Quantitative Trait Analysis Reveals Transforming Growth Factor-β2 as a Positive Regulator of Early Hematopoietic Progenitor and Stem Cell Function

Jessica C. Langer, Els Henckaerts, Jonathan Orenstein, and Hans-Willem Snoeck

Abstract

Elucidation of pathways involved in mouse strain–dependent variation in the hematopoietic stem cell (HSC) compartment may reveal novel mechanisms relevant in vivo. Here, we demonstrate genetically determined variation in the proliferation of lin$^-$/Sca1$^+$kit$^+$ (LSK) primitive hematopoietic progenitor cells in response to transforming growth factor-β (TGF-β) 2, the dose response of which was biphasic with a stimulatory effect at low concentrations. In contrast, the dose responses of TGF-β1 or -β3 were inhibitory and did not show mouse strain–dependent variation. A quantitative trait locus (QTL) for the effect of TGF-β2 was identified on chromosome 4 overlapping with a QTL regulating the frequency of LSK cells. These overlapping QTL were corroborated by the observation that the frequency of LSK cells is lower in adult Tgfb2$^{+/−}$ mice than in wild-type littermates, indicating that TGF-β2 is a genetically determined positive regulator LSK number in vivo. Furthermore, adult Tgfb2$^{+/−}$ mice have a defect in competitive repopulation potential that becomes more pronounced upon serial transplantation. In fetal TGF-β2–deficient HSCs, a defect only appears after serial reconstitution. These data suggest that TGF-β2 can act cell autonomously and is important for HSCs that have undergone replicative stress. Thus, TGF-β2 is a novel, genetically determined positive regulator of adult HSCs.

Key words: growth inhibitors • immunologic and biologic factors • hematopoiesis • autocrine communication • growth and embryonic development

Introduction

The lifelong production of blood cells is maintained by hematopoietic stem cells (HSCs) that display the capacity to differentiate into multiple lineages of mature cells and to self-renew. Although these characteristics of stem cells have been established for some time, the underlying regulatory mechanisms remain poorly understood (1, 2). The observation that HSC number and function vary in inbred stains of mice provides strong evidence that these mechanisms are, in part, genetically determined (3–11). A powerful approach to begin to dissect the regulation of HSCs is the investigation of genetically determined variation in the function and size of the HSC compartment by quantitative trait analysis. We have demonstrated previously genetic variation in the number and frequency of hematopoietic stem and progenitor cells as determined by the lin$^-$/Sca1$^+$kit$^+$ (LSK) phenotype and in their response to the early acting cytokines kit ligand (KL), flt3 ligand (flt3L), and thrombopoietin (TPO). Linkage analysis identified quantitative trait loci on chromosomes 2, 4, and 7 for the frequency of LSK cells, and on chromosome 2 and X for their response to early acting cytokines (9). The observation that differential responsiveness to these critical positive regulators of HSCs (12, 13) may be involved in the genetic variation in the HSC compartment raised the question whether there is also genetic variation in the responsiveness to negative regulators. One of the most potent negative regulators of hematopoiesis is TGF-β1 (14, 15), which inhibits the proliferative response of hematopoietic progenitors to early acting cytokines (16–18). TGF-β1 is expressed in primitive hematopoietic progenitor and stem
cells. Inhibition of TGF-β1 activity by antibodies or antisense oligonucleotides enhanced the proliferation of these cells in vitro (15), and increased the repopulating capacity of HSCs in vivo (19), suggesting a negative regulatory role that is, in part, cell autonomous. Both mice and humans express two other TGF-β isoforms, TGF-β2 and TGF-β3, that share significant sequence homology with and signal through the same receptor complex as TGF-β1 (20, 21). Although some in vitro analyses have described a potent inhibitory effect for TGF-β3 (22), and a weaker inhibitory effect for TGF-β2 (14, 23) on early hematopoietic progenitor cells, their role in hematopoietic regulation remains largely unknown. The role of the individual TGF-β isoforms in adult hematopoiesis in vivo is unclear as homozygous deletion of each is lethal at or shortly after birth (20).

The goal of the studies reported here was to determine whether there is genetically determined variation in the responsiveness of hematopoietic stem and progenitor cells to TGF-β in inbred mice, and, if so, how this genetic variation would impact the regulation of the hematopoietic stem and progenitor cell compartment in vivo. We show here that TGF-β2 is, in fact, a positive regulator of HSCs in vivo, and is likely involved in genetically determined variation in the function and size of the HSC compartment.

**Materials and Methods**

*Mouse.* 8-wk-old C57BL/6J, DBA/2J, BALB/cJ, BXD recombinant inbred (RI), and tgfβ2<sup>−/−</sup> mice were purchased from Jackson Laboratories. C57BL/6.SJL-Ptprc<sup>Pyb/Brej</sup> mice were purchased from the National Cancer Institute. Experiments and animal care were performed in accordance with the Mount Sinai Institutional Animal Care and Use Committee.

*Antibodies and Cytokines.* Unconjugated CD2, CD3, CD8, CD4, B220, Ly6G/Gr1, Mac1, PE-conjugated Sca1, FITC-conjugated CD45.1, PE-conjugated CD45.2, Spectral red-conjugated Gr1, B220 and Mac1, biotinylated Thy1, cychrome-conjugated streptavidin, and FITC-conjugated goat anti-rat antibodies (GARs), biotinylated anti-c-kits, and allophycocyanin-Cy7–conjugated streptavidin were purchased from Southern Biotechnology Associates, Inc. Unconjugated Ter119, alloploidy-conjugated GARs, biotinylated anti–c–kits, and alloploidy-conamin-Cy7–conjugated streptavidin were purchased from BD Biosciences. Hoechst 33342 was purchased from Sigma-Aldrich. Recombinant mouse flk3L, KL, TPO, TGF-β1, TGF-β2 and TGF-β3, polyclonal pan–anti–TGF-β, and anti–TGF-β1 antibodies were purchased from R&D Systems. Supernatants obtained from BHK/HM-5, BHK/MKL (both a gift from J. Matous, University of Washington, Seattle, WA), and WEHI 3B (a gift from S. Tsai, Mount Sinai School of Medicine, New York, NY) were used as a source of GM-CSF, KL, and IL-3, respectively.

