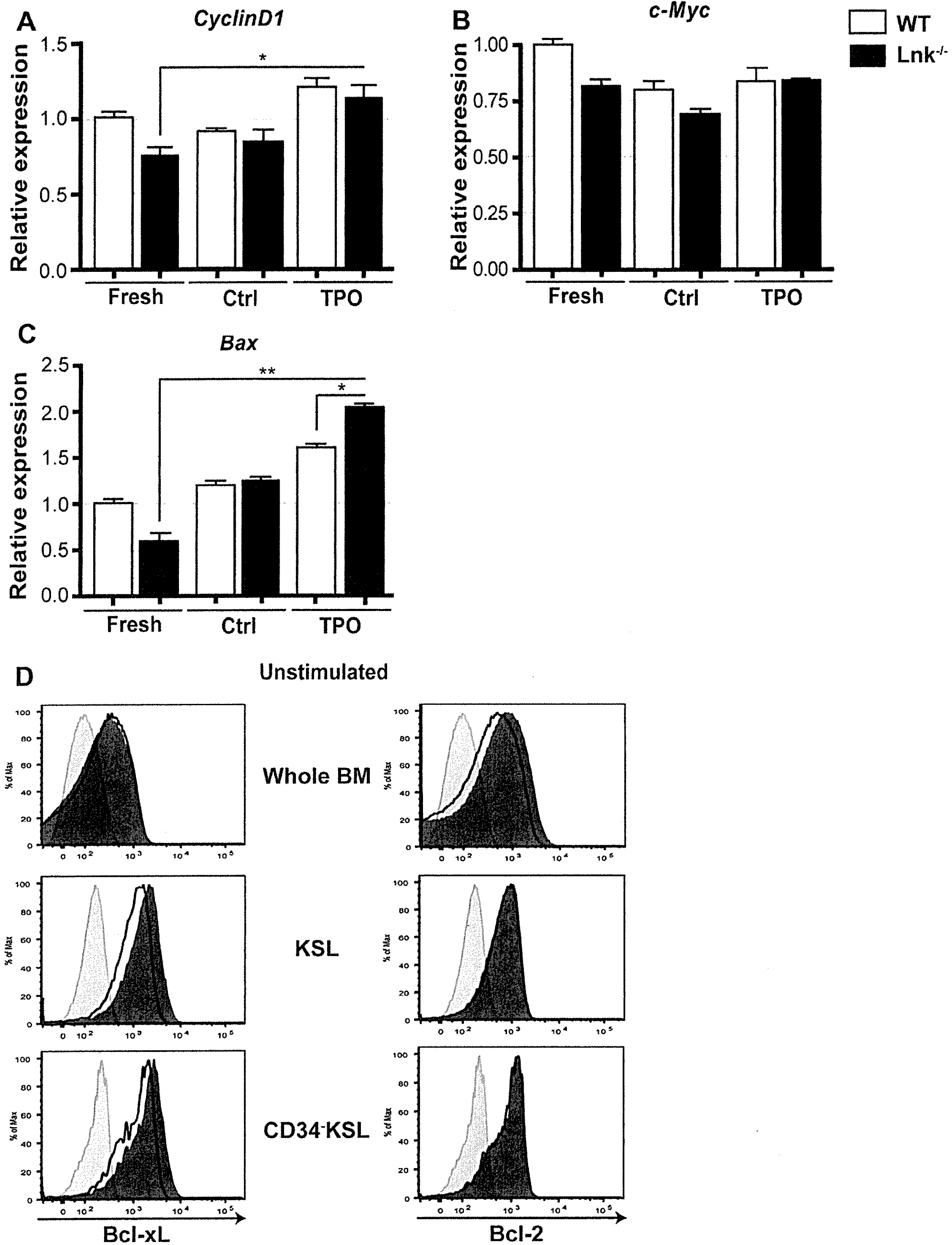
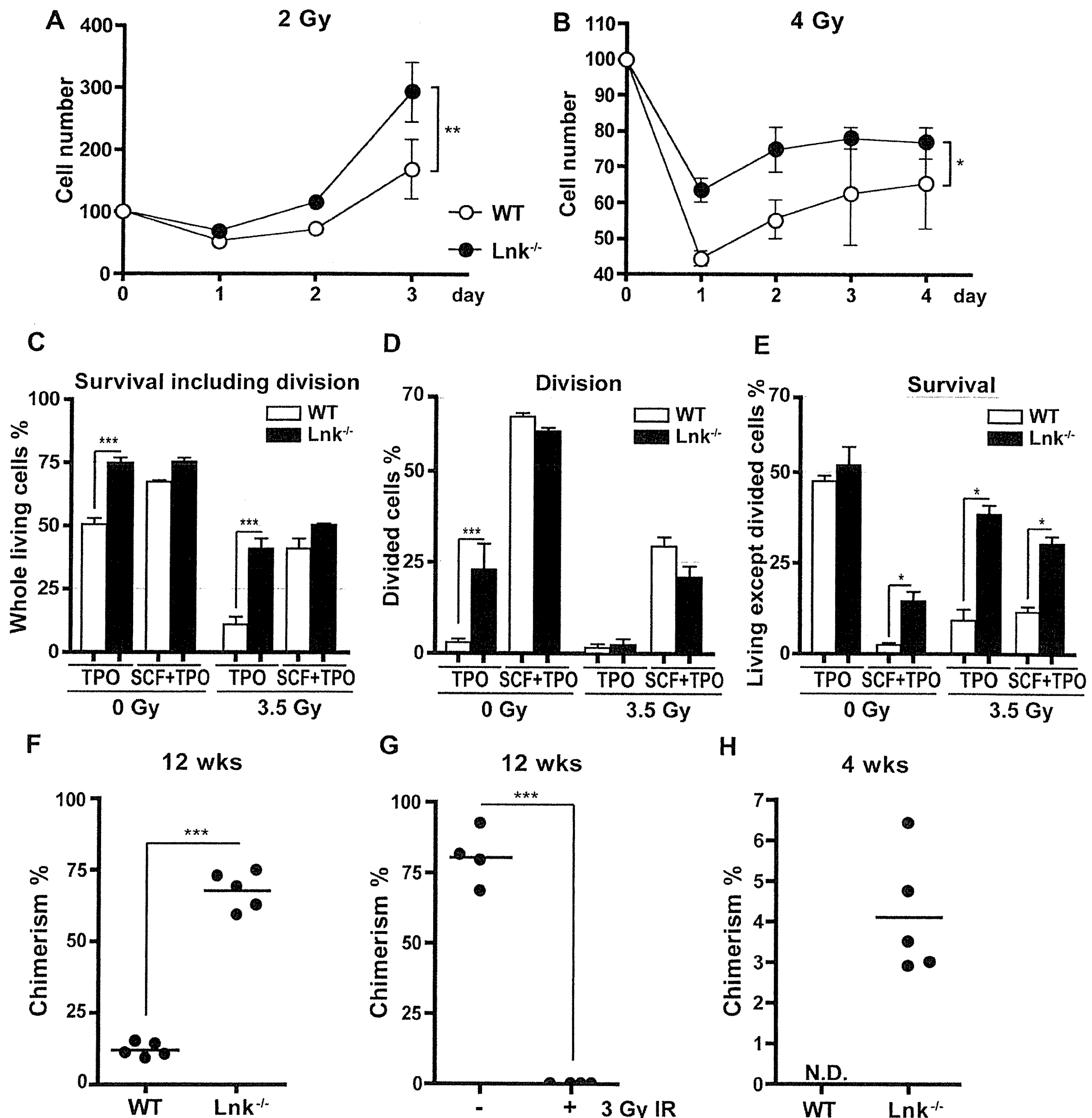
