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CD61 enriches long-term repopulating hematopoietic stem cells

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Abstract

Among the subsets that define hematopoietic stem cells (HSCs), $CD34^-$ c-kit^+ Sca-1^+ lineage marker$^-$ ($CD34^-$KSL) cells are regarded as one of the populations that have the highest enrichment of HSCs in adult mouse bone marrow. Here, we demonstrate that long-term repopulating hematopoietic stem cells (LTR-HSCs) have high expression of CD61 (integrin β_3) within the $CD34^-$KSL population. Approximately 60% of $CD34^-$KSL cells showed high expression of CD61. $CD61^{High}CD34^-$KSL populations also exhibited significantly greater properties of HSC, such as expression of HSC markers, the side population (SP) phenotype, and ability for long-term repopulation. In both SP cells and non-SP (NSP) cells, $CD61^{High}CD34^-$KSL cells also contained significantly more LTR-HSCs than $CD61^{Low/-}CD34^-$KSL cells. Our results indicate that CD61 is exploitable for HSC enrichment as a supportive positive cell surface marker.

Keywords: Hematopoietic stem cell; CD61; $CD34^-$KSL; Side population

Hematopoietic stem cells (HSCs) reside in a specialized microenvironment, known as the "niche" and govern the entire blood forming system by giving rise to all lymphoid, myeloid, and erythroid cells throughout adult life [1]. This extraordinary ability of HSCs for self-renewal and multi-differentiation was demonstrated by repopulation of the entire hematopoietic system with a single HSC [2,3]. Therefore, the identification of markers that can distinguish HSCs from their differentiated progeny is essential for a clearer understanding of stem cell-related properties. Although HSCs comprise an extremely small fraction of the total bone marrow, several markers to isolate HSCs have been established. For over a decade, the most common methods have involved combinations of surface markers such as $CD34^-$c-kit^+Sca-1^+lineage$^-$ ($CD34^-$KSL) or Thy-1^{Low}c-kit^+Sca-1^+lineage$^-$ [2,4]. More recently, CD150 and endoglin have also been reported as critical markers for HSCs [5,6]. Additionally, other methods for the isolation of HSCs have been established using the ability of HSCs to efflux various fluorescent dyes, such as Hoechst 33342 [7] and rhodamine [8]. In particular, the side population (SP) phenotype which exploits the ability of HSCs to efflux the DNA-binding dye Hoechst 33342 via ABCG2, has been developed [7,9]. Similarly, SP cells have also been identified in several organs and tissues other than the bone marrow [10–13], indicating that the SP phenotype may be a common feature of somatic stem cells.

CD61, also known as the integrin β_3 subunit, is the common β chain of two receptor structures: glycoprotein IIb–IIIa (CD41/CD61, integrin $\alpha_{IIb}\beta_3$) and the vitronectin receptor (CD51/CD61, integrin $\alpha_V\beta_3$) [14]. Glycoprotein

Abbreviations: HSC, hematopoietic stem cell; KSL, c-kit^+/Sca-1^+/lineage$^-$; LTR, long-term repopulating; SP, side population; FACS, fluorescence-activated cell sorting; MACS, magnetic cell sorting; NSP, non-side population; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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IIb–IIIa, which functions as a receptor for fibrinogen, von willebrand factor, and thrombospondin, is expressed on platelets and megakaryocytes. In contrast, the vitronectin receptor behaves as a receptor for vitronectin, osteopontin, and CD31 and participates in many biological processes in several cell types [15,16]. We recently showed that expression of CD61 is also correlated to the features of HSCs that possess the SP phenotype [17]. While the use of Hoechst 33342 exclusion has been established as a powerful tool for stem cell biology, it remains unclear whether SP cells can account for all HSCs in the bone marrow [18]. In this study, we investigated the relationship between CD61 and HSCs isolated using classical surface marker characterization, and not limited to the SP fraction.

Materials and methods

All animal experiments were performed according to the "Guidelines of Tokyo Women's Medical University on Animal Use", the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Antibodies. The following monoclonal antibodies were used for fluorescence-activated cell sorting (FACS): FITC conjugated anti-Sca-1 (E13-161.7), APC conjugated anti-c-kit (CD117) (2B8, BioLegend, San Diego, CA), biotin-conjugated anti-CD34 (RAM34) and anti-CD61 (2C9.G2), FITC conjugated anti-CD45.2 (104), and PE conjugated anti-CD45.1 (A20). All antibodies for fluorescence-activated cell sorting (FACS) and flow cytometric analysis were obtained from BD Biosciences (Pharmingen, San Jose, CA) unless otherwise noted.

