effect of $\beta3^{PY747}$ signaling on the LTR activity of HSCs during *ex vivo* expansion induced by the combination of SCF plus TPO. Application of 2C9.G2 or control IgG to 40 HSCs during 5-days of *ex vivo* culture in the presence of SCF plus TPO did not alter total cell numbers (Figure 5A), and levels of β3 integrin bound with 2C9.G2 were not affected (Figure 5B). By contrast, the number of $CD48^-$KSL cells was reduced by 2C9.G2 treatment (Figure 5C), suggesting that the frequency of HSCs after *ex vivo* culture was reduced by cellular manipulation via $\beta3^{PY747}$. Indeed, in cultures treated with 2C9.G2 for 5 days, the HSC frequency was lower and number of HSC in transplantation assays was reduced (Figure 5D, Table S2), suggesting outside-in β3 integrin signaling suppresses the amplification of HSCs. But interestingly, 2C9.G2-treated cells eventually showed a tendency toward increased HSC activity, as compared to cells cultured with IgG in long-term competitive repopulation assays (Figure 5E). This tendency was confirmed through *in vivo* evaluation of repopulating unit (RU) values, which imply LTR activity[38] and are based on the % donor-derived cells in the peripheral blood of recipients 20 weeks after transplantation (Table S2), and the mean activity of stem cell (MAS) values (RU divided by the number of detectable HSCs in recipient mice)[39] (Figure 5F). MAS values clearly showed that *ex vivo* culture with IgG reduced LTR activity per single HSC, as compared to uncultured fresh HSCs (Figure 5F). By contrast, 2C9.G2 treatment enabled purified HSCs to expand *ex vivo* without diminishing LTR activity (Figure 5F, Table S2). Thus, β3 integrin signaling may also be able to maintain LTR capability at the single-HSC level during *ex vivo* expansion.

The collaboration between β3 integrin signaling and TPO enhanced genes involved in the maintenance of HSC activity.

Finally, to determine the molecules involved in β3 integrin signaling and acting in concert with TPO, we carried out DNA microarray analyses using purified HSCs. In sorted $CD48^{-}$KSL cells following *ex vivo* culture, we selected genes showing elevated or reduced expression upon administration of 2C9.G2 (as compared to IgG) in the presence of only TPO (Figure 6A and 6B, Table S3 and S4). Eleven of the up-regulated gene sets and 5 of down-regulated sets were picked up by gene ontology (GO) enrichment analysis (Table 1). Interestingly, chromatin modification (GO ID: 0016568), one of up-regulated gene sets, contained Vps72, which is known to be a key gene involved in LTR activity, and Mll1 (Table 1A), which is involved in the maintenance of HSCs[40,41]. Similarly, Runx1 and Etv6, which are required for definitive HSC generation and maintenance, were also included among the up-regulated genes (Table S3)[42,43]. Subsequent real-time quantitative RT-PCR confirmed the 2C9.G2-induced enhancement of Vps72, Mll1 and Runx1 expression in the presence of TPO alone, although Etv6 was not apparent (Figure 6C), and that effect was reversed by Y747A mutation in fresh HSCs (Figure 6D). These results suggest that the contribution of β3 integrin signaling to the maintenance of LTR activity by HSCs is related at least to the enhanced expression of these three genes.

DISCUSSTION

In the present study, we showed that β3 integrin signaling contributed to HSC regulation by collaborating with TPO signaling. In Figure 7, we propose a model in which HSC maintenance is regulated by αvβ3 integrin-mediated bidirectional (inside-out and outside-in) signaling. Downstream signaling via $\beta3^{PY747}$ in the presence of TPO may also involve Vps72, Mll1 and Runx1, which contribute to the regulation of HSC LTR activity (Figure 6C and 6D). Perhaps through a still undetected mechanism, treatment with 2C9.G2 or a natural ligand not only contributes to enhanced LTR activity following culture in the presence of TPO alone (Figure 3, 4), but also to the maintenance of HSC activity at the single-cell level, even after *ex vivo* expansion induced with SCF plus TPO, by compensating for the reduction in activity that occurs during the culture (Figure 5F).