*Isolation of Hematopoietic Progenitor and Stem Cells from Bone Marrow and Fetal Liver.* Low-density bone marrow cells were stained with antibodies for lineage antigens, Sca1 and c-kit, and isolated by cell sorting as described previously (9) to obtain lineage negative (Lin<sup>−</sup>) Sca1<sup>+</sup>c-kit<sup>+</sup> cells (see Fig. 3 a for a representative example of sort windows). Fetal liver was dissected from E14 fetuses. Single cell suspensions were obtained by passing the cells through a nylon mesh. Labeling of fetal liver cells was identical to labeling of bone marrow cells, but the Mac-1 antibody (24) was omitted.

*Culture of LSK Cells.* Sorted cells were cultured in triplicate at 20–40 cells/well in flat-bottom 96-well plates in IMDM, 10% FCS, 100 μg/ml penicillin/streptomycin, and 50 ng/ml flt3L, TPO, and KL. 3 h after plating, the exact number of cells per well was determined by visually counting the cells at a magnification of 40. After 5 d of liquid culture at 37°C and 5% CO<sub>2</sub>, the cells were counted again; in some experiments, 500 cells were plated in methylcellulose cultures containing IMDM, IL-3 (10% of WEHI.3B supernatant), GM-CSF (10% of BHK/HM-5 supernatant), KL (10% of BHK/MKL supernatant), 2 U/ml erythropoietin, 10% FCS, 10 μg/ml anti-TGF-β, and 10<sup>−6</sup> M α-thioglycerol. After 8 d of incubation at 37°C in a humidified incubator with 5% CO<sub>2</sub>, the cultures were scored for colony formation. Most secondary colonies were myeloid, with 20–40% macroscopic colonies derived from high proliferative potential cells. Colony-forming cell (CFC) output correlated with cellular proliferation in the liquid culture phase in every experiment.

*Cell Cycle Analysis of LSK Cells.* Bone marrow samples were stained with lineage markers, followed by alloploidy-conamin–GAR, Sca1–PE, biotinylated anti–c–kit antibody, and finally with streptavidin–allophycocyanin-Cy7. The cells were fixed overnight in 1% paraformaldehyde and 0.2% Triton X-100. The following day, the cells were washed in PBS and labeled with Hoechst 33342 at 4°C for 60 min. Analysis was performed on a triple laser FACS Vantage<sup>TM</sup> Flow cytometer with DiVA software (Becton Dickinson). Doublet exclusion using pulse-shape analysis was done by plotting area versus height of the UV-excited Hoechst fluorescence. A live gate on singlet LSK cells was used to acquire up to 10<sup>5</sup> cells.

*semi-quantitative RT-PCR.* Total RNA from purified LSK cells was isolated using TRizol reagent (GIBCO BRL) according to the manufacturer's instructions and treated with DNaseI. RNA was reverse transcribed using Superscript II (GIBCO BRL), for 1 h at 42°C using oligo(dT) primers. PCR primers used were as follows: 5′-TGAACCTAAGGCCAACCGTG-3′ (nucleotide [nt] 409–429) and 5′-GCTCATAGCTCTTCT CCCAGG-3′ (nt 804–789) for actin; and 5′-ATGGCTTTGGATGCTGC-3′ (nt 2124–2143) and 5′-TTCCTGCGATCAAGA CTTGACCA-3′ (nt 2436–2459) for TGF-β2, respectively.

*Competitive Repopulation Assays.* 2 × 10<sup>6</sup> bone marrow cells or 10<sup>6</sup> E14 fetal liver cells from Tgfβ2<sup>+/−</sup>, Tgfβ2<sup>−/−</sup>, or WT mice (CD45.2<sup>+</sup>, competing cells), and equal numbers of heterozygous B6.SJL-Ptprc<sup>Pyb/Brej</sup> mice (CD45.1<sup>−</sup>, CD45.2<sup>+</sup>) or homozygous B6.SJL-Ptprc<sup>Pyb/Brej</sup> mice (CD45.1<sup>+</sup>; competitor cells) were mixed and injected in lethally (950 cG) irradiated C57BL/6.SJL-Ptprc<sup>Pyb/Brej</sup> mice (CD45.1<sup>+</sup>). Peripheral blood cells were analyzed for the expression of CD45.1, CD45.2, and lineage antigens (Thy-1, Gr-1, and B220) 12–24 wk after transplantation. Competitive serial transplantations were performed 12–24 wk after reconstitution by injecting 2 × 10<sup>6</sup> bone marrow cells from primary recipients into lethally irradiated secondary C57BL/6 (CD45.1<sup>+</sup>) recipients, and from secondary recipients into lethally irradiated tertiary C57BL/6 (CD45.1<sup>+</sup>) recipients. Three secondary or tertiary recipients were reconstituted with bone marrow from one donor. All transplant recipients survived. After 3 mo, the ratio between the competing cell populations cells was measured in the peripheral blood of the secondary or tertiary recipients. The data are presented as ratios between the two donor populations a determined by the expression CD45 alleles. Changes in reconstitution ratios between primary and secondary, or between secondary and tertiary recipients were analyzed by evaluating the difference in the logarithm of the reconstitution ratios Δ (log ratio) in the respective recipients. The rationale for
using ratios is that the change in the reconstitution ratio between primary and secondary recipients is a better measure of a shift in reconstitution capacity upon serial transplantation than a change in the percentage contribution of CD45.2+ cells between primary and secondary recipients. This is because a change in the percent CD45.2+ cells after serial transplantation from a 50% contribution level in the primary recipient reflects a smaller change in the function or the number of HSCs than the same change at higher or lower contribution levels in the primary recipient (i.e., a 5% change from 50 to 45% is a change in reconstitution ratio from 1 to 0.82, whereas a 5% change from 90 to 85% represents a much larger change in reconstitution ratio from 9 to 5.6). A better measure is the ratio between the CD45.2+/CD45.1+CD45.2+ ratio pre- (input, primary recipient) and postsecondary (output, secondary recipient) reconstitution. With ratiometric data, the difference between log CD45.2+/CD45.1+CD45.2+ ratios in primary and secondary recipients or in secondary and tertiary recipients can be used. An advantage of log transformation is that a ratio smaller than one will give a negative value, and negative ratios will extend over the same numerical ranges as positive ones (e.g., a ratio of 0.01 gives a log ratio of −2, a ratio of 100 gives a log ratio of +2). Thus, data are presented as the difference between input log CD45.2+/CD45.1+CD45.2+ (i.e., the log ratio in the first recipient) and the output log CD45.2+/CD45.1+CD45.2+ (i.e., the log ratio 3 mo after reconstitution of the serial recipients), and will be referred to as Δ (log ratio). Differences in the efficiency of serial repopulation were assessed by comparing the Δ (log ratio) for recipients of WT or TGF-β2-deficient bone marrow or fetal liver cells as competing cells.

