Silencing of a Targeted Protein in In Vivo Platelets Using a Lentiviral Vector Delivering Short Hairpin RNA Sequence

Tsukasa Ohmori, Yuji Kashiwakura, Akira Ishiwata, Seiji Madoiwa, Jun Mimuro, Yoichi Sakata

Objective—Because platelets are anucleate cells having a limited life span, direct gene manipulation cannot in principle be used to investigate the involvement of a specific signal transduction pathway in platelet activation. In this study, we examined whether the expression of a short hairpin RNA (shRNA) sequence in hematopoietic stem cells is maintained during megakaryocyte differentiation, thus resulting in inhibition of targeted protein in platelets.

Methods and Results—To identify platelets derived from transduced stem cells, we generated a lentiviral vector that simultaneously expresses the shRNA sequence driven by the U6 promoter and GFP under the control of the glycoprotein (GP) Ibα promoter. Transplantation of mouse bone marrow cells transduced with the vector facilitated specifically mark platelets derived from the transduced cells. Transplantation of cells transduced with shRNA sequence targeting integrin αIIb caused a significant reduction of integrin αIIbβ3 (αIIbβ3) expression in GFP-positive platelets. It also inhibited αIIbβ3 activation assessed by the binding of JON/A, an antibody that recognizes activated αIIbβ3. Talin-1 silencing by the same method resulted in normal αIIbβ3 expression but deficient inside-out αIIbβ3 signaling.

Conclusions—shRNA expression driven by the U6 promoter is preserved during megakaryopoiesis. This method facilitates functional analysis of targeted protein in platelet activation. (Arterioscler Thromb Vasc Biol. 2007;27:2266-2272.)

Key Words: shRNA ■ RNA interference ■ platelets ■ talin ■ integrin

Platelets are terminally-differentiated circulating anucleate cells whose adhesive and signaling functions are essential for normal hemostasis. Platelets are produced in the bone marrow from megakaryocytes as cytoplasmic fragments without genomic DNA. Although platelets contain mRNA within their cytoplasm and can respond to physiological stimuli using biosynthetic processes regulated at the protein translation level, application of direct genetic manipulation in platelets has not been reported. Alternatively, megakaryocyte lineage cells derived from embryonic or hematopoietic stem cells are amenable to genetic manipulation using gene transduction systems, enabling molecular studies of adhesion and signaling in megakaryocytes in a way not possible with platelets.

Because platelets and their precursor megakaryocytes have a finite lifespan, hematopoietic stem cells are preferable targets for genetic transfer to establish long-term in vivo expression of the targeted protein in platelets. When a retroviral vector containing the integrin β3 (β3) gene driven by the integrin αIIb (αIIb) promoter was transduced into CD34+ cells from a Glanzmann thrombasthenia patient with defects in the β3 gene, integrin αIIbβ3 (αIIbβ3) was detected after in vitro megakaryocyte differentiation. We have previously shown that transduction of hematopoietic stem cells with lentiviral vector harboring the glycoprotein (GP) Ibα promoter enables specific and efficient expression of the targeted protein in platelets in vivo. Further, the therapeutic expression of αIIbβ3 in β3-deficient mice using a lentivirus vector containing β3 complimentary DNA (cDNA) under the control of the αIIb promoter has been reported. These data indicate that gene expression driven by a platelet-specific promoter using the transduction of hematopoietic stem cells with lentiviral vector can be applied to investigations of the involvement of specific proteins in platelet signaling pathways. However, there has been no evaluation of whether the knock-down of targeted proteins in hematopoietic stem cells using a short hairpin RNA (shRNA) sequence results in sufficient protein reduction in platelets. In this study, we examined whether shRNA expression driven by the RNA polymerase III promoter is sustained during megakaryopoiesis, and whether gene silencing with shRNAs is applicable to analyzing the functions of αIIbβ3 in in vivo platelets.

Materials and Methods
Materials, cDNA cloning, transfection, construction of lentiviral vector, stem cell transplantation, flow cytometry, immunoblotting, RT–polymerase chain reaction (PCR), platelet adhesion assay, and

Arterioscler Thromb Vasc Biol. is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.107.149872

2266
immunohistochemistry are described in detail in the supplemental materials, available online at http://atvb.ahajournals.org.

