Cloning & Transgenesis

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Toward Gene and Cell Therapies Employing Human Artificial Chromosomes in Conjunction with Stem Cells

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Abstract

Various Stem cells including, induced Pluripotent Stem Cells (iPSCs) are expected to contribute to the development of personalized regenerative medicines. However, human diseases are often associated with large genomic lesions which are beyond the capacity of most conventional vectors. Also a native genomic environment is required to express right protein isoform in the right time and right cell type. Human Artificial Chromosome (HAC) vectors, which have no limits with respect to the size of inserts and can be independently maintained without integration into or potential disruption to host chromosomes, therefore are expected to play a significant role in gene and cell therapy in the future. Various applications and methods have been developed using HAC vectors; however, in this review we introduce current research focused towards gene and cell therapy of Duchenne muscular dystrophy and hemophilia A using stem cells. In addition, the use of HAC vectors to generate iPSCs with a safeguard system to eliminate tumorigenic cells is discussed.

Keywords: Human artificial chromosome; Stem cells; Gene therapy; Cell therapy

Abbreviations: Sgca: α-sarcoglycan; BACs: Bacterial artificial chromosomes; CMs: cardiomyocytes; CHO: Chinese hamster ovary; DMD: Duchenne muscular dystrophy; EB: embryoid body; ESC: Embryonic stem cell; FVIII: Factor VIII; GFP: Green fluorescent protein; HAC: Human artificial chromosome; MMCT: Microcell-mediated chromosome transfer; hiMSCs: human immortalized Mesenchymal Stem Cells; HIDEMs: Human iPSC-derived MAB-like stem/progenitors; IF : Immunofluorescence; iHAC: inducing human artificial chromosome; iPSCs: induced pluripotent stem cells; i.a : intra-arterially; i.m: intramuscularly; KO: knockout; LGMD2D: Limb-girdle muscular dystrophy; MABs; Mesoangioblasts; PACs: Phage artificial chromosomes; PF4: Platelet factor-4; RT-PCR: Reverse-transcription polymerase chain reaction; SMA: Spinal muscular atrophy; CRISPR : Clustered Regularly Interspaced Short Palindromic Repeat; TPO: Thrombopoietin; HSV-1: Herpes simplex virus type 1; PDL: Population doubling

Introduction

Various types of stem cell are thought to have great potential in the realization of personalized regenerative medicine. In particular, induced Pluripotent Stem Cells (iPSCs) can be conveniently generated from most tissue of patients with various disorders [1–3]. Recently, a clinical trial for cell transplantation in age-related macular degeneration using iPSCs has been approved in Japan [4,5]. Various iPSCs derived from patients with genetic disorders such as Spinal Muscular Atrophy (SMA) [6], Fanconi anemia [7] and Duchenne muscular dystrophy (DMD) [8,9] have been generated for the realization of personalized medicine.

The following conditions are ideally required for gene and cell transplantation therapies using iPSCs: (i) efficient generation of iPSCs from patients with genetic disorders, ideally by the use of technologies with minimal genetic alterations or small molecules only; (ii) repair of defective genes or chromosomal regions in stem cells from patients with minimal interference to the host genome before transplantation; (iii) differentiation of the cell types that patients need and optimized cell transplantation therapy techniques to treat the relevant disease gene; and (iv) development of a safeguard system to prevent tumor

formation by transplanted cells derived from iPSCs. In this review, we introduce the technology of Human Artificial Chromosome (HAC), and the current status and challenges of gene and cell transplantation therapies against DMD and hemophilia using stem cells and how HACs may help to develop personalized medicine.

HAC Vectors

The eukaryotic chromosome is linear, with one centromere and two telomeres at the ends of each chromosome. The chromosome replicates from the replication origins during the S-phase of the cell cycle. The centromere proteins, CENP-A, -B, and -C, located in the centromere sequence, assist with assembly of the kinetochore complex during the M phase. Then, the spindle fiber is attached to the kinetochore, and the sister chromosomes are distributed equally to both poles. In this way, replicated chromosomes are equally distributed to two daughter cells, and the genomic information of a mother cell is equally and stably transmitted to daughter cells. Physiologically, the same mechanisms are used for the replication and transmission of the HACs [10].

Generation of HAC vectors

Various HAC vectors have been developed using the bottom-up method [10,11], natural chromosome fragments [12] and the top-down method [13–15]. In our laboratory, we developed the 21HAC vectors [15], derived from human chromosome 21 in normal fibroblasts [16] (Figure 1). Current chromosome engineering technology for the generation of HAC vectors involves insertion of a loxP site and targeting telomere-associated chromosomal fragmentation at both

ends of telomeres by homologous recombination using chicken DT40 cells. Large inserts are transferred into recipient cells using a Microcell-Mediated Chromosome Transfer (MMCT) technique [17,18].

