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Lineage marker expression on mouse hematopoietic stem cells

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Whether hematopoietic stem cells (HSCs) express lineage markers is controversial. In this study, we highly purified HSCs from the adult bone marrow of C57BL/6 mice and examined their gene expression and reconstitution potential. We first focused on the integrin family. Single-cell reverse transcription polymerase chain reaction revealed that the expression of *ItgaM/Itgb2* (Mac-1) and *Itga2b/Itgb3* (CD41/CD61) gradually increased along HSC differentiation, whereas *Itga4, Itga5, Itga6*, and *ItgaV* (CD51) together with *Itgb1* were highly expressed in both HSCs and hematopoietic progenitor cells (HPCs). We next fractionated HSCs based on their expression of Mac-1, CD41, and CD51 by flow cytometry. We detected Mac-negative and Mac-low, but not Mac-high cells, in the HSC population. We also detected CD41-negative, -low, and -high cells in the HSC population. Competitive repopulation revealed that Mac-1-negative and -low HSCs were functionally similar, and CD41-negative and -low HSCs were functionally similar, at the single-cell level, but CD41-high HSCs were not detectable. We then found that the selection of Mac-1-negative HSCs or CD41-negative HSCs had no advantage in HSC purification. We moreover found that HSCs expressed more CD51 than CD41, and HPCs expressed more CD41 than CD51, suggesting that CD51 expression was gradually replaced by CD41 expression during megakaryocyte differentiation. We concluded that low levels of Mac-1 and CD41 expression are irrelevant to the self-renewal and differentiation potentials in HSCs. **© 2019 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.**

Hematopoietic stem cells (HSCs) reside at the top of the hematopoietic hierarchy and differentiate into all blood cell lineages [1]. It is generally accepted, based on an early study [2], that HSCs do not express lineage markers. Many studies, including our own, have used anti-Mac-1 (ItgaM/Itgb2, CD11b/CD18) antibody as one of the lineage antibodies in a lineage cocktail to remove myeloid lineage-committed cells for HSC purification. Mac-1 is known to be expressed in HSCs at the fetal liver stage [3], but its expression is presumed to be lost in adult bone marrow (BM) HSCs. However, it has been reported that some adult BM HSCs express Mac-1 [4–6].

Itga2b/Intgb3 (CD41/CD61) is expressed in platelets and plays a role in their aggregation [7]. This complex is also expressed in megakaryocytes and their progenitor cells [8–11]. Thus, CD41 can be used as one of the megakaryocyte lineage markers. It has been reported that some adult BM HSCs express CD41 [12]. CD41 expression may mark myeloid-biased HSCs [13]. Contrarily, it has also been reported that HSCs do not express CD41 in adult BM [14,15]. Whether CD41 is expressed on adult HSCs thus remains controversial. To address these issues, we examined Mac-1 and CD41 expression in highly purified mouse

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HSCs and found their low level of expression in a proportion of HSCs, which was functionally verified by transplantation assays. Here, we report the existence of Mac-1-negative or -low and CD41-negative or -low HSCs in adult BM. We further examine the relationship between CD41 (Intga2b) and CD51 (IntgaV) expression on HSCs because the expression of CD51/CD61 has been reported [16], and CD41 and CD51 may share CD61 (Intgb3) as β-subunit integrin. From the practical point of view, we recommend not using the depletion of Mac-1^+ cells or the selection of $CD41^-$ cells as a process of HSC purification.

Methods

Mice

C57BL/6 (B6-CD45.2) mice were purchased from Beijing HFK Bioscience Company (Beijing, China). CD45.1 congenic B6 mice (B6-CD45.1) and GFP transgenic B6 mice were bred and maintained at the State Key Laboratory of Experimental Hematology. Eight- to 12-week-old female mice were used for all experiments. All experimental protocols using mice were approved by the Institutional Animal Care and Use Committee at the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

HSC purification

BM cells were obtained from the femurs, tibias and iliac crests of B6-CD45.1 or B6-GFP mice. Prior to antibody staining, c-Kit-positive cells were enriched with anti-c-Kit antibody-conjugated MACS beads (Miltenyi Biotec). Lineage depletion by MACS beads was not performed. Cells were stained with allophycocyanin (APC)/eFluor 780 (eF780)-conjugated anti-Mac-1 (M1/70), CD3 (SK7), CD4 (Gk1.5), CD8a (53-6.7), B220 (RA3-6B2), Gr-1 (RB6-8C5), Ter-119 (TER-119), or all seven antibodies. Cells were also stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (RAM34), APC-conjugated anti-c-Kit (2B8), phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti Sca-1 (D7), Brilliant Violet (BV) 421-conjugated anti-CD48 (HM48-1), PE-conjugated anti-CD150 (TC15-12F12.2), and BV510 or PerCP/eF710-conjugated anti-CD41 (eBioMWReg30) antibodies. Cells were analyzed and sorted with a FACS Aria III (BD Biosciences). Data were exported with FCS 3.0 and analyzed by Flowjo. HSC1 was defined as $CD150^+CD41^-CD34^-Kit^+Sca\text{-}1^+Lineage^-$ ($CD150^+41^-34^-KSL$) cells. HSC2 was defined as $CD150^-41^-34^-$ KSL cells. HPC1 was defined as $CD150^+41^+34^-KSL$ cells. HPC2 was defined as $CD150^+41^+34^+$ KSL cells. HPC3 was defined as $CD150^-41^-34^+$ KSL cells (see the sorting gates for these populations in Figure 1A).

Transplantation

A competitive repopulation assay was performed with 5×10^5 BM cells from B6-CD45.2 mice as competitor cells. Single or multiple cells were mixed with competitor cells and transplanted into B6-CD45.1 mice that had been irradiated twice at a dose of 4.75 Gy. Secondary transplantation was performed by transferring 2×10^7 BM cells of primary recipient mice into lethally irradiated B6-CD45.2 mice.

Peripheral blood cells from the recipient mice were analyzed after the following procedures. Erythrocytes were lysed with buffer containing 0.15 mol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA in water (pH 7.2). Cells were stained with FITC-conjugated anti-CD45.1 (A20), PE-conjugated anti-CD45.2 (104), PE-Cy7-conjugated anti-CD4, APC-conjugated anti-CD8a, PerCP-Cy5.5-conjugated anti-B220, and APC/eF780-conjugated anti-Mac-1 and Gr-1 antibodies. Cells were analyzed on a Canto II (Becton Dickinson). The percentage of donor cells was calculated using the following formula: (% $CD45.1^+$ cells) $\times$ 100/(% $CD45.1^+$ test cells + % $CD45.2^+$ cells). When the percentage of donor cells was $\geq$0.1, the hematopoietic system was considered to be reconstituted with donor cells (positive mice). Mac-1/Gr-1^+ cells were considered to be of the myeloid lineage. $B220^+$ cells were considered to be of the B-lymphoid lineage. $CD4^+$ or $CD8^+$ cells were considered to be of the T-lymphoid lineage. ST-HSCs were defined as HSCs with myeloid reconstitution potential for <6 months after transplantation and with lymphoid reconstitution potential. LT-HSCs were defined as HSCs with myeloid reconstitution potential for >6 months after transplantation.

Single-cell colony assay

Single cells were sorted by a FACS Aria III into 96-well U-bottom plates, in which each well contained 200 μL α-MEM supplemented with 10% fetal bovine serum, 100 mg/mL penicillin/streptomycin, 50 ng/mL mouse stem cell factor, 50 ng/mL mouse thrombopoietin, 10 ng/mL mouse interleukin-3, and 1 IU/mL human erythropoietin, and cultured at 37°C in a humidified atmosphere with 5% CO_2. On day 14 of culture, colonies were scored if a well contained $\geq$50 cells. Colonies were individually subjected to centrifugation onto glass slides by a Shandon Cytospin 4 (Thermo Scientific). Glass slides were stained with May–Giemsa staining solution. Cells were morphologically classified as neutrophils, macrophages, erythroblasts, and megakaryocytes. Megakaryocyte colonies were scored by in situ observation of four or more cells per well. Some megakaryocyte colonies were stained with anti-CD41 antibody to confirm CD41 expression.