Results

Efficient GFP Expression in Platelets Using LentiLox Vector Harboring GPlbα Promoter In Vivo

We first substituted the CMV promoter of pLL3.7, a lentiviral gene transfer vector which simultaneously expresses shRNA and GFP (LentiLox-CMV),10 with the platelet-specific GPlbα promoter (LentiLox-GPlbα) (supplemental Figure I). To compare strengths and the specificities of the CMV and GPlbα promoters and to assess GFP transduction using these lentiviral vectors in vivo, bone marrow cells transduced with LentiLox-CMV or LentiLox-GPlbα were transplanted into the recipient mice. When bone marrow cells transduced with LentiLox-CMV were transplanted, GFP expression was observed in 14% to 32% of CD45+/H11001 cells and in 0.7 to 2.4% of platelets in peripheral blood (supplemental Figure II). As described previously, transduction with LentiLox-GPlbα resulted in efficient GFP gene marking in platelets (10% to 22%); however, only marginal GFP expression was observed in CD45+ and red blood cells (supplemental Figure II). These data suggested that the LentiLox-GPlbα
system enables specific GFP marking of platelets derived from transduced hematopoietic stem cells.

**Efficiency of Lentiviral shRNA for Silencing of Targeted Protein Expression**

We next validated the effects of shRNA sequence expression driven by the U6 promoter in LentiLox on expression of the targeted protein. We selected the shRNA sequences for integrin αIIbβ3 expression in platelets of peripheral blood are shown 30 days after transplantation. The plots represent the degree of GFP expression (horizontal) and specific antibody binding for integrin αIIb (vertical). The specific antibody binding in GFP-positive platelets is shown (right panel; white: Lentilox-scramble-GPIbα, gray: Lentilox-αIIbA-GPIbα). Columns and error bars represent the mean±SD of mean fluorescence intensity of antibody binding for αIIb in GFP-positive platelets (n=9 per group). The activation of αIIbβ3 assessed by JON/A binding and P-selectin expression in platelets stimulated with 150 ng/mL of convulxin. The plots represent the degree of P-selectin expression (horizontal) and JON/A binding (vertical) in GFP-positive platelets. Columns and error bars represent the mean±SD of mean fluorescence intensity (MFI) of antibody binding after stimulation with 6 μmol/L ADP or 150 ng/mL of convulxin to GFP-positive platelets (n=5 to 7 per group).

**Silencing of αIIbβ3 Expression In Vivo Platelets by Transplantation of Transduced Bone Marrow Cells With shRNA Lentiviral Vector**

We next examined the effects of shRNA driven by the U6 promoter during megakaryopoiesis in vivo. The bone marrow cells transduced with LentiLox-GPlbα containing the scramble...
The plots represent the degree of P-selectin expression (horizontal) and specific antibody binding (vertical). D, Activation of JON/A binding to platelets incubated with 150 ng/mL of convulxin. The plots represent the degree of GFP expression (horizontal) and specific antibody binding (vertical). JON/A binding to platelets incubated with 150 ng/mL of convulxin. The plots represent the degree of P-selectin expression.

**Figure 3.** Knockdown of talin-1 inhibits integrin αIIbβ3 activation and P-selectin expression in platelets. Bone marrow cells transduced with LentiLox-scramble-GPIbα or LentiLox-talinA-GPIbα were transplanted into recipient mice. A, Representative flowcytometry analyses of intracellular talin expression in GFP-positive (white: LentiLox-scramble-GPIbα; gray: LentiLox-talinA-GPIbα). B, After the sorting of GFP-positive platelets, the platelet lysates were immunoblotted with anti-talin MoAb (upper panel), anti-vinculin polyclonal antibody (middle panel), or anti-paxillin antibody (lower panel). C, Activation of αIIbβ3 was assessed by JON/A binding to platelets incubated with 150 ng/mL of convulxin. The plots represent the degree of GFP expression (horizontal) and specific antibody binding (vertical). D, Activation of αIIbβ3 assessed by JON/A binding and P-selectin expression in GFP-positive platelets stimulated with 150 ng/mL of convulxin. The plots represent the degree of P-selectin expression.

