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An induced pluripotent stem cell-mediated and integration-free factor VIII expression system

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ABSTRACT

Human artificial chromosome (HAC) has several advantages as a gene therapy vector, including stable episomal maintenance and the ability to carry large gene inserts. Induced pluripotent stem (iPS) cells also have a great potential for gene therapy, which can be generated from an individual's own tissues and contribute to any tissues when reintroduced. A Sendai virus (SeV) vector with reprogramming factors is a powerful tool for generating iPS cells because of the high infection efficiency without the risk of integration into host chromosomes. In this study, we developed an iPS cell-mediated and integration-free coagulation factor VIII (FVIII) expression system using non-integrating SeV- and HAC-vectors. Multiple human FVIII genes, which were under the control of the megakaryocyte-specific platelet factor-4 (PF4) promoter for development of a treatment for hemophilia A, were inserted into a HAC vector (PF4-FVIII-HAC). The PF4-FVIII-HAC was introduced into SeV vector-mediated iPS cells derived from a mouse model of hemophilia A. After *in vitro* differentiation of iPS cells with the PF4-FVIII-HAC into megakaryocytes/platelets, the PF4-FVIII-HAC resulted in expression of FVIII. This study has developed the iPS cell-mediated PF4-driven FVIII expression system using two non-integrating vectors; therefore, this system may be a promising tool for safer gene- and cell-therapy of hemophilia A.

1. Introduction

Hemophilia A is an X chromosome-linked hemorrhagic disorder caused by defects in the coagulation factor VIII (FVIII) gene [1]. Current treatment consists of factor replacement with plasma-derived or recombinant FVIII products. However, these therapies are limited by the risk of infectious disease, the need for frequent injections, and the high cost of treatment [2]. Alternatively, gene therapy is an attractive approach for the treatment of hemophilia A, because a relatively modest increase in FVIII levels results in a sufficient therapeutic effect and it may provide sustained levels of FVIII. Hence, many groups have previously developed various strategies for gene therapy of hemophilia [3–5]. However, the ultimate cure for hemophilia by gene therapy has not been achieved because of several issues with conventional vector systems as follows; (1) the limited packaging capacity of vector particles, (2) the risk of oncogene activation caused by insertional mutagenesis, (3) over-expression or silencing, and (4) immune responses to the viral capsid [6].

Human artificial chromosome (HAC) vectors show considerable promise for gene therapy applications because they are stably maintained independent of host chromosomes as a mini-chromosome, thus diminishing or eliminating the risk of insertional mutagenesis [7]. In addition, HACs have the capacity to deliver an extremely large genomic region, such as 5 Mb [8], and allow physiological regulation of the introduced gene in a manner similar to that of the native chromosome [9–11]. Therefore, the use of HACs as a vector for gene therapy can solve the problems of conventional vectors, and are expected to be used for future gene- and cell-therapy.

Induced pluripotent stem (iPS) cells also have a great potential for gene therapy, which can be generated from an individual's own

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tissues and contribute to the specialized function of any tissue when reintroduced. However, a problem with iPS cell induction is integration of transgenes into host chromosomes, which includes the risk of oncogene activation [12]. Sendai virus (SeV) exists as a form of negative-sense single-stranded RNA in the cytoplasm of infected cells. Therefore, a SeV vector with reprogramming factors has been a solution for induction of integration-free iPS cells [13].

In the present study, we used the megakaryocyte-specific platelet factor-4 (PF4) promoter [14], and established a HAC vector containing multiple FVIII expression cassettes under the control of the PF4 promoter. We transferred the HAC vector into hemophilia A model mouse iPS (FVIII KO-iPS) cells induced by the SeV vector with reprogramming factors, and examined whether the HAC vector is able to induce FVIII expression in megakaryocytes. Although we must perform *in vivo* experiments to demonstrate the safety and efficiency of this strategy, the present findings suggest that this approach may be a promising strategy for safe gene- and cell-therapy of hemophilia A.

2. Materials and methods

2.1. Vector construction

We previously developed a P1 bacteriophage artificial chromosome (pPAC)-FVIII vector with FVIII under the control of the cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter [15]. In this study, the CAG promoter was excised by *XhoI* and replaced by the human megakaryocyte-specific PF4 promoter [14]. Multiple tandem copies of the FVIII expression cassette were constructed using compatible restriction sites. The FVIII expression cassette was excised by *AscI* and *AvrII*, and cloned into the pPAC-PF4-FVIII vector digested by *AscI* and *NheI*. Using this strategy, we obtained pPAC-PF4-FVIII with two and four copies of the FVIII expression cassette.

