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Lab Resource: Stem Cell Line

Generation and characterization of the human iPSC line IDISi001-A isolated from blood cells of a CADASIL patient carrying a *NOTCH3* mutation

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ABSTRACT

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common form of hereditary stroke disorder. It is caused by mutations in *NOTCH3* that lead to progressive degeneration of the smooth muscle cells in blood vessels. There is currently no treatment for this disorder. We reprogrammed to pluripotency blood mononuclear cells isolated from a patient carrying a *NOTCH3* mutation by using a commercially available non-integrating system. The success in the generation of this iPSC line (IDISi001-A) suggests that the *NOTCH3* mutation did not limit cell reprogramming and offers an unprecedented opportunity for studying and modeling CADASIL pathology.

Resource table.

Unique stem cell line identifier	IDISi001-A
Alternative name(s) of stem cell line	N/A
Institution	Health Research Institute of Santiago de Compostela (IDIS)
Contact information of distributor	Héctor Fernández Susavila; Francisco Campos Pérez hector.fernandez.susavila@sergas.es francisco.campos.perez@sergas.es
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 67 Sex: male Ethnicity if known: Caucasian
Cell Source	Blood
Clonality	Clonal
Method of reprogramming	CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific). The episomal reprogramming vectors include the four Yamanaka factors OCT4, SOX2, KLF4, and c-Myc
Genetic Modification	No
Type of Modification	N/A
Associated disease	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)
Gene/locus	*NOTCH3* [c.3724C>T(p.Arg1242Cys)], germinal mutation/19p13.12
Method of modification	Not applicable
Name of transgene or resistance	Not applicable
Inducible/constitutive system	Not applicable
Date archived/stock date	N/A
Cell line repository/bank	N/A
Ethical approval	Written informed consent was obtained from the patient. The study was approved by the Scientific Ethics Committee for the Region of Santiago-Lugo (protocol number 2016/450) and the Commission on Guarantees for the Donation and Use of Human Cells and Tissues of Instituto de Salud Carlos III (protocol number 428-346-1)

Resource utility

To date, there is no effective treatment for cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). The research into CADASIL mechanisms, which has been limited mainly to the experiments in *Notch3* mutant animal models,

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has not generated successful results for the clinical practice so far. We believe that the generation of human iPSC-derived muscle cells from CADASIL patients will facilitate gene-editing approaches for developmental biology studies as well as for disease modeling and drug screening.

Resource details

CADASIL is defined as cerebral autosomal dominant arteriopathy produced by the mutation of the *NOTCH3* gene (Joutel et al., 2000). The main neurological symptoms of CADASIL include migraine with aura, psychiatric problems, ischemic episodes, and cognitive deficits. This disease causes degeneration of the smooth muscle due to the deposition of granular osmiophilic material within the basal lamina, progressive loss of smooth muscle cells, and fibrosis of the media layer of microarteries (Chabriat et al., 2009). Currently, the experimental modeling of CADASIL is restricted to the use of *Notch3* knockout mice, which, however, present important limitations in reproducing the phenotype of this disease (Ayata, 2010).

In this study, we report for first time the generation of a human iPSC line from a patient with CADASIL carrying a *NOTCH3* Arg > Cys mutation at codon 1242 of exon 23. Human peripheral blood mononuclear cells (PBMCs) from this patient were reprogrammed into iPSCs (IDISi001-A) using the non-integrative CytoTune iPS 2.0 Reprograming System (Thermo Fisher Scientific) to deliver Yamanaka's factors OCT4, SOX2, KLF4, and c-Myc. iPSCs colony formation was observed daily. Colonies had characteristic round-shaped morphology with refracting borders and contained small round-shaped cells with a large nucleus/cytoplasm ratio. After 3 months, the first colonies were frozen. During the reprogramming process, the alkaline phosphatase test was used to select the potential pluripotent colonies (Fig. 1A). Once the cell line was established, the following standard tests were carried out: A) PCR analysis for the absence of mycoplasma (Supplemental Fig. 1); B) PCR analysis to confirm the loss of the Sendai virus (SeV) used for the reprogramming (Supplemental Figs. 2 and 3); C) short tandem repeat (STR) analysis used to demonstrate that the generated iPSCs genetically matched donor's cells (Supplemental Fig. 4); and D) PCR analysis of the *NOTCH3* gene to confirm the presence of the mutation in the iPSCs (Supplemental Fig. 5). Subsequently, the expression of the membrane and nuclear pluripotency markers (Tra-1-60, SSEA4, SOX-2, OCT-4, and NANOG, using the antibodies showed in Table 1) was evaluated by immunofluorescence analysis (Fig. 1B). Cells from the new iPSC line were spontaneously differentiated in vitro into the three germ layers by the induction of embryoid body (EB) formation (Fig. 1C). Expression levels of genes encoding transcription factors essential to maintain pluripotent embryonic stem cell phenotype (*SOX2*, *OCT4*, and *NANOG*) were evaluated by qPCR in different clones (#42.2, #53.3, #76.2, #78B.2, #83.3, #80.3) derived from the same cell line. These results were compared to those obtained with a human foreskin fibroblast (HFF) cell line, used as SeV-uninfected cell control, and to those in already characterized iPSC positive control cells (iPSCs C+) (Fig. 1D). In addition, the presence of the mutation previously diagnosed in the patient in IDISi001-A cells was confirmed by forward and reverse sequencing of the corresponding *NOTCH3* fragment (Fig. 1E, panels 1 and 2, respectively) and compared to the results of similar sequencing of the same fragment from a healthy subject without *NOTCH3* mutation (Fig. 1E; panels 3 and 4, respectively). Finally, the karyotype of the new reprogrammed cell line showed a normal chromosomal profile, with no abnormalities (Fig. 1F). In conclusion, we believe that the generation of a human iPSC line from a CADASIL patient will facilitate studies of developmental biology and stimulate disease modeling for drug screening and therapeutic gene-editing approaches for this important cerebral arteriopathy (see Graphical abstract) (Table 2).

