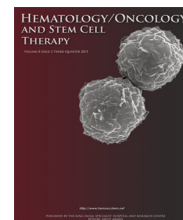




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ORIGINAL RESEARCH REPORT

Therapeutic approaches for treating hemophilia A using embryonic stem cells



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Abstract

Hemophilia A is an X-linked recessive bleeding disorder that results from *F8* gene aberrations. Previously, we established embryonic stem (ES) cells (tet-226aa/N6-Ainv18) that secrete human factor VIII (hFVIII) by introducing the human *F8* gene in mouse Ainv18 ES cells. Here, we explored the potential of cell transplantation therapy for hemophilia A using the ES cells. Transplant tet-226aa/N6-Ainv18 ES cells were injected into the spleens of severe combined immunodeficiency (SCID) mice, carbon tetrachloride (CCl₄)-pretreated wild-type mice, and CCl₄-pretreated hemophilia A mice. *F8* expression was induced by doxycycline in drinking water, and hFVIII-antigen production was assessed in all cell transplantation experiments. Injecting the ES cells into SCID mice resulted in an enhanced expression of the hFVIII antigen; however, teratoma generation was confirmed in the spleen. Transplantation of ES cells into wild-type mice after CCl₄-induced liver injury facilitated survival and engraftment of transplanted cells without teratoma formation, resulting in hFVIII production in the plasma. Although CCl₄ was lethal to most hemophilia A mice, therapeutic levels of FVIII activity, as well as the hFVIII antigen, were detected in surviving hemophilia A mice after cell transplantation. Immunolocalization results for hFVIII suggested that transplanted ES cells might be engrafted at

Abbreviations: Bry, brachyury; CCl₄, carbon tetrachloride; DAPI, 4',6-diamidino-2-phenylindole; Dox, doxycycline; EB, embryoid body; ES, embryonic stem; FVIII, factor VIII; FVIII:Ag, factor VIII antigen; FVIII:C, factor VIII activity; FIX, factor IX; GFP, green fluorescent protein; H&E, hematoxylin and eosin; hFVIII, human factor VIII; iPS, induced pluripotent stem; LSEC, liver sinusoidal endothelial cell; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; tet, tetracycline; VWF, von Willebrand factor; WT, wild type

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the periportal area in the liver. Although the development of a safer induction method for liver regeneration is required, our results suggested the potential for developing an effective ES-cell transplantation therapeutic model for treating hemophilia A in the future.

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Introduction

Hemophilia A is an inherited X-linked, recessive hemorrhagic disorder caused by a deficiency of clotting factor VIII (FVIII) and is commonly treated by FVIII replacement. Concentrated FVIII products are derived from human plasma or produced recombinantly. Advances in viral-screening and -inactivation methods have improved the safety of plasma-derived products. Furthermore, the infectious risk of recombinant FVIII products is lowered by excluding human- or animal-derived proteins from cell culture solutions [1]. However, FVIII concentrates remain extraordinarily expensive, and frequent injections are required for FVIII replenishment, especially with severe hemophilia A patients.

Since hemophilia A is a single-gene disorder and small increases in FVIII levels exert a curative influence, the disease is a good candidate for gene therapy. However, problems are associated with gene therapy, such as the development of leukemia and decreased target-protein expression following cytotoxic CD8 T-cell induction against viral vector-derived capsid epitopes [2]. Nevertheless, a single injection of an adeno-associated virus serotype 8 vector in patients with severe hemophilia B caused prolonged factor IX (FIX) overexpression and clinical improvement [3]. However, viral vectors employed in gene therapy cannot deliver the *F8* gene due to its large size. Therefore, truncated *F8* gene variants, such as a B-domain-deleted variant, have been used, but successful hemophilia A gene therapy using viral vectors has not been achieved clinically. A recent study using a hemophilia A mouse model demonstrated that transducing the entire *F8* gene via the piggyBac vector improved clotting activity [4]. However, as the vector preferentially integrates near transcriptional start sites [5], insertional mutagenesis and genotoxicity remain significant concerns.

As the liver is the major site of FVIII synthesis, liver transplantation is effective in hemophilia A patients [6]; however, the lack of donor organs hinders clinical applications. Recent studies revealed that the main source of FVIII is liver sinusoidal endothelial cells (LSECs), and transplantation experiments using murine or human LSECs in hemophilia A mice demonstrated symptomatic improvement [7]. However, such transplantation requires a donor liver from which LSECs can be isolated, and contamination with different donor cells remains a potential problem. Several therapies have been investigated using cells capable of differentiating into LSECs [8–11].