Cell preparation. Cell suspensions were prepared from the bone marrow of C57BL/6 and C57BL/6-Ly5.1 congenic mice (Sankyo Lab Service Corp., Tokyo, Japan). Bone marrow cells were obtained by flushing of excised femurs with Hank's balanced salt solution (Sigma, St. Louis, MO) containing 5% fetal bovine serum (FBS; Moregate Biotech, Queensland, Australia). Then, cells were washed two times with Dulbecco's phosphate buffered saline (PBS; Sigma) and suspended in PBS containing 2% FBS and 1 mM Hepes prior to staining and cell sorting.

Antibody staining for flow cytometric analysis. For the isolation of specific cell populations, lineage$^+$ cells were first eliminated by magnetic cell sorting (MACS) with Auto MACS system (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) using the Lineage cell Depletion Kit (Miltenyi Biotec), prior to staining with the corresponding antibodies for 30 min on ice. In cases of biotinylated antibodies, cells were incubated with streptavidin-conjugated PE-Texas Red (Pharmingen) for 30 min on ice, prior to flow cytometric analysis. Stained cells were then subjected to analysis and sorting by an EPICS® ALTRA FACS analysis system (Beckman Coulter, Fullerton, CA).

In vitro culture. For cell proliferation assay, one hundred cells of indicated fractions were sorted into 1-well of a flat-bottom 96-well plate and cultured in the StemSpan serum-free medium (StemCell technologies, Vancouver, BC) supplemented with 100 ng/ml of mouse stem cells factor, 20 ng/ml of mouse thrombopoietin, 20 ng/ml of mouse insulin like growth factor-2 (all from R&D Systems, Minneapolis, MN), and 10 ng/ml of human fibroblast growth factor (Invitrogen, Carlabad, CA). For colony assay, one cell of indicated fractions was sorted into 1-well of a flat-bottom 96-well plate and cultured in the same medium. After 7 days of culture, the total cell number was counted and the colony size was evaluated under a phase contrast microscope.

Gene expression analysis. Expression of mRNA was examined by real-time quantitative RT-PCR. Total RNA was obtained from 10,000 cells of each population using Isogen (Nippongene, Tokyo, Japan), followed by synthesis of single stranded cDNA with the Superscript First-strand System III for RT-PCR (Invitrogen, Carlsbad, CA). Primer pairs and Taqman® MGB probes were designed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ABCG2, CD150, and endoglin with the Taqman® gene expression assay™ (Applied Biosystems). Quantitative PCR was performed with a 7300 Real Time PCR System (Applied Biosystems). mRNA expression levels were normalized with the expression level of GAPDH.

Analysis of the SP phenotype. c-kit$^+$ cells were obtained from lineage$^-$ cells by MACS with anti-c-kit conjugated MicroBeads (Miltenyi Biotec), prior to staining with Hoechst 33342. Analysis and sorting of SP cells were performed as described previously [19,20]. Briefly, isolated c-kit$^+$ cells were stained with 5 μg/ml of Hoechst 33342 (Sigma) at a concentration of 10^6 cells/ml in staining medium (Dulbecco's modified Eagle's medium (DMEM) containing 2% FBS and 10 mM Hepes) for 90 min at 37 °C. The cells stained with Hoechst 33342 were then incubated with the corresponding antibodies as described above.

Long-term competitive repopulation assay. C57BL/6-Ly5.2 mice, irradiated at 10 Gy total, were transplanted with test cells prepared from C57BL/6-Ly5.1 mice and 2×10^5 whole bone marrow cells obtained from C57BL/6-Ly5.2 mice. Three months after transplantation, mononuclear cells isolated from the peripheral blood were analyzed by flow cytometry.

Results

We firstly examined CD61 expression within the CD34$^-$KSL fraction, which is considered one of the most potent HSC populations. Within CD34$^-$KSL cells, two different populations (peaks) were observed in the histogram (Fig. 1E) and approximately 60% of the cells showed high expression of CD61 (Fig. 1). In contrast, the majority of CD34$^+$KSL cells possessed low expression of CD61 (Fig. 1). Then, CD61^{High} and CD61$^{Low/-}$ fractions within CD34$^-$KSL cells were subjected to cell culture under a serum-free condition where the HSC subsets (CD34$^-$KSL and CD34$^-$KSL SP cells) showed much higher cell proliferation than differentiated cells and hematopoietic progenitors such as CD34$^+$KSL cells (Fig. 2C). After 7 days of culture, CD61^{High}CD34$^-$KSL cells exhibited much higher cell proliferation than CD61$^{Low/-}$CD34$^-$KSL cells and slightly higher than CD34$^-$KSL cells (Fig. 2C). Moreover, comparing the size distribution of colonies derived from single cells among each cell fraction, the colony size distribution was very similar between CD61^{High}CD34$^-$KSL and CD34$^-$KSL cells (Fig. 2D). Interestingly, CD61$^{Low/-}$ CD34$^-$KSL cells exhibited a similar phenotype to CD34$^+$KSL cells in the ability for proliferation and the colony size distribution (Fig. 2C and D). Therefore, these results suggested that the properties of HSCs were correlated with expression of CD61 within CD34$^-$KSL cells.

