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Lab Resource: Stem Cell Line

Derivation of the human embryonic stem cell line RCe009-A (RC-5)

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A B S T R A C T
The human embryonic stem cell line RCe009-A (RC-5) was derived from a frozen and thawed Day 2 embryo voluntarily donated as unsuitable and surplus to requirement for fertility treatment following informed consent under licence from the UK Human Fertilisation and Embryology Authority. RCe009-A carries the common DF508 mutation on the cystic fibrosis trans-membrane regulator gene associated with the disease cystic fibrosis. The cell line shows normal pluripotency marker expression and differentiation to the three germ layers in vitro. It has a normal 46XX female karyotype and microsatellite PCR identity, HLA and blood group typing data are available.

Resource Table

| Name of stem cell construct | RCe009-A |
|-----------------------------|----------|
| Alternative name            | RC-5, RC5 |
| Institution                 | Roslin Cells Ltd. |
| Person who created resource | B. Tye, K. Bruce, P. Dand, G. Russell, D. M. Collins, J. Gardner |
| Contact person and email    | Paul.desousa@roslincells.com; Paul.desousa@ed.ac.uk; Janet.downie@roslincells.com; Aidan.courtney@roslincells.com; Malcolm.bateman@roslinfoundation.com |
| Date archived/stock date    | 14 March 2008 |
| Type of resource            | Biological reagent: cell line |
| Sub-type                    | hESCs, research grade |
| Origin                      | Cleavage stage embryo cultured to blastocyst stage. |
| Key transcription factors   | Oct4 and Nanog, (confirmed by immunocytochemistry) |
| Authentication              | See Quality Control Test Summary, Table 1 |
| Link to related literature  | N/A |
| Information in public databases | http://hpscreg.eu/cell-line/RCe009-A |
| Ethics                      | http://dx.doi.org/10.1016/j.scr.2016.02.030; 1873-5061/© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/). |

RCe009-A (RC-5) was derived from a frozen and thawed Day 2 embryo voluntarily donated as unsuitable and surplus to requirement for fertility treatment following informed consent under licence from the UK Human Fertilisation and Embryology Authority. RCe009-A carries the common DF508 mutation on the cystic fibrosis trans-membrane regulator gene associated with the disease cystic fibrosis. The cell line shows normal pluripotency marker expression and differentiation to the three germ layers in vitro. It has a normal 46XX female karyotype and microsatellite PCR identity, HLA and blood group typing data are available.

Verification and authentication

The cell line was analysed for genome stability by G-banding (Fig. 4) and showed a normal 46XX female genotype. The cell line is free from mycoplasma contamination as determined by RC-qPCR. Microsatellite PCR DNA profiling for cell identity is available.

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Materials and methods

Ethics

Derivation of hESC from surplus to requirement and failed to fertilise/develop embryos was approved by The Scotland A Research Ethics Committee and local ethics board at participating fertility clinics and conducted under licence no. R0136 from the UK HFEA with informed donor consent.

Cell culture

Frozen embryos were thawed using Embryo Thawing Pack (Origio (Medicult), Denmark) using standard techniques and were cultured in EmbryoAssist (Origio) until Day 3 and BlastAssist (Origio) after Day 3 of development. Embryos were cultured at 36.5–37.5 °C, 5 ± 0.5% CO₂, 5 ± 0.5% O₂ in drops under paraffin oil (Origio) and transferred to fresh medium at least every 2–3 days.

By Day 8 of development, or when spontaneous hatching occurred, embryos were placed in derivation conditions consisting of mitotically inactivated neonatal human dermal fibroblasts (HDFs) (ThermoFisher Scientific (Cascade Biologics), Paisley, UK) on tissue culture plastic pre-coated with 2 μg/cm² human laminin (Sigma Aldrich, Dorset, UK) as per manufacturer’s recommendation. If required, assisted hatching was performed by removing the zona pellucidae mechanically using Swemed Cutting tools (Vitrolife, Göteborg, Sweden).

HDF cells were cultured in DMEM (Lonza, Slough, UK), 10% FCS (GE Healthcare (PAA), Buckinghamshire, UK) and 2 mM L-glutamine (ThermoFisher Scientific). HDF were mitotically inactivated using gamma irradiation at 50Gy using a Gammacell Elite 1000 machine. For use as a feeder layer, irradiated HDFs were plated at 2–50,000 cells/cm² in HDF conditioned medium (80% Knockout-DMEM, 20% Knockout serum replacement (KOSR), 1 mM glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/ml human bFGF (all ThermoFisher Scientific) over 24 h intervals over 7 days) supplemented with an additional 24 ng/ml human bFGF. Cells were cultured at 36.5–37.5 °C, 5 ± 0.5% CO₂, 5 ± 0.5% O₂ and 50% medium exchanged 6 days a week.

