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Minimizing the Inhibitory Effect of Neutralizing Antibody for Efficient Gene Expression in the Liver With Adeno-associated Virus 8 Vectors

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Neutralizing antibodies (NAbs) against adeno-associated viruses (AAVs) are known to interfere with AAV vector-mediated gene transfer by intravascular delivery. Evading the inhibitory effects of antibodies against AAV vectors is necessary for efficient transfer of therapeutic genes clinically. For this purpose, we tested the efficacy of saline flushing in order to avoid contact of vectors with NAbs present in blood. Direct injection of the AAV8 vector carrying the *factor IX (FIX)* gene into the portal vein of macaques using saline flushing achieved transgene-derived FIX expression (4.7 ± 2.10–10.1 ± 5.45% of normal human FIX concentration) in the presence of NAbs. Expression was as efficient as that (5.43 ± 2.59–12.68 ± 4.83%) in macaques lacking NAbs. We next tested the efficacy of saline flushing using less invasive balloon catheter-guided injection. This approach also resulted in efficient expression of transgene-derived FIX (2.5 ± 1.06–9.0 ± 2.37%) in the presence of NAbs (14–56× dilutions). NAbs at this range of titers reduced the efficiency of transduction in the macaque liver by 100-fold when the same vector was injected into mesenteric veins without balloon catheters. Our results suggest that portal vein-directed vector delivery strategies with flushing to remove blood are efficacious for minimizing the inhibitory effect of anti-AAV antibodies.

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INTRODUCTION

Gene and cell therapies are expected to be the next generation of therapies for a variety of inherited diseases. Hemophilia is thought to be an ideal target disease for these approaches as it is caused by a genetic abnormality in the factor VIII gene for hemophilia A, or the factor IX (FIX) gene for hemophilia B.[1–7] The current strategy of hemophilia gene therapy involves inducing expression of the normal coagulation factor gene or transplanting cells expressing the respective coagulation factor. The liver is normally the primary target of gene transfer for coagulation factors since the majority of these coagulation factors are synthesized in the liver with appropriate post-translation modifications before secretion into the circulatory system.

Substantial effort has been applied to express coagulation factor genes using various vector types. Among the viral vectors, recombinant adeno-associated virus (AAV) vectors are preferred for therapeutic gene transfer *in vivo* because they reside in the episome and rarely integrate into genomes. However, retrovirus vectors including lentivirus vectors require integration into the host cell genome.[6,7] In addition, AAV vectors can transfer genes to nondividing cells and allow long-term expression of transgenes in these cells.

Clinical trials for hemophilia gene therapy have recently been conducted using various types of vectors.[4–11] These trials were designed based upon data obtained from mouse models of hemophilia and hemophiliac dogs and proved to be more efficient in these models than for humans. Species differences between humans and these other animal models might partially account for the results observed. Therefore, gene transfer studies in non-\ human primates may well predict the efficacy of gene transfer in humans. Indeed, *FIX* gene transfer studies using a new type of vector have been conducted in rhesus macaques.[12,13] The results from these studies provided the basis for recent hemophilia B gene therapy clinical trials employing an AAV8 vector.[13–16] Gene transfer in mice using AAV vectors results in excellent transduction efficiency. This is especially so for AAV8 vector-mediated gene transfer in the mouse liver;[12–14,17] however, the efficacy of AAV8 vectors is modest in macaques.[13]

There are also difficulties associated with *FIX* gene expression when using AAV8 vectors in nonhuman primates. Growing evidence suggests that the presence of neutralizing antibodies (NAbs) against AAV8, due to previous natural infection by wild-type AAV, significantly inhibits transduction in the macaque liver. It is likely that antibodies against one serotype of AAV cross-react with other AAV serotypes.[18] A hemophilia B gene therapy

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Table 1 Expression of macaque T262A in nonhuman primates with AAV8-HCRHAAT-macFIXT262A

Macaque number	Age	Vector dose (vg/kg)	Route of vector injection	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#14	5.7	1×10^{12}	Mesenteric vein	0.02 ± 0.019	0.1	56×
#17	5.8	1×10^{13}	Mesenteric vein	0.13 ± 0.081	0.4	56×
#24	6.6	1×10^{12}	Mesenteric vein	0.09 ± 0.048	0.5	14×
#28	7.8	5×10^{12}	Saphenous vein	12.68 ± 4.83	38.2	Negative
#30	2.9	5×10^{12}	Saphenous vein	5.43 ± 2.59	48.2	Negative
#31	2.9	5×10^{12}	Saphenous vein	7.64 ± 2.32	49.6	Negative

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region.
The concentration of macaque FIX T262A is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 neutralizing antibody (NAb) titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

clinical study using an AAV8 vector was successfully conducted in hemophilia B patients negative for pre-existing antibodies against AAV8.[15] Because of the high prevalence of AAV infection in humans,[18] evading NAbs against this virus is an important hurdle to overcome before AAV8 vectors can be routinely and effectively employed for therapies.

The aim of our study was to develop an administration method of AAV8 vectors that assisted in minimizing the inhibitory effect of NAbs against AAV in macaques that were already seropositive for AAV8 antibodies.

RESULTS

The AAV8 vector carrying the macaque *FIX T262A* gene located downstream of the liver-specific chimeric promoter consisted of an enhancer element of hepatic control region (HCR) of the *ApoE/C-I* gene and the 5′ flanking region of the *α1-antitrypsin* (HAAT) gene (AAV8-HCRHAAT-macFIXT262A). This vector was used to express mutant macaque FIX containing a single amino acid substitution of Thr to Ala at the position 262 (macaque FIX T262A) in the following experiments. Macaque FIX T262A but not wild-type macaque FIX could be bound to human FIX-specific monoclonal antibody 3A6, thereby macaque FIX T262A expressed in macaques with AAV8-HCRHAAT-macFIXT262A could be precisely quantified by an enzyme immunoassay with 3A6.[17] The amino acid sequence of macaque FIX is highly homologous to the human FIX amino acid sequence. Twelve amino acid residues of human FIX are different at corresponding positions of macaque FIX, while only one amino acid of macaque FIX T262A is different from wild-type macaque FIX. Expression of macFIX T262A in a macaque would mimic a situation where normal human FIX is expressed in a hemophilia B patient with a missense mutation in the *FIX* gene.

Results corresponding to the expression of macaque FIX T262A following injection of AAV8HCRHAATmacFIXT262A can be seen in **Table 1**. When AAV8HCRHAATmacFIXT262A (5×10^{12} vector genome copies (vg)/kg) was injected into the saphenous veins of three AAV8 NAb-negative macaques (#28, #30, #31), expression of macFIX T262A in the therapeutic range (>5% of normal FIX concentration) was achieved. However, injection of the same vector (1×10^{12}–1×10^{13} vg/kg) into the mesenteric vein branches of AAV8 NAb-positive macaques (#14, #17, #24; inhibitory titers: 14–56×) resulted in subtherapeutic levels (<0.2%) of macFIX T262A expression. The amount of vector DNA in the liver of AAV8 NAb-positive macaques was ~1% of that seen in AAV8 NAb-negative macaques (**Table 1**). These data suggest that low titers of NAbs against AAV8 significantly inhibit transduction even when the vector is injected into the mesenteric vein branches. In addition, only short period of time may be required for NAbs in the blood to neutralize the AAV8 vector since the blood of the mesenteric vein rapidly goes to the liver through the portal vein after gathering with the blood from other viscera.

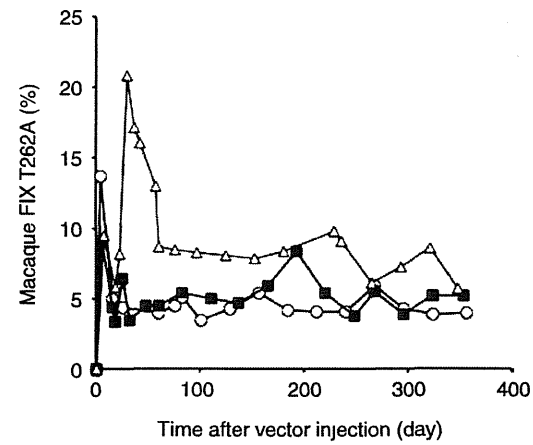

Figure 1 Expression of FIX T262A in macaques after direct vector injection into portal veins. Macaques ($n = 3$) were subjected to direct injection of AAV8 vector into the portal vein. Concentrations of FIX T262A in macaque plasma samples (macaque #26, open triangles; #27, open circles; #29, closed squares) were measured by ELISA. AAV, adeno-associated virus; ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Evading AAV8 NAbs could be achieved by ensuring the AAV8 vector and NAbs do not come into physical contact with each other in the blood. Blood enters the liver from the hepatic artery and portal vein. The hepatic artery accounts for ~20–30% of blood flow, while the portal vein supplies the remaining blood flow to hepatocytes.[19,20] Blood from the portal vein and hepatic artery are eventually mixed in the sinusoids of the liver; however, the blood from the portal vein mainly supplies hepatocytes. Therefore, direct injection of AAV8 vectors into the portal vein branch was

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Table 2 Expression of macaque T262A in nonhuman primates with direct, and balloon catheter-guided vector (AAV8-HCRHAAT-macFIXT262A) injection into the portal vein

Macaque ID	Age	Vector dose (vg/kg)	Injection method to portal vein branch	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#26	10.1	5×10^{12}	Direct	4.7 ± 2.10	77.9	28×
#27	7.4	5×10^{12}	Direct	10.1 ± 5.45	28.5	14×
#29	11.0	5×10^{12}	Direct	5.3 ± 1.40	64.3	14×
#37	7.5	5×10^{12}	Catheter-guided	9.0 ± 2.37	61.1	14×
#38	10.7	5×10^{12}	Catheter-guided	3.2 ± 1.21	13	56×
#42	7.7	5×10^{12}	Catheter-guided	2.5 ± 1.06	15.3	14×

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region; Nab, neutralizing antibody.
FIX T262A concentration is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 NAb titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

investigated to determine whether saline flushing to remove blood from the portal vein just before injection of the vector would diminish the inhibitory effects of anti-AAV8 NAbs. Three macaques (#26, #27, #29; inhibitory titers: 14–28×) were directly injected with vector (5×10^{12} vg/kg) into the left portal vein after flushing saline to remove blood (**Supplementary Table S1**). Expression of transgene-derived FIX (macaque FIX T262A) increased to therapeutic levels with the AAV8 vector carrying the macaque *FIX T262A* gene and persisted for greater than 1 year in the three macaques (**Figure 1**). Average FIX and vector genome levels in macaque liver tissues are presented in **Table 2**. Compared with the results of vector injection to the mesenteric vein of NAb-positive macaques #14, #17, and #24 (**Table 1**), the levels of macaque FIX T262A in the circulation of the macaques #26, #27, and #29 that received vector injection directly to the left portal vein with flushing to remove blood, were increased to therapeutic levels with significant amounts of vector genome detected (Table 2).

Blood chemistry analysis and liver biopsies were conducted following administration of the vector to determine whether there were any adverse effects induced by the injection. Moderate increases in liver enzymes, such as transaminases, were observed just after injection of the vector (**Supplementary Figure S1**). However, no significant pathological changes were seen in liver biopsy samples taken on days 14, 28 or 48 (data not shown). We did not observe an increase in the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)-positive hepatocytes in the liver biopsy specimens (data not shown).

