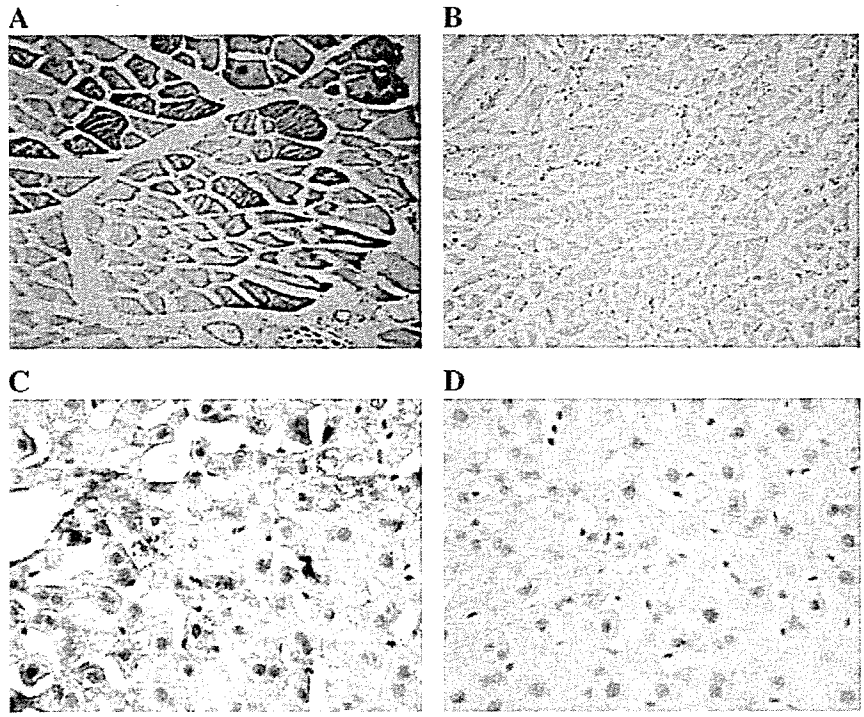

Figure 6 Immunohistochemical Analysis for cFVIII transgene products. Immunohistochemistry for cFVIII of the skeletal muscles of hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII vectors (A) and the liver of hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII vectors (C) is shown (positive stain: brown). For the control, sections of the skeletal muscles (B) and the liver (D) obtained from hemophilia A mice without vector injection were processed simultaneously with anti-FVIII antibodies.

with the traceable expression in the heart, lung, and spleen (not shown). In accordance with the data on cFVIII transcripts, cFVIII molecules were immunohistochemically detected in the skeletal muscles of AAV1-β-actin-cFVIII injected mice and in the liver of mice with intravenous injection of AAV8-β-actin-cFVIII (Fig. 6).

Discussion

Because of the size and nature of the FVIII gene (cDNA), there were difficulties in hemophilia A gene therapy compared with gene therapy for hemophilia B. These difficulties were solved by efforts of many investigators that allowed use of a modified FVIII gene such as BDD FVIII cDNA, improved vector systems, and new strategies. Based upon these studies, a few clinical trials of hemophilia A gene therapy were conducted [17–19]. Increase of FVIII activities in the circulation and clinical improvements were observed in patients who received vector injection or transplantation of genetically modified cells. However, long-term expression of FVIII from the transgenes was not achieved in these studies. Thus, reexamination of the vector systems, the target organs for transduction, and the promoters may be required.

The recombinant AAV vectors are thought to be one of the better vectors in terms of its capability to transduce non-dividing cells and long-term transgene expression, although delivery of the FVIII gene using AAV vectors were limited by its small packaging capacity [4]. The dual AAV vector system utilizing separate AAV2 vectors independently carrying the FVIII heavy chain gene and the FVIII light chain gene could express functionally active FVIII [5]. However, there was an imbalance in the expression levels of the FVIII heavy chain and FVIII light chain, suggesting that over-expressed free FVIII light chain molecules might be more immunogenic than the native molecules. The BDD FVIII gene could be packaged in AAV2 or AAV8 vectors in the previous studies and these vectors could efficiently transduce the liver with intraportal injection of the vectors [6]. Transduction of the liver with peripheral vein injection of AAV8 vectors was as efficient as portal vein injection of vectors, although that of AAV2 vectors was not [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ, so FVIII

synthesis in hepatocytes and its subsequent secretion into the circulation may be warranted. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs. AAV1 vector-based transduction of the skeletal muscles has beneficial characteristics of removing the transgenes. This is the first report of sufficient expression of FVIII in the skeletal muscles transduced with AAV vectors and suggests that skeletal muscle-directed FVIII expression has a potential for hemophilia A gene therapy.

Compared with synthesis and secretion of FVIII into the circulation from the liver, transport of sufficient FVIII into the circulation from the skeletal muscle fibers is not assured. Based upon our data, it is apparent that transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal muscles with AAV1-β-actin-cFVIII regarding FVIII production. The difference between FVIII levels in the peripheral blood of these vector-injected mice may be due to how the transduced cells secrete FVIII molecules into the circulation. Hepatocytes actively secrete a variety of molecules including FVIII into the circulation. Since recombinant cFVIII is in a BDD form, its expression in and secretion from hepatocytes is expected to be better than native FVIII [20], accounting for the high cFVIII expression in mice with intravenous injection of AAV8 vectors carrying the cFVIII gene though cFVIII expressing hepatocytes were not abundant. Although muscle fibers are surrounded by capillaries, transport of recombinant FVIII molecules from muscle fibers to capillaries would not be as efficient as that from hepatocytes.

In terms of the immune reaction to transgene products, muscle stem cells have been shown to function as antigen-presenting cells, suggesting that expression of the transgene by the ubiquitous promoter in the skeletal muscles might lead to development of antibodies against the transgene products if there is no immune tolerance to the transgene products [21]. This was confirmed by Wang et al. [22]. Neutralizing antibody formation was observed in 66.7% of mice with AAV1cFVIII injection even with administration of immunosuppressant, while it was not observed in mice with AAV8-β-actin cFVIII injection by week 12 after vector injection, supporting the potential advantage of AAV8 vector-based transduction of the liver over the muscle-directed transduction by AAV1 vectors.

Each vector system has advantages and disadvantages in these respects. We may need to confirm the results obtained in hemophilia mice using dogs and non-human primates that genetically are more close to humans because there may be differences in transduction efficiency of various serotypes between mice and humans [23]. Taken together, we may need to perform a comparative study using another animal models such as hemophilic dogs and non-human primates that are more genetically close to humans than mice to address these questions. Additionally, use of tissue-specific promoters to minimize neutralizing antibody formation may be a better strategy for expressing transgenes in a tissue- and organ-specific manner. These experiments will be performed in future studies.

In conclusion, our data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have the potential for hemophilia A gene therapy. Our present studies have provided important insight about selecting the appropriate target for delivery of the therapeutic genes and the vector system for the hemophilia A gene therapy.

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The FASEB Journal • FJ Express Full-Length Article

Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibα promoter: *in vivo* model for platelet-targeting gene therapy

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ABSTRACT **Platelets release several mediators that modify vascular integrity and hemostasis. In the present study, we developed a technique for efficient transgene expression in platelets *in vivo* and examined whether this targeted-gene-product delivery system using a platelet release reaction could be exploited for clinical applications. Analysis of luciferase reporter gene constructs driven by platelet-specific promoters (the GPIIb, GPIbα, and GPVI) revealed that the GPIbα promoter was the most potent in the megakaryoblastic cell line UT-7/TPO and human CD34$^+$-derived megakaryocytes. Transduction of UT-7/TPO; CD34$^+$-derived megakaryocytes; and c-Kit$^+$, *Sca*I$^+$, and Lineage$^-$ (KSL) murine hematopoietic stem cells with a simian immunodeficiency virus (SIV)-based lentiviral vector carrying *eGFP* resulted in efficient, dose-dependent expression of eGFP, and the GPIbα promoter seemed to bestow megakaryocytic-specific expression. Transplantation of KSL cells transduced with SIV vector containing eGFP into mice showed that there was preferable expression of eGFP in platelets driven by the GPIbα promoter [7–11% for the cytomeglovirus (CMV) promoter, 16–27% for the GPIbα promoter]. Furthermore, transplantation of *ex vivo*-transduced KSL cells by SIV vector carrying *human factorVIII (hFVIII)* driven by the GPIbα promoter induced the production of detectable transcripts of the *hFVIII* gene and the hFVIII antigen in bone marrow and spleen for at least 90 days and partially corrected the hemophilia A phenotype. Platelet-targeting gene therapy using SIV vectors appears to be promising for gene therapy approaches toward not only inherited platelet diseases but also other hemorrhagic disorders such as hemophilia A.—Ohmori, T., Mimuro, J., Takano, K., Madoiwa, S., Kashiwakura, Y., Ishiwata, A., Niimura, M., Mitomo, K., Tabata, T., Hasegawa, M., Ozawa, K., Sakata, Y. Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibα promoter: *in vivo* model for platelet-targeting gene therapy. *FASEB J.* 20, E769–E779 (2006)**

Key Words: hemophilia A · lentiviral vector · stem cell transplantation

BLOOD PLATELETS, the principal cells responsible for primary hemostasis, play major roles in thrombosis, atherosclerosis, tumor metastasis, and inflammation. At the site of vascular injury, activated platelets aggregate and release several mediators that modify vascular integrity and hemostasis (1). Platelet-derived bioactive products are released by exocytosis of the three types of granules (dense-core, alpha, and lysosome) within platelets; this process is mediated by soluble NSF attachment protein receptor proteins and VAMP-3 (2). Taking advantage of the platelet-release reaction as a delivery system for a specific factor would be a reasonable approach for therapy for individuals deficient in the factor because it provides a way to enhance the local concentration of target substances at the site of vascular injury, while minimizing the influence of plasma proteins that may inhibit their activities.

In transgenic settings, platelet-targeting gene transfer has been reported to enable the storage of the targeted substance within platelets. Platelet expression of urokinase-type plasminogen activator (u-PA) using a megakaryocyte-specific platelet factor 4 promoter enabled u-PA to be stored in platelets and then released within developing thrombi when the platelets became activated (3). Furthermore, platelet-specific expression of coagulation factor VIII (FVIII) could be achieved in

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a transgenic setting with the resultant FVIII predominantly or exclusively stored in platelet granules rather than being released into the plasma (4). When transgenic mice were crossed onto a FVIII null background, whole blood clotting time was partially corrected (4). These findings have facilitated the development of methods for gene therapy that use platelets to deliver therapeutic agents to the site of vascular injury.

The present study aimed at megakaryocyte- and platelet-directed gene transfer using a platelet-specific promoter that could be directly applicable to gene therapy for not only inherited platelet disorders but also coagulation factor deficiencies such as hemophilia. Since megakaryocytes have a finite life span, hematopoietic stem cells are preferable targets for genetic transfer to establish *in vivo* long-term expression of the target protein in platelets. When retroviral vector containing the *Glycoprotein (GP) IIIa* gene driven by the GPIIb promoter was transduced into $CD34^+$ cells from a Glanzmann thrombasthenia patient with defects in the GPIIIa gene, GPIIb/IIIa were detected after *in vitro* megakaryocyte differentiation (5). Furthermore, retrovirus transduction of FVIII driven by the virus promoter into human $CD34^+$ hematopoietic stem cells reportedly enabled FVIII-transduced megakaryocytes to store human FVIII with von Willebrand factor (VWF; ref 6). Although the concept of platelet-targeting gene therapy is very attractive and some reports have clinical implications as described above (3–6), there have been no evaluations of whether gene transduction of hematopoietic stem cells effectively results in sufficient genetic information being given in platelets so that they synthesize enough transgene products to correct the phenotype of the hemorrhagic disorder.

