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Generation of gene-corrected isogenic control cell lines from a DYT1 dystonia patient iPSC line carrying a heterozygous GAG mutation in *TOR1A* gene

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Abstract

Childhood-onset torsin dystonia (DYT1) is a rare hereditary movement disorder and usually caused by a heterozygous GAG deletion (c.907–909) in the *TOR1A* gene (ΔE, p.Glu303del). The neuronal functions of torsin proteins and the pathogenesis of ΔE mutation are not clear. Previously, we have generated a hiPSC line from DYT1 patient fibroblast cells. In this study, we genetically corrected GAG deletion and obtained two isogenic control lines. These hiPSC lines contain the wild-type *TOR1A* sequence, showed the normal stem cell morphology and karyotype, expressed pluripotency markers, and differentiated into three germ layers, providing a valuable resource in DYT1 research.

2. Resource utility

The patient derived DYT1 hiPSCs together with these genetically corrected isogenic controls will provide invaluable resources for DYT1 dystonia research. Neurons derived from hiPSCs will enable us to biochemically identify dysregulated factors in diseased neurons and potentially discover novel molecular targets for therapeutic interventions (Table 1).

3. Resource details

DYT1 dystonia is a movement disorder mainly caused by a heterozygous trinucleotide deletion (ΔGAG; c.907–909) in the *TOR1A* gene and manifests as sustained or intermittent

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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.2022.102807.

muscle contractions (Ding et al., 2021). It is often initiated or worsened by voluntary action and associated with overflow muscle activation. The specific mechanism by which the *TOR1A* mutation leads to the development of dystonic phenotype is still unknown. Even though animal models provide insights into disease mechanisms, significant species-dependent differences exist because animals with identical heterozygous mutation (ΔE) fail to show the pathology seen in human patients (Goodchild et al., 2005). In addition, the limited access to patient neurons greatly impedes the progress of research in dystonia. Excitingly, the generation of patient- specific neurons via human induced pluripotent stem cells (hiPSCs) provides an unprecedented approach for dystonia research (Akter et al., 2021; Akter et al., 2022). Previously, we generated a hiPSC line (CSUi002-A) from a DYT1 patient fibroblast cell line that is carrying a heterozygous mutation in the *TOR1A* gene (Ding et al., 2021; Wu et al., 2021). In this study, we genetically corrected the GAG deletion in this line with CRISPR/Cas9 method and obtained two hiPSC lines (LSUHSi003-A-2 and LSUHSi003-A-3), in which the GAG deletion in the *TOR1A* gene was restored. These isogenic controls together with the DYT1 patient hiPSC line provide a valuable resource in DYT1 dystonia research.

Both mutation-corrected hiPSC lines showed a typical pluripotent stem cell morphology with a high nucleus/cytoplasm ratio (Fig. 1A). DNA sequencing confirmed that the GAG deletion was restored in both newly generated hiPSC lines (LSUHSi003-A-2 and LSUHSi003-A-3) (Supp. Fig.S1A and B). Sanger DNA sequencing of polymerase chain reaction (PCR) products further verified that the DYT1 iPSC line (CSUi002-A) contains the heterozygous GAG deletion (c.907–909) in the *TOR1A* gene (one copy is wild-type and the sequence of the other copy is shifted due to GAG deletion), and this deletion was corrected in both newly generated lines (Fig. 1B). GTW banding method was used to analyze the chromosomes from both corrected hiPSC lines and demonstrated that they are normal male karyotype, 46, XY (Fig. 1C). Short tandem repeat (STR) analysis at 15 loci identified a 100% allele match between DYT1 and DYT1 correction clones (Supp. Fig.S1C). Immunostaining indicated that these iPSCs highly expressed pluripotency markers of *OCT4, NANOG, SOX2,* and *SSEA4* (Fig. 1D). Quantitative RT- PCR analysis demonstrated that the pluripotency markers of *OCT4*, SOX2, *NANOG*, and *KLF4* in DYT1 correction lines were expressed at similar levels as the parental line (CSUi002-A) (Fig. 1E). Following spontaneous differentiation, embryoid bodies (EBs) (Fig. 1F) derived from both corrected cell lines displayed dramatic upregulation of markers of the ectoderm (*PAX6, OTX1*), mesoderm (*DCN, IGF2, GATA2*), and endoderm (*SOX7, SOX17)* lineages. The expression levels of these trilineage markers were consistent with the parental line (CSUi002-A) and much higher than undifferentiated iPSCs (Fig. 1G). PCR screening demonstrated that both DYT1 correction hiPSC lines (LSUHSi003-A-2 and LSUHSi003-A-3) were negative for mycoplasma (Supp. Fig.S1 D).

