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Lab Resource: Stem Cell Line

Generation of GZKHQi001-A and GZWWTi001-A, two induced pluripotent stem cell lines derived from peripheral blood mononuclear cells of Duchenne muscular dystrophy patients

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ABSTRACT

Duchenne muscular dystrophy (DMD) is an X-linked disease caused by mutations in the DMD gene, which spans ~2.4 Mb of genomic sequence at locus Xp21. This mutation results in the loss of the protein dystrophin. DMD patients die in their second or third decade due to either respiratory failure or cardiomyopathy, as the absence of dystrophin leads to myofiber membrane fragility and necrosis, eventually resulting in muscle atrophy and contractures. Currently, there is no effective treatment for DMD, therefore induced pluripotent stem cells from DMD patients would be a powerful tool for studying disease mechanisms.

Resource Table

Unique stem cell lines identifier	GZKHQi001-A, GZWWTi001-A
Alternative names of stem cell lines	DMD-iPS1, DMD-iPS2
Institution	The Third Affiliated Hospital of Guangzhou Medical University
Contact information of distributor	xiaofangsun@gzhmu.edu.cn
Type of cell lines	iPSC
Origin	human
Cell source	Peripheral blood mononuclear cells (PBMCs)
Clonality	clonal
Method of reprogramming	Sendai virus
Multiline rationale	same disease non-isogenic cell lines
Gene modification	NO
Type of modification	NA
Associated disease	Duchenne muscular dystrophy
Gene/locus	DMD(exon51)/Xp21 DMD(exon50&56–60)/Xp21
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	September.2017
Cell line repository/bank	NA
Ethical approval	The generation of iPS cells from PBMCs donated by patients with informed consent was reviewed and approved by the IRB at The Third Affiliated Hospital of Guangzhou Medical University (No. 2016-001).

Resource utility

There is no effective treatment for DMD (Bushby et al., 2010). The generation of induced pluripotent stem cells from DMD patients represents a promising resource to be used as a tool for the discovery of novel drug treatments, and the development of new therapies for DMD (Young et al., 2017).

Resource details

Two 3-year-old patients who were diagnosed with Duchenne muscular dystrophy (DMD) at the Reproductive Medicine Centre of The Third Affiliated Hospital of Guangzhou Medical University each donated 5 ml of peripheral blood with informed consent. Clinical information of the PBMC donors is summarized in Table 1. Both samples of the

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
GZKHQi001-A (DMD-iPS1)	DMD-iPS1	male	3	Chinese	Exon 51 of DMD gene deletion	DMD
GZWWTi001-A (DMD-iPS2)	DMD-iPS2	male	3	Chinese	Exon 45–50&56–60 of DMD gene duplication	DMD

peripheral blood mononuclear cells (PBMCs) obtained exhibited a 46, XY karyotype (Fig. 1A). The first patient exhibited a DMD DelEx51 (deletion Exon 51 of DMD gene) detected by multiplex ligation-dependent probe amplification (MLPA). The second patient exhibited a DMD DupEx45–50&56–60 (Duplication Exon 45–50&56–60 of DMD gene) also detected by MLPA (Fig. S1C). To generate iPSCs, the PBMCs were infected with Sendai virus containing the reprogramming factors OCT4, SOX2, KLF4 and c-MYC, as previously described (Seki et al., 2012). The protocol for iPS cell generation is described in detail in the Materials and Methods section. Approximately 20 days after infection, the generated colonies exhibited typical human embryonic stem cell morphology (Fig. 1B). The colonies (GZKHQi001-A, GZWWTi001-A) described in this paper were picked and expanded for further characterization. Both cell lines were verified to be mycoplasma-negative (Fig. S1A). The cells maintained the 46, XY karyotype consistent with the donor cells (Fig. 1A), and the short tandem repeats (STR) analysis confirmed that these cell lines had the same genetic background as the donor cells (STR analysis, Fig. S1B). Both cell lines were positive for alkaline phosphatase (Fig. 1C), which is a primary marker of pluripotency (Fig. 1D). We also performed RT-PCR analysis for pluripotency markers (OCT4, NANOG and Lin28) (Fig. 1E). FACS analysis and immunostaining confirmed the expression of the pluripotency markers OCT4 and SSEA4 (Fig. 1F). To test the differentiation potential of GZKHQi001-A and GZWWTi001-A, we performed differentiation assays both *in vitro* and *in vivo*. The embryoid body formation assay showed that the cell lines could spontaneously differentiate into cells that highly expressed AFP (endoderm), SMA (mesoderm) and NESTIN (ectoderm) (Fig. 1G). The results showed that both cell lines retained the ability to differentiate into three germ layers *in vitro*. In teratoma sections generated from these two cell lines, haematoxylin-eosin staining revealed glands (endoderm), cartilage (mesoderm) and nerve bundles (ectoderm) (Fig. 1H). These results demonstrated that GZKHQi001-A and GZWWTi001-A cells were pluripotent and could differentiate into all three germ layers.

