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Generation of a Rhesus Macaque induced Pluripotent Stem Cell Line (riPSC05) under Feeder-Free Conditions

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Abstract

We generated and characterized a rhesus macaque induced pluripotent stem cell (iPSC) line using induced reprogramming of fibroblasts isolated from a rhesus macaque fetus. The fibroblasts were expanded and then reprogrammed using non-integrating Sendai virus technology. This line is available as riPSC05. The authenticity of riPSC05 was confirmed through the expression of pluripotent and self-renewal markers, *in vitro-directed* differentiation towards three germ layers (ectoderm, mesoderm, and endoderm), karyotyping, and STR analysis.

2. Resource utility

The rhesus macaque induced pluripotent stem cell line riPSC05 was generated and maintained under feeder-free conditions using a commercially available xeno-free matrix, serum-free media, and a non-enzymatic passaging reagent. These cells have the potential for use in the fields of regenerative medicine, disease modelling, or may serve as a valuable resource for testing new cell therapies in a relevant pre-clinical model system.

3. Resource details

Rhesus macaques (*Macaca mulatta*) are nonhuman primates which have commonly been used as a translational model to address human health concerns. Despite this, a relatively small number of well-characterized rhesus macaque induced pluripotent stem cell lines (riPSC) are currently available and even fewer have been generated in defined feeder-

Declaration of competing interest

None of the authors have any financial conflicts or interests to report regarding the publication of the contents of the manuscript.

free conditions. Previously established riPSC lines were derived and cultured on mouse embryonic fibroblasts (MEFs) (Sosa et al., 2018), however MEFs introduce risks of cross contamination (Llames et. al., 2015). Therefore, in this study we established a feeder-free rhesus iPSC line that was generated and maintained, in a chemically defined medium and a xeno-free matrix.

Rhesus macaque fibroblast cells called REF05 (Figure 1A) was derived from fetal skin obtained from the trunk of a male Day 100 rhesus macaque collected at the Oregon National Primate Research Center. Using the REF05 cells, riPSC05 lines were generated using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit containing the reprogramming factors OCT3/4, SOX2, KLF4, and c-MYC. Twenty-one days after transduction, individual colonies were manually picked onto vitronectin™ coated plates, and expanded. The riPSC05 line exhibited a typical iPSC morphology (Figure 1B) which was determined to have a normal 42, XY karyotype through G-band karyotyping (Figure 1C). Next, to confirm that the riPSC05 line originated from REF05 fibroblasts, a short tandem repeat (STR) analysis was performed which resulted in 30 matching loci (Supplementary Figure 1A). In order to quantitatively assess riPSC05's expression of markers of self-renewal, we performed Fluorescence-activated Cell Sorting (FACs) and confirmed that 99.5% of cells expressed the cell surface protein SSEA-4 (Figure 1D). We also performed quantitative RT-PCR for the pluripotency gene POU5F1, which was found to be highly expressed in riPSC05 as compared to the differentiated control (Figure 1E). To qualitatively access riPSC05's expression of markers of self-renewal, we performed immunofluorescence, and confirmed that riPSC05 cells expressed the transcription factors NANOG and OCT4 as well as the surface proteins TRA-1-81 and SSEA-4 (Figure 1F). Furthermore, to confirm elimination of the transgenes used for reprogramming, we performed RT-PCR on the riPSC05 line which was negative compared to newly transfected REF05 cells as a control which were positive (Figure 1G). To assess riPSC05's capacity for differentiation, we used an established protocol from cells cultured in E8™ (Lin and Chen, 2014) to create embryoid bodies (EBs). After 14 days of culture, EBs were collected and quantitative RT-PCR analysis was performed to confirm the expression of genes involved in ectoderm (*NES* and *OTX2*), endoderm (*FOXA2* and *SOX17*), and mesoderm (*EOMES* and *TBXT*) formation as compared to the undifferentiated riPSC05 controls (Figure 1H). Routine mycoplasma testing was done to confirm riPSC05 was negative for mycoplasma (Supplementary Figure 1B).

