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Lab Resource: Stem Cell Line

Generation of a human induced pluripotent stem cell (iPSC) line from a Bernard-Soulier syndrome patient with the mutation p.Asn45Ser in the *GPIX* gene

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ABSTRACT

Bernard Soulier Syndrome (BSS) is an inherited rare platelet disorder characterized by mutations in the platelet glycoprotein complex GPIb-IX-V. We generated an induced pluripotent stem cell (iPSC) line from a BSS patient with a mutation p.Asn45Ser in the *GPIX* locus (BSS2-PBMC-iPS4F24). Peripheral blood mononuclear cells were reprogrammed using non-integrative viral transduction. Characterization of BSS2-PBMC-iPS4F24 included mutational analysis of *GPIX* locus, analysis of conventional pluripotency-associated factors at mRNA and protein level and *in vitro* and *in vivo* differentiation studies. This iPSC line will provide a powerful tool to study the biology of BSS disease.

Resource table.

Name of Stem Cell line	BSS2-PBMC-iPS4F24
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	July, 2016
Origin	Human peripheral blood mononuclear cells (PBMCs)
Type of resource	Induced pluripotent stem cell (iPSC)
Sub-type	Cell line
Key transcription factors	Oct4, 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

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Resource details

Bernard-Soulier Syndrome (BSS) is an autosomal recessive rare platelet disorder characterized by thrombocytopenia, large platelets and frequent bleeding. This disease is caused by mutations in one of the three genes coding for the glycoprotein complex GPIb-IX-V which functions as a receptor for vWF (Berndt and Andrews, 2011; Andrews and Berndt, 2013). We have recently described the first human iPSC model for BSS (Lopez-Onieva et al., 2016). In this study, human BSS peripheral blood mononuclear cells (PBMCs) with the mutation p.AsnN45Ser in the *GPIX* gene (BSS2-PBMC) (Wright et al., 1993; Sachs et al., 2003; Clemetson et al., 1994) were reprogrammed into iPSCs (BSS2-PBMC-iPS4F24) using the non-integrative CytoTune iPS 2.0 Reprograming System (Life Technologies, Invitrogen).

Several genetic and functional assays were performed to determine the quality of the BSS2-PBMC-iPS4F24 line. Sequencing analysis of the *GPIX* locus confirmed the presence of c. 182 A>G change in exon 3 of the *GPIX* gene corresponding to a homozygous p.Asn45Ser mutation, identical to PBMCs-BSS2 (Fig. 1A). In addition, BSS2-PBMC-iPS4F24 line silenced the expression of exogenous reprogramming transgenes after 8 passages (Fig. 1B) and showed normal karyotype (46, XX) (Fig. 1C). Additionally, Short Tandem Repeat (STR) profiling confirmed same genetic identity between both samples (Table 1).

Pluripotency was assessed by alkaline phosphatase staining (ALP) (Fig. 1D), qRT-PCR analysis of endogenous pluripotent transcription

