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

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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

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Abbreviations used in this paper: 5-FU, 5-fluorouracil; CFC, colony-forming cell; flt3L, flt3 ligand; GAR, goat anti–rat antibody; HSC, hematopoietic stem cell; KL, kit ligand; LSK, $lin^{-}Sca1^{++}kit^{+}$; nt, nucleotide; QTL, quantitative trait locus; RI, recombinant inbred; TGF-RII, type II TGF receptor; TPO, thrombopoietin.

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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

Mice. 8-wk-old C57BL/6J, DBA/2J, BALB/cJ, BXD recombinant inbred (RI), and tgfb2^{tm1doe} mice were purchased from Jackson Laboratories. C57BL/6.SJL-*Ptprca*$^{Pep3b/BoyJ}$ 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) were purchased from Southern Biotechnology Associates, Inc. Unconjugated Ter119, allophycocyanin-conjugated GARs, biotinylated anti–c-kit, and allophycocyanin-Cy7–conjugated streptavidin were purchased from BD Biosciences. Hoechst 33342 was purchased from Sigma-Aldrich. Recombinant mouse flt3L, KL, TPO, TGF-β1, TGF-β2 and TGF-β3, polyclonal pan-anti–TGF-β, and anti–TGF-RII 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) cells 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−) Sca1^{++}kit^{+} 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 mg/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_2, 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^{-6} M α-thioglycerol. After 8 d of incubation at 37°C in a humidified incubator with 5% CO_2, 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 allophycocyanin-GAR, Sca-1-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 FACSVantage™ 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 10^4 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′-TGAACCCTAAGGCCAACCGTG-3′ (nucleotide [nt] 409-429) and 5′-GCTCATAGCTCTTCTCCAGGG-3′ (nt 804-789) for actin; and 5′-ATGGCTTTGGATGCTGCCTA-3′ (nt 2124-2143) and 5′-TTAGCTGCATTTACAAGACTTGACCA-3′ (nt 2436-2459) for TGF-β2, respectively.

Competitive Repopulation Assays. 2×10^6 bone marrow cells or 10^5 E14 fetal liver cells from *Tgfb2*$^{+/-}$, *Tgfb2*$^{-/-}$, or WT mice (CD45.2^{+}, competing cells), and equal numbers of heterozygous B6.B6.SJL-*Ptprca*$^{Pep3b/BoyJ}$ mice (CD45.1^{+}CD45.2^{+}) or homozygous B6.SJL-*Ptprca*$^{Pep3b/BoyJ}$ mice (CD45.1^{+}; competitor cells) were mixed and injected in lethally (950 cG) irradiated C57BL/6.SJL-*Ptprca*$^{Pep3b/BoyJ}$ mice (CD45.1^{+}). 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 2×10^6 bone marrow cells from primary recipients into lethally irradiated secondary C57BL/6 (CD45.1^{+}) recipients, and from secondary recipients into lethally irradiated tertiary C57BL/6 (CD45.1^{+}) 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.

