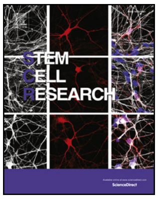
Stem Cell Research 41 (2019) 101603

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr



Lab resource: Stem Cell Line

GENYOi005-A: An induced pluripotent stem cells (iPSCs) line generated from a patient with Familial Platelet Disorder with associated Myeloid Malignancy (FPDMM) carrying a p.Thr196Ala variant

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ABSTRACT

Familial Platelet Disorder with associated Myeloid Malignancy (FPDMM) is a rare platelet disorder caused by mutations in *RUNX1*. We generated an iPSC line (GENYOi005-A) from a FPDMM patient with a non-previously reported variant p.Thr196Ala. Non-integrative Sendai viruses expressing the Yamanaka reprogramming factors were used to reprogram peripheral blood mononuclear cells from this FPDMM patient. Characterization of GENYOi005-A included genetic analysis of *RUNX1* locus, Short Tandem Repeats profiling, alkaline phosphatase enzymatic activity, expression of pluripotency-associated factors and differentiation studies *in vitro* and *in vivo*. This iPSC line will provide a powerful tool to study developmental alterations of FPDMM patients.

Resource table

Unique stem cell line identifier	*GENYOi005-A*
Alternative name(s) of stem cell line	*FPD/AML-PBMC-iPSC4F73*
Institution	*Gene Regulation, Stem Cells and Development Group, GENYO: Centre for Genomics and Oncological Research Pfizer-University of Granada-Junta de Andalucía, PTS, Granada 18016, Spain;*
Contact information of distributor	Pedro J. Real: pedro.real@genyo.es Jose M. Bastida: jmbastida@saludcastillayleon.es
Type of cell line	*iPSC*
Origin	*Human*
Additional origin info	*Age: 54* *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	*Familial platelet disorder with associated myeloid malignancy (FPDMM)*
Gene/locus	*p.Thr196Ala variant in RUNX1*
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*25/05/2018*
Cell line repository/bank	*hpscreg.eu/user/cellline/edit/GENYOi005-A*
Ethical approval	*Comisión de Garantías para la Donación y Utilización de Células y Tejidos Humanos. Junta de Andalucia. RC/003/2013 & RC/004/2013*

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https://doi.org/10.1016/j.scr.2019.101603
Received 15 July 2019; Received in revised form 23 September 2019; Accepted 24 September 2019

1. Resource utility

GENYOi005-A is an iPSC line generated from a Familial platelet disorder with associated myeloid malignancy (FPDMM) patient with this new variant p.Thr196Ala in *RUNX1*. This cell line will be a very useful tool to understand hematopoiesis development and leukemogenesis of this *RUNX1* variant.

2. Resource details

Familial platelet disorder with associated myeloid malignancy (FPDMM) is an autosomal dominant disease of the hematopoietic system caused by heterozygous mutations in *RUNX1* (OMIM#601399). FPDMM is characterized by thrombocytopenia, abnormal platelet function and an increased risk of developing other blood disorders or cancers such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Sakurai et al., 2016). iPSCs lines from this pathology have been previously obtained (Sakurai et al., 2014). In this study, we report the generation of a new cellular line from peripheral blood mononuclear cells (PBMCs) obtained from a FPDMM patient carrying a non-previously reported variant (p.Thr196Ala) in the *RUNX1* locus, which was identified by our next-generation sequencing panel (Bastida et al., 2018).

CytoTune iPS 2.0 Sendai Reprogramming kit (ThermoFisher Scientific) was used to reprogram PBMCs from the FDPMM patient. PBMCs were transduced with Sendai virus (SeV) vectors expressing and delivering the reprogramming factors OCT3/4, SOX2, KLF4 and c-MYC. 2–3 weeks after viral exposure several clones were selected and characterized as described below.

