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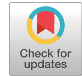
Lab Resource: Stem Cell Line

Copy number variations in healthy subjects. *Case study: iPSC line CSSi005-A (3544) production from an individual with variation in 15q13.3 chromosome duplicating gene CHRNA7*



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ABSTRACT

CHRNA7, encoding the neuronal alpha7 nicotinic acetylcholine receptor (a7nAChR), is highly expressed in the brain, particularly in the hippocampus. It is situated in the 15q13.3 chromosome region, frequently associated with a Copy Number Variation (CNV), which causes its duplication or deletion. The clinical significance of *CHRNA7* duplications is unknown so far, but there are several research data suggesting that they may be pathogenic, with reduced penetrance. We have produced an iPS cell line from a single healthy donor's fibroblasts carrying a 15q13.3 CNV, including CHRNA7 in order to study the exact role of this CNV during the neurodevelopment.

Resource table

Unique stem cell line identifier	*CSSi005-A (3544)*
Alternative name(s) of stem cell line	*COL03 cl.C3*
Institution	*Cellular Reprogramming Unit, IRCCS Casa Sollievo della Sofferenza – Viale dei Cappuccini, 71,013 San Giovanni Rotondo, Foggia, Italy*
Contact information of distributor	*Jessica ROSATIj.rosati@css-mendel.it*
Type of cell line	*iPSC*
Origin	*human*
Additional origin info	*Age: 38* *Sex: Female* *Ethnicity: Caucasian/Italian*
Cell Source	*Dermal Fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Non integrating episomal vectors*
Genetic Modification	*NO*
Type of Modification	*N/A*
Associated disease	*N/A*
Gene/locus	*15q11q13 CHRNA7*
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*April 2017*
Cell line repository/bank	*N/A*
Ethical approval	*Casa Sollievo della Sofferenza Ethical Committee, approval number: 75/CE*

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	*Fig. 1panel A*
Phenotype	Qualitative analysis *immunofluorescence*	*Staining of pluripotency markers: OCT4 (green) TRA-1-60 (red)*	*Fig. 1panel B*
	Quantitative analysis: *qPCR*	*Expression of pluripotency markers: LIN28, SOX2, L-MYC, OCT4*	*Fig. 1panel C*
Genotype	Karyotype (G-banding) and resolution	*46XX, Resolution 450–500*	*Fig. 1panel H*
Identity	Microsatellite PCR (mPCR) OR STR analysis	*N/A* *19 loci tested; all matched*	*N/A* *Data available with the authors*
Mutation analysis (IF APPLICABLE)	qPCR	*Increased copy number*	*Fig. 1panel I*
	Southern Blot OR WGS	*N/A*	*N/A*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by* N-Garde Mycoplasma PCR kit (EuroClone)*: Negative*	*Supplementary Figure S1*
Differentiation potential	*In vitro*: embryoid body formation	*Embryoid bodies.*	*Fig. 1panel E*
		Expression of genes in embryoid bodies: NESTIN, PAX6, SOX1, T, EOMES, FOXA2,GATA4, SOX 17.	*Fig. 1panel F*
	In vivo: teratoma formation	*Proof of three germ layers formation: Ectoderm, Mesoderm, Endoderm*	*Fig. 1panel 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

Resource utility

The generation of 15q13.3 CNV iPSC, including *CHRNA7*, may be useful in both scientific and clinical fields, because it allows us, on the one hand, to study the role of *CHRNA7* duplication on the development of the nervous system, and on the other hand, to eventually develop targeted therapeutics.

