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

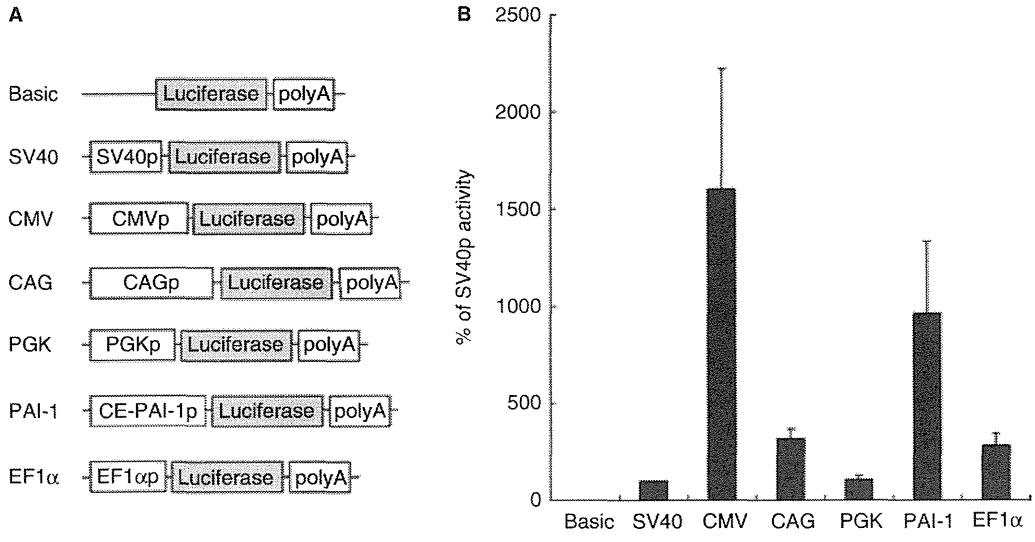

Fig. 1. Comparison of promoter activities in mesenchymal stem cells (MSCs). (A) Schematic diagrams of the promoters used in the experiments. (B) Each construct, along with a promoterless vector (basic) or a positive control vector (SV40/Enhancer), was transfected into MSCs. Luciferase activity was measured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

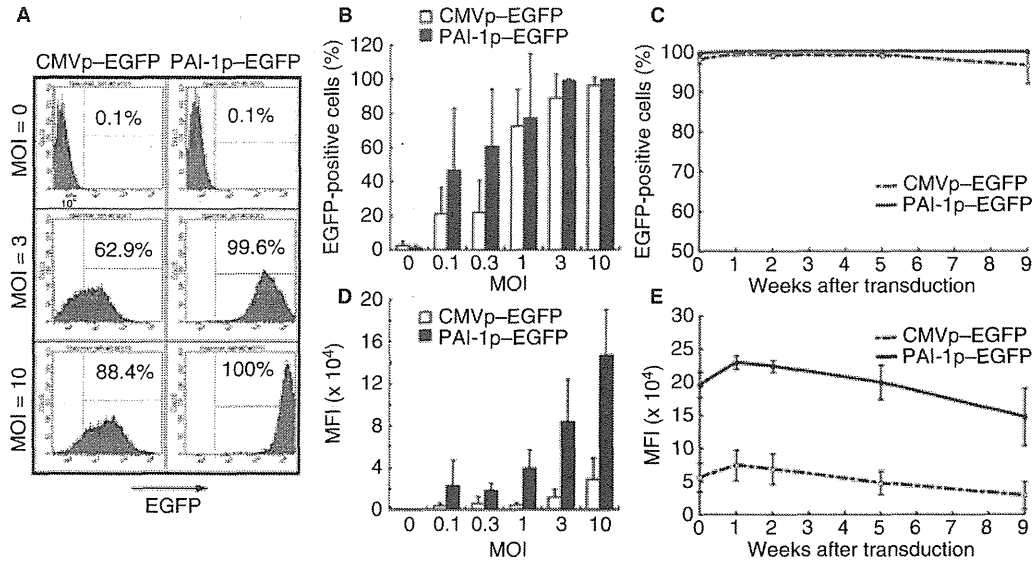

Fig. 2. Expression of enhanced green fluorescent protein (EGFP) in mesenchymal stem cells (MSCs) transduced with simian immunodeficiency virus (SIV) vectors carrying the EGFP gene driven by the cytomegalovirus (CMV) or plasminogen activator inhibitor-1 (PAI-1) promoter. MSCs were transduced with SIV–CMVp–EGFP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

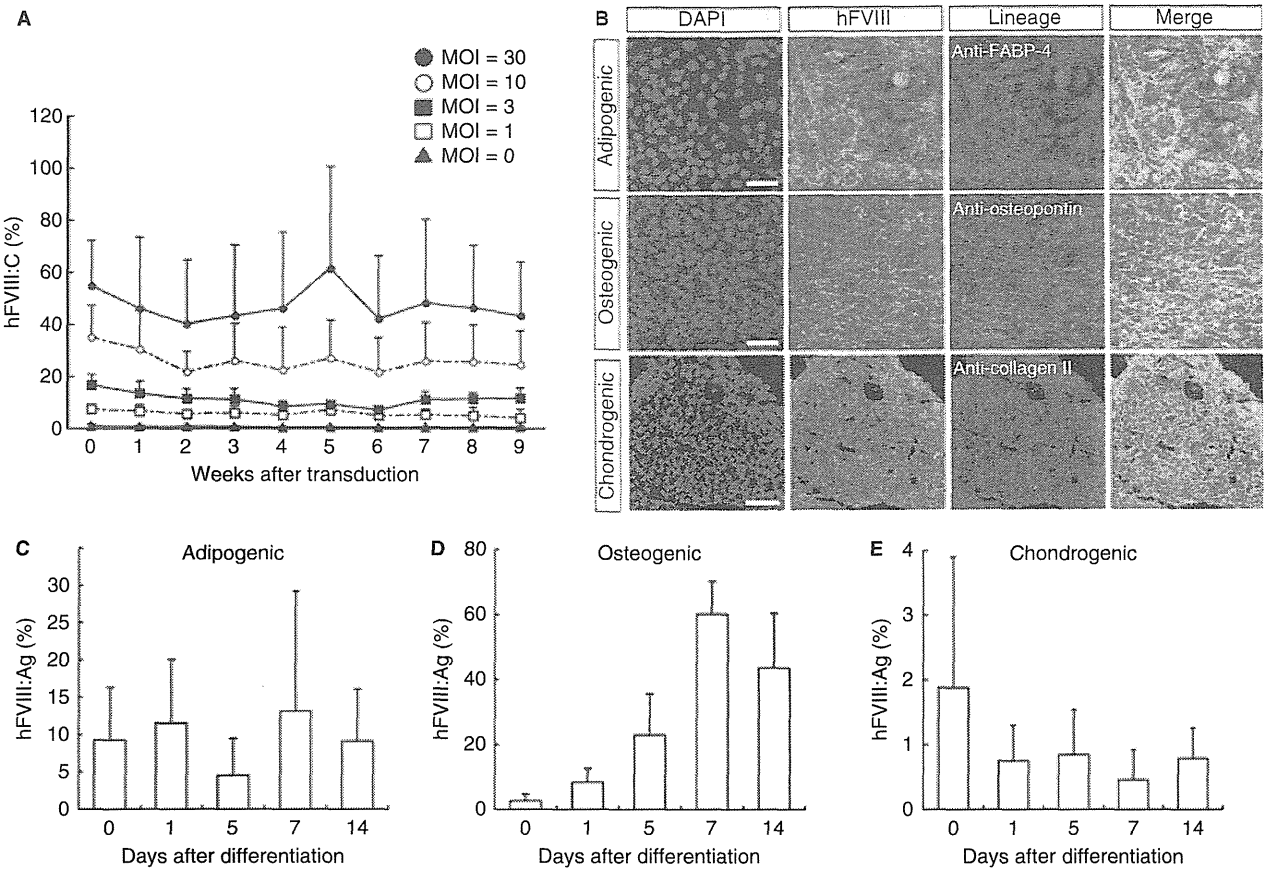

Fig. 3. Persistent expression of human FVIII (hFVIII) from transduced mesenchymal stem cells (MSCs) during maintenance and differentiation. (A) MSCs were transduced with the simian immunodeficiency virus (SIV) vector carrying the hFVIII gene driven by the plasminogen activator inhibitor-1 promoter at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

