

Table 4

Logistic regression analyses of molecular markers for 28-day mortality after diagnosis of sepsis-induced DIC (n = 117).

Molecular markers	28-Day Mortality after DIC Diagnosis		
	Hazard Ratio	95% CI	p
Platelet, $\times 10^3/\mu\text{L}$	0.872	0.778-0.978	0.019
Prothrombin time, s	1.136	0.974-1.325	0.105
Fibrinogen, mg/dL	0.999	0.995-1.003	0.648
TAT, ng/mL	0.985	0.960-1.010	0.229
SFMC, $\mu\text{g/mL}$	1.032	1.004-1.060	0.023
PAI-1, ng/mL	1.012	1.003-1.021	0.011
PlC, $\mu\text{g/mL}$	0.737	0.547-0.993	0.045
FDP, $\mu\text{g/mL}$	1.046	1.003-1.091	0.035
p-XDP, $\mu\text{g/mL}$	1.013	0.942-1.088	0.731
e-XDP, U/mL	0.957	0.917-0.999	0.045

procoagulant action of neutrophil elastase may allow lysis to progress or not progress, or even enhancement of coagulation in clinical situation [26]. However, coagulation-related and inflammation-associated data are required to elucidate the clear mechanisms which cause the insufficient or sufficient activation of leukocyte elastase-mediated fibrinolysis in sepsis-induced DIC patients.

The group with e-XDP levels less than 3 U/mL showed significantly lower survival rates to 28 days after DIC diagnosis than patients with e-XDPs levels of 3–10 U/mL or 10–30 U/mL by Kaplan-Meier analyses (Fig. 5), and the adjusted odds for the mortality rate of this group were 4.432 (95% CI, 1.557–12.615, $p = 0.005$, Table 3). Multivariate logistic regression analyses showed that plasma e-XDP levels at DIC diagnosis might be an independent factor for 28-day mortality in sepsis-induced DIC patients (Table 4). However, it might not biologically be very relevant, as the hazard ratio was modest (0.957, 95% CI, 0.917–0.999). Collectively, the degree of the local activation of leukocyte elastase might contribute to organ damage as well as the actual prognosis in sepsis-induced DIC patients. Poor activation of leukocyte elastase (e-XDPs levels, <3 U/mL) might result in massive fibrin deposition when the plasminogen activator-plasmin system is suppressed, balanced activation (e-XDPs levels, 3–10 U/mL) could effectively degrade fibrin thrombi to protect against ischemic organ damage, and excessive activation (e-XDPs levels, >10 U/mL) evading local inhibitors might result in organ injury with proteolytic cleavage of tissue and plasma components [10,19,45].

In conclusion, we have demonstrated that leukocyte elastase could contribute to the degradation of cross-linked fibrin, and that e-XDPs levels at the time of diagnosis for DIC might predict the prognosis of patients with sepsis-induced DIC. The evaluation of leukocyte elastase-mediated fibrinolysis and control of its activity by specific inhibitors such as sivelestat could improve the poor outcome of septic DIC [46,47].

Authorship contribution

S. Madoiwa designed and performed the research, analyzed data, and wrote the paper. H. Tanaka, Y. Nagahama, M. Dokai, Y. Kashiwakura, A. Ishiwata, A. Sakata, and A. Yasumoto performed experiments; S. Madoiwa, T. Ohmori, J. Mimuro, and Y. Sakata analyzed data and revised the paper.

Conflict of interest statement

The authors state that they have no conflict of interest.

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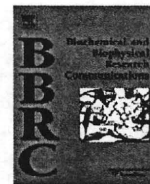
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Vinculin activates inside-out signaling of integrin α IIb β 3 in Chinese hamster ovary cells

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ABSTRACT

Although vinculin is used frequently as a marker for integrin-mediated focal adhesion complexes, how it regulates the activation of integrin is mostly unknown. In this study, we examined whether vinculin would activate integrin in Chinese hamster ovary (CHO) cells expressing human integrin α IIb β 3. Silencing of vinculin by lentiviral transduction with a short hairpin RNA sequence affected the binding of PAC-1 (an antibody recognizing activated human α IIb β 3) to a constitutively active form of α IIb β 3 (α 6B β 3) expressed on CHO cells, while its inhibitory effects were much weaker than those of talin-1. Overexpression of an active form of vinculin without intramolecular interactions, but not the full length one, induced PAC-1 binding to native α IIb β 3 expressed on CHO cells in a manner dependent on talin-1. On the other hand, silencing of talin-1, but not vinculin, failed to induce cell spreading of α 6B β 3-CHO cells on fibrinogen, even in the presence of PT 25-2, a monoclonal antibody that activates α IIb β 3. Thus, an active form of vinculin could induce α IIb β 3 inside-out signaling through the actions of talin-1, while vinculin was dispensable for outside-in signaling.

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1. Introduction

Vinculin is a highly conserved actin-binding protein that is frequently used as a marker for integrin-mediated focal adhesion complexes [1,2]. Although vinculin itself does not directly bind to integrins, it is thought to play key roles in focal adhesion assembly and in cell adhesion by connecting talin and α -actinin to the actin cytoskeleton [1,3]. Previous studies have suggested that vinculin contains a globular head domain, a flexible neck and a tail domain. The head and tail domain are connected by the flexible proline-rich neck region and interact to form a closed, autoinhibited conformation that masks the binding sites for many other cytoskeletal proteins [2,4]. Vinculin is also critical for proper embryonic development, because all homozygous vinculin^{-/-} embryos die be-

cause of brain and heart defects [5]. Despite extensive reports on the structure of vinculin and its associations with other cytoskeletal proteins, its precise roles in directly modulating the ligand-binding capacities of integrins remain to be elucidated.

Blood platelets are terminally differentiated anucleate cells that are responsible for primary hemostasis and pathological thrombosis. Platelets express members of the integrin β 1 subfamily that support platelet adhesion to extracellular matrix proteins, as well as expressing members of the integrin β 3 subfamily [6]. Among them, integrin α IIb β 3, a receptor for fibrinogen, von Willebrand factor, fibronectin and vitronectin, is an essential requirement for platelet aggregation [7]. This molecule is suitable for studies of integrin receptors because it is well characterized with respect to its ligand binding and signal transduction. In this study, we employed CHO cells expressing α IIb β 3 to investigate the roles of vinculin in modulating the ligand-binding capacities of integrins. We found that the activated form of vinculin can increase the affinity of α IIb β 3 through the actions of talin-1.

2. Materials and methods

2.1. Materials

The materials used in this study were obtained from the following suppliers: PAC-1 (a monoclonal antibody (mAb) recognizing

Abbreviations: CHO, Chinese hamster ovary; mAb, monoclonal antibody; APC, allophycocyanin; aa, amino acid; EGFP, enhanced green fluorescent protein; HIV, human immunodeficiency virus; sh, short hairpin; MOI, multiplicity of infection; LLK, integrin-linked kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate.

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activated human integrin α IIb β 3) and anti-paxillin mAb (clone 349; BD Biosciences Co., San Jose, CA); horseradish peroxidase-conjugated anti-green fluorescent protein (GFP) polyclonal antibody (Acris Antibodies GmbH, Himmelreich, Germany); allophycocyanin (APC)-conjugated anti-mouse IgM (eBioscience Inc., San Diego, CA); anti-talin mAb (clone 8D4; Sigma-Aldrich Co., St. Louis, MO); anti-vinculin mAb (clone V284; Chemicon International Inc., Billerica, MA); anti-human integrin α IIb β 3 mAb (clone 5B12; DakoCytomation, Glostrup, Denmark); PT 25-2 (a mAb that activates human integrin α IIb β 3; Takara Bio Inc., Otsu, Japan) [8].

2.2. cDNA cloning, construction of lentiviral vectors and virus production

cDNAs for mouse vinculin and talin-1 were cloned as described [9]. Truncated cDNA fragments of vinculin (amino acids (aa) 1–258, 1–880 and 881–1066) were amplified by polymerase chain reaction (PCR). A constitutively active form of vinculin (T 12) [3] was created using a Quick Change Site-directed Mutagenesis Kit (Stratagene Corp., La Jolla, CA). The primer pairs used in this study are shown in Supplementary Table 1. Enhanced green fluorescent protein (EGFP)-fusion proteins were created by insertion of various cDNAs into the sequence of pEGFP-C plasmid (Clontech Laboratories Inc., Mountain View, CA).

A gene transfer vector, pLL3.7, for constructing replication-defective self-inactivating human immunodeficiency virus (HIV) lentiviral vectors expressing short hairpin (sh) RNA sequences

(LentiLox vectors) was purchased from ATCC (Manassas, VA). We selected two shRNA sequences for mouse vinculin, designated Vin-B and Vin-C and cloned them into the pLL3.7 lentiviral vector plasmid (LentiLox) [10]. The shRNA sequences for control (Random) or talin-1 (Talin-A) were as reported [9]. To create HIV lentiviral vectors for protein expression, the pLL3.7 lentiviral vector was digested with XbaI and EcoRI to delete the sequence from the U6 promoter to EGFP and each EGFP-fusion protein sequence driven by the CMV promoter derived from the pEGFP-C plasmid was then inserted. The lentiviral vectors were essentially generated as described [9–11].

