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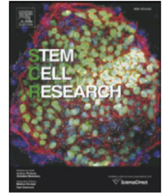

Lab resource: Stem cell line

Generation of an iPS cell line from bone marrow derived mesenchymal stromal cells from an elderly patient

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ABSTRACT

An induced pluripotent stem cell line was generated from primary human bone marrow derived mesenchymal stromal cells of a 74 year old donor using retroviruses harboring *OCT4*, *SOX2*, *KLF4* and *c-MYC* in combination with the following inhibitors TGFβ receptor-SB 431542, MEK-PD325901, and p53-Pifithrin α. Pluripotency was confirmed both *in vitro* and *in vivo.*

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1. Resource table:

Name of stem cell construct	pMXs-hOCT4 (Addgene, plasmid 17217), pMXs-hSOX2 (Addgene, plasmid 17218), pMXs-hKLF4 (Addgene, plasmid 17219), pMXs-hc-MYC (Addgene, plasmid 17220)
Institution	Max Planck-Institute for Molecular Genetics
Person who created resource	Matthias Megges
Contact person and email	James Adjaye, James.Adjaye@med.uni-duesseldorf.de
Date archived/stock date	June 12, 2011
Origin	Primary human bone marrow derived mesenchymal stromal cells of a 74 year old female donor
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); derived from primary human bone marrow derived mesenchymal stromal cells of a 74 year old female donor
Sub-type	Cell line
Key transcription factors	OC4, SOX2, c-MYC and KLF4
Authentication	Identity of cell line confirmed (Fig. 1 and Fig. 2.)
Link to related literature (direct URL links and full references)	
Information in public databases	no

2. Resource details

pMX vector-based retroviral delivery of the reprogramming factors OCT4, SOX2, KLF4 and c-MYC was used in combination with the TGFβ receptor inhibitor SB431542, MEK inhibitor PD325901 and the p53 inhibitor Pifithrin α to induce pluripotency in the parental primary bone marrow derived mesenchymal stromal cells (parental hBM MSC). The expression of pluripotency-associated proteins was confirmed in the iPS cell line generated from parental hBM MSC (MSC-iPSC (74)) (Fig. 1A). The expression of pluripotency-associated genes was comparatively confirmed in MSC-iPSC (74) and hESC H1 using real time PCR (Fig. 1B). In addition, microarray-based transcriptome profiling revealed a high similarity between MSC-iPSC (74), hESC H1 and hESC H9 compared to the parental hBM MSC (Fig. 1C and D). The somatic origin of MSC-iPSC 74 and the absence of cross-contamination with embryonic stem cell lines H1 and H9 were confirmed by DNA fingerprinting (Fig. 1E). Karyotype analysis revealed a normal female karyotype (Fig. 1F). In addition, pluripotency in MSC-iPSC (74) was confirmed *in vitro* using embryoid body based differentiation and. *in vivo* using the teratoma assay (Fig. 2A and B).

3. Materials and methods

3.1. Ethics statement

Mesenchymal stromal cells, used for generation of the induced pluripotent stem cell line, were isolated from the bone marrow of a 74-year-old female donor after written informed consent of the patient. The corresponding protocol was approved by the research ethics board of the Charite-Universitätsmedizin, Berlin (IRB approval EA2/126/07).

3.2. Cell culture

Parental hBM MSC were isolated from human bone marrow by density separation using Histopaque®-1077 (Sigma-Aldrich) and