Single-cell RT-PCR

Single cells were sorted into 48 wells of a 96-well plate, where each well contained 10 μL of a reverse transcription and specific target amplification mixture consisting of 2.5 μL 0.2$\times$ primers containing 18 sets of α-integrin primers, 8 sets of β-integrin primers, and *Gapdh* primers, 5.0 μL 2$\times$ reaction mix, 0.5 μL_{III}, and 2.0 μL Tris–EDTA buffer. Reverse transcription (RT) 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 sec and 60°C for 4 min. Five microliters from each of the samples was mixed with 20 μL of Tris– EDTA. Then, 2.7 μL of the diluted samples was mixed with 3.0 μL Taqman universal polymerase chain reaction PCR master mix (Applied Biosystems) and 0.3 μL sample loading buffer (for a total of 6.0 μL of sample loading mix). After 3.0 μL of 20$\times$ concentrations of each set of primers was mixed with 3.0 μL of assay loading reagent (a total of 6.0 μL of assay loading mix), 5 μL of the sample loading mix and 5 μL of the assay loading mix were applied to a 48-chip array, and 48 $\times$ 27 reactions were prepared by an integrated fluidics circuit controller. The chip was set on a Fluidigm BioMark and incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for

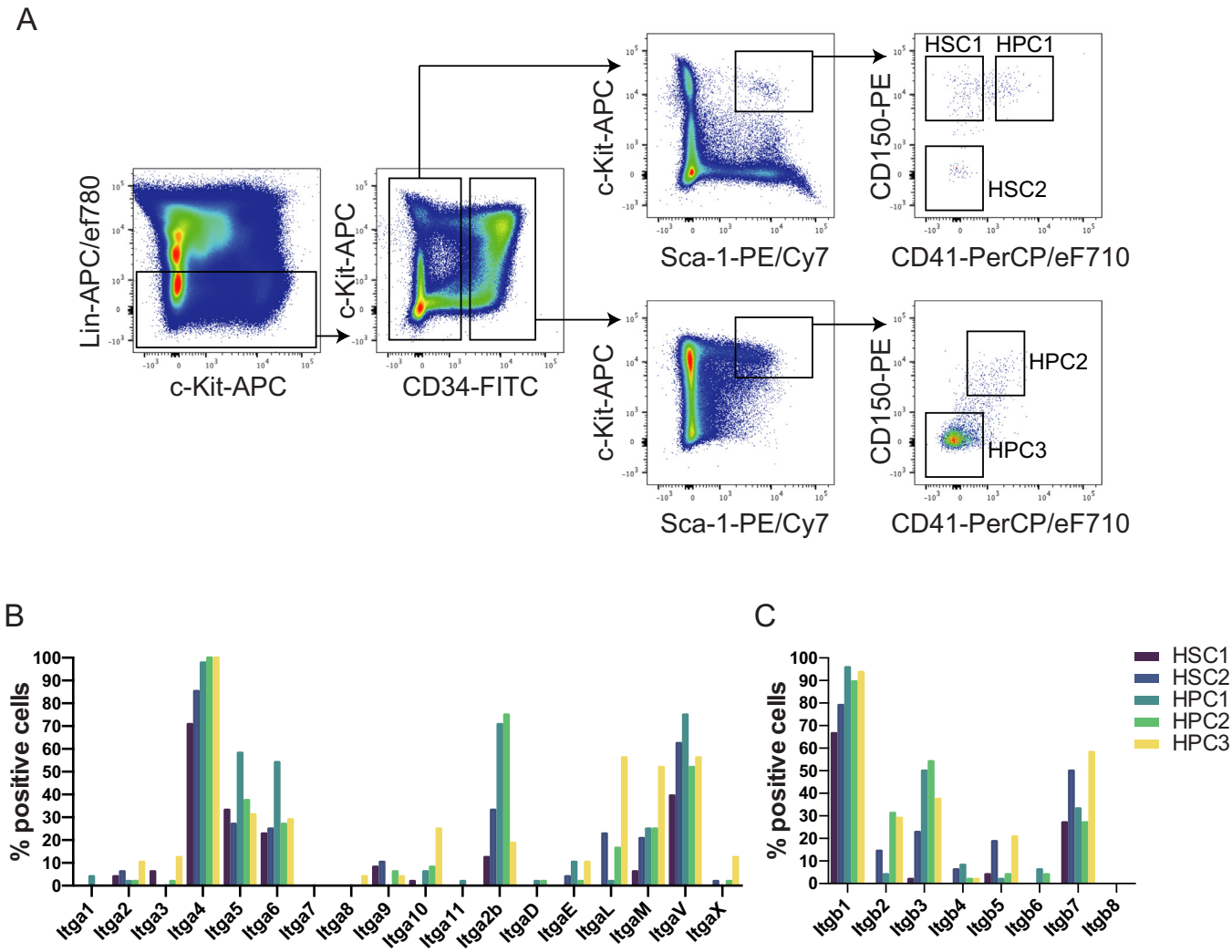

Figure 1. Expression of integrins in HSCs and HPCs. (**A**) Sorting gates for HSC1, HSC2, HPC1, HSC2, and HPC3. HSC1: $CD150^{+}41^{-}34^{-}KSL$ cells; HSC2: $CD150^{-}41^{-}34^{-}KSL$ cells; HPC1: $CD150^{+}41^{+}34^{-}KSL$ cells; HPC2: $CD150^{+}41^{+}34^{+}KSL$ cells; HPC3: $CD150^{-}41^{-}34^{+}KSL$ cells. Seven lineages were used for gating of Lin-negative cells. (**B**) Single-cell RT-PCR was performed on HSC1, HSC2, HPC1, HPC2, and HPC3 cells. The frequencies of integrin-expressing cells (positive cells with C_t <27.65) are illustrated.

60 sec. Data were analyzed with BioMark real-time PCR analysis software (Fluidigm). The PCR primers purchased from Thermo Fisher Scientific. When threshold cycle values (C_t) were <27.65, cells were considered to express the gene (positive cells).

Statistical analysis

The Mann–Whitney test and Fisher's exact test were performed.

Results

Integrin expression in HSCs

We first examined the expression of all members of the integrin family [17] in purified HSCs and HPCs by single-cell RT-PCR. We used two HSC populations: HSC1 and HSC2 (Figure 1A). HSC1 was enriched in long-term (LT >6 months) HSCs, and HSC2 was enriched in short-term HSCs (ST <6 months) [15,18,19]. We used three HPC populations: HPC1, HPC2, and HPC3 (Figure 1A). HPC1 was enriched in myeloid HPCs. HPC2 was enriched in day 12 colony-forming cells in spleen, and HPC3 was enriched in lymphoid-primed multipotent progenitors [15,18,20,21]. The relationship between Trampp's classification and our classification has previously been reported [21]. We used a lineage antibody cocktail consisting of seven lineage antibodies (see Methods) in this experiment. Mac-1^{+} cells were depleted from these purified HSCs and HPCs. Thus, the expression of integrin genes, particularly *ItgaM*/*Ingb2*, might be underestimated.

Consistent with previous work [22], the expression of *Itga4*, *Itga5*, and *Itga6,* together with that of *Itgb1*, was detected in HSC1 and HSC2 (Figure 1B). Interestingly, these integrins were also expressed in HPC1,

HPC2, and HPC3. *ItgaM,* and *Itga2b*-expressing cells were also detected at a low frequency in HSC1. The frequencies of these integrin-expressing cells, together with those of *Itgb2*- and *Itgb3*-expressing cells, gradually increased from HSCs to HPCs, suggesting that their expression is associated with cell differentiation. Of note was that the frequency of *Itgb2*-expressing cells in HSC2 remained very low. In addition, *ItgaV*-expressing cells were detected in both HSC and HPC populations. The frequencies of *Itgb3*- and *Itgb5*-expressing cells were very low in HSC1 and neither *Itgb6*- nor *Itgb8*-expressing cells were detected in HSC1 and HSC2. Thus, *ItgaV/Itgb1* as a heterodimer might be expressed in HSCs. From these results, we decided to focus on integrins αM (Mac-1) and αIIb (CD41), and αV (CD51) for the further study.