2.2. Cell culture

Hypoxanthine phosphoribosyl transferase (HPRT)-deficient Chinese hamster ovary (CHO) cells (JCR B0218) containing the HAC vector were cultured at 37 °C with 5% CO_2 in Ham's F-12 nutrient mixture (Invitrogen) plus 10% fetal bovine serum (FBS) with 8 μg/ml blasticidin S (BS; Funakoshi). Mouse iPS cells (see Section 2.8) were maintained on mitomycin C (Sigma)-treated mouse embryonic fibroblasts (MEFs) at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Wako) containing 20% FBS, 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1,000 U/ml leukemia inhibitory factor (Funakoshi).

2.3. Transient transfection

Human megakaryoblastic leukemia cell line UT-7/GM cells [16] were maintained in Iscove's modified Dulbecco's medium (Invitrogen) containing 10% FBS and 1 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF; Sigma). Cells were washed twice in PBS and resuspended in K-PBS buffer (31 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 10 mM $MgCl_2$) to a final concentration of 2×10^6 cells per 100 μl. These cells were mixed with each pPAC-PF4-FVIII vector, exposed to an exponential discharge of 150 V from a 25 μF capacitor using a Gene Pulser apparatus (Bio-Rad), and then transferred into culture medium. For induction of megakaryocytes/platelets differentiation, UT-7/GM cells were cultured for 72 h with 10 ng/ml thrombopoietin (TPO; a gift from Kirin Brewery) in place of GM-CSF.

2.4. Construction of PF4-FVIII-HAC

Modified pPAC-PF4-FVIII and Cre-recombinase expression vectors (pBS185; Invitrogen) were co-transfected into CHO cells containing the 21HAC2 vector [8] using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of culture in basic growth medium, cells were cultured in medium containing hypoxanthine–aminopterin–thymidine (HAT; Sigma). After 12 days of selection, HAT-resistant colonies were picked up and expanded for genomic PCR and fluorescence *in situ* hybridization (FISH) analyses.

2.5. Microcell-mediated chromosome transfer (MMCT)

MMCT was performed as described previously [17]. CHO cells containing PF4-FVIII-HAC were used as donor microcell hybrids. Briefly, mouse iPS cells were fused with microcells prepared from donor hybrid CHO cells, and then selected with BS (10 μg/ml). The transferred PF4-FVIII-HAC in each cell line was characterized by genomic PCR and FISH analyses.

2.6. Genomic PCR analyses

Genomic DNA was extracted from host cells containing PF4-FVIII-HAC using a Gentra Puregene Cell Kit (Qiagen), and PCR was performed using the following primers: FVIII-1 (sense, 5′-ggatcacctttttcaacatcg-3′; and antisense, 5′-tcttgaactgagggacactg-3′), FVIII-2 (sense, 5′-atacaacgctttctccccaa-3′; and antisense, 5′-gttcagtgtgttagtgtggc-3′), PF4 (sense, 5′-catatagttgtcagggaaggg-3′; and antisense, 5′-ggctgtttcttcattgtttcc-3′), and HPRT (sense, 5′-tggaggccataaacaagaagac-3′; and antisense, 5′-ccccttgacccagaaattcca-3′).

2.7. FISH analyses

FISH analyses were performed using either fixed metaphase or interphase spreads of each cell hybrid using digoxigenin-labeled (Roche) human Cot-1 DNA (Invitrogen) and biotin-labeled pPAC-PF4-FVIII DNA probes as described previously [17]. Chromosomal DNA was counterstained with DAPI (Sigma). Images were captured using an NIS-Elements system (Nikon) and Axio Imager-Z2 (Carl Zeiss).

2.8. Induction of iPS cells

Induction of iPS cells from hemophilia A model mouse embryonic fibroblasts (FVIII KO-MEFs; Jackson Laboratory, strain name: 129S4-F8tm1Kaz/J, stock number: 004424) was performed using a SeV vector system (Dnavec) as described previously [13]. Briefly, four SeV vectors containing Oct3/4, Klf4, c-MYC and Sox2 were used to infect FVIII KO-MEFs. At 6 days after infection, FVIII KO-MEFs were re-plated at 5×10^4 cells per 10-cm dish on MEF feeder cells. The next day, the medium was replaced with mouse iPS cell medium. At 24 days after transfection, mouse iPS-like colonies were selected and transferred onto feeder cells in 6-well plates.

2.9. Immunofluorescence staining

Immunofluorescence staining was performed using a primary anti-SeV polyclonal antibody (Medical and Biological laboratories) after fixation with 4% paraformaldehyde in PBS. A secondary anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (Molecular Probes) was used, followed by analysis with a fluorescence microscope (ECLIPSE Ti-U, Nikon).