

# TGF- $\beta$ 1 Negatively Regulates the Number and Function of Hematopoietic Stem Cells

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## SUMMARY

Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) plays a role in the maintenance of quiescent hematopoietic stem cells (HSCs) *in vivo*. We asked whether TGF- $\beta$ 1 controls the cell cycle status of HSCs *in vitro* to enhance the reconstitution activity. To examine the effect of TGF- $\beta$ 1 on the HSC function, we used an *in vitro* culture system in which single HSCs divide with the retention of their short- and long-term reconstitution ability. Extensive single-cell analyses showed that, regardless of its concentration, TGF- $\beta$ 1 slowed down the cell cycle progression of HSCs but consequently suppressed their self-renewal potential. Cycling HSCs were not able to go back to quiescence with TGF- $\beta$ 1. This study revealed a negative role of TGF- $\beta$ 1 in the regulation of the HSC number and reconstitution activity.

## INTRODUCTION

Hematopoietic stem cells (HSCs) have the potential of self-renewal and differentiation into all blood lineages (Seita and Weissman, 2010). Since their self-renewal potential is finite (Ema et al., 2005), adult HSCs stay in a quiescent state most of the time (Sun et al., 2014) and undergo self-renewal divisions only four times in the lifespan of a mouse (Bernitz et al., 2016), preventing them from exhaustion. Quiescence is an important property of HSCs, but how HSCs maintain the quiescent state and how cycling HSCs return to the quiescent state is poorly understood.

Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) is a candidate cytokine that regulates quiescent HSCs in the bone marrow niche (Sitnicka et al., 1996; Yamazaki et al., 2007, 2011; Zhao et al., 2014). TGF- $\beta$ 1 activates Smad/FoxO signaling (Seoane et al., 2004), which is required for the maintenance of the HSC pool (Karlsson et al., 2007; Miyamoto et al., 2007; Tothova et al., 2007).

Numerous studies have attempted to achieve the *ex vivo* expansion of HSCs for clinical applications but with little success. It is difficult to induce self-renewal alone in HSCs because the differentiation process is tightly associated with division in HSCs. It is likely that rapidly dividing HSCs have a greater probability of losing the self-renewal potential than do slowly dividing HSCs. Our serum-free culture system with stem cell factor (SCF) and thrombopoietin (TPO) supports the *in vitro* self-renewal division of HSCs for a limited period of time (Ema et al., 2000; Ieyasu et al., 2017; Saka et al., 2017). Using this culture system, we aimed to control the number and speed of HSC divisions by TGF- $\beta$ 1 and, accordingly, to enhance their reconstitution activity after division.

HSCs possess a functional heterogeneity (Benz et al., 2012; Challen et al., 2010; Dykstra et al., 2007; Ema et al., 2005; Jordan and Lemischka, 1990; Morita et al., 2010; Muller-Sieburg et al., 2002; Yamamoto et al., 2013). In the mouse, HSC subsets are classified as myeloid-biased, balanced, and lymphoid-biased HSCs (Cho et al., 2008; Muller-Sieburg et al., 2002, 2004). It was recently reported that TGF- $\beta$ 1 at a low concentration stimulates the proliferation of myeloid-biased HSCs but inhibits that of lymphoid-biased HSCs (Challen et al., 2010). In this study, we used transplantation assays to examine the effects of different concentrations of TGF- $\beta$ 1 on long-term (LT, >6 months) and short-term (ST, <6 months) HSCs, in which myeloid-biased HSCs and lymphoid-biased HSCs are enriched (Ema et al., 2014).

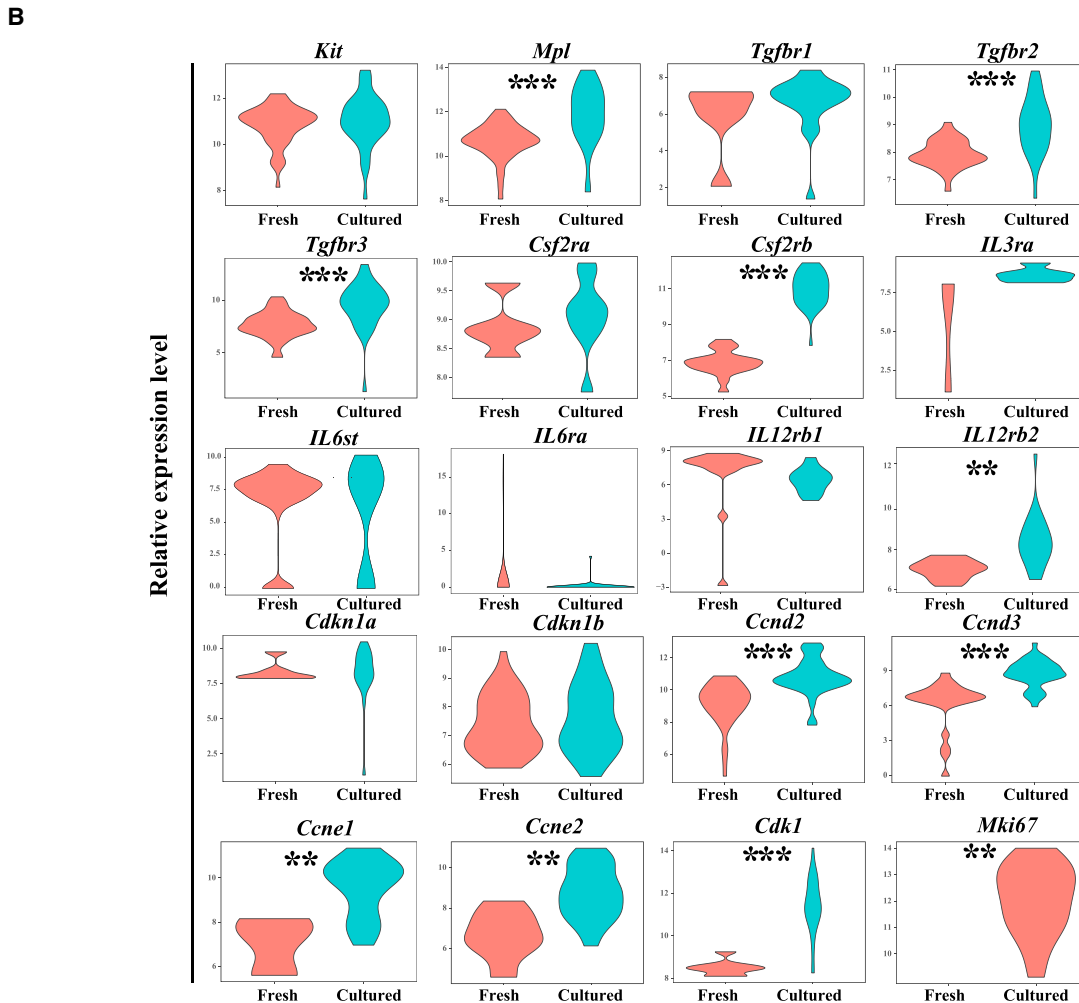
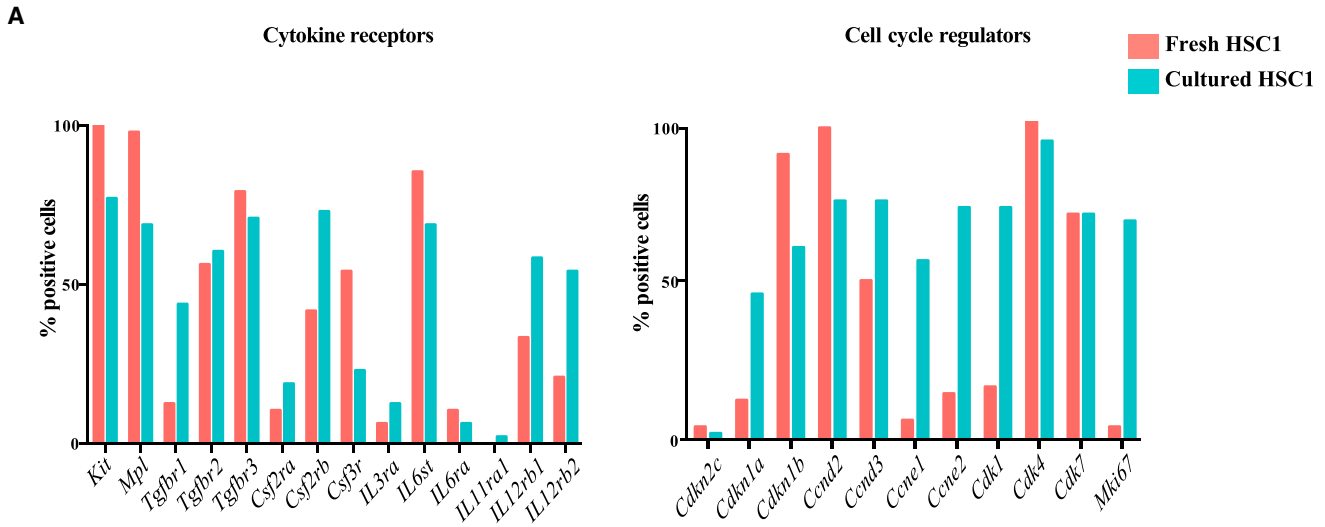
To overcome the problem of heterogeneity in the HSC population, we used single-cell culture, single-cell transplantation, and single-cell PCR to examine the direct effect of TGF- $\beta$ 1 on single HSCs. Here, we report that although TGF- $\beta$ 1 slowed down the cell cycle progression in HSCs, the self-renewal potential in both the LT- and ST-HSCs was reduced. Thus, we propose that TGF- $\beta$ 1 is a negative regulator of the number and reconstitution activity of HSCs that have entered the cell cycle (cycling HSCs).

