Stem Cell Research 21 (2017) 47–50

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Stem Cell Line

# Generation of a Sprague-Dawley-GFP rat iPS cell line

Giulia Coppiello [a], Gloria Abizanda [a], Natalia Aguado [a], Elena Iglesias [a], Olalla Iglesias-Garcia [c], Antonio Lo Nigro [d], Felipe Prosper [a,b,*], Xabier L. Aranguren [a,**]

[a] Cell Therapy Program, Center for Applied Medical Research (CIMA), University of Navarra, Health Research Institute of Navarra, Pamplona, Spain
[b] Cell Therapy Area, Clínica Universidad de Navarra, University of Navarra, Health Research Institute of Navarra, Pamplona, Spain
[c] Center of Regenerative Medicine in Barcelona (CMRB), Spain
[d] Ri.Med Foundation, Department of Laboratory Medicine and Advanced Biotechnologies, IRCCS-ISMETT (Istituto Mediterraneo per i Trapianti e Terapie ad Alta Specializzazione), Palermo, Italy

ARTICLE INFO

*Article history:*
Received 11 January 2017
Received in revised form 28 February 2017
Accepted 28 March 2017
Available online 31 March 2017

ABSTRACT

We generated a rat iPSC line called ATCi-rSD95 from transgenic Sprague-Dawley GFP fetal fibroblasts. Established ATCi-rSD95 cells present a normal karyotype, silencing of the transgenes and express pluripotency-associated markers. Additionally, ATCi-rSD95 cells are able to form teratoma with differentiated cells derived from the three germ-layers that maintain the GFP expression.



Resource table

| | |
|---|---|
| Name of stem cell line | ATCi-rSD95 |
| Institution | Center for Applied Medical Research (CIMA), University of Navarra |
| Person who created resource | Giulia Coppiello, Xabier L. Aranguren |
| Contact person and email | X.L. Aranguren, xlaranguren@unav.es; F. Prosper, fprosper@unav.es |
| Date archived/stock date | May 2015 |
| Origin | Sprague-Dawley rat fetal fibroblast |
| Type of resource | Biological reagent: rat induced pluripotent stem cell (iPSC) |
| Sub-type | Cell line |
| Key transcription factors | OCT3/4, KLF4, SOX2 |
| Authentication | Identity and purity of the cell line confirmed (Fig. 1) |
| Link to related literature | Not available |
| Information in public databases | Not available |

## Resource details

E15.5 embryos were obtained from the crossing of heterozygous Sprague-Dawley (SD)-Tg(GFP)1BalRrrc rats. Rat embryos' trunks were digested and fibroblasts were cultured and genotyped. Female GFP homozygous rat embryonic fibroblasts were transduced with the STEMCCA doxycycline-inducible lentivirus containing *Oct3/4*, *Klf4* and *Sox2* (and mCherry reporter gene) to generate induced pluripotent stem cells (IPSC) (Nakagawa et al. 2008) (Fig. 1A). Three-four weeks after transduction, individual ES-like colonies were picked up, dissociated using Accutase and re-plated on irradiated mouse embryonic fibroblasts (iMEFs). Established ATCi-rSD95 show transgene silencing, as tested by RT-PCR (Fig. 1A–B). Accordingly, cells do not show Cherry positive signal despite the presence of doxycycline, as analyzed under fluorescence microscope (Fig.1C). Cells were routinely splitted 1:3–1:4 every 3–5 days and tested for mycoplasma contamination. Results for B/A ratio of mycoplasma test as measured with MicoAlert R Sample Kit (Cambrex) run on cells at passage 23 are: CTR+ ratio 27.49; CTR- ratio 0.58; ATCi-rSD95 ratio 0.56. Rat iPSC ATCi-rSD95 possess normal karyotype (82% of the cells, in line with previously reported rat pluripotent cells characterization) (Li et al. 2008) (Fig. 1D). Cells express endogenous *Oct3/4* and *Klf4* as well as other pluripotency-associated markers like *Nanog* and *Esrrb1* as shown by qRT-PCR (Fig. 1E). OCT3/4, NANOG, SOX2 and SSEA1 protein expression was verified by immunofluorescence staining while SSEA-4, a primate (primed) pluripotent stem cells marker was not expressed, as expected (Fig. 1F). Furthermore, cells show alkaline phosphatase activity (AP, Fig. 1F). Finally, *in vivo* teratoma assay was used to determine the capacity of the cells to differentiate into tissues from the three germ layers (Fig. 1G). GFP signal was maintained upon differentiation both *in vitro* and *in vivo* (Supplementary Fig. 1).

