Generation of human induced pluripotent stem cells (NIHTVBi004-A, NIHTVBi005-A, NIHTVBi006-A, NIHTVBi007-A, NIHTVBi008-A) from 5 CADASIL patients with NOTCH3 mutation

Guibin Chen¹, Zhongwen Li²,³, Yangtengyu Liu¹,³, Daniel Chen¹, Jeanette Beers⁴, Cornelia Cudrici¹, Elisa A. Ferrante¹, Robin Schwartzbeck¹, Natalia Dmitrieva¹, Dan Yang¹, Jizhong Zou⁵, M. Luisa Iruela-Arispe⁶, Manfred Boehm¹

¹Translational Vascular Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, United States
²Guangdong General Hospital, Guangzhou, Guangdong 510080, China
³Current address: Department of Rheumatology and Immunology, Xiangya Hospital, Central South University, Changsha, China
⁴Induced Pluripotent Stem Cells (iPSC) Core, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, United States
⁵Department of Cell and Developmental Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL, 60611, United States

Abstract

We have successfully generated induced pluripotent stem cell (iPSC) lines derived from peripheral blood mononuclear cells of five patients with Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). These cells carry the genetic NOTCH3 mutation present in their parental cells. These iPSC cells exhibited normal karyotype and phenotype, which were sustained through propagation. Furthermore, these iPSCs displayed the capacity of differentiating toward the three germ layers in vitro.

Resource Utility

Human induced pluripotent stem cells (hiPSCs) from CADASIL patients may provide a powerful tool for studying the effects of NOTCH3 on human development and the pathophysiology of CADASIL disease.

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Corresponding author.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101821.
Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited disease caused by mutations in the NOTCH3 gene located on chromosome 19. These autosomal dominant mutations are nonsynonymous substitutions, resulting in the addition or deletion of a cysteine residue within the NOTCH3 extracellular domain coding sequences (Tournier–Lasserve et al., 1993; and Joutel et al., 1996). This disease is characterized by migraines, white matter changes, recurrent strokes and early onset dementia through a largely unknown mechanism that is thought to primarily affect the small blood vessels in the brain. (Opherk et al., 2004; Rutten et al., 2016).

The five CADASIL patients were enrolled into one of our NHLBI clinical protocols (16-H-0132). Information regarding clinical onset was obtained using a standard clinical interview format. All 5 patients have been found to have heterozygous missense mutations in the gene NOTCH3 located on chromosome 19. The mutations result in the addition or deletion of a cysteine residue within epidermal growth factor (EGF)-like repeats of NOTCH3 extracellular domain which is responsible for CADASIL disease. The details of genetic information are shown in Table 1, and Supplement file 1. The patient NIHHTVBi004-A (HT405A) has been found to have heterozygous missense mutations in the gene NOTCH3 (c.994C>T; p.Arg332Cys) which affects the calcium binding domain in EGF8; the patient NIHHTVBi005-A (HT406D) has been found to have heterozygous missense mutations in the gene NOTCH3 (c.505C>T; p.Arg169Cys) which affects the calcium binding domain in EGF4; the patient NIHHTVBi006-A (HT407D) has been found to have heterozygous missense mutations in the gene NOTCH3 (c.697T>G; p.Cys233Gly) which affects the protein binding domain in EGF5; the patient NIHHTVBi007-A (HT409A) has been found to have heterozygous missense mutations in the gene NOTCH3 (c.665G>A; p.Cys222Tyr) which affects the protein binding domain of EGF5; and the patient NIHHTVBi008-A (HT454D) has been found to have heterozygous missense mutations in the gene NOTCH3 (c.1364G>A; p.Cys455Tyr) which affects the calcium binding domain in EGF11. Blood samples were collected at the NIH Clinical Center. Using a Sendai viral vector delivery system expressing four transcription factors (OCT4, SOX2, KLF4 and C-MYC), we successfully generated hiPSC lines carrying each of the five NOTCH3 mutations. The hiPSCeCADASIL lines maintained typical morphologies and expressed the common pluripotency markers OCT4, NANOG, TRA-1–60, SSEA4 and SOX2, as shown by immunocytochemistry (Fig. 1A), flow cytometry (Fig. 1B) and/or real-time (RT)-qPCR (Fig. 1D). HiPSCeCADASIL genotyping confirmed NOTCH3 mutations corresponding to each parental cell line (Fig. 1C). To test the differentiation potential of these cell lines, we performed a monolayer differentiation assay to drive cells towards the three germ layers in vitro. We determined the marker gene expression for the endoderm (AFP), ectoderm (NES) and mesoderm (RUNX1) by RT-qPCR, which showed up-regulated expression levels in differentiating cells at day 7 compared to undifferentiated hiPSCeCADASIL cells (Fig. 1D). All five iPSC lines demonstrated chromosomal stability and normal karyotype with G-banding (Fig. 1E). Short tandem repeat (STR) profiles indicated that all hiPSCeCADASIL lines matched their parental blood cells completely in 15 amplified STR loci (Supplementary...
file 2). All cell cultures were routinely tested for *Mycoplasma* contamination and were found to be *Mycoplasma* free (Supplementary file 3). The iPSCs were free of Sendai virus after the 15th passage as shown by PCR (Supplementary file 4). In conclusion, hiPSCeCADASIL lines exhibited pluripotent potential for self-renewal, proliferation and differentiation. To the best of our knowledge, this is the first published study in which hiPSC lines were generated from individuals with the CADASIL carrying *NOTCH3* mutations (Table 2).

