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Stem Cell Research 16 (2016) 692–695

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Stem Cell Line

Generation of induced pluripotent stem cells (iPSCs) from a Bernard–Soulier syndrome patient carrying a W71R mutation in the *GPIX* gene

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ARTICLE INFO

Article history:
Received 12 April 2016
Accepted 13 April 2016
Available online 15 April 2016

ABSTRACT

We generated an induced pluripotent stem cell (iPSC) line from a Bernard–Soulier Syndrome (BSS) patient carrying the mutation p.Trp71Arg in the *GPIX* locus (BSS1-PBMC-iPS4F4). Peripheral blood mononuclear cells (PBMCs) were reprogrammed using heat sensitive non-integrative Sendai viruses containing the reprogramming factors Oct3/4, SOX2, KLF4 and c-MYC. Successful silencing of the exogenous reprogramming factors was checked by RT-PCR. Characterization of BSS1-PBMC-iPS4F4 included mutation analysis of *GPIX* locus, Short Tandem Repeats (STR) profiling, alkaline phosphatase enzymatic activity, analysis of conventional pluripotency-associated factors at mRNA and protein level and *in vivo* differentiation studies. BSS1-PBMC-iPS4F4 will provide a powerful tool to study BSS.

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Resource table.

Name of stem cell line	BSS1-PBMC-iPS4F4
Institution	Gene regulation, Stem Cells and Development Group, GENYO: Centre for Genomics and Oncological Research Pfizer-Universidad de Granada-Junta de Andalucía, PTS Granada
Person who created resource	Lourdes Lopez-Onieva, Pedro J. Real
Contact person and email	Lourdes Lopez-Onieva, lourdes.lopez@genyo.es; Pedro J. Real, pedro.real@genyo.es
Date archived/stock date	September 2015
Origin	Human peripheral blood mononuclear cells
Type of resource	Induced pluripotent stem cell (iPS) line
Sub-type	Cell line
Key transcription factors	Oct3/4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed
Link to related literature	Not available
Information in public databases	Not available
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Resource details

Bernard Soulier syndrome (BSS) is an inherited autosomal recessive rare platelet disorder caused by mutations in the genes coding for the membrane glycoprotein complex GPIb-IX-V (Berndt and Andrews, 2011). In this study we generated an iPSC line from PBMCs of a BSS patient containing the mutation p.Trp71Arg in the *GPIX* gene (PBMCs-BSS1) (Sánchez-Guiu et al., 2014). This new iPSC line was named BSS1-PBMC-iPS4F4.

We used CytoTune iPS 2.0 Reprograming System (Life Technologies, Invitrogen) that contains the vectors used for delivering and expressing the reprogramming factors Oct3/4, Sox2, Klf4 and c-Myc to reprogram PBMCs-BSS1 (Takahashi et al., 2007; Yu et al., 2007). Sequencing analysis of the *GPIX* locus confirmed the presence of c. 259 T > C change in exon 3 of the *GPIX* gene corresponding to a homozygous p.W71R mutation, identical to PBMCs-BSS1 (Fig. 1A). Additionally, Short Tandem Repeat (STR) profiling confirmed same genetic identity between both samples (Table 1). This iPSC line silenced the expression of exogenous reprogramming transgenes after 8 passages (Fig. 1B) and showed normal karyotype (46, XX) (Fig. 1C). BSS1-PBMC-iPS4F4 colonies displayed typical round shape morphology and they were positive for alkaline phosphatase activity (Fig. 1D).

BSS1-PBMC-iPS4F4 expressed the endogenous pluripotent transcription factors *OCT3/4*, *SOX2*, *REX1* and *NANOG*, assessed by RT-PCR (Fig. 2A) and the protein pluripotent markers SSEA3, SSEA4, Tra1–60,

