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Lab resource: Stem Cell Line

Generation of an iPSC line of a patient with Angelman syndrome due to an imprinting defect

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ABSTRACT

Angelman syndrome (AS) is a neurodevelopmental disorder with leading symptoms of happy demeanor, intellectual disability, ataxia and seizures. AS can be caused by genetic and epigenetic aberrations, resulting in the absence of functional UBE3A protein in the brain. *UBE3A* is an imprinted gene, which is, in neurons of the brain, expressed exclusively from maternal chromosome 15. The generated iPSC line was derived from skin fibroblasts of a patient with AS, who, due to an imprinting defect, lacked DNA methylation at the chromosome 15 imprinting center, which controls maternal-specific expression of *UBE3A*.

Resource table

Unique stem cell line identifier	ZIPi015-K
Alternative name(s) of stem cell line	AS_ID, ZIP15
Institution	Zentrum für integrative Psychiatrie, University Hospital Kiel, Kiel, Germany
Contact information of distributor	Franz-Josef Müller, franz-josef.mueller@uksh.de Laura Steenpass, laura.steenpass@uk-essen.de
Type of cell line	iPSC
Origin	human
Additional origin info	Age: 12 Sex: female Ethnicity if known: caucasian
Cell Source	skin fibroblasts
Clonality	clonal
Method of reprogramming	episomal/transgene-free
Genetic Modification	epigenetic aberration – imprinting defect
Type of Modification	lack of DNA methylation establishment or maintenance in the germ line of the patient's mother
Associated disease	Angelman syndrome (OMIM #105830)
Gene/locus	Prader-Willi/Angelman syndrome locus, chromosome 15q11q13
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	14.04.2017 (Essen)
Cell line repository/bank	NA
Ethical approval	Ethikkommission der medizinischen Fakultät der Christian-Albrechts Universität zu Kiel, Approval number A145/11 A145/11

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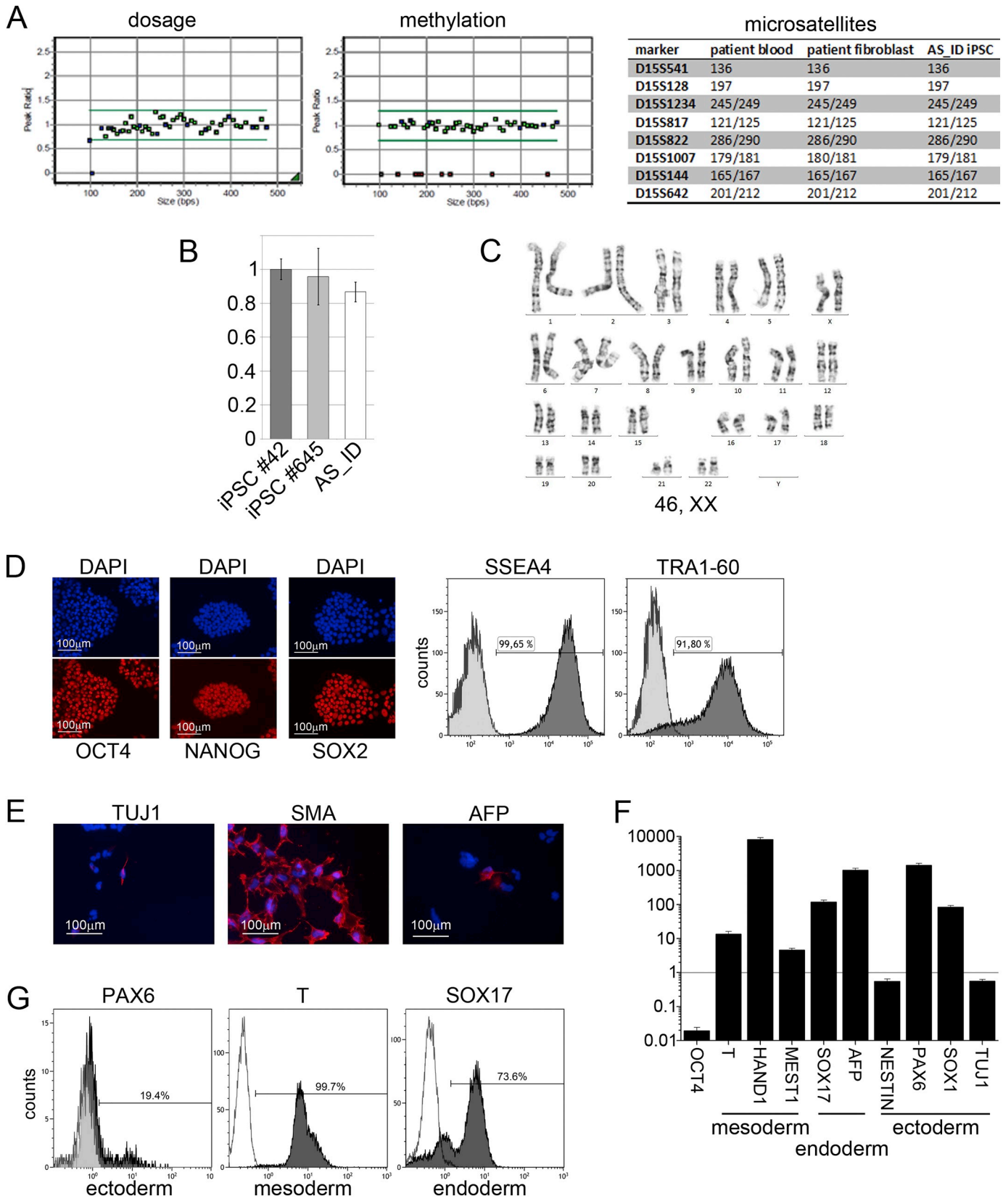