2.3. Immunoblotting

Immunoblotting with specific antibodies was performed as described previously [9,10].

2.4. PAC-1 binding to α IIb β 3-CHO cells

A chimera of the α IIb extracellular and transmembrane domains joined to the α 6B cytoplasmic domain was cotransfected with integrin β 3 into CHO-K1 cells [12]. The chimeric integrin (α 6B β 3) is a constitutively active form that spontaneously binds to PAC-1 [13]. We also cloned CHO cells expressing the native form of human integrin α IIb β 3. The oligonucleotide primer pairs used for cDNA cloning are shown in Supplementary Table 1. CHO cells ($2-4 \times 10^4$) expressing α 6B β 3 (α 6B β 3-CHO cells) or native human

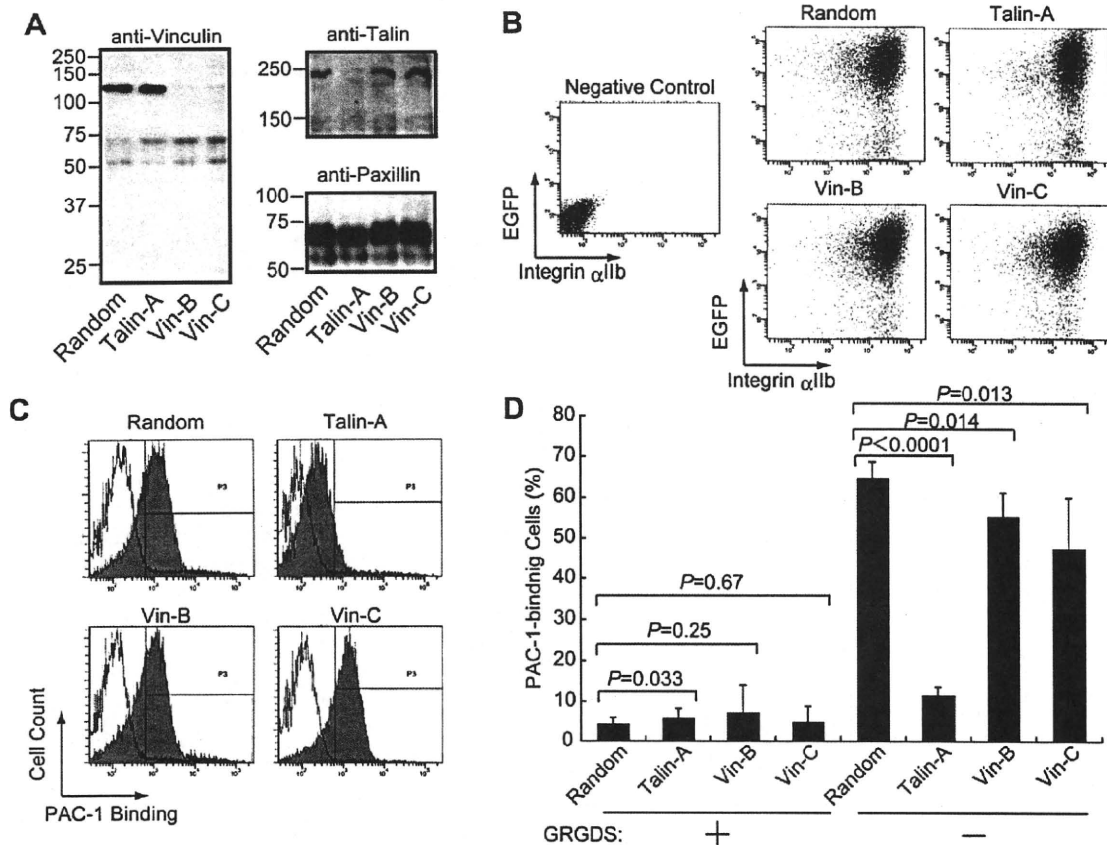


Fig. 1. Loss of vinculin marginally affects the integrin activation status in α 6B β 3-CHO cells. Cells were transduced with the indicated LentiLox vectors at a multiplicity of infection (MOI) of 3. (A) Lysates obtained from the transduced cells were immunoblotted with anti-vinculin, anti-talin and anti-paxillin monoclonal antibodies (mAbs). (B) Enhanced green fluorescent protein (EGFP) expression (vertical) and α IIb β 3 expression (horizontal) were examined by flow cytometry after transduction. (C) The affinity state of integrin α 6B β 3 was assessed by PAC-1 binding in the absence (solid gray) or presence (black line) of 1 mM GRGDS. (D) Columns and error bars represent the mean \pm SD of PAC-1 binding ($n = 7$). Differences between the two groups were analyzed statistically using Student's *t* test.

integrin α IIb β 3 (α IIb β 3-CHO cells) were allowed to adhere to 12- or 6-well plates for 16 h and then transduced with various LentiLox vectors or HIV vector at a multiplicity of infection (MOI) of 3. The PAC-1-binding activities and protein expressions were assessed after 72–96 h. Subsequently, the cells were resuspended in HEPES/Tyrode buffer containing 1.5 mM CaCl₂ and incubated with or without 1 mM GRGDS. The cells were then incubated with 1 μ g of PAC-1 and the antibody binding was detected with an APC-conjugated anti-mouse IgM antibody. To transduce the cells with two HIV vectors for a target EGFP-fusion protein expression vector and shRNA, the cells were simultaneously transduced with an HIV vector to overexpress the target protein at a MOI of 3 and with a LentiLox vector at a MOI of 6 (see Fig. 4A). For these experiments, we removed the DNA sequence for the EGFP gene driven by the CMV promoter from the LentiLox plasmid by digestion with NsiI and EcoRI.

2.5. Adhesion of CHO cells to fibrinogen

Cell adhesion to fibrinogen was assayed in 8-well Lab-Tek[®] Chamber Slides[™]. The chambers were coated with 500 μ g/ml of fibrinogen for 16 h at 37 °C, washed twice with phosphate buffered saline (PBS) and blocked with 10 mg/ml of bovine serum albumin (BSA) for 1 h. The transduced CHO cells were placed in the fibrino-

gen-coated chambers for 120 min at 37 °C. After two washes 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 an anti-paxillin mAb (1:500) for 16 h at 4 °C, washed in PBS and incubated with an Alexa 488-conjugated goat anti-mouse IgG polyclonal antibody (Molecular Probes Inc., Eugene, OR) for 2 h. Actin filaments were detected simultaneously by staining with 1 μ g/ml of rhodamine-conjugated phalloidin. After mounting in Vectashield[®] with DAPI (Vector Laboratories Inc., Burlingame, CA), the immunofluorescence staining was observed and photographed using a confocal microscope (FV1000; Olympus, Tokyo, Japan). When required, the cell area was quantified using NIH Image J Ver. 10.2 for Macintosh (NIH, Bethesda, MD; <http://rsbweb.nih.gov/ij/>) by a blinded observer.

3. Results

3.1. Expression of the activated form of vinculin induces inside-out signaling of integrin α IIb β 3

To examine the effects of vinculin silencing on integrin activation, we first employed CHO cells (α 6 β 3-CHO cells) expressing the chimeric integrin α 6 β 3 in a constitutively energy-dependent