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http://dx.doi.org/10.1016/j.scr.2016.04.013
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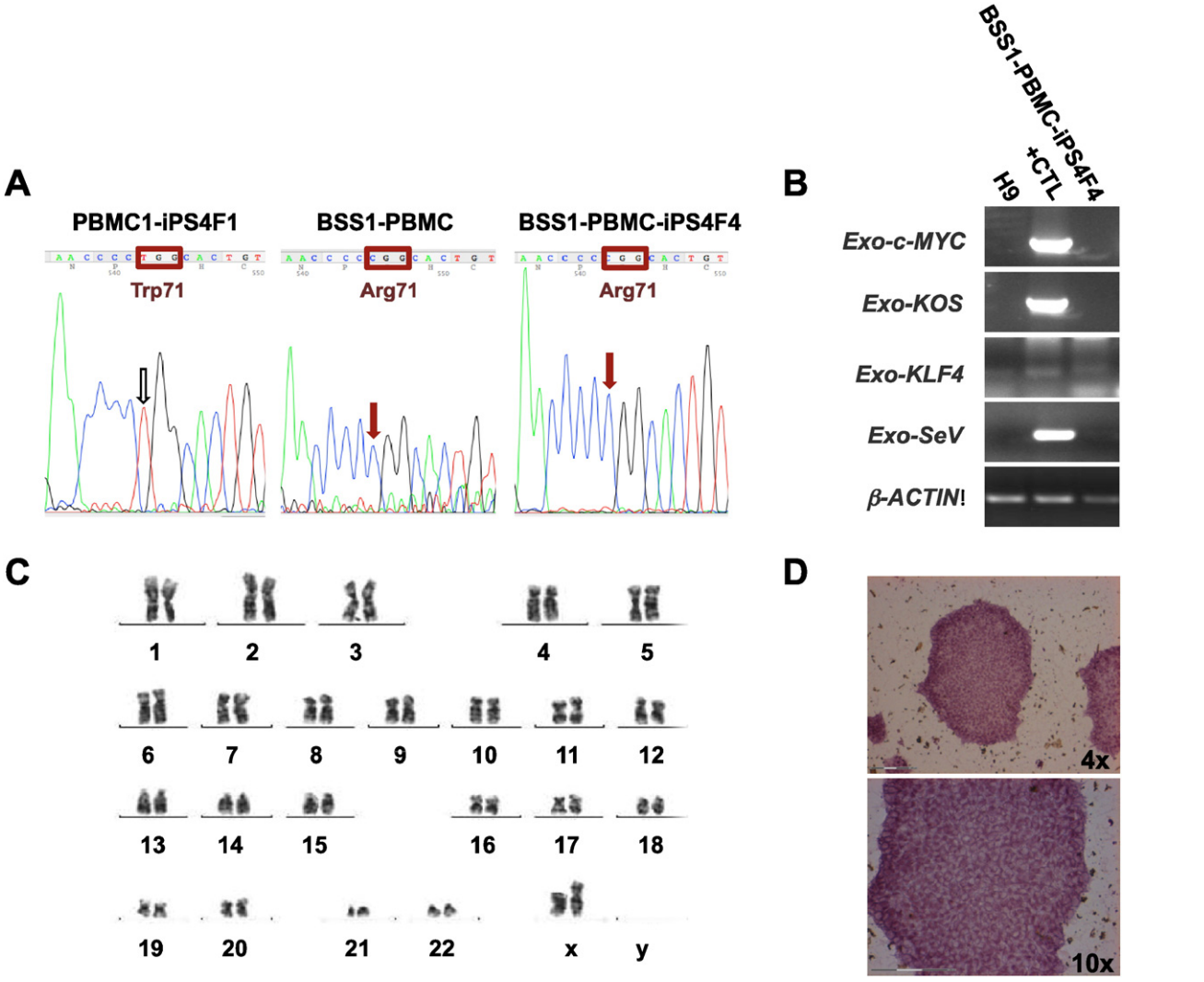

Fig. 1. Characterization of BSS1-PBMC-iPS4F4 cell line. (A) Sequence analysis of c. 259 T > C change in exon 3 of the *GPIX* gene from control iPSC line (PBMC1-iPS4F1) (left panel), BSS1-PBMC (middle panel) and BSS1-PBMC-iPS4F4 (right panel). (B) Silencing of exogenous reprogramming factors and SeV vector confirmed by RT-PCR. H9 cell line was used as a negative control and BSS1-PBMC-iPS4F4 at passage 2 was used as a positive control. (C) GTG-banding shows a normal karyotype in BSS1-PBMC-iPS4F4 cell line. (D) Alkaline phosphatase enzymatic activity staining.

Tra1–81 and Oct3/4, evaluated by flow cytometry analysis (Fig. 2B). To demonstrate the capacity of BSS1-PBMC-iPS4F4 to differentiate into the three germ layers *in vivo*, teratoma formation assays were accomplished (6). As shown in Fig. 2C, teratomas derived from this line showed expression of representative markers of ectoderm (β3-Tubulin), mesoderm (Vimentin) and endoderm (Cytokeratin CK AE1–AE) (Fig. 2C).

Table 1
Short Tandem Repeat (STR) profiling of the original patient cells (BSS1-PBMC) and the iPSC patient-derived cells (BSS1-PBMC-iPS4F4).