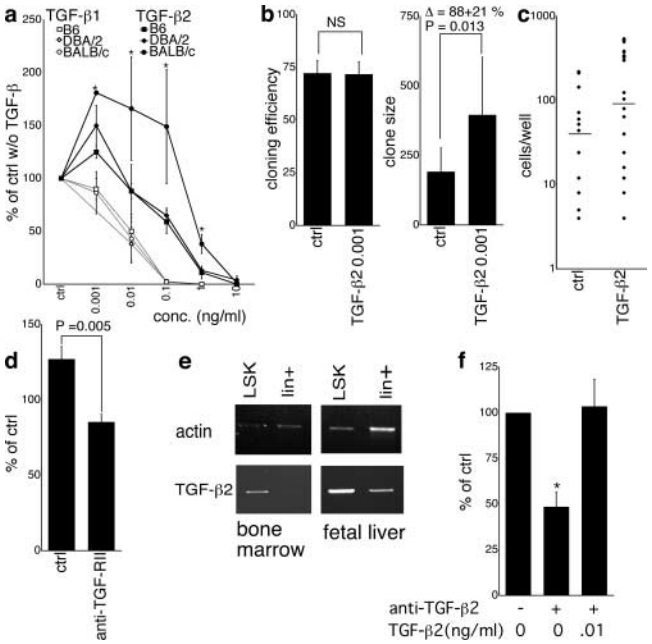

Figure 1. Effect of TGF-β2 in vitro. (a) Dose response of TGF-β1 and TGF-β2 on proliferation in 5-d cultures of LSK cells from the mouse strains listed (top), supported by KL, flt3L, and TPO (mean ± SEM; $n = 3$–13; *, significantly different DBA/2 and C57BL/6). (b) Effect of 0.001 ng/ml TGF-β2 on cloning 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 preincubation 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; $P = 0.04$).

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

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-RI, 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.

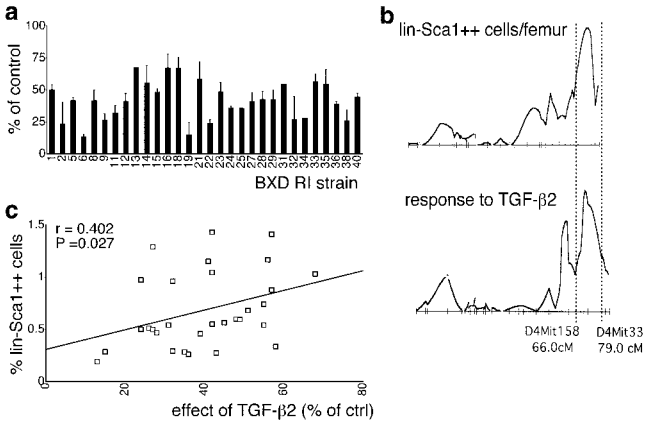

Figure 2. Linkage analysis in BXD RI strains. (a) Strain distribution pattern of the effect of 0.1 ng/ml TGF-β2 on the KL, flt3L, and TPO-supported proliferation of LSK cells in BXD mice (mean ± SEM; $n = 2–4$). (b) Pattern of likelihood ratio statistic (y axis) values along chromosome 4 for the absolute number of lin^-Sca1^{++} cells (reference 9) and their responsiveness to TGF-β2. The y axis scale is relative to normalize the size of both peaks (the maximum likelihood ratio statistic value is 15.9 for the number of lin^-Sca1^{++} cells, and 13.7 of the effect of TGF-β2). (c) Correlation between the effect of TGF-β2 on the proliferation of LSK cells (a) and the frequency of lin^-Sca1^{++} cells (reference 9).

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-

plantation experiments, *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, 10^5 fetal liver cells from *Tgfb2*$^{-/-}$, *Tgfb2*$^{+/-}$, or WT fetuses (CD45.2$^+$) were transplanted together with 2×10^5 bone marrow cells from congenic C57BL/6.SJL-*Ptprca*$^{Pep3b/BoyJ}$ (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-β2 plays no role in fetal hematopoiesis. To further evaluate the repopulation capacity of these cells, we performed serial transplantations by injecting 2×10^6 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 (Δ [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*$^{-/-}$,

