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Lab Resource: Multiple Cell Lines

Generation of a transgene-free iPSC line and genetically modified line from a facioscapulohumeral muscular dystrophy type 2 (FSHD2) patient with SMCHD1 p.Lys607Ter mutation

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A B S T R A C T

Facioscapulohumeral muscular dystrophy type2 (FSHD2), which constitutes approximately 5% of total FSHD cases and develops the same symptoms as FSHD type 1 (FSHD1), is caused by various mutations in genes including *SMCHD1*. We report the generation and characterization of an iPSC line derived from an FSHD2 patient carrying the SMCHD1 p.Lys607Ter mutation and its gene-corrected iPSC line which are free from transgene. These iPSC lines maintained normal karyotype, presented typical morphology, expressed endogenous pluripotency markers, and could be differentiated into ectodermal, mesodermal and endodermal cells, confirming their pluripotency.

1. Resource Table

Unique stem cell lines identifier	CIRAi006-ACIRAi006-A-1
Alternative names of stem cell lines	F2KU2#17 (CIRAi006-A)IsC41-F2KU2#17 (CIRAi006-A-1).
Institution	Center for iPS Cell Research and Application, Kyoto University
Contact information of distributor	Hidetoshi Sakurai; hsakurai@cira.kyoto-u.ac.jp
Type of cell lines	iPSC
Origin	Human
Cell Source	fibroblast
Clonality	Clonal
Method of reprogramming	Episomal vector
Multiline rationale	disease and gene corrected clones
Gene modification	YES
Type of modification	Gene Correction
Associated disease	facioscapulohumeral muscular dystrophy 2
Gene/locus	SMCHD1 c.1819A > T
Method of modification	CRISPR/Cas9 with ssODN as template
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	2020-05-29
Cell line repository/bank	the public repository: hPSCregthe accession number: CIRAi006-A, CIRAi006-A-1
Ethical approval	Ethical committee name: National Center of Neurology and PsychiatryApproval number: A2012-111

2. Resource utility

SMCHD1 mutations, combined with 4qA haplotype, allow DUX4 mis-expression, leading to skeletal muscle atrophy and weakness. The isogenic iPSC lines in the presence or absence of pathogenic SMCHD1 mutation described here will provide a valuable cell model for investigation of FSHD pathology and novel cure development.

3. Resource Details

Facioscapulohumeral muscular dystrophy (FSHD) is caused by reduction of D4Z4 macrosatellite repeat on the chromosome 4q35 region combined with cis-4qA haplotype (FSHD type1; FSHD1) or caused by gene mutations in chromatin regulators for the 4q35 region including SMCHD1 combined with 4qA haplotype not associated with abnormal D4Z4 reduction (FSHD type2; FSHD2) (Greco et al., 2020; Lemmers et al., 2012). Gene correction of SMCHD1 mutation in FSHD2 patient-derived cells theoretically convert the original clone into isogenic cells genetically unrelated to FSHD, which will provide a useful cell model for pathological studies of FSHD allowing precise genetic comparison. In this study, we report the generation and characterization of an iPSC clone derived from dermal fibroblasts of a female FSHD2 patient carrying a heterozygous SMCHD1 p.Lys607Ter mutation, whose clinical information is referred to the patient 2 in the previous report (Hamanaka et al., 2016) and its gene-corrected iPSC clone. The fibroblasts were isolated from a skin biopsy of the donor patient and transfected with the episomal vectors for reprogramming. Primary colonies were picked and expanded as individual clones. The iPSC clone

