Complete Genetic Correction of iPS Cells From Duchenne Muscular Dystrophy

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Human artificial chromosome (HAC) has several advantages as a gene therapy vector, including stable episomal maintenance that avoids insertional mutations and the ability to carry large gene inserts including the regulatory elements. Induced pluripotent stem (iPS) cells have great potential for gene therapy, as such cells can be generated from the individual's own tissues, and when reintroduced can contribute to the specialized function of any tissue. As a proof of concept, we show herein the complete correction of a genetic deficiency in iPS cells derived from Duchenne muscular dystrophy (DMD) model (mdx) mice and a human DMD patient using a HAC with a complete genomic dystrophin sequence (DYS-HAC). Deletion or mutation of dystrophin in iPS cells was corrected by transferring the DYS-HAC via microcell-mediated chromosome transfer (MMCT). DMD patient- and mdx-specific iPS cells with the DYS-HAC gave rise to differentiation of three germ layers in the teratoma, and human dystrophin expression was detected in muscle-like tissues. Furthermore, chimeric mice from mdx-iPS (DYS-HAC) cells were produced and DYS-HAC was detected in all tissues examined, with tissue-specific expression of dystrophin. Therefore, the combination of patient-specific iPS cells and HAC-containing defective genes represents a powerful tool for gene and cell therapies.

RESULTS
Characterization of iPS cells from mdx mice

First, we attempted genetic correction of iPS cells derived from mdx mouse, as a model for DMD (Figure 1). The mdx-iPS cells were induced from mdx mouse embryonic fibroblasts by retroviral infection of the three factors including Klf4, Sox2, and Oct4. Transduced fibroblasts from the mdx mice gave rise to ES cell–like colonies, and these colonies were isolated based on morphological criteria. Most mdx-iPS cells were positive for ES cell
markers, displayed a normal karyotype, and generated teratoma and chimeras (Supplementary Figure S1). Two randomly selected clones were used for the following experiments.

**Correction of mdx-iPS cells with DYS-HAC**

We have previously developed a novel HAC vector containing full-length genomic dystrophin, which was designated as DYS-HAC.21 This DYS-HAC, which contains a visualization marker gene GFP and suicide gene TK, was transferred to the two independent mdx-iPS clones via microcell-mediated chromosome transfer (MMCT). Eight GFP+ clones were selected and examined in the following experiments (Figure 2a). PCR analysis using primers for the detection of the DYS-HAC showed that six of the eight clones contained the intact dystrophin region in the HAC (Figure 2b). Reverse transcription (RT)-PCR analysis showed expression of dystrophin from the DYS-HAC was detected in six clones with the intact genomic dystrophin, whereas enhanced green fluorescent protein (EGFP) was expressed in all the clones examined (Figure 2d). Fluorescence in situ hybridization (FISH) analyses showed that the DYS-HAC was present as an individual chromosome in the mdx-iPS cells (Figure 2c). These results show that the DYS-HAC can be transferred to mouse iPS cells at a comparable efficiency to that in mouse ES cells.21 To test the stemness of the iPS cells, RT-PCR analyses using primers for ES cell–specific genes were performed (Figure 2d). Endogenous Rex1, Nanog, Oct4, and Sox2 were expressed in all mdx-iPS (DYS-HAC) cells, comparable to the parent mdx-iPS cells and Nanog-iPS cells generated previously with four Yamanaka’s factors, including c-Myc. Exogenous expressions of Klf4, Sox2, and Oct4 in most mdx-iPS (DYS-HAC) cells were lower than those of parent mdx-iPS cells, suggesting that expression of transgenes was more or less silenced after MMCT.