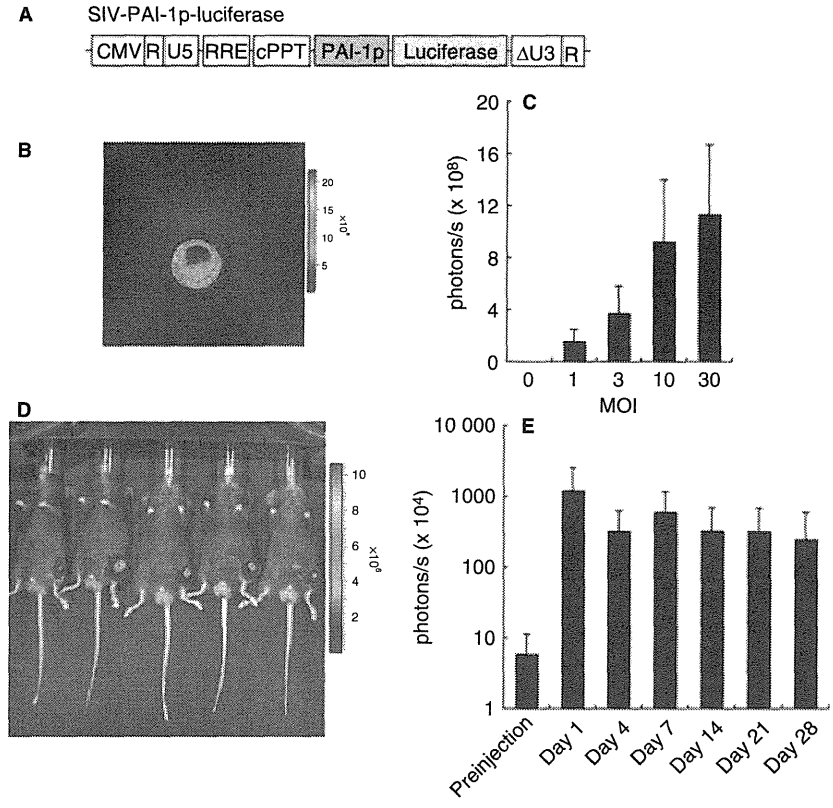

Fig. 4. Fate of transduced mesenchymal stem cells (MSCs) injected into the knee joint space *in vivo*. (A) Schematic diagram of the simian immunodeficiency virus (SIV) lentiviral vector used in this experiment. The SIV vector expresses a luciferase gene driven by the plasminogen activator inhibitor-1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

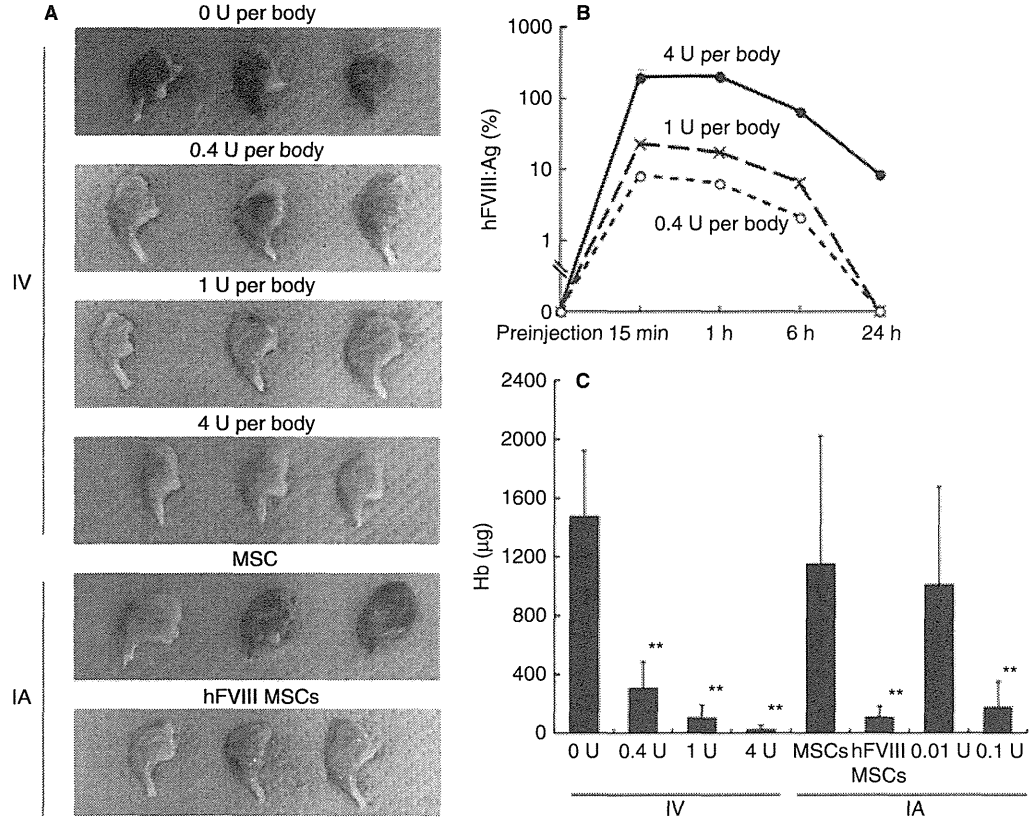

Fig. 5. Local injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) protects against hemarthrosis induced by joint puncture in FVIII-deficient mice. FVIII-deficient mice received an intravenous dose of recombinant hFVIII or an intra-articular injection of non-transduced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

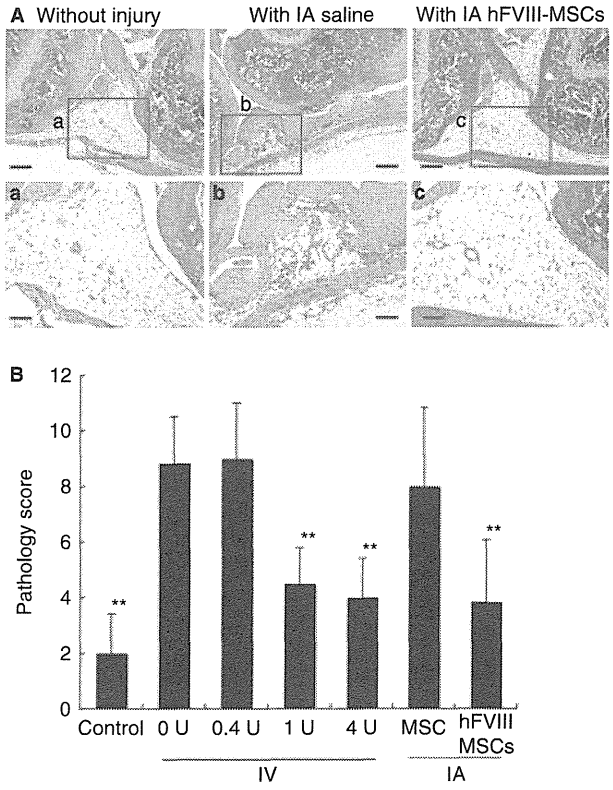

Fig. 6. Local injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) protects against hemophilic arthropathy in FVIII-deficient mice. (A) Representative histopathologic images taken 4 weeks after the joint challenge. Without injury: FVIII-deficient mouse knee joint without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