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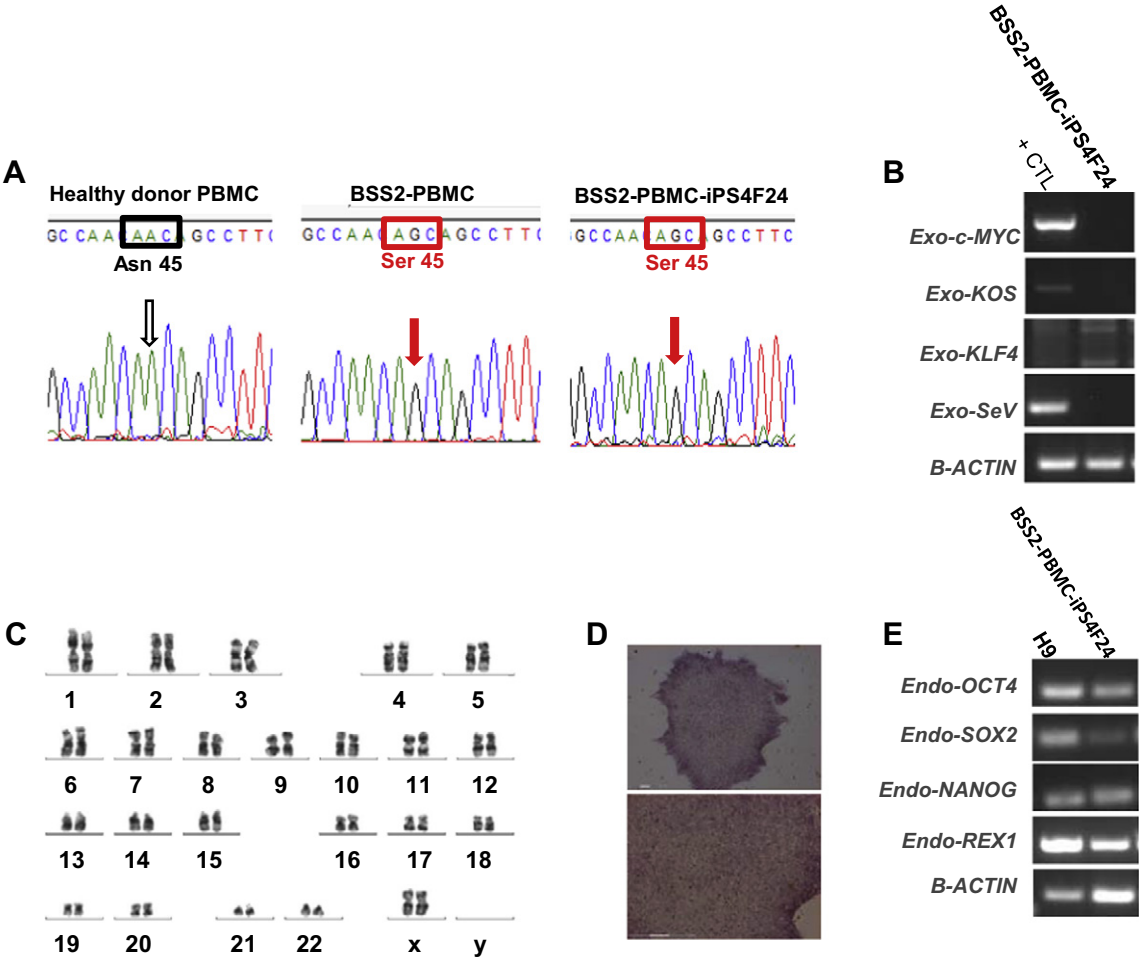

Fig. 1. Molecular characterization of BSS2-PBMC-iPS4F24 cell line. (A) Sequence analysis of c. 182 A>G change in exon 3 of the *GPIX* gene from healthy donor PBMCs (left panel), BSS2-PBMCs (middle panel) and BSS2-PBMC-iPS4F24 (right panel). (B) Silencing of exogenous reprogramming factors and SeV vector confirmed by RT-PCR. BSS2-PBMC-iPS4F24 at passage 4 was used as a positive control. (C) GTG-banding shows a normal karyotype in BSS2-PBMC-iPS4F24 cell line. (D) Alkaline phosphatase enzymatic activity staining. (E) Endogenous pluripotency markers *OCT4*, *SOX2*, *REX1* y *NANOG* were confirmed by RT-PCR.

factors OCT-4, SOX-2, NANOG and REX1 (Fig. 1E) and flow cytometry analysis of protein stem cell markers SSEA3, SSEA4, Tra1-60, Tra1-81 and Oct3/4 (Fig. 2A). Finally, we assessed the functionality of the BSS2-PBMC-iPS4F24 cells by differentiating them into the three germ layers both *in vitro* (by embryoid body (EB) formation) and *in vivo* (teratoma formation). Both EBs (Fig. 2B) and teratomas (Fig. 2C) showed expression of representative markers of ectoderm, mesoderm and endoderm.

Table 1
Short Tandem Repeat (STR) profiling of the original patient cells (BSS2-PBMC) and the iPSC patient-derived cells (BSS2-PBMC-iPS4F24).

