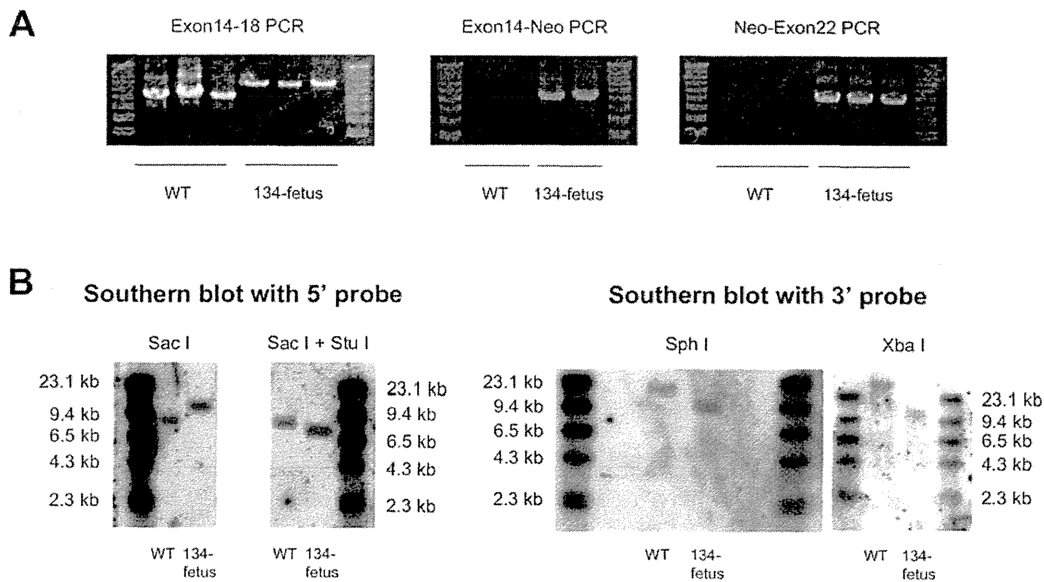

Figure 2. *F8* targeting and genetic analysis of the colony 134-derived fetus. PCR analysis of genomic DNA of 134-fetus was shown. **(A)** Two or three independent PCR reactions were carried out for detection of recombination in *F8* of 134-fetus. **(B)** Southern blotting with a 5′ exon 14 probe (on *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.

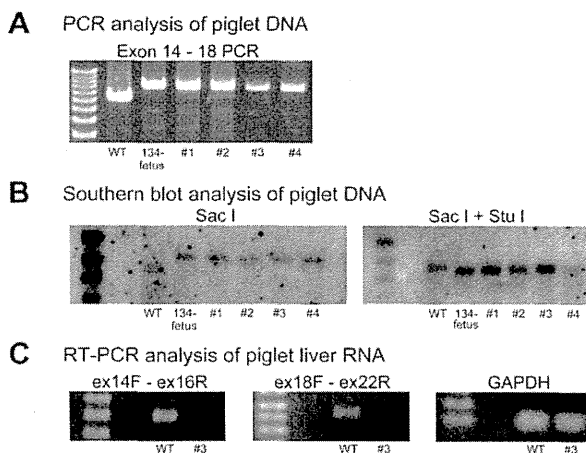

Figure 3. Analysis of the *F8* in cloned piglets. **(A)** PCR analysis of genomic DNA of piglet DNA was shown. Genomic DNA of wild-type, 134-fetus, piglet #1, piglet #2, piglet #3, and piglet #4 was subjected to PCR analysis with primers Exon 14 sF and Exon 18 sR as in Figure 1. The 8.3 kb exon 14–18 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).
doi:10.1371/journal.pone.0049450.g003

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.

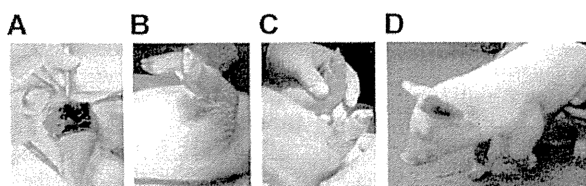

Figure 4. The bleeding phenotype of cloned *F8* KO piglets. (A) A part of macroscopic picture of cloned piglet #1, which died by day 2 after birth is shown. Ecchymosis was seen in the cheek, the forelimb, and the hind limb (not shown). Pathological examination revealed hematomas in these areas of piglet #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.