## RESULTS

### Single-Cell RT-PCR

We defined CD150<sup>+</sup>CD41<sup>-</sup>CD34<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lineage<sup>-</sup> (KSL) cells as the HSC1 population (Figure S1A). TGF- $\beta$ 1 binds to the receptor consisting of TGFBR1 and TGFBR2 (Massague, 1998). We performed a single-cell





**Figure 1. Gene Expression in Single HSCs**

Forty-eight single HSC1 cells were sorted by flow cytometry (fresh cells). Forty-eight single HSC1 cells were cultured in SCF + TPO for 24 hr (cultured cells). RT-PCR was performed on these single cells to compare the expression of the 48 genes.

(legend continued on next page)



gene expression analysis of freshly isolated HSC1 cells and cultured single cells. Single HSC1 cells were cultured in the presence of SCF + TPO for 24 hr. The cells that remained as single cells after culture were selected. The expression of 48 genes was examined for 48 single cells (Table S1). *Gapdh* and *Actb* were included as controls.

The gene expression data are shown in a heatmap format in Figure S1B. Figure 1A shows the percentage of gene-expressing cells (percentage positive cells, threshold cycle values [Ct] < 27.65). Figure 1B shows the violin plots of the relative gene expression level in the positive cells. *Gapdh*, *Actb*, *c-Kit*, and *c-Mpl* were detected in most cells. In the freshly isolated HSC1 cells, only six of the 48 cells (12.5%) expressed *Tgfb1*. However, after 24 hr in culture, 21 of the 48 cells (43.8%) expressed *Tgfb1*. The relative expression of *Tgfb1* was similar between the freshly isolated cells and the cultured cells. In the freshly isolated HSC1 cells, 27 of the 48 cells (56.3%) expressed *Tgfb2*, and 38 of the 48 cells (79.2%) expressed *Tgfb3*. The number of *Tgfb2*- and *Tgfb3*-positive cells did not change after culture. However, the relative expression of *Tgfb2* and *Tgfb3* became significantly higher after culture. Most importantly, both *Tgfb1* and *Tgfb2* were expressed in only 1 of 48 cells (2.1%) before culture and 16 of 48 cells (33.3%) after culture. Other cytokine receptor genes, with the exception of *Csf2rb*, *IL-12rb1*, and *IL-12rb2*, did not change in either the percentage of positive cells or the relative expression.

*Mki67*, a marker of proliferation, was dramatically increased after culture in both the percentage of positive cells and the relative expression. Similarly, *Ccne1*, *Ccne2*, and *Cdk1* increased after culture in both the percentage of positive cells and the relative expression. Interestingly, *Cdkn1b* and *Ccnd2* were already expressed in the freshly isolated cells. These data showed that upregulation of the TGF- $\beta$ 1 receptor was associated with cell cycle progression in HSCs.

#### Titration of TGF- $\beta$ 1 by *In Vitro* and *In Vivo* Assays

We next examined the dose effect of TGF- $\beta$ 1 on HSC1 cells by *in vitro* single-cell culture. Single HSC1 cells were directly sorted into 96-well plates containing SF-O3 serum-free medium with SCF + TPO in the presence or absence of human TGF- $\beta$ 1 at different concentrations, as follows: 0 and 0.1 pg/mL; 1, 10, and 100 pg/mL; 1 and 10 ng/mL. Based on the number of cells per well from each day of culture, the cell division kinetics were estimated (Figure S2A). Figure S2B shows the data. The first, second, and third divi-

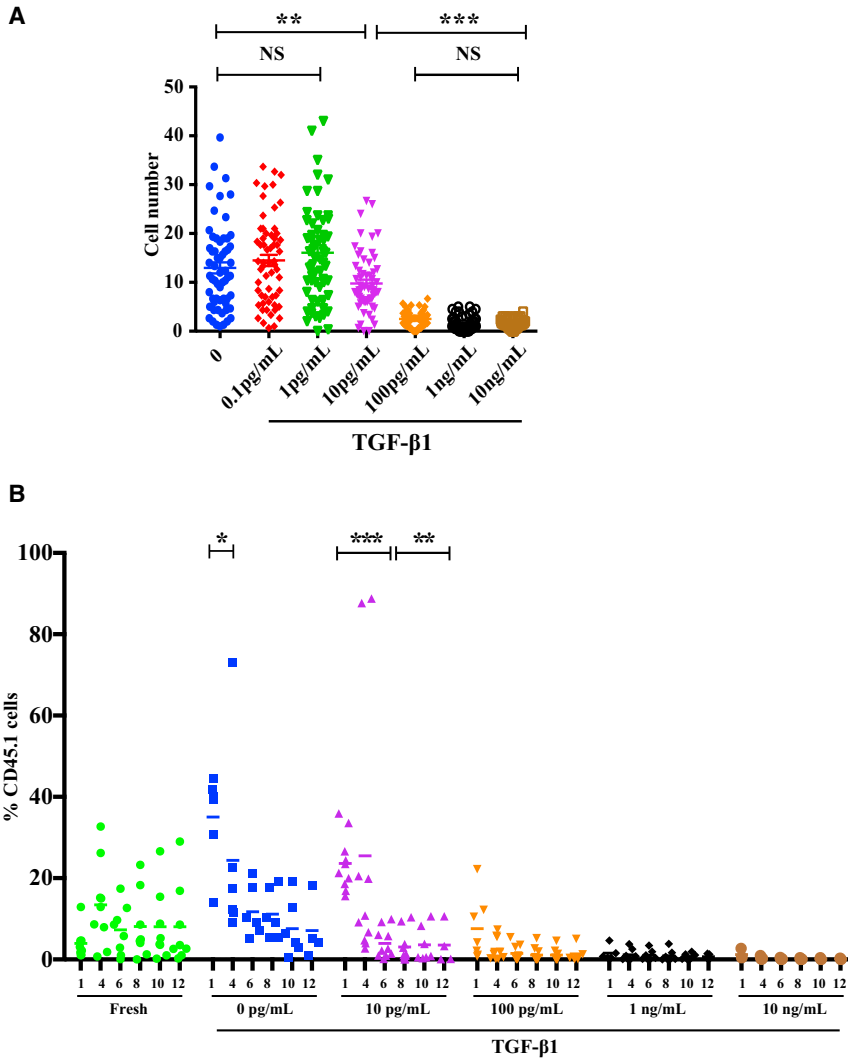
sions were similar between SCF + TPO and SCF + TPO + TGF- $\beta$ 1 cultures at 0, 0.1, 1, and 10 pg/mL of TGF- $\beta$ 1. In contrast, the second and third divisions slowed down in the SCF + TPO + TGF- $\beta$ 1 culture at 100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF- $\beta$ 1. Figure 2A shows the number of cells per well on day 5 of culture. Without TGF- $\beta$ 1, the number of cells per well was  $17.9 \pm 1.43$  (mean  $\pm$  SD,  $n = 180$ ). The numbers of cells per well with TGF- $\beta$ 1 at concentrations of 0.1 pg/mL, 1 pg/mL, 10 pg/mL, 100 pg/mL, 1 ng/mL, and 10 ng/mL were  $19.2 \pm 1.33$ ,  $22.0 \pm 1.6$ ,  $13.2 \pm 0.7$ ,  $3.7 \pm 0.2$ ,  $2.5 \pm 0.2$ , and  $2.6 \pm 0.1$ , respectively (mean  $\pm$  SD,  $n = 180$  in each culture). The number of cells in culture with 10 pg/mL of TGF- $\beta$ 1 was significantly smaller than those in culture with 0.1 pg/mL and 1 pg/mL of TGF- $\beta$ 1 ( $p = 0.0026$ ) and was significantly larger than those in culture with 100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF- $\beta$ 1 ( $p < 0.0001$ ). These data showed that 100 pg/mL of TGF- $\beta$ 1 was sufficient for the *in vitro* suppression of HSC proliferation. The representative pictures of day 7 colonies from each concentration of TGF- $\beta$ 1 are shown in Figure S2C.