STR Locus	Alleles	
	BSS2-PBMC	BSS2-PBMC-iPS4F24
Amelogenin	X, X	X, X
CSF1PO	12, 12	12, 12
D13S317	11, 11	11, 11
D16S539	9, 9	9, 9
D21S11	30, 32	30, 32
D5S818	12, 12	12, 12
D7S820	10, 10	10, 10
TH01	9, 9.3	9, 9.3
TPOX	9, 11	9, 11
vWA	16, 18	16, 18

Materials and methods

Reprogramming of BSS2-PBMCs

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. BSS2-PBMCs were isolated by centrifugation using Ficoll Paque ™ PLUS (GE Healthcare). Isolated BSS2-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-27632 (Sigma-Aldrich). Three days after transduction, cells were co-cultured in StemSpan™ SFEM (StemCell Technologies) in absence of cytokines on a layer of irradiated human mesenchymal

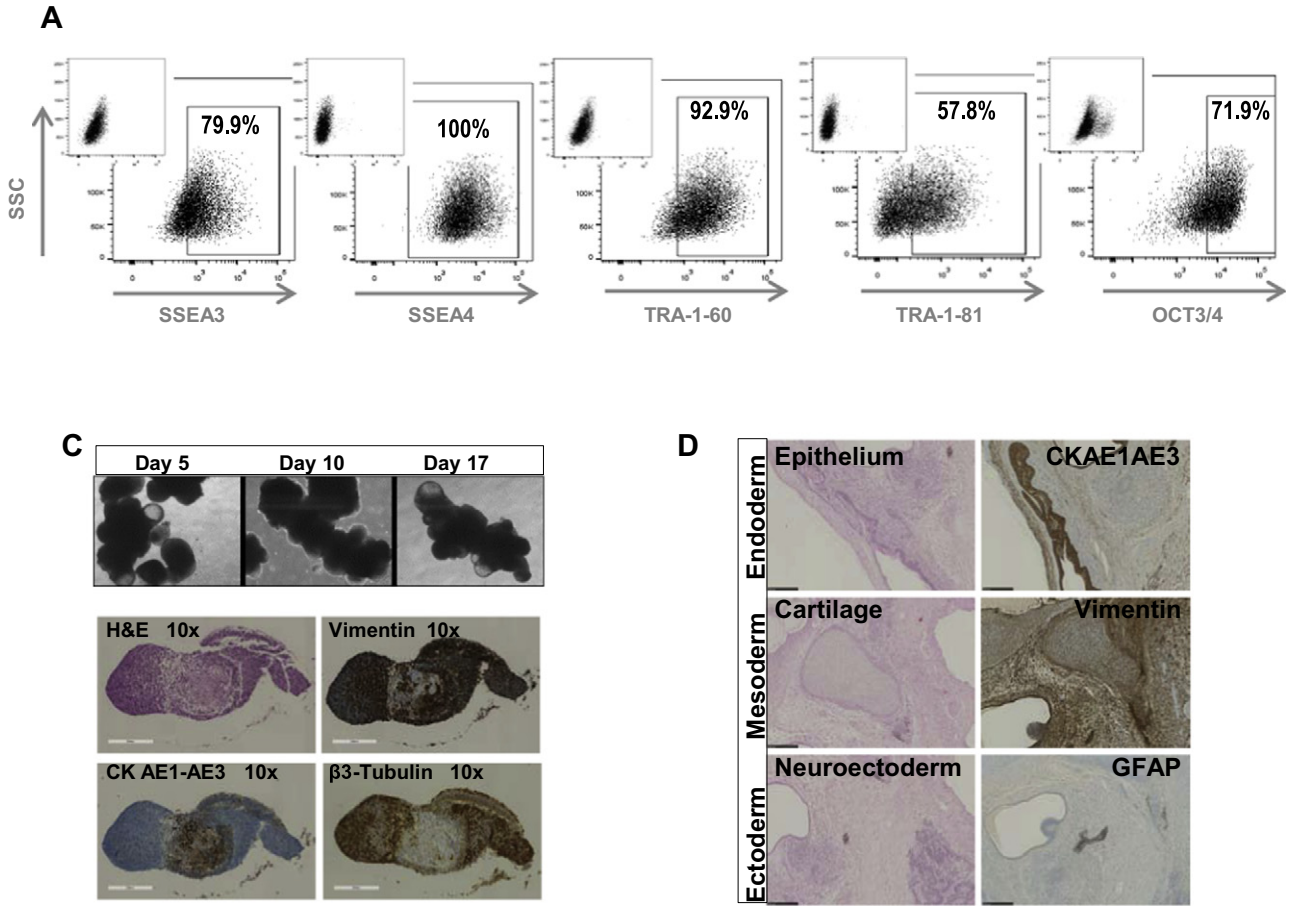

Fig. 2. Pluripotency markers and three germ layer differentiation capacity of BSS2-PBMC-iPS4F24 line. (A) Expression of pluripotency-associated markers SSEA3, SSEA4, TRA1-81, TRA 1-60 and OCT3/4 at protein level by FACS analysis. The inset shows the staining using the corresponding irrelevant isotype-matched antibody. (B) EB formation by *in vitro* spontaneous differentiation (upper panel). Histological sections from 21 day developed EBs (lower panel). Hematoxylin and eosin (H&E) and immunohistochemistry analysis for mesoderm (Vimentin), endoderm (CK AE1-AE3) and ectoderm (β3-Tubulin). (Scale bar = 200 μM) (C) *In vivo* differentiation test by teratoma formation assay. Histological sections from 13 week-teratomas. Hematoxylin and eosin (H&E) staining reveals characteristic tissues from the three germ layers (left pannel); immunohistochemistry analysis showed differentiation to mesoderm (Vimentin), endoderm (CK AE1-AE3) and ectoderm (GFAP). (Scale bar = 250 μM).

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.

Cell culture

One month after reprogramming, BSS2-PBMC-iPS4F24 cells growing on PSC medium were also adapted to grow in E8 based-medium (Chen et al., 2011) on Matrigel (BD Biosciences). Subsequent experiments were performed in E8 based-medium.

Mutational analysis

Genomic DNA was isolated from PBMCs from a healthy donor, BSS2-PBMCs and BSS2-PBMC-iPS4F24 line using a 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.

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

Short tandem repeat (STR) profiling

To verify the origin of the new iPSC line generated, DNA from BSS2-PBMCs patient and BSS2-PBMC-iPS4F24 were purified. GenePrint® 10 System PCR Amplification kit (Promega) was used to determine the genetic signature of both samples based on the multiplex analysis of 9 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, BSS2-PBMC-iPS4F24 conserves identical STR profile than the original BSS2-PBMC.