**Silencing of Talin in Platelets Decreases αIIbβ3 Activation**

We next examined whether talin-1 knockdown affects αIIbβ3 activation in vivo platelets, using shRNA silencing. Although talin is believed to be involved in the final common step of integrin αIIbβ3 activation, functional analysis in vivo platelets has not been performed. When bone marrow cells transduced with LentiLox-scramble-GPIbα or LentiLox-talinA-GPIbα at an MOI of 30 were transplanted, we confirmed the inhibition of talin expression in platelets derived from the cells transduced with LentiLox-talinA-GPIbα by intracellular flowcytometry (Figure 3A). In addition, talin reduction was also verified after sorting of GFP-positive platelets by immunoblotting (Figure 3B). On the other hand, the expressions of αIIbβ3, GPIbα, and GPVI were not affected (data not shown). As shown in Figure 3C through 3E, in talin-deficient platelets identified as GFP-positive cells, αIIbβ3 activation after ADP or convulxin stimulation was significantly decreased. Furthermore, talin-deficient platelet partly affected the expression of P-selectin after the platelet stimulation (Figure 3D and 3E). These data clarified that talin was involved not only in αIIbβ3-dependent platelet activation but also in the release reaction in actual platelets.

Finally, using a platelet adhesion assay we attempted to determine whether talin-deficient platelets influenced the spreading onto fibrinogen. Platelet adhesion to immobilized sequence (LentiLox-scramble-GPIbα) or αIIbA sequence (LentiLox-αIIbA-GPIbα) were transplanted into recipient mice. After transplantation, GFP expression was observed in 15% to 20% of platelets in both transplanted groups (data not shown). It is of note that αIIbβ3 expression in GFP-positive platelets was significantly reduced in the recipient mice transplanted with cells transduced with LentiLox-αIIbA-GPIbα (Figure 2A and 2B). As well, JON/A binding after ADP stimulation, which recognizes activated αIIbβ3, was reduced to a greater extent by the transduction with LentiLox-αIIbA-GPIbα (Figure 2B and 2D). The discrepancy in the results between αIIbβ3 expression and JON/A binding is thought to be partly the result of the expression of an incompetent αIIbβ3 complex that is recognized as antigen but does not act as functional receptor. Because αIIb is the most abundant protein in platelets, it is possible that mRNA degradation of αIIb by siRNA in platelets becomes incompletely. Under the same conditions, GPIb and GPVI expressions were not affected (data not shown). Additionally, P-selectin expression after platelet activation was hardly affected (Figure 2C and 2D). These data suggested that the expression of the shRNA sequence driven by the U6 promoter is maintained during megakaryopoiesis, and that this method can be applied to investigations of the involvement of specific proteins in platelet activation.

**Figure 3 (Continued).** (horizontal) and JON/A binding (vertical). E, Columns and error bars represent the mean ± SD of mean fluorescence intensity (MFI) of antibody binding after stimulation with 6 μmol/L ADP or 150 ng/mL of convulxin to GFP-positive platelets (n=7 to 11 per group).
fibrinogen by itself was not inhibited by the deficiency of talin (Figure 4). However, platelet spreading after stimulation with PMA was markedly suppressed (Figure 4), suggesting that talin is required for platelet spreading on fibrinogen.

**Discussion**

RNA interference using siRNAs to inhibit specific gene expression is a powerful and promising technology for both basic research and therapeutic intervention. A number of vector systems have been reported to mediate stable transduction and expression of shRNAs in mammalian cells. Among them, lentiviral vectors have been demonstrated to have the ability to stably transduce nondividing cells such as stem cells through integration of the vector DNA into the genome. RNA polymerase III promoters, most commonly the H1 and U6 promoters, have been incorporated into the lentiviral vectors for stable expression of shRNAs. The potential problem for functional genomics studies inherent in transfection with shRNA is variability in transfection efficiency. A solution to this problem is coexpression of reporter genes such as GFP, which facilitates the selection of transduced cells. To obtain efficient gene marking of platelets derived from transduced hematopoietic stem cells, we substituted the ubiquitous CMV promoter of LentiLox with the platelet-specific GPIbα/H925 promoter and showed that transplantation of bone marrow cells transduced with the vector enabled specific marking of platelets derived from transduced hematopoietic stem cells expressing shRNA sequence.

