Stem Cell Research 16 (2016) 128–132

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab resource: Stem cell line

Episomal plasmid-based generation of induced pluripotent stem cells from fetal femur-derived human mesenchymal stromal cells

Matthias Megges [a,b,d], Richard O.C. Oreffo [c], James Adjaye [a,b,*]

[a] Institute for Stem Cell Research and Regenerative Medicine, Heinrich Heine University Duesseldorf, Moorenstr. 5, 40225 Duesseldorf, Germany
[b] Max Planck Institute for Molecular Genetics, Ihnestraße 63-73, 14195 Berlin, Germany
[c] Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences, University of Southampton, Southampton SO16 6YD, UK
[d] Department of Biology, Chemistry and Pharmacy, Institute of Chemistry and Biochemistry, Freie Universität Berlin, Thielallee 63, 14195, Berlin, Germany

ARTICLE INFO

Article history:
Received 15 December 2015
Accepted 23 December 2015
Available online 28 December 2015

ABSTRACT

Human bone mesenchymal stromal cells derived from fetal femur 55 days post-conception were reprogrammed to induced pluripotent stem cells using episomal plasmid-based expression of *OCT4*, *SOX2*, *NANOG*, *LIN28*, *SV40LT*, *KLF4* and *c-MYC* and supplemented with the following pathway inhibitors — TGFβ receptor inhibitor (A-83-01), MEK inhibitor (PD325901), GSK3β inhibitor (CHIR99021) and ROCK inhibitor (HA-100). Successful induction of pluripotency in two iPS-cell lines was demonstrated in vitro and by the Pluritest.

Resource table.

Name of stem cell construct	pEP4 E02S EN2K (Addgene, #20925), pCEP4-M2L (Addgene, #20926), pEP4 E02S ET2K (Addgene, #20927)
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	May 22, 2012
Origin	Primary human mesenchymal stromal cells isolated from fetal femur 55 days post-conception
Type of resource	Biological reagent: induced pluripotent stem cell (iPS-cell); generated from primary human mesenchymal stromal cells isolated from the fetal femur 55 days post-conception
Sub-type	Cell line
Key transcription factors	OCT4, SOX2, NANOG, LIN28, SV40LT, KLF4 and c-MYC
Authentication	Identity of cell line confirmed (Fig. 1 and Fig. 2)
Link to related literature (direct URL links and full references)	http://onlinelibrary.wiley.com/doi/10.1634/stemcells.2005-0368/pdf
Information in public databases	No

1. Resource details

Episomal plasmids harboring the transgenes *OCT4*, *SOX2*, *NANOG*, *LIN28*, *SV40LT*, *KLF4* and *c-MYC* were introduced into human mesenchymal stromal cells derived from the fetal femur of the age 55 days post-conception (fetal hMSCs) by nucleofection. In addition, a combination of TGFβ receptor inhibitor — A-83-01, MEK inhibitor — PD325901, GSK3β inhibitor — CHIR99021 and ROCK inhibitor — HA-100 was used to enhance the efficiency of reprogramming (Yu et al., 2011). Two iPS-cell lines, MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 were characterized. The expression of pluripotency-associated markers was confirmed by immunofluorescence staining and microarray-based transcriptome profiling (Fig. 1A and B). The transcriptomes of MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 were more comparable to the transcriptome of the human embryonic stem cell line H1 (hESC H1) than to that of their parental cells with Pearson correlations of 0.953 and 0.952 respectively (Fig. 1C and D). In addition, the origin of the cell-types as well as the absence of episomal plasmids in the generated iPS cells was confirmed by PCR (Fig. 1D and E). Chromosome analyses of iPS(fetal)#1 and MSC-iPS(fetal)#2 revealed a normal male karyotype for both iPS-cell lines (Fig. 2A). Pluripotency in vitro was also confirmed by embryoid body-based assays (Fig. 2B). Both iPS-cell lines showed high transcriptome similarity with hESC H1 compared to the parental cells based on the in silico PluriTest (Fig. 2C) (Müller et al., 2011).

