Generation of Induced Pluripotent Stem Cells From Patients With Duchenne Muscular Dystrophy and Their Induction to Cardiomyocytes

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Summary

Duchenne muscular dystrophy (DMD) is caused by mutations in the DMD gene which encodes dystrophin protein. Dystrophin defect affects cardiac muscle as well as skeletal muscle. Cardiac dysfunction is observed in all patients with DMD over 18 years of age, but there is no curative treatment for DMD cardiomyopathy. To establish novel experimental platforms which reproduce the cardiac phenotype of DMD patients, here we established iPS cell lines from T lymphocytes donated from two DMD patients, with a protocol using Sendai virus vectors. We successfully conducted the differentiation of the DMD patient-specific iPS cells into beating cardiomyocytes. DMD patient-specific iPS cells and iPS cell-derived cardiomyocytes would be a useful in vitro experimental system with which to investigate DMD cardiomyopathy. (Int Heart J 2016; 57: 112-117)

Key word: Cardiomyopathy, Human

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting 1 in 3,500 male births. DMD is caused by mutations in the DMD gene which encodes dystrophin protein. Dystrophin protein constitutes a core element of dystrophin-glycoprotein complex, and plays essential roles in transmitting force and maintaining sarcolemma stability. Lack of dystrophin protein impairs membrane integrity and causes progressive degeneration and loss of skeletal muscle. Dystrophin defect also affects cardiac muscle and cardiac dysfunction is observed in all patients with DMD over 18 years of age. With recent advances in the management of respiratory failure for patients with DMD, increasing attention has been paid to cardiac dysfunction since the heart failure is becoming the most frequent cause of death among adult DMD patients. Although medical treatments using beta-blockers and angiotensin converting enzyme inhibitors have been reported to delay progression of DMD cardiomyopathy, the effects are limited. Exon skipping has been developed as a novel gene therapy for DMD and some effects have been observed in skeletal muscles, however, the recovery of dystrophin was only scarcely observed in heart muscle due to the difficulty of delivering antisense oligonucleotides to cardiomyocytes. Thus, we need to clarify the underlying mechanisms of DMD-associated cardiomyopathy and develop novel therapeutic strategies.

To date, mdx mice have been widely used as an animal model of dystrophin gene abnormality. Recent studies have reported various abnormalities in cardiomyocytes of mdx mice such as increased susceptibility to stretch-mediated calcium overload, abnormal activation in stretch-activated channels, diastolic calcium leak from sarcoplasmic reticulum, and hyperactivation of X-ROS signaling. Nevertheless, mdx mice have critical limitations as a model for DMD cardiomyopathy since they do not develop heart failure. On the other hand, using both in vitro and in vivo non-genetic rat models, a recent study demonstrated that microRNA-340-5p may be involved in the progression of heart failure through the interaction with DMD gene. Considering these issues, novel experimental platforms that reproduce the cardiac phenotype of DMD patients is critically required for the development of new drugs as...
well as elucidation of the pathogenesis.

Recently, a technique to establish patient-specific iPS cell lines has been developed and disease-specific iPS cells are now considered as a potential tool for the investigation of disease mechanisms and drug discovery. In the present study, we established iPS cell lines from T lymphocytes donated from two DMD patients, with a protocol using Sendai virus vectors, and we successfully conducted differentiation of the DMD patient-specific iPS cells into cardiomyocytes. Here, we demonstrate that the DMD patient-specific iPSCs (DMD-iPS cells) and iPSC cell-derived cardiomyocytes (DMD-iPS-CM) could be a useful in vitro experimental system with which to investigate DMD cardiomyopathy.

**METHODS**

**Ethical approval:** The present study was approved by the Institutional Review Board of Osaka University and written consent was obtained from all patients.

**Clinical diagnosis:** Patients were clinically suspected as having DMD and diagnosed by the lack of dystrophin staining of biopsied skeletal muscle. Genetic tests of dystrophin gene revealed that Patient 1 carried the deletion of exon 48-54 and Patient 2 carried the deletion of exon 46 and 47 (Figure 1). Before peripheral blood sampling, echocardiography and plasma brain natriuretic peptide (BNP) measurement were performed. The left ventricular ejection fraction (LVEF), left ventricular end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), and plasma BNP level of each patient were as follows:

- **Patient 1:** LVEF 44.2%, LVDd 59 mm, LVDs 46 mm, BNP 38.3 pg/mL.
- **Patient 2:** LVEF 58.9%, LVDd 49 mm, LVDs 36 mm, BNP 11.7 pg/mL.