Platelets are terminally-differentiated anucleate cells, and for this reason direct gene transfer and silencing using virus or plasmid vector have been thought to be impossible. Since the development of gene targeting technologies in embryonic stem cells, the “gold standard” for the analysis of gene function in platelets has been the creation of knockout mice. A large number of knockout studies have shown aberrant platelet phenotypes. However, one of the major drawbacks of conventional knockouts is that if the gene product is essential in many tissues, then it is quite likely that the consequence of homozygosity for the mutated allele will be lethality. Several experimental strategies are used or have the
potential to overcome the problem of lethality. First, improvements in the technological procedures have allowed for refined analyses of gene functions at specific developmental stages or in specific tissues, based on conditional knock-out strategies by means of Cre-lox regulated recombination.21

Another solution, which has been applied in a few cases, is to use hematopoietic cells from fetal liver of knockout embryos to reconstitute the hematopoietic system of lethally-irradiated wild-type animals.22,23 Despite these significant improvements, the creation of loss-of-function alleles in the mouse remains time-consuming and costly. Our demonstration that the expression of shRNAs driven by RNA polymerase III promoters can be used to functionally silence protein expression in platelets suggests that RNAi-based technologies might represent a convenient strategy for the study of platelet signal transduction. Our methods could in fact detect platelets derived from transduced stem cells as GFP-positive platelets, and so enable the examination of the involvement of target proteins in platelet signal transduction by flow cytometry and platelet aggregation assay. However, GFP-positive platelets after the transplantation were limited by up to 20% in our protocols. Hence, platelet aggregation testing and the analysis of intracellular signaling pathways including tyrosine phosphorylation are not thought impossible. Higher transfection efficiencies would be required to demonstrate genuine effects and to be a valid alternative to making gene knockout mice.

Cellular control of integrin activation is essential for normal development because it controls cell adhesion, migration, and assembly of the extracellular matrix.24,25 Platelets express members of the β1 subfamily (αvβ1, α2β1, and α6β1) that support platelet adhesion to the extracellular matrix proteins including collagen and laminins, as well as expressing members of the β3 subfamily (αvβ3 and αIIbb3).24 Among them, αIIbb3, a receptor for fibrinogen, von Willebrand factor (VWF), fibronectin, and vitronectin is an essential requirement for platelet aggregation. Integrin activation can be controlled by signaling pathways that are thought to act by regulating specific interactions between cytoplasmic proteins and the integrin—β subunit—cytoplasmic tail.25,26 Although many types of proteins interacting with integrin cytoplasmic tails have been reported to be involved in platelet aggregation,12,27–31 functional analysis of in vivo platelets has not been reported; embryos lacking these proteins, including talin, vinculin, FAK, and Cas do not normally grow in the uterus.32–35 Talin is a major cytoskeletal protein that colocalizes with activated integrins and binds to integrin β cytoplasmic domains; with the overexpression of the N-terminal region of talin results in activation of integrins.14,28,36 Additionally, binding of talin to integrin β tails has been shown to be a common final step in integrin activation.12 In this study, using a method involving RNA interference, we clearly demonstrated that talin is involved in αIIbb3-dependent platelet activation in vivo platelets. Furthermore, talin might partly participate in the α-granule release reaction. These results confirmed that this strategy could be useful as a convenient and powerful method to investigate the role of specific proteins in platelet activation in vivo.

Acknowledgments

We thank N. Matsumoto and M. Ito for their excellent technical assistance.

Sources of Funding


Disclosures

None.

References


Silencing of a Targeted Protein in In Vivo Platelets Using a Lentiviral Vector Delivering Short Hairpin RNA Sequence
Tsukasa Ohmori, Yuji Kashiwakura, Akira Ishiwata, Seiji Madoiwa, Jun Mimuro and Yoichi Sakata

Arterioscler Thromb Vasc Biol. 2007;27:2266-2272; originally published online September 13, 2007;
doi: 10.1161/ATVBAHA.107.149872
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/10/2266

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/09/20/ATVBAHA.107.149872.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Silencing of a Targeted Protein in \textit{in vivo} Platelets Using a Lentiviral Vector Delivering Short Hairpin RNA Sequence

\textbf{First Author:} Tsukasa Ohmori  
\textbf{Short Title:} Silencing of targeted protein in platelets

