10032

Summary

The *flk2* receptor tyrosine kinase has been implicated in hematopoietic development. Mice deficient in *flk2* were generated. Mutants developed into healthy adults with normal mature hematopoietic populations. However, they possessed specific deficiencies in primitive B lymphoid progenitors. Bone marrow transplantation experiments revealed a further deficiency in T cell and myeloid reconstitution by mutant stem cells. Mice deficient for both c-*kit* and *flk2* exhibited a more severe phenotype characterized by large overall decreases in hematopoietic cell numbers, further reductions in the relative frequencies of lymphoid progenitors, and a postnatal lethality. Taken together, the data suggest that *flk2* plays a role both in multipotent stem cells and in lymphoid differentiation.

Introduction

Hematopoiesis is a self-renewing process capable of continuously producing at least eight distinct blood cell lineages (reviewed by Dexter and Spooncer, 1987; Lemischka, 1992). At the heart of this system is a population of hematopoietic stem cells, which are multipotent in their ability to differentiate and which can self-renew. Transplantation experiments have demonstrated that single stem cells are both necessary and sufficient for lifelong blood cell production (Jordan and Lemischka, 1990b; Smith et al., 1991). Traditionally, hematopoietic stem cells have been studied indirectly; that is, as activities either in transplantation contexts or in complex in vitro culture systems. Efforts in a number of laboratories have yielded procedures for substantial purification of these cells, thus providing a means for direct mechanistic analyses of stem cell behavior (Visser et al., 1984; Spangrude et al., 1988; Ploemacher and Brons, 1989; Jordan et al., 1990). Numerous studies have implicated a panel of cytokines that act in vitro on purified stem cell populations (reviewed by Nicola, 1989; Ogawa, 1993). However, relatively little information is available regarding the mechanisms that regulate the proliferation and differentiation of stem cells in a normal in vivo context.

As part of an effort to identify signal transduction molecules involved in stem cell behavior, a cDNA encoding a novel receptor tyrosine kinase, called *flk2*, was identified in partially purified fetal liver cell populations that contain all primitive hematopoietic cell activities (Matthews et al., 1991). This receptor was independently identified in testis cDNAs and termed flt3 (Rosnet et al., 1991). It shows significant sequence and structural homology to the class III receptor tyrosine kinases, which includes the colony-stimulating factor 1 (CSF-1) receptor (c-*fms*), the stem cell factor (SCF) receptor (c-*kit*), and the α and β platelet-derived growth factor receptors. Two receptors in this subfamily of transmembrane tyrosine kinases, namely c-*fms* and c-*kit*, are known to function in hematopoiesis. The c-*fms* receptor and CSF-1 play a role in the development of monocyte–macrophages (Stanley and Heard, 1977; Das and Stanley, 1982). The c-*kit* receptor and its ligand SCF are the only tyrosine kinase receptor–ligand pair presently known to influence the behavior of very immature hematopoietic cells (Ogawa, 1993; Okada et al., 1991a; Kodama et al., 1992).

The in vivo roles of c-*fms* and c-*kit* are best understood due to the existence of mutant mice in which genes encoding the receptors or their respective ligands are defective (Chabot et al., 1988; Geissler et al., 1988; Copeland et al., 1990; Yoshida et al., 1990). In particular, mutations in the c-*kit* receptor and its ligand are well represented by numerous white-spotting (W) and Steel (Sl) mutant alleles, respectively (reviewed by Russell, 1979). The hematopoietic defects of homozygous W mice include a macrocytic anemia, reductions in tissue mast cells, and a decrease in hematopoietic stem cells (Murphy et al., 1973; Harrison and Astle, 1976; Harrison et al., 1979; Kitamura et al., 1979; Russell, 1979).

flk2 transcripts have been detected in murine and human cell populations enriched for hematopoietic stem and progenitor cells (Matthews et al., 1991; Small et al., 1994). Recently, *flk2* expressing fetal liver and adult bone marrow cells were shown to be capable of reconstituting the hematopoietic system of transplant recipients (Zeigler et al., 1994). *flk2* is also expressed in human pre-B cell lines and numerous lymphoid leukemias (Birg et al., 1992; Rosnet et al., 1993). Analysis of its downstream signaling pathways suggests that this receptor can function as a mitogenic signaling molecule in primitive B lymphoid cells (Dosil et al., 1993; Lyman et al., 1993). Two groups have reported the identification of the ligand for the *flk2* receptor (Lyman et al., 1993; Hannum et al., 1994). In combination with other cytokines, this molecule has potent in vitro activity on myeloid and lymphoid progenitors. Studies investigating the activities of the *flk2* ligand on the most primitive multipotent stem cell population have not been reported.

To analyze the role of *flk2* signaling in normal hematopoiesis, we generated mice lacking a functional *flk2* receptor via homologous recombination in embryonic stem (ES) cells. *flk2* mutant mice developed normally and had a largely normal hematopoietic system. However, very primitive B lymphoid progenitor cell populations were diminished. Long-term transplantation experiments revealed

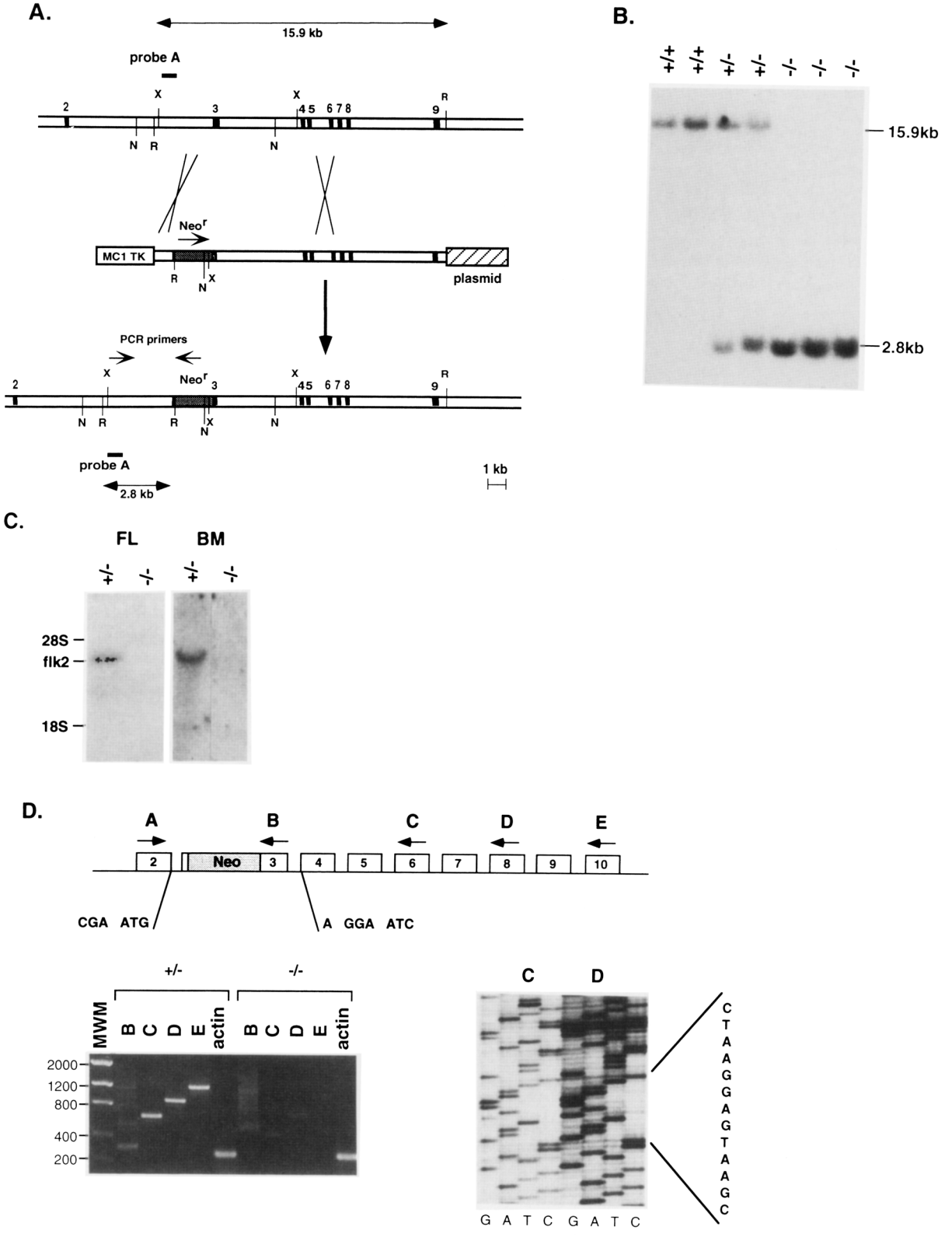

Figure 1. Generation of *flk2* Null Mutants

(A) Strategy for disruption of the *flk2* gene. Closed boxes represent exons. The targeting vector contained approximately 14 kb of genomic *flk2* sequence with the selectable *neo*^{r} gene inserted into exon 3. A *tk* gene was cloned at one end of the vector to allow for enrichment of homologous recombination events by positive–negative selection (Mansour et al., 1988). The length of diagnostic restriction fragments and the location of probe A used for Southern blot analysis are shown. Enzymes are EcoRI (R), XbaI (X), and NcoI (N). Arrows represent PCR primers or the direction of transcription of the *neo* gene.

that the mutation impaired the developmental capacity of primitive progenitor cells of all hematopoietic lineages, having the greatest impact on progenitors of the lymphoid lineage. These results suggest that *flk2* and its cognate ligand play a role in the origin, maintenance, or expansion of primitive lymphoid progenitors and potentially also have a function in precursor cells of the myeloid lineage and multipotent stem/progenitor cells.

