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Lab Resource: Genetically-Modified Single Cell Line

Generation of heterozygous *SAMD9* CRISPR/Cas9-edited iPSC line (ESi086-A-3), carrying p.I1567M mutation

Joan Pera [a], Julio Castaño [b], Joan Casamitjana [a,c,d], Alessandra Giorgetti [a,e,*], Damia Romero-Moya [a,*]

[a] Regenerative Medicine Program, Bellvitge Institute for Biomedical Research (IDIBELL) and Program for Clinical Translation of Regenerative Medicine in Catalonia (P-CMRC), 08908 L'Hospitalet de Llobregat, Spain
[b] Plataforma de Terapias Avanzadas. Banc de Sang i Teixits, Edifici Dr. Frederic Duran i Jordà, Passeig Taulat, 116, 08005 Barcelona, Spain
[c] Department of Physiological Science, School of Medicine, University of Barcelona (UB), L'Hospitalet de Llobregat, Barcelona, Spain
[d] Pancreas Regeneration: Pancreatic Progenitors and Their Niche Group, Regenerative Medicine Program, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), L'Hospitalet de Llobregat, Barcelona, Spain
[e] Department of Pathology and Experimental Therapeutics, Faculty of Medicine and Health Sciences, Barcelona University, Barcelona, Spain

A B S T R A C T

Germline *SAMD9* mutations are one of the most common alterations that predispose to pediatric myelodysplastic syndrome (MDS), a clonal disorder characterized by ineffective hematopoiesis, increasing the risk of developing acute myeloid leukemia (AML). Up to date, a disease model to study the role of *SAMD9* mutation in MDS is still lacking. Here, we have generated a human induced pluripotent stem cell (hiPSC) line carrying *SAMD9*^{mut} (p.I1567M), taking advantage of CRISPR/Cas9 system. As a result, the genetic engineered hiPSC line represent a new *in vitro* disease model to understand the impact of SAMD9 mutation at molecular and cellular level during hematopoiesis.

1. Resource Table:

Unique stem cell line identifier	*ESi086-A-3*
Alternative name(s) of stem cell line	*CBiPS8-3F-4 SAMD9 p.I1567M*
Institution	*Institut d'Investigació Biomèdica de Bellvitge (IDIBELL)*
Contact information of the reported cell line distributor	*Alessandra Giorgetti (agiorgetti@idibell.cat)*
Type of cell line	*iPSC*
Origin	*Human*
Additional origin info *(applicable for human ESC or iPSC)*	*Age: 0* *Sex: Male* *Ethnicity N/A*
Cell Source	*Cord Blood CD133+ cells*
Method of reprogramming	*Retrovirus: OCT4, SOX2, KLF4*
Clonality	*Clonal*
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*N/A*
Cell culture system used	*hiPSC line is maintained in a feeder-free culture system on Matrigel (BD Biosciences, MA)-coated 60-mm plates with mTeSR1 medium (StemCell Technologies) at 37 °C, 5% CO_2 & 20% O_2. Culture medium was changed daily, and the cell was passaged weekly by EDTA dissociation (PBS + 0.5mM EDTA).*
Type of Genetic Modification	*Monoallelic point mutation in exon 3 of SAMD9 gene*
Associated disease	Transient aplasia, cytopenia, MIRAGE syndrome and monosomy 7
Gene/locus	*SAMD9 (OMIM: 610456; NC_000007.14)*
Method of modification/site-specific nuclease used	*CRISPR/Cas9 system*
Site-specific nuclease (SSN) delivery method	*RNP*
All genetic material introduced into the cells	*HDR donor vector*
Analysis of the nuclease-targeted allele status	*Sequencing of the targeted allele*
Method of the off-target nuclease activity surveillance	*N/A*
Name of transgene	*N/A*
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	*N/A*
Inducible/constitutive system details	*N/A*
Date archived/stock date	*Date cell line archived or deposited in a repository (05/07/2022)*

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* Corresponding authors.
E-mail addresses: agiorgetti@idibell.cat (A. Giorgetti), dromero@idibell.cat (D. Romero-Moya).

