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Lab Resource: Single Cell Line

Generation of a human induced pluripotent stem cell line (INEUi001-A) from an amyotrophic lateral sclerosis/frontotemporal dementia patient with a C9ORF72 G4C2 genotype of <2 (GGGGCCG) and 10 repeats

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A B S T R A C T

Human induced pluripotent stem cell (hiPSC) line INEUi001-A was reprogrammed from peripheral blood mononuclear cells (PBMC) using the lentiviral-hSTEMCCA-loxP vector. PBMCs were obtained from a 75- year-old female ALS/FTD disease patient carrying a heterozygous deletion within the *C9ORF72* hexanucleotide repeat region resulting in a GGGGCCG sequence (~1.16 repeats). *C9ORF72* genotype was maintained and stemness and pluripotency confirmed in INEUi001-A hiPSC line.

1. Resource table:

Unique stem cell line identifier	INEUi001-A
Alternative name(s) of stem cell line	*FBDC9 1.3*
Institution	*Instituto de neurociencias, Fundación para la lucha contra las enfermedades neurológicas de la infancia. (INEU-Fleni – CONICET)*
Contact information of distributor	*Mariela Marazita (mmarazita@fleni.org.ar)*
Type of cell line	*Induced pluripotent stem cell (iPSC)*
Origin	*Human*
Additional origin info requiredfor human ESC or iPSC	*Age:75Sex: female Ethnicity if known: white latino*
Cell Source	*Peripheral blood mononuclear cells (PBMCs).*
Clonality	*Clonal*
Method of reprogramming	*Lentiviral EF1a-hSTEMCCA-loxP vector expressing OCT-4, SOX-2, c-MYC and KLF-4*
Genetic Modification	*NO*
Type of Genetic Modification	*N/A*
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*RT-/q-PCR.*
Associated disease	*Amyotrophic Lateral Sclerosis / Frontotemporal dementia*
Gene/locus	*C9ORF72*/NG_031977.2(C9ORF72): g.5328_5338del
Date archived/stock date	*08/07/2022*
Cell line repository/bank	https://hpscreg.eu/cell-line/INEUi001-A
Ethical approval	*The study was approved by local Ethics Committee (Comité de ética en investigaciones biomédicas del Instituto Fleni) (code number:3570). Written informed consent was obtained from the patient.*

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2. Resource utility

The ALS/FTD -patient-derived iPSC line harbours a heterozygous deletion within the *C9ORF72* hexanucleotide repeat region resulting in a GGGGCCG sequence (~1.16 repeats), in contrast to the most frequent 2-repeat allele in the general population. The generated iPSC line can help to uncover physiopathological mechanisms that lead to neuro-degeneration in ALS-FTD.

