Vinculin Is Indispensable for Repopulation by Hematopoietic Stem Cells, Independent of Integrin Function*12

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Tsukasa Ohmori1,†, Yuji Kashinakura†, Akira Ishiwata†, Seiji Madoiwa†, Jun Mimuro†, Yusuke Furukawa9, and Yoichi Sakata2†

From the 1Research Division of Cell and Molecular Medicine and the 2Division of Stem Cell Regulation, Center for Molecular Medicine, Jichi Medical University, Tochigi 329-0498, Japan

Vinculin is a highly conserved actin-binding protein that is localized in integrin-mediated focal adhesion complexes. Although critical roles have been proposed for integrins in hematopoietic stem cell (HSC) function, little is known about the involvement of intracellular focal adhesion proteins in HSC functions. This study showed that the ability of c-Kit+/Sca-1+/Lin- HSCs to support reconstitution of hematopoiesis after competitive transplantation was severely impaired by lentiviral transduction with short hairpin RNA sequences for vinculin. The potential of these HSCs to differentiate into granulocytic and monocytic lineages, to migrate toward stromal cell-derived factor 1α (SDF-1α), and to home to the bone marrow in vivo were not inhibited by the loss of vinculin. However, the capacities to form long term culture-initiating cells and cobblestone-like areas were abolished in vinculin-silenced c-Kit+/Sca-1+/Lin- HSCs. In contrast, adhesion to the extracellular matrix was inhibited by silencing of talin-1, but not of vinculin. Whole body in vivo luminescence analyses to detect transduced HSCs confirmed the role of vinculin in long term HSC reconstitution. Our results suggest that vinculin is an indispensable factor determining HSC reconstitution capacity, independent of integrin functions.

The development and maintenance of hematopoietic stem cells (HSCs),‡ which can self-renew and differentiate into all hematopoietic blood cell lineages, are thought to depend on their interactions with the microenvironment, referred to as their "niche" (1, 2). Direct molecular interactions of HSCs with bone marrow (BM) through N-cadherin and Tie2 reportedly sustain HSC maintenance and self-renewal (3, 4). Although the importance of integrins and their ligand interactions in the hematopoietic niche are not fully understood, critical roles have been proposed for integrins in HSC functions. HSCs mainly express integrins α4, α5, and α6, which can associate with integrin β1 (5). Integrin β1-null HSCs fail to engraft in irradiated recipient mice, because of sequestration of the HSCs into the circulation (failure of HSCs to home to the BM and spleen) (6). Conditional ablation of integrin α4 in hematopoietic cells results in sustained and significant increases in circulating progenitors (7), whereas administration of a function-blocking antibody (Ab) or blocking peptide against integrin α4 reduces HSC homing and leads to HSC release into the blood (8, 9). Furthermore, integrin α4β1-mediated attachment of HSCs to fibronectin promotes their proliferation and survival (10).

Integrins are heterodimeric receptors formed by noncovalent associations between α and β subunits. There are at least 24 different combinations of subunits, each of which can bind to a specific ligand, including extracellular matrix components (ECM) and soluble ligands, after their activation (11). The conformation of integrins is tightly controlled through interactions between cytoplasmic focal adhesion proteins and the integrin cytoplasmic tails, which regulate the transition from a low to a high affinity ligand-binding state (12, 13). Among these integrin-associated proteins, talin, the kindlin family, and integrin-linked kinase have emerged as essential for integrin activation and linkage to the actin cytoskeleton (14–18).

Vinculin is a highly conserved actin-binding protein that is frequently used as a marker for integrin-mediated cell-ECM interactions (19). Although vinculin itself does not bind directly to integrins, it is thought to play key roles in focal adhesion assembly and cell adhesion (19). The crystal structure of vinculin reveals that its N-terminal head domain forms a complex with the rod domain of talin, which contains three vinculin-binding sites (20, 21). Vinculin is autoinhibited by intramolecular interactions (22), but its conformation can be changed by interactions with talin and actin, making it functionally active (23). Although numerous studies have investigated the structure of vinculin and its associations with talin and other cytoskeletal proteins, the precise role of these cytoskeletal proteins in directly modulating the function of HSCs remains to be elucidated. In this study, we focused on the involvement of two well known focal adhesion proteins, vinculin and talin-1, in HSC function, and provide the first evidence that vinculin is

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† To whom correspondence may be addressed: Yakushiji 3311-1 Shimotsuke, Tochigi 329-0498, Japan. Tel.: 81-285-58-7397; Fax: 81-285-44-7817; E-mail: tohmori@jichi.ac.jp.

