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Lab Resource: Stem Cell Line

Derivation of the human embryonic stem cell line RCe008-A (RC-4)

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ABSTRACT

The human embryonic stem cell line RCe008-A (RC-4) was derived from a blastocyst voluntarily donated as unsuitable and surplus to fertility requirements following ethics committee approved informed consent under licence from the UK Human Fertilisation and Embryology Authority. The cell line shows normal pluripotency marker expression and differentiation to ectoderm and mesoderm *in vitro*. It has a mixed 46XX/45X female karyotype and microsatellite PCR identity and blood group typing data is available.

Resource table.

Name of stem cell construct	RCe008-A
Alternative name	RC-4, RC4
Institution	Roslin Cells Ltd.
Person who created resource	B. Tye, K. Bruce, P. Dand, 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	11 June 2008
Type of resource	Biological reagent: Cell line
Sub-type	hESC, research grade
Origin	Blastocyst with ICM and Trophoblast
Key transcription factors	Oct4 and Nanog (confirmed by immunocytochemistry)
Authentication	See Quality Control Test Summary, Table 1
Link to related literature (direct URL links and full references)	N/A
Information in public databases	http://hpscreg.eu/cell-line/RCe008-A
Ethics	Informed consent obtained. Scotland A Research Ethics committee approval obtained (07/MRE00/56). Conducted under the UK Human Fertilisation and Embryology Authority licence no R0136 to centre 0202.

Resource details

RCe008-A (RC-4) was derived from a fresh blastocyst that was surplus to requirement or unsuitable for clinical use. The cell line was derived by whole embryo outgrowth on a chemically defined matrix consisting of laminin, fibronectin, Collagen IV and vitronectin (Ludwig et al., 2006) using human fibroblast (HDF) conditioned medium and expanded under feeder free conditions.

RCe008-A (RC-4) was shown to be pluripotent by expression of Oct-4 and Nanog using immunocytochemistry (Table 1, Fig. 1). By flow cytometric analysis, the expression of pluripotency markers Tra-1-60, Tra-1-81 and SSEA-4 was 74%, 58% and 75%, respectively, whereas low expression of SSEA-1 (5%) was observed (Fig. 2). Germinal lineage marker expression is not available due to poor EB formation.

A microsatellite PCR profile has been obtained for the cell line and blood group genotyping gave the blood group AO_1 (Table 2).

Verification and authentication

The cell line was analysed for genome stability by G-banding (Fig. 3) and showed a mixture of a normal female karyotype (46XX in 16 cells) and monosomy X (45X in 3 cells). The cell line is free from mycoplasma contamination as determined by RT-qPCR.

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.

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Table 1
Summary of quality control testing and results for RCe008-A (RC-4).

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
	Immunocytochemistry	To assess levels of staining for the pluripotency markers.	Expression of Oct-4 and Nanog
Phenotype	Flow cytometry	Assess antigen levels & cell surface markers commonly associated with hESC	CD9: 84% Tra 1-60: 74% Tra 1-81: 58% SSEA-4: 75% SSEA-1: 5%
Genotype (details provided in Table 2)	Blood group genotyping (DNA analysis)	To establish blood group of the line	AO_1
	Karyology (G-banding)	Confirmation of normal ploidy by G-banding	46XX (16)/45X (3)
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-qPCR	Negative
	Endotoxin	Screening for endotoxin levels	1.87 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	Poor EB formation — germinal linage marker expression not available