The strong similarities in both sequence and expression pattern between the *flk2* and c-*kit* receptors suggest that the two molecules may play similar roles in many cells. To investigate this possibility, we analyzed the hematopoietic system of c-*kit*/*flk2* double-mutant mice. These studies revealed that the generation or maintenance of proper pools of lymphoid progenitors is dependent on these two receptors. Furthermore, combined mutations in both genes lead to a severe reduction in the overall size of the hematopoietic system and to postnatal lethality. These results suggest that the c-*kit* and the *flk2* receptors have additive functions in hematopoietic development.

Results

Generation of *flk2* Mutant Mice

The targeting strategy was designed to yield a null mutation of the *flk2* gene. Accordingly, the gene was disrupted by inserting a *neo* cassette into exon 3 at a position corresponding to amino acid 59 of the mature protein (Figure 1A). The targeting vector was introduced into the CCE ES cell line (Robertson et al., 1986). ES cells containing a disrupted *flk2* gene were identified by polymerase chain reaction (PCR) and by Southern blot analysis (data not shown). The targeting frequency was 1 in 5 × 10^6 electroporated cells or 1 in 28 G418^{r}FIAU^r colonies. Mice were generated and two were germline chimeras. Heterozygotes were intercrossed to generate mice homozygous for the mutant *flk2* allele (Figure 1B).

Northern blot analysis revealed an absence of *flk2* RNA in homozygous fetal liver and bone marrow cells (Figure 1C). However, RNAase protection showed that the mutated gene gave rise to low amounts of RNA, encoding at least a portion of the *flk2* sequence (data not shown). The structure of the residual *flk2* transcript was analyzed by reverse transcriptase PCR (RT-PCR) using primers in different exons flanking the *neo* insertion (Figure 1D). The sizes of the amplified products from the homozygous bone marrow cells were approximately 200 bp smaller than those from heterozygous control samples (Figure 1D). Because all intron–exon boundaries of the *flk2* gene have been mapped (K. M. and I. R. L., unpublished data), these data suggested a splicing-mediated deletion of the mutated exon 3. To verify this, the amplified PCR products were sequenced. The sequence confirmed splicing from exon 2 to exon 4 in the mutant *flk2* transcripts (Figure 1D). This shifts the translation frame after amino acid 56, which results in a stop codon after 51 aa. Therefore, a functional *flk2* protein cannot be produced from this mRNA.

B Lymphoid Precursor Compartments Are Reduced in *flk2* Mutants

Mice carrying two mutated alleles of *flk2* were normal, healthy, and fertile. Genotyping of 600 mice showed that the targeted allele was transmitted at predicted Mendelian frequencies (data not shown). The analysis of a cohort of 30 age-matched young adult *flk2* homozygous, heterozygous, and wild-type mice did not reveal any differences in the cellularities of their hematopoietic organs. Furthermore, peripheral blood smears and differential blood counts showed no morphological or quantitative abnormalities (data not shown). Using a variety of cell surface markers (see Experimental Procedures), the absolute numbers and frequencies of different myeloid and lymphoid cell populations were analyzed. The levels of myeloid and lymphoid cell populations in homozygous spleens were normal, as were the numbers and relative frequencies of thymocyte subpopulations (data not shown). In the bone marrow, no variations in monocyte, granulocyte, and erythrocyte levels were observed (data not shown). However, the percentage of bone marrow B220$^+$ cells in *flk2* mutant mice was reduced (20.6 ± 1.0 in homozygotes [n = 6] versus 30.8 ± 1.8 in heterozygotes [n = 6], p = 0.001), suggesting a defect in the B lymphoid population.

The B cell lineage in adult bone marrow can be divided into four developmental stages (Figure 2A) (Hardy et al., 1991). The earliest identifiable members of the B cell lineage, the pro-B cells, express B220 and CD43. Figure 2B shows a comparison between the B cell staining profile of a representative control and a *flk2* mutant mouse. Mutant marrow contained significantly less pro-B cells (Figure 2B, population 1 and fractions A–C) and somewhat reduced numbers of pre-B cells (fraction D), whereas the percentages of more differentiated B cells were normal (subpopu-

(B) Southern blot analysis of a litter containing wild-type (+/+), heterozygous (+/−), and homozygous (−/−) pups. Tail DNA was digested with EcoRI, blotted, and hybridized with probe A. Exon 3 of the *flk2* gene is contained within a 15.9 kb EcoRI fragment. Probe A hybridizes to an intron region upstream of exon 3 within this EcoRI fragment, but is not contained within the targeting vector. Insertion of the vector by homologous recombination alters the size of this fragment to 2.8 kb due to the introduction of an EcoRI site present in *neo*. We verified the structure of the targeted locus using two other restriction enzymes, NcoI and XbaI (see [A]).

(C) Northern blot analysis of total RNA isolated from day 14 fetal liver (FL) or from adult bone marrow (BM) cells of homozygous (−/−) and heterozygous (+/−) mice. Each lane contains 30–40 μg of total RNA. The blots were hybridized with a *flk2* cDNA probe. Ethidium bromide staining of the gels and hybridization with a β-actin probe verified equal amounts of RNA in mutant and control lanes (data not shown).

(D) RT-PCR performed on poly(A)-selected RNA isolated from bone marrow cells of heterozygous (+/−) or homozygous (−/−) mice. Primers used for RT-PCR and subsequent sequencing reactions are represented as arrows with primer designations on top. Primer A in exon 2 of the *flk2* gene was used as the upstream primer in combination with one of the downstream primers. Predicted PCR product sizes in base pairs were 295 (A plus B), 623 (A plus C), 884 (A plus D), and 1196 (A plus E). As a positive control, an actin fragment was amplified from each RNA preparation. The sizes of marker fragments (MWM) are given in base pairs. The PCR products obtained with the primer combinations A plus C and A plus D were sequenced. The nucleotide sequence at the splice junction (from exon 2 to exon 4) is shown.

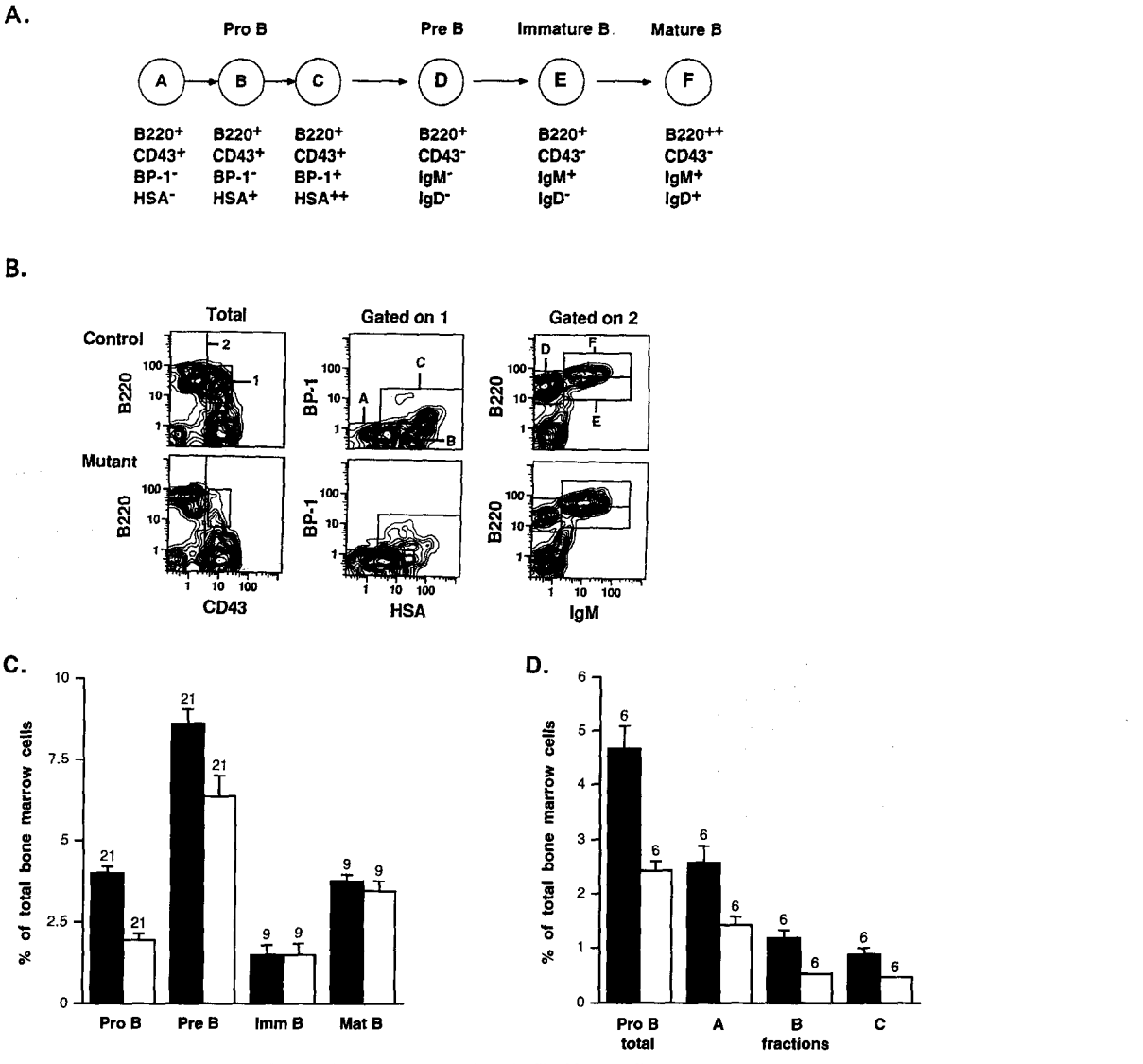

Figure 2. Selective Reduction of Early B Lymphoid Progenitors in *flk2* Mutant Mice

(A) Developmental pathway of B lymphocytes in bone marrow defined by cell surface markers.

