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Human induced pluripotent stem cells generated from a patient with a homozygous L272P mutation in the *OTULIN* gene (NIHTVBi014-A)

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Abstract

We have successfully generated induced pluripotent stem cells (iPSC) from dermal fibroblasts of a patient with a homozygous p.Leu272Pro mutation in the gene encoding the linear deubiquitinase OTULIN. Biallelic loss of function mutations in this gene are responsible for the OTULIN deficiency termed Otulipenia or OTULIN-related autoinflammatory syndrome (ORAS). The iPSC carrying homozygous L272P *OTULIN* gene mutations are phenotypically normal and they have capacity to differentiate toward the three germ layers. These iPSC have great potential to study the role of linear ubiquitination in the regulation of immune responses and other cellular pathways.

1. -25167335560Resource Table

Unique stem cell lines identifier	NIHTVBi014-A

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[1]These authors contributed equally to this work

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101921.

Alternative names of stem cell lines	iPSC p342
Institution	National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, Maryland, USA
Contact information of distributor	Manfred Boehm; boehmm@nhlbi.nih.gov
Type of cell lines	iPSC
Origin	Human
Cell Source	Dermal fibroblasts
Clonality	Clonal cell lines
Method of reprogramming	Sendai-virus vectors containing the transcription factors Oct-4, Klf4, Sox2 and c-MYC
Multiline rationale	Lines derived from the individual
Gene modification	Yes
Type of modification	Hereditary
Associated disease	OTULIN-related autoinflammatory syndrome (ORAS)
Gene/locus	OTULIN, 5p15.2
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	2015
Cell line repository/bank	N/A
Ethical approval	National Institutes of Health Ethics Committee (Approval Number: 15H0190)

2. Resource utility

The human induced pluripotent stem cells (hiPSC) carrying homozygous L272P *OTULIN* gene mutations possess the potential of differentiating into variety of cell types including immune cells. The derivatives sustaining the mutation could be a powerful platform allowing for investigating the molecular mechanisms of disease, and for drug screening to search the targets for treatment of these patients.

3. Resource details

The OUTLIN is the key deubiquitinase (DUB) with the role to remove linear also known as Methionine-1 (M1)-linked ubiquitin chains from various signaling pathways, including the canonical NF-κB pathway, which regulate immune homeostasis and responses to infection (Keusekotten et al., 2013). It has been reported that M1-specific deubiquitinase OTULIN is essential for preventing TNF-associated systemic inflammation in humans and mice. Biallelic hypomorphic mutation in human *OTULIN* gene causes a potentially fatal autoinflammatory condition variably termed Otulipenia, OTULIN-related autoinflammatory syndrome (ORAS), or Autoinflammation, panniculitis, and dermatosis syndrome (AIPDS). This monogenic autoinflammatory disease is characterized by neonatal-onset of recurrent fever, erythematous rash with painful nodules and lipodystrophy, joint and gastrointestinal inflammation (Damgaard et al., 2016, Zhou et al., 2016). OTULIN deficiency is a rare and potentially lethal disease and very few patients have been reported in the literature. Patients present with a severe inflammatory phenotype that is responsive to treatment

with corticosteroids or cytokine inhibitors. The hematopoietic stem cell transplantation (HSCT) could rescue the phenotype and has been successfully performed in one patient (Damgaard et al., 2019). However, the precise molecular mechanism of dysregulated M1-linked polyubiquitin signaling resulting in the disease is largely unknown.

