Stem Cell Research 50 (2021) 102127

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Multiple Cell Lines

Generation of an iPSC line (AKOSi004-A) from fibroblasts of a female adult NPC1 patient, carrying the compound heterozygous mutation p. Val1023Serfs*15/p.Gly992Arg and of an iPSC line (AKOSi005-A) from a female adult control individual

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A B S T R A C T

Niemann-Pick disease Type C (NPC) is a rare progressive neurodegenerative disorder with an incidence of 1:120,000 caused by mutations in the *NPC1* or *NPC2* gene leading to a massive cholesterol accumulation. Here, we describe the generation of induced pluripotent stem cells (iPSCs) of an affected female adult individual carrying the *NPC1* mutation p.Val1023Serfs*15/p.Gly992Arg and an iPSC line from an unrelated healthy female adult control individual. Human iPSCs were derived from fibroblasts using retroviruses carrying the four reprogramming factors *OCT4, SOX2, KLF4* and *C-MYC*. These lines provide a valuable resource for studying the pathophysiology of NPC and for pharmacological intervention.

1. Resource Table:

Unique stem cell lines identifier	1: AKOSi004-A 2: AKOSi005-A
Alternative names of stem cell lines	1: iPS DD-NPC1-12 2: iPS CTRL28-5
Institution	Translational Neurodegeneration Section "Albrecht-Kossel", Department of Neurology, University Medical Center Rostock, 18147 Rostock, Germany
Contact information of distributor	Dr. Moritz J. Frech; moritz.frech@med.uni-rostock.de
Type of cell lines	iPSC
Origin	Human
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Retrovirus
Multiline rationale	Age- and sex-matched control and NPC1-disease line
Gene modification	Yes
Type of modification	Hereditary
Associated disease	Niemann-Pick disease Type C1
Gene/locus	1: NPC1 / 18q11.12c.3066_3073delinsT /c.2974G > C 2: N/A
Method of modification	N/A
Name of transgene or resistance	*OCT4, SOX2, KLF4, C-MYC*
Inducible/constitutive system	N/A
Date archived/stock date	1: November 2019 2: March 2020
Cell line repository/bank	N/A
Ethical approval	Ethical by the Ethical Committee of the Technische Universität Dresden, Germany EK45022009.

2. Resource utility

NPC1 is a neurovisceral condition with progressive neuronal loss and hepatosplenomegaly. Since iPSCs are able to differentiate into every cell type of the human body, they display an excellent tool for studying multisystemic diseases. In this study we generated a hiPSC line from an

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https://doi.org/10.1016/j.scr.2020.102127
Received 26 October 2020; Received in revised form 20 November 2020; Accepted 13 December 2020
Available online 15 December 2020

NPC1 patient with compound heterozygous mutation p. Val1023Serfs*15/p.Gly992Arg and from an unaffected control individual (Tables 1 and 2), offering the opportunity for disease modelling, studying pathogenic mechanisms and drug development.

3. Resource Details

Niemann-Pick disease type C1 (NPC1, OMIM # 257220) is a rare lysosomal storage disorder affecting 1 in 120,000 live births (Vanier, 2010). The disease is transmitted in an autosomal-recessive manner by mutations in the *NPC1* gene (Millat et al., 2001). This gene encodes for a protein involved in the cholesterol transport. Mutations lead to a cholesterol accumulation in lysosomes (Vanier, 2010). Patients can display neurological symptoms, based on progressive neurodegeneration, as well as systemic symptoms like hepatosplenomegaly (Vanier, 2010). For such multisystemic diseases iPSCs are an excellent tool for studying pathogenic mechanisms as they can be differentiated in specific cell types of the human body.

Here, we describe the generation of a patient-specific induced pluripotent stem cell line of an NPC1 patient carrying the undescribed compound heterozygous mutation p.Val1023Serfs*15/p.Gly992Arg (AKOSi004-A) and a healthy control individual (AKOSi005-A). Both mutations are located in the cysteine-rich domain (Supplementary Fig. S1A). This domain harbors most of the disease-causing mutations (Li et al., 2017). The G992R mutation is known to lead to a variant phenotype, irrespective of the second allele (Millat et al., 2001). The female NPC1-patient, the donor of the fibroblasts used for the generation of the cell line AKOSi004-A, suffered from hepatosplenomegaly and developed severe neurological symptoms.