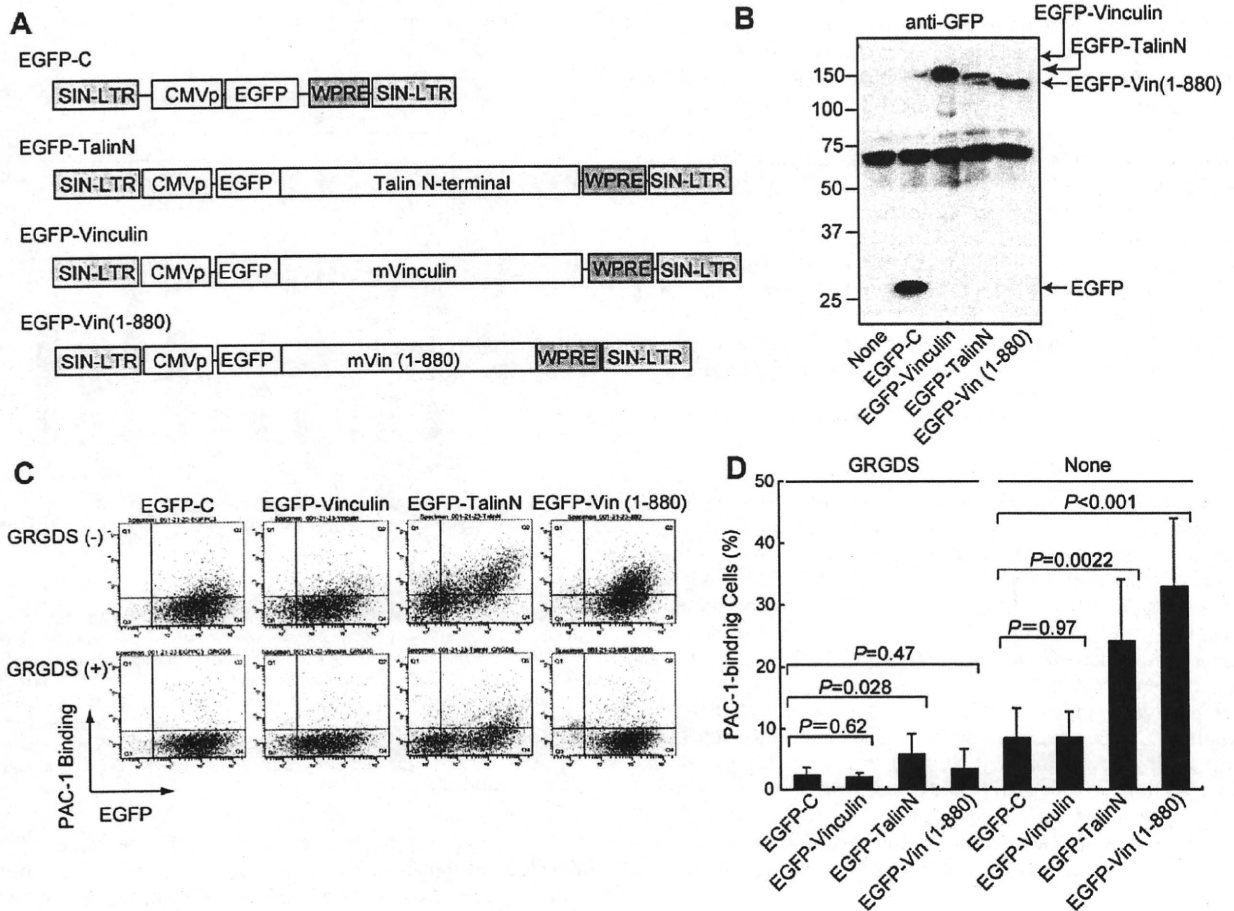


Fig. 2. Overexpression of the vinculin N-terminal domain, but not full-length vinculin, induces activation of native integrin α IIb β 3 in CHO cells. (A) Schematic representations of the HIV vector constructs used in this experiment. (B–D) α IIb β 3-CHO cells were transduced with an HIV vector encoding for EGFP, for EGFP-conjugated full-length vinculin, for the EGFP-conjugated N-terminal region of talin (EGFP-TalinN; aa 1–1072) and for the EGFP-conjugated vinculin head domain (EGFP-Vin (1–880); aa 1–880). (B) The expression levels of the target proteins were confirmed by immunoblotting with an anti-GFP antibody. (C) PAC-1 binding after transduction in the presence or absence of 1 mM GRGDS was assessed by flow cytometry. The data are representative of at least three independent experiments. (D) Columns and error bars represent the mean \pm SD of PAC-1 binding ($n = 3–6$). Differences between the two groups were analyzed statistically using Student's t test.

active form that binds to PAC-1 spontaneously [13]. The $\alpha 6\beta 3$ -CHO cells were transduced with the indicated LentiLox vectors and the protein expressions were determined by immunoblotting. As shown in Fig. 1A, the expression levels of vinculin and talin-1 were significantly inhibited by expression of their specific shRNA sequences. We further confirmed that EGFP and $\alpha 6\beta 3$ expression remained unchanged in these experiments (Fig. 1B). Although binding of PAC-1 to $\alpha 6\beta 3$ -CHO cells was significantly inhibited by loss of vinculin, the inhibitory effect was much weaker than that produced by silencing of talin-1 (Fig. 1C and D). These data indicate that loss of vinculin marginally affected the energy-dependent activation of $\alpha 6\beta 3$.

Next, we examined whether vinculin itself induced native $\alpha 6\beta 3$ activation. We created cDNAs for three EGFP-fusion proteins as follows: EGFP-conjugated N-terminal region of mouse talin-1 (EGFP-TalinN; aa 1–1072); EGFP-conjugated full-length vinculin (EGFP-vinculin) and an EGFP-conjugated N-terminal region of vinculin containing the head and neck domains (EGFP-Vin (1–880); aa 1–880) (Fig. 2A). The DNA fragments encoding these EGFP-fusion proteins driven by the CMV promoter were inserted individually into the HIV vector (Fig. 2A). The expression of the N-terminal region of talin (aa 1–1072) activated the native $\alpha 6\beta 3$ expressed on $\alpha 6\beta 3$ -CHO cells (Fig. 2C and D), as reported [14]. Interestingly, the expression of EGFP-Vin (1–880), but not full-length EGFP-vinculin, significantly induced the activation of $\alpha 6\beta 3$ expressed on $\alpha 6\beta 3$ -CHO cells (Fig. 2C and D).

To further assess the molecular mechanisms by which vinculin activates $\alpha 6\beta 3$, we created other fusion constructs to EGFP derivatives as follows: head domain of vinculin (EGFP-Vin (1–258); aa 1–258); C-terminal tail region of vinculin (EGFP-Vin (881–1066); aa 881–1066) and a constitutively active form of vinculin bearing mutations that inhibit the head–tail association (EGFP-Vin (T12)) (Fig. 3A) [3]. PAC-1 binding to CHO cells expressing native $\alpha 6\beta 3$ was significantly increased by the expression of EGFP-Vin (1–880) but also EGFP-Vin (1–258) and EGFP-Vin (T12), but was not affected by the expression of EGFP-Vin (881–1066) (Fig. 3C). The increase in PAC-1 binding elicited by EGFP-Vin (1–880) was much higher than those elicited by EGFP-Vin (1–258) or EGFP-Vin (T12) (Fig. 3C). These data suggest that the activated form of vinculin can increase the ligand-binding capacity of $\alpha 6\beta 3$.

3.2. Talin-1 is required for vinculin-mediated integrin $\alpha 6\beta 3$ activation

Because vinculin is unable to interact constitutively with integrin cytoplasmic tails, we focused on the role of talin-1 in vinculin-elicited $\alpha 6\beta 3$ activation. The $\alpha 6\beta 3$ -CHO cells transduced with HIV vectors expressing EGFP-TalinN or EGFP-Vin (1–880) were simultaneously transduced with LentiLox vectors containing the control (random), talin-1 (Talin-A) or vinculin (Vin-B) shRNA sequences, in which the CMV promoter and EGFP sequence in LentiLox had been removed (Fig. 4A). As shown in Fig. 4B, we confirmed the respective disappearances of the EGFP-conjugated Talin-N and vinculin constructs mediated by the Talin-A and Vin-B shRNA sequences using flow cytometry. Notably, PAC-1 binding by the vinculin head domain was significantly abolished by the silencing of talin-1 (Fig. 4C), suggesting that talin-1 is required for vinculin-induced integrin $\alpha 6\beta 3$ activation. On the other hand, talin-dependent activation of $\alpha 6\beta 3$ was not affected by the loss of vinculin (Fig. 4C).

3.3. Vinculin is not required for the spreading of $\alpha 6\beta 3$ -CHO cells

Finally, we investigated whether vinculin is involved in outside-in signaling of $\alpha 6\beta 3$ by examining cell spreading on a fibrinogen substrate. We employed $\alpha 6\beta 3$ -CHO cells treated with PT 25-2, a

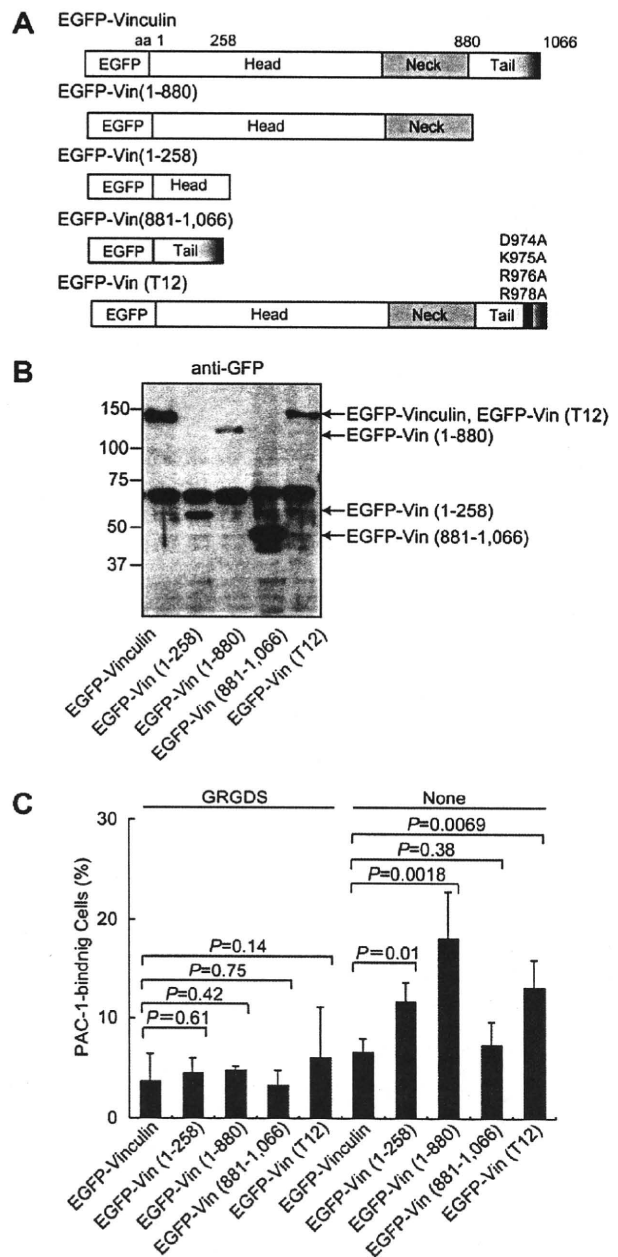


Fig. 3. Effects of various mutant forms of vinculin on the activation of integrin $\alpha 6\beta 3$ in CHO cells. (A) Schematic representations of the vinculin constructs expressed as fusion constructs with EGFP derivatives in $\alpha 6\beta 3$ -CHO cells using HIV vectors. (B) The protein expression levels after transduction of the HIV vectors were confirmed by immunoblotting using an anti-GFP antibody. (C) PAC-1 binding after transduction with the indicated HIV vectors in the presence or absence of 1 mM GRGDS was assessed by flow cytometry. Columns and error bars represent the mean \pm SD of PAC-1 binding (n = 4). Differences between the two groups were analyzed statistically using Student's *t* test.