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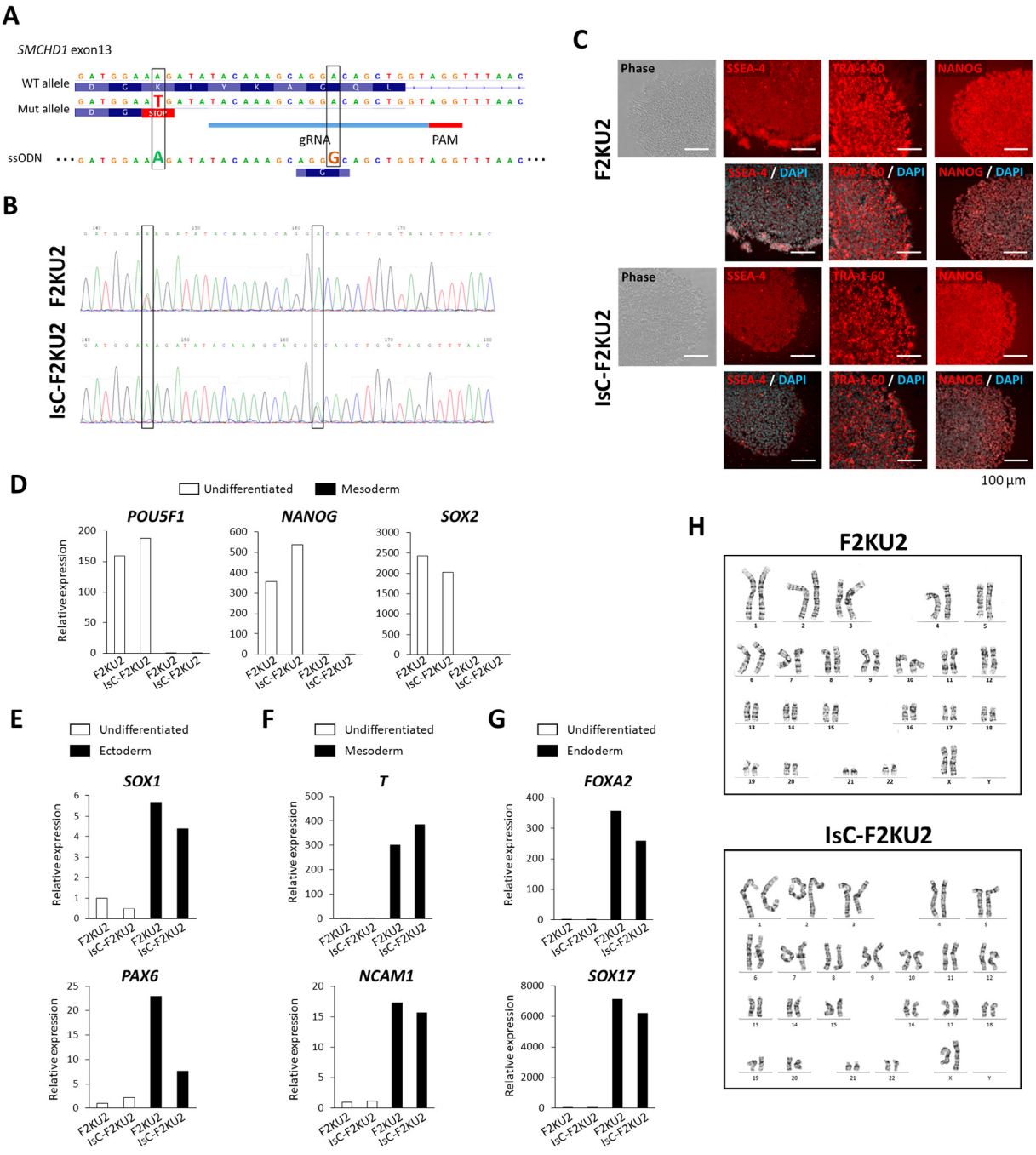

Figure 1.

F2KU2#17 (or shortly F2KU2 in Fig.1) was selected after series of characterization (Table 1). For gene correction, the F2KU2#17 cells were transfected with Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9) protein, synthesized sgRNA (single-guide RNA) targeting the site close to the mutation and template single-stranded oligodeoxynucleotide (ssODN) which contains the wild type sequence before and after the donor's mutated site with a single silent mutation within the sgRNA target region for indication of homologous recombination and blocking of secondary Cas9 access (Fig. 1A). After sub-cloning of transfected cells followed by sequence analysis, in the IsC41-F2KU2#17 clone (or shortly IsC-F2KU2 in Fig.1), one of the two successfully edited clones in total 48 clones, the substitution for pathogenic mutation and the heterozygous silent mutation were confirmed (Fig. 1B). TA cloning showed that the heterozygous silent mutation was induced on the originally pathogenic allele and wild type allele was intact (*data not shown*). These isogenic clones showed normal iPSC morphology (flat, round or polygonal with defined borders) by light microscopy observation and expressed the human pluripotency markers SSEA-4, TRA-1-60 and NANOG, as demonstrated by immunofluorescence (Fig. 1C) and POU5F1, NANOG and SOX2 by RT-qPCR (Fig. 1F). In order to demonstrate the pluripotency of these established iPSC clones, the cells were differentiated following three separate differentiation protocols that promote the induction of ectodermal, mesodermal and endodermal lineages. RT-qPCR analysis confirmed the gene expression of markers including SOX1 and PAX6 in the ectodermal induction (Fig. 1E), T and NCAM1 in the mesodermal induction (Fig. 1F) and FOXA2 and SOX17 in the endodermal induction (Fig. 1G). Short tandem repeat (STR) analysis of 10 different genomic loci demonstrated that the parental F2KU2#17 iPSC clone and its isogenic control IsC41- F2KU2#17 clone shared alleles with a 100% match