In this study, we used GPIbα promoter to achieve platelet-specific gene expression, based on analyses of the promoter activities of three platelet-specific genes, *GPIIb, GPIbα*, and *GPVI,* in megakaryocytes and then we assessed the gene transfer efficiency to platelets *in vivo* by transducing hematopoietic stem cells with simian immunodeficiency lentivirus vector (SIV). We also examined whether FVIII ectopically expressed in platelets corrected the hemorrhagic phenotype of FVIII-deficient mice by using the platelet release reaction.

MATERIALS AND METHODS

Mice

Hemophilic A mice with targeted destruction of exon 16 of the *FVIII* gene were kindly provided by H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA; ref. 7). 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 at Jichi Medical School, and animal care was performed in accordance with the guidelines of the committee (8).

Cytokines and antibodies

Recombinant human thrombopoietin (TPO) and recombinant human stem cell factor (SCF) were gifts from Kirin Brewery (Gunma, Japan). The following materials were obtained from the indicated suppliers: recombinant human interleukin (IL)-6, recombinant human soluble IL-6 receptor (sIL-6R), recombinant human basic fibroblast growth factor (b-FGF), and recombinant human Flt3-Ligand (Flt3-L; PeproTech EC, London, UK); recombinant human IL-3 (IL-3; TECHNE, Minneapolis, MN); antimouse c-Kit monoclonal antibody (MoAb; clone 2B8), antimouse Sca-1 MoAb (clone D7), antimouse Ly5 (CD45) MoAb (clone 30-F11), and antimouse Ly5.1 (CD45.1) MoAb (clone A20; BD Pharmingen, CA); antimouse GPIbα MoAb (clone Xia.G5; Emfret Analytics, Wurzberg, Germany); and anti-human GPIIb/IIIa MoAb (clone 5B12), anti-human GPIbα MoAb (clone AN51), and anti-human CD34 MoAb (clone BIRMA-K3; DakoCytomation, Glostrup, Denmark).

Cell culture

The human megakaryoblastic cell line UT-7/TPO was kindly provided by Dr. Norio Komatsu (Yamanashi University, Yamanashi, Japan; ref. 9). The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 10 ng/ml of TPO. Human umbilical vein endothelial cells (HUVEC) were obtained and maintained as described previously (10). U937, a myelomonocytic cell line, and K562, an erythroleukemic cell line, were obtained from the American Type Culture Collection and were cultured in RPMI 1640 supplemented with 10% FBS. 3T3 fibroblasts were cultured with Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% FBS. Human aortic smooth muscle cells (SMC) were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and maintained in DMEM/F-12 with 10% FBS and 10 ng/ml of b-FGF.

Megakaryocyte differentiation

Human umbilical cord blood was obtained during normal full-term deliveries. The institutional review board of Jichi Medical School approved the study protocols, and informed consent was obtained from all donors. Human $CD34^+$ cells derived from cord blood were isolated using the AutoMACS magnetic cell sorting system (Miltenyi Biotec., Auburn, CA) according to the manufacturer's instructions. The purity of isolated $CD34^+$ cells was >90% (data not shown). $CD34^+$ cells were expanded and differentiated into megakaryocytes with IMDM containing 1% fatty acid-free BSA, 200 μg/ml of human iron-saturated transferrin, and 10 μg/ml of human recombinant insulin, supplemented with 50 ng/ml of TPO and 10 ng/ml of IL-3 (11). After 14 days of culture, 75–90% of cells were positive for GPIIb/IIIa.

Construction of luciferase reporter plasmid, transient transfection, and luciferase assay

The DNA fragments for the promoter, which reportedly each had maximum promoter activity [GPIIb promoter: −554 to+33 (12), GPIbα promoter:- 254 to+330 (13), GPVI promoter: −320 to + 28 (14)], were amplified by polymerase chain reaction (PCR) using human genomic DNA as template. Since the region surrounding the translation start site of GPIbα gene at position −5 from the initiator ATG codon is important for transgene expression (15), we selected the DNA fragment just before the first ATG sequence, as reported previously (13). The oligonucleotide primer pairs used for

the cloning of the promoter sequence were as follows: 5′-CCATTCCAGAAGGTGTGAAG-3′ (sense) and 5′-GTTCCTCAGCCCTGTCCTG-3′ (antisense) for GPVI promoter (GenBank #AF521646); 5′-CTAAAGCTTGGCTCAAGACG-3′ (sense) and 5′-CTTCCTTCTTCCACAACCTC-3′ (antisense) for GPIIb promoter (GenBank #M33319); and 5′-GTTCTGGGATTACAGGCATGAG-3′ (sense) and 5′-GAGGACCTGTGGGCAAGGGAG-3′ (antisense) for GPIbα promoter (GenBank #M22403).

After being subcloned into pCR-Blunt-TOPO (Invitrogen, Carlsbad, CA), the fragment was subsequently cloned into pGL3-basic, a promoterless luciferase plasmid (Promega, Madison, CA). Five hundred thousand cells (UT-7/TPO, $CD34^+$-derived megakaryocytes, K562, and U937) plated in IMDM (without FBS or growth factors) or subconfluent cells (HUVEC and SMC) in each well of six-well plates were transfected with 4 μg of plasmid DNA using DMRIE-C Reagent (Invitrogen). After 4 h, an equal volume of IMDM supplemented with 2× growth factors was added to each well. Cells were incubated for 48 h at 37°C, and luciferase activities were assayed according to the manufacturer's instructions (Luciferase Assay System, Promega).

Construction and production of SIV vectors

Replication-defective self-inactivating (SIN) SIV vector was created by deletion of the U3 region of 3′ long terminal repeat (LTR), which contains two NF-κB sites, three Sp-1 sites, and a TATA box, as described previously (ref. 16; Gene Bank association number: #X07805). The transduction efficiency of the SIN vector did not differ from the intact U3 sequence-containing vector (16).

The full-length *human FVIII (hFVIII)* cDNA was a generous gift from Dr. J.A. van Mourik (Blood Coagulation, Sanquin, Amsterdam, Netherlands), and the *human B domain-deleted (BDD) FVIII (hBDD-FVIII)* cDNA was generated by PCR-based mutagenesis as described previously (17). *eGFP* or *hBDD-FVIII* driven by the CMV promoter (SIV-CMV-eGFP/hFVIII) or GPIbα promoter (SIV-GPIbα-eGFP/hFVIII) was inserted between the LTR-containing elements of an SIV-derived vector (see Results).

The gene transfer plasmid was transfected together with three packaging plasmids (encoding *gag-pol, rev,* and VSV-G *env*) into 293T cells using Lipofectamine Plus reagent (Invitrogen). After 12 h, the culture medium was replaced to start harvesting virus particles; harvesting was undertaken at 48 h and virus particles were concentrated by ultracentrifugation. Transduction units of SIV vectors carrying *eGFP* were measured by infection of 293 cells followed by measurement of eGFP expression by FACS analysis. The average infectivity of the SIV-CMV-eGFP vector was in the range of 2–5 × 10^8 TU/ml. To compare viral infectivity between different promoters or targeted genes, viral particle titer was simultaneously measured by real-time quantitative reverse transcriptase (RT)-PCR. Viral RNA was isolated using QIAamp viral RNA mini kit (QIAGEN, Valencia, CA), and the isolated RNA was reverse-transcribed using SuperScript II (Invitrogen). Quantification of vector particles was performed by measuring copies of vector-specific post transcriptional regulatory element derived from the wood-chuck hepatitis virus (WPRE) sequences by real-time quantitative PCR using the QuantiTect Probe PCR system (QIAGEN). The WPRE sequence was amplified with WPRE forward primer 5′-GCTTTCATTTTCTCCTCCTT-3′ and WPRE reverse primer 5′-GGCCACAACTCCTCATAA-3′. The FAM-labeled probe sequence was 5′-ATCCTGGTTGCTGTCTC-3′. The PCR started with an initial incubation step of 15 min at 95°C. Thermal cycling consisted of 45 cycles of 94°C for 15 s, 56°C for 30 s, and 76°C for 30 s. During the annealing phase of PCR, reporter fluorescent dye from a specific probe was detected with a ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA). The standard curve of the viral titer was estimated by serial dilution of the gene transfer vector plasmid. The average virus particle titer of the SIV-based vector was in the range of 1–2 × 10^{10} TU/ml. The infectivity of SIV vectors [multiplicity of infection (MOI)] was estimated from the ratio of particle titer to SIV-CMV-eGFP.

For the transduction of UT-7/TPO and $CD34^+$-derived megakaryocytes with SIV vectors, 1 × 10^5 cells were resuspended in 100 μl of culture medium containing 8 μg/ml of polybrene. Cells were transduced with SIV vectors at various MOIs indicated in the test for 24 h. Cells were then resuspended in 300 μl PBS containing 0.5% BSA and 2 mM EDTA, and eGFP-positive cells were subjected to FACS analysis. When $CD34^+$ cells were transduced with SIV containing *eGFP* followed by differentiation of megakaryocytes *in vitro*, we could not observe sustained eGFP expression during the differentiation. This was probably due to the short life span of the eGFP-expressing differentiating cells *in vitro*. Hence, $CD34^+$-derived megakaryocytes, at an indicated day after the start of differentiation, were transduced with SIV to examine the influence of megakaryopoiesis on promoter activity.

Hematopoietic stem cell isolation and viral transduction

Bone marrow cells obtained from mouse femur and tibia were depleted for cells expressing lineage cell markers B220, CD5, CD11b, Gr-1, and Ter-119 by magnetic sorting using a Lineage Cell Depletion kit (Miltenyi Biotec.) and then sorted for Sca-1^+ and c-kit^+ cells (KSL) by FACS (FACSAria Cell Sorter, Becton-Dickinson).

When freshly isolated KSL cells were directly infected with SIV vectors, PI-positive cells (dead cells) increased after the transduction and the efficacy of the transduction decreased (data not shown). Hence, we examined the culture conditions of KSL cells to improve SIV transduction and cell viability. When isolated KSL cells were cultured with IL-3, IL-6, and SCF, SIV transduction of the eGFP gene into KSL cells resulted in lower transduction efficiencies (25–35%: MOI of 30) and a significant increase in PI-positive cells (20–30%; data not shown). On the other hand, high proportions of eGFP positive cells were obtained by culturing the KSL cells with SCF, IL-6, sIL-6R, Flt-3L, and TPO for 3–7 days (see Results), and PI-positive cells after eGFP transduction were significantly decreased (8–12%). Hence, KSL cells were precultured with IMDM 1% fatty acid-free BSA, 200 μg/ml of transferrin, and 10 μg/ml of insulin, supplemented with 100 ng/ml of SCF, 10 ng/ml of TPO, 100 ng /ml of IL-6, 100 ng/ml of Flt-3L, and 400 ng/ml of sIL-6R before viral transduction in accordance with a method for human $CD34^+$ expansion (18). Infection of cells with SIV was carried out in a plate coated with 50 μg/ml of RetroNectin (TakaraBio, Tokyo, Japan). Cultured KSL cells (1×10^5 or 1×10^6 cells) were resuspended in 100 μl or 1 ml of IMDM with 10% FBS. The cells were transduced with SIV vectors at various MOIs as indicated in the text for 12 h in the presence of the same cytokine combination and incubated at 37°C. Since the use of polybrene (up to 4 μg/ml) *per se* did not significantly improve the transduction efficiencies of mouse KSL and human $CD34^+$ cells (data not shown), our procedure for stem cell transduction was performed without polybrene. Cells were then resuspended in 100 μl of PBS containing 1% BSA for stem cell transplantation or incubated for the indicated number of days for FACS analysis.