Targeted insertion of loxP sites by homologous recombination

To insert the loxP site [19] into chromosome 21, we generated a linearized plasmid vector harboring two homologous arms (5 kb) at both ends, a loxP site, and the first and second exons of the HPRT gene to isolate only cells containing recombinants using hypoxanthine-aminopterin-thymidine selection by Cre-loxP system. When the linearized plasmid vector was electroporated into DT40 cells containing human chromosome 21, the vector was inserted at the target site through homologous recombination (Figure 1).

Telomere truncation

Targeted telomere-associated chromosomal fragmentation is known as telomere truncation. Because original gene regions from human chromosome 21 were present from the introduced loxP sites, it was necessary to remove unwanted gene regions by telomere truncation so as to avoid unexpected gene expression. For this, we prepared a linearized plasmid vector harboring a 5 kb homologous arm at one side and a 1 kb artificial telomere (TTAGGG)n at the opposite side [10,13] ,and electroporated it into DT40 cells. Homologous recombination occurred at the target site and the downstream region of the chromosome was deleted and replaced with the telomere sequence. Thus, the 21HAC vector was generated, which harbors no endogenous gene, but permits to insert transgenes using the Cre-loxP system (Figure 1).

Gene Expression using 21HAC Vectors

Insertion of circular plasmid vectors into 21HAC

Transgenes can be easily inserted into the 21HAC vector by co-transfection with a Cre-expression vector and a circular plasmid vector containing the gene(s) of interest, a dominant selectable marker and loxP site, such as Phase Artificial Chromosomes (PACs) and Bacterial Artificial Chromosomes (BACs).

Cloning entire genomic regions by translocation in 21HAC vectors

A larger genomic region can be inserted into 21HAC vectors than conventional vectors (Figure 2). As 21HAC vectors have the same character with natural chromosomes, 21HAC vectors can carry similar size of DNA component of natural chromosomes. Thus, chromosome 1 contains more than 230 Mb [20]. Translocation-mediated insertion can be used for an extremely large genomic fragment (Figure 5). We have already succeeded to load and transfer 30 Mb DNA fragment concerning the Down syndrome critical region by this method (unpublished data). The introduction of a large insert and/or gene clusters into targeted cells allows for correct expression of transgenes by native regulatory elements [10,21]. Because the 21HAC vector has been generated in this manner, there are a number of advantages associated with its use (Figure 3). First, the 21HAC vector is maintained independently from host chromosomes without insertion; therefore, it does not disrupt a host gene and is not subject to positional effects. Second, the 21 HAC is stably maintained over long periods *in vivo* and *in vitro*. Third, there is no limitation with respect to the size of DNA that can be inserted. Physiological gene expression is expected, and it is thought to be resistant to gene silencing by epigenetic modification

[10]. These features are important for future clinical application of gene and cell therapies using HACs derived from the 21HAC vector.

Examples of Gene and Cell Therapies using HACs

Towards future gene and cell therapy, a number of genes have been loaded onto HACs (Table 1). Here some examples are introduced.

Duchene muscular dystrophy (DMD)

DMD is a genetic disorder of a progressive myopathy, involving paralysis and premature death, and affects 1 in 3,500 newborn males.
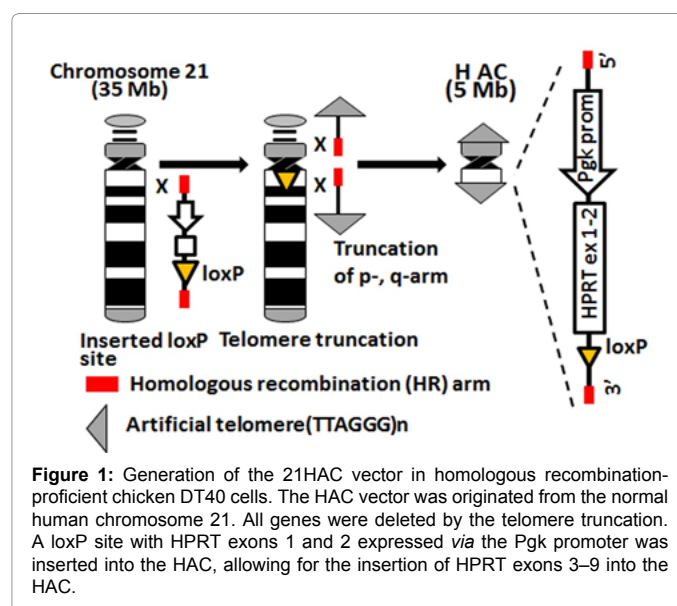

Figure 1: Generation of the 21HAC vector in homologous recombination-proficient chicken DT40 cells. The HAC vector was originated from the normal human chromosome 21. All genes were deleted by the telomere truncation. A loxP site with HPRT exons 1 and 2 expressed *via* the Pgk promoter was inserted into the HAC, allowing for the insertion of HPRT exons 3–9 into the HAC.