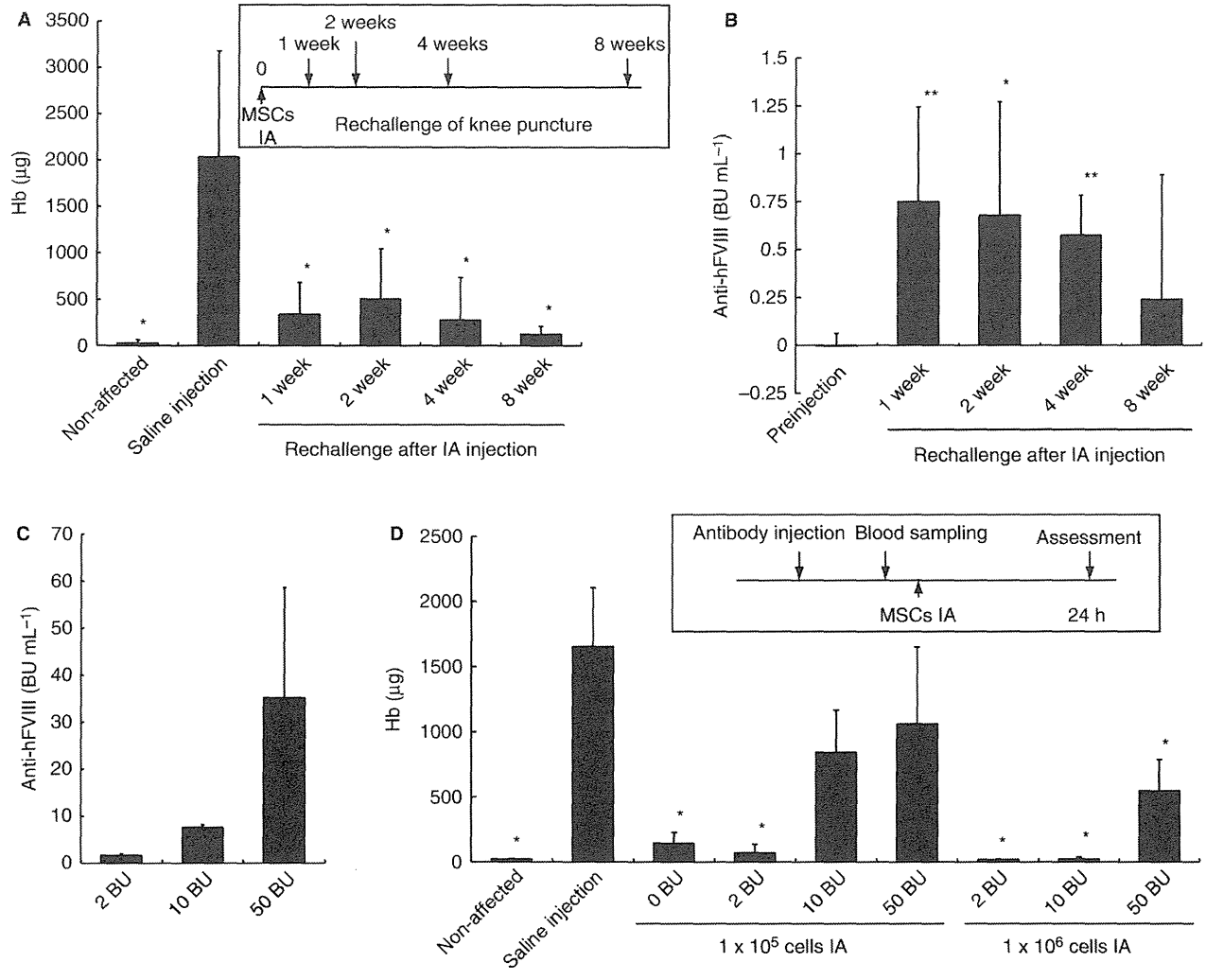

Fig. 7. Intra-articular (IA) injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) persistently inhibits hemarthrosis, and protects against hemarthrosis in the presence of low titers of neutralizing antibodies against hFVIII. (A) FVIII-deficient mice received an IA injection 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

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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Neurobiology

FTY720 Improves Functional Recovery after Spinal Cord Injury by Primarily Nonimmunomodulatory Mechanisms

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Spinal cord injury (SCI) is an incapacitating injury that can result in limited functional recovery. We have previously shown increases in the lysophospholipid mediator, sphingosine-1-phosphate (S1P), in the spinal cord after contusion injury. To apply S1P receptor modulation to the treatment of SCI, we examined the therapeutic effects of FTY720, an S1P receptor agonist, on locomotor recovery after SCI in mice. Oral administration of FTY720 shortly after contusion SCI significantly improved motor function recovery, as assessed by both Basso Mouse Scale scores and Rotarod Performance test results. FTY720 induced lymphopenia and reduced T-cell infiltration in the spinal cord after SCI but did not affect the early infiltration of neutrophils and the activation of microglia. In addition, plasma levels and mRNA expression of inflammatory cytokines in the spinal cord after SCI were not attenuated by FTY720. Vascular permeability and astrocyte accumulation were both decreased by FTY720 in the injured spinal cord. The therapeutic effects of FTY720 were not solely dependent on immune modulation, as confirmed by the demonstration that FTY720 also ameliorated motor function after SCI in mice with severe combined immunodeficiency. Finally, the $S1P_1$ receptor agonist, SEW2871, partly mimicked the therapeutic effect of FTY720. Our data highlight the importance of immune-independent functions of FTY720 in decreasing vascular permeability and astrogliosis in the injured spinal cord and promoting locomotor function recovery after SCI. *(Am J Pathol 2012, 180:1625–1635; DOI: 10.1016/j.ajpath.2011.12.012)*

Spinal cord injury (SCI) is a debilitating injury often sustained in accidents and ultimately results in marked poor neuropathological features and limited functional recovery, despite adequate existing surgical and medical treatments.[1] SCI primarily arises from mechanical disruption of the spinal cord, leading to rapid death of neurons and glia.[2,3] In addition to initial tissue damage, much of the post-traumatic degeneration of the spinal cord is due to a multifactorial secondary injury (eg, neuronal and glial apoptosis, inflammation, glial scar formation, local edema/ischemia, and oxidative stress).[2,3] The theoretical aim of SCI treatment is to counteract the mechanisms of secondary injury and/or to prevent their pathological consequences, because central nervous system (CNS) neurons have a limited capacity to self-repair and regenerate.[4,5]

Sphingosine-1-phosphate (S1P) is a bioactive lysophospholipid mediator that produces a variety of cellular responses, including proliferation, survival, motility, and cytoskeletal reorganization, through the action of the S1P subfamily of G-protein–coupled receptors.[6–8] We have previously shown that the S1P concentration in the spinal cord was significantly increased in the location of a contusion injury and that such changes stimulated $S1P_1$-mediated migration of *in vivo*–transplanted neural stem/progenitor cells.[9] Furthermore, in areas of brain infarction, $S1P_2$ receptor antagonism also enhances the migration of endogenous neural stem/progenitor cells.[10] Because S1P receptors are ubiquitously expressed in many organs, including the CNS, and S1P produces a variety of responses related to the function of the nervous sys-

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tem,[11–14] we hypothesize that targeting these receptors may become a candidate therapy for various refractory CNS disorders, including SCI.

FTY720 is an orally available S1P receptor modulator, known clinically as fingolimod.[15,16] It acts as a broad S1P receptor agonist after phosphorylation by sphingosine kinase *in vivo*[16] and has recently had clinical efficacy in phase 3 clinical trials for multiple sclerosis.[17,18] Also, FTY720 protects a rodent model of ischemia-reperfusion after cerebral ischemia[19,20] and improves functional outcomes in a rat model of SCI.[21] The efficacy of FTY720 in CNS disorders is believed to derive from immunological modulation.[19–21] However, other secondary responses that worsen functional outcome in CNS disorders have been identified, and the effect of the drug on these disorders has not been elucidated. In this study, we examine the therapeutic effects of FTY720 on a mouse model of SCI and highlight the nonimmunological mechanism by which FTY720 improves secondary injuries after SCI.

Materials and Methods

Animals

Female C57BL/6N and CB-17/Icr-*scid/scid* [SCID (severe combined immunodeficiency)] mice (aged 9 to 12 weeks) were obtained from Japan SLC, Inc. (Shizuoka, Japan) and CLEA Japan, Inc. (Tokyo, Japan), respectively. All animal procedures were approved by the Institutional Animal Care and Concern Committee of Jichi Medical University (Tochigi, Japan), and animal care was performed in accordance with the guidelines of the committee.

Mouse Model of Contusion SCI

Contusion SCI was induced using an Infinite Horizon Impactor (Precision Systems and Instrumentation, Lexington, KY).[9] After anesthesia with isoflurane, the spinal cord segment was exposed by removing the dorsal part of the vertebra, and a contusion injury of the tenth thoracic spinal cord was induced at a force of 60 kdyne. Postoperative care was performed as previously described.[9]

Drug Administration

FTY720 was kindly provided by Novartis Pharma AG (Basel, Switzerland) and dissolved in distilled water. SEW2871, a specific agonist for the $S1P_1$ receptor,[22] was obtained from Calbiochem (San Diego, CA). SEW2871 was dissolved in 100% dimethyl sulfoxide and diluted with a 25% (v/v) aqueous solution of Tween-20, because of its low solubility in pure water. FTY720 (3, 0.3, or 0.03 mg/kg), SEW2871 (10 mg/kg), or a corresponding control solvent was given orally to mice every 24 hours, immediately after SCI.

Assessment of Functional Recovery from SCI

Recovery was scored by the Basso Mouse Scale (BMS) open-field locomotor rating scale, developed specifically for mice and consisting of scores ranging from 0 (complete paralysis) to 9 (normal mobility).[23] Scoring was performed by two independent evaluators blinded to the group (Y.N. and A.K.), and analysis was performed on a mean value of two scores.

Recovery of motor function was also quantified by the Rotarod Performance test (MK-610A; Muromachi Kikai Co., Tokyo). The Rotarod treadmill consists of a computer-controlled stepper motor-driven drum (diameter, 30 mm) with either constant or accelerating speed modes; it automatically records the amount of time spent by the animal on the drum at the point of falling. We measured ride performance in acceleration speed mode (30 rpm/300 seconds) to assess motor function at the indicated times after SCI. Two trials were performed by each mouse, and analysis was performed on the average.

Blood Collection and Isolation of White Blood Cells

Whole blood (180 μL) was drawn from the right jugular vein using a 29-gauge syringe containing 20 μL of sodium citrate. After performing a complete blood cell count by automatic analyzer (MEK-6358 Celltac α; Nihon Kohden Corp, Tokyo, Japan), platelet-poor plasma was isolated by centrifugation and stored at −80°C. White blood cells isolated from the buffy-coat fraction by hemolysis were incubated with one of a panel of biotin-conjugated antibodies against cell-specific cell surface antigens (B220, CD3e, CD11b, and Gr-1 for B cells, T cells, macrophages, and granulocytes, respectively; BD Biosciences, San Jose, CA). Samples were then incubated with allophycocyanin-conjugated streptavidin, and antibody binding was analyzed by flow cytometry (FACSAria cell sorter; BD Biosciences).

