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Lab Resource: Stem Cell Line

Isolation and characterization of Sprague-Dawley and Wistar Kyoto GFP rat embryonic stem cells

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ABSTRACT

We generated two rat embryonic stem cell (ESC) lines: ATCe-SD7.8 from Sprague-Dawley strain and ATCe-WK1 from Wistar Kyoto strain. Cells were marked with enhanced green fluorescent protein (eGFP) by transduction with a lentiviral vector. Cells present a normal karyotype and express pluripotency-associated markers. Pluripotency was tested *in vivo* with the teratoma formation assay. Cells maintain eGFP expression upon differentiation to the three-germ layers. These cells can be a useful tool for cell therapy studies and chimera generation as they can be easily tracked by eGFP expression.

Resource table

Name of stem cell line	ATCe-rSD78; ATCe-rWK1
Institution	Cell Therapy Program, 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	February 2015
Origin	Blastocysts from SD-Tg(GFP)1BalRrrc Sprague-Dawley rats and wild type Wistar-Kyoto rats
Type of resource	Biological reagent: rat embryonic stem (rES) cells
Sub-type	Cell line
Key transcription factors	N/A
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

Blastocysts (E4.5) from the crossing of heterozygous Sprague-Dawley (SD) GFP rats (SD-Tg(GFP)1BalRrrc) and from wild type Wistar-Kyoto (WK) rats were used to isolate embryonic stem (ES) cells in N2B27 2i media. In this report we present the detailed characterization of one cell line from each rat strain: ATCe-rSD78 from SD strain and ATCe-rWK1 from WK strain. SD-Tg(GFP)1BalRrrc rats express the GFP under the control of the human ubiquitin promoter. Nevertheless, we observed that isolated ES cells did not show homogeneous GFP fluorescence signal and the penetrance of the GFP was variable in the different cell lines, therefore we selected the GFP-negative cell line ATCe-rSD78 and both ATCe-rSD78 and ATCe-rWK1 were transduced with pWPXL lentiviral construct driving eGFP expression under the control of the Ef1α promoter. Cells were transduced at early passages, FACS sorted, expanded and characterized. Both cell lines were splitted 1:3–1:4 every 3–5 days and displayed a typical small, round shape and tightly packed colony morphology (Fig. 1A). Cells were routinely tested for mycoplasma contamination and microsatellite analysis confirmed the different origin of the two cell lines (Supplementary Fig. 1). Both cell lines show normal karyotype in more than 80% of the cells, which is in accordance with previous publications related to rat ES cells isolation (Li et al. 2008), (Fig. 1B and Table 1). The expression of several pluripotency-associated markers such as OCT3/4, NANOG, SOX2, KLF4, ESRRB1, SSEA-1 was confirmed by qRT-PCR (Fig. 1C) and/or cell staining (Fig. 1D). SSEA-4, a primate (primed) pluripotent stem cells marker was not expressed, as expected. Additionally, cells possess alkaline phosphatase (AP) activity (Fig. 1E). To assay differentiation