Allele	Profile	
	BSS1-PBMC	BSS1-PBMC-iPS4F4
Amelogenin	X	X
CSF1PO	10	10
D3S1358	14, 15	14, 15
D5S818	11, 12	11, 12
D7S820	9, 10	9, 10
D8S1179	11, 16	11, 16
D13S317	8, 11	8, 11
D16S539	1, 12	1, 12
D18S51	13, 14	13, 14
D19S433	13, 14	13, 14
D21S11	30	30
FGA	19, 23	19, 23
TH01	8, 9.3	8, 9.3
TPOX	8, 9	8, 9
vWA	17, 18	17, 18

Materials and methods

Generation of BSS1-PBMC-iPS4 line from PBMCs-BSS1

Peripheral blood sample was obtained from a woman with BSS after informed consent according with the Andalusian Ethics Review Board for Cellular Reprogramming requirements and with Spanish and EU legislation. BSS1-PBMCs were isolated by centrifugation using Ficoll Paque™ PLUS (GE Healthcare). Isolated BSS1-PBMCs were cultured in StemSpan™ SFEM (StemCell Technologies) supplemented with 100 ng/ml hSCF, 100 ng/ml hFLT3L, 20 ng/ml hTPO, 10 ng/ml G-CSF and 2 ng/ml hIL3 (Peprotech) for four days. Then, mononuclear cells were transferred to a 12-well fibronectin coated plate (BD BioCoat™) and Sendai virus (SeV) (CytoTune®-iPS 2.0 Reprogramming kit, Life Technologies, Invitrogen) were added at a multiplicity of infection of three (MOI:3) in the presence of 8 μg/ml Polybrene (Sigma-Aldrich) and 10 μM Y-27,632 (Sigma-Aldrich). Three days after transduction, cells were co-cultured in StemSpan™ SFEM (StemCell Technologies)