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

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

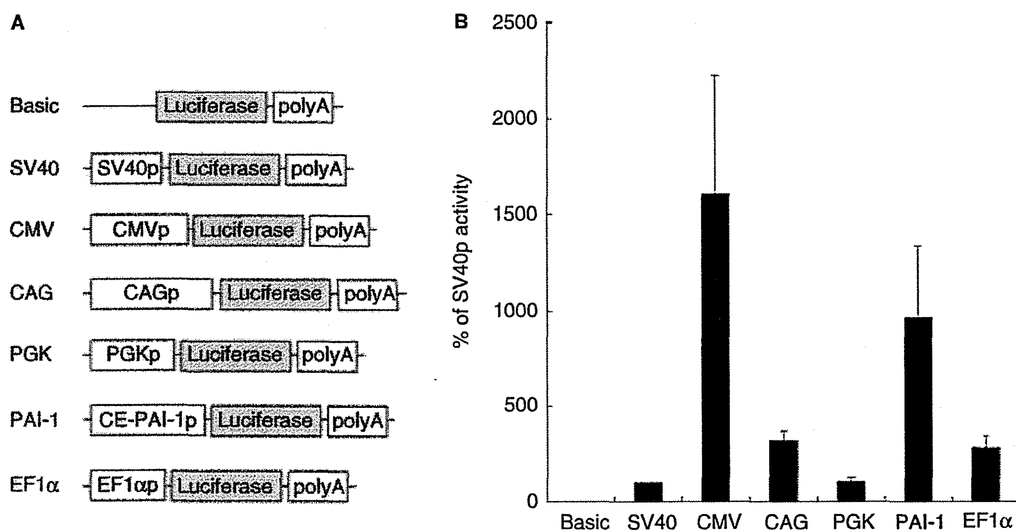

Fig. 1. Comparison of promoter activities in mesenchymal stem cells (MSCs). (A) Schematic diagrams of the promoters used in the experiments. (B) Each construct, along with a promoterless vector (basic) or a positive control vector (SV40/Enhancer), was transfected into MSCs. Luciferase activity was measured 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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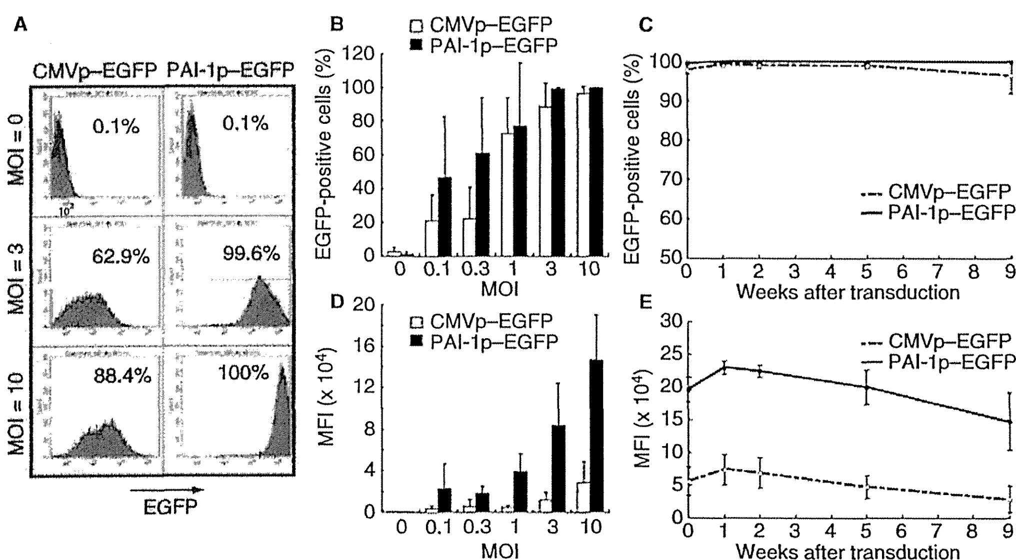

Fig. 2. Expression of enhanced green fluorescent protein (EGFP) in mesenchymal stem cells (MSCs) transduced with simian immunodeficiency virus (SIV) vectors carrying the EGFP gene driven by the cytomegalovirus (CMV) or plasminogen activator inhibitor-1 (PAI-1) promoter. MSCs were transduced with SIV–CMVp–EGFP (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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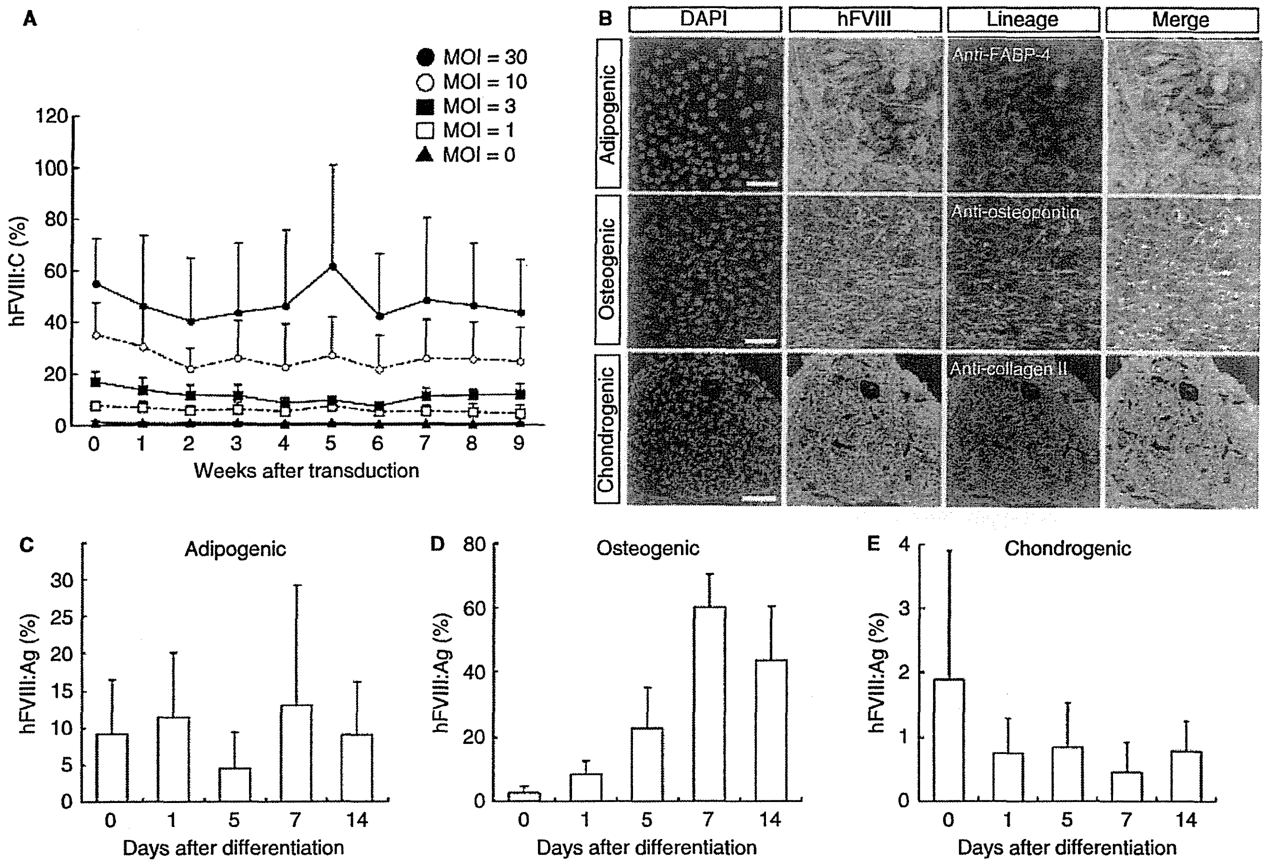