2. Materials and methods

2.1. Ethics statement

Fetal femur cells were derived from fetal tissue obtained at 55 days post-conception following informed, written patient consent obtained at the termination of pregnancy. Approval was obtained by the

* Corresponding author at: Institute for Stem Cell Research and Regenerative Medicine, Heinrich Heine University Duesseldorf, Moorenstr. 5, 40225 Duesseldorf, Germany.

http://dx.doi.org/10.1016/j.scr.2015.12.013

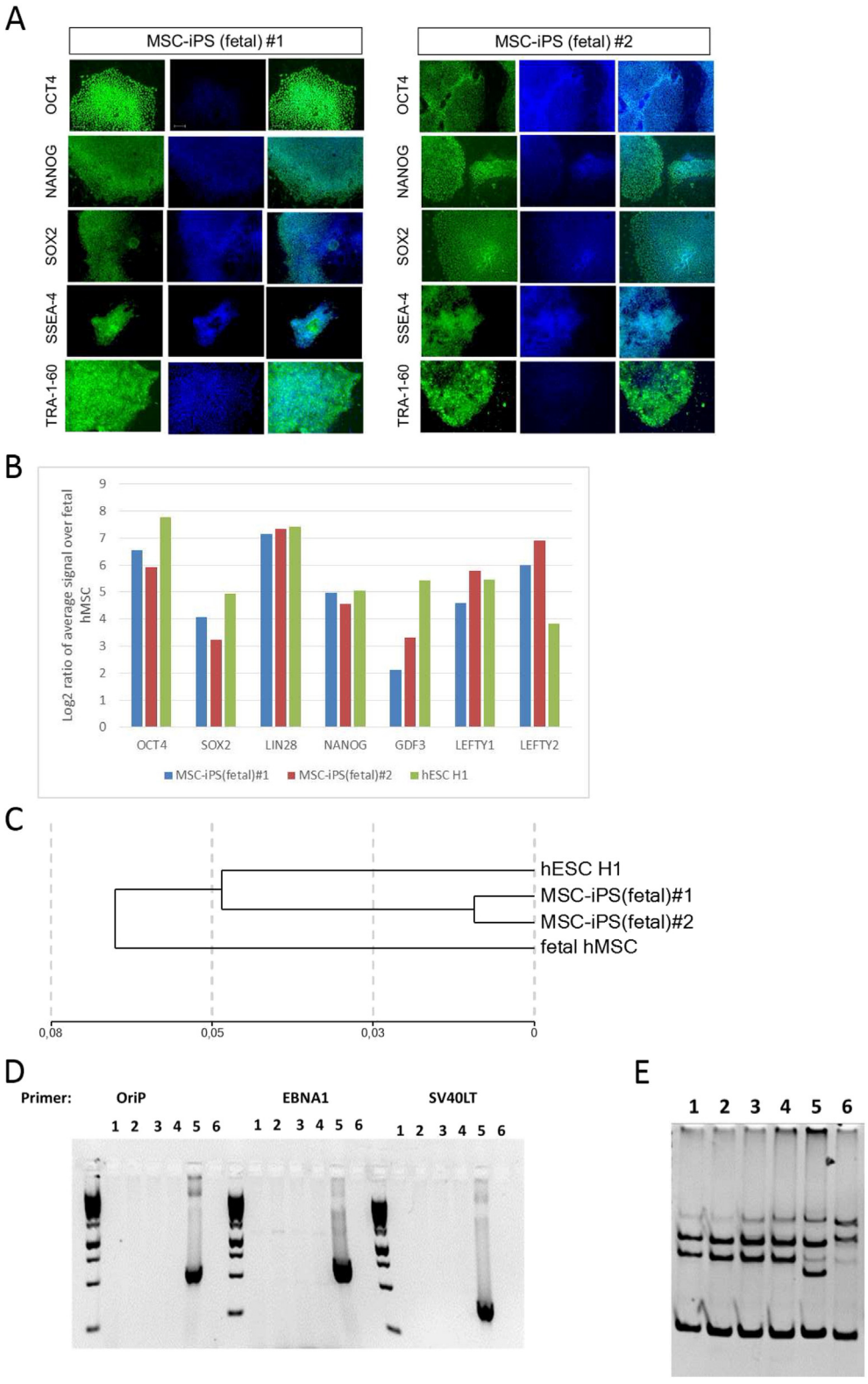

Fig. 1. Confirmation of pluripotency marker expression, absence of episomal plasmids and proof of the parental cell type. A) Expression of pluripotency-associated markers in MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 confirmed by immunofluorescence staining. B) mRNA expression derived from the microarray-based transcriptome profiling dataset. The diagram shows the Log2 ratio of the average signal detected in the respective iPS-cell line or hESC H1 as control over the average signal detected in parental fetal hMSCs. C) Correlation values between the transcriptomes of MSC-iPS(fetal)#1, MSC-iPS(fetal)#2, their parental primary hMSCs and hESC H1 based on expression values detected by microarray. Pearson correlation is shown between the samples calculated with the software GenomeStudio (Illumina). D) Clustering dendrogram based on Pearson correlation depicting the similarity between parental fetal hMSCs and the generated iPS-cell lines and hESC H1. The dendrogram was generated using the software Genome Studio (Illumina). E) DNA-fingerprinting to confirm the origin of MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2. Polymerase chain reaction with a microsatellite-specific primer. Acrylamide gel electrophoresis. Samples: 1: parental fetal hMSC; 2: MSC-iPS (fetal)#1; 3: MSC-iPS(fetal)#2; 4: iPS-cell line derived from fetal hMSC, not part of this study; 5: hESC H1; 6: hESC H9.). F) Confirmation of the absence of the episomal plasmids used for reprogramming. Polymerase chain reaction using