‡ To whom correspondence may be addressed: Yakushiji 3311-1 Shimotsuke, Tochigi 329-0498, Japan. Tel.: 81-285-58-7397; Fax: 81-285-44-7817; E-mail: yoisaka@jichi.ac.jp.

§ The abbreviations used are: HSC, hematopoietic stem cell; BM, bone marrow; Ab, antibody; ECM, extracellular matrix; EGFP, enhanced GFP; MOI, multiplicity of infection; KSL, c-Kit+/Sca-1+ Lineage; TPO, thrombopoietin; CFU-GM, colony-forming units of granulocytes/macrophages; CFU-G, colony-forming units of granulocytes; LTC-IC, long term culture-initiating cells.
involved in HSC repopulation through an integrin-independent mechanism.

**EXPERIMENTAL PROCEDURES**

An expanded “Methods” section including detailed information on materials, bone marrow transplantation, RT-PCR, and immunoblotting is provided in the supplemental materials.

**Construction of Lentiviral Vectors and Virus Production**—The gene transfer vector pLL3.7 (LentiLox) for constructing replication-defective self-inactivating HIV lentiviral vectors expressing shRNA sequences was purchased from ATCC (Manassas, VA) (see Fig. 1A) (24). Putative shRNA sequences were designed using the web software provided by Dharmacon RNA Technologies. Each shRNA sequence was cloned into the HpaI and XhoI sites of pLL3.7 (see Fig. 1A and supplemental Table S1) (24). The control (random) and talin-1 (Talin-A) shRNA sequences have been reported previously (25). We designed three shRNA sequences for mouse vinculin, designated Vin-B, Vin-C, and Vin-D, and found that ectopic expression of mouse vinculin in HEK293 cells was significantly inhibited by cotransfection with the constructs expressing the Vin-B and Vin-C shRNA sequences (data not shown). We therefore selected the Vin-B and Vin-C sequences for use in this study. The lentiviral vectors were essentially generated as described previously (26). The vector titers of LentiLox carrying enhanced green fluorescent protein (EGFP) were measured by infection of 1 × 10^5 UT-7/TPO cells (a megakaryoblastic cell line). EGFP expression was measured by FACS analysis at 48 h after transduction. Typically, 1 μl of concentrated vector solution was able to transduce EGFP in 50–60% of the cells, and the vector titers for UT-7/TPO cells were considered to be 5–6 × 10^7 cells/ml. Because the vector titers against HEK293 cells were much higher than those against UT-7/TPO cells, the multiplicity of infections (MOIs) used in this study were lower than those in our previous studies (25, 26).

**Isolation of c-Kit^+ Sca1^+ Lin^- (KSL) HSCs and Competitive Reconstitution Assay**—C57BL/6 mice (Ly5.2) and C57BL/6 mice congenic for the Ly5 locus (Ly5.1) were purchased from Japan SLC (Shizuoka, Japan) and Sankyo–Lab Service (Tsukuba, Japan), respectively. All of the animal procedures were approved by the Institutional Animal Care and Concern Committee of Jichi Medical University, and animal care was performed in accordance with the committee guidelines. BM cells were depleted of cells expressing the cell lineage markers B220, CD3e, CD11b, Gr-1, and Ter-119 (biotin-conjugated mouse lineage panel; BD Biosciences Co., San Jose, CA) by magnetic cell sorting using streptavidin microbeads (Miltenyi Biotec, Gladbach, Germany) and a magnetic cell sorter (autoMACSTM; Miltenyi Biotec). The remaining cells were sorted for KSL cells by FACS (FACSAria™ Cell Sorter; BD Biosciences) (see Fig. 1B). CD34^low^ KSL cells, comprising HSCs with long term BM repopulating ability, were defined as the lowest 20% of the KSL cell population in terms of CD34 expression. The isolated KSL cells were cultured in StemPro®-34 SFM medium (Invitrogen) supplemented with 100 ng/ml stem cell factor, 100 ng/ml TPO, 100 ng/ml IL-6, 100 ng/ml fms-like tyrosine kinase 3 ligand, and 200 ng/ml soluble IL-6 receptor for 16 h before lentiviral transduction. The cells were transduced with lentiviral vectors at a MOI of 20 in the presence of the same cytokines and polybrene (8 μg/ml). After the transduction with LentiLox vectors, EGFP expression was confirmed in at least 80% of the transduced KSL cells in the subsequent experiments (see Fig. 1C). When EGFP-positive cells comprised less than 80% of the cells, the EGFP-positive KSL cells were further sorted by FACS, and the sorted cells were then used in subsequent experiments on the following day.