Stem cell transplantation

Bone marrow cells were obtained from B6-Ly5.1 to allow us to distinguish between donor and recipient cells. Recipient mice

(8–12 wk old B6-Ly5.2) were irradiated with a single lethal dose of 9.5 Gy (^{60}Co, Gamma Cell; Norton International, Ontario, Canada). One hundred thousand cultured KSL cells from B6-Ly5.1 without or with SIV transduction were injected together with 5×10^5 freshly isolated Ly5.2 unfractionated bone marrow cells. Since the transplantation procedure without unfractionated bone marrow cells increased mortality to 60–80%, we simultaneously transplanted KSL cells with non-transduced bone marrow cells as competitor cells. To assess the reconstitution of bone marrow, peripheral blood was drawn from the retro-orbital sinus with heparin-coated micropipettes and analyzed for the percentages of Ly5.1 (donor-derived) lymphoid and myeloid cells by flow cytometry. In our transplantation procedure, engraftment by Ly5.1 cells was 40–55% in lymphoid and myeloid cells 4 months after transplantation. For secondary transplantations, 2×10^6 unfractionated marrow cells collected from primary recipient mice 4 months after primary transplantation were used to reconstitute lethally irradiated recipients (9.5 Gy). The animals with relatively high eGFP levels after transplantation were chosen as donors for the secondary bone marrow transplantation.

Detection of the transcripts of hFVIII transgene

Detection of *hFVIII* transgene transcripts was performed by RT-PCR (19). RNA was isolated from mouse organs using an RNA isolation kit (RNeasy Protect Kit; QIAGEN). RNA samples were subjected to RT-PCR using a pair of primers for *hFVIII* (19) and an RT-PCR kit (SuperScript One-Step RT-PCR System, Invitrogen). A primer pair for mouse *GAPDH* mRNA (R&D Systems, Minneapolis, MN) was used in the control RT-PCR experiments.

Quantification of SIV vector-derived mRNA expression was examined by real-time quantitative RT, using the QuantiTect Probe RT-PCR system (QIAGEN). Mouse GAPDH (mGAPDH) control reagents (QIAGEN) were used to estimate the amount of RNA analyzed. A standard curve was established by analyzing duplicate aliquots of serial dilutions of SIV gene transfer vector. Fifty nanograms of purified RNA from tissues as indicated in the text were used as a template. For each sample, the amounts of WPRE sequences compared with the standard control curve were estimated, and the quantities of the WPRE sequences were determined by dividing the copy number of the WPRE sequences by those of the mGAPDH sequences.

Integration of vector sequence into genomic DNA

Genomic DNA was extracted from bone marrow cells of mice 4–5 months after transplantation using a DNA isolation kit (DNeasy Tissue Kit; QIAGEN). WPRE sequence derived from the SIV vector was amplified using real-time quantitative PCR with a QuantiTect Probe PCR system as described above. For each sample, the amounts of WPRE sequences compared with the standard control curve were estimated, and the integration of the vector sequence was calculated from dividing the copy number by the cell count corresponding to the DNA quantity.

Immunohistochemistry

Bone marrow cells attached to glass slides using a Cytospin3 (Shandon, ThermoShandon) were fixed with 3% paraformaldehyde in PBS and then permeabilized with 0.2% Triton X-100. After being blocked with 1% BSA, samples were incubated with polyclonal anti-hFVIII antibody (Ab) conjugated with biotin at 4°C for 2 h, washed with PBS, and then incubated with streptavidin conjugated with Alexa 594 (Molecular Probes, Eugene, OR) and FITC-labeled ant-imouse GPIbα MoAb. Immunofluorescent staining was observed and photographed using a fluorescent microscope with an attached camera.

For detection of hFVIII molecules in mouse tissues by immunohistochemistry, the spleen was fixed with 4% paraformaldehyde in PBS for 2 h at 4°C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at −25°C and attached to polylysine-coated glass slides. For detection of hFVIII, tissue sections were blocked with 1% rabbit serum in PBS containing Triton-X 100 (0.1%) and incubated with sheep polyclonal anti-human FVIII Ab (Cedarlane Laboratories, Homby, Ontario, Canada) at 4°C for 16 h. After being washed with PBS, sections were incubated with biotin-conjugated rabbit anti-sheep IgG Ab followed by the avidin-biotin complex (ABC) reagents (Vectastain ABC Elite kit; Vector, Burlingame, CA) and a 3,3′-diaminobenzidine kit (Vector).

Measurement of hFVIII antigen, neutralizing antibodies, and phenotypic correction

hFVIII antigens were measured by an anti hFVIII-specific ELISA kit (Affinity Biological, Hamilton, Ontario, Canada) and compared with those in pooled human normal plasma. Two hundred and seventy microliters of whole blood were drawn from the superior vena cava of anesthetized mice using a syringe containing 30 μl of sodium citrate and 1 μM PGI_2 to inhibit platelet activation. When indicated, 1 μM phorbol myristyl acetate (PMA) and 50 μg/ml of collagen were added to the blood to activate platelets. After centrifugation, the platelet-poor plasma was frozen at −80°C until assayed for FVIII antigen. Analyses of neutralizing antibodies against hFVIII developed in mice were performed by the Bethesda method as described using FVIII deficient plasma and normal pooled plasma (8). Phenotypic correction was tested in some of the transplanted mice by anesthetizing them with diethyl ether and clipping 1.5 cm of their tails. The mice were then observed for survival after 24 h.

RESULTS

Comparison of luciferase reporter expression driven by platelet-specific promoters

To achieve efficient expression of genes targeted in platelets, we first compared the promoter activities of three platelet-specific genes, *GPIIb*, *GPIbα*, and *GPVI*, in megakaryocytic cells. **Figure 1*A*** shows a schematic diagram of the platelet-specific promoters used in this study with their unique regulatory elements. The luciferase reporter gene was used to compare promoter activities among different promoters. We used the nucleotide region in promoters that had the highest activity in previous studies (12–14). The GPIbα promoter directed the most powerful expression of luciferase in UT-7/TPO cells, a megakaryoblastic cell line (Fig. 1*B*), and its relative efficiency was even more marked in $CD34^+$-derived megakaryocytes (Fig. 1*C*). Since *GPIb* and *GPVI* were reportedly expressed in endothelial cells (20,21), we examined whether these platelet-specific promoter activities were stimulated in

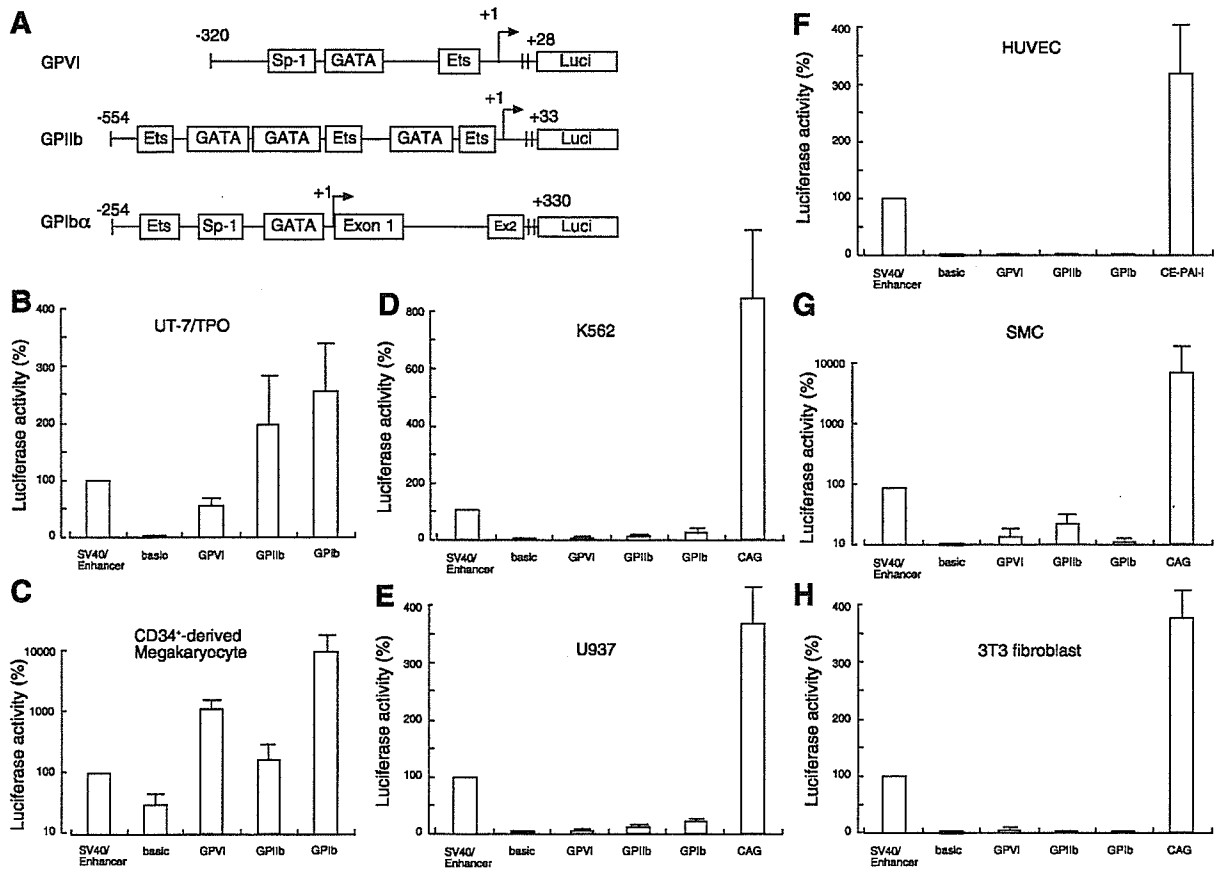

Figure 1. Comparison of platelet-specific promoter activities. *A)* A schematic presentation of platelet-specific promoters used in experiments is shown. Each construct along with a promoter-less vector (basic) or a positive control vector (SV40/Enhancer) was transfected into UT-7/TPO (*B*), $CD34^+$-derived megakaryocytes (*C*), K562 (*D*), U937 (*E*), HUVECs (*F*), SMC (*G*), and 3T3 fibroblasts (*H*). Luciferase activities were measured 48 h after transfection and are shown relative to the activity driven by the SV40 promoter (SV40/Enhancer). Each experiment was carried out 5 times with duplicate samples. Columns and error bars are mean ± SD.

endothelial cells. Under conditions in which the PAI-1 promoter (22) efficiently directed luciferase expression, the GPIIb, GPIbα, and GPVI promoters did not direct luciferase expression in HUVECs (Fig. 1). We further examined promoter specificity by using other cells including K562, an erythroleukemic cell line; U937, a myelomonocytic cell line; human aortic artery SMC and 3T3 fibroblast cell. In these cells, the platelet-specific promoters drove less reporter gene than SV40/Enhancer and CAG promoter (Fig. 1). Hence, the specificities of the platelet glycoprotein promoters did not differ, and the activity of GPIbα promoter was the strongest in megakaryocytes.

Efficient transformation of megakaryocytic cells by SIV-based vectors

Next, we constructed SIV-based lentiviral vectors containing the *eGFP* gene under the control of either the CMV promoter (SIV-CMV-eGFP), GPIbα promoter (SIV-GPIbα-eGFP), GPIIb (SIV-GPIIb-eGFP), or GPVI (SIV-GPVI-eGFP). The transgene located downstream of the promoter was inserted between the LTR-containing elements of a SIV-derived vector (**Fig. 2*A***). To increase gene expression in the transduced cells, a post-transcriptional regulatory element derived from woodchuck hepatitis virus (WPRE) was inserted downstream of the gene expressed (Fig. 2*A*). To investigate *eGFP* gene transduction of megakaryocytes, UT-7/TPO or $CD34^+$-derived megakaryocytes were cultured for 24 h in the presence of various concentrations of the indicated SIV vector. SIV-GPIIb-eGFP, SIV-GPIbα-eGFP, SIV-GPVI-eGFP, and SIV-CMV-eGFP efficiently transduced the eGFP gene into UT-7/TPO and $CD34^+$-derived megakaryocytes (Fig. 2*B*, C). There were no significant differences in eGFP expressions among cells transfected with SIV-GPIIb-eGFP and SIV-GPIbα-eGFP (Fig. 2*B* and *C*).

