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Lab Resource: Stem Cell Line

# Generation of MERRF patient-derived induced pluripotent stem cell line iMERRF-C7

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ABSTRACT

Human iPSC line iMERRF-C7 was generated from PBMCs of a patient with mitochondrial disorder MERRF. Using Sendai virus, the reprogramming factors Oct3/4, Sox2, Klf4, and cMyc were delivered non-integratively. The resulting iPSCs expressed pluripotency markers, could differentiate into the three germ layers in vivo, had normal genomic structure, and retained the disease-causing m.8344 mutation with similar heteroplasmic level.



Resource table.

| | |
|---|---|
| Name of stem cell line | iMERRF-C7 |
| Institution | Obstretrics and Gynecology Hospital Affiliated to Nanjing Medical University |
| Person who created resource | Dong Liang |
| Contact person and email | Dong Liang, liangdong@njmu.edu.cn |
| Date archived/stock date | Sep. 24, 2016 |
| Origin | Human PBMC |
| Type of resource | Biological reagent: induced pluripotent stem cell (iPS) derived from human MERRF patient |
| Sub-type | Cell line |
| Key transcription factors | Oct3/4, Sox2, cMyc, Klf4 |
| Authentication | Identity and purity of cell line confirmed (Fig. 1) |
| Link to related literature | None |
| Information in public databases | None |
| Ethics | Patient informed consent obtained<br>Ethics Review Board-competent authority approval obtained |

## Resource details

Myoclonic epilepsy associated with ragged-red fibers (MERRF) is a multisystem disorder caused by a mutation in the mitochondrial DNA (mtDNA) gene MT-TK. The most common pathogenic variant is an A-to-G transition at nucleotide 8344 (m.8344A>G). However, the occurrence of "heteroplasmy" can result in varying tissue distribution of mutated mtDNA, and lead to varying symptoms (Hammans et al., 1993; Enriquez et al., 1995). In this study, PBMCs were obtained from an 11-years-old MERRF patient with m.8344A>G mutation in the MT-TK allele. Using Sendai virus containing the reprogramming factors Oct3/4, Sox2, cMyc, Klf4, the human MERRF iPSC lines were generated from the PBMCs non-integratively (Fig. 1A). The iPSC line described in this publication was named iMERRF-C7. Five additional clones from the same patient were also isolated and characterized (data not shown). All iMERRF-C7 iPSCs stained positive for the pluripotency markers SSEA-4, TRA-1-60 and TRA-1-81 at passage of 10 (Fig. 1B), illustrating the purity of the iPSC line. Accordingly, the expression of the other pluripotency genes OCT4, SOX2, C-MYC, NANOG and TERT was upregulated in iPSCs compared to patient PBMCs, and the gene expression levels were comparable to those of a characterized positive control H9 line (Fig. 1C). Pluripotency was supported by the capability of iMERRF-C7 to differentiate into the three germ layers in vivo by teratoma assay (Fig. 1D). iMERRF-C7 had a normal karyotype (46, XX), and had no copy number variation larger than 300 kb analyzed by SNP-array (Fig. 1E). The co-existence of the m.8344A>G mutation and wild type allele in both iMERRF-C7 and patient PBMCs was confirmed by Sanger sequencing. The heteroplasmic level of iMERRF-C7 was measured by pyrosequencing the mitochondrial DNA flanking m.8344 loci, which is about the same level compared to the primary patient PBMCs (Fig. 1F). Additionally, there are variations of the m.8344 heteroplasmic levels in the other iPSC lines generated from the same patient, including one line with no mutant allele (Table S1), which is similar with the previous study (Fujikura et al., 2012).

## Materials and methods

### *Reprogramming of PBMCs to iPSCs*

Written informed consent was obtained from the MERRF patient and the study was approved by the scientific ethical committee in Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University. Peripheral blood was taken from the 11-years-old female MERRF patient with m.8344A>G mutation. PBMC was isolated by density gradient centrifugation with Histopaque Reagent (Sigma-Aldrich, USA).