Materials and methods

Reprogramming of CADASIL-PBMCs

A peripheral blood sample was taken from a patient diagnosed with CADASIL, whose mutation was located at codon 1243 of exon 23 of *NOTCH3* (genotype: Arg1242Cys/normal). This mutation had not been reported before. The mutation was analyzed by sequencing the entire coding region of the gene (new generation sequencing technique, SOLID 5500XL, Thermo Fisher Scientific).

To obtain PBMCs, the blood sample was mixed with PBS + 2% FBS and centrifuged in a SepMate-50 tube (StemCell Technologies) with Lymphoprep (StemCell Technologies) in a density gradient.

Before reprogramming, PBMCs were cultured for 3 days using Stempro-34 SFM (Gibco) with cytokines (StemCell Technologies). Reprogramming was performed using a CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific). Three hundred thousand cells were infected at the following multiplicity of infection (MOI) values: KOS = 5; c-MYC = 5; KLF4 = 3.

Cell culture

Cells were cultured in mTeSR1 medium (StemCell Technologies). On the first and second passage, cells were maintained in the wells of a 12-well plate. On the third passage, they were transferred into wells of a 6-well plate, and from passage four and thereafter, they were cultured in 35-mm Petri dishes. All wells and dishes were coated with Matrigel™ matrix (Corning). For the first and second passages, colonies were selected and picked with the help of an Origio Stripper (Origio) with Stripper tips (Origio) of 175 μm in diameter. Later passages were performed mechanically, using cell lifters.

PCR and qPCR

Total RNA from undifferentiated IDISi001-A cell line, human fibroblasts (considered as negative control), and a previously established iPSC line was isolated using a Quick-RNA™ MiniPrep kit (Zymo Research) and quantified with Nanodrop (Thermo Fisher Scientific). For RT-PCR, 1 μg of cDNA was generated using a iScriptTM cDNA Synthesis Kit (BIORAD) following the manufacturer's instructions. PCR to analyze the presence/absence of SeV was performed using a Ready Mix REDTaq PCR Reaction Mix (Sigma-Aldrich). For qPCR, iTaq Universal SYBR Green Supermix (BIORAD) was used. In order to check for the expression of pluripotency factors, qPCR for *NANOG*, *OCT4*, and *SOX2* was performed.

Table 1 shows all primer sequences used for PCR and qPCR. All primers (they came lyophilized but were diluted to a final concentration of 100 μM with nuclease-free H_2O) were designed in-house and ordered from Sigma-Aldrich.

Alkaline phosphatase

For the alkaline phosphatase (AP) test, an AP Live Stain Kit (Thermo Fisher Scientific) was used. This test was performed always after 3–5 days of cell culture based on plate confluence and according to the manufacturer's instructions. Colonies were not affected by the process and kept growing normally after the AP test.