Fig. 3. Persistent expression of human FVIII (hFVIII) from transduced mesenchymal stem cells (MSCs) during maintenance and differentiation. (A) MSCs were transduced with the simian immunodeficiency virus (SIV) vector carrying the hFVIII gene driven by the plasminogen activator inhibitor-1 promoter at 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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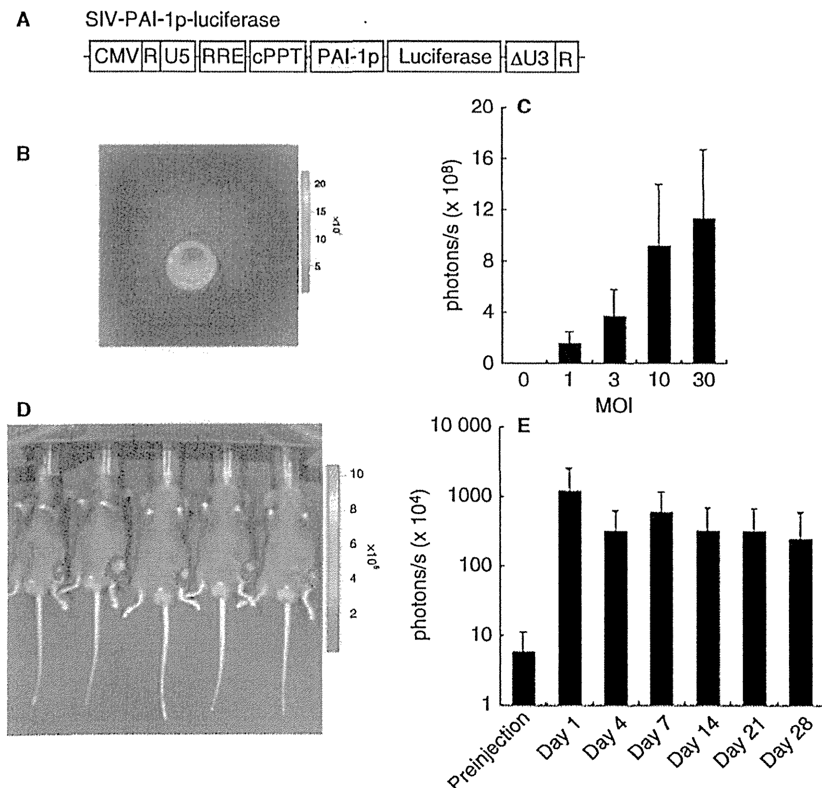

Fig. 4. Fate of transduced mesenchymal stem cells (MSCs) injected into the knee joint space *in vivo*. (A) Schematic diagram of the simian immunodeficiency virus (SIV) lentiviral vector used in this experiment. The SIV vector expresses a luciferase gene driven by the plasminogen activator inhibitor-1 (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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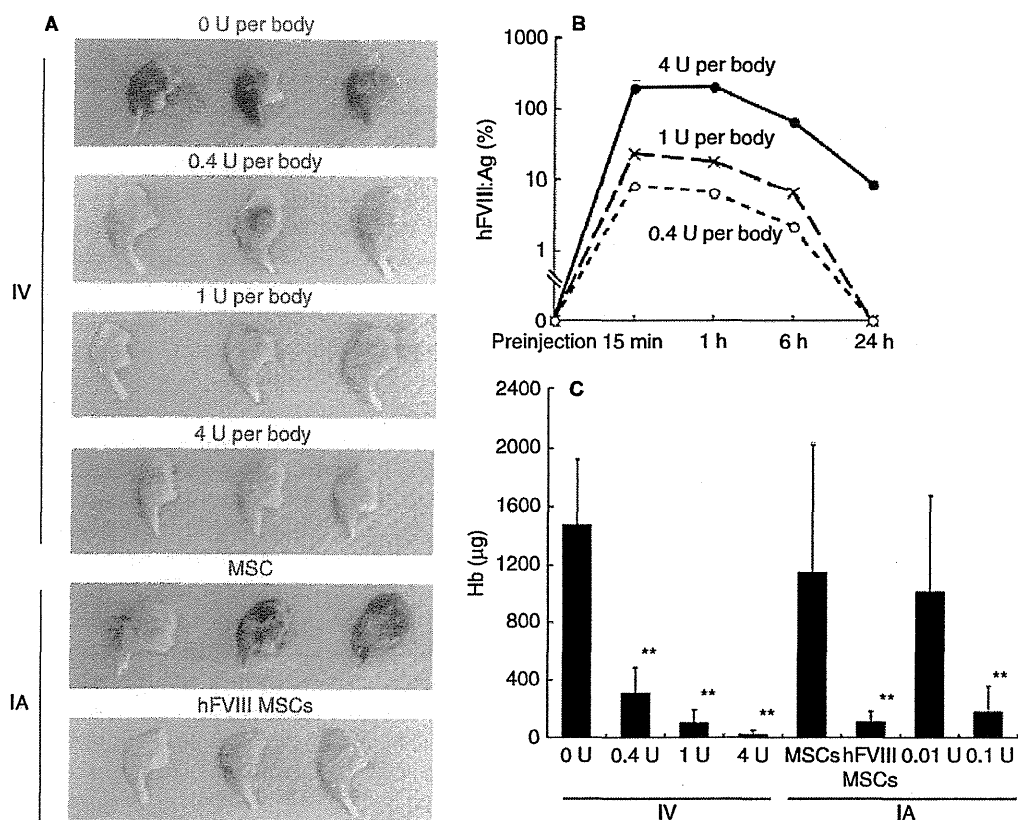

Fig. 5. Local injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) protects against hemarthrosis induced by joint puncture in FVIII-deficient mice. FVIII-deficient mice received an intravenous dose of recombinant hFVIII or an intra-articular injection of non-transduced 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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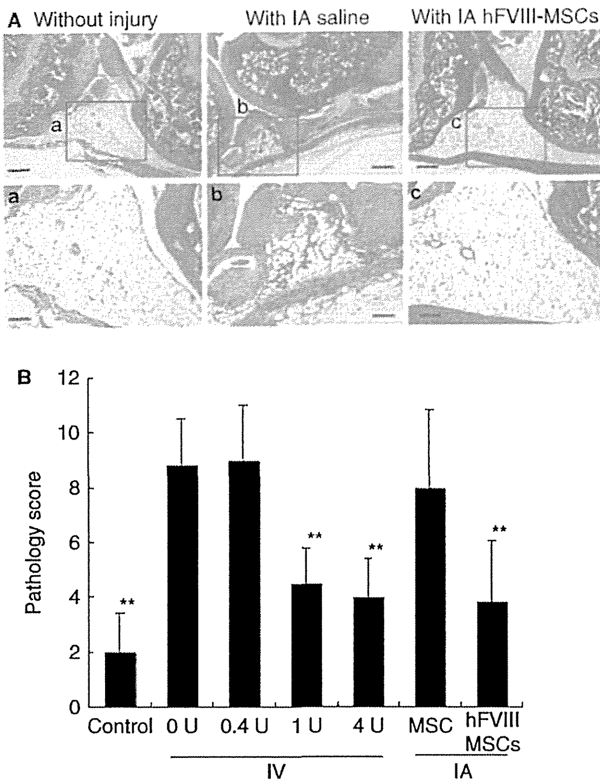

