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Lab resource

# Generation of human induced pluripotent stem cells in defined, feeder-free conditions

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ABSTRACT

Herein, we describe a modified protocol for the generation of human induced pluripotent stem cells (hiPS) and expansion under defined, serum free and feeder free conditions. These cells exhibit a high level of plasticity towards various differentiation pathways both in vitro and in vivo. Ultimately, hiPS-derived lines achieved high standards of three dimensional differentiations on biomaterial scaffolds and promoted in vivo regeneration of complex organs, such as Anterior Cruciate Ligament (in swine ACL-rupture models) and other tissues as well.



Resource table.

| | |
|---|---|
| Name of stem cell construct | FORTHi001A (hiPS line 1)<br>FORTHi001B (hiPS line 2)<br>FORTHi001C (hiPS line 3) |
| Institution | Department of Biomedical Research- Institute of Molecular Biology & Biotechnology, Foundation of Research and Technology-Hellas (BRI-FORTH), University Campus, 45110 Ioannina, Greece |
| Person who created resource | Athena Kyrkou |
| Contact person and email | Carol Murphy, c.e.murphy@bham.ac.uk |
| Date archived/stock date | 30 June 2014 |
| Origin | Human skin fibroblasts |
| Type of resource | Human induced pluripotent cells (hiPS) |
| Sub-type | Cell line |
| Key transcription factors | Human Oct3/4, Sox2, L-Myc, Klf4, Lin28, sh-p53 |
| Authentication | Identity and purity of cell line confirmed (Fig. 1) |
| Q3 Link to related literature (direct URL links and full references) | Kouroupis et al., 2016, Stem Cell Research-submitted |
| Information in public databases | Microarray data available in NCBI Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/<br>GEO#GSE58932 |

## 1. Resource details, materials and methods

Human skin fibroblasts (ATCC #CRL 2429) were plated at a density of 100,000 cells per 35 mm cell culture dish. The reprogramming strategy was based on a modification of the Y4 episomal approach (Okita et al.). Plasmids for the reprogramming pCXLE-hOCT3/4 -shp53pCXLE -hULpCXLE were purchased from Addgene, Middlesex, UK. In our modified protocol, subconfluent fibroblasts were transfected with the episomes using lipofectamine 2000 (LifeSciences, #11668, Invitrogen, Carlsbad, CA, USA), according to the manufacturers' protocol. 48 h post-transfection, cells were refreshed with fibroblast medium (IMDM #30–2005, supplemented with 10% fetal bovine serum/FBS #16000-044, Gibco, Gaithersburg, USA). On day 4 cells were fed with serum containing embryonic stem cell medium (Knockout DMEM/F12 #12660, 15% FBS #16000-044, 1% Non-Essential Amino Acids/NEAA #11140, 1% Glutamax #35050, 1 × 2-mercaptoethanol #21985023 all from Gibco, supplemented with 40 ng/ml bfgf #100-18B, Peprotech, Rochy Hill, USA) which was refreshed daily, for the next two days. On day 6, this cell population was trypsinized and reseeded 1:7 on irradiated fibroblasts(#GSC-6301G, GlobalStem, Oxfordshire, UK) to boost reprogramming. On day 8 cells were grown in serum free embryonic stem cell medium (Knockout DMEM/F12 #12660, 15% KnockOut Serum Replacement #10828 -Gibco, 1% NEAA, 1% Glutamax, supplemented with 1 × 2-mercaptoethanol and 20 ng/ml bfgf) and the medium was changed daily. From day 12 to Day 20 cells were fed with conditioned serum-free embryonic stem cell medium from feeders, to sustain pre-formed human induced pluripotent (pre-hiPS) colonies' growth. On day 20, pre-hiPS

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were manually picked using 1 mg/ml dispase (#17105, Gibco) and grown on matrigel (dilution 1:100, # 354277, BD Biosciences, Oxford, UK) with mTeSR medium (#05850, Stem Cell Technologies, Vancouver, Canada). Thereafter, hiPS colonies were manually dissected "en block" and expanded in feeder free and serum free conditions (Fig. 1A).