To determine the correlation between CD61 and features of HSCs within the well-established CD34$^-$KSL stem cell population, various properties, such as expression of HSC markers, the presence of the SP phenotype, and the ability for long-term repopulation were examined. Using quantitative real-time RT-PCR, expression of two critical markers for HSCs, CD150, and endoglin was examined. CD150, a member of the SLAM family of receptors that regulate lymphocyte signaling, is specifically expressed by LTR-HSCs [5]. Similarly, endoglin, a homolog of the type III TGF-β receptor, is differentially expressed by HSCs

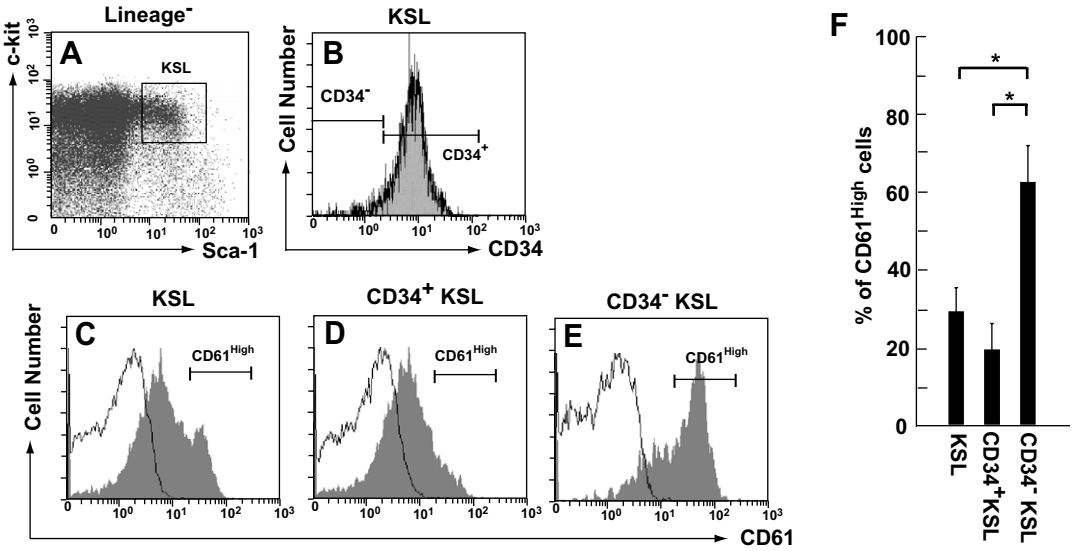
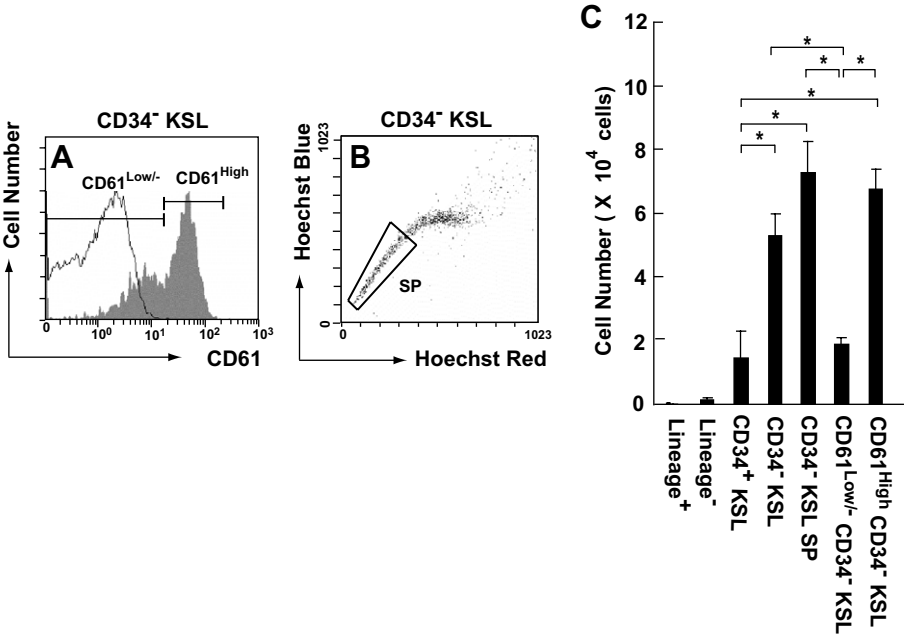


