Phenotypic correction of murine hemophilia A using an iPS cell-based therapy

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Hemophilia A is caused by mutations within the Factor VIII (FVIII) gene that lead to depleted protein production and inefficient blood clotting. Several attempts at gene therapy have failed for various reasons—including immune rejection. The recent generation of induced pluripotent stem (iPS) cells from somatic cells by the ectopic expression of 3 transcription factors, Oct4, Sox2, and Klf4, provides a means of circumventing the immune rejection barrier. To date, iPS cells appear to be indistinguishable from ES cells and thus provide tremendous therapeutic potential. Here we prepared murine iPS cells from tail-tip fibroblasts and differentiated them to both endothelial cells and endothelial progenitor cells by using the embryoid body differentiation method. These iPS cells express major ES cell markers such as Oct4, Nanog, SSEA-1, alkaline phosphatase, and SALL4. Endothelial/endothelial progenitor cells derived from iPS cells expressed cell-specific markers such as CD31, CD34, and Flk1 and secreted FVIII protein. These iPS-derived cells were injected directly into the liver of irradiated hemophilia A mice. At various times after transplantation (7–90 days) hemophilia A mice and their control mice counterparts were challenged by a tail-clip bleeding assay. Nontransplanted hemophilia A mice died within a few hours, whereas transplanted mice survived for more than 3 months. Plasma FVIII levels increased in transplanted hemophilia A mice during this period to 8% to 12% of wild type and corrected the hemophilia A phenotype. Our studies provide additional evidence that iPS cell therapy may be able to treat human monogenetic disorders in the future.

endothelial cell precursors | Factor VIII | Oct4 | Sox2 | Klf4

Hemophilia is a congenital coagulation disorder. Hemophilia A occurs in 1 or 2 individuals per 10,000 and represents roughly 90% of hemophilia cases (1, 2). Hemophilia B affects 1 in 30,000 individuals. Both hemophilia A and B are sex-linked disorders characterized by deficiencies of Factor VIII (FVIII) in hemophilia A and Factor IX (FIX) in hemophilia B. Clinically, severe hemophilia (<1% factor activity) is characterized by spontaneous and prolonged hemorrhage that can result in disability and death. The most common chronic condition is arthritis caused by bleeding within the joints. Traumatic injuries to individuals who have hemophilia cause rapid blood loss and, if not treated promptly, can be lethal. Current therapies include fixed-dose FVIII or FIX prophylaxis, factor replacement therapies, and most recently, gene therapy. Prophylaxis and FVIII or FIX replacement therapies are limited by incomplete efficacy, high cost, restricted availability, and the possible development of neutralizing antibodies in chronically treated individuals. However, much of the clinical presentation in hemophilia patients can be relieved by expression of only a small fraction of wild-type clotting protein, and in most cases an individual with 30% of wild-type clotting factor would be considered phenotypically normal (2, 3).

Hemophilia long has been recognized as a suitable disease for gene therapy (4). Expression of only a small percentage of wild-type factor VIII can be beneficial to the patient, the expression need not be tissue specific, and it has a broad therapeutic index (2). The overarching goal of gene therapy is to correct the defective gene sequence, resulting in a phenotypic

correction of the disease. Addition of exogenous genetic material through viral vectors has been the usual therapeutic approach, which supplements an insufficiency rather than replacing the defective copy of the endogenous gene. Limited success has been reported using gene therapy for the treatment of hemophilia, even though there have been several phase I clinical trials in humans. The failure is primarily caused by host immune responses to virally encoded proteins and lack of sustained production of the therapeutic gene products (5, 6).

Yamanaka and others (7–12) have reported techniques for generating induced pluripotent stem (iPS) cells from adult cells such as fibroblasts. Methods for creating iPS cells that circumvent the need for human embryos should quell the political controversy arising from opposition to stem cell research based on moral and religious premises. iPS cells have the same features as embryonic stem (ES) cells and are capable of self-renewal and differentiation into all adult cell types, offering potential for clinical cell therapies. iPS cells can be derived from the patient's somatic cells, thus avoiding potential side effects related to immune rejection. As a result, such patient-derived iPS cell therapy potentially could function better when implanted in diseased organs than either currently available ES cell lines or gene therapies.

