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Lab Resource: Multiple Cell Lines

Generation of three human induced pluripotent stem cell sublines (UCLAi005-A, UCLAi005-B and UCLAi005-C) for reproductive science research

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A B S T R A C T

We generated three human induced pluripotent stem cell (hiPSC) sublines from human dermal fibroblasts (HDF) (MZT05) generated from a skin biopsy donated from a previously fertile woman. The skin biopsy was broadly consented for generating hiPSC lines for biomedical research, including unique consent specifically for studying human fertility, infertility and germ cell differentiation. hiPSCs were reprogrammed using Sendai virus vectors and were subsequently positive for markers of self-renewal. Pluripotency was further verified using PluriTest analysis and in vitro differentiation was tested using Taqman Real-Time PCR assays. These sublines serve as controls for hiPSC research projects aimed at understanding the cell and molecular regulation of female fertility.

Resource Table

Unique stem cell lines identifier	UCLAi005-A UCLAi005-B UCLAi005-C
Alternative names of stem cell lines	MZT05-D MZT05-F MZT05-L
Institution	UCLA
Contact information of distributor	Dr. Amander Clark
Type of cell lines	hiPSC
Origin	Human
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai, OCT4, SOX2, KLF4, and c-MYC
Multiline rationale	Isogenic clones
Gene modification	No
Type of modification	N/A
Associated disease	None
Gene/locus	N/A
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	03/05/2019
Cell line repository/bank	https://hpscreg.eu/cell-line/UCLAi005-B https://hpscreg.eu/cell-line/UCLAi005-C https://hpscreg.eu/cell-line/UCLAi005-A
Ethical approval	UCLA Office of the Human Resource Protection Program- IRB#16-001176-CR-00002 and UCLA Embryonic Stem Cell Research Oversight Committee (ESCRO# 20016-003)

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1. Resource utility

Differentiation of the human germline is a process that links humanity from one generation to the next. Here, we generated hiPSC sublines specifically consented for fertility and infertility research including the differentiation of germ cells. These sublines were derived from a patient with previously known fertility, and can be used as controls for germline and reproductive science research.

2. Resource details

Germline cell research involving the specification and differentiation of germline cells from hiPSCs can raise ethical concerns due to the moral

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significance of reproduction. The strong opinions generated by some groups for reproductive science research, and creation of gametes using hiPSCs indicates that it could be important to inform tissue donors that their donation will be used specifically for creation of germ cells and gametes from hiPSCs. In addition to this specific consent, it is equally important to consider broad consent from donating patients to cover any potential future unanticipated research, and to facilitate the sharing of generated hiPSC lines and sublines. Thus, we created hiPSC sublines UCLAi005-A (MZT05-D), UCLAi005-B (MZT05-F), and UCLAi005-C (MZT05-L) to fill a void in the availability of verified control hiPSCs for fertility research where the reproductive status of the donor is known.

We generated three integration-free hiPSC sublines from human dermal fibroblasts (HDFs). Fibroblasts were derived from a skin punch biopsy from a 47-year-old woman who was previously fertile (Fig. 1). These fibroblasts (MZT05) were reprogrammed to hiPSCs using the non-integrating recombinant Sendai virus containing reprogramming factors OCT3/4, SOX2, KLF4 and c-MYC4 (Tokusumi et al., 2002). Twenty-seven days after the transduction, individual colonies were manually picked onto mouse embryonic fibroblast feeder cells to create the