Cell culture

Fresh embryos were cultured EmbryoAssist (Origio (Medicult), Denmark) until Day 3 or BlastAssist (Origio) after Day 3 of development. Embryos were cultured at 36.5–37.5 °C, 5 ± 0.5% CO_2, 5 ± 0.5% O_2 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 a mixture of laminin (5 μg/cm^2; Sigma Aldrich, Dorset, UK), fibronectin (5 μg/cm^2; BD, Oxford, UK), vitronectin (0.2 μg/cm^2; BD), and collagen IV (10 μg/cm^2; Sigma) (Ludwig et al., 2006) in human dermal fibroblast (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, Paisley, UK) over 24 h intervals over 7 days) supplemented with an additional 24 ng/ml human bFGF. If required, assisted hatching was performed by removing the zona pellucidae mechanically using Swemed cutting tools (Vitrolife, Göteborg, Sweden). Cells were cultured at 36.5–37.5 °C, 5 ± 0.5% CO_2, 5 ± 0.5% O_2 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). Passaging was performed mechanically using an EZ passage tool

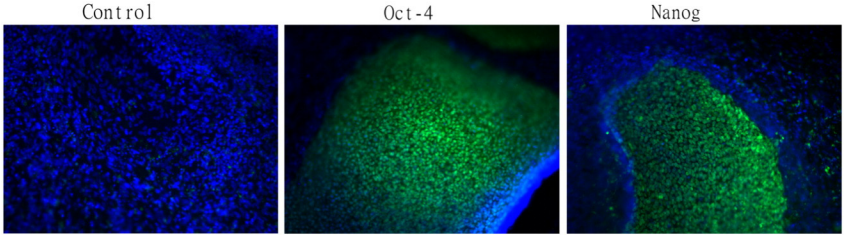

Fig. 1. RCe008-A (RC-4) expresses pluripotency markers Oct-4 and Nanog. Cell nuclei are counterstained with DAPI (blue).

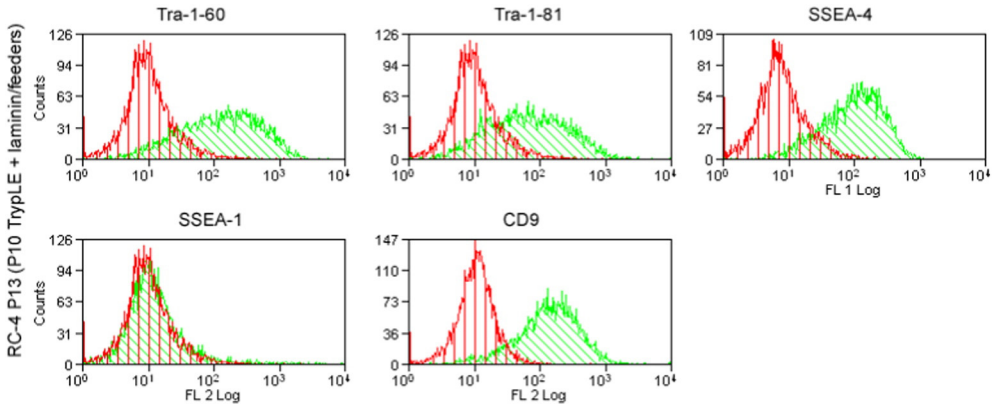

Fig. 2. Flow cytometry staining of RCe008-A (RC-4) at Passage 13. Isotype/negative control is shown in red, specific staining in green. Percentage staining is indicated in Table 1.

Table 2
Microsatellite PCR, blood group and HLA tissue typing results for RCe008-A (RC-4).

Microsatellite PCR results							
D3S1358 1	D3S1358 2	vWA 1	vWA 2	D16S539 1	D16S539 2	D2S1338 1	D2S1338 2
125	129	167	170	281	281	336	348
Amelogenin 1	Amelogenin 2	D8S1179 1	D8S1179 2	D21S11 1	D21S11 2	D18S51 1	D18S51 2
X	X	145	150	209	215	ND	ND
D19S433 1	D19S433 2	THO1 1	THO1 2	FGA 1	FGA 2	CSF1PO 1	CSF1PO 2
121	121	180	184	237	245	325	329
D5S818 1	D5S818 2	D7S820 1	D7S820 2	D13S317 1	D13S317 2	TPOX 1	TPOX 2
152	152	272	276	218	222	231	231
Blood group genotyping							
RhD	RhC	Rhc	RhE	Rhe	Fy a	Fy b	Fy GATA
pos	neg	pos	pos	pos	pos	pos	neg
Jka	Jkb	K	k	M	N	S	s
neg	pos	neg	pos	pos	pos	neg	pos
Kp a	Kp b	Do a	Do b	ABO			
neg	pos	neg	pos	AO1			

ND = not determined

(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 the manufacturer's instruction.