We also examined the dose effect of TGF- $\beta$ 1 on HSC1 cells using an *in vivo* repopulating assay. Ten HSC1 cells were sorted and injected into lethally irradiated mice along with competitor cells. Ten HSC1 cells were also cultured with SCF, TPO, and TGF- $\beta$ 1 at concentrations of 0, 10, and 100 pg/mL and 1 and 10 ng/mL for 5 days and were similarly transplanted. To detect repopulation by the test donor cells, peripheral blood was examined from 1 month to 12 months after transplantation. Figure 2B shows that the recipient mice transplanted with 10 freshly isolated HSC1 cells showed varying degrees of donor cell repopulation. The same was true for recipient mice transplanted with 10 HSC1 cells that were cultured with SCF + TPO for 5 days. After culture with SCF + TPO for 5 days, the ST reconstitution levels (1, 2, and 4 months after transplantation) were significantly greater than those in the freshly isolated cells ( $p = 0.026$ ). ST reconstitution levels (1, 2, and 4 months after transplantation) in the cells cultured with 10 pg/mL of TGF- $\beta$ 1 were significantly greater than those in the cells cultured with 100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF- $\beta$ 1 ( $p < 0.001$ ) but were not greater than those in the cells cultured without TGF- $\beta$ 1. LT reconstitution levels (6, 8, 10, and 12 months after transplantation) in the cells cultured with 10 pg/mL of TGF- $\beta$ 1 were significantly lower than those in the cells cultured without TGF- $\beta$ 1 ( $p = 0.006$ ) but not lower than those in the cells

(A) The percentage of gene-expressing cells in the 48 cells is shown for cytokine receptors and cell cycle regulators. Positive cells were defined as cells with threshold cycle values (Ct) < 27.65.

(B) Violin plots of the relative gene expression levels for positive cells. The relative expression level was defined as the (28-Ct) values. Among the genes presented, the relative expression levels of *Mpl*, *Tgfb2*, *Tgfb3*, *Csf2rb*, *IL-12rb2*, *Ccnd2*, *Ccnd3*, *Ccne1*, *Ccne2*, *Cdk1*, and *Mki67* significantly increased after culture. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  (unpaired t test).

See also Figure S1 and Table S1.



**Figure 2. In Vitro and In Vivo Effect of Different TGF-β1 Concentrations on HSCs**

(A) Sixty single HSC1 cells were cultured in the presence of SCF + TPO with TGF-β1 at concentrations between 0 and 10 ng/mL for 5 days. A total of 180 single cells from three independent experiments were analyzed for each culture condition. The number of cells in culture with 10 pg/mL of TGF-β1 was significantly smaller than for those in culture with 0, 0.1, and 1 pg/mL of TGF-β1 (\*\*p = 0.0026) and was significantly larger than for those in culture with 100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF-β1 (\*\*p < 0.001). The numbers of cells in culture with 0, 0.1, and 1 pg/mL of TGF-β1 or 100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF-β1 were not significantly different (ANOVA). NS, not significant.

(B) Ten HSC1 cells were transplanted into a lethally irradiated mouse with  $5 \times 10^5$  BM cells (fresh). Ten HSC1 cells were cultured with SCF, TPO, and TGF-β1 at concentrations of 0, 10, or 100 pg/mL and 1 or 10 ng/mL for 5 days and were transplanted into a group of 10 lethally irradiated mice. To detect the percentage of CD45.1<sup>+</sup> cells, the peripheral blood was examined 1, 2, 4, 6, 8, 10, and 12 months after transplantation. Seven to 10 mice survived for each group. ST reconstitution levels (1 and 4 months after transplantation) in the cells cultured with SCF + TPO were significantly higher than those in freshly isolated cells (\*p = 0.026). ST reconstitution levels in the cells cultured with 10 pg/mL of TGF-β1 were significantly higher than those in the cells cultured with

100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF-β1 (\*\*p < 0.001) but were not significantly different from those in the cells cultured without TGF-β1. LT reconstitution levels (6, 8, 10, and 12 months after transplantation) in the cells cultured with 10 pg/mL of TGF-β1 were significantly lower than those in the cells cultured without TGF-β1 (\*\*p = 0.006) but were not significantly different from those in the cells cultured with 100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF-β1 (ANOVA).

See also [Figure S2](#).

cultured with 100 pg/mL, 1 ng/mL, and 10 ng/mL of TGF-β1. These data showed that 100 pg/mL of TGF-β1 was sufficient for the suppression of ST and LT reconstitution activity. Interestingly, 10 pg/mL of TGF-β1 suppressed LT but not ST reconstitution activity in HSCs. From these *in vitro* and *in vivo* titration data, we decided to use 100 pg/mL of TGF-β1 for the rest of the study.

### Early Cell Division Kinetics of HSC1 Cells in the Presence of TGF-β1

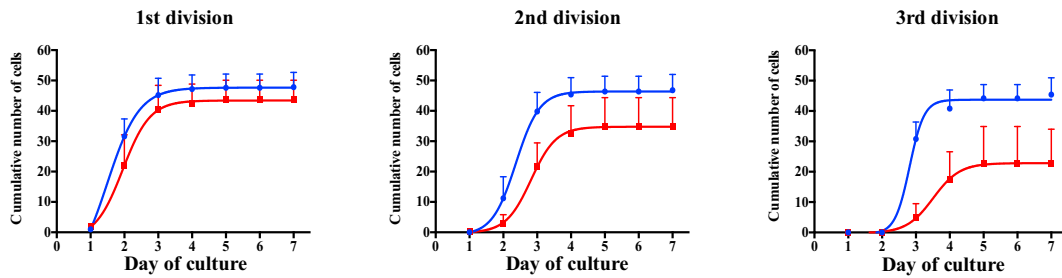
We performed single-cell cultures with the HSC1 cells. [Figure 3A](#) shows the first, second, and third divisions of the HSC1 cells in the presence of SCF + TPO or

SCF + TPO + TGF-β1. On average, 80.0% of the HSC1 cells underwent the first, second, and third divisions in the presence of SCF + TPO, showing that the cells continuously and rapidly divided. However, 76.7%, 50.0%, and 33.3% of the HSC1 cells underwent the first, second, and third division in the presence of SCF + TPO + TGF-β1. The division kinetics curves were significantly shifted to the right in the second and third divisions in the presence of SCF + TPO + TGF-β1, compared with those in the presence of SCF + TPO. These data showed that the cells slowly divided and that a portion of the cells stopped dividing in the presence of TGF-β1.

[Figure 3B](#) shows the number of cells per well for each of the cultures. The cell number in the SCF + TPO cultures



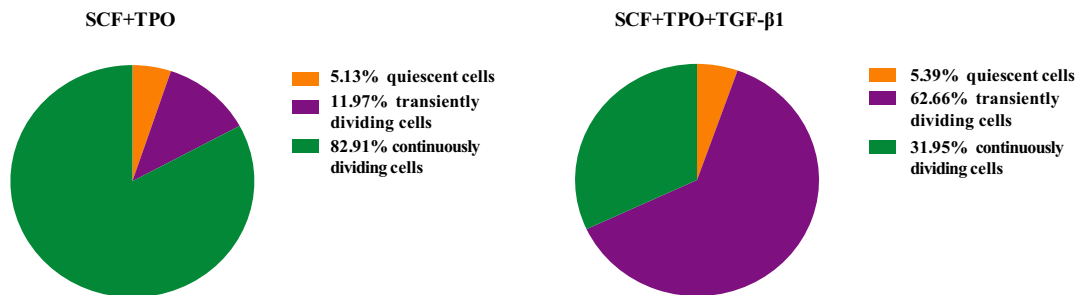
**A**



**B**

Culture condition	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
SCF+TPO	1.02±0.145	2.17±1.14 **	5.94±4.51 ***	11.7±9.71 ***	21.9±20.0 ***	49.9±49.2 ***
SCF+TPO+ TGF-β1	1.0±0.158	1.4±0.626	2.45±1.48	3.13±1.92	3.21±2.64	2.51±2.10

**C**



**Figure 3. Single-Cell Culture of HSCs with TGF-β1**

(A) Early division kinetics of HSC1 cells. Sixty single HSC1 cells were cultured with SCF + TPO or SCF + TPO + TGF-β1 for 7 days. The cumulative numbers of cells per 60 cells are shown for the first, second, and third divisions.

(B) The table shows the number of cells per well on days 1–7. Sixty single HSC1 cells were cultured in the presence of SCF + TPO with or without TGF-β1. The cell numbers in the SCF + TPO cultures were significantly greater than those in the SCF + TPO + TGF-β1 cultures on days 2–7. \*\**p* < 0.01; \*\*\**p* < 0.001 (unpaired *t* test). The wells without cells were excluded from the analysis. (A and B) Data are shown as mean ± SD from five independent experiments.