**Quantitative real-time polymerase chain reaction:** Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as described previously with minor modification. Briefly, RNA was extracted from cells using an RNeasy Plus Mini kit (Qiagen). An aliquot of total RNA was reverse transcribed using an oligo (dT) primer. For the thermal cycle reactions, the cDNA template was amplified (ABI PRISM 7900HT Sequence Detection System, Thermo Fisher Scientific) with gene-specific primer sets using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher Scientific) under the following reaction conditions: 40 cycles of PCR (95°C for 15 seconds and 60°C for 1 minute) after an initial denaturation at 95°C for 2 minutes. Fluorescence was monitored during every PCR cycle at the annealing step. The authenticity and size of the PCR products were confirmed by melting curve analysis (using software provided by Thermo Fisher Scientific). mRNA levels were normalized using GAPDH as a housekeeping gene.

The primers used were as follows:

- **OCT3/4:** forward: cagacattgccaagctctgga and reverse: ttcgagctctgcaagaacttct
- **NANOG:** forward: ttcagcgatgcaagaactcctg and reverse: tccagcgatgtgcctagatt
- **TERT:** forward: gagcaagttgcaagctctt and reverse: ttctctggcaagctctt

Human embryonic stem (ES) cells and the human fibroblast cell line MRC5 were used as positive and negative controls, respectively. These control cells were kindly provided by the National Center for Child Health and Development (Tokyo).

**Teratoma formation:** Teratoma formation was evaluated using a protocol described previously with minor modification. Briefly, clusters of DMD-iPS cells were dispersed by Dispase II (383-0228, Wako Pure Chemical Industries) treatment, collected into tubes, and centrifuged (114 g, 3 minutes). The same volume of Matrigel basement membrane matrix (356237, Corning) was added to the cell suspension. The cells (> 1 × 10⁶) were subcutaneously inoculated into immuno-deficient, BALB/cSlc-nu/nu mice (Sankyo Labo Corporation). After 12 to 14 weeks, the resulting tumors were dissected and fixed with phosphate buffered saline (PBS) containing 4% paraformaldehyde. Paraffin-embedded tissues were sliced and stained with hematoxylin and eosin (HE). The operation protocols were approved by the Laboratory Animal Care and Use Committee of the National Research Institute for Child and Health Development.

**Cardiac differentiation of iPS cell lines and purification of differentiated cardiomyocytes:** iPS cell lines were differentiated into cardiomyocytes using an embryoid body (EB) formation protocol. At day 0, iPS cell colonies were detached by CTK solution containing 1 mg/mL collagenase type IV (17104-019, Thermo Fisher Scientific), 0.25% trypsin (215240, BD), 20% Knockout Serum Replacement (10828-028, Thermo Fisher Scientific) and 1 mM of CaCl₂ and feeder cells were removed by washing with medium. Detached colonies were crushed into small clusters and floated in Cellstar cell-repellent surface dishes (Greiner Bio One) using FF2 medium (RCHEMD006, Reprocell) with 5 ng/mL basic fibroblast growth factor (bFGF) and 10 μM Y-27532. At day 1, the medium was replaced with differentiation medium (StemPro-34 medium with 2.5% Knockout Serum Replacement, 50 μg/mL ascorbic acid, 150 μg/mL transferrin, 0.45 mM monothioglycerol and penicillin/streptomycin), supplemented with 4 μM CHIR99021, 10 nM IDE1, and 5 ng/mL bFGF. At day 4, the medium was replaced with differentiation medium supplemented with 5 μM IWP-2, 10 ng/mL VEGF, 5 μM SB431542, and 0.5 μM dorsomorphin. At day 6, medium was replaced with differentiation medium supplemented with 5 μM IWP-2 and 10 ng/mL VEGF. At day 8, medium was replaced with differentiation medium supplemented with 5 ng/mL bFGF and 10 ng/mL VEGF. Until day 20, medium was changed with day 8 medium every 3 to 4 days.

**Figure 1.** Schema of dystrophin mutations. Patient 1 and Patient 2 carried deletion of exon 48-54 and deletion of exon 46 and 47, respectively.
days. Differentiated cardiomyocytes were purified by metabolic selection with slight modification. Briefly, on or after day 20, culture medium was changed to glucose-free DMEM (11966-025, Thermo Fisher Scientific) with 4 mM L-lactate (129-02666, Wako Pure Chemical Industries) and penicillin/streptomycin and the medium was changed every second day for 8 days. After day 28, beating EBs were selected, washed with PBS, and dissociated into single cells by incubating with 0.25% trypsin-EDTA solution for 10 minutes at 37°C and then passed through a 40 μm cell strainer and plated using IMDM (31980-030, Thermo Fisher Scientific) with 2% fetal bovine serum and penicillin/streptomycin.