4. Materials and methods

4.1 Reprogramming

Rhesus macaque fibroblasts called REF05 were culture in 6-well plates using fibroblast medium (DMEM (Gibco, cat. 11965092), 10% Fetal Bovine Serum (Gibco, cat. 26140079) 1X Penicillin-Streptomycin-Glutamine (Gibco, cat. 10378016), 1X Primocin® (InvivoGen, cat. ant-pm-05)). Using the CTS™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher, cat. A16517), 200,000-300,000 fibroblasts were transduced with Sendai viral vectors expressing human OCT3/4, SOX2, KLF4, and c-Myc based on manufacturer's instructions. The cells were cultured for 6 days, with media changes occurring every other day. On day 7, cells were dissociated using 0.05% trypsin/EDTA (Gibco, cat. 25300054) and seeded into

vitronectin (Thermo Fisher, cat. A31804) coated plates. After 24 hours, the cell culture medium was switched to Essential 8™ (E8) medium (Thermo Fisher, cat. A1517001) and changed every day thereafter. After 21 days, emerging colonies with stem cell-like morphology were manually isolated and seeded on a 6-well plate coated in vitronectin. After 7 days, the cells were passaged using ReLeSR™ and cultured in vitronectin coated plates with E8™ medium.

4.2 Stem cell culture

Rhesus iPS cells were culture on vitronectin in standard culture conditions (37°C and 5% CO_2) using E8™ medium. Rhesus iPS cells were passaged at 80% confluency after 5-7 days in culture using ReLeSR™.

4.3 Viral vector clearance

RNA was isolated using the RNeasy® Micro Kit (Qiagen, cat. 74004) from transduced fibroblasts at passage 0 (P0) and from riPSC05 after 16 passages (P16). cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, cat. 18064014) and PCR products generated using the primers provided by the manufacturer (Table 2) were analyzed by agarose gel electrophoresis.

4.4 Immunofluorescence and imaging

Immunofluorescence staining of the riPSC05 cell line was performed as previously published (Sosa et al., 2016). riPSC05 colonies (passage 11) were plated onto a 4-well Falcon™chambered cell culture slide (Thermo Fisher, cat. 08-774-25). Once cultures were 80% confluent (~day 3-4), colonies were fixed in 4% PFA, permeabilized with PBS plus 0.5% Triton™ X-100, then washed in PBST (PBS with 0.1% Tween-20). Primary antibodies consisted of OCT4, NANOG, SSEA4, TRA-1-81, and were incubated overnight at 4°C. Secondary antibodies consisted of AF594 and AF488 and were incubated for 1 hour at room temperature. Samples were mounted using ProLong™ Gold antifade reagent (Invitrogen P36934). Samples were imaged using an LSM880 (Zeiss) confocal laser scanning microscope and images were processed using the image analysis program IMARIS (Bitplane).

4.5 Flow cytometry

After 18 passages, cells were dissociated into a single cell suspension using 0.05% Trypsin™ (Thermo Fisher, cat. 25300054). riPSC05 were incubated with the SSEA-4 antibody for 30 minutes, washed, then resuspended in FACs buffer. Unstained riPSC05s were used as our control. Sorting was done on a BD FACSAria II cell sorter.

Flow plots were created using FlowJo™ software.

4.6 Karyotype analysis and STR analysis

riPSC05 was karyotyped after 8 passages using metaphase spreads and G-banding by Cell Line Genetics (Madison, WI). STR Analysis was performed on genomic DNA isolated from frozen cells of REF05(RES3809) and riPSC05(RES3810) lines by the UC Davis Veterinary Genetics Laboratory.

4.7 Embryoid body formation assay

After reaching 60-70% confluency, rhesus iPS cells were dissociated using EDTA/PBS for 10-15 minutes, then neutralized with E8™/Polyvinyl Alcohol (PVA), washed, then resuspended with E8™/PVA. Cells were then transferred to an AggreWell-800 plate (STEMCELL™ Technologies cat. 34811) at a concentration of 1.6 x 10^6 /1.5mL in each well (5000 cells/microwell) and cultured in DMEM/F12 at 37°C and with 5% CO_2. After 36 hours, embryoid bodies (EBs) were removed from the AggreWell-800 plate and transferred into low attachment dishes (Corning, cat. 3261). EBs were then cultured in Essential 6™ (E6) medium for 9-14 days, with medium changed every 2 days. cDNA was synthesized from undifferentiated riPSC05 cells and D14 EBs.

TaqMan™ gene expression Assays (Table 2) were used to detect the expression of marker genes for ectoderm, endoderm, and mesoderm differentiation on a Bio-Rad CFX96 Real-time detection system.