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Cell line repository/bank	*Instituto de Salud Carlos IIIhttps://hpscreg.eu/cell-line/ESi086-A-3*
Ethical/GMO work approvals	*La Comissió de Garanties per a la Donació i Utilització de Cèl·lules i Teixits Humans emet l'informe favorable al projecte de recerca. Title: Noves estratègies per a la identificació i caracterització funcional de marcadors genètics pronòstics en les síndromes mielodispàsiques/leucèmies agudes mioblàstiques hereditàries de la infància. Codigo: 557 457 1*
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	*N/A*

2. Manuscript section expected contents clarification

2.1. Resource utility

Induced Pluripotent Stem Cells (iPSCs) carrying heterozygous *SAMD9* mutation (p.I1567M), offer an unprecedent opportunity to generate unlimited iPSC-derived blood progenitors in vitro, bypassing the necessity of primary patient samples. Moreover, this human-based *SAMD9* model will allow a deep understanding of myelodysplastic predisposition susceptibility related to *SAMD9*-germline mutation. Table 1.

2.2. Resource Details

Sterile alpha motif domain protein 9 (*SAMD9*) is a highly conserve genes where missense mutations are associated with predisposition to familial MDS and increased the risk to develop acute myeloid leukemia (AML) (Schwartz et al., 2021). *SAMD9* have a common expression in the human tissue where it regulates cell proliferation and apoptosis (Li et al., 2007). *SAMD9* function is currently cryptic but it has been shown an antiproliferative potential as well as a tumour suppressor function, among others (Sahoo et al., 2021). Figure 1

Individuals carrying a germline heterozygous *SAMD9* mutation often suffer from a transient aplasia, cytopenia, MIRAGE syndrome and monosomy 7 among others (Davidsson et al., 2018). Nowadays, the hematopoietic stem cell transplantation (HSCT) is the only curative treatment, and the 5-year overall survival is ~84% in cases with normal karyotype. However, in patients with abnormal karyotype as monosomy 7 the overall survival is 70-80% (Sahoo et al. Nat Med 2021). The understanding of the role of germline SAMD9 mutations in familial MDS/AML will be critical to elucidate the molecular mechanisms of disease progression. Therefore, a reliable and trustworthy disease model is required. The identified *SAMD9* mutations are described as missense, in-frame deletion, frameshift, or nonsense. The germline mutations in *SAMD9* resulted in gain of function (GOF), promoting cell apoptosis and a cell growth-suppressive effect compared to the non-mutated cells (Sahoo et al., 2021). Consequently, taking advantage of precise gene editing tools, we have generated human iPSCs carrying the p.I1567M *SAMD9* mutations described in familial MDS patients (Sahoo et al., 2021).

Using a pre-designed sgRNA, targeting p.I1567M mutation, the CRISPR/Cas9 system was applied on healthy iPSC line (CBI08-3F-4). The iPSCs were nucleofected with Cas9 protein, the sgRNA together with a ssODN donor template carrying the mutation c.4701A>G. Right after, we performed a positive clone screening selection by Restriction Length Polymorphism (RFLP) by digestion with NlaIII and confirmed them by Sanger sequencing.

After the selection and expansion of the clone carrying the p.I1567M mutation, a karyotype analysis was performed to avoid chromosomic abnormalities. Concluding that the engineered hiPSC line ESi086-A-3 has a normal 46XY karyotype, showing alkaline phosphatase (AP)

