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Lab Resource: Stem Cell Line

GENYOi004-A: An induced pluripotent stem cells (iPSCs) line generated from a patient with autism-related ADNP syndrome carrying a pTyr719* mutation

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A B S T R A C T

ADNP syndrome is an intellectual disability associated with Autism spectrum disorder caused by mutations in *ADNP*. We generated an iPSC line from an ADNP syndrome pediatric patient harboring the mutation p.Trp719* (GENYOi004-A). Peripheral blood mononuclear cells were reprogrammed using a non-transmissible form of Sendai viruses expressing the four Yamanaka factors (Oct3/4, SOX2, KLF4 and c-MYC). Characterization of GENYOi004-A included mutation analysis of *ADNP* by allele-specific PCR, genetic identity by Short Tandem Repeats polymorphism profiling, alkaline phosphatase enzymatic activity, expression of pluripotency-associated factors and pluripotency studies *in vivo*. GENYOi004-A will be useful to evaluate ADNP syndrome alterations at early developmental stages.

Resource table.

Unique stem cell line identifier	GENYOi004-A
Alternative name(s) of stem cell line	ASD-PBMC-iPS4F2
Institution	Gene Regulation, Stem Cells and Development Group, GENYO: Centre for Genomics and Oncological Research Pfizer-University of Granada-Junta de Andalucía, PTS, Granada 18,016, Spain;
Contact information of distributor	Pedro J. Real: pedro.real@genyo.es Jose L. Fernández-Luna: joseluis.fernandez@scsalud.es
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 8 Sex: Female Ethnicity: Spaniard Caucasian
Cell Source	Blood
Clonality	Clonal
Method of reprogramming	Sendai Virus (Cytotune iPS 2.0 Reprograming System)
Genetic Modification	YES
Type of Modification	Spontaneous mutation
Associated disease	ADNP Syndrome
Gene/locus	pTyr719* mutation in ADNP gene
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	September 2017
Cell line repository/bank	hpscreg.eu/user/cellline/edit/GENYOi004-A
Ethical approval	Comisión de Garantías para la Donación y Utilización de Células y Tejidos Humanos. Junta de Andalucia. PR-05-2017

Resource utility

GENYOi004-A is the first iPSC line generated from ADNP syndrome patients. GENYOi004-A will be a very useful tool to understand ADNP syndrome alterations at early developmental stages and to test experimental treatments for these patients *in vitro*.