(C) The cells were classified into three categories as follows: quiescent cells (one cell per well throughout the culture period), transiently dividing cells (an increase and then a decrease in cell number during the culture period), and continuously dividing cells (a continuous increase in cell number during the culture period). The percentages of the classified cells are shown. The wells without cells were excluded from the analysis. (B and C) A total of 238 wells from five independent experiments were analyzed for SCF + TPO and a total of 245 wells from five independent experiments were analyzed for SCF + TPO + TGF-β1.

increased exponentially from day 1 to day 7. However, the cell number in the SCF + TPO + TGF-β1 cultures slightly increased until day 4. To compare the cell division dy-

namics between cultures with SCF + TPO and SCF + TPO + TGF-β1, the cells were classified into three groups as follows: quiescent cells (one cell per well throughout



the culture period), transiently dividing cells (an increase and then a decrease in cell number during the culture period), and continuously dividing cells (a continuous increase in cell number during the culture period). As shown in [Figure 3C](#), in the absence of TGF- $\beta$ 1, 5.1%, 12.0%, and 82.9% of the wells contained quiescent cells, transiently dividing cells, and continuously dividing cells, respectively. In the presence of TGF- $\beta$ 1, 5.4%, 62.7%, and 32.0% of the wells contained quiescent cells, transiently dividing cells, and continuously dividing cells, respectively. The quiescent cells persisted for 7 days in SCF + TPO, and the proportion of these cells did not change with the addition of TGF- $\beta$ 1. A total of 62.7% of the cells in the TGF- $\beta$ 1 culture divided only one or two times. Of note were the cells in the TGF- $\beta$ 1 culture that underwent the first division without delay. The remaining 32.0% of the cells in the TGF- $\beta$ 1 culture kept dividing slowly.

#### TGF- $\beta$ 1 Inhibits Dividing HSC1 Cells in Both the Early and Late Phases of Culture

We evaluated the effect of TGF- $\beta$ 1 on dividing HSC1 cells at different time periods using an *in vivo* repopulating assay. Ten HSC1 cells were sorted and injected into lethally irradiated mice along with competitor cells. Ten HSC1 cells were also cultured with SCF + TPO as controls and SCF + TPO+100 pg/mL of TGF- $\beta$ 1 for 3, 5, or 7 days and were then similarly transplanted. To detect repopulation by the test donor cells, the peripheral blood was examined from 1 month to 12 months after transplantation.

[Figure 4A](#) shows that the recipient mice transplanted with 10 freshly isolated HSC1 cells showed donor cell repopulation. The same was found for recipient mice transplanted with 10 HSC1 cells that were cultured with SCF + TPO for 3, 5, or 7 days. After culture with SCF + TPO for 3, 5, or 7 days, the ST and LT reconstitution levels were comparable with those of the freshly isolated cells. In contrast, ST and LT reconstitution levels in the cells cultured with TGF- $\beta$ 1 were significantly lower than those in the cells cultured with SCF + TPO ( $p < 0.001$ ). The reconstitution levels in the cells cultured with TGF- $\beta$ 1 for 7 days were significantly lower than those in the cells cultured with TGF- $\beta$ 1 for 3 or 5 days ( $p < 0.001$ ). These data showed that TGF- $\beta$ 1 reduced not only ST reconstitution but also LT reconstitution from the early to late phases of culture.

We next examined the effect of a delayed addition of TGF- $\beta$ 1 on cell division in the single-cell culture. [Figure S3A](#) shows that when TGF- $\beta$ 1 was added to cultures at day 0, the number of cells that underwent second and third divisions was reduced, and these divisions occurred slowly. When TGF- $\beta$ 1 was added to cultures at day 3, the number of third divisions was reduced. When TGF- $\beta$ 1 was added to cultures at day 5, most of the cells had gone through these divisions, and we did not see any effect on these divisions. [Figure S3B](#)

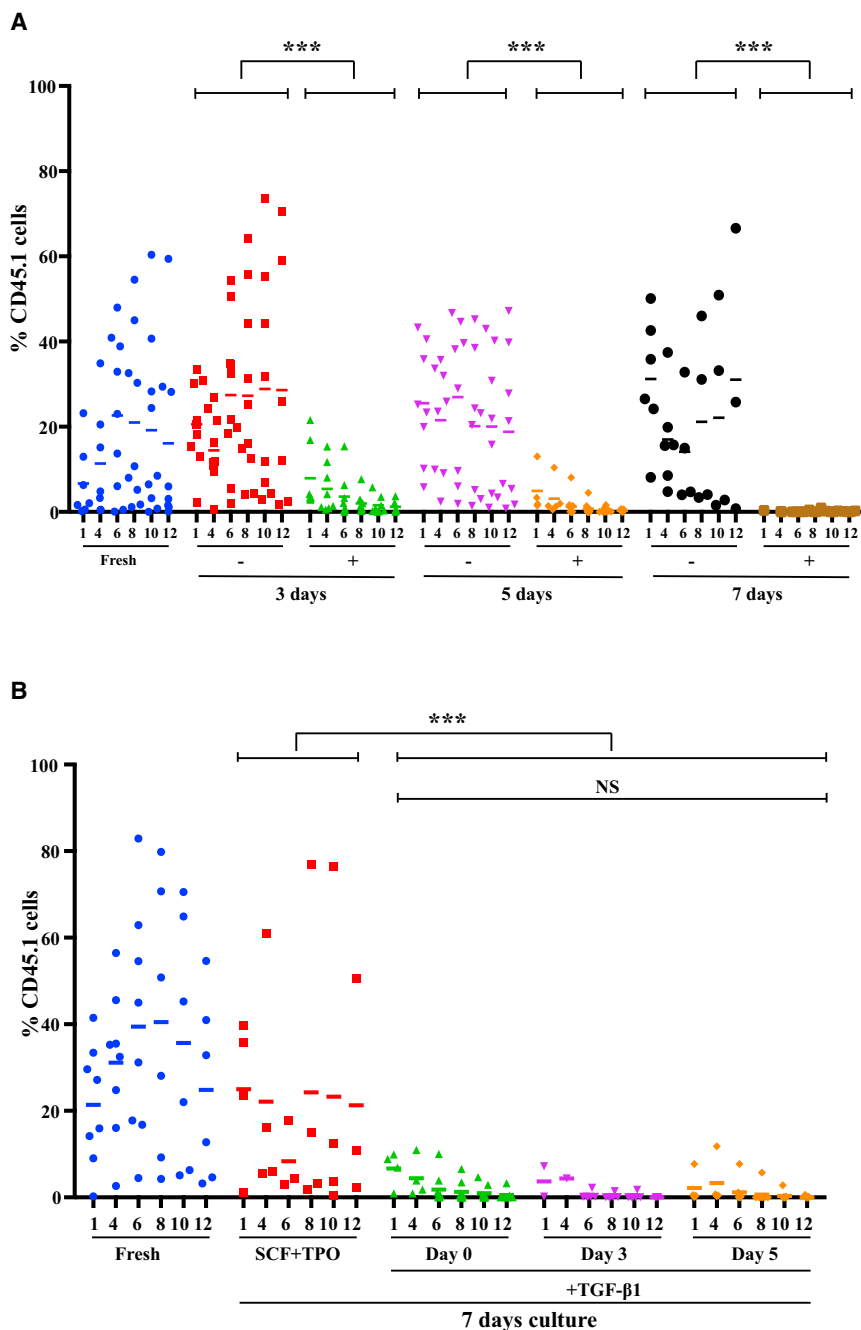
shows the number of cells per well at day 7. The number of cells with TGF- $\beta$ 1 from days 0 and 3, but not from day 5, were significantly different from those without TGF- $\beta$ 1.

We then examined the effect of a delayed addition of TGF- $\beta$ 1 on the repopulation activity in HSC1 cells. Ten HSC1 cells were sorted and injected into lethally irradiated mice along with competitor cells. Ten HSC1 cells were also cultured with SCF + TPO as a control, and TGF- $\beta$ 1 was added into the medium at day 0, 3, or 5 of culture. On day 7 of culture, the cells were transplanted. To detect repopulation by the test donor cells, the peripheral blood was examined from 1 month to 12 months after transplantation. [Figure 4B](#) shows that all the recipient mice transplanted with 10 freshly isolated HSC1 cells showed donor cell repopulation. The same was true for those transplanted with 10 HSC1 cells cultured with SCF + TPO for 7 days. Regardless of the day of the TGF- $\beta$ 1 addition, the ST and LT reconstitution levels were all significantly reduced ( $p < 0.001$ ). Interestingly, only the last 2 days of exposure were sufficient to make this reduction. These data suggested that, in the last 2 days of culture, the cells were more sensitive to TGF- $\beta$ 1.