The established cell line was expanded and banked using CellStart matrix and Stempro hESC Serum Free Medium (ThermoFisher Scientific) until the last passage before cryopreservation.

Table 1
Summary of quality control testing and results for RC-5 (RCe009-A).

| Classification | Test | Purpose | Result |
|---------------|------|---------|--------|
| Donor screening | HIV 1 + 2, Hepatitis B, Hepatitis C | Donor screening for adventitious agents | Negative |
| Identity | Microsatellite PCR (mPCR) | DNA Profiling to give cell line its signature, gender/species | Performed |
| Phenotype | Immunocytochemistry | To assess levels of staining for the pluripotency markers, SSEA-4, Tra-1-60 and Tra-1-81 | Expression of Oct4, Nanog, SSEA-4, Tra-1-60 and Tra-1-81 |
| Flow cytometry | | Assess antigen levels and cell surface markers commonly associated with hESC | Tra-1-60: 94.8%, Tra-1-81: 93.6%, SSEA-4: 94.8%, SSEA-1: 37.3% |
| Genotype | Blood group genotyping (DNA analysis) | To establish blood group of the line | AO1 |
| Karyology (G-banding) | | Confirmation of normal ploidy by G-banding | 46XX |
| HLA tissue typing | | To establish full HLA Type I and II genotype of the line | HLA typed Class I and Class II |
| Microbiology and Virology | Mycoplasma | Mycoplasma testing by RT-qPCR | Negative |
| Endotoxin | | Screening for endotoxin levels | 1.82 EU/mL |
| Morphology | Photography | To capture a visual record of the line | Normal |
| Differentiation potential | Embryoid body formation | To show differentiation to three germ layers | Expression of muscle actin, β-tubulin and α-fetoprotein |

Fig. 1. Immunostaining of RCe009-A (RC5) show expression of pluripotency markers Oct-4, Nanog, Tra-1-60, Tra-1-81 and SSEA-4, but not differentiation marker SSEA-1.
Scientific). Passaging was performed mechanically using an EZ passage tool (ThermoFisher Scientific). hESC lines were expanded to 25–30 wells of a 6-well plate and cryopreserved in 0.5-1 ml KOSR based cryopreservation solution (75% KO-DMEM, 15% Xeno-free KOSR (ThermoFisher Scientific) and 10% DMSO (Origen Biomedical, Texas, USA)) or Cryostor CS10 (Biolife Solution, Washington, USA).

**Mycoplasma**

Mycoplasma detection was performed using Applied Biosystems PrepSEQ™ Mycoplasma Nucleic Acid Extraction Kit and MicroSEQ™ Mycoplasma Real-Time PCR Detection Kit (ThermoFisher Scientific (Applied Biosystems)) according to manufacturer’s instruction.

**Endotoxin**

Endotoxin levels were determined using the Kinetic-QCL assay (Lonza) and an incubating plate reader (BioTek ELx808) according to manufacturer’s instructions. Briefly, an unknown sample was compared with a standard curve of known levels of control endotoxin. An assay was deemed valid if the coefficient of correlation, \( r \geq 0.980 \) and the CV (%) for the standard curve was \( \leq 10\% \).

**Flow cytometry**

Human embryonic stem cells were dissociated using Trypsin (ThermoFisher Scientific). Non-specific staining was blocked using 5% goat serum (Sigma) in PBS (Lonza) containing 0.01% Tween-20 (Sigma). Cells were stained with antibodies against SSEA-4, SSEA-1, Tra-1-60 and Tra-1-81 (all BD, Oxford, UK), at 250 ng per reaction followed by Goat F(ab)2 anti-mouse IgM-PE Goat F(ab)2 anti-mouse IgG3-FITC (1:200; Santa Cruz Biotechnology, Texas, USA). Cells were analysed using a FACS Aria flow cytometer (BD).

**Immunocytochemistry**

hESC were fixed in 4% paraformaldehyde (ThermoFisher Scientific (Alfa Aesar)), permeabilised using 100% ethanol (ThermoFisher Scientific), muscle-specific actin (1:100; DAKO, Glostrup, Denmark), Oct-4 (1:200; Santa Cruz Biotechnology, Texas, USA), Nanog (1:20; R&D Systems, Abingdon, UK), Tra-1-60, Tra-1-81, SSEA-1 and SSEA-4 (all 1:50; BD) and secondary antibodies anti-mouse IgG-FITC (1:200; Sigma), anti-mouse IgG-AlexaFluor 488, anti-goat IgG-AlexaFluor 488, anti-goat IgG-AlexaFluor-594 and anti-donkey polyclonal AlexaFluor-594 (all 1:200; ThermoFisher Scientific). Images were acquired using a Zeiss S100 Axiovert fluorescence microscope or Nikon eC1 confocal microscope.