* Correspondence to: F. Prosper, Cell Therapy Area, Clínica Universidad de Navarra, University of Navarra, Health Research Institute of Navarra, Av. Pío XII 36, Pamplona 31008, Spain.
** Correspondence to: X.L. Aranguren, Cell Therapy Program, Center for Applied Medical Research (CIMA), University of Navarra, Health Research Institute of Navarra, Av. Pío XII 55, Pamplona 31008, Spain.
*E-mail addresses:* fprosper@unav.es (F. Prosper), xlaranguren@unav.es (X.L. Aranguren).

http://dx.doi.org/10.1016/j.scr.2017.03.021

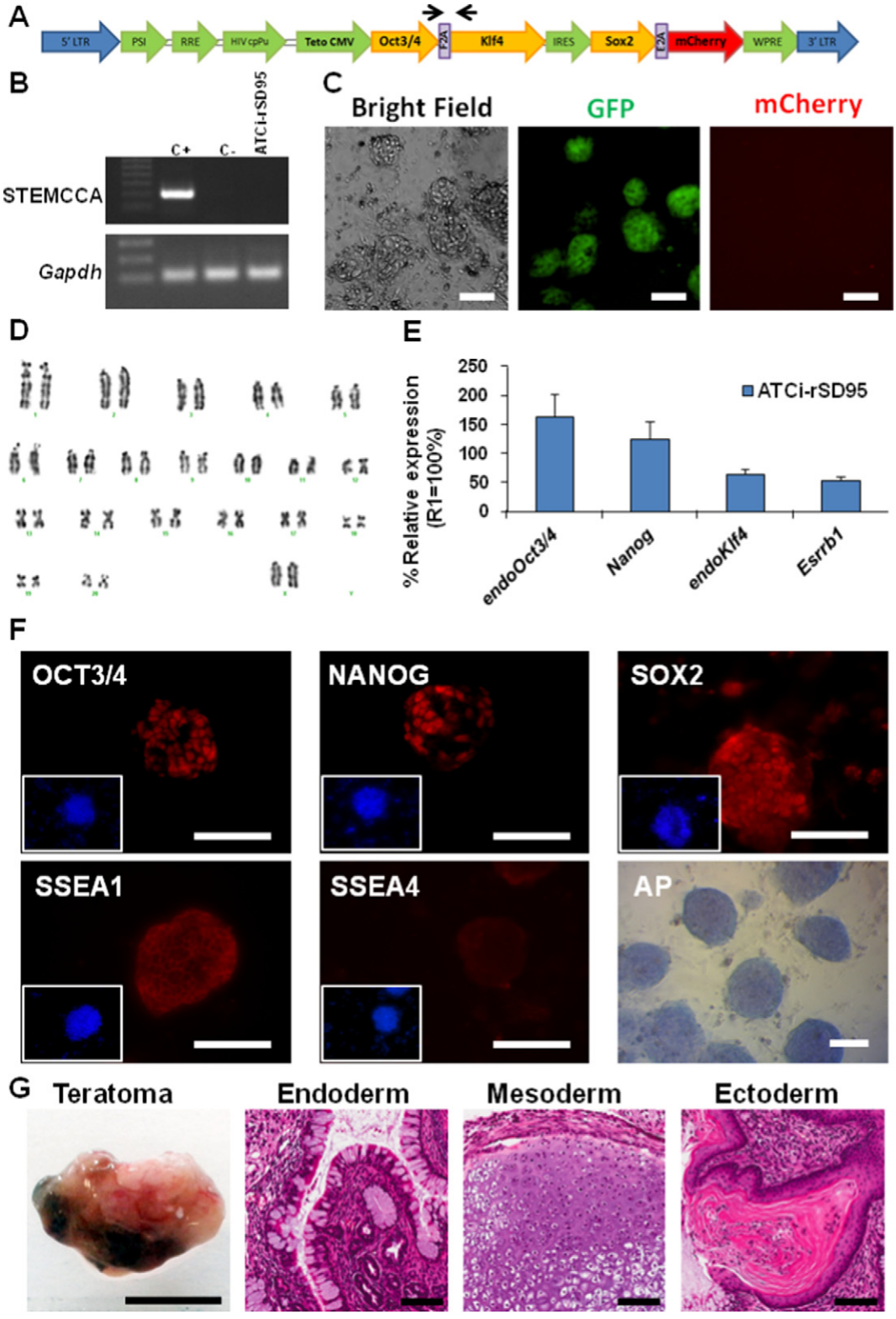


**Fig. 1.** Characterization of Rat iPS cell line ATCi-rSD95. A. Graphic representation of polycistronic doxycycline-inducible STEMCCA lentiviral vector used for iPS generation, carrying the expression of *Oct3/4; Klf4; Sox2* and mCherry reporter gene. Black arrows indicate the primers sequences used for determining transgenes silencing B. RT-PCR showing silencing of STEMCCA transgenes expression in established ATCi-rSD95 cells (CTR+: transduced fibroblasts two days after addition of doxycycline; CTR-: non transduced fibroblasts). C. Phase contrast and fluorescence microscopy images of established ATCi-rSD95 showing colony morphology, endogenous GFP positive signal and transgenic Cherry negative signal, due to the viral vector silencing (with presence of doxycycline in the culture media). D. Representative karyotype from ATCi-rSD95 chromosome analysis. E. qRT-PCR gene expression analysis of endogenous pluripotency-associated markersin ATCi-rSD95 cells: *Oct4*, *Nanog*, *Klf4* and *Esrrb1*. Data have been compared with expression in R1 mouse ES cells (100%) and are expressed as averages + SEM (n = 3). F. Immunofluorescence staining for pluripotency associated markers OCT4, NANOG, SOX2 and SSEA1 (in red) and negative signal for SSEA4. Hoechst nuclear counterstaining, in blue, is shown in the insert). Scale bar 100 μm. Alkaline phosphatase AP) enzymatic activity. Scale bar 100 μm. G. Macroscopic picture of the teratoma formed by ATCi-rSD95 cells *in vivo* (scale bar 10 mm) and H&E staining of teratoma sections, showing presence of differentiated tissues from the three germ layers (scale bar 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## Materials and methods

*Isolation and culture of Sprague-Dawley embryonic fibroblasts*