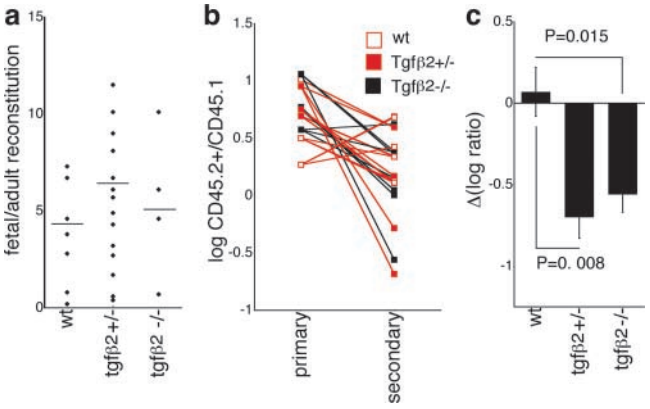

Figure 3. Hematopoietic phenotype of *Tgfb2*$^{-/-}$ and *Tgfb2*$^{+/-}$ E14 fetal mice. (a) Competitive repopulation of 10^5 *Tgfb2*$^{-/-}$, *Tgfb2*$^{+/-}$, and WT (wt) fetal liver cells against 2×10^5 C57BL/6 bone marrow cells analyzed 12–16 wk after transplantation. Data are expressed as ratio between fetal liver–derived (CD45.2$^+$) and C57BL/6 adult bone marrow–derived (CD45.1$^+$) cells in the peripheral blood. (b) Log ratio CD45.2$^+$/CD45.1$^+$ in individual primary and secondary recipients in serial repopulation experiments from primary donors reconstituted with fetal liver–derived cells (*Tgfb2*$^{+/-}$, *Tgfb2*$^{-/-}$, or WT) as competing cells. (c) Average Δ [log ratio = log(CD45.2/CD45.1) in primary recipients − log(CD45.2/CD45.1) in secondary recipients] for WT, *Tgfb2*$^{+/-}$, and *Tgfb2*$^{-/-}$ fetal liver cells upon serial transplantation (mean ± SEM; $n = 9$ for each genotype).