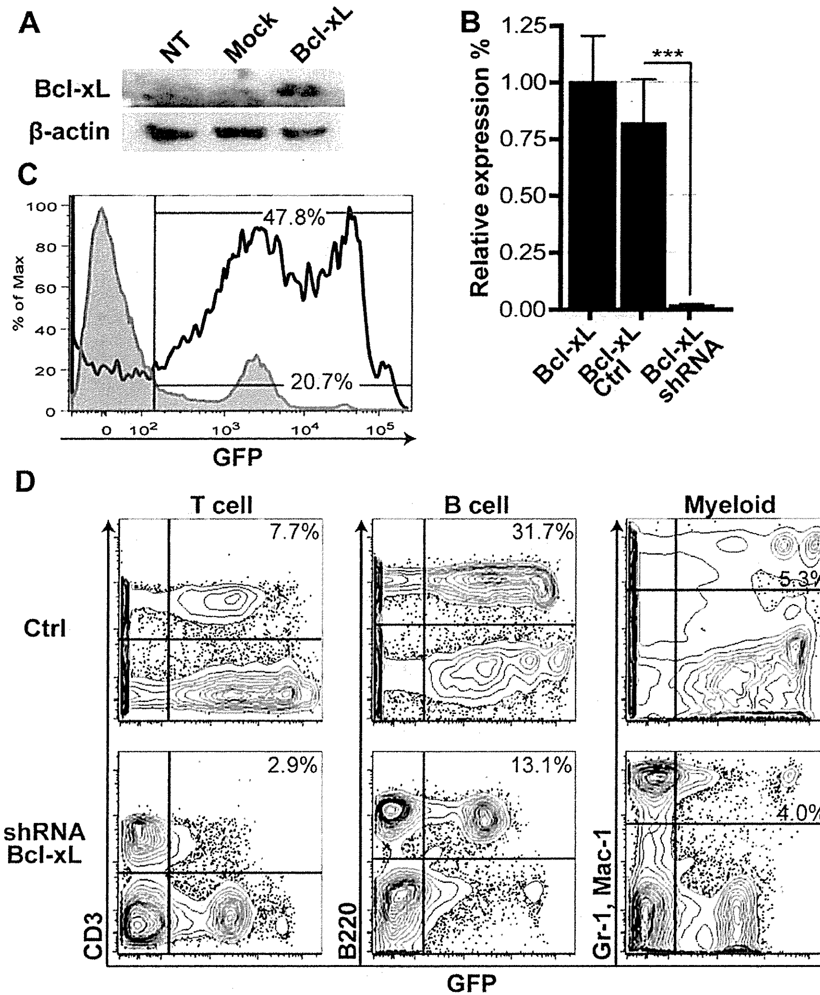