Animal procedures were in compliance with the Spanish and European legislation and were approved by the Ethical Committee of University of Navarra. Rat embryos were obtained from the crossing of heterozygous SD-Tg(GFP)1BalRrrc rats (Rat Resource & Research Center). At E15.5, pregnant females were euthanized with $CO_2$ overexposure and the embryos were retrieved from the uterus. The head and the limbs of the embryos were removed and fibroblasts were obtained by mincing and digesting the rest of the embryo with 1 mg/ml collagenase type I (Life technologies) for 1 h at 37 °C with regular shaking and pipetting. After washing with PBS, cells were filtered through a 40 μm mesh (Falcon), seeded on 0.1% gelatin-coated flasks and cultured in medium composed by DMEM high glucose (Gibco) 15% Fetal bovine serum (FBS) with 1% Penicillin/Streptomycin (P/S), 1% Glutamax (Gibco) and 0.1 mM β-mercaptoethanol (MEF media). After 2–3 days in culture, fibroblasts samples were retrieved and genotyped for GFP and gender. Female homozygous $GFP^+$ fibroblasts (primers in Table 1) were selected for iPS generation.

*iPS generation and culture*

50.000 rat embryonic fibroblasts plated the day before on 0.1% gelatin (5.000 cells/$cm^2$) with MEF media were transduced with polycistronic doxycycline-inducible STEMCCA lentiviral vector containing *Oct3/4*, *Klf4* and *Sox2* transgenes (and mCherry reporter gene). Two days later, cells were detached with Accutase (Gibco) and re-plated on iMEFs and media was shifted to iPSC media (N2B27 2i): N2B27 media containing rat LIF (Millipore) 1000 U/ml; CHIR99021 1 μM (Axon medchem BV) and PD0325901 1 μM (Axon medchem BV). For the preparation of N2B27 media, N2 100× stock was home made, while B27 50× supplement was obtained from Life technologies. N2 stock was diluted in DMEM/F12 (Sigma) to a concentration of 1× and B27 stock was diluted in Neurobasal media (Life technologies) to a concentration of 1×. The two solutions were then mixed in 1:1 proportion. β-mercaptoethanol was added to a final concentration of 0.1 mM and 1× Glutamax (Gibco) and 1× Antibiotic/antimycotic solution (Life technologies) were added. Two weeks later individual colonies were picked-up and placed onto 20 μl of Accutase, partially digested and re-plated onto iMEFs on iPS media at 37 °C, 5% $CO_2$, 20%$O_2$. Cells were routinely passed with partial digestion with Accutase or TrypLe (Gibco) every 3–5 days (1:3–1:4). After 3–4 passages A83-01 1 μM (Alk 4,5 and 7 inhibitor; Tocris) was added and glutamine was removed from the media. These changes allowed to maintain homogenous undifferentiated cells in culture. Cells were maintained for more than 30 passages without detecting decreased cell proliferation potential. Only cells at passages 5 to 20 were used for the experiments.