(B) Representative analysis of the B lineage subpopulations present in normal and *flk2* mutant bone marrow. Cells isolated from a homozygous mutant and a wild-type control mouse were stained with monoclonal antibodies and analyzed by flow cytometry. Boxes indicate various cell populations: population 1, pro B cells ($B220^{dull}CD43^{+}$); population 2 ($CD43^{-}$). Population 2 was subdivided into population D, pre-B cells ($B220^{+}IgM^{-}CD43^{-}$); population E, immature B cells ($B220^{dull}CD43^{-}$ IgM^{+}) and population F, mature B cells ($B220^{bright}CD43^{-}IgM^{+}$). The immature and mature B cells (subpopulations E and F) were further identified by staining for IgD expression (profiles not shown). Population 1 was subdivided into fraction A ($BP\text{-}1^{-}HSA^{-}$), fraction B ($BP\text{-}1^{-}HSA^{+}$), and fraction C ($BP\text{-}1^{+}HSA^{2+}$).

(C) Mean percentages of cell populations as determined by flow cytometry of mutant (open bars) and control (+/− or +/+, shaded bars) adult bone marrow. Values obtained for heterozygous and wild-type mice were comparable and thus pooled. All animals were age-matched outbred or inbred mice. The error bars represent one standard error of the mean. The number at the top of each bar indicates the number of mice analyzed. Statistical analysis (two-tailed t-test) gives $p < 0.0001$ for pro-B and $p < 0.01$ for pre-B cell experiments. The average total number of pro-B and pre-B cells per femur was 4.2×10^5 and 8.9×10^5 for heterozygotes ($n = 12$) versus 1.6×10^5 and 5.2×10^5 for homozygotes ($n = 12$), respectively. Imm B, immature B cells; Mat B, mature B cells.

(D) Mean percentages of pro-B cells in mutant (open bars) and control (+/− or +/+, shaded bars) bone marrow further subdivided into three fractions as defined by the expression of two additional antigens, BP-1 and HSA. All mice were age-matched outbreds. Data for heterozygous and wild-type animals were pooled because they did not differ. Statistical analysis (two-tailed t-test) gives $p < 0.005$ for fraction A, $p < 0.001$ for fraction B, and $p < 0.005$ for fraction C.

lations E and F). The sizes of the pro-B and pre-B cell compartments in *flk2* homozygotes were, on average, approximately 49% and 74% of those in control animals, respectively (Figure 2C). Whereas the pro-B cell compartment was reduced in all *flk2* knockout mice, the pre-B cell population ranged from reduced to normal levels among individual animals. This variability was not due to the genetic background, since similar results were obtained for outbred and 129/Sv/Ev inbred homozygotes (data combined in Figure 2C). The pro-B lymphocyte population can be further resolved into three distinct subpopulations (Figure 2A) (Hardy et al., 1991). The differentiation pathway is believed to proceed from fraction A to B to C. This progression is based on the decreasing stromal dependence

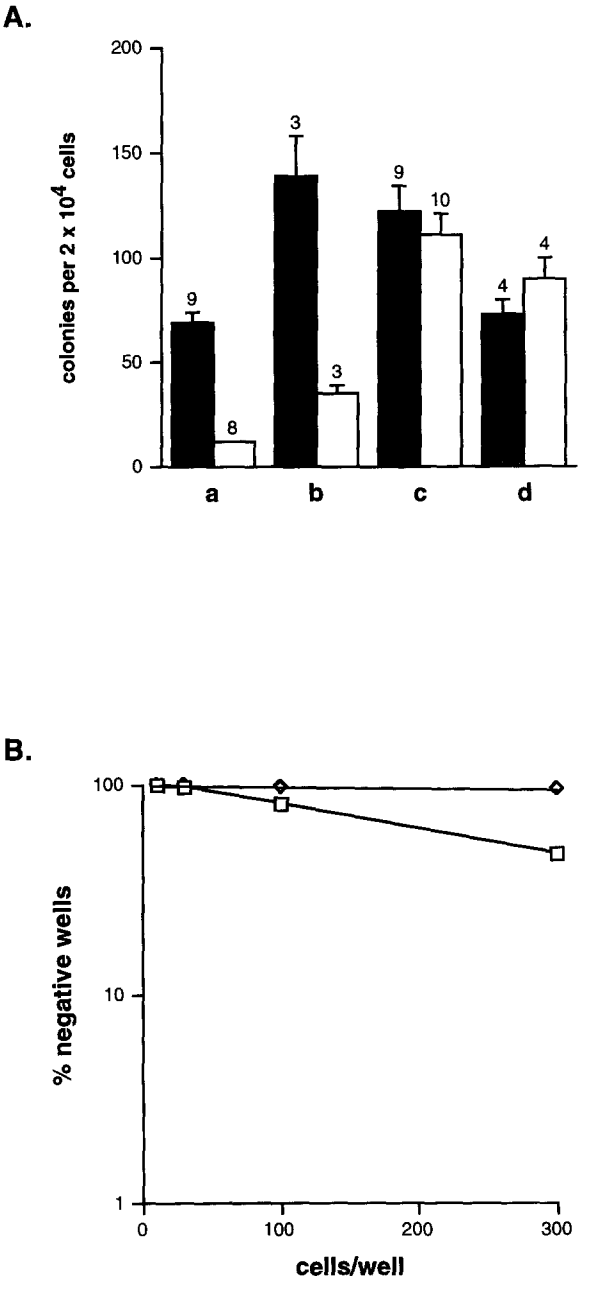

Figure 3. B Cell but Not Myeloid Progenitors Are Reduced in *flk2* Mutants

(A) In vitro analysis of B lymphocyte and myeloid precursors present in adult bone marrow of homozygous mutant (open bars) and heterozygous control (shaded bars) mice. Bone marrow cells were plated in semisolid media in the presence of cytokine combinations: a, IL-7 alone; b, IL-7 and SCF; c, IL-3, IL-6, SCF, and erythropoietin; d, IL-3, IL-6, GM-CSF, and erythropoietin. All mice were age-matched inbreds. Statistical analysis (two-tailed t test) gives $p < 0.0001$ for experiment a (IL-7) and $p < 0.05$ for experiment b (IL-7 plus SCF).

(B) Limiting-dilution analysis of stroma-dependent B cell progenitors in *flk2* mutant bone marrow. Bone marrow was harvested from age-matched homozygous (diamonds) and heterozygous (squares) mice and plated in limiting-dilution over 2018 stromal monolayers. The cultures were maintained in standard Whitlock-Witte conditions and B cell colonies were scored at two weeks. The results are from two separate experiments with 13 mice total, 6 heterozygous and 7 homozygous. The frequency of colony initiating progenitors at 37% negative wells was 1/350 for heterozygous marrow (linear regression coefficient $r = 0.998$) and 1/3000 for homozygous marrow ($r = 0.953$).

of these cells for growth and the rearrangement status of their immunoglobulin genes. Compared with control littermates, all three fractions in *flk2* mutants were reduced about 2-fold (Figure 2D).

To extend our analyses of primitive hematopoietic cells, in vitro colony assays for B lymphoid as well as myeloid progenitors were performed. Bone marrow cells from *flk2* mutants gave rise to about 6-fold fewer B cell colonies stimulated by interleukin-7 (IL-7) and about 4-fold fewer colonies stimulated by IL-7 and SCF. The numbers, sizes, and lineage composition of myeloid (neutrophil, macrophage, neutrophil/macrophage, erythroid, and mixed) colonies were not significantly different between the two groups (Figure 3A).

To quantitate more primitive B lymphoid progenitors, adult bone marrow cells were seeded in limiting dilution onto an established monolayer of 2018 stromal cells (Deryugina et al., 1994) and maintained in B lymphoid conditions (Whitlock and Witte, 1982). In these cultures, the frequency of colony-initiating progenitors in homozygous bone marrow populations was approximately 10-fold reduced (1/350 for the heterozygous controls, but only 1/3000 for the homozygotes) (Figure 3B).

To address a possible function of *flk2* in more primitive hematopoietic progenitors, a long-term culture assay was employed. Long-term Dexter cultures have been shown to maintain multipotential progenitor cells in vitro (Dexter et al., 1976). After 1 month, cultures initiated with either mutant or control cells contained similar numbers of myeloid progenitors (Figure 4A). However, as seen with fresh bone marrow, the frequency of *flk2* mutant stroma-dependent B cell progenitors in these Dexter cultures was 5-fold decreased (1/2600 for the heterozygous controls, but only 1/12000 for the homozygotes) (Figure 4B).

The colony-forming unit-spleen (CFU-S) and pre-CFU-S assays were used as initial measures of in vivo progenitor cell populations. The day 13 CFU-S assay measures myeloid progenitor cells that form colonies in spleens of transplant recipients (Till and McCulloch, 1961; Magli et al., 1982). The *flk2* mutation did not affect the frequency of these CFU-S progenitors. The pre-CFU-S assay measures a cell that is believed to be more closely related to the long-term repopulating stem cell (Ploemacher and Brons, 1989). The frequency of pre-CFU-S progenitors was also not affected by the *flk2* mutation (Figure 4C). In addition, myeloid and lymphoid progenitor cells in the bone marrow of the primary pre-CFU-S transplant recipients were measured. The myeloid progenitor contents were comparable, whereas the frequency of lymphoid precursors was significantly reduced (Figure 4D).