HSCs treated with 2C9.G2 in the presence of TPO exhibited enhanced expression of genes related to chromatin modification (GO ID: 0016568) (Table 1), and the positive effect of 2C9.G2 on the LTR activity of HSCs was dependent on the presence of TPO, but not SCF (Figure 3A). These results suggest that outside-in signaling via $\beta3^{PY747}$ contributes to the maintenance of HSC LTR activity, possibly via chromatin modification in the presence of TPO signal. Consistent with that idea, integrin signaling reportedly acts in concert with cytokine stimulation to influence chromatin modification for the maintenance of tissue-specific function in mammary gland cells[44]. We therefore propose that a change in the TPO concentration (possibly a large increase) eventually elicits outside-in signaling via $\beta3^{PY747}$ that mediates chromatin modification related to cell division and/or maintenance of the stemness of HSCs. GO enrichment analysis also revealed a number of down-regulated gene sets, two of which were 'cell

division' (GO: 0051301) and 'mitosis' (GO: 0007067) (Table 1B). Moreover, 4 genes, Apc, Rb, Rbl2 and Nf2, within the up-regulated gene set 'cell cycle' (GO: 0007507) also belonged to the GO term 'negative regulation of cell cycle' (GO ID: 0045786) (Table 1A). These data suggest that TPO and outside-in signaling via $\beta3^{PY747}$ act collaboratively to regulate expression of these genes, which may imply that HSC amplification is repressed during *ex vivo* expansion through a combination of SCF and TPO signaling (Figure 5D, Table S2). Indeed, single-cell culture of a HSC showed suppressed cell division in the presence of 2C9.G2 (Figure S4), again confirming the repressive effects of integrin β3 signaling on HSC division during the *ex vivo* expansion trial with SCF and TPO. However, neither BrdU uptake assays or Hoechst staining revealed a direct link between $\beta3^{PY747}$ and the regulation of the cell cycle in HSCs *in vivo* (Figure S5) or during *ex vivo* expansion (data not shown).

This suppressive action of integrin β3 signaling was apparent in HSCs undergoing rapid cell division (i.e., *ex vivo* expansion induced by SCF plus TPO) (Figure 5D, Table S2), but not in slowly dividing HSCs (i.e., in cultures exposed to TPO alone) (Figure 3C and D, Figure 4D and E) or quiescent HSCs (i.e., *in vivo*) (Figure 1D). Because the effect of integrin β3 signaling appears to be small, as discussed above, we suggest that the suppressive action may be masked by the state of the slowly dividing or quiescent HSCs. By contrast, rapidly dividing HSCs (i.e., during *ex vivo* expansion requiring by both SCF and TPO) may emphasize the suppressive effect of integrin β3 on HSC expansion (Figure 5D, Table S2). We therefore suggest that suppression of cell division via a $\beta3^{PY747}$-dependent mechanism could contribute to the maintenance of HSCs through the prevention of excess cell division. But the true mechanism by which β3 integrin ligation associates with TPO signaling and influences HSC function

remains to be elucidated.

Fresh L746A HSCs without *ex vivo* culture exhibited LTR activity similar to that of Wt HSCs in primary and secondary transplantation assays (Figure 1A). This confirms the dominant importance of outside-in signaling via $\beta3^{PY747}$, as L746A is only critical for inside-out signaling, which implies a "change in the integrin conformational change"[21]. But L746A mutants affected impaired outside-in based function (Figure 4B left), whereas ligation with 2C9.G2 in the presence of TPO plus Mn^{2+} augmented the LTR activity of HSCs (Figure 4B right). To explain this apparent discrepancy, we consider that at least outside-in signaling via $\beta3^{PY747}$ may require the fully activated form of αvβ3 integrin during *ex vivo* culture. Alternatively, stimulants other than TPO may be required for this action. An investigation of extrinsic factors that induce the fully active conformation of αvβ3 integrin in HSCs via a pathway not involving the talin-binding motif, particularly Leu746 of β3 integrin, or an unknown TPO-dependent ligand from surrounding cells, may enable us to suggest or clarify the "true" niche circumstances of BM.

