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Lab resource: Stem Cell Line

Generation of genomic-integration-free human induced pluripotent stem cells and the derived cardiomyocytes of X-linked dilated cardiomyopathy from *DMD* gene mutation

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A B S T R A C T

We derived an integration-free induced pluripotent stem cell (iPSC) line from the peripheral blood mononuclear cells (PBMCs) of a 23-year-old male patient. This patient carries a 5′ splice site point mutation in intron 1 (c.31 + 1G > A) of the dystrophin gene, a mutation associated with X-linked dilated cardiomyopathy (XLDCM). Sendai virus was used to reprogram the PBMCs and deliver OCT3/4, SOX2, c-MYC, and KLF4 factors. The iPSC line (HKUi002-A) generated preserved the mutation, expressed common pluripotency markers, differentiated into three germ layers *in vivo*, and exhibited a normal karyotype. Further differentiation into cardiomyocytes enables the study of the disease mechanisms of XLDCM.

1. Resource table

Unique stem cell line identifier	HKUi002-A
Alternative name(s) of stem cell line	HKUi002-A-AYC12, AYC12
Institution	Department of Paediatrics and Adolescent Medicine, The University of Hong Kong
Contact information of distributor	Sophelia Hoi Shan Chan sophehs@hku.hk
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 23 years old Sex: Male Ethnicity if known: N/A
Cell Source	Peripheral blood mononuclear cells
Clonality	Clonal
Method of reprogramming	Sendai Virus vectors (CytoTune™-iPS 2.0 Sendai Reprogramming Kit, Thermo Fisher Scientific).
Genetic Modification	Yes
Type of Modification	Spontaneous mutation
Associated disease	X-linked dilated cardiomyopathy
Gene/locus	NM_004006.2 (DMD): c.31 + 1G > A On chromosome Xp21.2-p21.1
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	06/01/2019
Cell line repository/bank	The cell line has been registered at https://hpscreg.eu/cell-line/HKUi002-A
Ethical approval	

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2. Resource utility

The iPSC line generated can be used to study the novel pathophysiological mechanisms of the splice site intron 1 mutation of the dystrophin gene, which is associated with X-linked dilated cardiomyopathy with skeletal muscle sparing. It can also be used as a disease model for rescue drug screenings.

3. Resource details

X-linked dilated cardiomyopathy (XLDCM) due to dystrophin gene mutations is a cardiac-specific phenotype of dystrophinopathy characterized by preferential cardiac involvement without overt skeletal muscle weakness. It is a serious condition with early mortality in adolescents and young adults due to severe heart failure. Previous case studies have found a strong association between point mutations in the first exon-intron boundary of the dystrophin gene and XLDCM with skeletal sparing attributed to the upregulation of the brain and Purkinje isoforms of the dystrophin protein in the skeletal muscles but not the heart muscles (Chan et al., 2018; Kimura et al., 2007).

Our patient with XLDCM had severe heart failure required heart transplantation. One-year post-transplantation, he developed exercise-induced transient muscle weakness with new onset of elevated creatine kinase levels. Muscle biopsy confirmed normal structures and normal dystrophin immunohistochemical labeling. Sanger sequencing showed a heterozygous c.31 + 1G > A (GeneBank accession no. of ref. sequence: NM_004006.2) mutation at the 5′ splice site of intron 1 of the *DMD* gene.

Following institutional ethical committee approval and informed consent, blood was taken from our 23-year-old male patient and a human iPSC (induced pluripotent stem cell) line was generated. We reprogrammed the patient's peripheral blood mononuclear cells (PBMCs) into iPSCs using Sendai viruses containing the reprogramming factors OCT 3/4, SOX2, KLF4, and c-MYC.