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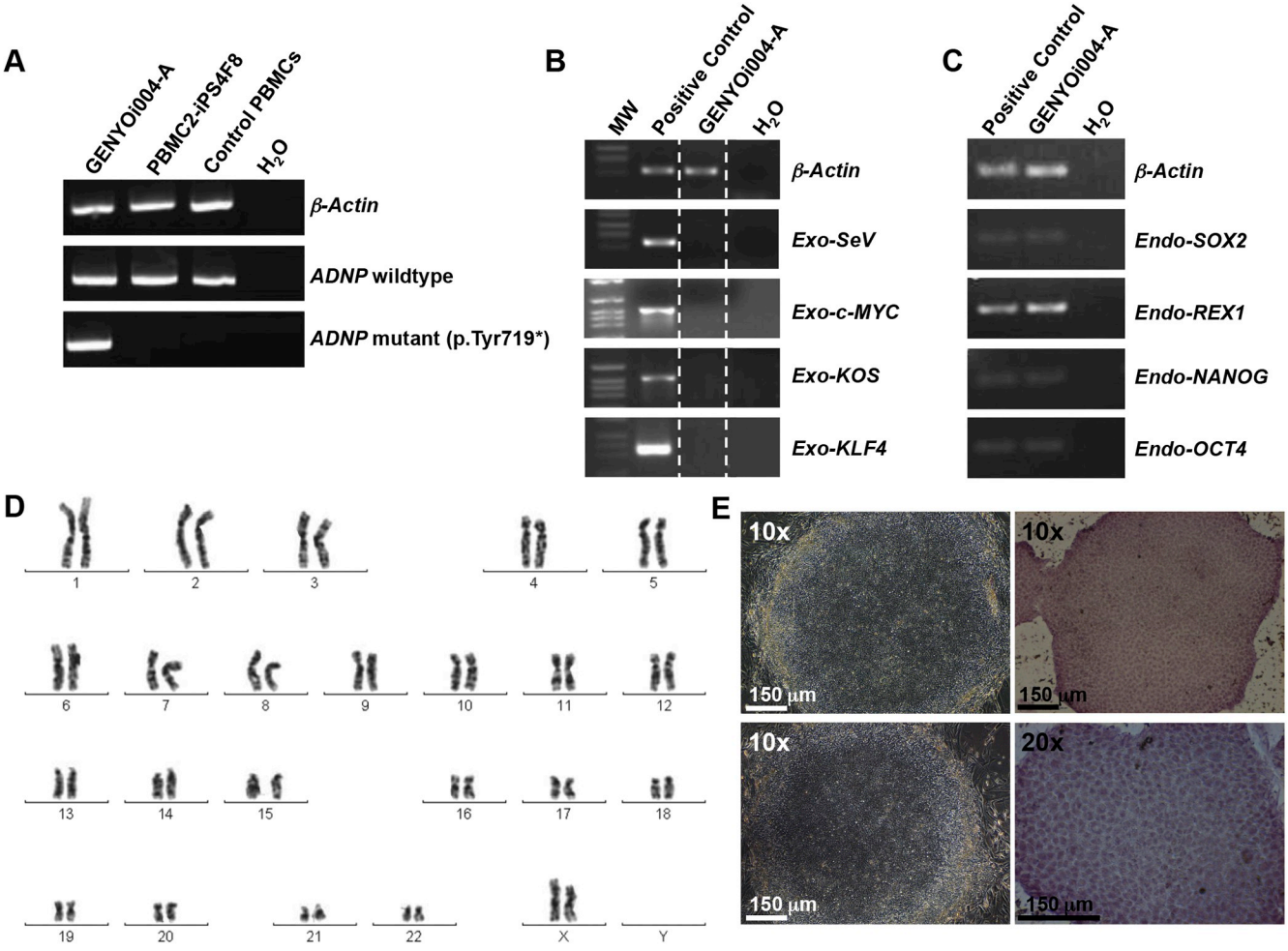

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Fig. 1. (A) Allele-specific PCR from GENYOi004-A, PBMC2-iPS4F8 and PBMCs from a healthy donor (Control PBMCs). Amplification of the b-Actin gene was used as a control. (B) Silencing of exogenous reprogramming factors and SeV vector confirmed by RT-PCR. PBMCs transduced cells from the patient at day 4 after Sendai virus exposure was used as a positive control. (C) Expression of the endogenous pluripotent transcription factors SOX2, REX1, NANOG and OCT 3 was assessed by RT-PCR. Human embryonic stem cell H9 was used as positive control. (D) GTG-banding shows a normal karyotype in GENYOi004-A cell line. (E) Alkaline phosphatase enzymatic activity staining. (Scale bar = 150 μm). (F) Expression of pluripotency-associated markers SSEA3, SSEA4, TRA1-81, TRA 1-60 and OCT3/4 at protein level by FACS analysis. The inset shows the staining of the isotype-matched antibody. (G) Embryo body (EB) differentiation assay. Immunohistochemistry analysis for endoderm (CKAE1-AE3), mesoderm (Vimentin) and ectoderm (β-III-Tubulin) from 3 week developed EBs. (Scale bar = 100 mm) (H) In vivo differentiation test by teratoma formation assay. Histological sections from 15 week-teratomas developed in the dorsal flanks of NOD/LtSz-scid interleukin-2Rγ_/_ mice following injection with ASD-PBMC-iPS4F2 cells. Hematoxylin and eosin (H&E) staining and immunohistochemistry analysis showed differentiation to endoderm (CK AE1-AE3), mesoderm (Vimentin) and ectoderm (GFAP). (Scale bar = 250 μm).

Resource details

Autism spectrum disorder (ASD) is a developmental disorder characterized by alterations in communication and behavior. Several ASD genes have been recently identified (De Rubeis et al., 2014; Iossifov et al., 2014). Mutations in Activity-Dependent Neuroprotective Protein gene (*ADNP*) have been associated with Helsmoortel-Van der Aa Syndrome (OMIM: 615873) (Helsmoortel et al., 2014), also known as ADNP syndrome that is considered an ASD. We have generated an iPSC line from peripheral blood mononuclear cells (PBMCs) obtained from an ADNP syndrome pediatric patient carrying a *de novo* p.Trp719*mutation (Mollinedo et al., 2019). This new iPSC line was named ASD-PBMC-iPS4F2 (registered as GENYOi004-A at www.hPSCreg.com). In parallel, we also generated an iPSC line from her mother (PBMC2-iPS4F8) that does not harbor any mutation in *ADNP* and will be consider as a negative control for genetic assays.