Tsukasa Ohmori, Yuji Kashiwakura, Akira Ishiwata, Seiji Madoiwa, Jun Mimuro, and Yoichi Sakata

\textbf{Supplemental Materials}
Materials and Methods

Cytokines and Antibodies

Recombinant human thrombopoietin (TPO) and recombinant human stem cell factor (SCF) were gifts from Kirin Brewery Co. Ltd. (Gunma, Japan). The following materials were obtained from the indicated suppliers: recombinant human IL-6 (IL-6), recombinant human soluble IL-6 receptor (sIL-6R), and recombinant human Flt3-Ligand (Flt3-L) (PeproTech EC, London, UK); anti-mouse Ly5 (CD45) monoclonal antibody (MoAb) (clone 30-F11), anti-mouse Ly5.1 (CD45.1) MoAb (clone A20), and anti-mouse P-selectin (clone RB40.34), APC-conjugated streptavidin and anti-paxillin MoAb (clone 349) (BD Biosciences Co., San Jose, CA); anti-mouse GPIbα MoAb (clone Xia.G5), anti-mouse GPVI MoAb (clone Six.E10), anti-mouse activated αIIbβ3 MoAb (clone JON/A), and anti-mouse αIIb MoAb (clone MWReg30 and clone Leo.D2) (Emfret Analytics GmbH & Co., Wurzberg, Germany); anti-talin MoAb (Clone 8D4) and anti-vinculin rabbit polyclonal antibody (Sigma-Aldrich Co. St. Louis, MO); anti-GFP sheep polyclonal antibody (AbD Serotec, Oxford, UK).

Complementary DNA Cloning and Transient Transfection

cDNA of mouse αIIb, β3, vinculin and GPVI was amplified by RT-PCR using RNA from mouse bone marrow cells as template. Total RNA of mouse bone marrow cells was prepared with an RNA isolation kit (RNeasy Mini kit; Qiagen Inc., Valencia, CA). The isolated RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen Co., Carlsbad, CA). Reverse transcribed cDNA was amplified using Pfu
Ultra High-fidelity DNA polymerase (Stratagene, La Jolla, CA), with 40 cycles of 30 s at 94°C, 30 s at 60°C, and 4 min at 72°C. Integrin αIIb and integrin β3 were cloned in two stages at the ClaI site and XmaI site, respectively. The cDNA of mouse talin-1 was cloned by PCR from RIKEN mouse FANTOM™ Clone (M421024C16). The oligonucleotide primer pairs used for the cDNA cloning are described in Supplemental Table I.

After subcloning into pCR-Blunt-TOPO (Invitrogen), the cDNA fragment was subsequently cloned into the pcDNA3 expression plasmid (Invitrogen). Sub-confluent HEK293 cells in each well of 12-well plates were transfected with 0.7 μg of plasmid(s) containing cDNA of mouse αIIb and β3, GPVI, vinculin, or talin-1, together with 1.3 μg of the shRNA vector, using Lipofectamine 2000 (Invitrogen). After 2 h, an equal volume of Dulbecco’s Modified Eagle’s Medium-Ham’s F12 medium (DMEM/F12; Invitrogen) supplemented with 20% fetal bovine serum (FBS) was added to each well. Cells were incubated for 48 h at 37°C, and expression was then examined by flow cytometry or immunoblotting, while mRNA levels were quantified by real time quantitative PCR, as described below.

Construction of Lentiviral Vector and Virus Transduction

A gene transfer vector, pLL3.7, for constructing replication-defective self-inactivating HIV shRNA vector was purchased from ATCC (LentiLox-CMV). The characteristics of this vector are that the cells expressing targeting shRNA can be distinguished by GFP expression driven by the cytomegalovirus (CMV) promoter. We previously showed
that transduction of hematopoietic stem cells with a lentiviral vector harboring the GPIbα promoter, but not the CMV promoter, resulted in preferential GFP expression in platelets in vivo. ³ To generate a vector enabling efficient identification of platelets derived from transduced hematopoietic stem cells, the CMV promoter was removed from pLL3.7 by digestion with NolI and NheI, followed by insertion of the GPIbα promoter (LentiLox-GPIbα).