In our limited studies, VN-coated plates favored TPO-dependent enhancement of LTR activity in HSCs without Mn^{2+} (Figure 4G), while OPN required Mn^{2+} for its effect (Figure 4G). This suggests that VN, a major component of the BM sinusoid[45], rather than OPN, a component of the osteoblastic niche[2], is preferentially involved in HSC division via αvβ3 integrin. As has been claimed previously, the osteoblastic niche may preferentially use the interaction of β1 integrin (α4β1, α9β1) with OPN[1,2]. Alternatively, there may be a TPO gradient within the BM microenvironment – i.e., the TPO concentration in the osteoblastic niche is lower than in the sinusoid. Since a higher TPO concentration would accelerate HSC division into progenitors and further

megakaryopoiesis, TPO-dependent activation of αvβ3 integrin via inside-out signaling may induce subsequent ligation with an appropriate ligand, leading to Tyr747 phosphorylation and, in turn, inhibition of cell division and maintenance of LTR activity on HSCs.

Our results showed that the phenotype of $\beta3^{-/-}$ HSCs in transplantation assay and hierarchal cluster analysis is much closer to that of Wt HSCs than Y747A HSCs (Figure 1A-C, Figure 2A). This suggests that in $\beta3^{-/-}$ HSCs an as yet undetected system exerts a compensatory effect in response to the deficiency in the β3 molecular component. By contrast, impairment of outside-in signaling through Y747A point mutation might effectively and negatively affect LTR activity without inducing compensation because the β3 molecular component is retained in Y747A HSCs. Identification of the mechanisms that redundantly compensate for the absence of integrin β3 signaling in $\beta3^{-/-}$ HSCs will require further investigation.

In sum, our findings demonstrate for the first time the critical role played by bidirectional β3 integrin signaling, especially outside-in signaling via $\beta3^{PY747}$, in the maintenance of LTR activity in HSCs *in vitro* and *in vivo*. Moreover, activation of integrin αvβ, but not inside-out signaling, is required for $\beta3^{PY747}$-mediated maintenance of HSC activity per each cell. This implies that the induction of activated integrin αvβ can be regarded as a novel function of the HSC niche. We also show that outside-in signaling via $\beta3^{PY747}$ plays an essential role in HSC maintenance mediated by TPO but not SCF, which suggests a novel mechanism for HSC maintenance by TPO. These results thus open a new area of inquiry into the link not only between integrin αvβ3 activation and the surrounding microenvironment within the BM niche, but also between integrin and cytokine signaling in the maintenance of hematopoietic

homeostasis.

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AUTHOR CONTRIBUTIONS

T.U. and K.E. designed the study. T.U. performed most of the cellular and molecular experiments. T.U. wrote the paper. K.E. edited manuscript. J. I. and M.U. helped with the cellular experiments. H.T. bred and kept the mice. Y.S., M.T. and T.S. helped with transplantation assays; B.P generated integrin knock-in mice. Y. M., M.Y., K.N., Y.K., H.N. and T.O. all supervised aspects of the project and helped with manuscript preparation.

CONFLICT of INTEREST

We have no disclosure of COI.

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FIGURE AND TABLE LEGENDS

Figure 1. Tyr747 of β3 integrin is essential for the long-term *in vivo* repopulating and self-renewal activities of mouse HSCs, independent of *ex vivo* expansion.

(A) HSCs from Wt or mutant mice were used for serial competitive repopulation assays. Forty sorted $CD34^-KSL$ cells (Ly5.2) were transplanted into lethally irradiated mice (Ly5.1) along with $2x10^5$ BM competitor cells (Ly5.1). Twelve weeks later, the % donor cells (Ly5.2) were determined in peripheral blood (B). 10^6 BM cells from primary recipient mice were then transplanted into other irradiated mice, followed by secondary analysis of peripheral blood (C). The plot indicates donor-derived cells (% of $Ly5.2^+$ cells) in the peripheral blood. In addition, recipient mice with donor cell chimerism of <1.0% for any lineage were considered not to be reconstituted (negative mice). Bars indicate mean values (*$p<0.01$). (D) The table shows the total cell number and frequency of HSC subsets among BMCs from both femurs and tibias. (E) Also shown are the frequencies or relative mean fluorescent intensity (MFI) in $CD150^+$ and integrin $\beta3^+$ ($CD61^+$) cells among the $CD34^-$ KSL population. The value of the MFI obtained in the presence of the isotype control IgG was used as the control. Data are means ± S.D. (n=6-8). The histograms depict the expression of β3 integrin in murine hematopoietic stem cells (HSCs, $CD34^-KSL$) or hematopoietic progenitors ($CD34^+KSL$) derived from wild-type (Wt), integrin $\beta3^{-/-}$, and Y747A or L746A mutants, all of which are shown in white. The isotype control is in gray.