In summary, analyses of hematopoietic cell populations in unperturbed mutant mice, in vitro culture assays, and short-term transplantation experiments have shown no apparent alterations in myelopoiesis but have revealed defects in primitive B lymphoid progenitors.

The *flk2* Mutation Affects the Long-Term In Vivo Repopulating Ability of Myeloid as well as Lymphoid Progenitors

The only completely unambiguous assay for primitive stem cells is long-term transplantation. Accordingly, we

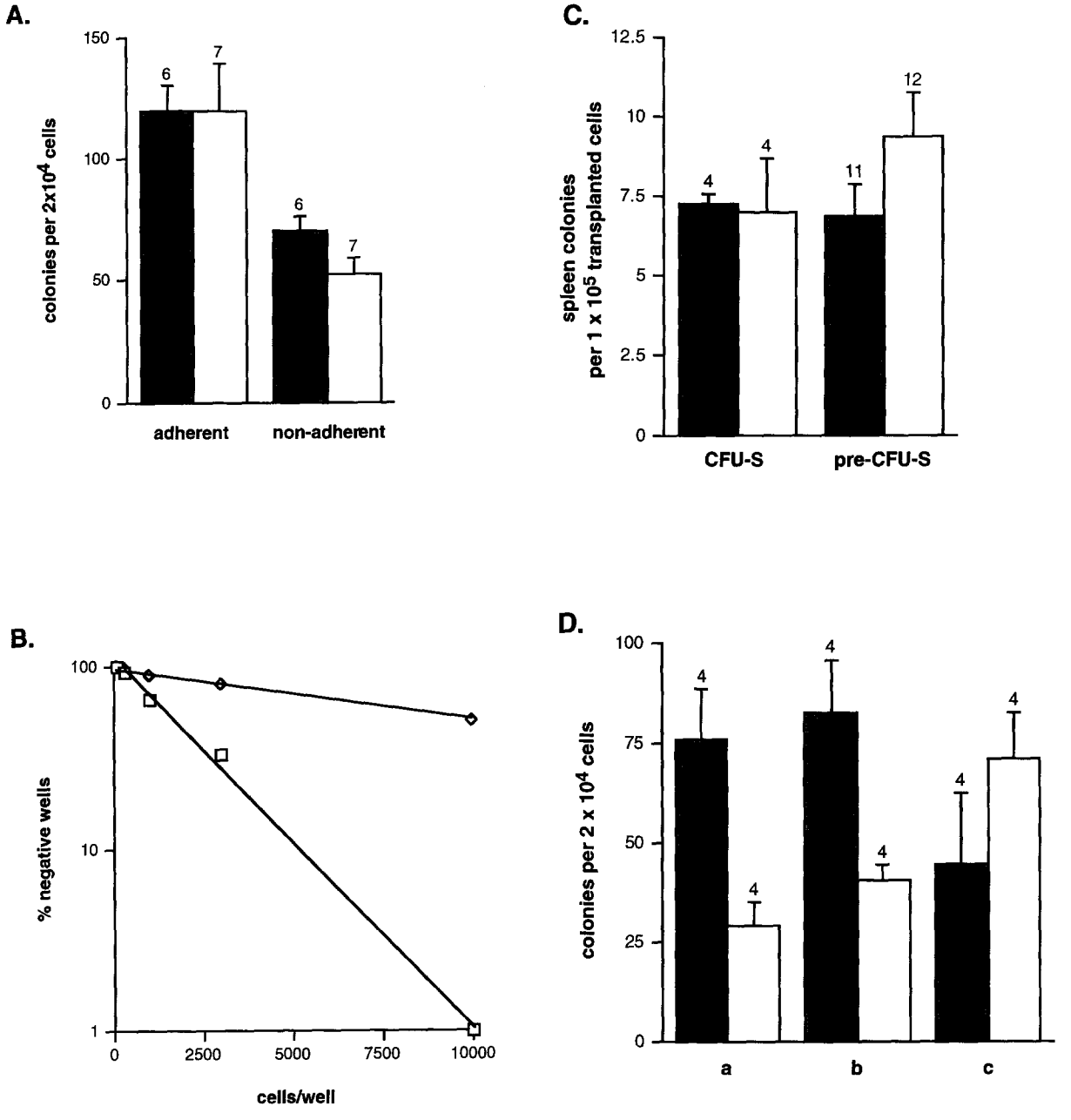

Figure 4. B Lymphoid but Not Myeloid Progenitors Are Reduced in Long-Term *flk2* Mutant Bone Marrow Cultures

(A) In vitro analysis of myeloid progenitors present in Dexter cultures of adult bone marrow of homozygous mutant (open bars) and heterozygous control (shaded bars) mice. Bone marrow was harvested from age-matched, inbred mice and was maintained for four weeks in Dexter cultures. The adherent and non-adherent cells were subsequently harvested and were plated in semisolid media for colony growth in the presence of IL-3, IL-6, SCF and erythropoietin.

(B) Limiting-dilution analysis of stroma-dependent B cell progenitors present in the adherent layer of Dexter cultures of *flk2* mutant bone marrow. The frequency of colony initiating progenitors at 37% negative wells was 1/2600 for heterozygous marrow ($r = 0.912$) and 1/12000 for homozygous marrow ($r = 0.987$).

(C) Day 13 spleen colonies derived from primary (CFU-S) or secondary (pre-CFU-S) transplanted adult bone marrow of homozygous (open bars) or heterozygous (shaded bars) mice.

(D) Colony assay for B cell and myeloid progenitors in *flk2* mutant transplanted bone marrow. Mean number of in vitro colonies derived from homozygous (open bars) or heterozygous (shaded bars) transplanted bone marrow cells are presented. Primary recipients received bone marrow cells from either mutant or control 129/Sv/Ev donors. The number of clonable progenitors in the bone marrow of the recipients was determined 2 weeks later by plating in semisolid media in the presence of a, IL-7 alone; b, IL-7 and SCF; c, IL-3, IL-6, SCF, and erythropoietin. Statistical analysis (two-tailed t-test) gives $p < 0.05$ for a (IL-7) and $p<0.05$ for b (IL-7 plus SCF).

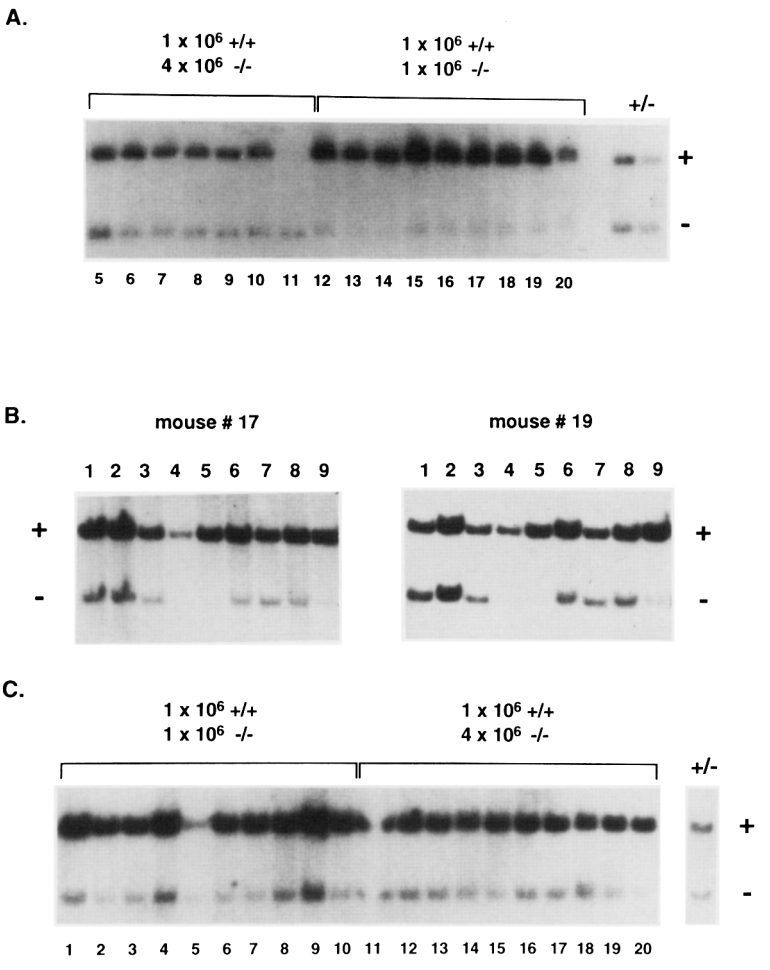

Figure 5. Competitive Repopulation with *flk2* Mutant Bone Marrow Cells

(A) Peripheral blood analysis of primary recipients 9 weeks after transplantation. The cell dosage is indicated and individual recipients are numbered. The wild-type XbaI restriction fragment detected with probe A (plus) is 5.7 kb and the mutant *flk2* allele (minus) is 4.0 kb (see Figure 1A).

(B) Lineage analysis of competitively repopulated mice. The reconstituted recipients were sacrificed 39 weeks after transplantation. Two representative mice are shown. Mice 17 and 19 had each received 1 × 10^6 mutant cells and 1 × 10^6 normal competitor cells. Cells isolated from their hematopoietic organs were separated into various hematopoietic lineages for Southern analysis: (1) total bone marrow, (2) bone marrow granulocytes, (3) bone marrow macrophages, (4) bone marrow B lymphoid cells, (5) total thymus, (6) total spleen, (7) spleen granulocytes, (8) spleen T cells, (9) spleen B cells. DNA analysis was performed as described above.