Considering their potential for multilineage development and illimitable proliferation potential, embryonic stem (ES) cells may enable cell transplantation therapy. Fair et al. [12] reported that injecting mouse ES cells into the livers of hemophilia B mice corrected FIX deficiency. However, there have been few reports describing cell therapy for hemophilia A using ES cells. Previously, we established

mouse Ainv18 ES cells (tet-226aa/N6-Ainv18), which secrete human FVIII (hFVIII) by introducing the human *F8* gene [13]. Here, we investigated ES-cell transplantation therapy against hemophilia A.

Materials and methods

Mice

All animal experiments were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and national regulations after approval from the institutional animal-care committee. Severe combined immunodeficiency (SCID) mice and wild-type (WT) C57/BL6 mice were purchased from CLEA Japan (Tokyo, Japan). *F8*-deficient (hemophilia A) mice were provided by Professor Yoichi Sakata (Jichi Medical University, Shimotsuke, Japan) and back-crossed for over eight generations with the C57/BL6 background as previously described [14]. Mouse blood was collected by retro-orbital bleeding at the indicated times and placed into 3.8% sodium citrate buffer. Immediately after blood sampling, plasma was isolated by centrifugation to evaluate FVIII activity (FVIII:C) and human FVIII-antigen (hFVIII:Ag).

FVIII assay

Mouse plasma samples were diluted with Owren's Veronal Buffer (Sysmex, Kobe, Japan). FVIII:C was measured in a one-stage aPTT clotting assay in a KC10A coagulometer (Amelung, Lemgo, Germany) using human FVIII-deficient plasma (George King Biomedical, Overland Park, KS, USA). Activated partial thromboplastin and CaCl₂ were purchased from bioMerieux (Durham, NC, USA). hFVIII:Ag was quantified using the VisuLize FVIII Antigen ELISA Kit (Affinity Biologicals, Ancaster, ON, Canada). For measurements of both FVIII:C and hFVIII:Ag, a standard curve was generated using normal human plasma (Sysmex). The detection limits of the FVIII:C and FVIII:Ag assays were 0.1% and 2 ng/mL, respectively.

ES cells

Production of tet-226aa/N6 ES cells was described previously [13]. Briefly, for efficient production of the active FVIII protein, we employed 226aa/N6-*F8* cDNA [15]. The 226aa/N6-*F8* construct encodes a shortened B-domain of 226 amino acids with six N-linked oligosaccharides. Green fluorescent protein (GFP)-brachyury (Bry) Ainv18 ES cells, established by targeting the GFP cDNA to the Bry locus in Ainv18 ES cells [16], were transfected with the 226aa/N6-plox targeting plasmids, yielding tet-226aa/N6 ES cells.

Tet-226aa/N6 ES cells drive *F8* gene overexpression via a tetracycline (tet)-inducible promoter following doxycycline (Dox) exposure. Undifferentiated tet-226aa/N6 ES cells differentiated into mesendodermal embryoid bodies (EBs) [13,16]. Day-3.5 EBs were dissociated with trypsin–EDTA, stained with anti-mouse c-kit-phycoerythrin (BD PharMingen, San Diego, CA, USA), and sorted in a FACSAria cell sorter (Becton Dickinson, San Jose, CA, USA). We prepared GFP-Bry⁺/c-kit⁺ cells for transplantation, because they contained a definitive endoderm population and produced active hFVIII more efficiently than pre-sorted populations [13]. The GFP-Bry⁺/c-kit⁺ population was subsequently used for all transplantations.

Cell transplantation into SCID mice

SCID mice were used, because they did not demonstrate rejection responses. Transplant cells (1×10^6 cells) were injected into the spleens of three SCID mice anesthetized with isoflurane. One control mouse was injected with culture media alone. At 1-week post-transplantation, *F8* expression was induced by replacing the normal drinking water with Dox-containing water (2 mg/mL). Subsequently, blood samples were collected weekly, and hFVIII:Ag was assessed. At 3-weeks post-transplantation, two of the three mice were sacrificed, and the livers and spleens were harvested, while the remaining mouse underwent a splenectomy. The resected livers and spleens were fixed with 10% formalin for hematoxylin and eosin (H&E) staining. Total RNA was extracted from the livers and spleens and reverse transcribed into cDNA [17]. *F8* gene expression in each organ was evaluated by polymerase chain reaction (PCR) using human *F8*-specific primers [13]. β -actin was used as an endogenous control.