#### TGF- $\beta$ 1 Reduces the Reconstitution Activity in Single HSC1s

In the dose response experiment, we showed that TGF- $\beta$ 1 at 10 pg/mL reduced only the LT reconstitution ability and at 100 pg/mL reduced both the ST and LT reconstitution ability in the culture cells. To verify these findings, we examined the ability of cells derived from single HSC1 cells in culture at 10 and 100 pg/mL of TGF- $\beta$ 1. As illustrated in [Figure 5A](#), single HSC1 cells were transplanted into lethally irradiated recipient mice along with  $5 \times 10^5$  competitor cells. The single HSC1 cells were cultured in SCF, TPO, and TGF- $\beta$ 1 (0, 10, or 100 pg/mL) for 5 days and were then transplanted into lethally irradiated mice.

As shown in [Figure 5B](#), the frequencies of the reconstitution were 36.7% for the freshly isolated HSC1 cells (11/30 mice), 36.7% for the SCF + TPO cultured cells (11/30 mice), 23.3% for the SCF + TPO+10 pg/mL TGF- $\beta$ 1 cultured cells (7/30 mice), and 13.3% for SCF + TPO+100 pg/mL TGF- $\beta$ 1 cultured cells (4/30 mice). There were eight LT-HSCs and three ST-HSCs detected in the fresh group. Six LT-HSCs and five ST-HSCs were detected in the SCF + TPO group. No LT-HSCs, but one ST-HSC and six B lymphoid progenitors were detected in the 10 pg/mL TGF- $\beta$ 1 culture. Neither the LT-HSCs nor the ST-HSCs were detected in the 100 pg/mL TGF- $\beta$ 1 culture. Four B lymphoid progenitors were detected in this group. Consistent with the *in vivo* dose response data, the LT-HSCs were more sensitive than the ST-HSCs in clonal transplantation. Taken together, both the LT and ST reconstitution and the myeloid and T



### Figure 4. Cycling HSCs Are More Sensitive to TGF-β1

(A) Different culture periods. Ten HSC1 cells were sorted from the BM cells of the B6-Ly5.1 mice; cultured with SCF + TPO or SCF + TPO + TGF-β1 for 3, 5, or 7 days; and transplanted with  $5 \times 10^5$  competitor cells from the B6-Ly5.2 mice into 10 lethally irradiated B6-Ly5.2 mice. The percentage of chimerism of the granulocytes/monocytes, B cells, and T cells in the peripheral blood was analyzed 1, 4, 6, 8, 10, and 12 months after the transplantation. Four to 10 mice survived for 12 months for each group. ST and LT reconstitution levels in the cells cultured with SCF + TPO + TGF-β1 were significantly lower than those in the cells cultured with SCF + TPO for 3, 5, and 7 days (\*\* $p < 0.001$ , ANOVA).

(B) Delayed addition of TGF-β1 in the HSC cultures with SCF + TPO. Ten HSC1 cells were sorted from the BM cells of the B6-Ly5.1 mice and were transplanted into 10 lethally irradiated B6-Ly5.2 mice. Ten HSC1 cells were cultured with SCF + TPO, to which 100 pg/mL of TGF-β1 was added on day 0, 3, or 5 of culture. On day 7 of culture, the cells were transplanted into the lethally irradiated mice. The percentage of chimerism of the granulocytes/monocytes, B cells, and T cells in the peripheral blood was analyzed 1, 4, 6, 8, 10, and 12 months after the transplantation. Three to 10 mice survived for 12 months for each group. ST and LT reconstitution levels in the cells cultured with SCF + TPO + TGF-β1, regardless of the day of TGF-β1 addition, were significantly lower than those in the cells cultured with SCF + TPO (\*\* $p < 0.001$ ). The ST and LT reconstitution levels in the cells cultured with SCF + TPO + TGF-β1 in the day 0, 3, and 5 groups were not significantly different (ANOVA).

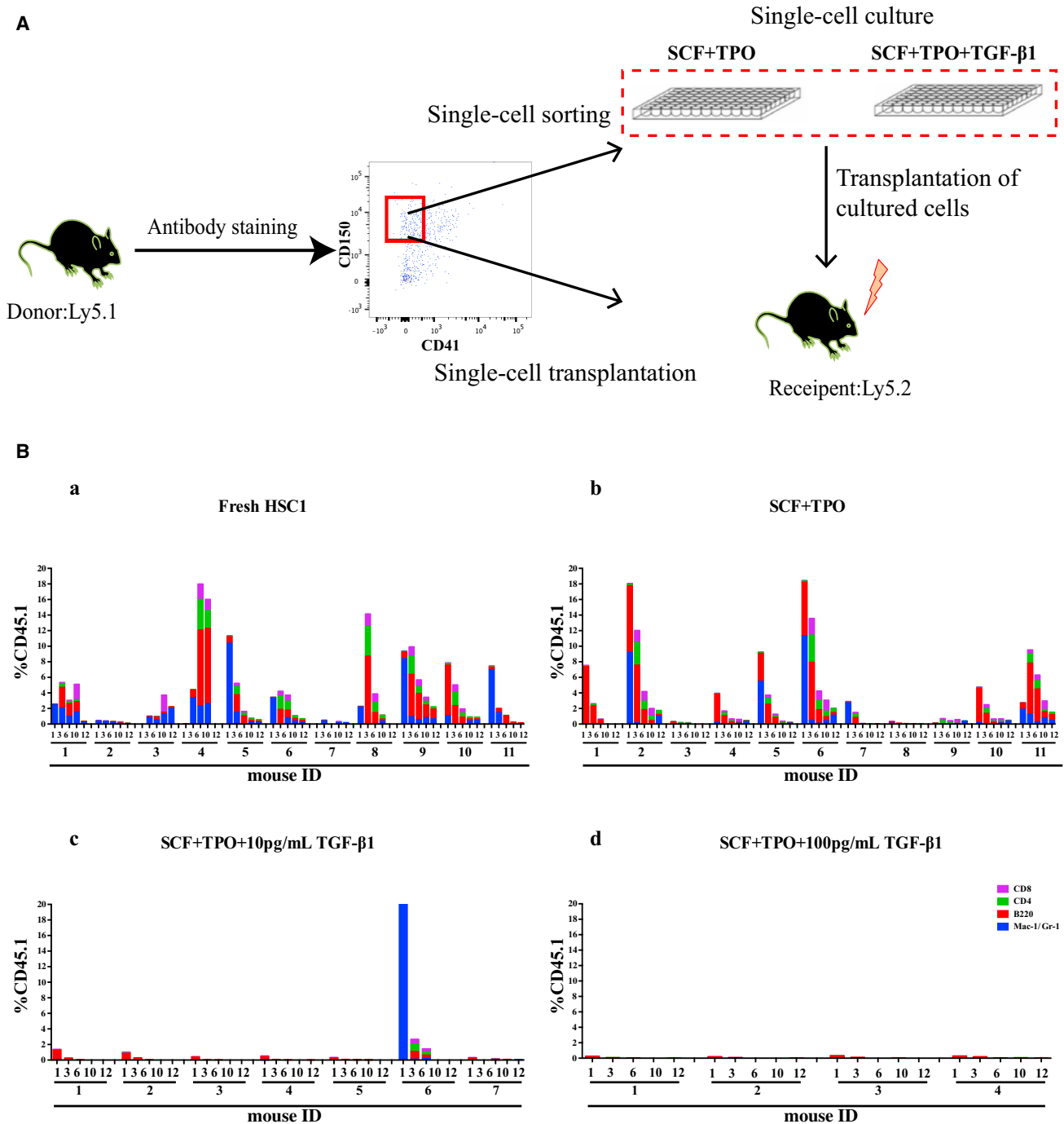
See also Figure S3.

lymphoid lineage differentiation potentials were markedly reduced by TGF-β1.

### Gene Expression Analysis of Slowly Cycling Cells with TGF-β1

We wished to see the effect of TGF-β1 on the expression of cytokine receptors and cell cycle regulators in cultured cells. Single HSC1 cells were cultured in serum-free media with SCF + TPO or SCF + TPO + TGF-β1 for 48 and

120 hr. One cell was picked from each of the 48 wells and subjected to single-cell RT-PCR. As shown in Figures 6A and S4A, the percentages of gene-expressing cells for *Kit*, *Mpl*, *Tgfb1*, *Tgfb2*, and *Tgfb3* were similar at 48 and 120 hr after culture between the SCF + TPO and SCF + TPO + TGF-β1 cultures. As shown in Figures 6B and S4B, the percentages of gene-expressing cells for *Ccnd2*, *Ccnd3*, *Ccne1*, *Ccne2*, *Cdk1*, *Cdk4*, and *Mki67* were similar at 48 and 120 hr after culture between the SCF + TPO and