The direct injection of the AAV8 vector into the left portal vein branch with saline flushing to remove blood from the portal vein just before injection of the vector was effective to minimize the inhibitory effects of anti-AAV8 NAbs. Therefore, we explored the possibility of utilizing a balloon catheter to perform the vector injection into the portal vein branch with saline flushing to remove blood, taking the concern about the safety of the procedures into consideration. Using a microballoon catheter, we injected the vector into the left portal vein of three anti-AAV8 antibody-positive macaques (#37, # 38, #42; inhibitory titers: 14–56×) (**Table 2**, **Supplementary Table S2**). Fluorography in macaque #37 representing angiography of the portal vein branch is shown in **Figure 2** and **Supplementary Video S1**. Increase of FIX T262A to therapeutic levels was achieved in the three macaques (#37, # 38, #42),

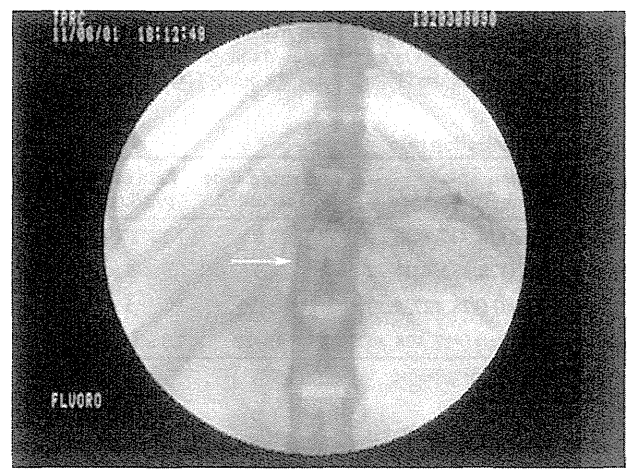

Figure 2 Fluorography in macaque #37. A balloon catheter was inserted into the portal vein of macaque #37 and contrast medium injected before vector administration. The left portal vein branches can be visualized. The arrow (white) indicates the tip of the catheter. See **Supplementary Video S1** which also recorded inflation of the balloon before the vector administration and deflation of the balloon after the administration.

and macaque FIX T262A expression in the circulation persisted (**Figure 3**, **Table 2**). The two portal vein vector delivery methods were successful in expressing macaque FIX T262A with the AAV8 vector in NAb-positive macaques (**Table 2**). The data suggest that the gene transfer efficiency using the catheter-guided vector injection method is similar to that of the direct vector injection into the portal vein branch with flushing.

Blood chemistry analysis and liver biopsies were conducted following injection of the vector. Increases in the levels of liver enzymes just after injection of the vector were not observed, suggesting that the ischemic effect of the temporary occlusion of the left portal vein branch was minimum compared with that of the direct vector injection procedure. Moderate increases in transaminases were observed following the vector injection, but did not persist (**Supplementary Figure S1**). Although the cause of the changes in the liver enzymes was not elucidated, no animals showed pathological changes, including histology of liver biopsy samples (data not shown).

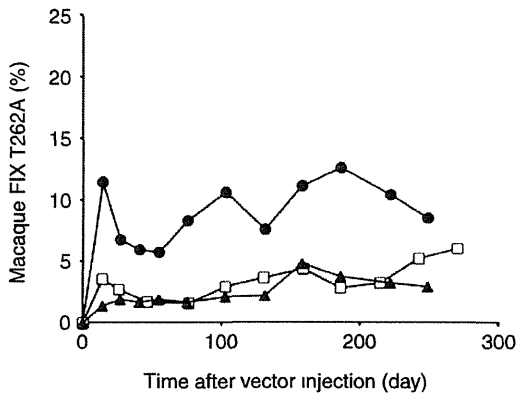

Figure 3 Expression of FIX T262A in macaques following balloon catheter-guided vector injection into portal veins. Three macaques (n = 3) were subjected to balloon catheter-guided vector injection into the portal vein. Concentrations of FIX T262A in macaque plasma samples (#37, closed circles; #38, open squares; #42, closed triangles) were measured by ELISA. ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Table 3 Vector injection rate

Route of injection (macaque number)	Elapsed time (seconds)	Rate of injection (vg/kg/second)
Mesenteric vein (#14, #17, #24)	5	2×10^{11}–2×10^{12}
Saphenous vein (#28, #30, #31)	5	1×10^{12}
Portal vein (direct) (#26, #27, #29)	8–10	5×10^{11}
Portal vein (catheter) (#37, #38, #42)	15–22	2.3×10^{11}–3.3×10^{11}

Abbreviation: vg, vector genome.

Vector injection rates of the four different vector injection procedures are listed in Table 3 for comparison. The vector injection rates of the portal vein-directed strategies were similar to those of bolus vector injection into the saphenous vein and the mesenteric vein. Thus, the effect of vector injection speed on the transduction efficiency of the vector was thought to be minimal.

DISCUSSION

There are many features that make recombinant AAV vectors attractive for transferring therapeutic genes into target organs, and many vectors have been tried for the treatment of various diseases.[6,7,11,15,21–23] However, lines of evidence suggest that NAbs against AAV interfere with AAV vector-mediated gene transfer by intravascular vector delivery.[7,23–26] A clinical gene therapy trial for hemophilia B using a self-complementary AAV8 vector carrying the FIX gene has been conducted and reported to be successful.[15] However, even the self-complementary AAV8 vector failed to express FIX in a subject with a relatively high anti-AAV8 antibody titer compared with other subjects with no or lower antibody titers.[15]

According to the previous reports on the prevalence of NAbs against various AAV serotypes in normal subjects, the seropositivity against AAV8 is 15–30%, which is lower than that against AAV2 (50–60%), although the technical details of the NAb assay is different.[27,28] These reports have also demonstrated that the antibody titer against AAV8 is generally lower than for AAV2. Our data suggest that a low titer of NAbs against AAV8 can interfere with transduction even if the vector is injected into the mesenteric vein. Therefore, the use of another serotype vector such as AAV5 vector could be the next approach for this type of gene therapy because of the divergence in capsid sequence of AAV5 from other AAV serotypes.[13,16] Although the prevalence of NAbs against AAV5 is much lower than those against AAV1 and AAV2, and the prevalence of NAbs against AAV5 is comparable to or even lower than that against AAV8 in humans,[25,27] it is possible that subjects of gene therapy may contain cross-reactive NAbs against various AAV serotypes.

Another approach for evading NAbs against AAV could be the use of chemically or genetically modified AAV variants. Such variants could include AAV vector mutants with amino acid substitutions, or chimeric AAV vectors made by serotype shuffling.[23] Approaches that enable evasion of NAb inhibitory effects are necessary if researchers and clinicians wish to effectively apply AAV vectors for gene therapy because of NAb cross-reactivity.

An alternative approach for overcoming the inhibitory effect of NAb against AAVs is to develop a vector injection method. In the current study, two portal vein vector delivery strategies were employed that ensured that the AAV8 vector and NAbs do not come into physical contact with each other in the blood. These strategies were investigated using macaques whether the strategies could efficiently transduce hepatocytes with the AAV8 vector in the presence of NAbs. The first approach was the direct injection of AAV8 vectors into the portal vein branch after flushing with saline to remove blood. This strategy proved to be successful for the vector expressing FIX T262A in anti-AAV8 antibody-positive macaques. Since there are safety concerns about the direct vector injection method, injection of the vector into the portal vein using a balloon catheter was investigated. The catheter-guided vector injection may be less invasive than the direct vector injection into the portal vein branch because exfoliation of hepatic hilum is not required. In addition, fine surgical skills, such as manipulation of the hepatic hilum and suturing the venotomy site of portal vein after the direct vector injection without causing stenosis, are required for the direct vector injection method into the left portal vein. Obviously, catheterization from the mesenteric vein branch is required for the balloon catheter-guided vector injection method but insertion of a catheter into the portal vein from a branch of the mesenteric vein is not difficult for a cardiologist and a radiologist familiar with angiography. In addition, suturing the venotomy site of the mesenteric vein branch is easier and safer than suturing the venotomy site of portal vein, and the ischemic effect of this procedure was expected to be less than that of the direct vector injection into the portal vein branch. Taken together, these studies suggested that both the direct vector injection into the left portal vein and the balloon catheter-guided vector injection into the left portal vein were similarly effective for hepatocyte transduction with the AAV8 vector in the presence of low titer NAbs but the balloon catheter-guided vector injection method into the left portal vein was thought to be safer than the direct vector injection into the left portal vein.

Considering that the antibody titer against AAV8 was generally lower than that against AAV2 and that NAbs at low titers could interfere with the AAV8 vector-mediated gene transfer to the liver significantly, we selected macaques with low NAb titers

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for the portal vein vector delivery strategies. However, the impact of the presence of high titer NAbs on the efficacy of these methods was not studied. Thus, the extent of AAV8 NAb titer, for that this approach is effective, needs to be investigated in the future.

In conclusion, we have provided the basis for an alternative approach for gene transfer to the liver that minimizes the deleterious effects of anti-AAV NAbs. Our result might expand the potential of the AAV vector-mediated gene delivery for medical application.

MATERIALS AND METHODS

AAV vector production. Construction of pAAV2-HCRHAAT-macFIX T262A and production of AAV8 carrying the macaque FIX T262A gene (AAV8HCRHAATmacFIXT262A) has been previously described.[17] Briefly, DNA fragments harboring the *macFIXT262A* gene located downstream of the chimeric promoter consisted of an enhancer element of HCR of the human ApoE/C-I gene and the 5′ flanking region of the human HAAT gene (HCRHAAT promoter), and the SV40 polyadenylation signal sequence flanked by AAV2 inverted terminal repeats in pAAV2-HCRHAAT-macFIX T262A. The genes were packaged by triple plasmid transfection of human embryonic kidney 293 cells (Avigen, San Diego, CA) to generate AAV8-HCRHAAT-macFIXT262A, with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), and the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA), as previously described.[17] The chimeric packaging plasmid for AAV8 capsid pseudotyping[29] was constructed by inserting the synthetic AAV8 *Cap* gene (Takara Bio, Otsu, Shiga, Japan) downstream of the AAV2 Rep gene of pHelp19. For virus vector purification, the DNase (Benzonase; Merck Japan, Tokyo, Japan)-treated viral particles containing samples were subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) supplemented with 25 mmol/l EDTA at 21 °C, as previously described.[17] Titration of recombinant AAV vectors was carried out by quantitative PCR using a real-time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan).[17] AAV8HCRHAATmacFIXT262A was previously shown to express macaque FIXT262A in mice efficiently.[17] Human FIX could be expressed in macaques and detected, however, macaques developed antibody against human FIX under certain experimental conditions. Only one amino acid residue at position 262 was humanized in macaque FIX T262A for detection with the human FIX-specific monoclonal antibody.

Animals. Cynomolgus macaques were bred and maintained at the Tsukuba Primate Research Center (Ibaraki, Japan). The animal experiments using macaques were performed at the Tsukuba Primate Research Center according to the guidelines of the Institutional Animal Care and Concern Committees at Jichi Medical University and the Tsukuba Primate Research Center. The use of macaques in animal experiments was approved by the Animal Care and Concern Committees. All surgical procedures were carried out under anesthesia, with vital signs and electrocardiogram monitoring conducted in accordance with the stipulated guidelines. Male macaques with low NAb titers (<56×) were used in this study.

Vector injection from peripheral and mesenteric vein. Injection of AAV8 vector to a saphenous vein (peripheral vein) was performed under intramuscular anesthesia. Injection of the AAV8 vector into a terminal branch of the superior mesenteric vein was carried out with laparotomy under anesthesia with isoflurane and electrocardiogram monitoring.

Direct portal vein vector injection with saline flushing. Direct injection of the vector solution into the left portal vein was carried out after induction of general anesthesia with isoflurane and sterilization. A right subcostal incision (5 cm) was made through the skin and the subcutaneous tissue. The abdominal cavity was explored and the soft tissue of hepatic hilum was exfoliated surgically, then the main portal vein and its right and left branches were exposed. The main portal vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo, Tokyo, Japan), which was advanced into the left portal vein branch. The left and right portal vein branches were then clamped with vascular forceps. After flushing the left portal vein with saline, the vector solution was injected, and then a second saline solution, for flushing, was injected. Volumes of solutions used in the experiments were determined by taking a standard liver volume, a hepatic vascular bed volume, and effects of solutions on the systemic circulation into consideration.[19,30] A standard liver volume of a macaque was estimated with the formula (standard liver volume = 706.2 × body surface area + 2.4)[31] and the vascular bed volume of the liver was estimated to 25–30% of the standard liver volume.[19] A hepatic vascular bed volume can increase to 60% of the liver volume upon infusion of solutions and this may function as a reservoir and reduce the effects of the solutions on the systemic circulation.[20,32,33] The forceps were then removed immediately and the venotomy site was closed with an 8-0 prolene suture.

Catheter-guided vector injection to the portal vein with saline flushing. Balloon catheter-guided injection of the vector into the left portal vein of AAV8 antibody-positive macaques was carried out after the induction of general anesthesia. A 5-cm right paramedian incision was made through the skin and subcutaneous tissue. The abdominal cavity was carefully entered, with a part of the ileum identified and brought out through the incision. A peripheral branch of the superior mesenteric vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo). A temporary occlusion microcatheter (Iliguman 3.3F; Fuji System, Tokyo, Japan) was advanced into the left portal vein using a guide-wire (run through 0.014 (0.36 mm); Terumo) under a fluoroscope. The positions of the catheter and the balloon were confirmed by imaging with contrast medium. Blood flow in the left portal vein was occluded with a silicone balloon catheter and 40 ml of saline, followed by the AAV8 vector solution, and another 20 ml of saline was injected sequentially through the microcatheter. Volumes of solutions used in the experiments were determined as above with taking the result of the experiment of direct vector injection to the left portal vein branch into consideration. Following deflation of the balloon, the microcatheter was withdrawn and the peripheral venotomy ligated. The abdominal wall was then closed in layers.