Statistical Analysis. Student’s t test for unpaired samples was used unless indicated otherwise.

Linkage Analysis in BXD RI Strains. Linkage analysis was performed in BXD RI strains. These are commercially available and were generated by repeated inbreeding of F2 mice derived from the inbred progenitor strains C57BL/6 and DBA/2. The genome of RI strains is composed of a patchwork of homozygous chromosome segments derived from either progenitor strain, with each of the RI lines having a unique combination of “patches” from the progenitors (25). Linkage analysis is performed by determining the relevant trait in each of the strains, which results in a strain distribution pattern. The data are analyzed using MapmanagerQTb29ppc software (26). This software statistically analyzes the linkage of a given trait with previously typed polymorphic loci in the RI strains, which are inherited from either parental strain. This allows assignment of a trait to a corresponding map position, and the calculation of statistical significance according to published criteria (27, 28). Mapping databases were downloaded from www.nervenet.org/papers/bxn.html (29).

Results

The Dose Response of TGF-β2 on Primitive Hematopoietic Progenitor Cells Is Biphasic and Subject to Mouse Strain-dependent Variation. TGF-β1, -β2, or -β3 was added to liquid cultures of LSK cells, a population highly enriched for hematopoietic stem and early progenitor cells (1, 2), supported by KL, flt3L, and TPO. LSK cells from C57BL/6, DBA/2, and BALB/c mice, three mouse strains that display genetically determined variation in the HSC compartment (5, 6), were used for these experiments. In these cultures, the dose response of TGF-β2 on the proliferation of LSK cells was significantly less inhibitory than the dose responses of TGF-β1 (Fig. 1 a) and TGF-β3 (not depicted). In addition, TGF-β2 had a significant (P < 0.05) net stimulatory effect on the proliferation of LSK cells at the lowest concentration tested (0.001 ng/ml) in the three mouse strains. These observations indicate that, in vitro, TGF-β2 has a biphasic dose response on the proliferation of LSK cells. Furthermore, the dose response of TGF-β2 showed genetically determined variation among the three mouse strains tested (Fig. 1 a). LSK cells from BALB/c mice were significantly less responsive to the inhibitory effect of TGF-β2 at higher concentrations, and more responsive to the stimulatory effect of TGF-β2 at low concentrations than LSK cells from DBA/2 and C57BL/6 mice. In contrast to the dose response of TGF-β2, the dose responses of TGF-β1 (Fig. 1 a) and TGF-β3 (not depicted) were similar in these three mouse strains.

To determine whether the stimulatory effect of low concentrations of TGF-β2 was direct, we deposited single LSK cells from C57BL/6 mice in individual wells of 96-well plates in the presence of KL, flt3L, and TPO with and

Figure 1. Effect of TGF-β2 in vitro. (a) Dose response of TGF-β1 and TGF-β2 on proliferation in 5-d cultures of LSK cells from the mouse strains listed (top), supported by KL, flt3L, and TPO (mean ± SEM; n = 3–13; *, significantly different DBA/2 and C57BL/6). (b) Effect of 0.001 ng/ml TGF-β2 on cloning efficiency (left) and on average clone size (right) of single cell–sorted LSK cells from C57BL/6 mice (mean ± SEM; n = 5). (c) Representative example of the colony-size distribution after 5 d of single cell culture supported by KL, flt3L, and TPO of fetal liver LSK cells in the presence of TGF-β2 at 0.001 ng/ml. (d) Effect of neutralizing anti-TGF-RII antibodies on the effect of 0.001 ng/ml TGF-β2 on the proliferation of LSK cells from C57BL/6 (mean ± SEM; n = 5). (e) Expression of TGF-β2 mRNA in purified LSK cells, lineage positive cells from fetal liver, and adult bone marrow as detected by RT PCR. (f) Effect of an affinity-purified polyclonal anti–TGF-β2 antibody on the proliferation of LSK cells supported by KL, flt3L, and TPO after preconditioning of the antibody with recombinant TGF-β2 at the depicted concentrations (bottom, mean ± SEM; n = 3; *, significantly different from control without TGF-β2 or anti–TGF-β2; P = 0.04).
without 0.001 ng/ml TGF-β2. The addition of TGF-β2 at this concentration did not affect the cloning efficiency of these progenitors, which was ~70% (Fig. 1 b). However, it did increase the average clone size by ~1.9-fold (Fig. 1 b, P = 0.013) after 5 d of culture. A representative example using fetal liver–derived LSK cells is shown in Fig. 1 c. These data demonstrate that TGF-β2 directly enhances the proliferative potential of LSK cells responsive to early acting cytokines, rather than initiating proliferation in nonresponsive cells.

TGF-β binds to the type II TGF receptor (TGF-RII), which associates with and phosphorylates the TGF-R1, thereby triggering a signaling cascade mediated by Smad proteins (20, 21). The fact that low concentrations of TGF-β2 are stimulatory, in contrast to the potent inhibitory effects of other TGF-β isoforms (14–19, 22) on the proliferation of primitive hematopoietic progenitor cells, might suggest that this effect is mediated by a different receptor. To address this question, we examined the effects of TGF-β2 in the presence of neutralizing antibodies to the TGF-RII. These antibodies blocked the stimulatory effect of TGF-β2 (at 0.001 ng/ml) on the proliferation of LSK cells (Fig. 1 d), suggesting involvement of the canonical TGF-RII in the observed stimulatory response.

TGF-β1 is produced in primitive progenitor cells, and inhibition of TGF-β1 activity enhances the proliferative capacity of these cells in vitro (15). Therefore, we tested whether TGF-β2 is also expressed in LSK cells. RT PCR analysis showed that TGF-β2 mRNA is expressed in purified fetal liver and bone marrow–derived LSK cells (Fig. 1 e). Expression of TGF-β3 was not detected (unpublished data). To investigate whether endogenous TGF-β2 affects the proliferative capacity of LSK cells, we tested the effect of anti–TGF-β2 antibodies. Affinity-purified anti–TGF-β2 antibodies significantly inhibited proliferation of LSK cells supported by the early acting cytokines flt3L, KL, and TPO by ~50% (Fig. 1 f, P = 0.04). Preincubation of the antibody with recombinant TGF-β2 alleviated the inhibitory effect of these antibodies (Fig. 1 f), indicating that this effect was not due to toxicity. In contrast, addition of anti–TGF-β1 antibodies enhanced the proliferation of LSK cells two- to threefold (unpublished data), a finding consistent with previously published data (15).

