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Generation of a transgene-free human induced pluripotent stem cell line (UNIPDi001-A) from oral mucosa epithelial stem cells

Gualtiero Alvisi, Marta Trevisan, Giulia Masi, Vanessa Canel, Luciana Caenazzo, Patrizia Nespeca, Luisa Barzon, Enzo Di Iorio, Vanessa Barbaro, Giorgio Palù

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Lab Resource: Single Cell Line - template

Title: *Generation of a transgene-free human induced pluripotent stem cell line (UNIPDi001-A) from oral mucosa epithelial stem cells.*

Authors: Gualtiero Alvisi[a], Marta Trevisan[a*], Giulia Masi[a], Vanessa Canel[a], Luciana Caenazzo[a], Patrizia Nespeca[a], Luisa Barzon[a], Enzo Di Iorio[a], Vanessa Barbaro[b], Giorgio Palù[a].

Affiliations: [a]Department of Molecular Medicine, University of Padova, 35121, Padua, Italy.

[b]Fondazione Banca degli Occhi del Veneto, 30174 Venice, Italy.

* Correspondence: marta.trevisan@unipd.it;

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Abstract

Human oral mucosa epithelial stem cells (hOMESCs) were obtained from a fresh oral biopsy collected from a healthy subject at the Fondazione Banca degli Occhi del Veneto (FBOV). An integration-free reprogramming protocol was applied exploiting episomal plasmids transfected into cells using a Nucleofector device. Around day 20 post transfection, several human induced pluripotent stem cell (hiPSC) colonies were manually picked and expanded. One of these (UNIPDi001-A-hiPSCs) expressed undifferentiated state marker alkaline phosphatase along with a panel of pluripotency state markers and was able to differentiate into the derivatives of all the three germ layers.

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Resource Table:

Unique stem cell line identifier	*UNIPDi001-A-hiPSC*
Alternative name(s) of stem cell line	*KeNu-C-hiPSCs*
Institution	*Department of Molecular Medicine, University of Padova, Padua, Italy*
Contact information of distributor	*Marta Trevisan, marta.trevisan@unipd.it*
Type of cell line	*iPSC*
Origin	*human*
Additional origin info	*Age: N/A* *Sex: female* *Ethnicity: caucasian*
Cell Source	*human oral mucosa epithelial stem cells (hOMESCs)*
Clonality	*Clonal*
Method of reprogramming	*Transgene free, episomal vectors*
Genetic Modification	*No modification*
Type of Modification	*No modification*
Associated disease	*N/A*
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	*N/A*
Cell line repository/bank	*N/A*
Ethical approval	*Patient informed consent obtained and approved by the Venetian Ethical Committee for Clinical Research Studies (Prot. 2009/77661, November 19, 2009*

Resource utility

This hiPSC line was generated from human oral mucosal epithelial stem cells (hOMESCs). This cell source can offer many advantages such as it share many features and a very similar phenotype with limbal stem cells and could therefore be applied to study the regeneration of ocular tissues from hiPSCs.

Resource Details

Human oral mucosa epithelial stem cells (hOMESCs) obtained from oral biopsy of a healthy donor were used for the generation of the human induced Pluripotent Stem cell (hiPSC) line UNIPDi001-A-hiPSC. hOMESCs were grown on irradiated 3T3-J2 feeder and expanded until sub-confluent. At this point, cells were detached and nucleofected with episomal vectors expressing the pluripotency genes OCT4, SOX2, KLF4, l-MYC and LIN28. A plasmid expressing a shRNA against p53 was also included [1]. Transfection was performed usin g the 4D Nucleofector device with a condition which proved the highest transfection efficiency. Transfected cells were seeded on mouse embryonic fibroblasts (MEFs)-coated plates, which were supplemented weekly hereafter. While cells progressed from somatic state towards pluripotency, morphology changes were gradually observed and round shaped hiPSCs colonies with defined edges and faint cytoplasm started to emerge from as early as day 15 post transfection (p.t.). Different colonies were picked starting from day 20 p.t. under a stereomicroscope. The UNIPDi001-A-hiPSC line was expanded and fully characterized. Analysis of fingerprinting confirmed its identity to the parental cell line (Fig. 1A). G-band karyotype evaluation showed no chromosomal abnormalities (Fig. 1D). PCR and RT-PCR analysis demonstrated the absence of residual episomal vectors