To determine whether the mdx-iPS (DYS-HAC) cells have the ability to differentiate into all three embryonic germ layers (endoderm, mesoderm, and ectoderm), cells were subcutaneously injected into nude mice. Transplanted mdx-iPS (DYS-HAC) gave rise to typical teratomas (n = 10), and GFP+ tissues were detected in these teratomas (Supplementary Figure S2a,b). Histological analyses showed that tumors contained all three embryonic germ layers (Supplementary Figure S2c). FISH analyses showed that DYS-HAC was detected in 90% of cells in tumor tissues (Supplementary Figure S2d). Immunohistochemical analyses detected the expression of human dystrophin in muscle-like tissues of teratomas derived from mdx-iPS (DYS-HAC) cells, but not in those derived from mdx-iPS cells (Figure 2e). These data suggest that loss of dystrophin expression in mdx-derived muscle tissue was restored by transferring the DYS-HAC into mdx-iPS cells.

**Expression in tissues from chimeric mice with DYS-HAC**

In order to determine developmental potential and expression of the transferred human dystrophin in various tissues, chimeric mice were produced from mdx-iPS (DYS-HAC) (Supplementary Figure S1). Two randomly selected clones were used for the following experiments.
Chimeras with various forms of coat-color chimerism were obtained, and GFP⁺ chimeric mice were used for the following analyses. The EGFP gene driven by the CAG promoter on the DYS-HAC was expressed in all tissues examined (Figure 3a), suggesting that DYS-HAC was stably maintained in vivo. To investigate whether the tissue-specific isoform of the human dystrophin on the DYS-HAC was expressed, total RNAs from various tissues of the chimeras were analyzed by RT-PCR using three pairs of specific primers to detect human dystrophin tissue-specific transcripts. Isoforms Dp427I and Dp427m were expressed in chimeric heart and skeletal muscle, and Dp140 was expressed in chimeric brain, comparable to the human expression profile (Figure 3b). FISH analyses showed that the DYS-HAC was detected in 50% of cells at least in the brain, liver, kidney, spleen, and skeletal muscle (Figure 3c). This detection correlated with coat-color chimerism, and the DYS-HAC was present as an individual chromosome in chimeric tail fibroblasts (Supplementary Figure S2e,f). Immunohistochemical analysis using a human dystrophin–specific antibody showed human dystrophin protein localized at the sarcolemmal membrane in skeletal muscle (Figure 3d). These results suggest that isoforms of human dystrophin on the DYS-HAC were expressed in a tissue-specific manner, and the human dystrophin protein was localized at the correct locus in iPS-derived chimeric skeletal muscle. These data were consistent with the results from our previous study using chimeric mice from normal mouse ES cells with the DYS-HAC.21

Correlation of DMD-iPS cells with DYS-HAC

Next, we attempted genetic correction of iPS cells derived from a DMD patient (Figure 1). We chose a DMD patient with deletion of exons 4–43 for iPS cell induction because a large deletion of this type cannot be corrected even using homologous recombination or other conventional vectors. The DYS-HAC was transferred to DMD patient–derived fibroblasts via MMCT, as we failed to directly transfer the HAC into human iPS or human ES cells due to the difficulty of cloning colonies derived from single cell following transfection or MMCT, which is an unsolved issue.

Figure S2e). Chimeras with various forms of coat-color chimerism were obtained, and GFP⁺ chimeric mice were used for the following analyses. The EGFP gene driven by the CAG promoter on the DYS-HAC was expressed in all tissues examined (Figure 3a), suggesting that DYS-HAC was stably maintained in vivo. To investigate whether the tissue-specific isoform of the human dystrophin on the DYS-HAC was expressed, total RNAs from various tissues of the chimeras were analyzed by RT-PCR using three pairs of specific primers to detect human dystrophin tissue-specific transcripts. Isoforms Dp427I and Dp427m were expressed in chimeric heart and skeletal muscle, and Dp140 was expressed in chimeric brain, comparable to the human expression profile (Figure 3b). FISH analyses showed that the DYS-HAC was detected in 50% of cells at least in the brain, liver, kidney, spleen, and skeletal muscle (Figure 3c). This detection correlated with coat-color chimerism, and the DYS-HAC was present as an individual chromosome in chimeric tail fibroblasts (Supplementary Figure S2e,f). Immunohistochemical analysis using a human dystrophin–specific antibody showed human dystrophin protein localized at the sarcolemmal membrane in skeletal muscle (Figure 3d). These results suggest that isoforms of human dystrophin on the DYS-HAC were expressed in a tissue-specific manner, and the human dystrophin protein was localized at the correct locus in iPS-derived chimeric skeletal muscle. These data were consistent with the results from our previous study using chimeric mice from normal mouse ES cells with the DYS-HAC.21
Genetic Correction of iPS Cells From DMD