ELISA, Quantitative RT-PCR, and Test for Blood Coagulation

Plasma levels of IL-6, IL-10, interferon-γ, monocyte chemotactic protein-1, and tumor necrosis factor (TNF)-α were measured by enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (eBioscience Inc., San Diego, CA). The ELISA kit for IL-1β was obtained from R&D Systems Inc. (Minneapolis, MN). Real-time quantitative RT-PCR was performed as previously described.[9] The oligonucleotide primer pairs used for RT-PCR were as follows: 5′-CCCAAGCAATACCCAAAGAA-3′ (sense) and 5′-GCTTGTGCTCTGCTTGTGAG-3′ (antisense) for IL-1β (GenBank NM_008361.3); 5′-CCGGAGAGGAGACTTCACAG-3′ (sense) and 5′-TCCACGATTTCCCAGAGAAC-3′ (antisense) for IL-6 (GenBank NM_031168.1); 5′-TAGCCAGGAGGGAGAACAGA-3′ (sense) and 5′-TTTTCTGGAGGGAGATGTGG-3′ (antisense) for TNF-α (GenBank NM_013693.2); 5′-ACAACTTTGGCCGACTTCAC-3′ (sense) and 5′-GGGTTCACTGGCACTTTGAT-3′ (antisense) for IL-18 (GenBank NM_008360.1); 5′-GTCGGTGTGAACGGATTT-3′ (sense) and 5′-CGTGAGTGGAGTCATACTGGAA-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GenBank NM_008084.2).

Plasma prothrombin time and activated partial thromboplastin time were measured using an automatic coagulation analyzer (CA500; Sysmex, Kobe, Japan).

Infiltration of Peripheral Blood Cells and Microglia in the Spinal Cord

Mice anesthetized with isoflurane were perfused with PBS, and the section of spinal cord (10 mm thick) was isolated. The extracted spinal cord was homogenized in 500 μL of dissociation medium (Dulbecco's modified Eagle's medium/Ham's F-12 containing 1.3 mg/mL trypsin, 0.7 mg/mL hyaluronidase, 0.2 mg/mL kynurenic acid, and 200 U/mL DNase) and incubated at 37°C for 30 minutes. After neutralizing trypsin with a trypsin inhibitor (Invitrogen Corp., Carlsbad, CA), the cells were washed twice with PBS, then simultaneously labeled with phosphatidylethanolamine-conjugated anti-mouse CD45 (clone 30-F11; BD Biosciences) and lineage-specific antibodies (Gr-1, CD11b, B220, and CD3e). Total numbers of CD45-positive cells and cells positive for each lineage marker were determined using Flow-Count Fluorospheres (Beckman Coulter, Miami, FL) and flow cytometry. To assess infiltration of a T-cell subset in the injured spinal cord, the cells were stained with mouse T-lymphocyte subset antibody cocktail (BD Biosciences) after the removal of myelin debris by Percoll-saline solution.[24]

Tissue Bleeding

Mice were sacrificed at 3 days after SCI and perfused with heparinized saline to remove intravascular blood. Segments (10 mm thick) of cord encompassing the lesion were homogenized in distilled water (250 μL) and processed to measure tissue bleeding, as previously described.[25] Briefly, 20 μL of supernatant containing hemoglobin was incubated with 80 μL of Drabkin's reagent (Sigma-Aldrich, St. Louis, MO), and the hemoglobin concentration was assessed by measuring the optical density of the solution at a 550-nm wavelength.

Vascular Permeability

To measure vascular permeability, 100 μL of saline containing 2.5% Evans blue dye (Sigma-Aldrich) was i.v. injected into each mouse. Animals were then anesthetized with isoflurane and perfused through the heart with 100 to 150 mL of PBS containing heparin sulfate (0.01 U/mL). Spinal cords (10 mm centered on the lesion site) were removed and dissociated in 150 μL of dimethylformamide using a homogenizer. After incubation at 37°C for 24 hours, Evans blue fluorescence was quantified by using a microplate spectrofluorometer (Gemini EM; Molecular Devices, Inc., Sunnyvale, CA) (excitation, 620 nm; emission, 680 nm). Sample values were compared with Evans blue dye standards in the same solvent. Results were expressed as a percentage of total injected Evans blue dye.

Histological Analysis

Mice anesthetized with isoflurane were perfused with 50 mL of PBS, followed by 50 mL of 4% paraformaldehyde. The isolated spinal cord was fixed with 4% paraformaldehyde in PBS for 2 hours at 4°C, incubated with PBS

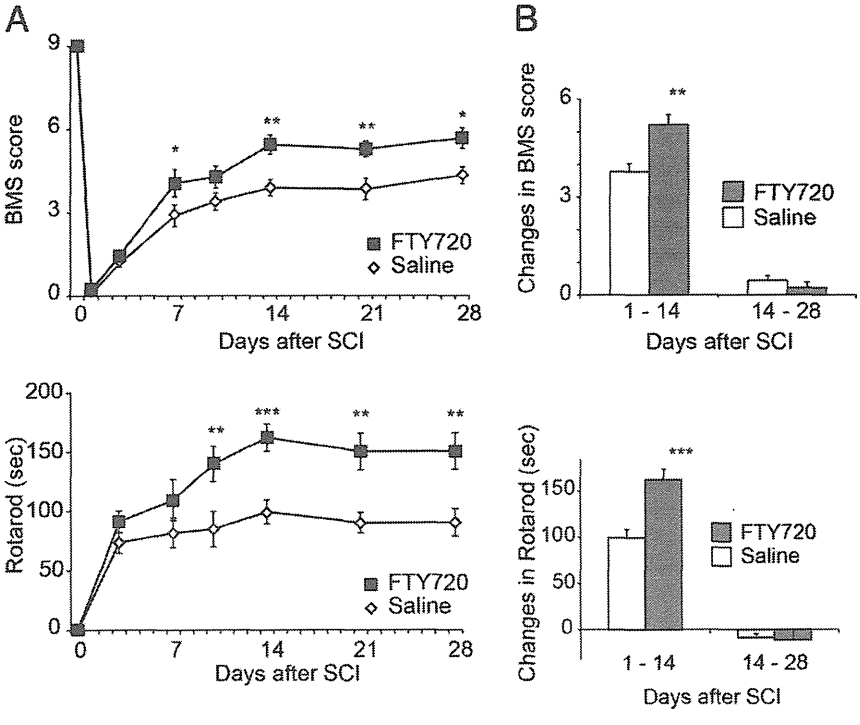

Figure 1. FTY720 promotes functional locomotor recovery after SCI in mice. **A:** FTY720 (3 mg/kg) or saline was given orally every 24 hours after a contusion SCI. The recovery of motor function was quantified by BMS score (**top panel**) and Rotarod Performance test result (**bottom panel**) at the indicated times after SCI. Data represent mean ± SEM (n = 9 in each group). Drug efficacy was statistically significant by two-way repeated-measures analysis of variance ($P < 0.0001$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the control experiment (post hoc Bonferroni test). **B:** The changes in recovery rate of motor function quantified by BMS score (**top panel**) and Rotarod Performance test result (**bottom panel**) were separately assessed in the early (0 to 14 days) or late (14 to 28 days) phase after SCI. Data represent the mean ± SEM (n = 9 in each group). The therapeutic effect was observed in the early phase after SCI. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control experiment (two-tailed Student's t-test).

Table 1. FTY720 Promotes Functional Locomotor Recovery after SCI in Mice

Time after SCI (days)	BMS score							*P* value
	1	3	7	10	14	21	28	
FTY720 (mg/kg)	0.111 ± 0.220	1.167 ± 0.354	2.889 ± 1.17	3.389 ± 0.928	3.889 ± 0.858	3.833 ± 1.146	4.333 ± 0.901	NS
Control, 0								
3	0.222 ± 0.264	1.444 ± 0.527	4.056 ± 1.467*	4.278 ± 1.176	5.444 ± 1.044**	5.278 ± 0.833**	5.667 ± 1.118*	0.0272
0.3	0 ± 0	1.250 ± 0.463	2.750 ± 0.655	3.250 ± 0.926	4.125 ± 1.302	4.125 ± 1.356	4.250 ± 1.165	0.9255
0.03	0 ± 0	1.188 ± 0.259	2.625 ± 0.518	3.063 ± 0.623	3.688 ± 0.753	4.188 ± 1.067	4.313 ± 0.799	0.8895