Fig. 1. Expression of CD61 in $CD34^{-}$KSL population. In $CD34^{-}$, c-kit+, Sca-1^{+}, lineage^{-} ($CD34^{-}$KSL) cells, expression of CD61 was investigated by flow cytometry. Lineage^{-} cells sorted by MACS were stained with antibodies for c-kit, Sca-1, CD34, and CD61. The dot plot for the parameter of c-kit and Sca-1 denotes the gate for c-kit^{+}, Sca-1^{+}, and lineage^{-} cells (KSL) (A). Gated KSL cells were separated into either $CD34^{+}$KSL or $CD34^{-}$KSL cells by strength of expression of CD34 (B). The histograms present expression of CD61 in KSL (C), $CD34^{+}$KSL (D), and $CD34^{-}$KSL (E) cells. White: isotype control, gray: CD61. The graph represents the percentage of the cells engulfed within the $CD61^{High}$ gate in each fraction (F). Data are presented as means ± SD ($^{*}p < 0.01$).



D

Colony Size (diameter)	$CD34^{+}$KSL	$CD34^{-}$KSL	$CD61^{Low/-}$ $CD34^{-}$KSL	$CD61^{High}$ $CD34^{-}$KSL
> 1000um	3.05% (5/164)	8.72% (15/172)	1.48% (2/135)	15.2% (30/198)
500 ~ 1000 um	19.5% (32/164)	44.2% (76/172)	14.1% (19/135)	44.4% (88/198)
200 ~ 500 um	42.7% (70/164)	36.6% (63/172)	43.0% (58/135)	34.8% (46/198)
< 200 um	34.8% (57/164)	10.5% (18/172)	41.5% (56/135)	5.56% (11/198)

Fig. 2. Serum-free culture. $CD34^{-}$KSL fraction was divided to $CD61^{High}CD34^{-}$KSL and $CD61^{Low/-}CD34^{-}$KSL with anti-CD61 antibody staining (A). White: isotype control, gray: CD61. $CD34^{-}$KSL SP fraction was also obtained with Hoechst 33342 staining (B). One hundred cells of each fraction were cultured for 7 days and total cell numbers were counted (C). Data are presented as means ± SD ($^{*}p < 0.01$). The size of colonies derived from single cells was measured and assorted (D). Data are presented as the percentage of the colonies within the indicated size (number of the colonies corresponded to indicated size/total colony number).

[6,21]. Gene expression of both HSC markers was significantly greater in CD61^{High}CD34^{-}KSL cells, compared to CD61$^{Low/-}$CD34^{-}KSL cells (Fig. 3A and B). We also found that expression of CD61 was correlated to increased frequency of SP cells (Fig. 4A–E) and expression of ABCG2; a critical mediator of the SP phenotype (Fig. 3C) within CD34^{-}KSL cells. Furthermore, we examined the correlation between expression of CD61 and the ability for long-term hematopoiesis. CD61^{High}CD34^{-}KSL cells contained significantly more LTR-HSCs than either CD61$^{Low/-}$CD34^{-}KSL cells or reasonably CD34^{-}KSL cells (Fig. 3D). Thus, these results demonstrated that high expression of CD61 was also correlated to properties of HSCs within the CD34^{-}KSL cell fraction.

Although expression of CD61 was observed in HSCs from CD34^{-}KSL fraction (Figs. 1–3), it remained unclear whether other LTR-HSCs within NSP fractions possessed high expression of CD61. Since CD61^{High}CD34^{-}KSL fraction was richly populated with SP cells, which can be regarded as a unique HSC pool (Fig. 4A–E), it was possible that the frequency of LTR-HSCs was dependent on the presence of SP cells and thus high expression of CD61 was a function of the SP phenotype. To elucidate this critical point, we next examined whether the NSP fractions from both CD61^{High}CD34^{-}KSL and CD61$^{Low/-}$CD34^{-}KSL cells contained LTR-HSCs. To determine whether CD61 should be regarded as only a specific marker of SP cells, rather than HSCs, we investigated expression of CD61 in LTR-HSCs from NSP cells within CD34^{-}KSL populations. We performed long-term competitive reconstitution assay using CD61^{High}CD34^{-}KSL SP, CD61^{High}CD34^{-}KSL NSP, and CD61$^{Low/-}$CD34^{-}KSL NSP cells, while CD61$^{Low/-}$CD34^{-}KSL SP cells were not examined, due to the severely limited cell number. Our results confirmed that CD61^{High}CD34^{-}KSL NSP cells contained a sub-fraction of LTR-HSCs but fewer HSCs than CD61^{High}CD34^{-}KSL SP cells (Fig. 4F). However, CD61$^{Low/-}$CD34^{-}KSL NSP cells showed much lower reconstitution abilities compared to CD61^{High}CD34^{-}KSL NSP (Fig. 4F). Expression of CD61 as a marker of LTR-HSCs was thus independent of the SP phenotype and could be used to support purification of LTR-HSCs using surface characterization.