Table 1
Characterization and validation

Classification (optional *italicized*)	Test	Result	Data
Morphology	*Photography Bright field*	*Typical embryonic stem cell-like morphology*	Figure 1 *C*
Pluripotency status evidence for the described cell line	*Qualitative analysis*	*Immunostaining for pluripotency markers: OCT3/4 and NANOG*	Figure 1 *C*
	Quantitative analysis	*Flow cytometry expression of SSEA.4 TRA-1-81 and TRA-1-60*	Figure 1 *D*
Karyotype	*Karyotype (G-banding) and resolution*	*46 XYResolution 300-500 bands*	Figure 1 *B*
Genotyping for the desired genomic alteration/allelic status of the gene of interest	*PCR across the edited site*	*Point mutation found in heterozygosis by PCR + Sequencing*	Figure 1 *panel A*
	Transgene-specific PCR	*N/A*	*N/A*
Verification of the absence of random plasmid integration events	*PCR/Southern*	*N/A*	*N/A*
Parental and modified cell line genetic identity evidence	*Microsatellite PCR (mPCR) OR*	*N/A*	*N/A*
	STR analysis	*STR analysis of TH01, D2S11, D5S818, D13S317, D7820, D16S539, CSF1PO, AMEL, vWA and TPOX. The edited cell line matched to individual host profile*	*Submitted in archive with journal*
Mutagenesis / genetic modification outcome analysis	*Sequencing*	*Point mutation found in heterozygosis by PCR + Sequencing*	Figure 1 *panel A*
	PCR-based analyses	*N/A*	*N/A*
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	*N/A*	*N/A*
Off-target nuclease analysis-	*PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing*		
Specific pathogen-free status	*Mycoplasma*	*Mycoplasma was tested by RT-PCR showing a negative result*	Figure 1 *Supplementary*
Multilineage differentiation potential	*Embryoid body formation*	*The cell line was differentiated into cells of mesoderm (CD43 and CD45), endoderm (FOXA2 and AFP) and ectoderm (GFAP and TUJ1)*	Figure 1 *F*
Donor screening (OPTIONAL)	*HIV 1 + 2 Hepatitis B, Hepatitis C*	N/A	N/A
Genotype - additional histocompatibility info (OPTIONAL)	*Blood group genotyping*	N/A	N/A
	HLA tissue typing	N/A	N/A