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(EBNA1) and of transgenes expression, respectively (Fig. 1E). Importantly, UNIPDi001-A-hiPSCs expressed the marker of undifferentiated state alkaline phosphatase (AP; Fig. 1B, BF: bright field; scale bar=100μm), along with a panel of pluripotency proteins such as OCT4, SSEA3, KLF4, Nanog and SSEA4, as shown by indirect immunofluorescence analysis (Fig. 1C, scale bars=100μm). Gene expression of pluripotency markers (i.e. OCT4, NANOG, and SOX2,) was also demonstrated by qRT-PCR (Fig. 1F). Finally, UNIPDi001-A-hiPSCs formed embryoid bodies (EBs) in vitro (Fig. 1G, scale bar=200μm) and expressed the markers belonging to the ectodermal (TUBB, PAX6), endodermal (AFP, GATA4) and mesodermal (FLK1, GATA2, PECAM, CDH5) germ layers, as demonstrated by RT-PCR (Fig. 1H). These results confirmed the pluripotent state of the UNIPDi001-A-hiPSC line.

Materials and Methods

Cell cultures:

hOMESCs were grown on irradiated 3T3-J2, in keratinocytes growth medium (KGM), as previously described [2]. The generated hiPSC line was grown on MEFs (MTI-Global Stem) in human embryonic stem (hES) cell medium as previously described [3].

Reprogramming of hOMESCs

hOMESCs (10^6) were re-suspended in 100 μl of Amaxa P3 solution (Lonza) together with 2μg of each of the episomal plasmids pCXLE-hOCT3/4, pCXLE-hSK and pCXLE-hUL (Addgene plasmids: # 27077, #27078, # 27080). The mixture was transferred to a cuvette and electroporated with a 4D Nucleofector device (Lonza). Cells were seeded on 10cm dishes previously coated with MEFs in KGM. Medium was changed every other day for one week gradually switching from KGM to hES medium [3]. From day 7 on, hES medium was changed on a daily basis.

Fingerprinting analysis:

DNA from the UNIPDi001-A-hiPSC line and from parental hOMESCs was isolated with the QIAamp DNA Mini Kit (Qiagen). The DNA profiling test was carried out with the AmpFlSTR Identifiler Plus kit (Thermo Fisher Scientific) through multiplex PCR. Amplification products were then analysed by capillary electrophoresis on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Karyotype analysis

G-banding karyotype analysis was performed by Cell Guidance system (Cambridge, CB22 3AT, UK).

Analysis of residual episomal plasmids and residual transgene expression

Episomal vectors possibly retained in UNIPDi001-A-hiPSC were investigated by PCR upon DNA isolation. For analysis of residual transgene expression, total RNA was isolated from UNIPDi001-A-hiPSCs line with the RNaeasy mini kit (Qiagen) and used for the RT-PCR analysis (Table 2).

AP live staining

The UNIPDi001-A-hiPSC cell line was incubated for 30 min at room temperature (RT) with 1X AP Live Stain Solution (Thermo Fisher Scientific) and observed using a Leica DFC420 inverted epifluorescence microscope.

Pluripotency marker expression by immunofluorescence

UNIPDi001-A-hiPSCs were treated with PFA 4% (Sigma Aldrich) for 20 min at RT and permeabilized for 15 min at RT with PBS/0.1% Triton X-100 (Sigma Aldrich). Cells were then saturated with 4% BSA/PBS (Sigma Aldrich) overnight at 4°C. The next day, cells were incubated for 1 hour at RT with primary antibodies (Table 2) and

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subsequently, with secondary antibodies (Table 2) for 1 hour at RT in the dark. Nuclei were stained by incubation with DRAQ5, 5μM in PBS (Thermo Fisher Scientific) for 10 min at RT.

Pluripotency gene expression by qRT-PCR

Total RNA was isolated from UNIPDi001-A-hiPSC line. cDNA was obtained by reverse transcription and used as template for the qRT-PCR analysis (Table 2).

Embryoid bodies (EBs) assay

UNIPDi001-A-hiPSCs were detached, moved to a ultra-low density attachment plate (Corning) and forced to grow in suspension with hES medium deprived of bFGF. After 7 days the EBs were transferred to a 0.1% porcine gelatine (Merck Millipore) coated plate and the medium was switched to regular 10% Foetal Bovine Serum (FBS) DMEM. After 7 days cells were harvested, total RNA was isolated, reverse transcribed and used as template for RT-PCR analysis of a panel of genes belonging to the ectoderm, endoderm and mesoderm germ layers (Table 2).