Resource details

The region 15q13.3, including *CHRNA7* gene, is a chromosome region particularly prone to rearrangements, such as microdeletion/microduplication (CNVs), due to an enrichment of low copy repeat (LCR) elements (De Jaco et al., 2017). Microdeletion of 15q13.3 has been found to cause neurological phenotypes with high penetrance, while gains encompassing *CHRNA7* is even more enigmatic, making the genetic counselling problematic because experimental and epidemiological data are still controversial (Gillentine et al., 2017a, 2017b). The influence of *CHRNA7* duplication can be studied directly on patients' cells through induced pluripotent stem cell (iPSCs) technology, facilitating the comprehension of its influence on brain development. Our donor is a subject with a normal working and family life, who has no dysmorphic features or other physical abnormalities, and no form of hyperactivity, distractibility, or social impairment. The sole cytogenetic feature of this donor is the presence of CNV, inherited from her father, affecting the cytogenetic region 15q13.3. The study was carried out, with the patient's consent, utilizing cells from a skin biopsy. We reprogrammed the fibroblasts into iPSCs, using non-integrative episomal vectors containing the reprogramming factors OCT4, SOX2, L-MYC, KLF4, LIN28, shp53. The clones with Embryonic Stem-like morphology (Fig. 1A) were characterized in indirect immunofluorescence (IIF) microscopy using specific antibodies against the surface marker TRA-1-60 and the transcription factor OCT4, confirming their expression: TRA-1-60 protein is present in cell surfaces and OCT4 protein is evident in the nuclear compartment of the iPS cells (Fig. 1B). Using qPCR we verified and confirmed the presence in the clones of endogenous expression of the pluripotency markers, *LIN28, SOX2, OCT4 and L-MYC* using, as negative control, the wild-type fibroblasts (Fig. 1C). After more than ten passages, we confirmed the suppression of exogenous reprogramming factor expression to negligible levels by qPCR using, as positive control, the nucleofected fibroblasts (Fig. 1D). Subsequently, pluripotency capacity of the iPSCs was tested, differentiating the iPSC both *in vitro* through embryoid bodies assay (Fig. 1E) and *in vivo* through a teratoma assay (on nude immune-deficient mice; NOD/SCID). The expression level of the markers of each germ layer was demonstrated, through qPCR, in the embryoid bodies using, as negative control, the iPSCs (Fig. 1F). Also in the iPSC-derived teratoma, hematoxylin-eosin images showed the presence of ectodermal derivatives: neuroepithelial structure with characteristic melanin deposits, mesodermal derivatives: adipose tissue, endodermal derivatives: secreting (dashed line) and batiprismatic lining (arrowheads) epithelia (Fig. 1G). Moreover, we confirmed that, after more than twenty passages, the iPSC line displayed a normal karyotype (46, XX) (Fig. 1H). Short tandem repeat analysis (STR) showed that the DNA profile of the donor's fibroblasts was identical to the derived iPSCs (data available from the authors). qPCR on genomic DNA verified that the *CHRNA7* CNV was retained in the iPSCs (Fig. 1I). All cells tested negative for mycoplasma contamination (Supplementary File S1). These data demonstrate that we have successfully generated a stable patient specific iPSC line, useful for studying 15q13.3 interstitial duplication encompassing *CHRNA7* at the molecular level (See Table 1).

Materials and methods

Primary skin fibroblast culture

Skin fragments were cut and plated on a 35 mm tissue-culture dish with Dulbecco's Modified Eagle Medium (DMEM) High Glucose supplemented with 20% FBS, 2 mM L-Glutamine, 100 U/ml Penicillin-Streptomicin and 1× Non-Essential Amino Acids (Sigma Aldrich) and cultured for 30 days to allow fibroblasts to grow out. Fibroblasts were split by Trypsin once a week, seeding $2*10^5$ cells into a 100 mm dish.

Cellular reprogramming

Approximately 1×10^5 fibroblasts were nucleofected with 4D-Nucleofector™ X unit (Lonza), using P2 primary cell nucleofector kit and FF113 program. A mix of three pCXLE-based episomal vectors were used: pCXLE-hOCT4-shp53, pCXLE-hUL and pCXLE-hSK (Addgene). Cells were cultured in fibroblasts medium for 6 days. On day 7, cells were plated on Matrigel (Corning) coated dishes (BD Biosciences) in NutristemXF medium (Biological Industries). The emerging iPS colonies were selected according to their hESC-like colony morphology and expanded under feeder-free conditions. Both fibroblasts and iPSCs were