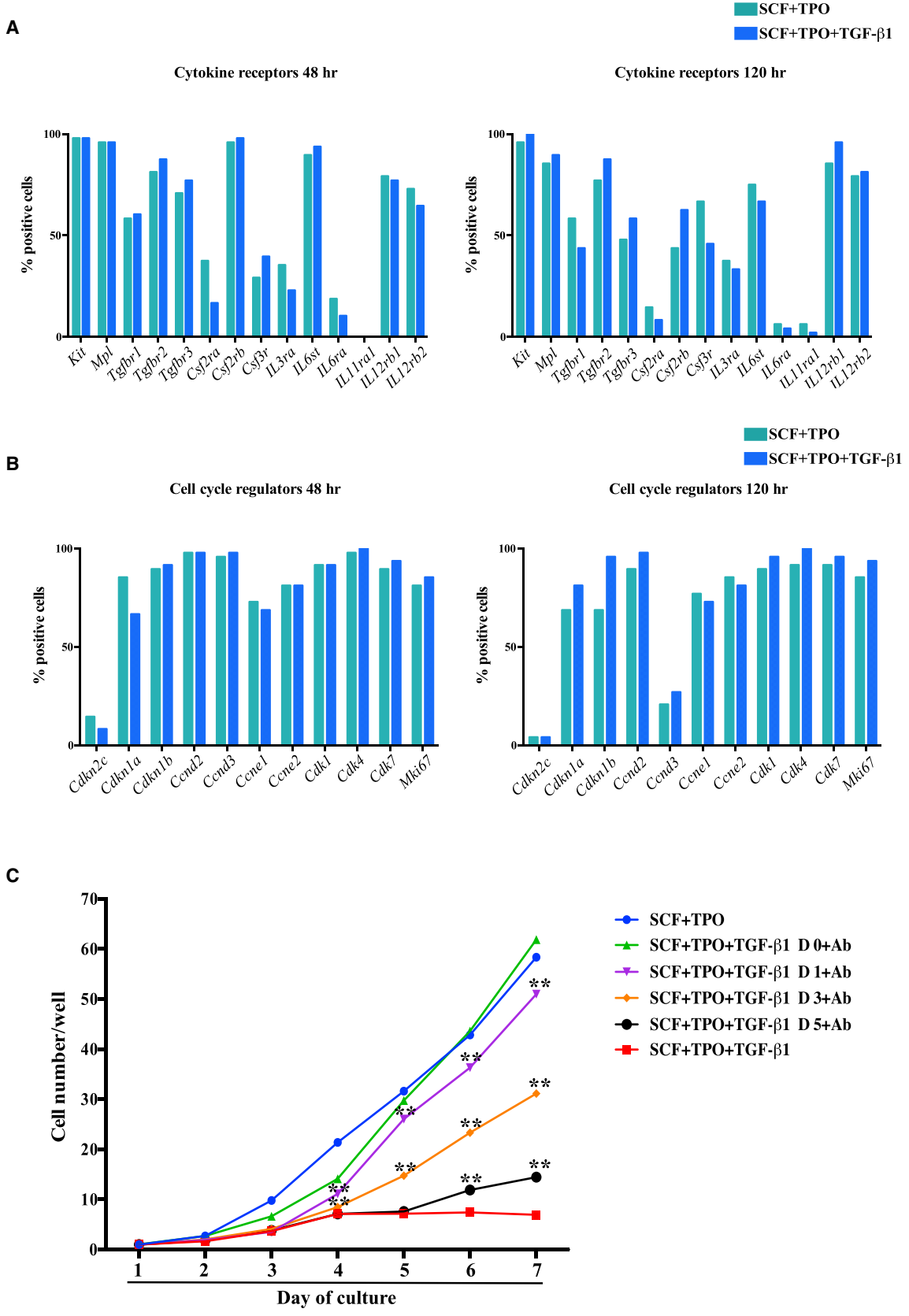


**Figure 5. TGF- $\beta$ 1 at Concentrations of 10 and 100  $\mu$ g/mL Reduces the Total and Myeloid Reconstitution Levels in a Single-Cell Culture**

(A) Single HSC1 cells, sorted from the BM cells of the B6-Ly5.1 mice, were transplanted into 30 lethally irradiated B6-Ly5.2 mice with  $5 \times 10^5$  competitor cells from the B6-Ly5.2 mice. The single cells were also cultured in serum-free medium with SCF + TPO or SCF + TPO + TGF- $\beta$ 1 (10 or 100  $\mu$ g/mL) for 5 days and were then transplanted into 30 lethally irradiated B6-Ly5.2 mice with  $5 \times 10^5$  competitor cells from the B6-Ly5.2 mice.

(B) Clonal cell transplantation. The percentage of chimerism in the granulocytes/monocytes, B cells, and T cells of the peripheral blood was analyzed 1, 3, 6, 10, and 12 days after transplantation. Two separate cohorts of single-cell transplantation experiments were performed, but the data from one experiment are shown. Individual mice were identified by numbering.





(legend on next page)



SCF + TPO + TGF- $\beta$ 1 cultures. To confirm that these data represented cultured cells, 48 single cells were picked from a colony consisting of 56 cells in the SCF + TPO culture, and 8, 20, and 20 single cells were picked from colonies consisting of 8, 20, and 26 cells, respectively, in the SCF + TPO + TGF- $\beta$ 1 culture. Single-cell gene expression analysis showed similar data (data not shown). These data indicate that cells surviving in the presence of TGF- $\beta$ 1 remained in the cell cycle and expressed cytokine receptors.

### Blockade of the Inhibitory Effect of TGF- $\beta$ 1

To rescue TGF- $\beta$ 1-treated cells in culture, a neutralizing antibody against TGF- $\beta$ 1 was used. Single HSC1 cells were cultured in SCF + TPO or SCF + TPO + TGF- $\beta$ 1 for 7 days. Anti-TGF- $\beta$ 1 neutralizing antibody was added to the culture on days 0, 1, 3, and 5. As shown in Figure 6C, the number of cells per well was similar to that in SCF + TPO when the antibody was added on day 0, showing a complete block of the effect of TGF- $\beta$ 1. When the antibody was added on days 1, 3, and 5, the numbers of cells per well were significantly greater than those in SCF + TPO + TGF- $\beta$ 1 on days 4–7, days 4–7, and days 6–7, respectively. These results suggested that the reduced cell division rate was reversible.

## DISCUSSION

TGF- $\beta$  signaling is mediated by the TGFBR1 and TGFBR2 complex, phosphorylating Smad2/Smad3 (Shi and Massague, 2003; ten Dijke and Hill, 2004). These receptor-regulated Smads bind to Smad4 and together translocate to the nucleus and regulate gene expression. TGFBR1, TGFBR2, Smad2 and Smad3, and Smad4 knockout mice all showed lethal inflammation disease mostly due to activated T cells (Billing et al., 2017; Karlsson et al., 2007; Larsson et al., 2003, 2005; Yamazaki et al., 2011). However, only TGFBR2 and Smad4 knockout mice showed a decrease in LT reconstitution activity (Karlsson et al., 2007; Yamazaki

et al., 2011). These data suggested that TGF- $\beta$ /Smad signaling differs between T cells and HSCs, and the signal pathways in HSCs are more complex than those in T cells. Together with our previous data, on average,  $13.8\% \pm 2.8\%$  (mean  $\pm$  SD,  $n = 5$ ) of the HSC1 cells expressed *Tgfr1*, whereas  $45.0\% \pm 7.8\%$  of the HSC1 cells expressed *Tgfr2*. These data are consistent with the quantitative RT-PCR data of CD34<sup>-</sup>KSL cells (Utsugisawa et al., 2006). Notably, single-cell RT-PCR showed that only  $6.7\% \pm 3.1\%$  of the cells expressed both *Tgfr1* and *Tgfr2*. During our single-cell culture, approximately 5% of the HSC1 cells were detected as single cells for 7 days (Figure 3C). These cells were likely to be in G0 phase, as previously reported (Yamazaki et al., 2009). After culture with SCF + TPO, 44% and 60% of the cells expressed *Tgfr1* and *Tgfr2*, respectively. As a result, 33% of the cells expressed both receptors (Figure 1A). The first division of the HSC1 cells in the SCF + TPO culture was not affected by TGF- $\beta$ 1 (Figure 3A). The HSC1 cells were ready to respond to TGF- $\beta$ 1 from their second division. Time seemed to be required for HSC1 cells to upregulate the expression of *Tgfr1* and respond to TGF- $\beta$ 1. These data suggest that a small number of HSC1 cells express both receptors *in vivo*, but a cytokine storm, which may occur in severe inflammation, for instance after bone marrow transplantation, likely induces the expression of TGF- $\beta$ 1 receptors.

Our data suggested that *Tgfr1* is not expressed in the majority of HSCs *in vivo*. This might be a reason why no abnormal function of HSCs was found in the TGFBR1 knockout mouse (Larsson et al., 2005). We may consider the possibility that *Tgfr2* is expressed with an unidentified type 1 receptor. Alternatively, different signals may be involved in Smad4 activation to maintain or induce the quiescence in HSCs. If this were the case, other mechanisms for the regulation of quiescent HSCs should be identified.