*Transgenes silencing analysis*

Total RNA was extracted using TRIzol® reagent (Life technologies). RNA was treated with Turbo DNase (Thermofisher) and then 0.5 μg were reverse transcribed using PrimeScript™ RT reagent Kit (Takara) following the manufacturer's recommended protocol. Complementary DNA (cDNA) underwent 35 cycles of amplification using KAPA2G PCR mix (Kapa Biosystems) and PCR products were run on a 2% agarose gel and visualized with ethidium bromide. Gene expression levels were normalized using *Gapdh* (primers in Table 1). STEMCCA forward primer is partially overlapping the very end of Oct3/4 transgene and part of F2A sequence of the STEMCCA viral vector, while the reverse primer is designed at the beginning of Klf4 transgene (Table 1, Fig. 1A).

*Mycoplasma test*

Cells were routinely screened for mycoplasma contamination using MicoAlert R Sample Kit (Cambrex) biochemical test following the manufacturer's protocol. Briefly, supernatant from 48 h cell culture was sequentially incubated with reagent and substrate kit components and bioluminescence was detected with a luminometer after addition of each component (read A and B respectively). A luminescence signal ratio B/A lower than one demonstrates absence of mycoplasma contamination.

*Karyotype analysis*

Rat ATCi-rSD95 cells were grown on a T25 flask on iMEF to 50–60% confluency. The day of harvest 20 μl of Colcemid (10 μg/ml) was added to the media for 1 h at 37 °C. Samples were washed with PBS and 2 ml of pre-warmed hypotonic solution (potassium chloride) was added drop by drop to the sample and incubated for 30 min at 37 °C. Finally, cells were fixed with Carnoy solution. Analysis of chromosomal stability was performed and analyzed by the Genetics Service at Policlínica Gipuzkoa (Spain). 22 metaphases of ATCi-rSD95 cells at passage 16 were photographed and quantified. Karyotype resolution is 9 MB.

*qRT-PCR gene expression analysis.*

Total RNA was extracted using TRIzol® reagent (Life technologies) and 0.5 μg were reverse transcribed using PrimeScript™ RT reagent Kit (Takara) following the manufacturer's recommended protocol. Complementary cDNA underwent 40 rounds of amplification on a 7500 Real-Time PCR system (Applied Biosystems) using SYBR-Green master mix (Applied Biosystems). Primers (Table 1) were designed in regions with 100% homology with mouse sequences, in order to directly compare the gene expression profile of ATCi-rSD95 with standard mouse ES cells (R1 cell line). Gene expression levels were normalized using *Gapdh* and data are represented as percentages in comparison with mouse R1 ES cell line expression (100%).

**Table 1**
Primers used in this study.

| Gene | Forward | Reverse |
|---|---|---|
| *Gapdh* | CAATGCATCCTGCACCAC | CAGTGATGGCATGGACTGTG |
| *Nanog* | TACCTCAGCCTCCAGCAGAT | GCAATGGATGCTGGGATACT |
| *Esrrb1* | TATCAAGGCCCTGACCACTC | GCGTATGCCAGCTTGTCATC |
| *Oct3/4* endogenous | TTCCCAACGAGAAGAGTATG | ATCCCCAGGGAGGGCTGGTGC |
| *Klf4* endogenous | ATTAATGAGGCAGCCACCTG | ACGCAGTGTCTTCTCCCTTC |
| STEMCCA | CATGCATTCAAACGGAAGTG | AGAGAGTTCCTCACGCCAAC |
| Y chromosome | GCATTTATGGTGTGGTCCCG | GGTATTTCTCTCTGTGTAGG |
| Rat 14q21 | AGCAATGAATAGCCTCTCTCC | CCCATATGTGCCAAGCACTTTACC |
| GFP/lentiviral transgene | GTCTGAAGGGATGGTTGTAGCTGT | |