4. Materials and methods

4.1. Correction and culture of DYT1 iPSCs

The GAG mutation in the *TOR1A* gene in DYT1 hiPSC was genetically corrected at the Genome Engineering and iPSC Center (GEiC) at Washington University in St. Louis.

Briefly, approximately 1×10^6 single cells were resuspended in P3 primary buffer (Lonza) with gRNA/Cas9 ribonuclease protein (RNP) complex (200 pmol synthetic gRNA and 80 pmol HiFi Cas9 protein) and *TOR1A* correction ssODN (Table 2). A silent mutation (C to T) was introduced in the donor oligo sequence (ssODN) to avoid the re-cutting of the edited sequence by CRISPR/Cas9. Subsequently, cells were electroporated with a 4DNucleofector (Lonza) using the CA-137 program. Following nucleofection, the editing efficiency was confirmed by targeted deep sequencing using primer sets specific to target regions and then the pool was single-cell sorted. Single cell clones were screened with targeted deep sequencing analysis. All iPSCs were cultured with mTeSR Plus (STEMCELL Technology) on Matrigel-coated plates at 37 °C in a humidified, 5% CO2 incubator and passage at a 1:6 ratio using gentle cell dissociation reagent (Versene, Gibco).

4.2. Embryoid bodies (EB) formation

As our previous report (Akter et al., 2021), cultured hiPSCs were dissociated with Versene and transferred to low attachment 10-cm petri dishes in KOSR medium (DMEM/F12 medium containing 20% KnockOut Serum Replacement, 1% GlutaMax, 1% non-essential amino acids, 50 μM β-mercaptoethanol and 1% penicillin–streptomycin) in the presence of 10 μM Y-27632 (STEMCELL Technologies). Changed the medium every other day and EBs gradually formed (Fig. 1F). After 7 days of suspension culture, EBs were digested with 0.25% Trypsin and cultured on gelatin coated plates with KOSR medium for another 7 days. The total RNAs were extracted for RT-PCR analysis of trilineage markers.

4.3. Immunostaining and confocal microscopy

Cultured iPSCs were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and incubated in blocking buffer (3% bovine serum albumin in PBS) with (for nuclear markers) or without (for a cell surface marker SSEA4) 0.2% Triton X-100 for 1 h. Cells were then sequentially incubated with primary and secondary antibodies (Table 2) as previously described (Akter et al., 2021). Hoechst 33,342 (Invitrogen) was used to stain nuclei. Images were obtained with a Leica SP5 confocal microscope.

4.4. Quantitative PCR analysis

As previously report (Akter et al., 2021), cultured iPSCs and EBs were collected and lysed in TRIzol (Invitrogen). Total RNAs were extracted using the phenol/chloroform method, and then reverse-transcripted into cDNAs using the SuperScript™ III Reverse Transcriptase (Invitrogen). Quantitative PCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems) and run on a StepOne qPCR machine (Applied Biosystems). The gene expression data were analyzed using the $\Delta\Delta C_T$ method and the values were normalized to the expression of the housekeeping gene GAPDH (Fig. 1E and G). Primers used in this study were listed in Table 2.

4.5. Karyotyping

Chromosomes from iPSC clones were analyzed using the GTW banding method at GEiC at Washington University in St. Louis.

4.6. STR analysis

Short tandem repeat (STR) analysis of 15 loci (Fig. S1C) were performed at GEiC at Washington University in St. Louis.