We identified a male patient carrying the homozygous missense mutation p.Leu272Pro (614712.0001) in the *OTULIN* gene by exome sequencing. The patient was enrolled into our NHLBI clinical protocols for further investigations. Information regarding clinical features of this patient were obtained using the standard clinical questionnaire (Table 1). Skin punch biopsy samples were collected at the NIH Clinical Center. Using a Sendai-OKSM delivery system expressing four transcription factors (OCT4, SOX2, KLF4, and C-MYC), we have successfully generated iPSC (p342) from skin fibroblasts from the patient with the homozygous mutation in the *OTULIN* gene. We have also derived iPSC lines from healthy volunteers (Control) who did not have the mutation (data did not shown). The iPSC (p342) maintained typical morphologies and expressed phenotypically the pluripotency markers OCT4, NANOG, TRA-160, SSEA4, and SOX2, as shown by immunocytochemistry (Fig. 1A) and/or FACS (Fig. 1B), and/or real-time (RT)-qPCR (Fig. 1E). The iPSC (p342) were free of Sendai virus confirmed by RT-PCR at 15th passage (Fig. 1C). Genotyping of the generated iPSC (p342) showed the point mutation L272P in the *OTULIN* gene that were the same as their parental fibroblasts (Fig. 1D). To test the differentiation potential of the cells, we performed a monolayer differentiation assay to drive the cells towards the three germ layers *in vitro.* We determined the marker gene expression for the mesoderm (*RUNX1*), endoderm (*AFP*), and ectoderm (*NESTIN*) by RT-qPCR, which showed comparable expression levels between the iPSC (p342) and control lines (Control) (Fig. 1E). Short tandem repeat (STR) profiles indicated that the iPSC (p342) matched with its parental fibroblast completely in 15 amplified STR loci (see Supplementary file 1). All cultures were routinely tested for *Mycoplasma* contamination and were found to be *Mycoplasma* free as shown in Supplementary file 2. The iPSC (p342) demonstrated chromosomal stability and a normal karyotype with G-banding (Fig. 1F). In conclusion, the hiPSC (p342) exhibited the pluripotent potential for self-renew and differentiation, suggesting the successful generation of iPSCs from ORAS patient. To the best of our knowledge, this is the first report of human iPSC that were generated from a patient with OTULIN deficiency (see Table 2).

4. Materials and methods

4.1. Subjects and derivation of fibroblasts

The fibroblasts were derived from skin punch biopsy samples obtained from the ORAS patient who carries homozygous *OTULIN* gene mutation. Those cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% penicillin–-streptomycin, as previously described (Jin et al., 2016). This study was approved by the NHLBI's institutional review board, and samples were collected after obtaining informed written consents.

4.2. Generation and culture of human iPSC from fibroblasts

Fibroblasts from the ORAS patient were reprogrammed for generating iPSC lines by using CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Invitrogen). The iPSC colonies were picked up at 21 days posttransduction, and expanded in a typical hESC/iPSC culture condition (Jin et al., 2016).

4.3. Immunofluorescent staining and Flow cytometry analysis (FACS)

iPSC colonies were fixed with 4% paraformaldehyde and stained following the previous protocol (Jin et al., 2016). In brief, cells were incubated with primary antibodies against NANOG, SOX2, SSEA4, and TRA-1–60 (Table 3) at 4 °C overnight. Following washing with PBS, they were incubated with appropriate fluorophore-tagged secondary antibodies at room temperature for 1 h. After washing with PBS, nuclei were stained with DAPI. Images were captured using a fluorescence microscope (Zeiss).

For FACS analysis, iPSC were dissociated into a single cell by using Trypsin-EDTA (Invitrogen). Following by fixation and permeabilization, the cells were stained with antibodies designed (Table 3). The data acquisition was performed on a MACSQuant Flow Cytometer (Miltenyi) and the results were analyzed with FlowJo software (FlowJo, LLC).

4.4. Monolayer differentiation assay

To assess differentiation ability of iPSC *in vitro*, cells were dissociated into small clumps with 0.5 μM EDTA and then cultured on Matrigel Precoated Plates (Corning) with differentiation medium consisting of 90% KnockOut DMEM, 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 0.1 mM 2-mercaptoethanol (Invitrogen). After seven days, cells were harvested for further analysis.

4.5. Gene expression analysis by RT-PCR

The total RNA was isolated by using RNeasy Mini Kits (Qiagen). cDNA was synthesized by reverse transcription (RT) using Super script™ III (Invitrogen).

After 15 passages, iPSC was tested for Sendai virus (SeV) residues. PCR was performed using the primers indicated in Table 3 and following the instructions as recommended by the manufacturer. The iPSC obtained at passage 1 served as positive control (PC) shown in Fig. 1C.

Endogenous mRNA expression levels of *NANOG*, *SOX2*, *AFP*, *NESTIN*, and *RUNX1* were determined in iPSC (iPSC) and in differentiating cells at day 7 (differentiated) shown in Fig. 1E. For this, RT-qPCR was performed by using SYBR Green Premix on a Real-Time PCR Detection System (Bio-Rad). Assays were run in triplicate and the results were normalized to 18S ribosomal RNA expression. Primers used for RT-qPCR are shown in Table 3.

4.6. Karyotyping assay

The karyotype of the iPSC was evaluated by the WiCell Research Institute using G-banding metaphase karyotype analysis.

4.7. DNA sequencing and STR

Genomic DNA was extracted by using DNeasy Blood & Tissue Kit (Qiagen). To amplify the corresponding deletion position in *OTULIN* gene, PCR was performed with specific primers (Table 3). Following purification, the PCR products were sent to Eurofins Scientific for sequencing.