Analysis of macaque FIX T262A expression in macaques. Macaque FIX T262A was bound to 3A6, a human FIX-specific monoclonal antibody for analyses. An enzyme-linked immunosorbent assay (ELISA) for the detection of macaque FIX T262A was carried out using 3A6, as previously described.[17,34]

NAb assay. An assay for the detection of anti-AAV8 NAbs was performed as previously reported, with some modifications.[35,36] Briefly, 5×10^4 2V6.11 cells/well were seeded in the wells of 96-well culture plates. Ponasterone A was added to the culture media the day before transduction to induce expression of the E4 gene. On the day of transduction, 10 µl of serum (undiluted, or subject to serial twofold dilutions) was incubated with the vector (AAV8-CMV-LacZ, 5×10^7 vg/10 µl) at 37 °C for 1 hour, and this mixture was added to a culture well. Sucrose was added to the culture medium such that the final concentration was 125 mmol/l. The culture medium was removed after a 48-hour incubation, and β-galactosidase activity quantified with a β-Gal assay kit (Invitrogen, Carlsbad, CA). Briefly, o-nitrophenyl-β-D-galactopyranoside was added to cell lysates, incubated for 30 minutes, and color change quantified with a microplate reader (Benchmark Plus; Bio-Rad, Hercules, CA). If β-galactosidase activity was inhibited with a test sample that contained more than 50% of control fetal bovine serum, it was judged as positive for neutralizing capacity. The inhibitory titer of the serum sample was expressed as the highest final dilution in the culture medium that showed inhibitory activity.

Quantitation of AAV8 vector DNA in macaque tissue. Quantitation of AAV8 vector DNA in macaque tissues was performed using quantitative

PCR assays using a StepOnePlus instrument (Applied Biosystems Japan). DNA was isolated from macaque tissues using a DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) and subjected to PCR using primers 5′-GAT AAC TGG GGT GAC CTT GG-3′ and 5′-GCC TGG TGA TTC TGC CAT GA-3′, and Cybergreen reagent (Applied Biosystems Japan).

SUPPLEMENTARY MATERIAL

Figure S1. Changes in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in macaques.
Table S1. Direct vector injection into the portal vein of macaques.
Table S2. Balloon catheter-guided vector injection into macaques.
Video S1.

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Porcine Model of Hemophilia A

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Abstract

Hemophilia A is a common X chromosome-linked genetic bleeding disorder caused by abnormalities in the coagulation factor VIII gene (*F8*). Hemophilia A patients suffer from a bleeding diathesis, such as life-threatening bleeding in the brain and harmful bleeding in joints and muscles. Because it could potentially be cured by gene therapy, subhuman animal models have been sought. Current mouse hemophilia A models generated by gene targeting of the *F8* have difficulties to extrapolate human disease due to differences in the coagulation and immune systems between mice and humans. Here, we generated a porcine model of hemophilia A by nuclear transfer cloning from *F8*-targeted fibroblasts. The hemophilia A pigs showed a severe bleeding tendency upon birth, similar to human severe hemophiliacs, but in contrast to hemophilia A mice which rarely bleed under standard breed conditions. Infusion of human factor VIII was effective in stopping bleeding and reducing the bleeding frequency of a hemophilia A piglet but was blocked by the inhibitor against human factor VIII. These data suggest that the hemophilia A pig is a severe hemophilia A animal model for studying not only hemophilia A gene therapy but also the next generation recombinant coagulation factors, such as recombinant factor VIII variants with a slower clearance rate.

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Introduction

Hemophilia A is an inherited X-linked bleeding disorder caused by abnormalities in the coagulation factor VIII (FVIII) gene (*F8*). The genetic abnormalities result in FVIII deficiency, which in turn creates a bleeding diathesis, such as life-threatening bleeding in the brain and harmful bleeding in joints and muscles. The morbidity of hemophilia A is one in 5,000 male live births [1]. The current standard therapy for hemophilia A is intravenous injection of recombinant FVIII or monoclonal antibody-purified FVIII from plasma. Prophylactic administration of FVIII is effective in preventing harmful bleeding; however, hemophilia A patients are still not free from the risks of life-threatening intracranial and other harmful bleeding [1] [2]. In addition, severe hemophilia A patients develop antibody against FVIII (inhibitor) upon infusion of FVIII frequently [1].

Gene therapy, that enables sustained elevation of coagulation factor levels, will provide the next-generation therapy for hemophilia [1,3,4]. In fact, gene and cell therapy for hemophilia clinical trials were conducted. Compared with clinical trials of the gene therapy for hemophilia B [5,6], gene and cell therapies for hemophilia A have had limited successes [7,8]. Upcoming therapeutic alternatives for hemophilia A are FVIII variants with a slower clearance rate. Therapeutic factors, such as recombinant activated factor VII and plasma-derived activated prothrombin complex, are used for the treatment of hemophilia A patients with inhibitors, and the second generation therapeutic factors for hemophilia A patients with inhibitors are also currently under development. For studying next-generation therapeutics, good animal models are required. Hemophilia A mice generated by targeted ablation of mouse *F8* [9] have been the mainstay for assessment of hemophilia A gene therapy and evaluation of FVIII variants. However, there are significant species differences between mice and humans. For example, the half-life of human FVIII in the mouse circulation is very short, making it difficult to analyze the efficacy of human FVIII-expressing vectors for gene therapy or novel FVIII variants. As alternatives, there are natural hemophilia A dogs and hemophilia A sheep. Hemophilia A dogs have been used for gene therapy studies [10,11,12]. Hemophilia A sheep would be an alternative model [13]. There may be interspecies differences, such as body size, physiology, disease

progression and chromosome structure homology, between these animal models and humans.

Pigs are excellent biomedical models of human diseases [14,15]. The porcine blood coagulation system is very similar to that in humans, because of the high homology between the coagulation factor amino acid sequences [16,17,18]. In addition, porcine FVIII has been used to treat hemophilia A patients with FVIII inhibitors [19,20,21]. Therefore, the hemophilia A pig could be a good animal model to study the next-generation therapeutics for hemophilia A. Moreover, a miniature pig strain exists, and thus, cloned pigs could be downsized to an adequate size, approximately 20–30 kg in weight. For these reasons, we decided to generate hemophilia A pigs by cloning technology.

Results

Firstly, we constructed a *F8* targeting vector (Figure 1**A**) and targeted *F8* in male porcine embryonic fibroblasts (PEF) with the *F8*-targeting vector as shown in Figure 1. The DNA fragment amplified from the non-transfected PEF DNA migrated at 6.5 kb on agarose gel electrophoresis, whereas two DNA fragments, migrating at 6.5 kb and 8.3 kb, were amplified from PEF colony 134. The 8.3 kb DNA was not amplified from genomic DNA of PEF colonies 135–137. The 8.3 kb fragment PCR-amplified from PEF colony 134 was cleaved into a 2.4-kb fragment and a 5.9-kb fragment by *Stu* I, whereas the PCR-amplified 6.5-kb DNA fragment was not susceptible to *Stu* I digestion. This supports that the PCR-amplified 8.3-kb fragment is derived from the *F8*-targeted genome because a *Stu* I recognition sequence present in the neo-resistant gene but not in the PCR amplified DNA fragment from the wild-type *F8*. The expected DNA fragments were amplified by PCR with Neo primers from genomic DNA from PEF colony 134, but not from wild-type genomic DNA (WT). PCR analysis of genomic DNA with three primer sets revealed a recombination event in *F8* of a colony, 134 (PEF-134). PEF-134 nuclei were then injected into enucleated oocytes. After an electrical pulse, the oocytes were transplanted into the oviduct of a female pig [22,23]. Transplantation of nucleus-transferred oocytes to the oviducts of female pigs was repeated four times. Three months later, a fetus was obtained by induced abortion. Dermal fibroblasts from this PEF-134-derived fetus (134-fetus) were isolated and cultured, and genomic DNA was isolated for analysis by PCR and by Southern blotting (Figure 2). The PCR amplified wild-type (WT) *F8* exon 14–18 fragment migrated at 6.5 kb, whereas the 8.3-kb targeted DNA fragment was amplified solely from 134-fetus fibroblast DNA. PCR-amplified DNA fragments using an *F8* exonic primer and a Neo primer were obtained only from 134-fetus DNA. The PCRs demonstrated insertion of the neomycin-resistance gene in *F8* (Figure 2**A**). Southern blotting showed that the 5′ probe hybridized with the 8.1 kb DNA fragment of Sac I-digested wild-type DNA while the 5′ probe hybridized with 9.9 kb DNA fragment of Sac I-digested 134-fetus DNA. Southern blotting with the 3′ probe confirmed recombination in the *F8* gene in the 134-fetus genome because a Sph I recognition sequence and a Xba I recognition sequence located in the 3′ end of the Neo resistant gene of the targeted allele (Figure 2**B**). Therefore, five transfers of fetal fibroblast nuclei to oocytes followed by oocyte transplantation were performed. Four females became pregnant and each produced a full-term delivery.

Four live offspring were obtained and PCR analysis and Southern blotting were carried out. As shown in Figure 3**A**, the 8.3 kb DNA fragments were PCR amplified from piglets DNA as same as that of 134-fetus (Figure 2). Similarly, Southern blotting of Sac I-treated and *Sac* I and *Stu* I-treated DNA of the piglets with

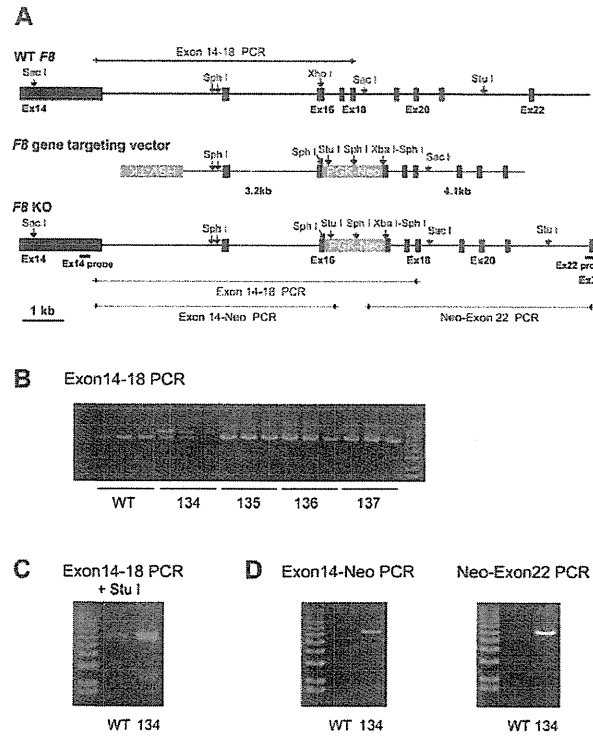

Figure 1. *F8* targeting of porcine fetal fibroblasts (PEF). (**A**) Schematic diagram of part of porcine *F8*, the positions of the restriction endonuclease sites, the *F8* targeting vector structure, and the targeted *F8* (*F8* KO) allele are shown. The neomycin-resistance gene (PGK-neo) was inserted in the exon 16 DNA fragment with deletion of a part of exon 16 and was flanked by two *F8* DNA fragments (5′ arm: 3.2 kb; 3′ arm: 4.1 kb) in *F8* targeting vector. The positions of PCR primers (arrowheads), expected amplified DNA fragments (bars), and restriction endonuclease sites used for the Southern blot analysis are indicated in the schema for *F8* KO. (**B**) *F8* exon 14–18 PCR on genomic DNA from non-transfected PEF (WT), PEF colony 134 (134), and three other PEF colonies (135–137) was shown. (**C**) The *F8* exon 14–18 PCR products were treated with *Stu* I and analyzed by agarose gel electrophoresis. (**D**) PCR analyses with two sets of primer pairs for exon 14 and the neomycin resistance gene and for the neomycin resistance gene and exon 22 were shown.
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the 5′ probe confirmed the recombination of F8 of piglets and showed that each piglet had a single copy of the targeted *F8* (Figure 3, **A** & **B**). RT-PCR analysis revealed that FVIII mRNA was not detected in the liver of piglet #3 (Figure 3**C**). Analysis of the blood of piglets #3 and #4 confirmed that the FVIII level was severely decreased to less than 1%, using an activated partial thromboplastin time (APTT)-based coagulation assay for human FVIII (Table 1). Other coagulation factors were moderately decreased (Table 1). The levels of albumin and cholinesterase of these piglet blood were also measured as the references to study whether the decreased level of coagulation factors II, V, VII, IX, and X were specific or not. The albumin levels of piglet #3 and #4 were decreased significantly compared with the wild type piglets. However, the cholinesterase activities of piglets #3 and #4 were not decreased. The data suggested that synthesis of some proteins in the liver of the cloned piglets was altered. The precise mechanism of the moderately decreased levels of coagulation factors II, V, VII, IX, and X, and albumin was not elucidated in this study.