Competitive reconstitution assays were performed using the Ly5 congenic mouse system. Recipient Ly5.2 mice (8–12 weeks of age) were irradiated with a single lethal dose of 9.5 gray (Gamma Cell; Norton International), followed by injection of 1 × 10^6 transduced KSL cells from Ly5.1 donor mice, together with 2 × 10^6 freshly isolated Ly5.2 unfractionated BM cells.

** Colony-forming Cell Assays**— Colony-forming cell assays were performed as described previously (27). Briefly, KSL cells were transduced with LentiLox vectors at a MOI of 20. Transduced cells (2 × 10^5) were then suspended in 1 ml of MethoCult™M3231 medium (StemCell Technologies) supplemented with 20 ng/ml of mouse granulocyte macrophage-colony stimulating factor or 20 ng/ml of granulocyte-colony stimulating factor (for colony-forming units of granulocytes/macrophages (CFU-GM) or colony-forming units of granulocytes (CFU-G), respectively) and plated on 3.5-cm dishes. Colonies containing at least 30 cells on day 5 were counted under a microscope in five random fields at a magnification of ×40 by a blinded observer. The mean value from the duplicate experiments was treated as the result of a single independent experiment.

**Cell Migration**—Cell migration was assessed by a modified Boyden chamber assay using Costar Transwell® cell culture inserts (Corning Inc., Canton, NY) with polycarbonate filters, with 8-μm pores separating the upper and lower chambers. Transduced KSL cells were added to the upper chamber at a density of 1 × 10^5 cells/100 μl of DMEM/Ham’s F-12 medium containing 1% fatty acid-free BSA and incubated for 4 h at 37 °C. The cells were allowed to migrate toward 100 ng/ml of stromal cell-derived factor 1α in the lower chamber. Following migration, the cells in the lower chamber were recovered by centrifugation and then lysed using 200 μl of CyQUANT® GR dye/cell lysis buffer (Invitrogen). The cell number was quantified by measuring the fluorescence with excitation at 480 nm and emission detection at 520 nm.

**HSC Homing to the BM and Spleen**—HSC homing assays were essentially performed as described previously (28). Briefly, transduced KSL cells (1 × 10^5 cells) were injected into lethally irradiated recipient mice (9.5 gray). At 16–20 h after transplantation, the cells from femurs and spleen were harvested, and 50% of a femur and 10% of the spleen homogenate/dish were used for CFU-GM assays, as described above. The CFU-GM colonies were counted manually by a blinded observer and considered to represent homing KSL cells in the BM or spleen after transplantation. Two percent of KSL cells were directly assessed for CFU-GM assays in each experiment to estimate the total number of CFU-GM colonies derived from the transplanted KSL cells. The mean values of duplicate experiments were calculated. The number of recovered CFU-GM was corrected to represent the whole BM, based on the estimate...
that one femur represents 5.9% of the total BM (28, 29). The data are presented as the percentages of homing KSL cells; the total number of CFU-GM colonies from the BM or spleen was divided by the estimated number of CFU-GM colonies from injected KSL cells (number of CFU-GM from 2% of KSL cells multiplied by 50). One mouse for each experiment was irradiated but did not receive KSL cells to assess the number of residual host-derived CFU-GM; only 0–1 CFU-GM were observed in these BM and spleen samples.

**Cell Proliferation Assay and Cell Cycle Analysis in Liquid Culture**—Transduced KSL cells (1 × 10^5 cells) were suspended with 1 ml of StemPro-34 SFM medium supplemented with 50 ng/ml stem cell factor, 50 ng/ml TPO, 50 ng/ml IL-6, 50 ng/ml fms-like tyrosine kinase 3 ligand, and 100 ng/ml soluble IL-6 receptor in 12-well plates. The cell numbers were determined at specific time points using Flow-Count fluorospheres (Beckman Coulter, Miami, FL). All of the experiments were performed in triplicate, and the mean value was considered to represent the result of a single independent experiment. For cell cycle analysis, the cultured KSL cells (1–2 × 10^5 cells) were suspended in 1 ml of StemPro-34 SFM medium supplemented with the same cytokine combination at 48–72 h after lentiviral transduction. The cells were incubated with 10 μl of 1 μm BrdU for 30 min. The cell cycle positions and active DNA synthetic activities of the cells were determined by analyzing the correlated expression of total DNA (7-aminoactinomycin D) and incorporated BrdU levels (anti-BrdU antibody) by FACS, in accordance with the manufacturer’s instructions (BrdU flow kit; BD Biosciences).

