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Lab Resource: Multiple Cell Lines

Generation of the induced human pluripotent stem cell lines CSSi009-A from a patient with a *GNB5* pathogenic variant, and CSSi010-A from a CRISPR/Cas9 engineered *GNB5* knock-out human cell line

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A B S T R A C T

GNB5 loss-of-function pathogenic variants cause IDDCA, a rare autosomal recessive human genetic disease characterized by infantile onset of intellectual disability, sinus bradycardia, hypotonia, visual abnormalities, and epilepsy. We generated human induced pluripotent stem cells (hiPSCs) from skin fibroblasts of a patient with the homozygous c.136delG frameshift variant, and a *GNB5* knock-out (KO) line by CRISPR/Cas9 editing. hiPSCs express common pluripotency markers and differentiate into the three germ layers. These lines represent a powerful cellular model to study the molecular basis of *GNB5*-related disorders as well as offer an *in vitro* model for drug screening.

Resource utility

hiPSC lines offer the opportunity to produce large amount of patient-specific and GNB5 knock-out cells, and to differentiate them into cell types relevant to the GNB5 disorders, like cardiomyocytes and neurons. Their characterization will give insights on the disorder pathogenesis and offer a powerful *in vitro* model for drug screening.

Resource details

Bi-allelic non-sense and frameshift mutations in the *GNB5* gene, encoding the β5 subunit of heterotrimeric G-proteins (Gβ5), are associated with the autosomal-recessive multisystem syndrome named Intellectual Developmental Disorder with Cardiac Arrhythmia (IDDCA; MIM#617173) (Lodder et al., 2016). This syndrome is characterized by intellectual disability, developmental and epileptic encephalopathy, retinal abnormalities and early-onset sinus node dysfunction (with bradycardia). Bi-allelic *GNB5* missense variants correlate with a milder manifestation of the disorder, characterized by language delay, attention-deficit/hyperactivity disorder, and mild cognitive impairment with or without cardiac arrhythmia (LADCI; MIM#617182) (Shamseldin et al., 2016). The biological mechanisms underlying this syndrome are unclear. In order to elucidate how loss of Gβ5 activity affects functional properties of excitable cells involved in the syndrome, such as cardiomyocytes and neurons, the availability of an unlimited source of these fully differentiated cell types is a major requirement.

Here we report the generation of: (i) a hiPSC line from a 10-years-old male proband carrying the c.136delG (p.E46fs8X) homozygous frameshift *GNB5* mutation and (ii) an hiPSC line, from a healthy donor, in which we selectively knocked-out (KO) *GNB5*.

hiPSCs were generated from skin fibroblasts by the mRNA-based reprogramming method to generate integration free, virus-free hiPSCs, using a single transfection step (Simplicon™ RNA Reprogramming Kit; Merck-Millipore). The first colonies appeared 10 days after transfection and displayed the classical pluripotent stem cell morphology (Supplementary Fig. 1A). The hiPSC control line was handled for genome editing by CRISPR/Cas9; a clone with the homozygous GNB5 frameshift variant c.204_208delCATGG was selected and amplified (Table 1).

The expression of pluripotency genes (NANOG, OCT4, LIN28, REX1, SOX2, and GAPDH as reference gene), assessed by RT-qPCR, was higher in both the hiPSC lines than in the fibroblast of origin (Fig. 1A). Protein

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
	CSSi009-A	Male	10	Asian	Hom c.136delG	IDDCA
	CSSi010-A	Female	48	Caucasian	Hom c.204_208delCATGG	Induced homozygous *GNB5* gene Knock-out

expression of NANOG, OCT4, SOX2 and TRA-1-60 was also assessed by Immunocytochemistry (Fig. 1B). Moreover, quantitative flow cytometry analysis revealed that between 85.3 %and 99.4% of the cells of both hiPSC lines expressed the pluripotency markers SOX2 and SSEA-4 (Fig. 1C).