Endotoxin

Endotoxin levels were determined using the Kinetic-QCL assay (Lonza) and an incubating plate reader (BioTek ELx808) according to the 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 ≤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) and stained with AFP (1:500; Sigma), β-tubulin III (1:1000; Sigma), muscle-specific actin (1:50; DAKO, Glostrup, Denmark), Oct-4 (1:200; Santa Cruz Biotechnology, Texas, USA), Nanog (1:20; R&D Systems, Abingdon, UK) 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 donkey polyclonal AlexaFluor-594 (all 1:200; ThermoFisher Scientific). Images were

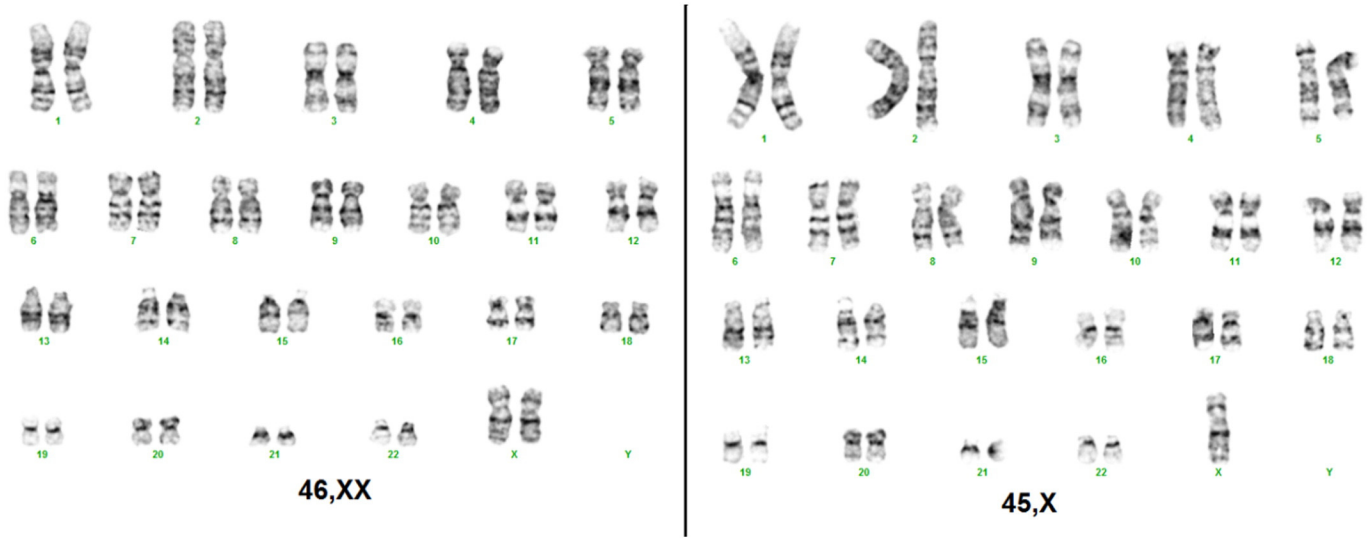

Fig. 3. RCe008-A (RC-4) was analysed by Giesma staining of 20 metaphase spreads and showed a mixed karyotype. The majority of cells analysed (16) showed a normal 46XX karyotype, but a subpopulation (3 cells) showed 45X (monosomy X).

acquired using a Zeiss S100 Axiovert fluorescence microscope or Nikon eC1 confocal microscope.

In vitro differentiation

hESCs 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/cm^2 for 14 days.

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 the 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, D75820, D135317 and TPOX.

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

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.

Acknowledgments

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References

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