After 14 days of reprogramming, iPSC colonies were manually picked. Three clones (HKUi002-A-AYC12, HKUi002-A-AYC47, HKUi002-A-AYC48) were selected for further expansion and characterization. In Table 1, we present the detailed characterization of clone HKUi002-A-AYC12 (AYC12). The iPSCs were evaluated based on their distinctive iPSC-like morphology (Fig. 1A), the expression of pluripotency markers by immunocytochemistry (Fig. 1B) and flow cytometry (Fig. 1C). Functional pluripotency was illustrated by teratoma formation assay. Hematoxylin-eosin staining revealed glands (endoderm), cartilage (mesoderm) and nerve endings (ectoderm) after iPSC transplantation and demonstrated the capacity of the iPSCs to differentiate into the three germ layers *in vivo*. (Fig. 1D).

HKUi002-A presented a normal 46 XY karyotype (Fig. 1E) with correct ploidy and no major chromosomal abnormalities. Fig. 1F shows the whole genome view of the chromosomal microarray of patient's PBMCs and HKUi002-A. The upper and lower panels show the log2 ratio (weighted) of the intensity of oligo probes, and allele difference plot of SNP probes for copy number analysis on each chromosome. No aberrant copy gain and loss was detected in HKUi002-A when comparing its genomic profile with that of the PBMCs. Sanger sequencing confirmed HKUi002-A had preserved the disease-causing mutation (Fig. 1G). HKUi002-A was also negative for mycoplasma contamination.

HKUi002-A were differentiated into cardiomyocytes (Burridge et al., 2015). Spontaneously beating cardiomyocytes were observed on Day 10 of differentiation. They expressed cardiomyocyte-specific markers, as revealed by immunofluorescence staining for the sarcomere proteins cardiac troponin T (cTnT) and α-actinin (Fig. 1H), and by RT-PCR of troponin I (*TNNI*), ventricular myosin light chain (*MYL2*), and myosin heavy chains *MYH6* and *MYH7* (Fig. 1I). The HKUi002-A-derived cardiomyocytes demonstrated absence of the dystrophin expression in Western blot analysis, with the cardiomyocytes derived from healthy control-derived iPSCs had full length dystrophin expression (Fig. 1J).

4. Materials and methods

4.1. Generation of patient-derived iPSCs

PBMCs from the patient's blood were isolated using the Ficoll–Paque centrifugation method. $5x10^5$ of the isolated PBMC were plated to a 24-well plate (Day −4) in StemPro-34 medium supplemented with 100 ng/ml SCF, 100 ng/ml FLT-3 Ligand, 20 ng/ml IL-3 and 20 ng/ml IL-6 (Thermo Fisher Scientific). From Day −3 to Day −1, cells were fed daily with a half-change of the same medium. On Day 0, transduction and reprogramming were performed using the CytoTune-

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography (Phase contrast microscopy)	IPSCs exhibit pluripotent stem cell-like morphology	Fig. 1 panel A
Phenotype	Qualitative analysis Immunocytochemistry	Positive staining for pluripotency markers: OCT4, NANOG, SOX2, TRA-1–60, TRA-1–81, SSEA4	Fig. 1 panel B
	Quantitative analysis Flow cytometry	83.2% $SOX2^+Oct3/4^+$ 87.2% $NANOG^+Oct3/4^+$	Fig. 1 panel C
Genotype	Karyotype (G-banding) and chromosomal microarray analysis	Normal karyotype 46, XY	Fig. 1 panel E and F
Identity	STR analysis	33 STR markers from 5 autosomes were analyzed with 28 and 23 markers being heterozygous, respectively, in PB-AY original and their iPSC-derived cells. Results of the STR markers confirmed the identity of the iPSC-derived cells.	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	X-linked mutations: DMD c.31 + 1G > A in intron 1	Fig. 1 panel G
Microbiology and virology	Mycoplasma	Mycoplasma testing by qPCR. Negative.	Submitted in archive with journal
Differentiation potential	Teratoma formation and direct differentiation into cardiomyocytes	Teratoma pathological analysis proofed differentiation to 3 germ layers: ectoderm, mesoderm and endoderm. Direct differentiation into cardiomyocytes resulted in beating iPSC-CMs	Fig. 1 panel D, H, I and J
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