TGF- $\beta$ 1 is a pleiotropic factor that has positive or negative effects on a variety of cells (Dybedal et al., 1997; Larsson and Karlsson, 2005). It is suggested that the effect of TGF- $\beta$ 1 differs depending on its concentrations (Challen

### Figure 6. Gene Expression in Single Cells From Culture

- (A) Percentage of gene-expressing cells in the 48-hr and 120-hr cultured cells for cytokine receptors.
- (B) Percentage of gene-expressing cells in the 48-hr and 120-hr cultured cells for cell cycle regulators. (A and B) Single HSC1 cells were cultured in SCF + TPO or SCF + TPO + TGF- $\beta$ 1 for 48 and 120 hr. One cell was picked from each of 48 wells at 48 and 120 hr of culture by a micromanipulator.
- (C) Blockade of the inhibitory effect of TGF- $\beta$ 1 by the neutralizing antibody (1D11). Sixty single HSC1 cells were cultured in SCF + TPO or SCF + TPO + TGF- $\beta$ 1 for 7 days. Anti-TGF- $\beta$ 1 neutralizing antibody was added to the culture on days 0, 1, 3, and 5 (D0, D1, D3, and D5 + Ab). The mean numbers of cells per well from three independent experiments are plotted. The number of cells analyzed for the SCT + TPO, SCF + TPO + TGF- $\beta$ 1, D0, D1, D3, and D5 + Ab cultures were 144, 135, 140, 143, 130, and 152, respectively (the wells without cells were excluded). The numbers of cells in the SCF + TPO + TGF- $\beta$ 1 D1 + Ab, D3 + Ab, and D5 + Ab cultures were significantly greater on days 4–7, days 4–7, and days 6–7, respectively, than those in the SCF + TPO + TGF- $\beta$ 1 culture (\*\* $p < 0.001$ ; unpaired t test).

See also Figure S4 and Tables S2 and S3.



et al., 2010; Kale and Vaidya, 2004). This study was the first to examine its dose response effects on HSCs by transplantation assays. Data from an *in vitro* single-cell culture and *in vivo* competitive repopulation assay (Figures 2A and 2B) consistently showed that 100 pg/mL of TGF- $\beta$ 1 was sufficient to exert its effect. This concentration was much lower than those in previous studies (Challen et al., 2010; Sitnicka et al., 1996; Soma et al., 1996; Wiesmann et al., 2000; Yamazaki et al., 2009). ST and LT reconstitution activities in the SCF + TPO culture were significantly reduced by 100 pg/mL of TGF- $\beta$ 1 (Figure 4), supporting the previous study (Soma et al., 1996; Wiesmann et al., 2000). We further confirmed this finding by single-cell transplantation. Single-cell transplantation has an advantage over bulk-cell transplantation to identify different types of HSCs. We wished to see the effect of TGF- $\beta$ 1 on individual ST- and LT-HSCs. Consistent with 10-cell transplantation data, we did not see any positive effect on either ST-HSC or LT-HSCs (myeloid-biased HSCs) by single-cell transplantation (Figure 5). These data suggested that the self-renewal potential was markedly reduced in HSCs. Our transplantation data did not support the data from *in vitro* colony assays (Challen et al., 2010). The effect of TGF- $\beta$ 1 on the *in vivo* reconstitution potential may simply differ from that of the *in vitro* colony formation.

*In vivo* and *in vitro* studies are equally important because an *in vivo* study is suitable for examining physiological effects, while an *in vitro* study is suitable for examining direct effects. Perhaps, it is extremely difficult to clarify the mechanisms by only an *in vivo* study. If data from *in vivo* and *in vitro* studies are consistent, the finding should be affirmative. In this study, we focused on the *in vitro* effect of TGF- $\beta$ 1 on HSCs. We used a serum-free culture system, which is essential for maintaining the self-renewal potential in HSCs. Then, we were able to examine the effect of TGF- $\beta$ 1 on HSCs. We believe that a similar effect should be seen *in vivo*, at least in certain circumstances.

The biological activity of TGF- $\beta$  family members is regulated in a complex manner. We are far from understanding how and where TGF- $\beta$ 1 is produced, activated, and degraded *in vivo*. Another advantage of our *in vitro* study was that TGF- $\beta$ 1 could be tested at a range of low concentrations, such as 10 pg/mL to 10 ng/mL (Figures 2B and 5). It is difficult to examine the precise concentrations of TGF- $\beta$ 1 *in vivo*. If TGF- $\beta$ 1 regulates cycling HSCs *in vivo* as well, some HSCs may undergo cell cycle arrest and apoptosis during severe inflammation. It was reported that anti-TGF- $\beta$  neutralizing antibody can enhance the hematopoietic recovery after 5-fluorouracil treatment or bone marrow transplantation in mice (Brenet et al., 2013). The authors of this paper claimed that anti-TGF- $\beta$ 1 antibody prevents HSCs from returning to the G0 state. The antibody may also have rescued HSCs from apoptosis in severe inflammation.

Cell cycle regulation plays a critical role in controlling HSC function (Pietras et al., 2011). TGF- $\beta$ 1 slowed down the cell cycle progression of HSC progeny (Figure 3). Some HSCs stopped dividing, which resulted in cell cycle arrest with the loss of LT-HSC and ST-HSC activities (Figures 4 and 5). In particular, these activities were promptly lost in the last 2 days during 7 days of culture. We assumed that the HSCs were losing their self-renewal potential during this period of time and that TGF- $\beta$ 1 enhanced this event. Analysis of the expression of cell cycle regulators in surviving cells on day 5 of culture with TGF- $\beta$ 1 showed that all cells were still cycling. The expression of cytokine receptors on these cells was not changed by TGF- $\beta$ 1. These cells rapidly divided again after TGF- $\beta$ 1 was blocked by the antibody. It was likely that cells that had died before day 5 of culture were not detected by these assays. Importantly, quiescent cells were not detected either.

Nevertheless, TGF- $\beta$ 1 negatively regulated cycling HSCs in culture with SCF + TPO. If the G0 phase is the status in which HSCs can maintain their potential, TGF- $\beta$ 1 does not seem to be an appropriate cytokine to inhibit cycling HSCs for the purpose of HSC maintenance. In this regard, the TGF- $\beta$ 1-induced cell cycle status *in vitro* differs from the G0 status *in vivo*. Supporting the first study of TGF- $\beta$ 1 as a negative regulator in hematopoiesis (Ohta et al., 1987), this work provides a revised view of the action of TGF- $\beta$ 1 on HSCs.

## EXPERIMENTAL PROCEDURES

### Mice

C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) and C57BL/6 (B6-Ly5.2) mice were obtained from the animal facility of the State Key Laboratory of Experimental Hematology. The mice were housed in individual cages in a specific-pathogen-free-grade animal facility. All the experimental protocols were approved by the Animal Care and Use Committee of the Institute of Hematology and Blood Diseases Hospital.

### Flow Cytometry

Bone marrow (BM) cells were harvested from the tibiae, femora, and pelvis in phosphate buffered solution supplemented with 2% fetal calf serum (HyClone, US). For staining, the BM cells were incubated with anti-c-Kit microbeads (catalog no. 130-091-224, Miltenyi Biotec, Germany). The cells expressing c-Kit were enriched by columns. The cells were stained with a cocktail of biotinylated monoclonal antibodies (mAbs; BD Biosciences) specific for the antigens CD3 (clone 145-2C11, catalog no.13-0031-85), CD4 (clone GK1.5, catalog no.13-0041-85), CD8 (clone 53-6.7, catalog no. 13-0081-85), B220 (clone RA3-6B2, catalog no. 13-0452-85), Mac-1 (clone M1/7, catalog no. 13-0112-85), Gr-1 (clone RB6-8C5, catalog no. 13-5931-85), Ter-119 (clone TER-119, catalog no. 13-5921-85); PE/cyanin7 (PE-cy7) conjugated anti-Sca-1 (clone D7, catalog no. 25-5981-82, eBioscience), APC-conjugated



anti-c-Kit (clone 2B8, catalog no. 17-1171-82, eBioscience), FITC-conjugated anti-CD34 (clone RAM34, catalog no. 11-0341-85, eBioscience), PE-conjugated anti-CD150 (clone TC15-12F12.2, catalog no. 115904, BioLegend), and PercP-eFluor710-conjugated anti-CD41 (clone MWReg30, catalog no. 46-0411-82, eBioscience) mAbs. The biotinylated mAbs were developed with APC/cyanin7 (APC-cy7)-conjugated streptavidin (catalog no. 47-4317-82, eBioscience). The cells were sorted on a FACSAria III cell sorter (BD Biosciences) under the single-cell sorting mode. CD150<sup>+</sup>CD41<sup>-</sup>CD34<sup>-</sup>KSL cells enriched in LT-HSCs (Yamamoto et al., 2013) were designated as HSC1 cells.