Figure 2: Advantages of the 21HAC vector. Conventional vectors are limited to the size of the gene that can be inserted [44–45]. The limitation of viral vectors depends on the kind of virus. The largest one is HSV-1 vectors [46]. The 21HAC vector can carry genes and genomic regions of millions nucleotide base pare size. (Modified from Oshimura et al. [10]).
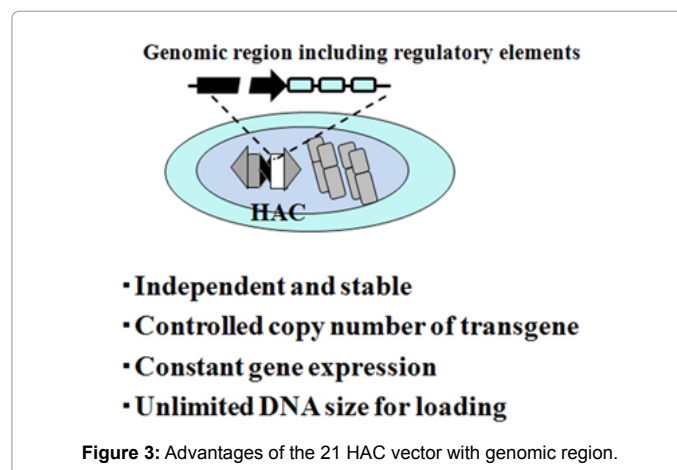

Figure 3: Advantages of the 21 HAC vector with genomic region.

Inserted genes	Aim	References
Ubc-hTERT-IRES-GFP	Extended lifespan of normal fibroblasts	[44]
PGK-ScFv-gp130-IRES-EGFP	Antigen-mediated growth control	[45,46]
TR-DNA-PKcs	Tetracycline-mediated inducible gene expression system	[47]
Mouse CD40L	BAC-PAC-mediated gene expression system for gene therapy	[48]
Human HPRT	TAR cloning-mediated or ready made PAC-mediated gene insertion	[49,50]
HSP70-Insulin	Heat-regulated gene expression system	[51]
Human TP53	Genetic correction in mGS cells	[50]
OPN-EGFP	Lineage-specific gene expression	[52]
CMV-human EPO	Therapeutic protein expression in normal fibroblasts	[53]
OC-GFP	Evaluation system for bioactive substances	[54]
MC1-HSV-TK	Suicide gene- and MSC-mediated treatment of glioma	[55]
Human dystrophin	Genetic correction in MABs and differentiation of iPSCs to MABs	[8,9,23,26]
Yamanaka factors and p53shRNA	Generation of iPSCs	[32]
CAG-human FVIII (1-16copies)	Copy number-dependent gene expression system	[28]
PF4-human FVIII (1-4copies)	Platelet-specific FVIII expression	[31]

Table 1: 21HAC vectors used for gene delivery toward gene- and cell-therapy.