**Linkage Analysis in BXD RI Strains.** To further investigate mouse strain–dependent variation in the dose response of TGF-β2, we performed linkage analysis using BXD RI strains. Although the TGF-β2 dose response in LSK cells from C57BL/6 and DBA/2 mice (the progenitors of the BXD RI set) was very similar, linkage analysis was attempted using these strains because a relatively large number are readily available in this set. If a trait is multigenic, phenotypic variation can be more pronounced among RI strains than between their progenitor strains because the balance between positive-acting and negative-acting alleles present in the progenitor strains may be completely unbalanced in some of the RI strains (25). This may allow quantitative trait locus (QTL) identification, even when phenotypic variation between the progenitor strains is not significant. The effect of TGF-β2 at 0.1 ng/ml on the proliferation of LSK cells from 30 BXD RI strains was measured because this concentration revealed the most pronounced difference in the TGF-β2 dose response between the inbred strains (Fig. 1 a). The individual BXD strains showed wide variation in this assay (Fig. 2 a), indicating that the responsiveness to TGF-β2 is indeed a multigenic trait. One QTL contributing to the effect of TGF-β2 was identified on chromosome 4 between 67 (D4Mit158) and 79 (D4MIt33) cM (maximum likelihood ratio statistic 13.7; P = 0.00021). The level of significance was suggestive according to permutation analysis (27, 28). Although suggestive QTL require confirmation (28), the location of this QTL was compelling because this interval overlaps with the interval containing a previously identified QTL contributing to genetic variation in the pool size of lin–Sca1++ and of LSK cells in vivo (Fig. 2 b; reference 9). Furthermore, a significant correlation was found between the effect of TGF-β2 and the number of lin–Sca1++ cells in BXD RI strains (Fig. 2 c). One interpretation of these findings is that TGF-β2 signaling regulates the size of the hematopoietic stem and progenitor cell compartment as defined by phenotype in vivo.

**Studies in Tgfb2−/− and Tgfb2+/− Fetal Liver.** The QTL analysis suggested that TGF-β2 may be a regulator of the hematopoietic stem and progenitor cell compartment relevant in vivo. To test this hypothesis, we investigated the hematopoietic phenotype of TGF-β2-deficient mice. As Tgfb2−/− mice die at birth from multiple malformations (30), we examined the hematopoietic phenotype of fetal livers from Tgfb2−/− and Tgfb2+/− mice. To allow trans-
planted pregnancy), Tgfb2+/− mice were backcrossed onto the C57BL/6 background for at least six generations.

The frequency of LSK cells and the proliferative capacity of these cells in vitro did not differ in mutant and WT fetal livers (unpublished data). To assess the repopulation potential of mutant HSCs, 10⁶ fetal liver cells from Tgfb2−/−, Tgfb2+/−, or WT fetuses (CD45.2+) were transplanted together with 2 × 10⁵ bone marrow cells from congenic C57BL/6.SJL-PtprcaPep3b/J (CD45.1+) mice into CD45.1+ recipients (31)). As expected (32), fetal liver cells displayed a significantly higher repopulation potential than adult bone marrow cells (Fig. 3 a, mean fetal/adult reconstitution ratio >1 after 12–24 wk) (21). There appeared to be no significant difference in the repopulation capacity of Tgfb2−/−, Tgfb2+/−, and WT fetal liver cells (Fig. 3 a). Together, these data suggest that TGF-B2 plays a role in fetal hematopoiesis. To further evaluate the repopulation capacity of these cells, we performed serial transplantations by injecting 2 × 10⁶ bone marrow cells from primary recipients into secondary C57BL/6 (CD45.1+) recipients 12–24 wk after reconstitution. After 3 mo, the ratio of CD45.2+/CD45.1+ cells was measured in the peripheral blood of the secondary recipients. We used the difference between log CD45.2+/CD45.1+ ratios in primary and secondary recipients (∆log ratio; Materials and Methods) for statistical analysis (Fig. 3 b). Although WT fetal liver cells maintained their repopulating capacity in the secondary recipients, Tgfb2−/− and Tgfb2+/− fetal liver cells showed a marked reduction in this capacity after transplantation. The lower secondary repopulating capacity in Tgfb2−/− and Tgfb2+/− mice affected all lineages (unpublished data). The difference in serial reconstitution capacity between Tgfb2+/− or Tgfb2−/−,
and WT fetal liver HSCs was approximately fourfold (Fig. 3c). The serial reconstitution capacity of Tgfb2+/− and Tgfb2−/− fetal liver cells was similar (Fig. 3c), indicating dominance of Tgfb2 for this particular biological effect. These findings indicate that TGF-β2 is important for maintaining the serial reconstitution capacity of fetal liver HSCs and suggest a positive regulatory role of TGF-β2 in vivo. Given that Tgfb2−/− and Tgfb2−−/− HSCs were exposed to a WT microenvironment in the primary recipients, the observed deficiency in secondary reconstitution potential strongly suggests that TGF-β2 may act in a cell autonomous fashion. The finding that TGF-β2 mRNA is expressed in purified LSK cells (Fig. 1e) supports this contention.

**TGF-β2 Is a Positive Regulator of Adult Hematopoiesis.** Because Tgfb2+/− mice are viable, and because Tgfb2+/− fetal liver HSCs showed a defect relative to WT cells, we were interested in determining whether adult Tgfb2+/− HSC were also defective. Peripheral white and red cell counts were similar in Tgfb2+/− mice and in their WT littersmates (unpublished data). However, bone marrow cellularity in Tgfb2−/− mice was slightly, but significantly lower than in WT littersmates (2.012 ± 0.61 × 10^7 vs. 1.86 ± 0.60 × 10^7 cells/femur, P = 0.03 by paired Student’s t test, using bone marrow pooled from at least three mice in each experiment; unpublished data). In contrast to end-stage cells, the numbers of early progenitor/stem cells were reduced in heterozygous animals. The fraction of lin−Sca1+/+ cells and of LSK cells were 34 ± 6% (P = 0.03) and 41 ± 30% (P = 0.01) lower in Tgfb2+/− mice than in WT mice, respectively (paired Student’s t test, n = 4, using bone marrow pooled from at least three mice in each experiment; Fig. 4 depicts a representative example). These findings suggest that TGF-β2 positively regulates the number of adult LSK cells in vivo, and corroborate the overlapping intervals for QTL contributing to genetic variation in the frequency of LSK cells on one hand, and responsiveness of LSK cells to TGF-β2 on the other hand. In culture assays, proliferation and CFC generation in response to KL, flt3L, and TPO of purified LSK cells from Tgfb2+/− mice were significantly lower than from WT littersmates (Fig. 4b), again supporting a positive regulatory role for TGF-β2 in adult early hematopoietic progenitors.

