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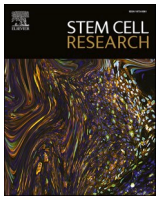

Lab Resource: Animal Multiple Cell lines

Generation and characterization of three fibroblast-derived Rhesus Macaque induced pluripotent stem cell lines

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A B S T R A C T

Cross-species comparisons using pluripotent stem cells from primates are crucial to better understand human biology, disease, and evolution. An important primate model is the Rhesus macaque (*Macaca mulatta*), and we reprogrammed skin fibroblasts from a male individual to generate three induced pluripotent stem cell (iPSC) lines. These cells exhibit the typical ESC-like colony morphology, express common pluripotency markers, and can differentiate into cells of the three germ layers. All generated iPSC lines can be cultured under feeder-free conditions in commercially available medium and are therefore valuable resources for cross-species comparisons.

1. Resource Table

Unique stem cell lines identifier	MPC-MacMul-C00001 (83Ab1.1) MPC-MacMul-C00002 (83D1) MPC-MacMul-C00003 (87B1)
Alternative name(s) of stem cell lines	83Ab1.1 83D1 87B1
Institution	Faculty of Biology, Ludwig-Maximilians-Universität München
Contact information of distributor	Prof. Dr. Wolfgang Enard: enard@bio.lmu.de Jessica Jocher: jocher@bio.lmu.de
Type of cell lines	iPSCs
Origin	Rhesus Macaque (*Macaca Mulatta*)
Additional origin info	Sex: Male
Cell Source	iPSCs were derived from Rhesus macaque skin fibroblasts, kindly provided by the DPZ Göttingen
Clonality	Clonal
Method of reprogramming	Integration-free sendai virus based OSKM vectors (CytoTune-iPSC 2.0 Sendai Reprogramming Kit, Thermo Fisher Scientific) were used for reprogramming
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	PCR analysis for transgene detection (negative)
Associated disease	N/A
Gene/locus	N/A
Date archived/stock date	November 2020
Cell line repository/bank	N/A
Ethical approval	The study was ethically approved by the Animal Welfare Committee at DPZ which is registered and authorized by the local and regional veterinary governmental authorities (Ref. no. 122910.3311900, PK 36674).

2. Resource utility

The three iPSC lines derived from one male Rhesus macaque skin sample can be used for cross-species comparisons investigating e.g., the molecular and cellular evolution of early primate development. Thereby, the three lines can help to assess clonal variation within one Rhesus macaque genetic background.

3. Resource details

Comparative analyses of human and non-human primates (NHP) can leverage unique information to understand evolutionary and developmental mechanisms and bridge the phylogenetic gap between humans and mice for translationally and biomedically relevant questions (Enard, 2012). Among NHPs, the Rhesus macaque (*Macaca mulatta*) is probably the most important model across biological disciplines and comprises 65 % of all NHP subjects used in the United States (Cooper et al., 2022). However, ethical, and practical limitations make it difficult to obtain

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comparable cells especially during development. Induced pluripotent stem cells (iPSCs) can be used to overcome these challenges (Juan et al., 2023).