Firstly, the presence of the variant p.Thr196Ala in the exon 6 of the *RUNX1* locus in the GENYOi005-A cell line was confirmed by Sanger sequencing**.** As shown in Fig. 1A, PBMCs from the patient (FPD-PBMCs) and GENYOi005-A cells share the same heterozygous variant. Short Tandem Repeat polymorphism (STR) analysis confirmed the same genetic identity between both samples. GENYOi005-A 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). Importantly, GENYOi005-A cells showed normal karyotype (46, XX) (Fig. 1D) and alkaline phosphatase activity (Fig. 1E). In addition, expression of the pluripotent markers SSEA3, SSEA4, Tra1-60, Tra1-81 and Oct3/4 was confirmed by flow cytometry analysis (Fig. 1F). Moreover, mycoplasma analysis was negative for GENYOi005-A cell line.

Finally, to demonstrate the capacity of GENYOi005-A to differentiate into the three germ layers *in vitro* and *in vivo*, embryoid bodies (EBs) formation assays (Fig. 1G) and teratoma formation assays were accomplished (Fig. 1H). EBs and teratomas derived from this cell line showed specific structures from endoderm (ciliated epithelium), mesoderm (cartilage) and ectoderm (neural rosettes). Besides, we also detected the expression of representative markers of the three germ layers: ectoderm (β3-Tubulin), mesoderm (Vimentin) and endoderm (Cytokeratin CKAE1-AE) (Table 1).

3. Materials and methods

3.1. Reprogramming of GENYOi005-A line

The peripheral blood sample was obtained from a woman with FPDMM after informed consent according to the Local Ethical Committee of the Instituto de Investigación Biomédica (IBSAL, Salamanca, Spain), the Andalusian Ethics Review Board for Cellular Reprogramming requirements and with Spanish and EU legislation. PBMCs from the patient were isolated by centrifugation using Ficoll Paque™ PLUS (GE Healthcare) and cultured for four days in StemSpan™ SFEM (StemCell Technologies) supplemented with hSCF, hFLT3L, hIL6 and hIL3 (Peprotech). Then, 3 million PBMCs were transduced with Sendai virus (SeV) using CytoTune®-iPS 2.0 Reprogramming kit (Life Technologies, Invitrogen) and plated over 12-well fibronectin-coated plate (BD BioCoat™) as formerly described (Lopez-Onieva et al., 2016). Four weeks after reprogramming, GENYOi005-A cells were accommodated to grow in Essential 8 medium (E8) on Matrigel (BD Bioscience). GENYOi005-A cells were split weekly at a ratio of 1:8 using PBS/EDTA (0.5 mM) and cultured at 37 °C, 5% CO_2.

3.2. Mutational analysis

Genomic DNA was isolated from PBMCs of the FPDMM patient, GENYOi005-A line and a healthy control iPSC line (PBMC1-iPS4F1) using the DNA extraction kit (Qiagen). PCR amplification was performed in all samples using a set of primers that recognizes *RUNX1* exon 6 (Set RUNX1, Table 2) following the manufacturer's instructions in a SureCycler 8800 thermal cycler (Agilent). Amplified fragments were run and sequenced using RUNX1 Forward primer, in an ABI 3130 genetic analyzer (Applied Biosystems, Life Technologies).

3.3. Short Tandem Repeat polymorphism (STR) profiling

The genetic signature of FPDMM-PBMCs and GENYOi005-A cell line was determined as previously described (Lopez-Onieva et al., 2016). This information is available with the authors.

3.4. RT-PCR analysis

Total RNA from GENYOi005-A cell line, the control PBMC1-iPS4F1line and PBMCs from FPDMM patient was isolated with the High pure RNA isolation kit (Roche) and cDNA was produced with the Transcription First Strand c-DNA synthesis kit (Roche) according to the manufacturer's instructions. PCR was performed using GoTaq Flexi DNA Polimerase kit (Promega) in a SureCycler 8800 thermal cycler (Agilent). PCR fragments were visualized in an agarose gel. SeV*, KLF4, c-MYC* and *KOS* primer sets were used to analyse the presence of exogenous genes and *Oct3/4, SOX2, NANOG, REX1* and *β-ACTIN* primer sets used to verify the expression of pluripotency markers as previously described (Montes et al., 2019). FPDMM-PBMCs transduced cells at day 4 subsequent to SeV exposure was used as a positive control and PBMC1-iPS4F1line as a negative control for exogenous reprogramming factors. For endogenous pluripotent transcription factors, PBMC1-iPS4F1 iPSC line was used as a positive control and FPDMM-PBMCs as a negative control. Primer sequences used are shown in Table 2.