Cell transplantation into WT mice

For cell transplantations into WT mice, liver injury was induced by carbon tetrachloride (CCl₄) administration with the expectation of better engraftment of transplanted cells. CCl₄ diluted in corn oil [10% (v/v), 0.5 mL/kg] was injected intraperitoneally on Day-4 and Day-1. On Day 0, 1×10^5 cells (one-tenth of the cell number used with SCID mice) were injected into the spleen. After transplantation, intraperitoneal CCl₄ injection was continued twice/week until Day 30. Dox-containing water was given to mice at 1-week post-transplantation. Blood samples were collected on Day 0, Day 10, Day 20, and Day 30, after which the obtained plasma were frozen at -80°C until hFVIII:Ag was evaluated (Fig. 1, upper panel).

Cell transplantation into hemophilia A mice

Liver injury was induced in hemophilia A mice using a method similar to that described for WT-mice. For perioperative hemostatic management, all hemophilia A mice were intraperitoneally administered with 500 μL normal mouse plasma 30 min before cell transplantation [18]. Mice were sacrificed on Day 10, and plasma FVIII:C and hFVIII:Ag levels were evaluated (Fig. 1, lower panel). Liver samples were harvested on Day 10 and embedded in Tissue-Tek OCT

compound (Sakura Finetek, Tokyo, Japan) with liquid nitrogen. Liver tissue sections were incubated with a human-specific polyclonal anti-FVIII antibody (Affinity Biologicals) and then with an Alexa Fluor 488-conjugated anti-sheep immunoglobulin G (Molecular Probes, Eugene, OR, USA). Cell nuclei were counterstained using the ProLong-Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Negative controls were prepared using untreated mouse livers from hemophilia A mice. Fluorescent detection of the hFVIII protein and cell nuclei was performed using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

Effect of CCl₄ administration on plasma coagulation-factor levels

To address how CCl₄-induced liver injury affected coagulation-factor production, a single dose of CCl₄ was intraperitoneally injected into WT mice. We injected CCl₄ at a dose of 2 mL/kg (4-fold higher than the dose used in transplantation experiments) to observe coagulation-factor differences after inducing greater liver injury. Before injection and at 24-h post-injection, we measured the plasma levels of coagulation factors, including fibrinogen and von Willebrand factor (VWF) antigen, and the activities of prothrombin and factors V, VII, VIII, IX, X, XI, and XII [17,19].

Results

FVIII expression in SCID mice

Before Dox administration beginning at 1-week post-transplantation, hFVIII:Ag was undetectable in the plasma of treated mice. hFVIII:Ag was also undetectable in untransplanted mice, even after Dox administration. At 1-week post-Dox administration (2-weeks post-transplantation), we detected elevated levels of hFVIII:Ag in all three mice.

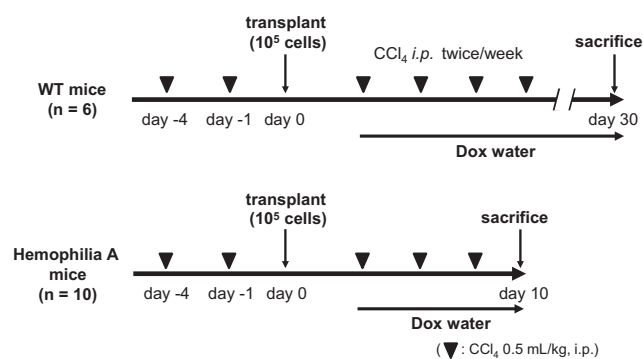


Figure 1 Transplanting ES cells into WT/hemophilia A mice with CCl₄-hepatic injury. Intraperitoneal injection of CCl₄ (0.5 mL/kg) was performed twice weekly in WT mice ($n = 6$) and hemophilia A mice ($n = 10$). One day after the second intraperitoneal CCl₄ injection, 1×10^5 ES cells were transplanted into the spleens. hFVIII:Ag levels were assessed every 10 days through post-transplantation Day 30 in WT mice, or post-transplantation Day 10 in hemophilia A mice. ES = embryonic stem; hFVIII:Ag = human factor VIII antigen.