It is caused by a loss of the dystrophin gene located on Xp21. The dystrophin gene contains 14 kb of complementary DNA, 79 exons, and encodes the dystrophin protein, which is a key structural protein of the nuclear membrane. The dystrophin gene is 2.5 Mb including regulatory elements, and contains many promoters and transcriptional start sites to express specific isoforms for each organ [22]. Given that 60% of DMD patients have multiple disruptions of exons, the introduction of the entire dystrophin gene is required to cure DMD. Some attempts to replace this gene have been made; however, conventional therapeutic methods using viruses are only able to insert around 20 kb. It has also been shown that therapeutic virus vectors containing dystrophin cDNA are unable express the various dystrophin isoforms [22]. Because of these problems, a vector that can carry a large gene, including the regulatory elements, is required. It is expected that the 21HAC vector could be used to clone and introduce the entire dystrophin gene in DMD patients for their treatment [8,9].
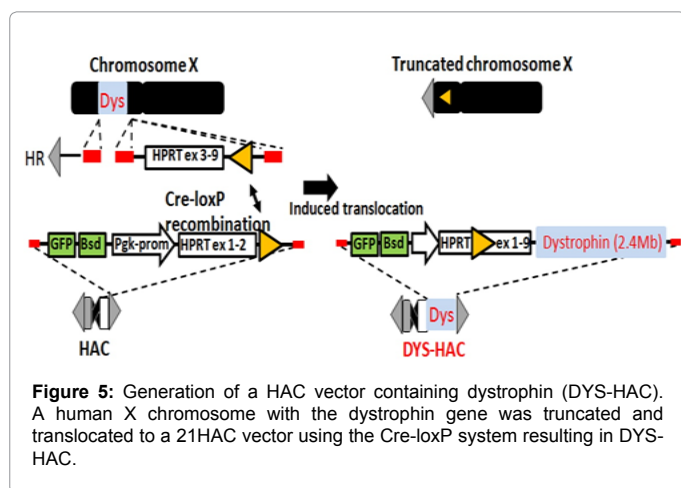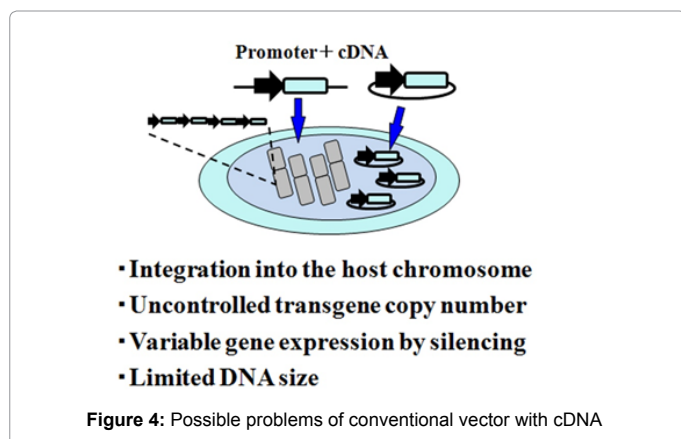
Translocation cloning of the dystrophin gene: A human X chromosome containing the dystrophin gene was transferred to DT40 cells and a loxP site inserted downstream of the dystrophin gene by homologous recombination (Figure 5) [8,9]. Exons 3–9 of the HPRT gene and a hygromycin gene (as a positive selectable marker) were simultaneously inserted near the dystrophin gene. Using telomere seeding, unwanted genes upstream of the dystrophin gene were deleted from the X chromosome. The modified X chromosome fragment was transferred to CHO cells containing the 21HAC vector with a loxP site, and exons 1–2 of the HPRT gene, to allow for selection of the correctly oriented HPRT gene. The dystrophin gene locus was then translocated to the 21HAC vector using Cre-loxP recombination at two sites in the two chromosomes. Thus, the HAC vector containing the dystrophin gene locus was generated and designated DYS-HAC. It was investigated whether DYS-HAC could compensate for genetic disruption in iPSCs derived from a DMD mouse model, and future DMD patients (Figure 6).

Introduction of DYS-HAC to iPSCs derived from a DMD mouse model: Fibroblasts from *mdx* mice, a murine model of DMD, were used to generate iPSCs (mdx-iPSCs) [8]. MMCT was used to introduce the DYS-HAC into the mdx-iPSCs, thereby correcting aberrant gene expression. Chimeric mice were generated using the corrected mdx-iPSCs. By monitoring the expression of GFP, a marker protein, DYS-HAC was observed to be maintained stably and independently. RT-PCR analyses showed tissue-specific expression of the Dp427l,

Dp427m, and Dp140 isoforms, along with sarcolemma localization of the dystrophin protein, demonstrating that genetic correction of the dystrophin gene using DYS-HAC was achieved.

Cell therapy in skeletal muscle using mesoangioblasts (MABs) derived from SCID/mdx-iPSCs containing DYS-HAC: Mesoangioblasts (MABs) were isolated from *mdx* mice and transferred along with DYS-HAC by MMCT to replace the dystrophin gene [22]. Prior to transplantation, MyoD was introduced into MABs using lentiviral transduction. The MABs were then transplanted into SCID/mdx mice intra-arterially (i.a.) or intramuscularly (i.m.). Transplanted cells were monitored by GFP expression to determine whether they were engrafted in skeletal muscle. Skeletal muscles of SCID/mdx mice were stained with Evans Blue, which shows damage in skeletal muscles. The results indicated a spread of GFP-positive engrafted MABs containing the DYS-HAC, and these cells exhibited reduced damage. Immunofluorescence (IF) analysis of skeletal muscle fibers showed that the dystrophin protein was localized to the sarcolemma. Treadmill tests showed that the treated SCID/mdx mice had about 1.7-fold (177%) of exercise tolerance compared with before cell transplantation. Around 250–270 days following i.m. administration of SCID/mdx MABs, exercise tolerance capacity was decreased 33% compared with the starting point. The treated group maintained over 80% of exercise tolerance capacity. The distance run for 24h among mice in the treated group was increased 2.5–4-fold compared with that for the untreated group [22]. These results showed that DYS-HAC could compensate for the mutated dystrophin gene in SCID/mdx MABs without disruption of the host genome. The corrected MABs could improve the function of skeletal muscle without tumor formation. The MABs that fused with the skeletal muscle fiber of the SCID/mdx mice expressed the dystrophin protein, and also prevented membrane fragility and progressive paralysis in mice.

