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Lab Resource: Multiple Cell Lines

Generation of an iPSC line (AKOSi006-A) from fibroblasts of an NPC1 patient, carrying the homozygous mutation p.I1061T (c.3182 T > C) and a control iPSC line (AKOSi007-A) using a non-integrating Sendai virus system

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A B S T R A C T

Niemann-Pick disease type C1 (NPC1) is a rare inherited lipid storage disorder caused by mutations in the *NPC1* gene. Mutations lead to impaired lipid trafficking and subsequently to accumulation of cholesterol and sphingolipids. NPC1-patients present variable multisystemic symptoms, including neurological deficits. Here, we describe the generation of human iPSC lines obtained from fibroblasts of a male individual, carrying the homozygous mutation p.I1061T, and an unrelated and healthy male individual. A non-integrating Sendai virus system, containing KLF4, OCT3/4, SOX2 and C-MYC, was used for reprogramming. These cell lines provide a valuable resource for studying the pathophysiology of multisystemic NPC1-disease.

1. Resource Table

Unique stem cell lines identifier	1: AKOSi006-A 2: AKOSi007-A
Alternative names of stem cell lines	1: iPS GM18453-S4 2: iPS GM08398-S14
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	Transgene free (CytoTune-iPSC 2.0 Sendai Reprogramming kit). This system includes three vector preparations: polycistronic KOS (KLF4–OCT3/4–SOX2), C-MYC, and KLF4
Multiline rationale	Control and disease pair
Gene modification	Yes
Type of modification	Hereditary
Associated disease	Niemann-Pick disease Type C1
Gene/locus	1: NPC1 / 18q11.12 c.3182T > C/c.3182T > C 2: –
Method of modification	N/A
Name of transgene or resistance	KOS (KLF4, OCT3/4, SOX2), C-MYC, KLF4
Inducible/constitutive system	N/A
Date archived/stock date	1: December 2019 2: December 2019
Cell line repository/bank	N/A
Ethical approval	Fibroblasts were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research.

2. Resource utility

The herein presented hiPSC line from a NPC1 patient with the most frequent NPC1 mutation found in Western Europe and the US, accounting for approximately 20% of all mutations (Vanier, 2010), p.I1061T, and the hiPSC line from an unaffected healthy control individual offer the opportunity for characterizing the NPC disease phenotype and studying pathologic mechanisms (Tables 1 and 2).

3. Resource details

Niemann-Pick disease type C1 (NPC, OMIM # 257220) is a rare

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Table 1
Summary of lines.

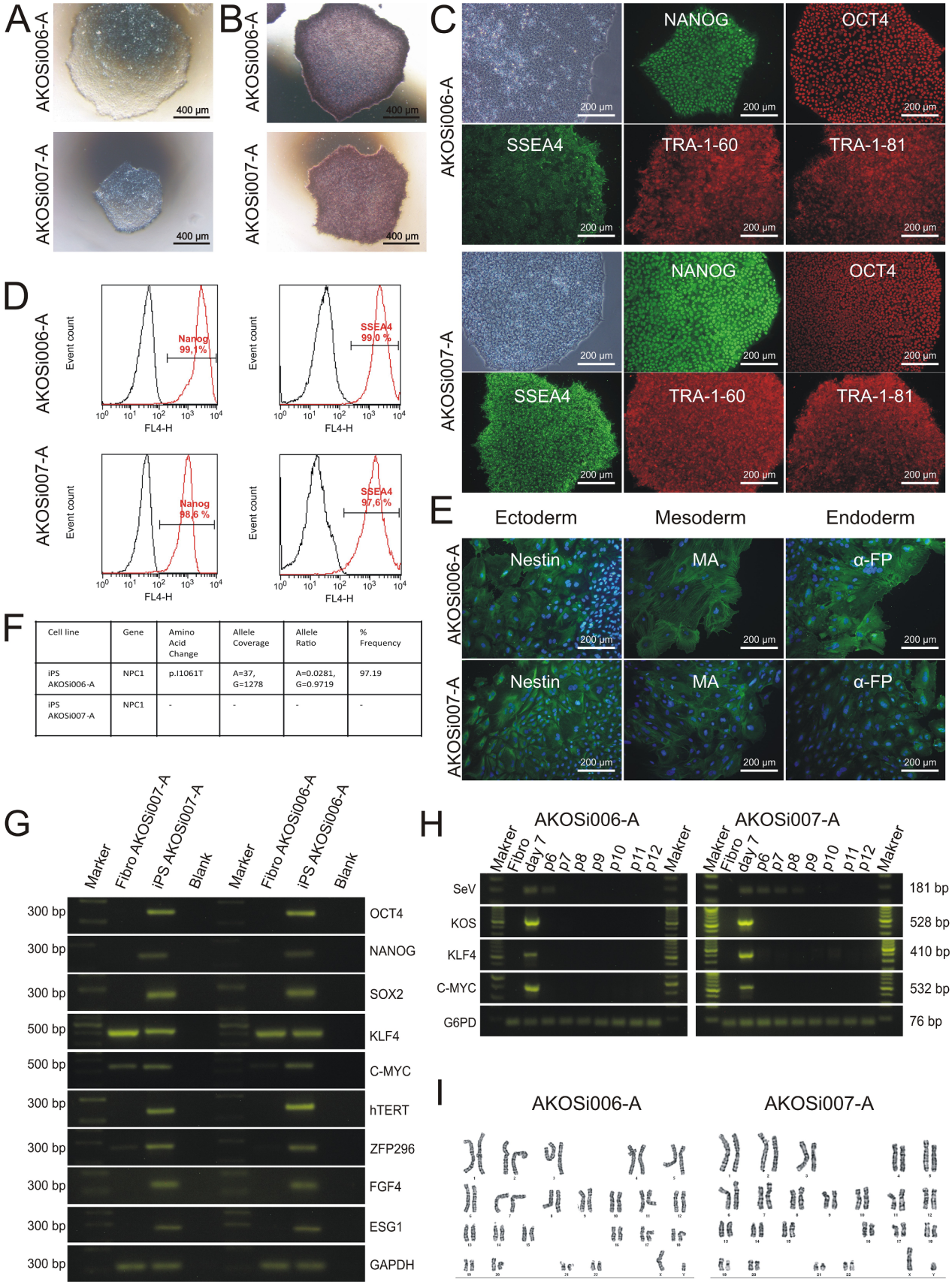
iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
AKOSi006-A (iPS GM18453-S4)	iPS 006-A	Male	unknown	Caucasian	NM_000271.5:c.3182 T > C /NM_000271.5:c.3182 T > C	Niemann-Pick disease Type C1
AKOSi007-A (iPS GM08398-S14)	iPS 007-A	Male	8	Caucasian	–	–