To analyze the reconstitution capacity of adult Tgfb2+/− HSCs, we transplanted lethally irradiated C57BL/6 recipients (CD45.1) with 2 × 10^6 bone marrow cells from either Tgfb2+/− mice or from WT littersmates (CD45.2+), and equal numbers of C57BL/6-derived bone marrow cells (CD45.1+CD45.2−). These experiments revealed a defect in adult Tgfb2+/− HSCs in primary recipients. The ratio of Tgfb2+/− to competitor cells in the peripheral blood of recipients mice was 1.32 ± 0.11. This was significantly lower than the ratio of WT to competitor cells (Fig. 4c, 1.77 ± 0.14; n = 10). We found the same ratio in all lineages (unpublished data). As observed in fetal liver, the difference in the reconstitution capacity between WT and Tgfb2−/− HSCs was more pronounced after transplantation into secondary recipients after 12 wk (Fig. 4d). The mean difference in Δ (log ratio) between WT and Tgfb2+/− HSC was 0.476, corresponding to a threefold loss in repopulating capacity of Tgfb2+/− HSCs upon transplantation into secondary hosts (Fig. 4e). After transplantation into tertiary recipients, the mean difference in Δ (log ratio) between WT and Tgfb2+/− HSCs increased further to 1.024, representing a 10-fold difference in repopulating capacity (Fig. 4d and e). Regardless of the expression level of TGF-β2, bone marrow cells from both the WT and the Tgfb2−−/− backcrossed mice outcompeted bone marrow cells from C57BL/6 mice in primary recipients (Fig. 4c), whereas in secondary and tertiary recipients, the competitive advantage of the bone marrow of WT backcrossed mice increased even further (Fig. 4d). A relative defect in the competitive repopulation capacity of CD45.1+ compared with CD45.2+ bone marrow cells has been observed by other investigators as well (see Fig. 5 in reference 33). Although the underlying reason for this is not clear, it is possible that the allelic difference at the Cd45 locus could play a role, given the role of Cd45 in cytokine signal transduction as a tyrosine phosphatase (34). Irrespective of this phenomenon, the repopulation studies clearly demonstrate that Tgfb2+/− bone marrow has a significantly lower serial reconstitution capacity than the bone marrow from WT littersmates. As in the experiments using fetal liver cells, the loss of repopulation capacity of adult Tgfb2+/− HSCs upon serial transplantation may suggest that TGF-β2 acts in a cell autonomous fashion.

HSCs have been shown to cycle slowly. In C57BL/6 mice, ~8% of HSCs enter cycle per day (35). Furthermore, after reconstitution of a lethally irradiated recipient, HSCs display enhanced cycling activity for at least 4 mo (36). HSCs in cell cycle may engraft less efficiently (37). Therefore, it is possible that the decreased serial reconstitution capacity of Tgfb2−/− HSCs compared with WT HSCs is due to enhanced cycling of the former. This would imply that TGF-β2 is, in fact, a negative regulator of HSCs cycling in vivo. To investigate cycling activity within the HSC compartment of Tgfb2+/− mice, we compared the cell cycle of Tgfb2+/− and WT LSK cells by four-color flow cytometry. Fixed bone marrow samples from Tgfb2+/− mice and from WT littersmates were stained with the DNA-binding dye Hoechst 33342 and by antibodies against lineage markers, Sca-1, and c-kit. As shown in Fig. 4f, significantly fewer LSK cells were in S/G2/M phase of the cell cycle in Tgfb2+/− mice, as determined by Hoechst 33342 staining. The fraction of cells in the S/G2/M phase of the cell cycle was similar in the total bone marrow population of Tgfb2+/− mice and WT littersmates, indicating that the positive regulatory effect of TGF-β2 is specific for the LSK compartment. A difference in the cell cycle activity of WT and Tgfb2+/− HSCs would confer differential sensitivity of these HSCs to drugs that selectively target cycling cells, such as 5-fluorouracil (5-FU) (38). Therefore, primary recipients of mixtures of bone marrow from CD45.1+CD45.2+C57BL/6 mice, and CD45.2+ WT or Tgfb2−/− mice were injected with 150 mg/kg 5-FU 3 mo
after reconstitution. 6 wk later, $2 \times 10^6$ bone marrow cells of these mice were injected into lethally irradiated secondary CD45.1+ recipients, which were analyzed 3 mo later. After injection of primary recipients with 5-FU, Tgfb2+/– HSCs gained repopulating capacity, whereas WT HSCs lost repopulating capacity compared with C57BL/6-derived competitor cells in secondary recipients (Fig. 4, g and h). This repopulating potential was opposite of that observed with bone marrow from untreated mice (Fig. 4, d and e), indicating that 5-FU differentially affects WT and Tgfb2+/– HSCs. Because the Tgfb2+/– HSCs are less sensitive to the effects of 5-FU, these data are consistent with the interpretation that Tgfb2+/– HSC cycle less frequently than WT HSCs. Together, our data demonstrate that TGF-β2 enhances the cell cycle activity of the LSK compartment, including repopulating HSCs.

Discussion

The key observations of this work are, first, that TGF-β2 is a positive regulator of HSC function in vivo; second, that TGF-β2 may act in a cell autonomous fashion; and third, that the dose response of TGF-β2 on LSK cells is subject to quantitative genetic variation.