Generation of human iPSCs from DMD patients: DYS-HAC was introduced into fibroblasts derived from a patient who had a large gene deletion from exons 4–43. Human iPSCs were generated from the fibroblasts containing DYS-HAC through the introduction of reprogramming factors (KLF4, OCT4, c-MYC, and SOX2) using retrovirus vectors (Figure 7) [9]. Pluripotency of the iPSCs was shown by the detection of pluripotency markers, and through the generation of teratomas. In the teratomas of immunodeficient nude mice, differentiation of skeletal muscle cells was observed and normal human dystrophin localized to sarcolemma [9].

Figure 4: Possible problems of conventional vector with cDNA

Figure 5: Generation of a HAC vector containing dystrophin (DYS-HAC). A human X chromosome with the dystrophin gene was truncated and translocated to a 21HAC vector using the Cre-loxP system resulting in DYS-HAC.

Zatti et al. verified expression of DYS-HAC isoforms in Cardiomyocytes (CMs) induced from a healthy iPSC clone, DYS-HAC iPSCs, and a DMD patient (DMD-iPSCs) [24]. They were differentiated into CMs *via* Embryoid Body (EB) formation and subjected to a staged differentiation protocol [25]. Over 16 days of differentiation, spontaneously contracting EBs were observed. To promote further maturation of CMs in the EBs, EBs were cultured in suspension for 20 days and then seeded on a hydrogel substrate for 4 days. The proportion of cTnT-positive CMs obtained from the healthy iPSC clone was $9.8 \pm 4.6\%$, $8.2 \pm 1.5\%$ for DYS-HAC, and $7.6 \pm 0.7\%$ for DMD-iPSCs. These findings show that the iPSC clones could be differentiated into mature CMs [24].

Differentiation of MABs from human iPSCs: To extrapolate the results of gene and cell therapy from a mouse model towards human DMD patients through the use of autologous iPSCs, a procedure is required to differentiate iPSCs into MABs (Figure 6). Tedesco et al. demonstrated cell therapy for Limb-Girdle Muscular Dystrophy (LGMD2D) [26]. Human iPSC-derived MAB-like stem/progenitor (HIDEM) cells were differentiated from LGMD2D-iPSCs. FACS analysis of HIDEMs revealed expression of the following differentiation markers: CD13+, CD44hi, CD49b+ and CD146+. HIDEMs had the ability to proliferate *in vitro*, but did not exhibit tumorigenicity *in vivo*.

To show that HIDEMs could treat DMD model mice, they were transplanted into α-sarcoglycan (Sgca)-null/scid mice. The HIDEMs introduced α-sarcoglycan as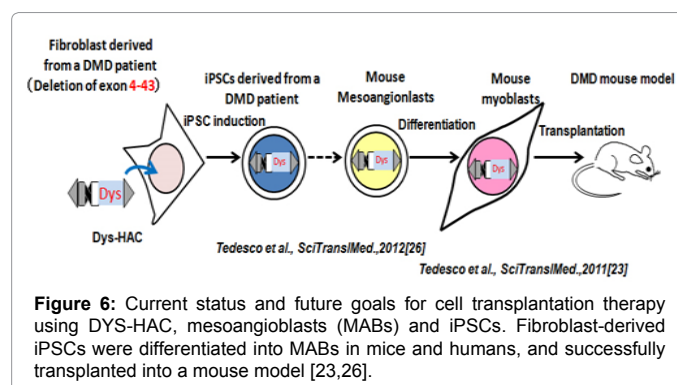 the therapeutic gene and were induced using tamoxifen. Between 12–24h post-transplantation, HIDEM migration was observed using GFP. Various PCR assays confirmed the presence of transplanted HIDEMs in skeletal muscle. Expression of SCGA was also detected in skeletal muscle for up to 1 month after transplantation. To verify exercise tolerance capacity, treadmill tests were conducted and revealed that mice showed enhanced exercise tolerance capacity after treatment. The treated mice ran 48–62% more compared with their baseline performance, and ran 12–22% more compared with untreated animals 35 days after transplantation. Differentiation of HIDEMs with human iPSCs containing DYS-HAC was also successful, suggesting that iPSCs with DYS-HAC can be used for cell therapy with MABs (Figure 6).
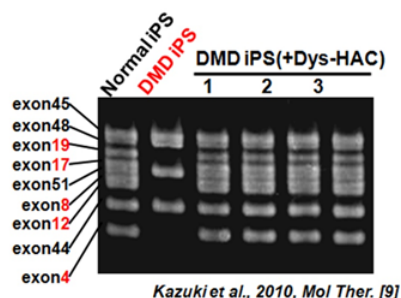
Gene and cell therapy of hemophilia A using HAC