**Long Term Culture-initiating Cell (LTC-IC) Assay**—LTC-IC assays were carried out using Myelocult™ M5300 medium (StemCell Technologies), according to the manufacturer’s recommendations, with some modifications. Confluent C3H/10T1/2 cells (Health Science Research Resources Bank, Osaka, Japan) plated on 96-well plates were irradiated at 50 grays. Transduced KSL cells resuspended in 150 μl of Myelocult™ M5300 medium in limiting dilution solutions from 300 to 37.5 cells were seeded in 12 wells each and cultured for 4 weeks at 33 °C under 5% CO2 with weekly half-medium changes. All of the cells were subsequently resuspended in 500 μl of methylcellulose medium (MethoCult™ M3434; StemCell Technologies), and CFU colonies were counted on day 12. The frequencies of LTC-IC were expressed as percentages of the positive wells (≥1 CFU/well) among 12 wells.

**Cobblestone-like Area Forming Assay**—Cobblestone-like area forming assays were essentially performed as reported previously (30). C3H/10T1/2 cells were cultured in 96-well plates and then irradiated with a single dose of 50 grays. Transduced KSL cells (62 cells) were suspended in 2 ml of Myelocult™ M5300 medium (StemCell Technologies). 150 μl of cell suspension was plated onto the irradiated C3H/10T1/2 cells and then cultured at 33 °C in 5% CO2. Half of the medium was changed every week. The presence of cobblestone-like areas per well was manually assessed by a blinded observer at 28 days after seeding. The frequency of positive wells (containing one or more cobblestone areas) was expressed as a percentage of the positive wells among 12 wells.

**Assessment of Transplanted KSL Cell Fates by Bioluminescence Studies**—The fates of transduced KSL cells in identical recipient mice in vivo were assessed using bioluminescence imaging to assess directly the luciferase activities derived from the transduced KSL cells. KSL cells (Ly5.1) were transduced with LentiLox vectors expressing luciferase (instead of EGFP) and then transplanted into lethally irradiated recipient mice. Luciferase activities derived from the KSL cells were determined after anesthetizing the mice with isoflurane and intraperitoneally injecting them with the luciferin substrate (1.5 mg/body). Photons transmitted through the body were collected for a specified length of time and analyzed using an IVIS Imaging System and Living Image software (Xenogen Corp., Alameda, CA). Quantitative data were expressed as photon units (photons/s). To confirm the transduction efficacies of KSL cells, the transduced KSL cells were directly assessed by the addition of the luciferin substrate (300 μg/ml) before transplantation, and the bioluminescence activity was measured for 3 min.

**Cell Adhesion and Immunofluorescence Microscopy**—Twenty-four-well tissue culture plates were coated with 10 μg/ml of fibronectin (Sigma-Aldrich) for 16 h at 4 °C or for 2 h at room temperature. After blocking of the plates with 2% BSA, 1 × 10^5 transduced KSL cells were suspended in 250 μl of StemPro-34 SFM medium, added to the plates with or without 2 mM EDTA or 2 mM MnCl2, and incubated for 3 h. After washing with PBS, the numbers of adherent cells were quantified by the fluorescence of the CyQUANT® GR dye (Invitrogen), as described above. To observe adherent KSL cells by microscopy, dishes (4-well Lab-Tek® Chamber Slide™) were coated with 10 μg/ml fibronectin for 16 h at 4 °C, washed twice with PBS, and blocked with 2% BSA for 1 h. Transduced KSL cells (2 × 10^4) were placed onto the fibronectin-coated dishes for 3 h at 37 °C. After washing twice with PBS, adherent cells were fixed with 3% paraformaldehyde in PBS for 40 min and then permeabilized with PBS containing 0.3% Triton X-100 and 5% donkey serum for 2 h. After washing with PBS, the cells were incubated with biotin-conjugated anti-GFP polyclonal Ab (200 molecules/ml rhodamine-conjugated phallolidin (Sigma-Aldrich). The samples were mounted in Vectashield with DAPI (Vector Laboratories) and observed by confocal microscopy (FV1000; Olympus, Tokyo, Japan). When indicated, the cell area was quantified using Image J Ver. 10.2 for Macintosh (National Institutes of Health, Bethesda, MD) by a blinded observer.

