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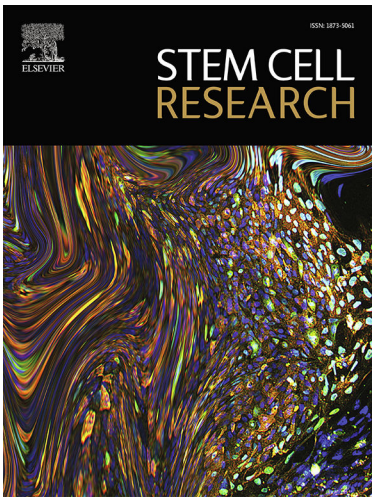

Generation of five induced pluripotent stem cells lines from four members of the same family carrying a *C9orf72* repeat expansion and one wild-type member

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Title: Generation of five induced pluripotent stem cells lines from four members of the same family carrying a *C9orf72* repeat expansion and one wild-type member

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Abstract: The most common genetic cause of Amyotrophic Lateral Sclerosis (ALS) is the expansion of a G4C2 hexanucleotide repeat in the C9orf72 gene. The size of the repeat expansion is highly variable and a cut-off of 30 repeats has been suggested as the lower pathological limit. Repeat size variability has been observed intergenerationally and intraindividually in tissues from different organs and within the same tissue, suggesting instability of the pathological repeat expansion. In order to study this genomic instability, we established iPSCs from five members of the same family of which four carried a C9orf72 repeat expansion and one was wild-type.

***Resource Table**:*

Unique stem cell lines identifier	IAIi005-A IAIi006-A IAIi007-A IAIi008-A IAIi009-A
Alternative name(s) of stem cell lines	AC52 (IAIi005-A) BC6 (IAIi006-A) CC5 (IAIi007-A) DC2 (IAIi008-A) EC1 (IAIi009-A)
Institution	IRCCS Istituto Auxologico Italiano, Milan, Italy

Type of cell lines	iPSC
Origin	Human
Additional origin info required	Ethnicity: Caucasian (IAIi005-A), Age:89, Sex: Male Ethnicity: Caucasian (IAIi006-A), Age:65, Sex: Female Ethnicity: Caucasian (IAIi007-A), Age:57, Sex: Female Ethnicity: Caucasian (IAIi008-A), Age:51, Sex: Female Ethnicity: Caucasian (IAIi009-A), Age:65, Sex: Female
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Genetic Modification	No
Type of Genetic Modification	N/A
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-PCR
Associated disease	Amyotrophic lateral sclerosis (ALS)
Gene/locus	C9orf72 gene/chromosome 9p21.2
Date archived/stock date	
Cell line repository/bank	https://hpscreg.eu/cell-line/IAIi005-A https://hpscreg.eu/cell-line/IAIi006-A https://hpscreg.eu/cell-line/IAIi007-A https://hpscreg.eu/cell-line/IAIi008-A https://hpscreg.eu/cell-line/IAIi009-A
Ethical approval	Ethical committee of IRCCS Istituto Auxologico Italiano, approval number 2022_03_15_12

1. Resource utility

Hexanucleotide repeat expansions in *C9orf72* cause ALS and Frontotemporal Dementia (FTD), two neurodegenerative diseases in a clinical *continuum*. Generation of iPSCs from four individuals with different repeat expansions and clinical history (3 ALS and 1 asymptomatic) and a wild-type member of the same family will enable study of *C9orf72*-related pathomechanisms.

2. Resource Details

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease affecting spinal, bulbar and cortical motor neurons and leading to a progressive muscular atrophy with rapid death of patients, usually due to respiratory failure. Expansion of the G_4C_2 hexanucleotide repeat in the first intron of *C9orf72* gene is the most common genetic cause of ALS and Frontotemporal Dementia (FTD) (1,2).