We next investigated whether *ex vivo* megakaryocyte differentiation affects gene expression. eGFP expression in $CD34^+$ hematopoietic progenitor cells transduced with SIV-GPIbα-eGFP or SIV-GPVI-eGFP was $<10\%$, whereas $\approx 20\%$ of the cells transduced with SIV-GPIIb-eGFP were positive for eGFP (day 0 in Fig. 2*D*), indicating that the GPIIb promoter worked at an

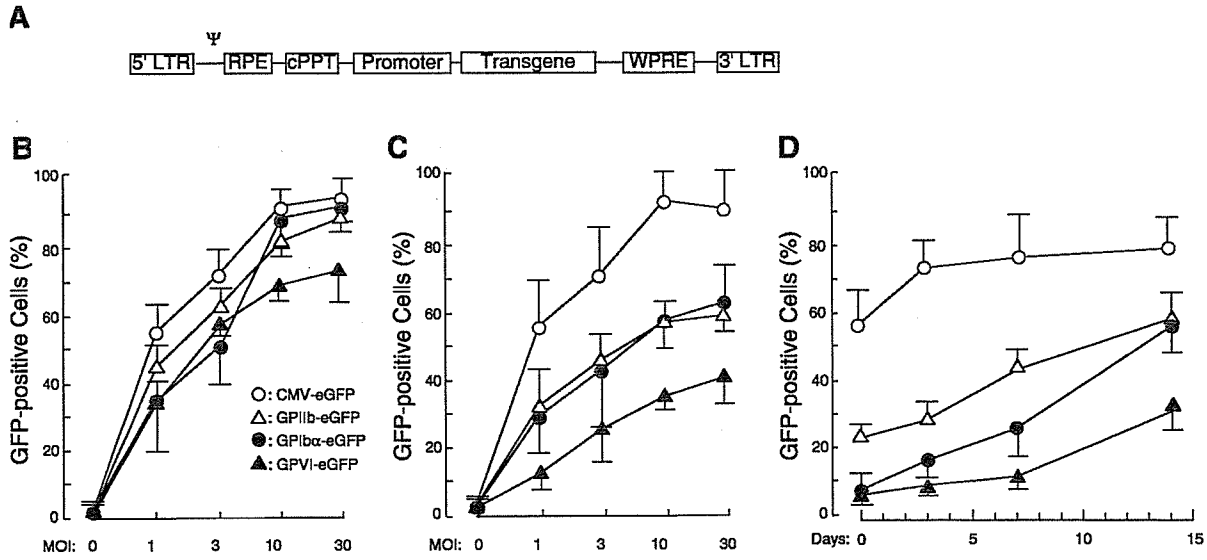

Figure 2. Expression of eGFP in UT-7/TPO cells and $CD34^+$-derived megakaryocytes transduced with SIV vectors carrying the *eGFP* gene driven by the CMV, GPIIb, GPVI, or GPIbα promoter. *A)* A schematic diagram of SIV constructs used in this study is shown. UT-7/TPO (*B*) and $CD34^+$-derived megakaryocytes (*C*) were infected with SIV-*CMV*-eGFP (open circle) SIV-GPIbα-eGFP (closed circle), SIV-GPIIb-eGFP (open triangle), or SIV-GPVI-eGFP (closed triangle) at indicated MOI. Expression of eGFP in cells was analyzed by flow cytometry. Columns and error bars are mean ± SD (n=3). *D)* $CD34^+$-derived megakaryocytes at day 0, 3, 7, and 14 after start of differentiation were transduced with SIV-*CMV*-eGFP (open circle) SIV-GPIbα-eGFP (closed circle), SIV-GPIIb-eGFP (open triangle), or SIV-GPVI-eGFP (closed triangle) at MOI of 30. Columns and error bars are mean ± SD (n=3).

earlier stage of megakaryopoiesis. Furthermore, the GPIbα compared with the GPIIb promoter seemed to work during a later phase of megakaryocyte maturation, and the percentages of eGFP expression did not change after differentiation (day 14 in Fig. 2*D*). eGFP expression driven by the CMV promoter was not affected by megakaryocyte differentiation (Fig. 2*D*). We selected the GPIbα promoter as the platelet-specific promoter for *in vivo* experiments because the promoter activity of GPIbα was the strongest in differentiated megakaryocytes (Fig. 1) and the promoter drove in the later phase of megakaryopoiesis (Fig. 2).

Establishment of efficient transduction of KSL cells with SIV vector

We next optimized the transduction protocol for KSL cells by using an SIV vector containing the *eGFP* gene driven by the CMV promoter. The transduction efficiency of eGFP in cultured KSL cells reached 60–80% (**Fig. 3*A***). The plateau value for transduction was observed with a MOI of 10–30. One day (24 h) after incubation with the viral vector was sufficient to achieve the efficient expression of the transduced gene (Fig. 3*B*). eGFP expression then gradually declined (Fig. 3*B*); the decrease might have been due to the reduction in cell viability, because PI-positive cells (dead cells) increased with time (data not shown). Although lentiviral vectors can express transgenes for long periods even in the absence of integration in $CD34^+$ cells (23), KSL cells could not maintain eGFP expression for long periods *in vitro*. Accordingly, we cultured KSL at an MOI of 30 for 24 h and transplanted the cells into recipient mice in the following experiments.

Preferential eGFP expression in platelets using SIV vectors harboring GPIbα promoter *in vivo*

To compare the strength and the specificity of the CMV and GPIbα promoters and to assess eGFP transduction by SIV vectors *in vivo*, KSL cells transduced with SIV-CMV-eGFP or SIV-GPIbα-eGFP were transplanted to recipient mice (Ly5.2). One hundred thousand cultured KSL cells (Ly5.1) transduced with SIV-CMV-eGFP or SIV-GPIbα-eGFP (MOI of 30) were transplanted together with 5×10^5 competitor cells (Ly5.2). When KSL cells transduced with SIV-CMV-eGFP were transplanted, eGFP expression was observed in 35–45% of $CD45^+$ cells and 7–11% of platelets in peripheral blood (**Fig. 4*A*** and ***B***). Interestingly, transduction of the SIV vector harboring the GPIbα promoter resulted in efficient gene marking to platelets (16–27%); however, only marginal eGFP expression was observed in $CD45^+$ and red blood cells (Fig. 4*A*, *B*). We next analyzed bone marrow cells from transplanted mice using specific markers to identify macrophages, granulocytes, B lymphocytes, T lymphocytes, and erythroblasts. Whereas eGFP was expressed in these lineages of cells of mice that received KSL cells transduced with SIV-CMV-eGFP, the GPIbα promoter drove just marginal eGFP expression in these cell lineages, confirming the specificity of its activity in megakaryocytes and platelets *in vivo* (Fig.

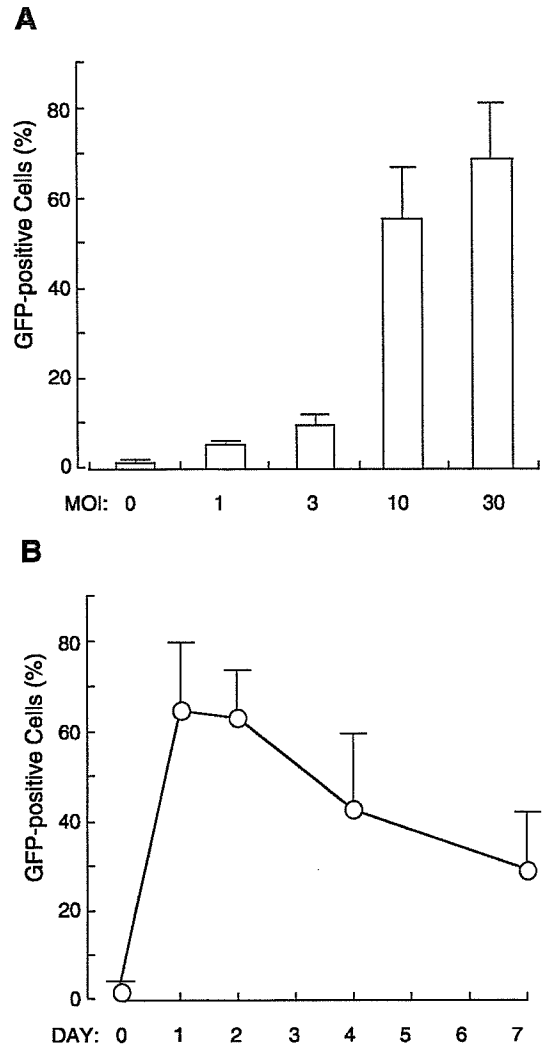

Figure 3. Transduction of KSL cells with SIV-*CMV*-eGFP. Cultured KSL cells were transfected with increasing concentrations of SIV-*CMV*-eGFP for 24 h (*A*) or with a fixed concentration (MOI=30) for various incubation times (*B*). After indicated incubations, expression of eGFP in KSL cells was analyzed by flow cytometry. Percentages of transduced cells expressing eGFP are shown. Columns and error bars are mean ± SD (n=3).

4*C*). We next performed a second bone marrow transplantation using marrow cells obtained from mice that had been transplanted 4 months earlier. As shown in Fig. 4*D*, eGFP expression driven by the CMV and GPIbα promoters in hematopoietic cells was sustained after the second stem cell transplantation, indicating that the promoter maintains transgene expression during differentiation of hematopoietic stem cells.

Expression of hFVIII and phenotypic correction in hemophilia A mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII

To determine whether platelet-directed gene therapy enables sustained expression of FVIII, we constructed two SIV-based vectors containing *hBDD-FVIII* cDNA under the control of either the CMV (SIV-CMV-hFVIII) or GPIbα promoter (SIV-GPIbα-hFVIII). We transplanted mice with 1×10^5 transduced KSL cells after lethal γ-irradiation. We first analyzed the presence of the *hFVIII* gene transcripts in organs of the transplanted recipients at 3 months after transplantation. Analyses for *hFVIII* transcripts driven by the CMV promoter revealed that the *hFVIII* gene was expressed mainly in bone marrow and to a lesser extent in the spleen (**Fig. 5*A*, *middle panel***). Interestingly, *hFVIII* mRNA was predominantly found in both the spleen and bone marrow in the recipients of KSL cells transduced with SIV-GPIbα-hFVIII (Fig. 5*A*, *lower panel*). To quantify the mRNA expression concentration in each organ, vector-specific WPRE expression was measured by real-time quantitative RT-PCR. As expected, based on the results from RT-PCR, bone marrow and spleen are the major sites for mice transplanted with KSL cells transduced with SIV vectors (Fig. 5*B*). Furthermore, we examined vector integration into the genome of bone marrow hematopoietic cells after transplantation and detected that 0.19–2.3 vector copies/genome were integrated in cells of transplanted mice (CMV promoter: 1.07±0.95; n=4; GPIbα promoter: 0.98±0.62; n=4). In accordance with the data on *hFVIII* transcripts, hFVIII molecules were immunohistochemically detected in bone marrow and the spleen in both types of transduced mice (**Fig. 6**). Interestingly, cells expressing GPIbα concurrently expressed hFVIII in bone marrow obtained from mice transduced with SIV-GPIbα-hFVIII (Fig. 6*A*).

