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Lab resource: Stem Cell Line

Generation of an OCT3/4 reporter cynomolgus monkey ES cell line using CRISPR/Cas9

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A B S T R A C T

Cynomolgus monkey ES (Cyn ES) cells can be generated in a similar manner as human ES cells. However, Cyn ES cells are difficult to maintain in an undifferentiated state by untrained researchers. For easier culture, we generated an OCT3/4-P2A tdTomato IRES Zeocin^R Cyn ES cell line using CRISPR/Cas9 genome editing technology. The stop codon of the endogenous *OCT3/4* locus was replaced with the P2A tdTomato IRES Zeocin^R pA cassette by homologous recombination. This cell line enables us to isolate pluripotent stem cells and exclude differentiated cells by addition of zeocin, especially for culture without feeder cells.

Resource table.

Unique stem cell line identifier	CynESC-OTZ4
Alternative name(s) of stem cell line	OTZ4
Institution	Research Center for Animal Life Science, Shiga University of Medical Science
Contact information of distributor	Masatsugu Ema, mema@belle.shiga-med.ac.jp
Type of cell line	ESC
Origin	CynESC-3X (Seita et al., 2019)
Additional origin info	N/A
Cell Source	N/A
Clonality	Clonal
Method of reprogramming	N/A
Genetic Modification	YES
Type of Modification	Transgene expression (resistance, reporter)
Associated disease	N/A
Gene/locus	*POU5F1*/Chromosome 4, NC022275.1 _139812901
Method of modification	CRISPR/Cas9
Name of transgene or resistance	P2A tdTomato, zeocin resistance
Inducible/constitutive system	N/A
Date archived/stock date	N/A
Cell line repository/bank	N/A
Ethical approval	N/A

1. Resource utility

We generated an OCT3/4-P2A tdTomato IRES Zeocin^R cynomolgus monkey ES cell line for easier culture by untrained researchers. This cell line enables us to visualize the localization of undifferentiated cells and exclude differentiated cells by addition of zeocin, especially for culture without feeder cells.

2. Resource details

Cynomolgus monkeys are considered as a useful animal model because they are closer to humans than other experimental animal models. Cynomolgus monkey ES cells (Cyn ES cells) can be generated in a similar manner as human ES cells and we isolated wild type (WT) Cyn ES Cells line named CynESC-3X (Seita et al., 2019). However, Cyn ES cells are more difficult to maintain in an undifferentiated state, because Cyn ES cells are unstable and tend to differentiate in comparison with mouse and human ES cells. OCT3/4 is indispensable to maintain pluripotency of pluripotent stem cells (Niwa, 2015). For easier culture, we generated an OCT3/4-P2A tdTomato IRES Zeocin^R Cyn ES cell line using CRISPR/Cas9 genome editing technology. The stop codon of the endogenous *OCT3/4* locus was replaced with the P2A tdTomato IRES Zeocin^R pA cassette by homologous recombination (Fig. 1A: Blue