**Western blotting:** Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 substitute, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with protease inhibitor cocktail (539134, Merck Millipore Corporation) and phosphatase inhibitor cocktail (78420, Thermo Fisher Scientific) and BCA protein assay kit (23225, Thermo Fisher Scientific). Cell lysates were incubated for 1 hour at 37°C with Laemmli buffer and fractionated with SDS polyacrylamide gel electrophoresis using 5-20% gradient gels (11955-54, Nacalai Tesque). Fractionated samples were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris buffered saline and Tween-20 (TBS-T) for 1 hour, and then incubated with primary antibody overnight at 4°C. After incubation, the membrane was washed with TBS-T, and incubated with secondary antibody in 5% skim milk for 1 hour at room temperature. The membrane was again washed with TBS-T and treated with Pierce ECL Plus Western Blotting Substrate (32132, Thermo Fisher Scientific) for 5 minutes. Chemiluminescent signals were developed with Amersham Hyperfilm ECL (28906838, GE Healthcare Life Sciences). The following antibodies were used: anti-dystrophin (1 μg/mL, ab15277, Abcam), anti-GAPDH (1:1500, #2118, Cell Signaling Technology), and Peroxidase AffiniPure donkey anti-rabbit IgG (H+L) (1:5000, 711-035-152, Jackson ImmunoResearch Laboratories), and Alexa Fluor 555 goat anti-mouse IgM mu chain (1:300, ab150121, Abcam), Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (1:200, A-21206, Thermo Fisher Scientific), and Alexa Fluor 594 donkey anti-mouse IgG (H+L) (1:200, A-21203, Thermo Fisher Scientific).

**RESULTS**

**Generation of DMD-iPS cell clones:** Peripheral blood samples used in the present study were obtained from Patient 1 and Patient 2 at the ages of 31 and 13 years old, respectively. DMD patient-specific iPS cell clones were generated from peripheral blood T lymphocytes as described previously. Briefly, T lymphocytes were separated from peripheral whole blood using Ficoll-Paque and a Pan T Cell Isolation Kit II (130-091-15, Miltenyi Biotec). Isolated T lymphocytes were then expanded by CD3 and CD28 with Dynabeads (11131D, Thermo Fisher Scientific) in KBM502 medium (16025020, Kohjin Bio). Reprogramming was performed by Sendai virus vectors with OCT3/4, SOX2, KLF4 and c-MYC (CytoTune-iPS, Dnavec). Multiplicity of infection was set to 20. Twenty-four hours after infection, T lymphocytes were seeded on CF-1 feeder cells. Approximately 20 days later, 90-100 colonies per sample were selected. iPS cells were maintained on mouse embryonic fibroblast feeder layers using KnockOut D-MEM/F-12 medium (12660-012, Thermo Fisher Scientific) with 20% KnockOut Serum Replacement, MEM non-essential amino acids, GlutaMAX (35050-061, Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol, and penicillin/streptomycin. We also established the control iPS cell line from a 36 year-old female healthy volunteer using the same method.

**Characterization of DMD-iPS cell lines:** We generated 18 and 53 clones from DMD Patient 1 and Patient 2, respectively. For further evaluation, we selected one clone for each patient, based on its differentiation efficiency. Quantitative PCR showed that undifferentiated cell marker genes OCT3/4, NANOG and TERT were expressed in the selected clones of Patient 1 and Patient 2 as in control human ES cells, whereas the expression of those genes was not detected in fibroblasts (Figure 2A). In addition, immunofluorescent staining demonstrated that these clones were positively stained for stem cell markers (NANOG, OCT3/4, Sox2, SSEA4, TRA-1-60) (Figure 2B). These results suggest that reprogramming of blood T lymphocytes was successfully conducted through the protocol in the present study. To further validate the pluripotency of the clones, we examined teratoma formation. We observed various tissues that originated from three germ layers including neural crest, mesoderm, and ectoderm in the clone from each patient (Figure 2C). Based on these data, we verified that iPS cells were established from DMD patients through our protocol of reprogramming.