Figure 2. The gene expression profile in Y747A-mutant HSCs differed from that in Wt and integrin $\beta3^{-/-}$ HSCs.

(A) Following whole transcriptome analysis of Wt, integrin $\beta3^{-/-}$ and Y747A HSCs using SOLiD sequencing, hierarchical cluster analysis was performed after filtration based on ANOVA ($p< 0.05$) and a >2-fold change against Wt (at least 1 pair). Up-regulated and down-regulated genes are shown in red and blue, respectively. (B) Gene set enrichment analysis was performed using the whole transcriptome of Wt and Y747A HSCs. The pie chart depicts the distribution of 102 gene sets up-regulated in Wt HSCs, compared to Y747A HSCs, into the indicated categories. The threshold was set at $p<0.05$ and FDR ($q<0.25$).

Figure 3. β3 integrin-mediated maintenance of long term HSC repopulating activity during *ex vivo* expansion is dependent upon TPO, but not SCF.

(A) To assess the influence of β3 integrin signaling, 40 $CD34^-KSL$ cells (Ly5.2) derived from Wt (A) or Y747A mice (B) were cultured for 5 days in the presence of 2C9.G2 or IgG in serum-free medium supplemented with 50 ng/ml SCF or 50 ng/ml TPO. After the culture, whole cultured cells were transplanted with $2x10^5$ BM competitor cells (Ly5.1) into lethally irradiated Ly5.1 mice. The plots depict the % donor (Ly5.2)-derived cells in the peripheral blood of individual mice 12 weeks or 20 weeks after transplantation. Bars represent mean values (*$p<0.01$). Recipient mice with donor cell chimerism of <1.0% for any lineage were considered not to be reconstituted (negative mice). (C) Following culture of 1000 sorted Wt $CD34^-KSL$ cells (Ly5.1) for 5 days with 2C9.G2 or hamster IgG (isotype control) in the presence of TPO, the percentages of KSL and $CD48^-KSL$ cells were determined by flow cytometric analysis.

The values in the dot plots are means ± S.D. (D) After culture, the total cell number was counted. The graph shows the fold-increase in total cell number after 5 days of culture. Data are means ± S.D.

Figure 4. TPO changes the activation status of β3 integrin through inside-out signaling, and post-ligation outside-in signaling via $\beta3^{PY747}$, is indispensable for maintenance of HSC function during *ex vivo* expansion.

(A) $CD34^-KSL$ cells derived from Wt and $\beta3^{-/-}$ mice were cultured with Alexa Flour 647-labeled fibrinogen in S-Clone SF-03 medium, with or without SCF or TPO. The fluorescence intensity of the bound fibrinogen was analyzed by flow cytometry: white, no cytokine; gray, stimulation of cytokine. The graphs depict the relative mean fluorescence intensity (MFI); binding in the absence of cytokine served as the control. Data are means ± S.D. (*$p<0.01$, $n>3$). (B and C) Forty $CD34^-KSL$ cells obtained from BM of L746A (Ly5.2)(B)or Y747A mice (Ly5.2)(C) were cultured with TPO for 5 days in the presence of 2C9.G2 or IgG, and examined using transplantation assays, as described above. To exogenously induce integrin activation (change the structure to the activated state), Mn^{2+} was added to TPO-containing medium. (D) Following culture of 1000 sorted L746A-mutant $CD34^-KSL$ cells (Ly5.1) for 5 days with 2C9.G2 or hamster IgG (isotype control) in the presence of TPO and Mn^{2+}, the percentages of KSL and $CD48^-KSL$ cells were determined by flow cytometry. The values in the dot plots are means ± S.D. (E) Following the culture, the total cell number was counted. The graph shows the fold-increase in total cell number after 5 days of culture. Data are means ± S.D. (F) Forty Wt $CD34^-KSL$ cells (Ly5.1) were also cultured for 5 days

in medium containing SCF and Mn^{2+} along with 2C9.G2 or IgG. (G) Using plates coated with vitronectin (VN), osteopontin (OPN) or BSA, 40 Wt $CD34^-KSL$ cells (Ly5.1) were cultured with TPO for 5 days in the absence or presence of Mn^{2+}. After the culture, cells were transplanted along with $2x10^5$ BM competitor cells into irradiated recipient mice. The plots depict the % donor (Ly5.2 or Ly5.1)-derived cells in the peripheral blood of individual mice 12 weeks after transplantation (*$p<0.01$, **$p<0.05$). Recipient mice with donor cell chimerism of <1.0% for any lineage were considered not to be reconstituted (negative mice).