	Rotarod performance test score (seconds)							
	0	3	7	10	14	21	28	
FTY720 (mg/kg)	ND	73.39 ± 26.37	81.67 ± 35.13	84.67 ± 42.04	99.22 ± 28.52	89.83 ± 24.37	90.39 ± 32.32	NS
Control, 0								
3	ND	91.06 ± 27.75	109.50 ± 52.18	139.78 ± 44.81**	162.00 ± 4.33**	150.17 ± 46.47**	150.63 ± 43.60**	0.0257
0.3	ND	61.38 ± 10.87	86.12 ± 31.04	103.81 ± 34.28	114.63 ± 47.61	122.19 ± 72.00	109.88 ± 57.98	0.6002
0.03	ND	61.56 ± 15.00	93.38 ± 22.51	103.88 ± 30.06	96.50 ± 35.15	104.81 ± 41.29	103.69 ± 31.06	0.7884

Daily doses of control and FTY720 are indicated.
*$P < 0.05$; **$P < 0.01$ compared with the control experiment (post hoc Bonferroni test).
ND, data could not be detected because all mice became completely paraplegic and were unable to stay on the rotary drum; NS, not significant.

containing sucrose (10% to 30%), and then frozen in the presence of optimal cutting temperature (OCT) compound (Sakura Finetek Inc., Torrance, CA). Sections were prepared from frozen tissues at −25°C and attached to polylysine-coated glass slides. To assess for myelin sparing, staining with eriochrome cyanine was performed as previously described,[26] and the area of myelin sparing in the SCI lesion epicenter was quantified. The accumulation of astrocytes was assessed by immunostaining with anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (Dako Denmark, Glostrup). Antibody binding was detected by the Dako REAL EnVision Detection System (Dako Denmark). Image analysis was performed using Scion Image for Windows (Scion Corp, Frederick, MD).

Statistical Analysis

Unless otherwise stated, values are expressed as the mean ± SEM. Statistical analysis was performed by a Student's *t*-test or a two-way repeated-measures analysis of variance with a post hoc Bonferroni test, as indicated in the figures.

Results

FTY720 Improves Functional Outcome after SCI in Mice

We first examined whether FTY720, an S1P receptor–activating drug, improves locomotor performance after contusion SCI in mice. We administered FTY720 or control solvent daily immediately after SCI and assessed the mouse hind limb locomotor function by BMS locomotor scoring and the Rotarod Performance test result for up to 4 weeks. All mice became almost completely paraplegic and were unable to stay on the rotary drum after the SCI but gradually began to display partial recovery of locomotor activity (Figure 1A). The administration of FTY720 significantly improved recovery of hind limb motor function assessed by both BMS score and Rotarod Performance test result after SCI, and the effect continued to the end of the analysis (Figure 1A). The beneficial effects of FTY720 were dose dependent and became significant at 7 to 10 days after SCI (Figure 1A and Table 1). We next assessed whether FTY720 exerted therapeutic effects in the early stage after SCI. We measured the recovery rate of BMS score and the Rotarod Performance test result between each of the observation points and found statistical significance in the recovery rate from 0 to 14 days, but not 14 to 28 days, suggesting that FTY720 improves the early phase of secondary injury after SCI (Figure 1B). The improvement of motor function was accompanied with histological amelioration, as evidenced by changes in the myelin-sparing area (Figure 2, A and B).

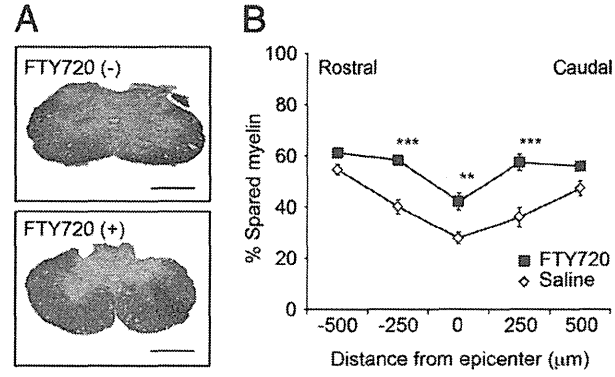

Figure 2. FTY720 ameliorates the myelin-sparing area in the spinal cord after SCI. **A:** Representative micrographs showing eriochrome cyanine staining of tissue sections at 28 days after SCI, with or without FTY720. Scale bar = 500 μm. **B:** Quantification of myelin sparing in the dorsal column in serial tissue sections rostral and caudal to the lesion epicenter. Data represent the mean ± SEM ($n = 6$ in each group). Drug efficacy was statistically significant by two-way repeated-measures analysis of variance ($P < 0.0001$). **$P < 0.01$, ***$P < 0.001$ compared with the control experiment (post hoc Bonferroni test).

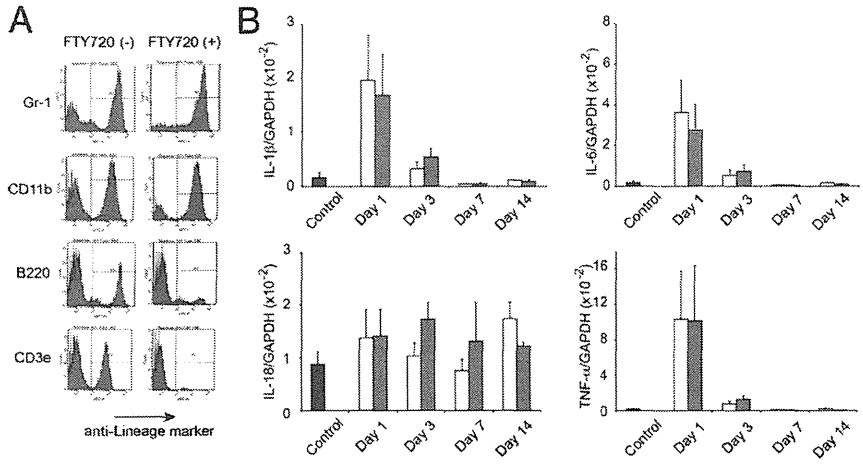

Figure 3. Failure of FTY720 to suppress early inflammatory responses in the spinal cord after SCI. FTY720 (3 mg/kg) or saline was given orally every 24 hours after contusion SCI. **A:** Surface antigens on peripheral blood cells (Gr-1, CD11b, B220, and CD3e) were assessed by flow cytometry at 3 days after SCI. The results shown are representative of at least five independent experiments. **B:** mRNA expression of IL-1β, IL-6, IL-18, and TNF-α in contused spinal cord at the indicated number of days after SCI. Black bars indicate control mice without SCI; white bars, mice with SCI treated with saline; and gray bars, mice with SCI treated with FTY720. Data represent the mean ± SEM (n = 4 to 10 in each group).

Failure of FTY720 to Ameliorate Early Inflammatory Response after SCI

Early inflammation after the SCI is one of a series of important downstream events termed secondary injuries, which culminate in progressive degenerative events in the spinal cord after SCI.[27,28] Because FTY720 acts as an immunosuppressant through its actions on the S1P receptor, we next examined whether FTY720 modulates inflammatory responses after SCI. As shown in Figure 3A, we confirmed that FTY720 induces lymphopenia, with CD3e-positive T lymphocytes and B220-positive B lymphocytes disappearing from peripheral blood after treatment with FTY720. However, mRNA expression levels of inflammatory mediators, including IL-1β, IL-6, IL-18, and TNF-α, in contused spinal cord were not changed by FTY720 (Figure 3B). Furthermore, plasma levels of IL-6, IL-10, IL-1β, monocyte chemotactic protein-1, interferon-γ, and TNF-α, which represent systemic inflammation, were unaffected by treatment with FTY720 (Figure 4).

FTY720 Reduces Infiltration of T Cells, but not Neutrophils and Microglia, at the Site of SCI

We next assessed infiltration of peripheral blood cells and activation of microglia in the spinal cord after SCI. Blood cells constitutively expressing CD45 antigen in the spinal cord were assessed by flow cytometry. In this method, CD45/CD11b double-positive cells are clearly characterized into blood-derived cells or microglial cells by the expression pattern of CD45 (Figure 5A). Gr-1– and CD11b-positive neutrophils rapidly accumulated within the lesion at day 1 after SCI and were gradually cleared (Figure 5, A and B). In contrast, T-lymphocyte entry and microglial accumulation were delayed for several weeks after injury (Figure 5B). FTY720 significantly suppressed