Supplementary Figure E1. Expression of genes downstream from JAK/STAT in WT or *Lnk*$^{-/-}$ HSCs with or without TPO stimulation. (**A–C**) messenger RNA expression of *Cyclin D1* (**A**), *c-Myc* (**B**), and *Bax* (**C**) in CD34^{-}KSL cells determined by quantitative reverse transcription PCR. Fresh: complementary DNA (cDNA) synthesized from freshly isolated CD34^{-}KSL cells. Ctrl: cDNA synthesized from CD34^{-}KSL cells incubated for 6 hours without cytokine stimulation. TPO: cDNA synthesized from CD34^{-}KSL cells incubated for 6 hours in the presence of TPO. Results are shown as mean ± standard deviation of triplicate samples (*$p < 0.05$; **$p < 0.01$). (**D**) Expression of Bcl-xL (left), Bcl-2 (right) in BM cells and KSL cells, and CD34^{-}KSL cells by flow cytometry. Cells were incubated for 6 hours in the serum-free medium without any cytokine. Y axis: cell frequency. X axis: fluorescence intensity as the expression level of Bcl-xL or Bcl-2. Gray-filled histogram: isotype control; blank histogram: WT; black-filled histogram: Lnk$^{-/-}$.



Supplementary Figure E2. $Lnk^{-/-}$ HSCs were resistant to irradiation-induced apoptosis. (**A**, **B**) Growth of WT or $Lnk^{-/-}$ CD34$^-$KSL HSCs after irradiation. One hundred freshly isolated CD34$^-$KSL cells were exposed to 2 Gy (**A**) or 4 Gy (**B**) x-rays and cultured in the presence of SCF and TPO for 3 to 4 days ($*p < 0.05$; $**p < 0.01$). (**C–E**) In vitro survival and division of single WT or $Lnk^{-/-}$ CD34$^-$KSL cells in the presence of cytokines. WT or $Lnk^{-/-}$ CD34$^-$KSL cells (n = 96) underwent single-cell serum-free culture in the presence of TPO or SCF and TPO with or without irradiation (3.5 Gy). At 72 hours of culture, the number of cells in each well was counted. Wells containing one or more cell(s) were judged to exhibit "survival including division" (**C**) and wells containing two or more cells were judged to exhibit "division" (**D**), and wells containing one cell were judged to exhibit "survival" (**E**). Frequency of each group from three independent experiments are shown as mean ± standard deviation ($*p < 0.05$; $***p < 0.001$). (**F**) Fifty CD34$^-$KSL cells from WT or $Lnk^{-/-}$ mice were injected into lethally irradiated recipients along with 2×10^5 competitor BM cells. The graph shows chimerism of donor-derived cells in the peripheral blood (PB) of recipient mice 12 weeks post-BM transplantation. Results are shown as mean ± standard deviation (SD) for n = 5 ($***p < 0.001$). (**G**) One thousand WT CD34$^-$KSL HSCs with or without 3 Gy irradiation were transferred into lethally irradiated recipient mice with 2×10^5 competitor WT BM cells. Graph shows chimerism of donor-derived cells in PB of recipient mice 12 weeks post-BM transplantation. Results are shown as mean ± SD of n = 4 ($***p < 0.001$). (**H**) One thousand WT or $Lnk^{-/-}$ CD34$^-$KSL HSCs with irradiation (3.5 Gy) were transferred into lethally irradiated recipient mice with 2×10^5 competitor WT BM cells. Graph shows chimerism of donor-derived cells in PB of recipient mice 4 weeks post-transplantation. Results are shown as mean ± SD of n = 5.