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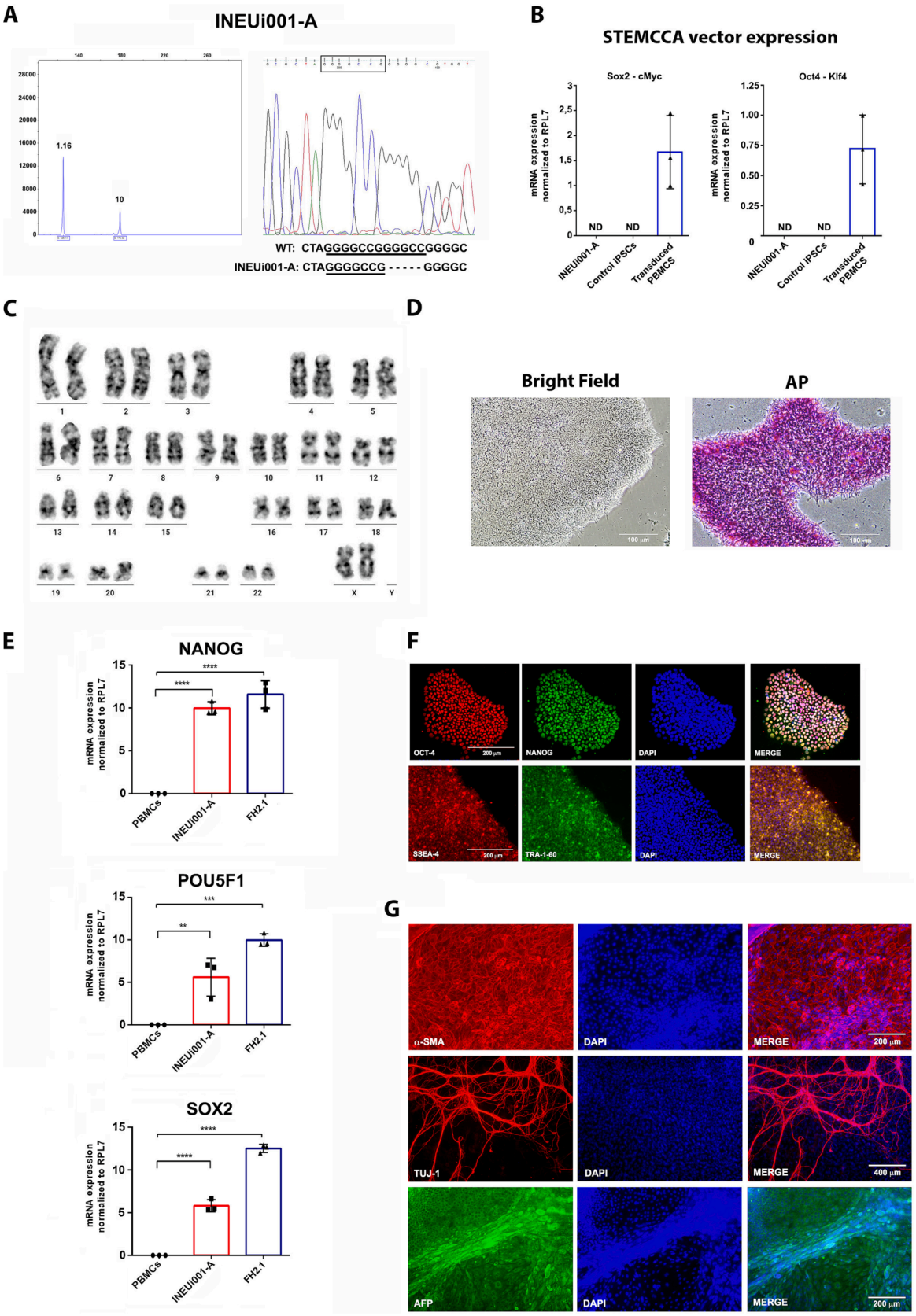

Fig. 1. Characterization of the INEUi001-A cell line with a C9ORF72 G4C2 genotype of <2 (GGGGCCG) and 10 repeats.
A) Amplified fragment length polymorphism (AFLP) for C9ORF72 hexanucleotide repeat genotyping and Sanger sequencing **B)** Quantitative PCR (qPCR) analysis of stemcca vector expression **C)** Karyotype of the INEUi001-A cell line by G-banded chromosome analysis **D)** Alkaline phosphatase staining **E)** Pluripotency markers analysis by qPCR (NANOG, POU5F1 and SOX2) **F)** Pluripotency markers verified by immunostaining (OCT-4, NANOG, SSEA-4 and TRA 1-60). **G)** Differentiation capacity into the three germ layers (Endoderm, AFP; Mesoderm, α-SMA; Ectoderm, TUJ-1)