Discussion

Our findings indicate that expression of CD61 was also correlated to the properties of HSCs within the CD34^{-}KSL population, which is regarded as one of the most enriched HSC populations. Moreover, our results also indicated that the behavior of CD61$^{Low/-}$CD34^{-}KSL cells was similar to that of CD34^{+}KSL cells that comprised short-term repopulating HSCs and hematopoietic progenitors [22] in gene expression of HSC markers (Fig. 3) and *in vitro* assays (Fig. 2). The CD34^{-}KSL cell population is currently acknowledged as a definitive benchmark for puri-

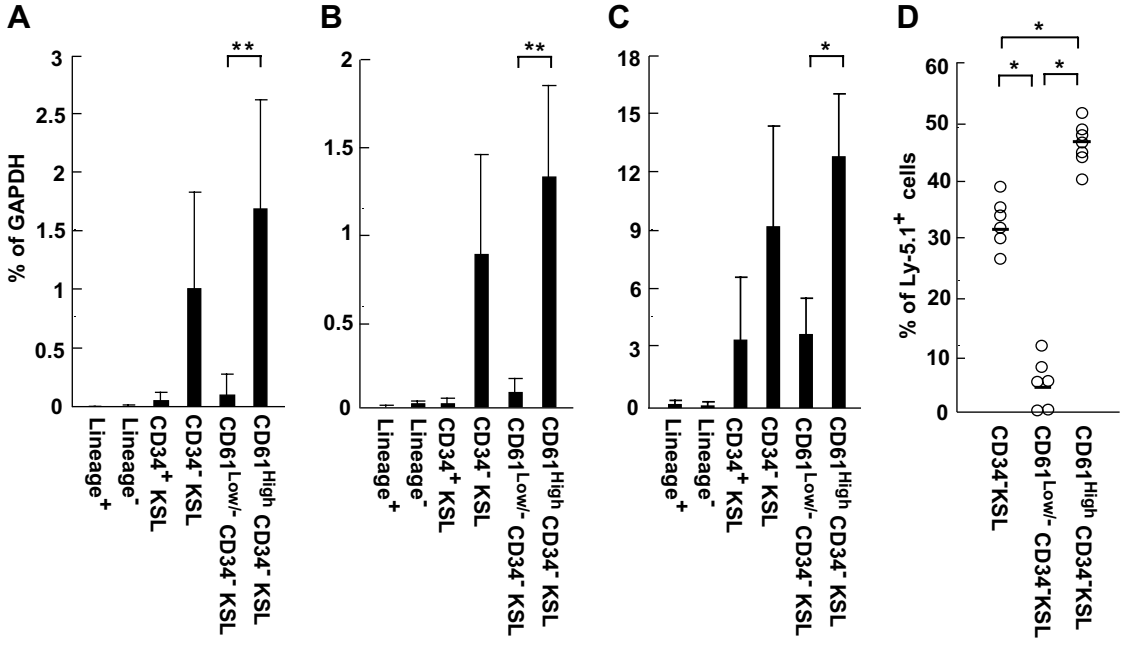


Fig. 3. Expression of CD61 was correlated to the features of HSCs in CD34^{-}KSL population. CD61^{High}CD34^{-}KSL cells were analyzed for expression of HSC markers. mRNA was extracted from sorted 10,000 cells of indicated fractions, followed by synthesis of cDNA. Graphs represent mRNA expression of CD150 (A), endoglin (B), and ABCG2 (C) in the various cell fractions, as determined by real-time quantitative RT-PCR. Data are presented as means ± SD ($^{*}p < 0.01$, $^{**}p < 0.05$). (D) Long-term competitive repopulation assay performed with CD34^{-}KSL and CD61$^{Low/-}$CD34^{-}KSL and CD61^{High}CD34^{-}KSL. Two hundred cells of the indicated fractions derived from C57BL/6-Ly5.1 mice and 2×10^5 mononuclear bone marrow cells from C57BL/6-Ly5.2 mice were transplanted into C57BL/6-Ly5.2 mice irradiated total 10 Gy of X-ray. The plot represents the percentage of donor-derived cells (% of Ly5.1^{+} cells) in the peripheral blood of each recipient animal, 3 months after bone marrow transplantation. Bars represent mean values ($^{*}p < 0.01$).

