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Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of two heterozygous GATA2 CRISPR/Cas9-edited iPSC lines, R398W and R396Q, for modeling GATA2 deficiency

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ABSTRACT

Germline heterozygous GATA2 mutations underlie a complex disorder characterized by bone marrow failure, immunodeficiency and high risk to develop myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Our understanding about GATA2 deficiency is limited due to the lack of relevant disease models. Here we generated high quality human induced pluripotent stem cell (iPSC) lines carrying two of the most recurrent germline GATA2 mutations (R389W and R396Q) associated with MDS, using CRISPR/Cas9. These hiPSCs represent an *in vitro* model to study the molecular and cellular mechanisms underlying GATA2 deficiency, when differentiated into blood progenitors.

Resource Table

Unique stem cell lines identifier	1. *ESi086-A-1* 2. *ESi086-A-2*
Alternative name(s) of stem cell lines	1. *CBiPS8-3F-4 GATA2-R396Q-12* 2. *CBiPS8-3F-4 GATA2 R398W-8*
Institution	*Intitut d'Investigació Biomédica de Bellvitge (IDIBELL)*
Contact information of the reported cell line distributor	*Alessandra Giorgetti (agiorgetti@idibell.cat)*
Type of cell lines	*iPSC*
Origin	*Human*
Additional origin info *(applicable for human ESC or iPSC)*	*Age: 0* *Sex: male*
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*

(*continued on next column*)

(*continued*)

Cell culture system used	*Cells have been grown in Matrigel and mTeSR-1 (Stem Cell Technologies)*
Type of Genetic Modification	*Monoallelic point mutations in exon 6 of GATA2 gene*
Associated disease	*GATA2 deficiency (OMIM:137295)*
Gene/locus	*GATA2 (OMIM:137295; NM_001145661.2)*
Method of modification/ site-specific nuclease used	*CRISPR/Cas9*
Site-specific nuclease (SSN) delivery method	*RNP*
All genetic material introduced into the cells	*ssODN*
Analysis of the nuclease-targeted allele status	*RFLP + Sequencing of the targeted allele*
Method of the off-target nuclease activity surveillance	*N/A*
Name of transgene	*N/A* *N/A*

(*continued on next page*)

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(*continued*)

Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	
Inducible/constitutive system details	*N/A*
Date archived/stock date	*April 2021*
Cell line repository/bank	*Cell lines registered at Banco Nacional de Líneas Celulares at Instituto de Salud Carlos III (SPAIN)* *https://www.isciii.es/QueHacemos/Servicios/BIOBANCOS/BNLC/Paginas/LineasiPS.aspx* *Nomenclature:* - *CBiPS8-3F-4 GATA2-R396Q-12* - *CBiPS8-3F-4 GATA2 R398W-8*
Ethical/GMO work approvals	*Comisíon de Seguimiento y Control de Donación y Utilizacion de Células y Tejidos Humanos" (approval number 206 173 1, Title: Generacion y banqueo de celulas humanas pluripotentes inducidas (ips) a partir de celulas madre de cordon umbilical para la producción de células madre sanguíneas and approval number 525 428 1 Title: Human disease modelling of GATA2- related Myelodysplastic Syndromes and Acute Myeloid Leukemia)*
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	*N/A*

1. Manuscript section expected contents clarification

1.1. Resource utility

The iPSCs carrying heterozygous GATA2 mutations, R398W and R396Q, give an unprecedent opportunity to provide unlimited number of iPSC-derived blood progenitors *in vitro*, overcoming the restrictions in accessing primary patient samples. This human-based GATA2 deficiency model will allow a better understanding of how GATA2-germline mutations promotes susceptibility to myeloid neoplasia. Table 1.

2. Resource details

GATA2 deficiency belongs to a newly established World Health Organization (WHO) group of hereditary syndromes with predisposition to myeloid malignancies (Smith et al., 2004).