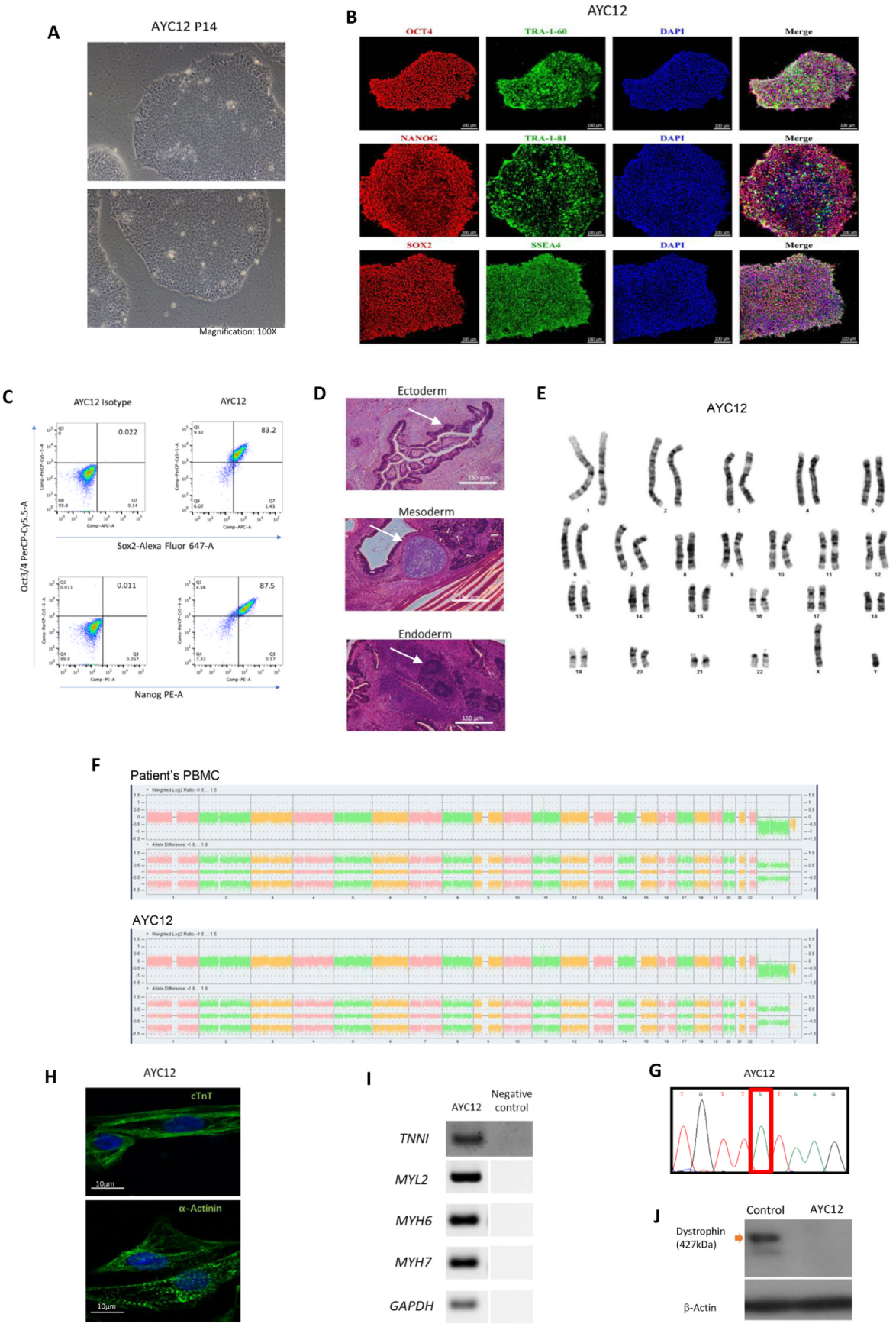

Fig. 1. Characterization of iPSC line HKUi002-A derived from a X-linked dilated cardiomyopathy patient carrying *DMD* gene mutation.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	rabbit anti-SOX2	1:500	Abcam Cat# ab97959, RRID:AB_2341193
Pluripotency markers	rabbit anti-OCT4	1:500	Abcam Cat# ab19857, RRID:AB_445175
Pluripotency markers	rabbit anti-NANOG	1:500	Abcam Cat# ab21624, RRID:AB_446437
Pluripotency markers	Mouse anti-SSEA4	1:500	Santa Cruz Biotechnology Cat# sc-21704, RRID:AB_628289
Pluripotency markers	Mouse anti-TRA-1–60	1:500	Abcam Cat# ab16288, RRID:AB_778563
Pluripotency markers	Mouse anti-TRA-1–81	1:500	Thermo Fisher Scientific Cat# 41–1100, RRID:AB_2533495
Secondary antibodies	Goat Anti-Mouse IgG H&L (Alexa Fluor® 488)	1:200	Abcam Cat# ab150113, RRID:AB_2576208
Secondary antibodies	Goat Anti-Rabbit IgG H&L (Alexa Fluor® 555)	1:200	Abcam Cat# ab150078, RRID:AB_2722519
Differentiation markers	Mouse anti-cTnT	1:500	Abcam Cat# ab8295, RRID:AB_306445
Differentiation markers	Mouse anti-α-actinin	1:500	Abcam Cat# ab9465, RRID:AB_307264
Western blot Primary antibodies	Rabbit anti-dystrophin	1:1000	Abcam, Cat# AB154168, RRID:AB_2858227
Western blot Primary antibodies	Mouse anti-β-actin	1:1500	Ambion Cat# AM4302, RRID:AB_437394
Western blot Secondary antibodies	Anti-Mouse IgG	1:10000	GE Healthcare Cat# NA931, RRID:AB_772210
Western blot Secondary antibodies	Anti-Rabbit IgG	1:10000	GE Healthcare Cat# NA934, RRID:AB_772206
Primers			