The DYS-HAC was successfully transferred to DMD-fibroblasts, as shown by PCR and multiplex PCR analyses (Figure 4a and Supplementary Figure S3a). FISH analyses showed that the DYS-HAC was present as an individual chromosome in DMD patient–derived fibroblasts (data not shown). The iPS cells were generated from the DMD-fibroblasts with the DYS-HAC using a combination of lenti-viral infection with mouse Slc7a1 and retro-viral infection with KLF4, SOX2, OCT4, and c-MYC, as reported previously.6 Thirty GFP+ and human ES–like DMD-iPS (DYS-HAC) cells derived from three independent DMD-fibroblast clones were selected, and randomly selected nine clones were analyzed in the following experiments. To test the stemness of iPS cells, RT-PCR analyses using primers for ES cell–specific genes were performed (Figure 4c). Endogenous REX1, NANOG, OCT4, and SOX2 were differentially expressed in iPS cells. Exogenous OCT4, KLF4, SOX2, and c-MYC were expressed in some iPS cells. These findings show that DMD-iPS (DYS-HAC) cells were comparable to DMD-iPS and normal human iPS cells. PCR and multiplex PCR analyses showed that the DYS-HAC was maintained in all examined iPS cells, comparable to parent DMD-fibroblast (DYS-HAC) clones (Supplementary Figure S3a,b). FISH analyses showed that the DYS-HAC was present as an individual chromosome in the DMD-iPS (DYS-HAC) cells (Figure 4d). To examine mitotic stability of the DYS-HAC in DMD-iPS (DYS-HAC) cells, iPS cells were cultured for about 4 months without selection. FISH analyses revealed that the DYS-HAC was independently and stably maintained in DMD-iPS (DYS-HAC) cells (Figure 4e). These data suggest that the DYS-HAC could be maintained stably during iPS generation and even after long-term culture in vitro.

To determine whether DMD-iPS (DYS-HAC) cells could differentiate into all three embryonic germ layers, cells were injected into testes of severe combined immunodeficiency mice. Transplanted DMD-iPS (DYS-HAC) gave rise to typical teratomas (n = 12), and GFP+ tissues were detected in these teratomas (Supplementary Figure S4a). Histological analyses revealed all three embryonic germ layers in all teratomas (Supplementary Figure S4b). FISH analyses showed that the DYS-HAC was detected in 90% of cells in tumor tissues (Supplementary Figure S4c). Immunohistochemical analysis showed expression of human dystrophin in muscle-like tissues of teratomas derived from DMD-iPS (DYS-HAC) cells, but not in those derived from...
Genetic Correction of iPS Cells From DMD

DMD-iPS cells (Figure 4f). These data were comparable to the results of the mdx-iPS (DYS-HAC) experiments mentioned above. These data suggest that loss of dystrophin expression in DMD-derived muscle tissue was restored by transferring the DYS-HAC into DMD-iPS cells.

DISCUSSION

Previously, mouse iPS cells derived from sickle cell anemia have been corrected using the homologous recombination approach, with the model mice treated using iPS-derived hematopoietic progenitors.9 Most recently, Fanconi anemia patient–derived iPS cells were corrected using the viral vector, and the iPS cells could give rise to hematopoietic progenitors of the myeloid and erythroid lineages, showing the correction of the disease phenotype.24 However, genetic restoration by the homologous recombination and gene transfer using the conventional vectors, including viral and plasmid vector, cannot be applied in DMD patients with large deletions in the gene. The present results have shown the first complete genetic correction of a human genetic disorder, DMD with deletion of exon 4–43, using a novel HAC as an episomal vector. As Kimura et al. recently reported myogenic conversion from fibroblasts by transducing the inducible myogenic regulator, MyoD, the DMD-fibroblast (DYS-HAC) developed in this study may also represent a source for gene therapy.25