**RESULTS**

**Lentiviral Vector-mediated shRNAs Efficiently Inhibit Expression of Target Proteins in KSL HSCs**—A lentiviral vector, in which the U6 RNA polymerase III promoter drives the expression of an shRNA for the target protein and the CMV promoter drives EGFP expression, was applied to inhibit target protein expression in KSL HSCs (Fig. 1A; LentiLox). A nonspecific control shRNA (random sequence), an shRNA directed against mouse talin-1 (Talin-A sequence), and two independent
shRNAs against mouse vinculin (Vin-B and Vin-C sequences) were designed. These sequences were confirmed to effectively inhibit ectopic expression of the target proteins in HEK293 cells (data not shown). The efficacies of these lentiviral constructs for inhibiting expression of the target mRNAs and proteins in HSCs were also examined. KSL HSCs isolated by FACS (Fig. 1B) were transduced with LentiLox vectors containing the random, Talin-A, Vin-B, or Vin-C sequences at MOIs of 20. We confirmed that the transduction efficiencies were as high as 80% of the KSL HSCs (Fig. 1C). The expression levels of the target proteins and their mRNAs in KSL cells were significantly inhibited by expression of the shRNA after transduction (Fig. 1D–F and supplemental Fig. S1). Vinculin expression in HSCs was inhibited more strongly by expression of the Vin-B sequence than by expression of the Vin-C sequence. Although levels of vinculin mRNA were marginally inhibited by the Talin-A sequence (~15%) (Fig. 1D), its protein levels were not significantly inhibited (Fig. 1F). The talin-1 shRNA sequence was confirmed to include five or more mismatch sequences for other mouse mRNAs in a BLAST search.

Expression of shRNAs against Vinculin Reduce Short Term Hematopoietic Engraftment—BM cells transduced with LentiLox vectors equipped with the random, Talin-A, Vin-B, or Vin-C shRNA sequences were transplanted to investigate the roles of vinculin and talin-1 in hematopoietic cell reconstitution after BM transplantation. After transplantation of the transduced BM cells at a MOI of 5, vinculin silencing resulted in fewer EGFP-positive peripheral blood cells, including CD45-positive white blood cells, red blood cells, and platelets, compared with control experiments (supplemental Fig. S2). Although talin-1 silencing in BM cells did reduce EGFP expression in peripheral blood cells, its inhibitory effects were weaker than those of vinculin silencing (supplemental Fig. S2). Despite the fact that not all blood cells were labeled with EGFP in this procedure, the method could still be used to determine the role of these proteins in HSC fate because the lentiviral vector used was able to simultaneously express the shRNA sequences and EGFP in identical transduced cells.

KSL cells transduced with the LentiLox vectors at a MOI of 20 were then transplanted together with nontransduced com-
petitor cells into lethally irradiated recipient mice to examine the contribution of vinculin to HSC engraftment. As predicted based on the results of BM cell transplantation, vinculin silencing significantly reduced EGFP expression not only in white blood cells but also in platelets and red blood cells after transplantation of the transduced KSL HSCs (Fig. 2, A and B). Similar results were obtained at 2 months after transplantation (supplemental Fig. S3). EGFP expression in BM cells was also examined. c-Kit is expressed on HSCs and progenitor cells, whereas the KSL cell fraction contains more primitive stem cells (31). KSL cells were further subdivided according to their surface expression of CD34. CD34lowKSL cells are highly enriched for HSCs with long term marrow repopulating ability, whereas CD34+ c-Kit+Sca-1−Lin− (CD34highKSL) cells are progenitors with short term reconstitution capacity, suggesting that CD34lowKSL cells have a higher rank in the hematopoietic hierarchy than CD34highKSL cells (32). The proportion of EGFP-positive cells was reduced by vinculin silencing not only in the progenitor fraction (c-Kit+Sca-1− cells) but also in more primitive HSCs (CD34lowKSL cells), thus strengthening the initial hypothesis that vinculin controls HSC fate after transplantation (Fig. 2C). EGFP expression in Lin− BM cells was also inhibited after transplantation (supplemental Fig. S4). The chimerism percentage (Ly5.1/Ly5) in peripheral white blood cells after transplantation represents the relative reconstitution ability of transduced KSL cells (Ly5.1) compared with nontransduced competitor cells (Ly5.2). The Ly5.1/Ly5 ratio after transplantation was reduced by vinculin silencing (Fig. 2D). In contrast, the CFU-GM and CFU-G colony forming capacities of KSL cells were not affected by expression of the vinculin shRNA sequences (Fig. 3), suggesting that vinculin silencing did not affect the capacities of KSL cells to differentiate into granulocytic lineage cells. These results indicate that KSL HSCs lacking vinculin do not engraft efficiently after transplantation.
liferation was investigated to distinguish between these possibilities. Transduced KSL cells were cultured in vitro in serum-free medium containing a cytokine combination, and the cell numbers were assessed by FACS. As shown in Fig. 4A, cell proliferation in response to the cytokine combination was impaired in KSL HSCs expressing shRNA sequences for vinculin but not talin-1. The cell cycle of KSL cells in liquid culture was further investigated by FACS. As shown in Table 1, vinculin silencing was associated with an increase in the apoptotic cell fraction and a decrease of the proportion of S phase cells. These results suggest that the loss of vinculin, but not talin-1, affects cell survival through modulation of the cell cycle and induction of apoptosis in HSCs. LTC-IC and cobblestone-like area formation assays were used to examine the self-renewal and proliferative abilities of HSCs on a stromal cell layer. The number of LTC-IC in vinculin-silenced KSL cells was significantly reduced compared with controls (Fig. 4B). The loss of talin-1 also affected the frequencies of LTC-IC, although this effect was weaker than that of vinculin (Fig. 4B). In addition, cobblestone-like area-forming cells on the stromal cell layer were significantly abolished in KSL HSCs transduced with shRNA sequences for vinculin (Fig. 4C).