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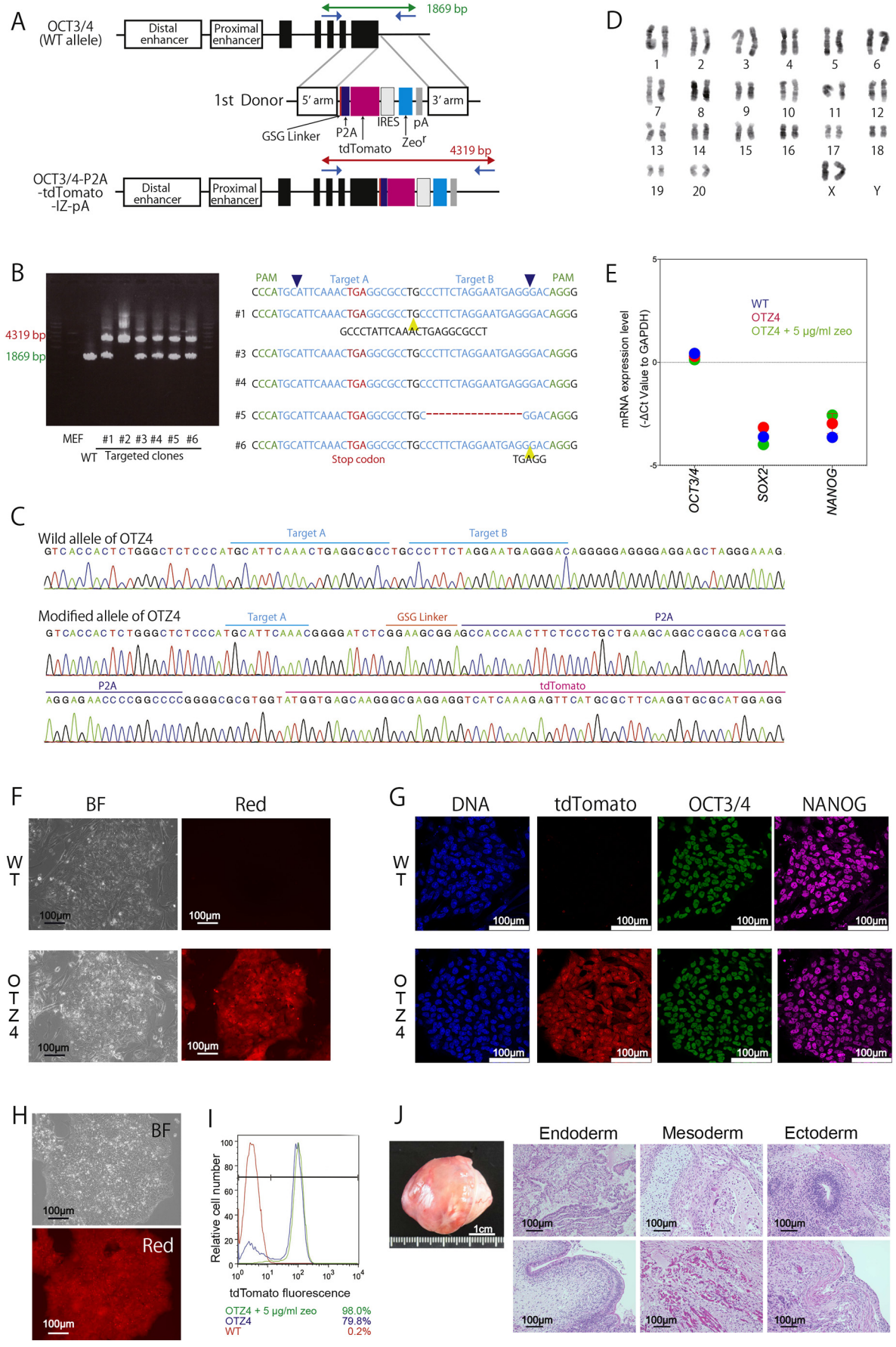

Fig. 1. Generation and characterization of CynESC-OTZ4 line.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel F and H
Phenotype	Qualitative analysis: Immunocytochemistry	Positive for pluripotency markers: OCT4 and NANOG	Fig. 1 panel G
	Quantitative analysis: Immunocytochemistry	% of positive cells	Fig. 1 panel G
		OCT3/4: 99%	Fig. 1 panel I
		NANOG: 99%	
	Flow cytometry	OCT3/4 represented by tdTomato: 98%	
Genotype	Karyotype Q-banding	42 XX Resolution 450	Fig. 1 panel D
Identity	Microsatellite PCR	N/A	N/A
	STR analysis	Four STR sites were tested, and matched.	Additional file1
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous reporter line	Fig. 1 panel B and C
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence: Negative	Supplemental file 1
Differentiation potential	Teratoma formation	Teratoma formation showed the three germ layers formation.	Fig. 1 panel J
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