Table 1
Summary of lines

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
F2KU2#17 (CIRAi006-A)	F2KU2	Female	55	Japanese	Heterozygous,c.1819A > T(p.Lys607Ter)	facioscapulohumeral muscular dystrophy type2 (FSHD2)
IsC41-F2KU2#17(CIRAi006-A-1)	IsC-F2KU2	Female	55	Japanese	Heterozygous,c.1835A > G(p.Gly612Gly)	facioscapulohumeral muscular dystrophy type2 (FSHD2)

(archived at SCR journal). These iPSC clones exhibited a normal karyotype (46, XX) (Fig. 1H) and were free from integration of episomal vectors (Supplementary Fig. 1A) and mycoplasma (Supplementary Fig. 1B).

4. Materials and Methods

4.1. Reprogramming of fibroblasts and maintenance of iPSCs

Fibroblasts were isolated from the FSHD2 patient and maintained in DMEM supplemented with 10% FBS. For reprogramming, fibroblasts are transfected with episomal vectors pCE-hOCT3/4 (addgene #41813), pCE-hSK (addgene #41814), pCE-hUL (addgene # 41855), pCE-mp53DD (addgene #41856) and pCXB-EBNA1 (addgene #41857) with Neon Transfection System (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were cultured in DMEM supplemented with 10% FBS, which was replaced gradually by primate embryonic stem (ES) cell medium (ReproCELL, Japan) supplemented with 4 ng/mL recombinant human basic fibroblast growth factor (Oriental Yeast, Japan). Colonies were picked and separately transferred into single wells with inactivated mouse feeder cells and after two passages transferred and maintained in StemFit® AK02N (AJINOMOTO) on a 12-well or 6-well plate coated with Easy iMatrix-511 silk (TAKARA). Karyotype was evaluated by G-banding method in LSI Medience.

4.2. Transfection and sub-cloning

iPS cells were harvested and resuspended in MaxCyte buffer. sgRNA was designed and synthesized according to the previous report (Xu et al., 2019). For RNP electroporation, 10 μg of recombinant SpCas9 protein (Thermo Fisher Scientific) and 2.5 μg of sgRNA were incubated for 5 minutes to make complex and then 1.25 x 10^6 cells with 50 μL of MaxCyte buffer and 12 μg of ssODN were added to the RNP complex and electroporation was processed with MaxCyte ATX system and OC-100 processing assemblies according to the manufacture's instruction. Electroporated cells were cultured in bulk, separated into single clones on 96 well plates at the first passage, cultured to form colonies and expanded on 24 well plates at the second passage for cell stocks and genome DNA extraction.

4.3. In vitro trilineage differentiation

hiPSCs were differentiated to each germ layer lineage using STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies) according to the manufacturer's protocol.

4.4. Immunocytochemistry

Immunostaining was performed following our standard procedures (Sasaki-Honda et al., 2018) using specific primary and secondary antibodies (Table 2). Cells were imaged on a BZ9000 system (Keyence, Japan).

4.5. RNA extraction and real-time reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from the iPSCs and processed by RT-qPCR following our standard procedures (Sasaki-Honda et al., 2018). Real-time RT-qPCR was performed using specific primers (Table 3), SYBR Green (Applied Biosystems) and a Step One Plus thermal cycler (Applied Biosystems) and relative expression level was calculated with the 2-ΔΔCT method. RPLP0 was used as the internal control.