The homing capacities of the transduced KSL cells were also assessed. Although the expression of CXCR4 was unaffected by either talin-1 or vinculin silencing (Fig. 5A), the chemotactic responses of the transduced HSCs to mouse stromal cell-derived factor 1α in vitro were significantly higher in vinculin-silenced HSCs (Fig. 5B). In addition, the homing of KSL cells to the BM and spleen were unaffected by silencing of either vinculin or talin-1 (Fig. 5C). Taken together, these data suggest that vinculin is not required for HSC homing to the BM but is required for the self-renewal potential and proliferation of HSCs on BM stromal cell layers.

Visualization of Long Term in Vivo Hematopoietic Reconstitution of KSL HSCs—Long term HSC reconstitution after transplantation in identical recipient mice was investigated by replacing the EGFP gene in Lentilox with a luciferase gene, and luciferase activities derived from the transduced HSCs were observed directly using an IVIS	extsuperscript{®} imaging system in vivo (Fig. 6A). The luciferase activities of the transduced KSL cells were similar in all of the groups prior to transplantation (Fig. 6B). Luciferase activities derived from the transduced KSL cells were detected from 7 days after transplantation (Fig. 6B). Consistent with the data for the KSL cell homing experiments (Fig. 5C), early engraftment of HSCs lacking vinculin was achieved.
TABLE 1
Quantitative cell cycle analysis of cultured KSL cells

<table>
<thead>
<tr>
<th>Cell cycle</th>
<th>shRNA sequence</th>
<th>Means ± S.D. (n = 5)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1 phase</td>
<td>Random</td>
<td>31.72 ± 3.53</td>
<td>0.070</td>
</tr>
<tr>
<td></td>
<td>Talin-A</td>
<td>34.12 ± 2.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vin-B</td>
<td>35.02 ± 2.39</td>
<td>0.016*</td>
</tr>
<tr>
<td></td>
<td>Vin-C</td>
<td>32.86 ± 4.21</td>
<td>0.46</td>
</tr>
<tr>
<td>S phase</td>
<td>Random</td>
<td>59.0 ± 3.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Talin-A</td>
<td>51.5 ± 11.0</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Vin-B</td>
<td>45.9 ± 5.35</td>
<td>0.0013*</td>
</tr>
<tr>
<td></td>
<td>Vin-C</td>
<td>53.3 ± 3.83</td>
<td>0.0384*</td>
</tr>
<tr>
<td>G2/M phase</td>
<td>Random</td>
<td>2.82 ± 1.93</td>
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<td></td>
<td>Talin-A</td>
<td>3.06 ± 1.71</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Vin-B</td>
<td>3.16 ± 1.59</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Vin-C</td>
<td>3.06 ± 1.67</td>
<td>0.76</td>
</tr>
<tr>
<td>Apoptotic cells</td>
<td>Random</td>
<td>5.44 ± 1.43</td>
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</tr>
<tr>
<td></td>
<td>Talin-A</td>
<td>6.44 ± 3.73</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Vin-B</td>
<td>14.08 ± 3.71</td>
<td>0.0026*</td>
</tr>
<tr>
<td></td>
<td>Vin-C</td>
<td>7.44 ± 1.41</td>
<td>0.0036*</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with the control experiment (random) under the same conditions (Student’s t test).

until 2 weeks after the transplantation (Fig. 6, C and D). The early luciferase activity derived from KSL cells expressing the Vin-B sequence was slightly attenuated (Fig. 6C). This was expected from the results of the cell proliferation experiments (Fig. 4A). It was notable that the luciferase activities derived from HSCs lacking vinculin seemed to gradually decrease, compared with the activities derived from control and talin-silenced cells (Fig. 6, C and D). These results support the idea that vinculin is an indispensable factor for HSC repopulation in the BM.