Supplementary Figure E3. Knockdown of *Bcl-xL* genes in HeLa cells. (**A**) Western blotting of Bcl-xL in HeLa cells transduced with mock or *Bcl-xL* in HeLa cells. NT = not transduced. β-actin was an internal control. (**B**) Messenger RNA expression of *Bcl-xL* in HeLa cells expressing Bcl-xL (Bcl-xL) transduced with control sequence (Bcl-xL ctrl) or shRNA-*Bcl-xL* (Bcl-xL shRNA) by quantitative reverse transcription PCR. Results are shown as mean ± standard deviation of triplicate samples (***$p < 0.001$). (**C, D**) Fifty CD34$^-$KSL cells from Ly5.1 *Lnk*$^{-/-}$ mice were transduced with either control sequence (Ctrl) or shRNA-*Bcl-xL* (shBcl-xL). Five days after transduction, cells were injected into lethally irradiated Ly5.2 recipient mice along with Ly5.1/5.2 competitor BM cells. (**C**) Histogram of Ctrl or shBcl-xL transduced *Lnk*$^{-/-}$ HSC-derived CD45$^+$ cells in peripheral blood (PB) of recipient mice at 12 weeks post-BM transplantation. Y axis: cell frequency. X axis: fluorescence intensity as the expression level of GFP (transgene). Gray-filled histogram: shBcl-xL; blank histogram: Ctrl. (**D**) Flow cytometric analysis of hematopoietic lineages in PB of a recipient mouse at 12 weeks after BM transfer.

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Integrin αvβ3 regulates thrombopoietin-mediated maintenance of hematopoietic stem cells

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Short Title: Role of αvβ3 integrin in HSCs

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ABSTRACT

Throughout life, one's blood supply depends upon sustained division of hematopoietic stem cells (HSCs) for self-renewal and differentiation. Within the bone marrow microenvironment, an adhesion-dependent or -independent niche system regulates HSC function. Here, we show that a novel adhesion-dependent mechanism via integrin β3 signaling contributes to HSC maintenance. Specific ligation of β3 integrin on HSCs using an antibody or extracellular matrix protein prevented loss of long-term repopulating (LTR) activity during *ex vivo* culture. The actions required activation of αvβ3 integrin 'inside-out' signaling, which is dependent upon thrombopoietin (TPO), an essential cytokine for activation of dormant HSCs. Subsequent 'outside-in' signaling via phosphorylation of Tyr747 in the β3 subunit cytoplasmic domain was indispensable for TPO-dependent, but not stem cell factor-dependent, LTR activity in HSCs *in vivo*. This was accompanied with enhanced expression of Vps72, Mll1 and Runx1, three factors known to be critical for maintaining HSC activity. Thus, our findings demonstrate a mechanistic link between β3 integrin and TPO in HSCs, which may contribute to maintenance of LTR activity *in vivo* as well as during *ex vivo* culture.

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INTRODUCTION

Hematopoietic stem cells (HSCs) are clonogenic cells capable of both self-renewal and multilineage differentiation, enabling life-long maintenance of blood cell generation. To maintain HSCs, at least one HSC daughter cell must always be capable of self-renewal and multilineage differentiation, but the mechanism by which HSCs retain that capability is not yet defined. It has been proposed that there is a specialized microenvironment, called 'niche', within the bone marrow (BM), where the balance among self-renewal, differentiation and quiescence is regulated by both adhesion-dependent (osteopontin/integrin α9β1 or α4β1, Tie-2/Angiopoetin-1 interaction, etc.) and -independent machinery (thrombopoietin [TPO], transforming growth factor [TGF]β, etc.)[1-5].

Recently, we found that the mouse $CD34^-$KSL HSC fraction, but not the $CD34^+$KSL hematopoietic progenitor fraction, dominantly expresses the integrin β3 subunit (CD61) bound to the αv (CD51) or αIIb (CD41) subunit[6]. In particular, the αvβ3 complex, but not the αIIbβ3 complex, appears to be involved in the function of HSCs, as αv positivity, but not αIIb positivity, enhances long-term repopulating (LTR) activity in $CD34^-$KSL HSCs after transplantation[7]. This led us to conclude that αvβ3 is crucial for HSC function.

Integrins are heterodimeric receptors consisting of an α and β subunit, and their active and inactive conformations (forms with higher and lower affinities for ligands) are tightly regulated by "inside-out" signaling, mediated through external stimulation of several receptors on the cell surface[8]. Following integrin activation, specific ligand binding to the protein initiates "outside-in" signaling, which coordinates with signaling cascades initiated through growth factor-, cytokine- and G protein-coupled receptors to

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regulate actin reorganization, cell survival and proliferation[9-11]. With regard to β3 integrin, inside-out signaling results in the binding of talin to the specific binding amino acids within the intracellular tail of the β3 subunit, which is essential for integrin activation[12]. Of these sequences, tyrosine phosphorylation of the β3 subunit, at least on Tyr747 (pY747), appears to be required for the outside-in signaling cascade, such as that seen during stable thrombus formation in platelets[13]. Although the roles of bidirectional integrin signaling by β3 integrin in other hematopoietic cells remains unclear, evidence suggests that an interaction between integrins α4β1 and α9β1 and osteopontin is required for regulation of HSC proliferation, which is indicative of the crucial contribution made by integrins to HSC maintenance[2]. Integrin αvβ3 reportedly interacts with osteopontin, as well as with vitronectin, fibronectin and CD31. Interestingly, CD31-null mice exhibit greater numbers of KSL cells but with less functionality than wild-type (Wt) mice, which could reflect the absence of interaction with αvβ3 integrin[14]. From these results, it seems apparent that although integrin αvβ3 appears to be involved in the regulation of HSC function, its precise role in that process remains unclear.