Materials and methods

Reprogramming of peripheral blood mononuclear cells (PBMC)

PBMCs obtained from peripheral blood were reprogrammed to iPSs using a previously described protocol (Seki et al., 2012). The Invitrogen CytoTune ® - iPS 2.0 Sendai reprogramming kit (A16517, purchased from Life Technologies), which includes four transcription factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, was used. 5×10^5 isolated PBMCs were seeded into one well of a 24-well plate and cultured for four days in StemSpan™ SFEM II PBMC complete medium (STEMCELL Technology, 09655) supplemented with 100 ng/ml SCF(PeproTech, 300–07), 100 ng/ml FLT3 (PeproTech, 300–19), 20 ng/ml IL-3 (PeproTech, 200–03) and 20 ng/ml IL-6 (PeproTech, 200–06). Immediately after plating, the cells were infected with Sendai virus for 48 h at 37 °C. The infected cells were transferred onto MEF feeders and cultured in StemSpan™ SFEM II. Following 21–28 days of culture, individual iPS colonies were picked and transferred to MEF feeder layers for expansion. After the fifth passage, cells were maintained in E8 medium.

Karyotype analysis

Karyotype analysis was performed on the cells using conventional G-banding techniques according to standard cytogenetic protocols based on the International System for Human Cytogenetic Nomenclature. At least 25 metaphases were read for each sample (550-band resolution).

RNA extraction and RT-PCR

Total RNA from the cells was isolated by Trizol Reagent (Invitrogen). cDNA was synthesized from the RNA using SYBR® Premix Ex Taq™ II (TaKaRa, RR820A) and PrimeScript™ RT reagent Kit (TaKaRa, RR047A). PCR was performed for each of the genes using the primers listed in Table 2.

Alkaline phosphatase staining

Induced iPS cells of the 10th generation were stained with alkaline phosphatase staining fluid (SiDanSai, 1101-050/100), and the results were observed under a microscope (Nikon, TS 100).

Immunofluorescence staining

The expression of pluripotency markers was analyzed using IF staining Primary antibodies against SSEA-4, Tra-1-81, Sox2, and Oct4 were used (Table 3). Samples were analyzed using a fluorescence microscope and a confocal microscope (Nikon, Ti).

Teratoma formation

5×10^6 iPSCs suspended in 100 μl Matrigel were injected intramuscularly into the back of NOD/SCID mice (4–8 weeks old), and the resulting teratomas grew to 1–2 cm. Tumors stained with haematoxylin-eosin, and were examined for endoderm, mesoderm and ectoderm tissues under a microscope (Nikon, TS 100).

Embryoid body (EB) in vitro differentiation

iPSCs were dispersed into small clumps using 0.1 g/ml Dispase (Sigma, D4818) and resuspended in Knockout DMEM/F12 (Gibco, 12660012) supplemented with 15% KSR (Gibco, 10828028), 5% FBS (Gibco, 26140079), 1% NEAA (Gibco, 11140050), 1% Glutamax (Gibco, 35050061) and 0.1 mM β-Mercaptoethanol (Gibco, 21985-02). Cells were then transferred into Ultra Low Attachment Plates (Corning, 3471) where they aggregated to form EBs. EBs were cultured in suspension for seven days, and the medium was changed every two days. Following this, EBs were gently resuspended and plated on gelatin-coated coverslips in a 4-well plate, and cultured for an additional 11 days. Three primary antibodies, anti-AFP, anti-SMA, and anti-Nestin, were used for IF staining of EBs using a confocal microscope (Nikon, Ti) to identify the inner and outer ectoderm layers.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.01.028.