primer specific for *OriP*, *EBNA1* or *SV40LT* on the episomal plasmids. Genomic DNA was used as template. hESC H1 and hESC H9 were analyzed in parallel to rule out cross contamination. Samples: 1: parental fetal hMSC; 2: iPS-cell line derived from fetal hMSC, not part of this study; 3: MSC-iPS(fetal)#1; 4: MSC-iPS(fetal)#2; 5: episomal plasmid DNA of pEP4 E02S ET2K; 6: no template. Size marker: GeneRuler 1 kb DNA Ladder (Life Technologies).

Southampton and South West Hampshire Local Research Ethics Committee (LREC 296100).

2.2. Cell culture

Primary mesenchymal stromal cells were derived from human fetal femur isolated 55 days post-conception as previously described (Mirmalek-Sani et al., 2006). In brief, femurs were dissected from connective tissue and skeletal muscle and treated with a solution of 1 mg/ml collagenase B (Roche) in αMEM overnight at 37 °C. Isolated cells were passed through a 70 μm nylon mesh filter (Fisher Scientific) to remove remaining tissue and centrifuged for 5 min at 1000 rpm. Pellets were resuspended in αMEM, supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Life Technologies), and seeded in 25 cm^2 asks. Cells were maintained in a monolayer culture under standard conditions at 37 °C and 5% CO_2. The fetal MSCs were characterized as MSCs based on the minimal criteria described by the International Society for Cellular Therapy (Dominici et al., 2006). Pluripotent stem cells were cultured in a medium consisting of Knock-Out DMEM supplemented with 20% Knockout Serum Replacement, 0.1 mM β-mercaptoethanol, non-essential amino acids, L-glutamine, sodium pyruvate and penicillin/streptomycin (all purchased from Life Technologies). 8 ng/ml basic fibroblast growth factor (bFGF) (Peprotech) were added to the medium. Cell culture was carried out at 37 °C and 5% CO_2 in a humidified atmosphere. The cells were seeded onto cell culture dishes coated with Matrigel (Becton Dickinson) when cultured as feeder-free or on mitomycin-c inactivated mouse embryonic fibroblasts (MEFs).