3.5. Karyotyping

Chromosomal analysis of GENYOi005-A line at passage 13 was accomplished by GTG-banding analysis at the Andalusian Public Health System Biobank (Spain). 20 metaphases were evaluated following the International System Cytogenetics Nomenclature recommendations.

3.6. Alkaline phosphatase

After five days in culture on a 24-well plate (Corning), GENYOi005-A colonies were assayed for phosphatase alkaline enzymatic activity using Alkaline Phosphatase detection kit (Merck-Millipore) following manufacturer's instructions.

3.7. Flow cytometry analysis

GENYOi005-A cell line colonies were dissociated with Tryple Express (Life Technologies) and cell suspension was stained with SSEA3 (PE, BioScience), SSEA4 (Alexa Fluor® 647, BD Pharmingen), Tra1-60 (PE, BioScience) and Tra1-81 (Alexa Fluor® 647, BD Pharmingen) antibodies for 20 min. Intracellular staining for Oct3/4 was performed by

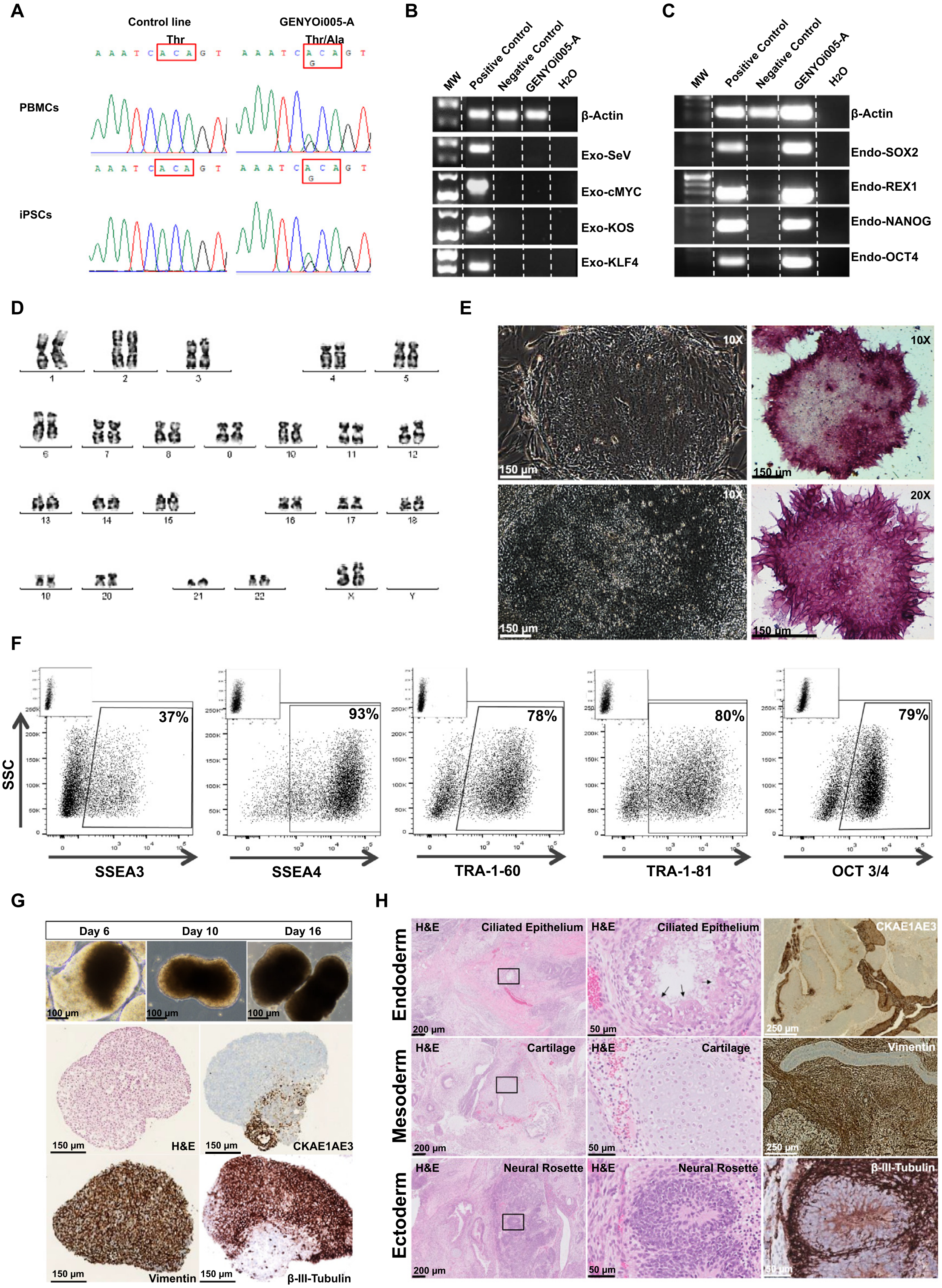

Fig. 1. Characterization of GENYOi005-A cell line (A) Sequence analysis of the variant p.Thr196Ala in exon 6 of the *RUNX1* from healthy control PBMCs and iPSC line (PBMC1-iPS4F1) (left panels) and FPDMM patient PBMCs and GENYOi005-A cell line (right panels). (B) RT-PCR analysis confirmed the silencing of exogenous reprogramming factors and SeV vector. PBMCs transduced cells from the patient at day 4 after Sendai virus exposure were used as a Positive Control. Non-transduced PBMCs from the patient were used as a Negative Control. (C) Expression of the endogenous pluripotent transcription factors SOX2, REX1, NANOG and OCT 3 was assessed by RT-PCR. PBMC1-iPS4F1 cell line was used as