arrows indicate the binding sites for screening PCR primers; the red bar indicates the band size (4319 bp) of PCR product for the knock-in allele; the green bar indicates the WT band size, 1869 bp). To improve the cleavage efficiency of P2A, a glycine-serine-glycine sequence was inserted before P2A (Kim et al., 2011). To promote homologous recombination, two gRNAs were designed before (target A) and after (Target B) the *OCT3/4* stop codon sequence. We used double nicking by Cas9 nickase to reduce off-target effects, instead of double strand breaks induced by Cas9. The reporter cassette donor template was delivered by transfection as a closed circular plasmid, together with Cas9 nickase-expressing plasmids containing the gRNA transcriptional cassette. Six zeocin-resistant clones were individually picked up and screened by PCR (Fig. 1B). The #2 clone was rejected by genotyping PCR analysis of the reporter knock-in because of homozygous insertion, and #1, #5, and #6 clones were rejected by sequencing analysis of the WT allele because of insertion or deletion of the normal allele (Fig. 1B). Sequencing analysis of the modified allele confirmed that genetic modification occurred properly in #4 clone (Fig. 1C), whereas an extra insertion of a plasmid fragment was found in #3 clone (data not shown). We named the #4 clone OTZ4 (Table 1). Karyotype analysis showed that this clone had a normal 42,XX karyotype (Fig. 1D), and STR genotyping demonstrated its derivation from monkeys, CE1235F and CE0049M whose oocytes and sperm, respectively, were used for the derivation of the original ES line, CynESC-3X (Additional file 1)(Penedo et al., 2005). Quantitative RT-PCR analysis revealed that expression of pluripotent stem cell markers, such as *OCT3/4*, *SOX2*, and *NANOG*, was comparable to that in a WT cell line (Fig. 1E). OTZ4 presented a red fluorescent signal (Fig. 1F), and immunocytochemistry showed overlapping of the tdTomato signal with OCT3/4 proteins (Fig. 1G). OTZ4 was able to be maintained without feeder cells (Fig. 1H). Zeocin selection was effective to maintain undifferentiated cells without feeder cells, because addition of zeocin eliminated tdTomato-negative differentiated cells under feeder-free conditions (Fig. 1I). An in vivo differentiation assay by injection of the cells into immunodeficient mice showed the presence of differentiated tissues from all three germ layers in teratomas (Fig. 1J).

3. Materials and methods

Cyn ES cell maintenance: Cells were cultured on MEF-coated 6-well plates in "hESC medium" [DMEM/F-12, GlutaMAX with 20% KnockOut Serum Replacement, 1% penicillin and streptomycin, 1% non-essential amino acids, 0.1% 2-mercaptoethanol (all from Thermo Fisher Scientific), and 10 ng/ml bFGF (Wako)] containing 10 μM ROCK inhibitor (Y-27632) and 10 μM XAV 939 (Millipore) (Sumi et al., 2013). The medium was changed every day.

Knock-in of the reporter cassette: Donor template OCT3/4-P2A tdTomato IRES Zeocin^R was cloned by PCR and inserted into pBRBlueII with homology arms. We used pX460 plasmid (Addgene plasmid #48873)(Ran et al., 2013) as the Cas9n plasmid, and 'Optimized CRISPR Design' (crispr.mit.edu/) was used to design guide RNAs. Donor template plasmid and Cas9n plasmids were transfected into 5×10^5 WT ES cells by Lipofectamine 2000 (Thermo Fisher Scientific). Transfected cells were seeded on 6-well plates coated with MEF feeder cells in hESC medium containing 10 μM ROCK inhibitor and 10 μM XAV 939. Selection with 5 μg/ml zeocin was started at 48 h after transfection. Colonies were picked after 9 days of selection.

Karyotyping analysis: The Q-banding technique was used for karyotyping analysis by Chromosome Science Labo Inc. (Sapporo, Japan; https://wx28.wadax.ne.jp/~chromoscience-jp/index_e.html). Twenty metaphase chromosomes were counted, and the Q-band resolution was 300–500.