	Target	Forward/Reverse primer (5′-3′)	Product size
Targeted mutation analysis/ sequencing	DMD c.31 + 1G > A at 5′ splice site of intron 1	TGCTGAAGTTTGTTGGTTTCTCA/ ACAAACTATCTCACAGCAATCAAA	476 bp
Cardiomyocytes markers	MYL2	TATTGGAACATGGCCTCTGGAT/ GGTGCTGAAGGCTGATTACGTT	382 bp
Cardiomyocytes markers	MYH6	AGATCATCAAGGCCAAGGCA/ CGCTGGGTGGTGAAATCATT	121 bp
Cardiomyocytes markers	MYH7	AGACTGTCGTGGGCTTGTATCAG/ GCCTTTGCCCTTCTCAATAGG	101 bp
Cardiomyocytes markers	TNNI	AGTCACCAAGAACATCACGGAGAT/ GCAGCGCCTGCATCATG	125 bp

iPS 2.0 Sendai Reprogramming Kit with feeder-free culture conditions (Thermo Fisher Scientific). By Day 15–21, colonies of reprogrammed cells were transferred to plates coated with Geltrex (Thermo Fisher Scientific). The cells were then expanded in Essential 8 Medium (Thermo Fisher Scientific) with a humidified atmosphere of 5% CO_2 at 37 °C. Cells were passaged at a 1:6 ratio with 0.5 mM EDTA every 3–4 days when 70–80% confluence was reached, the cells were passaged until vector-free iPSCs were obtained (Seki et al., 2012).

4.2. Pluripotency marker immunocytochemistry

HKUi002-A were fixed and permeabilized with cytofix/cytoperm (BD Biosciences), and incubated with anti-OCT4, anti-NANOG, anti-SOX2, anti-TRA-1–60, anti-TRA-1–81 and anti-SSEA4 primary antibodies. They were then stained with secondary antibodies [Goat Anti-Mouse IgG H&L Alexa Fluor® 488 (Abcam) or Goat Anti-Rabbit IgG H&L Alexa Fluor® 555 (Abcam)] (Table 2). Images were obtained using laser scanning confocal microscopy (Zeiss LSM 880).