Although the TGF-β system is believed to play a role in the maintenance of HSCs in quiescence (15), our findings show that at least one TGF-β isoform, TGF-β2, is a positive regulator of HSCs in vivo and in vitro. In vitro, a direct stimulatory effect of TGF-β2 on the proliferation of LSK cells could be detected only at low concentrations, whereas at higher concentrations, TGF-β2 was a significantly weaker inhibitor of proliferation than TGF-β1 and TGF-β2. The phenotype of TGF-β2–deficient mice suggests that it is the stimulatory effect of TGF-β2, detected in vitro at low concentrations, that is relevant in vivo. The three known TGF-β isoforms in mice and humans have clearly distinct roles in vivo, as evidenced by the wide variation in the phenotypes of mice with a homozygous deletion of the genes encoding the individual TGF-β isoforms. Because all TGF-β isoforms activate the same Smad-mediated signaling pathway, these data are likely explained, at least in part, by their different expression patterns (20, 21).

However, a role for intrinsically different effects of TGF-β isoforms on specific cell types cannot be excluded. For example, in vivo and in embryonic skin organ cultures in vitro, hair follicle morphogenesis is enhanced by TGF-β2 and inhibited by TGF-β1 (39). The data presented here indicate that also in the regulation of renewal and differentiation of HSCs, TGF-β1, and TGF-β2 play different, and even opposing roles. A molecular explanation for these data could lie in the differences between TGF-β2 on one hand and TGF-β1 and -β3 on the other hand with respect to receptor binding. The TGF-RII (40), as well as accessory receptors including endoglin (41), which is expressed on repopulating HSCs (42), TGF-RIII (43), and some less well-characterized proteins (44, 45) display differential binding affinities for TGF-β1 or -β3 compared with TGF-β2.

Therefore, these proteins may be involved in the divergent hematopoietic effects of TGF-β2 compared with TGF-β1 and TGF-β3.

Despite a defect in the hematopoietic stem and progenitor cell compartment, and even a small decrease in bone marrow cellularity, peripheral blood counts were normal in Tgfb2+/– mice. These observations indicate that different and independent homeostatic mechanisms operate in the regulation of the early and late stages of hematopoiesis. The defect in serial repopulation capacity of TGF-β2–deficient HSCs together with their lower sensitivity to the toxicity of 5-FU indicates that TGF-β2 acts on the most primitive HSCs. However, TGF-β2 induced a general increase in the clone size of growing LSK cells. LSK cells are still a functionally heterogeneous population, and only a minority of these cells are repopulating HSCs (1, 2). Furthermore, the proliferation of LSK cells in vitro is mainly differentiative. Therefore, our data indicate that TGF-β2 positively regulates a primitive progenitor cell population that includes, but is not limited to, the renewing HSCs.

The defect in the serial repopulation capacity of TGF-β2–deficient HSCs suggests that TGF-β2 positively regulates HSCs in a cell autonomous, and likely autocrine, fashion in vivo. Although this contention is supported by our in vitro data showing a lower proliferative capacity of Tgfb2+/– LSK cells and an inhibitory effect of anti–TGF-β2 antibodies on the proliferation of LSK cells, it cannot be entirely excluded that this defect is caused by the disappearance of a critical cellular element required for HSC proliferation and engraftment. However, the fact that in the serial transplantation experiments, WT C57BL/6 bone marrow cells were always cotransplanted makes this possibility unlikely. Autocrine regulation of HSCs has been demonstrated recently for vascular endothelial growth factor (46), and has been proposed for several other cytokines (47). An autocrine negative regulatory role of TGF-β1 has been suggested based previously on in vitro data, although its significance in vivo is unknown thus far (15). Thus, the findings reported here may support the emerging concept that cell-autonomous, intrinsic mechanisms mediated by soluble factors participate in the regulation of HSCs.

The observation that TGF-β2 plays no role in fetal hematopoiesis in vivo is consistent with the finding that TGF-β2 regulates the responsiveness of LSK cells to KL, flt3L, and TPO. These cytokines and their respective receptors (c-kit, flt3, and c-mpl) are most critical in postnatal hematopoiesis (12, 13), although KL plays a role in late-stage fetal hematopoiesis as well (48, 49). However, in TGF-β2–deficient fetal HSCs, a defect appeared upon serial transplantation, whereas in adult Tgfb2+/– HSCs, the existing repopulation defect increased upon serial transplantation. Fetal and adult HSCs differ in their proliferative history (50), whereas repopulation of a lethally irradiated recipient represents a significant proliferative stress for HSCs (36). Therefore, these findings suggest that TGF-β2 is mainly important for the function of long-term repopulating HSCs that have undergone replicative stress. An al-
ternative explanation for these data is that because the stimulatory effect of TGF-β2 on LSK cells is subtle in vitro, a deficiency in TGF-β2 only gives rise to detectable phenotype after multiple renewal divisions (i.e., in adult HSCs or after serial transplantation). It is anticipated that the phenotype of Tgfb2−/− mice will increase with aging. However, it has been proposed by several authors that a decrease in reconstitution capacity upon serial transplantation is not necessarily or not only due to replicative senescence (51, 52). Our data suggest that, because WT HSCs are more efficient at serial reconstitution that TGF-β2–deficient HSCs, TGF-β2 may be implicated in this phenomenon.

An alternative explanation for the apparent reconstituting defect of TGF-β2–deficient HSCs may be that TGF-β2–deficient mature cells have a shorter life span, so that they will contribute less to the peripheral blood, without a real defect in stem cell function. However, our data argue against this contention. First, the defects in reconstitution were found in all lineages to a similar extent. Given the vastly different turnover rates of different types of blood cells, it would be difficult to explain these data by a generalized effect of TGF-β2 on the life span of mature cells. Second, our in vivo 5-FU suicide data suggest that Tgfb2−/− HSCs cycle more slowly than WT HSCs, indicative of a stem cell phenotype. The comparative cell cycle analysis of LSK cells from Tgfb2−/− mice and from WT littermates in vivo again supports the idea that TGF-β2 is a specific positive regulator of the cycling activity in the stem cell compartment. Finally, the serial transplantation data strongly support the contention that the effect of TGF-β2 is situated at the level of the stem cell compartment, and not at the level of survival of mature cells. Mature cells do not contribute to the peripheral blood 3 mo after transplantation. An increasing defect in repopulation capacity after each round of serial transplantation can only be due to a defect in the HSCs because that is the only cell that is responsible for the serial reconstitution (1, 2). If only life spans of mature cells were affected, we would expect to see a similar small defect after each round of transplantation.