Figure 5. Integrin β3-mediated signaling leads to the suppression of expansion and cell division on HSCs during *ex vivo* culture.

Forty sorted Wt $CD34^-KSL$ cells (Ly5.1) were cultured for 5 days with 2C9.G2 or hamster IgG (isotype control) in S-Clone SF-03 serum-free medium supplemented with 50 ng/ml SCF plus 50 ng/ml TPO. (A) After the culture, total cell number was counted. Graph shows fold increase of total cell number after 5 days of culture. Data are means ± S.D. (B) To confirm 2C9.G2 binding to cells during culture, cells were stained with fluorescently-labeled hamster IgG and analyzed by flow cytometry: white, IgG; gray, 2C9.G2. (C) The percentages of KSL and $CD48^-KSL$ cells were also determined by flow cytometry after culture (**$p<0.05$). (D) HSC frequency among the cultured cells was determined using limiting dilution assays. After groups of 10, 30, 50, 100 or 500 whole cultured cells were counted exactly and sorted, the groups were individually transplanted into lethally irradiated Ly-5.2 mice along with $2x10^5$ BM cells from Ly5.2 mice. This was followed by analysis for chimerism 20 weeks after the transplantation. The table shows the rate of positive mice (multi-lineage reconstituted mice); the

numbers in parenthesis are the positive mice/tested mice. In the case of fresh $CD34^-KSL$ cells, a single cell was transplanted. After determining the % reconstructed mice (Table), the % unreconstructed mice (% negative mice on Y axis) were plotted vs. the number of input cells, leading to a theoretical HSC frequency based on a Poisson distribution. Inputting 500 cells resulted in 0% negative mice, and these data are not plotted. (E) Fresh or whole cultured cells (Ly5.1) were transplanted into lethally irradiated mice (Ly5.2) along with $5x10^5$ BM cells (Ly5.2). Twenty weeks later, peripheral blood from the recipient mice was analyzed by flow cytometry. The plots show the % donor-derived cells (% $Ly5.1^+$ cells) in the peripheral blood of individual recipients. Bars indicate the mean values (*$p<0.01$). (F) In addition, the mean activity of stem cell (MAS) reflects the repopulating ability of single HSCs, as estimated from the Repopulating unit (RU) value (Table S2) and HSC number (Table S2). Data are presented as means ± S.D (*$p<0.01$, **$p<0.05$).

Figure 6. Integrin β3-mediated signaling enhanced expression of stemness-related genes by cooperating TPO presence.

(A) DNA array analysis was performed using $CD48^-KSL$ cells after culture. The cells were sorted after culturing $CD34^-KSL$ cells for 5 days with 2C9.G2 or IgG in the presence of SCF and/or TPO. (B) Genes whose expression had changed from the DNA microarray data, genes that showed >1.4-fold up-regulation (with TPO: 2231 genes or SCF + TPO: 3354 genes) or down-regulation (with TPO: 4349 genes or SCF + TPO: 2630 genes) in 2C9.G2-treated cells were selected for extraction. This was followed by extraction of genes included in both populations (up-regulation: 605 genes, down-regulation: 695 genes). In addition, to clearly focus on the effect of the

combination by TPO and β3 integrin signaling, genes only showing >1.0-fold up-regulation (362 genes) or down-regulation (336 genes) in the presence of SCF was excluded. This left 243 genes that were up-regulated and 359 that were down-regulated in HSCs by 2C9.G2 in the presence of TPO. (C) Expression of candidate genes involved in the maintenance of LTR activity of HSCs was assessed using real-time RT-PCR with 2C9.G2- or IgG-treated $CD34^-$KSL cells cultured in the presence of TPO. The graphs depict mRNA expression of the indicated genes. Data are presented as means ± S.D. (**$p<0.05$, $n>3$). (D) Fresh (uncultured) $CD34^-$KSL cells obtained from the BM of Wt or Y747A mice was also subjected to real-time RT-PCR to examine expression of these genes. The graphs depict mRNA expression of the indicated genes. Data are presented as mean ± S.D. (**$p<0.05$, $n>3$).