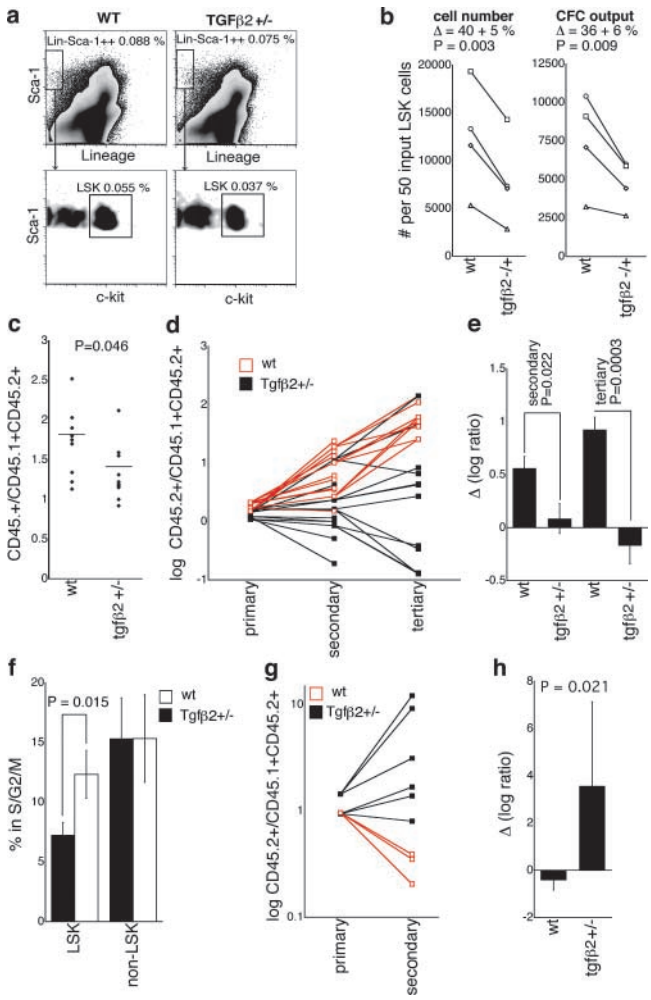

Figure 4. Hematopoietic phenotype of adult *Tgfb2*$^{+/-}$ mice. (a) Representative example of flow cytometric analysis of the frequency of lin$^-$Sca1^{++} and LSK cells in bone marrow of *Tgfb2*$^{+/-}$ mice and WT (wt) littermates. Bone marrow was pooled from three mice for each genotype. 10^6 events were recorded. (b) Proliferation and CFC output in pre-CFC assays of LSK cells from *Tgfb2*$^{+/-}$ mice or WT littermates. In each experiment, bone marrow was pooled from three mice for each genotype. (c) Competitive repopulation of 2×10^6 WT or *Tgfb2*$^{+/-}$ bone marrow cells with 2×10^6 C57BL/6-derived bone marrow cells in C57BL/6 recipients. Data are expressed as ratio for each individual recipient between WT or *Tgfb2*$^{+/-}$-derived (CD45.1$^+$CD45.2$^+$) and C57BL/6 competitor–derived (CD45.1$^+$CD45.2$^+$) reconstitution at 12 wk. (d) Log ratio CD45.2$^+$/CD45.1$^+$CD45.2$^+$ in individual primary, secondary, and tertiary recipients in serial repopulation experiments from primary donors reconstituted with *Tgfb2*$^{+/-}$ or WT bone marrow cells as competing cells. (e) Average Δ (log ratio) for WT and *Tgfb2*$^{+/-}$ bone marrow cells upon serial transplantation (mean ± SEM; $n = 9$ for each genotype). (f) Fraction of LSK cells and of cells falling outside the LSK window (non-LSK) in S/G2/M phase of the cell cycle in *Tgfb2*$^{+/-}$ mice and WT littermates ($n = 4$ experiments using mice from different litters). (g) Log (CD45.2$^+$/CD45.1$^+$CD45.2$^+$) in individual recipients in serial competitive repopulation experiments from primary donors reconstituted with adult bone marrow–derived cells (*Tgfb2*$^{+/-}$ or WT; CD45.2$^+$) as competing cells and with CD45.1$^+$CD45.2$^+$ C57BL/6-derived bone marrow cells as competitor cells. In contrast to the primary recipients shown in Fig. 4 d, the primary recipients in this experiment were treated with 5-FU 12 wk after primary reconstitution and 6 wk before secondary transplantation. (h) Average Δ (log ratio) for WT and *Tgfb2*$^{+/-}$ bone marrow cells upon serial transplantation after treatment of primary recipients with 5-FU (mean ± SEM; $n = 3$ for WT; $n = 6$ for *Tgfb2*$^{+/-}$).

and WT fetal liver HSCs was approximately fourfold (Fig. 3 c). The serial reconstitution capacity of *Tgfb2*$^{+/-}$ and *Tgfb2*$^{-/-}$ fetal liver cells was similar (Fig. 3 c), indicating dominance of *Tgfb2* for this particular biological effect. These findings indicate that TGF-β2 is important for maintaining the serial repopulation 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 repopulation 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. 1 e) 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 littermates (unpublished data). However, bone marrow cellularity in *Tgfb2*$^{+/-}$ mice was slightly, but significantly lower than in WT littermates ($2.012 \pm 0.61 \times 10^7$ vs. $1.86 \pm 0.60 \times 10^7$ 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 littermates (Fig. 4 b), 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 littermates (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. 4 c, 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 repopulation capacity between WT and *Tgfb2*$^{+/-}$ HSCs was more pronounced after transplantation into secondary recipients after 12 wk (Fig. 4 d). 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. 4 e). 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. 4, d 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. 4 c), whereas in secondary and tertiary recipients, the competitive advantage of the bone marrow of WT backcrossed mice increased even further (Fig. 4 d). 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 littermates. 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 littermates were stained with the DNA-binding dye Hoechst 33342 and by antibodies against lineage markers, Sca-1, and c-kit. As shown in Fig. 4 f, 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 cells cycle was similar in the total bone marrow population of *Tgfb2*$^{+/-}$ mice and WT littermates, 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, 2×10^6 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 than *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).

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