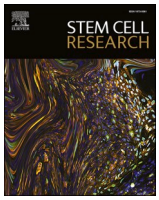
Stem Cell Research 67 (2023) 103016

Contents lists available at ScienceDirect

Stem Cell Research

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



Lab Resource: Multiple Cell Lines

Generation of four human induced pluripotent stem cells derived from ADHD patients carrying different genotypes for the risk SNP rs1397547 in the ADHD-associated gene ADGRL3

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A B S T R A C T

Single nucleotide polymorphisms (SNPs) in the *ADGRL3* gene have been significantly associated with the development of ADHD, the aetiology of which remains poorly understood. The rs1397547 SNP has additionally been associated with significantly altered *ADGRL3* transcription. We therefore generated iPSCs from two wild type ADHD patients, and two ADHD patients heterozygous for the risk SNP. With this resource we aim to facilitate further investigation into the complex and heterogenous pathology of ADHD. Furthermore, we demonstrate the feasibility of using magnetic activated cell sorting to allow the unbiased selection of fully reprogrammed iPSCs.

1. Resource table

Unique stem cell lines identifier	UKWi002-A https://hpscreg.eu/cell-line/UKWi002-AUKWi002-A-1 https://hpscreg.eu/cell-line/UKWi002-A-1UKWi003-A https://hpscreg.eu/cell-line/UKWi003-AUKWi003-A-1 https://hpscreg.eu/cell-line/UKWi003-A-1UKWi004-A https://hpscreg.eu/cell-line/UKWi004-AUKWi004-A-1 https://hpscreg.eu/cell-line/UKWi004-A-1UKWi005-A https://hpscreg.eu/cell-line/UKWi005-AUKWi005-A-1 https://hpscreg.eu/cell-line/UKWi005-A-1
Alternative name(s) of stem cell lines	awr925 CL28 (UKWi002-A)awr925 CLM+ (UKWi002-A-1)jem839 CL5 (UKWi003-A)jem839 CL7 (UKWi003-A-1)wya376 CL4 (UKWi004-A)wya376 CL6 (UKWi004-A-1)rnk730 CL20 (UKWi005-A)rnk730 CLM+ (UKWi005-A-1)
Institution	Department of Psychiatry, Psychosomatics and Psychotherapy, University Hospital, University of Würzburg, D-97080 Würzburg, Germany
Contact information of distributor	Dr Rhiannon McNeill (McNeill_R@ukw.de)
Type of cell lines	iPSC
Origin	Human
Additional origin info required *for human ESC or iPSC*	awr925 - Age: 27 Sex: Male Ethnicity if known: Caucasianrnk730 – Age: 46 Sex: Male Ethnicity if known: Caucasianjem839 – Age: 39 Sex: Male Ethnicity if known: Caucasianwya376 – Age: 39 Sex: Male Ethnicity if known: Caucasian
Cell Source	Fibroblast cells
Clonality	Clonal (manually picked) and polyclonal (cell sorting)
Associated disease	Attention-deficit/hyperactivity disorder (ADHD)
Gene/locus	*ADGRL3* SNP rs1397547 (wildtype = C allele, risk = G allele)chr4:61979670
Date archived/stock date	December 2021
Cell line repository/bank	https://hpscreg.eu/
Ethical approval	Ethics committee University of Würzburg, 06.11.2020, Ethical approval number 188/19-meEthics committee University of Frankfurt, 04.3.2015, Ethical approval number 425/14

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https://doi.org/10.1016/j.scr.2023.103016
Received 14 December 2022; Received in revised form 21 December 2022; Accepted 3 January 2023
Available online 5 January 2023

2. Resource utility

SNPs in the *ADGRL3* gene have been associated with ADHD, and the rs1397547 SNP has been identified as a significant independent predictor of *ADGRL3* transcription (McNeill et al., 2020). The generated cell lines can therefore be used to further investigate the role of *ADGRL3* genetic variation in ADHD aetiology. (49/50).

3. Resource details

Attention-deficit/hyperactivity disorder (ADHD) is an early-onset, chronic neurodevelopmental condition characterised by inattention, hyperactivity, and emotional dysregulation (American Psychiatric Association, 2013). ADHD has one of the highest heritability estimates among neuropsychiatric disorders (~74 %), and up to 20 % variance between case-control status has been attributed to single nucleotide polymorphisms (SNPs) (Rovira et al., 2020). However, ADHD pathogenesis remains poorly understood. The adhesion G protein-coupled receptor L3 (*ADGRL3*) gene, encoding the latrophilin-3 receptor, is thought to play roles in both glutamatergic synapse development and calcium signalling (Arcos-Burgos et al., 2010). Candidate gene association studies and animal models have repeatedly implicated *ADGRL3* in ADHD pathogenesis, with a recent *meta*-analysis confirming the role of *ADGRL3* SNPs in ADHD susceptibility (Bruxel et al., 2020). We recently revealed a potential functional mechanism for *ADGRL3* SNPs in ADHD pathogenesis for the first time, whereby dysregulated *ADGRL3* expression was found to be associated with the rs1397547 SNP in human fibroblast cells from both healthy controls and ADHD patients (McNeill et al., 2020). However, there is still a lack of both physiological and pathophysiological data regarding the ADGRL3 receptor. We therefore generated iPSC lines from four ADHD patients; two rs1397547 wildtype carriers, and two heterozygous risk allele carriers.