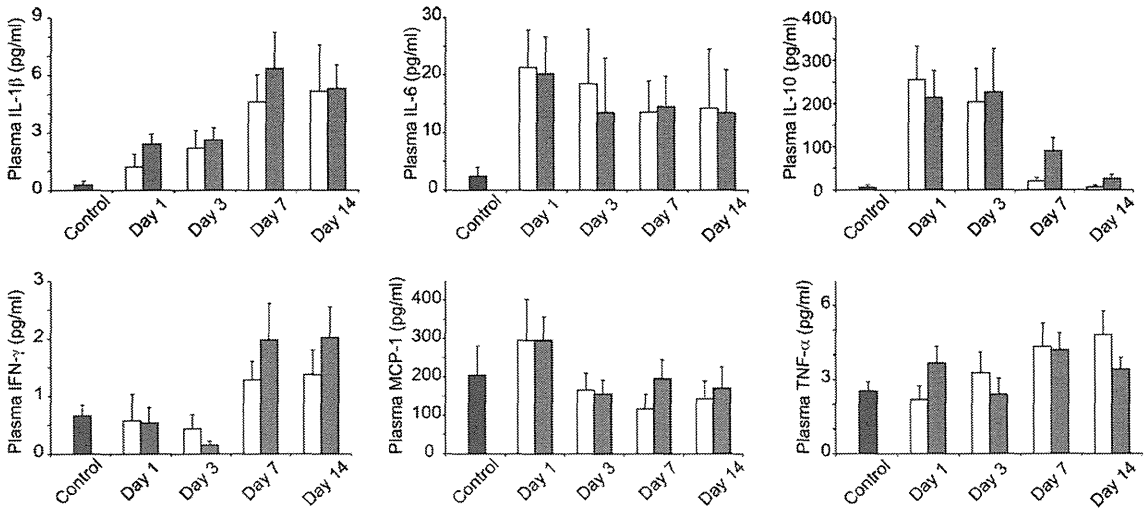

Figure 4. FTY720 does not ameliorate systemic inflammatory response after SCI. FTY720 (3 mg/kg) or saline was given orally every 24 hours after contusion SCI. Plasma levels of IL-1β, IL-6, IL-10, interferon (IFN)-γ, monocyte chemotactic protein (MCP)-1, and TNF-α were measured by ELISA on the indicated number of days after SCI. Black bars indicate control mice without SCI; white bars, mice with SCI treated with saline; and gray bars, mice with SCI treated with FTY720. Data represent the mean ± SEM (n = 10 in each group).

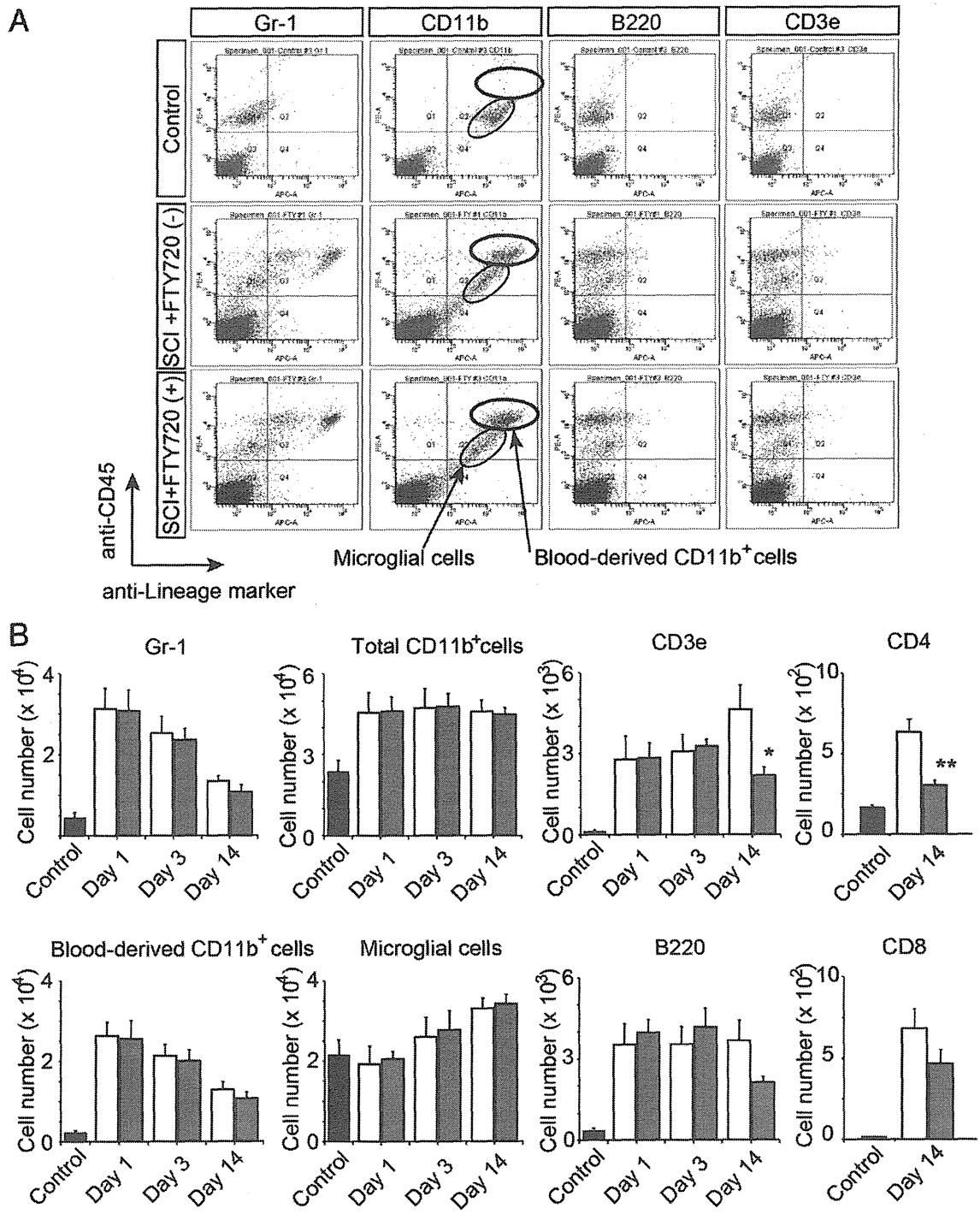

Figure 5. FTY720 reduces infiltration of T cells, but not neutrophils and microglia, in contused spinal cord. FTY720 (3 mg/kg) or saline was given orally every 24 hours after contusion SCI. **A:** CD45 expression (vertical) and lineage marker (horizontal; Gr-1, CD11b, B220, and CD3e) in cells obtained from the spinal cord were examined by flow cytometry. Representative data at 1 day after SCI, with or without FTY720, are shown. The data obtained from mice without SCI are also shown as control. $CD45^+$ and $CD11b^+$ cells are clearly characterized, and noted, as blood-derived macrophage or microglial cells by the expression pattern of CD45. **B:** The number of each cell type was quantified at the indicated days after SCI. Black bars indicate control mice without SCI; white bars, mice with SCI treated with saline; and gray bars, mice with SCI treated with FTY720. Data represent the mean ± SD ($n = 5$ in each group). $^*P < 0.05$, $^{**}P < 0.01$ compared with the control experiment (two-tailed Student's *t*-test).

the late infiltration of CD3e-positive T lymphocytes but not the early accumulation of peripheral neutrophils and microglial activation in the injured spinal cord (Figure 5B). The reduction of T-cell infiltration in the injured spinal cord by FTY720 was observed only at 14 days after SCI. The administration of FTY720 also significantly reduced the infiltration of CD4-positive helper T cells but not the CD8-positive cytotoxic T-cell population (Figure 5B).

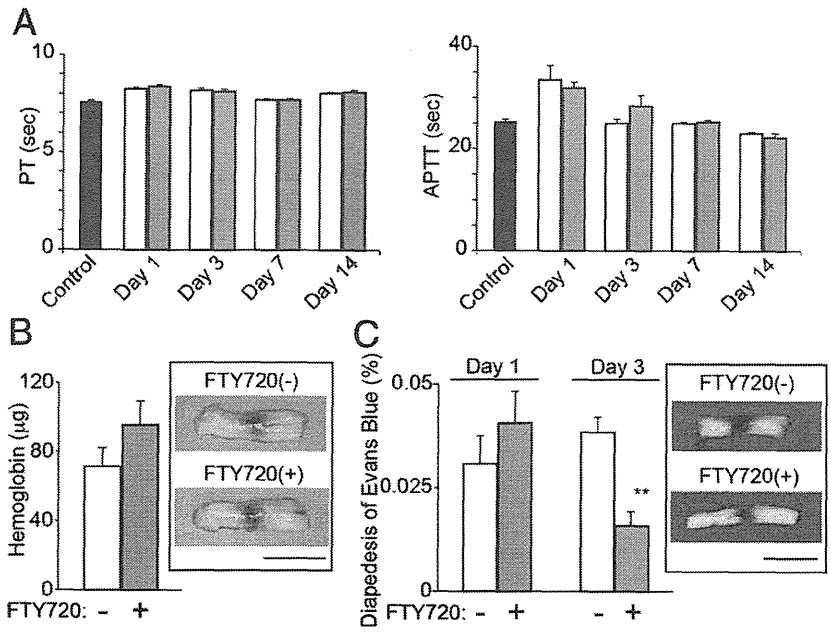

Figure 6. FTY720 decreases vascular permeability after SCI. **A:** Blood coagulation was assessed by the measurement of prothrombin time (PT) and activated partial thromboplastin time (APTT) at the indicated times after SCI. Black bars indicate control mice without SCI; white bars, mice with SCI treated with saline; and gray bars, mice with SCI treated with FTY720. Data represent the mean ± SEM (n = 5 in each group). **B:** Tissue bleeding in contused spinal cord was measured at 3 days after SCI. White bars indicate mice with SCI treated with saline; and gray bars, mice with SCI treated with FTY720. Data represent the mean ± SEM (n = 7 to 8). **Right panel:** Representative macroscopic findings showing bleeding in the spinal cord at 3 days after SCI, with or without FTY720, are shown. Scale bar = 5 mm. **C:** Vascular permeability in the spinal cord was quantified at 1 or 3 days after SCI, as described in *Materials and Methods*. White bars indicate mice with SCI treated with saline; and gray bars, mice with SCI treated with FTY720. Data represent the mean ± SEM (n = 5 in each group). $^{**}P < 0.01$ compared with the control experiment (two-tailed Student's t-test). **Right panel:** Representative macroscopic findings of the spinal cord after the injection of Evan's blue dye at 3 days after SCI, with or without FTY720, are shown. Scale bar = 5 mm.

