Generation and characterization of six human induced pluripotent stem cell lines (iPSC) from three families with AP4B1-associated hereditary spastic paraplegia (SPG47)

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Abstract

Bi-allelic variants in the subunits of the adaptor protein complex 4 lead to childhood-onset, complex hereditary spastic paraplegia (AP-4-HSP): SPG47 (AP4B1), SPG50 (AP4M1), SPG51 (AP4E1), and SPG52 (AP4S1). Here, we describe the generation of induced pluripotent stem cells (iPSCs) from three AP-4-HSP patients with compound-heterozygous, loss-of-function variants in AP4B1 and sex-matched parents. Fibroblasts were reprogrammed using non-integrating Sendai virus. iPSCs were characterized according to standard protocols including karyotyping, embryoid body formation, pluripotency marker expression and STR profiling. These first iPSC lines for SPG47 provide a valuable resource for studying this rare disease and related forms of hereditary spastic paraplegia.

Resource utility

These iPSC lines are the first human disease model of AP-4-HSP and provide a valuable resource to study adaptor protein complex 4 biology, disease mechanisms and therapeutic interventions.

Resource details

The hereditary spastic paraplegias are a group of > 80 neurodegenerative diseases and the most common cause of inherited spasticity and associated disability (Blackstone, 2018). Here, we focus on prototypical yet poorly understood forms of complex hereditary spastic paraplegia in children caused by bi-allelic variants in genes that encode subunits

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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.2019.101575.
of the adaptor protein complex 4 (AP-4-HSP): SPG47 (AP4B1); SPG50 (AP4M1), SPG51 (AP4E1), and SPG52 (AP4S1) (Ebrahimi-Fakhari et al., 2018b). AP-4 is a heterotetrameric protein complex that selectively incorporates transmembrane cargo proteins into vesicles and mediates their intracellular transport. Recently, several groups identified the core autophagy protein ATG9A as the major cargo of AP-4 (Davies et al., 2018; De Pace et al., 2018), linking loss of AP-4 function to defective autophagy. To understand AP-4 deficiency in patient derived cells, we created iPSC lines from three well-characterized patients with AP4B1-associated AP-4-HSP (or SPG47) (Ebrahimi-Fakhari et al., 2018a) and sex-matched parents as controls. The AP4B1 variants present are nonsense, missense, frameshift or canonical splice site mutations leading to no functional protein. Fibroblasts were obtained by standard punch biopsy and reprogrammed using non-integrating Sendai virus to overexpress OCT4, SOX2, KLF4 and hc-MYC. All iPSC lines recovered well after thawing (Fig. 1A, scale bar 400 μm). For each line, one clone was selected based on expression of pluripotency markers and differentiation potential. Pluripotency was assessed by immunofluorescence staining for OCT4, Nanog, SSEA4 and Tra-1–60 (Fig. 1B, scale bar 300 μm) as well as qRT-PCR of NANOG, OCT4, REX1, and SOX2 (Fig. 1D). All iPSC lines showed robust expression of pluripotency markers. To examine the potential to differentiate into all three germ layers, embryoid bodies from iPSC lines were tested for expression of ectodermal (EN1, MAP2 and NR2F2), mesodermal (SNAI2, RGS4 and HAND2) and endodermal (SST, KLF5 and AFP) markers using qRT-PCR (Fig. 1E). Karyotype analysis showed normal karyotypes and no clonal abnormalities (Fig. 1C). STR analysis for 16 short tandem repeat markers (Table 2) showed identical profiles for iPSC lines with their respective fibroblast line. To verify AP4B1 variants in iPSC lines, Sanger sequencing was performed (Fig. 1F). Mycoplasma testing using a standard assay (MycoAlert™) was negative.

Materials and methods

Generation of iPSC

Skin punch biopsies (2–3 mm) were incubated in 0.5% Dispase Solution (STEMCELL Technologies) to remove epidermis. Samples were placed in gelatin-coated wells under growth conditions (37 °C, 5% CO2), and a coverslip was added to prevent lifting. Fibroblast media consisted of DMEM with 10% FBS and 1% Penicillin/Streptomycin (Thermo Fisher Scientific). The CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) was used to transduce cells (Tables 1 & 2). Eight days after transduction, iPSCs were re-plated on Geltrex™-coated dishes and maintained in StemFlex medium (Thermo Fisher Scientific). Emerging stem cell colonies were picked and re-plated on Geltrex™-coated dishes for expansion. Cells were passaged weekly using Gentle Cell Dissociation Reagent (STEMCELL Technologies).