STR analysis was performed by WiCell Research Institute, which generated a STR profile via the Promega Powerplex® 16 System to verify STR polymorphisms for 15 loci plus amelogenin in genomic DNA extracted from iPSCs and their parental fibroblasts.

4.8. Mycoplasma detection

To validate the cultures of iPSC were *Mycoplasma* free, supernatant were collected after culturing for 48 h while the confluency of cells was at last 80%. The mycoplasma analysis was performed using the MycoAlert™ Mycoplasma Detection Kit (Lonza, LT27–224). The absence of mycoplasma contamination was confirmed in the culture tested (Supplement file 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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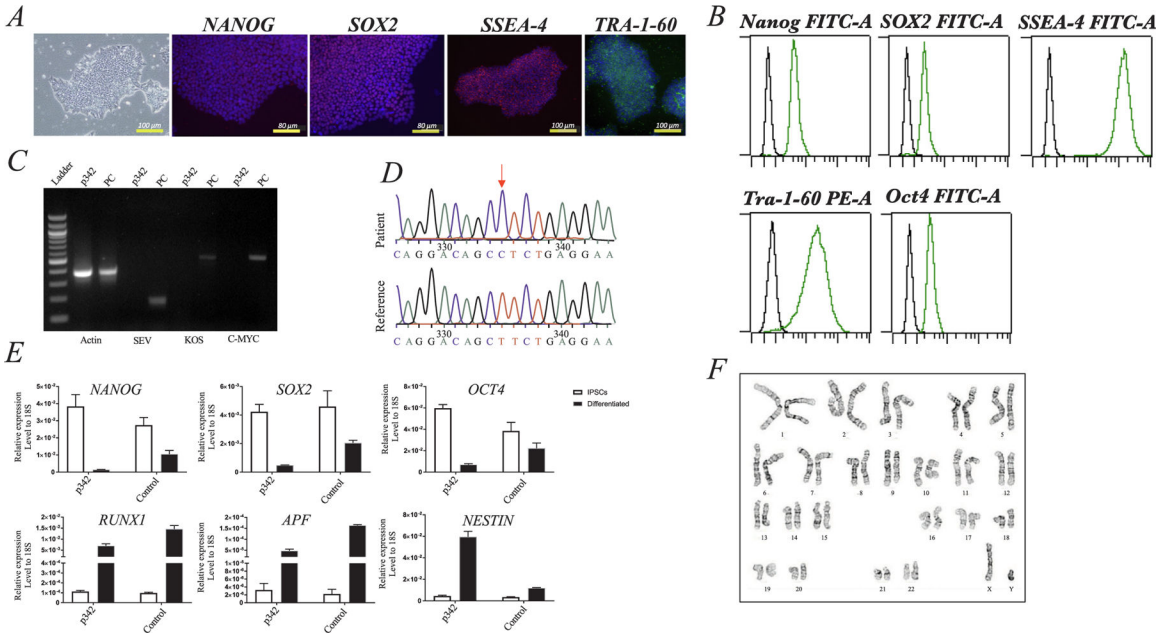

Fig. 1. Validation of human induced pluripotent stem cells (iPSC) generated from a patient with a homozygous L272P mutation in the *OTULIN* gene.

(A) Phase contrast images of iPSC clones were growing on passage 15 on a feeder-free plate. Expression of pluripotent markers (Nanog, Sox2, SSEA4, and Tra-1–60) were analyzed by immunofluorescence assay; DAPI staining of cell nuclei in blue. (B) The expression level of pluripotent markers (Nanog, Sox2, SSEA4, TRA-1–60, and Oct4) was quantitative analysis by Flow Cytometry Analysis. (C) qPCR demonstrated Sendai Virus free in iPSC at 15th passage. (D) PCR and DNA sequencing identified L272P mutation in the *Otulin* gene (red arrows). (E) Expression of pluripotent genes (*NANOG*, *SOX2*, and *OCT3/4*) was confirmed in iPSC derived from a ORAS patient as assessed by RT-qPCR (open bar). The iPSC from a ORAS patient were able to differentiate into three germ layers using monolayer differentiation *in vitro* at day 7, as shown by gene expression of *AFP*, *NESTIN* and *RUNX1* (black bar). The All genes tested shown comparable expression levels between the iPSC from a ORAS patient and control lines (Control). Data are represented as means ± SEM relative to mRNA levels. (F) G-Banding assay showed normal chromosomal stability in a ORAS patient derived iPSC.