Dermal fibroblasts were reprogrammed into iPSCs by retroviral transduction with SOX2, OCT4, C-MYC and KLF4. Before colonies were picked and passaged as clonal lines on a feeder layer of irradiated mouse embryonic fibroblasts (MEF), the absence of retroviral activity in the iPSCs was confirmed by loss of GFP signal (Supplementary Fig. S1B). Subsequently, the iPSC lines were transferred onto Matrigel-coated plates and the silencing of the virally expressed OCT4, SOX2, KLF4 and C-MYC transcription factors was verified by specific reverse transcription PCR (RT-PCR) (Supplementary Fig. S1C) at passage 24 of both cell lines. Clones AKOSi004-A and AKOSi005-A were selected for further characterization. Both iPSC lines displayed a stem cell-like morphology (Fig. 1A). Pluripotency of the iPSC lines was validated by high levels of alkaline phosphatase activity (Fig. 1B) and expression of pluripotency markers NANOG, OCT4, SSEA4, TRA-1-60 and TRA-1-81 as shown by immunocytochemistry (Fig. 1C) and flow cytometry analysis (Fig. 1D). The differentiation potential of the iPSC lines into three embryonic germ layers was tested by spontaneous *in vitro* differentiation of embryoid bodies, at passage 8 for AKOSi004-A and passage 4 for AKOSi005-A. Immunocytochemistry confirmed expression of α-fetoprotein (α-FP, endoderm), nestin (ectoderm) and muscle actin (MA, mesoderm) (Fig. 1E). Chromosome analysis revealed a normal karyotype (46,XX) for both iPSC lines (Fig. 1F). An apparently identical structural cytogenetic abnormality was noted in two metaphases, found in one culture vessel of AKOSi004-A, and was considered representing a pseudomosaicism. However, CNV analysis revealed a normal karyotype without obvious genetic abnormalities. Mutation analysis using targeted NGS sequencing confirmed the genetic mutation of fibroblasts and the corresponding iPSC line (Fig. 1G). To confirm cell line identity, short tandem repeat (STR) analysis of 18 genomic loci was performed and showed identical polymorphisms of the parental fibroblasts and the corresponding iPSC line (archived at journal; available with authors). Furthermore, RT-PCR for pluripotency-related genes was performed, wherein the expression of *OCT4*, *NANOG*, *SOX2*, *KLF4*, *C-MYC*, *hTERT*, *ZFP296*, *FGF4* and *ESG1* was shown (Fig. 1H). Primer pairs used for *OCT4*, *SOX2*, *KLF4* and *C-MYC* amplified endogenous but not transgenic transcripts. Both iPSC lines were shown to be free of mycoplasma contamination (Supplementary Fig. S1D). In conclusion, these generated iPSC lines support *in vitro* functional studies aimed to investigate the pathogenic mechanisms of NPC1 and to develop and characterize new drugs for the disease.

4. Materials and methods

4.1. Reprogramming of fibroblasts

Patient-derived fibroblasts were transduced using retroviral vectors for *OCT4, SOX2, KLF4* and *C-MYC* following the protocol previously published by Peter et al. (2017).

4.2. Cell culture

Fibroblasts and MEF feeder cells were cultured in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, GE Healthcare) and 1% penicillin–streptomycin (10000 U/ml, Gibco). IPSCs cultured on feeder cells were kept in iPSC medium (DMEM/F12, 20% Knockout serum replacement, 0.1 mM MEM non-essential amino acids, 1% GlutaMax, 0.1 mM 2-mercaptoethanol, 1% penicillin–streptomycin (all Gibco) supplemented with 12.5 ng/ml FGF-2 (Amsbio)). Passaging of iPSCs on feeder cells was done mechanically with glass hooks. IPSC colonies cultured on Matrigel (Corning) were maintained in mTeSR1 (STEMCELL Technologies) and 0.25% penicillin–streptomycin (Gibco). Feeder cell-free iPSCs on Matrigel were passaged using Dispase (STEMCELL Technologies). Cells were incubated at 37 °C in a humidified 5% CO_2 incubator.