Our group has a broad experience on the use of CytoTune iPS 2.0 Reprograming System (Life Technologies, Invitrogen) to reprogram PBMCs from donors and patients. PBMCs from the ADNP patient and her mother were exposed to Sendai virus vectors (SeV) expressing the reprogramming factors Oct3/4, SOX2, KLF4 and c-MYC. 3 weeks after transduction several clones were selected and characterized as described below.

Firstly, we determined the presence of the mutation p.Trp719* in *ADNP* by allele specific PCR from genomic DNA in both cell lines, GENYOi004-A and PBMC2-iPS4F8 (Mollinedo et al., 2019) (Fig. 1A). Next, Short Tandem Repeat polymorphism (STR) analysis confirmed the same genetic identity between PBMCs from the patient (ASD-PBMCs) and GENYOi004-A (Table 1). GENYOi004-A cells silenced the expression of exogenous reprogramming transgenes (Fig. 1B) and activated the expression of the endogenous pluripotent transcription factors, *SOX2*, *REX1*, *NANOG* and *OCT4* (Fig. 1C) at passage 4. Importantly, GENYOi004-A cells showed normal karyotype at passage 7 (46, XX) (Fig. 1D). This new cell line grows forming typical round-shaped colonies and shows alkaline phosphatase activity (Fig. 1E).

Moreover, expression of the pluripotent markers SSEA3, SSEA4, Tra1–60, Tra1–81 and OCT3/4 was evaluated by flow cytometry analysis (Fig. 1F). Finally, functional pluripotency was assessed *in vitro* (by embryo body formation) (Fig. 1G) and *in vivo* (teratoma formation assays) (Fig. 1H). In both cases, GENYOi004-A cells generate embryo bodies (EBs) and teratomas containing all three germ layers: ectoderm (GFAP or β3-Tubulin), mesoderm (Vimentin) and endoderm (Cytokeratin CK AE1-AE) as shown in Fig. 1G and H.

Materials and methods

Generation of GENYOi004-A and PBMC2-iPS4F8 lines

Peripheral blood sample was obtained from a pediatric patient with ADNP syndrome and her mother after informed consent according with the Andalusian Ethics Review Board for Cellular Reprogramming requirements and with Spanish and EU legislation. PBMCs were isolated by centrifugation using Ficoll Paque™ PLUS (GE Healthcare). Isolated mononuclear cells were cultured in StemSpan™ SFEM (StemCell Technologies) supplemented with hSCF, hFLT3L, hTPO, G-CSF and hIL3 (Peprotech) for four days, transferred to a 12-well fibronectin coated plate (BD BioCoat™) and exposed to Sendai virus (SeV) (CytoTune®-iPS 2.0 Reprogramming kit, Life Technologies, Invitrogen) as formerly described (Lopez-Onieva et al., 2016). One month after reprogramming GENYOi004-A and PBMC2-iPS4F8 lines were adapted to grow in Essential 8 growth medium on Matrigel (BD Bioscience). Cells were split at a ratio from 1:6 to 1:8 every 4–5 days using PBS/EDTA (0.5 M) and cultured in a conventional incubator (37 °C, 5% CO_2).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Fig. 1 panel E
Phenotype	Qualitative analysis (RT-PCR)	Positive for SOX2, REX1, NANOG and OCT4	Fig. 1 panel C
	(Alkaline Phosphatase staining)	Positive	Fig. 1 panel E
	Quantitative analysis (Flow cytometry)	Oct3/4: 80%Tra 1–60: 96%; Tra1–81:30%; SSEA-4: 99%; SSEA-3: 37%	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 450–500	Fig. 1 panel D
Identity	STR analysis	16 loci tested, all matched	Available with the authors
Mutation analysis (IF APPLICABLE)	Allele specific PCR Southern Blot OR WGS	Specific amplication of mutant ADNP in ASD-PBMC-iPS4F4	Fig. 1 panel A
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR Negative	Supplementary file 2
Differentiation potential	Embryoid body formation	Immunohistochemistry for Vimentin, CK-AE1-AE3, β-III-tubulin	Fig. 1 panel G
	Teratoma formation	Immunohistochemistry for Vimentin, CK-AE1-AE3, GFAP	Fig. 1 panel H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	Not shown but available with author
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	HLA-I A* 03/24 - B* 35/55 - C* HLA-II DRB1* 1401/1401 - DQA1* 01/01 - DQB1* 0503/0503	Not shown but available with author