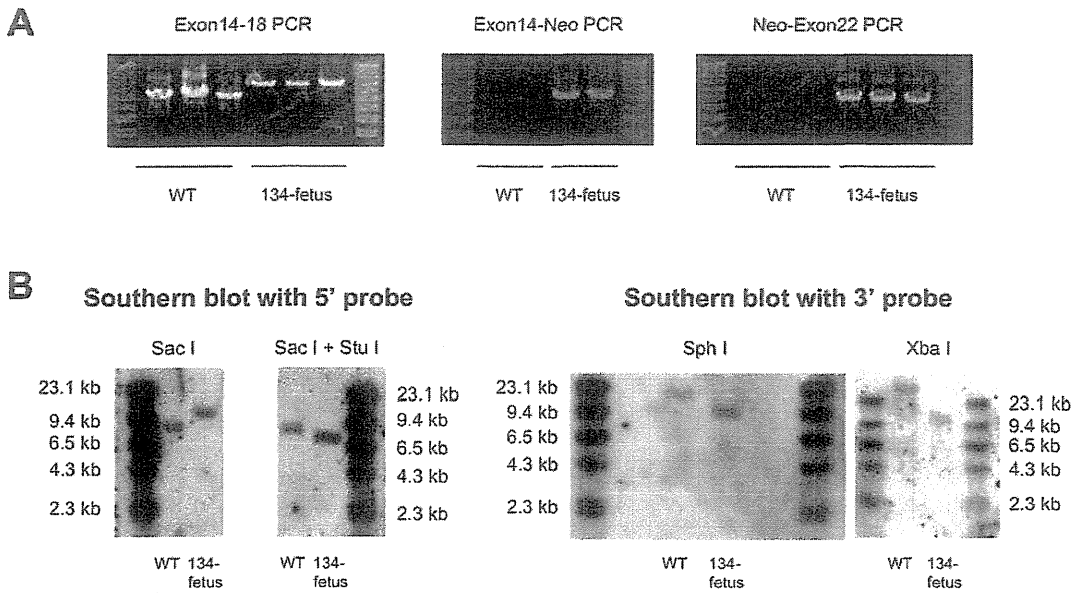

Figure 2. *F8* targeting and genetic analysis of the colony 134-derived fetus. PCR analysis of genomic DNA of 134-fetus was shown. (**A**) Two or three independent PCR reactions were carried out for detection of recombination in *F8* of 134-fetus. (**B**) Southern blotting with a 5′ exon 14 probe (on *Sac* I− or *Sac* I + *Stu* I-digested DNA) and with a 3′ exon 22 probe (on *Sph* I− or *Xba* I-digested DNA) showed correct targeting of the *F8* in 134-fetus.
doi:10.1371/journal.pone.0049450.g002

Two of the piglets (#1 and #2) found dead the next day (day 2) after delivery. The cause of death of these two piglets was not certain. Early deaths of cloned piglets after birth are not uncommon as described [24,25]. Accidental bleeding might affect the condition of piglet #1 since large hematomas were observed in piglet #1 (Figure 4). Massive traumatic intramuscular bleeding was thought to affect the death of piglet #3 on day 3 because the general condition of piglet #3 became severe immediately after the bleeding took place and piglet #3 died. Piglet #4 was born with bleeding in the left forelimb, thus, human FVIII concentrate (150 U/kg) was injected intravenously on day 2 after delivery, which cured the bleeding in the limb (Figure 4). However, because this piglet still showed a bleeding in the limbs and the tongue, which was cured with human FVIII infusion, it was given a prophylactic infusion of human FVIII (150 U/kg) twice a week, which was effective in reducing the bleeding frequency. The human FVIII activity at 12.1% (average of two points; day 10 and day 23 after birth) was detected in the piglet #4 plasma obtained two days after the injection. However, spontaneous bleeding still occurred in piglet #4, in particular repeated bleeding in the left forelimb, causing limping (Figure 4 and video S1). Piglet #4 died due to gastric bleeding from a gastric ulcer on day 38 after birth. Inhibitor (856 BU/mL) against human FVIII was detected in the plasma obtained on the day when piglet #4 died. The development of inhibitor might explain why human FVIII injected two days before was not effective to reduce bleeding from the gastric ulcer.

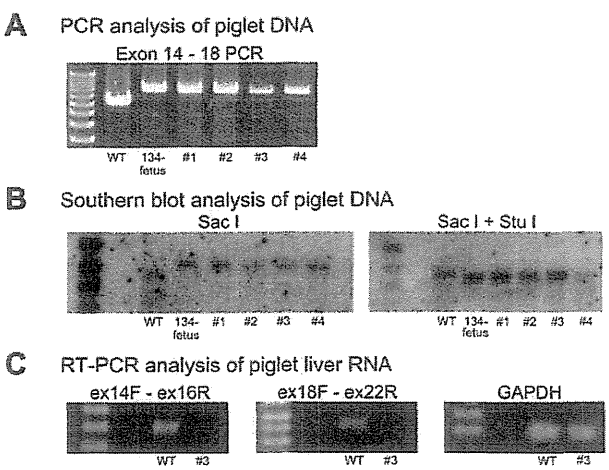

Figure 3. Analysis of the *F8* in cloned piglets. (**A**) PCR analysis of genomic DNA of piglet DNA was shown. Genomic DNA of wild-type, 134-fetus, piglet #1, piglet #2, piglet #3, and piglet #4 was subjected to PCR analysis with primers Exon 14 sF and Exon 18 sR as in Figure 1. The 8.3 kb exon 14–18 band was amplified from the 134-fetus DNA and the cloned piglet DNA. (**B**) Southern blotting with a 5′ exon 14 probe (on *Sac* I− or *Sac* I + *Stu* I-digested DNA) showed the same mobility shifts of the bands as those in Figure 2B and confirmed the insertion of the Neo resistant gene in *F8* of the cloned piglets. (**C**) RT-PCR analysis of piglet liver RNA was shown. Two independent PCRs (exons 14–16 and exons 18–22) revealed the absence of FVIII mRNA from the liver of cloned piglet #3. Control GAPDH mRNA was detected in the liver RNA of piglet #3 as in the wild type (WT).
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Discussion

Advances in cloning technology have allowed us to generate genetically modified animals [22,26,27]. Among these, a few gene-targeted pigs have been reported, such as cystic fibrosis pigs [28] and heterozygous fumarylacetoacetate hydrolase deficient pigs [29]as a disease model, and α1, 3-galactosyltransferase gene-knockout (KO) pigs [30] for organ transplantation [30,31]. Considering the limitations in studying human disease in murine models, gene-targeted pigs are thought to be preferred for studying

Table 1. Coagulation factor activity of piglets #3 and #4.

Coagulation factor	Wild type (n=4)	Piglet #3	Piglet #4
Fibrinogen (μmol/L)	2.67±1.39	1.56	ND
Factor II (%)	75.7±3.9	53	47
Factor V (%)	>200	118	168
Factor VII (%)	68.5±3.4	19	19
Factor VIII (%)	>200	1>	1>
Factor IX (%)	>200	96	69
Factor X (%)	134±7.0	72	64
	Wild type (n=4)	**Piglet #3**	**Piglet #4**
von Willebrand Factor (%)	174.7±25.9	124	251
Albumin (g/dL)	2.8±0.08	1.0	1.9
Cholinesterase (IU/L)	3.75±1.50	15	3

The coagulation factor levels of piglet #3 and #4 are shown with the control coagulation factor levels of wild-type piglets. Each coagulation factor activity was calculated from the standard curve generated with normal human plasma and expressed as the percentage of the respective coagulation factor activity in normal human plasma.
ND: not determined.
doi:10.1371/journal.pone.0049450.t001

human diseases and for translational research. We explore the possibility of *F8* KO pigs (hemophilia A pigs) for studying the next generation therapy for hemophilia A in the current study. The genotype of cloned pigs showed the proper recombination in the *F8* of the pigs and the blood coagulation factor levels of cloned pigs confirmed severe FVIII deficiency. The precise mechanism of moderately decreased other coagulation factor levels in piglets #3 and #4 was not elucidated yet, these changes may not be specific to the coagulation factors since the level of albumin was decreased but the cholinesterase level was not decreased (both albumin and cholinesterase are synthesized in the liver). One possible mechanism of the changes could be the epigenetic effect genome DNA methylation and histone acetylation, which alter gene expression in cloned pigs [24,32,33,34]. Hemophilia A pigs generated by the nuclear transfer technology did show a severe bleeding phenotype that is in contrast to *F8* KO mice that rarely exhibit spontaneous bleeding into the muscles and joints under standard breed conditions [9]. Therefore, hemophilia A pig can be used to evaluate an efficacy of novel therapy such as gene therapy for hemophilia A in a standard breed condition. Moreover, prophylactic infusion of human FVIII was effective in reducing bleeding in *F8* KO piglet #4 thought its therapeutic effect was not perfect. This suggests that the *F8* KO pig is a subhuman animal model of severe hemophilia A for the study of upcoming therapeutic factors, such novel FVIII variants. Piglet #4 died because of bleeding from a gastric ulcer. Since inhibitor against human FVIII was detected in the plasma sample obtained on the day when piglet #4 died, the therapeutic effect of human FVIII no longer existed at the time, resulting in severe bleeding from the gastric ulcer. It is possible that *F8* KO pigs might develop antibodies against porcine FVIII as against human FVIII. The possibility of the use of *F8* KO pigs as a model for studying immune tolerance induction therapy for FVIII inhibitor remains to be studied.

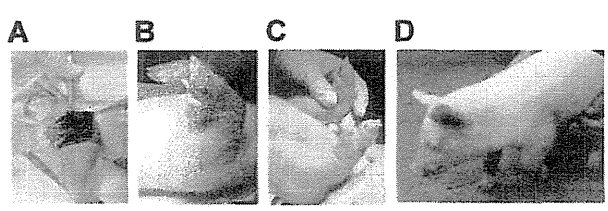

Figure 4. The bleeding phenotype of cloned *F8* KO piglets. (**A**) A part of macroscopic picture of cloned piglet #1, which died by day 2 after birth is shown. Ecchymosis was seen in the cheek, the forelimb, and the hind limb (not shown). Pathological examination revealed hematomas in these areas of piglet #1. (**B**) Forelimb of cloned piglet #4 on day 1 after delivery was shown. Ecchymosis had been seen in the left forelimb of cloned piglet #4 since delivery. (**C**) On day 5 after administration of human FVIII (150 U/kg), the bleeding in the left forelimb was not observed. Macroscopic picture of cloned piglet #4 on day 28 after birth showed that the left forelimb was swollen because of the repeated bleeding (**D**), causing the piglet to limp (also see video 1).
doi:10.1371/journal.pone.0049450.g004

Methods

Construction of the F8 targeting vector

Porcine genomic DNA was isolated from porcine embryonic fibroblasts (LW; Landrace – Large White, ED65). The *F8* targeting vector was constructed by inserting two genomic DNA fragments into the plasmid vector pHSV-TK/PGK-Neo. The *F8* targeting vector was designed by referring to the *F8* exon 16 gene-targeting vector used to generate hemophilia A mice [9]. *F8* DNA fragments from exons 14–22 were isolated by PCR using primers (Table S1) based on the *Sus scrofa* coagulation factor VIII mRNA sequence (accession number: NM_214167) and sequenced. The two homologous arms of the gene-targeting vector were generated by reference to this sequence. The 5′ DNA fragment spanning intron 15 to exon 21 of *F8* was PCR-amplified, digested with *Xho* I to generate an 11-nucleotide deletion of exon 16, and inserted into pHSV-TK/PGK-Neo. The 3′ DNA fragment was PCR-amplified from exon 16 to intron 21, and cloned into pHSV-TK/PGK-Neo containing the 5′ *F8* DNA fragment. The herpes simplex virus thymidine kinase gene was located in the opposite orientation on the 5′ end of the 5′ arm. The targeting vector was linearized with *Not* I before transfection.