**In vitro differentiation**

hESC cells were pre-treated for 1 h with 10 μM ROCK inhibitor in Stempro hESC SFM (ThermoFisher Scientific) and embryoid bodies EBs generated in ultra low attachment plates (Corning) for 7 days before being transferred into EB medium (20% FBS (GE Healthcare (PAA)), 80% KO-DMEM 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids (all ThermoFisher Scientific), on glass slide tissue culture chambers (Nunc, ThermoFisher Scientific) coated with 0.5% gelatin (Sigma) at 0.1 ml/cm² for 14 days.

**Fig. 2.** RCe009-A (RC-5) was subjected to flow cytometry analysis for markers of pluripotency with isotype controls (left hand column) or specific antibodies for SSEA-1, Tra-1-60 and Tra-1-81 (top row) or SSEA-4 (bottom row). Percentage staining is indicated in Table 1.

**Fig. 3.** In vitro differentiation of RCe007-A (RC-3) to ectoderm (β-tubulin III), mesoderm (muscle actin), and endoderm (α-fetoprotein). Specific staining shown in green, cell nuclei are counterstained with DAPI (blue).
Genomic analysis

All outsourced assays were carried out under a Quality and Technical Agreement. DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Manchester, UK) according to manufacturer’s recommendations and provided in recommended quantities to the service providers.

Microsatellite PCR, or Short Tandem Repeat analysis, was used to determine cell line identity and was carried out by Public Health England. A profile was obtained for the following core alleles: vWA, D16S539, Amelogenin, THO1, CSF1PO, D5S818, D7S820, D13S317 and TPOX.

Human Leukocyte Antigen (HLA) tissue typing was carried out by the Scottish National Blood Transfusion Service.

Table 2
Microsatellite PCR, blood group and HLA tissue typing results for RCe009-A (RC-5).

| Microsatellite PCR results |
|---------------------------|
| D3S1358 1 18              |
| D3S1358 2 18              |
| vWA 1 16                  |
| vWA 2 17                  |
| D16S539 1 9               |
| D16S539 2 11              |
| D2S1338 1 17              |
| D2S1338 2 21              |
| Amelogenin 1 X            |
| Amelogenin 2 X            |
| D8S1179 1 13              |
| D8S1179 2 14              |
| D21S11 1 29               |
| D21S11 2 30               |
| D18S51 1 10               |
| D18S51 2 15               |
| D19S433 1 14.2            |
| D19S433 2 15              |
| THO1 1 8                  |
| THO1 2 9                  |
| FGA 1 21                  |
| FGA 2 25                  |
| CSF1PO 1 10               |
| CSF1PO 2 10               |
| DSS818 1 11               |
| DSS818 2 12               |
| D7S820 1 10               |
| D7S820 2 11               |
| D13S317 1 8               |
| D13S317 2 13              |
| TPOX 1 8                  |
| TPOX 2 11                 |

Blood group genotyping

| Blood group genotyping                   |
|-----------------------------------------|
| RhD pos                                 |
| neg                                     |
| RhC pos                                 |
| Rhc pos                                 |
| RbE pos                                 |
| Rhe pos                                 |
| Fy a pos                                |
| Fy b pos                                |
| Fy GATA pos                             |
| Jka pos                                 |
| Jkb pos                                 |
| K neg                                   |
| k pos                                   |
| M pos                                   |
| N pos                                   |
| S neg                                   |
| S pos                                   |
| Kp a pos                                |
| Kp b pos                                |
| Do a pos                                |
| Do b pos                                |
| ABO pos                                 |
| AO1 pos                                 |
| HLA Tissue Typing                      |
| HLA Class I Type                       |
| HLA Class II Type                      |
| Comment                                 |
| HLA-A*01, A*32; B*40, B*57; C*02, C*06 |
| HLA-DRB1*13; DRB3*02; DQB1*06           |
| B*40 is expressed serologically as B61 |

Fig. 4. RCe009-A (RC-5) was analysed by Giesma staining of 20 metaphase spreads and showed a normal 46XX female karyotype.
Blood group genotyping was carried out by the Molecular Diagnostics laboratory at NHSBT.

Karyotype analysis was carried out by The Doctors Laboratory (London, UK) or the Western General Cytogenetics Laboratory (Edinburgh, UK). Live cells at 60–70% confluency were shipped overnight in warm containers, fixed and analysed by standard G-banding analysis. For research grade lines, 20 spreads were analysed.

Acknowledgements

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