Although TGF-β2 responsiveness is a multigenic trait, only one suggestive QTL was identified in our paper. The lower frequency of lin-Sca1++ and of LSK cells in Tgfb2−/− mice corroborates the overlapping QTL on chromosome 4 for the dose response to TGF-β2 and for the number and frequency of lin−Sca1++ and of LSK cells in BXD RI strains. Because Tgfb2−/− mice show a defect in the serial reconstitution capacity of HSCs, it is likely that genetically determined variation in the responsiveness to TGF-β2 also affects the function of HSCs in vivo. However, direct linkage analysis for the serial reconstitution capacity of HSC in BXD RI strains is impossible because of their immunological heterogeneity. Given the potential role of TGF-β2 in maintaining the function of HSCs that have undergone replicative stress, it will be of interest to determine whether TGF-β2 also plays a role in the significant genetic variation in the age-related decline in HSC function in inbred mice (3, 4, 11, 53), which has been shown to be caused by cell intrinsic mechanisms (3, 4).

The authors would like to thank Ms. P. Rebollo for her excellent technical assistance, and Dr. G. Keller for his critical reading of this manuscript.

This work was supported by National Institutes of Health grant RO1 AG16327 to H.-W. Snoeck.

Submitted: 16 June 2003
Accepted: 5 November 2003

References
1. Morrison, S.J., and I.L. Weissman. 1995. The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. 11:35–71.
2. Weissman, I.L., D.J. Anderson, and F. Gage. 2001. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu. Rev. Cell Dev. Biol. 17:387–403.
3. Van Zant, G., P.W. Eldridge, R.R. Behringer, and M.J. Dewey. 1983. Genetic control of hematopoietic kinetics revealed by analysis of allogeneic mice and stem cell suicide. Cell. 35:639–645.
4. Van Zant, G., B.P. Holland, P.W. Eldridge, and J.J. Chen. 1990. Genotype-restricted growth and aging patterns in hematopoietic stem cell populations of allogeneic mice. J. Exp. Med. 171:1547–1565.
5. Muller-Sieberg, C.E., and R. Riblet. 1996. Genetic control of the frequency of hematopoietic stem cells in mice: mapping of a candidate locus to chromosome 1. J. Exp. Med. 183:1141–1150.
6. de Haan, G., and G. Van Zant. 1997. Intrinsic and extrinsic control of hematopoietic stem cell numbers: mapping of a stem cell gene. J. Exp. Med. 86:529–536.
7. Chen, J., C.M. Astle, C.E. Muller-Sieburg, and D.E. Harrison. 2000. Primitive hematopoietic stem cell function in vivo is uniquely high in the CXB-12 mouse strain. Blood. 96:4124–4131.
8. Morrison, S.J., D. Qian, L. Jerabek, B.A. Thiel, I.K. Park, P.S. Ford, M.J. Kiel, N.J. Schork, I.L. Weissman, and M.F. Clarke. 2002. A genetic determinant that specifically regulates the frequency of hematopoietic stem cells. J. Immunol. 168:635–642.
9. Henckaerts, E., H. Geiger, J.C. Langer, P. Rebollo, G. Van Zant, and H.W. Snoeck. 2002. Genetically determined variation in the number of phenotypically defined hematopoietic progenitor and stem cells and in their response to early-acting cytokines. Blood. 99:3947–3954.
10. de Haan, G., S.J. Szilvassy, T.E. Meyerrose, B. Donjte, B. Grime, and G. Van Zant. 2000. Distinct functional properties of highly purified hematopoietic stem cells from mouse strains differing in stem cell numbers. Blood. 96:1374–1379.
11. Geiger, H., J. True, G. de Haan, and G. van Zant. 2001. Age- and stage-specific regulation patterns in hematopoietic stem cell hierarchy. Blood. 98:2966–2972.
12. Lyman, S.D., and S.E. Jacobsen. 1998. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood. 91:1101–1134.
13. Kimura, S., A.W. Roberts, D. Metcalf, and W.S. Alexander. 1998. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. Proc. Natl. Acad. Sci.
14. Ohta, M., J.S. Greenberger, P. Anklesaria, A. Bassols, and J. Massagué. 1987. Two forms of transforming growth factor-beta distinguished by multipotent haematopoietic progenitor cells. Nature. 329:539–541.

15. Fortunel, N.O., A. Hatzfeld, and J.A. Hatzfeld. 2000. Transforming growth factor-β: pleiotropic role in the regulation of hematopoiesis. Blood. 96:2022–2036.

16. Sitnicka, E., F.W. Ruscetti, G.V. Priestley, N.S. Wolf, and S.H. Bartelmez. 1996. Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. Blood. 88:88–88.

17. Jacobsen, S.E., O.P. Veiby, J. Myklebust, C. Okkenhaug, and S.D. Lyman. 1996. Ability of flt3 ligand to stimulate the in vitro growth of primitive murine hematopoietic progenitors is potently and directly inhibited by transforming growth factor-beta and tumor necrosis factor-alpha. Blood. 87:5016–5026.

18. Ramsfjell, V., O.J. Borge, L. Cui, and S.E. Jacobsen. 1997. Thrombopoietin directly and potently stimulates multilineage growth and progenitor cell expansion from primitive (CD34+CD38−) human bone marrow progenitor cells: distinct and key interactions with the ligands for c-kit and flt3, and inhibitory effects of TGF-β and TNF-α. J. Immunol. 158:5167–5177.

19. Soma, T., J.M. Yu, and C.E. Dunbar. 1996. Maintenance of murine long-term repopulating stem cells in ex vivo culture is affected by modulation of transforming growth factor-beta but not macrophage inflammatory protein-1 alpha activities. Blood. 87:4561–4567.

20. Piek, E., C.H. Heldin, and P. Ten Dijke. 1999. Specificity, diversity, and regulation in TGF-β superfamily signaling. FASEB J. 13:2105–2124.

21. Massagué, J. 1998. TGF-β signal transduction. Annu. Rev. Biochem. 67:753–791.

22. Jacobsen, S.E., J.R. Keller, F.W. Ruscetti, P. Kondaiah, A.B. Roberts, and L.A. Falk. 1991. Bidirectional effects of transforming growth factor beta (TGF-beta) on colony-stimulating factor-induced human myelopoiesis in vitro: differential effects of distinct TGF-beta isoforms. Blood. 78:2239–2247.

