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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).

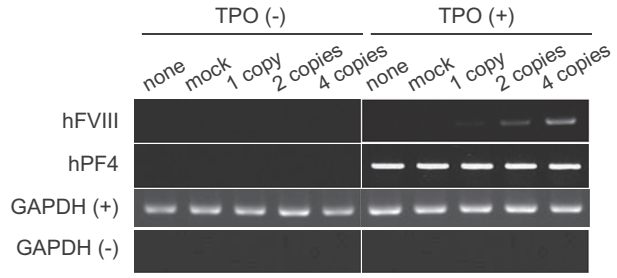

Fig. 1. UT-7/GM cells were transiently transfected with each of the pPAC-PF4-FVIII vectors (one, two and four copies) and cultured for 72 h with or without 10 ng/ml TPO. Total RNA was collected from these cells, and the levels of hPF4 and hFVIII mRNA were detected by RT-PCR. GAPDH was used as an internal control.

2.10. Differentiation of iPS cells

For differentiation into megakaryocytes/platelets, 1×10^4 iPS cells were seeded into each well of a 6-well plate containing confluent OP9 stromal cells (Riken BioResource Center) and cultured in α-MEM (Sigma) supplemented with 20% FBS and 1% L-glutamine [18]. After 5 days in culture, the cells were seeded onto a fresh OP9 layer in the same culture medium supplemented with 20 ng/ml human TPO. After 2 days of culture, the medium was changed every day. On day 12 of culture, FVIII expression was examined by RT-PCR.

2.11. RT-PCR

Total RNA was isolated from cells using Trizol (Invitrogen) and treated with a Turbo DNA-free kit (Ambion) to remove genomic DNA contamination. First-strand cDNA synthesis was performed using ReverTra Ace (Toyobo). After cDNA synthesis, cDNA was amplified by PCR using ExTaq (Takara). GAPDH and Nat1 were used as internal controls. PCR primers for amplification of each gene were as follows: Oct3/4 (SeV) (sense, 5′-cccgaaagagaaagcgaaccag-3′; and antisense, 5′-aatgtatcgaaggtgctcaa-3′), Sox2 (SeV) (sense, 5′-acaagagaaaaaacatgtatgg-3′; and antisense, 5′-atgcgctggttcacg cccgcgcccagg-3′), Klf4 (sense, 5′-acaagagaaaaaacatg tatgg-3′; and antisense, 5′-cgcgctggcagggccgctgctcgac-3′), c-MYC (sense, 5′-taac tgactagcaggcttgtcg-3′; and antisense, 5′-tccacatacagtcctggatgatgatg-3′), Oct4 (sense, 5′-tctttccaccaggcccccggctc-3′; and antisense, 5′-tgcgggcggacatggggagatcc-3′), Sox2 (endo) (sense, 5′-tagagctagactc cgggcgatga-3′; and antisense, 5′-ttgccttaaacaagaccacgaaa-3′), Nanog (sense, 5′-caggtgtttgagggtagctc-3′; and antisense, 5′-cggttcatcatgg-tacagtc-3′), Rex1 (sense, 5′-acgagtggcagtttcttcttggga-3′; and antisense, 5′-tatgactcacttccaggggcact-3′), hFVIII (sense, 5′-tctgccac actaacacactg-3′; and antisense, 5′-gggtcttgatgccgtgaata-3′), hPF4 (sense, 5′-agcatgagctccgcagccgggttct-3′; and antisense, 5′-cttccat tcttcagcgtggctatca-3′), mPF4 (sense, 5′-gtagaactttatctttgggt-3′; and antisense, 5′-aattttcctcccattcttca-3′), GAPDH (sense, 5′-ctcact-caagattgtcagca-3′; and antisense, 5′-gagttgggatagggcctc-3′), and Nat1 (sense, 5′-attcttcgttgtcaagccgccaaagtggag-3′; and antisense, 5′-agttgtttgctgcggagttgtcatctcgtc-3′). Amplified DNA fragments