Hemophilia A is an X-linked hemorrhagic disorder associated with aberrant expression of coagulation factor VIII (FVIII), which is encoded on chromosome Xq28. General treatment of hemophilia A involves FVIII replacement therapy. Once in the blood, the half-life of FVIII is short. It has also been reported that neutralizing antibodies against FVIII can be produced, ameliorating its effects. FVIII replacement therapy improves the quality of life for patients. Gene therapy for hemophilia A using virus vectors has been trialed; however, there are issues such as production of antibodies against viral antigens and tumorigenesis and disruption of host genome by transgenes [27]. Because the 21HAC vector can introduce transgenes stably without the disruption of the host genome, Kurosaki et al. attempted to develop a therapeutic vector that can achieve higher and more stable expression of FVIII.

Generation of a FVIII-expressing HAC vector (CAG-FVIII-HAC): When performing cell transplantation therapy, high expression levels of the FVIII protein are required to achieve systemic hemostatic effect. A circular vector derived from a PAC that included a loxP site, a HPRT gene and a FVIII cDNA cassette with a CAG promoter (CAG-FVIII-PAC) was generated [28]. To increase protein expression levels, multiple copies (2, 4, 8, 16 copies) of the FVIII cassette were made to produce CAG-FVIII (1–16) PAC. These were then inserted into the 21HAC vector by co-transfection with a Cre-recombinase-expressing vector using lipofection in CHO cells [CAG-FVIII (1–16)-HAC] (Figure 8).

Comparison of FVIII mRNA expression levels showed a correlation between mRNA expression level and the copy number of the FVIII cassette. CAG-FVIII (4)-HAC and CAG-FVIII (16)-HAC were introduced, by MMCT, into human immortalized mesenchymal stem cells (hiMSCs). There was FVIII activity in the released FVIII from hiMSCs containing CAG-FVIII-HACs. The CAG-FVIII-HACs were

Figure 6: Current status and future goals for cell transplantation therapy using DYS-HAC, mesoangioblasts (MABs) and iPSCs. Fibroblast-derived iPSCs were differentiated into MABs in mice and humans, and successfully transplanted into a mouse model [23,26].

Figure 7: Gene correction of iPSCs derived from a DMD patient [9]. Genomic multiplex PCRs were used to detect the dystrophin genome in DYS-HAC of DMD-iPSCs. Red lines indicate exons deleted in DMD-iPSCs.
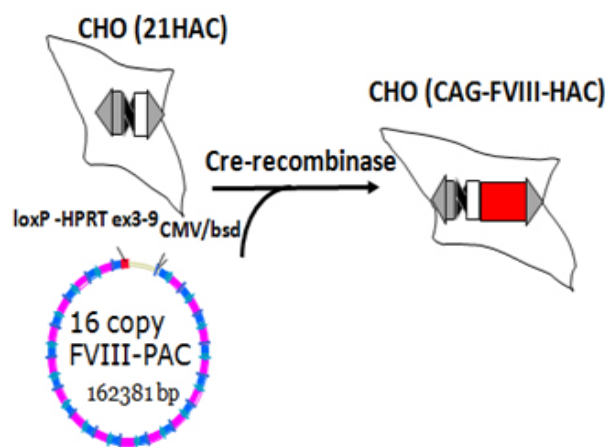

Figure 8: Toward gene and cell therapy for hemophilia A using a 21HAC vector. Insertion of genes into 21HAC using a PAC vector with a loxP site and 16 copies of FVIII-HAC. HPRT-deficient Chinese hamster ovary clones with a 21HAC vector was previously established. This 21HAC vector contained a loxP site and exons 1–2 of the HPRT gene.

resistant to gene silencing. CAG-FVIII PAC-derived expression was silenced during propagation of cells in most clones (Figure 9). These data show that the 21HAC vector has advantages for high protein production and long-term stability.