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photomicrography	Normal	Fig. 1 panel D
Phenotype	Immunocytochemistry	Positive for pluripotency markers OCT-4, NANOG, TRA1-60, and SSEA-4.	Fig. 1 panel F
	RT-qPCR	cells express OCT-4, SOX-2 and NANOG	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46,XX, resolution: 440	Fig. 1 panel C
Identity	Microsatellite PCR (mPCR) OR	N/A	N/A
	STR analysis	24 sites tested, matched	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Amplified fragment length polymorphism (AFLP) for C9ORF72 hexanucleotide repeat genotyping and Sanger sequencing – AFLP PCR resulted in two peaks: 125 bp and 178 bp (Fig. 1A, left). A new PCR reaction was run on a 12% polyacrylamide gel and the 125 bp band was excised, eluted and sequenced (Fig. 1A, right). A deletion was confirmed in this allele resulting in a GGGGCCG sequence (~1.16 repeats)present in INEUi001-A	Fig. 1 panel A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR (Negative) passage 16	Supplementary file 1
Differentiation potential	Embryoid body formation	Expression of α-smooth muscle actin (SMA), α-feto protein (AFP) and NESTIN was used as a proof of three germ layers formation	Fig. 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

3. Resource details

Amyotrophic lateral sclerosis (ALS) and Frontotemporal dementia (FTD), are part of a clinical-pathological continuum. ALS is characterized by the progressive loss of motor neurons and weakness of voluntary muscles, while FTD is the second most prevalent form of early-onset dementia.

The expansion of the hexanucleótido G4C2 in the *C9ORF72* gene accounts for almost 40% of familial ALS patients, 25% of familial FTD patients, and as high as 88% of familial ALS/FTD patients. The number of G4C2 repeats in healthy subjects ranges between 2 and 24, with most people harbouring two to eight repeats. Peripheral blood mononuclear cells were obtained from a blood sample of a 75-year-old female ALS/FTD patient harbouring a heterozygous deletion within the *C9ORF72* hexanucleotide repeat region resulting in a GGGGCCG sequence (~1.16 repeats), in contrast to the most frequent 2-repeat allele in the general population. The other allele corresponds to a 10-repeat G4C2 hexanucleotide. The EF1a-hSTEMCCA-loxP lentiviral vector expressing OCT-4, SOX-2, c-MYC and KLF4 pluripotency genes was used to generate the iPSC line INEUi001-A as described previously (Somers et al., 2010) using a feeder- and xeno-free reprogramming protocol. (Chen et al., 2011). The deletion within the G4C2 hexanucleotide is present in the INEUi001-A iPSC line as in the parental PBMCs (Fig. 1A, left panel: electropherogram; right panel: chromatogram). Short tandem repeat (STR) analysis confirmed that iPSCs profiles matched those of the donor PBMCs. Transgenes inserted by STEMCCA lentiviral vector silencing was confirmed by RT-qPCR using specific primers for exogenous expression (Fig. 1B and Table 2). iPSC-FH2.1 line and transduced PBMCs harvested on day 6 of reprogramming protocol were used as negative and positive control respectively (Fig. 1B). INEUi001-A iPSCs exhibit normal karyotype (46, XX) (30 metaphases were studied at a 440-band resolution) (Fig. 1C), show typical iPSCs morphological characteristics (formation of compact multicellular colonies with a high nucleus/cytoplasm ratio and distinct colony borders), and high Alkaline Phosphatase (AP) activity (Fig. 1D). Quantification of the mRNA expression levels of SOX-2, POU5F1 (OCT-4), and NANOG genes by RT-qPCR confirmed stemness, iPSC-FH2.1 line was used as positive control (Fig. 1E). Furthermore, immunofluorescence staining showed robust expression of stemness-associated markers OCT-4, NANOG, SSEA-4 and TRA1-60 (Fig. 1F). Finally, *in vitro* spontaneous differentiation through embryoid bodies-based method proved the pluripotent potential of INEUi001-A iPSC line to differentiate into the three germ layers as shown by immunofluorescence analysis of Smooth muscle actin (SMA, mesoderm), Alpha-fetoprotein (AFP, endoderm) and NESTIN (ectoderm) expression (Fig. 1G).