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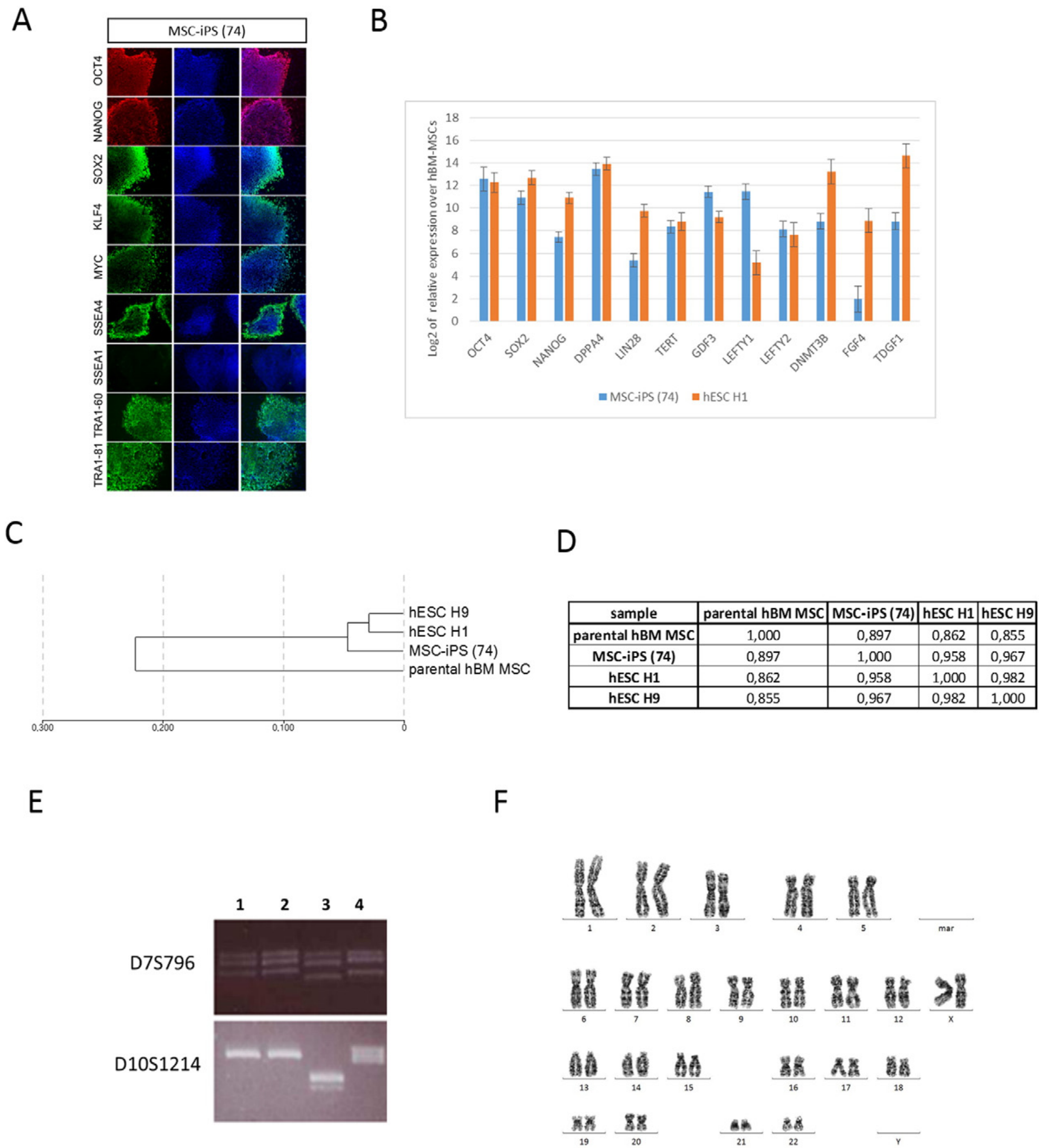

sample	parental hBM MSC	MSC-iPS (74)	hESC H1	hESC H9
parental hBM MSC	1,000	0,897	0,862	0,855
MSC-iPS (74)	0,897	1,000	0,958	0,967
hESC H1	0,862	0,958	1,000	0,982
hESC H9	0,855	0,967	0,982	1,000

Fig. 1. Characterization of the iPS line. A) Confirmation of pluripotency marker expression and the absence of the differentiation marker SSEA1 in MSC-iPSC (74). Immunofluorescence staining, red/green: detected marker; blue: DAPI to visualize nuclei. B) Confirmation of the mRNA expression of pluripotency-associated genes in MSC-iPSC (74) and hESC H1, which served as the control. The bars represent the Log_2 of the mean value of the detected relative expression in MSC-iPSC (74) or hESC H1 over the detected relative expression in the parental hBM-MSC. The error bars represent the standard deviation of three technical replicates. C) Clustering dendrogram generated with the software GenomeStudio (Illumina) based on the Pearson correlation between the transcriptomes of parental hBM MSC, MSC-iPSC (74), hESC H1 and hESC H9. The mRNA expression values were detected using an Illumina Bead Chip microarray. D) Values of the Pearson correlation co-efficient between the transcriptomes of the same samples compared in the clustering dendrogram. The correlation values were calculated using the software GenomeStudio (Illumina). E) Confirmation of the somatic origin of MSC-iPSC (74) by DNA fingerprinting using microsatellite-specific primers in a PCR with genomic DNA of 1: MSC-iPSC (74), 2: parental hBM MSC, 3: hESC H1 and 4: hESC H9. F) Karyotyping analysis of MSC-iPSC (74). The analysis revealed a normal female karyotype.