mAb that induces $\alpha 6\beta 3$ activation, to completely remove the role of inside-out signaling. PAC-1 binding to $\alpha 6\beta 3$ -CHO cells was significantly enhanced by the addition of PT 25-2, even in the absence of talin-1 (Supplementary Fig. 1A and B). Under the same conditions, silencing of talin-1 failed to induce cell spreading on fibrinogen (Supplementary Fig. 1A and B), confirming the role of talin-1 in $\alpha 6\beta 3$ outside-in signaling. On the other hand, loss of vinculin in $\alpha 6\beta 3$ -CHO cells only marginally affected cell spreading (Supplementary Fig. 1A and B).

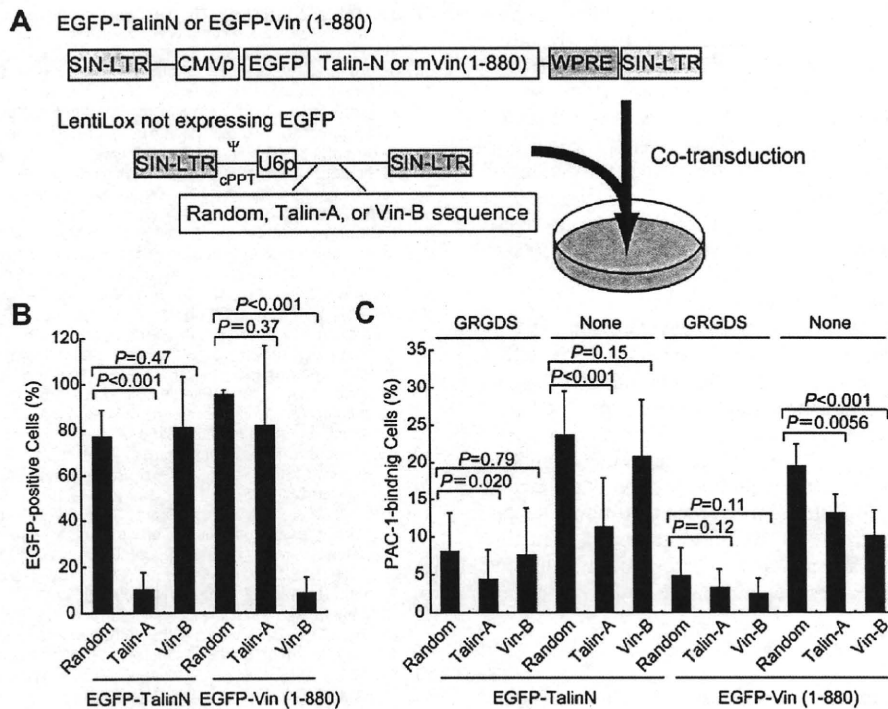


Fig. 4. Talin-1 is required for integrin activation elicited by vinculin. (A) α IIb β 3-CHO cells transduced with HIV vectors expressing EGFP-TalinN or EGFP-Vin (1-880) were simultaneously infected with the indicated LentiLox vectors (not expressing EGFP). (B) PAC-1 binding after transduction in the presence or absence of 1 mM GRGDS was assessed by flow cytometry. Columns and error bars represent the mean \pm SD of EGFP expression after the transduction. The expression of each EGFP-fusion protein was reduced significantly by the corresponding shRNA sequence. (C) Columns and error bars represent the mean \pm SD of PAC-1 binding ($n = 4$). Differences between the two groups were analyzed statistically using Student's *t* test.

4. Discussion

Although vinculin is used as a general marker for integrin-mediated formation of focal adhesion complexes, its importance in modulating the ligand-binding capacities of integrins has not been well addressed. The signal transduction pathways that lead to conformational changes of integrins are referred to as "inside-out signaling" pathways [7]. Cellular control of integrin activation requires transmission of a signal from the small cytoplasmic tails to the large extracellular domains. To date, more than 20 proteins have been identified to interact with either one or both cytoplasmic tails participating in inside-out signaling of α IIb β 3 [15]. Among these, it is well established that the binding of talin to β -integrin tails represents a final common step in integrin activation [16]. Kindlins and integrin-linked kinase (ILK) have now emerged as novel factors for integrin activation that can directly associate with β -integrin tails [12,17–19]. Although vinculin cannot bind directly to integrin cytoplasmic tails, our data suggest that it may have a role for inside-out signaling of α IIb β 3.

In the present study, the activation status of vinculin was found to be essential for increasing its affinity for integrins, because all the constructs that inhibited the intramolecular interaction also induced PAC-1 binding to α IIb β 3. Vinculin comprises three major domains that interact with other molecules, namely an N-terminal head domain, a flexible proline-rich neck domain (hinge) and a C-terminal tail domain [4,20]. The head domain can bind to talin and α -actinin, while the proline-rich region within the neck domain binds to vasodilator-stimulated phosphoprotein, vinexin, ponsin and the Arp2/3 complex [1]. Furthermore, other molecules including paxillin, F-actin and phosphatidylinositol (4,5)-bisphosphate (PIP₂) were reported to bind to the tail domain [1]. The crystal structure of vinculin has recently provided a molecular

explanation for the two conformations. In the closed state (inactivated form), the tail domain interacts with the N-terminal head domain, burying most of the binding sites for other molecules within vinculin [4,21,22]. Our data revealed that constitutively activated forms of vinculin that cannot assume the autoinhibited conformation induced the ligand-binding capacity of α IIb β 3. Of note, a truncated form containing the head and neck domains (aa 1–880) displayed more powerful α IIb β 3 activation than the activation status induced by the expression of the head domain only or the T 12 mutant form. These data suggest that the head and neck domains of vinculin both play important roles in increasing its ligand-binding capacity, while the tail domain's interactions with PIP₂ and/or F-actin negatively regulate vinculin to activate integrin inside-out signaling.

The molecular mechanism by which vinculin modulates integrin affinity seems to differ from those of talin-1 and ILK, because loss of vinculin marginally affected the ligand-binding capacity of α B β 3, a constitutively active form of α IIb β 3. One possible explanation is that vinculin stabilizes talin-mediated integrin activation after ligand binding. Talin was reported to activate vinculin by provoking helical bundle conversion [22]. Recently, it was reported that increased tension at focal adhesions stretches the talin rod domain, thereby triggering conformational changes that reveal new vinculin-binding sites in talin [23]. These alterations might enhance the affinities of integrins and enable stronger binding of the integrins to the extracellular matrix [24]. In support of this idea, vinculin-null cells can form focal adhesion complexes, but are less adherent and more motile, suggesting that vinculin stabilizes the interactions of integrins and their ligands [5,25].

During the preparation of this manuscript, successful generation of megakaryocyte/platelet lineage-specific vinculin-deficient mice by breeding *Vcl fl/fl* mice with *Pf4-Cre* mice was reported

[26]. Although the tail bleeding times were prolonged in mice producing vinculin-deficient platelets, platelet functions including platelet aggregation and adhesion were similar to wild-type mice [26]. These phenotypes differ from those obtained from talin-1- or kindlin-3-deficient platelets [18,27,28] and these results could not fully explain the mechanism whereby the bleeding time is prolonged in vinculin-deficient mice. Considering that the activation status of vinculin proved important to increase the ligand binding of α IIb β 3 in this study, the mechanism to activate vinculin might exist at the site of vascular injury *in vivo*, thereby stabilizing thrombus formation.

In a study of vinculin-deficient embryos, vinculin was shown to be necessary for normal neural and cardiac development [5]. Furthermore, transgenic mice with cardiac myocyte-specific deletion of the gene for vinculin exhibit sudden death and dilated cardiomyopathy [29]. Although these abnormalities are supposed to result from perturbation of integrin-dependent cell functions, the effects of specific functions of vinculin that are independent of integrins cannot be ruled out. We recently reported that vinculin also plays roles in hematopoietic stem cell reconstitution independently of its integrin function [10]. We are interested in further investigating the integrin-independent functions of vinculin and corresponding experiments are now under way in our laboratory.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.056.