**Characterization of DMD-iPS cardiomyocytes:** With the differentiation/purification protocol of cardiomyocytes, spontaneously beating EBs were efficiently obtained in most series of experiments. At day 42, enzymatically-dissociated cells from the beating EBs were subjected to further experiments for characterization. In Western blot experiments, total cell lysates obtained from day 42 beating EBs of DMD-iPS cells from Patient 1 and Patient 2 lacked dystrophin expression, whereas dystrophin was abundantly expressed in EBs of iPS cells from healthy volunteer using the same method.
the healthy volunteer (control) (Figure 3A). Immunostaining of cells dispersed from beating EBs showed that cardiac specific proteins including alpha-sarcomeric actinin and MLC2v were abundantly expressed in cells obtained from DMD-iPS cell-derived beating EBs from Patient 1 and Patient 2 as well as the control (Figure 3B and 3C). This result suggests that cells obtained from both control- and DMD-iPS cell-derived beating EBs demonstrated the properties of cardiomyocytes. On the other hand, cardiomyocytes derived from DMD-iPS cells lacked the expression of dystrophin, whereas cardiomyocytes from the healthy volunteer showed abundant expression of dystrophin (Figure 3B). These results suggest that patient-specific iPS cells were successfully differentiated into cardiomyocytes, which recapitulate the patient phenotype as DMD-iPS-CM.

**Discussion**

iPS cells have been considered as a potential cell source for regeneration therapy for cardiovascular diseases, and furthermore, it is hoped patient-specific iPS cells will become experimental platforms to investigate these diseases. In the present study, we established DMD patient-specific iPS cell lines using peripheral blood T cells and Sendai virus vectors based on a previous report. As for the establishment of iPS cells, the method used in the present study has advantages over conventional protocols which employed the combination of biopsied fibroblasts and retroviral infection, since our procedures are less invasive and interfere with the chromosomes of somatic cells is negligible.

![Figure 2](image1.png)

**Figure 2.** Characterization of established DMD-iPS cells. A: qRT-PCR of undifferentiated DMD-iPS cells. All of OCT3/4, NANOG and TERT (undifferentiated cell marker genes) were abundantly expressed in iPS cells as in ES cells (positive control) but not in fibroblasts (negative control). Data are presented as fold change (mean ± SD). B: Immunofluorescence of undifferentiated DMD-iPS cells. Cells generated from both DMD Patient 1 and Patient 2 were positive for stem cell markers NANOG, OCT3/4, SOX2, SSEA4 and TRA-1-60. Scale bar: 200 μm. C: Teratoma formation in BALB/cSlc-nu/nu mice after injection of undifferentiated DMD-iPS cells. Note the presence of 3 germ layer-derived tissues, neuroepithelium (ectoderm), cartilage (mesoderm), and intestine (endoderm and mesoderm). Scale bar: 200 μm.

![Figure 3](image2.png)

**Figure 3.** Characterization of cardiomyocytes derived from DMD-iPS cells. A: Expression of dystrophin in beating EBs. Total cell lysates were obtained from beating EBs. B: Immunofluorescence of cardiomyocytes. Dystrophin and sarcomeric α-actinin are presented in green and red, respectively. Note the lack of dystrophin in Patient 1 and Patient 2, but not in control, while sarcomeric α-actinin is expressed in all samples. Scale bar: 50 μm. C: Immunofluorescence of cardiomyocytes. MLC2v and sarcomeric α-actinin are presented in green and red, respectively. Note that MLC2v are expressed in all the samples as well as sarcomeric α-actinin. Scale bar: 50 μm.
The cells obtained from beating EBs from DMD-iPS cells showed the expression of cardiac specific markers but lacked the expression of dystrophin, whereas those from control-IPS-CM showed expression of both cardiac specific markers and dystrophin, suggesting that we successfully made DMD-iPS-CM. Despite numerous attempts, the underlying mechanisms of development of cardiomyopathy in DMD patients still need to be elucidated. To date, mdx mice have been most widely used as an animal model of DMD, however, mdx mice do not show severe cardiac dysfunction due to the expression of redundant protein utrophin in the heart of mdx mice. In this regard, DMD-iPS-CM could be a useful tool to investigate the disease progress.

Since cardiomyocytes of mdx mice contain high levels of calcium ion, it is conceivable that elevated levels of calcium ion activate calpain, a cysteine protease, leading to myocyte necrosis. Actually, overexpression of the calpain inhibitor calpain activate calpain, a cysteine protease, leading to myocyte