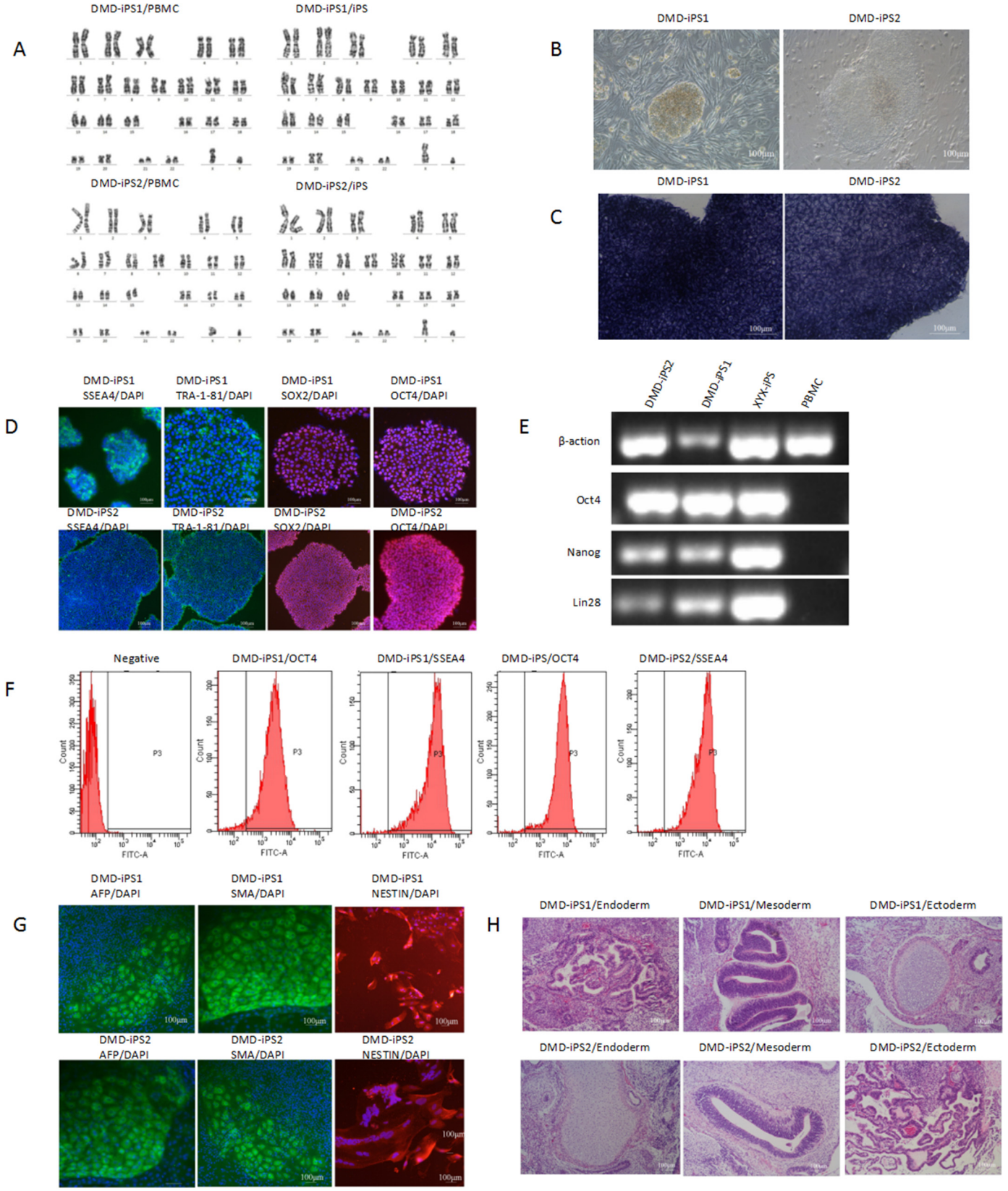

Fig. 1. Generation and chaaracterization of two DMD iPSC lines。in this figure are labelled with their names. (A) Karyograms of iPS lines. Karyotype resolution is 500 to 550 bands per haploid set. (B) Generation of induced pluripotent stem cells from the patient's peripheral blood. (C) Alkaline phosphatase staining. (D) Pluripotency of iPSCs confirmed by immunostaining with SSEA4, SOX2, TRA-1-60 and OCT-4, nuclei was counterstained with DAPI. (E) RT-PCR analysis showing that two lines generated express pluripotency markers at mRNA level. (F) FACS analysis for pluripotient markers. (G) Representative immunofluorescence images illustrating the ability of all iPSC lines generated to give rise to the three germ layers via the EB formation assay . AFP(alpha fetoprotein) to mark the endodermal, SMA(smooth muscle actin) to mark the mesodermal layer and NESTIN to mark the ectodermal. (H) All three germ layers are shown in teratoma derived from the cell lines with H&E staining.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	normal	Fig. 1 panel B and C
Phenotype	Qualitative analysis Immunocytochemistry	Assess staining of pluripotency markers: Oct4, Sox2,SSEA4, TRA-1-81	Fig. 1 panel D and E
	Quantitative analysis, Flow cytometry	DMD-ips1 Oct4: 94.7%, SSEA-4: 98.4%; DMD-ips2 Oct4: 98.4%, SSEA-4 98.8%	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	DMD-iPS1-PBMC:46, XY, DMD-iPS1:46, XY, DMD-iPS2-PBMC:46, XY, DMD-iPS2:46, XY (550-band resolution)	Fig. 1 panel A
Identity	STR analysis	DNA Profiling performed specific 25 sites tested, matched	Fig. S1 panel B submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing Southern Blot OR WGS	DMD-iPS1:45–50, 56–60 exon repeat, DMD-iPS2:51 exon is absent: Not performed	Fig. 1 panel F