subsequent selection by plastic adherence. For MSC cultivation, Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum (FCS; Biochrom), GlutaMAX™, and 100 U/mL penicillin + 100 μg/mL streptomycin (all from Life Technologies) were used. Isolated MSC were routinely characterized according the minimal criteria of the International Society for Cellular Therapy (Dominici et al., 2006). In this regard, parental hBM MSC were positive (>95%) for CD73, CD105, and CD90, and negative (<2%) of CD14, CD19, CD34, CD45, CD79α, and human leukocyte antigen-DR (HLA-DR). The capacity of parental hBM MSC to differentiate into osteogenic, adipogenic, and chondrogenic lineage was confirmed by addition of appropriate media as previously described (Glaeser et al., 2010).

Human embryonic stem cell (hESC) lines H1 and H9 (WiCell (#WA01 and #WA09, respectively)) as well as the generated MSC-

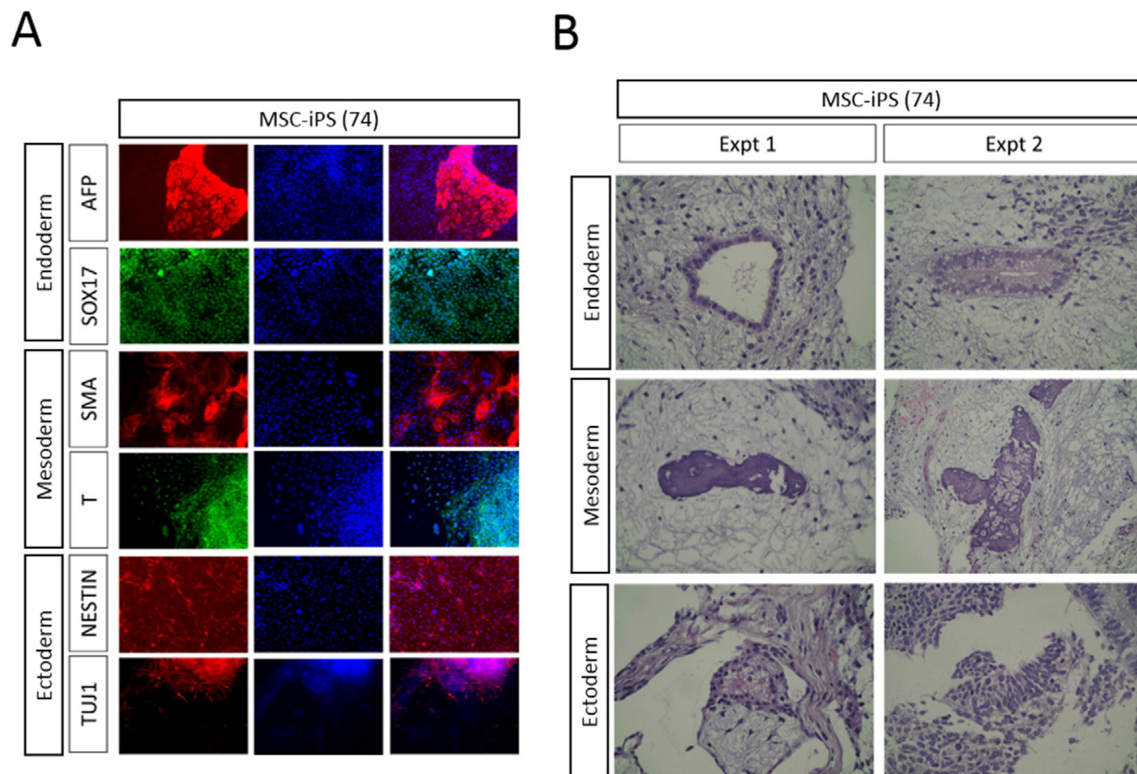

Fig. 2. Confirmation of ability to give rise to lineages of all three germ layers. A) Embryoid body-based *in vitro* test of pluripotency. MSC-iPSC (74) gave rise to cell lineages expressing endodermal markers (SOX17) and α Fetoprotein (AFP), mesodermal markers Brachyury (T) and Smooth-Muscle-Actin (SMA) and ectodermal markers NESTIN and β-TubulinIII (TUJ1). Detection by immunofluorescence staining. red/green: detected marker; blue: DAPI to visualize nuclei. B) Pluripotency confirmation by teratoma assay. MSC-iPSC (74) gave rise to structures representative of the three germ layers. Expt 1: endoderm: tubular gland-like structure; mesoderm: bone; ectoderm: mucous gland. Expt 2: endoderm: gut-like epithelium; mesoderm: bone; ectoderm: neuroepithelial structure. Hematoxylin and eosin staining of the teratoma tissue.

iPSC (74) were expanded in pluripotent stem cell medium containing KO-DMEM with 20% knockout serum replacement, L-glutamine, nonessential amino acids, sodium pyruvate, penicillin/streptomycin, 0.1 mM β-mercaptoethanol (all from Life Technologies). Basic fibroblast growth factor (bFGF) (Preprotech) was added with a final concentration of 8 ng/mL. The cultures were passaged with a split ratio of 1:3 using a syringe needle, pipette and binocular microscope. The pluripotent cells were cultured on mitomycin-C-inactivated mouse embryonic fibroblasts (MEFs) seeded on cell culture dishes coated with Matrigel (Beckton Dickinson). All cultures were carried out in a humidified atmosphere at 37 °C as well as 5% O_2 and 5% CO_2.