(C) Peripheral blood analysis of secondary recipients 7 weeks after transplantation. Bone marrow cells obtained from primary recipients were transplanted into lethally irradiated secondary recipients. The cell dosage for the primary recipients is indicated. DNA analysis was performed as described above.

analyzed the ability of mutant stem cells to reconstitute permanent hematopoiesis in irradiated hosts. Specifically, a competitive repopulation experimental design was chosen (Harrison et al., 1993). In this assay, two cell populations are transplanted into the same host to compare directly their developmental potentials. Our experiment was designed to reveal deficiencies of *flk2* mutant cells when they are in competition with wild-type stem cells. Two different doses of bone marrow from *flk2* mutant 129/Sv/Ev mice (1 × 10^6 or 4 × 10^6 cells/animal) were mixed with a constant dose of bone marrow from wild-type 129/SvEMSJ mice (1 × 10^6 cells/animal) and engrafted into lethally irradiated 129/SvEMSJ recipients. At two timepoints after engraftment, DNA samples from peripheral blood of transplant recipients were analyzed by Southern blotting. Hematopoietic reconstitution by mutant and wild-type stem cells was determined by the relative intensities of the targeted and normal *flk2* alleles.

At 9 weeks postengraftment, this analysis revealed a 5-fold reduction in repopulation potential of *flk2* mutant bone marrow cells compared with wild-type competitor cells (Figure 5A; Table 1). The two transplanted cell populations contributed approximately equally when the dosage of mutant cells was four times higher than that of competitor cells. Another blood sample, taken 26 weeks after transplantation, showed a similar reduction in contribution by *flk2* mutant cells (Table 1). At both timepoints little variation was seen between individual recipients, except for recipient number 11, which was reconstituted primarily by the mutant bone marrow cells (Figure 5A).

To determine the differentiation potential of the transplanted progenitor cells, the relative contribution of *flk2* mutant cells to different hematopoietic lineages was analyzed. Recipient mice were sacrificed 39 weeks after transplantation and representative hematopoietic lineages were isolated. In recipients that had received an equal dosage of mutant and wild-type cells, the majority of all hematopoietic cell types was derived from the wild-type marrow (Figure 5B; Table 1). The contribution by *flk2* mutant cells to myeloid cell populations was reduced by 2- to 3-fold. Mutant contribution to the bone marrow B cell population was reduced approximately 4-fold. Thymic T cells and splenic B cells of the recipients were derived almost exclusively from wild-type competitor cells. The contribution of the *flk2* mutant cells to the T cell population in the spleen was not as severely reduced as in the thymus. Even a 4-fold increase in the injected dosage of mutant marrow cells did not allow these cells to compete equally with the wild-type marrow. In fact, the transplantation of larger numbers of *flk2* mutant cells resulted in an almost

Table 1. Competitive Repopulating Ability of *flk2* Mutant Bone Marrow

		1×10^6(−/−) plus 1×10^6(+/+)			4×10^6(−/−) plus 1×10^6(+/+)		
Tissue	Weeks	Percent −/−[a]	Decrease[b]	N	Percent −/−[a]	Decrease[b]	N
Blood 1^0	9	11	4.5	9	43	1.2	7
Blood 1^0	26	11	4.5	9	34	1.5	7
BM	39	22	2.3	8	46	1.1	6
granulocyte		22	2.3		46	1.1	
macrophages		16	3.1		32	1.6	
B cells		14	3.6		17	2.9	
Thymus		6	8.3		2	25	
Spleen		24	2.1		33	1.5	
macrophages		20	2.5		44	1.1	
T cells		18	2.8		34	1.5	
B cells		3	16.7		3	16.7	
Blood 2^0	7	17	2.9	10	25	2.0	10

The contribution of the flk2 mutant bone marrow cells to the reconstitution of hematopoietic lineages at various times after transplantation of irradiated recipients is tabulated. The cell dosage indicates the number of flk2 mutant (−/−) and wild-type (+/+) cells injected into each recipient animal. Two mice died prior to the 39 week analysis. Blood 1^0 or 2^0, blood sample taken from primary or secondary recipients, respectively. N, number of recipients that survived the experimental time period; BM, bone marrow.

[a] Percent of mean contribution by −/− cells.

[b] Fold reduction in mean contribution of −/− cells compared with the contribution by wild-type cells.

complete repopulation of thymocytes by wild-type cells. Collectively, these results demonstrated that the *flk2* mutation not only impaired the development of B cells after transplantation but also had an effect on the generation of T lymphoid and myeloid cells.

To monitor further the proliferative potential of *flk2* mutant hematopoietic stem cells, bone marrow cells from the primary transplant recipients were transferred into a set of secondary mice. These recipients were analyzed after 7 weeks. The relative abilities of the mutant and wild-type stem cells to repopulate secondary recipients were similar to their activities in the primary mice (compare blood 1^0 and 2^0 in Table 1 and Figure 5).

The Entire Hematopoietic System of W/W^v *flk2*−/− Mutant Mice Is Diminished and Lymphoid Populations Are Severely Affected

To address the possibility of a functional redundancy between the *flk2* and *c-kit* receptors, mice with mutations in both of these genes were generated. Mating pairs consisted of a W^v/+ parent and a W/+ parent, one of which was heterozygous and the other homozygous for the *flk2* mutation. A smaller than expected fraction of the obtained W/W^v progeny was homozygous for the targeted *flk2* locus (17 W/W^v *flk2*−/− pups versus 31 W/W^v *flk2*+/− pups, $p < 0.05$). This distortion was not observed among W^v/+ or W/+ mice (data not shown). All live-born W/W^v *flk2*−/− and W/W^v *flk2*+/− mice appeared to develop normally into white-coated adults with a slightly reduced body weight when compared with black littermates at 3 weeks of age (Figure 6A). However, even though W/W^v *flk2*−/− and W/W^v *flk2*+/− mice were of equivalent average size and weight, the overall cellularities of the bone marrow and thymus of W/W^v *flk2*−/− mice were approximately 5-fold and 6-fold decreased, respectively (Figure 6B).

Flow cytometric analysis of different hematopoietic cell lineages revealed that the reduced cellularities in W/W^v *flk2*−/− mice encompassed both lymphoid and myeloid cell lineages (data not shown). In addition, a 2- to 3-fold further reduction in the frequencies of B lymphoid cells in the bone marrow of the W/W^v *flk2*−/− mice was evident (Table 2). The double mutants also had a slight decrease in the frequency of splenic B cells (Table 2). Taking into account the reduction in cellularity, the total number of B lineage cells in W/W^v *flk2*−/− bone marrow was reduced 10-fold relative to W/W^v *flk2*+/− mice bone marrow.

In vitro colony assays revealed no difference in the frequencies of myeloid lineage progenitors between double-mutant and control marrow cells (Table 2), nor did myeloid cell colonies differ in type or size. However, the absolute number of myeloid progenitors was diminished 8-fold in W/W^v *flk2*−/− mice. We observed a 16-fold reduction in the frequency of in vitro colony-forming progenitors of the B cell lineage, which is more severe than the 2-fold reduction in precursor B cell levels observed in *flk2* single-mutant littermates (Table 2) (the phenotype of *flk2*−/− single-mutant mice was not as severe in this 129/Sv/Ev × C57BL/6J outbred strain background as previously seen in the 129/Sv/Ev inbred strain background; see Figure 3A). Because of the reduction in bone marrow cellularity, the absolute number of clonogenic B lymphoid progenitor cells in W/W^v *flk2*−/− mice was reduced 56-fold. B lymphoid progenitors were also analyzed by flow cytometry. As expected, the *flk2*−/− single-mutant mice revealed a 2-fold reduction in their pro-B cell compartment and no consistent decrease in their levels of pre-B cells (Table 2). However, compared with *flk2* single-mutant animals, the pro-B and pre-B cell pools of W/W^v *flk2*−/− mice were reduced by another 2-fold (Table 2). It appears, therefore, that the diminished B lymphoid cell pools in W/W^v *flk2*−/−

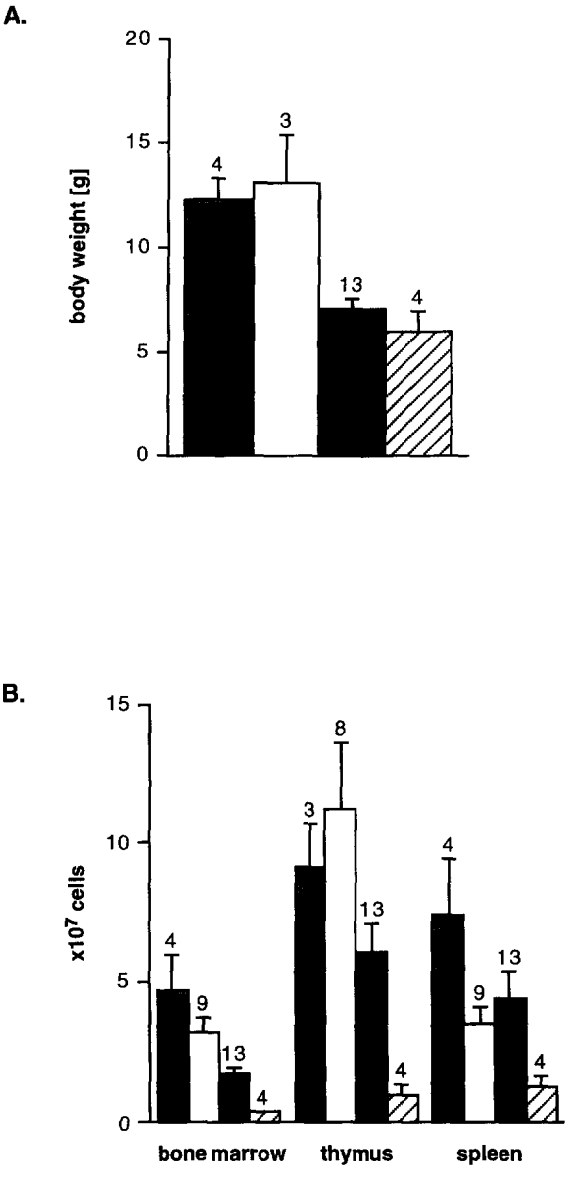

Figure 6. The Cellularity of Hematopoietic Organs in W/W^v *flk2–/–* Mutant Mice is Reduced

(A) Average weight of 3-week-old *flk2+/–* (shaded bars), *flk2–/–* (open bars), W/W^v *flk2+/–* (closed bars), and W/W^v *flk2–/–* (striped bars) mice.