Semiquantitative RT-PCR

Total RNA from undifferentiated BSS2-PBMC-iPS4F24 line and a control iPSC line was isolated using the High pure RNA isolation kit (Roche) and cDNA was generated using the Transcription First Strand c-DNA synthesis kit (Roche) according to the manufacturer's instructions. PCR was performed using Taq DNA Polimerase kit (Invitrogen). PCR products were visualized in an agarose gel. Primers used are shown in Table 2. *KLF4*, SeV, *c-MYC* and *KOS* primer sets were used to determine the presence of exogenous reprogramming factors (primer sequence provided by CytoTune iPS 2.0 Reprograming System kit (Life Technologies, Invitrogen)). *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, colonies were assayed for phosphatase alkaline enzymatic activity using Alkaline phosphatase detection kit (Merck-Millipore) according to the manufacturer's instructions.

Flow cytometry analysis

BSS2-PBMC-iPS4F24 colonies were dissociated using Tryple Express (Life Technologies), washed with PBS 10% FBS and 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 (BD BioScience)). 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 isotype-match antibody was used as a negative control. Live cells were analyzed using a FACS verse (BD Bioscience).

In vitro embryo body (EB) formation

BSS2-PBMC-iPS4F24 colonies were treated with Dispase solution (Stem Cell Technologies) and gently scraped off the flask. After centrifugation, cells were resuspended in E6 based- medium (E6 is similar to E8 medium but without FGF2 and TGF) and transferred into Corning low attachment dishes. EBs were culture in a 37 °C incubator with humidified atmosphere of 5% CO_2. The medium was changed every other day. After 21 days EBs were collected, 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.

In vivo teratoma formation

BSS2-PBMC-iPS4F24 colonies were dissociated with collagenase IV (Gibco) and resuspended in PBS. 2-5 × 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 14 weeks post implantation. Teratomas were collected, fixed in formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histological analysis. Immunocytochemistry analysis for GFAP (ectoderm), Vimentin (mesoderm) and CKAE1-AE3 (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.

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