Finally, we evaluated whether platelet-specific gene transduction using SIV-GPIbα-hFVIII resulted in phenotypic correction of FVIII-deficient hemophilia A mice. The plasma FVIII antigen concentration with or without platelet activation was measured in transplanted FVIII-deficient mice at 30 and 60 days after transplantation. We detected FVIII activity in the transplanted mice in which 1–2% correction was noted in the plasma of mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII (**Fig. 7*A***). When platelets were stimulated with collagen and PMA, the plasma FVIII concentration increased to 2–3.5% (Fig. 7*A*). The mortality rate after tail clipping was significantly improved in transduced mice (Fig. 7*B*). Furthermore, ectopically expressed hFVIII levels had not attenuated, and the appearance of inhibitor against hFVIII was not detected in mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII at day 60 after the transplantation (data not shown). We simultaneously performed transplantation experiments using SIV-*CMV*-hFVIII. Plasma levels of hemophilia A mice transplanted with KSL transduced with SIV-CMV-hFVIII were 3–6% after the transplantation, and phenotypic correction was also observed, as reported previously (19, 24, 25).

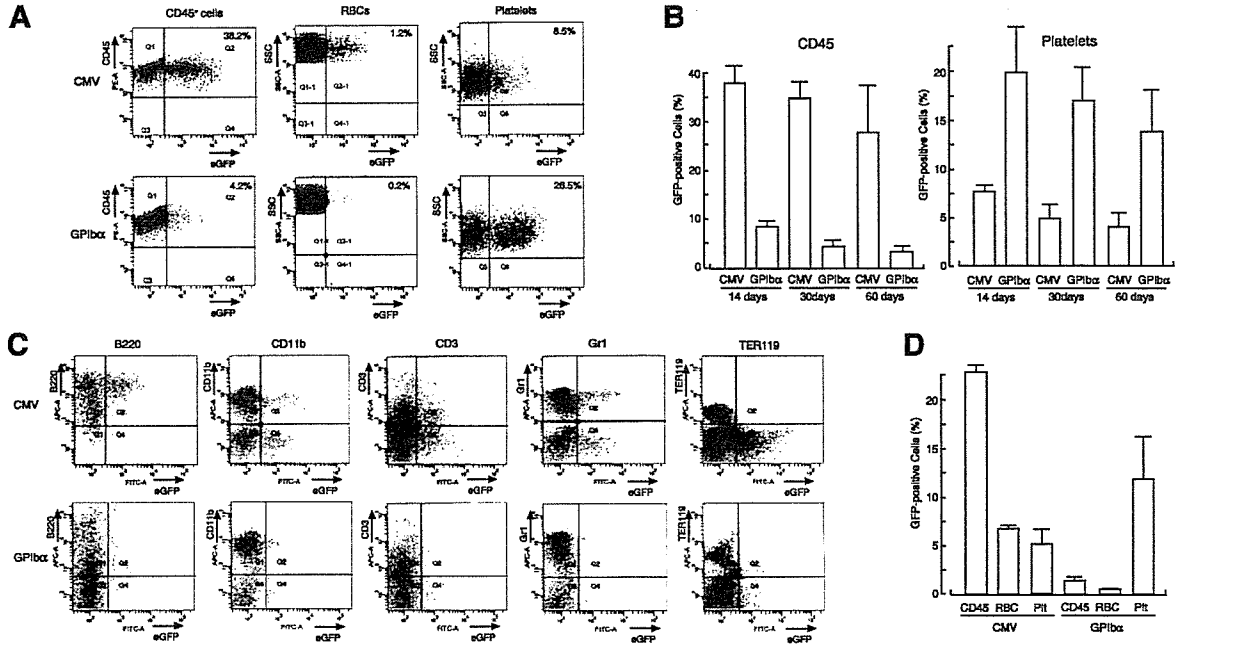

Figure 4. Effect of promoter differences on eGFP expression in blood cells *in vivo*. Cultured KSL cells were transduced with SIV-*CMV*-eGFP or SIV-GPIbα-eGFP at a MOI of 30. Each irradiated mouse received 100,000 transduced cells together with 5×10^5 unfractionated whole marrow cells. *A)* Representative flow cytometry analyses of eGFP-positive cells in $CD45^+$ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood are shown. *B)* Percentages of eGFP-positive cells in $CD45^+$ cells (*left*) and platelets (*right*) 14, 30, and 60 days after transplantation are shown. Columns and error bars are mean ± SD (n=5 per group). *C)* 60 days post-transplantation, bone marrow cells were stained using antibodies to detect B lymphocytes (B220), T lymphocytes (CD3), granulocytes (Gr1), macrophages (CD11b), and erythroblasts (TER119). GFP-positive cells in each lineage cells are measured by flow cytometry. Data represent 3 experiments. *D)* Flow cytometric analyses of $CD45^+$ cells, RBCs, and platelets in peripheral blood obtained from mice 30 days after second bone marrow transplantation. Columns and error bars mean ± SD (n=5 per group).

DISCUSSION

In this study, we examined the gene transduction of platelets and megakaryocytes by using an SIV lentiviral vector harboring a platelet-specific promoter *in vivo*. Since the strategy of using platelets as potential targets for producers of transgene products has already been proposed in transgenic mice (3, 4), it was shown that it is possible to apply this strategy to correct hemorrhagic disorders including hemophilia by efficient platelet-directed gene transduction *in vivo*. However, detailed comparisons of platelet-specific promoters and the efficiency of the transduction of transgenes *in vivo* have not previously been reported. In our system, the transduction of hematopoietic stem cells with an SIV lentiviral vector resulted in the expression of the transgene

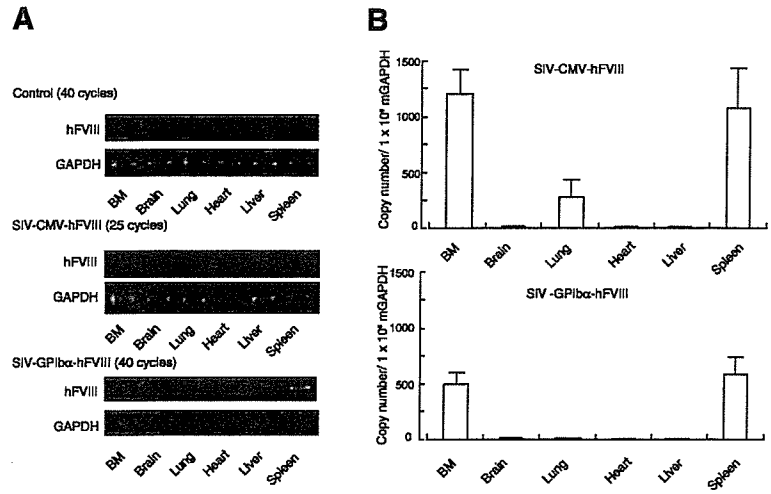

Figure 5. Expression of hFVIII in organs obtained from mice transplanted with hFVIII-transduced KSL cells. *A)* KSL cells not transduced (control) or transduced with SIV-*CMV*-hFVIII or SIV-GPIbα-hFVIII vector were injected into lethally irradiated CL57/B6 mice as described in Materials and Methods. RT-PCR analyses for the transcripts derived from the *hFVIII* gene in the indicated organs are shown. For control, RT-PCR for mouse GAPDH of RNA was performed simultaneously. Data represent 4 experiments. *B)* mRNA expression derived from SIV vectors was quantified by real time quantitative RT-PCR as described in Materials and Methods. Columns and error bars are mean ± SD (n=4 per group).

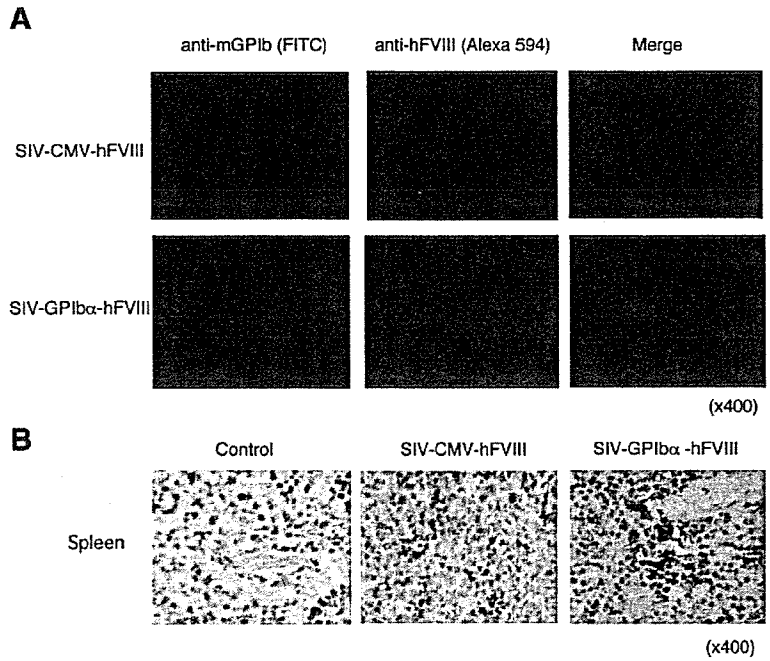

Figure 6. Expression of hFVIII in bone marrow and spleen in transplanted mice. *A)* Isolated bone marrow cells from mice transplanted with KSL cells transduced with SIV-*CMV*-hFVIII or SIV-GPIbα-hFVIII were immunostained for mouse GPIbα (*left*) and hFVIII (*middle*); 2 images are overlapped in right column, showing that in SIV-GPIbα-hFVIII-transduced bone marrow cells, GPIbα and FVIII expression overlapped. *B)* Immunohistochemistry for hFVIII in spleen of each transplanted mice (positive stain: brown). For control, sections of spleen obtained from mice transplanted with KSL cells without vector infection were processed simultaneously with anti-FVIII antibodies. Original magnification ×400.

in ≈20% of platelets and also resulted in a phenotypic correction of hemophilia A mice, suggesting that platelet-targeting gene therapy has the potential for further clinical applications. This is a first study to achieve a phenotypic correction of a coagulation abnormality such as hemophilia A by using platelet-directed gene transduction.

Megakaryocytes have a finite life span of ≈10–21 days (26); therefore, hematopoietic stem cells are a more practical target than megakaryocytes for genetic transfer to establish long-term expression of a target protein in platelets. Because lentiviruses are capable of infecting certain types of quiescent cells, there has been significant interest in the application of lentivirus-derived vectors to the transduction of hematopoietic cells; indeed, it has been shown that lentiviral vectors can efficiently transduce hematopoietic stem cells (27). We used the SIV lentiviral system for efficient platelet-targeting gene transduction because of its probable safety. The SIV lentiviral system was derived from SIVagmTYO1 and is nonpathogenic to its natural host and to experimentally infected Asian macaques (16). Replication-competent virus particles were not detected in vector-infected cells, and the risk of development of replication-competent lentivirus particles in HIV carrier patients may be significantly lower than that for the HIV-based vectors (19). Accordingly, SIV vectors have a safety advantage for clinical applications of gene therapy.

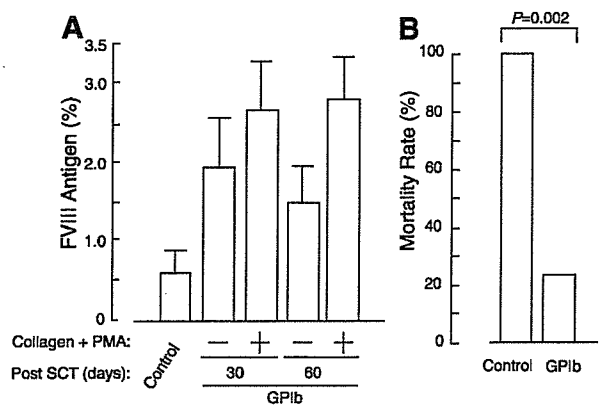

Figure 7. Phenotypic correction of hemophilia A mice by platelet-targeting gene delivery. *A)* Blood from FVIII-deficient mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII was stimulated without or with 50 μg/ml of collagen and 1 μM PMA for 15 min. After centrifugation, platelet-poor plasma was obtained, and hFVIII antigen levels were measured by ELISA. Columns and error bars are mean ± SD (n=4 per group). *B)* Mortality rate within 24 h after tail clipping in mice transplanted with KSL cells transduced with control or SIV-GPIbα-hFVIII (n=10 for control; n=8 for GPIbα). Mortality rate was statistically evaluated by a χ^2 test.