Mac-1-negative and -low HSCs in adult BM

We examined the expression of seven lineage markers individually and simultaneously by flow cytometry on $CD201^+CD150^+CD48^-CD41^-c\text{-}Kit^+Sca\text{-}1^+$ ($CD201^+150^+48^-41^-$ KS) cells, which are highly enriched in LT-HSCs (Figure 2A) [15,23]. A significant proportion of the cells expressed low levels of Mac-1 but not of CD3, CD4, CD8, B220, Gr-1, and Ter119 (Figure 2B). Notably, we never detected a high level of Mac-1 expression in $CD201^+150^+48^-41^-$ KS cells. We next performed competitive repopulation using the sorting gates illustrated in Supplementary Figure E1 (online only, available at www.exphem.org). Transplantation of 20 Mac-1-negative or -low $CD201^+150^+48^-41^-$ KS cells similarly revealed LT reconstitution activity in both populations (Figure 2C). After single-cell transplantation, two LT-HSCs and one

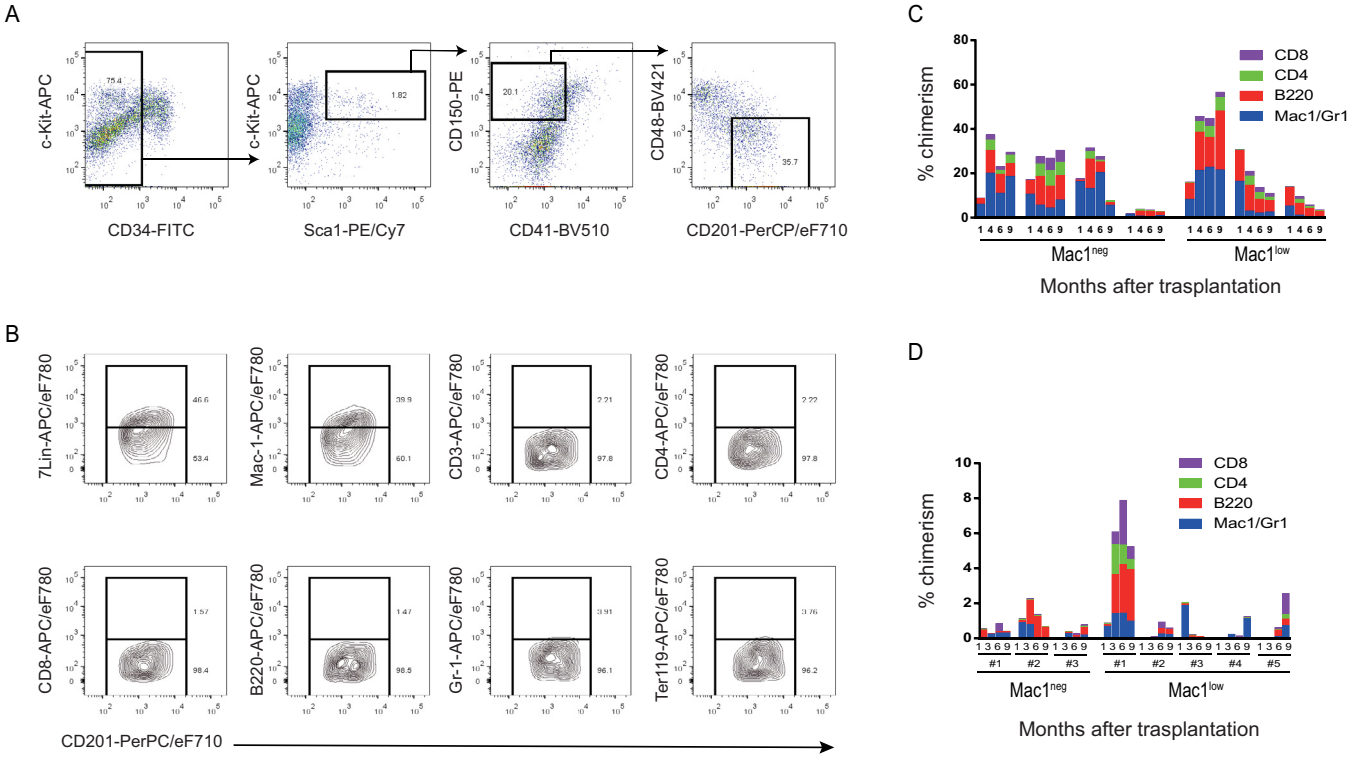

Figure 2. Mac-1-negative or -low HSCs. (**A**) Gating for $CD201^+150^+41^-48^-34^-$KS cells. (**B**) $CD201^+150^+41^-34^-$KS cells were further stained with one or seven lineage antibodies (Mac-1, CD3, CD4, CD8, B220, Gr-1, and Ter-119-APC/eF780). CD3 was used as a negative control. Seven lineage-positive cells represented 46.8 ± 4.3%, Mac-1^+ cells represented 32.7 ± 5.3%, $CD3^+$ cells represented 1.4 ± 0.5%, $CD4^+$ cells represented 1.5 ± 0.9%, $CD8^+$ cells represented 1.2 ± 0.5%, $B220^+$ cells represented 1.1 ± 0.4%, and $Ter119^+$ cells represented 1.1 ± 0.3% (mean ± SD, n = 5) of $CD201^+150^+CD41^-CD34^-$KS cells. (**C**) Twenty Mac-1^{neg} or Mac-1^{low} $CD201^+150^+41^-34^-$ KS cells were transplanted into five lethally irradiated mice with 5×10^5 competitor cells. The percentage of chimerism in the peripheral blood of recipient mice was examined 1, 4, 6, and 9 months after transplantation. One and two mice died early after transplantation with Mac-1^{low} and Mac-1^{neg} cells, respectively. No significant difference was found in the percentage of chimerism between Mac-1^{neg} and Mac-1^{low} cells (Mann–Whitney test). (**D**) Single Mac-1^{neg} or Mac-1^{low} $CD201^+150^+41^-34^-$KS cells were transplanted into 30 lethally irradiated mice with 5×10^5 competitor cells. Blood of recipient mice was analyzed 1, 3, 6, and 9 months after transplantation. The percentages of chimerism in Mac-1/Gr-1^+ myeloid, $B220^+$ B, $CD4^+$ T, and $CD8^+$ T cells are illustrated for individual recipient mice (#1–3 and #1–5). Four mice and one mouse died by 9 months after transplantation with Mac-1^{neg} and Mac-1^{low} cells, respectively. No significant difference was found in the frequency of HSCs between Mac-1^{neg} and Mac-1^{low} cells (Fisher's exact test).

ST-HSC were detected in the 26 recipients of Mac-1^{neg}CD201^{+}150^{+}48^{-}41^{-}KS cells. Four LT-HSCs and one ST-HSC were detected in the 29 recipients of Mac-1^{low}CD201^{+}150^{+}48^{-}41^{-}KS cells (Figure 2D). According to our definition, myeloid lineage reconstitution persisted <6 months after transplantation for ST-HSCs and >6 months after transplantation for LT-HSCs, respectively. ST-HSCs are almost equivalent to lymphoid-biased HSCs, and LT-HSCs are almost equivalent to myeloid-biased or balanced HSCs [19].

Three-lineage depletion as a standard procedure for HSC purification

To evaluate whether depletion of Mac-1^{+} cells is effective for HSC purification, we chose B220, Ter-119, and Gr-1 as the major three lineage markers and compared the degree of HSC purification between three- and seven-lineage depletion (the three lineages were B220, Ter-119, and Gr-1; the seven lineages consisted of B220, Ter-119, Gr-1, Mac-1, CD4, CD8, and CD3). Competitive repopulation with 10 CD150^{+}41^{-}34^{-} KSL$_3$ cells(three-lineage depletion) and 10 CD150^{+}41^{-}34^{-} KSL$_7$ cells (seven-lineage depletion) resulted in similar LT reconstitution activity in both populations (Figure 3A). Single-cell transplantation was also performed with 30 mice in each group. Three LT-HSCs and three ST-HSCs were detected in the three-lineage depletion, and four LT-HSCs and two ST-HSCs were detected in the seven-lineage depletion (Figure 3B). These data suggested that regardless of whether three- or seven-lineage antibodies are used for lineage depletion, the quality of purified HSCs is similar, but a proportion of HSCs may be lost during the purification procedures when seven lineages, including Mac-1, are used.

