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An integration-free, virus-free rhesus macaque induced pluripotent stem cell line (riPSC90) from embryonic fibroblasts

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Abstract

Rhesus macaque induced pluripotent stem cell (riPSC) line, UCLAi090-A (riPSC90), was generated from rhesus embryonic fibroblast (REF) cells called REF90. REF90 cells and the riPSC90 line were authenticated by short tandem repeat analysis and had a normal male (42, XY) karyotype. The riPSC90 line expressed markers of self-renewal including OCT4, NANOG, TRA-1-81 and SSEA4, and generated teratomas after transplantation into immunocompromised mice. riPSC90 could be used in parallel with riPSC89, which was derived from REFs cultured from a different rhesus macaque embryo (Sosa et al. 2016).

Key transcription factors

OCT4; KLF4; SOX2; GLIS1

Resource details

An integration-free, virus-free rhesus macaque (*Macacca mulata*) induced pluripotent stem cell (riPSC) line was generated from rhesus embryonic fibroblasts (REFs). REFs were expanded from samples of embryonic skin, which was removed from the torso of an embryonic day 47 rhesus embryo at Carnegie stage 23 (embryo # 34990). The REFs in this study were called REF90, and were reprogrammed using the non-infectious, non-viral self-

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The following is the supplementary data related to this article.

replicating RNA originally derived form the Venezuelan equine encephalitis (VEE) virus (Yoshioka *et al.*, 2013) and now commercially available as the product called Simplicon™ RNA reprogramming technology. The RNA replicon produces a synthetic RNA containing the reprograming factors *OCT4, KLF4, SOX2*, and *GLIS1* (OKS-iG) as a polycistronic transcript. Using this technology, we reprogrammed REF90 cells with *VEE-OKS-iG* RNA and *B18R* RNA in the presence of human recombinant B18R protein for eighteen days (Fig. 1A). Pioneering riPSC colonies first appeared between day 14–18, and were picked on day 21 (Fig 1A). Picked colonies were expanded in iPSC media on mytomycin C inactivated mouse embryonic fibroblasts (MEFs) and split every 5–7 days. The established riPSC line which originated from a single colony was called riPSC90 to reflect its origin from REF90 (Fig 1B). The picked colony was not grown from a single cell. riPSC90 (poor resolution) and REF90 (good resolution) were both normal male 42, XY karyotype (Fig. 1C). STR profiles for the parent fibroblast line, REF90 and the generated riPSC90 line were identical (Fig. S1A), but differed from the REF89/riPSC89 pair, which instead were identical to each other. (Fig. S1B). Non-integration was confirmed by genomic PCR, through the absence of the virus-specific nonstructural protein 2 (*NSP2*) (Fig. 1D). riPSC90 expressed markers of self-renewal, such as OCT4, NANOG, SSEA4 and TRA-1-81 (Fig. 1E). To assess pluripotency, we performed a teratoma analysis by injecting undifferentiated colonies of riPSC90 into severe combined immunodeficient-beige (SCID-bg) mice. Using this assay, it was discovered that riPSC90 was competent to form teratomas, with evidence of ectoderm, mesoderm and endoderm by histology (Fig.1F).