Immunocytochemistry for markers of pluripotency

For immunocytochemistry, iPSC were grown on coverslips. After washing with PBS, cells were fixed in 4% PFA at room temperature for 20 min. After three washes with PBS containing 0.05% Tween 20 (PBST), cells were permeabilized with PBS containing 0.1% Triton X-100 for 15 min and subsequently washed with PBST. Blocking was performed overnight at 4 °C with 4% donkey serum in PBS. After washing with PBS, cells were
incubated with primary antibodies (Table 3) for 1 h at room temperature, washed with PBST and incubated with secondary antibodies for 1 h at room temperature and stained with DAPI. Imaging was performed using the Olympus IX71 Inverted Microscope.

**RNA isolation and qRT-PCR**

Total RNA isolation was done using RNeasy Mini Kit (Qiagen), and RNA quantification was done using the qScript® cDNA Synthesis Kit (Quanta Bio). qRT-PCR cycles were performed using the QuantStudio 12 K Flex System (Thermo Fisher Scientific).

**Embryoid body formation**

To assess their ability to form three germ layers, iPSCs were lifted in clumps to allow for spontaneous EB formation in suspension. iPSC cultures were washed with DPBS and incubated with Accutase (STEMCELL Technologies) for 3 min at 37 °C. Cell clumps were lifted with a cell scraper in EB formation medium, consisting of DMEM, 5% KnockOut™ Serum Replacement and 0.5% Penicillin/Streptomycin (Thermo Fisher Scientific). Clumps were added to a 15 ml tube and settled for 10 min before removing the supernatant. EB formation medium with ROCK inhibitor (STEMCELL Technologies) was added, and cell clumps were plated on ultra-low attachment plates (Corning Costar) for 24 h. EB formation medium was replaced every other day until replating to Gelatin-coated-plates on day 8. Cells were then cultured until day 15 in DMEM with 10% FBS. RNA isolation and qRT-PCR was performed, as described above.

**Karyotyping**

Karyotyping was performed at WiCell.

**Mycoplasma detection**

Testing for mycoplasma contamination was done using the MycoAlert™ Detection Kit (Lonza).

**STR profiling**

Genomic DNA was isolated from fibroblasts and iPSCs using the DNeasy Blood & Tissue Kit (Qiagen). STR analysis was performed at Genetica DNA Laboratories. Sixteen loci and an additional mouse marker for the detection of mouse DNA contamination were analyzed using the PowerPlex® 16 HS System (Promega).

**Mutation verification**

To verify variants in *AP4B1*, PCR was performed on gDNA samples using Platinum PCR SuperMix High Fidelity and the SimpliAmp Thermal Cycler (Thermo Fisher Scientific). PCR products were verified on 1% agarose gel before purification using DNA Clean & Concentrator (Zymo Research) and submitted to Eton Bioscience and Genewiz for Sanger Sequencing.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Figure 1
| iPSC line names | Abbreviation in figures | Gender | Age   | Ethnicity  | Genotype of locus | Disease |
|----------------|-------------------------|--------|-------|-----------|------------------|---------|
| BCHNEx001-A    | AP4B1 [LoF/LoF]         | Male   | 2 years | Mixed     | c.1345A > T/c.1160_1161delCA | SPG47   |
| BCHNEx002-A    | AP4B1 [WT/LoF]          | Male   | 38 years | Mixed     | c.1160_1161delCA | Unaffected control |
| BCHNEx003-A    | AP4B1 [LoF/LoF]         | Female | 3 years  | Caucasian  | c.530_531insA & c.533_535delACT/c.114-2A > C | SPG47   |
| BCHNEx004-A    | AP4B1 [WT/LoF]          | Female | 33 years | Caucasian  | c.114-2A > C | Unaffected control |
| BCHNEx005-A    | AP4B1 [LoF/LoF]         | Female | 3 years 9 months | Caucasian  | c.1216C > T/c.1224T > C | Unaffected control |
| BCHNEx006-A    | AP4B1 [WT/LoF]          | Female | 39 years | Caucasian  | c.1228T > C | Unaffected control |
## Table 2