Attenuation of Vascular Permeability and Astrocyte Accumulation in the Injured Spinal Cord by FTY720

Because sphingolipids may affect thrombin generation *in vitro*,[29] we next focused on the effect of FTY720 on the blood coagulation cascade and extravasation of blood in contused spinal cord after SCI. Blood coagulation (assessed by the measurement of prothrombin time and activated partial thromboplastin time at the indicated times after SCI) was not modified by treatment with FTY720 (Figure 6A). Furthermore, FTY720 failed to improve the amount of extravasated blood in the contused spinal cord (Figure 6B).

Microvascular permeability after contusion injury may affect local edema and functional deterioration. We assessed vascular permeability in the spinal cord at 1 or 3 days after SCI by measuring extravasation of Evans blue dye injected i.v. (Figure 6C). The diapedesis of Evans blue was markedly suppressed with FTY720 at 3 days after SCI.

We next assessed astrocyte accumulation in the injured spinal cord, a condition that can lead to glial scar formation. The area of GFAP immunostaining that represents astrocytic reactivity in the injured spinal cord was significantly reduced after treatment with FTY720 (Figure 7, A and B). These data suggest that treatment with FTY720 negatively regulates vascular permeability and astrocyte accumulation in the injured spinal cord.

FTY720 Promotes Functional Recovery after SCI Even in SCID Mice

To investigate whether FTY720 improves functional outcome of SCI through immune modulation, we next used mice homozygous for the *scid* spontaneous mutation that is characterized by an absence of functional T and B cells and hypogammaglobulinemia (SCID mice). FTY720 significantly improved recovery of hind limb motor function, assessed by both BMS score and the Rotarod Performance test result after SCI (Figure 8A). The recovery rate of motor function showed statistical significance from 0 to 14 days but not from 14 to 28 days (Figure 8B). FTY720 reduced vascular permeability and astrocyte accumulation in the lesion after SCI in SCID mice (Figure 8, C and D). These data clearly indicate the importance of immune-independent functions of FTY720 in the amelioration of functional deficits after SCI in mice.

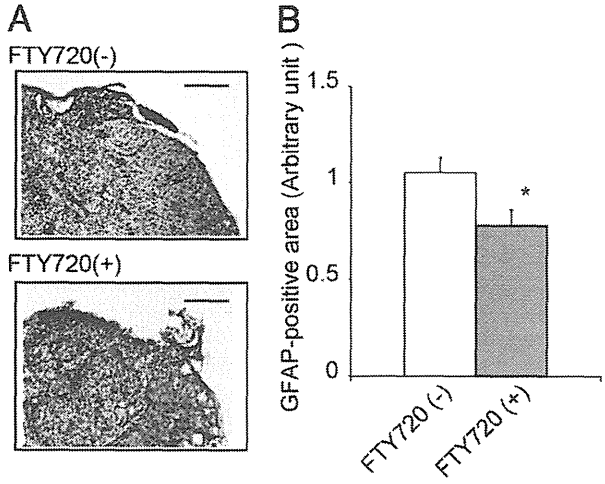

Figure 7. FTY720 regulates astrocyte accumulation in the injured spinal cord. **A:** Representative micrographs showing anti-GFAP staining of tissue sections at 28 days after SCI, with or without FTY720. Scale bar = 200 μm. **B:** Quantification of the GFAP-positive area in serial tissue sections in the lesion epicenter. Data represent the mean ± SEM (n = 6). $^{*}P < 0.05$ compared with the control experiment (two-tailed Student's t-test).

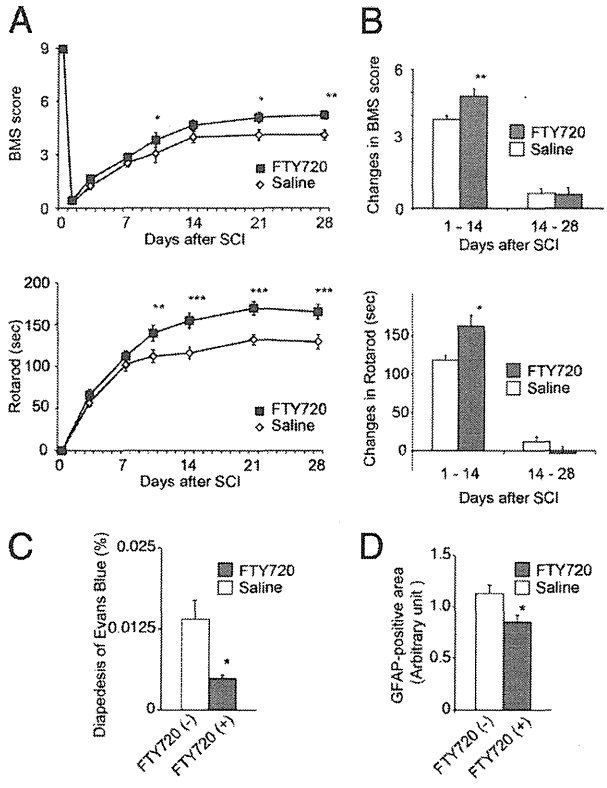

Figure 8. FTY720 promotes functional recovery after SCI in SCID mice. FTY720 (3 mg/kg) or saline was given orally every 24 hours in SCID mice after a contusion SCI. **A:** The recovery of motor function was quantified by BMS score (**top panel**) and Rotarod Performance test result (**bottom panel**) at the indicated times after SCI. Data represent the mean ± SEM ($n = 10$ in each group). Drug efficacy was statistically significant, as calculated by two-way repeated-measures analysis of variance ($P < 0.0001$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the control experiment (post hoc Bonferroni test). **B:** The changes in recovery rate of motor function quantified by BMS score (**top panel**) and Rotarod Performance test result (**bottom panel**) were separately assessed in the early (0 to 14 days) or late (14 to 28 days) phase after SCI. Data represent the mean ± SEM. The therapeutic effect was observed in the early phase after SCI. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control experiment (two-tailed Student's *t*-test). **C:** Vascular permeability in the spinal cord was quantified 3 days after SCI, as described in *Materials and Methods*. Data represent the mean ± SEM ($n = 5$). $^{*}P < 0.05$ compared with the control experiment (two-tailed Student's t-test). **D:** Quantification of the GFAP-positive area in serial tissue sections in the lesion epicenter at 28 days after SCI. Data represent the mean ± SEM ($n = 6$). $^{*}P < 0.05$ compared with the control experiment (two-tailed Student's *t*-test).

SEW2871, an $S1P_1$ Receptor Agonist, Partially Mimics the Effects of FTY720

An active phosphorylated metabolite of FTY720 activates all S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$), except for $S1P_2$.[30] To distinguish whether $S1P_1$ is specifically involved in the actions of FTY720 after SCI, we used SEW2871, an $S1P_1$ receptor–specific agonist. Similar to our results with FTY720, the administration of SEW2871 ameliorated motor function after SCI (Figure 9A). As was the case with FTY720, the improvement in recovery rate was observed from day 0 to day 14 after SCI (Figure 9B), and vascular permeability and astrocyte accumulation were also significantly reduced (Figure 9, C and D). However, the efficacy of SEW2871 was considerably lower than that of FTY720 (Figure 1), suggesting that the therapeutic effect of FTY720 after SCI requires other S1P receptors in addition to $S1P_1$.