Here, skin fibroblast obtained from Rhesus macaque were reprogrammed to iPSCs using Sendai viruses to introduce the Yamanaka factors OCT3/4, SOX2, KLF4 and C-MYC. Following reprogramming, colonies were picked, accustomed to feeder-free culture conditions and further characterized (Table 1). The established colonies exhibited a typical ESC-like morphology with defined borders, tight cellular packaging, and prominent nucleoli (Fig. 1A). A primate-specific SINE based PCR demonstrated that the iPSCs show the same Macaque-specific *ALU* element insertions as the parental skin fibroblasts, confirming that they were derived from the same species (Herke et al., 2007) (Supplementary Fig. S1A). In addition, single nucleotide polymorphisms (SNPs) were called from single-cell RNA-sequencing (scRNA-seq) data to profile the genotype of the cell lines. Around 4000 high quality SNPs with high coverage in all three clones were retrieved (Supplementary Fig. S1B). After some passages, karyotype analysis was performed and revealed no recurrent numerical or structural aberrations (Fig. 1B). In addition, one cell line was used for a detailed high resolution validation of numerical and structural chromosome integrity by FISH using human chromosome specific painting probes (Supplementary Fig. S1C). Immunofluorescence (IF) staining was performed, confirming the expression of the pluripotency associated proteins OCT3/4, SOX2 and NANOG, as well as the presence of the cell surface markers SSEA4 and EpCAM (Fig. 1D). Quantification of the IF staining for OCT3/4, SOX2 and NANOG showed that $>$ 95 % of cells were positive for these pluripotency markers (Figure C). All three iPSCs were negative for mycoplasma DNA (Fig. 1E) and negative for the Sendai-based reprogramming vectors (Fig. 1F). Moreover, all iPSC lines had the ability to differentiate into cells of the three germ layers, confirmed by positive immunofluorescence staining of germ layer-specific markers. Endodermal cells were positively stained for alpha-fetoprotein (AFP), mesodermal cells expressed alpha-smooth muscle actin (SMA) and ectodermal cells displayed neuron-specific beta-III tubulin expression (Fig. 1G). Additionally, scRNA-seq of Embryoid bodies (EBs) confirmed their potential for trilineage differentiation and the expression of germ layer-specific marker genes (Supplementary Fig. S1D). In summary, these characteristics suggest the successful reprogramming of three lines to mycoplasma free, integration free and feeder-free iPSCs from Rhesus macaque (*Macaca mulatta*).

4. Materials and methods

4.1. Reprogramming of fibroblasts and iPSC maintenance

Fibroblasts were cultured on 0.2 % Gelatin-coated dishes in DMEM/F12 (Fisher Scientific) supplemented with 10 % FBS and 100 U/mL Penicillin and 100 µg/mL Streptomycin (Thermo Fisher Scientific) at 37 °C with 5 % CO_2. For reprogramming, a CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) was used at a MOI of 5, using a modified protocol. Fibroblasts were incubated with the virus mix for 1 h at 37 °C in suspension, followed by seeding on feeder cell-coated wells. Cells were switched to mTesR1™ medium (STEMCELL Technologies) on day 5 after transduction. Emerging colonies were manually

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal iPSC colony morphology	Fig. 1A Scale bar represents 500 µm
Phenotype	Qualitative analysis by immunocytochemistry	iPSCs were positively stained for OCT4, NANOG, SOX2, SSEA4 and EpCAM	Fig. 1D Scale bar represents 100 µm
	Quantitative analysis by immunocytochemistry counting	% total cells positive for pluripotency markers (mean ± SD): 83Ab1.1 OCT4: 97.6 % ± 1.1 % NANOG: 94.4 % ± 2.8 % SOX2: 98.3 % ± 0.7 % 83D1 OCT4: 97.8 % ± 2 % NANOG: 98.4 % ± 0.3 % SOX2: 98.7 % ± 0.5 % 87B1 OCT4: 98.7 % ± 0.2 % NANOG: 98.7 % ± 0.9 % SOX2: 98.9 % ± 0.6 %	Fig. 1C
Genotype	Karyotype (G-banding and FISH)	3x inconspicuous male karyotype, 42,XYNo recurrent numeric or structural aberrations, after G-banding analysis of 46 to 48 cells per cell line with up to approximately 400 bphs (bands per haploid set)	Fig. 1B and Supplementary Fig. S1C
Identity	SINE-based genotyping PCR SNP analysis	DNA profiling performed, matched between iPSCs and parental fibroblasts Variant calling performed resulting in 4000 high quality SNPs	Supplementary Fig. S1A Submitted in archive with journal Summary: Supplementary Fig. S1B
Mutation analysis (IF APPLICABLE)	N/A N/A		
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: negative	Fig. 1E
	Sendai virus	PCR analysis for Sendai virus presence: negative	Fig. 1F
Differentiation potential	Embryoid body formation - IF staining	iPSCs are capable of differentiating into the three germ layers. Mesoderm: Smooth muscle actin (SMA) Endoderm: α-feto protein (AFP) Ectoderm: β-III Tubulin	Fig. 1G Scale bar represents 100 µm
	Embryoid body formation - scRNA-seq	Expression of multiple cell type and germ layer specific marker genes	Supplementary Fig. S1D
Donor screening (OPTIONAL)	N/A		
Genotype additional info (OPTIONAL)	N/A N/A		