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Vinculin Is Indispensable for Repopulation by Hematopoietic Stem Cells, Independent of Integrin Function^{*S}

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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⁺Sca1⁺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 1 α , 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⁺Sca1⁺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 repopulation capacity, independent of integrin functions.

The development and maintenance of hematopoietic stem cells (HSCs),³ 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 in the

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 $\alpha 4$, $\alpha 5$, and $\alpha 6$, which can associate with integrin $\beta 1$ (5). Integrin $\beta 1$ -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 $\alpha 4$ 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 $\alpha 4$ reduces HSC homing and leads to HSC release into the blood (8, 9). Furthermore, integrin $\alpha 4\beta 1$ -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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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental text, Table S1, and Figs. S1–S5.

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³ 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⁺Sca1⁺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.

Vinculin and HSC Repopulation

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 μ l 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 $5\text{--}6 \times 10^7$ 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 (FACSariaTM 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 μ g/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 grays (Gamma Cell; Norton International), followed by injection of 1×10^5 transduced KSL cells from Ly5.1 donor mice, together with 2×10^5 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 (2×10^3) were then suspended in 1 ml of MethoCultTM 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 $\times 40$ 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- μ m 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 μ l 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 1 α in the lower chamber. Following migration, the cells in the lower chamber were recovered by centrifugation and then lysed using 200 μ l 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, 29). Briefly, transduced KSL cells (1×10^5 cells) were injected into lethally irradiated recipient mice (9.5 grays). 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 (1×10^4 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 specified 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 ($1-2 \times 10^5$ 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 μ l of 1 mM 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 MyelocultTM 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 μ l of MyelocultTM 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 μ l of methylcellulose medium (MethoCultTM 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 MyelocultTM M5300 medium (StemCell Technologies). 150 μ l 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 μ g/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 μ g/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 μ l 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 SlideTM) were coated with 10 μ g/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 (1:200) (Abcam plc., Cambridge, UK) for 16 h at 4 °C, washed in PBS, and then incubated for 2 h with streptavidin conjugated with Alexa488 (1:200) (Molecular Probes). Actin filaments were detected by staining with 10 μ g/ml rhodamine-conjugated phalloidin (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

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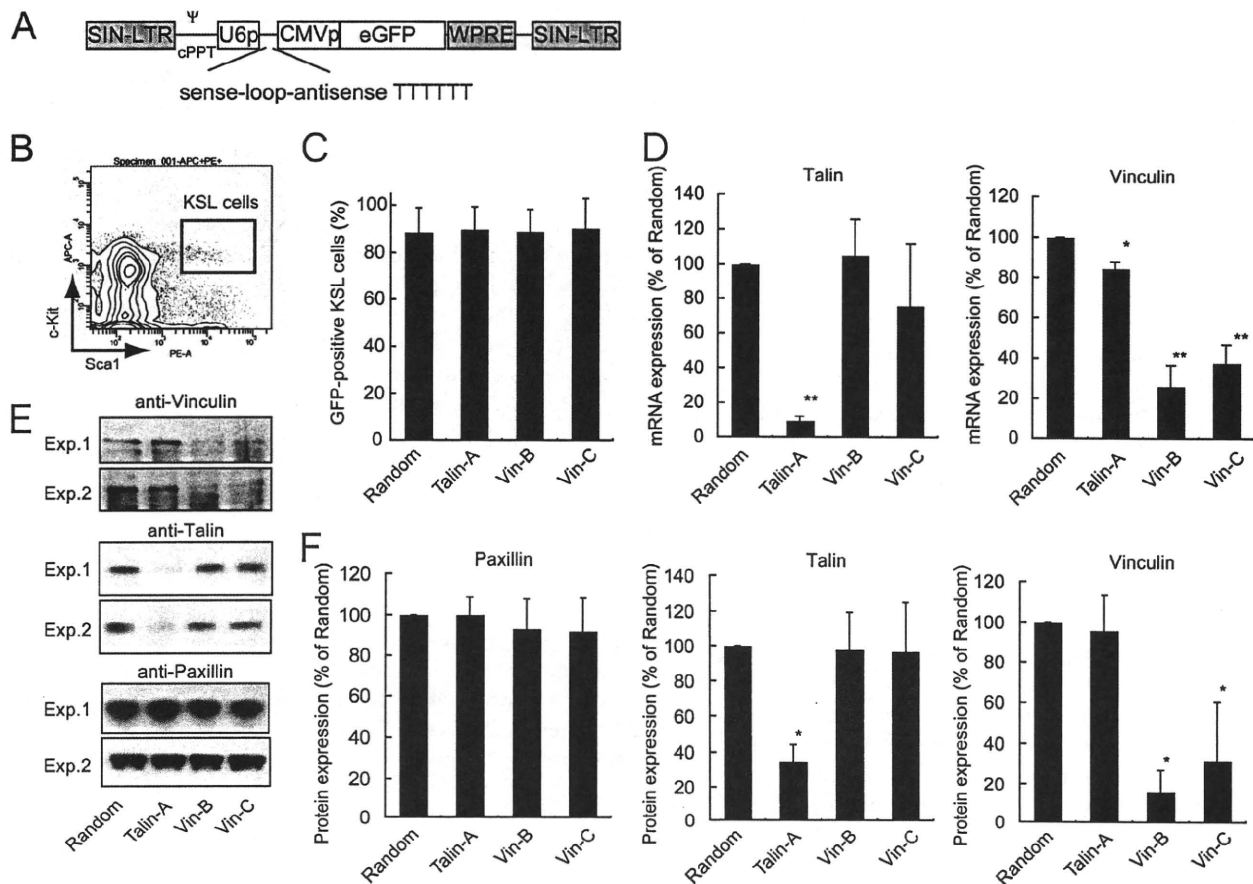


FIGURE 1. Silencing of target protein and mRNA expression in KSL cells transduced with LentiLox vectors. *A*, schematic presentation of the lentiviral vector (LentiLox). The shRNA sequences inserted into LentiLox are shown in supplemental Table S1. *B*, representative flow cytometric data for KSL cell sorting. *C*, expression of EGFP in KSL cells after transduction with LentiLox vectors expressing the indicated shRNA sequences at a MOI of 20 ($n = 8-10$). *D*, mRNA expression of talin (left panel) and vinculin (right panel) examined by real time quantitative PCR. The quantity was determined by dividing the copy number of the target sequence by that of the mouse glyceraldehyde-3-phosphate dehydrogenase sequence. Each value was expressed as a percentage relative to the value in the random shRNA experiment. The columns and error bars represent the means \pm S.D. ($n = 3$). *E*, expression of vinculin, talin, and paxillin proteins in KSL cells transduced with the indicated LentiLox vectors examined by immunoblotting. Two representative data are shown. *F*, expression of paxillin (left panel), talin (middle panel), and vinculin (right panel) proteins was quantified using ImageJ software. Each value was expressed as a percentage relative to the value in the random experiment. The columns and error bars represent the means \pm S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$, compared with the control experiment (random) under the same conditions (two-tailed Student's *t* test). Exp., experiment.

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. 1*B*) 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. 1*C*). The expression levels of the target proteins and their mRNAs in KSL cells were significantly inhibited by expression of the corresponding shRNA after transduction (Fig. 1*D-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 ($\sim 15\%$) (Fig. 1*D*), its protein levels were not significantly inhibited (Fig. 1*F*). 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-