23. Ottmann, O.G., and L.M. Pelus. 1988. Differential proliferative effects of transforming growth factor-beta on human hematopoietic progenitor cells. J. Immunol. 140:2661–2665.

24. Morrison, S.J., H.D. Hemmati, A.M. Wandycz, and I.L. Weissman. 1995. The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. USA. 92:10302–10306.

25. Moore, K.J., and D.L. Nagle. 2000. Complex trait analysis in the mouse: the strengths, the limitations and the promises yet to come. Annu. Rev. Genet. 34:653–686.

26. Manly, K.F., and J.M. Olson. 1999. Overview of QTL mapping software and introduction to map manager QT. Mamm. Genom. 10:327–334.

27. Churchill, G.A., and R.W. Doerge. 1994. Empirical threshold values for quantitative trait mapping. Genetics. 138:963–971.

28. Lander, E., and L. Kruglyak. 1995. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat. Genet. 11:241–247.

29. Williams, R.W., J. Gu, S. Qi, and L. Lu. 2001. The genetic structure of recombinant inbred mice: high-resolution consensus maps for complex trait analysis. Genome Biol. 2:RESEARCH0046. Epub. 2001. Oct. 22.

30. Sanford, L.P., I. Ormsby, A.C. Gittenberger-de Groot, H. Sariola, R. Friedman, G.P. Boivin, E.L. Cardell, and T. Doetschman. 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development. 124:2659–2670.

31. Harrison, D.E. 1992. Evaluating functional abilities of primitive hematopoietic stem cell populations. Curr. Top. Microbiol. Immunol. 177:13–23.

32. Rebêl, V.I., C.L. Miller, C.J. Eaves, and P.M. Lansdorp. 1996. The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their adult bone marrow counterparts. Blood. 87:3500–3507.

33. de Haan, G., E. Weersing, B. Donjte, R. van Os, L.V. Bystryk, E. Vellenga, and G. Miller. 2003. In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. Dev. Cell. 4:241–251.

34. Hermiston, M.L., Z. Xu, and A. Weiss. 2003. CD45: a critical regulator of signaling threshold in immune cells. Annu. Rev. Immunol. 21:107–137.

35. Cheshier, S.H., S.J. Morrison, X. Liao, and I.L. Weissman. 1999. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc. Natl. Acad. Sci. USA. 96:3120–3125.

36. Alhopp, R.C., S. Cheshier, and I.L. Weissman. 2001. Telomere shortening accompanies increased cell cycle activity during serial transplantation of hematopoietic stem cells. J. Exp. Med. 193:917–924.

37. Lanibert, J.-F., M. Liu, G.A. Colvin, M. Dooner, C.I. McAuliffe, P.S. Becker, B.G. Forget, S.M. Weissman, and P.J. Quesenberry. 2003. Marrow stem cells shift gene expression and engraftment phenotype with cell cycle transit. J. Exp. Med. 197:1563–1572.

38. Hodgson, G.S., and T.R. Bradley. 1979. Properties of hematopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell? Nature. 281:381–382.

39. Foitzik, K., R. Paus, T. Doetschman, and G.P. Dottore. 1999. The TGF-beta2 isoform is both a required and sufficient inducer of murine hair follicle morphogenesis. Dev. Biol. 212:278–289.

40. Rodriguez, C., F. Chen, R.A. Weinberg, and H.F. Lodish. 1995. Cooperative binding of transforming growth factor (TGF)-beta 2 to the types I and II TGF-beta receptors. J. Biol. Chem. 270:15919–15922.

41. Cheifetz, S., T. Bellon, C. Cales, S. Vera, C. Bernabeu, J. Massague, and M. Letarte. 1992. Endoglin is a component of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. USA. 92:10302–10306.

42. Moore, K.J., and D.L. Nagle. 2000. Complex trait analysis in the mouse: the strengths, the limitations and the promises yet to come. Annu. Rev. Genet. 34:653–686.

43. Manly, K.F., and J.M. Olson. 1999. Overview of QTL mapping software and introduction to map manager QT. Mamm. Genom. 10:327–334.

44. Churchill, G.A., and R.W. Doerge. 1994. Empirical threshold values for quantitative trait mapping. Genetics. 138:963–971.

45. Lander, E., and L. Kruglyak. 1995. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat. Genet. 11:241–247.

46. Williams, R.W., J. Gu, S. Qi, and L. Lu. 2001. The genetic structure of recombinant inbred mice: high-resolution consensus maps for complex trait analysis. Genome Biol. 2:RESEARCH0046. Epub. 2001. Oct. 22.
pase C. J. Biol. Chem. 266:20767–20772.

46. Gerber, H.P., A.K. Malik, G.P. Solar, D. Sherman, X.H. Liang, G. Meng, K. Hong, J.C. Marsters, and N. Ferrara. 2001. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature. 417:954–958.

47. Majka, M., A. Janowska-Wieczorek, J. Rajtajczak, K. Ehrenman, Z. Pietrzkowski, M.A. Kowalska, A.M. Gewirtz, S.G. Emerson, and M.Z. Rajtajczak. 2001. Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood. 97:3075–3085.

48. Ogawa, M., S. Nishikawa, K. Yoshinaga, S. Hayashi, T. Kunisada, J. Nakao, T. Kina, T. Sudo, H. Kodama, and S. Nishikawa. 1993. Expression and function of c-Kit in fetal hematopoietic progenitor cells: transition from the early c-Kit-independent to the late c-Kit-dependent wave of hematopoiesis in the murine embryo. Development. 117:1089–1098.

49. Ikuta, K., and I.L. Weissman. 1992. Evidence that hematopoietic stem cells express mouse c-kit, but do not depend on steel factor for their generation. Proc. Natl. Acad. Sci. USA. 89:1502–1506.

50. Lansdorp, P.M. 1995. Developmental changes in the function of hematopoietic stem cells. Exp. Hematol. 23:187–191.

51. Harrison, D.E., and C.M. Astle. 1982. Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number, and transplantation procedure. J. Exp. Med. 156:1767–1779.

52. Allsopp, R.C., G.B. Morin, J.W. Horner, R. DePinho, C.B. Harley, and I.L. Weissman. 2003. Effect of TERT overexpression on the long-term transplantation capacity of hematopoietic stem cells. Nat. Med. 9:369–371.

53. Chen, J., C.M. Astle, and D.E. Harrison. 2000. Genetic regulation of primitive hematopoietic stem cell senescence. Exp. Hematol. 28:442–450.