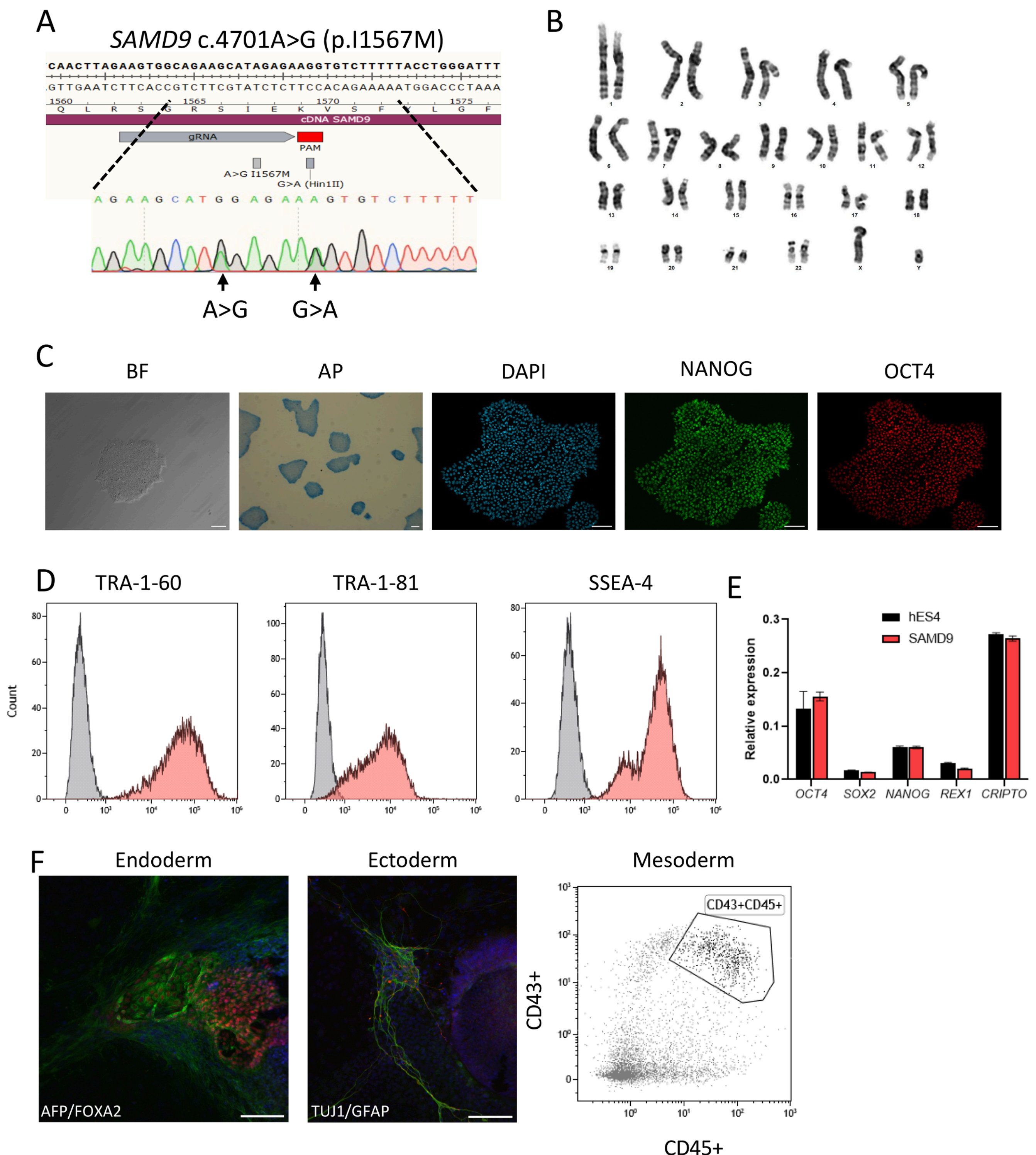

Figure 1. Characterization of *SAMD9* p.I1567M mutated iPSC line

activity which is a marker to identify pluripotent stem cells. Immunofluorescence and flow cytometry analysis revealed expression of pluripotency markers such as, NANOG, OCT4, SSEA4, TRA-1–81 and TRA-1–60. Additionally, the gene expression of OCT4, SOX2, NANOG, CRIPTO and REX1 was confirmed by RT-PCR. The *SAMD9* p.I1567M line has high capacity to generated embryoid bodies (EBs), which could be differentiated into derivatives of the three embryonic germ layers: being positive for TUJ1 and GFAP in ectoderm, α-fetoprotein (AFP) and FoxA2 in endoderm, and CD45/CD34 in mesoderm The iPSC line identity was confirmed by short tandem repeat analysis (STR) and compared with the original parental CBiPS8-3F-4 clone. The cell line was free from mycoplasma contamination as determined by PCR.

3. Materials and Methods

3.1. Human iPSC cultures

hiPSC line is maintained in a feeder-free culture system on Matrigel

Table 2
Reagents details *RRID Requirement for antibodies: use http://antibodyregistry.org/ to retrieve RRID for antibodies and include ID in the table as shown in examples.*