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Positive	Fig. S1 panel A
Differentiation potential	Embryoid body formation	Proof of three germ layers formation, SMA,AFP and NESTIN, endoderm, mesoderm and ectoderm	Fig. 1 panel G and H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	MLPA analysis Not performed	Fig. S1 panel C

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat# and RRID
Pluripotency markers	Mouse anti-OCT4	1:500	Abcam ab19857, RRID: AB_445175
Pluripotency markers	Mouse anti-SSEA4	1:100	Abcam ab16287, RRID: AB_778073
Pluripotency markers	Mouse anti-SOX2	1:100	ABGENT AM2048a, RRID: AB_1278243
Pluripotency markers	Mouse anti-TRA-1-81	1:100	Abcam ab16289, RRID: AB_2165986
Differentiation markers	Rabbit anti-AFP	1:200	Abcam ab3980, RRID: AB_304203
Differentiation markers	Rabbit anti-SMA	1:100	Abcam ab5694, RRID: AB_2223021
Differentiation markers	Rabbit anti-Nestin	1:50	ABGENT AP2020b, RRID: AB_2151009
Secondary antibodies	Goat anti-Mouse IgG Fluorescein (488)	1:1000	Biotium inc Cat# 20010, RRID:AB_10559812
Secondary antibodies	Goat anti-Rabbit IgG Fluorescein (488)	1:1000	ThermoFisher A-11034, RRID: AB_2576217

Primers

	Target	Forward/reverse primer (5′-3′)
Mycoplasma detection	Mycoplasma 16S rDNA	GGGAGCAAACAGGATTAGATACCCT TGCACCATCTGTCACTCTGTTAACCTC
Pluripotency markers (RT-PCR)	OCT4	CCTCACTTCACTGCACTGTA CAGGTTTTCTTTCCCTAGCT
Pluripotency markers (RT-PCR)	NANOG	TGAACCTCAGCTACAAACAG TGGTGGTAGGAAGAGTAAAG
Pluripotency markers (RT-PCR)	LIN28	AGCCATATGGTAGCCTCATGTCCGC TCAATTCTGTGCCTCCGGGAGCAGGGTAGG
Pluripotency markers (RT-PCR)	β-Actin	GGGAAATCGTGCGTGACATTAAGG CAGGAAGGAAGGCTGGAAGAGTG

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