Most reported studies have used the GPIIb promoter for megakaryocyte- and platelet-specific gene transduction. We used the GPIbα promoter as a platelet-specific promoter in this study because the promoter activity of GPIbα was more potent than that of GPIIb in UT-7/TPO and $CD34^+$-derived megakaryocytes. Another reason we selected this platelet-specific promoter was that the GPIbα promoter works at a late stage of megakaryopoiesis. Although the *GPIIb* gene is expressed in platelets and megakaryocytes, it is an early gene for megakaryopoiesis (28). In conditional knockout mice in which the thymidine kinase gene was driven by the GPIIb promoter, the administration of gancyclovir led to a dramatic reduction in the platelet count (29). In bone marrow, erythroid and myeloid progenitors were also affected, which indicated the presence of *GPIIb* in progenitor cells (29). Indeed, 18% of human $CD34^+$

hematopoietic stem cells already expressed GPIIb, and so the appearance of GPIb was markedly delayed as compared with that of GPIIb, indicating that GPIb is a later marker of megakaryocytic maturation. Platelet-targeting gene therapy using the GPIbα promoter was therefore expected to allow more specific and restricted expression of gene products in platelets than that using the GPIIb promoter.

Another important finding here was that the eGFP gene driven by the CMV promoter showed significantly decreased expression in platelets, despite the high transduction efficiencies of $CD45^+$ cells *in vivo*. Generally, the reduction of transgene expression caused by a shortened protein half-life is even more pronounced in terminally differentiated blood cells (30). The decreased expression might have been mediated by the down-regulation of the transgene during differentiation; the stability of the encoded protein is at least as relevant for the expression of a transgene as the choice of the promoter or *cis*-elements influencing RNA processing in differentiated cells (30). In this context, the use of the GPIbα promoter, which drives expression in late megakaryocyte differentiation, might be important for gene transduction of terminally differentiated anucleate platelets.

Our strategy of platelet-directed gene transduction has potential for not only inherited platelet disorders (such as Glanzmann's thrombasthenia and Bernard-Sourlier syndrome) but also other hemorrhagic disorders. Hemophilia A is an X chromosome-linked bleeding disorder caused by defects in the *FVIII* gene and affecting ≈1:5000 males (31). Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well vary over in a broad range (5–100%; ref 31). Although sustained therapeutic expression of FVIII has been achieved in preclinical studies using a wide range of gene transfer technologies targeted at different tissues (32), emergence of neutralizing Ab often limits their clinical applications (33). The targeting of hematopoietic stem cells is not an exception. Although lentiviral FVIII gene transduction of hematopoietic stem cells is able to produce therapeutic levels of FVIII (19, 24, 25, 34), the emergence of neutralizing antibodies to FVIII has resulted in decreased levels of FVIII activity (34). Platelet-directed gene therapy for hemophilia A has a possible advantage for therapeutic applications, because the use of the platelet-specific system may limit the development of inhibitors by preventing the expression of FVIII in antigen presenting cells. Furthermore, 10–30% of populations with hemophilia A develop inhibitors to infusion products, which leads to the disruption of coagulation and severe bleeding (31). Under these conditions, platelet-directed gene therapy of hemophilia A is very attractive because platelets could specifically store the protein in the bloodstream and then specifically release it at sites of thrombus formation, thereby minimizing the influence of any circulating inhibitors. For further clinical application, the long-term observations are required to substantiate long-term *in vivo* gene expression because our observation periods were limited in this study.

During the course of this study, the therapeutic expression of GPIIb/IIIa in GPIIIa-deficient mice using HIV-lentivirus vector containing GPIIIa cDNA under the control of the GPIIb promoter was reported (35). That study used a heterogeneous population of bone marrow cells as a source for stem cell transplantation and gene transduction. We demonstrated efficient transduction of KSL murine hematopoietic cells by a SIV vector harboring the GPIbα promoter and phenotypic correction of hemophilia A mice. Primitive KSL cells are a nearly homogeneous population, and a single KSL cell frequently can provide long-term multilineage engraftment of lethally irradiated mice (36). Targeting of primitive hematopoietic stem cells is thought to be a safer approach, because the number of transduced cells needed for reconstitution is much lower than that needed when using a heterogeneous bone marrow population. The development of leukemia in two children with severe combined immunodeficiency disease who were transplanted with retroviral vector-transduced bone marrow cells caused renewed concern about the risks associated with the integration of proviral sequences into chromosomal DNA (37). One way to possibly reduce the risks of insertional mutagenesis would be to use transduction protocols that minimize the total number of genetically modified cells (38). From this aspect, our procedure using KSL cells transduced with SIV lentiviral system is a practical approach for platelet-specific gene modification in clinical applications.

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Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibα promoter: *in vivo* model for platelet-targeting gene therapy

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SPECIFIC AIMS

Platelets release a number of mediators that modify vascular integrity and hemostasis. The goals of this work were *1)* to develop a technique for efficient transgene expression in platelets *in vivo*; and *2)* to examine whether this targeted-gene-product delivery system using the platelet release reaction was directly applicable to gene therapy for coagulation factor deficiency hemophilia A.

PRINCIPAL FINDINGS

1. Comparison of luciferase reporter expression driven by platelet-specific promoters

We first compared the promoter activities of three platelet-specific genes, *Glycoprotein (GP) IIb, GPIbα*, and *GPVI*, in megakaryocytic cells. The GPIbα promoter directed the most powerful expression of luciferase in UT-7/TPO cells, a megakaryoblastic cell line, and $CD34^+$-derived megakaryocytes. The platelet-specific promoters drove less reporter gene compared with SV40/enhancer in endothelial cells, smooth muscle cells, and other hematopoietic cell lines.

2. Efficient expression of transgenes in platelets *in vivo*

We constructed simian immunodeficiency virus (SIV)-based lentiviral vectors containing the *eGFP* gene under the control of either the cytomegalovirus [cytomeglovirus (CMV)] promoter (SIV-CMV-eGFP), GPIbα promoter (SIV-GPIbα-eGFP), GPIIb promoter, or GPVI promoter. Transduction of $CD34^+$-derived megakaryocytes with the SIV-based lentiviral vectors resulted in efficient, dose-dependent expression of eGFP, and the GPIbα promoter seemed to bestow megakaryocytic-specific expression. We selected the GPIbα promoter as the platelet-specific promoter for *in vivo* experiments, because the promoter activity of GPIbα was the strongest in megakaryocytes and the promoter drove in the later phase of megakaryopoiesis. We next optimized the transduction protocol of c-Kit^+, $Sca1^+$, and $Lineage^-$ (KSL) murine hematopoietic stem cells using SIV-CMV-eGFP. The transduction efficiency of eGFP in cultured KSL cells reached 60–80%. The plateau value of transduction was observed with a multiplicity of infection (MOI) of 10–30.

To compare the specificity of the CMV and GPIbα promoters and to assess eGFP transduction by SIV vectors *in vivo*, KSL cells transduced with SIV-CMV-eGFP or SIV-GPIbα-eGFP were transplanted to recipient mice (Ly5.2). One hundred thousand cultured KSL cells (Ly5.1) transduced with SIV-CMV-eGFP or SIV-GPIbα-eGFP (MOI of 30) were transplanted together with 5×10^5 competitor cells (Ly5.2) after lethal γ-irradiation (9.5 Gy). When KSL cells transduced with SIV-CMV-eGFP were transplanted, eGFP expression was observed in 35–45% of $CD45^+$ cells and 7–11% of platelets in peripheral blood (**Fig. 1*A*** and ***B***). Interestingly, transduction of SIV vector harboring the GPIbα promoter would be more likely to result in efficient gene marking to platelets (16–27%; Fig. 1*A* and *B*). We next analyzed eGFP expression of bone marrow cells

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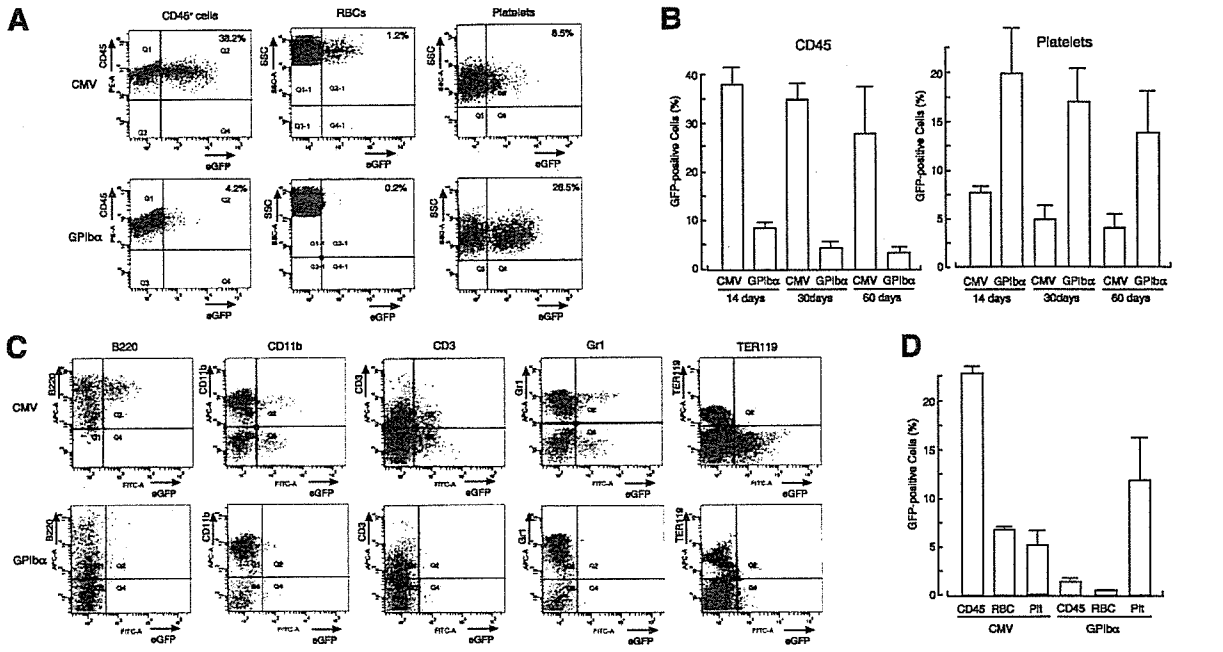

Figure 1. Effect of promoter differences on eGFP expression in blood cells *in vivo*. Cultured KSL cells were transduced with SIV-CMV-eGFP or SIV-GPIbα-eGFP at a MOI of 30. Each irradiated mouse received 100,000 transduced cells together with 5×10^5 unfractionated whole marrow cells. *A)* Representative flow cytometry analyses of eGFP-positive cells in $CD45^+$ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood are shown. *B)* Percentages of eGFP-positive cells in $CD45^+$ cells (*left*) and platelets (*right*) 14, 30, and 60 days after transplantation are shown. Columns and error bars are mean ± SD (n=5 per group). *C)* 60 days post-transplantation, bone marrow cells were stained using antibodies to detect B lymphocytes (B220), T lymphocytes (CD3), granulocytes (Gr1), macrophages (CD11b), and erythroblasts (TER119). GFP-positive cells in each lineage cells are measured by flow cytometry. Data represent 3 experiments. *D)* Flow cytometric analyses of $CD45^+$ cells, RBCs, and platelets in peripheral blood obtained from mice 30 days after second bone marrow transplantation. Columns and error bars are mean ± SD (n=5 per group).

from transplanted mice. Whereas eGFP was expressed in the lineage cells of mice that received KSL cells transduced with SIV-CMV-eGFP, the GPIbα promoter drove a marginal eGFP expression in these cell lineages, confirming the specificity of its activity in megakaryocytes and platelets (Fig. 1*C*). Next, we performed second bone marrow transplantations using marrow cells obtained from mice that had been transplanted 4 months earlier. As shown in Fig. 1*D*, eGFP expression driven by the CMV and GPIbα promoters in hematopoietic cells was sustained after the second stem cell transplantation.