marker	patient blood	patient fibroblast	AS_ID iPSC
D15S541	136	136	136
D15S128	197	197	197
D15S1234	245/249	245/249	245/249
D15S817	121/125	121/125	121/125
D15S822	286/290	286/290	286/290
D15S1007	179/181	180/181	179/181
D15S144	165/167	165/167	165/167
D15S642	201/212	201/212	201/212

Fig. 1. Characterisation of patient-derived AS_ID iPSCs (ZIPi015_K) iPSCs carrying an imprinting defet.

1. Resource utility

The unavailability of neuronal tissue of patients hampers AS research. iPSC lines reprogrammed from patient cells and differentiated into neurons in vitro are an alternative. AS can arise from different genetic and epigenetic causes and it is of interest to establish iPSC lines covering all possible causes, including imprinting defects.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal, high nucleus/cytoplasmic ratio	Fig. 1D
Phenotype	Qualitative analysis	Immunofluorescence for nuclear pluripotency markers: OCT4, NANOG, SOX2	Fig. 1D
	Quantitative analysis	Surface pluripotency marker expression (FACS analysis) SSEA4: 100%; Tra1–60: 92% positive	Fig. 1D
Genotype	Karyotype (G-banding) and resolution	46XX, Resolution 450	Fig. 1C
Identity	Microsatellite PCR (mPCR)	Primer pool for chromosome 15	Fig. 1A
	STR analysis	Powerplex 16 HS system	submitted in archive with journal
Mutation analysis (IF APPLICABLE)	MLPA, Microsatellite PCR for chromosome 15	Imprinting defect: dosage normal DNA methylation absent biparental origin	Fig. 1A
	Southern Blot OR WGS	NA	NA
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary file
Differentiation potential	Embryoid body formation and directed differentiation	EB: immunofluorescence for TUJ1, smooth muscle actin (SMA), α-fetoprotein (AFP), qRT_PCR directed differentiation: ectoderm (PAX6): 19.4% endoderm (SOX17): 99.7% mesoderm (T): 73.6%	EB: Fig. 1E, F directed differentiation: Fig. 1G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	NA	NA
Genotype additional info (OPTIONAL)	Blood group genotyping	NA	NA
	HLA tissue typing	NA	NA