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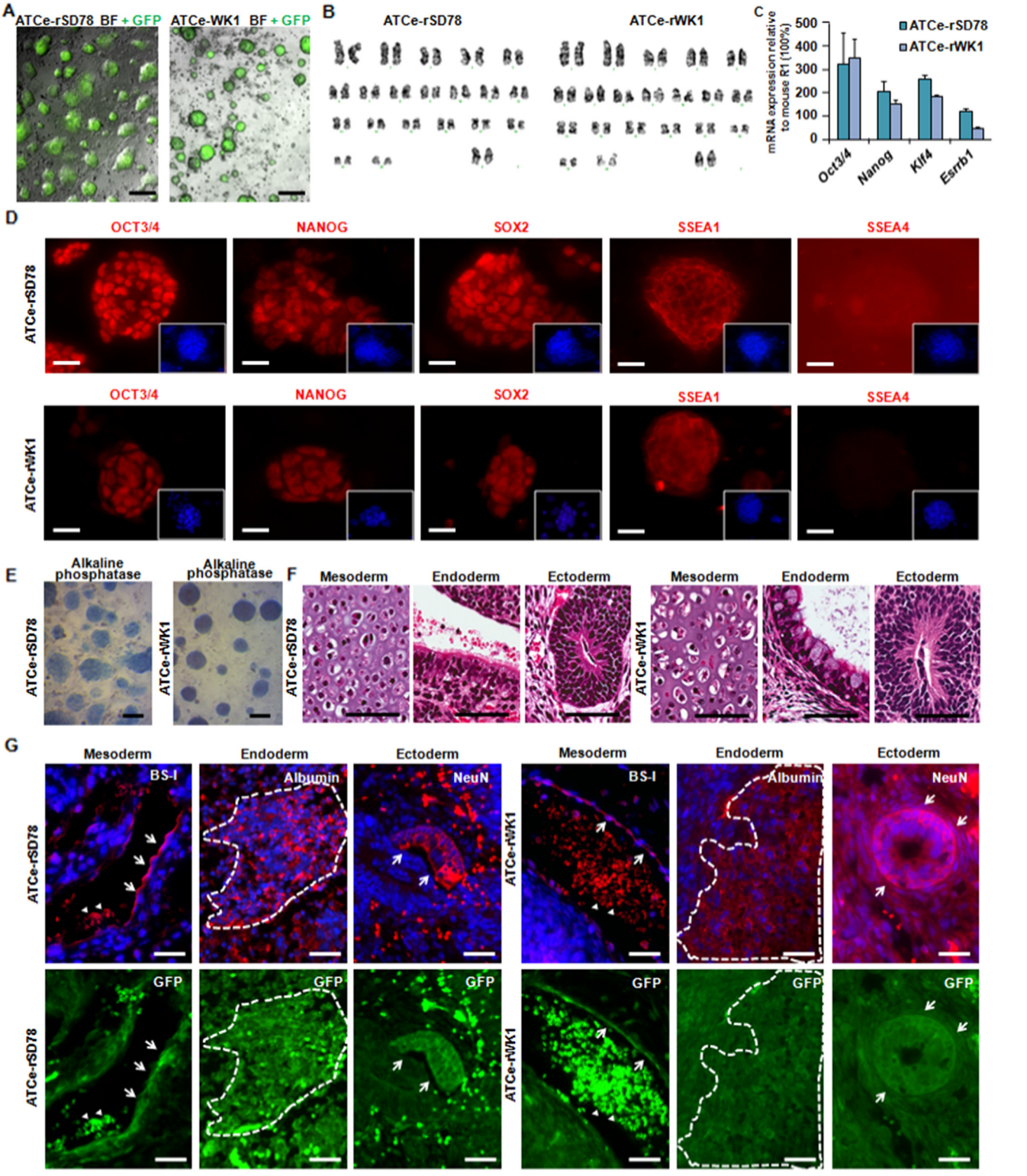

Fig. 1. Characterization of ATCe-rSD78 and ATCe-rWK1 rat ES cell lines. A. Fluorescence micrographs showing ATCe-rSD78 and ATCe-rWK1 colony morphology and homogeneous positive signal for GFP reporter. Scale bar 200 μm. B. Representative karyotypes of ATCe-rSD78 and ATCe-rWK1 cell lines. C. Gene expression analysis by qRT-PCR of pluripotency-associated markers *Oct3/4*; *Nanog*; *Klf4*; *Esrrb1* on ATCe-rSD78 and ATCe-rWK1 cells. Data have been compared with expression in R1 mouse ES cells (100%) and are expressed as averages + SEM ($n = 3$). D. Immunofluorescence staining on ATCe-rSD78 and ATCe-rWK1 cell lines show positive signal for pluripotency-associated markers: OCT3/4; NANOG; SOX2; SSEA1 (in red) and negative signal for SSEA4. Hoechst nuclear counterstaining in blue, is shown in the insert. Scale bar: 50 μm. E. Alkaline phosphatase enzymatic reaction of ATCe-rSD78 and ATCe-rWK1 cell lines. Scale bar: 100 μm. F. Hematoxylin-eosin staining of teratomas showing differentiation to representative tissues from the three germ layers. Scale bar: 50 μm. G. Immunofluorescence staining of teratoma sections derived from ATCe-rSD78 (on the left) and ATCe-rWK1 (on the right). Co-staining of GFP with Albumin (hepatocytes), NeuN (neurons) and BSI-lectin (endothelial cells) shows that cells differentiated to the endodermal, ectodermal and mesodermal lineages, respectively, maintain the GFP reporter expression. Arrows indicate positive cells, arrowheads indicate autofluorescent erythrocytes. Nuclei have been counterstained with Hoechst. Scale bars 30 μm.