Primary fibroblast cell cultures were reprogrammed using Sendai virus-mediated transduction of the Yamanaka factors. Initial iPSC colonies were isolated from mouse embryonic fibroblast (MEF) feeder cells via either manual picking (clonal) or magnetic activated cell sorting (MACS; polyclonal). Resulting colonies demonstrated iPSC-like morphology; tightly packed cells, high nuclear to cytoplasmic ratio, and round colonies with a distinct border (Fig. 1A). Pluripotency was confirmed using immunolabelling for OCT4, SSEA4 and TRA-1-60 protein expression (Fig. 1B) and RT-PCR for *DPPA5*, *NANOG*, *OCT4*, and *SOX2* transcript expression (Fig. 1E.). Colocalisation of pluripotency-associated proteins with cell nuclei was quantified and the coefficient was consistently above 0.8 (Fig. 1C). The ability of the cells to differentiate into all 3 germ layers was confirmed using directed trilineage differentiation, using *TUBB3* as an ectodermal marker, *AFP* as an endodermal marker, and *SMA* as a mesodermal marker (Fig. 1D). Absence of viral vectors was confirmed for all clones after passage 12, using RT-PCR for viral transcripts (Fig. 1E). Molecular karyotyping was performed using Infinium Global Screening Array-24 + v3.0, GSA v3.0 + MD and no changes to chromosomal structure or number were detected. Reprogramming-induced CNVs were detected in 2/4 hiPSC lines, consistent with findings from the ForIPS Consortium, which should be taken into account when designing experiments (Supp. Table 1). rs1397547 SNP genotype of parental fibroblasts and derived iPSCs was confirmed using KASP™ assay (Fig. 1F). STR analysis and SNP-Arrays confirmed a high positive genetic correlation between fibroblast cells and iPSCs generated from the same patient (Supp. Fig. 1A). All cell lines were negative for mycoplasma contamination (Supp. Fig. 1B). In summary, we have generated four new iPSC lines derived from ADHD patients carrying different genotypes for the *ADGRL3* SNP rs1397547. These cells will allow researchers to further investigate the complex aetiology of ADHD, and role that *ADGRL3* may play in disease susceptibility (Tables 1 and 2).

4. Materials and methods

4.1. Derivation of primary fibroblast cells from skin biopsies

Skin biopsies were performed under local anaesthetic by a trained clinician (SKS). Biopsies were incubated in dispase solution (2.4 U/ml – PAN Biotech) for 16 h at 4 °C. The epidermis was then physically removed, and the sample further incubated with collagenase (Serva) for 45 mins at 37 °C. Fibroblast cells were detached and extracted using trypsin-EDTA (PAN Biotech). Resulting fibroblast cultures were maintained in DMEM + 10 % FBS (Life Technologies) and medium changed twice weekly.

4.2. Fibroblast cell reprogramming

Fibroblast cells (passage < 5) were reprogrammed using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher). Briefly, fibroblast cell cultures were incubated with the virus for 24 h. Cells were then further cultured for 7 days before seeding onto Mouse Embryonic Fibroblasts (MEF) feeder cells. A full medium changed was performed daily until the emergence of colonies.

4.3. Isolation of iPSC colonies – Manual picking and MACS

Multiple colonies were manually picked and transferred to new wells. After picking, cultures were left to recover for 48 h, and Magnetic Activated Cell Sorting (MACS) was then performed. Briefly, the entire well of MEF feeder cells and iPSC colonies was dissociated using Accutase (StemCell), and labelled with Pluripotent Stem Cell Microbeads (Miltenyi Biotech). Cells were then magnetically separated based on their expression of the CD326/EpCAM antigen and seeded into new wells.

4.4. iPSC maintenance

Clones were expanded on Matrigel™ (Corning)-coated cultureware (diluted according to manufacturer's instructions) and fed daily with StemMACS™iPS-BrewXF (Miltenyi Biotech). Cells were routinely passaged as clumps at a 1:6 ratio using ReLeSR™ (StemCell) every 4–5 days, and medium supplemented with 10 μM Y27632 for the first 24 h after passaging. iPSC cells were cultured in an incubator at 37 °C, 5 % CO_2 and 20 % O_2. All cells were characterised between passages 12–13.

4.5. RT-PCR

Total RNA was extracted using the RNeasy-Plus Mini Kit (Qiagen) and reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). PCR was then performed, products separated by electrophoresis, and images obtained using the Fusion FX system (Vilber). A previously established hiPSC line at an early passage number was used as a positive control for the detection of SeV genome and viral vectors.