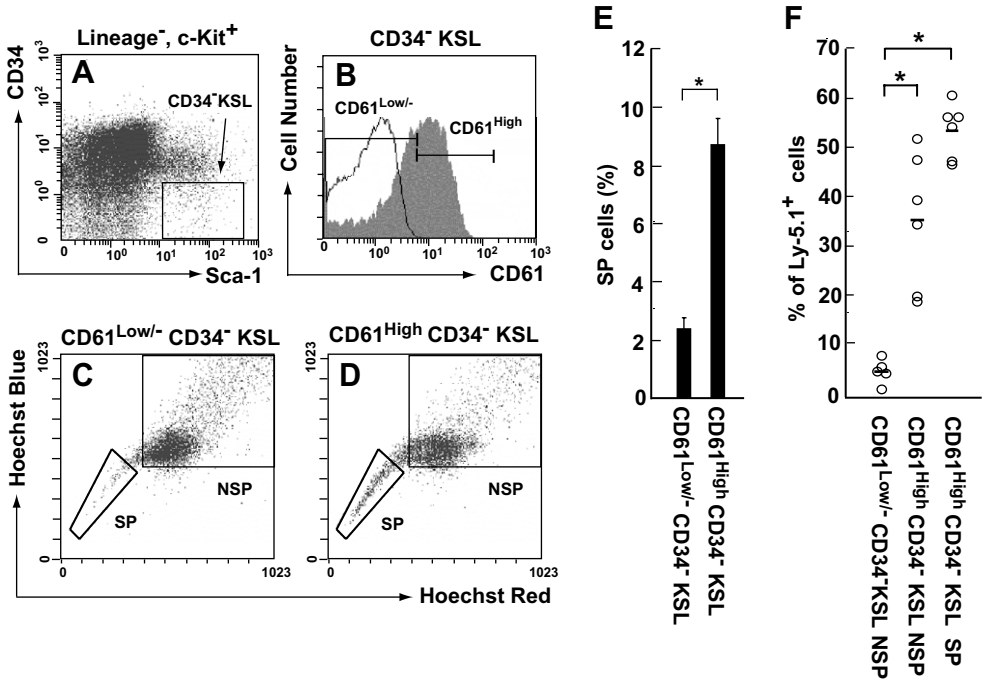


Fig. 4. Expression of CD61 was correlated to frequency of long-term repopulating HSCs within the NSP population. Hoechst 33342 staining in the $CD34^{-}$KSL population. $lineage^{-}$, $c\text{-}Kit^{+}$ cells sorted by MACS were stained with antibodies for Sca-1, CD34, and CD61, to determine the gates of $CD34^{-}$KSL (A), $CD61^{Low/-}CD34^{-}$KSL, and $CD61^{High}CD34^{-}$KSL (B) cell fractions. White: isotype control, gray: CD61. The SP phenotype was analyzed by staining with Hoechst 33342 in $CD61^{Low/-}CD34^{-}$KSL cells (C) or $CD61^{High}CD34^{-}$KSL cells (D). The graph shows the percentage of SP cells in the indicated fractions (E). Data are presented as means ± SD ($^{*}p < 0.01$). (F) Long-term competitive repopulation assay performed with $CD61^{Low/-}CD34^{-}$KSL NSP, $CD61^{High}CD34^{-}$KSL NSP, and $CD61^{High}CD34^{-}$KSL SP cells. 200 $CD61^{Low/-}CD34^{-}$KSL NSP, 200 $CD61^{High}CD34^{-}$KSL NSP, or 100 $CD61^{High}CD34^{-}$KSL SP cells derived from C57BL/6-Ly5.1 mice and 2×10^{5} mononuclear bone marrow cells from C57BL/6-Ly5.2 mice were transplanted into C57BL/6-Ly5.2 mice irradiated total 10 Gy of X-ray. The plot represents the percentage of donor-derived cells (% of $Ly5.1^{+}$ cells) in the peripheral blood of each recipient animal, 3 months after bone marrow transplantation. Bars represent mean values ($^{*}p < 0.01$).

fied HSC fractions, with approximately one in three cells possessing long-term repopulation abilities [2]. However, previous reports have never established that $CD34^{-}$KSL cells are composed of a homogeneous population. Therefore, extraction of $CD61^{High}$ fraction from the $CD34^{-}$KSL cells allows for enhanced purification of LTR-HSCs by excluding short-term progenitor cells and any contaminant $CD34^{+}$KSL cells, supported by our result that $CD61^{High}CD34^{-}$KSL cells contained a reasonably greater concentration of LTR-HSCs than the $CD34^{-}$KSL fraction (Fig. 3D). In addition, $CD34^{-}$KSL cells exhibited much higher expression of CD61, when compared to $CD34^{+}$KSL cells (Fig. 1), and our results demonstrated that CD61 can be used as supportive positive marker to improve the effectiveness of HSCs by combination with $CD34^{-}$KSL subset (Fig. 3D) or SP fraction [17]. However, total $CD61^{High}$ cells actually comprised a less purified population of HSCs, when compared to $CD34^{-}$ cells in the KSL fraction (data not shown). CD61 is also expressed on several hematopoietic cell types, including megakaryocytes and macrophages, which indicates that CD61 is present on the surface of some differentiated cell types and can account for this discrepancy. Nevertheless, although the parameter of CD61 expression is not able to individually distinguish LTR-HSCs, CD61 is exploitable for increased purification of LTR-HSCs as a supportive marker. Moreover, the identification of CD61 as a positive marker for the purification of HSCs from progenitor cells, such as $CD34^{+}$KSL fraction, is a significant discovery among the number of critical negative makers for HSCs that are currently applied.