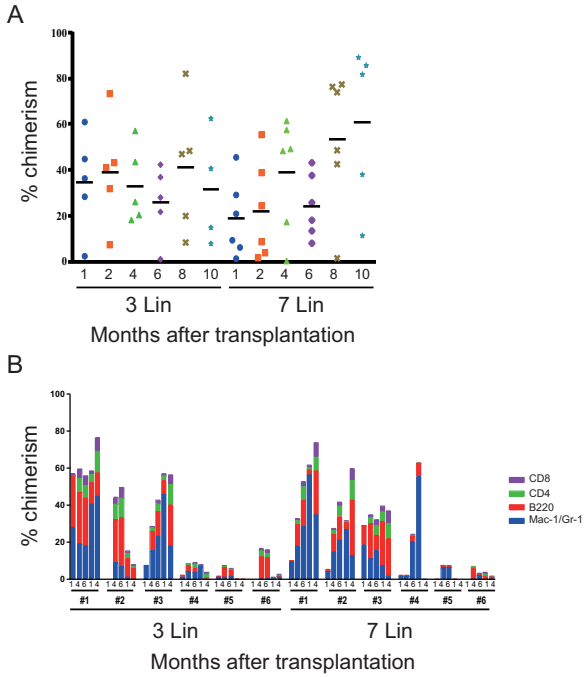

Figure 3. Transplantation of CD150^{+}41^{-}34^{-}SK cells after lineage depletion with either three or seven antibodies. (**A**) Ten three lineage−negative or seven lineage−negative CD150^{+}41^{-}34^{-}KS cells (CD150^{+}41^{-}34^{-}KSL$_3$ or CD150^{+}41^{-}34^{-}KSL$_7$ cells) were transplanted into five or six lethally irradiated mice with 5×10^5 competitor cells. Blood of recipient mice was analyzed 1, 4, 6, 8, and 10 months after transplantation. No significant difference was found in the percentage of chimerism between the two groups of mice (Mann−Whitney test). (**B**) Single three lineage−negative and seven lineage−negative CD150^{+}41^{-}34^{-}KS cells were transplanted into 30 lethally irradiated mice with 5×10^5 competitor cells. Blood of recipient mice was analyzed 1, 4, and 6 after primary transplantation and 1 and 4 months after secondary transplantation. The percentages of chimerism in Mac-1/Gr-1+ myeloid, B220+ B, and CD4/8+ T cells are illustrated for individual mice (#1−6 for each group). No significant differences were observed in the frequencies of HSCs between three- and seven-lineage depletion (Fisher's exact test).

CD41-negative and -low HSCs in adult BM

For this experiment, three-lineage depletion (L$_3$) was used. We operationally separated CD150^{+}34^{-}KSL$_3$ cells into CD41-negative (CD41^{neg}), CD41-low (CD41^{low}), and CD41-high (CD41^{high}) fractions (Figure 4A). CD41^{neg}, CD41^{low}, and CD41^{high} cells accounted for approximately 40%, 40%, and 20% of CD150^{+}34^{-}KSL$_3$ cells, respectively. Competitive repopulation was performed on 30 CD41^{neg}, CD41^{low}, or CD41^{high} CD150^{+}34^{-}KSL$_3$ cells. LT reconstitution activity was similarly detected in CD41^{neg} and CD41^{low} CD150^{+}34^{-}KSL$_3$ cells. However, only a low level of ST reconstitution activity was detected in CD41^{high}CD150^{+}34^{-}KSL$_3$ cells (Figure 4B). After single-cell transplantation, six LT-HSCs and two ST-HSCs were detected in the 19 recipients of CD41^{neg}CD150^{+}34^{-}KSL$_3$ cells, and five LT-HSCs and one ST-HSC were detected in the 16 recipients of CD41^{low}CD150^{+}34^{-}KSL$_3$ cells. In contrast, no HSCs were detected in the 19 recipients of CD41^{high} CD150^{+}34^{-}KSL$_3$ cells (Figure 4C).

CD41 and CD51 expression in HSCs

We examined CD41 and CD51 expression by flow cytometry on CD150^{+}48^{-}KL$_3$ cells (Figure 5A). These cells expressed varying amounts of CD41 and CD51. We operationally fractionated CD150^{+}48^{-}KL$_3$ cells into CD51^{+} CD41^{-}, CD51^{+}CD41^{+}, CD51^{-}CD41^{-}, and CD51^{-}CD41^{+} cells (Figure 5A). Competitive repopulation with 20 cells revealed LT reconstitution activity in CD51^{+}CD41^{-}, CD51^{+}CD41^{+}, and CD51^{-}CD41^{-}CD150^{+}48^{-}KL$_3$ cells, but not in CD51^{-}CD41^{+}CD150^{+}48^{-}KL$_3$ cells (Figure 5B). To confirm this, we fractionated CD150^{+}48^{-}KL$_3$ cells into CD51^{+} and CD51^{-}CD41^{+} cells (Figure 5C) and performed competitive repopulation with 20 cells. LT reconstitution