To the best of our knowledge, iPS cells have been generated from human primary fibroblast populations, but not from cloned cells.6 In this study, we could generate patient-derived genetically corrected iPS cells even from fibroblasts cloned after MMCT. Although several dystrophin isoforms cannot be expressed using previously reported vector systems, our HAC vector system containing an entire genomic dystrophin enabled isoform expressions in iPS-derived various tissues with the DYS-HAC in a tissue-specific manner. As the integrating viral vectors were used for iPS generation in this study, however, iPS induction using a nonintegrating vector system, protein introduction system, or chemical small molecules may be needed for safe gene therapy.26–33 The HAC vector may also be a promising tool for safe iPS generation: the HAC is a nonintegrating vector, and the elimination of a HAC, with a conditional centromere, from the cells can also be performed.34
In the MMCT methods, it is possible that host chromosomes (e.g., A9 and CHO) can be transferred along with the HAC at once. However, we have seldom observed the co-transfer of host chromosomes during MMCT (~1/100 clones). In this study, host chromosomes were not detected in mdx-iPS cells and DMD-fibroblasts after MMCT (data not shown).

Furthermore, advances in efficient methods for differentiation and purification of stem cells, including ES and iPS cells, are anticipated. Barberi et al. reported engraftable skeletal myoblasts from human ES cells. Darabi et al. also reported the generation of functional skeletal muscle from mouse ES cells by induction of Pax3 and the treatment of mdx mice using ES cell–derived cells. Application of these methods to iPS cells combined with our HAC vector system may open a way to more sophisticated DMD gene therapies. Taken together, genetic correction of patient-specific iPS cells by MMCT of the DYS-HAC, efficient differentiation from iPS cells into muscle stem cells in vitro, and transplantation of genetically corrected autologous cells into the same patient are needed for the gene therapy of DMD (Figure 1). Thus, stem cells derived from multiple potential sources combined with HAC-mediated gene delivery should allow safe treatment of various genetic defects, with elegant differentiation technology to come.

MATERIALS AND METHODS

Cell culture. Fibroblasts from a DMD patient (GM05169) containing deletion of exons 4–43 in the dystrophin gene were obtained from Coriell Institute (Camden, NJ). Fibroblasts were grown in α-MEM plus 15% fetal bovine serum (FBS) and 2 mmol/l L-glutamine. DMD model mice (mdx) were obtained from Charles River (Yokohama, Japan), and mouse embryonic fibroblasts (MEFs) were isolated from 13.5 days postcoitum embryos. The mdx-MEF and PLAT-E cells were grown in Dulbecco’s modified Eagle’s medium (Sigma, St Louis, MO) plus 10% FBS. Chinese hamster ovary (CHO) or A9 cells containing the DYS-HAC were used as microcell hybrids. Briefly, mdx-iPS and DMD-fibroblast cells were fused with microcells prepared from donor hybrid CHO (DYS-HAC) or A9 (DYS-HAC) cells, and selected with blasticidin S (3 µg/ml). The transferred DYS-HAC in each line was characterized by PCR, RT-PCR, and FISH analyses.

Genetic correction of iPS cells from DMD

Generation of iPS cells. Generation of iPS cells from mdx-MEFs was performed using a retroviral system as described previously. Briefly, retroviruses were generated with Plat-E packaging cells. Three retroviruses containing Oct4, Klf4, and Sox2, were infected into mdx-MEFs. Four days after transfection, mdx-MEFs were replated at 3.5 × 10^5 cells per 100-cm dish on MEF feeder cells. The next day, medium was replaced with mouse ES cell medium. Thirteen days after infection, mouse ES–like colonies were selected up to the MEF feeder cells on 24-well plates. Generation of iPS cells from human DMD-fibroblasts was performed using the retrovirus system combined with the lentivirus system as described previously.