4.8 Mycoplasma testing

Mycoplasma contamination was assessed using MycoAlert™ Mycoplasma Detection Kit (Lonza, cat. LT07-318) at Passage 11 according to manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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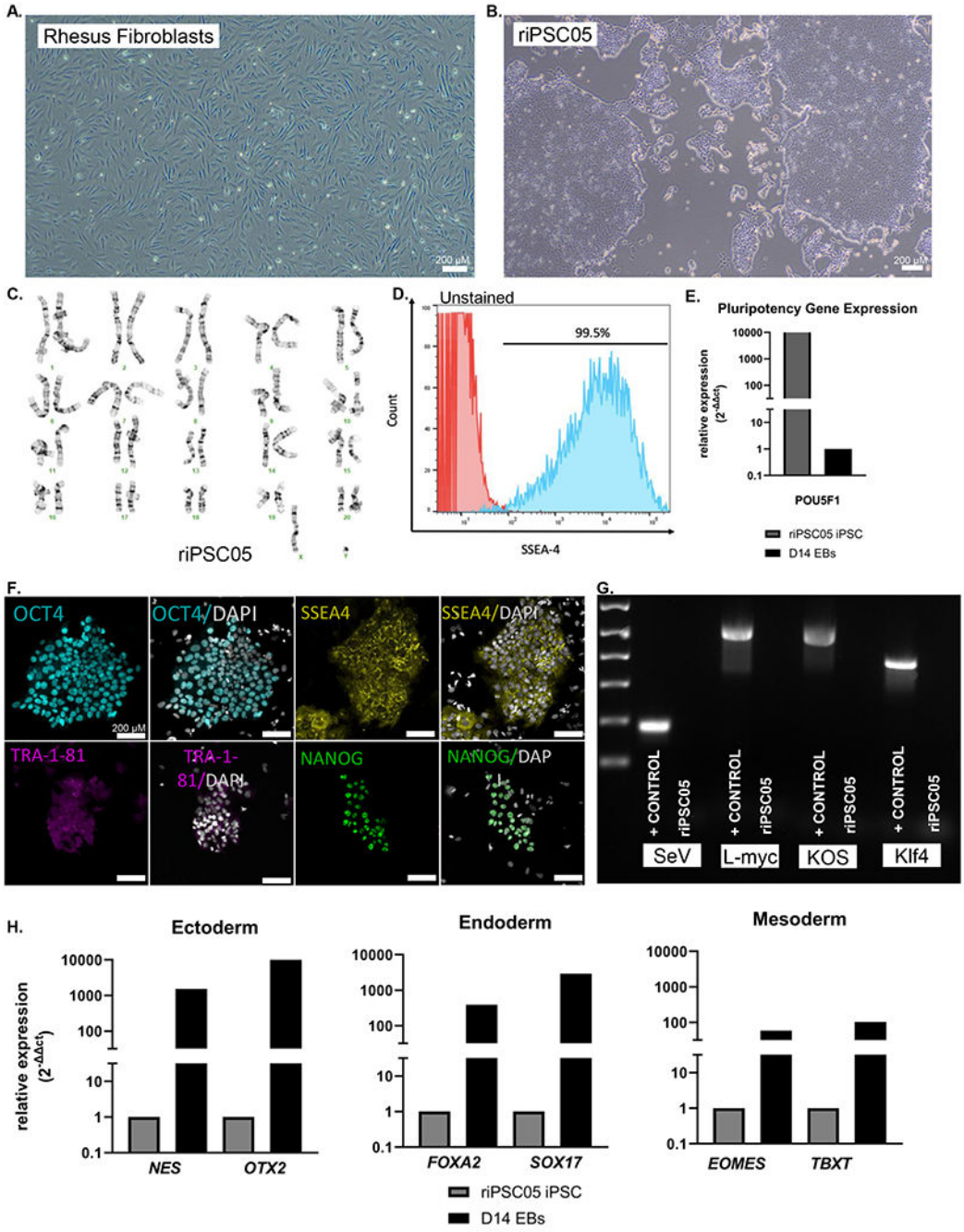

Fig. 1.
Characterization of the rhesus iPSC (riPSC05) line.