Allele-specific PCR

Genomic DNA was isolated from GENYOi004-A and PBMC2-iPS4F8 lines using the DNA extraction kit (Qiagen). DNA purified from PBMCs from a healthy donor not related to the patient was used as a wildtype control. PCR amplification with a set of primers that specifically recognizes wildtype or mutant alleles (Set *ADNP*, see Table 2) was performed in all three samples following the manufacturer's instructions (KapaBiosystems) in a SureCycler 8800 thermal cycler (Agilent). The PCR conditions were: 5 min (95 °C) initial denaturation, 35 cycles (30 s 95 °C denaturation, 30 s 55 °C annealing, 30 s 72 °C extension) followed by a 7-min final extension segment. The resulting PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

Short Tandem Repeat polymorphism (STR) profiling

The genetic identity of PBMCs from the ASD patient (ASD-PBMC) and GENYOi004-A was determined as previously described (Lopez-Onieva et al., 2016) (Table 1).

Semiquantitative RT-PCR

Total RNA from undifferentiated GENYOi004-A line and the human embryonic stem cell H9 (positive control) line was isolated using the High pure RNA isolation kit (Roche). cDNA was generated using the Transcription First Strand c-DNA synthesis kit (Roche) following the manufacturer's instructions. PCR was performed using GoTaq Flexi DNA Polymerase kit (Promega). The PCR conditions were optimized to get semiquantitative data within the linear range of amplification in a SureCycler 8800 thermal cycler (Agilent). The PCR conditions were: 2 min (95 °C) initial denaturation, 30–35 cycles (30 s 95 °C denaturation, 30 s 55–60 °C annealing, 30 s 72 °C extension) followed by a 5-min final extension segment. PCR products were electrophoresed in an agarose gel. For exogenous gene clearance analysis (Fig. 1B), PBMCs transduced cells from the patient at day 4 after Sendai virus exposure was used as a positive control. H9 hESCs cells were used as a positive control for endogenous pluripotent transcription factors.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-SSEA3-PE	1:100	eBioscience Cat# 12–8833-42 RRID:AB_10854121
Pluripotency Markers	Rabbit anti-SSEA4-PE	1:100	BD Pharmingen Cat# 560128 RRID:AB_1645533
Pluripotency Markers	Rabbit anti-Tra1–60-PE	1:100	eBioscience Cat# 12–8863-82 RRID:AB_891602
Pluripotency Markers	Rabbit anti-Tra1–81-PE	1:100	BD BioScience Cat# 560161 RRID:AB_1645540
Pluripotency Markers	Mouse anti-OCT4	1:100	BD BioScience Cat# 611203 RRID:AB_398737
Secondary antibodies	Goat Anti-Mouse IgG/IgM FITC	1:200	BD BioScience Cat# 554001 RRID:AB_395197
Isotype control PE	Mouse IgM, PE conjugated	1:100	BD BioScience Cat# 555584 RRID:AB_395960
Differentiation Markers	Mouse Anti-CKAE1-AE3	1:50	DAKO Cat # M3515 RRID:AB_2132885
Differentiation Markers	Mouse Anti-Vimentin (V9)	Ready to use	Roche Tissue Diagnostics Cat # 790–2917 RRID: N/A
Differentiation Markers	Mouse Anti-β-III-Tubulin	1:50	Millipore Cat # MAB1637 RRID:AB_2210524
Differentiation Markers	Mouse Anti-GFAP	1:200	DAKO Cat # M0761 RRID:AB_2109952
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Sendai Virus Plasmids (RT-PCR)	SeV (181 bp)	Forward: GGATCACTAGGTGATATCGAGC Reverse:ACCAGACAAGAGTTTAAGAGATATGTATC	
Sendai Virus KOS Plasmid (RT-PCR)	KOS (528 bp)	Forward: ATGCACCGCTACGACGTGAGCGC Reverse: ACCTTGACAATCCTGATGTGG	
Sendai Virus c-MYC Plasmid (RT-PCR)	c-MYC (532 bp)	Forward: TAACTGACTAGCAGGCTTGTCG Reverse: TCCACATACAGTCCTGGATGATGATG	
Sendai Virus KLF4 Plasmid (RT-PCR)	KLF4 (410 bp)	Forward: TTCCTGCATGCCAGAGGAGCCC Reverse: AATGTATCGAAGGTGCTCAA	
Targeted mutation analysis: Allele specific PCR	ADNP (206 bp)	Forward (wildtype): CACCTGTGAAGCGCACTTAC Forward (Mutant): CACCTGTGAAGCGCACTTAA Reverse: GGGATAGGGCTGTTTGTTGAA	
House-Keeping Genomics (RT-PCR)	β-ACTIN (232 bp)	Forward: GCGGGAAATCGTGCGTGACATT Reverse: GATGGAGTTGAAGGTAGTTTCGTG	
Pluripotency Markers (RT-PCR)	NANOG (96 bp)	Forward: TGCAGTTCCAGCCAAATTCTC Reverse: CCTAGTGGTCTGCTGTATTACATTAAGG	
Pluripotency Markers (RT-PCR)	OCT4 (110 bp)	Forward: AGTGAGAGGCAACCTGGAGA Reverse: ACACTCGGACCACATCCTTC	
Pluripotency Markers (RT-PCR)	REX1 (306 bp)	Forward: CAGATCCTAAACAGCTCGCAGAAT Reverse: GCGTACGCAAATTAAAGTCCAGA	
Pluripotency Markers (RT-PCR)	SOX2 (80 bp)	Forward: TCAGGAGTTGTCAAGGCAGAGAAG Reverse: CTCAGTCCTAGTCTTAAAGAGGCAGC	
House-Keeping Gene (RT-PCR)	β-ACTIN (165 bp)	Forward: CTGGAACGGTGAAGGTGACA Reverse: AAGGGACTTCCTGTAACAATGCA	