Isolation of porcine embryonic fibroblasts and isolation of F8-targeted cells

Porcine embryonic fibroblasts (PEF) were isolated from a male fetus of the LW strain as described [22]. PEFs (1×10^7 cells) were transfected with the *F8* targeting vector by electroporation (Gene Pulser II; Bio-Rad, Hercules, CA) at 278 V and 950 μF. After transfection, cells were cultured in Dulbecco's modified Eagle's medium with low-glucose (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum. After 48 h incubation, cells were selected with 800 μg/ml G418 (Nacalai Tesque, Inc., Kyoto, Japan) and 2 μM gancyclovir (Tanabe-Mitsubishi Pharma, Tokyo, Japan). On the eighth day following selection, G418-resistant colonies had grown. Cells from these colonies were grown in 24-well plates (Corning) in medium containing 4 ng/ml bFGF, and expanded for genomic DNA extraction and storage. DNA isolated from three wells of each colony was analyzed by three independent PCR reactions for recombination in the porcine *F8* (Table S1).

Southern blotting

Southern blotting for the *F8* recombination was performed by the standard procedure. Digoxigenin (DIG)-labeled 5′ and 3′ probes were generated by PCR (497 bp from exon 14 and 469 bp from exon 22, respectively) (Table S1). Signals were visualized using a DIG detection module (anti-DIG-alkaline phosphatase and a CSPD) (Roche Diagnostics GmbH., Mannheim, Germany).

RT-PCR of porcine FVIII mRNA

Total RNA was isolated from piglet liver using an RNeasy Mini kit (Invitrogen), converted to cDNA and PCR amplified using the SuperScript One-Step RT-PCR System (Invitrogen) with primer pairs specific for FVIII mRNA (Table S1) [35,36].

Nuclear transfer and transplantation of manipulated embryos to recipients

Production of clone piglets by nuclear transfer was performed as described previously [22,23]. In brief, metaphase II oocytes were enucleated by gentle aspiration of the first polar body and adjacent cytoplasm using a beveled pipette (25 to 30 μm) in PZM3 medium containing 5.0 μg/ml cytochalasin B. Enucleated oocytes were washed in PZM3 medium lacking cytochalasin B and nuclei of the *F8*-targeted cells introduced by direct intracytoplasmic injection using a piezo-actuated micromanipulator (Prime Tech., Tsuchiura, Japan). Oocytes were then stimulated with a direct current pulse of 1.5 kV/cm for 100 μS using a somatic hybridizer (SSH-10, Shimadzu, Kyoto, Japan) and transferred to PZM3 supplemented with cytochalasin B to prevent extrusion of a pseudo-second polar body. The nuclear transferred oocytes were then cultured in PZM3 medium in an atmosphere of 5% CO_2, 5% O_2 and 90% air at 38.5°C for 2 days until reaching the two-to-eight-cell stage. Cleaved embryos were transferred to the oviducts (200 embryos per recipient) of an anesthetized pseudopregnant surrogate mother (matured LWD; a Landrace×Large White× Duroc triple cross). Following embryo transfer, mother pigs were observed daily to confirm pregnancy by checking estrus. Farrowing was synchronized by injection of the prostaglandin F2α analog, (1)-cloprostenol (Planate, Osaka, Japan) on day 113–116 of gestation.

Coagulation factor activity measurement

Activities of porcine coagulation factors were measured at a clinical laboratory (SRL, Tokyo, Japan) by the standard clotting time method with respective coagulation factor-deficient human plasma. Normal human plasma was used as the standard for each test. The coagulation factor activity in piglet plasma was expressed as the percentage of the coagulation factor activity in normal pooled plasma, except for fibrinogen. The fibrinogen concentration was determined by the thrombin time method. von Willebrand factor levels in pig plasma were measured with an enzyme immunoassay with latex particle conjugated antibody (performed at SRL, Tokyo, Japan) since the von Willebrand factor activity (Ristocetin cofactor activity) in pig plasma was unable to be measured with human platelets. The von Willebrand factor antigen levels in pig plasma were expressed as percentages of the normal human plasma. An inhibitor assay for human FVIII was performed as described [36].

Blood chemistry analysis

The levels of albumin and choline esterase of piglet blood were measured at the Nagahama Life-science Laboratory of Oriental East Co. Ltd (Hagahama, Shiga-ken, Japan). Choline esterase activities of blood samples were measured with p-hydroxy benzoyl choline iodide as the substrate [37].

Animal experiments

All the animal experiments and surgical procedures were carried out in accordance with guidelines approved by the Institutional Animal Care and Concern Committees of Jichi Medical University and the National Institute of Agrobiological Sciences. Protocols for the use of animals in this study were approved by the review boards of Animal Care Committees of Jichi Medical University and the National Institute of Agrobiological Sciences. Wild type pigs used in this study were bred under a standard condition according to the institutional guideline of Animal Care Committee of National Institute of Agrobiological Sciences. After delivery, cloned F8KO pigs were separated from mother pigs and each cloned F8KO pig was bred by artificial suckling in a cage with protection of soft buffers to avoid traumas. All the experimental procedures including injection of FVIII were carried out under inhalation anesthesia with isoflurane and monitoring of body temperature. The endpoint of this study was to generate F8KO pigs and analyze the genotype and the phenotype of the F8KO pig precisely to investigate whether the F8KO pig can be a subhuman model of severe hemophilia A.

Supporting Information

Table S1 Sequences of primers used in this study.
(DOC)

Video S1 Piglet #4 (day 28 after birth) to limp in the left forelimb.
(MOV)

Author Contributions

Wrote the paper: JM. Senior investigator and supervised this study: YS. Acted as a senior investigator, planned, generated constructs for F8 gene targeting, and conducted the study: JM. Postdoctoral fellow, conducted most of the F8 targeting work and injected human FVIII into a cloned piglet: YK. Performed nuclear transfer of targeted cells to oocytes and transplantation of the oocytes, and managed care of cloned pigs: AO MI. Injected human FVIII into a cloned piglet on day 1 after birth: SM. Cared for cloned piglets and did pathological examination of cloned piglets: DF SS MS SS MH SY. Performed cell culture experiments: AI AY AS TO.

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ORIGINAL ARTICLE

Intra-articular injection of mesenchymal stem cells expressing coagulation factor ameliorates hemophilic arthropathy in factor VIII-deficient mice

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Summary. *Background:* Transplantation of cells overexpressing a target protein represents a viable gene therapeutic approach for treating hemophilia. Here, we focused on the use of autologous mesenchymal stem cells (MSCs) expressing coagulation factor for the treatment of coagulation factor VIII (FVIII) deficiency in mice. *Methods and Results:* Analysis of luciferase gene constructs driven by different promoters revealed that the plasminogen activator inhibitor-1 (PAI-1) gene promoter coupled with the cytomegalovirus promoter enhancer region was one of the most effective promoters for producing the target protein. MSCs transduced with the simian immunodeficiency virus (SIV) vector containing the FVIII gene driven by the PAI-1 promoter expressed FVIII for several months, and this expression was maintained after multiple mesenchymal lineage differentiation. Although intravenous injection of cell supernatant derived from MSCs transduced with an SIV vector containing the FVIII gene driven by the PAI-1 promoter significantly increased plasma FVIII levels, subcutaneous implantation of the MSCs resulted in a transient and weak increase in plasma FVIII levels in FVIII-deficient mice. Interestingly, intra-articular injection of the transduced MSCs significantly ameliorated the hemarthrosis and hemophilic arthropathy induced by knee joint needle puncture in FVIII-deficient mice. The therapeutic effects of a single intra-articular injection of transduced MSCs to inhibit joint bleeding persisted for at least 8 weeks after administration. *Conclusions:* MSCs provide a promising autologous cell source for the production of coagulation factor. Intra-articular injection of MSCs expressing coagulation factor may offer an attractive treatment approach for hemophilic arthropathy.

Keywords: arthropathy, gene therapy, hemophilia, lentiviral vector, mesenchymal stem cells.

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Introduction

Hemophilia is a recessive X-linked genetic bleeding disorder involving a lack of functional coagulation factor VIII (FVIII) or FIX. Hemophilia is considered to be suitable for gene therapy, because it is caused by a single gene abnormality, and therapeutic coagulation factor levels may vary across a broad range [1]. Although the objective of gene therapy is to correct a defective gene sequence responsible for a disease phenotype, recent studies have focused on ectopic expression of the target gene by viral or non-viral gene transfer [2,3]. Most gene therapy strategies for hemophilia are now exploiting two basic approaches: direct administration of a viral or plasmid vector for *in vivo* gene transfer, or transplantation of cells transduced *ex vivo* [2,3]. Adeno-associated virus (AAV) vectors have been extensively used for the former approach, and have shown dramatic efficacy in some animal models [4]. In fact, therapeutic levels of coagulation factor have also been achieved in patients with hemophilia B by use of the AAV8 serotype in a phase I clinical trial [5].

The other gene therapy strategy for hemophilia involves transplantation of cells transduced *ex vivo* to ectopically express coagulation factor [3]. We and others reported that transplantation of hematopoietic stem cells transduced with lentiviral vector expressing coagulation factor corrected the phenotype of mouse models of hemophilia [6–9]. In these studies, a blood cell lineage-specific promoter enabled the expression of coagulation factors in specific lineages of blood cells, including platelets [7,9], red blood cells [6], and

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lymphocytes [10]. The use of blood cells to deliver coagulation factor is particularly attractive, as it avoids interference from circulating inhibitors [6,11]. Autologous hematopoietic stem cell-based gene therapy now represents an emerging therapeutic option for several immunodeficiency diseases [12]. However, the requirement for a conditioning regimen, including irradiation and/or chemotherapy, for successful transplantation means that this approach is not realistic for hemophilic patients.

Local implantation of cells expressing coagulation factor without conditioning treatments has been proposed as an alternative approach for cell-based therapy for hemophilia [13–15]. The advantage of locally implanting *ex vivo* transduced cells is that it avoids unexpected side effects caused by systemic influx of a viral vector. Many different types of cell have been reported to effectively express coagulation factor in cell-based therapy for hemophilia [13–16]. However, the emergence of a neutralizing antibody against coagulation factor or the loss of viability of the transplanted cells often limits their clinical applications, even if transient therapeutic expression of FVIII has been achieved [13,14]. Indeed, the transplantation of autologous fibroblasts expressing high levels of FVIII onto the omentum failed to achieve long-term expression of the coagulation factor in human clinical trials [16]. Clearly, further development of transplantation procedures is necessary before cell-based therapy can be successfully applied for hemophilic patients.

In this study, we focused on the use of mesenchymal stem cells (MSCs) as an autologous cell source to produce coagulation factor for cell-based gene therapy of a mouse model of hemophilia A. MSCs can be easily expanded *in vitro*, and effectively produce FVIII following lentiviral transduction. We found that the plasminogen activator inhibitor-1 (PAI-1) promoter was one of the most effective promoters for producing the target protein. Although we failed to consistently increase the plasma levels of coagulation factor after subcutaneous transplantation of the transduced cells in FVIII-deficient mice, we did confirm that the transduced MSCs elicited therapeutic effects by acting as a local hemostatic biomaterial in hemarthrosis and the resultant hemophilic arthropathy.

Materials and methods

The methods for construction of luciferase reporter plasmids, the luciferase reporter assay, the isolation of murine MSCs, the differentiation of MSCs, the subcutaneous transplantation of MSCs, histologic analysis and analysis of circulating FVIII inhibitors are described in detail in Data S1.

Mice

FVIII-deficient mice (B6;129S4-*F8*^{tm1Kaz}/J) [17] were kindly provided by H. H. Kazazian Jr (University of Pennsylvania, Philadelphia, PA, USA). C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). All animal procedures were approved by the Institutional Animal Care and Concern Committee at Jichi Medical University, and animal care was in accordance with the committee's guidelines.

cDNA cloning, construction of lentiviral vectors, and virus production

The cDNAs for human B-domain-deleted FVIII (hBDD-FVIII) were generated as previously described [18]. The cDNAs for enhanced green fluorescent protein (EGFP), luciferase or hBDD-FVIII under the control of the indicated internal promoter were cloned into a self-inactivating simian immunodeficiency virus (SIV) lentiviral vector plasmid [19]. The SIV lentiviral vectors were generated essentially as previously described [7]. The transduction units of the lentiviral vector, transgene expression and proviral integration into the genomic DNA were measured as previously described [7,20].