Individuals with germline heterozygous GATA2 mutations show a very complex and multisystemic phenotype with hematologic cytopenia leading to MDS, immunodeficiency involving B, NK, monocytic, CD4+, DC cell lineages, deafness and lymphedema(Hahn et al., 2011). Based on literature at least 75% of GATA2 mutation carriers develop MDS/AML at an estimated median age of 20 years (Wlodarski et al., 2016). Nowadays, chemotherapy and allogenic hematopoietic stem cell (HSC) transplantation remains the only treatment with favorable response. A mechanistic understanding of how GATA2 haploinsufficiency affects hematopoietic development is hindered by the lack of faithful disease model systems. Germline GATA2 mutations are either truncating loss-of-function (LOF) mutations, missense mutations in ZF2, or mutations disrupting Intron 4 enhancer site (Wlodarski et al., 2016). These mutations are thought to result in reduced/loss of GATA2 function, specifically abolishing the DNA-binding function of ZF2 (Chong et al., 2018). To date only few germline GATA2 mutations have been functionally studied. Therefore, using precise gene editing strategy we generated human induced pluripotent stem cells (hiPSCs) carrying two of the most recurrent germline GATA2 mutations associated with MDS.

CRISPR/Cas9 system was applied on healthy iPSC line (CBiPS8-3F-4), by using self-designed sgRNA targeting each mutation (R396Q and R398W). iPSCs were nucleofected with Cas9 protein and sgRNA together with an ssODN donor carrying the desired mutation. A 1187 G > A was introduced for the R396Q edited cell line, and 1198C > T was introduced for R398W edited cell line. After nucleofection single cell clones were expanded and screened by Restriction Fragment Length Polymorphism (RFLP) by digestion with XmnI. Positives clones were Sanger sequenced to verify the presence of the monoallelic mutations (Fig. 1A). One representative clone was selected for each mutation.

Both hiPSC lines maintained a normal 46XY karyotypes (Fig. 1B) and showed strong alkaline phosphatase (AP) activity (Fig. 1C). Immunofluorescence and flow cytometry analysis revealed expression of pluripotency markers such as NANOG, OCT4, SSEA4, TRA-1–81 and TRA-1–60 (Fig. 1C and D; scale bar = 100 μm). Quantitative RT-PCR showed that both iPSC lines expressed a set of pluripotency genes, including OCT4, SOX2, NANOG, and REX1, uncovering a gene-expression profile comparable to hESC line, (Fig. 1E). Both hiPSC lines were able to form embryoid bodies (EBs) with high efficiency, which could be differentiated into derivatives of the three embryonic germ layers, including TUJ1 and GFAP-positive ectoderm, α-fetoprotein (AFP) and FoxA2-positive endoderm, and CD45/CD34-positive mesoderm (Fig. 1F, scale bar = 25 μm). The iPSC line identity was confirmed by short tandem repeat analysis (STR) and compared with the original parental CBiPS8-3F-4 clone (Castano et al., 2019) (Supplementary Table S1). All cell lines were free from mycoplasma contamination as determined by PCR. (Supplementary Fig. S1)

3. Materials and methods

3.1. Human iPSC cultures

Human iPSC lines were 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 and 20% O_2. Culture medium was changed daily and cells will be passaged weekly by EDTA dissociation (EDTA 0.5 μM + PBS).

3.2. CRISPR/Cas9 gene editing

The CRISPR tool (https://bioinfogp.cnb.csic.es/tools/breakingcas) was used to design GATA2 sgRNA (Table 2). A guide sequence with a high probability to target the region of interest and low probability to generate off-targets was chosen. Rock inhibitor (Y-27632; 10uM) was added to the iPSCs 3 h before nucleofection. 100 pmol Alt-R® S.p. 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 72 h 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 analyze the gene mutation.

3.3. Karyotyping

Genomic integrity of the iPSC lines (passages 29 & 34) was evaluated by G-banded metaphase analysis with a resolution of 300–500 bands (Sant Joan de Déu, Barcelona). 70% confluent iPSC colonies were incubated with KaryoMax colcemid (Invitrogen), trypsinized, treated with hypotonic solution and fixed in Carnoy solution (75% methanol,

Table 1
Characterization and validation.