Platelet-specific FVIII expression using the 21HAC vector: When patients were treated using FVIII replacement therapy, 20–30% of patients produced neutralizing antibodies [29]. To circumvent this, a platelet factor-4 (PF4) promoter was used for specific expression of platelet-localizing damaged blood vessels [30] (Figure 10). A PAC vector containing a loxP site, the HPRT gene, and FVIII expression cassettes expressed via the PF4 promoter was generated (PF4-FVIII-PAC); expression cassettes contained 2 or 4 copies of PF4-FVIII [31]. To confirm FVIII expression, the PAC vectors were transfected into the megakaryocytic cell line UT-7/GM. Transfected cells were stimulated with human Thrombopoietin (TPO), which induces megakaryocyte/platelet differentiation in the UT-7/GM cell line. As expected, the transfected cells expressed human FVIII mRNA, with levels increasing in a copy number-dependent manner. PF4-FVIII (4)-PAC was inserted into the 21HAC vector using the Cre-loxP system (PF4-FVIII-HAC) in CHO cells. PF4-FVIII-HAC was transferred into mouse iPSCs derived from FVIII knockout (KO) mice. PF4-FVIII-HAC was maintained

independently from the host chromosomes, and the karyotype of the FVIII KO-iPSC-harboring PF4-FVIII HAC was normal. *In vitro* differentiation into megakaryocytes and platelets with FVIII KO-iPSC-harboring PF4-FVIII HAC showed that human FVIII expression was induced in megakaryocytes and platelets [31]. Treatment of FVIII KO mice with PF4-FVIII-HAC is expected to verify its therapeutic effects.

Generation of iPSCs using the 21HAC vector

Inducing HAC (iHAC) vectors for reprogramming: iPSCs have been generated from various types of somatic cells through the
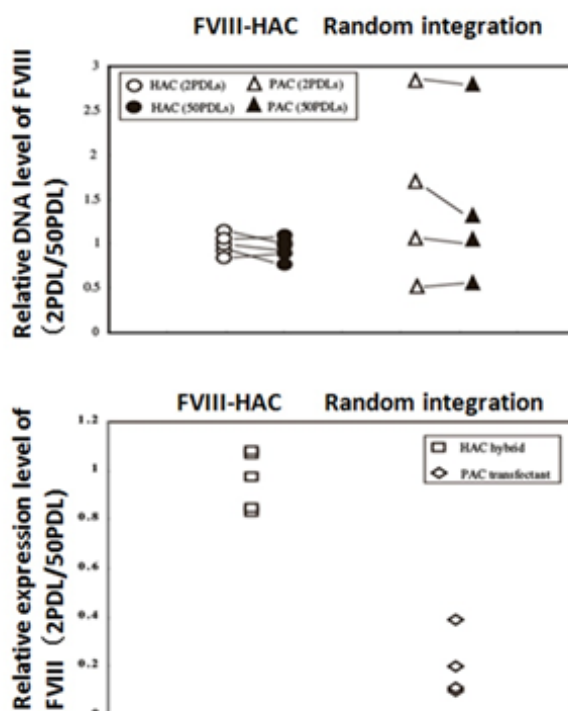

Figure 9: Comparison of gene expression stability between CAG-FVIII (16)-HAC and randomly integrated CAG-FVIII (16)-PAC (modified from Kurosaki *et. al.* [28]). Graphic representation of the FVIII transgene and its expression in hiMSC hybrids and transfectants after 2 (initial stage of cloning) and 50 population doublings (PDLs) (long-term culture after selection withdrawal). Genomic PCR analysis of FVIII and the b-actin control (top). The number of FVIII sequences in one of the five hiMSC hybrids clones at 2PDLs was used as a standard. Analysis of relative FVIII expression by RT-PCR at 50 PDLs to 2 PDLs (bottom). FVIII expression levels at 50 and 2 PDLs were standardized to GAPDH expression, and the relative FVIII expression in each clone at 2PDLs was used as the control.
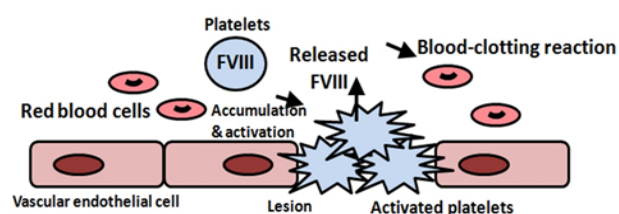

Figure 10: Hemostasis model of local FVIII expression with a platelet-specific PF4 promoter. Local expression and release of FVIII surrounding a lesion are expected. Only activated platelets released FVIII.

introduction of viral vectors containing reprogramming factors such as KLF4, SOX2, OCT4, c-MYC. Virus vectors tend to disrupt the host genome when transgenes are introduced. Two copies of Sox2, Klf4, and c-Myc, four copies of Oct4, and a p53 knockdown cassette were introduced into 21HAC (iHAC). Multiple copies of the reprogramming factors were used to increase mRNA expression levels [32].