Fig. 6. Local injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) protects against hemophilic arthropathy in FVIII-deficient mice. (A) Representative histopathologic images taken 4 weeks after the joint challenge. Without injury: FVIII-deficient mouse knee joint without 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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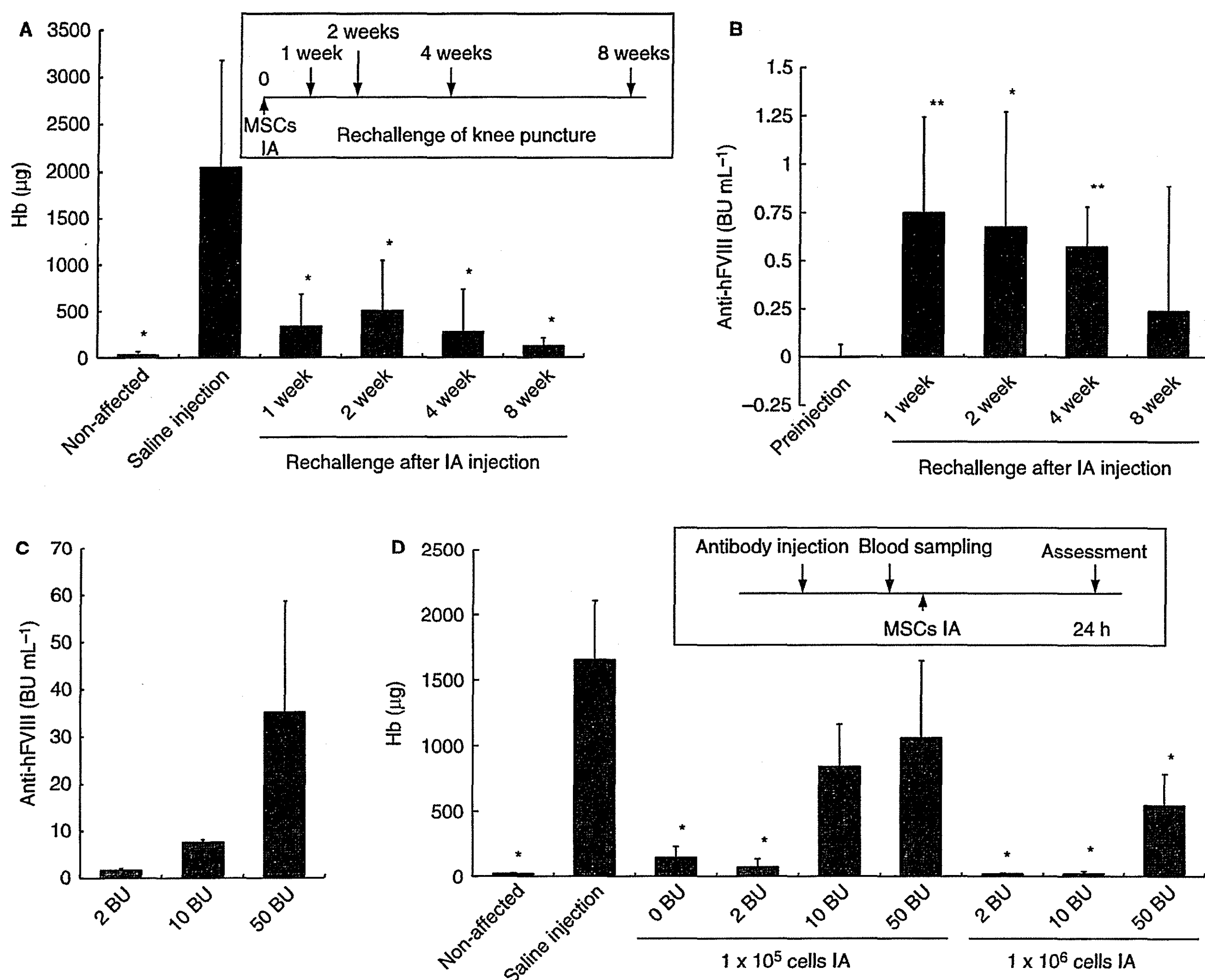

Fig. 7. Intra-articular (IA) injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) persistently inhibits hemarthrosis, and protects against hemarthrosis in the presence of low titers of neutralizing antibodies against hFVIII. (A) FVIII-deficient mice received an IA injection of transduced MSCs expressing hFVIII (1×10^5 cells), and this was followed by joint capsular needle puncture at the indicated times after IA injection. A schematic diagram of the procedure is shown in the box. Hemarthrosis at 24 h after knee puncture was quantified as the hemoglobin (Hb) concentration. Values are means ± standard deviations (SDs) (n = 4–9). *$P < 0.05$ as compared with the saline-injected control group (two-tailed Student's t-test). (B) Circulating plasma inhibitors were assessed as Bethesda Units (BU) mL^{-1} at the indicated times after IA injection. Values are means ± SDs (n = 4–10). (C) Neutralizing antibodies against hFVIII (2, 10 or 50 BU per mouse) were intravenously administrated into FVIII-deficient mice. Circulating plasma inhibitor concentrations were assessed as BU mL^{-1} (n = 6). (D) FVIII-deficient mice received an IA injection of transduced MSCs expressing hFVIII (1×10^5 cells or 1×10^6 cells), and this was followed by joint capsular needle puncture. Hemarthrosis at 24 h after knee puncture was quantified as the Hb concentration. A schematic diagram of the procedure is shown in the box. Values are means ± SDs (n = 3). *$P < 0.05$ as compared with the saline-injected control group (two-tailed Student's t-test).

conditions further enhances coagulation factor expression to ameliorate hemarthrosis.

Several studies have focused on cell-based therapy with MSCs expressing coagulation factor to treat hemophilia by increasing the plasma levels of coagulation factor [14,38–40]. However, long-term protein production from MSCs was not achieved *in vivo* after transplantation, because of the loss of cell viability and/or the emergence of inhibitory antibodies [14]. Recently, Coutu *et al.* [39] successfully achieved long-term expression of FIX by implanting a three-dimensional porous scaffold containing gene-modified MSCs to increase graft survival. They used the murine R333Q model of hemophilia B, which avoids the development of inhibitory antibodies [39]. In addition, Porada *et al.* [40] described the interesting treatment effect of MSCs in a sheep model of severe hemophilia A. They intraperitoneally transplanted MSCs expressing porcine FVIII into sheep with hemophilia A [40]. An increase in plasma FVIII activity could not be detected, and titers of the inhibitory antibody against hFVIII and porcine FVIII dramatically increased [40]. Nevertheless, transplantation of MSCs expressing coagulation factor resolved hemarthrosis and improved joint function [40]. The authors also observed the migration of

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transduced MSCs into a number of organs, including the synovium [40]. In our study, the level of circulating inhibitors of hFVIII induced by intra-articular injection of transduced MSCs was much lower than that following subcutaneous transplantation of MSCs, although a low titer of BUs was observed. Our results also suggest that implanting engineered MSCs expressing coagulation factor into the synovial joint space ameliorates hemarthrosis, even in the presence of inhibitory antibodies. Furthermore, a small number of transduced cells might be sufficient to achieve therapeutic effects, as compared with systemic transplantation of transduced cells. Accordingly, we believe that intra-articular injection of transduced MSCs represents a more realistic approach to ameliorate hemarthrosis and arthropathy, because of several advantages, including minimally invasive surgical procedures, the need for a small number of transduced cells, and a lower titer of inhibitory antibodies following treatment.