Quantitative RT-PCR: Total RNA was extracted from cells using RNeasy Mini kits (Qiagen). For reverse transcription, ReverTra Ace (Toyobo) and oligo (dT) 20 primer were used. For real-time PCR, THUNDERBIRD SYBR qPCR Mix (Toyobo) was used. Transcript levels were determined in triplicate reactions and normalized against the corresponding levels of *GAPDH*. All primers used in this study are listed in Table 2.

Fluorescence imaging of tdTomato: Red fluorescence of tdTomato was imaged by a Biorevo (Keyence) with the TRITC filter.

Immunofluorescence staining: The samples were fixed in 4% PFA/PBS for 20 min at room temperature, and permeabilized with 0.2% Triton-X/PBS for 30 min at room temperature. Then, they were blocked with 0.5% normal goat serum/0.1% Triton-X/PBS for 1 h at 4 °C and incubated with primary antibodies in the blocking solution overnight at 4 °C (Table 2). They were incubated with secondary antibodies in the blocking solution for 1 h at room temperature. Images were captured under a TCS-SP8 confocal microscope (Leica Microsystems). OCT3/4- and NANOG-positive cells were counted with ImageJ. A cell with a higher signal intensity than the background was defined as positive.

Flow-cytometry analysis: 20,000 cells were resuspended in 2 μg/ml propidium iodide and 2% FBS in PBS. FACS analysis was performed with a FACS Calibur (BD).

Teratoma assay: A total of 3×10^5 ES cells were injected into bilateral testes of 8-week-old SCID mice. At 8 weeks after injection, abdominal swelling was observed. Histological examination was performed by hematoxylin and eosin staining.

Mycoplasma test: Cells were screened for mycoplasma contamination using a Mycoalert detection kit (Lonza, #LT07-118, https://lonza.picturepark.com/Website/?Action=downloadAsset&AssetId=30191), following the manufacturer's protocol. Briefly, bioluminescence of the culture supernatant incubated with the reagent and substrate kit components was detected by a luminometer after addition of each

Table 2
Reagent details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-OCT3/4	1:100	Santa Cruz Cat# sc-5279, RRID:AB_628051
Pluripotency markers	Rabbit anti-NANOG	1:250	ReproCELL Cat# RCAB0004PF, RRID: AB_567470
Secondary antibodies	Alexa Fluor 488 Goat Anti-Mouse IgG	1:500	Thermo Fisher Scientific Cat# A11001, RRID:AB_2534069
Secondary antibodies	Alexa Fluor 405 Goat Anti-Rabbit IgG	1:500	Thermo Fisher Scientific Cat# A31556, RRID:AB_221605

Primers		
	Target	Forward/Reverse primer (5′-3′)
genotyping	*OCT3/4*	ATTGAACTTCACCTTCCCTCCAACCAG/GTCCAGGGTCTCTCTGTCCTTTCATGG
sequence	OCT3/4-P2A-tdTomato	ATGTCCAAGCAGAGTCAGGC
Pluripotency markers (RT-qPCR)	*OCT3/4*	CGAAACCCACCCTGCAGCAG/GCTCGATCGCTTGCCCTTCTG
Pluripotency markers (RT-qPCR)	*SOX2*	CATCACCCACAGCAAATGAC/AAGTTTTCTTGTCGGCATCG
Pluripotency markers (RT-qPCR)	*NANOG*	GGCCAAAGAATAGCAATGGTG/AAGGTTCCCAGTCGGGTTTAC
STR	*D6S2741*	AGACTAGATGTAGGGCTAGC/CTGCACTTGGCTATCTCAAC
STR	*D6S2876*	GGTAAAATTCCTGACTGGCC/GACAGCTCTTCTTAACCTGC
STR	*MICA*	CCTTTTTTTCAGGGAAAGTGC/CCTTACCATCTCCAGAAACTGC
STR	*D6S1961*	AGGACAGAATTTTGCCTC/GCTGCTCCTGTATAAGTAATAAAC

component (A and B). A luminescence signal ratio (B/A) lower than 0.9 demonstrated the absence of mycoplasma contamination.

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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.2019.101439.

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