Materials and methods

Rhesus Embryonic Fibroblast Derivation

The rhesus macaque embryo (embryo # 34990) was obtained from the time mated breeding (TMB) program at the Oregon National Primate Research Center (ONPRC) following Institutional Animal Care and Use Committee Approval. Hormone-guided time mated breeding was performed as previously described (Sosa et al., 2016), the adult male and female animals were housed together for a total of four days (day 8–11 of the menstrual cycle). Estrodial was measured in the female from day 5 to 11 of her menstrual cycle with the estrodiol peak occurring at day 9. The estimated day of fertilization (day 1 of embryo development) was predicted to occur 72 hours after the estrodiol peak. On embryonic day 47, the embryo was removed by cesarean section, and carnegie staging was performed according to Gribnau and Geijsberts (1981). Skin samples were removed from the torso and back of the embryo to generate rhesus embryonic fibroblasts (REFs). Freshly isolated skin samples were digested with 2mg/ml Collagenase IV (Life Technologies) at 37°C, 5.0% CO_2 for 2 hours. Digested samples were washed in DMEM/F12 (Life Technologies) media prior to plating on 0.1% gelatin (Sigma) coated plates in Rhesus Fibroblast (RhF) media (DMEM/F12 (Life Technologies), 15% Fetal bovine serum (GE Healthcare), Non-Essential Amino Acids (Invitrogen), Glutamax™ (Gibco), Penicillin-Streptomycin-Glutamine (Gibco), and Primocin (Invivogen)) at 37°C, 5.0% CO_2. Outgrowths were monitored for up to 2 weeks (passage 0), with media changes every 2–3 days. REFs were passaged using 0.25% Trypsin (Gibco) and re-plated at a density of 1.5×10^5 cells. The resulting REF cells were called REF90.

Generating the riPSC90 line

Reprogramming was performed as previously described (Sosa et al. 2016). In short, the Simplicon™ Reprogramming Kit (EMD Millipore) was used to reprogram 1.5×10^4 REF90 cells (passage 3). Fibroblast were pre-treated with the Human Recombinant B18R protein, then transfected once with both *B18R* RNA and *VEE-OKS-iG* RNA and allowed to recover overnight in Stage 1 media (Advanced DMEM (Gibco), 10% FBS (GE Healthcare), 2mM L-glutamine (Life Technologies) supplemented with 200ng/mL human recombinant B18R protein (EMD Millipore). Puromycin (Invivogen), at a 0.1ug/mL concentration, was added fresh to Stage 1 media after daily media changes until day 9 post-transfection. Ten days after transfection, REF90 cells were detached using Accumax™ (Sigma) and 1.0×10^4 transfected cells were plated on inactivated MEFs in Stage 2 media which consists of MEF conditioned medium (R & D Systems) plus10ng/mL bFGF (R&D system), TGF-β RI Kinase Inhibitor IV, Sodium Butyrate and PS48 (all from Simplicon), and 200 ng/mL B18R protein (EMD Millipore). Stage 2 media was changed every other day. Small colonies appeared on day 14 after transfection and by day 18, B18R protein was removed from the media. Putative riPSC colonies were manually picked on day 21 and re-plated on inactivated MEFs in iPSC media (DMEM/F-12 (Life Technologies), 20% KSR (Life Technologies), 10ng/mL bFGF (R & D Systems), 1% nonessential amino acids (Life Technologies), 2mM L-Glutamine (Life Technologies), Primocin™ (Invivogen), and 0.1mM β-mercaptoethanol (Sigma)) on mytomicin C treated MEFs (passage 0). Seven days later, riPSC colonies were manually picked and expanded in iPSC media (passage 1). The riPSC90 line was expanded from a single colony starting at passage 2. Mycoplasma testing was conducted using the Mycoalert Detection Kit (Lonza), confirming that the riPSC90 line is mycoplasma free.

Karyotyping Analysis and STR Analysis

REF90 and riPSC90 were karyotyped using metaphase spreads and G-banding by Cell Line Genetics (Madison, WI). STR analysis was performed on REF89, REF90, riPSC89, and riPSC90 lines by Cell Line Genetics (Madison, WI).

Teratoma Formation Assay

Teratomas were formed as previously detailed (Sosa et al. 2016). This involved the digestion of riPSC90 colonies with 1mg/mL collagenase IV (Life Technologies), and re-suspending in cold Matrigel (Corning). riPSC90 colonies in Matrigel were injected into the left and right testis of n=2 CB17/Icr.Cg-Prkdcscid Lystbg/Cr (SCID-bg) mice using survival surgery (n=4 testes total). Each testis was injected with 40uL of matrigel containing ~2.5×10^6 riPSC90 cells as small clumps. Two months after the transplant, the mice were euthanized and the testes containing tumors were excised and fixed in 4% Paraformaldehyde (PFA) in phosphate buffered saline (PBS). The tumors were processed to paraffin for hematoxylin and Eosin (H&E) staining. Diagnosis of teratoma was made using an Olympus BX-61 light microscope. All animal work was first approved by the UCLA Office of Animal Research Oversight.