One of the main barriers to implementing clinical trials of gene and cell-based therapy is concern over the safety of viral vectors. We used the third generation of the SIV lentiviral vector to express coagulation factor in MSCs, because it has a better safety profile than gamma retroviral vectors (γRVs) [41]. As compared with γRVs, lentiviral vectors preferentially integrate within active transcription units without an obvious bias for proliferation-associated genes or transcriptional start sites, suggesting that lentiviral vectors are less likely to trigger oncogenic events [42]. Self-inactivating vector systems, in which the promoter activity in the U3 region of the viral long-terminal repeat (LTR) is deleted, have been used in many studies because the promoter activity of the viral LTR is associated with transcriptional activation of oncogenes in γRVs [43,44]. It is possible that the use of a physiologic promoter, such as the PAI-1 promoter in a self-inactivating vector, may be safer than using a ubiquitous viral promoter. We did not observe any tumorigenesis in the transplanted sites or abnormal proliferation of the transduced MSCs during the observation period. We believe that the safety of cell-based therapies could be further enhanced by several approaches. First, we can investigate the proviral integration sites of the transduced cells before using cell-based therapy, but not after direct injection of a viral vector. Second, we can improve the safety of cell-based therapy by blocking the cell cycle of transduced MSCs before transplantation by irradiation or pretreatment with a cytotoxic agent such as mitomycin C, if repeated injections of transduced MSCs are possible.

Some limitations of this study merit discussion before the clinical application of this procedure. The main limitation of our work is the relatively modest improvement in prevention of hemarthrosis. Although the local concentration of hFVIII achieved by intra-articular injection of the transduced cells should be higher than that reaching the joint following intravenous infusion, needle puncture-induced hemarthrosis was not completely abolished (Fig. 5). Second, our procedure induced a low neutralizing antibody titer, suggesting the possibility that our procedure would enhance the immune responses to hFVIII in patients expressing the inhibitor, particularly those with high responder inhibitor levels. Although intra-articular injection might be effective in the presence of low circulating inhibitor titers, our procedure may be more appropriate for adults who have already undergone replacement therapy several times, and might not develop inhibitory antibodies after intra-articular injection of the transduced MSCs. Furthermore, we could not fully assess the duration of transgene expression required to inhibit hemarthrosis or the fate of the transplanted MSCs. As we could recover very little RNA from around the knee joint from mice, we could not detect transgene mRNA in the joint space (data not shown). Although we believe that the therapeutic range of FVIII expression would be maintained for at least for 8 weeks, on the basis of the results shown in Fig. 7, it is important to confirm the long-term therapeutic effect and safety of this procedure. In addition, it is important to assess transgene expression and cell fate in larger animals to determine how frequently this procedure should be conducted.

In conclusion, we have proposed a new treatment strategy for hemophilic arthropathy in which MSCs expressing coagulation factor are directly injected into the target tissue. Considering that intra-articular injection is a minimally invasive procedure and that the MSCs can facilitate repair of the damaged joint structure, the procedure described here may become an attractive approach to prevent and/or treat blood-induced joint disease in hemophilic patients. Further evaluations of cell-based therapy in larger animals (e.g. cynomolgus monkey) and of the long-term safety of lentivirally transduced cells after transplantation are necessary before these procedures can be tested in clinical trials.

Addendum

Y. Kashiwakura and T. Ohmori: designed and performed the experiments, analyzed the data, and wrote the manuscript; J. Mimuro: performed experiments, analyzed the data, and revised the manuscript; A. Yasumoto, A. Sakata, and A. Ishiwata: performed experiments; M. Inoue and M. Hasegawa: provided vital reagents and critically reviewed the manuscript; S. Madoiwa, K. Ozawa, and Y. Sakata: analyzed data and revised the manuscript.

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Disclosure of Conflict of Interests

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Activity and antigen levels of hFVIII produced from MSCs, MEFs and HepG2 cells transduced with the SIV vector.
Figure S2. Association between hFVIII activity and proviral integration into the genome in MSCs transduced with the SIV vector.
Figure S3. Increases in plasma FVIII antigen after direct injection of supernatant from transduced MSCs or subcutaneous implantation of transduced MSCs.
Figure S4. Immunohistochemical staining of luciferase.
Data S1. Supplemental methods.

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Il2rg Gene-Targeted Severe Combined Immunodeficiency Pigs