Putative short interfering RNA (siRNA) sequences were designed against targeted mouse genes using web software provided by Dharmacon RNA Technologies (http://www.dharmacon.com/). Sequences were determined to be unique to the gene by BLAST searches of the GenBank database. Oligonucleotide sequences were designed corresponding to the sense and antisense sequences of the siRNA target sites of interest separated by a hairpin loop sequence (Supplemental Figure I). The oligonucleotide pairs were then annealed and cloned into the HpaI/XhoI site of pLL3.7. The oligonucleotide pairs used to create the construct are described in Supplemental Table II.

Lentiviral vectors were generated essentially as described. ⁴ Briefly, the lentiviral shRNA vector and each packaging vector (gag/pol, rev, and VSVG) were cotransfected into HEK293T cells using Lipofectamine PLUS reagent (Invitrogen). Supernatants were collected 48 h after transfection, and filtered through a 0.4-µm filter. The transduction units of lentiviral vector were measured as described previously. ³

Bone marrow cells obtained from mouse femur and tibia were pre-cultured for 24 h before viral transduction with Iscove’s Modified Dulbecco’s Medicum (IMDM;
Invitrogen) containing 1% fatty acid-free bovine serum albumin (BSA), 200 \( \mu \text{g/mL} \) transferrin, and 10 \( \mu \text{g/mL} \) insulin, supplemented with 100 ng/mL SCF, 10 ng/mL TPO, 100 ng/mL IL-6, 100 ng/mL Flt-3L, and 400 ng/mL sIL-6R. Cultured cells (1 x 10^7 cells) were then resuspended in 10 mL of IMDM with 10% FBS. The cells were transduced with lentiviral vectors at a multiplicity of infection (MOI) of 30 in the presence of the same cytokine combination and incubated at 37°C.

**Stem Cell Transplantation**

C57BL/6 (B6-Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) were purchased from Sankyo-Lab Service (Tsukuba, Japan). All 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’s guidelines. Bone marrow cells were obtained from B6-Ly5.1 mice to allow us to distinguish between donor and recipient cells. Recipient mice (8 to 12-week-old B6-Ly5.2) were irradiated with a single lethal dose of 9.5 Gy (Gamma Cell; Norton International, ON, Canada), and then received 2 million cultured lentivirus-transduced bone marrow cells obtained from B6-Ly5.1 mice.

**Flow Cytometry**

Citrate whole blood was obtained from the superior vena cava of mice, and immediately diluted 1:20 with modified HEPES-Tyrode buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM NaHCO_3, 20 mM HEPES [N-2-
hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.4) containing 5 mM glucose, 0.4% BSA, 4 U/mL heparin, and 1 mM CaCl₂. For expression of glycoprotein and GFP in platelets, blood samples were incubated with appropriate fluorophore-conjugated monoclonal antibodies for 15 min at room temperature and directly analyzed on a FACS Aria (Becton Dickinson). When indicated, platelets were stimulated with 500 μM GRGDS, 6 μM ADP, or 150 ng/ml of convulxin (Alexis Biochemicals), and stained with fluorophore-labeled antibodies for 15 min at room temperature and then directly analyzed. To assess intracellular talin expression, washed mouse platelets were fixed with 0.5% paraformaldehyde for 60 min and then permealized with PBS containing 0.05% saponin and 0.2% BSA. After washing with PBS, the cells were incubated with 1 μg of biotin-conjugated anti-talin MoAb (8D4) for 30 min. Intracellular talin expression was detected by the binding to APC-labeled streptavidin.

**Immunoblotting**

Transfected HEK293 cells (1 x 10⁶ cells) were lysed in 250 μL of 1 x ice-cold lysis buffer [100 mM Tris (pH 7.4), 2% NP-40, 100 mM NaCl, 2 mM EDTA, and Complete Protease Inhibitor Cocktail (Roche Diagnostics Co., Basel, Switzerland)]. Lysates were centrifuged at 15,000 x g for 10 min, and the resultant supernatants used as whole cell lysate. To assess protein expression in GFP-positive platelets, 5.0 x 10⁶ GFP-positive platelets were sorted by FACS Aria, and then lysed by 50 μL of 1 x ice-cold lysis buffer. The proteins were resolved by SDS-PAGE and then electrophoretically transferred to a PVDF membrane. Membranes were blocked with 1% BSA in
phosphate buffered saline (PBS) for 3 h. After extensive washing with PBS containing 0.4% Tween-80, the membrane was incubated with anti-talin MoAb (1 µg/mL), anti-vinculin polyclonal antibody (1 µg/mL) or anti-paxillin MoAb (1:10,000) for 2 h. Antibody binding was detected using peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG and visualized with ECL PLUS reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RT-PCR