4.3. Alkaline phosphatase staining

IPSC colonies cultured on feeder cells were fixed with ice-cold methanol for 10 min and incubated with staining solution (75% distilled water, 10% 1 M sodium chloride, 10% 1 M Tris (pH 9.8), 5% 1 M magnesium chloride, 1:50 NBT/BCIP stock solution (Roche)) for 15 min.

4.4. Karyotyping

IPSC colonies were treated with colcemid (KaryoMAX, Gibco, 0.15 μg/ml in mTeSR1) for 90 min at 37 °C, then harvested by Accutase treatment (5 min) and centrifuged. Subsequently, the cells were treated with 0.075 M KCl for 30 min at 37 °C, sedimented and fixed in methanol: acetic acid (3:1). Chromosome analysis was performed using G-banding technique. The chromosome number was determined for at least 20 metaphases of each cell line. Of these, 12 metaphases each were fully karyotyped at a resolution of 300–550 bands per haploid set.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
AKOSi004-A (iPS DD-NPC1-12)	iPS 004-A	Female	33	Caucasian	NM_000271.5:c.2974G > C/ NM_000271.5: c.3066_3073delinsT	Niemann-Pick disease Type C1
AKOSi005-A (iPS CTRL28-5)	iPS 005-A	Female	28	Caucasian	N/A	N/A

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Fig. 1 panel A
Phenotype	Qualitative analysis: Alkaline phosphatase staining	Positive	Fig. 1 panel B
	Qualitative analysis: Immunocytochemistry	Expression of pluripotency markers: OCT4, NANOG, SSEA4, TRA-1-60, TRA-1-81	Fig. 1 panel C
	Qualitative analysis: RT-PCR	Expression of pluripotency genes: *OCT4*, *NANOG*, *SOX2*, *KLF4*, *C-MYC*, *hTERT*, *ZFP296*, *FGF4*, *ESG1*	Fig. 1 panel H
	Quantitative analysis: Flow cytometry	Percentage of positive cells iPS 004-A: NANOG: 98.9% OCT4: 98.0% SSEA4: 97.7% TRA-1–60: 95.7% TRA-1–81: 97.0% iPS 005-A: NANOG: 97.0% OCT4: 97.7% SSEA4: 98.4% TRA-1–60: 96.6% TRA-1–81: 94.7%	Fig. 1 panel D (representative)
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 300–550	Fig. 1 panel F
Identity	Microsatellite PCR (mPCR) OR STR analysis	Not performed 18 STR loci tested, 100% matched	N/A archived at journal; available with authors
Mutation analysis (IF APPLICABLE)	Sequencing	iPS 004-A: compound heterozygous p. Val1023Serfs*15/p. Gly992Arg	Fig. 1 panel G
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary Fig. S1 panel D
Differentiation potential	Embryoid body formation	Expression of genes in embryoid bodies: Muscle actin (MA), nestin and α-fetoprotein (α-FP)	Fig. 1 panel E
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

4.5. Copy number variation analysis (CNV)

CNV was performed using premade CytoScan HD Arrays (Affymetrix, Santa Clara, USA). Labelling of 250 ng DNA and hybridization were done following the manufacturer's instructions. Arrays were scanned by an Affymetrix 3000 7G scanner and analyzed through the Affymetrix Chromosome Analysis Suite software (ChAS analysis files for CytoScan® HD Array version NA33.2).

4.6. Immunocytochemistry

IPSC colonies were seeded onto Matrigel-coated glass cover slips. The cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Blocking was carried out using PBS containing 10% normal goat serum (NGS) and 0.1% Triton-X 100 for 45 min at room temperature. Primary antibodies were added overnight at 4 °C. Secondary antibodies were incubated for 1 h at room temperature. Finally, DAPI was added for 5 min at room temperature and iPS cells were mounted with Fluoromount-G® (SouthernBiotech). Images were acquired using a Keyence BZ-8000 K microscope (Keyence).

4.7. RT-PCR

Total RNA from iPSCs and corresponding fibroblasts was extracted using the Quick-RNA Miniprep kit (Zymo Research) according to manufacturer's instructions. One-step Reverse Transcriptase PCR (QIAGEN) was performed in an Eppendorf 5331 MasterCycler Gradient Thermal Cycler. 50 ng of total RNA were used for each reaction. Cycle number and annealing temperatures were optimized for each primer pair. Primers are listed in Table 3. To proof the viral vector silencing, the PCR was performed for 30 cycles using the respective viral vector as the positive control. PCR products were run on TBE agarose gels.