## 2. Verification and authentication

### 2.1. Phenotypic characterization of hiPS

Successful reprogramming of initial fibroblasts towards induced pluripotency was evaluated by immunostaining with key pluripotency

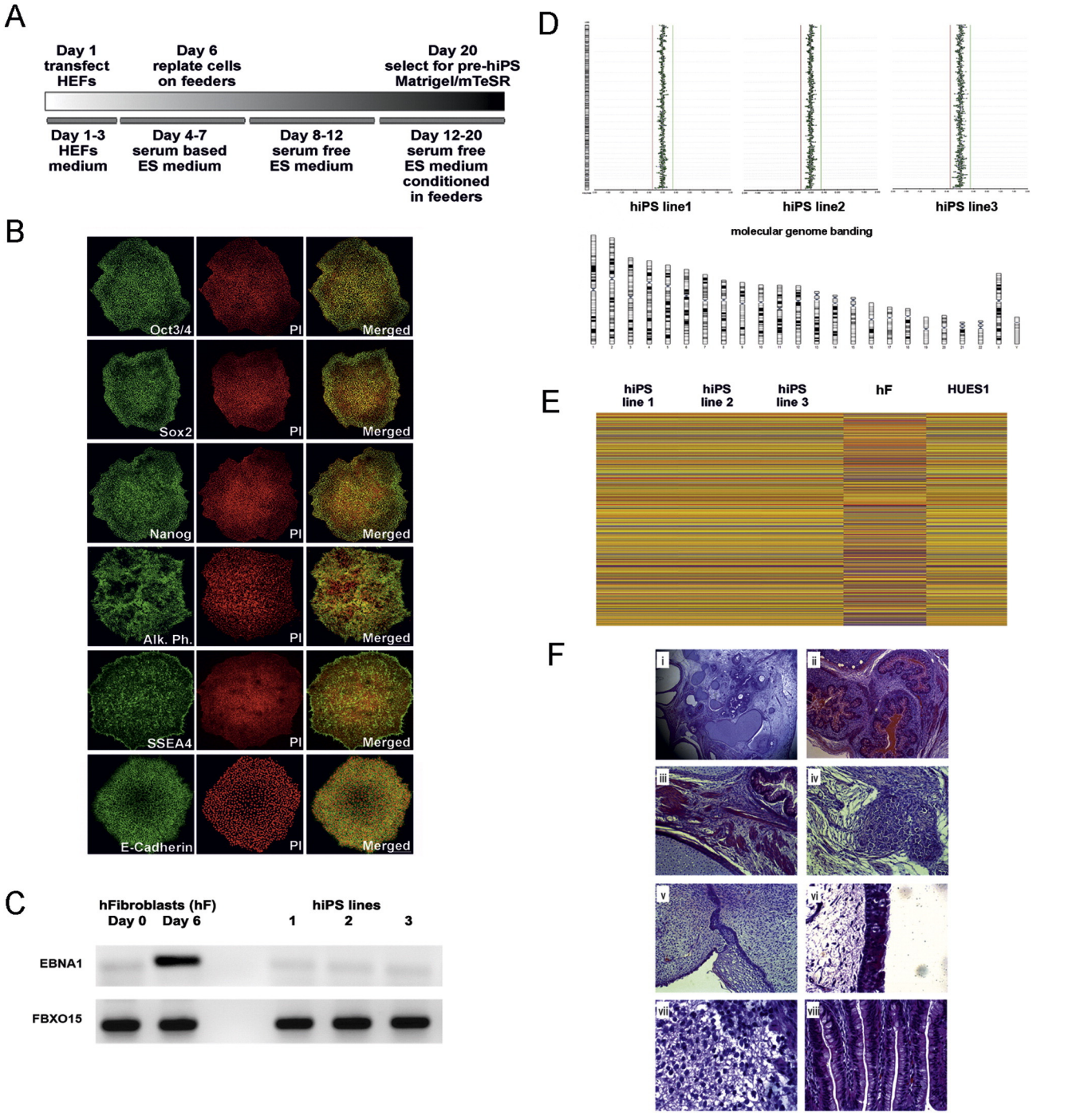


**Fig. 1.** Generation and validation of hiPS. A) Reprogramming strategy, B) immunofluorescence analysis of generated hiPS for embryonic stem cell markers. hiPS colonies were positive for Oct3/4, Sox2, Nanog, Alkaline phosphatase, SSEA4, and E-Cadherin stem cell markers. Propidium iodide was used to stain cell nucleus. C) Footprint analysis of generated hiPS lines. A PCR-based test for the validation of integration-free hiPS lines was performed targeting the EBNA-1 sequence derived from the Epstein-Barr virus to calculate the copy number of the episomal vector and another primer set for the endogenous locus FBXO15. The generated hiPS lines show no episomal footprint. D) Comparative genomic hybridisation analysis shows genetic stability with no evidence of chromosomal abnormalities, E) Heat map analysis of the differentially regulated genes following reprogramming among hiPSC lines, original human fibroblasts (hFs) and embryonic HUES1 stem cell line. All hiPS lines show heat map genetic profile identical to embryonic HUES and different from original hFs. F) Histological

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markers (Fig. 1B). Cells were manually seeded (en bloc) on a matrigel coated chamber-slide system (LabTek #177437). Cells were fixed with either ice cold Methanol or paraformaldehyde 3.7% (followed by permeabilization with 0.1% Triton for 4 min). After blocking with 5% donkey serum, cells were stained with antibodies recognizing stem markers for 1 h at the following concentrations: Oct3/4 (1:300), Nanog (1:100), Sox2 (1:100), Alkaline Phosphatase (1:100), SSEA4 (1:100) and E cadherin (1:300) (Suppl. Table 1). After washing, secondary antibodies FITC donkey α-mouse or FITC donkey α-goat were added for 45 min, followed by 30 min incubation with 1 mg RNAse (Sigma-Aldrich, Hertfordshire, UK) and 20 min with propidium iodide (Sigma-Aldrich, dilution 1:5000). Samples were mounted with Prolong antifade reagent (Invitrogen, Life Technologies) and imaging was performed with a Leica TCS SP5 (10× dry objective).