Table 1

Summary of a patient with a homozygous L272P mutation in the *OTULIN* gene.

iPSC line names	Abbreviation in figures	Gender	Age (years)	Ethnicity	Genotype of locus	Disease
NIHTVBi014-A	p342	M	11	Pakistani	*OTULIN*, 5p15.2	ORAS

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Table 2

Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase-contrast microscope	Normal	Fig. 1A
Phenotype	Qualitative analysis (immunofluorescence staining)	Expression of pluripotency markers: OCT4, NANOG, SSEA4 and TRA-1–60	Fig. 1A
	Quantitative analysis (RT-qPCR)	Expression of pluripotency markers: *SOX2* and *NANOG*	Fig. 1E
	Qualitative analysis (FACS)	Expression of pluripotency markers: NANOG, SOX2, SSEA4, TRA-1–60, OCT4	Fig. 1B
Genotype	Karyotype (G-banding) and resolution	46, XY; resolution 450–500 bands	Fig. 1F
Identity	Microsatellite PCR OR STR analysis	Not performed	N/A
		15 sites tested, 100% match	Supplementary file 1
Mutation analysis (IF APPLICABLE)	DNA sequencing	Homozygous, *OTULIN gene point mutation*	Fig. 1D
	Southern blot OR WGS	Not performed	N/A
Microbiology and virology	*Mycoplasma* testing by luminescence	Negative	Supplementary file 2
Differentiation potential	Monolayer differentiation assay	Differentiating cells are expression of *RUNX1*, *AFP*, and *NESTIN*; iPSC were able to differentiate into three germ layers	Fig. 1E
Donor screening (OPTIONAL)	HIV1 + HIV2, hepatitis B virus, hepatitis C virus	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

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Table 3

Reagents.

Antibodies used for immunocytochemistry					
	Antibody	**Dilution**	**Company**	**Cat#**	**RRID**
Primary antibodies	Rabbit anti-SOX2	1:100	Cell Signaling Technology	3579	AB_2722343
	Mouse anti-NANOG	1:100	Cell Signaling Technology	4893	AB_10548762
	Mouse anti-SSEA4	1:100	MilliporeSigma	MAB4304	AB_177629
	Mouse anti-TRA-1–60	1:150	MilliporeSigma	MAB4360	AB_2119183
	Alexa Fluor 488 anti-SSEA4 Antibody	1:10	BioLegend	330412	AB_1089198
	PE anti-TRA-1–60 Antibody	1:10	BioLegend	330610	AB_2119065
	Alexa Fluor 488 anti-SOX2 Antibody	1:10	BioLegend	656110	AB_2563957
	Alexa Fluor 488 anti-OCT4 Antibody	1:10	BioLegend	653708	AB_2563184
	Alexa Fluor 647 anti-NANOG Antibody	1:10	BioLegend	674210	AB_2650619
Secondary antibodies	Alexa Fluor 594 Donkey anti-rabbit	1:300	Life Technologies	A21207	AB_141637
	Alexa Fluor 594 Donkey anti-mouse	1:300	Life Technologies	A21203	AB_141633
	Alexa Fluor 488 Donkey anti-mouse	1:300	Life Technologies	A21202	AB_141607
	Alexa Fluor 555 Goat anti-mouse	1:300	Life Technologies	A21426	AB_2535847

Primers used for RT-qPCR and PCR	
Target	**Forward/reverse primer (5′–3′)**
NANOG	AGG GAA ACA ACC CAC TTC T/CCT TCT GCG TCA CAC CAT T
SOX2	CCC AGC AGA CTT CAC ATG T/CCT CCC ATT TCC CTC GTT TT
AFP	AGC TTG GTG GAT GAA AC/CCC TCT TCA GCA AAG CAG AC
NESTIN	GCG TTG GAA CAG AGG TTG GA/TGG GAG CAA AGA TCC AAG AC
RUNX1	CTG CCC ATC GCT TTC AAG GT/GCC GAG TAG TTT TCA TTG CC
OTULIN	TGT AAA ACG ACG GCC AGT GGA AAC CTG AAT GTT GTG AGC/AGG AAA CAG CTA TGA CCA TTA GAT CTT CCA GCC CCA GTC
Sev	GGA TCA CTA GGT GAT ATC GAG C/ACC AGA CAA GAG TTT AAG AGA TAT GTA TC
Sev c-MYC	TAA CTG ACT AGC AGG CTT GTC G/TCC ACA TAC AGT CCT GGA TGA TGA TG
Sev Kos	ATG CAC CGC TAC GAC GTG AGC GC/ACC TTG ACA ATC CTG ATG TGG
β-ACTIN	GAG AAG ATG ACC CAG ATC ATG TTT/GGC AGC TCG TAG CTC TTC TCC A