3. Phenotypic correction of hemophilia A mice (factor VIII-deficient mice)

To determine whether platelet-directed gene therapy enables the sustained expression of coagulation factor VIII (FVIII), we constructed two SIV-based vectors containing the *human FVIII (hFVIII)* cDNA under the control of either CMV (SIV-CMV-hFVIII) or GPIbα promoter (SIV-GPIbα-hFVIII). We first analyzed the presence of the *hFVIII* gene transcripts in organs of the transplanted recipients 3 months after transplantation. Real-time quantitative reverse transcriptase-polymerase chain reaction revealed that bone marrow and spleen are the major expression sites in mice transplanted with KSL cells transduced with SIV vectors. In accordance with the data on *hFVIII* transcripts, hFVIII molecules were immunohistochemically detected in bone marrow and the spleen in both types of transduced mice.

We finally evaluated whether platelet-specific gene transduction using SIV-GPIbα-hFVIII resulted in phenotypic correction of FVIII-deficient hemophilia A mice. The plasma hFVIII antigen concentration without or with platelet activation was measured in transplanted FVIII-deficient mice at 30 and 60 days after transplantation. We detected FVIII activity in transplanted mice; 1–2% correction was noted in the plasma of mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII (**Fig. 2*A***). When platelets were stimulated with collagen and PMA, the plasma FVIII concentration increased to 2–3.5% (Fig. 2*A*). The mortality rate after tail clipping was significantly improved in transduced mice (Fig. 2*B*). Furthermore, ectopically expressed hFVIII levels did not attenuate with time, and the appearance of inhibitor against hFVIII was not detected in mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII at day 60 after transplantation.

CONCLUSION AND SIGNIFICANCE

In this study, we examined gene transduction of platelets and megakaryocytes using an SIV lentiviral vector harboring a platelet-specific promoter *in vivo*. Since the strategy of using platelets as potential targets for producers of transgene products has already been proposed in transgenic mice, it was possible to apply this strategy to correct coagulation abnormalities including hemophilia A by efficient platelet-directed gene transduction *in vivo*. In our system, the transduction of hematopoietic stem cells with the SIV lentiviral vector resulted in expression of the transgene in ≈20% of platelets, and ectopically expressed FVIII in platelets resulted in phenotypic correction of hemophilia A mice.

Blood platelets, the principal cells responsible for primary hemostasis at the site of vascular injury, activated platelets aggregate and release several mediators that modify vascular integrity and hemostasis. Taking advantage of the platelet-release reaction as a delivery system for a specific factor would be a reasonable approach for treatment of individuals deficient in the factor, because it provides a way to enhance the local concentration of target substances at the site of vascular injury, while minimizing the influence of plasma proteins that may inhibit their activities (**Fig. 3**).

Megakaryocytes have a finite life span; therefore, hematopoietic stem cells are a more practical target than megakaryocytes for genetic transfer to establish long-term expression of a target protein in platelets. Because lentiviruses are capable of infecting certain types of quiescent cells, there has been significant interest in the application of lentivirus-derived vectors to the transduction of hematopoietic cells. We used the SIV lentiviral system for efficient platelet-targeting gene transduction, because it is potentially safe. The SIV lentiviral system was derived from SIVagmTYO1 and is nonpathogenic to its natural host and to experimentally infected Asian macaques. Replication-competent virus particles were not detected in vector-infected cells, and the risk of development of replication-competent lentivirus particles in HIV carrier patients may be significantly lower than that for the HIV-based vectors. Accordingly, SIV vectors have an advantage in respect to safety issues and in clinical applications of hematopoietic stem cell-directed gene therapy. Furthermore, we used the GPIbα promoter for efficient transgene expression in platelets because the promoter activty of GPIbα was more potent than that of GPIIb and GPVI in megakaryocytes, and the GPIbα promoter works at a later stage of megakaryopoiesis. Platelet-targeting gene therapy using the GPIbα promoter was expected to allow more specific and restricted expression in platelets.

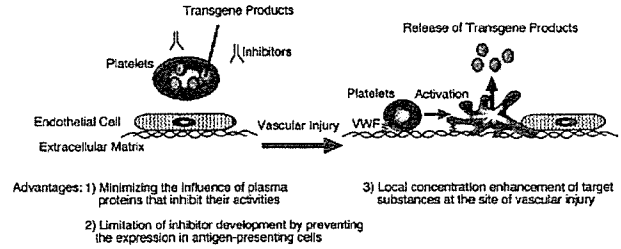

Figure 3. Expected advantages of platelet-directed gene therapy.

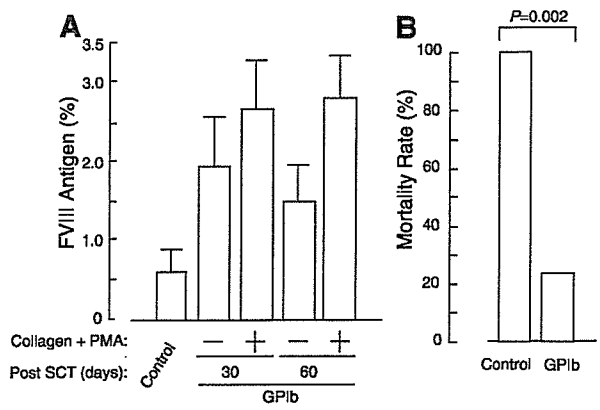

Figure 2. Phenotypic correction of hemophilia A mice by platelet-targeting gene delivery. *A)* Blood from FVIII-deficient mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII was stimulated without or with 50 μg/ml of collagen and 1 μM PMA for 15 min. After centrifugation, platelet-poor plasma was obtained, and hFVIII antigen levels were measured by ELISA. Columns and error bars are mean ± SD (n=4 per group). *B)* Mortality rate within 24 h after tail clipping in mice transplanted with KSL cells transduced with control or SIV-GPIbα-hFVIII (n=10 for control; n=8 for GPIbα). Mortality rate was statistically evaluated by a χ^2 test.

Hemophilia A is an X chromosome-linked bleeding disorder caused by defects in the *FVIII* gene. Hemophilia is considered suitable for gene therapy, because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well vary over in a broad range (5–100%). Although sustained therapeutic expression of FVIII has been achieved in preclinical studies using a wide range of gene transfer technologies targeted at different tissues, the emergence of neutralizing antibodies often limits their clinical applications. The transduction of hematopoietic stem cells is not an exception. Although lentiviral FVIII gene transduction of hematopoietic stem cells is able to produce therapeutic levels of FVIII, the emergence of neutralizing antibodies to FVIII has resulted in decreased levels of FVIII activity. Platelet-directed gene therapy for hemophilia A has a possible advantage for therapeutic applications, because the use of the platelet-specific system may limit the development of inhibitors by preventing the expression of FVIII in antigen presenting cells. Furthermore, 10–30% of populations with hemophilia A develop inhibitors to the infusion products, which leads to the disruption of the coagulation factor and severe bleeding. Under these conditions, platelet-directed gene therapy of hemophilia A is very attractive because platelets could specifically store the protein in the bloodstream and then specifically release it at sites of thrombus formation, thereby minimizing the influence of circulating inhibitors. FJ

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rAAV-mediated shRNA ameliorated neuropathology in Huntington disease model mouse

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Abstract

Huntington disease (HD) is a fatal progressive neurodegenerative disorder associated with expansion of a CAG repeat in the first exon of the gene coding the protein huntingtin (htt). Although the feasibility of RNA interference (RNAi)-mediated reduction of htt expression to attenuate HD-associated symptoms is suggested, the effects of post-symptomatic RNAi treatment in the HD model mice have not yet been certified. Here we show the effects of recombinant adeno-associated virus (rAAV)-mediated delivery of RNAi into the HD model mouse striatum after the onset of disease. Neuropathological abnormalities associated with HD, such as insoluble protein accumulation and down-regulation of DARPP-32 expression, were successfully ameliorated by the RNAi transduction. Importantly, neuronal aggregates in the striatum were reduced after RNAi transduction in the animals comparing to those at the time point of RNAi transduction. These results suggest that the direct inhibition of mutant gene expression by rAVV would be promising for post-symptomatic HD therapy.

Keywords: Polyglutamine; Huntington disease; DARPP-32; Adeno-associated virus; RNAi

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder, characterized by cognitive abnormalities and involuntary movements. Selective loss of brain neurons and the formation of intranuclear aggregates were observed [1]. HD is resulting from polyglutamine repeat (CAG repeat: polyQ) expansion in the protein huntingtin (htt). Expanded polyQ alters the protein conformation and then recruits many essential proteins such as transcription-regulating proteins, molecular chaperones, and ubiquitin-binding proteins [2–6]. Mutant htt also impairs the function of the ubiquitin-proteasome system [4,7] and also induces mitochondrial calcium defects [8].

For therapeutic treatment of HD, several substances, such as Congo red and trehalose, have beneficial effects to inhibit oligomerization or stabilize polyQ bearing molecules [9,10]. These approaches targeted downstream of mutant protein expression. In contrast, gene silencing by RNA interference (RNAi) targets mRNA of mutant protein in a sequence-specific manner and reduces mutant protein expression [11]. Introduction of 21-nt short interfering RNAs (siRNAs) into mammalian cells effectively inhibits endogenous genes without a non-specific viral response. Vector-based synthesis of siRNAs became available to use in various cells and tissues [12–14]. Taking advantage of this approach, gene silencing of mutant mRNA through RNAi provides a direct approach in the treatment of neurodegenerative diseases. Lentiviral vector-mediated delivery of short hairpin siRNAs (shRNAs) targeting SOD1 (superoxide dismutase 1) to the SOD1 mutant mouse resulted in the delayed onset, improvement of behavioral defects, and protection from neuronal degeneration in the spinal cord [15]. shRNAs targeting ataxin-1 were also delivered to the cerebellum by recombinant adeno-associated virus (rAAV) to improve pathological abnormalities, reduc-

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ing the intranuclear inclusions and restoring the cerebellar morphology [16]. In HD, many studies have suggested toxic gain-of-function by mutant htt plays a role, thus a gene silence strategy is likely a promising therapy for HD. More recently, injection of vector-based shRNA against huntingtin improved motor disturbance and neuropathological abnormalities [17,18]. Furthermore, introduction of synthesized siRNA against htt delayed disease onset with the improvement of motor disturbance, neuropathological abnormalities, and longevity in the HD model mouse [15,19]. However, these RNAi treatments were provided at the early pre-symptomatic stage of neurodegenerative disorder in each transgenic model.

Previously, a conditional model of HD using a tetracycline-regulation system showed that mice expressing a mutated htt fragment demonstrated neuronal inclusions and progressive motor dysfunction [20]. In this model, blocking expression in symptomatic mice led to the disappearance of inclusions and amelioration of the behavioral phenotype. Thus, reduction of htt expression by RNAi may attenuate HD-associated symptom progression even if treatment is carried out after the onset of symptoms. In this study, taking advantage of truncated htt-EGFP transgenic mouse in which the aggregates are visualized by EGFP fluorescence, we investigated the neuropathological changes in HD model mice by suppression of transgene with shRNAs delivered by rAAV.

