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Lab Resource: Stem Cell Line

Establishment of a rabbit induced pluripotent stem cell (RbiPSC) line using lentiviral delivery of human pluripotency factors

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ABSTRACT

Rabbit Embryonic Fibroblast (RbEF) cells (from Hycole hybrid rabbit foetus) were reprogrammed by lentiviral delivery of a self-silencing hOKSM polycistronic vector. The pluripotency of the newly generated RbiPSC was verified by the expression of pluripotency-associated markers and by in vitro spontaneous differentiation towards the 3 germ layers. Furthermore, the spontaneous differentiation potential of the iPSC was also tested in vivo by teratoma assay. The iPSC line showed normal karyotype. The advantages of using RbiPSC are the easy access to primary material and the possibility to study the efficacy and safety of the iPSC-based therapies on a non-rodent animal model.

Resource table

Name of Stem Cell line	RbiPSC
Institution	BioTalentum Ltd.
Person who created resource	András Dinnyés
Contact person and email	andras.dinnyes@biotalentum.hu
Date archived/stock date	December 2014
Origin	Rabbit Embryonic Fibroblast
Type of resource	Rabbit induced pluripotent stem cell
Sub-type	Induced pluripotent stem cells
Key transcription factors	hOCT4, hSOX2, hC-MYC, hKLF4
Authentication	Identity and purity of the cell line was confirmed by the following analyses: karyotyping, expression analysis of pluripotency markers, in vitro differentiation potential and teratoma assay
Link to related literature	http://dx.doi.org/10.1016/j.theriogenology.2012.06.017 http://dx.doi.org/10.1016/j.gene.2015.04.034
Information in public databases	N/A
Ethics	The protocols for animal care and handling were approved by the Regional Animal Health Authorities, Pest County, Hungary, and by the Joint Local Ethics and Animal Welfare Committee of Biotalentum Ltd./Immunogenes Ltd./Soft Flow Biotechnology Ltd.

Resource details

RbEF was isolated from day 12 foetuses and maintained under standard conditions. The RbEFs were reprogrammed with lenti-viral delivery using the pRRL.PPT.SF.hOKSMco.idTomato.preFRT (Voelkel et al., 2010; Warlich et al., 2011) polycistronic vector, containing human reprogramming factors (OCT4, SOX2, C-MYC, and KLF4), driven by SFFV retroviral promoter. The construct was introduced into RbEF cells by transduction. The newly generated rabbit iPSCs closely resembled to that of the human iPSC's morphology and culture requirements: they formed flattened colonies and proliferated indefinitely in the presence of bFGF. RbiPSC-like colonies were picked 18–21 days post-transduction. Stable lines were maintained and based on morphology criteria were chosen for further examination. The mycoplasma-free status of the cell line was confirmed regularly by the Venor GeM Advance detection kit (Minerva Biolabs, Berlin, Germany).

The pluripotency of the RbiPSC line was examined by immunocytochemistry staining, using antibodies against human OCT4, SOX2, and SSEA4 (Fig. 1A). The in vitro spontaneous differentiation potential towards the three germ layer of RbiPSC line was demonstrated by the expression of ectodermal (βIII-TUBULIN), mesodermal (SMOOTH MUSCLE ACTIN) and endodermal (GATA4) markers (Fig. 1A). Moreover, RbiPSC formed teratomas in vivo when injected into SCID mice (Fig.1B). The karyotype of the RbiPSC line was determined with Giemsa-banding,