Total RNA was isolated from the indicated tissues using an RNA isolation kit (RNeasy Mini kit; QIAGEN). Quantification of mRNA expression was performed by real-time quantitative RT-PCR using a SuperScript III Platinum SYBR® Green One-Step qRT-PCR Kit (Invitrogen), as described previously. The oligonucleotide primer pairs used in this experiment are shown in Supplemental Table I. Aliquots of 10 ng of purified RNA were used as the templates. RT-PCR amplification was initiated by incubations at 55°C for 30 min and then 95°C for 5 min. The thermal cycling profile consisted of 40 cycles of 94°C for 15 s and 60°C for 30 s. A standard curve was established by analyzing double aliquots of serial dilutions of each cDNA-containing plasmid. For each sample, the amount of the target sequence was estimated by reference to a standard curve, and the quantity determined by dividing the copy number of the sequence by that of the human GAPDH sequence.

Platelet adhesion assay and immunohistochemistry
Platelet adhesion to fibrinogen was assayed in 8-well Lab-Tek® Chamber Slide™. Dishes were coated with 200 μg/mL of fibrinogen for 16 h at 37°C, washed two times with PBS, and blocked with 1 mg/mL of BSA for 1 h. Washed mouse platelets were prepared as previously described. Two hundred μl of washed platelets (2 x 10⁵/μL) stimulated without or with 1 μM PMA were placed onto the fibrinogen-coated dishes for 1 h at 37 °C. After washing twice with PBS, adherent platelets 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 anti-GFP sheep polyclonal antibody (1:200), for 16 h at 4°C, washed in PBS, and then incubated for 2 h with goat anti-sheep polyclonal antibody conjugated with Alexa488 (Molecular Probes). Actin filaments were detected by staining with 5 μg/mL of rhodamine-conjugated phalloidin. Immunofluorescent staining was observed and photographed using a confocal microscope (FV1000, Olympus, Tokyo, Japan).
References


5. Leng XH, Hong SY, Larrueca S, Zhang W, Li TT, Lopez JA, Bray PF. Platelets of female mice are intrinsically more sensitive to agonists than are platelets of males. Arterioscler Thromb Vasc Biol. 2004;24:376-381.
### Supplemental Table I  
The oligonucleotide primer pairs used in this study