| Classification       | Test                                     | Result                                                                 | Data                        |
|----------------------|------------------------------------------|------------------------------------------------------------------------|-----------------------------|
| Morphology           | Photography                              | Normal                                                                 | Fig. 1 panel A              |
| Phenotype            | Qualitative analysis by immunocytochemistry | Immunocytochemistry for pluripotency markers OCT4, Nanog, SSEA4 and Tra-1–60 | Fig. 1 panel B              |
| Phenotype            | Quantitative analysis by RT-qPCR          | qRT-PCR for expression of Nanog OCT4, REX1, SOX, HTERT and DNMT3B       | Fig. 1 panel B              |
| Genotype             | Karyotype (G-banding) and resolution      | BCHNEUi001-A: 46,XY
              | Band Resolution: 425–500
              | BCHNEUi002-A: 46,XY
              | Band Resolution: 400–425
              | BCHNEUi004-A: 46,XX
              | Band Resolution: 375–475
              | BCHNEUi005-A: 46,XX
              | Band Resolution: 425–475
              | BCHNEUi005-A: 46,XX
              | Band Resolution: 425–500
              | BCHNEUi006-A: 46,XX
              | Band Resolution: 450–500                                    | Fig. 1 panel E              |
| Identity             | STR analysis                             | Performed                                                             | Archived with the journal   |
|                      |                                          | 16 loci tested, all matched (D8S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, PentaD, vWA, D8S1179, TPOX, FGA, Amelogenin) | Archived with the journal   |
| Mutation analysis    | Sanger sequencing                        | Confirmed variants listed in Table 1.                                  | Fig. 1 panel F              |
|                      | Southern Blot OR WGS                     | N/A                                                                   | N/A                         |
| Microbiology and     | Mycoplasma                               | Mycoplasma testing by luminescence (MycoAlert™): Negative              | Archived with the journal   |
| virology             |                                          | N/A                                                                   | N/A                         |
| Differentiation      | Embryoid body formation                  | Expression of ectodermal (EN1, MAP2 and NR2F2), mesodermal (SNAI1L2, RGS4 and HAND2) and endodermal markers (SST, KLF5 and AFP) | Fig. 1 panel D              |
| potential            |                                          | N/A                                                                   | N/A                         |
| Donor screening      | HIV 1 + 2 Hepatitis B, Hepatitis C       | N/A                                                                   | N/A                         |
| (Optional)           |                                          | Blood group genotyping                                               | N/A                         |
| Genotype additional  |                                          | HLA tissue typing                                                     | N/A                         |
| info (Optional)      |                                          | N/A                                                                   | N/A                         |
## Table 3

### Reagents details.

### Antibodies used for immunocytochemistry

| Antibody                  | Dilution | Company Cat # and RRID                                      |
|---------------------------|----------|-----------------------------------------------------------|
| Pluripotency markers      |          |                                                           |
| Rabbit anti-OCT4          | 1:100    | Abcam Cat# ab19857, RRID:AB_445175                       |
| Rabbit anti-NANOG         | 1:50     | Abcam Cat# ab21624, RRID:AB_446437                       |
| Rat anti-SSEA3            | 1:200    | MiUipore Cat# MAB 4303, RRID:AB_177628                   |
| Mouse anti-SSEA4          | 1:200    | MiUipore Cat# MAB 4304, RRID:AB_177629                   |
| Mouse anti-TRA-1–60       | 1:200    | MiUipore Cat# MAB 4360, RRID:AB_10917470                 |
| Secondary antibodies      |          |                                                           |
| AlexaFluor 488 Donkey Anti-Rabbit IgG | 1:500   | Thermo Fisher Scientific Cat# A-21206, RRID:AB_2535792   |
| AlexaFluor 488 Donkey Anti-Mouse IgG | 1:500   | Thermo Fisher Scientific Cat# A-21202, RRID:AB_141607    |
| AlexaFluor 555 Goat Anti-Mouse IgM | 1:500   | Thermo Fisher Scientific Cat# A-21426, RRID:AB_2535847   |