3.3. Derivation of iPSCs

Parental hBM MSC were seeded at a density of 2×10^5 cell per well of a six well plate for transduction with retroviruses expressing OCT4, SOX2, KLF4 and c-MYC. Viral particles were generated in 293T cells using a conventional $CaCl_2$ transfection protocol. After transduction, parental hBM MSC were split 1:3 onto Matrigel-coated cell culture dishes on which inactivated MEFs were seeded as a feeder layer. The transduced cells were further cultured for seven days in pluripotent stem cell medium supplemented with 8 ng/mL bFGF. After day seven, the cells were further cultured in a medium pre-conditioned with inactivated MEF and supplemented with 0.5 μM of the MEK inhibitor PD325901 (Stemgent, 04–0006), 2 μM of the TGFβ receptor inhibitor SB431542 (Sigma, S4317) and 10 μM of the p53 inhibitor Pifithrin α (Stemgent, 04–0038) (Hong et al., 2009; Lin et al., 2009) and with daily medium changes. Colonies with hESC-looking morphology were mechanically detached and transferred to a new Matrigel and feeder cell coated culture dish for expansion and characterization as previously described. (Hossini et al., 2015; Takahashi et al., 2007).

3.4. DNA fingerprinting analysis

To confirm the somatic origin of MSC-iPSC (74) and rule out cross contamination with hESCs, 50 ng of genomic DNA was amplified by PCR 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, for 40 cycles, in a Dyad thermal cycler (BioRad) as previously described (Prigione et al., 2010). PCR products were resolved in 3% agarose gels to visualize

Table 1
Primer sequences used for DNA fingerprinting and real time PCR.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
D7S796	TTTTGGTATTGGCCATCCTA	GAAAGGAACAGAGAGACAGGG
D10S1214	ATTGCCCCAAAACTTTTTTG	TTGAAGACCAGTCTGGGAAG
DNMT3B	GCTCACAGGGCCCGATACTT	GCAGTCCTGCAGCTCGAGTTTA
DPPA4	TGGTGTCAGGTGGTGTGTGG	CCAGGCTTGACCAGCATGAA
FGF4	CCCTTCTTCACCGATGAGTGC	CATTCTTGCTCAGGGCGATG
GDF3	TTGGCACAAGTGGATCATTGC	TTGGCACAAGTGGATCATTGC
LEFTY1	AATGTGTCATTGTTTACTTGTCCT GTC	CAGGTCTTAGGTCCAGAGTGGTG
NANOG	CCTGTGATTTGTGGGCCTG	GACAGTCTCCGTGTGAGGCAT
OCT4	GTGGAGGAAGCTGACAACAA	ATTCTCCAGGTTGCCTCTCA
SOX2	GTATCAGGAGTTGTCAAGGCAGAG	TCCTAGTCTTAAAGAGGCAGCA AAC
TERT	ACGGCGACATGGAGAACAAG	GAGGTGTCACCAACAAGAAATC ATC
LIN28	GCACCAGAGTAAGCTGCACA	ATGGATTCCAGACCCTTGGC
LEFTY2	CTCCCGCGGAAAGAGGTTC	CAGCGCGGCCTCCGA
TDGF1	AGGGAACAATGACAGAGTGTGA	CCCGGCAACTAATCCCAGTT