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 G
	Qualitative analysis: RT-PCR	Negative for Sendai vectors	Fig. 1 panel H
	Quantitative analysis: Flow cytometry	Percentage of positive cells iPS 006-A: NANOG: 99.1% OCT4: 94.4% SSEA4: 99.0% TRA-1–60: 89.7% TRA-1–81: 88.3% iPS 007-A: NANOG: 98.6% OCT4: 97.4% SSEA4: 97.6% TRA-1–60: 95.4% TRA-1–81: 95.3%	Fig. 1 panel D (representative)
Genotype	Karyotype (G-banding) and resolution	46, XY Resolution 300–550	Fig. 1 panel I
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 006-A: homozygous p.I1061T/ p.I1061T	Fig. 1 panel F
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary Fig. S1 panel A
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

neurodegenerative disorder. It is classified as a lysosomal storage disease. The prevalence of NPC is 1/120,000 per live births (Vanier, 2010). The disease is transmitted in an autosomal-recessive manner by mutations in the *NPC1* gene leading to a decrease of intracellular cholesterol trafficking which results in the accumulation of unesterified cholesterol and other lipids, like gangliosides. NPC1 displays a highly variable age of onset and disease progression. First symptoms of classic NPC typically occur during mid-to-late childhood and include hepatosplenomegaly as well as severe, progressive neurodegeneration of the CNS. However, both age of onset and disease progression are highly variable among NPC patients. For multisystemic diseases, like NPC, iPS cells represent an excellent tool for studying pathologic mechanisms as these cells can specifically differentiate into different cell types of the human body. Here, we describe the generation of a patient-specific induced pluripotent stem cell line of a NPC1 patient carrying the homozygous *NPC1* mutation p.I1061T (AKOSi006-A) and a healthy control individual (AKOSi007-A).

Patientś dermal fibroblasts were reprogrammed into iPSCs using non-integrating Sendai virus, transiently overexpressing *KLF4*, *OCT3/4*, *SOX2* and *C-MYC*. Colonies were picked and passaged as clonal lines on Matrigel-coated plates. Clones AKOSi006-A and AKOSi007-A were selected for full characterization. Both iPSC lines grew in typical colonies, displaying a stem cell-like morphology with a high nuclei-to-cell-size ratio (Fig. 1A). Pluripotency of the iPSC lines was validated by high levels of alkaline phosphatase activity (Fig. 1B) and expression of the 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 was proven by embryoid body formation and differentiation into cells of all three germ layers. Immunocytochemistry confirmed expression of α-fetoprotein (α-FP, endoderm), nestin (ectoderm) and muscle actin (MA, mesoderm) (Fig. 1E). Consistency of mutation in the *NPC1*-deficient iPSC line was confirmed by targeted NGS sequencing (Fig. 1F). Short tandem repeat (STR) analysis of 18 genomic loci proved 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. 1G). Primer pairs used for *OCT4*, *SOX2*, *KLF4* and *C-MYC* amplified endogenous but not transgenic transcripts. The loss of



Cell line	Gene	Amino Acid Change	Allele Coverage	Allele Ratio	% Frequency
iPS AKOSi006-A	NPC1	p.I1061T	A=37, G=1278	A=0.0281, G=0.9719	97.19
iPS AKOSi007-A	NPC1	-	-	-	-

Fig. 1. Characterization of AKOSi006-A and AKOSi007-A iPSC lines.

exogenous reprogramming vectors was validated by RT-PCR. IPSCs were negative for Sendai virus vector backbone and for transduced transgenes after 9 passages at the latest (Fig. 1H). IPSC lines demonstrated a normal male karyotype (46, XY) without any obvious aberrations (Fig. 1I). Both iPSC lines were free of mycoplasma contamination (Supplementary Fig. S1A). In conclusion, the generated iPSC lines support *in vitro* functional studies aimed to identify molecular features of NPC and drug development.