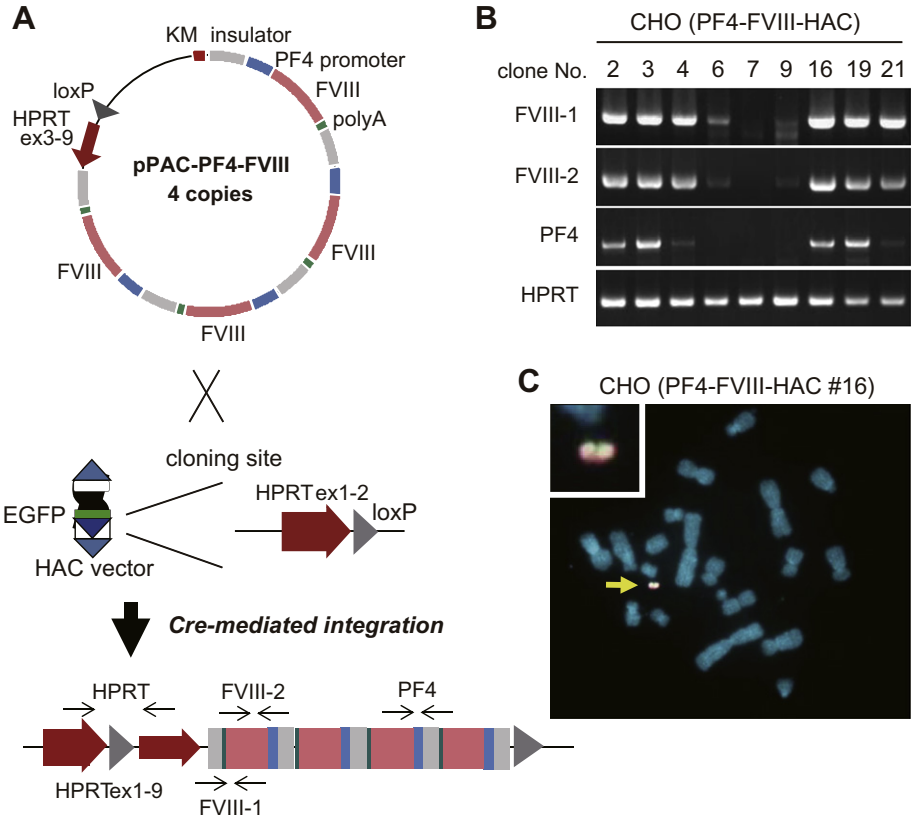

Fig. 2. (A) Schematic diagram of insertion of the FVIII expression cassette into the HAC vector. (B) Genomic PCR data of detecting PF4-FVIII-HAC in CHO cells. Each amplification site is mapped in (A, arrows). Representative data are shown. (C) FISH analysis of CHO cells. A digoxigenin-labeled human Cot 1 DNA probe (red) was used to detect the HAC vector. Biotin-labeled pPAC-PF4-FVIII (green) was used to detect the transgene on the HAC vector. Chromosomal DNA was counterstained with DAPI. The inset shows an enlarged image of the HAC vector (arrow). Representative data are shown.

were resolved by electrophoresis on a 2% agarose gel and visualized by ultraviolet fluorescence.

3. Results

3.1. Construction of the PF4-FVIII-HAC vector

To construct a HAC vector for gene expression of human FVIII (hFVIII) in megakaryocytes, the hFVIII gene was ligated to the human PF4 (hPF4) promoter. First, to demonstrate the ability of our construct to induce FVIII expression, human megakaryoblastic leukemia cell line UT-7/GM cells [16] was transiently transfected with each of the pPAC-PF4-FVIII vectors (one, two and four copies, see Section 2) (Fig. 1). Transfected cells were cultured with or without TPO, which has been shown to induce megakaryocytes/platelets differentiation of UT-7/GM cells [16], and differentiation into megakaryocytes/platelets was confirmed by detection of hPF4 mRNA expression (Fig. 1). As expected, hFVIII mRNA expression increased in a copy number-dependent manner.

The HAC vector contained a HPRT exon1-2-loxP cloning site into which circular DNA can be inserted using the Cre-loxP system. Eight micrograms of the pPAC-PF4-FVIII vector (four copies) was co-transfected with 1 μg of the Cre-recombinase expression plasmid into CHO cells containing an empty HAC vector (Fig. 2A). Twenty-eight HAT-resistant colonies were expanded and screened by genomic PCR analyses with primers for the FVIII transgene, PF4 promoter and reconstructed HPRT gene (Fig. 2A, arrow). Thirteen out of the twenty-eight HAT-resistant clones contained an intact PF4-FVIII-HAC (Fig. 2B), and then three clones were randomly selected for FISH analyses. FISH analyses with a biotin-labeled FVIII cDNA probe and digoxigenin-labeled human Cot1 DNA probe revealed that the presence of PF4-FVIII-HAC resulted in neither insertion nor translocation to the host chromosomes in two of the three clones (Fig. 2C).

3.2. Production of FVIII KO-iPS cells with the PF4-FVIII-HAC vector

FVIII KO-iPS cells were induced from FVIII KO-MEFs by infection with a SeV vector carrying four reprogramming factors, i.e., Oct3/4, Klf4, c-MYC and Sox2. Infected MEFs gave rise to iPS cell-like colonies that were isolated and expanded to cell lines. Two clones displaying a 40,*XY* normal metaphase were used for the following experiments (Fig. 3A).