Measurement of coagulation factor activity and antigen expression

Human hFVIII (hFVIII) antigen (hFVIII:Ag) was measured with an anti-hFVIII-specific ELISA kit (ASSERACHROM VIII:Ag; Diagnostica Stago, Seine, France). The functional activity of hFVIII (hFVIII:C) was measured with an activated partial thromboplastin time-based, one-stage clotting-time assay on an automated coagulation analyzer (Sysmex CA-500 analyzer; Sysmex, Kobe, Japan). We used pooled normal human plasma as a reference to measure both hFVIII:C and hFVIII:Ag.

Bioluminescence studies

The fates of transduced cells in identical recipient mice *in vivo* were directly assessed by measuring luciferase activities derived from the transduced cells (IVIS Imaging System and LIVING IMAGE software; Xenogen, Alameda, CA, USA), as previously described [20].

Hemarthrosis model and intra-articular injection

The mouse model of hemophilic hemarthrosis was established by single needle puncture of the knee joints of FVIII-deficient mice, as previously described [21,22]. Briefly, 6–8-week-old mice were anesthetized with isoflurane, and the hair covering the left knee joint was shaved. The knee joint capsule was punctured with a 30-G needle below the patella to induce intra-articular bleeding. MSCs (1×10^5 cells per 5 μL) or vehicle alone (5 μL of saline) were directly injected into the affected knee joint with a Hamilton syringe (Hamilton, Bonaduz, Switzerland). The mice were allowed to recover. Then, at specified times after surgery, the mice were anesthetized with isoflurane, perfused with 50 mL of saline, and killed. Knee joints were collected by sectioning the femur and tibia, and macroscopic bleeding was photographed. Some knee sections were fixed and decalcified by the use of routine histologic procedures.

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Quantification of hemoglobin content

Soft tissue around the knee joint was homogenized in distilled water, and processed for the measurement of tissue bleeding as previously described [23]. Briefly, 20 μL of supernatant containing hemoglobin was incubated with 80 μL of Drabkin's reagent (Sigma Aldrich Co., St. Louis, MO, USA), and the hemoglobin concentration was assessed by measuring the OD of the solution at 550 nm.

Grading of arthropathy pathology

Hemophilic arthropathy was graded according to a verified scoring system [22]. In this system, evidence of synovial overgrowth (0–3), neovascularity (0–3), the presence of blood (0 or 1), discoloration by hemosiderin (0 or 1), synovial vilus formation (0 or 1) or cartilage erosion (0 or 1) is scored from 0 to 10. Independent reviewers blinded to the experimental conditions examined the entire joint space and articular surfaces of the sections from each knee. The area of greatest synovial thickening and vascularity was identified, and mean total synovitis scores at the region from each knee were determined. Images were captured with a charge-coupled device camera by the use of NIS-ELEMENTS software (Nikon, Tokyo, Japan).

Results

The PAI-1 promoter enables efficient transgene expression in MSCs

We first examined whether MSCs could release functional hFVIII. MSCs, mouse embryonic fibroblasts (MEFs), and HepG2 cells, a hepatocellular carcinoma cell line, were transduced with the SIV lentiviral vector expressing hBDD-FVIII driven by a cytomegalovirus (CMV) promoter. MSCs efficiently produced functional hFVIII, as compared with other cell types (Fig. S1). The relative coagulant activities (hFVIII:C/hFVIII:Ag) were 1.44 ± 0.297, 0.90 ± 0.246, and 1.420 ± 1.041 in MSCs, MEFs, and HepG2 cells, respectively (Fig. S1C).

To achieve efficient expression of the transgenes in MSCs, we compared the activities of several promoters in MSCs by lipofection. Figure 1A shows a schematic diagram of the promoters used in the experiment. The luciferase reporter gene was used to compare the promoter activities, and the luciferase activity of the promoter was normalized for the luciferase activity of the SV40 promoter with an enhancer sequence. We chose the PAI-1 promoter as a candidate promoter in MSCs, because the PAI-1 gene is a highly inducible gene in MSCs exposed to hypoxia [24]. The DNA fragments for the promoter region of the human PAI-1 gene were fused with the early enhancer element of the CMV promoter, as previously described [25]. As shown in Fig. 1B. the CMV and PAI-1 promoter enabled efficient expression of luciferase in MSCs.

Next, we constructed SIV-based lentiviral vectors containing the EGFP gene under the control of either the CMV promoter (SIV–CMVp–EGFP) or the PAI-1 promoter (SIV–PAI-1p–EGFP) to confirm that gene expression is efficiently driven by the candidate promoters in MSCs with the SIV vector. Both vectors efficiently transduced the EGFP gene into MSCs (Fig. 2). It is of note that the mean fluorescence intensity (MFI) of EGFP expression driven by the PAI-1 promoter was much greater than the MFI of expression driven by the CMV promoter (Fig. 2). EGFP expression was maintained for at least 9 weeks after transduction (Fig. 2). Therefore, we used

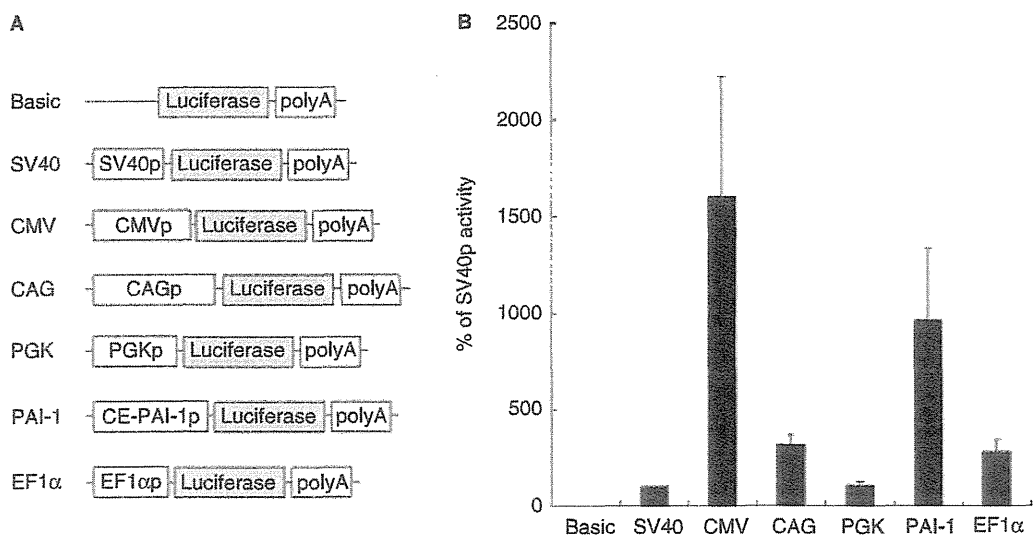

Fig. 1. Comparison of promoter activities in mesenchymal stem cells (MSCs). (A) Schematic diagrams of the promoters used in the experiments. (B) Each construct, along with a promoterless vector (basic) or a positive control vector (SV40/Enhancer), was transfected into MSCs. Luciferase activity was measured 48 h after transfection, and is shown relative to the activity driven by the SV40 promoter (SV40/Enhancer). Each experiment was carried out four to six times with duplicate samples. Values are means ± standard deviations. CE–PAI-1, PAI-1 promoter coupled with CMV promoter enhancer region; CMV, cytomegalovirus; EF1α, elongation factor-1α; PAI-1, plasminogen activator inhibitor-1; PGK, phosphoglycerate kinase 1.

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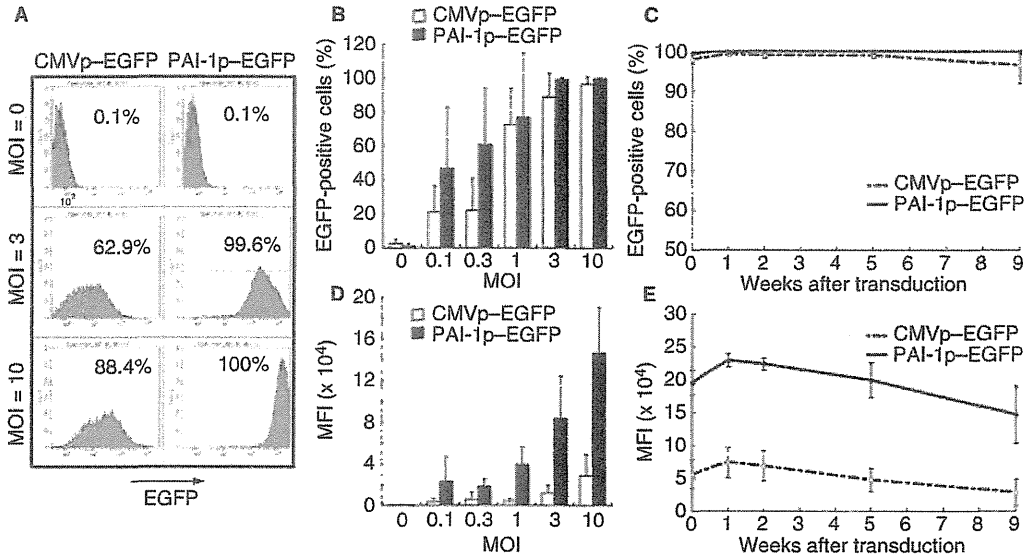

Fig. 2. Expression of enhanced green fluorescent protein (EGFP) in mesenchymal stem cells (MSCs) transduced with simian immunodeficiency virus (SIV) vectors carrying the EGFP gene driven by the cytomegalovirus (CMV) or plasminogen activator inhibitor-1 (PAI-1) promoter. MSCs were transduced with SIV–CMVp–EGFP (CMVp–EGFP) or SIV–PAI-1p–EGFP (PAI-1p–EGFP). Cellular expression of EGFP was analyzed by flow cytometry. (A) Representative data showing EGFP expression after transduction at the indicated multiplicity of infection (MOI). (B–E) The percentage of EGFP-positive cells (B, C) or the mean fluorescence intensity (MFI) of EGFP (D, E) was quantified in cells transduced with an increasing MOI for 48 h (B, D), or with a fixed MOI (30) for various times (C, E). Values are means ± standard deviations (n = 5).

the PAI-1 promoter in subsequent experiments because of its efficient expression of the transgene in MSCs by SIV.

hFVIII expression in MSCs during passage and differentiation

We next examined the maintenance of hFVIII production after transduction during passage. MSCs were transduced with the SIV vector containing hBDD-FVIII under the control of the PAI-1 promoter (SIV–PAI-1p–hFVIII) at an indicated multiplicity of infection (MOI). The activity of hFVIII produced from the cells over 24 h was assessed every week before each passage. As shown in Fig. 3A, the cells produced hFVIII in a vector dose-dependent manner, and the production was stably maintained *in vitro* for at least 9 weeks. Proviral integration into the genome was significantly correlated with hFVIII:C after transduction in a linear regression model ($P < 0.0001$) (Fig. S2).

To investigate whether differentiation of MSCs affects hFVIII production, MSCs transduced with SIV–PAI-1p–hFVIII at an MOI of 30 were differentiated into adipocytes, osteocytes and chondrocytes *in vitro*. We confirmed the expression of each lineage-specific marker and hFVIII:Ag after differentiation (Fig. 3B). Under the same conditions, the production and secretion of hFVIII:Ag persisted during adipogenic and osteogenic differentiation (Fig. 3C,D). On the other hand, although hFVIII:Ag was secreted from the cells during chondrogenic differentiation, the level was somewhat lower (Fig. 3E). This was probably because of the culture conditions, as the cells were cultured as aggregate cell pellets in chondrogenic differentiation medium (see Data S1). These results suggest that the release of hFVIII is maintained during cell division in undifferentiated MSCs, and that lineage differentiations are unlikely to influence the production of hFVIII.

Subcutaneous transplantation of MSCs expressing hFVIII does not improve the phenotype of FVIII-deficient mice

We next investigated the therapeutic effects of transplanting engineered MSCs expressing hFVIII on systemic bleeding in FVIII-deficient mice with hemophilia A. Direct intravenous injection of concentrated supernatant from 0.4–4 × 10^6 MSCs transduced with SIV–PAI-1p–hFVIII significantly increased plasma hFVIII:Ag levels (Fig. S3A), indicating that the autologous MSCs could be an attractive cell source for the production of functional FVIII. However, subcutaneous implantation of transduced MSCs mixed with Matrigel resulted in a marginal increase in plasma FVIII levels, and the expression of hFVIII:Ag was not persistent, even if the number of cells was increased to 3 × 10^7 (Fig. S3B). As expected from previous reports [14], we detected the emergence of circulating plasma inhibitors of hFVIII after transplantation (Fig. S3C). Thus, we concluded from these results that subcutaneous transplantation of transduced MSCs was unable to significantly improve systemic bleeding in mice with hemophilia A.