At 2-weeks post-Dox administration (3-weeks post-transplantation), hFVIII:Ag levels increased to 13–43% of normal human plasma levels (Fig. 2Ai). However, masses had formed in the abdomens of all mice. Laparotomies revealed spleen tumors, which were histologically confirmed as teratomas, consisting of muscle cells, chondrocytes, glial-like cells, and hair-root cells (Fig. 2Aii). In one mouse that underwent a splenectomy, hFVIII:Ag became undetectable within 3-weeks post-surgery (6-weeks post-transplantation). Reverse transcription-PCR analysis showed detectable human *F8* mRNA only in spleens with teratomas, but not in the livers (Fig. 2Aiii), indicating that plasma hFVIII:Ag was derived primarily from spleens with teratomas.

FVIII expression in WT mice

hFVIII:Ag was undetectable in WT mice before Dox administration. Dox administration began at 1-week post-transplantation, and hFVIII:Ag was detected in the plasma from three of the six transplanted mice (0.4–1.5% hFVIII:Ag of the normal hFVIII:Ag level) on Day 10. Although hFVIII:Ag became undetectable in one mouse, two mice continued to produce FVIII on Day 20. One mouse died on Day 20 after blood collection, but the other mouse produced

FVIII until Day 30 (Fig. 2B). CCl₄-induced liver injury did not cause fatal liver failure in WT mice. Tumor formation was not observed by laparotomy in transplanted mouse spleens or the livers.

Hemophilia A mice

Contrary to the WT mice, CCl₄-induced liver injury was lethal to *F8*-KO mice: six of the ten transplanted mice died within 7-days post-transplantation. Four surviving mice were subjected to blood collection on Day 10, and FVIII:C and hFVIII:Ag were detected in each surviving mouse (Table 1). One mouse (#780) demonstrated markedly higher levels of FVIII:C and hFVIII:Ag (both >10%). Teratoma formation was not found in the spleens or livers of hemophilia A mice. Liver samples were collected on Day 10 from transplanted mice, and hFVIII antigen was detected in the periportal area by immunofluorescence staining using an hFVIII-specific antibody (Fig. 2C).

Effect of CCl₄ on plasma coagulation-factor levels

In WT mice, the plasma levels of most coagulation factors significantly decreased 24 h post-CCl₄ injection, including