It is well known that TPO is essential for megakaryopoiesis[15] and also contributes to the maintenance and expansion of HSCs[16-18]. Mice deficient in TPO or its receptor (c-mpl) not only show impaired megakaryopoiesis, but also reduced HSC number and function[16,19,20]. Moreover, recent reports indicate that TPO is required for the maintenance of HSCs in a quiescent state within the BM[3,4].

Here, we demonstrate that integrin αvβ3 on HSCs plays essential roles in maintaining their stem cell activity. Furthermore, we show that specific ligation of β3 integrin contributes to the maintenance of LTR activity in HSCs through collaboration

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with TPO/c-mpl-mediated signaling, which inhibits the loss of LTR activity during *ex vivo* culture. Our approach to clarifying integrin function in HSCs entailed the use of knock-in mutant mice that display defective β3 integrin inside-out or outside-in signaling due to blockade of talin-binding to specific amino acids in the β3 integrin tail[21]. This enabled us to show that outside-in signaling via pY747 of integrin β3 ($\beta3^{PY747}$) following activation of αvβ3 integrin by TPO-mediated inside-out signaling is indispensable for TPO-mediated maintenance of HSC activity *in vitro*. In addition, outside-in signaling via $\beta3^{PY747}$ is also essential for maintenance of LTR activity *in vivo* within the BM niche.

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MATERIALS AND METHODS

Animals

C57BL/6-Ly5.2 and C57BL/6-Ly5.1 mice were from Sankyo Labo Service Corporation (Tokyo, Japan), and β3 integrin-deficient mice were from The Jackson Laboratory (Bar Harbor, ME) unless otherwise noted. β3 integrin Y747A and L746A knock-in mutant mice were described previously[21]. Each strain was back-crossed for more than 5 generations and used at 8-10 weeks of age. All animal experiments were approved by the Institutional Review Board for Animal Care and Use at the University of Tokyo.

Antibodies

The following monoclonal antibodies were used for cell sorting and flow cytometric analysis: anti-c-Kit (2B8, BioLegend, San Diego, CA), anti-CD34 (RAM34, eBiosciences, San Diego, CA), anti-CD150 (TC15-12F12.2, BioLegend, San Diego, CA) and anti-CD48 (HM48-1, BioLegend). Anti-integrin β3 (2C9.G2), anti-Sca-1 (E13-161.7), anti-CD45.2 (104), anti-CD45.1 (A20), anti-B220/CD45R (RA3-6B2), anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8C5), anti-CD4 (RM4-5) and anti-CD8 (53-6.72) antibodies were from BD Biosciences (Pharmingen, San Jose, CA) unless otherwise noted. For *in vitro* assays, low endotoxin and azide free (LEAF) anti-integrin β3 (2C9.G2, BioLegend) and its isotype control IgG (HTK888, BioLegend) were used.

Cell preparation

Suspensions of BM cells were prepared from mice as described previously[6,7].

Cell sorting and flow cytometric analysis

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We used an EPICS® ALTRA (Beckman Coulter, Fullerton, CA) or MoFlo™ XDP (Beckman Coulter) for cell sorting and flow cytometric analysis, as described previously[6,7].

Long-term competitive repopulation (LTCR) assays

LTCR assays were performed by transplantation of the indicated cells into lethally irradiated (9.5 Gy) C57BL/6-Ly5.2 or C57BL/6-Ly5.1 congenic mice, as described previously[6]. Twelve or 20 weeks after transplantation, recipient mice with donor cell chimerism (>1.0% for myeloid and B- and T-lymphoid lineages) were considered to be multilineage-reconstituted mice (positive mice). For serial transplantation, 10^6 whole BM cells were obtained from primary transplanted mice and transplanted into other irradiated recipient mice.

Whole transcriptome analysis using a SOLiD system

After sorting 1000-1500 $CD34^-$KSL cells, SOLiD sequencing was performed as described in the Supplemental Materials and Methods. The whole transcriptomes obtained through SOLiD sequencing were analyzed using GeneSpring (Agilent Technologies). Following filtration based on a significant ($p<0.05$ ANOVA) and >2-fold changes in expression, the selected genes were subjected to hierarchical cluster analysis. In addition, whole transcriptomes were subjected to gene set enrichment analysis using GSEA v2.06 software available from the Broad Institute (http://www.broad.mit.edu/gsea). Changed gene sets were selected based on a threshold set at a p-value <0.05 and FDR (q-value) <0.25.