An iHAC-containing GFP was introduced into Mouse Embryonic Fibroblasts (MEFs), which were transfected with miR293/294 to increase reprogramming efficiency. After 12 days, cells with an Embryonic Stem Cell (ESC)-like morphology appeared, were GFP-positive, and strongly proliferative. The iHACs were maintained independently from the host chromosome in these cells. 21HAC vectors are spontaneously lost at a low frequency during host cell division [32]. Therefore, a GFP-negative fraction of MEFs containing iHAC was harvested. The various reprogramming factors were not detected in these iHAC-free iPSCs, and they possessed a normal mouse karyotype. Various pluripotency markers in iHAC-free iPSCs were expressed at higher levels compared with those in iHAC-containing iPSCs. These findings indicate that reprogramming factors are obstacles following the reprogramming event, and that iHACs could generate iPSCs without genomic disruption.

For the next step, as there are a number of methods to generate iPSCs, an extensive comparison of various reprogramming methods is important to determine superiority or inferiority among the methods [33–35].

Issues surrounding gene and cell therapy

Safety: There are safety issues surrounding the use of undifferentiated cells because of their proliferating ability and tumorigenicity in patients. It is also possible that iPSCs can obtain instability of the chromosome and unexpected mutations that increase their proliferating ability during *in vitro* culture [33–37]. Current reprogramming method can't avoid adverse events. Therefore, safeguards are required to avoid tumor formation of iPSCs with suicide genes [38,39]. As conventional methods can cause transgene insertion in transplanted cells, 21HAC vectors could provide a greater level of safety. Additionally, the 21HAC vector can be used as a barrier against tumor formation. It is possible to construct a dual system, using allogeneic major histocompatibility complex and cancer testis antigen, that functions *in vitro* and *in vivo* to eliminate tumorigenic cells and driven by promoters of undifferentiated markers and tumor specific expression markers (Figure 11). Multiple safety barriers to prevent tumor formation may be useful to realize regenerative medicine using stem cells, even if the iPSCs are themselves genetically unstable, which may or may not result in tumors [33–35].

Improving microcell-mediated chromosome transfer efficiency: A major issue with chromosome engineering technology is microcell-mediated chromosome transfer efficiency. General efficiencies for microcell-mediated chromosome transfer efficiency are around 1×10^{-6}; however, using a cell fusion method with viral proteins increases efficiency about 100-fold (1×10^{-4}) [40]. For the practical use of HAC vectors, further improvement of chromosomal transfer efficiency is essential.

Genome editing and chromosome engineering technology: Artificial nucleases such as Zinc Finger Nuclease [41], Tale Effecter Nuclease [42] and CRISPR [43] have recently been attracting attention regarding genetic modification technology. Using the genome editing techniques, we attempt to develop a novel chromosome engineering technology to knock out selectable drug markers and GFP in DYS-
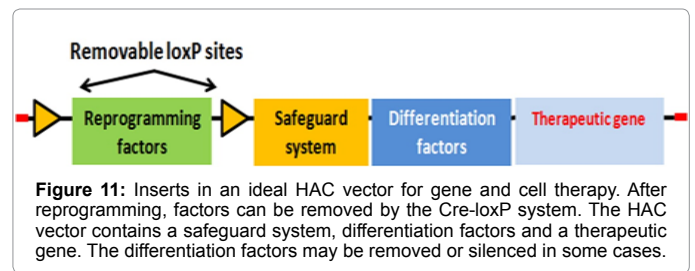

Figure 11: Inserts in an ideal HAC vector for gene and cell therapy. After reprogramming, factors can be removed by the Cre-loxP system. The HAC vector contains a safeguard system, differentiation factors and a therapeutic gene. The differentiation factors may be removed or silenced in some cases.

HAC. Because, if such transgenes are expressed in a patient *via* transplanted cells, they might cause an immune response. Namely, the genome editing technology might enable direct chromosome editing without DT40 cells in normal human cells; this will also ensure that gene therapy with 21HAC vectors will be safer.

The ideal HAC vector: Effective gene and cell therapy with a 21HAC vector, will be ideal to be used at all stages of the process towards clinical application of regenerative medicines through the step of generation of personal iPSCs from patients, the genetic correction of the iPSCs, the production of functional differentiated cells from the iPSCs and the follow-up after the transplantation (Figure 11). Currently, we are going to construct an all-in-one HAC vector that is ideal for gene and cell therapy (Figure 11).

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