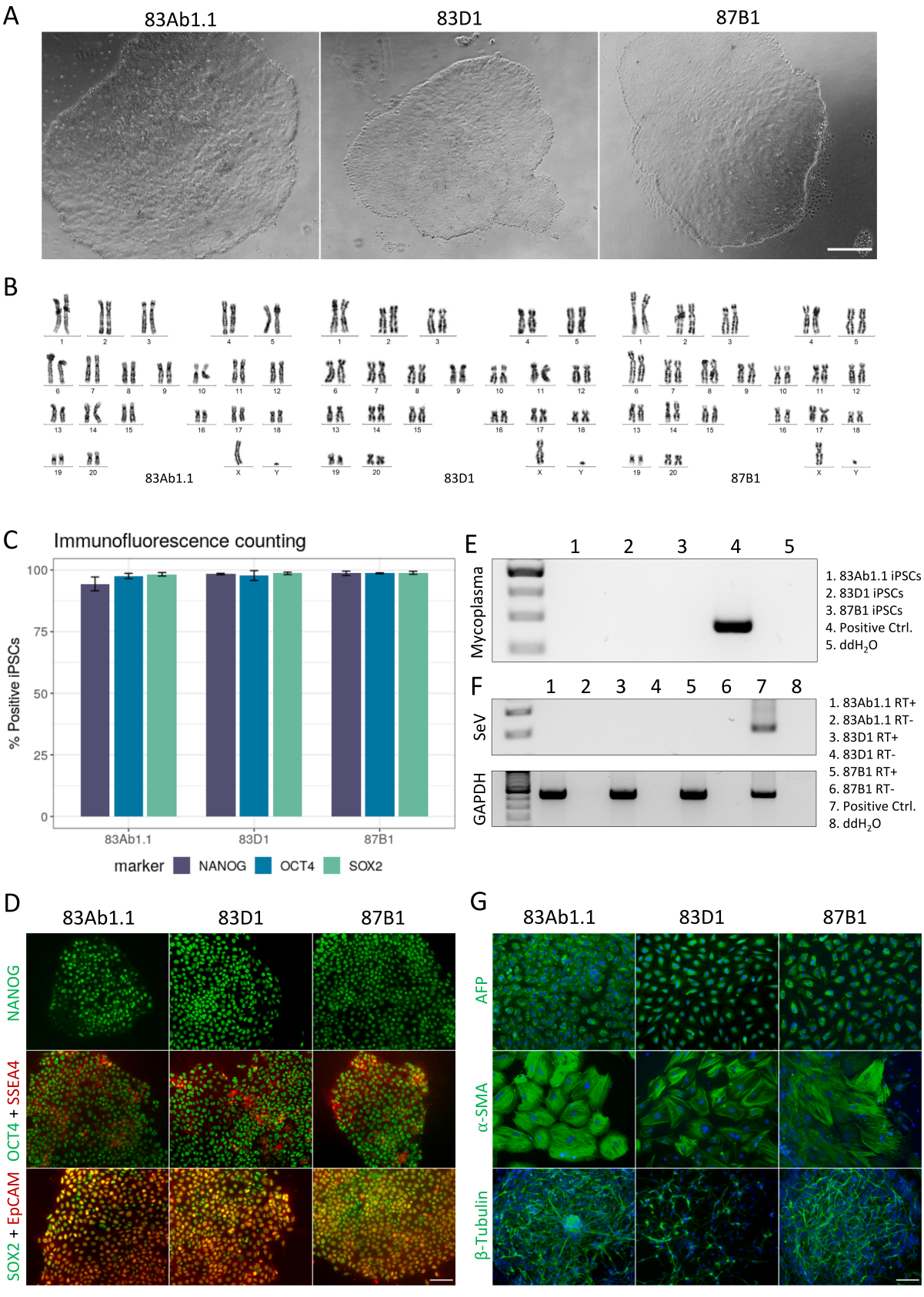

Fig. 1. Characterization of the three Rhesus macaque iPSC lines. (A) Phase contrast microscopy images of iPSC colonies. Scale bar represents 500 μm. (B) Karyotype analysis. (C) Immunofluorescence counting results for NANOG, OCT4 and SOX2. (D) Immunofluorescence staining for pluripotency markers. Scale bar represents 100 μm. (E) Mycoplasma test. (F) PCR for Sendai-based reprogramming vectors. (G) Immunofluorescence staining for germ layer-specific markers. Scale bar represents 100 μm.

picked on feeder cells and cultured in StemFit® Basic02 (Ajinomoto) supplemented with 100 ng/mL bFGF (Peprotech) and 100 U/mL Penicillin and 100 µg/mL Streptomycin. For generating feeder-free iPSCs, colonies were split using 0.5 mM EDTA on 1 % Geltrex™ (Thermo Fisher Scientific) -coated wells in feeder-conditioned StemFit. The ratio of feeder-conditioned to normal StemFit was reduced by 25 % after every second passage, until iPSCs could be cultured under feeder-free conditions. iPSCs were passaged using 0.5 mM EDTA at a ratio of 1:10–1:50 every 5 days, with medium changes every other day.

4.2. Immunocytochemistry

Attached cells (passage 15–20) were fixed for 15 min with 4 % PFA, permeabilized with 0.3 % Triton X-100 (Sigma Aldrich) and blocked with 5 % FBS for 30 min. Cells were incubated with primary antibodies (Table 2) diluted in staining buffer (PBS containing 1 % BSA and 0.3 % Triton X-100) overnight at 4 °C. The next day, cells were washed with PBS and incubated with secondary antibodies (Table 2) diluted in staining buffer for 1 h at room temperature. Nuclei were counterstained with 1 µg/mL DAPI. Positively-stained cells were quantified using the ImageJ software with the Cell Counter plugin.