Positive Control. (D) GTG-banding shows a normal karyotype in GENYOi005-A cell line. (E) Representative colonies of GENYOi005-A cell line growing in E8 medium (left pictures). Alkaline phosphatase enzymatic activity staining in GENYOi005-A colonies (right pictures) (Scale bar = 150 μm). (F) Protein expression of pluripotency-associated markers SSEA3, SSEA4, TRA1-81, TRA 1-60 and OCT3/4 by FACS analysis. The inset shows the staining of the isotype-matched antibody. (G) Embryo body (EB) differentiation assay for pluripotency. Immunohistochemistry analysis for endoderm (CKAE1-AE3), mesoderm (Vimentin) and ectoderm (β-III-Tubulin) from 3-week EBs. (Scale bar = 150 μm) (H) *In vivo* differentiation test by teratoma formation assay. Histological sections from 10-week teratomas developed in the dorsal flanks of NOD/LtSz-scid interleukin-2Rγ-/- mice after injecting 2 million GENYOi005-A cells. Hematoxylin and eosin (H&E) staining allowed us to identify several morphological structures characteristics for each germ layer: Ciliated Epithelium (Endoderm), Cartilage (Mesoderm) and Neural Rosette (Ectoderm). In the right panels, immunohistochemistry analysis confirmed differentiation to endoderm (CK-AE1-AE3), mesoderm (Vimentin) and ectoderm (GFAP). (Scales bar from 50–250 μm).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*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: 79%* *Tra1-60: 78%* *Tra1-81: 80%* *SSEA-4: 93%* *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)	Sequencing	*Heterozygous mutation*	Fig. 1 *panel A*
	Southern Blot OR WGS		
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	*N/A*	