Karyotyping

Chromosomal analysis from GENYOi004-A at passage 7 was performed by GTG-banding analysis at the Andalusian Public Health System Biobank, Spain. 20 metaphases were analyzed according to the International System Cytogenetics Nomenclature recommendations.

Alkaline phosphatase

GENYOi004-A colonies were assayed for phosphatase alkaline enzymatic activity using a commercial detection kit (Merck-Millipore) following manufacturer's instructions.

Flow cytometry analysis

GENYOi004-A colonies were dissociated using Tryple Express (Life Technologies) and the cell suspension was stained for SSEA3 (PE, BioScience), SSEA4 (Alexa Fluor® 647, BD Pharmingen), Tra1–60 (PE, BioScience), Tra1–81 (Alexa Fluor® 647, BD Pharmingen) and OCT3/4 (BD BioScience) as previously published (Lopez-Onieva et al., 2016). A relevant isotype-match antibody was always used as a negative control. Live cells were identified by 7-aminoactinomycin D exclusion and were analyzed using a FACS verse (BD Bioscience).

Embryo body differentiation assay

GENYOi004-A cells were gently scraped off centrifuged, resuspended into Essential 6 medium and plated over low attachment 6-well plates (Corning) and embryo bodies were formed spontaneously. Three weeks later, embryo bodies were centrifuged, fixed and embedded in paraffin. Immunocytochemistry analysis were completed as previously described (Lopez-Onieva et al., 2016).

In vivo teratoma formation

GENYOi004-A cells were dissociated with collagenase IV (Invitrogen) and resuspended in PBS supplemented with 30% matrigel. 2 million cells were subcutaneously injected into the dorsal flanks of NOD/LtSz-scid interleukin-2Rγ$^{-/-}$ mice (The Jackson Laboratory). At week 15, teratomas were removed, fixed in formaldehyde and embedded in paraffin. Immunocytochemistry analysis confirmed the presence of ectodermal (β3-Tubulin), mesodermal (Vimentin) and endodermal (CKAE1-AE3) tissues.

Mycoplasma testing

Mycoplasma detection analysis from GENYOi004-A cells was performed by quantitative PCR analysis (Venor GeM-qEP (Minerva Biolabs)) at the Genomics and Genotyping Unit in GENyO, Spain.

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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.2019.101446.

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