amplicon size patterns for each primer set: D7S796, D10S1214. The primer sequences are listed in Table 1.

3.5. Quantitative real-time polymerase chain reaction

Expression analysis of pluripotency marker genes was carried out using SYBR® Green PCR Master Mix (Life Technologies). The PCR was performed with an ABI PRISM® 7000 Sequence Detection System (Life Technologies). The software ABI PRISM SDS was used for data analysis. Primer sequences are listed in Table 1. The data were analyzed according to the ΔΔCt method. The Log_2 of relative expression measured in MSC-iPSC (74) or hESC H1 over the relative expression measured in parental hBM MSC with three technical replicates was calculated. The three samples were pooled before reverse transcription of the RNA was carried out.

3.6. Embryoid body based in-vitro test of pluripotency

MSC-iPSC (74) was split and seeded onto a 60 mm ultra low attachment culture dish (Corning) and cultured in DMEM supplemented with 10% FCS, nonessential amino acids, L-glutamine, penicillin/streptomycin and sodium pyruvate (all from Life Technologies) without additional bFGF to generate embryoid bodies (EBs). After 10 days EBs were seeded onto gelatine-coated culture dishes and grown another 10 days in the same medium. Subsequently, the cells were fixed with 4% paraformaldehyde and germ layer specific markers were visualized by immunofluorescence-based staining.

3.7. Immunofluorescence-based analyses of pluripotency associated proteins

To detect pluripotency-specific or germ layer-specific marker expression, MSC-iPSCs (74) were fixed using phosphate-buffered saline (PBS) with 4% paraformaldehyde (Science) for 20 min at room temperature. The pluripotency markers OCT4, SOX2, KLF4, c-MYC, SSEA1, SSEA4, TRA-1-60, TRA-1-81, and NANOG and the germ layer specific markers α Fetoprotein (AFP) Smooth-Muscle-Actin (SMA), SOX17, PAX6, NESTIN, β-TubulinIII (TUJ1) and Brachyury (T) were visualized by immunofluorescence labeling as previously described (Hossini et al., 2015).

3.8. Confirmation of pluripotency in vivo

To test the pluripotency of MSC-iPSC (74) *in vivo*, teratoma assays were carried out by EPO-Berlin GmbH, Berlin-Buch, Germany. Undifferentiated cells were detached by trypsinization, washed with PBS and subsequently injected into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice, which are known as NOD scid gamma (NSG) mice. The tumor was harvested and analyzed for the presence of structures of endodermal, ectodermal and mesodermal origin. The analysis was carried out at the Institute for Animal Pathology, Berlin, Germany.

3.9. Microarray based gene expression analysis

Total RNA was isolated using a combined DNA, RNA, and Protein extraction Kit (Roboklon, Berlin Germany). The RNA quality was analyzed using a Nanodrop spectrophotometer (Nanodrop). 500 ng of total RNA was used to produce biotin-labeled cRNA with a linear amplification kit (Ambion). The Illumina BeadStation 500 platform (Illumina) was used to further process the samples following the manufacturer's instruction for hybridization and Cy3-streptavidin staining. cRNA samples were hybridized onto HumanHT-8 v3.0 Gene Expression BeadChips. The clustering dendrogram comparing the expression data and the correlation values were calculated using the Gene Expression Module of the software GenomeStudio (Illumina).

3.10. Karyotype analysis

GTG banding analysis was performed at Human Genetic Centre, Berlin, Germany. 33 metaphases of MSC-iPSC (74) were analyzed.

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