Classification (optional *italicized*)	Test	Result	Data
Morphology	Photography	*Typical embryonic stem cell-like morphology*	Fig. 1*C*
Pluripotency status evidence for the described cell line	Qualitative analysis *(Immunofluorescence*	*Immunostaining for pluripotency markers: OCT3/4 and NANOG*	Fig. 1*C*
	Quantitative analysis *(Flow cytometry and qPCR)*	*Flow cytometry: Expression of SSEA-4, TRA-1–60 and TRA-1–81* *R396Q* *SSEA-4: 100%TRA-1–60: 98%TRA-1–81: 63%* *R398W* *SSEA-4: 100%* *TRA-1–60: 80%* *TRA-1–81: 95%* *qPCR: Expression of OCT4, SOX2, NANOG and REX1*	Fig. 1*D and E*
Karyotype	Karyotype (G-banding*)*	*R396Q* *46, XY* *R398W* *46, XY*	Figure 1*B*
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR and sequencing of the amplicon surrounding point mutations	*Point mutations found in heterozygosis*	Fig. 1*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	STR analysis	*STR analysis of TH01, D21S11, D5S818, D13S317, D7S820, D16S539, CSP1PO, AMEL, vWa, TPOX. Both edited iPSCs lines match to the individual host profile*	*Supplementary file 2, submitted in the archive with journal*
Mutagenesis / genetic modification outcome analysis	PCR and sequencing of the amplicon surrounding point mutations	*Point mutations found in heterozygosis*	Fig. 1*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	*No analysis was performed due to low probability in intergenic regions*	*N/A*
Specific pathogen-free status	Mycoplasma	*Mycoplasma testing by RT-PCR . Both clones are negative*	Supplementary Fig. 1
Multilineage differentiation potential	Embryoid body formation	*Both iPSC line were differentiated into cells of mesoderm (CD43 and CD45), endoderm (FOXA2 and AFP) and ectoderm GFAP and TUJ1)*	Fig. 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

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) until colonies turned blue.

3.5. Flow cytometry

iPSCs at passage 33 were dissociated as singles cells using Accutase (Gibco). Cells were stained with FACS antibodies (Table 2) for 15 min at RT in the dark. Gallios Flow Cytometer (Beckman Coulter) with the appopiate laser and filters sets was used to ran samples. The positive population was gated using Kaluza Analysis Software (Beckman Coulter).

3.6. Immunocytochemistry for pluripotency and in vitro differentiation

iPSCs at passage 34 were fixed with 4% PFA for 20 min at RT, blocked and permeabilized with TBS + 0.5% Triton X-100 + 6% donkey serum for 1 h 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 (Table 2). 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% N2, 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 decribed (Castano et al. 2019). 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 33 using the standard RNAeasy MiniKit (QIAGEN) following manufacturer's protocol. 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 analyzed 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; 95C for 5 min, 35 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 60 s and 72 °C for 7 min. PCR products