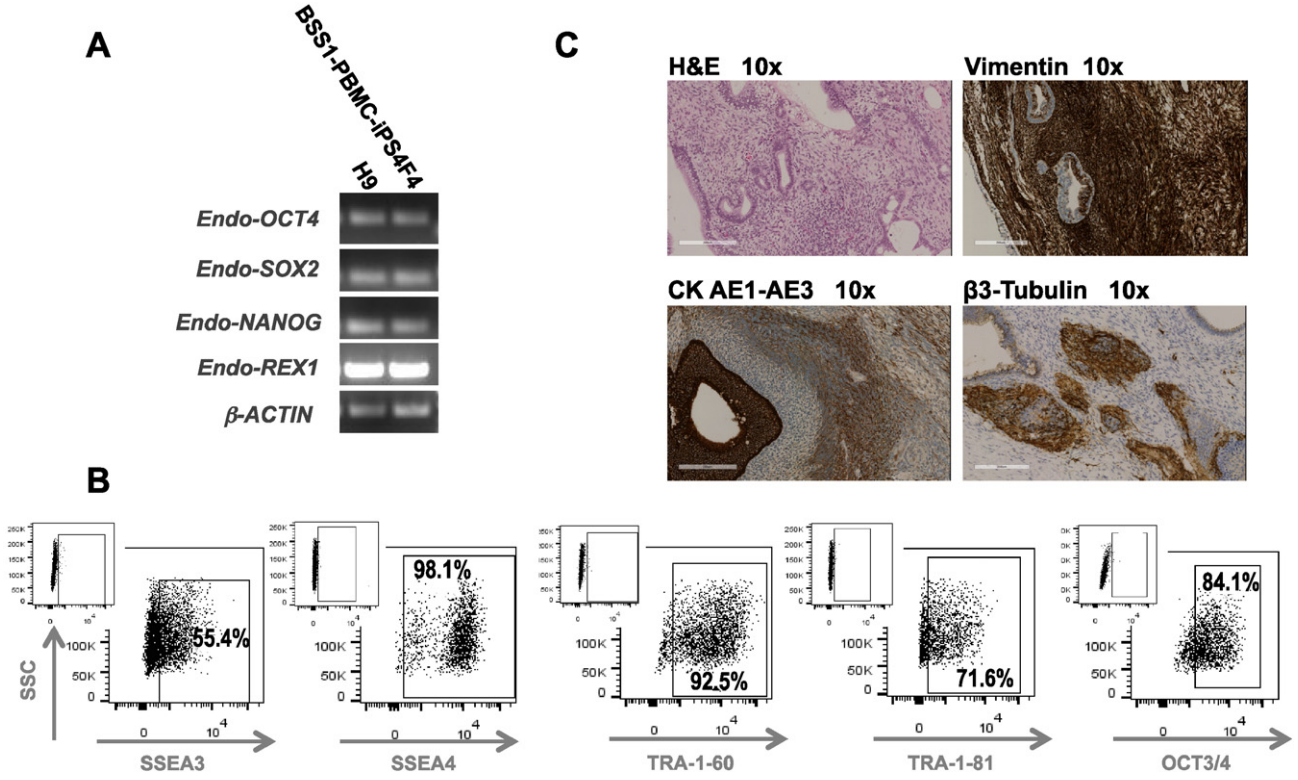

Fig. 2. Pluripotency markers and *in vivo* differentiation capacity of BSS1-PBMC-iPS4F4 line. (A) Endogenous pluripotency markers *OCT4*, *SOX2*, *REX1* y *NANOG* were confirmed by RT-PCR. (B) Expression of pluripotency-associated markers SSEA3, SSEA4, TRA1–81, TRA 1–60 and OCT3/4 at protein level by FACS analysis. The inset shows the staining using the corresponding irrelevant isotype-matched antibody. (C) *In vivo* differentiation test by teratoma formation assay. Histological sections from 13 week-teratomas developed in the dorsal flanks of SCID mice following injection with BSS1-PBMC-iPS4F4. Hematoxylin and eosin (H&E) staining reveals characteristic tissues from the three germ layers (left panel); immunohistochemistry analysis showed differentiation to mesoderm (Vimentin), endoderm (CK AE1–AE3) and ectoderm (β3-Tubulin).

in absence of cytokines on a layer of irradiated human mesenchymal stem cells (hMSCs, Inbiobank). Six days after transduction cells were adapted to pluripotent stem cell (PSC) medium consisting in KO-DMEM (Life Technologies) supplemented with 10% knockout serum replacement (Life Technologies), 8 ng/ml bFGF2 (Milteny Biotec), 10 μM Y-27632 (Sigma-Aldrich), 1 mM glutamine, 1% MEM non-essential amino acids and 0.1 mM 2-mercaptoethanol (all from Life Technologies). From day seven onwards cells were cultured in PSC medium. On day 12 after transduction emerging iPSC colonies were picked individually and expanded for characterization. From day 3 to day 21 after transduction, cells were cultured in a dedicated 37 °C chamber with 5% CO_2 and 5% O_2.

Mutational analysis

Genomic DNA was isolated from PBMC of BSS1 patient (BSS1-PBMCs), BSS1-PBMC-iPS4 line and a healthy control iPSC line (PBMC1-iPS4F1 line (Montes et al., 2015)) using the DNA extraction kit (Qiagen). PCR amplification with a set of primers flanking the mutation site (Set GPIX-4P, see Table 2) was performed in all three samples following the manufacturer's instructions. PCR products were sequenced using primer GPIX-4P Forward by Sanger sequencing on an ABI 3130 Genetic Analyzer.

Short tandem repeat (STR) profiling

To verify the origin of the new iPSC line generated, DNA from peripheral blood mononuclear cells from the BSS1 patient (BSS1-PBMC) and BSS1-PBMC-iPS4F4 were purified. AmpFlSTR® Idenfifiler® PCR Amplification kit (Applied Biosystems, Life Technologies) was used to determine the genetic signature of both samples based on the multiplex analysis of 15 loci and the Amelogenin gender-determining marker. PCR products were run in the ABI3130 genetic analyzer (Applied Biosystems, Life Technologies) and analyzed using the GeneMapper® ID Software (Applied Biosystems, Life Technologies) following the manufacturer's recommendations. As shown in Table 1, BSS1-PBMC-iPS4F4 conserves identical STR profile than the original BSS1-PBMC.

Semiquantitative RT-PCR

Total RNA from undifferentiated BSS1-PBMC-iPS4 and H9 (Thomson et al., 1998) lines was isolated using the High pure RNA isolation kit

Table 2
Primers sets used for DNA sequencing and RT-PCR.