4.7. Mycoplasma test

Mycoplasma test was performed by MycoAlert PLUS kit (Lonza) at GEiC at Washington University in St. Louis (Fig. S1D).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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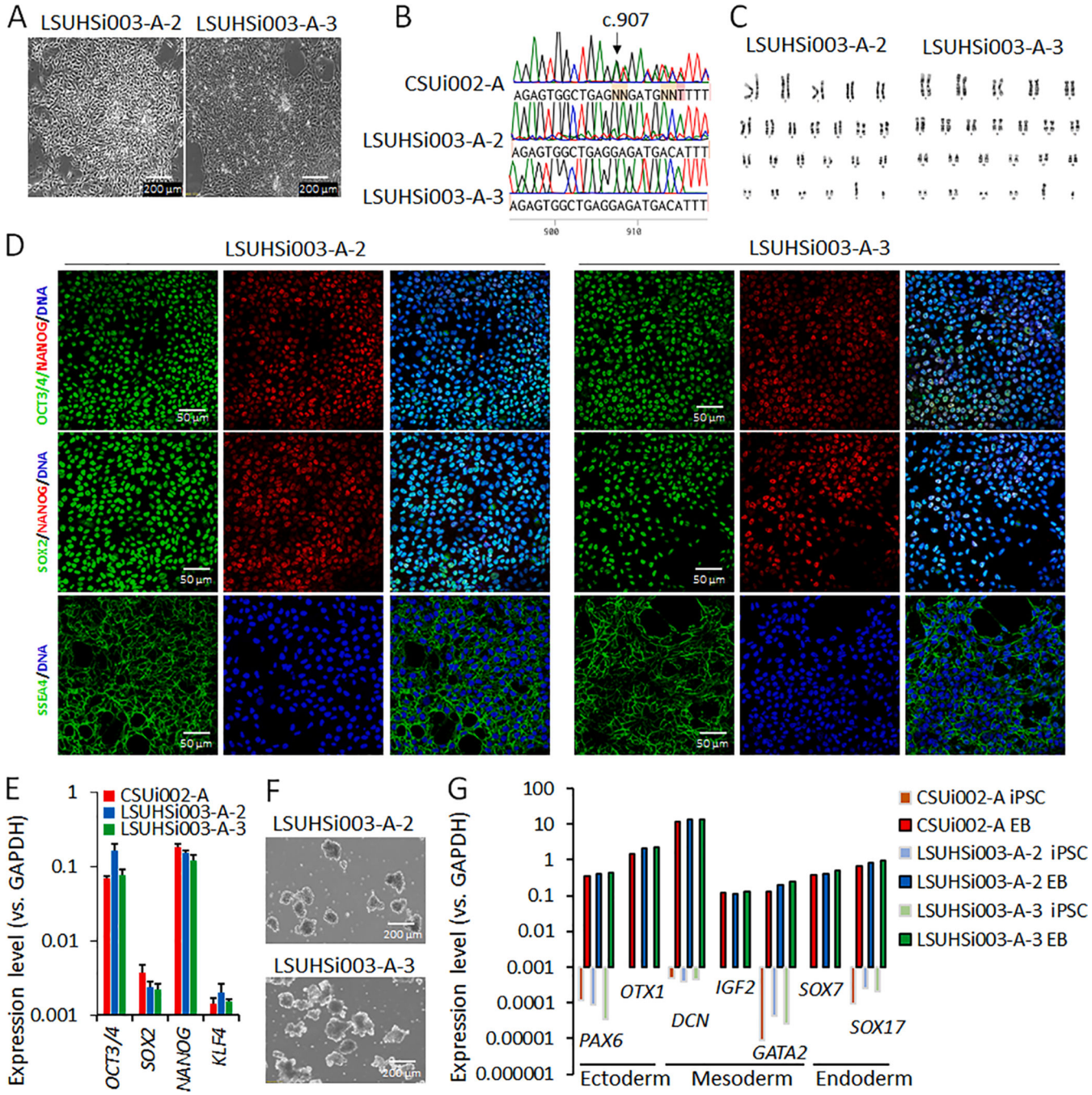

Fig. 1.
Characterization of DYT1 correction iPSC lines.