4. Materials and methods

4.1. Reprogramming and cell culture

PBMCs were isolated from blood using a Ficoll density gradient procedure (HISTOPAQUE®SIGMA, #10771). A total of 2.10^6 cells were cultured in 2 mL erythrocytes expansion media containing StemPro™-34-SFM (Gibco™ #10639011), 50 µg/mL ascorbic acid, 50 ng/mL SCF, 10 ng/mL IL-3, 2U/mL EPO, 40 ng/mL IGF-1 and 1 µM Dexamethasone, in 1 well of a 12-well dish at 37 °C, 5% CO_2. Media was replaced on days 3 and 6. On day 9, EF1a-hSTEMCCA-loxP lentiviral reprogramming vector was used to transduce cells at MOI = 7. Cells were plated on Geltrex-coated dishes (1%, Gibco™, #A1413202), and further culture according to TeSR™-E7™ manufacturers instructions (STEMCELL-technologies™, #05914). At day 30 post-transduction, iPSC colonies were mechanically isolated and expanded on Geltrex-coated dishes in mTeSR™ Plus medium (STEMCELL-technologies™, #17187501). Cell cultures were passage every 3 to 4 days using Versene (GIBCO, #15040066) at 1:8 ratio and adding 10 µM Y-27632 ROCK inhibitor (Cell-Signalling-Technology, #13624) for up to 24 h.

4.2. Genotyping and STR analysis

Genomic DNA was isolated using the Wizard-Genomic-DNA-

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	mouse anti-OCT-4 IgG	1:200	Santa Cruz Biotechnology Cat# sc-5279, RRID: AB_628051
Pluripotency Markers	rabbit anti-NANOG IgG	1:400	Cell Signaling Technology Cat#4903, RRID: AB_10559205
Pluripotency Markers	mouse anti-SSEA4 IgG	1:200	Santa Cruz Biotechnology Cat# sc-21704, RRID: AB_628289
Pluripotency Markers	mouse anti-TRA1-60 IgM	1:200	Santa Cruz Biotechnology Cat# sc-21705, RRID: AB_628385
Differentiation Markers	mouse anti-AFP IgG	1:200	Santa Cruz Biotechnology Cat# sc-166325, RRID: AB_2305278
Differentiation Markers	mouse anti-SMA IgG	1:400	Invitrogen Cat# PA5-87638, RRID: AB_2804309
Differentiation Markers	rabbit anti-Neuronal Class III β-tubulina (TUJ1) IgG	1:1000	Covance Antibody Products Cat# MMS-435P, RRID: AB_2313773
Secondary antibodies	Goat anti-Mouse IgG Alexa Fluor 594	1:400	Thermo Fisher Scientific Cat# A-11005, RRID: AB_2534073
Secondary antibodies	Goat anti-Mouse IgG Alexa Fluor 488	1:400	Thermo Fisher Scientific Cat# A-11001, RRID: AB_2534069
Secondary antibodies	Goat anti-Mouse IgM Alexa Fluor 488	1:400	Thermo Fisher Scientific Cat# A-21042, RRID: AB_2535711
Secondary antibodies	Goat anti-Rabbit IgG Alexa Fluor 594	1:400	Thermo Fisher Scientific Cat# A-11012, RRID: AB_2534079
Secondary antibodies	Goat anti-Rabbit IgG Alexa Fluor 488	1:400	Thermo Fisher Scientific Cat# A-11008, RRID: AB_143165
Primers			
	Target	**Amplicon size**	**Forward/Reverse primer (5′-3′)**
Exogenous factors (RT-PCR)	STEMCCA plasmid OCT-4/KLF4	561 bp	CAACGAGAGGATTTTGAGGC/ ATCGTTGAACTCCTCGGTCTCTCT
Exogenous factors (RT-PCR)	STEMCCA plasmid SOX-2/c-MYC	550 bp	TTGGCTCCATGGGTTCGGTG/ AAGGGTGTGACCGCAACGTAGG
Pluripotency Markers (qPCR)	POU5F1 (OCT-4)	105 bp	GCAGGCCCGAAAGAGAAAGCGA/ TGGCTGATCTGCTGCAGTGTGG
Pluripotency Markers (qPCR)	SOX-2	110 bp	AGCATGGAGAAAACCCGGTACGC/ CGTGAGTGTGGATGGGATTGGTGT
Pluripotency Markers (qPCR)	NANOG	120 bp	TCCTTCCTCTCCCCTCCTCCCAT/ TAGGCTCCAACCATACTCCACCCTC
House-Keeping Genes (qPCR)	RPL7	138 bp	AATGGCGAGGATGGCAAG/TGACGAAGGCGAAGAAGC
AFLP PCR-Forward Sequencing	*C9ORF72*	Information in Table 1, Mutation analysis section.	CAAGGAGGGAAACAACCGCAGCC
AFLP PCR-Reverse	*C9ORF72*	Information in Table 1, Mutation analysis section.	GCAGGCACCGCAACCGCAG