We then confirmed that both hiPSC lines were able to generate cells belonging to the three germ layers, as demonstrated by the positive expression of mesodermal (*CD31*, *SMA*), endodermal (*SOX7*, *AFP*), and ectodermal (*KTR14*, *NCAM1*) markers (Fig. 1D), achieved by RT-qPCR using β-actin as reference gene to normalize the expression of differentiation markers. The hiPSC lines showed a normal karyotype (Supplementary Fig. 1B) and presented the expected mutations in *GNB5* gene (Fig. 1E). Genetic DNA fingerprinting was performed for both hiPSC lines and their fibroblast counterparts, confirming their genetic identity (Table 2). All hiPSC lines were negative for mycoplasma contamination (Supplementary Fig. 1C).

Materials and methods

Cell culture and reprogramming

Skin biopsies from an IDDCA patient and a healthy individual were used to establish primary dermal fibroblast cultures. For primary fibroblasts reprogramming, the Oct4, Klf4, Sox2, and Glis1 transgenes were expressed by Simplicon™ RNA Reprogramming Kit (Merck-Millipore), following the manufacturer's instructions. On day 28, individual hiPSC colonies were picked and growth on Matrigel™ (Corning) in TeSR-E8 medium and propagated using ReLeSR (Stem Cell Technologies) medium at 37 °C and 5% CO_2.

Generation of hiPSC GNB5 Knock-out through CRISPR/Cas9

Healthy control hiPSCs were transiently transfected with 2 μg of Gβ5 CRISPR/Cas9 KO Plasmids (Santa Cruz). After 48 h, GFP positive transfected cells were enriched by FACS analysis and dispersed at low density into Matrigel-coated 10 cm^2 dishes in TeSR-E8 Medium containing 5 μM ROCK inhibitor. After approximately 20 days, largest colonies were picked and expanded. Each clone was analysed for the *GNB5* variants by Sanger Sequencing. A positive clone was selected and further analysed.

Mycoplasma test

Mycoplasma contamination was ruled out by PCR using primers able to recognize most of the Mycoplasma species (Supplementary Fig. 1C). The 500 bp PCR product size was carried out by iCycler (Bio-Rad, USA) using the following PCR steps: 1. Denaturation at 95 °C for 5 min; 2. Denaturation at 95 °C for 30 s; 3. Primer annealing at 38 °C for 30 s; 4. Primer extension at 72 °C for 1 min; 5. Primer extension at 72 °C for 10 min. Steps 2 to 4 was repeated 35 times.

Karyotype analysis

Cytogenetic G-banding analysis was performed on indicated cells (Supplementary Fig. 1B) at passage number 12, as previously described (Drets and Shaw, 1971). Twenty metaphases were counted and three karyotypes were visualized with a 1000× objective (Zeiss, Germany) and analysed by G-banding at GAG 300–400 band resolution on average, using Applied Imaging Cito-Vision (Version 7.5).

Genotyping, sequencing, and STR analysis

Genomic DNA was extracted using Allprep DNA/RNA Mini kit (Qiagen) and *GNB5* confirmed by Sanger sequencing (Fig. 1E; Table 3). For STR analysis, we used the Promega PowerPlex 16 PCR kit (Promega, USA) that analysed 16 loci (Table 2).

RNA isolation and quantitative RT-PCR (RT-qPCR)

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany). QuantiTect Reverse Transcription Kit (Qiagen) was used to carry out cDNA synthesis. cDNA was amplified using Power SYBR Green PCR Master Mix (Applied biosystem) on ABI PRISM 7009HT System (Table 3).

Immunofluorescence staining

The hiPSCs were fixed in 4% paraformaldehyde for 15 min at room temperature (RT). Fixed cells were permeabilized and blocked for 30 min at RT with PBS containing 10% donkey serum (Jackson ImmunoResearch) and 0.1% TritonX-100 (VWR). Cells were then incubated with primary antibodies for 2 h at RT in 10% donkey serum in PBS. The secondary antibodies were thereafter added for 1 h at RT in the dark, followed by nuclei counterstain with DAPI. Images were acquired on an Inverted Fluorescence Microscope (Axiovert 200 M, Zeiss Carl; Software: AxioVision release 4.7.2 Dec 2008).

Flow cytometry

Flow Cytometry analysis was performed using Multi-Color Flow Cytometry Kit (R&D System) following manufacturer's instruction. Samples were analysed using FACSAria™ flow cytometry (BD Biosciences).