Intra-articular injection of MSCs expressing hFVIII ameliorates hemarthrosis and arthropathy in FVIII-deficient mice

We next examined whether transduced autologous MSCs could serve as a local hemostatic biomaterial. The most significant morbidity resulting from congenital FVIII

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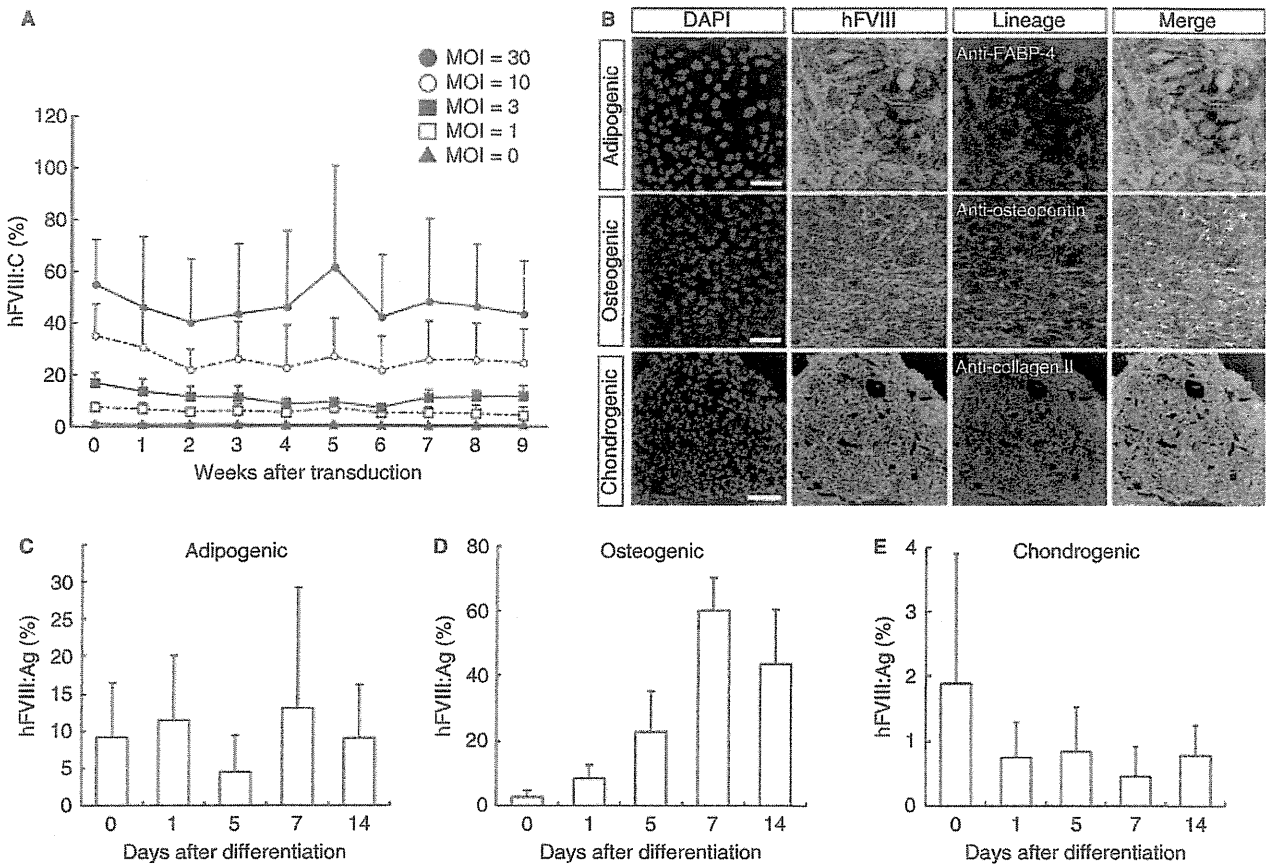

Fig. 3. Persistent expression of human FVIII (hFVIII) from transduced mesenchymal stem cells (MSCs) during maintenance and differentiation. (A) MSCs were transduced with the simian immunodeficiency virus (SIV) vector carrying the hFVIII gene driven by the plasminogen activator inhibitor-1 promoter at the indicated multiplicity of infection (MOI). hFVIII activity (hFVIII:C) in the supernatant derived from 5×10^5 cells for 24 h in 1 mL was assessed at the indicated times after transduction with a one-stage clotting-time assay on an automated coagulation analyzer. Values are means ± standard deviations (SDs) (n = 4). (B) MSCs transduced with the SIV vector carrying the hFVIII gene at an MOI of 30 were differentiated into multiple mesenchymal lineages, as described in Data S1. Immunocytochemistry was performed to detect differentiation into adipocytes (anti-FABP-4), osteocytes (anti-osteopontin), or chondrocytes (anti-collagen II) (red), and hFVIII antigen (hFVIII:Ag) (anti-hFVIII polyclonal antibody; green). Nuclear localization was simultaneously examined by 4′,6-diamidino-2-phenylindole (DAPI) staining. The merged images show colocalization of lineage marker and hFVIII antigen. Scale bars: 60 μm. (C–E) The supernatants were isolated at the indicated times after adipogenic (C), osteogenic (D) or chondrogenic differentiation (E). The antigen levels of hFVIII in the supernatant were quantified by ELISA. Values are means ± SDs (n = 4). FABP-4, fatty acid binding protein-4.

deficiency is the progressive destruction of joints resulting from recurrent intra-articular hemorrhage. The coagulation factor derived from transduced MSCs may prevent local hemorrhage, and the ability of MSCs to differentiate into osteocytes and chondrocytes might promote repair of the affected joints. Therefore, we examined the effects of intra-articular injection of transduced MSCs in preventing intra-articular hemorrhage and hemophilic arthropathy.

We first examined the biodistribution of transduced MSCs after injection into the knee. The MSCs were efficiently transduced with the SIV vector having a luciferase gene under the control of the PAI-1 promoter (Fig. 4B,C). We next injected 100 000 transduced MSCs into the left knee articular space in C57BL/6J mice (0.33% of the cells used in subcutaneous transplantation). The intensity and biodistribution of luciferase expression derived from the cells were imaged at the indicated times after injection. As shown in Fig. 4D,E, luciferase derived from the transduced MSCs was detected in the injected knee, and was maintained for at least 4 weeks. In contrast, no luciferase activity was detected in other organs (Fig. 4D). Furthermore, real-time RT-PCR could not detect transgene expression in other organs, including the heart, lung, liver, kidney, spleen, and bone marrow, after transplantation (data not shown). Immunohistochemical staining for luciferase in the knee revealed that luciferase was mainly expressed in chondrocytes in the joint structure (Fig. S4), suggesting that the transduced MSCs differentiated into chondrocytes after injection.

To examine the possibility that locally administered MSCs expressing hFVIII could protect against hemarthrosis in the absence of circulating FVIII, we injected the MSCs transduced with SIV–PAI-1p–hFVIII into the knee space (1×10^5 cells) after single needle puncture in FVIII-deficient mice. Macroscopic bleeding around the affected knee was observed, and the

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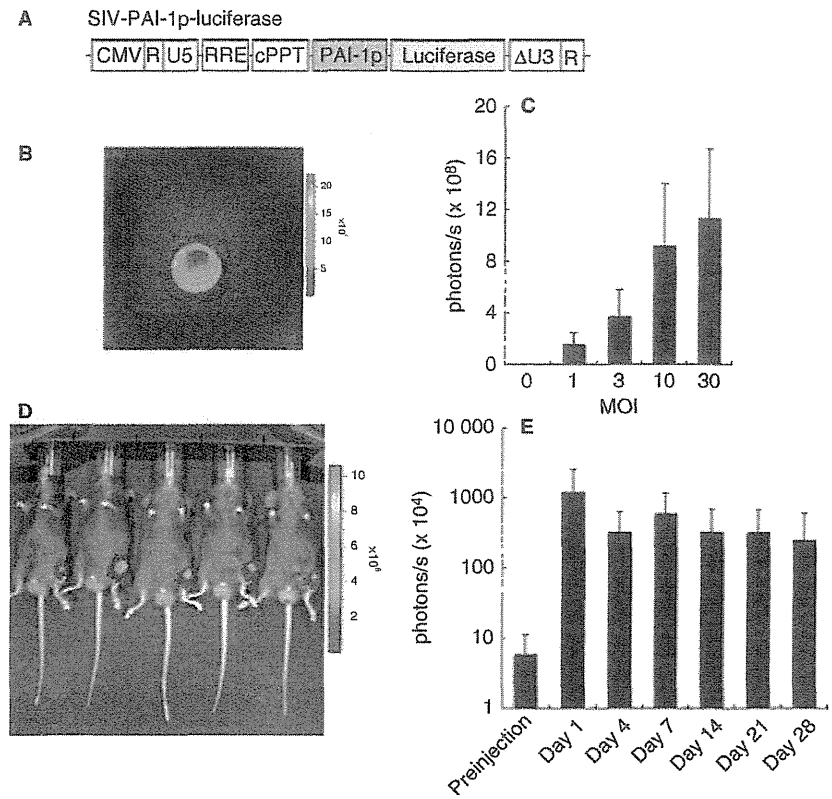

Fig. 4. Fate of transduced mesenchymal stem cells (MSCs) injected into the knee joint space *in vivo*. (A) Schematic diagram of the simian immunodeficiency virus (SIV) lentiviral vector used in this experiment. The SIV vector expresses a luciferase gene driven by the plasminogen activator inhibitor-1 (PAI-1) promoter. (B) MSCs were transduced with the SIV lentiviral vector at a multiplicity of infection (MOI) of 30. *Ex vivo* bioluminescence images of transduced MSCs were obtained with the IVIS Imaging System. (C) MSCs were transduced with the SIV lentiviral vector at the indicated MOI, and *ex vivo* bioluminescence of the transduced cells was quantified (photons s^{-1}). Values are means ± standard deviations (SDs) (n = 3). (D) *In vivo* bioluminescence images of transduced MSCs after transplantation. Photons transmitted through the body were recorded with the IVIS Imaging System 1 day after injection of the transduced MSCs (1×10^5 cells) into the left knee joint space. (E) *In vivo* bioluminescence of the mice was quantified for the indicated times after injection (photons s^{-1}). Values are means ± SDs (n = 5). CMV, cytomegalovirus; cPPT, central polypurine tract; PAI-1p, plasminogen activator inhibitor-1 promoter; RRE, rev response element.

blood leakage was quantified as the amount of hemoglobin measured 24 h after the knee challenge. Knee joint needle puncture resulted in massive bleeding in the joint space and in peripheral tissues (Fig. 5A,C). Intravenous injection of recombinant hFVIII significantly and dose-dependently improved joint bleeding (Fig. 5A–C). Interestingly, injection of MSCs transduced with SIV–PAI-1p–hFVIII, but not of non-transduced MSCs, had hemostatic effects that were equivalent to those in mice with hemophilia A intravenously treated with 1 U per mouse of recombinant hFVIII (Fig. 5A,C). The plasma concentration of recombinant hFVIII after intravenous injection of 1 U per mouse was 20–30% of that in normal human pooled plasma (Fig. 5B). On the other hand, we could not detect hFVIII:Ag in plasma after intra-articular injection of MSCs transduced with SIV-PAI-1p-hFVIII (data not shown). The estimated hFVIII:C produced by transplanted MSCs (1×10^5 cells) was 0.025–0.05 U per 24 h. We also found that intra-articular injection of 0.1 U of recombinant hFVIII, but not 0.01 U, significantly inhibited hemarthrosis (Fig. 5C). The peak concentrations seemed to be higher after direct injection of recombinant hFVIII than those produced by the transduced MSCs, suggesting that both hFVIII and MSCs in the synovial space are essential for the therapeutic effects of our procedure.