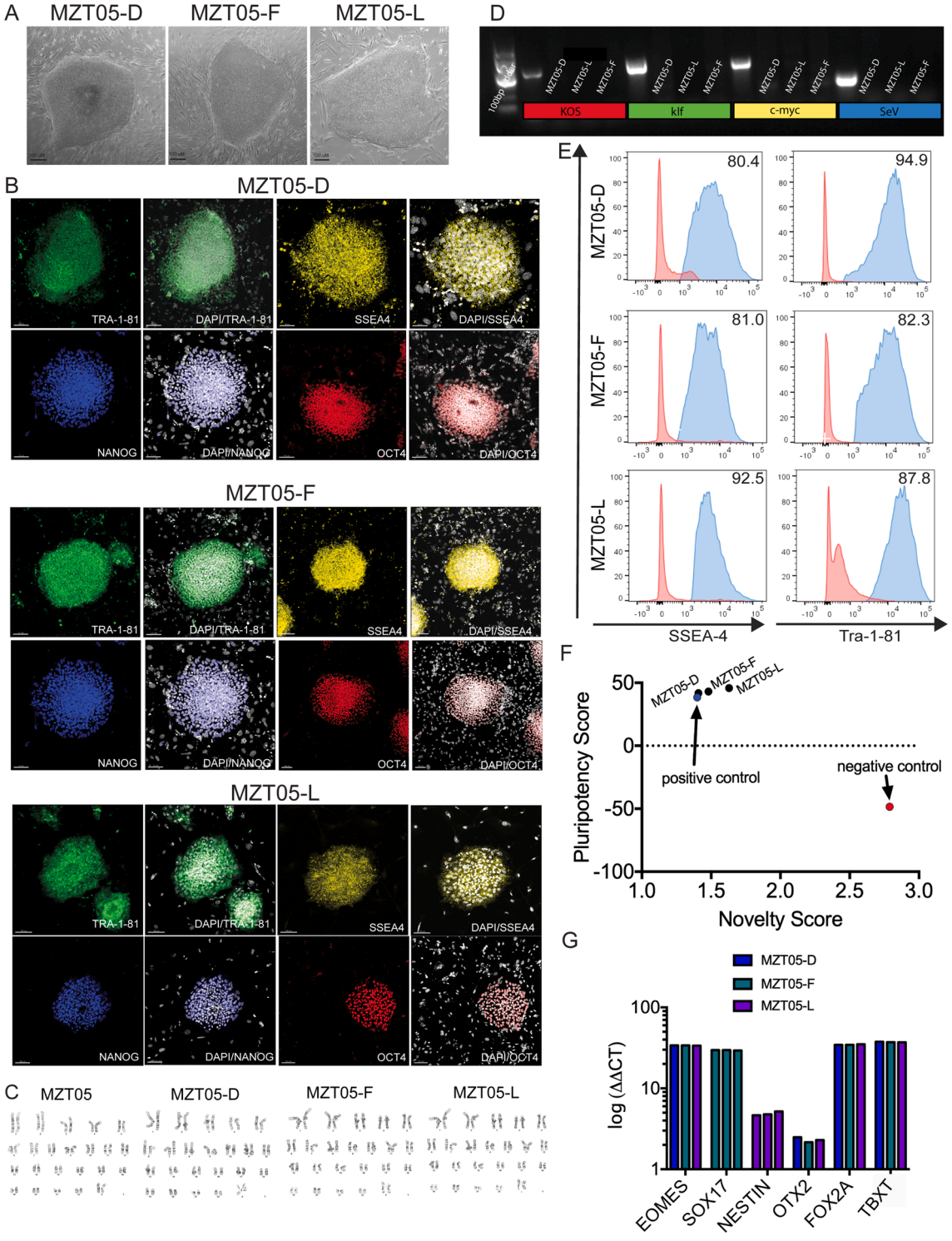

Fig. 1.

sublines. We selected three sublines called MZT05-D, MZT05-F and MZT05-L and characterized their self-renewal and pluripotency (Tables 1 and 2). All hiPSC sublines exhibited typical pluripotent stem cell morphology (Fig. 1A) and markers of self-renewal, as confirmed through immunofluorescence staining for NANOG, OCT4 and TRA-1-81 and SSEA-4 (Fig. 1B) and flow cytometry (Fig. 1E). The reprogrammed cells and the initial fibroblasts displayed a normal 46, XX karyotype (Fig. 1C), and did not express the exogenous reprogramming factors from passage 10 (Fig. 1D) (expected band size SeV: 181 bp, c-MYC: 532 bp, klf: 410 bp, KOS: 501 bp). To confirm that the hiPSC sublines originated from the donated fibroblasts, short tandem repeat (STR) analysis was conducted demonstrating that each of the three hiPSC lines were identical to the original HDFs (MZT05). We also confirmed that MZT05-D, MZT05-F, and MZT05-L were negative for mycoplasma through routine mycoplasma testing (Supplementary figure 1). To evaluate pluripotency of these lines, MZT05-D, MZT05-F, MZT05-L were assessed using a PluriTest analysis (Müller et al., 2011) (Fig. 1F). The ability of each hiSPC subline to differentiate into the three germ layers was assessed using Taqman Real-Time PCR assays for markers of ectoderm, mesoderm, and endoderm (Fig. 1G). This resource is a complement to two previous publications involving the derivation of three sublines from an unrelated woman who was previously fertile (Pandolfi et al., 2019) and six sublines from a pair of monozygotic twins discordant for ovarian failure (Pandolfi et al., 2021).