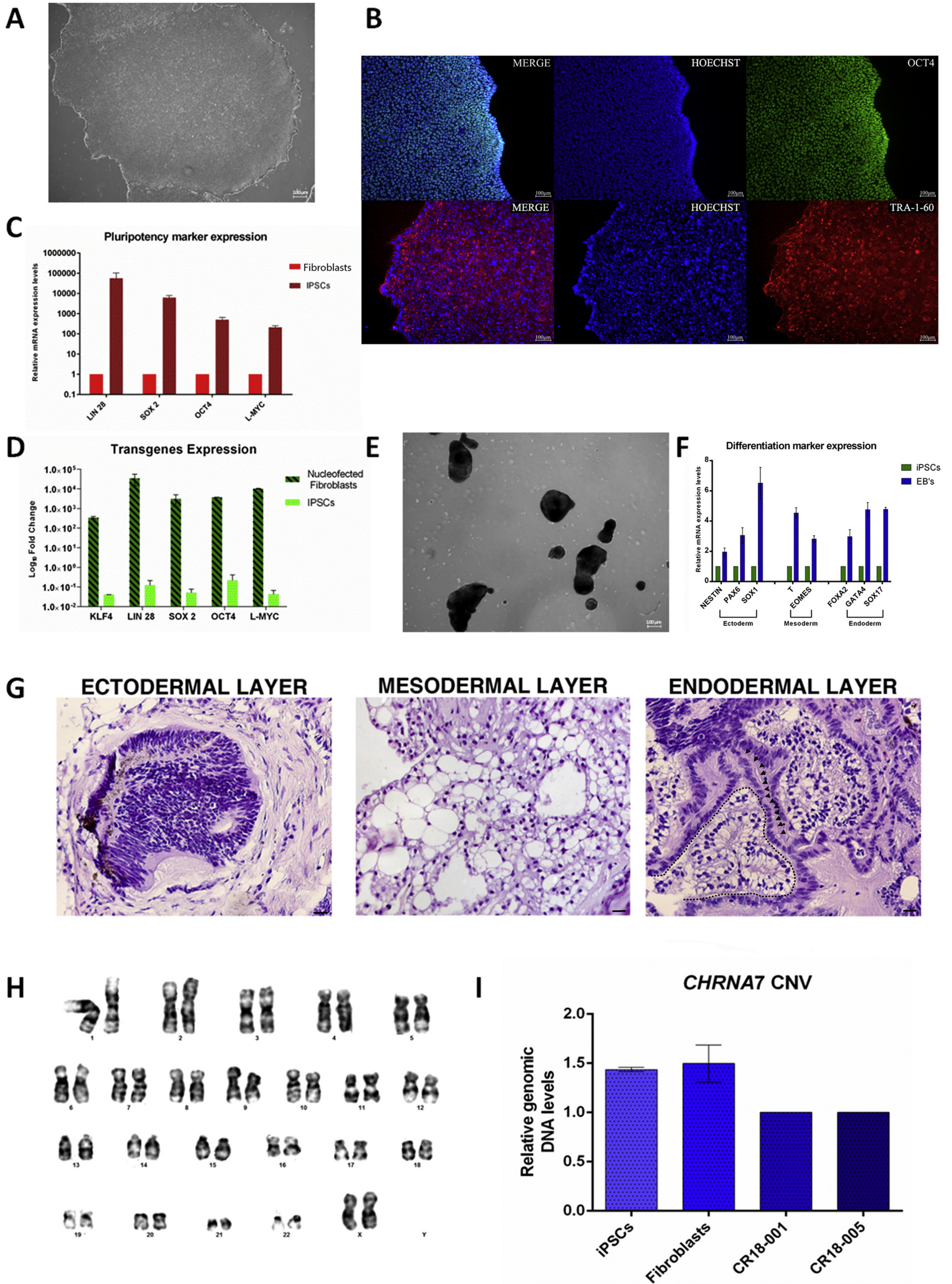

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Fig. 1. Characterization of COL03 iPSC.