Hanna, *et al.* recently demonstrated that bone marrow cells derived from iPS cells could reverse the sickle cell anemia phenotype in a suitable mouse model (12). In this report, we evaluated another therapeutic application using endothelial cells derived from iPS cells for treatment of a preclinical mouse model of hemophilia A. We demonstrate that iPS cells derived from tail-tip fibroblasts (TTFs) can be differentiated into endothelial progenitor cells that express FVIII both in vitro and in vivo. The endothelial progenitor cells derived from iPS cells engraft into the liver of a hemophilia mouse model and provide phenotypic correction of the bleeding disorder. This finding provides an additional proof of principle for potential clinical applications of reprogrammed somatic cells in the treatment of hemophilia and other genetic disorders.

Results

Production and Characterization of iPS Cells. Following the seminal work of Yamanaka and co-workers (9, 10), we and other laboratories (11) have been able to derive iPS cells successfully from fibroblasts of both mouse and human origin (data not shown). To reduce potential immune rejection, the mouse fibroblasts used for this study were derived from the tail tip of a C57BL/6 mouse strain, a close relative of the hemophilia A mouse model. Like other investigators, we note that iPS cells can

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Fig. 4. 3F-iPS-derived endothelial cells express endothelial cell markers as determined by quantitative real-time PCR. Quantitative real-time PCR analysis of 3F-iPS-derived endothelial cells differentiated for 18 days express FVIII and common endothelial cell markers. The identity of the amplification products was established by specific sense and anti-sense primers for each target gene. The 2 sets of mRNA were prepared from differentiated iPS-derived endothelial cell pools, and the data represent 2 independent experiments run concurrently. The cells used in this test were cultured with media containing VEGF. Normal TTFs were used as the negative control and were used as the calibrator for analysis. Melting curve analysis (data not shown) was performed for each test to verify single-product formation and contamination-free test. All tests were performed at an optimum condition (see *Methods*). For each primer set we checked that these conditions were in the nonsaturating range of PCR amplification.

transplanted cells by immunofluorescent staining of liver tissue with antibodies against GFP and CD31. This immunostaining revealed that the transplanted GFP-positive cells co-expressed CD31, a characteristic endothelial membrane marker (Fig. 7), and that these cells appeared to reconstitute structurally the sinusoidal endothelial compartment in the liver.

Discussion

Hemophilia A is a sex-linked bleeding disorder characterized by the deficiency of coagulation FVIII causing prolonged bleeding because of the inability to clot efficiently. Treatment for hemophilia generally includes either fixed-dose prophylaxis or factor replacement therapy on an as-needed basis. With either approach, neutralizing antibodies to the replacement protein have been reported and present a unique problem when treating hemophiliacs (4, 19).

The monogenic nature of hemophilia makes it an ideal target for gene therapy. However, attempts at gene therapy so far have failed because of the lack of an adequate delivery system or sustained FVIII expression or because of immune rejection. Cellular therapies for hemophilia present a unique delivery

method for FVIII protein. By generating iPS cells that express wild-type endogenous FVIII protein and transplanting them into the hepatic parenchyma, we demonstrated an effective phenotypic correction of hemophilia. This proof-of-principle study shows that endothelial progenitor cells derived from iPS cells can express the FVIII protein effectively, engraft within the hepatic parenchyma, and functionally integrate to provide the therapeutic benefit necessary for phenotypic correction of hemophilia. It is interesting that although the iPS-derived endothelial cells were injected into the liver, higher levels of FVIII mRNA were detected in spleen, heart, and kidney tissues of injected animals. Additional studies of GFP-tagged epithelial cells will be required to establish the complete whole-body distribution. Our study provides additional proof that correction of a single-gene disorder using iPS cell technology is feasible and expands on the work of Hanna, *et al.* (12) on correcting the sickle cell phenotype. It is clear the iPS cells will have tremendous therapeutic benefit for the treatment of monogenic disorders once several technical challenges are overcome.