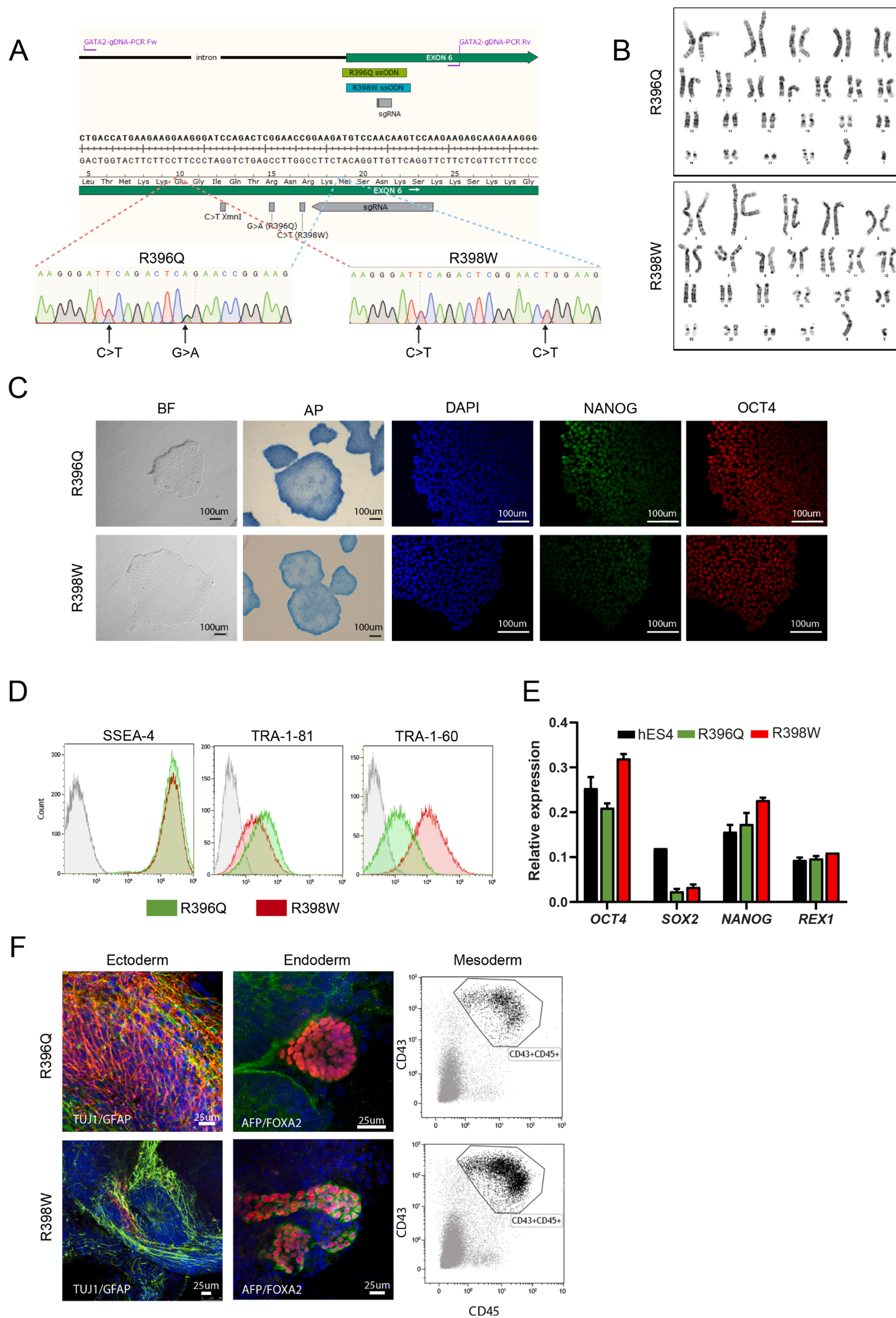

Figure 1: Generation and characterization of GATA2 (R396Q & R398W) mutated iPSC lines

Fig. 1. .