(B) Average white blood cell numbers of hematopoietic organs of 3-week-old *flk2+/–* (shaded bars), *flk2–/–* (open bars), W/W^v *flk2+/–* (closed bars), and W/W^v *flk2–/–* (striped bars) mice. The *flk2+/–* and *flk2–/–* mice were wild-type at the c-*kit* locus, as determined by coat color. For the reduction in bone marrow and thymus cell counts in W/W^v *flk2–/–* mice compared with W/W^v *flk2–/–* mice, statistical analysis (two-tailed t test) gives $p < 0.01$ and $p < 0.05$, respectively.

double-mutant mice are due to more pronounced deficiencies in primitive cell populations.

Frequency variations in thymocyte subpopulations of 3-week-old W/W^v *flk2–/–* pups were also detected. Compared with W/W^v *flk2+/–* mice, the proportion of CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ thymocytes, which represent mature T lymphocytes, was increased by approximately 2-fold (Ta-

Table 2. Abnormal Frequencies of Lymphoid Cell Populations in W/W^v *flk2–/–* Mice

	BM B220$^+$	SP B220$^+$	BM CFU-B	BM CFU-C	BM Pro-B	BM Pre-B	BM Imm+Mat B	Th CD4$^-$CD8$^-$	th CD4$^+$CD8$^+$	Th CD4$^+$CD8$^-$	Th CD4$^-$CD8$^+$
W/W^{v}flk2+/–	37.2 ± 5.4 (13)	57.0 ± 4.1 (13)	69.7 ± 16.4 (13)	72.6 ± 14.5 (8)	8.6 ± 1.1 (10)	15.1 ± 2.6 (10)	9.6 ± 1.6 (8)	2.2 ± 0.3 (13)	76.3 ± 2.7 (13)	7.8 ± 0.7 (13)	2.1 ± 0.5 (13)
W/W^{v}flk2–/–	16.4 ± 3.8 (4)	38.4 ± 6.8 (4)	4.5 ± 2.3 (4)	58.0 ± 21.5 (3)	2.5 ± 0.3 (3)	10.4 ± 3.8 (3)	10.1 ± 0.5 (3)	2.0 ± 0.6 (4)	69.0 ± 9.9 (4)	18.9 ± 8.1 (4)	5.1 ± 1.6 (4)
flk2+/–	45.2 ± 3.0 (4)	56.4 ± 2.6 (4)	96.3 ± 9.6 (4)	69.0 ± 3.4 (4)	10.4 ± 0.9 (2)	23.8 ± 1.1 (2)	12.1 ± 0.2 (2)	3.2 ± 0.6 (3)	80.5 ± 2.0 (3)	6.8 ± 1.1 (3)	1.1 ± 0.4 (3)
flk2–/–	41.9 ± 2.0 (9)	54.7 ± 1.1 (9)	43.8 ± 10.8 (9)	63.8 ± 20.5 (5)	5.0 ± 0.8 (5)	19.8 ± 2.0 (5)	9.5 ± 0.7 (5)	3.2 ± 0.4 (8)	81.7 ± 1.3 (8)	6.1 ± 0.4 (8)	3.8 ± 1.1 (8)

Mean percentages with standard errors of hematopoietic cell populations are presented. The sample size of each group is given in parenthesis. All animals were 3 weeks old and outbred. Comparison of W/W^{v}flk2+/– and W/W^{v}flk2–/– mice gives $p = 0.0573$ for B220$^+$ bone marrow cells and $p < 0.05$ for splenic B220$^+$ cells, thymic CD4$^+$CD8$^-$ cells, thymic CD4$^-$CD8$^+$ cells, CFU-B, and pro-B cells. Comparison of W/W^{v}flk2–/– and flk2–/– mice gives $p < 0.0001$ for bone marrow B220$^+$ cells, $p < 0.005$ for splenic B220$^+$ cells, $p = 0.0577$ for pro-B cells, and $p < 0.05$ for thymic CD4$^+$CD8$^-$ cells and CFU-B; the slight difference in CD4$^-$CD8$^+$ levels was not statistically significant. Comparison of flk2+/– and flk2–/– mice give $p < 0.05$ for *pro-B cells*. BM, bone marrow; Th, thymus; Sp, spleen; pro, progenitor; pre, precursor; imm, immature; mat, mature.

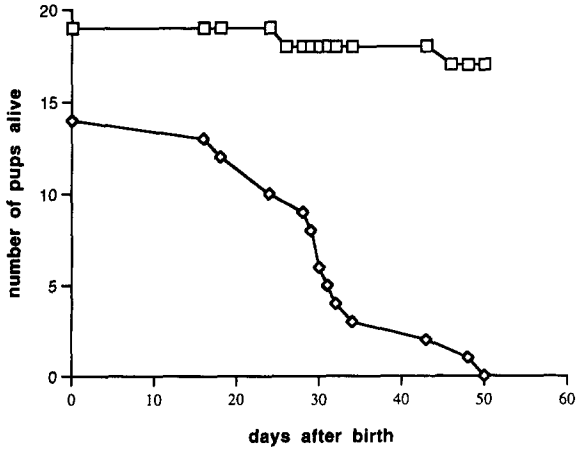

Figure 7. Viability Curve for W/W^v *flk2–/–* Pups

Comparison between the viability of *flk2–/–* (triangles) and flk2+/– (squares) W/W^v pups. The number of living pups (y-axis) is plotted against the number of days after birth (x-axis). All mice were in an outbred strain background.

ble 2). However, taking into account the reduction in thymus cellularity, W/W^v *flk2–/–* mice had approximately 4-fold fewer thymic CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ cells. Since the frequency of less mature thymocytes, namely the CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ T cells was not elevated, their total numbers were more severely affected by the reduction in thymus cellularity, resulting in 6- and 9-fold reductions, respectively.

After the age of 3 weeks, W/W^v *flk2–/–* mice exhibited severely reduced viability; none have survived for longer than 6 weeks (Figure 7). As they approached death, some animals appeared thin and weak, but overall, W/W^v *flk2–/–* mice showed no overt signs of disease. Postmortem analysis did not reveal obvious alterations. Detailed pathological examinations should help identify the cause of death.

Discussion

Mice carrying a loss-of-function mutation at the *flk2* locus were generated. Animals homozygous for the *flk2* mutation were viable, healthy, and fertile. The frequency of newborn homozygotes did not deviate from the predicted Mendelian frequency. The expression pattern of the *flk2* gene had suggested a function for this receptor in hematopoiesis. Accordingly, analyses of the mutant mice were focused on their hematopoietic system. Mature peripheral blood and tissue-derived hematopoietic cell populations in mutants were qualitatively and quantitatively identical to those in wild-type or heterozygous littermates. However, a statistically significant decrease was observed in the overall number of B lymphoid cells in bone marrow.

The *flk2* receptor is predominantly expressed in developmentally primitive cell populations (Matthews et al., 1991). Accordingly, stem/progenitor cells in homozygous mutant mice were analyzed. A flow cytometric dissection of the B cell developmental pathway in bone marrow revealed depletions in three primitive pro-B cell compartments. These populations are the earliest phenotypically identifiable precursors of this lineage in the adult mouse. The absolute numbers and relative frequencies of the more differentiated pre-B, immature-B, and mature B cells were at levels largely identical to those in heterozygous or wild-type littermates. In vitro stromal-dependent and stromal-independent colony assays confirmed the reduction in primitive B cell populations. These data suggest a potential role(s) for the *flk2* receptor in the maintenance, expansion, generation, or some combination of the three, of the primitive portion of the B lymphoid lineage. In long-term myeloid cultures, which support the survival of multipotential progenitors but not that of committed B cell precursors (Jones-Villeneuve and Phillips, 1980; Schrader and Schrader, 1978; Dorshkind and Phillips, 1983), the frequency of progenitor cells that can initiate subsequent stromal-dependent B lymphopoiesis was severely decreased. Therefore, *flk2* may play a role in the commitment of a more primitive progenitor to the B cell lineage.

The defect in B cell development in *flk2* mutant mice was cell intrinsic. *flk2* mutant bone marrow contained fewer B lymphoid precursors irrespective of whether the bone marrow cells were isolated directly from the mutant mice, recovered from transplant recipients, or maintained in cultures on wild-type stromal cell lines for extended periods of time. Therefore, the phenotype of *flk2* mutant mice is not due to insufficiencies in the microenvironment but is instead due to a cell intrinsic defect in hematopoietic progenitor cells.

Analysis of three successive pro-B cell stages showed consistent reductions in *flk2* mutant mice; however, by the pre-B cell stage more normal levels were observed. One possible explanation of these observations is that there exist balancing mechanisms within the B lymphoid lineage that compensate for deficiencies in primitive progenitor cell compartments by stimulating the generation, proliferation, or survival of more differentiated B cells.