2.3. Non-viral generation of iPS-cells from fetal hMSCs

Fetal hMSCs were nucleofected with episomal plasmids using the Human MSC (Mesenchymal Stem Cell) Nucleofector® Kit (Lonza, VPE-1001) and the Amaxa Nucleofector II® Device (Lonza) following the manufacturer's instructions. The combination of episomal plasmids 7F-2 was used (Yu et al., 2011) to initiate reprogramming. Primary fetal hMSCs were expanded until passage 2, detached by trypsin, and washed. 1×10^6 fetal hMSCs were mixed with 3 μg of pEP4 EO2S EN2K, 3.2 μg of pEP4 EO2S ET2K, 2.4 μg of pCEP4-M2L and 100 μl Human MSC Nucleofactor solution warmed to room temperature. After mixing, the program U-23 of the Amaxa Nucleofector II® Device was used to nucleofect the fetal hMSCs. Nucleofected cells were seeded onto a 150 cm^2 cell culture dish in a hMSC maintenance medium consisting of MEM α, nucleosides, GlutaMAX™ (Life technologies), 10% fetal bovine serum (FBS) (Biochrom AG), penicillin/streptomycin (Life Technologies) and non-essential amino acids. After 6 days of culturing, the cells were trypsinized and seeded onto cell culture plates coated with Matrigel and inactivated MEFs at a density of 6×10^4 per well of a 6 well plate. The seeded cells were cultured in an N2B27 medium supplemented with 8 ng/ml bFGF and TGFβ receptor inhibitor A-83-01, MEK inhibitor PD325901, GSK3β inhibitor CHIR99021 and ROCK inhibitor HA-100 as previously described (Yu et al., 2011). The media were changed every other day until cell colonies with hESC-like morphology were visible. These colonies were mechanically isolated and transferred to new cell culture dishes coated with Matrigel and inactivated MEFs as described previously (Hossini et al., 2015; Takahashi et al., 2007).

2.4. Detection of episomal plasmids in iPS-cells

To test for the absence or presence of the episomal plasmids in the generated iPS-cells, polymerase chain reaction (PCR) using plasmid-specific primer sequences was employed (Table 1). The following program was used: Initial denaturation 94 °C for 5 min, denaturation 94 °C for 15 s, primer annealing 55 °C for 30 s, primer extension 68 °C for 1 min, final extension 68 °C for 7 min for 35 cycles followed by a final hold at 4 °C. 50 ng of the plasmid pEP4 E02S ET2K was used as positive control. 100 ng of genomic DNA of the respective samples was used as template. The PCR products were analyzed by agarose gel electrophoresis on a 2% agarose gel.

2.5. DNA fingerprinting analysis

The origin of MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 was confirmed by DNA fingerprinting PCR with a primer specific for the microsatellite D7S796. The primer sequences are described in Table 1. 50 ng of genomic DNA was used as template. The samples were analyzed using a previously described PCR program in a Dyad thermal cycler (BioRad) (Megges et al., 2015). To compare the amplicon sizes of the respective samples, the PCR products were analyzed by acrylamide gel electrophoresis using a 4% acrylamide gel.

2.6. Embryoid body-based in-vitro test of pluripotency

MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 were cultured until confluent, split and transferred to a 60 mm ultra low attachment culture (Corning) containing medium consisting of DMEM 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine and penicillin/streptomycin (Life Technologies) without bFGF and the medium was changed every other day. The EBs were seeded onto gelatin-coated cell culture dishes after 10 days and cultured further in the same media with the same frequency of media change. After 10 days differentiated cells were stained by immunofluorescence to analyze the presence of makers of endoderm, ectoderm and mesoderm.

2.7. Immunofluorescence staining

MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 were analyzed for the presence of pluripotency-associated markers or germ-layer-specific markers by fixation for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. Subsequently the cells were washed using PBS and incubated in 1% Triton X-100 (Sigma) in PBS at room temperature for 10 min. Next, the cells were blocked by incubation in 10% FBS in 0.1% Triton X-100 in PBS. The cells were incubated with the necessary concentration of primary antibody at 4 °C overnight. This was followed by three washes with PBS and a 1 h-incubation with the secondary antibody at room temperature. Next, the cells were washed three times with PBS and incubated in 200 ng/ml DAPI (Invitrogen). The types and concentrations of primary and secondary antibodies that were used to characterize MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 were previously described (Megges et al., 2015). The immunofluorescence staining was visualized with a confocal microscope type LSM510 (Zeiss).