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	*Mouse anti-OCT4 Goat anti-NANOG AF-647 anti-SSEA-4 PE anti-TRA-1–60 AF-647 anti-TRA-1–81*	*1:2 1:5 1:40 1:20 1:20*	*Santa Cruz Biotechnology, SC-5279; RRID: AB_628051 R&D; AF1997; RRID: AB_355097 BD Bioscience; 560219; RRID:AB_1645442 BD Bioscience; 560850; RRID: AB_10565983 Invitrogen; 12–8883-82; RRID: AB_891606*
Differentiation Markers	*Rabbit anti-Alpha-1-fetoprotein Goat anti-FOXA2 Mouse anti-TUJ1 Rabbit anti-GFAP APC-H7 anti-CD45 APC anti-CD43*	*1:200 1:50 1:500 1:500 1:40 1:100*	*DAKO; A0008; RRID: AB_2650473 R&D; AF2400; RRID:AB_2845414 Covance; MMS-435P; RRID: AB_2313773 DAKO; Z0334; RRID:AB_10013382 BD Bioscience; 560178; RRID: AB_1645479 BD Bioscience; 560198; RRID:AB_1645460*
Secondary antibodies	*Cy3 Donkey anti-Mouse IgG AF488 Donkey anti-Goat IgG AF488 Donkey anti Rabbit IgG Cy3 Donkey anti-Goat IgG AF488 Donkey anti-Mouse IgG Cy3 Donkey anti-Rabbit IgG*	*1:200 1:200 1:200 1:200 1:200 1:200*	*Jackson Immuno Research; 715–165-151; RRID: AB_2315777 Jackson Immuno Research; 705–545-147; RRID:AB_2336933 Jackson Immuno Research; 711–545-152; RRID:AB_2313584 Jackson Immuno Research; 705–165-147; RRID: AB_2307351 Jackson Immuno Research; 715–545-151; RRID:AB_2341099 Jackson Immuno Research; 711–165-152; RRID:AB_2307443*
Nuclear stain	*DAPI*	*1:10000*	*Thermo Fisher Scientific*
Site-specific nuclease			
Nuclease information	*Alt-R® S.p. Cas9 Nuclease V3*	*IDT, Cat# 1,081,059*	
Delivery method	Nucleofection	*Lonza; 4D-NucleofectorTM Core Unit + X Unit (AAF-1002B + AAF-1002X)*	
Selection/enrichment strategy	N/A	*N/A*	

Primers and Oligonucleotides used in this study

	Target	Forward/Reverse primer (5′-3′)
GATA2-gDNA-PCR	*GATA2 (585 bp)*	*CTTGGCTTGGCTTGGGAAG / ACAGGTGCCATGTGTCCAG*
GATA2-R396Q-ssODN	*GATA2*	*CTCTAGGTTAACAGGCCACTGACCATGAAGAAGGAAGGGATTCAGACTCAGAACCGGAAGATGTCCAACAAGTCCAAGAAGAGCAAGAAAGGGGCGGAGT*
GATA2-R398W-ssODN	*GATA2*	*GTTAACAGGCCACTGACCATGAAGAAGGAAGGGATTCAGACTCGGAACTGGAAGATGTCCAACAAGTCCAAGAAGAGCAAGAAAGGGGCGGAGTGCTTCG*
Alt-R® CRISPR-Cas9 sgRNA	*GATA2*	*TGGACTTGTTGGACATCTTC*
Pluripotency Markers (qPCR)	*OCT4 (64 bp) SOX2 (72 bp) NANOG (111 bp) REX1 (61 bp)*	*GGAGGAAGCTGACAACAATGAAA/ GGCCTGCACGAGGGTTT TGCGAGCGCTGCACAT/ TCATGAGCGTCTTGGTTTTCC ACAACTGGCCGAAGAATAGCA/ GGTTCCCAGTCGGGTTCAC CCTGCAGGCGGAAATAGAAC/ GCACACATAGCCATCACATAAGG*
House-Keeping Gene (qPCR)	*GAPDH (75 bp)*	*GCACCGTCAAGGCTGAGAAC/ AGGGATCTCGCTCCTGGAA*
Genomic target sequence(s)	*GATA2 exon 6*	*Homo sapiens chromosome 3, GRCh38.p13 128481318–128481019*

were purified with DNA Clean & Conentratior-5 (Zymo Research) and sent for sequencing to Eurofins. Genetic alteratios were identified using Snap Gene v5.0.6

3.9. Mycoplasma test

Supernatand of confluent iPSCs at passage 33 was harvested. Venor GeM Classic Mycoplasma Detection Kit (Minerva Biolabs) was used following manufacturer's protocol.

Author contributions

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

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102445.

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