Gene	Primer sequence	Product size
GPIX-P4	Forward: GATGGGGTCTCTGCTAAGGG Reverse: AGCCCCAACTGATGTCTGGT	1070 bp
β-ACTIN	Forward: CTGGAACGGTGAAGGTGACA Reverse: AAGGGACTTCCTGTAACAATGCA	165 bp
OCT4	Forward: AGTGAGAGGCAACCTGGAGA Reverse: ACACTCGGACCACATCCTTC	110 bp
SOX2	Forward: TCAGGAGTTGTCAAGGCAGAGAAG Reverse: CTCAGTCCTAGTCTTAAAGAGGCAGC	80 bp
REX1	Forward: CAGATCCTAAACAGCTCGCAGAAT Reverse: GCGTACGCAAATTAAAGTCCAGA	306 bp
NANOG	Forward: TGCAGTTCCAGCCAAATTCTC Reverse: CCTAGTGGTCTGCTGTATTACATTAAGG	96 bp
SeV	Forward: GGATCACTAGGTGATATCGAGC Reverse:ACCAGACAAGAGTTTAAGAGATATGTATC	181 bp
KOS	Forward: ATGCACCGCTACGACGTGAGCGC Reverse: ACCTTGACAATCCTGATGTGG	528 bp
KLF4	Forward: TTCCTGCATGCCAGAGGAGCCC Reverse: AATGTATCGAAGGTGCTCAA	410 bp
c-MYC	Forward: TAACTGACTAGCAGGCTTGTCG Reverse: TCCACATACAGTCCTGGATGATGATG	532 bp

(Roche) and cDNA was generated using the Transcription First Strand cDNA synthesis kit (Roche) according to the manufacturer's instructions. PCR was performed using Taq DNA Polimerase kit (Invitrogen, Life Technologies). PCR products were electrophoresed in an agarose gel. The primers used are listed in Table 2. *KLF4*, SeV, *c-MYC* and *KOS* primer sets were used to determine the presence of exogenous reprogram factors. *Oct3/4*, *SOX2*, *NANOG* and *REX1* and β-*ACTIN* primer sets were used to confirm the expression of pluripotent markers (Ramos-Mejía et al., 2012).

Karyotyping

Chromosomal analysis was performed by GTG-banding analysis at the Andalusian Public Health System Biobank, Spain, following the International System Cytogenetics Nomenclature recommendations (Simons et al., 2013; Catalina et al., 2007).

Alkaline phosphatase

After five days in culture, BSS1-PBMC-iPS4 colonies were assayed for phosphatase alkaline enzymatic activity using a commercial detection kit (Merck-Millipore) according to the manufacturer's instructions.

Flow cytometry analysis

BSS1-PBMC-iPS4 colonies were dissociated using Tryple Express (Life Technologies) and the cell suspension was stained with SSEA3 (PE, BioScience), SSEA4 (Alexa Fluor® 647, BD Pharmingen), Tra1-60 (PE, BioScience), Tra1-81 (Alexa Fluor® 647, BD Pharmingen) surface antibodies for 30 min. Intracellular staining for Oct3/4 was performed by sequential incubations with fixation and permeabilization solutions (A and B Fix & Perm Solutions, Invitrogen). Then cells were incubated first with Oct3/4 (BD BioScience) primary antibody, and subsequently with FITC-conjugated secondary antibody (BD BioScience). After washing, cells were stained with 7-aminoactinomycin D (7-AAD) (BD Bioscience) for 5 min at RT. An irrelevant isotype-match antibody was always used as a negative control. Live cells were identified by 7-AAD exclusion and were analyzed using a FACS verse (BD Bioscience).

In vivo teratoma formation

Feeder free BSS1-PBMC-iPS4 colonies were dissociated with collagenase IV (Gibco) and resuspended in PBS. $2 - 5 \times 10^6$ cells per mouse were subcutaneously implanted into the dorsal flanks of SCID mice (NOD/LtSz-scid interleukin-2Rγ$^{-/-}$ mice, The Jackson Laboratory). Teratoma growth was monitored weekly and mice were sacrificed at 13 weeks post implantation. Teratomas were collected, fixed in formaldhyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin ((H&E) for histological analysis. Immunocytochemistry analysis for β3-Tubulin (ectoderm), Vimentin (mesoderm) and CKAE1-EA3 (endoderm) was performed on sectioned slides (Gutierrez-Aranda et al., 2010). The Animal Care Ethics Committee of the University of Granada approved all animal protocols.

Author disclosure statement

There are no competing financial interests in this study.

Acknowledgments

This work was supported by the Marie Curie Intra-European Fellow (FP7-MC-IEF-623806) to LLO; Postdoctoral Subprogramme Juan de la Cierva founded by the Ministry of Economy and Competitiveness (JCI_2012_12666) to RM; the Instituto de Salud Carlos III-FEDER (CP12/03175 and PI14/01412) to V.R-M.; (CP09/0063, PI12/1598 and CPII15/00018) to PJR and (PI14/01956 and CB15/00055) to MLL, VV & JR; TerCel (RD12/0019/0006) and the Telemaraton Todos Somos Raros, Todos somos Únicos (IP91-2014) to PJR.

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