Immunocytochemistry

Immunocytochemistry tests for the pluripotency nuclear markers SSEA4 and TRA1–60 and for the pluripotency membrane markers SOX2 and OCT4 were performed using a Pluripotent Stem Cell 4-Marker Immuno Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

To analyze NANOG (Thermo Fisher Scientific) expression, a standard immunocytochemistry protocol from our lab was

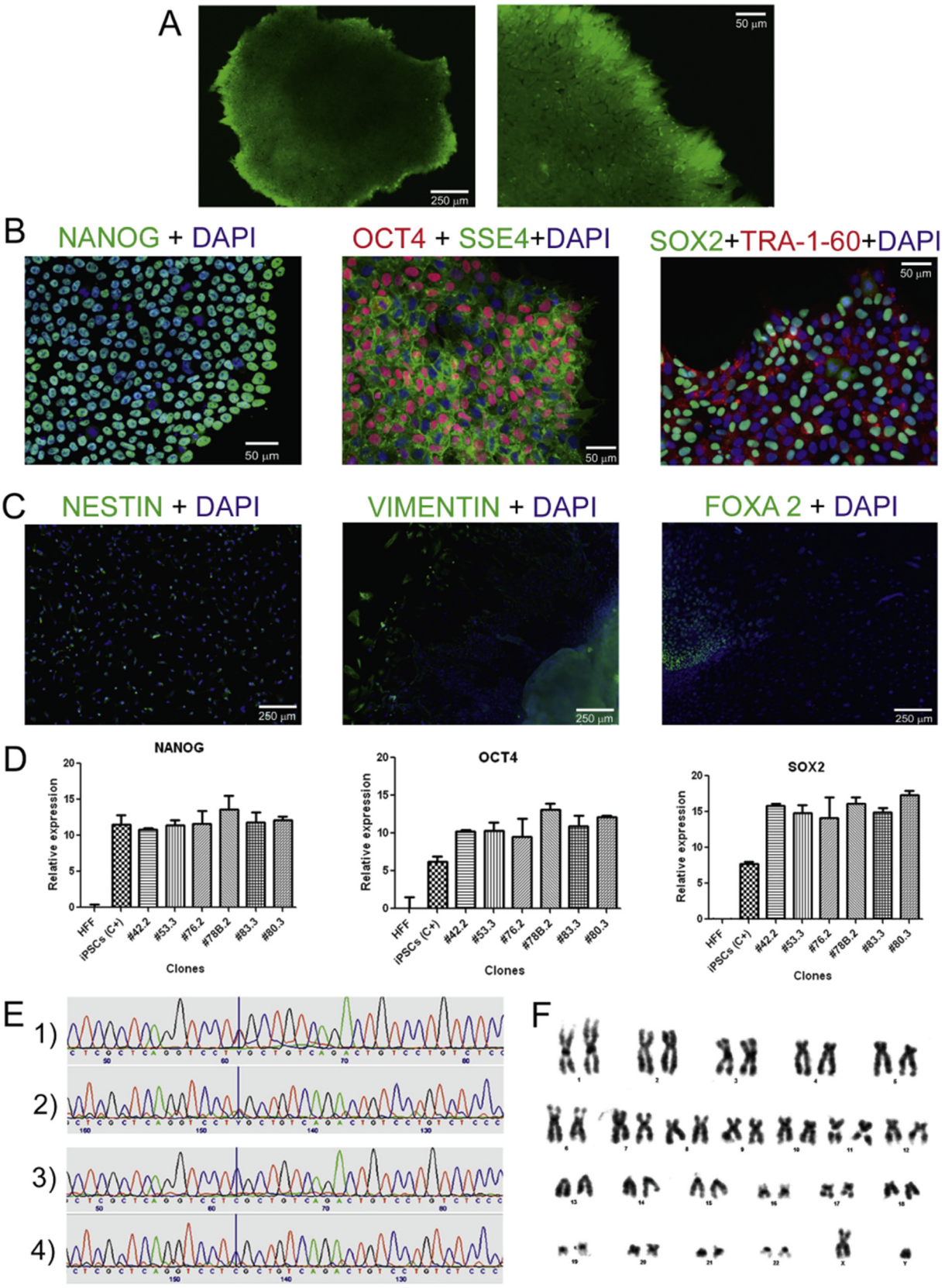

Fig. 1. Representation of IDISi001-A iPSC line characterization.

employed. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Karyotyping

Cells were blocked in their metaphase by incubating them with Colcemid (Gibco) for 3 h. Then, the cells were detached by adding