**Loss of Talin-1, but Not Vinculin, Impairs Adhesion of KSL Cells to the ECM—**The effect of vinculin silencing on HSC function, through integrin expression and adhesion to the ECM, was investigated. As shown in Fig. 7A, the expression levels of integrins β1 and β3 in KSL cells were unaffected after transduction of LentilOx vectors equipped with shRNA sequences for talin-1 and vinculin. Interestingly, adhesion to the ECM in the static condition was significantly inhibited in talin-deficient transduced KSL cells but not in vinculin-deficient transduced KSL cells (Fig. 7B). Cell spreading onto fibronectin was also investigated by confocal microscopy. The silencing of talin-1, but not of vinculin, resulted in a failure of cells to spread on fibronectin after adhesion (Fig. 7C and supplemental Fig. 5). These results indicate that vinculin is not involved in the attachment and spreading of KSL cells to the ECM in the static condition and that its role in HSC repopulation is independent of integrin function.

**DISCUSSION**

HSCs are the most thoroughly characterized type of adult stem cells, and the hematopoietic system has served as the principal model for stem cell biology. Transplantation of HSC populations has been shown to be sufficient for long term multilineage reconstitution, not only in experimental animal models but also in clinical patients with hematological malignancies. HSCs must undergo several steps to achieve engraftment after transplantation, including transendothelial migration into the BM (homing), settling in the BM niche (lodging and retention), and intra-BM proliferation and multilineage differentiation (repopulation) (30). The present study showed that HSCs lacking vinculin were unable to reconstitute the hematopoietic sys-
Vinculin and HSC Repopulation

A

Random sequence
Talin-A sequence
Vin-B sequence
Vin-C sequence

B

Random Talin-A Vin-B Vin-C

C

3 days 5 min
1 week 4 min
2 weeks 3 min
3 weeks 30 sec
5 weeks 15 sec
17 weeks 30 sec
26 weeks 30 sec
31 weeks 30 sec

D

Luciferase activity (x 10^{7} photon/s)

Random
Talin-A
Vin-B
Vin-C

Times after transplantation (Weeks)
tem efficiently and failed to maintain their self-renewal capacity on BM stromal cell layers. However, the loss of vinculin did not inhibit the potential of HSCs to differentiate into granulocytic and monocytic lineages, in vitro migration toward stromal cell-derived factor 1/H9251, and in vivo homing to the BM. In addition, HSC adhesion to the ECM was abolished by expression of an shRNA sequence against talin-1, but not vinculin. This suggests that vinculin controls repopulation by HSCs in the BM micro-environment, independent of integrin function.

Cell-cell and cell-ECM interactions have been reported to be crucial for a number of HSC functions in the BM microenvironment (1, 2). Two different niches have been identified in the BM: the endosteal stem cell niche at the endosteum of the bone and the vascular niche in close proximity to the blood vessels. N-cadherin/\(\beta\)-catenin, Tie2/Ang-1, vascular cell adhesion molecule/integrin, and osteopontin/\(\beta\)1 integrin represent important adhesion molecules for the functions of these niches (1, 33). These molecules play roles either in the attachment of HSCs to the niche or in the migration of HSCs. Roles for integrins in HSC homing have been investigated in \(\beta\)1 integrin-knock-out mice and by blocking integrin function with anti-integrin antibodies (6–8). Osteopontin was recently found to contribute to HSC migration toward the endosteal region through its interaction with \(\beta\)1 integrin (34). Interactions between an integrin and the ECM are required for its transition from a low to a high affinity state via signaling referred to as “inside-out signaling” pathways (35). The interaction between an integrin and its ligand triggers signaling that promotes cytoskeletal changes, leading to cell spreading and stabilization through the process termed “outside-in signaling” (36, 37). Proteins that can directly bind to \(\beta\)-integrin cytoplasmic tails are important for these signaling pathways (see the Introduction) (12, 13). Inside-out and outside-in signaling of integrins are often considered separately, but some \(\beta\) integrin cytoplasmic proteins, including talin and cytoplasmic phospholipase A2, may function in both (38, 39). The reconstitution of HSCs after