1. **Materials and methods**

1.1. **Subjects and study approval**

iPSCs were generated from blood samples obtained from five CADASIL patients carrying a *NOTCH3* mutation. This study was approved by the NHLBI’s Institutional Review Board, and samples were collected after obtaining informed written consent.

1.2. **Generation and culture of human iPSCs from peripheral blood mononuclear cells (PBMCs)**

The PBMCs were isolated from blood samples through density gradient centrifugation via Ficoll-Paque following the manufacturer’s protocol. Approximately 3 to 5 × 10^5 PBMCs were plated into one well of a 6-well plate within Erythroid Expansion Medium (Stemcell Technologies) for expansion of erythroid progenitor cells. Half of the medium was replaced with fresh medium every other day. At day 9~10, the cells were transduced with CytoTune™ 2.0 Sendai reprogramming vectors (Invitrogen). After three days in culture, the cells were collected and seeded into Matrigel (Corning) pre-coated plates supplemented with TesR-E7 medium (Stemcell Technologies) for 10~15 days. After which the medium was replaced with TesR-E8 medium (Stemcell Technologies) until day 21. Once iPSC colonies emerged in culture, they were picked up and transferred onto fresh Matrigel pre-coated plate for further expansion (Jin et al., 2016).

1.3. **Immunofluorescent staining and flow cytometry analysis**

iPSCs were fixed with 4% paraformaldehyde and stained for immunohistochemistry. Briefly, cells were incubated with primary antibodies against SSEA4 (Table 3) at 4 C overnight. After washes in PBS, they were incubated with fluorophore-tagged secondary antibodies at room temperature for 1 h. After PBS washes, the nuclei were then stained with DAPI and images were captured using a fluorescence microscope (Zeiss).

For FACS analysis, iPSCs were digested to a single cell suspension by incubation with Trypsin-EDTA (Invitrogen). Following by fixation and permeabilization, the cells were stained with antibodies. Analysis was performed on a MACSQuant Flow Cytometer (Miltenyi) and the results were analyzed using FlowJo software (FlowJo, LLC).

1.4. **Monolayer differentiation assay**

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

1.5. Gene expression analysis

The total RNA was isolated by using RNeasy Mini Kits (Qiagen). Endogenous mRNA expression levels of *NANOG, SOX2, AFP, NES,* and *RUNX1* were determined in iPSCs and in differentiating cells at day 7 (Fig. 1D). 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. After 15 passages, iPSC lines were tested for Sendai virus (SeV) residues by using RT-PCR with the primers indicated in Table 2.

1.6. Karyotyping assay

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

1.7. DNA sequencing and STR analysis

Genomic DNA was extracted by using DNeasy Blood & Tissue Kit (Qiagen). To amplify the corresponding mutation position in *NOTCH3*, 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.