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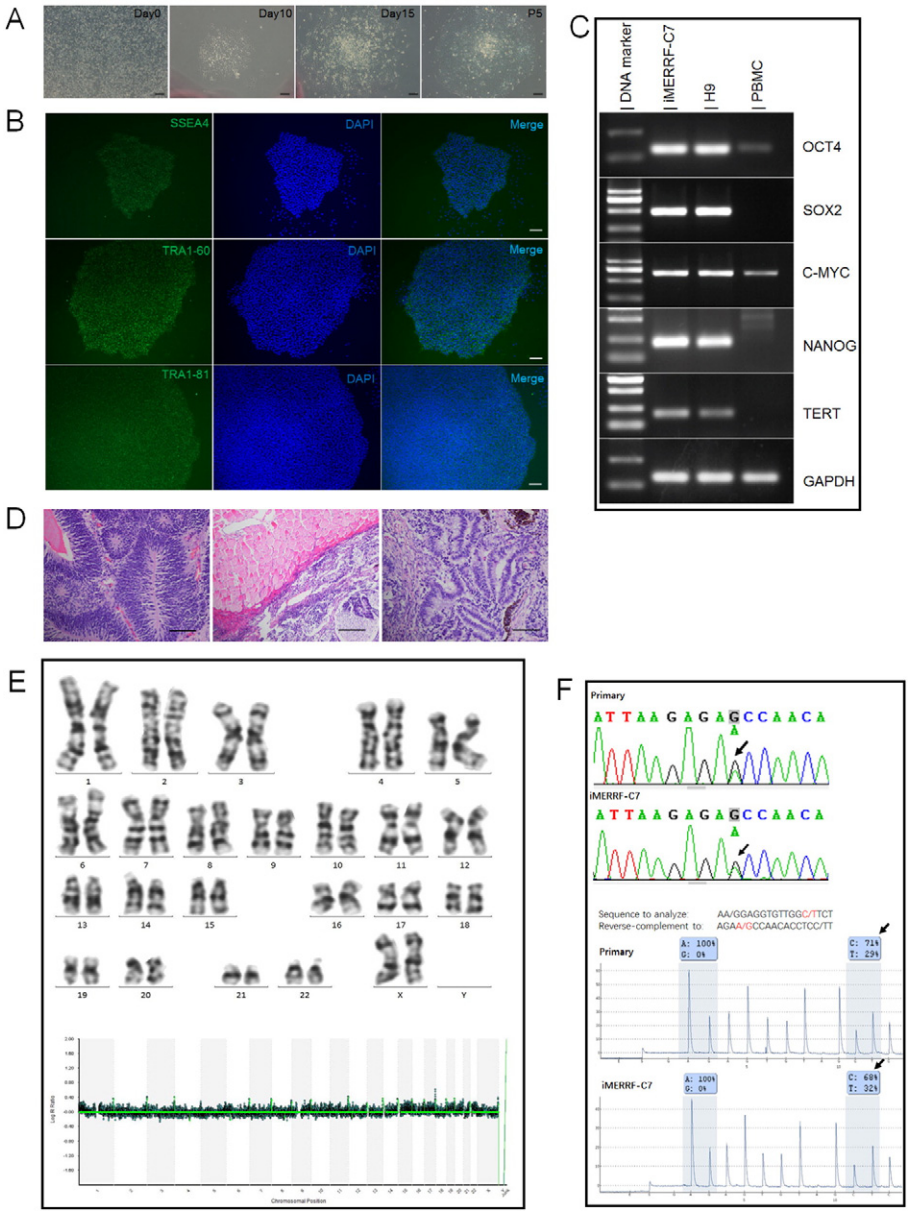


**Fig. 1.** Molecular and functional characterization of the iMERRF iPSC line. A. Reprogramming of the MERRF patient derived PBMC. Representative cells under a phase-contrast microscope. Scalebars: 100 μm. B. Protein expression of pluripotency markers. Fluorescent immunocytochemistry for pluripotency markers was performed on iMERRF-C7 iPSCs. Scalebars: 100 μm. C. Gene expression of pluripotency markers. Pluripotency gene expression was identified using RT-PCR. GAPDH was used as a loading control. D. The differentiation potential to three germ layers identified by in vivo teratoma assay. From left to right: ectoderm (neural tube), mesoderm (muscle), and endoderm (glands). Scalebars: 100 μm. E, Karyotyping and SNP-array. Representative karyotype and SNP-array result of iMERRF-C7 iPSCs. F. The mitochondrial mutation and heteroplasmic level. Upper, Sanger sequencing result showing the A to G mutation at the m.8344 loci in both primary cells and iMERRF-C7 iPSCs indicated by the arrows. Lower, Pyrosequencing result showing the similar mitochondrial heteroplasmic levels before and after reprogramming, while arrows indicate the heteroplasmic levels.

The reprogramming method was adopted from the protocol of CytoTune 2.0 Sendai Reprogramming Kit (ThermoFisher Scientific, USA). Briefly, the isolated PBMCs were cultured for 4 days at 37 °C and 5% $CO_2$ using StemPro-34 Medium (ThermoFisher Scientific) added with 100 ng/ml SCF, 100 ng/ml FLT-3, 20 ng/ml IL-3 and 20 ng/ml IL-6 (ThermoFisher Scientific). On the day of transduction, $3 \times 10^5$ cells were harvested and transduced using the CytoTune 2.0 Sendai reprogramming vectors (KOS MOI = 6, hc-Myc MOI = 6, hKlf4 MOI = 4, ThermoFisher Scientific). The transducted cells were cultured in StemPro-34 Medium for 2 days, and then seeded on hESC-qualified matrigel (BD Biosciences, USA) coated plates in Essential 8 medium (ThermoFisher Scientific). On days 21–28 after transduction, single iPSC colonies were manually cut and passaged. From passage 5, iPSCs were split every 4–7 days with 0.5 mM EDTA (ThermoFisher Scientific). The iPSC lines were frozen in Essential 8 medium with 10% DMSO or cultured for further tests.