Materials and methods

HD model mouse. The HD190QG transgenic mouse was used as a HD model in this study. The HD190QG transgenic mouse harbors mutant truncated N-terminal htt containing 190 CAG repeats fused with EGFP in its genome. This animal shows progressive motor abnormality, and neuropathology such as formation of aggregates in brain, and shorter viability [21]. All the experiments with mice were approved by the Animal Experiment Committee of the RIKEN Brain Science Institute.

Construction and production of rAAV. Ten candidate sequences for short hairpin RNAs targeting EGFP mRNA were ligated into a pSilencer A plasmid (Ambion, Inc., Austin, TX). Neuro2a cells were co-transfected with each shRNA in pSilencer and EGFP expression vector pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA). The effect of gene silencing was evaluated by Western blot analysis using GFP antibody (Roche Molecular Biochemicals, Indianapolis, IN) to choose an effective sequence of shRNA (shEGFP) and non-effective sequence as a control (shEGFPcontrol). The shEGFP expression cassette containing U6 promoter was obtained by PCR with primers containing a *Hind* III restriction site, forward primer: 5′-CCCAAGCTTGGGATCTTACCGCTGTTGAGA-3′ and reverse primer: 5′-CCCAAGCTTGGGCCACACTTCAAGAACTC-3′. The monomeric red fluorescence protein (mRFP) cDNA was derived from mRFP1 in $pRSET_B$ [22] and ligated into pcDNA3 (Invitrogen Corporation, Carlsbad, CA). The shEGFP expression cassette was ligated into a proviral vector plasmid bearing inverted terminal repeats derived from AAV2 or AAV5 (pAAV-LacZ or pAAV5-RNL) to create pAAV2-shEGFP or pAAV5-shEGFP. mRFP expression cassette driven by CMV promoter was also inserted into the vector plasmid to visualize transduction (Fig. 1A). rAAV-shEGFP and rAAV-shEGFPcontrol were prepared according to three-plasmid transfection protocol described previously [23,24]. The viral stock was titrated by dot-blot hybridization with plasmid standards to make a stock of 1×10^{10} genome copies/μl.

In vitro assay of shRNA effect. HEK 293 cells was transfected with the pEGFP-N1 or plasmids expressing N-terminal htt exon 1 gene containing 16, 60, and 150 CAG repeats fused with EGFP (Nhtt16QG, Nhtt60QG, and Nhtt150QG, respectively). Four hours after transfection, rAAV2-shEGFP or shEGFPcontrol was added to the culture medium at 1×10^5 genome copies/cell. The EGFP fluorescence intensity was analyzed by the Cellomics™ Array Scan®V™ System (Beckman Coulter Inc., Fullerton, CA) after 48 h of viral transduction. The relative level of GFP intensity within the RFP-positive area transduced with rAAV-shEGFP or rAAV-shEGFPcontrol was estimated.

Virus injection into the mouse brain. Virus injection was performed by using the following coordinates with respect to the bregma; 0.5 mm anterior, 2 mm lateral, 3 mm depth, 0.3 μl/min infusion rate, and 3 μl per site. An equal amount of buffer was simultaneously injected into the contralateral side of the brain. The viral injection was carried out at the age of 8 weeks or 12 weeks, and analysis was performed at the age of 24 weeks.

Detection of virus transduction and aggregates by fluorescent imager. Mouse brains were perfused and fixed overnight with 4% paraformaldehyde. Serial-cut 40-micrometer sections were analyzed with a laser-scanning imaging system (Molecular Imager FX; Bio-Rad Laboratories, Hercules, CA) with an external laser (Bio-Rad Laboratories). Sections were imaged using the 488-nm laser with the standard 530 bandpass emission filter for detection of GFP fluorescence and 532-nm laser with the standard 640 nm bandpass emission filter for detection of RFP fluorescence as described previously [21].

Immunohistochemistry and aggregate count. Serial-cut 40-micrometer free-floating sections were used for immunohistochemistry. Sections were treated with anti-RFP antibody (Clontech) followed by AlexaFluor 568-labeled anti-rabbit secondary antibody (Molecular Probes). Aggregates were counted using images of immunohistochemistry with antibodies against EGFP (Nacalai Tesque, Inc., Kyoto, Japan), htt (Chemicon International, Inc., Temecula, CA), and ubiquitin (Dako, Glostrup, Denmark) followed by detection using ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA). The number of aggregates was calculated using MacSCOPE (Mitani, Tokyo, Japan) after normalizing the contrast and brightness of the digital images as described previously [10].

Filter trap assay. The striatum, cortex, and hippocampus were sampled and homogenated in 5 volumes of IMAC buffer (20 mM Hepes, pH 7.4, 140 mM potassium acetate, 1 mM magnesium acetate, and 1 mM EGTA with EDTA-free complete protease inhibitor cocktail tablets; Roche) with seven strokes using the digital homogenizer (As One, Osaka, Japan) at 1000 rpm. Homogenate containing 10 μg of protein was diluted with 0.2 ml of 2% SDS and filtered through a 0.2 μm cellulose acetate membrane (Advantec Toyo Roshi Kaisha Ltd., Tokyo Japan). Captured insoluble protein was detected by incubation with antibodies against GFP (Roche) and htt (Chemicon) followed by incubation with secondary antibodies and fluorescence substrates. Insoluble protein was quantified using LAS-1000plus/Image Gauge software (FUJIFILM, Tokyo, Japan).

In situ hybridization. For in situ hybridization, serial-cut 40-micrometer sections and non-radioactive digoxigenin-labeled cRNA probe against DARPP-32 (the dopamine- and cAMP-regulated phosphoprotein, Mr 32,000) were used as described previously [21].

Quantitative RT-PCR. Total RNA was extracted from the striatum using TRIZOL® Reagent (Invitrogen). Contaminating genomic DNA was removed with RQ1 RNase free DNase (Promega, Madison, WI) and 2 μg of total RNA was used for RT-PCR using Superscript™III First-Strand Synthesis System (Invitrogen). TaqMan PCR was performed using the TaqMan primer and probe sets as described [21]. Expression of GAPDH was estimated in each sample using the same methods for normalization.

Statistical analysis. Statistical significance was determined by Student's *t* test using StatView 5.0 (SAS Institute Inc., Cary, NC).

Results

Gene silencing by rAAV-shRNA

In vitro screening was used to identify the efficiency of mRNA ablation of shRNAs directed to EGFP and

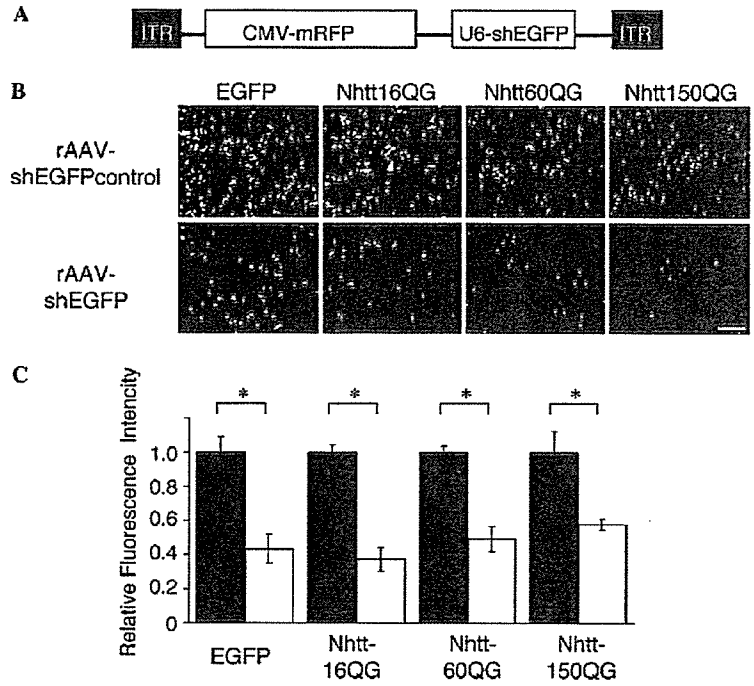

Fig. 1. rAAV-shEGFP reduced GFP expression in vitro. (A) AAV-shEGFP viral vector construct. ITR, inverted terminal repeat. CMV and U6 promoters were used for RFP and shEGFP. (B) Fluorescence photomicrographs of HEK-293 cells transfected with EGFP, Nhtt16QG, Nhtt60QG, and Nhtt150QG expression vectors and transduced with rAAV-shEGFP or rAAV-shEGFPcontrol, respectively. The photograph was taken after 48 h after viral transduction. Scale bar refers to all panels, 100 μm. (C) The relative level of GFP fluorescence intensity of rAAV-shEGFP transduced cells was compared to that of shEGFPcontrol transduced cells. The relative level of GFP fluorescence intensity of rAAV-shEGFPcontrol transduced cells (black bars); rAAV-shEGFP transduced cells (white bars). Values are given as means ± SEM ($n = 5$). $^{*}p < 0.001$.

EGFP-fused truncated htt-polyQ. EGFP-fused truncated htt-190Q is identical to the pathogenic transgene present in the HD190QG mouse [21]. The gene silencing function of 10 candidate shRNA sequences targeting EGFP was evaluated by the EGFP expression of co-transfected Neuro2A cells with shRNA and EGFP (data not shown). An shRNA targeting EGFP sequence 5′-GCAAGCTG ACCCTGAAGTTCAT-3′ (shEGFP) successfully reduced EGFP and EGFP-fused truncated htt expression significantly. Another shRNA targeting EGFP sequence 5′-GT TCATCTGCACCACCGGCTT-3′ had no gene silencing effect and was therefore used as a control (shEGFPcontrol). We next constructed an AAV-based vector (Fig. 1A). To test whether rAAV-mediated delivery of shEGFP could silence gene expression from EGFP or EGFP-fused truncated htt-polyQ, HEK293 cells were first transfected with EGFP or EGFP-fused truncated htt-polyQ expression vectors (EGFP, Nhtt16QG, Nhtt60QG, and Nhtt150QG, respectively), and subsequently transduced with rAAV2-shEGFP or rAAV2-shEGFPcontrol. shEGFP, but not shEGFPcontrol, significantly decreased GFP fluorescence intensity (Fig. 1B). The GFP intensity levels of shEGFP compared to those of shEGFPcontrol transduced cells were 0.43 ± 0.014, 0.37 ± 0.033, 0.50 ± 0.032, and 0.58 ± 0.027 (mean ± SEM, $n = 5$), in EGFP, Nhtt16QG, Nhtt60QG, and Nhtt150QG, respectively (Fig. 1C).

Expression and effect of shRNA in the mouse brain

rAAV5-shEGFP was injected into one side of the striatum and the same amount of buffer was injected into the other side of the striatum at 12 weeks old. The treated mice were sacrificed at 24 weeks old and serial-cut 40-micrometer sections were observed with laser-scanning imaging system. RFP fluorescence was expressed in the rAAV5-shEGFPinjected region and could show the infected cells (Fig. 2A). GFP fluorescence intensity was preferentially detected in the striatum, whereas the signal was decreased in the RFP-positive area. Further analysis at a higher magnification revealed that GFP fluorescent aggregates were absent in the RFP-positive neuronal cells up to three months after transduction of shEGFP in the striatum (Fig. 2B). In contrast, aggregates were abundantly observed in the contralateral side of the striatum. RFP expression was detected in the striatum as well as the cortex, lateral globus pallidus, hippocampus, and substantia nigra. However, reduction of GFP-positive aggregates was preferentially observed in the striatum, cortex, and hippocampus (data not shown).

Reduction of aggregate formation by shEGFP

Immunohistochemistry was performed using antibodies against GFP, htt, and ubiquitin. GFP and htt antibodies