Briefly, lentivirus production for Slc7a1 expression was performed using 293T cells, and lentivirus was infected into human fibroblasts. Four retroviruses containing OCT4, KLF4, Sox2, and MYC were transfected into Slc7a1-expressing human DMD-fibroblasts. The four retroviral expression vectors were obtained from Addgene (Cambridge, MA). Six days after transfection, fibroblasts were replated at 5 × 10^5 cells per 100-cm dish on SNL feeder cells. The next day, medium was replaced with primate ES cell medium supplemented with 4 ng/ml basic fibroblast growth factor. Thirty days after transduction, human ES–like colonies were selected up to the SNL feeder cells on 24-well plates.

MMCT. MMCT was performed as described previously. CHO or A9 cells containing the DYS-HAC were used as donor microcell hybrids. Briefly, mdx-iPS and DMD-fibroblast cells were fused with microcells prepared from donor hybrid CHO (DYS-HAC) or A9 (DYS-HAC) cells, and selected with blasticidin S (3 µg/ml). The transferred DYS-HAC in each line was characterized by PCR, RT-PCR, and FISH analyses.

Genomic PCR analyses. Genomic DNA was extracted from cell lines and chimeric tissue specimens using a genomic extraction kit (Genta Systems, Minneapolis, MN), and PCR was performed using primers as follows. Primer pairs for the detection of the region of human dystrophin were as follows: DYS1L/3R (163 base pairs (bp)), 5′-ACACGAGCCGGTGA-3′ and 5′-GGGTGGTGGTTGATTTT-3′; DYSL4/5R (128 bp), 5′-GCAAGACAAAAATGGCCCTC-3′ and 5′-AGCTTCTTGAGGTCTTCCCA-3′; DYS5L/5R (132 bp), 5′-ACTCTACGACCCCGAGGAAC-3′ and 5′-AGGGACCTCTTCTCTGACTCC-3′; DYS6L/6R (170 bp), 5′-TGGAGCCATTGTTGTTGTTT-3′ and 5′-AAACAATGCGCTGTCCAAA-3′; DYS7L7R (151 bp), 5′-TTTGCTCCCTTTTTGCTGTAT-3′ and 5′-AAACATGAAACCTGCCCCACT-3′; CHO (DYS-HAC) cell line and microcell hybrid clones, mdx-iPS (DYS-HAC), were maintained in Dulbecco’s modified Eagle’s medium plus 2 mmol/l L-glutamine. DMD model mice (mdx) were obtained from Charles River (Yokohama, Japan). The A9 (DYS-HAC) cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% FBS with 2 mmol/l L-glutamine. Human iPS cells were maintained as described previously. The control iPS cell line, Nanog-iPS (APS0001, iPS-MEF-Ng-20D-17), was obtained from the RIKEN BRC Cell Bank (Tsukuba, Japan). The SNL cell line for feeder layer was obtained from the SANGER Institute (Cambridge, UK).

Generation of iPS cells. Generation of iPS cells from mdx-MEFs was performed using a retroviral system as described previously. Briefly, retroviruses were generated with Plat-E packaging cells. Three retroviruses containing Oct4, Klf4, and Sox2, were infected into mdx-MEFs. Four days after transfection, mdx-MEFs were replated at 3.5 × 10^5 cells per 100-cm dish on MEF feeder cells. The next day, medium was replaced with mouse ES cell medium. Thirteen days after infection, mouse ES–like colonies were selected up to the MEF feeder cells on 24-well plates. Generation of iPS cells from human DMD-fibroblasts was performed using the retrovirus system combined with the lentivirus system as described previously.