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HSC cultures

$CD34^-KSL$ cells were sorted and cultured for 5 days in S-Clone SF-03 medium (Sanko-Junyaku Co., Tokyo, Japan) supplemented with 0.5% bovine serum albumin (Sigma, St. Louis, MO) and 50 ng/ml mouse stem cell factor (SCF) and/or 50 ng/ml mouse TPO (all from R&D systems., Minneapolis, MN). To induce integrin signaling via $\beta 3^{PY747}$, 2C9.G2 (50 μg/ml) (BioLegend, San Diego, CA) was added to the medium; hamster IgG (BioLegend) was used as a control. Activation of β3 integrin was also exogenously induced by adding to the medium 0.015 mM $MnCl_2$, an activator of integrin receptors. Total cell numbers were then counted under a light-phase microscope after the culture.

Estimation of integrin activation (inside-out signaling)

$CD34^-KSL$ cells were cultured for 18 h with Alexa Fluor 647-conjugated human fibrinogen (Invitrogen) in S-Clone SF-03 medium supplemented with 50 ng/ml SCF or 50 ng/ml TPO. Samples without cytokine stimulation served as the control. The cells were washed twice in PBS and analyzed for fluorescence intensity using flow cytometry to assess the degree of fibrinogen binding to cells.

Ligand-coated plates

Ninety-six-well plates were coated with 5 μg/ml vitronectin (Molecular Innovations, Novi, MI) or 5 μg/ml osteopontin (R&D Systems) overnight at 4°C. They were then blocked with 1% BSA for 1 h at 37°C, after which they were used as ligand-coated plates. BSA-coated plates served as the control.

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Limiting-dilution assay

After culture, exactly 10, 30, 50, 100 or 500 whole cultured cells were counted and sorted using a cell sorter and transplanted along with $2x10^5$ BMCs from Ly5.2 mice as competitor cells. Twelve weeks after transplantation, HSC frequency was estimated from the input cell number and the percentage of multi-lineage reconstructed mice (positive mice), based on the Poisson distribution[22]. As a control, single fresh (uncultured) $CD34^-KSL$ cells were also assessed using the same procedure.

Calculation of parameters on HSCs after ex vivo expansion

HSC numbers were estimated based on the total cell number counted using a phase-contrast microscope and the HSC frequency determined from limiting dilution assays. Repopulating unit (RU) values were calculated from the results of LTRC assays using 40 fresh $CD34^-KSL$ cells or their progeny with $5x10^5$ Ly5.2 whole bone marrow competitor cells with the following formula.

RU= (% donor chimerism / % competitor chimerism) x 5

In addition, MAS values, which reflect the repopulation ability of single HSCs, were estimated by dividing the RU values by the calculated HSC number.

DNA microarray

Using $CD48^-KSL$ cells that were sorted after culture of $CD34^-KSL$ cells for 5 days with 2C9.G2 antibody or control IgG under the indicated conditions, RNA extraction, amplification and microarray analyses were performed as described in the Supplemental

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Materials and Methods. All microarray data are available for viewing at the Gene Expression Omnibus (GEO) under accession number GSE33696.

Real-time quantitative RT-PCR

Using 5000 sorted cells from each sample, mRNA expression was assessed using real-time quantitative RT-PCR as described previously[6].

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RESULTS

Outside-in signaling via pY747 of β3 integrin, but not inside-out signaling, is required for the maintenance of the TLR activity on HSCs *in vivo*.

We previously reported that CD34⁻KSL cells expressed higher levels of β3 integrin than CD34⁺KSL cells, while other reports suggested β1 integrin is involved in the maintenance of HSCs[1,2]. Our aim was to investigate why HSCs, and not progenitor cells, dominantly express β3, and whether this integrin has a distinct function in HSCs that differs from its function in other KSL cell populations.

We initially focused on intracellular signaling by β3 integrin in HSCs. It has been demonstrated that $\beta3^{-/-}$ mice exhibit osteopetrosis induced by a lack of interaction between the tail of the β3 cytoplasmic domain and c-Src, leading to impaired osteoclast differentiation[23]. Although it has been proposed that dysregulation of osteoclasts leads to impairment of HSCs[24,25], evaluation of the long-term repopulating (LTR) activity of $\beta3^{-/-}$ HSCs after transplantation into irradiated mice *in vivo* showed that β3 integrin might be involved in HSC function (Figure 1A-C). To clearly rule out the effects of osteoclast dysregulation on specific integrin signaling in HSCs, we simultaneously utilized L746A (leucine-to-alanine substitution) and Y747A (tyrosine-to-alanine) knock-in mice, which have normal osteoclasts (data not shown and refs 21 and 23). β3 integrin 'inside-out' signaling is mediated by talin binding to the Leu746 and Tyr747 residues in the β3 tail[12]. Consequently, Y747A mutation disrupted both signals, while L746A mutation disrupted inside-out signaling without affecting outside-in signaling, for which pY747 is indispensable[21]. Neither mutation altered the total cell number or the frequency of the HSC population (Figure 1D). In addition, there was also no difference between the expression profiles of integrin β3 and CD150, a reliable maker

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of mouse HSCs[26], within $CD34^{-}KSL$ cells derived from both mutant mice (Figure 1E).