4.8. Flow cytometry

IPSCs were harvested using Gentle Cell Dissociation Reagent (STEMCELL Technologies). For the intracellular pluripotency markers OCT4 and NANOG, the True-Nuclear™ Transcription Factor Buffer Set (Biolegend) was used for fixation and permeabilization. Cells were incubated with fluorophore-conjugated antibodies for 1 h at room temperature. $5x10^4$ cells were analyzed using FACSCalibur (BD). Data analysis was performed with software FCSalyzer version 0.9.18-alpha.

4.9. Embryoid body (EB) formation

IPSC colonies were mechanically detached from the feeder cell layer and transferred to a low attachment plate. EBs were cultured in suspension for 5 days in EB medium containing 78% Knockout DMEM, 0.1 mM MEM non-essential amino acids, 1% GlutaMax, 0.1 mM 2-mercaptoethanol, 0.25% penicillin–streptomycin (all Gibco) and 20% FBS (GE Healthcare). After 5 days EBs were seeded onto gelatin-coated cover slips and allowed to differentiate for 10 days. EBs were fixed in 4% paraformaldehyde for 15 min at room temperature and stained with antibodies against α-fetoprotein, nestin and muscle actin.

4.10. Short tandem repeat (STR) analysis

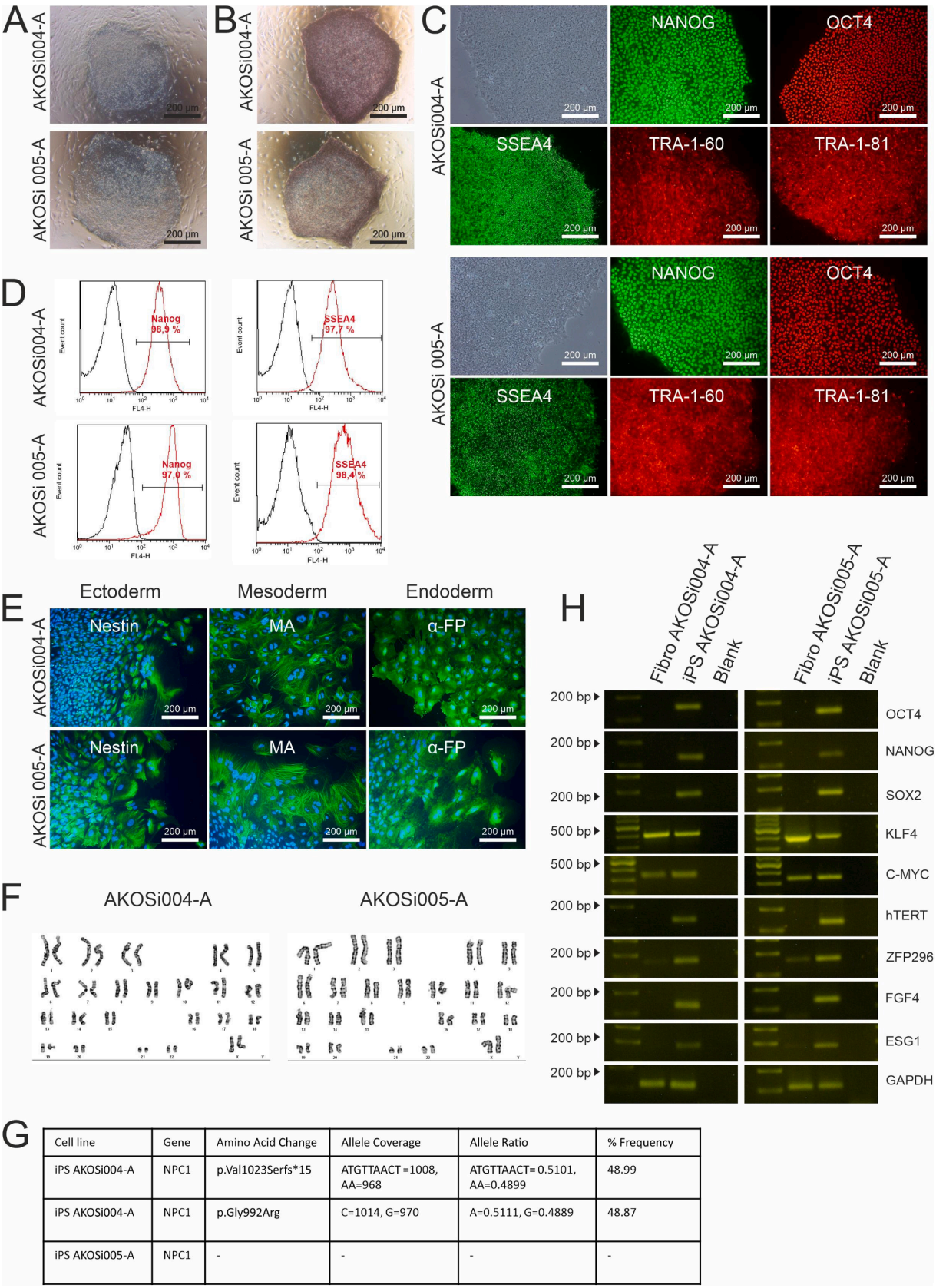
Short Tandem Repeat (STR) Analysis was done using the FTA Sample Collection Kit for Human Cell Authentication Service provided by ATCC. Cell suspensions of fibroblasts and iPSCs ($1x10^6$ cells/ml) were collected on Whatman® FTA® cards according to the manufacturer's protocol. In total 18 STR loci were analyzed.