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SUMMARY

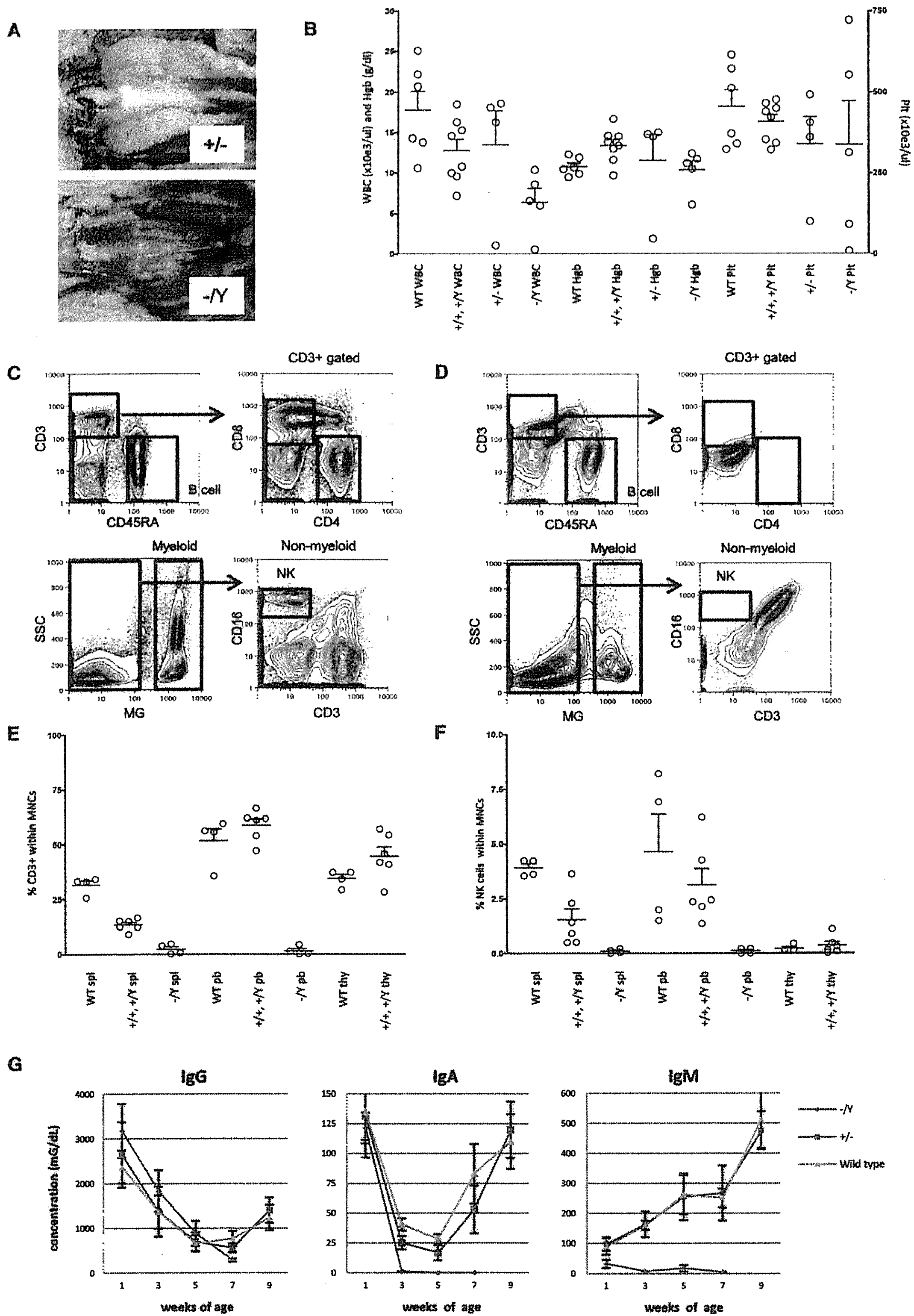
A porcine model of severe combined immunodeficiency (SCID) promises to facilitate human cancer studies, the humanization of tissue for xenotransplantation, and the evaluation of stem cells for clinical therapy, but SCID pigs have not been described. We report here the generation and preliminary evaluation of a porcine SCID model. Fibroblasts containing a targeted disruption of the X-linked interleukin-2 receptor gamma chain gene, *Il2rg*, were used as donors to generate cloned pigs by serial nuclear transfer. Germline transmission of the *Il2rg* deletion produced healthy *Il2rg*$^{+/-}$ females, while *Il2rg*$^{-/Y}$ males were athymic and exhibited markedly impaired immunoglobulin and T and NK cell production, robustly recapitulating human SCID. Following allogeneic bone marrow transplantation, donor cells stably integrated in *Il2rg*$^{-/Y}$ heterozygotes and reconstituted the *Il2rg*$^{-/Y}$ lymphoid lineage. The SCID pigs described here represent a step toward the comprehensive evaluation of preclinical cellular regenerative strategies.

The common gamma chain, IL2RG, is an IL-2 receptor subunit (Takeshita et al., 1992) shared by IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Kondo et al., 1993; Noguchi et al., 1993a; Russell et al., 1993; Giri et al., 1994; Kimura et al., 1995; Asao et al., 2001). IL2RG is present in T, NK, NKT, and dendritic cells (Ishii et al., 1994) and plays an essential role in lymphoid development by activating, through its cytoplasmic domain, Janus kinase 3 (Nakamura et al., 1994; Nelson et al., 1994, 1997).

Mammalian *IL2RG* orthologs are typically located on the X chromosome; in humans, *IL2RG* mutations result in X-linked severe combined immunodeficiency (XSCID) in which T and NK cells are absent or profoundly reduced in number, while B cells are numerically normal (or increased) but functionally impaired (Noguchi et al., 1993b; Leonard, 1996; Fischer et al., 1997). Gene-targeted mice lacking *Il2rg* also exhibit immunological defects (Cao et al., 1995; Ohbo et al., 1996) including the ablation of NK cell activity. NOD/SCID/*Il2rg*^{null}, NOG, and *Rag2*^{null}/*Il2rg*^{null} mice permit the functional reconstitution of human hematopoietic and immune systems following the injection of purified human hematopoietic stem cells (Traggiai et al., 2004; Ishikawa et al., 2005; Shultz et al., 2005). Unfortunately, phenotypic differences exist between XSCID humans and *Il2rg* null mice, including a pronounced numerical B cell reduction in the latter. Two dog breeds develop SCID caused by *Il2rg* mutations (Felsburg et al., 1999; Perryman, 2004), but dogs are poorly characterized research models.

In contrast, the pig more closely resembles humans regarding anatomy, hematology, physiology, size, and longevity. By enabling long-term follow-up, pig models will permit the evaluation of human cancer and stem cell transplantation over clinically relevant time frames. We here describe disruption of the porcine *Il2rg* gene to generate SCID pigs, their phenotypic characterization, and proof-of-principle transplantation studies.

A conventional positive-negative selection *Il2rg* gene targeting vector (TV) (Figure S1A) enabled the functional inactivation of porcine *Il2rg* by removing exon 6 (Kanai et al., 1999). Following fetal fibroblast transfection, selection, and PCR screening, 1 of 3 TV-targeted cell lines was expanded for nuclear transfer (Table S1). Even after screening, PCR-positive colonies often contain a substantial proportion of nontargeted cells (not shown) that would result in a corresponding proportion of nontargeted cloned pigs following nuclear transfer. To ensure that all clones harbored a targeted *Il2rg* locus, we adopted a serial cloning strategy. Nine embryonic day 35 (E35) or E39 nuclear transfer embryos were collected and screened by PCR and Southern blotting. PCR (not shown) and Southern blotting (Figure S1B) revealed that six contained the genomic configuration predicted for a single-targeted *Il2rg* allele. Fibroblasts were cultured from one targeted embryo and used in secondary nuclear transfer to


A
+/-
-/Y
B
WBC (x10e3/ul) and Hgb (g/dl)
Plt (x10e3/ul)
WT WBC
+/+, +/Y WBC
+/- WBC
-/Y WBC
WT Hgb
+/+, +/Y Hgb
+/- Hgb
-/Y Hgb
WT Plt
+/+, +/Y Plt
+/- Plt
-/Y Plt
C
D
CD3+ gated
CD3
CD45RA
CD8
CD4
B cell
Myeloid
Non-myeloid
NK
SSC
MG
CD16
E
% CD3+ within MNCs
F
% NK cells within MNCs
WT spl
+/+, +/Y spl
-/Y spl
WT pb
+/+, +/Y pb
-/Y pb
WT thy
+/+, +/Y thy
G
IgG
IgA
IgM
concentration (mG/dL)
weeks of age
-/Y
+/-
Wild type

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produce 31 cloned F_0 piglets; all were females heterozygously targeted at their *Il2rg* genes as judged by PCR (not shown) and Southern blotting (Figure S1C).