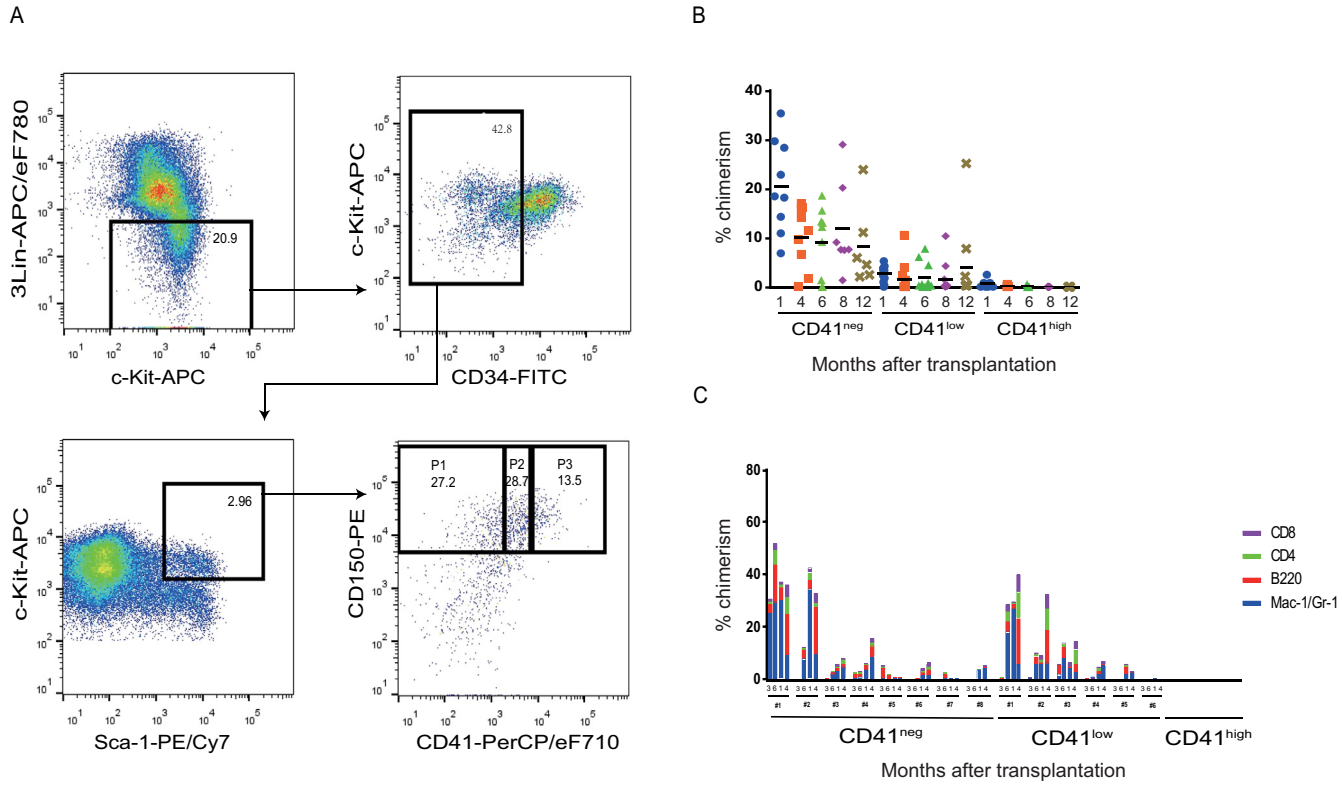

Figure 4. CD41-negative or -low HSCs. (**A**) Gating for $CD41^{neg}$, $CD41^{low}$, and $CD41^{high}$ $CD150^{+}34^{-}KSL_3$ cells. Three antibodies (anti-Gr-1, B220, and Ter-119) were used in this sorting (L_3). (**B**) Thirty $CD41^{neg}$, $CD41^{low}$, and $CD41^{high}$ $CD150^{+}34^{-}KSL_3$ cells were transplanted into 10 lethally irradiated mice with 5×10^5 competitor cells. Percentages of chimerism in the peripheral blood of recipient mice 1, 4, 6, 8, and 12 months after transplantation are illustrated. Four, one, and three mice died after transplantation with $CD41^{neg}$, $CD41^{low}$, and $CD41^{high}$ cells, respectively. No significant difference in the percentage of chimerism was found between the $CD41^{neg}$ and $CD41^{low}$ groups (Mann–Whitney test). (**C**) Single $CD41^{neg}$, $CD41^{low}$, and $CD41^{high}$ $CD150^{+}34^{-}KSL_3$ cells were transplanted into 30 lethally irradiated mice with 5×10^5 competitor cells. The blood of recipient mice is shown individually with the numbers of mice that were measured 3 and 6 months after primary transplantation and 1 and 4 months after secondary transplantation. The percentages of chimerism for Mac-1/Gr-1^{+} myeloid, $B220^{+}$ B, $CD4^{+}$ T, and $CD8^{+}$ T cells are shown for individual mice (#1–8 for $CD41^{neg}$ cells and #1–6 for $CD41^{low}$ cells). Eleven, fourteen, and eleven mice died after transplantation with $CD41^{neg}$, $CD41^{low}$, and $CD41^{high}$ cells, respectively. No reconstitution was observed after transplantation with $CD41^{high}$ $CD150^{+}34^{-}KSL_3$ cells. No significant difference was observed in the frequencies of HSCs between $CD41^{neg}$ and $CD41^{low}$ cells (Fisher's exact test).

activity was again detected in $CD51^{+}CD150^{+}48^{-}KL_3$ cells but not in $CD51^{-}CD41^{+}CD150^{+}48^{-}KL_3$ cells (Figure 5D), suggesting that $CD51^{-}CD41^{+}CD150^{+}48^{-}KL_3$ cells were more differentiated cells. To characterize these cells, we performed a single-cell colony assay. In this assay 59.7 ± 21.0% (mean ± SD, n = 6) of $CD51^{+}CD150^{+}48^{-}KL_3$ cells formed colonies, of which the frequencies of multilineage colonies consisting of neutrophil/macrophage/erythroblast/megakaryocyte (nmEM), nmE, and nmM colonies were 32.5 ± 17.0% (n = 6) and that of megakaryocyte colonies was 10.3 ± 10.7% (n = 6). On the other hand, 61.1 ± 11.0% (mean ± SD, n = 6) of $CD51^{-}CD41^{+}CD150^{+}48^{-}KL_3$ cells formed colonies, of which multilineage colonies accounted for 3.3 ± 6.6% (n = 6) and megakaryocyte colonies accounted for 56.1 ± 8.6% (n = 6) (Figure 5E). The frequency of multipotent colonies was significantly greater in $CD51^{+}CD150^{+}48^{-}KL_3$ cells than in $CD51^{-}CD41^{+}CD150^{+}48^{-}KL_3$ cells ($p = 0.0077$), whereas the frequency of megakaryocyte colonies was significantly greater in $CD51^{-}CD41^{+}CD150^{+}48^{-}KL_3$ cells than in $CD51^{+}CD150^{+}48^{-}KL_3$ cells ($p = 0.0050$). These data provide support that $CD51^{-}CD41^{+}CD150^{+}48^{-}KL_3$ cells are enriched in megakaryocyte progenitors.

Anti-CD41-neutralizing antibody does not reduce the reconstitution activity in HSCs

We used anti-CD41 antibody (clone: MWReg30) in cell staining for flow cytometry sorting. It has been reported that this antibody has a neutralizing effect and reduces the reconstitution activity when HSCs are reacted with a certain amount of this antibody and transplanted [13]. To examine whether the amount of anti-CD41 antibody used for staining procedure reduces the reconstitution activity of HSCs in our experiments, 20 $CD150^{+}48^{-}34^{-}KSL_3$ cells were transplanted with or without anti-CD41 antibody staining. In this