4.3. Embryoid body formation

iPSCs at passage 15–20 were dissociated into clumps and cultured in sterile bacterial dishes containing StemFit Basic02 w/o bFGF at 37 °C with 5 % CO_2. A medium change was performed every other day during the first 8 days of floating culture. Afterwards, EBs were seeded into 6-wells coated with 0.2 % Gelatin for 8 days of attached culture. On day 16, differentiated cells were analyzed with specific antibodies for mesoderm, endoderm, and ectoderm (Table 2) using immunocytochemistry. In addition, cells were also sampled for scRNA-seq on day 16. Briefly, EBs were dissociated using Accumax, and sequencing libraries were generated using the 10x Genomics Chromium Next GEM Single Cell 3′Kit V3.1 workflow. Cluster analysis was performed in R using the package Seurat v5 and clusters were assigned to germ layers based on the expression of known marker genes.

4.4. Karyotyping

For Metaphase preparation, cells (passage 16–23) at 80 % confluency were incubated with 0.1 mg/mL Colcemid (Gibco) for 13 h and harvested using Accumax (Sigma Aldrich). Cells were treated with hypotonic Na-Citrate/NaCl for 35 min at 37 °C, followed by a subsequent fixation with methanol/acetic acid glacial (3:1) for 20 min at -20 °C. After pelleting, cells were washed twice with methanol/acetic acid as stated above. Differentially stained mitotic chromosome spreads were prepared using the G-banding technique and fluorescence in situ hybridization (FISH) was performed using human chromosome specific painting probes, following standard procedures.