Fourteen of the 31 were stillborn and 3 of the 17 live-born died neonatally of unknown cause(s) (Table S1). Ten survivors died from pneumonia and severe arthritis (five were euthanized) between postnatal day 7 (P7) and P70. The remaining four (#5, 9, 15 and 20) survived for >1 year.

Stillborn and neonatal fatalities often had spleens with hypoplastic lymphoid aggregations (Figure S1D). Most (24/31, 77%) had undetectable or severely hypoplastic thymi (Figure S1E and Table S2). F_0 clones that died within 10 weeks also lacked detectable thymi and had few, if any, T cells either in their spleens or circulating (Figures S1F and S1G). In contrast, levels of $CD4^+$ and $CD8^+$ T cells in four long-lived F_0 lines were comparable to those of WT controls. Analysis of peripheral blood (PB) mononuclear cell (PBMC) RNA corroborated this: *Il2rg*, *CD4*, and *CD8* transcript levels were reduced in athymic *Il2rg*$^{+/-}$ clones that perished relative to respective levels in long-lived clones and WT controls (Figure S1H).

Thus, most *Il2rg*$^{+/-}$ clones exhibited SCID-like phenotypes, albeit that they had one WT allele. We attributed this high proportion to aberrant X-inactivation, a previously observed epigenetic cloning phenotype (Senda et al., 2004; Nolen et al., 2005; Jiang et al., 2008). However, epigenetic cloning phenotypes are corrected by germline transmission (Shimozawa et al., 2002). To confirm this and isolate the *Il2rg*$^{+/-}$ phenotype, we analyzed progeny derived by fertilization from *Il2rg*$^{+/-}$ cloned female #9.

Female *Il2rg*$^{+/-}$ #9 inseminated with WT sperm produced 19 F_1 (12 m, 7 f) offspring, and of these F_1 offspring, two *Il2rg*$^{+/-}$ females produced 21 F_2 (13 m, 8 f) when inseminated with WT sperm. Autopsies of representative F_1 and F_2 progeny revealed that, as expected, all *Il2rg*$^{-/Y}$ males had undetectable thymi, whereas *Il2rg*$^{+/-}$ females had thymi of normal size (Figure 1A and Table S3).

Hematological parameters in PB exhibited a significantly ($p = 0.0041$) reduced white blood cell (WBC) count in F_1 *Il2rg*$^{-/Y}$ males ($6.4 \pm 1.6 \times 10^3/\mu l$, $n = 5$) compared to WT controls ($17.8 \pm 2.3 \times 10^3/\mu l$, $n = 6$), while hemoglobin levels and platelet counts were unaffected (Figure 1B). F_1 *Il2rg*$^{+/-}$ females and WT littermates yielded comparable PB T, NK, and B cell numbers, indicative of intact acquired and innate immunity (Figures 1C, 1E, and 1F). In contrast, *Il2rg*$^{-/Y}$ males harbored significantly reduced PB T cells (*Il2rg*$^{-/Y}$ males, 1.5% ± 1.0%; *Il2rg*$^{+/Y}$ males, 57.3% ± 4.3%; n = 4 each, $p < 0.0001$) and NK cells (*Il2rg*$^{-/Y}$ males, 0.1% ± 0.1%; *Il2rg*$^{+/Y}$ males, 3.6% ± 1.1%; n = 4 each, $p = 0.0162$) (Figures 1D, 1E, and 1F). In proportion to the PB reductions, *Il2rg*$^{-/Y}$ spleens exhibited significant numerical reductions of T cells (*Il2rg*$^{-/Y}$ males, 2.3% ± 1.1%; *Il2rg*$^{+/Y}$ males, 13.0% ± 1.4%; n = 4 each, $p = 0.0011$) and NK cells (*Il2rg*$^{-/Y}$ males, 0.1% ± 0.0%; *Il2rg*$^{+/Y}$ males, 0.8% ± 0.2%; n = 4 each, $p = 0.0162$) (Figures 1E and 1F). B cells and myeloid cells accounted for the majority of $CD45^+$ leukocytes in *Il2rg*$^{-/Y}$ males, indicating that their immune deficiency was limited to T and NK cell lineages. The presence in *Il2rg*$^{-/Y}$ males of $CD33^+$ myeloid cells with both mononuclear and polynuclear properties suggests the differentiation of both granulocyte lineages and antigen-presenting cells, including monocytes and dendritic cells (Figures 1C and 1D).

We next evaluated humoral immune status in *Il2rg*-targeted pigs (Figure 1G). Serum IgG and IgA levels were high at 1 week (P7) and decreased gradually from 3 to 5 weeks in *Il2rg*$^{-/Y}$ (males), WT littermates, and *Il2rg*$^{+/-}$ female controls. After 7 weeks, IgG and IgA levels re-elevated in WT controls, while levels of both remained low in *Il2rg*$^{-/Y}$ males. Serum IgM was low at 1 week and increased gradually in controls but remained low in *Il2rg*$^{-/Y}$ males. Because both IgG and IgA at 1 week of age are entirely transferred via the colostrum in pigs, these results indicate that there had been no de novo Ig production in *Il2rg*$^{-/Y}$ males after weaning at 4 weeks. Impaired antibody production by *Il2rg*$^{-/Y}$ B cells is likely due to the absence of critical $CD4^+$ T helper cells. Consistent with their impaired immunity, all F_1 *Il2rg*$^{-/Y}$ males became systemically ill in the conventional housing conditions used, while F_1 *Il2rg*$^{+/-}$ females appeared healthy.

Collectively, this shows that when produced by conventional breeding, *Il2rg*$^{-/Y}$ F_1 males, but not *Il2rg*$^{+/-}$ females, present SCID phenotypes. SCID-like phenotypes observed in many *Il2rg*$^{+/-}$ female clones are attributable to aberrant, nonrandom X-inactivation during somatic cell cloning. Following germline transmission, *Il2rg*-targeted phenotypes resembled those of X-linked SCID in other species, with greatly reduced T and NK cell development and function (Cao et al., 1995; Puck et al., 1987). *Il2rg*-targeted pigs harbor B cells and thereby recapitulate human XSCID more closely than do *Il2rg*-targeted mice.