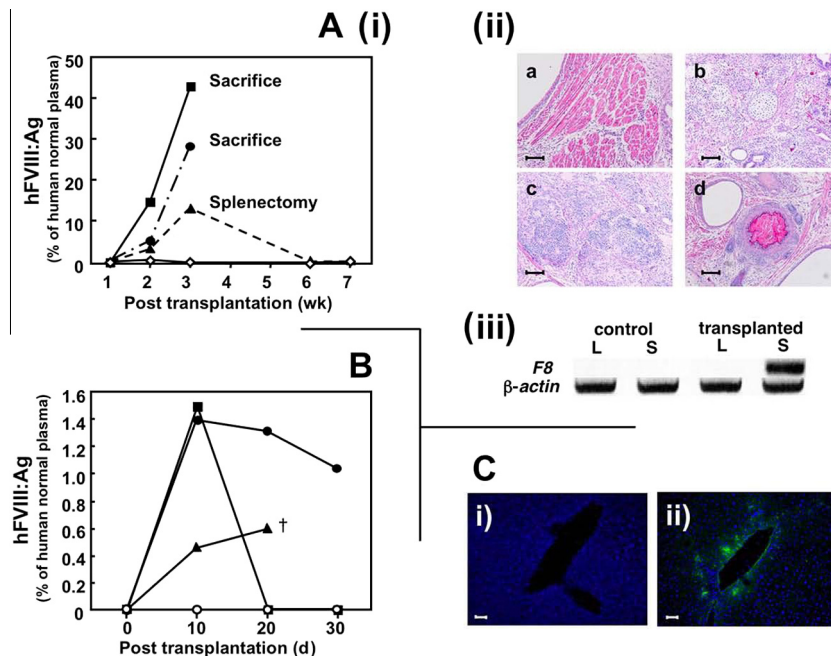


Figure 2 (A) ES-cell transplantation into SCID mice. (i) Plasma hFVIII:Ag levels after ES-cell transplantation. Splenic hFVIII production was suggested as the hFVIII:Ag level became undetectable after a splenectomy. (ii) Splenic teratoma formation in a SCID mouse after ES-cell transplantation. H&E staining of splenic tumor sections showed differentiation into various tissues, including (a) muscle (mesoderm), (b) chondrocytes (mesoderm), (c) glial-like cells (ectoderm), and (d) hair-root cells (ectoderm). Scale bars: 100 μ m. (iii) Expression of human *F8* mRNA after ES-cell transplantation. Expression of human *F8* mRNA was confirmed only in a mouse spleen following ES-cell transplantation. L: liver; S, spleen. (B) Plasma hFVIII:Ag levels after ES-cell transplantation. Closed circles, triangles, and squares indicate ES cell-transplanted WT mice with detectable hFVIII:Ag. hFVIII:Ag was undetectable in the other three ES cell-transplanted WT mice. Open circles indicate results from control mice. (C) FVIII immunolocalization in the liver of a hemophilia A mouse by confocal microscopy. Liver sections were stained for FVIII (green), and nuclei were counterstained with DAPI (blue). Representative images of FVIII staining (bar, 100 μ m) in the liver of hemophilia A mice without ES-cell transplantation (i) and 10 days post-ES-cell transplantation (ii) are shown. DAPI = 4',6-diamidino-2-phenylindole; ES = embryonic stem; FVIII = factor VIII; H&E = hematoxylin and eosin; hFVIII:Ag = human factor VIII antigen; SCID = severe combined immunodeficiency.

fibrinogen, prothrombin, and factors V, VII, IX, and X. However, the plasma levels of FVIII:C and VWF antigen significantly increased following injection (Fig. 3). Injection of the same CCl₄ dose into hemophilia A mice caused death within 24 h in five of 16 hemophilia A mice, whereas no hemophilia A mice died after administration with the same volume of vehicle control ($n = 6$). WT mice ($n = 16$) showed no mortality under the same conditions.

Discussion

Here, we investigated whether tet-226aa/N6-Ainv18 ES cells integrate and produce hFVIII at the transplant site. Cell infusion into the spleens of SCID mice caused increased hFVIII:Ag levels, ranging from ~10% to ~40% of the normal hFVIII:Ag level. However, teratomas developed in the spleens, even though the transplanted cells differentiated into liver EBs. Neoplastic transformation would facilitate the production of enormous amounts of hFVIII. Nevertheless, successful synthesis of hFVIII was encouraging, although few mice were used. Next, cell-transfer

experiments were conducted using hemophilia A-model mice. While transplanted cells might show low survival due to the hemophilia A mice having normal immunocompetence, it was expected that some of the engrafted cells would potentially produce hFVIII with low teratoma incidence. However, hFVIII was undetectable in hemophilia A mice using the same cell transplantation procedure as that used for SCID mice (not shown). Therefore, anticipating increased survival rates of transplanted cells in the presence of liver-regeneration stimuli, cell transplantation was performed in WT mice after intraperitoneal CCl₄ administration. hFVIII was detected without teratoma formation in some, but not all mice administered a reduced number of transplant cells. Although intraperitoneal CCl₄ administration was lethal to most hemophilia A mice, hFVIII:Ag was detected in circulating blood and the periportal area of the liver of surviving hemophilia A mice, suggesting that tet-226aa/N6-Ainv18 ES cells are potentially useful for treating hemophilia A mice.

To establish ES cells, early embryos (blastocyst stage) are required as a source. Because ethical issues are associated with using human ES cells experimentally, practical

Table 1 FVIII activity and antigen levels 10 days after cell transplantation in hemophilia A mice.

Mouse No.	FVIII:C (%)	FVIII:Ag (%)
727	0.96	2.74
738	1.34	4.84
777	1.07	2.99
780	10.9	10.9

Note. FVIII:Ag = factor VIII antigen; FVIII:C = human factor VIII activity.