In vitro differentiation by embryoid body (EB) formation

Embryoid bodies (EBs) were formed from iPSCs detached and grown in ultra-low attachment plates (Corning) for 7 days in DMEM-F12 with 20% fetal bovine serum, 1 mM NEAAs, 2 mM L-Glutamine, 1% penicillin-Streptomycin and 0.1 mM β-mercaptoethanol. EBs were then seeded onto 0.1% gelatin-coated dishes for further 10 days of differentiation and analysed by RT-qPCR for endodermal, mesodermal, and ectodermal genes.

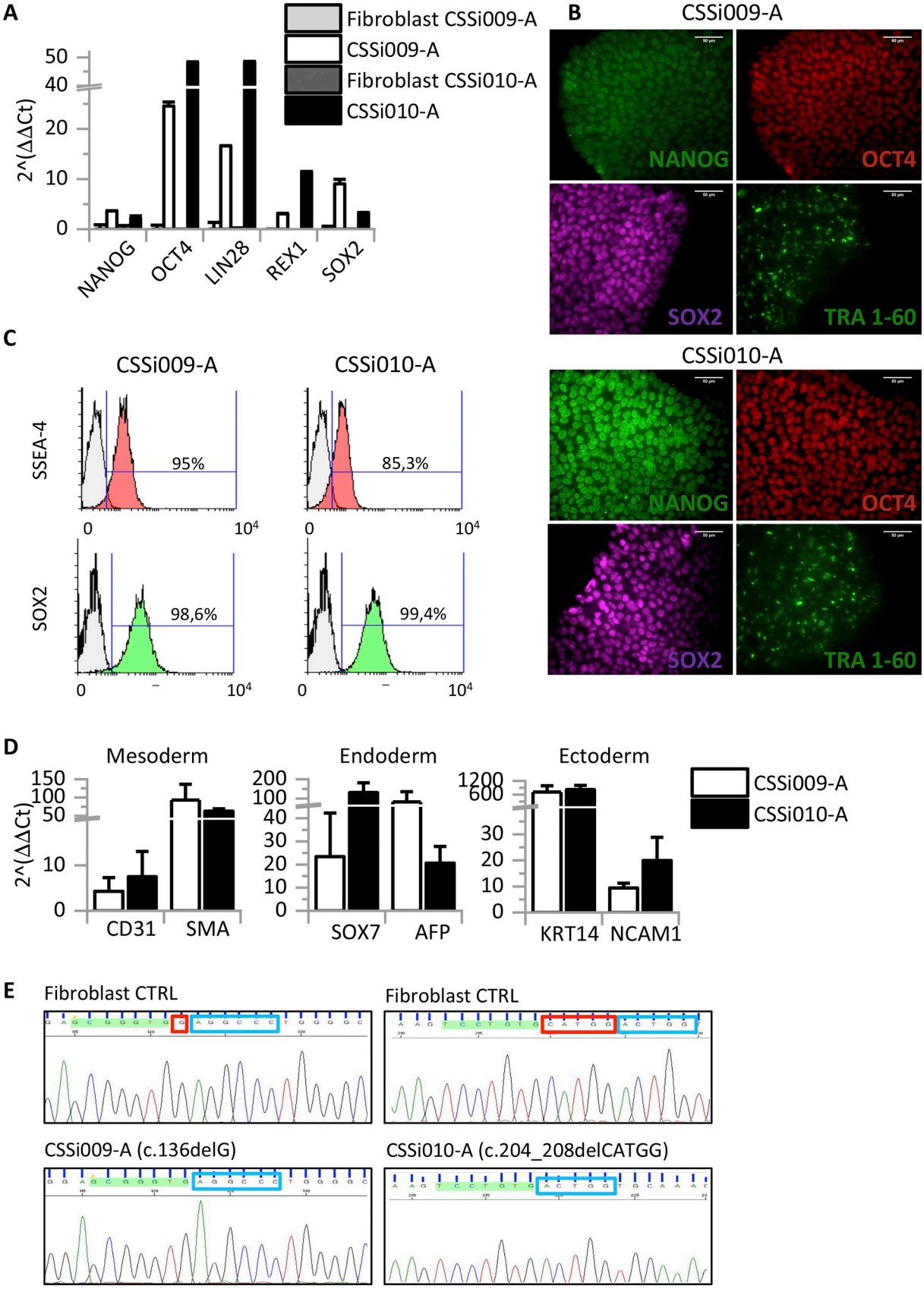

Fig. 1. Characterization of CSSi009-A and CSSi010-A lines.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase contrast bright field microscopy	Normal morphology	Supplementary Fig. 1A
Phenotype	Quantitative analysis by RT-qPCR	Expression of high levels of the pluripotency markers: *OCT4*, *SOX2*, *NANOG*, *LIN28* and *REX1*. Reference genes: *βactin* and *GAPDH*	Fig. 1 panel A
	Qualitative analysis by Immunocytochemistry	Both hiPSC lines expressed the pluripotency markers: *OCT4*, *SOX2*, *NANOG*, *TRA-1-60*	Fig. 1 panel B
	Flow cytometry	CSSi009-A showed positivity to: SSEA4 99.4 ± 0.05%; SOX2 = 85.3 ± 0.90%; CSSi010-A showed positivity to: SSEA4 96.9% ± 0.15%; SOX2 95% ± 0.56%	Fig. 1 panel C
Genotype	Karyotype (G-banding) and resolution	Normal karyotype 46XY GDB1307_Z2#1 (CSSi009-A); Normal karyotype 46XX GNB5_KO#5A (CSSi010-A); Resolution 300–400 bands	Supplementary Fig. 1B
Identity	Microsatellite PCR (mPCR)	Not performed	
	STR analysis	The STR profiles of both cell lines matched with that of the parental fibroblast cells. 16 loci analysed: Amelogenin (for gender identification), D3S1358, TH01, D21S11, D18S51, Penta_E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta_D, Vwa, D8S1179, TPOX and FGA (Promega PowerPlex 16 kit)	Submitted in archive with journal