We next performed proof-of-principle allogeneic transplantation experiments using *Il2rg*-targeted pigs as recipients. Preliminary conditioning with orally administered fludarabine and busulfan produced significantly decreased WBC and platelet counts (not shown), which might promote the engraftment of transplanted cells. However, 2 of 6 *Il2rg*$^{-/Y}$ males died within 2 weeks postadministration, suggesting that the regimen was lethal for some piglets. Bone marrow (BM) cells from WT siblings were intravenously transplanted to four P11-12 *Il2rg*$^{-/Y}$ males with (#113, #115) or without (#605, #610) conditioning. Ubiquitously GFP-expressing (#184) BM cells (Watanabe et al., 2005)

Figure 1. Phenotypes of F_1 and F_2 Progeny Derived from *Il2rg*$^{+/-}$ Clone #9 by Germline Transmission
(A) Thymic phenotype in an *Il2rg*$^{+/-}$ female at 10 weeks and an *Il2rg*$^{-/Y}$ male at 9 weeks.
(B) Peripheral blood (PB) white blood cell (WBC), hemoglobin (Hgb), and platelet count (Plt) at 2 months of age in WT controls, *Il2rg*$^{+/+}$ female littermates, *Il2rg*$^{+/-}$ female littermates, and *IL2rg*$^{-/Y}$ males.
(C) Identification of acquired and innate immune subsets in an *Il2rg*$^{+/+}$ female by surface phenotype of $CD45RA^+CD3^-$ B cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD3^-CD16^+$ NK cells among the nonmyeloid fraction and myeloid cells.
(D) Analysis as for (C), but of an *IL2rg*$^{-/Y}$ male.
(E) Proportion of $CD3^+$ T cells in the spleen (spl), PB, and thymus (thy) in control *Il2rg*$^{+/+}$ females and male *Il2rg*$^{+/Y}$ littermates and nonlittermates (WT).
(F) Analysis as for (E), except showing the proportion of $CD3^-CD16^+$ NK cells.
(G) Changes with time postpartum in serum IgG (left), IgA (middle), and IgM levels. All error bars indicate SEM. See also Figure S1 and Tables S1–S3.

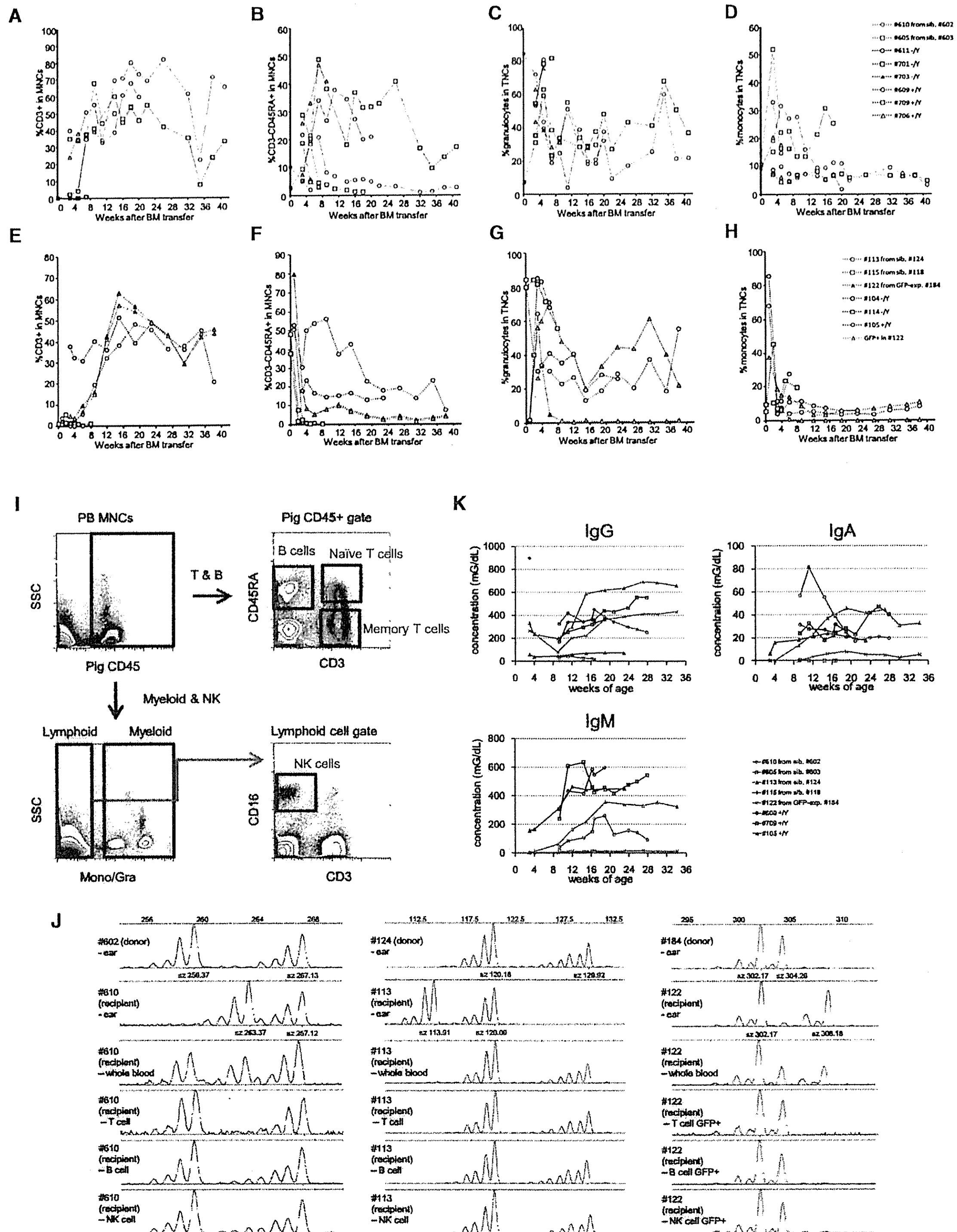

Figure 2. Allogeneic Bone Marrow Transfer into *Il2rg*$^{-/Y}$ Males

(A–H) Flow cytometric quantification of each subtype of leukocytes in *Il2rg*$^{-/Y}$ males (red lines) at different times after BM transfer. Controls are WT littermates (blue lines) and *Il2rg*$^{-/Y}$ males (black lines) without BM transfer. (A)–(D) correspond to BMT without conditioning, the case for #605 and #610. (E)–(H) correspond to BMT with conditioning, the case for #105, #113, and #122. (A) and (E) show the proportion of CD3^{+} T cells in PB at different times after BM transfer. (B) and (F) show proportion of CD45RA^{+}CD3^{-} B cells in PB at different times after BM transfer. (C) and (G) show proportion of granulocytes in PB at different times after BM transfer. (D) and (H) show proportion of monocytes in PB at different times after BM transfer.