Table 1
Karyotype analysis.

ES Cell line	ATCe-rSD78	ATCe-rWK1
Passage	20	19
Analyzed metaphases	29	29
Normal (%)	25 (86%)	24 (83%)
Abnormal (%)	4 (14%)	5 (17%)

potential we performed the *in vivo* teratoma-formation assay in Rag2$^{-/-}$ γC$^{-/-}$ immunodeficient mice. After 4 weeks, formed teratomas were excised and analyzed histologically, revealing presence of differentiated tissues derived from the three germ layers (Fig. 1F). By immunofluorescence staining we confirmed the expression of GFP reporter in differentiated cells from the three germ layers within the teratoma (Fig. 1G).

Materials and methods

ES cells isolation and culture

All animal procedures described in this work were approved by the University of Navarra Ethical Committee and were in compliance with institutional and European Union guidelines for animal care and welfare. Sprague-Dawley GFP rats (SD-Tg(GFP)1BalRrrc) were obtained from Rat Resource & Research Center and Wistar-Kyoto rats from Charles River Laboratories. Animals were housed with 12 h light-dark cycle and fed with standard chow *ad libitum*. Rats were mated naturally in the afternoon and the mating was confirmed by plug check next morning, considering this time point 0.5 days post-coitum (E0.5). Plugged females were sacrificed by CO_2 overexposure on day E4.5, the uteruses were removed and perfused with 1 ml of M2 media (Sigma). The isolated rat blastocysts were briefly treated with Tyrode's Solution (Sigma) to remove the zona pellucida and then seeded on irradiated mouse embryonic fibroblasts (iMEFs) in N2B27 2i: N2B27 media containing rat LIF 1000u/ml (Millipore); CHIR99021 1 μM (Axon medchem BV) and PD0325901 1 μM (Axon medchem BV) as described in (Buehr et al. 2008; Li et al. 2008). For 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 medium (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 Glutamax (Gibco) and Antibiotic/antimycotic solutions (Life technologies) were added to a final 1× concentration. 4–7 days after plating, the outgrowths of blastocysts were manually detached and individually placed onto 20 μl drop of Accutase (Gibco). After partial digestion, embryos' fragments were placed onto iMEFs on N2B27 2i and cultured at 37 °C, 5% CO_2, 20%O_2. After 3–4 passages A83-01 1 μM (Alk 4,5 and 7 inhibitor, Tocris) was added to N2B27 2i, while glutamine was removed, in order to avoid spontaneous cell differentiation. Cells were passed every 3–5 days (1:3–1:4) by partial digestion with Accutase or TrypLe (Gibco) and were maintained for more than 30 passages without showing decreased proliferation capacity. Nevertheless, only cells at passages 5 to 20 were used for the experiments.

Cell labeling

Although the SD rat strain used was transgenic for eGFP, we observed that isolated ES cells did not show homogeneous GFP fluorescence signal and the penetrance of the GFP signal was variable in the different cell lines, therefore, we selected a GFP-negative cell line which we called ATCe-rSD78, and both ATCe-rSD78 and ATCe-rWK1 were transduced with pWPXL lentiviral construct driving eGFP expression under the control of the Ef1α promoter. Lentiviral particles were produced in HEK 293 cell line after co-transfection of pWPXL plasmid (Addgene) with psPax2 and pMD2.G plasmids. Lentiviral particles were concentrated 10× by ultracentrifugation at 20.000 g for 2 h and 100 μl were added onto ATCe-rSD78 and ATCe-rWK1 cell lines. One week later, cells were dissociated with Accutase and GFP-positive cells were sorted using FACS Aria (BD) and subcultured.