1.8. Mycoplasma detection

To validate that the hiPSCeCADASIL cultures were *Mycoplasma* free, mycoplasma analysis was performed using the MycoAlert™ Mycoplasma Detection Kit (Lonza, LT27–224). Briefly, the culture media was collected after culturing for 48 h while the confluency of cells was at last 80%. As according to manufacturer’s protocol, 100 μl of clearer supernatant was used as the test sample to screen for mycoplasma contamination. The absence of mycoplasma contamination was confirmed in all hiPSCeCADASIL culture tested (Supplement file 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

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Fig. 1.

Characterization of human iPSC lines derived from five CADASIL patients with a NOTCH3 mutation. (A) iPSCs were cultured to passage 15 on a feeder-coated plate. Phase contrast images of iPSC lines derived from five CADASIL patients with a NOTCH3 mutation (HT405A, HT406D, HT407D, HT409A and HT454D) (top column). Expression of pluripotent markers SSEA4 was analyzed by immunofluorescence; DAPI staining of cell nuclei in blue (bottom columns) (all scale bars: 100 μm). (B) The expression level of pluripotent markers (SSEA4, TRA-1–60, Oct4, Sox2, and Nanog) was quantitative analysis by Flow Cytometry Analysis. (C) PCR and DNA sequencing identified mutations in NOTCH3 in the iPSC lines from the five patients (red arrows). (D) Expression of pluripotent genes (NANOG, SOX2, and OCT3/4) was confirmed in all hiPSC lines derived from CADASIL patients as assessed by RT-qPCR (black bar). All five hiPSC lines from CADASIL patients 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 (gray bar). Data are represented as means ± SEM relative to mRNA levels. (E) All five hiPSC lines from CADASIL patients showed a normal karyotype with G-band analysis.
### Table 1

Summary of patients with a CADASIL disease.

| iPSC line names | Abbreviation in figures | Gender | Age (years) | Ethnicity | Genotype of locus | Disease |
|-----------------|-------------------------|--------|-------------|-----------|-------------------|---------|
| NIHTVBi004-A    | HT405A                  | M      | 43          | White/non-hispanic | Notch 3, C.994C > T; p.Arg332Cys | CADASIL |
| NIHTVBi005-A    | HT406D                  | F      | 61          | White/non-hispanic | Notch 3, C.505C > T; p.Arg169Cys | CADASIL |
| NIHTVBi006-A    | HT407D                  | M      | 43          | White/non-hispanic | Notch 3, C.697T > G; p.Cys233Gly | CADASIL |
| NIHTVBi007-A    | HT409A                  | F      | 62          | White/non-hispanic | Notch 3, C.665G > A; p.Cys222Tyr | CADASIL |
| NIHTVBi008-A    | HT454D                  | M      | 50          | White/non-hispanic | Notch 3, C.1346G > A; p.Cys452Tyr | CADASIL |
## Table 2

| Classification               | Test                                              | Result                                                                 | Data          |
|------------------------------|---------------------------------------------------|------------------------------------------------------------------------|---------------|
| Morphology Phenotype         | Phase-contrast microscope                         | Normal                                                                | Fig. 1A       |
|                              | Qualitative analysis (immunofluorescence staining) | Expression of pluripotency markers: Oct4, Nanog, SSEA4 and Tra-1–60    | Fig. 1A       |
|                              | Quantitative analysis (flow cytometry analysis, RT-qPCR) | Expression of pluripotency markers: SOX2 and NANOG                     | Fig. 1B, 1D   |
| Genotype                     | Karyotype (G-banding) and resolution               | 46,XX or 46,XY; resolution 450–500 bands                              | Fig. 1E       |
| Identity                     | Microsatellite PCR OR STR analysis                 | Not performed 15 sites tested, 100% match                              | N/A Supplementary file 2 |
| Mutation analysis (IF APPLICABLE) | DNA sequencing                                      | Notch 3, Chromosome 19, GRch38                                        | Fig. 1C       |
|                              | Southern blot OR WGS                              | Not performed                                                         | N/A           |
| Microbiology and virology    | Mycoplasma testing by luminescence                 | Negative                                                              | Supplementary file 3 |
| Differentiation potential    | Monolayer differentiation assay                    | Differentiating cells are expression of RUNX1, AFP, and NES; iPSCs were able to differentiate into three germ layers | Fig. 1D       |
| Donor screening (OPTIONAL)   | HIV1 + HIV2, hepatitis B virus, hepatitis C virus  | Not performed                                                         | N/A           |
| Genotype additional info     | Blood group genotyping                             | Not performed                                                         | N/A           |
| (OPTIONAL)                   | HLA tissue typing                                  | Not performed                                                         | N/A           |
### Table 3