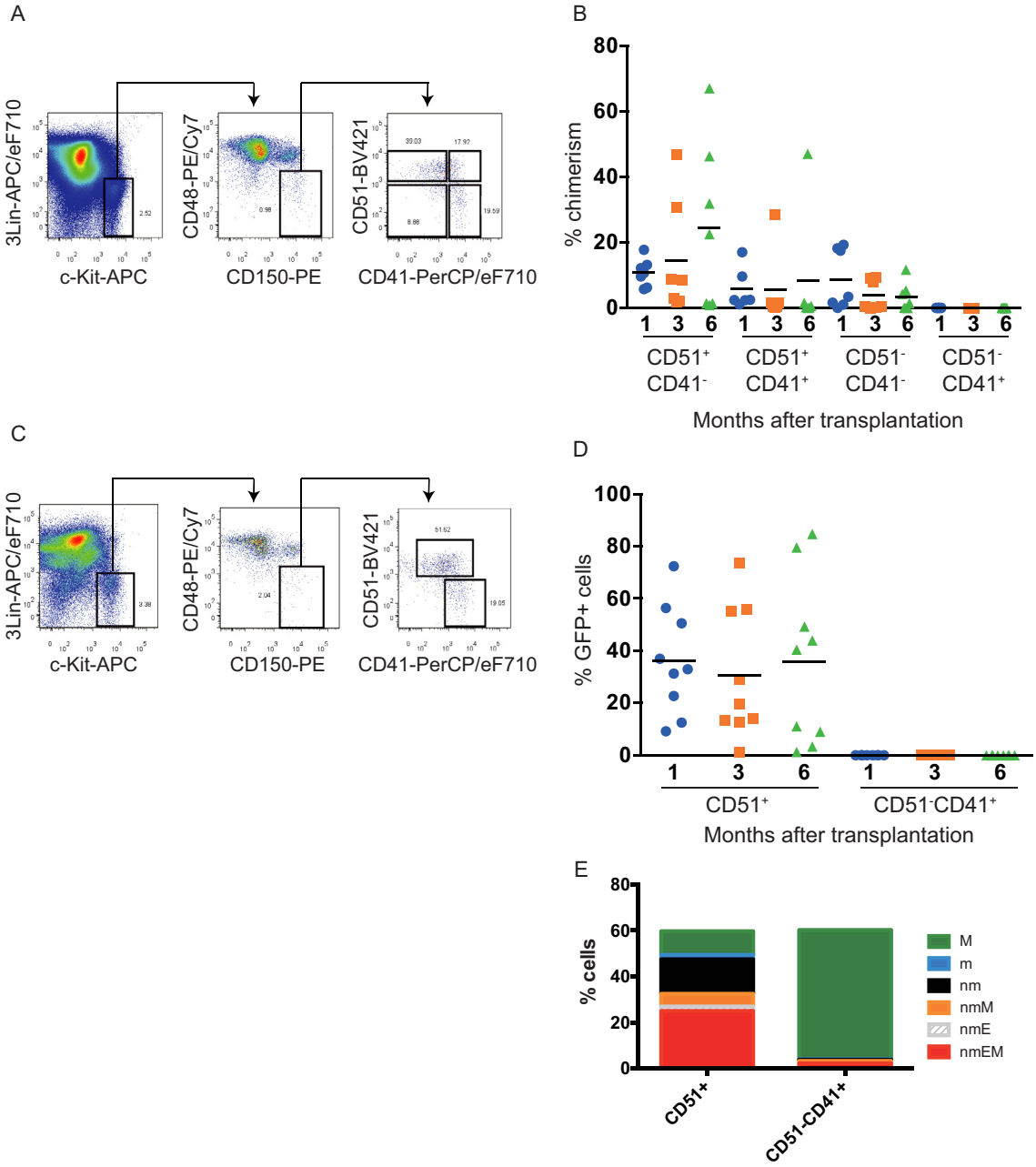

Figure 5. CD51 expression on HSCs. (**A**) Gating for $CD51^+CD41^-$, $CD51^+CD41^+$, $CD51^-CD41^-$, and $CD51^-CD41^+CD150^+48^-$ KL_3 cells. Three antibodies (anti-Gr-1, B220, and Ter-119) were used in this sorting (L_3). (**B**) Twenty $CD51^+CD41^-$, $CD51^+CD41^+$, $CD51^-CD41^-$, and $CD51^-CD41^+CD150^+48^-$ KL_3 cells were transplanted into 10 lethally irradiated mice with 5×10^5 competitor cells. The percentage of chimerism in the peripheral blood of recipient mice 1, 3, and 6 months after transplantation is shown. Three, three, four, and three mice died after transplantation with $CD41^{neg}$, $CD41^{low}$, $CD41^{high}$, and $CD150^+48^-$ KL_3 cells, respectively. No significant difference was found in the percentage of chimerism among $CD51^+CD41^-$, $CD51^+CD41^+$, and $CD51^-CD41^-$ groups (nonparametric analysis of variance). (**C**) Gating for $CD51^+$ and $CD51^-CD41^+CD150^+48^-$ KL_3 cells is shown. (**D**) Twenty $CD51^+$ and $CD51^-CD41^+$ $CD150^+48^-$ KL_3 cells from GFP transgenic mice were transplanted into 10 lethally irradiated B6 mice with 5×10^5 competitor cells. The percentages of GFP+ cells in the peripheral blood of recipient mice 1, 3, and 6 months after transplantation are illustrated. One and two mice died after transplantation with $CD51^+$ and $CD51^-CD41^+$ $CD150^+48^-KL_3$ cells, respectively. (**E**) Lineage compositions in colonies formed by $CD51^+$ and $CD51^-CD41^+CD150^+48^-KL_3$ cells. Single-cell colony assays were performed on 60 cells each. Colonies comprised neutrophils (n), macrophages (m), erythroblasts (E), and megakaryocytes (M). The mean from six independent experiments is shown.

experiment, the CD41 gate was not used for sorting, regardless of whether anti-CD41 antibody was used (Figure 6A–D). There was no significant difference in reconstitution activity 6 months after transplantation between the groups in which antibody was added and the groups in which antibody was not added (Figure 6F).

Anti-CD41 antibody is not essential for HSC purification

We finally examined how CD41 gating was effective for further purification of HSCs. Single $CD150^{+}48^{-}34^{-}KSL_3$ cells (Figure 6A–D) and $CD41^{-}CD150^{+}48^{-}34^{-}KSL_3$ cells (Figure 6A–E) were sorted from the same stained sample and transplanted into a group of 20 lethally irradiated mice with competitor cells. No significant difference was found in the frequencies of repopulating cells between these two groups (Figure 6G). In this single-cell transplantation, the percentage of chimerism was not taken into account because the number of repopulating cells in competitor cells appeared to influence the results more than the reconstitution potential level in single test donor cells [24]. As $CD41^{high}$ cells accounted for a small proportion of $CD150^{+}48^{-}34^{-}KSL_3$ cells, the selection of CD41-negative cells did not increase the purity of LT-HSCs.

Discussion

Over the last decades, flow cytometry instruments have been significantly improved mainly because of the development of new laser and fluorescent dyes. As a result, the stable sorting of very rare cells with high speed and efficiency has become possible. We were able to address the controversial issues with a small number of sorted cells or even at the single-cell level. We detected single Mac-1-negative and -low HSCs in adult BM, consistent with previous studies [4–6]. A low level of Mac-1 expression is detected in fetal liver HSCs [3]. Mac-1^{low} HSCs in the adult BM might have arisen from the fetal liver. We detected single CD41-negative and -low HSCs, but not CD41-high HSCs, in adult BM. A low level of CD41 expression is detected in pre-HSCs at the aorta–gonad–mesonephros region [25,26]. The proportion of $CD41^{low}$ HSCs in BM increases in aged mice [13,27]. These data suggest that the expression of these integrin molecules on HSCs is developmentally regulated. Hence, knowing their functional role in the regulation of HSCs is of great interest.

In general, the percentage of chimerism is taken into account for transplantation of bulk cells, but not for single-cell transplantation. In the case of bulk cell transplantation, the percentage of chimerism depends on the numbers of repopulating cells in both test donor cells and competitor cells. To compare the reconstitution potentials in different test donor cells, we may be able to compare the means of chimerism if the numbers of repopulating cells in both test donor cells and competitor cells and the number of recipient mice are sufficient. However, in the case of single-cell transplantation, the percentage of chimerism depends only on the number of repopulating cells in competitor cells. Therefore, the frequency of positive mice is more meaningful than the level of reconstitution in positive mice [24]. The time length and lineage contribution after transplantation are also important parameters in single-cell transplantation.

Competitive repopulation has served as the most reliable assay for HSCs. However, it is often difficult to obtain the same percentages of chimerism from different experiments with this in vivo assay. It is likely that the sorting efficiency, the number of repopulating cells in test donor cells and competitor cells, and the conditions of donor and recipient mice may somewhat differ from experiment to experiment. Therefore, it is important to repeat experiments to have consistent data. In this study, we instead performed both bulk-cell transplantation and single-cell transplantation, providing complementary data to compare HSC activity in two or more samples.

Both ST lymphoid-biased HSCs and LT myeloid-biased HSCs were detected in Mac-1-negative and -low fractions (Figure 2). Similarly, both ST lymphoid-biased HSCs and LT myeloid-biased HSCs were detected in CD41-negative and -low fractions (Figure 4). These data suggested that the expression of Mac-1 and CD41 is not associated with the self-renewal potential and lineage-biased differentiation potentials in HSCs. Platelet-biased HSCs have been reported [28–30]. These HSCs were marked with von Willebrand factor (vWF) expression by using vWF-reporter transgenic mouse. vWF^{+} platelet-biased HSCs were detected in both CD41-negative and -low $CD150^{+}48^{+}34^{-}KSL$ cells [29]. These data together with our data suggested that CD41 expression level is not related to the potentials of LT-HSCs and platelet-biased HSCs.