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*
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: exon specific PCR	*RUNX1*	*Forward: GGCCACCAACCTCATTCTGT* *Reverse:ACTTTTTGGCTTTACGGGGG*	
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*	

several incubations of 15 min using fixation and permeabilization solutions (A and B Fix and Perm Solutions (BD BioScience)). Then, GENYOi005-A cells were incubated with Oct3/4 (BD BioScience) primary antibody, and subsequently with FITC-conjugated secondary antibody (BD BioScience). An isotype-match antibody was used as a negative control. Viable cells were identified with 7-aminoactinomycin D staining and were analyzed in the BD FACSVerse™ flow cytometer (BD Bioscience) using BD FACSuite™ software (BD Bioscience) for acquisition and FlowJo™ for analysis (FlowJo, LLC-BD Bioscience).

3.8. *Embryo body (EB) differentiation assay*

GENYOi005-A colonies were gently scraped off the flask after treatment with collagenase IV (Invitrogen), re-suspended into Essential 6 medium (E6) after centrifugation, transferred into low attachment 6-well plates (Corning) and cultured in an incubator at 37 °C, 5% CO_2. After 16 days, EBs were collected, fixed, embedded in paraffin and sectioned for histological and immunocytochemistry analysis. Sectioned EBs were stained with hematoxylin and eosin (H&E) for histological analysis. Immunocytochemistry analysis was performed in the Pathology Department of the University Hospitals of Granada. Briefly, antigen retrieval was done by boiling sections for 20 min at 95 °C in citrate buffer (pH = 6) in a PT-Module (ThermoScientific). The Master Polymer Plus Detection System (Peroxidase) (Master Diagnostica) was used as a visualization system following manufacturer's instructions, in an Autostainer 480 S-2D automatic immunostainer (ThermoScientific). Diaminobenzidine (DAB) was used as the chromogen. Primary antibodies for β-III-Tubulin (ectoderm), Vimentin (mesoderm) and CKAE1-EA3 (endoderm) are included in Table 2.

3.9. *In vivo teratoma formation*

GENYOi005-A cells were dissociated with collagenase IV (Invitrogen) and resuspended in PBS with 30% Matrigel. 2 million cells were subcutaneously injected into the dorsal flanks of NOD/LtSz-scid interleukin-2R$\gamma^{-/-}$ mice (The Jackson Laboratory). At week 10 post-implantation, teratomas were collected, fixed in formaldehyde, embedded in paraffin, sectioned and stained with H&E for histological analysis. Immunocytochemistry analysis for CKAE1-AE3 (endoderm), Vimentin (mesoderm) and β-III-Tubulin (ectoderm) was performed on sectioned slides as described above.

3.10. *Mycoplasma analysis*

Mycoplasma test for GENYOi005-A cell line was performed by quantitative PCR analysis (Venor GeM-qEP, Minerva Biolabs) at the Genomics and Genotyping Unit in GENyO, Spain.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

This work was supported by the Ramon y Cajal (RYC-2015-18382) to PJR founded by the Ministry of Economy and Competitiveness; the Instituto de Salud Carlos III-FEDER (CP12/03175 and CPII17/00032) to V.R-M., (PI17/01311) to M.L.L and J.R., (PI17/01966; Fundación Mutua Madrileña AP172142019; Premio Lopez Borrasca SETH 2019; GRS2061/A/19) to J.M.B. and (CPII15/00018 and PI16/01340) to PJR; by the Chair "Doctors Galera-Requena in cancer stem cell research" (CMC-CTS963) to J.A.M. and C.G-L. GM-N is supported by the predoctoral program from Instituto de Salud Carlos III (F17/00178) and the Research Initiation Grants for Official Master Students program from the University of Granada (2017). ML, IS and GM-N are PhD students from the Doctoral Programme in Biomedicine from University of Granada. IS is supported by the Young Researcher program from University of Granada (Joven Personal Investigador-Fondo Social Europeo. Universidad de Granada (2018-19)). L.L-O. is supported by PhD Reincorporation Grants from University of Granada (2017) (Programa de Incorporación de Doctores. Plan Propio. Universidad de Granada).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101603.

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