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Bright Field	Normal	Fig. 1 panel A
Phenotype	Immunofluorescence	Positive for self-renewal markers: OCT4, NANOG, SSEA-4, Tra-1-81	Fig. 1 panel B
	Flow cytometry	MZT05D: Tra 1-81: 94.9%, SSEA-4: 80.4% MZT05F: Tra 1-81: 82.3%, SSEA-4: 81.0% MZT05L: Tra 1-81: 87.8%, SSEA-4: 92.5%	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46,XX	Fig. 1 panel C
Identity	Microsatellite PCR (mPCR) OR	Performed	Supplementary Fig. 2
	STR analysis	16 sites tested, all three lines match each other, and the HDFs	Supplementary Fig. 2
Mutation analysis (IF APPLICABLE)	Sequencing	*N/A*	
	Southern Blot OR	*N/A*	
	WGS		
Microbiology and virology	Mycoplasma	Mycoplasma testing by Luminescence	Supplementary Fig. 1
Differentiation potential	PluriTest	Pluripotent	Fig. 1 panel F
	In vitro Differentiation	Ectoderm, Mesoderm, Endoderm potential	Fig. 1 Panel G
Donor screening (OPTIONAL)	N/A		
Genotype additional info (OPTIONAL)	N/A		

3. Materials and methods

3.1. Maintenance of hiPSC lines

Undifferentiated hiPSC subline cells were cultured on a feeder layer of mitomycin C-treated murine embryonic fibroblasts (MEFs) in hESC media (DMEM/F-12) (Life Technologies), 20% KSR (Life Technologies), 10 ng/mL bFGF (R&D Systems), 1% nonessential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies), Primocin™ (Invivogen), and 0.1 mM β-mercaptoethanol (Sigma). Media was changed daily and colonies were passaged as clumps with collagenase (ThermoFisher, 17104019) 1:4–1:6 every 7 days without rock inhibitor . Cells were cultured in an incubator at 37°, 5.0% CO_2.

4. Fibroblast derivation

A 1 mm skin punch biopsy was dissected and then digested in Collagenase IV (Life Technologies) for 1 h at 37 °C, 5.0% CO_2. The digested pieces were then plated down on 0.1% gelatin (Sigma) coated (Millipore) plates in human fibroblast media, 15% Fetal bovine serum (GE Healthcare), 1% Non-Essential Amino Acids (Invitrogen), 1% Glutamax, (GibcoTM), 1% Penicillin-Streptomycin-Glutamine (Gibco), and Primocin (Invivogen), at 37°, 5.0% CO_2. Outgrowths of fibroblasts were monitored for two weeks and the media was changed every three days. Fibroblasts were passaged using 0.05% Trypsin (Gibco) and re-plated, the derived cells were termed MZT05.

5. Reprogramming the fibroblasts

Fibroblasts were thawed and cultivated in human fibroblast medium. When ~80% confluent, the MZT05 cells were transfected with Sendai virus (SeV) based non-integration CytoTune™ iPS Reprogramming Kit (Life Technologies) according to manufacturer's instructions. Colonies began to appear after 11 days and were picked after three weeks. Three colonies were manually picked and expanded onto mouse embryonic fibroblast feeder cells in hiPSC media (DMEM/F-12 (Life Technologies), 20% KSR (Life Technologies), 10 ng/mL bFGF (R & D Systems), 1% nonessential amino acids (Life Technologies), 1% Penicillin-Strepromyocin-Glutamine (Gibco), Primocin™ (Invivogen), and 0.1 mM β-mercaptoethanol (Sigma)).

6. Flow cytometry

Single cell suspension was obtained using 0.05% Tryspin (Gibco). hiPSCs were then resuspended in PBS with 1% BSA. Antibody incubation lasted 30 min at 4 °C with conjugated antibodies. An LSRII machine was used to process samples and analysis was conducted using FlowJo software. Flow Cytometry analysis was conducted at P17 for MZT05-D, P17 for MZT05-F, and P17 for MZT05-L.