Although our previous study demonstrated the correlation between high expression of CD61 and HSCs within the SP cell fraction [17], it remained unclear whether HSCs without the SP phenotype have high expression of CD61. Recent findings have indicated that LTR-HSCs from the NSP fraction were also present within $CD34^{-}$KSLs [18], indicating that the NSP fraction also contains HSCs that are distinct from cells with the ability to efflux Hoechst 33342 via ABCG2. In this study, our results indicated that LTR-HSCs were more richly collected in the $CD61^{High}CD34^{-}$KSL NSP fraction than in the $CD61^{Low/-}CD34^{-}$KSL NSP (Fig. 4). However, this percentage of SP cells was lower in $CD34^{-}$KSL population, compared to previous reports [18,23]. In our experiments, we performed cell sorting for the indicated fractions from $lineage^{-}$ $c\text{-}kit^{+}$ cells that were obtained by MACS, because of the shortage of fluorescent detectors for the eight parameters (Hoechst red, Hoechst blue, propidium iodide, lineage, Sca-1, c-kit, CD34, and CD61) that were used in our FACS system. Thus, it appears that these experimental differences can account for the rougher selectiveness of the $lineage^{-}$ $c\text{-}kit^{+}$ population, compared to the defined gates

in flow cytometric analysis. However, even when these differences in cell sorting such as slight contamination during magnetic cell sorting or differential sorting gates for lineage$^-$ c-kit$^+$ cells, it was plausible that our CD34$^-$KSL population contained the pure CD34$^-$KSL cells as well as some contaminations. Thus, our results strongly showed that the CD61^{High} population was more highly enriched for LTR-HSCs than CD61$^{Low/-}$ population in NSP cells of the fraction containing the pure CD34$^-$KSL cells (Fig. 4F). Moreover, although the CD34$^-$KSL population exhibited two peaks in Figs. 1E and 2A, the population had only one peak in Fig. 4B, suggesting that the elimination of the second during Hoechst staining may be a result of the selection of lineage$^-$ c-kit$^+$ cells by MACS. Nevertheless, the CD61^{High} population contained significantly greater numbers of LTR-HSCs than the CD61^{Low} population in NSP cells (Fig. 4F), as well as in SP cells as previously described [17]. Therefore, our results indicate that the expression of CD61 in HSCs is not limited to SP cells, but rather a distinct marker of HSCs.

Interestingly, much lower expression of CD61 was observed in CD34$^+$KSL cells than in CD34$^-$KSL cells (Fig. 1). In addition to expression of CD61, there were other differences between the CD34$^+$KSL population (the fraction containing hematopoietic progenitors) and CD34$^-$KSL cells (the fraction containing HSCs), such as cell cycle state and the ability for long-term hematopoiesis. Moreover, previous studies have indicated that CD61 on LTR-HSCs was likely heterodimerized with CD51 and not CD41 [5,17,24,25]. CD51/CD61, also known as integrin $\alpha_V\beta_3$ or vitronectin receptor, is related to adhesion, movement, and apoptosis in several cell types [15,16]. Therefore, the elucidation of CD51/CD61 signal transduction in LTR-HSCs may clarify mechanisms involved in the maintenance of stem-ness, as well as distinctions between HSCs and hematopoietic progenitors.

In conclusion, our findings demonstrate that CD61 is exploitable as supportive positive marker to further enrich LTR-HSCs without being limited to the SP fraction. These results suggest that high expression of CD61 may contribute to the general determination of stem-ness such as niche interactions, self-renewal, and quiescence.