*Fluorescent immunocytochemistry*

Cells were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature, followed by three washes with PBS and permeabilization with 1% Triton X-100 (Sigma) for 30 min. Cells were blocked in a solution containing 1% bovine serum albumin (BSA) in PBST for 1 h at room temperature. Incubate with the primary antibody diluted in the blocking buffer overnight at 4 °C. Primary antibodies were used as follows: SSEA4, TRA1-60, TRA1-8 (1:100 dilution, ThermoFisher Scientific). After washing 3 × 10 min with PBS, appropriate fluorescence-labeled secondary antibodies were diluted at 1:1000 in the blocking

**Table 1**
PCR primers.

| | Target | Forward/reverse primer (5′–3′) |
|---|---|---|
| Pluripotency markers | *OCT4* | GGGAGGAGCTAGGGAAAGAAAACCT<br>GAACTTCACCTTCCCTCCAACCAGT |
| | *SOX2* | CCCCCGGCGGCAATAGCA<br>TCGGCGCCGGGGAGATACAT |
| | *C-MYC* | GGAACAAGAAGATGAGGAAG<br>TGATTGCTCAGGACATTTC |
| | *NANOG* | ATGGAGGGTGGAGTATGGTTGG<br>AGGCTGAGGCAGGAGAATGG |
| | *TERT* | TGTGCACCAACATCTACAAG<br>GCGTTCTTGGCTTTCAGGAT |
| House-keeping gene | *GAPDH* | GCACCGTCAAGGCTGAGAAC<br>TGGTGAAGACGCCAGTGGA |
| Pyrosequencing | *MT-TK* (m.8344) | [Btn]-TGGAGCAAACCACAGTTTCAT<br>TGGTGGGCCATACGGTAGTATT |
| | Probe | GGGGCATTTCACTGTA |

buffer and incubated for 1 h at room temperature while protected from light. After washing 3 × 10 min with PBS, the nuclei were counterstained with DAPI (Invitrogen) staining solution for 10 min at room temperature. Results were visualized and imaged under the DMi8 fluorescent microscope (Leica, Germany).

*Reverse transcript polymerase chain reaction (RT-PCR)*

RNA of the iPS cells were isolated using Trizol LS Reagent (Invitrogen) according to standard protocol. 1 μg RNA/reaction was reverse transcribed using RevertAid First Strand cDNA Sytnthesis Kit (ThermoFisher Scientific) according to manufacturer's protocol. Primers are listed in Table 1.

*Teratoma assay*

The in vivo differentiation experiments (teratoma formation) were performed by Cellapy Company (China, http://www.cellapy2013.bioon.com.cn/). Briefly, 3 aliquots of approximately $1 \times 10^7$ cells of the P10 iPSCs were collected by 0.5 mM EDTA treatment. Cells were resuspended in PBS and immediately injected subcutaneously into 3 immunodeficient NOD-SCID mice (Charles River, China). The teratomas were collected after 2 months, and processed using standard procedures for paraffin embedding, followed by paraffin section and hematoxylin and eosin staining. Histological analysis was performed by a pathologist.

*Karyotyping and SNP-array*

For cytogenetic analysis of the iPSC lines generated from the MERRF patient, GTG banding at the 400 to 550-band level was performed according to a standard protocol. Twenty metaphases were counted and karyograms were analyzed using cytovision software.

To detect any potential genomic abnormalities at a higher resolution, SNP-array was performed. Briefly, genomic DNA was extracted from the cells using the QIAamp DNA Mini Kit (Qiagen, Germany). The human cyto12 SNP-array (Illumina, USA) comprising around 300,000 SNPs was applied for the whole genome scan. Scanned images were then analyzed and quantified by an algorithm with fixed settings in BlueFuse Multi Software (Illumina) (available protocol at www.cytochip.com).

*Sanger sequencing and pyrosequencing*

Point mutation in the mitochondrial DNA 8344 were identified Sanger Sequencing. The fragments flanking the m.8344 loci were amplified by PCR from the total DNA of primary cells or iPSCs, then PCR products were purified and sequenced on an ABI 3730xl DNA analyzer.

The heteroplasmy level of the primary PBMCs or iPSCs was determined by pyrosequencing. The primers for PCR amplification and sequencing were designed using the software PryoMark Assay Design (version 2.0, Qiagen). PCR was performed with primers biotinylated to convert the PCR product to single-stranded DNA templates, using the Vacuum Prep Tool. After PCR amplification, pyrosequencing reactions quantification were performed using PyroMark Q96 ID (Qiagen) reagents, equipment, and software (version 2.0, Qiagen), according to the manufacturer's instructions.

All the primers used are listed in Table 1.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.11.008.

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