Purification-kit (Promega, # A1120). The G4C2 repeat was genotyped from iPSCs (passage 15) as previously described (Itzcovich et al., 2016). STR analysis from PBMC (passage 1) and iPSC (passage 11) was performed at Laboratorio de Huellas Digitales Genéticas (FFyB-UBA).

4.3. Karyotyping

G-banded chromosome analysis of 30 metaphases from iPSCs (passage 12) was performed by Kromos Laboratory.

4.4. Alkaline phosphatase assay

iPSCs were subjected to alkaline phosphatase staining following manufacturer's instructions (Sigma, #86R).

4.5. RNA isolation and RT-qPCR

RNA was extracted with TRIzol (ThermoFisher-Scientific, #15596026) and cDNA was synthesized from total RNA with Oligo(dT) primers and M−MLV Reverse Transcriptase (Promega, #M170B) following manufactureŕs instructions. qPCR amplification (95 °C/15 s, 60 °C/1 min, 40 cycles) and analysis were performed with StepOnePlus-Real-Time-PCR-System (PE-Applied-Biosystems). The FastStart-Universal-SYBR-Green-Master Mix(ROX) (Roche, #04913850001) was used for all reactions. Transgenes expression was assessed using primers that bridge over two of the four transgenes on the STEMCCA lentiviral vector sequence. This design allows amplification of only cDNA generated from the exogenous mRNA expression of these genes. Cells were analysed at passages 12/14/16. Values were normalized to a housekeeping (RPL7) gene.

4.6. In vitro differentiation

iPSCs (passage 12) were detached with Dispase (Gibco™, #17105041) and transferred to non-adherent Petri dishes in DMEM/F12 + 1x GlutaMAX (Gibco, #35050061), 20% knock-out serum replacement (Gibco™, #10828), 1x non-essential amino acids (Sigma, #M7145), 0.1 μM 2-mecaptoethanol and 100U/ml penicillin and 50 μg/ml streptomycin to induce formation of embryoid bodies (EBs). On day 4, EBs were plated onto 0.1% gelatin coated 24-well plates and cultured for additional 17 days in DMEM/F12 supplemented with 20% FBS (Gibco, #10270106), 1x GlutaMAX, 100U/ml penicillin and 50 μg/ml streptomycin.

4.7. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde (30 min) and permeabilized with 0.1% Triton X-100f (30 min). Primary antibody incubation was performed overnight (4 °C) in PBS/BSA-1%. Fluorescent-dye conjugated secondary antibodies were incubated in PBS/BSA-1% (1 h, room temperature). Cells were counterstained with DAPI and examined under a NIKON-Eclipse-TE2000-S inverted microscope. Pluripotent markers were analysed at passage 9.

4.8. Statistical analysis

Results are expressed as mean ± SEM (n = 3). Comparisons were analysed by One-Way ANOVA followed by Dunnetś-test (**$p < 0.01$; ****$p < 0.0001$).

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.

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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.2023.103076.

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