4.5. Mycoplasma testing

The medium of a confluent 6-well with iPSCs at passage 15–20 was collected and pelleted, followed by resuspension in 100 µL PBS. After a 5 min incubation at 95 °C, 1 µL was used for a screening PCR with specific primers for the Mycoplasma 16S rRNA (Table 2).

4.6. Genotyping PCR

Total gDNA was isolated from cell pellets using the DirectPCR Lysis Reagent (VWR) supplemented with 20 mg/mL Proteinase K (Life Technologies), and a PCR (36 cycles) was performed with primers for the primate-specific *Alu* SINE (Table 2).

4.7. Variant calling

10x scRNA-seq data of day 16 EBs were used to call SNPs against the reference genome rheMac10 using GATK (Genome Analysis Tool Kit). High quality, biallelic SNPs were retained by joint genotyping of all three clones followed by quality filtering of the variants for high coverage (DP > 99) and quality by depth (QD > 2).

4.8. SeV detection

Total RNA was isolated from iPSCs at passage 10–15 using the Direct-zol RNA Microprep Kit (Zymo Research). After cDNA synthesis using the Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific), the cDNA was used to perform a PCR (36 cycles) with specific primers for SeV and GAPDH (Table 2).

Declaration of competing interest

The authors declare the following financial interests/personal

Table 2
Reagents details.

Antibodies used for immunocytochemistry

	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-OCT4	1:400	Cell Signaling Technology, Cat# 2750S	RRID: AB_823583
	Mouse anti-SOX2	1:400	Cell Signaling Technology, Cat# 4900S	RRID: AB_10560516
	Rabbit anti-Nanog	1:400	Cell Signaling Technology, Cat# 4903S	RRID: AB_10559205
	Mouse anti-SSEA4	1:500	NEB, Cat# 4755S	RRID: AB_1264259
	Rabbit anti-EpCAM	1:500	Thermo Fisher Scientific, Cat# 710524	RRID: AB_2532731
Differentiation Markers	Mouse anti- α-Smooth Muscle Actin	1:100	R&D Systems, Cat# MAB1420	RRID: AB_262054
	Mouse anti- Neuron-specific beta-III Tubulin	1:100	R&D Systems, Cat# MAB1195	RRID: AB_357520
	Mouse anti-alpha Fetoprotein	1:100	R&D Systems, Cat# MAB1368	RRID: AB_357658
Secondary Antibodies	Alexa Fluor 488 donkey anti-mouse IgG (H + L)	1:500	Thermo Fisher Scientific, Cat# A-21202	RRID: AB_141607
	Alexa Fluor 594 donkey anti-rabbit IgG (H + L)	1:500	Thermo Fisher Scientific, Cat# A-21207	RRID: AB_141637

Primers

	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**
Reprogramming factor clearance	Sendai Virus	180 bp	GGATCACTAGGTGATATCGAGC/ ACCAGACAAGAGTTTAAGAGATATGTATC
	GAPDH (housekeeping gene)	450 bp	ACCACAGTCCATGCCATCAC/TCCACCACCCTGTTGCTGTA
Mycoplasma testing	Mycoplasma 16S	270 bp	TGCACCATCTGTCACTCTGTTAACCTC/ GGGAGCAAACAGGATTAGATACCCT
Genotyping PCR	*Alu* (primate-specific SINE)	548 bp	CTCTCAGCTCCCTGTTTCTGTT/CATGGACATCAGACTAGCCACT

relationships which may be considered as potential competing interests: Wolfgang Enard reports financial support, article publishing charges, and travel were provided by German Research Foundation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103277.

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