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. Results for B/A ratio of mycoplasma test run on cells at passage 17–18 are: CTR+ ratio 36.34; CTR− ratio 0.54; ATCe-rSD78 ratio 0.31; ATCe-rWK1 ratio 0.51.

Microsatellite analysis

The origin of ATCe-rSD78 and ATCe-rWK1 cells from different parental rat strains was confirmed using 8 microsatellite markers described in Serikawa et al. 1992. Genomic DNA from both cell lines was extracted using Nucleospin tissue (Macherey-Nagel) following manufacturer's suggested protocol. Primers in Table 2 were used to amplify microsatellites loci by 40 cycles of PCR amplification using KAPA2G PCR mix (Kapa Biosystems) and PCR products were run on 4% agarose gel and visualized using ethidium bromide.

Karyotype analysis

Rat ES cell 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. After 1 h at 37 °C cells were washed with PBS and 2 ml of pre-warmed hypotonic potassium chloride solution was added drop by drop and incubated for 30 min at 37 °C, followed by the fixation in Carnoy solution. Analysis of chromosomal stability was performed and analyzed by the Genetics Service at Policlinica Gipuzkoa (Spain). For each cell line, 29 metaphases were photographed and the number of chromosomes were quantified. Karyotype resolution is 9 MB.

qRT-PCR gene expression analysis

Total RNA was extracted using TRIzol® reagent (Life Technologies) and 0.5 μg of total RNA was reverse-transcribed using PrimeScript™ RT reagent Kit (Takara). Complementary DNA (cDNA) underwent 40 rounds of amplification on a 7500 Real-Time PCR system (Applied Biosystems) with SYBR-Green master mix (Applied Biosystems). Primer sequences, which were designed to be 100% homologous between mouse and rat are listed in Table 3. Gene expression levels were normalized using *Gapdh* and data are presented as percentages in comparison with mouse R1 ES cell line expression (100%).

Table 2
Primers used for microsatellite analysis.

Name	Forward	Reverse
R2	CCAGAGCCTTCACTTACACG	CATCCACTTCAGTCCTGCAC
R6	TCATCTGGTGGGGACATAAC	GATGAACCAGCACATGGAAG
R36	GATTTCTCGAAAGGCTCCAC	GACAGTGAAACGGCTTTGG
R40	TTTTCGTAGTAACGGAAGCC	TAAGGATTCTCAGATGCAAATG
R47	GCACCATGCAACTTCTTCAG	CTGCTGCCTTCGGATATTAC
R76	TCACGTGTGTTCTCAAGATCC	TCATCGCCATGTTCAACTATTG
R100	TTGTAGGGCTGAAAACACTAAAG	GTGAACTGTGGGTTGACAATAAT
R122	TCCTTGATGATTTCTCATTGTG	TTATGCCTCACATGTGTGAGAC

Table 3
Primers used for qRT-PCR.

Gene	Forward	Reverse
Gapdh	CAATGCATCCTGCACCAC	CAGTGATGGCATGGACTGTG
Nanog	TACCTCAGCCTCCAGCAGAT	GCAATGGATGCTGGGATACT
Oct-4	CAGGGTCTCCGATTTGCAT	GCAGCTCAGCCTTAAGAACA
Klf4	CCAAAGAGGGGAAGAAGGTC	CGTCCCAGTCACAGTGGTAA
Esrrb1	TATCAAGGCCCTGACCACTC	GCGTATGCCAGCTTGTCATC

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). After rinsing three times with PBS to remove the unbound antibodies, samples were 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. Alkaline phosphatase activity was assayed with Sigma AB0300 kit following the manufacturer's instructions on fixed cells. Pictures were taken with Olympus CKX41 contrast phase inverted microscope.

In vivo teratoma formation assay

One million 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 (blocking solution) 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.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.03.020.

Author disclosure statement

There are no competing financial interests in this study.

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