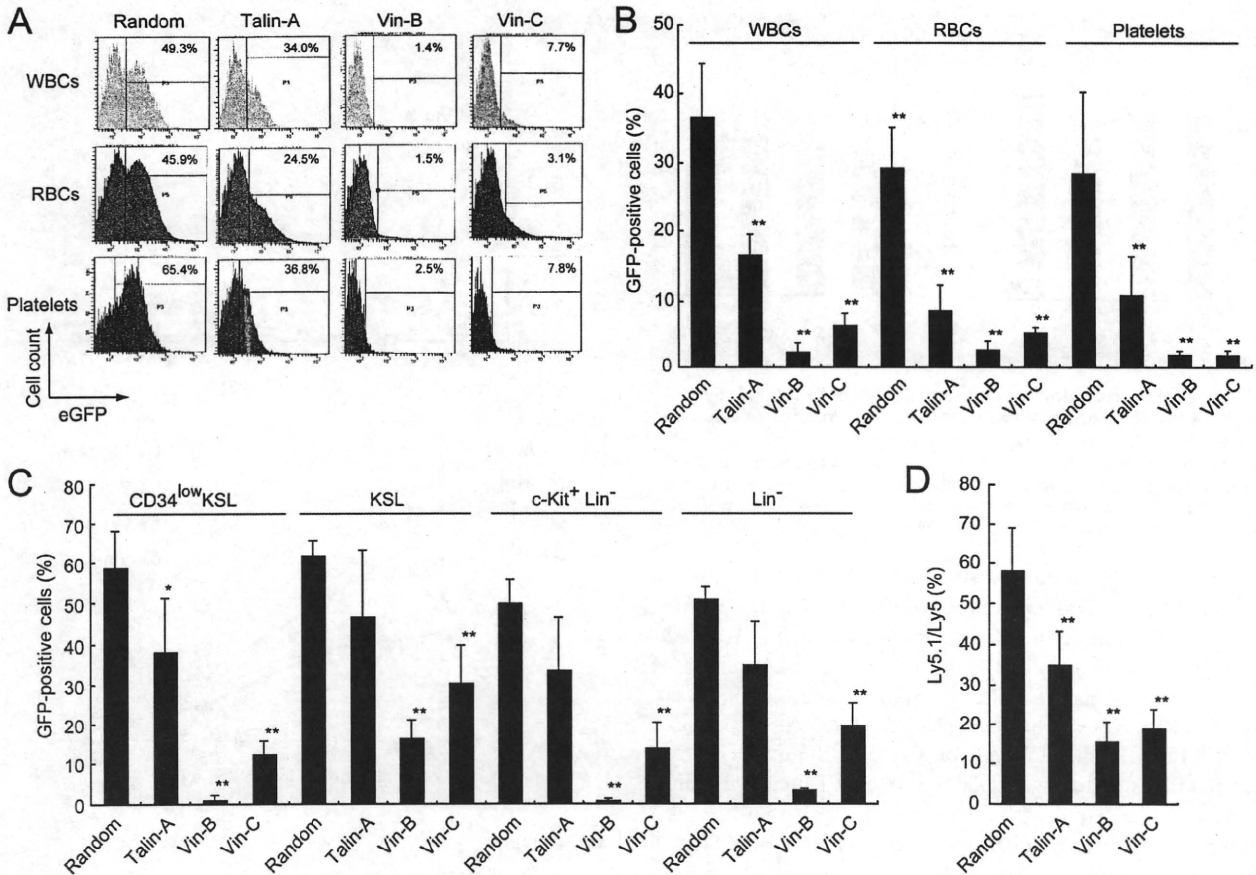


FIGURE 2. Reconstitution capacities of EGFP-positive KSL cells transduced with LentiLox vectors. Transduced KSL cells (1×10^5) isolated from Ly5.1 congenic mice together with nontransduced Ly5.2 BM cells (2×10^5) were transplanted into lethally irradiated recipient mice. EGFP expression in peripheral blood cells and BM cells was assessed at 1 month after transplantation. *A*, representative data for EGFP expression in CD45-positive white blood cells (WBCs), red blood cells (RBCs), and platelets in each experiment. *B*, quantification of EGFP expression in peripheral blood cells. The columns and error bars represent the means \pm S.D. ($n = 6-11$). *C*, EGFP expression in the indicated BM cells. The columns and error bars represent the means \pm S.D. ($n = 3-4$). *D*, chimerism after transplantation was calculated by the expression of Ly5.1-positive cells (%) among the total Ly5-positive white blood cells. The columns and error bars represent the means \pm S.D. ($n = 6-11$). *, $p < 0.05$; **, $p < 0.01$, compared with the control experiment (random) under the same conditions (two-tailed Student's *t* test).

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. CD34^{low} c-Kit⁺ Sca-1⁺ Lin⁻ (CD34^{low}KSL) cells are highly enriched for HSCs with long term marrow repopulating ability, whereas CD34⁺ c-Kit⁺ Sca-1⁺ Lin⁻ (CD34⁺KSL) cells are progenitors with short term reconstitution capacity, suggesting that CD34^{low}KSL cells have a higher rank in the hematopoietic hierarchy than CD34⁺KSL cells (32). The proportion of EGFP-positive cells was reduced by vinculin silencing not only in the progenitor fraction (c-Kit⁺Lin⁻ cells) but also in more primitive HSCs (CD34^{low}KSL cells), thus strengthening the initial hypothesis

that vinculin controls HSC fate after transplantation (Fig. 2*C*). 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. 2*D*). 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.

Frequencies of LTC-IC and Cobblestone-like Area Forming Capacities on BM Stromal Cells, but Not Homing to the BM, Are Impaired in Vinculin-silenced KSL HSCs—The observed reduction in HSC engraftment by vinculin silencing could be a consequence of a reduced ability of HSCs either to home to the BM or to reconstitute hematopoiesis (repopulating capacity of HSCs in the BM). The effect of vinculin silencing on HSC pro-

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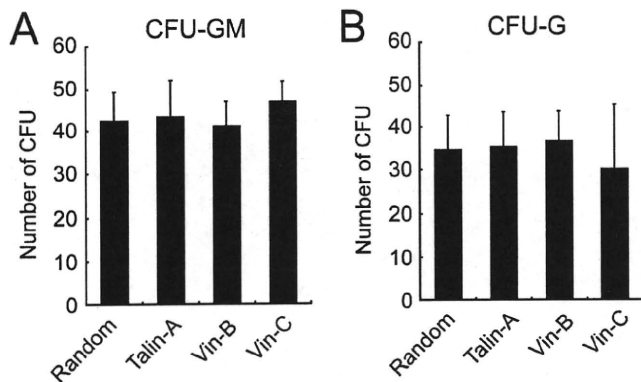


FIGURE 3. Loss of vinculin does not impair CFU-GM or CFU-G colony formation. KSL cells were transduced with the indicated LentiLox lentiviral vectors at a MOI of 20. Colony forming assays for CFU-GM and CFU-G were performed as described under "Experimental Procedures." A and B, the numbers of CFU-GM (A) and CFU-G (B) per 2×10^3 transduced KSL cells were assessed. The columns and error bars represent the means \pm S.D. ($n = 3$). There were no significant differences between the groups (two-tailed Student's *t* test).

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 1 α *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[®] imaging system *in vivo* (Fig.

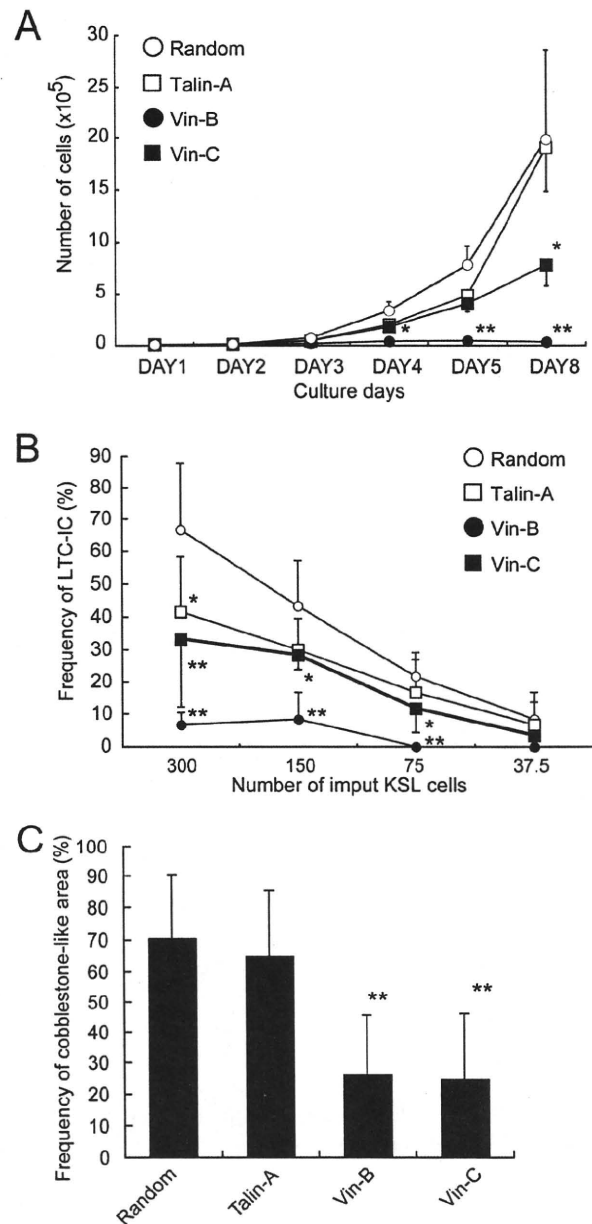


FIGURE 4. Loss of vinculin impairs the frequencies of LTC-IC and cobblestone-like area-forming cells. A, transduced KSL cells (1×10^4) were cultured in StemPro-34 SFM medium with a cytokine combination, as described under "Experimental Procedures." The cell numbers were quantified by FACS at the indicated time points. Values represent the means \pm S.E. ($n = 5$). B, the frequencies of LTC-IC among KSL cells (300 to 37.5 cells) transduced with the indicated LentiLox vectors were examined as described under "Experimental Procedures." Values represent the means \pm S.D. ($n = 5$). C, the frequency of cobblestone-like areas formed on irradiated C3H/10T1/2 cells was assessed at 28 days after seeding, as described under "Experimental Procedures." The columns and error bars represent the means \pm S.D. ($n = 6$). *, $p < 0.05$; **, $p < 0.01$, compared with the control experiment (random) under the same conditions (two-tailed Student's *t* test).