4.3. Flow cytometry analysis

The iPSCs were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and then stained using the Human Pluripotent Stem Cell Transcription Factor Analysis Kit (BD Biosciences) and analyzed with BD LSR II flow analyzer.

4.4. Teratoma formation

5×10^6 iPSCs were injected into the thigh muscles of 4-week-old severe combined immunodeficient mice. After 8 to 12 weeks, teratomas were observed. Formalin-fixed and hematoxylin-and-eosin stained teratoma sections were examined for endoderm, mesoderm, and ectoderm tissues under a microscope (Nikon, TS 100) (Seki et al., 2012).

4.5. Karyotyping by G-banding and chromosomal microarray analysis

Metaphase arrest was performed with 0.1ug/ml colcemid for 1 h. Cells were harvested and metaphase slides were prepared. G-banded metaphases were imaged and karyotyped by CytoVision Image Analysis and Capture Systems (Leica Microsystems). For chromosomal microarray analysis, genomic DNA (250 ng) extracted from HKUi002-A was tested for genome-wide copy number variation and the absence of heterozygosity (AOH) analyses using CytoScan 750 k SNP array (Affymetrix, Thermo Fisher Scientific). The DNA was subjected to restriction enzyme digestion, ligation, amplification, fragmentation, and labelling before loading onto the array for hybridization. After hybridization, the array was washed in GeneChip Fluidics Station 450 (Affymetrix) before being scanned by GeneChip Scanner 3000 7G (Affymetrix), then examined independently by two trained scientists using Chromosome Analysis Suite (ChAS) version 4.0 (Affymetrix) (D'Antonio et al., 2017).

4.6. Genotyping

Genomic DNA was extracted from HKUi002-A by alkaline lysis. PCR was performed to amplify the region containing the mutation site in the *DMD* gene by primers listed in Table 2 for Sanger sequencing.

4.7. iPSCs differentiation into cardiomyocytes

iPSCs were differentiated into cardiomyocytes via modulation of the WNT signaling pathway using the CDM3 media (Burridge et al., 2015). The iPSCs were plated onto Matrigel-coated surfaces. Between Day 10–16, CDM3 medium was replaced with CDM3-L (no D-glucose and

with L-lactic acid) to select for cardiomyocytes. Daily microscopic observations were performed to detect beating cells.

4.8. Reverse transcription-PCR for cardiac markers

RNA from the cells was isolated using Trizol Reagent (Invitrogen). cDNA was synthesized using PrimeScript™ RT Master Mix (TaKaRa). The reverse-transcription reaction was performed at 37 ˚C for 15 min, and then 85 ˚C for 5 sec. PCR was performed using AmpliTaq Gold™ 360 Master Mix (Thermo Fisher Scientific) with cycling parameters of 3 min initial denaturation at 93 ˚C, followed by 40 cycles of 93 ˚C for 30 sec, 60 ˚C for 30 sec, and 72 ˚C for 30 sec, in an Applied Biosystems SimpliAmp Thermal Cycler.

4.9. Western blot analysis

After lysing the cells with RIPA buffer, protein samples were run on a precast mini-protean TGX 4–15% gel (Bio-Rad). Proteins were transferred to a nitrocellulose membrane. Full length dystrophin protein of 427 kDa was detected by anti-dystrophin antibody (Abcam). β-Actin was used as a loading control (see Table 2).

4.10. Cardiac markers immunostaining

Immunofluorescence staining was performed for the cardiac-specific markers. Cells were fixed and permeabilized with the cytofix/cytoperm (BD Biosciences). They were then washed with Perm/Wash buffer (BD Biosciences), blocked with normal goat serum. Cells were incubated with primary antibodies for 1 h at 37 ˚C. The primary antibodies mouse anti-cTnT and mouse anti-alpha-actinin at 1:500 dilutions were used. Cells were then incubated with secondary antibody Goat Anti-Mouse IgG H&L Alexa Fluor® 488 (Abcam) (1:200) at room temperature in dark for 1 h. Images were taken using a laser scanning confocal microscope (Zeiss LSM 880).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.102040.

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