$CD150^{+}201^{+}48^{-}34^{-}KS$ cells (Supplementary Figure E2E, online only, available at www.exphem.org) are enriched in LT-HSCs, whereas $CD150^{-}201^{+}48^{-}34^{-}KS$ cells (Supplementary Figure E2F) are enriched in ST-HSCs [18,31]. The proportion of Mac-1^{low} cells increased and the proportion of $CD41^{low}$ cells decreased in the $CD150^{-}$ ST-HSC population compared with that of the $CD150^{+}$LT-HSC population. It was most likely that CD51 was expressed with CD29 (Itgb1) in HSCs (Figure 1). The expression of CD51/CD29 and possibly CD51/CD61 also was gradually replaced by that of CD41/CD61 in the megakaryocyte

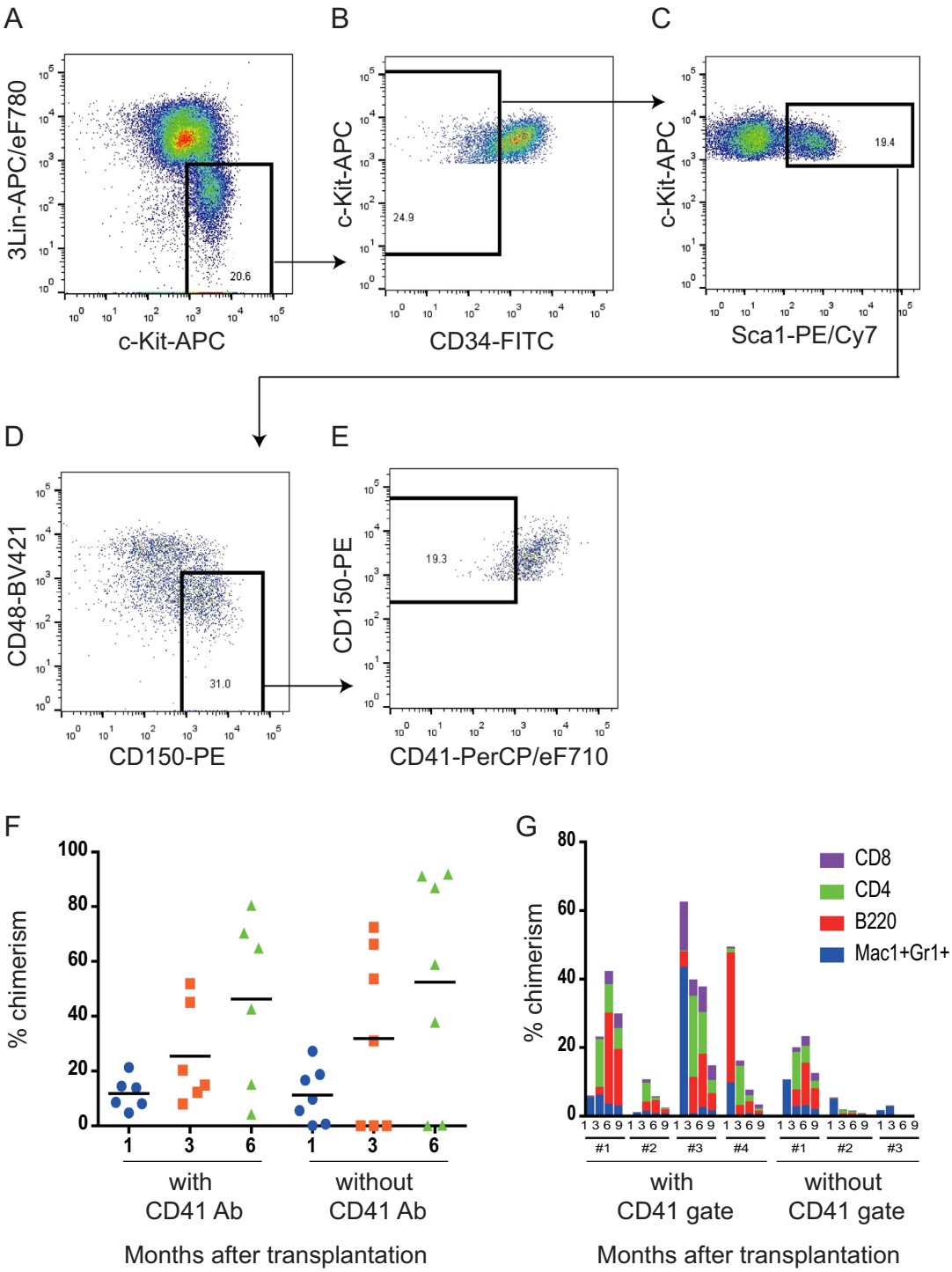

Figure 6. HSC purification with or without use of anti-CD41 antibody or the CD41 gate. (**A–D**) Sequential gating for $CD150^{+}48^{-}34^{-}$ KSL_3 cells is shown. (**E**) CD41-negative gate. (**F**) Effect of anti-CD41 antibody. Twenty $CD150^{+}48^{-}34^{-}$ KSL_3 cells were transplanted into lethally irradiated mice together with 5×10^5 BM competitor cells after staining with anti-CD41 antibody but without use of CD41 gating (shown as with CD41 Ab). Twenty $CD150^{+}48^{-}34^{-}$ KSL_3 cells were transplanted into lethally irradiated mice together with 5×10^5 BM competitor cells without use of anti-CD41 antibody (indicated as without CD41 Ab). No significant difference was found in the percentage of chimerism between groups with and those without antibody (Mann–Whitney test). (**G**) Single $CD150^{+}48^{-}34^{-}$ KSL_3 cells were transplanted into 20 lethally irradiated mice with 5×10^5 competitor cells (shown as with CD41 gate). Single $CD150^{+}41^{-}48^{-}34^{-}$ KSL_3 cells after gating CD41-negative cells (shown in E) were transplanted into 20 lethally irradiated mice with 5×10^5 competitor cells (indicated as without CD41 gate). After transplantation with $CD150^{+}48^{-}34^{-}$ KSL_3 and $CD150^{+}41^{-}48^{-}34^{-}$ KSL_3 cells, respectively, three and four mice died by 9 months. The percentages of chimerism in Mac-1/Gr-1^{+} myeloid, $B220^{+}$ B, $CD4^{+}$ T, and $CD8^{+}$ T cells are given for individual mice (#1–4 with CD41 gate and #1–3 without CD41). No significant difference was observed in the frequencies of HSCs between groups with and those without the CD41 gate (Fisher's exact test).

differentiation pathway (Figure 5). CD41 expression increased while a high level of CD150 expression was maintained along the megakaryocyte differentiation pathway [8–10,32]. We used anti-CD41 antibody, which has a neutralizing effect. However, we did not detect any neutralizing activity in this study, presumably because the amount of antibody we used for staining was insufficient to exert such an effect. In vitro megakaryocyte colony formation by $CD41^{high}$ $CD150^{+}48^{-}KL$ cells was consistent with previous studies [8–10]. Because $CD150^{+}201^{+}48^{-}34^{-}KSL_3$ cells uniformly expressed CD51 (data not shown), the use of CD51 as an additional marker appeared to be not useful for further purification of HSCs.

The expression of Mac-1 and CD41 seemed almost mutually exclusive in HSC populations (Supplementary Figure E2). We speculated the existence of two distinct differentiation pathways. In one pathway, LT-HSCs give rise to ST-HSCs, and this differentiation pathway is at least in part marked by Mac-1 expression. The other pathway is the myeloid and megakaryocyte differentiation pathway, which is marked by CD41 expression. Because a low-level expression of Mac-1 and CD41 in HSC1 is not associated with HSC function, we might consider their expression as "footprints" of HSCs at this very early stage of hematopoiesis.

Finally, concerning the HSC purification protocols, this study suggested that the use of anti-Mac-1 antibody should be excluded from lineage depletion and that the use of anti-CD41 antibody has little advantage with respect to the degree of purification of HSCs.

Acknowledgements

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Conflict of interest disclosure

The authors declare no competing financial interests.