4.6. Sequencing analysis and transgene integration check

Genome DNA was extracted from each iPSC clone and used as a PCR

Table 2
Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	*Visual record of the line: normal iPSC morphology*	Fig. 1 C
Phenotype	Qualitative analysis	Wide expression of pluripotency markers: SSEA-4, TRA-1-60, and NANOG	Fig. 1 C
	Quantitative analysis by RT-qPCR	The lines express OCT4, NANOG and SOX2.	Fig. 1 D
Genotype	Karyotype (G-banding) and resolution	46, XX,Resolution 400	Fig. 1 H
Identity	STR analysis	N/A 10 sites tested, identity verified (100% match)	N/A submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	F2KU2: heterozygous, c.1819A > TIsC-F2KU2: heterozygous, c.1835A > G	Fig. 1 A and B
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence.Negative	Supplementary Fig. 1B
Differentiation potential	In vitro trilineage differentiation	proof of three germ layers formation by RT-qPCR for gene markers of each lineage	Fig. 1 E-G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	not shown but available with author
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3
Reagents details

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Mouse Anti-TRA-1-60	1:100	Millipore Cat# MAB4360, RRID: AB_2119183
Pluripotency Markers	Mouse Anti-SSEA-4	1:100	Millipore Cat# MAB4304, RRID: AB_177629
Pluripotency Markers	Mouse Anti- Nanog	1:200	Cell Signaling Technology Cat# 4893, RRID: AB_10548762
Secondary antibodies	Goat Anti-Mouse IgG	1:500	Thermo Fisher Scientific Cat# A-11031, RRID: AB_144696
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Pluripotency Markers (qPCR)	POU5F1	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC	
Pluripotency Markers (qPCR)	NANOG	CAGTCTGGACACTGGCTGAA/ CTCGCTGATTAGGCTCCAAC	
Pluripotency Markers (qPCR)	SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG	
Ectodermal Markers (qPCR)	SOX1	ACCAGGCCATGGATGAAG/ CTTAATTGCTGGGGAATTGG	
Ectodermal Markers (qPCR)	PAX6	TGTCCAACGGATGTGTGAGT/TTTCCCAAGCAAAGATGGAC	
Mesodermal Markers (qPCR)	T	ACCCAGTTCATAGCGGTGAC/ CATTGGGAGTACCCAGGTTG	
Mesodermal Markers (qPCR)	NCAM1	TGAGTGGAGAGCAGTTGGTG/ CCTTACGGCGTACGTTGTTT	
Endodermal Markers (qPCR)	FOXA2	TGGGAGCGGTGAAGATGGAAGGGCAC/ TCATGCCAGCGCCCACGTACGACGAC	
Endodermal Markers (qPCR)	SOX17	CGCACGGAATTTGAACAGTA/ GGATCAGGGACCTGTCACAC	
House-Keeping Genes (qPCR)	RPLP0	AAACGAGTCCTGGCCTTGTCT/ GCAGATGGATCAGCCAAGAAG	
Targeted mutation analysis/sequencing	SMCHD1	GAACAGCGAATGAAAATTGA/ TTCATTCTTACCTAGCTCCT	
ssODN	SMCHD1	taaaaagcaaggtccctgggcaacatatgcagcaatagaatgggatggaAagatatacaaagcaggGcagctggtaggtttaacttattgtcacttttt	
sgRNA	SMCHD1	TACAAAGCAGGACAGCTGGT AGG (PAM)	
Integration check PCR	Episomal vectors	TTCCACGAGGGTAGTGAACC/ TCGGGGGTGTTAGAGACAAC	
Integration check PCR	DLX5	TTCCAAGCTCCGTTCCAGAC/ CCCCGTAGGGCTGTAGTAGT	

template. The SMCHD1 mutation and induced silent mutation was confirmed by PCR amplification (primers listed in Table 3) and Sanger sequencing (Eurofins Genomics) by using the forward primer of the PCR reaction. For integration check, PCR amplification with primers for episomal vectors and endogenous DLX5 gene locus were performed and the products were checked by electrophoresis.

4.7. Mycoplasma detection

The absence of mycoplasma in the culture medium was tested using MycoAlert™ Mycoplasma Detection Kit (LONZA).

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101884.

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