For the peripheral blood analysis, the mice were bled from the tail, the red blood cells were lysed with red blood cell lysis buffer, and the cells were incubated with the following antibodies on ice for 30 min: FITC-conjugated anti-CD45.1 (clone A20, catalog no. 11-0453-85), PE-conjugated anti-CD45.2 (clone 104, catalog no. 12-0454-82), PE/cyanin7 (PE-cy7)-conjugated anti-CD4 (clone GK1.5, catalog no. 12-0454-82), APC-conjugated anti-CD8 (clone 53-6.7, catalog no. 17-0081-82), Percp-cy5.5-conjugated anti-B220 (clone RA3-6B2, catalog no. 45-0452-82), APC-eFluor780-conjugated anti-Mac-1 and Gr-1 (clone M1/70, catalog no. 47-0112-82 and clone RB6-8C5, catalog no. 47-5931-82), and DAPI, all purchased from eBioscience. The cells were then spun down and resuspended in a PBS solution, and the analysis was accomplished on live cells with an FACS Canto II system (Becton Dickinson, US).

### Single-Cell Culture

Single HSC1 cells were deposited into 96-well microtiter plates containing 200  $\mu$ L of SF-O3 (Sanko Junyaku, Japan) supplemented with  $5 \times 10^{-5}$  M 2- $\beta$ -mercaptoethanol, 0.5 mg/mL of recombinant human serum albumin (catalog no. 1001, Albumin Bioscience, Japan), 10 mM HEPES (catalog no. H0887, Sigma, US), 10  $\mu$ M nonessential amino acids (catalog no. 11,140-050, Gibco, US), 50 U of penicillin/streptomycin (catalog no. 15,140-122, Life Technology, US), and 2 mM L-glutamine (catalog no. 25,030-081, Gibco, US) as a serum-free medium (Ieyasu et al., 2017). The cells were cultured in 50 ng/mL of SCF and 50 ng/mL of TPO with different concentrations (0, 0.1 pg/mL, 1 pg/mL, 10 pg/mL, 100 pg/mL, 1 ng/mL, and 10 ng/mL) of human TGF- $\beta$ 1 (PeproTech, US). The cells were cultured at 37°C with 5% CO<sub>2</sub> in the air. The number of cells per well was monitored daily by microscopy.

### Ten-Cell Transplantation

Ten HSC1 cells were sorted from the BM cells of the B6-Ly5.1 mice and were injected into lethally irradiated B6-Ly5.2 mice with  $5 \times 10^5$  competitor cells from the B6-Ly5.2 mice. Ten HSC1 cells were cultured in serum-free medium with SCF + TPO as a control, and 100 pg/mL of TGF- $\beta$ 1 was added into the medium at day 0, 3, or 5 of the culture. On day 7 of the culture, the cells were transplanted with  $5 \times 10^5$  competitor cells from the Ly5.2 mice into the lethally irradiated B6-Ly5.2 mice. The percentage of test donor cells (CD45.1<sup>+</sup> cells) of granulocytes/monocytes, B cells, and T cells in the peripheral blood was analyzed 1, 2, 4, 6, 8, 10, and 12 months after the transplantation.

Ten HSC1 cells were sorted from the BM cells of the B6-Ly5.1 mice and were later injected into lethally irradiated B6-Ly5.2

mice with  $5 \times 10^5$  competitor cells from the B6-Ly5.2 mice. Ten HSC1 cells were sorted from the BM cells of the B6-Ly5.1 mice, were cultured in serum-free medium with SCF + TPO as a control or SCF + TPO+100 pg/mL of TGF- $\beta$ 1 for 3–7 days, and were transplanted with  $5 \times 10^5$  competitor cells from the Ly5.2 mice into the lethally irradiated B6-Ly5.2 mice. The percentage of test donor cells (CD45.1<sup>+</sup> cells) of granulocytes/monocytes, B cells, and T cells in peripheral blood was analyzed 1, 2, 4, 6, 8, 10, and 12 months after the transplantation.

### Single-Cell Transplantation

Single HSC1 cells, sorted from the BM cells of the B6-Ly5.1 mice, were transplanted into lethally irradiated B6-Ly5.2 mice with  $5 \times 10^5$  competitor cells from the B6-Ly5.2 mice. The single cells were also cultured in serum-free medium with SCF + TPO or SCF + TPO + TGF- $\beta$ 1 (10 or 100 pg/mL) for 5 days and were then transplanted. The percentage of test donor cells (CD45.1<sup>+</sup> cells) in the granulocytes/monocytes, B cells, and T cells of the peripheral blood was analyzed 1, 3, 6, 8, 10, and 12 months after transplantation. LT-HSCs were defined by a myeloid lineage reconstitution for 6 months or more. Lymphoid lineage reconstitution may not be essential for LT-HSCs, as recently suggested (Carrelha et al., 2018). ST-HSCs were defined as myeloid lineage reconstitution for less than 6 months, with B and T lymphoid reconstitution at one time. When the percentage of CD45.1<sup>+</sup> cells was greater than 0.1%, reconstitution was considered to have occurred (positive mouse).

### Single-Cell RT-PCR

Single HSC1 cells were sorted into 48 wells of a 96-well plate where each well contained 10  $\mu$ L of a reverse-transcription and specific-target amplification mixture (RT buffer) consisting of 2.5  $\mu$ L of 0.2 $\times$  primers, containing all 48 sets of primers (Table S1), 5.0  $\mu$ L of a 2 $\times$  reaction mix, 0.5  $\mu$ L of Superscript III, and 2.0  $\mu$ L of Tris-EDTA buffer. Single HSC1 cells were cultured in serum-free media with SCF + TPO for 24 hr. Single cells were identified under an inverted microscope and were picked up by a micromanipulator (Narishige, Japan) and plated into the RT buffer. Single HSC1 cells were cultured in serum-free media with SCF + TPO or SCF + TPO + TGF- $\beta$ 1 for 48 and 120 hr. One cell was picked from each of 48 wells by the micromanipulator and plated into the RT buffer. On the other hand, single HSC1 cells were cultured in serum-free media with SCF + TPO or SCF + TPO + TGF- $\beta$ 1 for 120 hr. In the case of the SCF + TPO culture, 48 single cells were picked from a colony consisting of 56 cells. In the case of SCF + TPO + TGF- $\beta$ 1, 8, 20, and 20 single cells were picked from colonies consisting of 8, 20, and 26 cells, respectively, and plated into the RT buffer.

Reverse transcription was performed at 50°C for 15 min. The samples were incubated at 95°C for 2 min, followed by 22 cycles of 95°C for 15 s and 60°C for 4 min. Five microliters, taken from the samples, was mixed with 20  $\mu$ L of Tris-EDTA and was used for real-time PCR. Then, 2.7  $\mu$ L, taken from the diluted samples, was mixed with 3.0  $\mu$ L TaqMan universal PCR master mix (Applied Biosystems) and 0.3  $\mu$ L of sample loading buffer (a total of 6.0  $\mu$ L sample loading mix). On the other hand, 3.0  $\mu$ L of each set of 20 $\times$  primers was mixed with 3.0  $\mu$ L of the assay loading reagent (a total of 6.0  $\mu$ L assay loading mix). Then, 5  $\mu$ L of the sample



loading mix and 5  $\mu$ L of the assay loading mix were applied to a 48 chip, and 48  $\times$  48 reactions were prepared by an integrated fluidic circuit controller. The chip was set on a Fluidigm Biomark system and was incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The data were analyzed by Biomark real-time PCR analysis software (Fluidigm). The sets of PCR primers, as listed in Tables S1–S3, were purchased from Thermo Fisher Scientific (Peixoto et al., 2004).

### Neutralization of TGF- $\beta$ 1

Anti-TGF- $\beta$ 1 neutralizing antibody (1D11) was purchased from R&D Systems. HSC1 cells were cultured in SCF + TPO + TGF- $\beta$ 1 (100 pg/mL) with different concentrations (0, 5, 50, and 500 ng/mL, and 5  $\mu$ g/mL), and 500 ng/mL was found to block the negative effect of TGF- $\beta$ 1 sufficiently. Single HSC1 cells were cultured in SCF + TPO or SCF + TPO + TGF- $\beta$ 1 for 7 days. Anti-TGF- $\beta$ 1 neutralizing antibody was added at the concentration of 500 ng/mL to the culture on days 0, 1, 3, and 5.

### Statistics

An unpaired t test was used to compare the means from two groups, and ANOVA was used to compare the means from three groups or more, using GraphPad Prism 5.0 (GraphPad) and SPSS 17.0 statistical software (IBM).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at <https://doi.org/10.1016/j.stemcr.2018.05.017>.

### AUTHOR CONTRIBUTIONS

X.W. performed the experiments, analyzed the data, and wrote the manuscript. F.D., S.Z., Z.W., and Sh.Z. helped with the transplantation experiments. W.Y., W.Y., and S.M. helped with the single-cell gene expression analysis. J.W. helped with the flow cytometry operation. P.W., Y.G., J.D., and F.T. helped with data analysis. T.C. and H.E. directed the research programs, designed the study, and wrote the manuscript.

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