4. Materials and methods

4.1. Generation of human induced pluripotent stem cells

Patient-derived fibroblasts were cultured in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, GE Healthcare) and 1% penicillin-streptomycin (10000 U/ml, Gibco). Fibroblasts were transduced using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) according to the manufactureŕs instructions. At day 8 post transduction, cells were re-seeded on 6-well dishes coated with Matrigel (Corning) and cultured in E8 medium (Thermo Fisher Scientific). Stem cell colonies were picked and expanded on Matrigel coated dishes in E8 or mTeSR1 (STEMCELL Technologies) with daily medium changes. Cells were passaged every 5 to 7 days using 0.5 mM EDTA in PBS (Life Technologies). Cells were incubated at 37 °C in a humidified 5% CO_2 incubator.

4.2. Alkaline phosphatase staining

IPSC colonies were fixed with ice-cold methanol for 10 min and incubated for 15 min with staining solution containing 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).

4.3. Karyotyping

IPSC colonies were treated with colcemid (KaryoMAX, Gibco, 0.15 μg/ml in mTeSR1) for 2 h 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). G-banding was performed following routine procedures (accutase treatment followed by Giemsa-staining). The chromosome number was determined for at least 20 metaphases of each cell line. For each cell line 7 metaphases were fully karyotyped at a resolution of at least 300 bands per haploid set.

4.4. 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. The samples were incubated with primary antibodies overnight at 4 °C and with secondary antibodies for 1 h at room temperature. 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).

4.5. RT-PCR

Total RNA from iPSCs and corresponding fibroblasts was obtained using the Quick-RNA Miniprep kit (Zymo Research) according to manufacturer's instruction. An Eppendorf 5331 MasterCycler Gradient Thermal Cycler was used to perform a One-step Reverse Transcriptase PCR (QIAGEN). 50 ng of total RNA were used for each reaction. Cycle number and annealing temperatures were optimized for each primer pair. Primers are given in Table 3. PCR products were run on TBE agarose gels.

4.6. Control of transgene expression silencing

Total RNA was extracted from iPSCs using the Quick-RNA Miniprep kit (Zymo Research) according to manufacturer's instructions. cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen). Absence of transgene was detected by PCR using LightCycler® FastStart DNA MasterPLUS SYBR Green I Kit (Roche) and transgene-specific primers (Table 3).

4.7. Flow cytometry

IPSCs were collected using Gentle Cell Dissociation Reagent (STEMCELL Technologies). For the analysis of intracellular pluripotency markers OCT4 and NANOG, cells were prepared with the True-Nuclear™ Transcription Factor Buffer Set (Biolegend). Fluorophore-conjugated antibodies were incubated for 1 h at room temperature. $5x10^4$ cells were measured with FACSCalibur (BD) and analysis was done with the FCSalyzer software version 0.9.18-alpha.

4.8. Embryoid body formation

Formation of embryoid bodies (EBs) was achieved by spontaneous differentiation of iPSCs. Colonies were treated with 0.5 mM ETDA/PBS and detached with a cell scraper and subsequently transferred to a low attachment plate. EBs were cultured in suspension for 5 days in mTeSR1 supplemented with 4 mg/ml polyvinylalcohol. After 5 days EBs were seeded onto gelatin-coated cover slips and allowed to differentiate for 10 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). EBs were fixed in 4% paraformaldehyde for 15 min at room temperature and stained with antibodies against α-fetoprotein, nestin and muscle actin.

4.9. Short tandem repeat (STR) analysis

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

4.10. 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).

4.11. Mycoplasma detection

PCR Mycoplasma Test Kit I/C (PromoCell) was used to detect contamination, following manufacturer's instructions.

Declaration of Competing Interest

The authors declare that they have no known competing financial

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	
Sendai reprogramming vector (RT-PCR)	*SeV*	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTT AAGAGATATGTATC	
Sendai reprogramming vector (RT-PCR)	*KOS (KLF4, OCT4, SOX2)*	ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTGATGTGG	
Sendai reprogramming vector (RT-PCR)	*KLF4*	TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA	
Sendai reprogramming vector (RT-PCR)	*C-MYC*	TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCT GGATGATGATG	
House-Keeping Gene (RT-PCR)	*G6PD*	TGCCCCCGACCGTCTAC/ATGCGGTTCCAGCCTATCTG	

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.102056.

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