4.11. Targeted sequencing

Genomic DNA was extracted using the Quick-DNA™ Miniprep Kit (Zymo Research). Targeted sequencing library construction was performed using a custom designed Ion AmpliSeq™ NPC1 Panel (Thermo Fisher Scientific). 10 ng of genomic DNA were used for library construction covering the complete coding sequence. Sequencing was carried out on an Ion Torrent™ Personal Genome Machine™ System, using an Ion Torrent 318 V2 chip. Sequence analysis was performed using the hg19 assembly of the human genome using Torrent Suite™ software and the variant caller plugin version 5.12.V2 (Thermo Fisher Scientific).

4.12. Mycoplasma detection

Mycoplasma detection was performed using PCR Mycoplasma Test Kit I/C (PromoCell) following manufacturer's instructions.



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Cell line	Gene	Amino Acid Change	Allele Coverage	Allele Ratio	% Frequency
iPS AKOSi004-A	NPC1	p.Val1023Serfs*15	ATGTTAACT =1008, AA=968	ATGTTAACT= 0.5101, AA=0.4899	48.99
iPS AKOSi004-A	NPC1	p.Gly992Arg	C=1014, G=970	A=0.5111, G=0.4889	48.87
iPS AKOSi005-A	NPC1	-	-	-	-

Fig. 1. Characterization of AKOSi004-A and AKOSi005-A iPSC lines.