In vitro colony assays failed to demonstrate a deficiency in any population of myeloid colony-forming progenitors, including multipotential cells. Similarly, short-term CFU-S and pre-CFU-S in vivo transplantation studies revealed normal numbers of these progenitors in the *flk2* homozygous animals. Mutant bone marrow cells were also fully capable of sustaining long-term in vitro myelopoiesis in cultures on preestablished stromal monolayers. Taken together, these data do not support a critical role(s) for the *flk2* receptor in myelopoiesis. A function for the *flk2* receptor in myelopoiesis had been previously proposed on the basis of antisense oligonucleotide in vitro culture experiments (Small et al., 1994). Similarly disparate results from antisense and gene-targeting approaches have been observed in studies of the *vav* gene (Wulf et al., 1993; Zhang et al., 1994).

All of the above studies focused on steady-state hematopoiesis in *flk2* mutants or on the ability of mutant cells to function in the absence of competition from wild-type cells. Moreover, none of these studies addressed a potential role for *flk2* in the behavior of the most primitive stem cell

population. To increase the resolution and scope of mutant analyses, long-term competitive repopulation studies were performed. The ability of mutant bone marrow cells to contribute to myeloid and lymphoid cell lineages and to total nucleated peripheral blood cell populations was measured relative to wild-type marrow. The long postreconstitution times of analysis insured that all mature cell populations in the transplant recipients were derived from primitive stem cells rather than from more committed progenitors.

The competitive reconstitution experiment magnified and extended the *flk2* mutant phenotype. In contrast with the experiments discussed above, this study revealed an impaired ability of mutant stem cells to contribute to both myeloid and lymphoid cell populations. However, mutant stem cell contribution to B and T cell lineages was more severely affected than contribution to myeloid cell populations. The long-term nature of these studies suggests a possible role for *flk2* in multipotent stem cells or their immediate clonal progeny.

Of particular significance is the marked deficiency in repopulation of the T cell lineage by mutant cells. This result complements our previous finding that *flk2* is expressed in a primitive thymocyte subset defined as $CD4^-CD8^-Thy\text{-}1^{lo}IL\text{-}2R^-$ (Matthews et al., 1991). A statistically significant, though subtle, decrease in this thymocyte population in *flk2* mutant mice at postnatal days 0–2 was also observed (data not shown). The levels of all other thymocyte subsets were normal, as were the levels of this very primitive subset in mice of different pre- or postnatal stages. It is possible that a more elaborate analysis of $CD4^-CD8^-$ cell subsets will reveal additional immature progenitor decreases at other developmental stages. Taken together, our results clearly demonstrate a role for *flk2* in T cell development, possibly analogous to its role in the B cell lineage.

The *flk2* receptor was recently shown to be expressed on a subset of long-term reconstituting stem cells. Moreover, *flk2*-expressing stem cells appear to be predominantly in active cell cycle (Zeigler et al., 1994). Within the population of stem cells, there is a direct correlation between cell cycle activity and commitment to differentiation (Lemischka, 1992). Therefore, the *flk2* receptor could function within a subpopulation of stem cells poised for active hematopoiesis. As such, it could function in commitment/differentiation processes rather than in self-renewal events. Accordingly, the mutant stem cells should display no defect in their ability to self-renew. The observation, that the hematopoietic activity of transplanted mutant stem cells is stable over long time intervals, is consistent with this. A more stringent measure of self-renewal is the ability of stem cells to function upon serial retransplantation. Retransplantation of marrow from the reconstituted animals did not reveal a further quantitative impairment in the ability of mutant stem cells to contribute to mature blood cells. This is a strong indication that the *flk2* mutation does not impair the self-renewal process. Importantly, the observed similarities for mutant stem cell activity in primary and secondary recipient animals also suggest that the *flk2* receptor does not play a major role in stem cell engrafting parameters such as homing and seeding of bone marrow microenvironments.

Although our studies suggest the importance of *flk2* in lymphoid development and in multipotential stem cell differentiation pathways, the viability of the mutants and the subtle nature of the phenotype suggest the existence of at least one compensatory pathway. Owing to similarities in structure and expression pattern, an obvious candidate for such a role is the c-*kit* receptor. Both receptors are expressed in multipotent hematopoietic progenitor cells capable of long-term reconstitution of irradiated mice (Matthews et al., 1991; Ogawa et al., 1991; Okada et al., 1992). The c-*kit* receptor and its ligand are also thought to be involved in lymphopoiesis (Rolink et al., 1991; Billips et al., 1992; Palacios and Samaridis, 1992). Accordingly, the consequences of combining the *flk2* mutation with mutations at the c-*kit* locus were investigated. The W and the W^v alleles of c-*kit* were chosen, because this combination (W/W^v), although severely anemic, is viable (Russell, 1979). Surprisingly, W/W^v animals homozygous for the *flk2* mutation were obtained, albeit at lower than expected frequency. This indicates that hematopoietic development can occur in the absence of the *flk2* receptor and severely reduced c-*kit* receptor activity.

In contrast with c-*kit* or *flk2* single-mutant animals, mice homozygous for both c-*kit* and *flk2* mutations had severely reduced myeloid and lymphoid cellularities in their primary hematopoietic organs. Thus, both receptors have functions that together provide for normal overall levels of blood cells. *flk2* may therefore function together with c-*kit* in the progenitor cells of all hematopoietic cell lineages. Alternatively, the two receptors could function separately at distinct stages of hematopoiesis and their coordinated activities may insure the production of normal numbers of hematopoietic cells.

The two receptors also appear to function together in lymphoid development. The absolute numbers of bone marrow B cells and thymic T cells were decreased in the double mutants. Furthermore, W/W^v animals homozygous for the *flk2* mutation possessed a greater reduction in progenitor B cell frequency than *flk2* or c-*kit* single-mutant animals. In addition, the relative frequencies of T lymphoid cells were altered in favor of mature T lymphocytes. Since neither c-*kit*, nor *flk2*, are expressed in mature T cells or in their immediate $CD4^+CD8^+$ precursors (Matthews et al., 1991; Palacios and Nishikawa, 1992), this increase in frequency may reflect compensatory mechanisms in this lineage, which function to correct deficiencies in primitive cell pools. Alternatively, it is possible that the lack of functional *flk2* and c-*kit* receptors causes an acceleration of differentiation in the T cell lineage, leading to a relative increase in the percentages of mature cells.

In summary, it appears that signaling through two structurally related molecules, the c-*kit* and *flk2* receptors, insures the proper production or expansion of hematopoietic progenitors, leading ultimately to the generation of adequate populations of mature hematopoietic cells. Because of the striking reduction in the viability of double-mutant mice, it seems likely that their combined functions in the

hematopoietic system are vital to the survival of the organism.

Experimental Procedures

flk2 Homologous Recombination Constructs

To construct the targeting vectors, four fragments were ligated: the upstream and downstream *flk2* gene sequences, the PGK-1–*neo* gene, and the MC1–*tk* gene. From cosmid clone 16–1 (isolated from a 129 isogenic mouse genomic library [gift from Dr. L. Silver, Princeton University]), which contains the 5′ half of the *flk2* gene, a 2.8 kilobase (kb) EcoRI–BamHI fragment was isolated and subcloned into the pSport1 vector (GIBCO BRL). The insert was sequenced from the BamHI side to verify that this restriction site was located in exon 3 of the *flk2* gene. A 1.0 kb fragment reaching from this BamHI site to a PstI site located further upstream was isolated, HindIII linkers were added to the PstI site, and the BamHI site was blunted. This fragment was then cloned into the PGK-1–*neo* plasmid that had been linearized with EcoRV and HindIII to yield subclone III-1. A 13.2 kb BamHI fragment containing *flk2* exons 4–9 as well as part of exon 3 was isolated from cosmid clone 16–1 and modified with NotI linkers. The fragment was ligated in the correct orientation into subclone III-1 cut with NotI to derive subclone III-2. Finally, a 1.86 kb ClaI–SalI fragment containing the MC1–*tk* gene was isolated from the pIC19R–MC1-TK plasmid and ligated with subclone III-2 cut with the same enzymes to yield the *flk2* targeting construct III. The vector was linearized with SalI before introduction into ES cells. The pPGK–*neo* bpA and pIC19R–MC1-TK plasmids were a gift from Dr. E. Robertson (Harvard University).

Transfection and Selection of ES Cells

CCE ES cells (Robertson et al., 1986) were grown as previously described (Robertson, 1987). Confluent plates of ES cells were harvested and electroporated with 10 μg/ml of DNA using a Gene Pulser (Bio-Rad). The cells were selected in G418 (0.6 mg/ml, GIBCO BRL) and (1-[2-deoxy, 2-fluoro-β-D-arabinofuranosil-]-5-iodouracil) FIAU, 0.2 μM final concentration (Oclassen Pharmaceutical, Incorporated). Colonies were picked and expanded.

PCR Screening and Southern Blot Analysis of Recombinant Clones

DNA was extracted from ES cells as previously described (Laird et al., 1991). PCR analysis was performed with primers derived from the *flk2* genomic sequence upstream of the PstI site preceding exon 3 (5′-ACAGTGCTCAGTACCTATAG-3′) and from the *neo* gene (5′-TAAAGCGCATGCTCCAGACT-3′). The size of the *flk2/neo* PCR product diagnostic for a homologous recombination event is 1.0 kb. DNA from PCR-positive ES clones was digested with XbaI, EcoRI, or NcoI, Southern blotted, and hybridized with a 150 bp PCR-amplified fragment (PCR primer sequences were 5′-CAGATGTGCCATGTGGTTGA-3′ and 5′-CTATAGGTACTGAGCACTGT-3′) that contains *flk2* intron sequence (probe A). Hybridization conditions were as previously described (Jordan and Lemischka, 1990). Probes were labeled by the hexamer extension method (Feinberg and Vogelstein, 1983).