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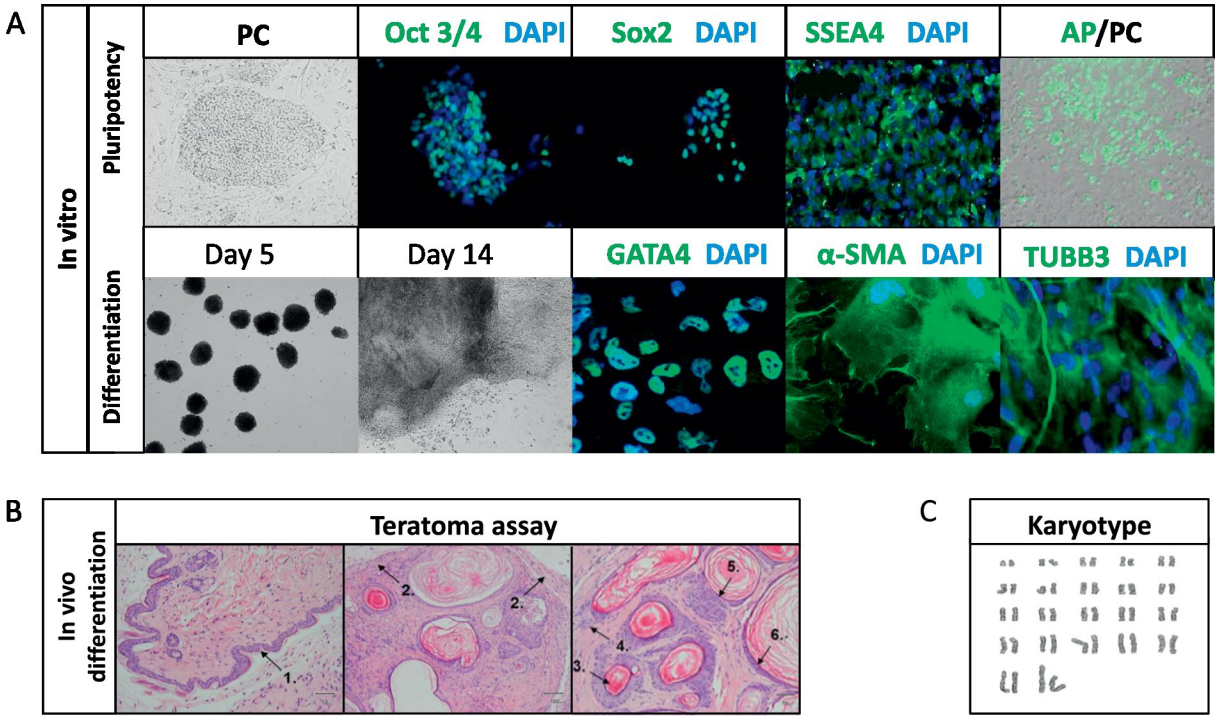

Fig. 1. Characterisation of RbiPSCs line A) Above: Morphology of generated RbiPSCs (4×) and representative immunofluorescent micrographs of iPSCs positive for stem cells markers OCT4, SOX2 and SSEA4 (in green), Nucleus is labelled with DAPI (in blue). Alkaline phosphatase (AP) activity was detected with live staining. Below: Spontaneously formed EBs (Day 5) and their further differentiation (Day 14). Immunostainings for endodermal (GATA4), mesodermal (α-SMA, SMOOTH MUSCLE ACTIN) and ectodermal (TUBB3, βIII-TUBULIN) germ layer markers (in green). Nucleus is labelled with DAPI (in blue). B) In vivo differentiation potential by Teratoma assay: Hematoxylin-eosin staining revealed endoderm (1 respiratory ciliated epithelium), mesoderm (skeletal muscle) and ectoderm (3 keratinized epithelium, 4 sebaceous, 5 stratum corneum, 6 keratohialin granules) tissues. C) Karyogram of RbiPSC is showing normal 44 chromosomes (XX) (Magnification: 120×; resolution: 300 dpi).

proving normal diploid 44, XX karyotype, without any detectable abnormalities (Fig. 1C).

Materials and methods

Experimental procedures

The chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and all the culture reagents from Thermo Fisher Scientific (Waltham, MA, USA), unless otherwise specified.

Cell culture

All cells were cultured at 38 °C in humidified atmosphere containing 5% CO_2. The embryonic day 12 RbEF cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 40 ng/ml bFGF. The fibroblast cells were passaged by 0.05% trypsin-EDTA and seeded in 1×10^5 cells/cm^2 density 1 day before transduction. The RbiPSCs were cultured in iPSM (DMEM/Ham's F-12 supplemented with 20% knock-out serum replacement (KSR), 2 mM GlutaMax, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 4 ng/ml human bFGF and 10^3 units/ml ESGRO LIF (murine leukemia inhibitory factor; Millipore) supplemented with 4 ng/ml bFGF, changed daily and passaged by 0.05% trypsin- EDTA every 3–4 days.