2.8. Confirmation of pluripotency by PluriTest

Pluripotency of the generated iPS-cells was tested with the in silico test, PluriTest — www.pluritest.org. (Müller et al., 2011).

2.9. Microarray-based transcriptome analysis

Total RNA was extracted using the Universal RNA Purification Kit (Roboklon). 500 ng of RNA was used to generate biotin-labeled cRNA using a linear amplification kit (Ambion). The cRNA samples were processed with the Illumina BeadStation 500 platform (Illumina) following the manufacturer's instructions. HumanHT-12 v4.0 Gene Expression BeadChips were used to hybridize the cRNA samples. The gene expression module of the software Genome Studio (Illumina) was used to analyze and process the gene expression data detected with this system and to generate the clustering dendrogram.

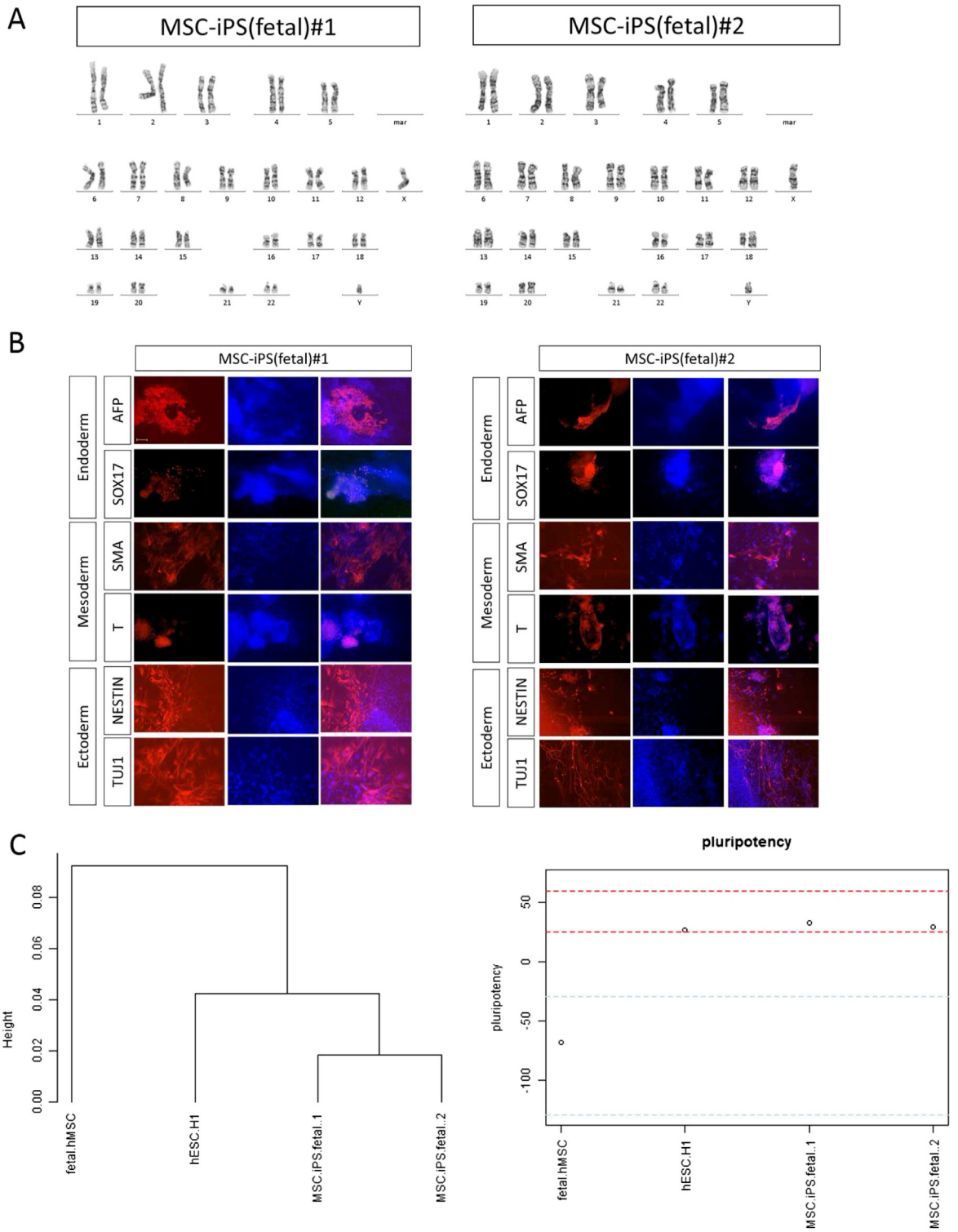

Fig. 2. Karyotype analysis and confirmation of pluripotency in vitro and by PluriTest. A) Chromosomal analysis based on GTG-banding revealed a normal male karyotype in the iPS-cells generated from fetal hMSC in 20 metaphases. B) Confirmation of pluripotency in vitro by embryoid body based differentiation. MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 were able to differentiate into cells expressing ectodermal markers β-TubulinIII (TUJ1) and NESTIN, into cell expressing mesodermal markers Smooth-Muscle-Actin (SMA) and Brachyury (T) and into cells expressing the endodermal markers SOX17 and α Fetoprotein (AFP). Immunofluorescence staining. Red: expression of marker. Blue: DAPI visualizing the nuclei. C) Confirmation of pluripotency with the bioinformatics tool PluriTest based on the transcriptome detected by microarray. Parental fetal hMSC with their respective iPS-cells and hESC H1 was compared. A dendrogram based on Pearson correlation of the calculated pluripotency between the samples (left) and the comparison of the calculated pluripotency measure based on PluriTest. (www.pluritest.org) (Müller et al., 2011).