A. COL03 iPSC colonies morphology **B.** Immunofluorescence staining shows expression of pluripotency markers: OCT4 (green), TRA-1-60 (red), cell nuclei with HOECHST (blue). **C.** qPCR analysis for pluripotency markers verified the expression levels of endogenous transcripts in COL03 iPSC. This experiment was repeated twice in triplicate using independently prepared cDNAs. **D.** qPCR analysis for transgenes transcripts, after 10 passage, in COL03 iPSC compared to nucleofected fibroblasts. This experiment was repeated twice in triplicate using independently prepared cDNA. **E.** COL03 EBs in floating condition. **F.** qPCR analysis of all three germ layer markers (Ectoderm, Mesoderm and Endoderm) from COL03 Embryoid Bodies. Data denote actin-normalized fold changes relative to undifferentiated parental iPSC. **G.** Histological sections of all three germ layer (Ectoderm, Mesoderm and Endoderm), derived from teratoma stained with hematoxylin and eosin. **H.** iPSC karyogram shows normal ploidy of COL03 female subject. **I.** qPCR analysis on genomic DNA verified that the CHRNA7 CNV was retained in the iPSCs.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	*Rabbit anti-OCT4*	*1:100*	*Life technologies (A13998)*
	Mouse anti-TRA-1-60	*1:100*	*Life technologies (411000)*
Differentiation Markers	*N/A*	*N/A*	*N/A*
Secondary antibodies	*anti-Rabbit AlexaFluor 488*	*1:10000*	*Invitrogen (A11034)*
	anti-Mouse AlexaFluor 555	*1:10000*	*Invitrogen (A21422)*

Primers		
Sybr Green Primers used for qPCR	Target	Forward/Reverse sequence (5′-3′)
Exogenous Episomal genes	*eOCT4*	*Fwd: CAT TCA AAC TGA GGT AAG GG*
		Rev: TAG CGT AAA AGG AGC AAC ATA G
	eKLF4	*Fwd: CCA CCT CGC CTT ACA CAT GAA GA*
		Rev: TAG CGT AAA AGG AGC AAC ATA G
	eLIN28	*Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C*
		Rev: TAG CGT AAA AGG AGC AAC ATA G
	eL-MYC	*Fwd: GGC TGA GAA GAG GAT GGC TAC*
		Rev: TTT GTT TGA CAG GAG CGA CAA T
	eSOX2	*Fwd: TTC ACA TGT CCC AGC ACT ACC AGA*
		Rev: TTT GTT TGA CAG GAG CGA CAA T
Pluripotency Markers (qPCR)	*OCT4*	*Fwd: CCC CAG GGC CCC ATT TTG GTA CC*
		Rev: ACC TCA GTT TGA ATG CAT GGG AGA GC
	LIN28	*Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C*
		Rev: TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG
	L-MYC	*Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC*
		Rev: CAG GGG GTC TGC TCG CAC CGT GAT G
	SOX2	*Fwd: TTC ACA TGT CCC AGC ACT ACC AGA*
		Rev: TCA CAT GTG TGA GAG GGG CAG TGT GC
House-Keeping Genes (qPCR)	*β-ACTIN*	*Fwd: GGC ATC CTC ACC CTG AAG TA*
		Rev: GGG GTG TTG AAG GTC TCA AA
Genotyping		
Targeted mutation analysis	*CHRNA7*	*Fwd: GCAGGAAGTGCTGGCCTATG*
		Rev: GGTCCAAACTGTGGGCTCC
TaqMan primers used for qPCR	Target	Forward/Reverse sequence (5′-3′)
Differentiation markers	*SOX1*	*Hs01057642_s1*
	NESTIN	*Hs04187831_g1*
	PAX6	*Hs00240871_m1*
	T	*Hs00610080_m1*
	EOMES	*Hs00172872_m1*
	GATA4	*Hs00171403_m1*
	FOXA2	*Hs00232764_m1*
	SOX17	*Hs00751752_s1*
House-Keeping Gene	*βActin*	*Hs 99999903_m1*

cultured in 5% CO_2 at 37 °C. The iPSC colonies were mechanically-detached and amplified once a week, the split ratio of iPSCs was usually 1:6. N-Garde Mycoplasma PCR kit (EuroClone) was used to verify the absence of mycoplasma contamination.