PF4-FVIII-HAC vector was transferred into the two FVIII KO-iPS clones by MMCT. Because the HAC vector contained a green fluorescence protein (GFP) marker gene (Fig. 2A), after BS selection, GFP-positive clones were selected and examined in the following experiments (Fig. 3B). Thirteen of the resulting GFP-positive clones

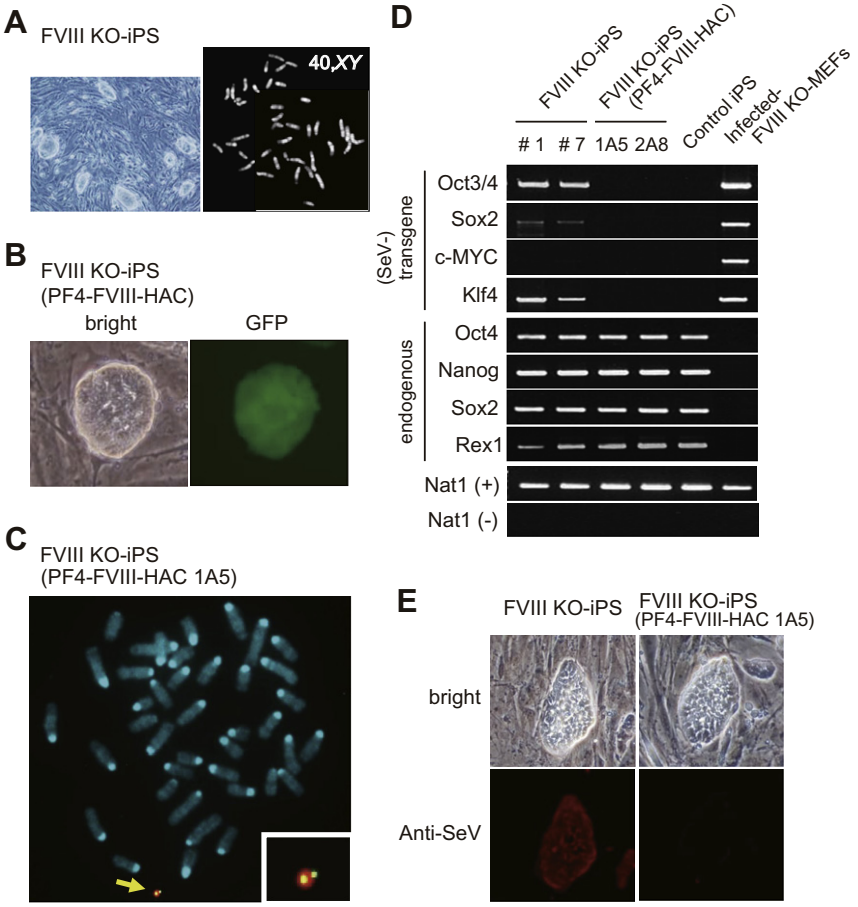

Fig. 3. Characterization of mouse iPS cells. (A) Morphology of FVIII KO-iPS clones (left panel). Q-banding metaphase are shown (right panel). (B) Morphology of FVIII KO-iPS (PF4-FVIII-HAC) cells. Bright field (left panel) and GFP fluorescence (right panel) microscopy images are shown. (C) FISH analysis of FVIII KO-iPS (PF4-FVIII-HAC) cells. A digoxigenin-labeled human Cot 1 DNA probe (red) was used to detect the HAC vector. Biotin-labeled pPAC-PF4-FVIII (green) was used to detect the transgene on the HAC vector. Chromosomal DNA was counterstained with DAPI. The inset shows an enlarged image of the HAC vector (arrow). Representative data are shown. (D) RT-PCR analyses of ES cell marker genes and four exogenous transcription factors. Nat1 was used as an internal control. Representative data are shown. (E) Immunostaining of parent FVIII KO-iPS cells and iPS cells containing PF4-FVIII-HAC. Bright field (top panel) and fluorescence (bottom panel) microscopy images are shown. Representative data are shown.