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Table 1: Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	*Visual record of the line: normal*	*Figure 1 B*
Phenotype	Qualitative analysis *Immunocytochemistry*	*Pluripotency markers: OCT4, SSEA3, NANOG, SSEA4*	*Figure 1 panel C*
	Quantitative analysis (qPCR) Immunocytochemistry counting	*Oct4, Nanog, Sox2* *Oct4: 99%; Nanog: 98%; SSEA3: 97%; SSEA4: 98%*	*Figure 1 panel F*
Genotype	Karyotype (G-banding) and resolution	*46XX, Resolution 450-500*	*Figure 1 panel D*
Identity	STR analysis	*DNA Profiling: N/A*	*N/A*
		15 sites tested, all matched	*Figure 1 panel A, Figure2/ supplementary; Submitted in archive with journal*
Mutation analysis (IF APPLICABLE)	Sequencing	*N/A*	*N/A*
	Southern Blot OR WGS	*N/A*	*N/A*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by PCR. Negative*	*Figure 1/supplementary*
Differentiation potential	Embryoid body formation	*ectoderm(TUBB, PAX6), endoderm (AFP, GATA4) and mesoderm (FLK1, GATA2, PECAM, CDH5)*	*Figure 1 panel G and H*
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

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Table 2: Reagents details

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	*-Goat anti-OCT3/4 [N-9]*	*1:200*	*-Santa Cruz Cat#: sc-8628, RRID:AB_653551*
	-Rat anti-SSEA3[MC631]	*1:50*	*-Abcam Cat#: ab16286, RRID:AB_882700*
	-Mouse a*nti-SSEA4 [MC813]*	*1:50*	*-Abcam Cat#: ab16287, RRID:AB_778073*
	-Mouse anti-NANOG[NNG-811]	*1:50*	*-Abcam Cat#: ab62734, RRID:AB_956161*
Differentiation Markers	*N/A*	*N/A*	*N/A*
Secondary antibodies	*-Goat anti-Rat IgG H&L (FITC)*	*1:250*	*-Abcam Cat#: ab6840, RRID:AB_955326*
	-Donkey anti-goat IgG-R	*1:250*	*-Santa Cruz Cat# sc-2094, RRID:AB_641161*
	-Goat anti-Mouse IgG	*1:500*	*- Millipore Cat# AP124R, RRID:AB_90563*

Primers		
	Target	**Forward/Reverse primer (5'-3')**
Episomal Plasmids	*OCT4 Plasmid*	*CAT TCA AAC TGA GGT AAG GG* *TAG CGT AAA AGG AGC AAC ATA G*
	KLF4 Plasmid	*CCA CCT CGC CTT ACA CAT GAA GA* *TAG CGT AAA AGG AGC AAC ATA G*
	SOX2 Plasmid	*TTC ACA TGT CCC AGC ACT ACC AGA* *TTT GTT TGA CAG GAG CGA CAA T*
	l-MYC Plasmid	*GGC TGA GAA GAG GAT GGC TAC* *TTT GTT TGA CAG GAG CGA CAA T*
	LIN28 Plasmid	*AGC CAT ATG GTA GCC TCA TGT CCG C* *TAG CGT AAA AGG AGC AAC ATA G*
	EBNA1	*ATC AGG GCC AAG ACA TAG AGA TG* *GCC AAT GCA ACT TGG ACG TT*
Pluripotency Markers	*OCT4*	*GGTGCCTGCCCTTCTAGGAATGGGGGA* *CAAAAACCCTGGCACAAACTC* *Probe: CTAGGGAAAGAAAACC*
	SOX2	*GAGAAGTTTGAGCCCCAGG* *AGAGGCAAACTGGAATCAGG* *Probe: ATCATCGGCGGCGGCA*
	NANOG	*GGTCTCGATCTCCTGACCTTGT* *GCCTGTAAATCCCAGCTGTTAGG* *Probe: TCCACCCGCCTCGG*
House-Keeping Genes	*ACTIN*	*CGGGACCTGACTGACTACCTC* *CCATCTCTTGCTCGAAGTCCAG* *Probe: TCCTTAATGTCACGCACGATTTCCCGCT*
Differentiation Markers	*TUBB*	*CAGATGTTCGATGCCAAGAA* *TGCTGTTCTTGCTCTGGATG*
	PAX6	*TCTAATCGAAGGGCCAAATG*