2. Resource details

The patient whose fibroblasts were used for reprogramming was diagnosed with AS caused by an imprinting defect. An imprinting defect constitutes as biparental origin of genetically intact chromosomes 15 but lack of DNA methylation at the relevant imprinting center (Horsthemke and Buiting, 2008). Patient fibroblasts were derived from a skin biopsy and reprogrammed by introduction of episomal vectors expressing L-MYC, LIN28, SOX2, KLF4, OCT3/4 and shRNA against p53. A total of 7 clonal iPSC lines were derived, and one line was characterized in detail. Consistent with the patient's initial diagnosis, chromosome 15 specific MS-MLPA and microsatellite analysis of genomic DNA from patient blood and fibroblasts and the derived iPSCs confirmed absence of deletions, DNA methylation at the imprinting center and biparental origin of chromosomes 15 (Fig. 1A). MS-MLPA allows simultaneous analysis of copy-number changes and DNA methylation. Briefly, MLPA probes hybridize to genomic DNA and adjacent probes are ligated before they can be amplified by PCR. For detection of DNA methylation, genomic DNA is first digested with a methylation-sensitive restriction enzyme. Absence of DNA methylation results in failure of amplification. Normal levels of gene dosage display with a peak ratio of around one (Fig. 1A). A normal DNA methylation result on the chromosome 15 imprinting center is indicated by a peak ratio of respective probes of 0.5, indicating a level of 50% DNA methylation. Fig. 1A shows loss of DNA methylation at chromosome 15, indicated by the probes residing on baseline. RNA expression of *UBE3A* was proven by quantitative real-time PCR and comparable to published control iPSC lines #42 and #645 (Fig. 1B; expression normalized to *GAPDH* and calibrated to iPSC line #42; Stanurova, 2016). Karyotype analysis in passage 27 revealed a female karyotype of 46, XX (Fig. 1C). Pluripotency of the generated AS_ID iPSC line was demonstrated by qualitative immunofluorescence for expression of nuclear marker proteins OCT4, SOX2 and NANOG and by quantitative FACS analysis of SSEA4 and TRA1–60 surface marker protein expression (Fig. 1D; IF: marker proteins in red, nuclei in blue, scale bar 100 µm; FACS: isotype control (grey), sample (black), percentage of positive cells is indicated). Differentiation potential of AS_ID iPSCs was confirmed by embryoid body formation and immunofluorescence for expression of germ line marker proteins, TUJ1 (ectoderm), smooth muscle actin (SMA, mesoderm) and α-fetoprotein (AFP, endoderm) (Fig. 1E, marker proteins in red, nuclei in blue, scale bar 100 µm). Expression of several marker genes in embryoid bodies was additionally analysed by qRT-PCR (Fig. 1F; expression normalized to *GAPDH* and calibrated to d0 of differentiation). Single-cell quantitative analysis of directed differentiation was analysed by FACS for expression of PAX6 (ectoderm), T (mesoderm) and SOX17 (endoderm) (Fig. 1G; isotype control (grey), sample (black), percentage of positive cells is indicated). 19% of cells were positive for ectodermal, almost 100% for mesodermal and 73% for endodermal marker protein expression. Although success of ectodermal differentiation was low, we successfully use these cells for neuronal differentiation (data not shown), proving that this cell line is able to differentiate into the ectodermal lineage.

3. Materials and methods

3.1. Reprogramming and cell culture

Human dermal fibroblasts (< P5) isolated from patient's skin biopsy were reprogrammed as described (Okita et al., 2011). 1.5 µg of each episomal plasmid (Addgene; pCXLE-hUL (#27080), pCXLE-hSK (#27078), pCXLE-hOCT3/4-shp53F #27077) were electroporated into 1×10^6 cells using the Neon Transfection System (100 µl tips; 10 ms, 1650 V, 3 pulses; Thermo Fisher). Transfected cells were seeded onto mouse feeder cells in fibroblast medium (DMEM/10% FBS, 2 mM L-glutamine). Medium was switched to TeSR-E7 (Stemcell Technologies) two days post transfection. Established cell line was cultured on Vitronectin-coated plates in StemMACS™ iPS-Brew (Miltenyi Biotec). Cells were passaged every 5 to 6 days using Cell Dissociation Buffer or ReLeSR (Stemcell Technologies).

3.2. MS-MLPA

Gene dosage and DNA methylation analysis was performed by methylation-sensitive multiplex-ligation probe amplification using the SALSA MLPA ME028 Prader-Willi/Angelman probemix, according to instructions (MRC Holland). Fragment length analysis was performed on an ABI Genetic Analyzer 3130XL using the program GeneMarker (Softgenetics) Table 1.