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Not shown but available from the authors upon request
Phenotype	Immunocytochemistry	OCT4, NANOG, SOX2, SSEA4, TRA-1-60	Fig. 1, panel B
	RT-qPCR	Relative gene expression: *SOX2*, *OCT4*, *NANOG*	Fig. 1, panel D
Genotype	Karyotype (G-Banding)	46XY, Resolution 400	Fig. 1, panel F
Identity	Microsatellite PCR (mPCR) OR	Short tandem repeat analysis	Fig. 4, supplementary file
	short tandem repeat analysis	Not performed	N/A
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous	Diagnostic available from the authors upon request
	Southern blot OR whole genome sequencing	Substitution	Fig. 1, panel E
Microbiology and virology	Mycoplasma	Negative	Fig. 1, supplementary
Differentiation potential	Embryoid body formation	FOXA-2, vimentin, nestin	Fig. 1, panel C
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	Not available
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	Not available
	HLA tissue typing	Not performed	Not available

trypsin-EDTA for 8–10 min and washed 2–3 times with DMEM +5% PBS. After that, they were centrifuged and exposed to a hypotonic 0.075 M KCL solution for 10 min at 37 °C. Cells were then fixed by the triple exposure to methanol/glacial acetic acid mixture (3:1). To check the karyotypes, Giemsa staining was performed. A total of 29 metaphases were analyzed, all of which were normal without any kind of chromosomal anomalies.

Analysis of the mutation

The *NOTCH3* fragment containing the mutation was amplified by PCR. The samples were sequenced by New Generation Sequencing using a SOLID 5500XL genetic analyzer.

In vitro embryoid body formation and differentiation

Once a Petri dish reached approximately 85% confluence, colonies were washed carefully with DMEM-F12 and after that, Embryoid Body Medium with 20% FBS (EBM20%) was added. This medium contained DMEM-F12 with Glutamax, 20% FBS, non-essential aminoacids 1× (Gibco), P/S 1×, and 50 mM β-mercaptoethanol (Gibco).

Colonies were cut making a grid pattern with the aid of a sterile 200-μL pipette and then, they were detached from the plate either mechanically or enzymatically (Dispase solution, StemCell Technologies). Once detached, clusters were transferred to a well of an ultra-low 6-well attachment plate (Corning) with EBM20% plus 10 μM ROCK inhibitor (StemCell Techonologies). EBs already formed at day 1. EBs were maintained for 7 days with medium replacement every other day. On day 7, EBs were transferred into a chamber slide (Sarstedt) previously coated with Matrigel™, 6–7 EBs per well, to allow their attachment and differentiation. EBs inside the chamber slides were maintained for 14 days in EBM20% (plus ROCK inhibitor just the first plating day), before performing immunocytochemistry experiments.

For the characterization of the three germ layers, the endoderm was identified using an anti-FOXA2 antibody (Thermofisher; 1:100 dilution), the mesoderm was identified using an anti-vimentin antibody (Bioss Antibodies; 1:100 dilution), whereas the ectoderm was identified using an anti-nestin antibody (Abcam; 1:200 dilution).

Acknowledgements

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Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency markers	Rabbit anti-OCT4	1:100	Thermo Fisher, A24867
	Mouse anti-SSEA4	1:100	Thermo Fisher, A24866
	Rat anti-SOX2	1:100	Thermo Fisher, A24759
	Mouse anti-TRA-1-60	1:100	Thermo Fisher,24868
	Rabbit anti-NANOG	1:200	Thermo Fisher, PA-1-097X
Differentiation markers	Rabbit anti-FOXA2	1:100	Thermo Fisher, 720061
	Rabbit anti-vimentin	1:100	BIOSS Antibodies, bs-0756R
	Rabbit anti-nestin	1:100	Abcam, ab93157
Secondary antibodies	Alexa Fluor 488 Goat anti-mouse IgG3	1:250	Thermo Fisher, A24877
	Alexa Fluor 594 Donkey anti-rabbit	1:250	Thermo Fisher, A24870
	Alexa Fluor 488 Donkey anti-rat	1:250	Thermo Fisher, A24876
	Alexa Fluor 594 Goat anti-mouse IgM	1:250	Thermo Fisher,A24872
	DyLight 488 anti-rabbit IgG	1:200	Vector Laboratories, DI-1488
	Biotinylated anti-mouse IgG	1:200	Vector Laboratories, BA-2001

Primers

	Target	Forward/Reverse primer (5′-3′)
Episomal plasmids (qPCR)	SeV plasmid	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC
Pluripotency markers (qPCR)	*NANOG*	AGGAAGACAAGGTCCCGGTCAA/TCTGGAACCAGGTCTTCACCTGT
	OCT4	GGGTTTTTGGGATTAAGTTCTTCA/GCCCCCACCCTTTGTGTT
	SOX2	CAAAAATGGCCATGCAGGTT/AGTTGGGATCGAACAAAAGCTATT
Housekeeping genes (qPCR)	*GAPDH*	GAAGGTCGGAGTCAACGGATT/TGACGGTGCCATGGAATTTG

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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.2018.01.023.

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