Table 1

Characterization and validation.

Classification	Test	Result	Data
Morphology	Brightfield microscopy	Typical primed pluripotent human stem cell morphology	Fig. 1 Panel A
Pluripotency status evidence for the described cell line	Qualitative analysis	Immunocytochemistry showed expression of pluripotency markers: OCT4, SOX2, NANOG, SSEA4	Fig. 1 Panel D
	Quantitative analysis	Compared to DAPI, % of positive cell (LSUHSi003-A-2, LSUHSi003-A-3) OCT4: 97%, 96%; SOX2: 98%, 97%; NANOG: 95%, 96%; SSEA-4: 96%, 97%. RT-PCR showed highly express OCT4, SOX2, NANOG, KLF4	Fig. 1 Panel D and E
Karyotype	Karyotype (G-banding) and resolution	46, XY, Resolution 400	Fig. 1 Panel C
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site and deep sequencing analysis Transgene- specific PCR	Heterozygous GAG deletion (c.970–909) in *TOR1A* gene was restored. N/A	Fig. 1 Panel B and Supplementary Fig. S1A and B N/A
Verification of the absence of random plasmid integration events	PCR/Southern	Off Target Analysis of gRNA showed 100% minus a weighted sum of off target hit-scores in the target genome.	N/A
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	STR analysis of 15 loci, all matched.	Supplementary Fig. S1C
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product) PCR-based analyses	The sequencing results of genomic DNA all matched with parent line. The sequencing results PCR products all matched with parent line.	Fig. 1 Panel B and Supplementary Fig. S1A and B Fig. 1 Panel B and Supplementary Fig. S1A and B
	Southern Blot or WGS; western blotting (for knockouts, KOs)	N/A	N/A
Off-target nuclease analysis	PCR across top 5/10 predicted top likely off target sites, whole genome/ exome sequencing	N/A	N/A
Specific pathogen-free status	Mycoplasma	Tested by MycoAlert PLUS kit: Negative	Fig. S1D
Multilineage differentiation potential	Embryoid body formation, RT-PCR	Upregulation of trilineage markers *PAX6*, *OTX1* (ectoderm), *DCN*, *IGF2*, *GATA2* (mesoderm), and *SOX7*, *SOX17* (endoderm).	Fig. 1 Panel F and G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A N/A	N/A N/A

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Table 2

Reagents details.