The major obstacles that must be overcome are the challenges surrounding the generation of iPS cells using multiple factors and retroviral transduction. In this study, we have produced iPS cells by using the retroviral transduction of Oct4, Sox2, and Klf4

Fig. 5. mRNA expression of FVIII in various organs obtained from hemophilia A mice 1 month after transplantation with FVIII-expressing endothelial cells. Hemophilia A mice were transplanted with endothelial progenitor cells, and quantitative real-time PCR analysis was performed on major organs (liver, spleen, heart, and kidney) to test FVIII expression. Transplanted mice have a significant increase in FVIII expression in the major organs tested, demonstrating successful engraftment of the progenitor cells in major organs, usually with normal FVIII expression. Tissues from wild-type mice were run as positive controls and showed very high FVIII expression (data not shown).

Fig. 6. Plasma FVIII level in hemophilia A mouse model transplanted with endothelial progenitor cells. FVIII levels in plasma were monitored sequentially at 7, 30, 60, and 90 days after transplantation. Transplanted mice survived the tail-clip challenges at the various time points, and the level of plasma FVIII in 3 hemophilia A mice (Hemo A 1, 2, 3) increased significantly compared with normal plasma controls collected from C57BL/6 mice. Hemo A 3 was used 60 days after transplantation for histologic studies of GFP-labeled cells injected into the liver (see Fig. 7).

Fig. 7. Immunofluorescence of liver tissue section from transplanted hemophilia A mice. Purified cells (2×10^6) expressing CD31 and FVIII-expressing cells tagged with GFP were transplanted into the hemophilia mice model via liver parenchyma injection to monitor the location of the cells in vivo 60 days after transplantation. Immunofluorescence staining of liver tissue sections showed coexpression of CD31- and GFP-expressing FVIII cells (pseudo-colored red) in the liver sinusoid of the treated hemophilia A mice. Liver tissue section from untreated mice showed expression of CD31- but absence of GFP-expressing cells. Secondary antibody-only staining was done to check the specificity of antibodies. DAPI was used to stain the nuclei.

into mouse TTFs. We have observed no tumor formation or other induced pathological problems in our study to date. However, long-term follow-up throughout their lifetimes will be needed to determine whether down-stream adverse effects will emerge in the mice. Ideally, one would like to remove the integration of viral vectors altogether. Toward this end, recent studies have demonstrated the ability to generate murine iPS cells by using nonintegrating adenovirus vectors expressing Oct4, Sox2, Klf4, and c-MYC (20, 21). Adequate expression of 3 or 4 transcription factors using nonviral means may be difficult. Small molecules are another alternative to viral introduction of transcription factors, and recent studies (22, 23) have suggested that this option may be a viable one, although theoretically a small molecule may be necessary for each transcription factor in the reprogramming mixture. There is little doubt that reducing the number of reprogramming factors would ease these problems, and this reduction may be possible in the near future (24).

Methods

Retroviral Transfection and Fibroblast Infection. Three pMXs-based retroviral vectors encoding Oct-4, Sox2, or Klf-4 were purchased from Addgene. Vectors were transfected into a Plat-E packaging cell line (Clontech) by using Lipofectamine 2000 (Invitrogen) according to manufacturer's directions. Twelve h after transfection, the medium was replaced with medium containing DMEM high glucose (Invitrogen) supplemented with 10% FBS (Invitrogen), 1X Pen-Strep (Invitrogen), and 1 mM sodium pyruvate (Sigma). After 48 h, virus-containing supernatant was collected for each transcription factor and combined with supplemental 8 μ l/ml Polybrene (Chemicon). Medium was replenished, and the transfected packaging cells were allowed to generate virus for an additional 24 h. Filtered virus-containing supernatant was used to infect 2×10^5 TTFs from C57BL/6 mice at passage 3–4.

iPS Cell Generation and EB Formation. Seven days after infection, fibroblasts were harvested by trypsinization and replated at 5×10^4 cells per 100-mm dish with a mitomycin-C-treated feeder layer (Chemicon). Cells were plated by using mouse ES medium containing knockout DMEM (KDMEM, Invitrogen), 15% ES-qualified FBS (PAA Laboratories), 2 mM L-glutamine (Invitrogen), 1×10^{-4} M nonessential amino acids (Invitrogen), 1×10^{-4} M 2-mercaptoethanol (Sigma), 1X Pen-Strep (Invitrogen), and 12.5 ng/ml LIF (Chemicon). The medium was changed every other day. Fifteen days after infection, single colonies were picked and transferred into 50 μ l 0.25% trypsin in 1X PBS. After 3 min of incubation at 37°C, 100 μ l mouse ES medium was added, and the colony was

dissociated by pipetting up and down. The cell suspension was transferred to a feeder layer in 24-well plates and then was passaged to 6-well plates and then into 100-mm tissue culture dishes. The iPS cells were designated 3F-iPS, denoting their derivation from a 3-factor retro-differentiation.

