Generation of gene-corrected isogenic control cell lines from a DYT1 dystonia patient iPS 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...
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 $1 \times 10^6$ 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 \textit{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 ΔΔC\textsubscript{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.                                                                                                                                   | Fig. 1 Panel B and Supplementary Fig. S1A and B |
| 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 |
|                                                     | 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                           |
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 | Delivery method | Selection/enrichment strategy |
|----------------------------------------|----------------------|-----------------|-------------------------------|
|                                        | HiFi Cas9 nuclease   | electroporation | 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 | CGAGAGGATTGGGAGGCTGC/CGAGAGGATCAGTGAGTGA |
| Pluripotency marker                           | SOX 2   | AGGATAAGTACACGCTGCC/TTCTAGTGCGGTAACGTC |
| Pluripotency marker                           | NANOG   | GTTCTTCTGCTGAGATGGCTT/CAGAAGTGGTTTGTGCT |
| Pluripotency marker                           | KLF4    | TCTCCAATTCGATACCCAT/CGGATCGATAGTGAGGCT |
| Differentiation marker                        | PAX6    | GCCTCTTCTTCTTCTACGCATGTTGGTTGAGTATG |
| Differentiation marker                        | OTX1    | TACGCCCTTCTCTCTTCTACGATGTTGGTTGAGTATG |
| Differentiation marker                        | DCN     | CTGAAGAACCCTTACGATGTA/GGCAATCCCTTCAGCTGATT |
| Differentiation marker                        | IGF2    | CAATATGCACTGCTGGAGTACAGTGAGA/ATCAGGAGGAGGAGG |
| Differentiation marker                        | GATA2   | ACCGTGTTGCAAAATTGTGAG/ATCCCTTCTTCTTCTATTG |
| Differentiation marker                        | SOX7    | ACTACCTCCAACTTCCAGT/TTCAATTGGCTGATACGTTCAC |
| Differentiation marker                        | SOX17   | ATGGGAGGAGATGGAAGTTGA/TTCAATTGGCTGATACGTTCAC |
| Housekeeping Genes                            | GAPDH   | CAATATGCACTGCTGGAGTACAGTGAGA/ATCAGGAGGAGGAGG |
| Genotyping-PCR                                | TOR1A   | ACAGCACTTAAATTGAGC/ATCCCTTCTTCTTCTTCTT |
| Sequencing                                   | TOR1A   | GTGATCCCGATGGGAATG |

Table 2

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| Antibody                        | Dilution                        | Company Cat # and RRID                          |
|--------------------------------|--------------------------------|-----------------------------------------------|
| TOR1A gRNA (IDT)               | TOR1A                          | TGAAGACATTGTAAGCAGAG                           |
| TOR1A correction ssODN (IDT)   | TOR1A                          | AATGTGTATCCGAGTAGGAAATGCGAGTCCGAGGAATTGATGAAGA|
|                                |                                | GACATTGTAAGTAGTGGAGGGAGTAGACATTTTCTCCAAAAGAGAGA|
|                                |                                | GAGTTTTCTCCAGAAAAAGGCTGCA                      |
| Resource Table |
|----------------|
| **Unique stem cell lines identifier** | LSUHS003-A-2  LSUHS003-A-3 |
| Alternative names of stem cell lines | DYT1-CR-4B2 (CSU002-A-2)  DYT1-CR-A6 (CSU002-A-3) |
| Institution | Louisiana State University Health Science 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 (CSU002-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 |