Immunofluorescence staining

Immunofluorescence staining of the riPSC90 cell line was performed as previously published (Sosa et al., 2016). In short, riPSC90 colonies were fixed in 4% PFA, permeabilized with PBS plus 0.5% Triton™ X-100 (Sigma) then washed in PBST (PBS with 0.1% Tween-20 (Sigma)) Primary antibodies included goat-anti-human OCT4 (1:100, Santa Cruz), goat-anti-human NANOG (1:100, R&D Systems), mouse-anti-human SSEA4 (1:100, Developmental Studies Hybridoma Bank), and mouse-anti-human TRA-1-81 (1:100, eBiosciences). For detecting primary antibodies raised in goat, AF594-conjugated donkey anti-goat (1:100, Life Technologies) secondary antibodies were used. For detecting primary antibodies raised in mice, AF488- or AF594-conjugated donkey-anti-mouse (1:100, Life Technologies) secondary antibodies were used. Images of cells were acquired with a Zeiss LSM 880 confocal laser-scanning microscope. Images were analysed using ImageJ version 1.51d (NIH).

Non-integration confirmed by PCR of genomic DNA

Genomic DNA was isolated from riPSC90 using Quick-gDNA™ miniprep kit (Zymogen). *T7-VEE-OKS-iG* plasmid DNA (Addgene), was isolated from bacteria using the plasmid DNA miniprep kit (Qiagen). At least 500ng of gDNA was used to confirm non-integration of VEE-OKS-iG RNA. Primers sets and PCR conditions used for the amplification of the gene NSP2 have been previously reported (Yoshioka *et al.*, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Resource Table

Name of Stem Cell line	UCLAi090-A (riPSC90)
Institution	UCLA
Person who created resource	Enrique Sosa
Contact person and email	Amander T. Clark, clarka@ucla.edu
Date archived/stock date	March 1, 2016
Origin	Carnegie Stage 23 (embryonic day 47) rhesus macaque (*Macaque mulatta*) embryonic fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell line
Sub-type	cell line
Key transcription factors	OCT4, KLF4, SOX2, GLIS1
Authentication	Identity and purity of cell line confirmed (Fig. 1; Fig. S1)

Link to related literature	https://www.emdmillipore.com/US/en/product/Simplicon%E2%84%A2-RNA-Reprogramming-Kit-(OKSG),MM_NF-SCR550 http://www.ncbi.nlm.nih.gov/pubmed/23910086 http://dx.doi.org/10.1016/j.scr.2016.09.015
Information in public databases	N/A
Ethics	The time mated rhesus embryo (#34990) was obtained following Institutional Animal Care and Use committee approval (ONPRC). Teratoma formation was performed following Office of Animal Research Oversight Approval (UCLA)