*Immunofluorescence and alkaline phosphatase staining on cultured cells*

Cells were fixed with 4% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS) for 10 min, washed 3 times in PBS, permeabilized with PBS 0.1% TritonX-100 for another 10 min and then blocked in PBS 1% BSA (blocking solution) for 30 min. Samples were stained with the following primary antibodies in blocking solution overnight at 4 °C: OCT3/4 (1:50 sc-9081 Santa cruz), NANOG (1:100 ab80892 Abcam) SOX2 (1:100 ab5603 Abcam), SSEA1 (1:100 90230 Millipore) and SSEA4 (1:100 90231 Millipore). Next day, samples were washed 3 times in PBS and were incubated with Alexa Fluor 594 secondary antibodies (Life technologies) for 45 min at room temperature (dilution 1:500 in blocking solution). Nuclear counterstain was performed with Hoechst 33342 trihydrochloride (Life technologies; dilution 1:1000) for 10 min at room temperature. Images were taken with Leica DM IL epifluorescence microscopy. Alkaline phosphatase activity was assayed with Sigma AB0300 kit following the manufacturer's instructions on fixed cells. Pictures were taken with CKX41 microscope (Olympus).

*In vivo teratoma formation assay*

One million ATCi-rSD95 cells were mixed with a cold solution of matrigel: PBS (1:1 proportion) and were injected subcutaneously into the hind-leg of anesthetized 8 weeks old immunodeficient Rag2$^{-/-}$ γC$^{-/-}$ males. Four weeks later, mice were sacrificed by cervical dislocation and teratomas were dissected, photographed and fixed overnight in 4% PFA. Samples were paraffin-embedded, sectioned and stained with Hematoxylin/Eosin (H&E) by the Imaging Core at CIMA. Histological evaluation was performed using Aperio CS2 Digital Pathology Scanner (Leica) and Aperio Scan Scope software (Leica).

*Immunofluorescence staining on teratoma samples*

After deparaffinization, antigen retrieval was performed with citrate buffer pH 6 in a microwave (10 min maximum power and 10 min minimum power), rinsed in water and blocked in PBS 1% BSA for 1 h. Primary antibodies against GFP (1:200 A11122 Life technologies), NeuN (1:100 MAB377 Chemicon) and albumin (1:100 sc-46293 Santa-cruz), and rhodamine labeled BS-I lectin (20 μg/ml RL-1102 Vector Laboratories) were incubated overnight at 4 °C in blocking solution. Next day, samples were rinsed 3 times with PBS and exposed to Alexa Fluor 594 secondary antibodies (Life technologies) for 45 min at room temperature (dilution 1:500 in blocking solution). Nuclear counterstain was performed with Hoechst 33342 trihydrochloride (1:1000 Life technologies) for 10 min at room temperature. Images were taken with Leica DM IL epifluorescence microscope.

*In vitro differentiation by formation of embryoid bodies*

Embryoid body (EB) formation was performed as described in Li et al., 2008. Briefly, dissociated rat IPSCs were plated onto "AggreWell 400" plates at an approximate density of 1000 cells/EB, following manufacturer's instructions (Stemcell technologies) in MEF media containing 0.5 μM CHIR99021 and 0.5 μM PD0325901. After 48 h the media was switched to MEF-conditioned media for additional 48 h to improve embryoid body formation. After this period, embryoid bodies were gently collected and plated on cell culture-treated plate in MEF media for additional 3 days, moment at which GFP and mCherry signal was analyzed by fluorescence microscopy.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.03.021.

## Author disclosure statement

There are no competing financial interests in this study.

## Acknowledgement

We thank Gustavo Mostoslavsky, Boston University School of Medicine for providing the doxycycline inducible STEMCCA plasmid. This work was supported by the Marie-Curie Fellowship (FP7-PEOPLE-2013-IEF 626220) and Ramón y Cajal State Program (MINECO, RYC-2015-17233) personal grants to XLA; by "Retos de la Sociedad" State Program (MINECO SAF2015-64224-R) project grant, "Fundación Bancaria Caja Navarra" (project grant no 70278) and Health Department of Government of Navarra 2015 (project grant no 031-2015) to XLA by Juan de la Cierva Formación (FJCI-2014-22909) personal grant to GC and by the Institute of Health Carlos III RETIC (RD12/0019/0031) project grant to FP.

## References

Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R.E., Schulze, E.N., Song, H., Hsieh, C.L., Pera, M.F., Ying, Q.L., 2008. Germline competent embryonic stem cells derived from rat blastocysts. Cell 135 (7), 1299–1310.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., Yamanaka, S., 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnol. 26 (1), 101–106.