### 2.2. Genotyping

High-resolution array-based comparative genomic hybridization (CGH) (Centre of molecular biology and cytogenetics AlphaLab, Greece) showed that hiPSC lines had no indication for copy number variations and were therefore chromosomally stable (Fig. 1C). Moreover, PCR screening of the genomic DNA, using primers specific for the EBNA1 amplicon of the episomes used, verified footprint-free generation (Fig. 1D).

### 2.3. Whole transcriptome analysis

To validate the comparable transcriptional profile of the generated hiPS lines versus human embryonic stem cells (HUES1 line, NIH repository), we proceeded with whole transcriptome analysis, accessible in the Gene Expression Omnibus (GEO) of NCBI (#GSE58932). Quality-assessed RNA samples were hybridized to Affymetrix ExonExprChip.HuGene-1 arrays (EMBL-GeneCore, Heidelberg, Germany). Normalized data underwent statistical analysis (GeneSpring Software) to determine differentially expressed genes ($p \leq 0.05$ and fold change $\geq 2$). A differential expression of approximately 3300 genes was observed between hiPSCs and control embryonic stem cell line HUES1 versus the initially transduced fibroblasts (heatmap in Fig. 1E).

### 2.4. Differentiation

To ensure full pluripotency we carried out teratoma assays in immune-deficient NOD/SCID mice, maintained under SPF conditions (Animal facility of the Academy of Athens, Greece). After manual dissection of hiPS and HUES1 cultures (each from a subconfluent 35 mm dish), cell clumps were centrifuged and resuspended in 100 μl of hES-qualified matrigel, just prior to injection. The cell-matrigel mix was injected through 25G 7/8 needle (BD Biosciences) subcutaneously in the hind legs of the mice. Transplanted animals developed tumors within a month and histopathological examination yielded tissue derivatives from all three germ lines: ectoderm, mesoderm and endoderm (Fig. 1F).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.05.006.

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