7. PluriTest

Cryopreserved pelleted cells were sent to Life Sciences Solutions.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
UCLAi005-A	MZT05-D	Female	47	Asian	N/A	None
UCLAi005-B	MZT05-F	Female	47	Asian	N/A	None
UCLAi005-C	MZT05-L	Female	47	Asian	N/A	None

Transcriptional profiles of the hiPSC lines were compared to an extensive reference set. The Pluripotency Score is an indication of how strongly a model-based pluripotency signature is expressed in the samples analyzed. The Novelty Score indicates the general model fit for a given sample (Müller et al., 2011).

8. Taqman Real-Time PCR

At Day 7 of self-renewal, the hiPSC subliness were trypsinized (0.05% trypsin, Life Technologies) and the MEFs were depleted by plating the cell suspension in tissue culture dishes, two times, for 5 min each. The resulting cell suspensions were pelleted and resuspended in media containing (GMEM) (Life Technologies), 15% KSR (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), penicillin/streptomycin/L-glutamine (Life Technologies), Primocin™ (Invivogen), 0.1 mM β-mercaptoethanol (Sigma), sodium pyruvate (Life Technologies), activin A (PeproTech), CHIR99021 (Stemgent), Y-27632 (Stemgent), filtered through a 40 μm cell strainer (Falcon). The cell suspension was plated at a density of 2.0×10^5 cells per well of a human plasma fibronectin (Invitrogen)-coated 12-well plate. After 24 h of incubation at 37 °C with 5.0% CO^2 the cells, now called incipient mesoderm-like cells (iMELCs) were trypsinized (0.05% trypsin) and resuspended in media containing (GMEM) (Life Technologies), 15% KSR (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), penicillin/streptomycin/L-glutamine (Life Technologies), Primocin™ (Invivogen), 0.1 mM β-mercaptoethanol (Sigma), sodium pyruvate (Life Technologies), 10 ng/mL human LIF (EMD Millipore), 200 ng/mL BMP4 (R&D Systems), 50 ng/mL EGF (Fisher Scientific), 10 μM Y-27632 (Stemgent). The cells were plated at a density of 3.0×10^3 cells per well of a low adherence spheroid forming 96-well plate (Corning) and cultured for four additional days. Testing was conducted on MZT05-D (p30), MZT05-L (p31), MZT05-F (P28).

At day 4 of differentiation in low adherence plates, the differentiated cells were harvested in RLT buffer and RNA was extracted using qiagen RNAeasy microkit (qiagen, 74004). RNA was converted to cDNA using SuperScript II reverse transcriptase (Thermofisher, 18064014) and random hexamers (Thermofisher, N8080127). Taqman probes (Table 3) were used to identify two markers from each germ layer including; ectoderm: *OTX2* (72 bp product) (Thermofisher, Hs00222238_m1), *NESTIN* (58 bp product) (Thermofisher, Hs04187831_g1); endoderm: *SOX17* (149 bp product) (Thermofisher, Hs00751752_s1), *FOXA2* (144 bp product) (Thermofisher, Hs00610080_m1); mesoderm: *TBXT* (132 bp product) (Thermofisher, Hs00610080_m1), *EOMES* (81 bp product) (Thermofisher, Hs00610080_m1). The Taqman assays were run with the following conditions, 50° C for 2 min, 95° C for 10 min, and 50 cycles of 95° C for 15 sec followed by 60° C for 1 min. Three technical replicates were used to examine gene expression in each of the three MZT05 hiPSC sublines. qPCR was performed using CFX Connect Real-Time PCR Detection System. Averages of each hiPSCs subline were normalized to GAPDH expression for the six target genes. hESC line UCLA2 (Diaz Perez et al., 2012) was used to as a control, and delta delta CT was calculated relative this line.

9. Karyotyping and STR analysis

The three hiPSC sublines, and the HDF primary culture that they were derived from, were karyotyped using metaphase spreads and G-banding by Cell Line Genetics (Madison, WI). Karyotyping on hiPSCs was conducted at P6 for MZT05 HDFs primary cell cultures, P14 for MZT05-D, P11 for MZT05-F, and P11 for MZT05-L. Twenty metaphase spreads were counted for each Karyotype analysis. Cell Line Genetics also performed STR analysis on the three hiPSC sublines and one HDF primary culture using the PowerPlex 16 System (cat# DC6531, Promega).