However, when we performed a serial competitive repopulation assay, chimerism in primary recipients 12 weeks after transplantation showed defective reconstitution with Y747A HSCs, but not with L746A HSCs (Figure 1B). This finding was further confirmed by secondary transplantation (Figure 1C). Given the input HSC number used in the primary transplantation assays, these results indicate that stem cell activity was diminished in Y747A HSCs, as compared to Wt HSCs, which suggests outside-in signaling via at least pY747 of $\beta3^{PY747}$, but not inside-out signaling or formation of an $\alpha v\beta3$/c-Src complex[27], contributes to both the LTR and self-renewal activities of HSCs *in vivo*.

Given the specificity of the effects of $\beta3^{PY747}$, we wondered why $\beta3^{-/-}$ HSCs did not exhibit impaired LTR activity after primary transplantation (Figure 1A-C). Cluster analysis of the whole transcriptome revealed that the gene expression pattern of Y747A HSCs differed from that of Wt or $\beta3^{-/-}$ HSCs (Figure 2A). Gene set enrichment analysis (GSEA) identified 102 gene sets up-regulated (and 0 down-regulated gene sets) in Wt HSCs, as compared to Y747A HSCs, based on thresholds set at a p-value <0.05 and FDR (q-value) <0.25. (Table S1). On the other hand, there was no differential set between Wt and $\beta3^{-/-}$ HSCs (data not shown). These results indicate that the phenotype of $\beta3^{-/-}$ HSCs is significantly closer to that of Wt HSCs than Y747A HSCs, suggesting that an as yet undetected molecule (perhaps other integrin receptors) might exert a compensatory effect mitigating the β3 deficiency.

In addition, our GSEA study also showed that Y747A mutation significantly reduced enrichment of a gene set "DORSAM_HOXA9_UP" which are up-regulated by Hoxa9 (Table S1), while the level of Hox-a9 expression did not significantly differ

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between Y747A and Wt HSCs (data not shown). Hoxa9 is well-known to be an essential factor involved in the maintenance of HSCs[28], and TPO signaling reportedly promotes its transfer into the nucleus[29]. Moreover, Y747A HSCs also exhibited less enrichment of gene sets involved in "signal transduction" and "cytokine responses," than Wt HSCs (Figure 2B, Table S1). Thus, our results suggest that impaired cytokine-mediated maintenance of HSC activity in the TPO/c-mpl axis leads to reduced LTR activity in Y747A HSCs, probably resulting in a diminished capacity for engraftment in transplantation assays (Figure 1A-C).

β3 integrin signaling contributes to HSC maintenance, which is dependent on TPO.

Because outside-in signaling, at least via $\beta3^{PY747}$, appeared to be involved in the TPO-mediated effects on HSCs, we next examined whether this signaling would also mediate the effects of TPO on *ex vivo* cultures of HSCs. For exogenous manipulation of $\beta3^{PY747}$, we utilized 2C9.G2, a β3 integrin antibody that acts as a ligand stimulating transduction of αvβ3 integrin-mediated intracellular signaling in smooth muscle cells and neutrophils[30,31]. We also confirmed that 2C9.G2 ligation to β3 integrin induced phosphorylation of $\beta3^{PY747}$ as well as c-Src^{PY418} and Syk, hallmarks of outside-in signaling from β3 integrin in mouse platelets[9,32](Figure S1). Following culture of HSCs in the presence of TPO alone, we assessed the effect of 2C9.G2 on the LTR activity of HSCs 12 and 20 weeks after transplantation. Interestingly, 2C9.G2 treatment in the presence of TPO positively influenced HSC LTR activity (Figure 3A), and this effect was blocked by Y747A mutation (Figure. 3B). By contrast, this positive action of 2C9.G2 disappeared during culture in the presence of SCF, a crucial cytokine

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involved in the maintenance and proliferation of HSCs (Figure 3A). These results indicate that the $\beta3^{PY747}$-mediated effects on LTR activity in HSCs and the positive action by 2C9.G2 on the LTR activity of HSCs are both TPO-dependent. Interestingly, 2C9.G2 treatment had little effect on the frequency of KSL or CD48^{-}KSL cells, a population enriched in HSCs after culture[33](Figure 3C), or on total cell number (Figure 3D) after culture in the presence of TPO. Outside-in signaling via $\beta3^{PY747}$ *per se* appears to be independent of HSC expansion in the presence of TPO. This suggests that given the effects of 2C9.G2 on LTR activity and HSC expansion, outside-in signaling via $\beta3^{PY747}$ may contribute to the enhanced LTR activity of individual HSCs only by collaborating with TPO, rather than through HSC amplification.