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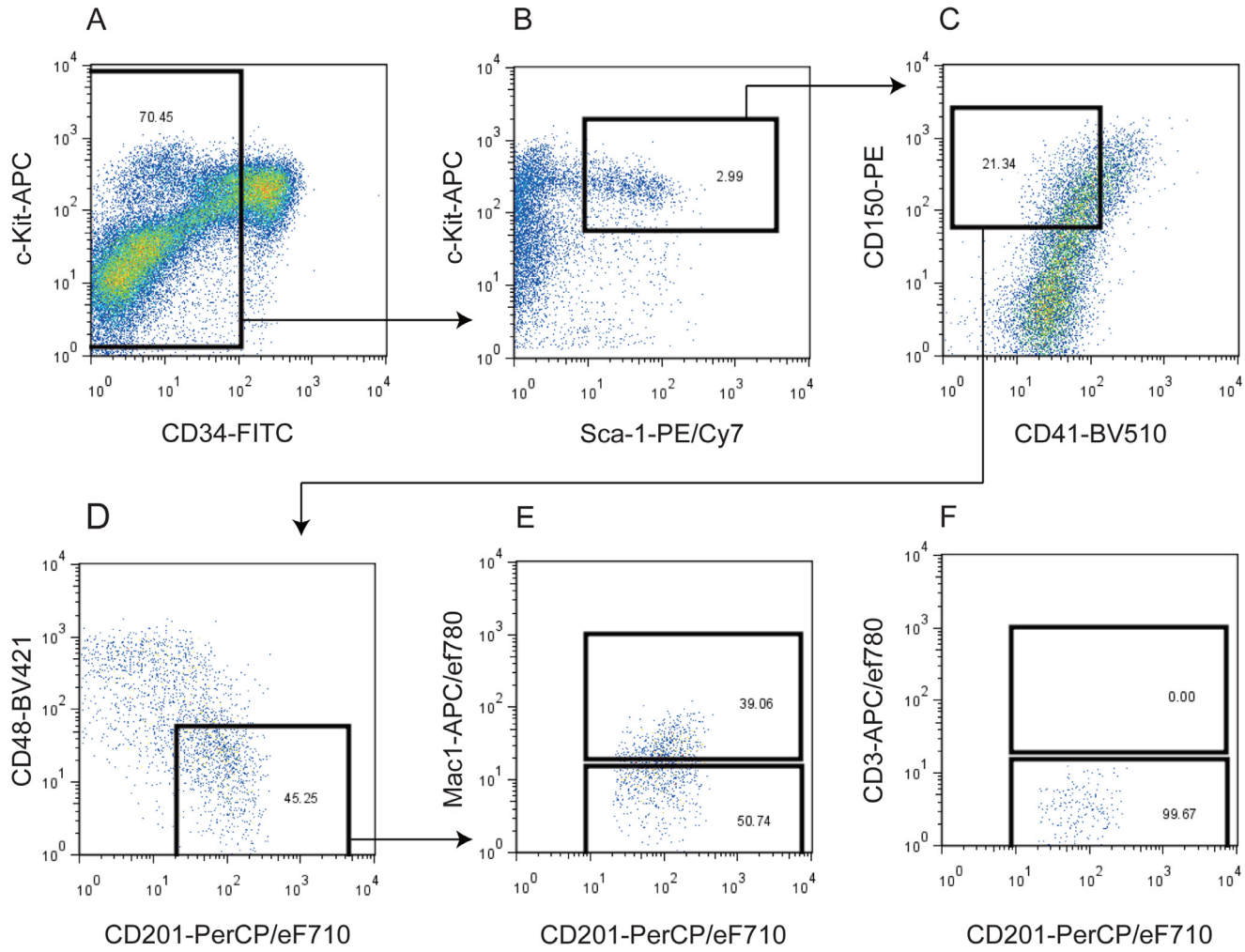
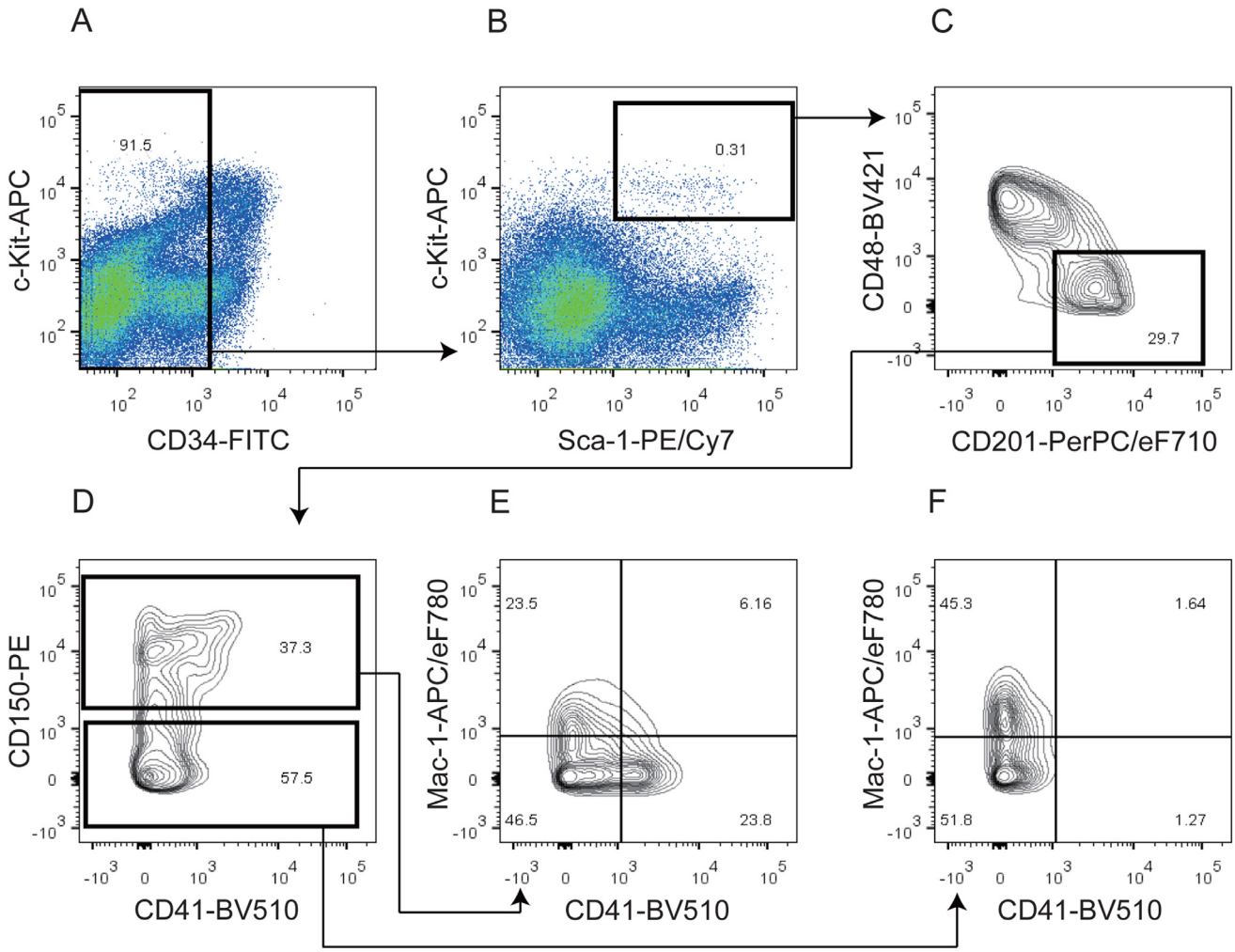


Supplementary Figure E1. Sorting gates for Mac-1-negative or -low $CD201^{+}150^{+}41^{-}34^{-}$ KS cells. (**A–D**) Sequential gating for $CD201^{+}150^{+}41^{-}34^{-}$ KS cells. (**E**) Sorting gates of Mac-1-negative and Mac-1-low $CD201^{+}150^{+}41^{-}34^{-}$ KS cells. (**F**) CD3 was used as a negative control.



Supplementary Figure E2. Mac-1 and CD41 expression in LT- and ST-HSCs. (**A**) $CD34^{-/low}$ cells were gated. (**B**) $c\text{-}Kit^{+}Sca\text{-}1^{+}$ cells were gated. (**C**) $CD201^{+}CD48^{-}$ cells were gated. (**D**) Either $CD150^{+}$ cells or $CD150^{-}$ cells were gated. (**E**) CD41 and Mac-1 expression on $CD150^{+}201^{+}48^{-}34^{-}$KS cells. (**F**) CD41 and Mac-1 expression on $CD150^{-}201^{+}48^{-}34^{-}$ KS cells.