EBs from 3F-iPS were formed by a hanging drop method (25) by using EB differentiation medium containing KDMEM, 15% ES qualified FBS, 2 mM L-glutamine, 1×10^{-4} M nonessential amino acids, 1×10^{-4} M 2-mercaptoethanol, and 1X Pen-Strep. After 2 days in hanging drop culture, EBs were collected and transferred to an ultra-low-binding dish (Corning) for another 2 days in EB differentiation medium.

Characterization of iPS Cells. Alkaline phosphatase staining was performed by using an ES-alkaline phosphatase detection kit (Cat # SCR004, Chemicon) according to the manufacturer's instructions. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde (EMD Chemicals) diluted in 1X PBS (Mediatech) for 10 min at room temperature. After washing with PBS, the cells were blocked with 5% normal goat and 5% BSA (Sigma), in 0.1% Triton X-100 (Fisher Scientific) for 1 h at room temperature. Primary antibodies included mouse anti-SSEA-1 (1:25 Chemicon), mouse anti-Oct-4 (1:25, Chemicon), mouse anti-Sox2 (1:50, Santa Biotechnology), mouse anti-Nanog (1:50, Santa Biotechnology), and rabbit anti-SALL4 (1:400, developed in house). The cells then were stained with fluorescent-labeled secondary antibodies including FITC-labeled anti-mouse IgG (Sigma) and FITC-labeled anti-rabbit IgG (Southern Biotech). Nuclei were counterstained with 1 mg/ml DAPI (Sigma) for 10 min.

Teratoma Formation. 3F-iPS cells were harvested by 0.25% trypsin treatment, collected into tubes, centrifuged, and the pellets were resuspended in EB differentiation medium. A 10^6 -cell suspension in 100 μ l was mixed with an equal volume of Geltrex (Invitrogen) and injected s.c. into SCID mice (Jackson Laboratory). A total of 5 mice were injected. One month after injection, teratomas were dissected and fixed with formalin (Fisher Scientific). Paraffin-embedded tissue was sectioned and stained with hematoxylin and eosin.

Endothelial Cell Differentiation. The EBs were transferred to 35-mm tissue culture dishes after 4 days of growth in EB differentiation medium and were allowed to differentiate into endothelial progenitor cells using medium containing KDMEM, 15% FBS, 2 mM L-glutamine, 1×10^{-4} M nonessential amino acids, 1×10^{-4} M 2-mercaptoethanol, 1X Pen-Strep, 20 ng/ml basic FGF (bFGF) (Invitrogen), 20 ng/ml EGF (Invitrogen), 50 ng/ml VEGF (R&D Systems), 20 ng/ml IGF (Sigma), 50 μ g/ml ascorbic acid (Sigma), and 1 μ g/ml hydrocortisone (Sigma). For endothelial progenitor cell differentiation, cells were collected after 10 days of differentiation using collagenase IV (Invitrogen) for identification and characterization. For mature endothelial cell differentiation, cells

were passaged by using 0.25% trypsin (Invitrogen) and replated on gelatin-coated dishes by using endothelial cell culture medium containing EGM-2 (Lonza), 15% FBS, 2 mM L-glutamine, 1×10^{-4} M nonessential amino acids, 1×10^{-4} M 2-mercaptoethanol, 1X Pen-Strep, 20 ng/ml bFGF, 20 ng/ml EGF, 50 ng/ml VEGF, 20 ng/ml IGF, 50 μ g/ml ascorbic acid, and 1 μ g/ml hydrocortisone. Cells were passed when dishes were 90% confluent.