Mutation analysis (if applicable)	Sanger Sequencing	c.136delG (CSSi009-A); c.204_208delCATGG (CSSi010-A)	Fig. 1 panel E
	Southern Blot OR WGS	Not performed	Not performed
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR: Negative	Supplementary Fig. 1C
Differentiation potential	Spontaneous differentiation through Embryoid body (EB) formation	All cell lines expressed genes of the three germ layers when subjected to spontaneous differentiation in EBs. (SOX7 and AFP for endoderm; CD31, SMA for mesoderm; KRT14 and NCAM1 for ectoderm)	Fig. 1 panel D
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

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers (Immunocytochemistry)	Mouse anti-OCT4	1:100	Santa Cruz Biotech; Cat# sc-5279; RRID:AB_628051
	Rabbit anti-SOX2	1:500	Abcam; Cat# ab97959; RRID:AB_2341193
	Goat anti-NANOG	1:100	Everest Biotech; Cat# EB06860; RRID:AB_2150379
	StainAlive TRA-1-60 (DyLight™488)	1:200	Stemgent; Cat# 09-0068; RRID:AB_2233143
Pluripotency markers (flow cytometry)	PE-SOX2 Mouse IgG2A	1:20	R&D System; Cat# IC2018P; RRID:AB_357273
	CFS- SSEA-4 Mouse IgG3	1:20	R&D System; Cat# FAB1435F; RRID:AB_952015
	PE Isotype control- mouse IgG2A	1:20	R&D System; Cat# IC003P; RRID:AB_357245
	CFS Isotype control- Mouse IgG3	1:20	R&D System; Cat# IC007F; RRID:AB_952037
Differentiation markers	N/A	N/A	
Secondary antibodies (Immunocytochemistry)	Alexa Fluor®568 Goat Anti-Mouse	1:400	Thermo Fisher Scientific; Cat#A11004, RRID AB_2534072
	Alexa Fluor®488 Goat Anti-Rabbit	1:400	Thermo Fisher Scientific; Cat#A11008, RRID AB_143165
	Alexa Fluor®488 Donkey Anti-Goat	1:400	Thermo Fisher Scientific; Cat#A11055, RRID AB_2534102