| Antibodies used for immunocytochemistry and Flow Cytometry Analysis | Antibody                          | Dilution | Company     | Cat#      | RRID      |
|---------------------------------------------------------------|-----------------------------------|----------|-------------|-----------|-----------|
| Primary antibodies                                            | Mouse anti-SSEA4                 | 1:100    | MilliporeSigma | MAB4304   | AB_177,629|
|                                                              | Alexa Fluor 488 anti-SSEA4 Antibody | 1:10     | BioLegend   | 330,412   | AB_1,089,198|
|                                                              | PE anti-TRA-1–60 Antibody         | 1:10     | BioLegend   | 330,610   | AB_2,119,065|
|                                                              | Alexa Fluor 488 anti-SOX2 Antibody | 1:10     | BioLegend   | 656,110   | AB_2,563,957|
|                                                              | Alexa Fluor 488 anti-OCT4 Antibody | 1:10     | BioLegend   | 653,708   | AB_2,563,184|
|                                                              | Alexa Fluor 647 anti-NANOG Antibody | 1:10    | BioLegend   | 674,210   | AB_2,600,619|
| Secondary antibodies                                          | Alexa Fluor 488 Donkey anti-mouse | 1:300    | Life Technologies | A21202   | AB_141,607|

| Primers used for RT-qPCR and PCR                              | Target                           | Forward/reverse primer (5’ – 3’) |
|---------------------------------------------------------------|-----------------------------------|----------------------------------|
|                                                              | NANOOG                            | AGG GAA ACA ACC CAC TTC T/CCT TCT TCG TCA CAC CAT T |
|                                                              | S0X2                              | CCC AGC AGA CTT CAC AIG T/CCT CCC ATT TCC CTC GTT TT |
|                                                              | AFP                               | AGC TTG GTG GAT GAA AC/CAC 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 TCC AAG GT/GCC GAG TAG TTT TCA TGG CC |
|                                                              | Sev                               | GGA TCA CTA GGT GAT ATC GAG C/ACC AC JA CAA GAG TTT AAG AGA TAT GTA TC |
|                                                              | Sev c-MYC                         | TAA CTG ACT AGC AGG CTT GTC G/TCC AC A TAC AGT CCT GGA TGA TG |
|                                                              | N0TCH3 HT405A/407D                | ACG ACT GTG CCT GTG TTC CT/TGG CCA GCC TAG CAT AAT CT |
|                                                              | N0TCH3                            | GGG GTG TGG TCA GTG CTA AA/CTG ACC CTC AAA CCC TAG CA |
|                                                              | HT406D/409A                       | GGA GCT CCA TCG TCT GTG A/ACC TCC TTC CAG GCT TCA GT |
|                                                              | N0TCH3                            | GAG AAG AIG ACC CAG ATG TTT/GGC AGC TCG TAG TTC TCC A |

β-ACTIN
## Resource Table

| Unique stem cell lines identifier | NIHTVBi004-A  
|----------------------------------|----------------|
|                                  | NIHTVBi005-A  
|                                  | NIHTVBi006-A  
|                                  | NIHTVBi007-A  
|                                  | NIHTVBi008-A  
| Alternative names of stem cell lines | HT405A  
|                                   | HT406D  
|                                   | HT407D  
|                                   | HT409A  
|                                   | HT454D  
| 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 | Peripheral blood mononuclear cells  
| Clonality | Clonal cell lines  
| Method of reprogramming | Sendai vectors containing the transcription factors Oct-4, Klf4, Sox2 and c-MYC  
| Multiline rationale | Lines derived from the five individuals  
| Gene modification | Yes  
| Type of modification | Hereditary  
| Associated disease | None  
| Gene/locus | NOTCH3, Chromosome 19, GRch38.pl2  
| Method of modification | N/A  
| Name of transgene or resistance | N/A  
| Inducible/constitutive system | N/A  
| Date archived/stock date | December 2013  
| Cell line repository/bank | N/A  
| Ethical approval | National Institutes of Health Ethics Committee (Approval Number: 16-H-0132)  

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