Table 1
Sequences of the primers used for DNA fingerprinting and PCR-based detection of episomal plasmids.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
D7S796	TTTTGGTATTGGCCATCCTA	GAAAGGAACAGAGAGACAGGG
OriP	TTCCACGAGGGTAGTGAACC	TCGGGGGTGTTAGAGACAAC
EBNA	ATCGTCAAAGCTGCACACAG	CCCAGGAGTCCCAGTAGTCA
SV40LT	AGTTTGTGCCAGGGTTTTTG	ACTTCACCTTCCCTCCAACC

2.10. Karyotype analysis

The karyotype of 20 metaphases of MSC-iPS(fetal)#1 and MSC-iPS(fetal)#2 were analyzed by GTG banding by the Human Genetic Centre, Berlin, Germany.

Acknowledgments

JA acknowledges support from the Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany and the BMBF grant number 01GN1005. RO is supported by grants from the BBSRC (LO21072/1) and MRC (MR/K026682/1). We thank Prof David Wilson, University of Southampton for access to fetal tissue and Dr Kelvin Cheung, University of Southampton for derivation of fetal femur explant cultures.

References

Dominici, M., et al., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8 (4), 315–317.
Hossini, A.M., et al., 2015. Induced pluripotent stem cell-derived neuronal cells from a sporadic Alzheimer's disease donor as a model for investigating AD-associated gene regulatory networks. BMC Genomics 16.
Megges, M., et al., 2015. Generation of an iPS-cell line from bone marrow derived mesenchymal stromal cells from an elderly patient. Stem Cell Res. 15 (3), 565–568.
Mirmalek-Sani, S.-H., et al., 2006. Characterization and multipotentiality of human fetal femur-derived cells: implications for skeletal tissue regeneration. Stem Cells 24 (4), 1042–1053.
Müller, F.-J., et al., 2011. A bioinformatic assay for pluripotency in human cells. Nat. Methods 8 (4), 315–317.
Takahashi, K., et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861–872.
Yu, J., et al., 2011. Efficient feeder-free episomal reprogramming with small molecules. PLoS One 6 (3), e17557.