Antibodies and stains used for immunocytochemistry/flow-cytometry	**Antibody**	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	*Mouse IgG anti-OCT4 Goat IgG anti-NANOG AF-647 SSEA-4 PE*	*1:2 1:5 1:40*	*Santa Cruz Biotechnology, SC- 5279; R&D; AF1997; BD Bioscience; 560219;*
Differentiation Markers	*Rabbit IgG anti-alpha-1-fetoprotein Goat IgG anti-FOXA2 Mouse IgG anti-Beta III-Tubulin TUJ1 Rabbit IgG anti-GFAP APC-H7 anti-CD45 APC anti-CD43*	*1:200 1:50 1:500 1:500 1:40 1:100*	*DAKO; A0008; 2650473 R&D; AF2400; Covance; MMS-435P; DAKO; Z0334; BD Bioscience; 560178; BD Bioscience; 560198*
Secondary antibodies	*Anti-rabbit IgG Cy2 anti-goat IgG Cy3 anti-mouse IgG Cy2 anti-rabbit IgG Cy3*	*1:200 1:200 1:200 1:200*	*Jackson Immuno Research; 715–165-151; Jackson Immuno Research; 705–545-147; Jackson Immuno Research; 711–545- 152; Jackson Immuno Research; 705–165-147; Jackson Immuno Research; 715–545-151; Jackson Immuno Research; 711–165- 152; Thermo Fisher Scientific*
Nuclear stain	7AAD	*1μg/mL*	*Invitrogen # 00-6993-50*
Site-specific nuclease			
Nuclease information	*Nuclease type/nomenclature*	*Alt-R® S.p. Cas9 Nuclease V3*	
Delivery method	*Nucleofection*	Nucleofected with 4-D Nucleofector System (Lonza) using the CA-137 program.	
Selection/enrichment strategy			
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids (qPCR)	*N/A*	*N/A*	
Pluripotency Markers (qPCR)	*OCT4 (64 bp) SOX2 (72 bp) NANOG (111 bp) REX1 (61 bp) CRIPTO (163 bp)*	*GGAGGAAGCTGACAACAATGAAA/ GGCCTGCACGAGGGTTT TGCGAGCGCTGCACAT/ TCATGAGCGTCTTGGTTTTCC ACAACTGGCCGAAGAATAGCA/ GGTTCCCAGTCGGGTTCAC CCTGCAGGCGGAAATAGAAC/ GCACACATAGCCATCACATAAGG CGGAACTGTGAGCACGATGT/ GGGCAGCCAGGTGTCATG*	
House-Keeping Gene (qPCR)	*GAPDH (75bp)*	*GCACCGTCAAGGCTGAGAAC/ AGGGATCTCGCTCCTGGAA*	
Genotyping	*SAMD9 exon 3 (446bp)*	*TGCATCGTACAAAGCAACCA/ ACCTTGCCGGTTTAAAGCAT*	
Targeted mutation analysis/ sequencing	*SAMD9 exon 3 c.4701A>G (446bp)*	*TGCATCGTACAAAGCAACCA/ ACCTTGCCGGTTTAAAGCAT*	
Potential random integration-detecting PCRs	*e.g. plasmid backbone, for targeting events- vector/homology arm end PCRs*	*N/A*	
gRNA oligonucleotide	*SAMD9 Exon 3 (OMIM: 610456; NC_000007.14)*	*AAGTGGCAGAAGCATAGAGAAGG*	
Genomic target sequence(s)	*PAM: AGG*	*CAACTTAGAAGTGGCAGAAGCATAGAGAAGGTGTCTTTTTACCTGGGATT (chr7:93,101,371-93,101,420)*	
e.g. Top off-target mutagenesis predicted site sequencing (for CRISPR/ Cas9 and TALENs) primers	*OT1- F&ROT2-F&R…*	*N/A*	
ODNs	*SAMD9 Exon 3 (OMIM: 610456; NC_000007.14)*	*ATACCCATCACTCCCGCTTTTTAGGTCAACTTAGAAGTGGCAGAAGCATgGAGAAaGTGTCTTTTTACCTGGGATTTTCCATTGGAGGCCCACTTGCTT*	

(BD Biosciences, MA)-coated 60-mm plates with mTeSR1 medium (StemCell Technologies) at 37 °C, 5% CO_2 & 20% O_2. Culture medium was changed daily, and the cell was passaged weekly by EDTA dissociation (PBS + 0.5mM EDTA).

3.2. CRISPR/Cas9 gene editing

The CRISPR tool (https://bioinfogp.cnb.csic.es/tools/breakingcas/) was used in the sgRNA design. A high probability to target the region of interest and low probability to generate off-targets gRNA sequence was selected. Rock inhibitor (Y-27632, 10μM) was added to the iPSCs 3 hours before nucleofection. 100 pmol Alt-R® CRISPR-Cas9 (IDT) was incubated with 120 pmol Alt-R® CRISPR-Cas9 sgRNA (IDT) at 25 °C for 10 min. 4uM of ssODN was added to the RNP complex prior the nucleofection. 200.000 cells were dissociated with Accutase (Gibco), washed twice with PBS without Ca and Mg and resuspended with 20ul of P3/S1Buffer. RNP complex + ssODN was added to the cell pellet and transferred to the 20ul cuvette. Cells were nucleofected with 4-D Nucleofector System (Lonza) using the CA-137 program. Nucleofected cells were cultured in a 12 well plate, with mTSR1 and 10uM of Y-27632. After 72h of recovery, 1000 cells were seeded at a single cell level in a 100 mm plate to form single-cell colony. Genotyping was performed by PCR, RFLP and Sanger sequencing in single cell colonies to analyse the gene mutation.