Figure 7. Model depicting the role of β3 integrin in TPO-dependent regulation of HSC division leading to the maintenance of LTR ability

Integrin β3 bidirectional signaling and TPO were dependent on each other in the maintenance of HSCs. (A) TPO/c-mpl signaling leads to conformational change of integrin αvβ3 into high affinity form for their ligands (the activation of integrin αvβ3) by inducing inside-out signaling. (B) Outside-in signaling via Tyr747 phosphorylation of integrin β3 induces enhanced expression of stemness-related genes following their ligation.

Table 1. Gene ontology (GO) enrichment analysis using genes extracted based on a change in their expression level

The genes extracted from DNA arrays were subjected to gene ontology (GO) enrichment analysis. The tables list the up-regulated (A) and down-regulated (B) GO terms. P-values were corrected by FDR (Benjamini-Hochberg), and the threshold was set at the corrected p-value <0.1.

Figure 1
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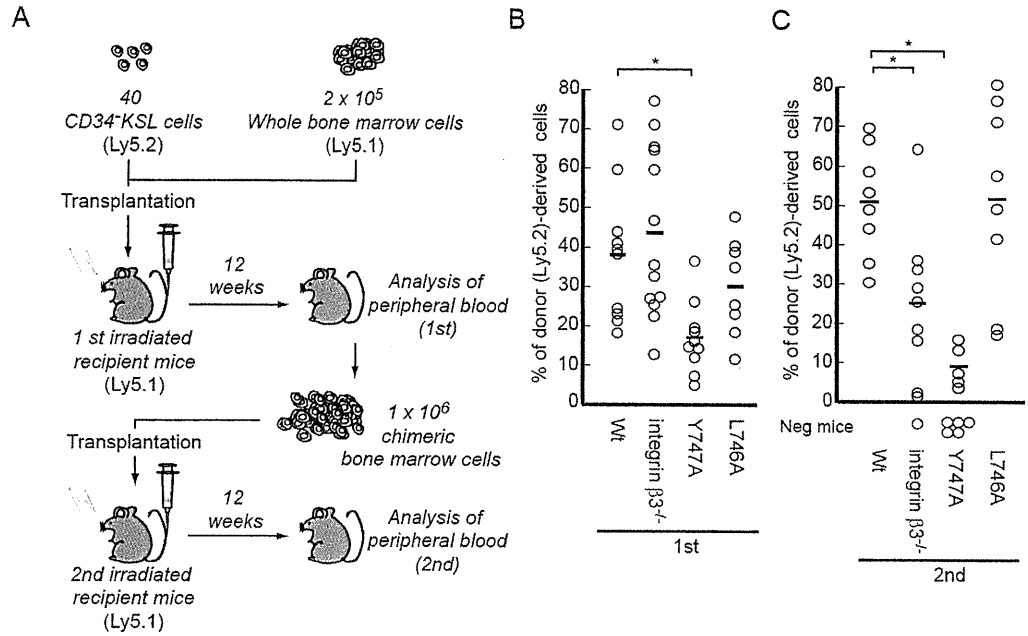

D

	Wt	integrin β3$^{-/-}$	Y747A	L746A
Total cell number (x 10^{-8} cells)	1.32±0.07	1.32±0.14	1.27±0.17	1.26±0.27
KSL (%)	0.075±0.014	0.085±0.016	0.089±0.042	0.062±0.020
CD34$^-$KSL (x 10^{-3} %)	6.01±2.12	7.95±2.68	6.70±1.16	7.15±1.67

in whole bone marrow cells

E

	Wt	integrin β3$^{-/-}$	Y747A	L746A
CD150$^+$ cells (%)	74.8±2.94	75.1±10.8	73.3±9.74	79.0±2.74
Relative MFI of CD150	66.9±18.8	63.2±11.4	62.4±11.5	69.9±8.99
integrin β3$^+$ cells (%)	85.7±6.47	N.D.	88.1±4.39	86.1±1.12
Relative MFI of integrin β3	48.4±7.90	N.D.	55.2±8.47	50.3±9.67

in CD34$^-$KSL cells