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tissues were used as negative controls. Total RNA from cultured cells was purified with Trizol reagent (Invitrogen) and treated with a Turbo DNA-free kit (Ambion/Applied Biosystems, Tokyo, Japan) to remove genomic DNA contamination. First-strand cDNA synthesis was undertaken using a oligo(dT)_18 primer and ReverTraAce-α (Toyobo, Osaka, Japan). In analyses of mouse iPSCs, Nanog-iPS and mouse ES (TT2) cells were used as positive controls, whereas mdx-MEF was used as a negative control. Nat1 was used as an internal control. In analyses of human iPSCs, DMD-fibroblasts were used as negative controls. Nat1 was used as an internal control. PCR was performed with CDNA using ExTaq (Takara Bio, Otsu, Japan) or AmpliTaQ Gold (PerkinElmer, Waltham, MA). Amplifications were performed with an annealing temperature of 58°C for 30–35 cycles, then amplified fragments were resolved by electrophoresis on a 2% agarose gel, followed by staining with ethidium bromide. Primer sequences were as follows: for the dystrophin isoform Dp427m, DYS 427me1L/DYS 427me1R (211 bp), 5′-TTCCCCCTACAGGACTCAGA-3′ and 5′-TCTT CCCACCAAAGCATTTT-3′; for dystrophin isoform Dp427, DYS 427te1L/DYS 427te3R (150 bp), 5′-CTCATGATGAAGAGAGATGTCAAA-3′ and 5′-CGTCCGAGCTGCATTGAGA-3′; for dystrophin isoform Dp140, DYS 140e1L/DYS e45R (189 bp), 5′-TGGCTGCGTCTCTGAACTA-3′ and 5′-GGGCTTCCAAATTTTTCTGT-3′; for EF1G, EF1GFL/R (479 bp), 5′-CCTGAAGTTCATCTGACCA-3′ and 5′-TGTCAGGATGTTGCTGGTGTCG-3′; for GAPDH (mice and human), RPC1/2, 5′-CCATCTTCCAG GAGCGA-3′ and 5′-TGTCATAACAGAAATGAGCC-3′. Primers for DYS6L/6R, DYS7L/7R, and DYS8L/8R were the same as in genomic PCR analyses. RT-PCR to detect endogenous ES markers and exogenous genes were performed using primers as described previously. Primer pairs for the detection of transgenes of cMyC and SOX 2 were as follows: hMYC-S3148/PMXs-AS8095, 5′-CAGAGGAGGACGCTGAAAC-3′ and 5′-AGACCACTGTTAATGTTAGCG-3′; hSOX2-S691/pMXs-AS3206, 5′-GCGACCCTTGATAGCTGGTGC-3′ and 5′-TTATC CGGAGACATTCTGGG-3′.  

**Generation of Chimeric Mice.** Chimeric mice were produced from the two mdx-iPS and three mdx-iPS (DYS-HAC) cell lines. Chimera performance was performed as described previously. Briefly, iPSC cells were injected into blastocyst-stage embryos derived from ICR mouse (CLEA Japan) and then transferred into pseudopregnant ICR females. Three chimeric mice showing 50% coat-color chimerism were used for expression analyses in various tissues. All chimeric mice used for analyses were 1–4 weeks old. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.  

**Teratoma formation and histology.** To produce teratomas, 2 × 10⁶ mdx-iPS (DYS-HAC), mdx-iPS, and mdx-fibroblast cells were subcutaneously injected into CD-1 (ICR)-nu mice (Charles River), and 1 × 10⁶ DMD-iPS (DYS-HAC), DMD-iPS, and DMD-fibroblast (DYS-HAC) cells were injected into testes of severe combined immunodeficient mice (Charles River). After 5–6 weeks in the mdx-iPS series and 9–13 weeks in the DMD-iPS series, resected teratomas were fixed in 20% formalin and processed for paraffin sectioning, then stained with hematoxylin and eosin.  

**Immunohistochemical analyses.** Formalin-fixed and paraffin-embedded specimens and OCT (optimal cutting temperature) compound-embedded frozen specimens were used for immunohistochemistry. Primary antibodies used in this study were as follows: rabbit polyclonal antibody against both human and mouse dystrophin (1:100; Lab Vision, Fremont, CA); and mouse monoclonal antibody against human dystrophin (MANDYS106, diluted 1:4; a gift from Glenn E. Morris, Keele University, Shropshire, UK). Immunoreaction was developed by using the SAB (streptavidin–biotin) peroxidase complex method with the Histofine SAB-PO Kit (Nichirei, Tokyo, Japan) for rabbit polyclonal antibody and the Histofine Mouse Stain Kit (Nichirei) for mouse monoclonal antibody, or visualized with Alexa Fluor 555 goat anti-mouse conjugate (diluted 1:1,000; Molecular Probes, Eugene, OR).
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