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Marker (IF)	Rabbit anti-OCT4	1:100	Stemgent Cat# 09-0023, RRID: AB_2167689
Pluripotency Marker (IF)	Rabbit anti-NANOG	1:100	Stemgent Cat# 09-0020, RRID: AB_2298294
Pluripotency Marker (IF)	Mouse anti-SSEA4	1:100	Stemgent Cat# 09-0006, RRID: AB_1512169
Pluripotency Marker (IF)	Mouse anti-TRA-1-60	1:100	Stemgent Cat# 09-0010, RRID: AB_1512170
Pluripotency Marker (IF)	Mouse anti-TRA-1-81	1:100	Stemgent Cat# 09-0011, RRID: AB_1512171
Pluripotency Marker (FC)	Alexa Fluor 488 anti-OCT4, mouse IgG2b	1:20	BioLegend Cat# 653705, RRID: AB_2562250
Pluripotency Marker (FC)	Alexa Fluor 647 anti-NANOG, mouse IgG1	1:50	BioLegend Cat# 674210, RRID: AB_2650619
Pluripotency Marker (FC)	Alexa Fluor 647 anti- SSEA-4, mouse IgG3	1:500	BioLegend Cat# 330407, RRID: AB_1089201
Pluripotency Marker (FC)	PE anti-human TRA-1-60-R, mouse IgM	1:20	BioLegend Cat# 330609, RRID: AB_1279447
Pluripotency Marker (FC)	Alexa Fluor 488 anti-TRA-1-81, mouse IgM	1:20	BioLegend Cat# 330709, RRID: AB_2561741
Differentiation Marker (IF)	Mouse anti-Muscle actin	1:50	Agilent Dako Cat# M0635, RRID: AB_2242301
Differentiation Marker (IF)	Mouse anti-Nestin	1:100	R and D Systems Cat# MAB1259, RRID: AB_2251304
Differentiation Marker (IF)	Mouse anti-Alpha fetoprotein	1:20	R and D Systems Cat# MAB1368, RRID: AB_357658
Secondary antibody	Alexa Fluor 488, Goat anti-mouse IgG	1:500	Thermo Fisher Scientific Cat# A-11029, RRID: AB_2534088
Secondary antibody	Alexa Fluor 568, Goat anti-mouse IgM	1:500	Thermo Fisher Scientific Cat# A-21043, RRID: AB_2535712
Secondary antibody	Alexa Fluor 488, Goat anti-rabbit IgG	1:500	Thermo Fisher Scientific Cat# A-11034, RRID: AB_2576217
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Pluripotency marker (RT-PCR)	*C-MYC*	GCGTCCTGGGAAGGGAGATCCGGAGC/ TTGAGGGGCATCGTCGCGGGAGGCTG	
Pluripotency marker (RT-PCR)	*NANOG*	TGTGTTCTCTTCCACCCAGC/ ACCAGGTCTTCACCTGTTTGT	
Pluripotency marker (RT-PCR)	*OCT4*	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC	
Pluripotency marker (RT-PCR)	*SOX2*	AGGGAGAGAAGTTTGAGCCC/ GCGAGGAAAATCAGGCGAAG	
Pluripotency marker (RT-PCR)	*KLF4*	ACGATCGTGGCCCCGGAAAAGGACC/ TGATTGTAGTGCTTTCTGGCTGGGCTCC	
Pluripotency marker (RT-PCR)	*ZFP296*	CTGGACCGACAAACACCCAG/ CTTCAGCTCCTCTCGTTCTGAG	
Pluripotency marker (RT-PCR)	*ESG1*	ATATCCCGCCGTGGGTGAAAGTTC/ ACTCAGCCATGGACTGGAGCATCC	
Pluripotency marker (RT-PCR)	*FGF4*	CAAGCTCTATGGCTCGCCCT/ TCTTCCCATTCTTGCTCAGGG	
Pluripotency marker (RT-PCR)	*hTERT*	GAGCTGACGTGGAAGATGAGC/ CATCAGCCAGTGCAGGAACTT	
House-Keeping Gene (RT-PCR)	*GAPDH*	CATGTTCCAATATGATTCCACCC/ GGGATCTCGCTCCTGGAAGAT	
Viral OCT4 expression (RT-PCR)	*OCT4_pMIG/ IRES*	GTACTCCTCGGTCCCTTTCC/ GCATTCCTTTGGCGAGAG	
Viral SOX2 expression (RT-PCR)	*SOX2_pMIG/ IRES*	CATGTCCCAGCACTACCAGA/ GCATTCCTTTGGCGAGAG	
Viral KLF4 expression (RT-PCR)	*KLF4_pMIG/IRES*	CCCACACAGGTGAGAAACCT/ GCATTCCTTTGGCGAGAG	
Viral C-MYC expression (RT-PCR)	*C-MYC_MSCV/ IRES*	AAGAGGACTTGTTGCGGAAA/ GCATTCCTTTGGCGAGAG	

Acknowledgements

The authors thank Heike Janecek-Meyer (Institute for Medical Genetics, University Medical Center Rostock) for assistance in genetic analyses and Dr. Sabine Bartnitzke, Human Genetics, University of Bremen, for second-look evaluation of karyotypes. JP and JL are supported by the European Union (grant number ESF/14-BM-A55-0046/16). AH is supported by the Hermann und Lilly Schilling-Stiftung für medizinische Forschung im Stifterverband. CV and ML are supported by a grant of the Landesgraduiertenförderung Mecklenburg-Vorpommern.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.102127.

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