Generation of Chimeric Mice

Chimeras were generated as described (Bradley, 1987). Approximately 10–15 ES cells were injected into C57BL/6J blastocysts at 3 days postcoitum. The embryos were transplanted surgically to the uteri of pseudopregnant C57BL/6J × DBA F1 recipients 2.5 days postcoitum. Pups were scored for chimerism on the basis of agouti coat color. Chimeric males were bred to female C57BL/6J or 129/Sv/Ev mice, the strain from which the CCE line was derived (Robertson et al., 1986). Germline transmission was scored by the presence of agouti coat color in the F1 animals. 129/Sv/Ev mice carrying the mutation were bred back to 129/Sv/Ev mice to generate inbred mutant mice.

Animals

Animals were housed under nonsterile conditions in a conventional animal facility. They were provided with standard lab chow and water ad libidum. C57BL/6J, 129/SvJ, and 129/SvEMSJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The C57BL/6J × DBA F1 blastocyst recipients as well as the vasectomized males of the same genetic background were obtained from Charles River. Breeding pairs of W/+ and W^v/+ mice in the C57BL/6J background were the gift of Dr. P. Besmer (Sloan Kettering Institute, New York). Their W/W^v, +/+, W/+, and W^v/+ offspring were identified by their coat colors. All mice were genotyped for the *flk2* mutation by Southern blotting.

RNA Analysis

Total RNA from day 14 fetal liver or adult bone marrow was purified by the guanidinium–isothiocyanate protocol followed by cesium chloride centrifugation (Ausubel et al., 1987). For Northern analysis, 30–40 μg of RNA was blotted and hybridized with a ^{32}P-labeled *flk2* cDNA probe (a 3.5 kb fragment covering the entire coding region). Final washes were at 65°C in 0.1× SSC, 0.1% SDS. For PCR analysis of RNA, reverse transcription was performed with the Superscript II kit (GIBCO BRL) on poly(A)-selected RNA isolated from adult bone marrow cells. The resulting cDNA was amplified by PCR using the following *flk2*-specific primers: primer A, bp 96 (5′-TCTTGAGACCGTTACAAACC-3′); primer B, bp 391 (5′-AATCAAAGTGCGGCTGGC-3′); primer C, bp 719 (5′-ATGTCTGTTCCGAACAACTC-3′); primer D, bp 980 (5′-AAGGCCA-AGAGAATCCGAAT-3′); primer E, bp 1292 (5′-GCATAGAATATGTAC-TCTCC-3′). PCR conditions were optimized using the Opti-Prime PCR kit (Stratagene). For subsequent sequencing, single-stranded template was generated from the PCR products by asymmetric PCR as described (McCabe, 1990). The Sequenase kit (US Biochemical) was used for sequence analysis.

Flow Cytometry Analysis

Single cell suspensions were prepared from individual tissues, including bone marrow, spleen, and thymus, by standard procedures (Parks et al., 1986). The antibodies RA3–6B2 (rat anti-mouse CD45R [B220], allophycocyanin-conjugated), R6–60.2 (rat anti-mouse immunoglobulin M [IgM], biotin-conjugated), rat anti-mouse Mac-1 (R-phycoerythrin-conjugated), rat anti-mouse Gr-1 (fluorescein isothiocyanate–conjugated), and streptavidin (Texas red–, fluorescein isothiocyanate–, or R-phycoerythrin-conjugated) were purchased from PharMingen. Rat anti-mouse Ter119 was a gift from Dr. T. Kina (Kyoto University, Japan) and monoclonal antibodies M1/69 (rat anti-mouse HSA), 6C3 (rat anti-mouse 6C3/BP-1), S7 (rat anti-mouse leucosialin (CD43), and rat anti-mouse IgD were a gift from Dr. A. Stall (Columbia University). All antibodies were titrated on tissues (normal spleen, thymus, and bone marrow) with distinct positive and negative subpopulations and used at saturating concentrations and were compared with isotype controls. Approximately 1 × 10^6 cells were used for most stainings. Generally, the cells were first blocked with normal rat serum. They were then incubated on ice with the first antibody, washed three times in PBS plus 3% fetal calf serum (FCS) and resuspended in PBS plus 3% FCS. The next antibody or a secondary antibody was added for another incubation on ice followed by another three washes in PBS plus 3% FCS before FACS analysis. Background controls were treated identically except that primary antibodies were omitted. Cells were initially gated by size and by scatter to identify live cells. In some experiments, cell viability was also monitored by propidium iodide staining. Data from 1–5 × 10^4 gated cells was collected. The statistical evaluation of the data was done with the StatView 4.02 software package (Abacus Concepts, Incorporated).

In Vitro CFU Assays

CFU assays were performed as previously described (Okada et al., 1992). For each sample, 2 × 10^4 cells were plated in triplicate in 1 ml cultures. The culture mixture consisted of 1.2% methylcellulose (Fischer-Scientific) in αMEM containing 30% FCS, 0.1 mmol/l β-mercaptoethanol, and 1% deionized bovine serum albumin and was supplemented with growth factors (5 U/ml recombinant human [rh] erythropoietin [Epo] [R and D Systems, Incorporated, Minneapolis, Minnesota], 2 ng/ml recombinant mouse [rm] IL-7 [UBI, Lake Placid, New York], 100 ng/ml rh IL-6 [UBI], 10 ng/ml rm IL-3 [UBI], 10 ng/ml rm SCF [Genzyme, Cambridge, Massachusetts]). The cultures were maintained at 5% CO_2 and 37°C. The CFU-B (rm IL-7) cultures were counted using an inverted microscope 5 days after plating, whereas the CFU-C (rh IL-6, rm IL-3, rh EPO, rm SCF) and CFU-B (rm IL-7, rm SCF) plates were scored after 7 days. Experiments involving young W/W^v mice were done as described above except that 4 × 10^4 bone marrow cells were plated per dish and SCF was omitted.

Stromal Cultures

Stromal cocultures were established with two different day 14 fetal liver stromal cell lines. The 2018 stromal line has been shown to support B cell progenitors in Whitlock–Witte conditions (Deryugina et al., 1994; R. Wager et al., unpublished data). The AFT024 stromal line has been shown to efficiently support high levels of myeloid–erythroid progenitors, late-appearing cobblestone area cells, and long-term in vivo repopulating stem cells in Dexter conditions (R. Wager et al., unpublished data). The Dexter–myeloid culture media was 10% FCS, 10% horse serum, 50 μM β-mercaptoethanol, 0.1 μM hydrocortisone in DMEM (modified from Dexter et al., 1976). Cultures were maintained at 33°C in 5% CO_2, 100% humidity. Whitlock–Witte culture media was 5% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 50 μg/ml streptomycin in RPMI 1640 (Whitlock and Witte, 1982). Cultures were maintained at 37°C in 5% CO_2, 100% humidity. Stromal cells were plated on gelatin coated dishes and irradiated (2000 rads) when subconfluent. Dexter cocultures were initiated with 10^6 bone marrow cells/ml and maintained for 4 weeks with weekly half-volume media changes. Limiting-dilution Whitlock–Witte cultures were performed in 96-well trays, 24 wells per cell dilution. Media was changed weekly and colonies were counted at 2 weeks. The limiting-dilution data was evaluated by linear regression analysis.

CFU-S and Pre-CFU-S Assays

Assays were performed as previously described (Till and McCulloch, 1961; Ploemacher and Brons, 1989; Magli et al., 1982). The recipients were irradiated with 950 rads (137 Cs source) administered in two equal doses 3 hr apart. This dose is sufficient to eliminate endogenous spleen colony formation completely. For day 13 CFU-S, they were injected intravenously with 1×10^5 bone marrow cells. All recipients were maintained in microisolator cages on sterilized food and acidified sterile water containing 10^6 U/liter polymyxin B sulfate and 1.1 g/liter neomycin sulfate. The recipients were killed after 13 days, their spleens were fixed in Teleyesniczky's solution (70% ethanol:acetic acid:formalin, 20:1:1), and macroscopic colonies were counted. For the pre-CFU-S assay, four primary 129/SvEMSJ recipients for each group were transplanted with 1×10^6 femoral marrow cells isolated from either three mutant or control donor mice. Bone marrow cells obtained from these primary recipients 14 days later were pooled and then injected into secondary 129/SvJ mice, 1×10^5 cells per recipient. Spleen colonies were enumerated at 13 days.

Competitive Repopulation

129/SvEMSJ recipient mice were irradiated and maintained as described above. Bone marrow cell suspensions were prepared from femurs and tibias of three *flk2* homozygous 129/Sv/Ev donor mice or three wild-type 129/SvEMSJ competitor mice. The cell suspensions were then mixed at 1:1 and 4:1 (mutant:wild-type) ratios. Doses of 2×10^6 and 5×10^6 cells were then injected intravenously. Repopulation of the hematopoietic system after transplantation was determined by Southern blotting. Blood was obtained from the retro-orbital sinus and genomic DNA was extracted as described (Jordan and Lemischka, 1990). After 9 months the primary recipients were sacrificed, their hematopoietic organs were harvested, and cell lineages were fractionated as described (Jordan and Lemischka, 1990). The purity of the myeloid and lymphoid fractions was 80%–90% as assessed by staining for lineage-specific antigens (Gr-1, Thy-1.2, B220, Mac-1) and flow cytometry. Monocyte–macrophages were grown in L cell–conditioned medium for 6–10 days as described (Jordan and Lemischka, 1990). Secondary recipients were injected with bone marrow cells pooled from the primary recipients (2×10^6 cells/animal). DNA was extracted from all cell fractions. The intensity of individual bands on filters representing wild-type or targeted *flk2* alleles was measured using a PhosphoImager (Molecular Dynamics) and quantitated using the ImageQuant software packet. The efficiency of transfer and hybridization of the two bands was controlled for by inclusion of DNA samples from heterozygous *flk2* mice on each blot.

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