### Primers

| Target                  | Forward/Reverse primer (5’−3’)               |
|-------------------------|----------------------------------------------|
| Pluripotency markers (qPCR) |                                              |
| NANOG                   | CAGTCTGGA/ACTGCGCTGAA/CTCGCTGATTAGGCTCCAAC  |
| OCT4                    | TGTACTCTCGTCCCTTTTCTCAGGTGTTTTCTTCCCCAGTCG |
| SOX2                    | GCTAGTCCTCAAGGCGAAGCAAGAAGCCCTCTCCTGAAAA    |
| DNMT3B                  | ATGAAGTCGAGTTGCTGCTGGCAACATCTGAAGCCATTT     |
| HTERT                   | TGTGCAACCAACATCTACAAG/AGCGTTCTGCTTTGCAGATT |
| REX1                    | TGGACACGTCCTGCTTCTCGATCTGCTGCGTCTTGCAGAAG  |
| House-keeping genes (qPCR) |                                              |
| ACTB                    | GGACTTTGCGAGAAGAGATGG/AGCAGCTGTGGTGCGTGTAAG |

### Targeted sequencing of AP4B1

| Target | Forward/Reverse primer (5’−3’)               |
|--------|----------------------------------------------|
| AP4B1  | BCHEU0001-A & BCHEU0002-A:                  |
| 1)     | GTCAAGTGTCCTCCACAAAAA/AAAGGCAGGCATTACCTGTG  |
| 2)     | ACACCTCTCTCTCGTGCGACTGCGAGCGTCCATTCTGTTCBCHNEU0003-A & BCHEU0004-A:|
| 1)     | AATCTCGCTGGCTACCTCTGATGAGCCAGGAGGCC        |
| 2)     | CTCTTAGTGGCATCTTCTTGATGAGCCAGGAGGCCAGG    |
| 1)     | TTGACACGACTCCAAAAACC/ACAGGGTCGTACATAACAGCTT |
| 2)     | ACACCTCTCTCTCGTGCGACTGCGAGGCCTACATTTCTCTT  |
| Key resource table |  |
|--------------------|---|
| **Unique stem cell lines identifier** | BCHNEUi001-A  
BCHNEUi002-A  
BCHNEUi003-A  
BCHNEUi004-A  
BCHNEUi005-A  
BCHNEUi006-A  |
| **Alternative names of stem cell lines** | HNDS_0052–01  
HNDS_0052–03  
HNDS_0054–01  
HNDS_0054–02  
HNDS_0058–01  
HNDS_0058–02  |
| **Institution** | Boston Children's Hospital, Harvard Stem Cell Institute  |
| **Contact information of distributor** | Darius Ebrahimi-Fakhari darius.ebrahimi-fakhari@childrens.harvard.edu  |
| **Type of cell lines** | iPSC  |
| **Origin** | Human  |
| **Cell Source** | Fibroblasts  |
| **Clonality** | Clonal cell lines  |
| **Method of reprogramming** | Sendai Virus, non-integrating (OCT4, SOX2, KLF4 and hc-MYC)  |
| **Multiline rationale** | Three lines from patients with AP-4-HSP due to compound-heterozygous variants in AP4B1 and control lines from sex-matched parents who are clinically unaffected heterozygous carriers.  |
| **Gene modification** | 3 cell lines with homozygous variants  
3 cell lines with heterozygous variants  |
| **Type of modification** | N/A  |
| **Associated disease** | AP4B1, Hereditary Spastic Paraplegia type 47, SPG47  |
| **Gene/locus** | AP4B1; Reference sequences: NM_001253852.1  
BCHNEUi001-A: c.1345A > T/c.1160_1161delCA  
BCHNEUi002-A: c.1160_1161delCA  
BCHNEUi003-A: c.530_531insA/c.114-2A > C  
BCHNEUi004-A: c.114-2A > C  
BCHNEUi005-A: c.1216C > T/c.1328T > C  
BCHNEUi006-A: c.1328T > C  |
| **Method of modification** | N/A  |
| **Name of transgene or resistance** | N/A  |
| **Inducible/constitutive system** | N/A  |
| **Date archived/stock date** | May 3rd 2018  |
| **Cell line repository/bank** | N/A  |
| **Ethical approval** | This study was approved by the Institutional Review Board at Boston Children’s Hospital (IRB#: P00016119). Written informed consent was obtained.  |