Table 1:

Characterization and validation

Classification	Test	Result	Data
Morphology	Brightfield Imaging	Normal Morphology	Figure 1 Panel A and B
Phenotype	Qualitative Analysis: Immunocytochemistry	Positive staining of markers: OCT4, NANOG, SSEA4, TRA-1-81 relative to controls at Passage 14	Figure 1 Panel F
	Quantitative Analysis: Flow cytometry; RT-qPCR	SSEA-4: 99.5% Expression of *POU5F1*	Figure 1 Panel D; Figure 1 Panel E
Genotype	Karyotype (G-banding) and resolution	42XY, Resolution 460 bands	Figure 1 Panel C; Supplementary Figure 2
Identity	STR Analysis	DNA Profiling Performed	Supplementary Figure 1 Panel A
Mutation analysis (IF APPLICABLE)	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence, Negative	Supplementary Figure 1 Panel B
Differentiation potential	Embryoid body Formation	Expression of genes in embryoid bodies: ectoderm markers *(NES and OTX2)*, endoderm markers *(FOXA2* and *SOX17*) , and mesoderm markers (*EOMES* and *TBXT*)	Figure 1 Panel H
Donor screening (OPTIONAL)	i.e. Testing done for any adventitious pathogen found in this species.	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2:

Reagents details

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency Markers (Immunostaining)	Rabbit anti-NANOG	1:100	R&D Systems Cat# AF1997	RRID:AB_355097
Pluripotency Markers (Immunostaining)	Mouse anti-OCT4	1:100	Santa Cruz Biotechnology Cat# sc-5279	RRID:AB_628051
Pluripotency Markers (Immunostaining)	Mouse anti-SSEA4	1:100	Developmental Studies Hybridoma Bank Cat# MC-813-70	RRID:AB_528477
Pluripotency Markers (Immunostaining)	Mouse anti-TRA-1-81	1:100	Invitrogen Cat# 14-8883-82	RRID:AB_891614
Rabbit Secondary Antibody	AF488-conjugated donkey-anti-mouse	1:200	Thermo Fisher Cat# A-21206	RRID:AB_2535792
Mouse Secondary Antibody	AF488-conjugated donkey-anti-rabbit	1:200	Life Technologies Cat# A-21131	RRID:AB_2535771
Pluripotency Markers (Flow Cytometry)	APC anti-human SSEA-4	1:100	Stem Cell Technologies Cat# 330417	RRID:AB_2616818
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
Sendai Viral Vector (RT-PCR)	SeV	181bp	Forward: GGA TCA CTA GGT GAT ATC GAG C Reverse: ACC AGA CAA GAG TTT AAG AGA TAT GTA TC	
Sendai Viral Vector (RT-PCR)	KOS	528bp	Forward: ATG CAC CGC TAC GAC GTG AGC GC Reverse: ACC TTG ACA ATC CTG ATG TGG	
Sendai Viral Vector (RT-PCR)	Klf4	410bp	Forward: TTC CTG CAT GCC AGA GGA GCC C Reverse: AAT GTA TCG AAG GTG CTC AA	
Sendai Viral Vector (RT-PCR)	c-Myc	532bp	Forward: TAA CTG ACT AGC AGG CTT GTC G Reverse: TCC ACA TAC AGT CCT GGA TGA TGA TG	
	Taqman™ Gene Expression Assays			
	Target	**Amplicon Length**	**Assay ID**	
Pluripotency Marker (RT-qPCR)	*POU5F1*	64	Hs03005111_g1	
Ectoderm Differentiation Marker (RT-qPCR)	*NES*	125	Rh02861378_m1	
Ectoderm Differentiation Marker (RT-qPCR)	*OTX2*	74	Rh04256253_m1	
Endoderm Differentiation Marker (RT-qPCR)	*FOXA2*	63	Rh02819217_m1	
Endoderm Differentiation Marker (RT-qPCR)	*SOX17*	53	Rh02976917_s1	
Mesoderm Differentiation Marker (RT-qPCR)	*EOMES*	83	Rh01015625_m1	
Mesoderm Differentiation Marker (RT-qPCR)	*TBXT*	132	Rh00610080_m1	
House-keeping Gene (RT-qPCR)	*GAPDH*	86	Rh02621745_g1	

1.

Resource table:

Unique stem cell line identifier	CVCL_C8D3
Alternative name(s) of stem cell line	riPSC05
Institution	University of California, Los Angeles
Contact information of distributor	Amander Clark, clarka@ucla.edu
Type of cell line	iPSC
Origin	Rhesus macaque
Additional origin info (optional)	Age: Day 100 post-fertilization Sex: Male
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	CTS™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-PCR
Associated disease	N/A
Gene/locus	N/A
Date archived/stock date	03 December 2020
Cell line repository/bank	This cell line has been registered with Cellosaurus (https://www.cellosaurus.org/CVCL_C8D3)
Ethical approval	The study has been approved by the UCLA Institutional Animal Care and Use Committee (Approval Number: ARC-2022-055).