Antibodies used for immunocytochemistry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Mouse anti-OCT4	1:200	Santa Cruz Cat# sc-5279, RRID: AB_628051
	Mouse anti-SOX2	1:200	Santa Cruz Biotechnology Cat# sc-365823, RRID:AB_10842165
	Mouse anti-SSEA4	1:200	Abcam Cat# ab16287, RRID:AB_778073
	Rabbit anti-Nanog	1:100	Abcam Cat# ab21624, RRID:AB_446437
Secondary antibodies	Donkey anti-Mouse IgG (H + L), Alexa Fluor 488	1:500	Jackson ImmunoResearch Labs Cat# 715–545150, RRID:AB_2340846
	Donkey Anti-Rabbit IgG (H + L), Alexa Fluor 594	1:500	Jackson ImmunoResearch Labs Cat# 711–585152, RRID:AB_2340621
Nuclear stain	Hoechst33342	1 μg/mL	Invitrogen Cat # H3570. RRID: NOT FOUND
Site-specific nuclease			
Nuclease information	HiFi Cas9 nuclease	HiFi Cas9 nuclease (IDT, Cat #1081061)	
Delivery method	electroporation	4D-Nucleofector (Lonza, Cat # AAF-1002B)	
Selection/enrichment strategy	sorted into 96-well plates with one cell per well	Single cell clones were screened and expanded	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′–3′)**	
Pluripotency marker	*OCT 3/4*	CGAGAGGATTTTGAGGCTGC/CGAGGAGTACAGTGCAGTGA	
Pluripotency marker	*SOX 2*	AGGATAAGTACACGCTGCCC/TTCATGTGCGCGTAACTGTC	
Pluripotency marker	*NANOG*	TGTCTTCTGCTGAGATGCCT/CAGAAGTGGGTTGTTTGCCT	
Pluripotency marker	*KLF4*	TCTCCAATTCGCTGACCCAT/CGGATCGGATAGGTGAAGCT	
Differentiation marker	*PAX6*	GGGCGGAGTTATGATACCTACA/ATATCAGGTTCACTTCCGGGAA	
Differentiation marker	*OTX1*	TACGCCCTCCTCTTCCTACT/GCATGTGGGTGGTGATGATG	
Differentiation marker	*DCN*	CTGAAGAACCTTCACGCATTGA/GGCAATTCCTTCAGCTGATTCT	
Differentiation marker	*IGF2*	CAATATGACACCTGGAAGCAGT/GTAGAGCAATCAGGGGACGG	
Differentiation marker	*GATA2*	ACCTGTTGTGCAAATTGTCAGA/ATCCCTTCCTTCTTCATGGTCA	
Differentiation marker	*SOX7*	ACTCCACTCCAACCTCCAAG/TTCATTGCGATCCATGTCCC	
Differentiation marker	*SOX17*	ATCGGGGACATGAAGGTGAA/TCCTTAGCCCACACCATGAA	
Housekeeping Genes	*GAPDH*	CAAATTCCATGGCACCGTCA/GGACTCCACGACGTACTCAG	
Genotyping-PCR	*TOR1A*	ACAGCAGCTTAATTGACCGGA/ATCATGAGCCCTGCGATGAG	
Sequencing	*TOR1A*	GTGTATCCGAGTGGAAATGC	

Antibodies used for immunocytochemistry			
	Antibody	**Dilution**	**Company Cat # and RRID**
TOR1A gRNA (IDT)	*TOR1A*	TGAAGACATTGTAAGCAGAG	
TOR1A correction ssODN (IDT)	*TOR1A*	AATGTGTATCCGAGTGGAAATGCAGTCCCGAGGCTATGAAATTGATGAA GACATTGTAAGTAGAGTGGCTGAGGAGATGACATTTTTCCCAAAGAGGAGA GAGTTTTCTCAGATAAAGGCTGCA	

1.

Resource Table

Unique stem cell lines identifier	**LSUHSi003-A-2 LSUHSi003-A-3**
Alternative names of stem cell lines	DYT1-CR-4B2 (CSUi002-A-2)
	DYT1-CR-A6 (CSUi002-A-3)
Institution	Louisiana State University Health
	Sciences Center in Shreveport, LA USA
Contact information of the reported cell line distributor	Baojin Ding (baojin.ding@lsuhs.edu)
Type of cell lines	iPSC
Origin	Human
Additional origin info *(Applicable for human ESC or iPSC)*	Age: 30 YR Sex: Male Ethnicity: White
Cell Source	hiPSC (CSUi002-A)
Method of reprogramming	N/A
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT/q-PCR
Cell culture system used	Serum-free and feeder-free medium
Type of Genetic Modification	Gene correction of pathogenic mutation
Associated disease	DYT1 dystonia
Gene/locus	*TOR1A* c.907_909delGAG (p.Glu303del)/9q34.11
Multiline rationale	Isogenic clones with corrected GAG mutation
Method of modification/site specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Electroporated with a 4D-Nucleofector (Lonza) using CA-137 program.
All genetic material introduced into the cells	Synthetic gRNA (IDT)
	HiFi Cas9 nuclease V3 (IDT)
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity surveillance	Targeted PCR/sequencing
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	January 21, 2022

Unique stem cell lines identifier	**LSUHSi003-A-2 LSUHSi003-A-3**
Cell line repository/bank	https://hpscreg.eu/cell-line/LSUHSi003-A-2 https://hpscreg.eu/cell-line/LSUHSi003-A-3
Ethical/GMO work approvals	Genetic modification was performed at Genome Engineering and iPSC Center (GEiC) at Washington University in St. Louis.
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A