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. 6C). 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

Cell cycle	shRNA sequence	Means ± S.D. (n = 5)	p value
%			
G ₀ /G ₁ phase	Random	31.72 ± 3.53	
	Talin-A	34.12 ± 2.13	0.070
	Vin-B	35.02 ± 2.39	0.016 ^a
	Vin-C	32.86 ± 4.21	0.46
S phase	Random	59.0 ± 3.29	
	Talin-A	51.5 ± 11.0	0.13
	Vin-B	45.9 ± 5.35	0.0013 ^a
	Vin-C	55.3 ± 3.83	0.0384 ^a
G ₂ /M phase	Random	2.82 ± 1.93	
	Talin-A	3.06 ± 1.71	0.66
	Vin-B	3.16 ± 1.59	0.64
	Vin-C	3.06 ± 1.67	0.76
Apoptotic cells	Random	5.44 ± 1.43	
	Talin-A	6.44 ± 3.73	0.49
	Vin-B	14.08 ± 3.71	0.0026 ^a
	Vin-C	7.44 ± 1.41	0.0036 ^a

^a 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-

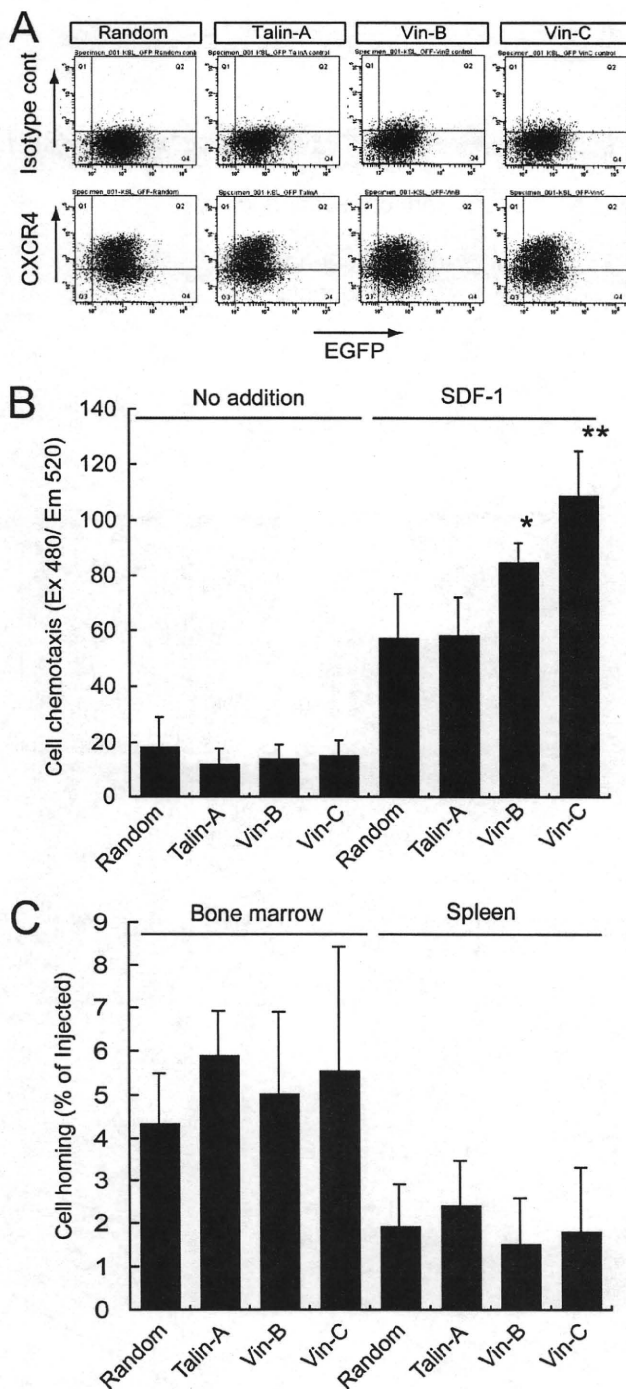
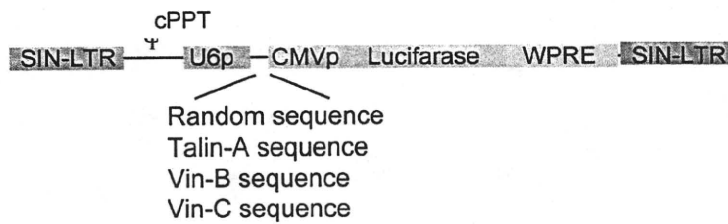


FIGURE 5. Roles of talin and vinculin in the homing of KSL cells. A, the expression of CXCR4 in KSL cells transduced with the indicated LentiLox vector was evaluated by FACS (lower panels). The results for an isotype-matched control Ab are also shown (upper panels). B, transduced KSL cell migration *in vitro* was assessed using a modified Boyden chamber assay. Stromal cell-derived factor 1 (100 ng/ml) was placed in the lower chamber (or not added), and the cells were allowed to migrate for 4 h. The columns and error bars represent the means ± S.D. (n = 4). C, transduced KSL cells were injected into lethally irradiated recipient mice. The cells from the BM and spleen were collected for CFU-GM assays at 16–20 h after injection. The total numbers of CFU-GM recovered from the BM and spleen were counted manually and expressed as percentages of the number of CFU-GM from the injected KSL cells. The columns and error bars represent the means ± S.D. (n = 4). *, p < 0.05; **, p < 0.01, compared with the control experiment (random) under the same conditions (two-tailed Student's t test).