3.3. Karoytyping

Genomic integrity of the iPSC line (passage 36) was evaluated by G-banded metaphase analysis with a resolution of 300–500 bands (Sant Joan de Deu, Barcelona). 70% confluent iPSC colonies were incubated with KaryoMax colcemid (Invitrogen), trypsinized, treated with hypotonic solution and fixed in Carnoy solution (75% methanol, 25% acetic acid). A minimum of 20 metaphases were examined.

3.4. Alkaline Phosphatase

iPSCs were fixed with 4% paraformaldehyde (PFA) for 1 min, washed with PBS and incubated with AP solution (Sigma) for 20 minutes.

3.5. Flow Cytometry

iPSCs at passage 38 were dissociated as single-cell using Accutase (Gibco). Cells were stained with FACS antibodies for 15 min at RT in the dark. Gallios Flow Cytometer (Beckman Coulter) with the appropriate laser and filters sets was used to ran samples. The positive population was gated using Kaluza Analysis Software (Beckman Coulter).

3.6. Immunohistochemistry for pluripotency and in vitro differentiation

iPSC at passage 38 were fixed with 4% PFA for 20 at RT, blocked and permeabilized with TBS + 0.5% Triton X-100 + 6% donkey serum at 1h at RT. Primary antibodies were incubated overnight in TBS + 0.1% Triton X-100 + 6% donkey serum at 4 °C, and secondary antibodies were incubated 2 h at 37 °C. Nuclei were stained using 4,6-diamino-2-fenilindol (DAPI).

To evaluate the differential potential of our iPSC lines, in vitro differentiation based on EB generation was performed at 37 °C, 5% CO2 and 20% O2. iPSC colonies at passage 35 were incubated with dispase and colonies manually lifted, and incubated in ultra-low attachment plates in human embryonic stem (hES; Knockout DMEM, 10% KSR, 1% P/S) media for 24 h. EBs were then cultured with specific mediums for 48 h and seeded on matrigel coated slideflasks for 15–20 days: Ectoderm medium containing 50% Neurobasal, 50% DMEM/F12, 1% N_2, 1% B27, 1% Glutamax and 1% Penicilin-Streptomycin; Endoderm containing Knockout-DMEM, 10% FBS (Hyclone), 1% NEAA, 0.1% β-mercaptoethanol, 1% Glutamax and 1% P/S; and Mesoderm as previously described (Castaño et al., 2021 Aug). Differentiated cells were analysed by immunocytochemistry or Flow cytometry as described above. Confocal images were taken using Leica TSC SPE or Leica SP5 microscopes.

3.7. Quantitative RT-PCR

mRNA was isolated from iPSCs at passage 39 using the standard Maxwell® RSC Instrument for automated RNA extraction and following manufacturer's protocol. SuperScript III (Invitrogen) was used to generate cDNA. For qRT-PCR, SYBR green (Life technologies) was used. Primer sequences are listed in Table 2. Ct values were normalized using GAPDH as housekeeping gene. Assays were run on the ABI PRISM 7900HT system (Applied Biosystems) and data were analysed with the 2-ΔCt method.

3.8. Sequencing

Genomic DNA was isolated using QIAamp DNA Mini Kit (QIAGEN) according to manufacturer's protocol. PCR was performed with the primers *GATA2-gDNA-PCR (*Table 2) with GoTaq Flexi DNA Polymerase (Promega) with the following protocol: 95 °C for 5 min, 35 cycles of 95 °C for 30s, 61 °C for 30s, 72 °C for 60s and 72 °C for 7min. PCR products were purified with DNA Clean & Concentrator-5 (Zymo Research) and sent for sequencing to Eurofins. Genetic alterations were identified using Benchling.

3.9. Mycoplasma

Supernatant of confluent iPSCs at passage 37 was harvested. Venor GeM Classic Mycoplasma Detection Kit (Minerva Biolabs) was used following manufacturer's protocol.

Author contributions

J.P. designed the study, carried out the experiments, analyzed the data and wrote the manuscript; J.C. designed the study; J.C. carried out the experiments; A.G. and D.R.M analyzed and discussed the data and wrote the manuscript; all authors approved the final version of the manuscript

5. Abnormal karyotype

The cell line presents a normal karyotype

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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