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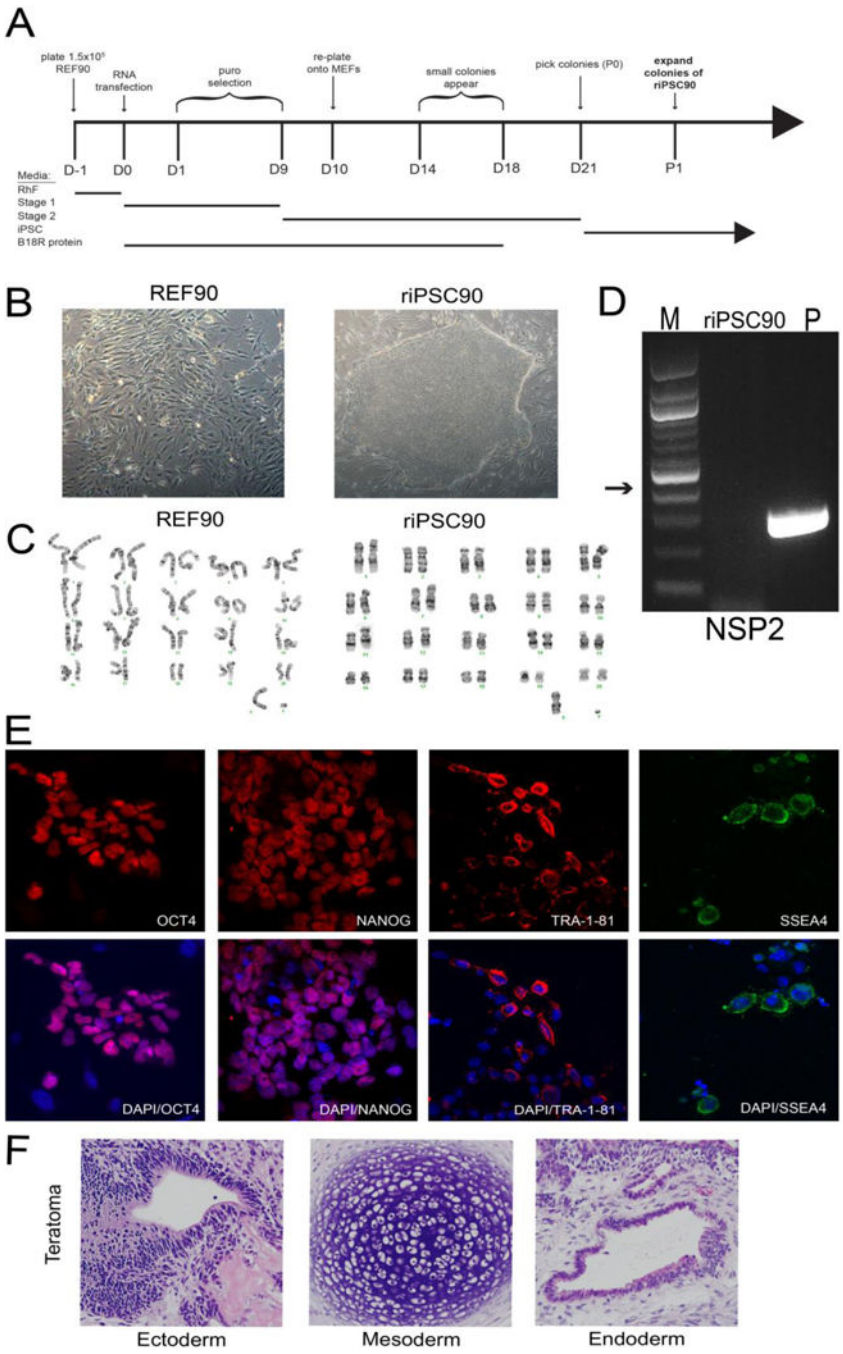

Fig 1. Characterization of riPSC90

(A) Timeline of events during the reprogramming REF90 fibroblasts and subsequent establishment and characterization of the riPSC90 line from a single colony. (B) Morphology of the REF90 (Fibroblasts) and a colony of riPSC90 (iPSCs). (C) REF90 and riPSC90 were karyotypically normal male (42, XY). (D) Non-integration after reprogramming confirmed by PCR of riPSC90 genomic DNA (500ng). NSP2 is only present in plasmid DNA and not riPSC90 gDNA (M=100 base pair marker with arrow pointing to 500bp, P=plasmid DNA). (E) riPSC90 express markers of self-renewal such as OCT4 and

NANOG, as well as, the cell surface proteins SSEA4 and TRA-1-81. (F) Pluripotency of riPSC90 was confirmed using a teratoma assay, which contained cell types representing endoderm, mesoderm and ectoderm.