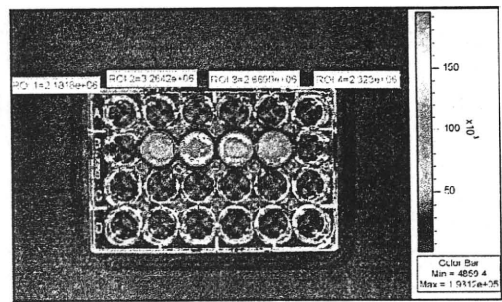
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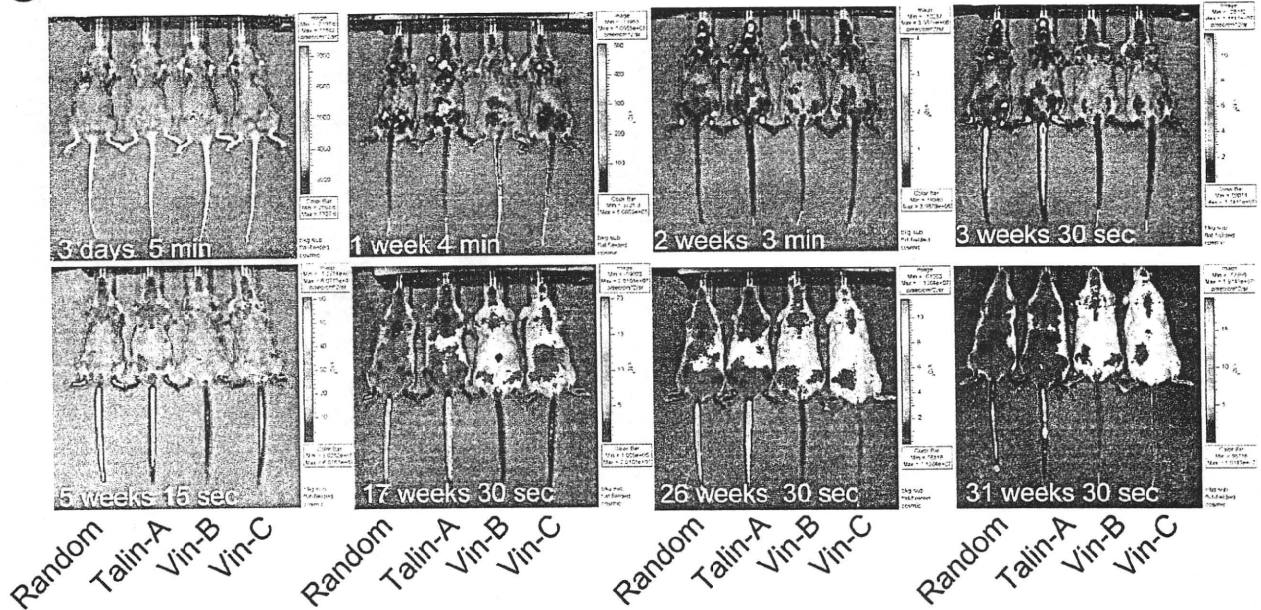
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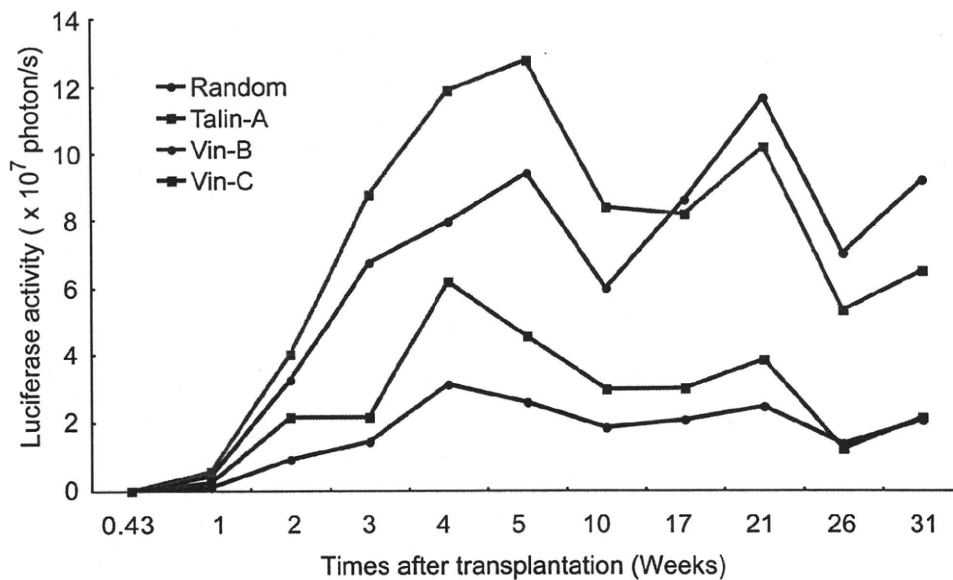
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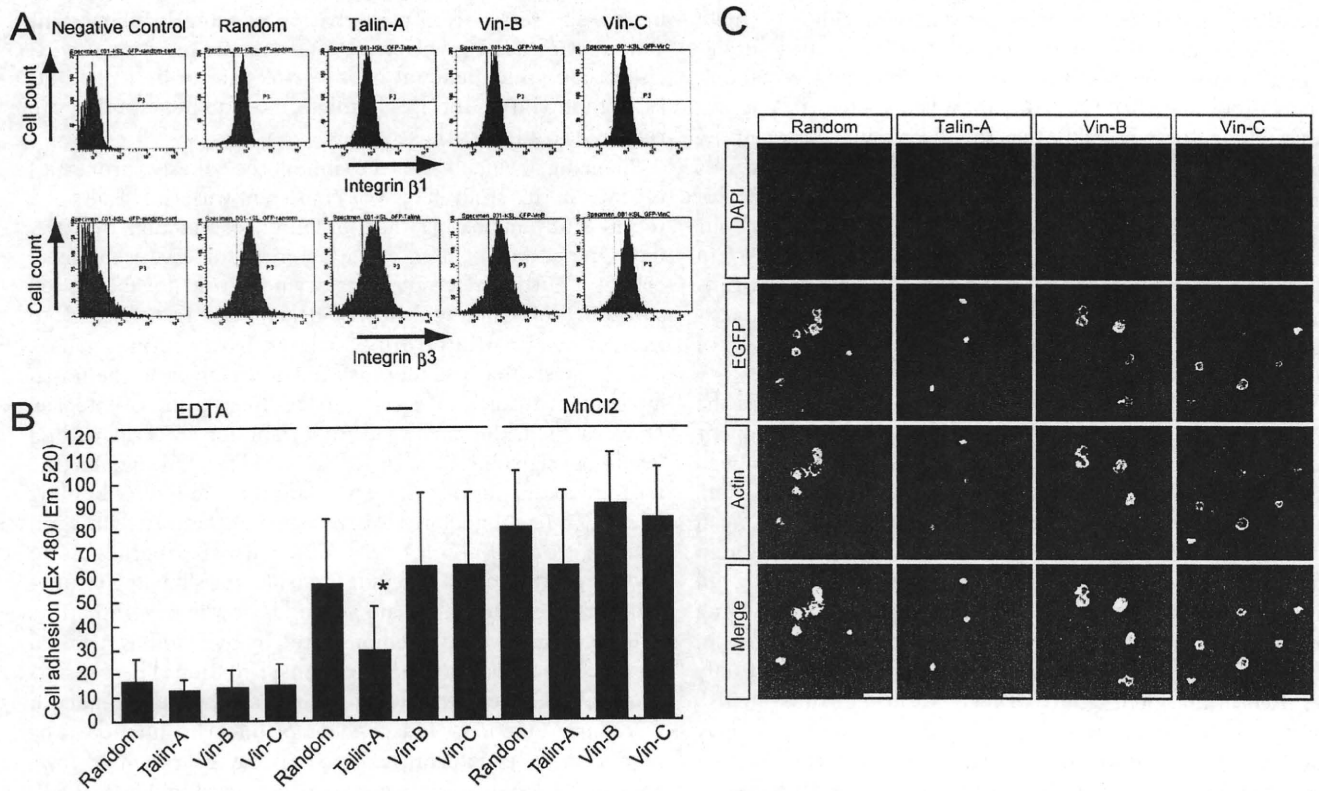


FIGURE 7. Loss of vinculin does not affect integrin expression and adhesion of KSL cells to the ECM. KSL cells were transduced with the indicated LentiLox vectors. *A*, the expression levels of integrin $\beta 1$ (upper panels) and integrin $\beta 3$ (lower panels) were examined by FACS. The results for an isotype-matched control Ab are also shown. *B*, cell adhesion to fibronectin (10 $\mu\text{g}/\text{ml}$) in the absence or presence of 2 mM EDTA or 2 mM MnCl_2 were assessed as described under "Experimental Procedures." The columns and error bars represent the means \pm S.D. ($n = 4$). *, $p < 0.05$, compared with the control experiment (random) under the same conditions (two-tailed Student's *t* test). *C*, the transduced KSL cells were placed on immobilized fibronectin (10 $\mu\text{g}/\text{ml}$) for 3 h. The cells were fixed and then stained with DAPI (blue), anti-GFP antibody (green), and rhodamine-conjugated phalloidin (red). Bar, 40 μm . The data are representative of three independent experiments.

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 α , 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/ β -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" (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 β -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 β integrin cytoplasmic proteins, including talin and cytoplasmic phospholipase A_2 , 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). *B*, *ex 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.

Vinculin and HSC Repopulation

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 cellular 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 α IIB β 3 (α IIB β 3-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 α IIB β 3 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 α IIB β 3) to α IIB β 3-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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Regular Article

Mutant Macaque Factor IX T262A: A Tool for Hemophilia B Gene Therapy Studies in Macaques

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ABSTRACT

Introduction: Gene therapy is expected to be the next generation therapy for hemophilia, and a good animal model is required for hemophilia gene therapy preclinical studies.

Methods: Taking advantage of the human factor IX (FIX) specificity of monoclonal antibody 3A6, the epitope of which resides in the amino acid polypeptide segment including Ala 262 of human FIX, mutant macaque FIX with an amino acid substitution of Thr 262 to Ala (macaque FIX T262A) was generated and its reactivity to monoclonal antibody 3A6, biological activity and expression *in vivo* were studied.

Results: Enzyme-linked immunosorbent assays (ELISAs) and Western blot analyses showed that monoclonal antibody 3A6 bound to human FIX and macaque FIX T262A but not to wild-type macaque FIX. Recombinant macaque FIX T262A exhibited a comparable coagulation activity to wild-type macaque FIX and human FIX. High expression of macaque FIX T262A was achieved in mice by injection of AAV8 vectors carrying the macaque FIX T262A gene and reached levels of up to 31.5 µg/mL (1050% of the normal human FIX concentration). Macaque FIX T262A expressed in the liver of mice was as biologically active as that expressed *in vitro*. In addition, the macaque FIX T262A concentrations determined by a 3A6-based ELISA were not influenced by the presence of normal macaque plasma.

Conclusions: The results of the present study suggest that macaque FIX T262A may be processed appropriately *in vivo* and that the macaque FIX T262A concentration in the macaque circulation can be quantified precisely by a monoclonal antibody 3A6-based ELISA.

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A wide variety of disorders are caused by genetic abnormalities, thereby giving rise to enthusiasm for gene therapy as the next generation therapeutics for many diseases [1–4]. Indeed, many gene therapy clinical trials have been conducted, and some have achieved great successes [5–7]. However, others have been unsuccessful. Furthermore, unpredicted adverse effects occurred in some trials [8,9]. To establish gene therapy technologies, good animal models are required. Advances in developmental biotechnology have allowed us to create a variety of mouse disease models, transgenic mice and gene-targeted mice. However, there are significant species differences

between humans and mice, thus making it difficult under certain circumstances to extrapolate data obtained in mice to human patients [10]. Factor IX (FIX)-deficient mice (hemophilia B mice) and natural hemophilia B dogs have been used to study gene therapy approaches for the treatment of hemophilia B [3,11–13]. However, better animal models may be required because of the limited success in human trials [9,10]. Primates are used successfully as models in disease applications, but there are presently no hemophilic primates available for gene therapy studies. If one can distinguish human molecules from primate molecules *in vivo*, non-human primates may be used for hemophilia gene therapy preclinical studies [11,14,15], despite the fact that this genetic abnormality is not indigenous to these species.

Rhesus macaques have been proposed as a good primate model for hemophilia B gene therapy studies because of the amino acid sequence similarity between human FIX and macaque FIX and the low immunogenicity of human FIX in rhesus macaques [16]. However,

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