<table>
<thead>
<tr>
<th>cDNA (Gen Bank Accession Number)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For cDNA cloning</strong></td>
<td></td>
</tr>
<tr>
<td>The first part of mαIIb</td>
<td>sense 5'-CCTGTTTAGGACGTTTGGAAG-3'</td>
</tr>
<tr>
<td>(NM_010575.1)</td>
<td>antisense 5'-CACAATCACGTTGTTGATCCA-3'</td>
</tr>
<tr>
<td>The last part of mαIIb</td>
<td>sense 5'-CTTCGTTGGGCGTTGACATC-3'</td>
</tr>
<tr>
<td></td>
<td>antisense 5'-CTGCTGTCATTTCCTCTCT-3'</td>
</tr>
<tr>
<td>The first part of mβ3</td>
<td>sense 5'-TGAAGGCGCGGACAGGAT-3'</td>
</tr>
<tr>
<td>(AF_026509.1)</td>
<td>antisense 5'-CTCCAAATCTTGAGGCCACAC-3'</td>
</tr>
<tr>
<td>The last part of mβ3</td>
<td>sense 5'-CTCAACAACGAGGTTATCCCA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense 5'-CATTCCTGCCCCGAGTGG-3'</td>
</tr>
<tr>
<td>murine GPVI</td>
<td>sense 5'-GTTGCAGAACCATGTTCCA-3'</td>
</tr>
<tr>
<td>(XM_975576)</td>
<td>antisense 5'-GTATTCTTAGCCAGTGAG-3'</td>
</tr>
<tr>
<td>murine Vinculin</td>
<td>sense 5'-ATGCCGCGGTTTCACACCGCTACA-3'</td>
</tr>
<tr>
<td>(NM_009502)</td>
<td>antisense 5'-CTACTGAGTACGAGATTTCTCT-3'</td>
</tr>
<tr>
<td>murine Talin-1</td>
<td>sense 5'-CATGGTGTTGCGTTTCGCTGAA-3'</td>
</tr>
<tr>
<td>(AK_147228)</td>
<td>antisense 5'-TAGTGTCTCTCGTCAAGCTGAG-3'</td>
</tr>
<tr>
<td><strong>For real time qPCR</strong></td>
<td></td>
</tr>
<tr>
<td>murine αIIb</td>
<td>sense 5'-GTCAAGAGGACGACTGGAGCC-3'</td>
</tr>
<tr>
<td>(NM_010575.1)</td>
<td>antisense 5'-TCCTGTGCTCATCCCTGAGTC-3'</td>
</tr>
<tr>
<td>murine GPVI</td>
<td>sense 5'-GATATGCCTTGGTGCCACAGAT-3'</td>
</tr>
<tr>
<td>(XM_975576)</td>
<td>antisense 5'-TGCAAAGCTTCATCTCCCTGT-3'</td>
</tr>
<tr>
<td>murine Vinculin</td>
<td>sense 5'-CAGGGCTCTGTTGAAGACAGCC-3'</td>
</tr>
<tr>
<td>(NM_009502)</td>
<td>antisense 5'-AATAGCCCTGTCACCAGCC-3'</td>
</tr>
<tr>
<td>murine Talin-1</td>
<td>sense 5'-TGCCCTCCCAACGACTTTC-3'</td>
</tr>
<tr>
<td>(AK_147228)</td>
<td>antisense 5'-GCATGTCTGTGACGGTCTTGG-3'</td>
</tr>
<tr>
<td>human GAPDH</td>
<td>sense 5'-TCAACGTGTGGTCATCTCCG-3'</td>
</tr>
<tr>
<td>(NM_014364.4)</td>
<td>antisense 5'-AATGTTCATGGAGCCAGGTAA-3'</td>
</tr>
<tr>
<td>siRNA</td>
<td>sence</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Scramble</td>
<td>sence</td>
</tr>
<tr>
<td></td>
<td>antiseence</td>
</tr>
<tr>
<td>αIIb-A</td>
<td>sence</td>
</tr>
<tr>
<td></td>
<td>antiseence</td>
</tr>
<tr>
<td>αIIb-B</td>
<td>sence</td>
</tr>
<tr>
<td></td>
<td>antiseence</td>
</tr>
<tr>
<td>Talin-A</td>
<td>sence</td>
</tr>
<tr>
<td></td>
<td>antiseence</td>
</tr>
<tr>
<td>Talin-B</td>
<td>sence</td>
</tr>
<tr>
<td></td>
<td>antiseence</td>
</tr>
</tbody>
</table>
Supplemental Figure I  Schematic representation of the lentiviral vector used in this study.

(A) Characteristics of the lentiviral shRNA vector.  (B) Locations of oligonucleotides of the genes encoding shRNA for mouse integrin αIIb (ITGA2IIB) and mouse talin-1 (TLN-1).
Supplemental Figure II  The effects of promoter differences on GFP expression in blood cells in vivo. (A) Bone marrow cells were transduced with LentiLox-CMV or LentiLox-GPlbα at an MOI of 30. Each irradiated mouse received 2,000,000 transduced cells. Representative flow cytometry analyses of GFP-positive cells in CD45+ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood 30 days after transplantation. (B) Percentages of GFP-positive cells in CD45+ cells, RBCs, and platelets 30 days after transplantation. Columns and error bars represent the mean ± SD (n=9 in LentiLox-CMV; n=10 in LentiLox-GPlbα).
Supplemental Figure III  Silencing of mRNA expression by LentiLox plasmid containing shRNA sequence. HEK293 cells were trasfected with expression plasmid containing cDNA of integrin αIIbβ3 (A), GPVI (B), Talin (C), or vinculin (D) together with an Lenti-Lox plasmid without (Empty) or with shRNA sequence containing scramble, αIIb-A, αIIb-B, Talin-A, or Talin-B. At 2 days after the transduction, silencing of mRNA expression was quantified by real-time quantitative RT-PCR. Columns and error bars represent