Discussion

We demonstrated herein a therapeutic effect of the S1P receptor agonist, FTY720, in a mouse model of SCI and examined the mechanism by which FTY720 improves secondary degeneration after SCI. FTY720 is phosphorylated by sphingosine kinase,[16] and this phosphorylated form acts as a nonselective S1P receptor agonist for four of the five S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$) *in vivo*.[30] The most recognized systemic effect of FTY720 administration is an immunosuppressive action, mediated by the $S1P_1$ receptor, which prevents lymphocyte egress from lymphoid organs.[30] FTY720 has been effective in several animal models of autoimmune disorders and allograft survival[31–33]; reperfusion injury, including

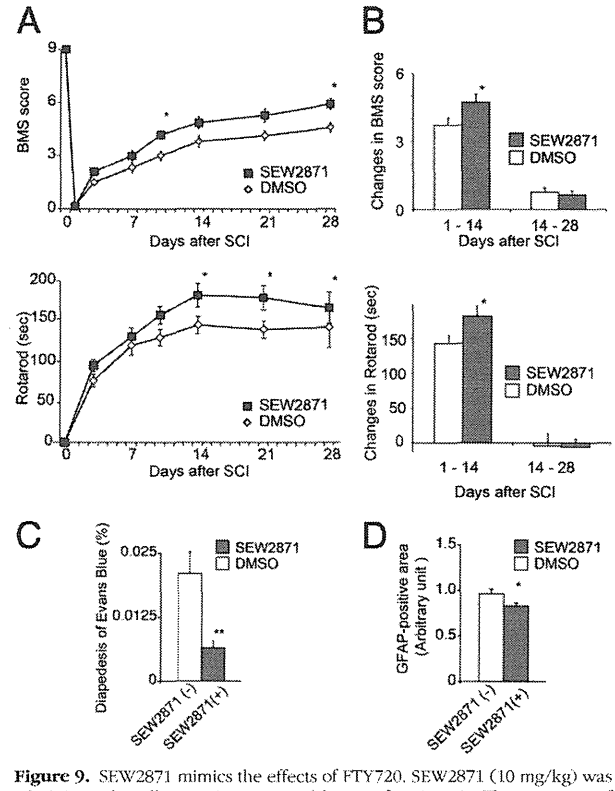

Figure 9. SEW2871 mimics the effects of FTY720. SEW2871 (10 mg/kg) was administered orally to mice every 24 hours after SCI. **A:** The recovery of motor function was quantified by BMS score (**top panel**) and Rotarod Performance test result (**bottom panel**) at the indicated times after SCI. Data represent the mean ± SEM ($n = 12$). Drug efficacy was statistically significant by two-way repeated-measures analysis of variance ($P < 0.0001$). $^{*}P < 0.05$ compared with the control experiment (post hoc Bonferroni test). **B:** The changes in recovery rate of motor function quantified by BMS score (**top panel**) and Rotarod Performance test result (**bottom panel**) were separately assessed in the early (0 to 14 days) or late (14 to 28 days) phase after SCI. Data represent the mean ± SEM. The therapeutic effect was observed in the early phase after SCI. $^{*}P < 0.05$ compared with the control experiment (two-tailed Student's *t*-test). **C:** Vascular permeability in the spinal cord was quantified 3 days after SCI, as described in *Materials and Methods*. Data represent the mean ± SEM ($n = 5$). $^{**}P < 0.01$ compared with the control experiment (two-tailed Student's t-test). **D:** Quantification of the GFAP-positive area in serial tissue sections in the lesion epicenter at 28 days after SCI. Data represent the mean ± SEM ($n = 5$). $^{*}P < 0.05$ compared with the control experiment (two-tailed Student's *t*-test). DMSO, dimethyl sulfoxide.

cerebral ischemia[19,20]; and renal injury.[34] The main biological activity responsible for these actions is believed to be immunological, but our data suggest that nonimmunological role(s) of FTY720 are also important in the treatment of SCI.

SCI induces a complex cascade of secondary injury that further promotes tissue destruction and results in clinical deterioration.[3] The cascade of secondary injury for SCI involves vascular disturbances, ischemia, propagation of free radicals, excitotoxicity, and inflammation, which leads to necrotic and apoptotic death of neurons and glia and subsequently to demyelination and axonal damage.[3,4] A complex inflammatory response is believed to be a prominent component of secondary tissue damage, which increases functional loss.[2,3,35] FTY720 reportedly attenuated cytokine production and inflammatory response in a mouse model of multiple sclerosis and cerebral infarction in previous studies.[19,36] However, we saw no decrease of any accumulation of neutrophils or microglia nor changes in the expression of inflammatory cytokines in the earlier phases after SCI. The discrepancy between previous reports is probably due to differences in disease models or animal species. Early inflammation in the spinal cord after traumatic SCI is mainly initiated by a transient increase in inflammatory cytokines and neutrophil accumulation within the lesion, which are rapidly cleared. On the other hand, lymphocyte entry after SCI is delayed in humans and mice.[3] The modulation of late T-cell infiltration by FTY720 in mouse models of SCI would not explain the early therapeutic response to FTY720. More important, FTY720 maintained its efficacy, even in SCID mice, confirming the importance of immune-independent mechanisms of action of FTY720 in this mouse model of SCI.

Because S1P receptors exist in many types of cells, including the CNS, and S1P plays a role in many cellular processes,[12,13] it is not surprising that various aspects of the response to SCI have been targeted by FTY720. Among the secondary injury events other than inflammation, we observed that FTY720 decreased vascular permeability and astrocyte accumulation in the injured spinal cord. These changes were also observed in SCID mice, suggesting that they are not dependent on modulating lymphocyte function. In addition, $S1P_1$ receptors are a likely target, because the administration of SEW2871 produced similar effects. Increased vascular permeability can lead to destruction of the blood-brain barrier in the spinal cord and causes local edema that adversely affects functional outcomes after SCI.[37] Furthermore, astrocyte accumulation is the main cellular component of the glial scar after CNS injury.[38] Although the formation of the glial scar has had both beneficial and detrimental effects,[39] the proliferation of astrocytes modifies the extracellular matrix by secretion of laminin, fibronectin, and proteoglycans, which inhibit the regeneration process after SCI.[40,41] FTY720 might counteract these secondary injuries and, thereby, prevent their pathological consequences.

The issue remaining unresolved is whether FTY720 behaves as an agonist or antagonist for S1P receptors in the treatment of SCI. Several reports[42,43] implicate the importance of the $S1P_1$ receptor in endothelial function, including endothelial barrier function, migration, cell survival, and angiogenesis. It is possible that the modification of vascular permeability in the injured spinal cord may result from $S1P_1$ stimulation by phosphorylated FTY720, as is the case with allergen-induced plasma leakage.[44] On the other hand, the reduction of vascular leakage in this study may be dependent on unknown compensatory mechanisms, because FTY720 also inhibits vascular permeability independently of $S1P_1$.[45]

It is also possible that phosphorylated FTY720 acts as a functional antagonist for the $S1P_1$ receptor to inhibit astrocyte accumulation in the injured spinal cord. It was reported that S1P receptors bound to FTY720 are irreversibly internalized and degraded.[46,47] Recently, in a mouse model of autoimmune encephalomyelitis, phosphorylated FTY720 induced the loss of the $S1P_1$ receptor on astrocytes through functional antagonism, and the functional loss of $S1P_1$ receptors from the astrocyte cell surface is a primary locus for FTY720 efficacy.[48] Although specific $S1P_1$ receptor modulation by SEW2871 reproduced some treatment effects, including reducing vascular permeability and astrocyte accumulation, treatment with SEW2871 led to weaker improvement in locomotor function compared with FTY720, suggesting a requirement for the additional activity of other S1P receptors in obtaining full efficacy with FTY720 in SCI. Additional studies will be required using mice genetically engineered to perturb other S1P receptors.

Our present study focused mainly on secondary injury after SCI, but direct beneficial effects of FTY720 on other neurological cells, including neurons and oligodendrocytes, cannot be excluded. S1P receptors are also expressed on many CNS cell types and have influenced CNS cell proliferation, morphological characteristics, and migration.[11–13] FTY720 easily crosses the blood-brain barrier, resulting in high concentrations of free phosphorylated FTY720 in the spinal fluid.[49] FTY720 rescues oligodendrocyte progenitors from death induced by growth factor depletion and exposure to activated microglia[50] and directly enhances remyelination through $S1P_3$ and $S1P_5$ receptors.[51] Furthermore, S1P acts as a chemoattractant for neural progenitor cells toward CNS injury,[9,10] suggesting that neurogenesis could be directly modified by FTY720 treatment.

Some limitations in this study merit discussion. Our data suggest that FTY720 has pleiotropic actions, in addition to modulating early inflammation and lymphocyte sequestration, to improve neuronal outcomes after SCI. However, we could not completely exclude the importance of the immunosuppressive actions of FTY720 and the role of marginal immune responses in SCID mice to improve functional outcomes. In addition, we could not show evidence that these pleiotropic actions modulate vascular permeability and astrocyte migration to directly improve motor function after SCI.

In summary, our data suggest that targeting S1P receptors with FTY720 is an attractive therapeutic approach for SCI. FTY720 is an oral drug that has shown efficacy in clinical trials for human multiple sclerosis[17,18]; it was recently approved by the Food and Drug Admin-

istration. The safety profile of FTY720 in the treatment of multiple sclerosis encourages us to apply it in a clinical trial for SCI. However, further evaluation using larger animals, such as nonhuman primates, will be necessary to confirm its efficacy in treating SCI. Furthermore, strategies targeted at modulating the S1P concentration in the injured CNS may lead to new therapeutic approaches toward repairing various CNS disorders.

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