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Self-renewal markers	goat-anti-human OCT4	1:100	Santa Cruz, sc8628 RRID: AB_653551
Self-renewal markers	goat-anti-human NANOG	1:40	R&D Systems, AF1997 RRID: AB_355097
Self-renewal markers	mouse-anti-human SSEA-4	1:100	Developmental Studies Hybridoma Bank, MC-813–70 RRID: AB_528477
Self-renewal markers	mouse-anti-human TRA-1–81	1:100	eBiosciences, 14–8883-82 RRID: AB_891614
Pluripotency markers	SSEA-4-Allophycocyanin	1:30	R&D Systems, FAB1435A RRID: AB_494994
Pluripotency markers	TRA-1–85-Phycoerythrin	1:60	R&D Systems, FAB3195P RRID: AB_2066683
Pluripotency markers	TRA-1–81, Alexa Fluor 488	1:60	Stemcell Technologies, 60065AD RRID: AB_2721032
Nuclear marker	Dapi	1:100	BioVision, B1098-25 RRID: AB_2336790
Secondary antibodies	AF488-conjugated donkey-anti-goat	1:200	JacksonImmunoResearch, 705–546-147 RRID: AB_2340430
Secondary antibodies	AF488-conjugated donkey-anti-mouse	1:200	Life Technologies, A-21131 RRID: AB_2535771
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Reprogramming virus	SeV	GGA TCA CTA GGT GAT ATC GAG C/ ACC AGA CAA GAG TTT AAG AGA TAT GTA TC	
Reprogramming virus	KOS	ATG CAC CGC TAC GAC GTG AGC GC/ ACC TTG ACA ATC CTG ATG TGG	
Reprogramming virus	Klf4	TTC CTG CAT GCC AGA GGA GCC C/ AAT GTA TCG AAG GTG CTC AA	
Reprogramming virus	c-Myc	TAA CTG ACT AGC AGG CTT GTC G/ TCC ACA TAC AGT CCT GGA TGA TGA TG	
Taqman assay	EOMES	Hs00610080_m1	
Taqman assay	SOX17	Hs00751752_s1	
Taqman assay	FOXA2	Hs00610080_m1	
Taqman assay	OTX2	Hs00222238_m1	
Taqman assay	TBXT	Hs00610080_m1	
Taqman assay	NESTIN	Hs04187831_g1	
Taqman assay	GAPDH	Hs02786624_g1	

10. Immunofluorescence staining

Immunofluorescence staining was performed by fixing the hiPSCs in 4%PFS for 15 min at room temperature, and then permeabilizing the cells with PBS plus 0.5% Triton™ X-100 (Sigma). The hiPSCs were then blocked in 10% donkey serum (Jackson Immunoresearch) for 30 min at room temperature. Cells were incubated overnight at 4 °C with primary antibodies and then were incubated in secondary antibodies for 1 h at room temperature (Table 3). Cells were incubated with DAPI nuclear stain for 15 min. Immunofluorescence was imaged using a Zeiss LSM 880 confocal laser-scanning microscope. Immunofluorescence analysis was conducted at P15 for MZT05-D, P13 for MZT05-F, and P14 for MZT05-L.

11. Absence of the reprogramming virus

RNA was isolated according to manufacturer's instructions (cytotune) from reprogrammed fibroblasts at P0 before hiPSCs were picked and cultured to function as the positive control. cDNA was synthesized from the RNA and RT-PCR was performed using primers provided from the manufacturer (Table 3). All hiPSC sublines were tested for absence of the reprogramming virus at P10.

12. Mycoplasma detection

Mycoplasma was regularly tested using MycoAlert kit from Lonza - Catalog #LT07-318. We used the Mycoalert kit to calculate the presence of mycosomal enzymes within hiPSC test sample. Through measurement of ADP to ATP conversion both before and after addition of the MycoAlertTM PLUS Substrate, a ratio can be constructed that indicates presence of absence of the virus. Ratios below 1.0 indicate that the sample is negative for mycoplasma, a value over 1.2 indicates presence of mycoplasma in the sample. Mycoplasma analysis was conducted at P20 for MZT05-D, P23 for MZT05-F, and P29 for MZT05-L.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2021.102409.

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