Acknowledgments

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References

[1] G.J. Spangrude, S. Heimfeld, I.L. Weissman, Purification and characterization of mouse hematopoietic stem cells, Science 241 (1988) 58–62.
[2] M. Osawa, K. Hanada, H. Hamada, H. Nakauchi, Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell, Science 273 (1996) 242–245.
[3] C.T. Jordan, I.R. Lemischka, Clonal and systemic analysis of long-term hematopoiesis in the mouse, Genes Dev. 4 (1990) 220–232.
[4] S.J. Morrison, I.L. Weissman, The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype, Immunity 1 (1994) 661–673.
[5] M.J. Kiel, O.H. Yilmaz, T. Iwashita, O.H. Yilmaz, C. Terhorst, S.J. Morrison, SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells, Cell 121 (2005) 1109–1121.
[6] C.Z. Chen, M. Li, D. de Graaf, S. Monti, B. Gottgens, M.J. Sanchez, E.S. Lander, T.R. Golub, A.R. Green, H.F. Lodish, Identification of endoglin as a functional marker that defines long-term repopulating hematopoietic stem cells, Proc. Natl. Acad. Sci. USA 99 (2002) 15468–15473.
[7] M.A. Goodell, K. Brose, G. Paradis, A.S. Conner, R.C. Mulligan, Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo, J. Exp. Med. 183 (1996) 1797–1806.
[8] J.M. Zijlmans, J.W. Visser, K. Kleiverda, P.M. Kluin, R. Willemze, W.E. Fibbe, Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term or long-term bone marrow-repopulating ability, Proc. Natl. Acad. Sci. USA 92 (1995) 8901–8905.
[9] S. Zhou, J.D. Schuetz, K.D. Bunting, A.M. Colapietro, J. Sampath, J.J. Morris, I. Lagutina, G.C. Grosveld, M. Osawa, H. Nakauchi, B.P. Sorrentino, The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype, Nat. Med. 7 (2001) 1028–1034.
[10] K.A. Jackson, T. Mi, M.A. Goodell, Hematopoietic potential of stem cells isolated from murine skeletal muscle, Proc. Natl. Acad. Sci. USA 96 (1999) 14482–14486.
[11] K. Shimano, M. Satake, A. Okaya, J. Kitanaka, N. Kitanaka, M. Takemura, M. Sakagami, N. Terada, T. Tsujimura, Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1, Am. J. Pathol. 163 (2003) 3–9.
[12] R. Summer, D.N. Kotton, X. Sun, B. Ma, K. Fitzsimmons, A. Fine, Side population cells and Bcrp1 expression in lung, Am. J. Physiol. Lung Cell. Mol. Physiol. 285 (2003) L97–L104.
[13] N. Uchida, F.Y. Leung, C.J. Eaves, Liver and marrow of adult mdr-1a/1b(−/−) mice show normal generation, function, and multi-tissue trafficking of primitive hematopoietic cells, Exp. Hematol. 30 (2002) 862–869.
[14] K. Switala-Jelen, K. Dabrowska, A. Opolski, L. Lipinska, M. Nowaczyk, A. Gorski, The biological functions of beta3 integrins, Folia Biol. (Praha) 50 (2004) 143–152.
[15] K.R. Fath, C.J. Edgell, K. Burridge, The distribution of distinct integrins in focal contacts is determined by the substratum composition, J. Cell Sci. 92 (Pt. 1) (1989) 67–75.
[16] J. Savill, I. Dransfield, N. Hogg, C. Haslett, Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis, Nature 343 (1990) 170–173.
[17] T. Umemoto, M. Yamato, Y. Shiratsuchi, M. Terasawa, J. Yang, K. Nishida, Y. Kobayashi, T. Okano, Expression of Integrin beta3 is correlated to the properties of quiescent hemopoietic stem cells possessing the side population phenotype, J. Immunol. 177 (2006) 7733–7739.
[18] Y. Morita, H. Ema, S. Yamazaki, H. Nakauchi, Non-side-population hematopoietic stem cells in mouse bone marrow, Blood 108 (2006) 2850–2856.
[19] T. Umemoto, M. Yamato, K. Nishida, J. Yang, Y. Tano, T. Okano, Limbal epithelial side-population cells have stem cell-like properties, including quiescent state, Stem Cells 24 (2006) 86–94.
[20] T. Umemoto, M. Yamato, K. Nishida, J. Yang, Y. Tano, T. Okano, p57Kip2 is expressed in quiescent mouse bone marrow side population cells, Biochem. Biophys. Res. Commun. 337 (2005) 14–21.
[21] C.Z. Chen, L. Li, M. Li, H.F. Lodish, The endoglin(positive) sca-1(positive) rhodamine(low) phenotype defines a near-homogeneous

population of long-term repopulating hematopoietic stem cells, Immunity 19 (2003) 525–533.
[22] Y. Zhao, Y. Lin, Y. Zhan, G. Yang, J. Louie, D.E. Harrison, W.F. Anderson, Murine hematopoietic stem cell characterization and its regulation in BM transplantation, Blood 96 (2000) 3016–3022.
[23] Y. Matsuzaki, K. Kinjo, R.C. Mulligan, H. Okano, Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells, Immunity 20 (2004) 87–93.
[24] M.J. Ferkowicz, M. Starr, X. Xie, W. Li, S.A. Johnson, W.C. Shelley, P.R. Morrison, M.C. Yoder, CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo, Development 130 (2003) 4393–4403.
[25] H.K. Mikkola, Y. Fujiwara, T.M. Schlaeger, D. Traver, S.H. Orkin, Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo, Blood 101 (2003) 508–516.