Primers

	Target	Forward/Reverse primer (5′-3′)
Pluripotency markers (RTqPCR)	OCT4	CGACCATCTGCCGCTTTG/GCCGCAGCTTACACATGTTCT
Pluripotency markers (RT-qPCR)	SOX2	CGCGATGCCGACAAGAA/AAAAATAGTCCCCCAAAAAGAAGTC
Pluripotency markers (RT-qPCR)	NANOG	AAATCTAAGAGGTGGCAGAAAAACA/CTTCTGCGTCACACCATTGC
Pluripotency markers (RT-qPCR)	LIN28	CCGGACCTGGTGGAGTATTCT/CGCTTCTGCATGCTCTTTCC
Pluripotency markers (RT-qPCR)	REX1	CCTGCAGGCGGAAATAGAAC/GCACACATAGCCATCACATAAGG
Three germ layer markers (endoderm) (RT-qPCR)	SOX7	TGAACGCCTTCATGGTTTG/AGCGCCTTCCACGACTTT
Three germ layer markers (endoderm) (RT-qPCR)	AFP	GTGCCAAGCTCAGGGTGTAG/CAGCCTCAAGTTGTTCCTCTG
Three germ layer markers (mesoderm) (RT-qPCR)	CD31	ATGCCGTGGAAAGCAGATAC/CTGTTCTTCTCGGAACATGGA
Three germ layer markers (mesoderm) (RT-qPCR)	SMA	GTGATCACCATCGGAAATGAA/TCATGATGCTGTTGTAGGTGGT
Three germ layer markers (ectoderm) (RT-qPCR)	KRIT14	CACCTCTCCTCCTCCCAGTT/ATGACCTTGGTGCGGATTT
Three germ layer markers (ectoderm) (RT-qPCR)	NCAM1	CAGATGGGAGAGGATGGAAA/CAGACGGGAGCCTGATCTCT
House-keeping genes (RT-qPCR)	GAPDH	GAAGGTGAAGGTCGGAGTC/GAAGATGGTGATGGGATTTC
	ACTB	CACTCTTCCAGCCTTCCTTC/AGTGATCTCCTTCTGCATCCT
Genotyping		
Targeted mutation analysis (Sequencing)	GNB5	TGTGGCTTGTTATGGAGGAA/ACCCGCTCACCTGTTATGC
Mycoplasma detection		TGCACCATCTGTCATTCTGTTAACCTC/ACTCCTACGGGAGGCAGCAGTA

Key resources table

Unique stem cell lines identifier	CSSi009-A CSSi010-A
Alternative names of stem cell lines	GDB1307_Z2#1 (CSSi009-A) GNB5_KO#5A (CSSi010-A)
Institution	Division of Medical Genetics, Fondazione IRCCS Casa Sollievo della Sofferenza, Italy
Contact information of distributor	Giuseppe Merla g.merla@operapadrepio.it
Type of cell lines	hiPSC
Origin	Human
Cell Source	Skin fibroblasts
Clonality	Clonal
Method of reprogramming	mRNA-based reprogramming method
Multiline rationale	Generate hiPSC from IDDCA patient with *GNB5* mutation (hereditary homozygous variant c.136delG) along with hiPSC cell line *GNB5* knock-out (homozygous variant c.204_208delCATGG)
Gene modification	Yes
Type of modification	Induced homozygous *GNB5* gene Knock-out
Associated disease	Intellectual Development Disorder with Cardiac Arrhythmia (IDDCA; MIM#617173)
Gene/locus	G Protein Subunit Beta 5-*GNB5*/chromosome 15q21.2
Method of modification	Gβ5 CRISPR/Cas9 KO Plasmid
Name of transgene or resistance	GFP
Inducible/constitutive system	N/A
Date archived/stock date	November 2018 (CSSi009-A) June 2018 (CSSi010-A)
Cell line repository/bank	Genomic and Genetic Disorders Biobank (GGDB) (http://biobanknetwork.telethon.it/)
Ethical approval	Fibroblasts were obtained from skin biopsies of a patient and of a healthy control after signing the appropriate informed consent, provided by Genomic and Genetic Disorders Biobank, member of the Telethon Network of Genetic Biobanks. The generation and use of hiPSCs was reviewed and approved by Ethical Committee at Fondazione IRCCS Casa Sollievo della Sofferenza (14/11/2018, 156/CE)

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101547.

Declaration of Competing Interest

None

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