Preparation of GFP and Labeling of Endothelial Cells. The GFP lentivirus (pSSI13772, a kind gift from Dr Bill Andrews, Sierra Sciences LLC) was prepared as described (26). Before transplantation, endothelial progenitor cells were infected with GFP lentivirus overnight with supplemental 8 μ g/ml Polybrene. Forty-eight hours postinfection, the endothelial cells were collected by trypsinization, suspended into single cells, and injected into the hemophilia A mice.

Characterization of Endothelial Cells by Immunofluorescence and Immunocytochemistry. Immunofluorescence methods used to characterize endothelial cells derived from 3F-iPS cells were similar to those used initially to characterize the 3F-iPS. Primary antibodies used included mouse anti-FLK1 (1:50, Alpha Diagnostic International), mouse anti-CD31 (1:25, BD PharMingen), rabbit anti-CD31 for histology (Biacore), mouse anti-GFP (Chemicon), and mouse anti-Factor VIII (1:50, Diagnostic Biosystems). Cells or tissue sections then were incubated with fluorescent-labeled secondary antibodies including rhodamine-labeled anti-mouse IgG, phycoerythrin-labeled anti-rabbit-IgG (Southern Biotech), FITC-labeled anti-mouse-IgG (Sigma), and FITC-labeled anti-rabbit-IgG (Southern Biotech). Nuclei were counterstained with 1 mg/ml 4',6'-diamidino-2-phenylindole DAPI (Sigma) for 10 min.

Real-Time PCR. RNA was extracted from samples by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. One μ g of RNA from each sample was reverse transcribed by using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed by using the Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. GAPDH (Eurogentec) was used as an endogenous control. Primer sequences used are listed in [supporting information \(SI\) Table S1](#). Analysis was done by using the Applied Biosystems SDS 7500 FAST software v. 1.4.

Plasma FVIII Activity Assay. FVIII activity was measured by chromogenic methodology by using the Chromogenix Coamatic Factor VIII kit (Instrumentation

Laboratory). Assays were performed in duplicate according to the manufacturer's instructions by using the microplate method and end-point detection as described for measuring extremely low levels of FVIII activity. A high (0%–150% FVIII activity) and low (0%–5% FVIII activity) standard curve was constructed by using serial dilutions of normal human reference plasma (Precision Biologic) which was calibrated against the 5th International World Health Organization standard Factor VIII and von Willebrand factor in plasma (NIBSC code 02/150). Murine FVIII protein shows 74% amino acid sequence homology with human FVIII, and the murine protein demonstrates conservation of functionally important domains (27). Wild-type C57BL/6 mice have FVIII levels $\approx$ 100% that of a normal human plasma standard.

Mouse Model. All animal studies were performed following National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee at Nevada Cancer Institute. Six hemophilia A mice (Jackson Laboratory, strain name: B6; 129S4-F8tm1Kaz/J, stock number: 004424) 6–8 weeks old were used for this study (28). Hemophilia A mice were produced originally by disruption of the FVIII gene through insertion of a targeting vector containing a neomycin gene cassette in the 3' end of exon 16 (28). The targeting vector was introduced into 129S4/SvJae-derived J1 ES cells and was injected into C57BL/6 blastocysts. Genotype of each hemophilia A mice was verified by PCR as described (28). Hemophilia A mice used in this study were irradiated with 200 cGy radiation 1 day before transplantation. We administered 4 U of FVIII recombinant protein by i.p. injection (Baxter Health Care) before transplantation to ensure survival of the hemophilia A mice. All mice tested also were anesthetized with 0.5 mg/g Avertin (Sigma) via i.p. injection before transplantation. Each mouse was injected with 2×10^6 endothelial progenitor cells derived from 3F-iPS cells via liver injection using 27-gauge needles. Brief pressure was applied to injection sites to ensure proper hemostasis. At various times (7, 30, 60, and 90 days) after transplantation, a tail-clip bleeding challenge was performed by cutting 1 cm from the end of the mouse tail and was used to assess correction of the hemophilia phenotype, as previously described (29). C57BL/6J control mice ($n = 6$), the closest relative of the hemophilia A mice model, and irradiated hemophilia A mice ($n = 6$) were injected with PBS as controls. Concurrently, plasma from each mouse tested was collected for FVIII level measurement at the indicated times of tail-clip challenge.

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