Reprogramming

For transduction 3×10^4 RbEFs were seeded into a well of a 24-well plate on the day of transduction. 6 h later the virus-containing supernatant was diluted in 1 ml iPSM medium supplemented with polybrene (final concentration: 8 μg/ml). The MOI (Multiplicity of Infection) was between 1 and 3. Two days post- transduction the RbEF cells were treated with 0.05% trypsin-EDTA and plated on mitomycin C-treated MEFs in iPSM medium (6000 cells/cm^2). The medium was refreshed daily. The appearing ES-like colonies were manually picked (Day 25–30) onto MEF and cultured as iPSCs thereafter.

Immunocytochemistry and AP staining

Cells were fixed in 4% paraformaldehyde (PFA) for 30 min at RT, then permeabilized in PBS containing 0.1% Triton X-100 (Sigma) for 5 min and blocked by 3% bovine serum albumin for 1 h at RT. The cells were incubated with primary antibodies (overnight at 4 °C) and visualized with secondary antibodies. For nuclei counterstaining 0.2 μg/ml DAPI (20 min, RT) was used. The antibodies and applied dilutions are listed in (Table 1). For detecting alkaline phosphatase (AP) activity, RbiPSCs were stained using AP live stain kit (Life Technologies) according to the manufacturer's protocol. The cells were observed under fluorescent microscope equipped with 3D imaging module, (Axio Imager system with ApoTome; Carl Zeiss) controlled by AxioVision 4.8.1 microscope software (Carl Zeiss).

Karyotyping

Cells were treated with KaryoMAX® Colcemid™ Solution (Thermo Fisher Scientific) and processed with standard methods. Standard Giemsa-banded karyotype was performed and minimum of 20 metaphases were analyzed.

In vitro differentiation

The RbiPSCs were treated with 0.05% trypsin-EDTA, resuspended in differentiation medium (DMEM/Ham's F-12, 20% KSR, 2 mM GlutaMax, 1% nonessential amino acids, and 0.1 mM β-mercaptoethanol

Table 1
Antibodies used for immunocytochemistry.

	Antibody	Dilution	Company (Cat #)
Pluripotency	mouse anti-SOX2	1:200	Santa Cruz Biotechnologies; (sc-365823)
	mouse anti-OCT4	1:50	Santa Cruz Biotechnologies; (sc-5279)
	mouse anti-SSEA4	1:100	Hybridoma Bank; (MC-813-70)
In vitro differentiation	rabbit anti-β-III-TUBULIN	1:2000	Covance (PRB-435P)
	rabbit anti-SMOOTH MUSCLE ACTIN	1:100	Abcam (ab5694)
	mouse anti-GATA4	1:50	Santa Cruz Biotechnologies; (sc-25310)
Secondary antibodies	Alexa Fluor 488 donkey anti-mouse IgG	1:2000	Thermo Fisher Scientific (A-21202)
	Alexa Fluor 488 donkey anti-rabbit IgG	1:2000	Thermo Fisher Scientific (A-21206)

supplemented freshly with 4 ng/ml bFGF, and 50 ng/ml activin A (R & D systems), and cultured in low cell binding Petri-dishes (Nunc). After 5 days the Embryoid Bodies (EBs) were plated on 0.1% gelatin covered cover slips in differentiation medium and fixed with 4% PFA on Day 14 of differentiation.

In vivo differentiation

For teratoma assay 1–2 × 10^6 RbiPSCs were injected under the kidney capsule of five- to eight-week-old SCID mice. After four to eight weeks teratomas were dissected and fixed in 4% PFA (overnight at 4 °C), then embedded in paraffin with standard methods. Paraffin wax sections were stained with hematoxylin and eosin. Tissue sections were analyzed histologically.

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