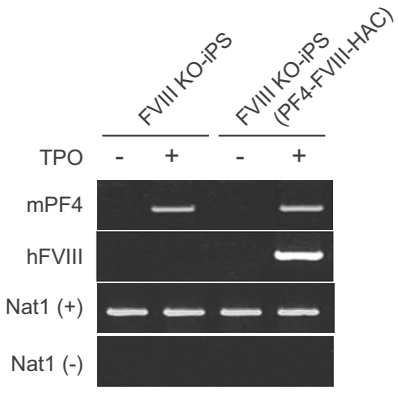

Fig. 4. *In vitro* differentiation of FVIII KO-iPS cells with or without PF4-FVIII-HAC. RT-PCR analyses of mPF4 and the hFVIII gene. Nat1 was used as an internal control.

were screened by genomic PCR analyses using primers to detect PF4-FVIII-HAC. Ten out of thirteen clones contained an intact PF4-FVIII-HAC (data not shown), and then FISH analyses showed that PF4-FVIII-HAC was present as an individual chromosome and normal karyotype in four clones (Fig. 3C). These four clones showed that the PF4-FVIII-HAC can be transferred to FVIII KO-iPS cells without affecting host chromosomes. Next, to test the stemness or undifferentiated state of these iPS cells, RT-PCR analyses using primers for embryonic stem (ES) cell-specific genes were performed. Endogenous Oct4, Nanog, Sox2 and Rex1 were expressed in two parent FVIII KO-iPS cell lines (#1 and #7) and all FVIII KO-iPS containing PF4-FVIII-HAC [FVIII KO-iPS (PF4-FVIII-HAC)] clones, comparable with that in control iPS cells (Fig. 3D). Exogenous SeV-transgenes, Oct3/4, Sox2, c-MYC and Klf4, were strongly or weakly expressed in two parent FVIII KO-iPS cell lines. However, expression of these genes was undetected in two FVIII KO-iPS (PF4-FVIII-HAC) clones (1A5 and 2A8) (Fig. 3D and E), suggesting that SeV-transgene expression was more or less decreased during cell division or after MMCT re-cloning. These results indicated that two clones (1A5 and 2A8) were SeV-free pluripotent stem cells and displayed a normal karyotype and independent PF4-FVIII-HAC vector.

3.3. Analyses of FVIII expression

We investigated whether *in vitro* megakaryocytes/platelets differentiation would result in FVIII expression. We cultured FVIII KO-iPS (PF4-FVIII-HAC) cells with TPO to induce differentiation, and then megakaryocytes/platelets differentiation was confirmed by detection of mPF4 mRNA expression (Fig. 4). FVIII expression was detected in megakaryocytes/platelets derived from FVIII KO-iPS (PF4-FVIII-HAC) cells, whereas undifferentiated cells showed no FVIII expression (Fig. 4). Taken together, these results show that PF4-FVIII-HAC vector accomplishes ectopic expression of FVIII in megakaryocytes/platelets derived from FVIII KO-iPS cells.

4. Discussion

Gene- and cell- therapy requires the development of an efficient and safe gene delivery system to be acceptable for clinical applications. Previously, both non-viral and viral vectors have been considered for the development of gene therapy of hemophilia in various animal models [3,19–21]. HAC vectors also support the correction of defective genes because expression from and transmission of these vectors are stable throughout many cell divisions [7,15,22]. In addition, because of their episomal nature, silencing of the introduced gene or oncogenesis resulting from integration into host chromosomes should be minimized. Another advantage of the HAC vector is transference into various cell types by MMCT methods without insertional mutagenesis. Nonetheless, the transfer rate of HAC via MMCT method is low, i.e., 10^5. Nevertheless, the MMCT frequency needs to improve. Moreover, in MMCT methods, it is possible to simultaneously transfer host chromosomes (e.g., CHO) along with the HAC. However, using FISH, karyotyping and RT-PCR, we have seldom observed co-transfer of host chromosomes during MMCT. Use of the HAC vector for FVIII expression is therefore expected to contribute to the development of new treatments for hemophilia A.

Integration of transgenes into host chromosomes is a major technical hurdle for clinical application. Although several methods for induction of iPS cells have been developed using adenoviruses [23] or plasmids [24], the risk of integration still remains while DNA vectors are used. In this study, we used a SeV vector, an RNA virus with no risk of altering host chromosomes, for iPS cell generation and successfully obtained exogenous transgene-free iPS cells. In addition, the HAC vector has a major advantage by being maintained independently of host chromosomes. Application of these methods to iPS cells combined with the HAC vector may be a powerful tool for safe gene- and cell- therapy.

In conclusion, we have developed HAC vectors containing multiple FVIII expression cassettes for gene therapy of hemophilia A, and transferred PF4-FVIII-HAC into SeV-free iPS cells, which is ectopically expressed in megakaryocytes/platelets. By combining integration-free iPS cells derived from patients [11,25,26], this HAC vector may be a promising strategy for novel and safe gene- and cell-therapy of hemophilia A. However, for such validation of the expression level or the therapeutic effect, *in vivo* experimental confirmation remains to be performed. Furthermore, several groups have reported that ectopic expression of the FVIII gene in megakaryocytes/platelets is therapeutic towards hemophilia A with inhibitory antibodies [27,28]; therefore, we need to verify the effect of inhibitory antibodies.

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