thousands in ALS/FTD patients. The correlation between repeat expansion length and disease severity or phenotype still needs to be fully clarified in particular for small expansions (<100 repeats). Similarly, how these expansions may have occurred by genome instability is still under debate. We generated iPSCs lines from five members of the same *C9orf72* family (PED25) already described (3,4). Previous Southern Blot analysis on both peripheral blood and fibroblasts revealed a small expansion for the asymptomatic father (AC52) (70 repeats), while the three daughters with ALS (BC6, CC5, DC2) had a larger expansion (~1,750 repeats) and one unaffected daughter (EC1) was wild-type (3,4). Reprogramming was performed on fibroblasts of all these family members using a non-integrating Sendai virus commercial kit. One clone from each subject was fully characterized. All clones displayed an iPSC-like morphology (Fig. 1A), were positive both by immunocytochemistry (Fig. 1B) and qPCR (Fig. 1C) for the pluripotent markers Oct3/4, Nanog and Sox2 and exhibited a normal karyotype (Fig. 1D). Short tandem repeat (STR) analysis confirmed matching of all 22 STR markers between fibroblasts and iPSC, indicating cell identity. Maintenance of a small repeat expansion (47 repeats) in iPSCs of the father, a larger expansion in iPSCs of three ALS daughters and the absence of the expansion in the wild-type daughter's iPSCs was confirmed by Repeat-primed PCR (Fig. 1E). Absence of Mycoplasma contamination was verified by PCR (Suppl. Fig. 1). All clones were able to spontaneously differentiate into the three germ layers *in vitro* as revealed by positivity to specific markers by immunocytochemistry (endoderm: alpha-fetoprotein (AFP); mesoderm: desmin; ectoderm: βIII Tubulin (βIIITub)) (Fig. 1F). Absence of Sendai vector transcripts (Klf4, KOS, c-myc, and Sev) was confirmed by semi-quantitative RT-PCR (Fig. 1G).

In summary, we generated clonal cell lines from five members of the same family, fulfilling all the criteria to be considered iPSCs and representing a useful *in vitro* model to study genetic instability of the *C9orf72* repeat expansion. Indeed, in this family, the small repeat expansion of the unaffected father jumped to a larger pathogenic length in the three daughters presenting with ALS (3,4).

Materials and Methods

3.1 Fibroblast reprogramming

Fibroblasts from the five family members were obtained and propagated as previously described (3). Fibroblasts below passage six were frozen and shipped to the laboratory of Neurosciences (Istituto Auxologico Italiano IRCCS, Italy) where they were reprogrammed using the CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific). At day 7, transduced cells were harvested and plated onto Matrigel (Corning) coated dishes. Medium was switched to Essential 8 medium (Thermo Fisher Scientific) until emerging colonies reached a suitable size to be picked. Colonies were grown at 37°C, 5% CO2 and passaged 1:10 using 0.5mM EDTA solution.

3.2 Stemness evaluation

Expression of stemness markers was evaluated by immunocytochemistry and by qPCR. iPSCs grown for 6 passages on coverslips were fixed in 4% paraformaldehyde (Santa Cruz Biotechnology), permeabilized with 0.3% Triton X-100 and incubated for 20 min in blocking buffer containing 10% normal goat serum (Gibco) in PBS. Cells were incubated with primary antibodies (Table 2) for 90 min at 37°C and then with fluorescently-labelled secondary antibodies (Table 2) for 45 min at room temperature, both antibodies diluted in blocking buffer. Nuclei were stained with DAPI (Sigma-Aldrich). Images were acquired with Eclipse C1 confocal microscope and NIS-elements software (Nikon). For qPCR, total RNA was extracted from iPSCs and fibroblasts using TRIzol Reagent following manufacturer instructions and reverse transcribed using SuperScript II reverse transcriptase.

mix (All from ThermoFisher Scientific) using QuantStudio 12k Flex instrument (Applied Biosystems). Target gene expression data (Ct) were normalized to *RPL10a* gene Ct values and fold change was calculated as $2^{-\Delta\Delta Ct}$.

3.3 Karyotyping

Standard cytogenetic procedures were used to analyse iPSC karyotype. Following overnight addition of Colcemid solution (KaryoMAX™, Thermo Fisher Scientific), chromosome analysis was achieved by Q-Band staining.

3.4 STR analysis

Genomic DNA from the 5 iPSCs cell lines and from the parental fibroblasts was extracted using Wizard Genomic DNA Purification kit (Promega). The genetic STR profile was obtained using ChromoQuant SuperSTaR Optima QF-PCR Kit (CyberGene AB) detecting 22 STR loci (mix solution 1) according to the manufacturer instructions. Amplicons were run on ABI Prism 3500 (Applied Biosystems) and analyzed using Gene Mapper v.4 software (Applied Biosystems).

3.5 Mutation analysis

The presence of the *C9orf72* repeat expansion in iPSCs and fibroblasts was evaluated by Repeat-primed PCR using a commercial kit (Asuragen). Amplicons were analyzed on ABI 3500 Genetic Analyzer and by using Gene Mapper v.4 software. The kit allows detection of repeat expansions up to 145 units.

3.6 Mycoplasma detection

Absence of mycoplasma contamination was evaluated by PCR using a commercial Kit from Minerva biolabs.