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		TGTGAGGGCTGTGTCTGTTC
	AFP	*AGCTTGGTGGTGGATGAAAC* *CCCTCTTCAGCAAAGCAGAC*
	GATA4	*CTAGACCGTGGGTTTTGCAT* *TGGGTTAAGTGCCCCTGTAG*
	FLK1	*AGTGATCGGAAATGACACTGGA* *GCACAAAGTGACACGTTGAGAT*
	GATA2	*GCAACCCCTACTATGCCAACC* *CAGTGGCGTCTTGGAGAAG*
	PECAM	*CCCAGCCCAGGATTTCTTAT* *ACCGCAGGATCATTTGAGTT*
	CDH5	*CAGCCCAAAGTGTGTGAGAA* *TGTGATGTTGGCCGTGTTAT*
Genotyping	*N/A*	*N/A*
Targeted mutation analysis/sequencing	*N/A*	*N/A*

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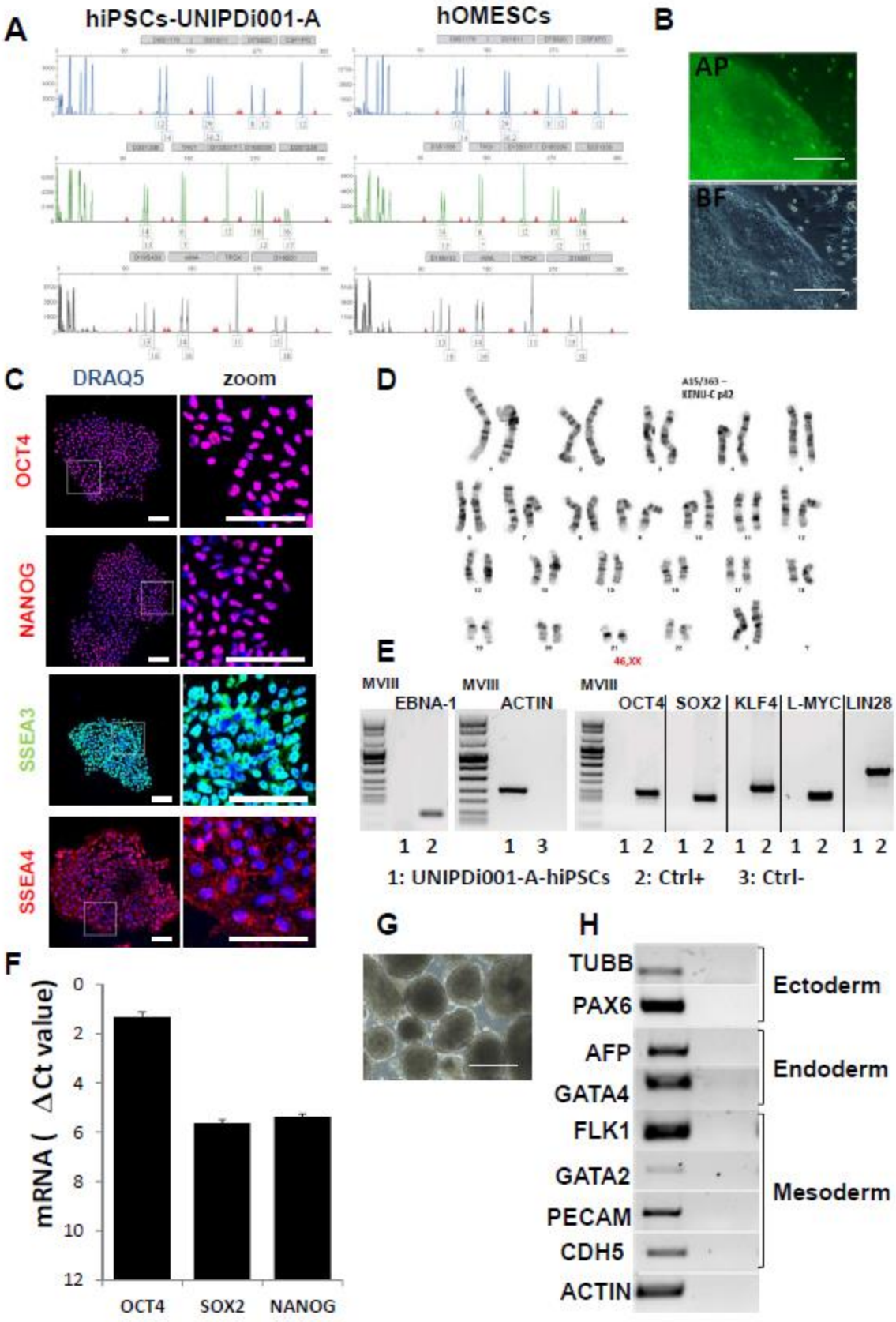

Fig. 1