FIGURE 6. Visualization of KSL cell fates in vivo in the absence of talin or vinculin. A, schematic presentation of the lentiviral vector used in this experiment. The EGFP gene of the LentiLox lentiviral vector was replaced with a luciferase gene. B–D, KSL cells were transduced with a lentiviral vector expressing a control shRNA sequence (random) or shRNA sequences against talin (Talin-A) or vinculin (Vin-B and Vin-C). E, in vivo bioluminescence images of transduced KSL cells before transplantation. C, transduced KSL cells together with competitor cells were transplanted into lethally irradiated recipient mice. The photons transmitted through the body were collected for the indicated lengths of time at the indicated days after transplantation using an IVIS Imaging System. D, quantitative data for in vivo bioluminescence imaging of the mice expressed as photon units (photons/s). The data are representative mean results obtained from two mice after transplantation in two independent experiments.
transplantation was partially impaired under conditions where the adhesion and spreading of HSCs onto ECM (outside-in signaling) were significantly inhibited by silencing of talin-1, whereas homing of HSCs to the BM was not affected. We cannot rule out the possibility that the discrepancy between the current and previous results (6) regarding the role of integrins in HSC homing could be the result of incomplete inhibition of integrin function by silencing of talin-1. However, our results suggest that signaling through integrins expressed in HSCs is involved in reconstitution in the BM microenvironment, although the contribution of talin-1 to integrin activation and HSC reconstitution was much weaker than that of vinculin.

The most important finding of the present study was the ability of vinculin to control HSC repopulation independently of integrin function. Activation of vinculin has been reported to be triggered by talin and actin polymerization, and this activation may strengthen the interactions between integrins and their ligands (40, 41). However, a reduction in the direct interaction between an integrin and its ligand alone cannot readily explain the phenotype of HSCs lacking vinculin, because although the loss of talin expression in HSCs caused severe impairment of integrin function, it only resulted in marginal failure of reconstitution. Vinculin also exists in cadherin-mediated cell-cell contacts and may therefore play an important role in cadherin-mediated cell-cell attachments in the niche, although conflicting results regarding the role of N-cadherin in HSC maintenance have been reported (42, 43). It is also possible that vinculin-deficient HSCs are unable to achieve a balance between self-renewal and differentiation; HSCs maintain the balance between stem cell and differentiated cell populations by choosing between several alternative fates in the BM, such as self-renewal and commitment to differentiation (44, 45). The former ensures preservation of the HSC fate upon cell division, whereas the latter enables differentiation into multiple lineages (44). In the present study, the expression of shRNA sequences against vinculin significantly abolished the frequency of LTC-IC and the formation of cobblestone-like areas but not the ability of HSCs to differentiate into granulocytic lineage cells, indicating that vinculin may regulate the self-renewal potential of HSCs. A self-renewal division implies that an HSC is permissive in terms of cell cycle entry but restricted from engaging in differentiation, apoptosis, or senescence pathways (44). A number of genes involved in the cell cycle machinery and HOX proteins (INK4A, HoxB4, and HoxA9) have been shown to regulate intrinsic programs in HSCs during the self-renewal process (46). It is possible that vinculin is directly involved in these spatial and temporal control processes responsible for maintaining the intrinsic balance of HSC self-renewal. Indeed, silencing of vinculin induced cell cycle modulation and the induction of apoptosis in KSL HSCs. In a study of vinculin-deficient embryos, vinculin was found to be necessary for normal neural and cardiac development (47). Furthermore, a mouse model involving cardiac myocyte-specific deletion of the vinculin gene resulted in sudden death and dilated cardiomyopathy (48). Although these abnormalities are believed to result from perturbation of integrin-dependent cell functions, the involvement of a specific function of vinculin that is independent of integrins cannot be ruled out, as demonstrated for HSC functions. Further studies are required to address these issues.

Silencing of vinculin failed to inhibit the adhesive properties of HSCs in this study. This was consistent with the results of a recent study indicating that inhibition of vinculin by RNA interference did not affect cell attachment to ECM in epithelial cells (49). Furthermore, silencing of vinculin did not abolish cell spreading of Chinese hamster ovary cells transformed to express integrin αIIβ3 (αIIβ3-CHO cells) on fibrinogen (50). This suggests that vinculin may not be involved in the initial attachment to the ECM and outside-in signaling of integrin. However, vinculin might be important for integrin αIIβ3 inside-out signaling, because the activated form of vinculin (the form inhibiting head-to-tail association) could induce binding of PAC-1 (a monoclonal Ab recognizing activated integrin αIIβ3) to αIIβ3-CHO cells (50). Further experiments to investigate the role of vinculin in inside-out signaling of integrins in platelet activation are now underway in our laboratory.

In summary, we have demonstrated that vinculin is required for HSC repopulation after HSC transplantation. These results provide the first evidence for vinculin as an important regulator of cellular functions, independent of integrin function. The manipulation of vinculin expression may represent a novel approach for not only regenerative and developmental medicine but also for the treatment of hematological malignancies. Further studies are needed to determine the precise cellular mechanisms whereby vinculin modulates HSC repopulation independently of integrin function and to apply vinculin-targeting therapies to the treatment of a variety of refractory disorders.

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REFERENCES