3.7 In vitro spontaneous differentiation

To evaluate the spontaneous differentiation potential of iPSCs into the three germ layers, we generated embryoid bodies (EBs) cultured on low adhesion plates in for 7 days (HUES medium). EBs were seeded onto Matrigel-coated plates in Essential 8 medium for an additional 10 days. Immunocytochemical analysis were performed to evaluate the expression of mesodermal, ectodermal and endodermal specific markers (Table 2).

Acknowledgments

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Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1A
Phenotype	Qualitative analysis: immunocytochemistry	Expression of the pluripotency markers: Oct3/4, Nanog, Sox2	Fig. 1B
	Quantitative analysis: qPCR	Expression of the pluripotency markers: Oct3/4, Nanog, Sox2	Fig. 1C
Genotype	Karyotype (Q-banding) and resolution	AC52: 46,XY BC6: 46,XX CC5: 46,XX DC2: 46,XX EC1: 46,XX	Fig. 1D
Identity	STR analysis	22 loci analyzed, 22 matched	Available from the authors
Mutation analysis (IF APPLICABLE)	Sequencing	Number of GGGGCC repeats in C9orf72 AC52: 2/47 BC6: 2/> 145 CC5: 2/> 145 DC2: 2/> 145 EC1: 2/2	Fig. 1E
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Venor®GeM OneStep Mycoplasma detection: all negative	Suppl. Fig. 1
Differentiation potential	Embryoid body derived germ layers	Expression of specific markers: endoderm: AFP, mesoderm: desmin and ectoderm: βIIITub	Fig. 1F
List of recommended germ layer markers	Expression of the markers has to be demonstrated at mRNA (RT PCR) or protein (IF) levels, at least 2 markers need to be shown per germ layer	Expression of specific markers: endoderm: AFP, mesoderm: desmin and ectoderm: βIIITub	Fig. 1F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency marker	Mouse anti-Oct-3/4	1:200	Santa Cruz Biotechnology cat#sc-5279	RRID:AB_628051
	Rabbit anti-Nanog	1:200	Abcam cat#ab21624	RRID:AB_446437
	Rabbit anti-Sox2	1:70	Abcam cat#ab15830	RRID:AB_443255
Differentiation Markers	Rabbit anti-β III tubulin	1:500	Abcam cat#ab-52623	RRID:AB_869991
	Rabbit anti-Desmin	1:10	Chemicon Millipore cat#AB907	RRID:AB_2092609
	Mouse anti-Alpha-fetoprotein	1:125	Invitrogen cat#14-6583-80	RRID:AB_2865213
Secondary antibodies	Alexa FluorTM 488 goat anti-mouse IgG (H+L)	1:500	Life Technologies cat# A-11001	RRID:AB_2534069
	Alexa FluorTM 488 goat anti-rabbit IgG	1:500	Life Technologies cat#A-11008	RRID:AB_143165
	Alexa FluorTM 555 goat anti-rabbit IgG (H+L)	1:500	Life Technologies cat# A-21428	RRID:AB_2535849
Nuclear stain	4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	2 µg/mL	Sigma-Aldrich D9542	
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5'-3')**	
Pluripotency Markers (q-PCR)	OCT4	81 bp	Fwd: AGTGCCCGAAACCCACACTG Rev: CCACACTCGGACCACATCCT	
	NANOG	154 bp	Fwd: TGAACCTCAGCTACAAACAG Rev: TGGTGGTAGGAAGAGTAAAG	
	SOX2	151 bp	Fwd: GGGAAATGGGAGGGGTGCAAAAGAGG Rev: CACCAATCCCATCCACACTCACGCAA	

House-Keeping Genes (q-PCR)	RPL10a	51 bp	Fwd: GAAGAAGGTGTTATGTCTGG Rev: TCTGTCATCTTCACGTGAC
Sendai virus detection (RT-PCR)	KOS	528 bp	Fwd: ATGCACCGCTACGACGTGAGCGC Rev: ACCTTGACAATCCTGATGTGG
	Klf4	410 bp	Fwd: TTCCTGCATGCCAGAGGAGCCC Rev: AATGTATCGAAGGTGCTCAA
	c-myc	532 bp	Fwd: TAACTGACTAGCAGGCTTGTCG Rev: TCCACATACAGTCCTGGATGATGATG
	Sev	181 bp	Fwd: GGATCACTAGGTGATATCGAGC Rev: ACCAGACAAGAGTTTAAGAGATATGTATC
	RPL10a	228 bp	Fwd: CAAGAAGCTGGCCAAGAAGTATG Rev: TCTGTCATCTTCACGTGAC
Genotyping	C9orf72 expansion	From 129bp	AmplideX® PCR/CE C9orf72 Kit - Asuragen
Targeted mutation analysis/sequencing	Not performed	N/A	N/A