3.3. qRT-PCR

RNA was isolated using the RNeasy mini kit and 500 ng of RNA was

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers			
IF: StemLight Pluripotency antibody kit	Rabbit anti-Oct4 Rabbit anti-Nanog Rabbit anti-Sox2	1:200	Cell Signaling Technology Cat# 9656S, RRID:AB_10692662
FACS	mouse anti-human SSEA4	5 µl/10^6 cells	BioLegend Cat# 330408 RRID: AB_1089200
FACS	mouse IgG3κ, SSEA4 isotype	5 µl/10^6 cells	BioLegend Cat# 401321 RRID: AB_10683445
FACS	mouse anti-human TRA1–60	10 µl/10^6 cells	BioLegend Cat# 330610 RRID: AB_2119065
FACS	mouse IGMκ, TRA1–60 isotype	10 µl/10^6 cells	BioLegend: Cat# 401611 RRID: not given
Differentiation Markers			
IF	Mouse anti-beta III tubulin (TUJ1)	1:200	Sigma Cat# T8660, RRID: AB_477590
IF	Mouse anti-SMA clone 14A	1:200	Agilent Cat# M085129–2, RRID: AB_86452
IF	Mouse anti-AFP	1:200	Sigma Cat# WH0000174M1, RRID: AB_1839587
FACS	anti-human PAX6	10 µl/10^6 cells	Miltenyi Biotec Cat# 130–107-775 RRID: AB_2653167
FACS	REA(I) control, PAX6 istoype	10 µl/10^6 cells	Miltenyi Biotec Cat# 130–104-613 RRID: AB_2661678
FACS	anti-human T	5 µl/10^6 cells	R&D Systems Cat# IC2085G RRID: not listed
FACS	normal goat IgG, T isotype	5 µl/10^6 cells	R&D Systems Cat# IC108G RRID: AB_10890944
FACS	anti-human SOX17	2 µl/10^6 cells	Miltenyi Biotec Cat# 130–111-032 RRID: AB_2653493
FACS	REA(I) control, SOX17 isotype	2 µl/10^6 cells	Miltenyi Biotec Cat#130–104-613 RRID: AB_2661678
Primers			
	Target	Forward/Reverse primer (5′-3′)	
RT-PCR	UBE3A	CTCTTCTTGCAGTTTACAACGG CTTGAGTATTCCGGAAGTAAAAGC Tm: 60 °C; product size: 152 nt	
	POU5F1 (OCT4)	GAAGGTGAAGTTCAATGATGCTC ATTCCCATCCCTACCTCAGTAAC Tm: 60 °C; product size: 139 nt	
	T (BRACHYURY)	TGCTTCCCTGAGACCCAGTT GATCACTTCTTTCCTTTGCATCAAG Tm: 60 °C; product size: 121 nt	
	HAND1	TTCAAGGCTGAACTCAAGAAGG CTTTAATCCTCTTCTCGACTGGG Tm: 60 °C; product size: 115 nt	
	MEST1	TAGTGATGTGGTCTCGGTTTGT GAGATAGTTGTGCTTTTACACGGTT Tm: 60 °C; product size: 146 nt	
	SOX17	GAAGCTGTTTTGGGACACATTC ATTTTGTCTGCCACTTGAACAG Tm: 60 °C; product size: 142 nt	
	AFP	AGAAATACATCCAGGAGAGCCA TTTGTGTAAGCAACGAGAAACG Tm: 60 °C; product size: 106 nt	
	NESTIN	CTTGGTCCTTCTCCACCGTA GGAAGAGAACCTGGGAAAGG Tm: 60 °C; product size: 122 nt	
	PAX6	AACAGACACAGCCCTCACAAACA CGGGAACTTGAACTGGAACTGAC Tm: 60 °C; product size: 275 nt	
	SOX1	CAATGCGGGGAGGAGAAGTC CTCGAAACATTTTGGGTGGGG Tm: 60 °C; product size: 95 nt	
	TUBB3 (TUJ1)	CTCAGGGGCCTTTGGACATC CAGGCAGTCGCAGTTTTCAC Tm: 60 °C; product size: 160 nt	

reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen). qRT-PCR was performed with Quantitect SYBR-Green and analysed with BioRad CFX-Manager Software. Mean and standard deviation of technical triplicates are shown. Primer sequences are listed in Table 2.

3.4. Karyotype analysis

Metaphase chromosomes were prepared as described (Stanurova et al., 2016), chromosome number was analysed in eleven metaphase spreads and three detailed karyotypes were made.

3.5. Immunofluorescence

Cells were grown on Vitronectin-coated cover slips (Matrigel for EBs), fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton-X-100 for 10 min, blocked in PBS/5% BSA for 60 min (all at room temperature) and incubated with primary antibodies on 4 °C over night. Secondary antibody staining was performed for 60 min at room temperature. Images were taken with a Zeiss Axiovert A1 microscope with Axiovision software. Antibodies (Table 2) were diluted in PBS/5% goat serum.

3.6. FACS analysis

A single cell suspension was passed through a 40 µm cell strainer. For intracellular epitopes, cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton-X-100 for 15 min. Antibody staining was performed in PBS/10% FCS. Cells were measured on a CytoFLEX flow cytometer using the program CytExpert (Beckman Coulter), plots: X-axis: fluorescence intensity, y-axis: counts. Antibodies are listed in Table 2.

3.7. Germ layer in vitro differentiation

Cells were dissociated with ReLeSR (Stemcell Technologies) as multicellular clusters and seeded on a 6-well-ultra low attachment plate in 50% StemMACS™ iPS-Brew/50% DMEM-F12 + GlutaMax. Next day, medium was changed to DMEM-F12 + Glutamax, 20% FBS, 1% MEM-NEAA, 0,2% β-Mercaptoethanol. On day 7, embryoid bodies were transferred to matrigel-coated dishes or coverslips and cultivated for another 7 days. Medium was changed every other day. For directed monolayer differentiation the STEMDiff Trilineage Kit (Stemcell Technologies) was used according to manufacturer's instructions.

3.8. STR and GeneScan analysis

For STR analysis, the Powerplex 16 HS system (Promega) was used

according to instructions.

Fragments of eight microsatellites on chromosome 15 were amplified from genomic DNA and fragment length was analysed on an ABI Genetic Analyzer 3130XL using the program Gene Marker (Softgenetics). For primer sequences refer to www.ncbi.nlm.nih.gov/probe.

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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.2018.09.015.

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