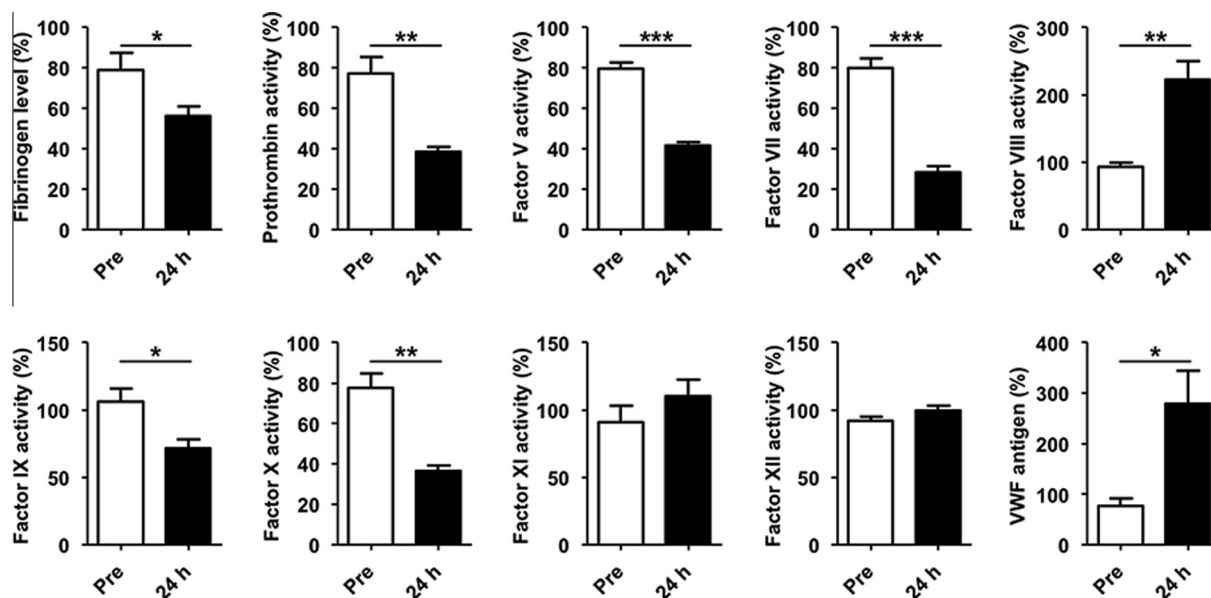


Figure 3 Plasma levels of coagulation factors before (open bars) and after CCl₄ administration (closed bars). WT mice ($n = 4$) were injected intraperitoneally with CCl₄ (2 mL/kg), and plasma samples were obtained before and 24-hours post-injection. Activity levels of prothrombin and factors V, VII, VIII, IX, X, XI, and XII were measured in a one-stage clotting assay, fibrinogen levels were measured by the Clauss method, and VWF antigen levels were measured by ELISA. Data were analyzed by Student *t* test, and statistical significance is shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. ELISA = enzyme-linked immunosorbent assay; VWF = von Willebrand factor.

applications with human ES cells are limited. Ethical issues regarding the source of ES cells and rejection responses are resolved by the establishment of patient-derived induced pluripotent stem (iPS) cells. Xu et al. [20] reported successful iPS-cell therapy using a hemophilia A mouse model. These authors differentiated iPS cells into endothelial cells through EBs in the presence of a vascular endothelial growth factor. Injection of iPS cell-derived endothelial cells into the livers of X-ray-irradiated hemophilia A mice resulted in successful engraftment into the sinusoids and increased FVIII:C. However, this strategy cannot be directly applied for treating human hemophilia A, because immunologically mismatched iPS cells from other donors will be rejected, and iPS cells from hemophilia A patients are unsuitable for cell therapy due to *F8* gene aberrations. For human applications, regardless of the type of stem cells used, introduction of the *F8* gene into stem cells is, therefore, essential, unless an iPS cell bank possessing sufficient iPS-cell variations to match all patients immunologically is established.

The genetic risks of cell therapy have been reduced by genome-editing technology, including transcription activator-like effector nucleases and the CRISPR/Cas system [21,22], which facilitates *in situ* correction of mutant genes, reduced risks for random genome insertions, and *ex vivo* selection of genetically safe cells. The survival rate of transplanted cells remains unsatisfactory; therefore, methods were developed to promote hepatic regeneration, such as CCl₄ treatment, X-ray irradiation, and partial hepatectomy. As shown following CCl₄-induced liver damage, coagulation-factor activities decreased, except for FVIII and VWF, which likely increased due to excessive release from storage granules in damaged endothelial cells. Because hemophilia A mice are intrinsically deficient in FVIII, CCl₄ treatment resulted in reduced activities in most key coagulation factors, resulting in a tendency for severe or sometimes lethal bleeding. Instead of using these invasive methods clinically, novel methods for the effective engraftment of transplanted cells should be explored.

Conclusion

In conclusion, our data showed that hFVIII-secreting ES cells were capable of engraftment at the periportal area in the liver and synthesizing hFVIII. Although development of a safer induction method for liver regeneration is required, our results showed the potential for developing an effective cell transplantation therapeutic model for treating hemophilia A.

Conflicts of interest

None.

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