We next assessed the progression of hemophilic arthropathy after intra-articular injection of transduced MSCs. Four weeks after injection, the joints were harvested, and histopathologic grading of arthropathy was performed. Results were compared between mice treated with or without intravenous injection of recombinant hFVIII or with intra-articular injection of non-transduced MSCs. As shown in Fig. 6A, intra-articular injection of MSCs transduced with SIV–PAI-1p–hFVIII significantly reduced the extent of hemorrhage-induced synovitis, including synovial hyperplasia, vascularity, and discoloration. The pathologic score of mice given an intra-articular injection of transduced MSCs was equivalent to that in mice intravenously treated with 1–4 U per mouse of recombinant hFVIII (Fig. 6B).

We also investigated the long-term treatment effects of a single intra-articular injection of transduced MSCs in inhibiting joint bleeding. FVIII-deficient mice received an intra-articular

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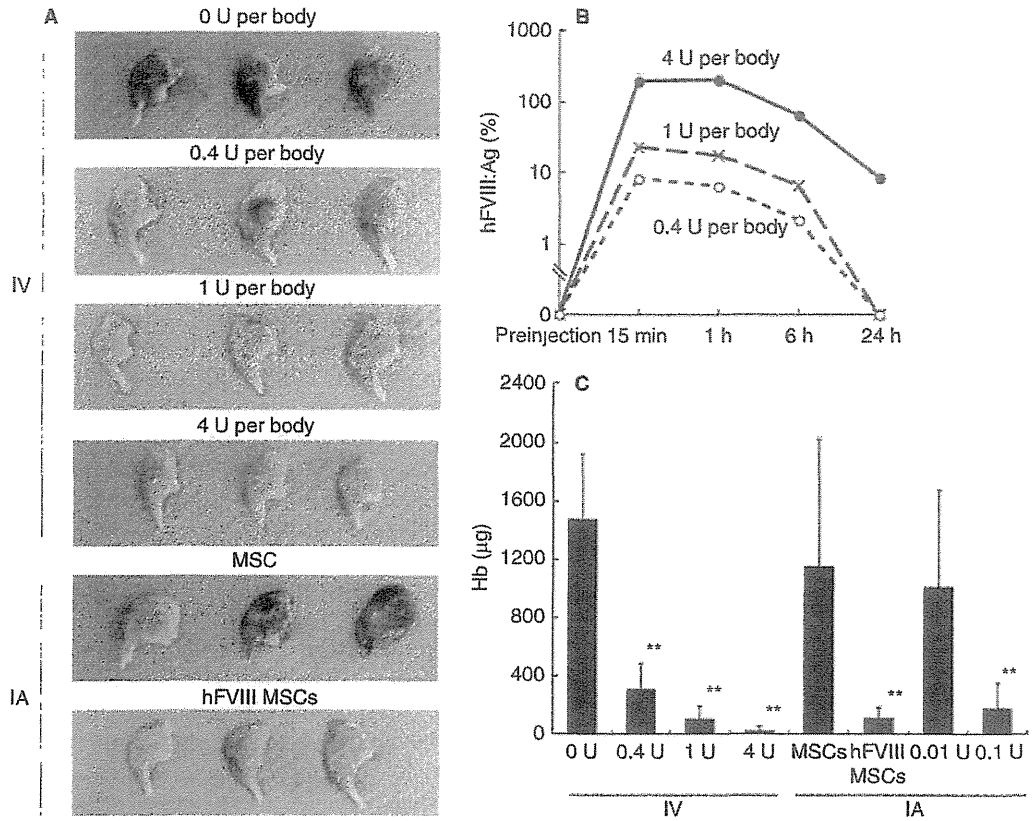

Fig. 5. Local injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) protects against hemarthrosis induced by joint puncture in FVIII-deficient mice. FVIII-deficient mice received an intravenous dose of recombinant hFVIII or an intra-articular injection of non-transduced MSCs or transduced MSCs expressing hFVIII (1×10^5 cells), and this was followed by joint capsular needle puncture injury. (A) Macroscopic findings of hemarthrosis at 24 h after knee puncture. IV: intravenous injection of the indicated dose of recombinant hFVIII. IA: intra-articular administration of MSCs transduced without (MSCs) or with (hFVIII MSCs) SIV–PAI-1p–hFVIII. (B) Plasma hFVIII antigen (hFVIII:Ag) levels at the indicated times after intravenous administration of recombinant hFVIII. Values are means ± standard deviations (SDs) ($n = 3$). (C) Bleeding around the knee joint quantified as the hemoglobin (Hb) concentration. Values are means ± SDs ($n = 5$). Data are also shown for mice receiving an intra-articular injection of recombinant hFVIII (0.01 U or 0.1 U) ($n = 5$). $^{**}P < 0.01$ as compared with the untreated control (two-tailed Student's *t*-test). PAI-1p, plasminogen activator inhibitor-1 promoter; SIV, simian immunodeficiency virus vector.

injection of transduced MSCs expressing hFVIII, followed by rechallenge (i.e. needle puncture of the affected knee). As shown in Fig. 7B, hemarthrosis at 24 h after the rechallenge was significantly improved by intra-articular injection of transduced MSCs. The therapeutic effects persisted for at least 8 weeks after administration (Fig. 7A). Low titers of circulating inhibitors of hFVIII could be detected after intra-articular injection of transduced MSCs, but these were much lower than those detected after subcutaneous transplantation of MSCs (Figs 7B and S3C).

We finally examined whether intra-articular injection of transduced MSCs ameliorates hemarthrosis in the presence of circulating inhibitors. FVIII-deficient mice were immunized by weekly injection of recombinant hFVIII (4 U per mouse). We obtained pooled plasma containing a high titer of hFVIII inhibitor after six doses (1110 Bethesda Units [BU] mL^{-1}), and intravenously injected the indicated volume of plasma into naïve FVIII-deficient mice. The plasma neutralizing antibody titer increased to 1.62 ± 0.387, 7.58 ± 0.577 and 35.14 ± 23.460 after the injection of 2, 10 and 50 BU per mouse, respectively (Fig. 7C). Intra-articular injection of transduced MSCs (1×10^5 cells) reduced the hemarthrosis elicited by needle puncture in the presence of a low titer of the inhibitors (2 BU per mouse), although the effects were weaker in the presence of a higher titer of circulating inhibitor (10 or 50 BU per mouse) (Fig. 7D). Increasing the number of transplanted cells (1×10^6 cells) partly overcame the attenuated treatment effects caused by a higher neutralizing antibody titer (Fig. 7D).

Discussion

Hemophilic arthropathy – the progressive destruction of the joint structure resulting from recurrent intra-articular hemorrhage – is a frequent and serious complication experienced by patients with severe hemophilia [26]. Despite advances in treatment and the delivery of comprehensive care, joint bleeding and hemophilic arthropathy are still the most common complications of hemophilia, and are associated with

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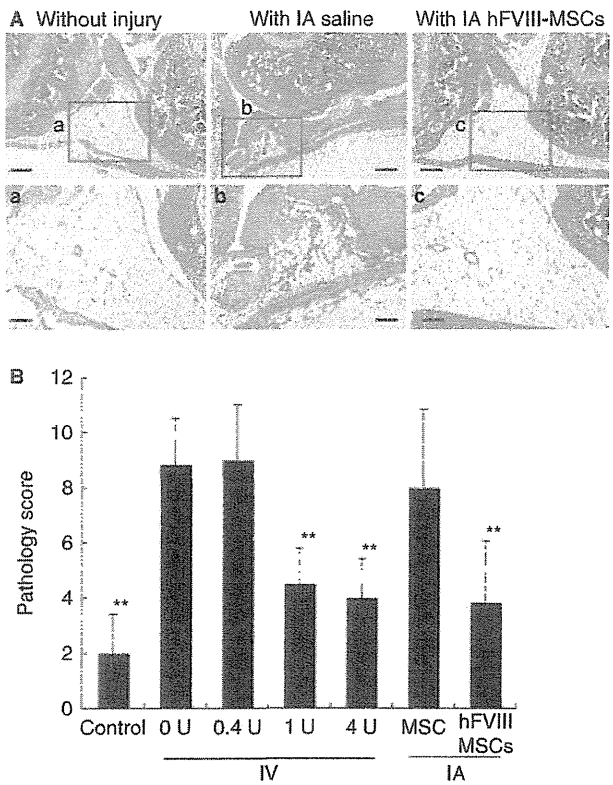

Fig. 6. Local injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) protects against hemophilic arthropathy in FVIII-deficient mice. (A) Representative histopathologic images taken 4 weeks after the joint challenge. Without injury: FVIII-deficient mouse knee joint without knee puncture. With IA saline: the knee joint after knee puncture and treatment with intra-articular saline. With IA hFVIII MSCs: the knee joint after knee puncture and treatment with intra-articular MSCs transduced with SIV–PAI-1p–hFVIII. Higher magnifications of the numbered boxed regions are shown in the lower panel. Scale bars: 125 μm in upper panel; 50 μm in lower panel. (B) Histologic changes were assessed with a validated mouse hemophilic synovitis grading system. The severity of synovial hyperplasia, vascularity, or the presence of blood, synovial villus formation, discoloration by hemosiderin or cartilage erosion are graded from 0 to 10. Control: FVIII-deficient mouse knee joint without knee puncture. IV: the knee joint after knee puncture and treatment with intravenous injection of the indicated dose of recombinant hFVIII. IA: knee joint after knee puncture with intra-articular injection of MSCs transduced without (MSCs) or with (hFVIII MSCs) SIV–PAI-1p–hFVIII. Values are means ± standard deviations (SDs) (n = 4). $^{**}P < 0.01$ as compared with untreated control (two-tailed Student's t-test).

a very poor quality of life [27]. It was shown that episodic prophylactic treatment with recombinant coagulation factor could prevent joint damage in young children with severe hemophilia [28], although this approach did not prevent the progression of joint damage in adolescence, after the joint damage had fully developed [29]. The costs to the healthcare system of treating hemophilia are substantial, because of the need for prophylactic treatment with recombinant coagulation factor. Patients also experience significant loss of productivity and greatly diminished quality of life, as a result of bleeding into the joints and arthropathy [30]. Therefore, there is a need for new adjunctive treatments or prophylactic strategies that are specific for joint bleeding and the prevention of hemophilic arthropathy.

Here, we found that intra-articular transplantation of autologous MSCs expressing hFVIII ameliorated acute joint bleeding and the resultant hemophilic arthropathy in FVIII-deficient mice with hemophilia A. Intra-articular injection of transduced MSCs effectively inhibited acute joint bleeding, even in conditions where the plasma FVIII levels did not increase. It was also reported that direct injection of an AAV vector expressing FIX into the joint space improved hemophilic arthropathy in FIX-deficient mice [22]. As compared with intravenous administration of recombinant hFVIII, the main advantage of cell-based therapy and gene therapy is consistent production of the functional coagulation factor by the transduced cells. The major mechanism by which transduced MSCs affect hemophilic arthropathy seems to involve hemostasis by targeting of acute bleeding through extracellular production of hFVIII. There are also several reasons why MSCs should be selected to treat hemophilic arthropathy. MSCs can be expanded *in vitro* as autologous cells, can differentiate into chondrocytes and osteoblasts, and can produce a number of bioactive mediators with regenerative effects. These functions of MSCs can be exploited therapeutically to repair degenerative joints, as MSC-based strategies can be used to repair chondral and osteochondral lesions, or to modulate endogenous factors that enhance regenerative processes in degenerative joints [31]. In addition, it has been reported that MSCs can modulate immune responses and control inflammation by targeting T lymphocytes [32]. Inflammatory responses, including cytokine release and inflammatory cell invasion, caused by the response to blood in the joint space play key roles in the pathophysiology of hemophilic arthropathy [33]. The injection of MSCs into the joint space is likely to ameliorate the inflammatory response, which would otherwise promote destruction of the joint structure [31,34].

MSCs offer a promising autologous cell source for the production of coagulation factor. We found that the PAI-1 promoter was one of the most effective promoters for producing the target protein by lentiviral transduction. The different results obtained with transient transduction with a plasmid vector and lentiviral transduction may be attributable to post-transcriptional silencing of the CMV promoter after lentiviral transduction. It is well known that CMV promoter silencing limits its usefulness in many research applications and in gene therapy [35,36]. The PAI-1 promoter stably and effectively drove transgene expression even after multiple mesenchymal lineage differentiation *in vitro*, and luciferase expression from the transduced cells was detected at least 4 weeks after injection of the cells into the joint space. PAI-1 was reported to be an inducible factor whose expression is consistently upregulated by ischemic conditions in MSCs [24]. As hypoxia is an important event in the perpetuation of joint destruction [37], it is possible that the increase in transgene expression driven by the PAI-1 promoter under hypoxic

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