TPO promotes activation of at least β3 integrin possibly via inside-out signaling

We have so far proposed a strong link between TPO and $\beta3^{PY747}$ signaling. As for why $\beta3^{PY747}$-mediated function in HSCs is dependent on TPO, we initially wondered whether TPO enhanced expression of integrin β3 on HSCs. We found that, in fact, TPO-treated HSCs showed slightly less expression of integrin β3 than fresh HSCs or SCF-treated HSCs (Figure S2). We next hypothesized that TPO might regulate integrin affinity via a conformational change for activation. Accordingly purified CD34^{-}KSL cells (from Wt or $\beta3^{-/-}$ mice as negative control) were treated with TPO or SCF for 18 h, after which the binding of Alexa Flour® 647-labeled fibrinogen, a ligand for αvβ3, αIIbβ3, and/or αMβ2 (Lishko et al., J Biol Chem, 2004), was assessed. As shown in Figure 4A, TPO, but not SCF, increased fibrinogen binding to Wt HSCs detected with flow cytometry. However, fibrinogen also bound to $\beta3^{-/-}$ HSCs, indicating an interaction with another receptor, probably αMβ2 integrin[34] (Lishko et al.,

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J Biol Chem, 2004). After subtracting the binding to $\beta3^{-/-}$ HSCs from that to Wt HSCs, we were likely left with the binding to αvβ3 rather than αIIbβ3 integrin (Figure 4A). This is because we used Mg^{2+}-containing Ca^{2+}-free medium, which was previously shown to exclude binding to αIIbβ3 integrin (data not shown)[35,36]. Thus, TPO apparently contributes to the activation of at least αvβ3 integrin via the inside-out intracellular machinery in HSCs.

Extracellular circumstances affect integrin activation status and full integrin activation is required for LTR activity of HSCs.

Although it has already been shown that disrupting inside-out signaling without affecting outside-in signaling through L746A mutation has little effect on the capacity of HSCs for reconstitution *in vivo* (Figure 1A-C), we again tested whether the L746A mutation is independent of 2C9.G2-mediated $\beta3^{PY747}$ effects on the LTR activity of HSCs. Interestingly, L746A mutation did not appear to increase chimerism by 2C9.G2 treatment in a manner similar to Y747A mutation, even in the presence of TPO (Figure 4B left). Whereas the addition of Mn^{2+}, a strong external inducer of integrin activation that acts independently of intracellular signaling, ameliorated the effect of 2C9.G2 on LTR activity in L746A HSCs (Figure 4B right), it had no such effect on Y747A HSCs (Figure 4C). There was also no effect of 2C9.G2 administration on the frequency of KSL or $CD48^{-}$KSL cells (Figure 4D) or on total cell number (Figure 4E) after culture of L746A HSCs in the presence of TPO plus Mn^{2+}, again indicating that the collaboration between TPO and outside-in signaling via $\beta3^{PY747}$ is independent of HSC expansion *in vitro*. Taken together, these results indicate that activation of integrin αvβ3 and subsequent outside-in signaling via $\beta3^{PY747}$ are required to enhance LTR capability per

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individual HSC via a intergin-mediated function, and suggest these results are consistent with the *in vivo* study summarized in Figure 1A-1C. On the other hand, forcing integrin activation with Mn^{2+} did not restore 2C9.G2 activity in the presence of SCF in Wt HSCs (Figure 4F). We therefore concluded that not only outside-in signaling via $\beta3^{PY747}$, but also activation of integrin $\alpha v\beta3$ *per se* is required for TPO-dependent, but not SCF-dependent, full LTR maintenance in HSCs. We also sought to identify endogenous $\alpha v\beta3$ integrin ligands involved in the HSC function that maintains LTR. Vitronectin (VN) and osteopontin (OPN) are well-known $\alpha v\beta3$ ligands. Interestingly, LTR activity was enhanced when HSCs were cultured with TPO for 5 days on plates coated with VN alone (Figure 4G). On the other hand, OPN exerted a positive effect on LTR activity only when Mn^{2+} was simultaneously administered (Figure 4G). Moreover, these natural ligands had no effect on HSC expansion, as evidenced by the similar frequency of HSC subsets (Figure S3A) and total cell numbers (Figure S3B) under all culture conditions. These findings again allowed us to consider the possibility that factors in the microenvironment other than TPO might influence integrin activation status, and that full activation of integrin bound to extracellular matrix or other ligands might be required for $\beta3^{PY747}$ -mediated enhancement of LTR activity per individual HSC.

β3 integrin signaling maintains HSC activity during *ex vivo* expansion.

We found that bidirectional β3 integrin signaling contributed to the maintenance of HSC activity at the single-cell level through collaboration with TPO. Ema *et al.* previously reported that under various conditions SCF and TPO act together to effectively induce *ex vivo* expansion of HSCs through rapid cell division [37]. We therefore examined the