Immunofluorescence staining

Cells were fixed using 4% paraformaldehyde for 20 min at room temperature and blocked in PBS containing 20% Normal Goat Serum. 0.1% Triton X-100 was used for 30 min for only OCT4 staining. Next, primary antibodies, listed in Table 2, diluted in 5% BSA, were added and incubated O/N at 4 °C. After washing, Alexa-Fluor-conjugated secondary antibodies were added for 1 h at room temperature. Cellular nuclei were counterstained with Hoechst. Microphotographs were taken using a Nikon C2 fluorescence microscope.

RNA extraction and reverse-transcription

Total RNAs were isolated using TRIzol reagent (Life Technologies) according to manufacturer's instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Real-time PCR (qPCR)

qPCR was performed on cDNA using SYBR Green primers for pluripotency markers and TaqMan primers for differentiation markers

(Table 2) in a 7900HT Fast Real-Time PCR system (Applied Biosystem). Each reaction was performed in triplicate with *β-ACTIN* gene as reference. $2^{-\Delta\Delta CT}$ method was adopted to calculate the relative expression levels.

The same method was used to confirm the presence of *CHRNA7* duplication on DNA extracted from iPSC line using column-based extraction kit (DNeasy Blood & Tissue it; QIAGEN, Hilden, Germany). DNAs extracted from blood and previously tested negative by SNP-array were used as controls. *TERT* gene was used as a reference. Data analysis was performed as described in Carbone et al. (2008).

Teratoma formation

Approximately 3×10^6 cells were detached using Dispase (Gibco). Cellular suspension was collected at 800 rpm for 5 min and then resuspended in 100 μl of Matrigel. Cells were injected into the right flank of nude immune-deficient mice (NOD/SCID) following ethical guidelines. Teratomas formed in 4–8 weeks. Upon removal, tumors were histologically analyzed to check for their *in vivo* differentiation capacity into derivatives of all three germ layers: ectodermal, mesodermal and endodermal.

Embryoid body in vitro differentiation

Mechanically-detached iPSCs were plated in a Petri dish in floating condition. Nutristem-XF medium was then gradually switched with DMEM F-12, 20% Knock-out serum replacement (Gibco), 0.1 mM β-mercaptoethanol, 1× NEAA, 50 U/ml Penicillin-Streptomicin, 2 mM L-glutamine in 3 days. EBs were cultured in suspension for 14 days, changing the medium every two days, then pelletted and RNAs were extracted for qRT-PCR analysis.

Karyotype analysis

Cells were cultured in chamber slides (Thermo Fisher Scientific) and treated with a 0.1 μg/ml COLCEMID solution (Thermo Fisher Scientific) for 60 min at 37 °C. Metaphases were obtained by adding 30 mM KCl in 10% FBS at 37 °C for 6 min and by fixation using cold fresh-made 3:1 ethanol:acetic acid solution. Karyotype analysis was carried out on GTG-banded metaphases. Fifteen metaphases were counted and three karyotypes analyzed (450–500 resolution). Only clonal aberrations were considered, following the ISCN recommendations.

STR analysis

Fibroblasts and iPSCs DNA were extracted by Dneasy blood and tissue kit (QIAGEN) following manufacturer's suggestions. PCR amplification of 19 distinct STRs (D13S252, D13S305, D13S634, D13S800, D13S628, D18S819, D18S535, D18S978, D18S386, D18S390, D21S11, D21S1437, D21S1409, D21S1442, D21S1435, D21S1446, DXS6803, XHPRT, DXS1187) was carried out using the QST*Rplusv2 kit (Elucigene Diagnostics), PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper version 4.0 (Applied Biosystems).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.09.002.

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