4.6. Trilineage differentiation

iPSCs underwent directed differentiated into the 3 germ layers using the Trilineage Differentiation Kit (Miltenyi Biotech). iPSCs were seeded as single cells at specific cell densities for each germ layer, and were subsequently differentiated using germ layer-specific media for 7 days.

4.7. Immunofluorescent labelling

Cells were fixed with 4 % paraformaldehyde (Roth) for 15 mins, then permeabilised and blocked with PBS + 5 % FBS + 1 % BSA + 0.2 % Triton X-100 for 45 mins (Sigma). Primary antibodies were incubated at 4 °C overnight, and secondary antibodies were incubated at RT for 1 h. Coverslips were mounted using Prolong Diamond Antifade Mountant

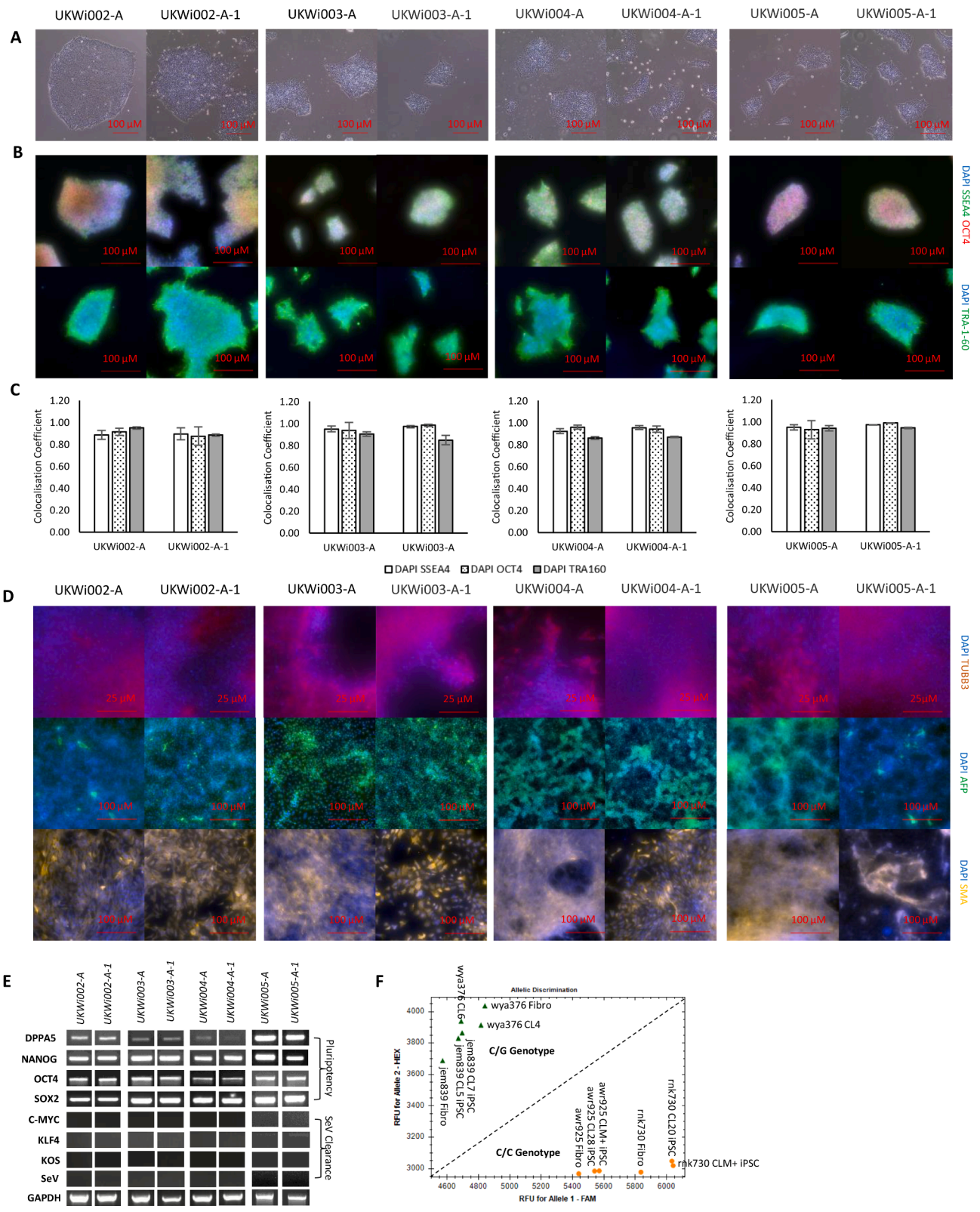

Fig. 1. Quality control of generated iPSC cell lines. A) Light microscopy images confirming the cells demonstrate hESC-like morphology. B) Immunofluorescence labelling for the pluriplotency markers OCT4, SSEA4 and TRA-1-60. C) Colocalisation analysis of immunofluorescence for the nuclear stain DAPI and each pluripotency marker. D) Immunofluorescence of markers for the three different germ layers after directed differentiation of iPSCs: TUBB3 for ectoderm, SMA for mesoderm and AFP for endoderm. E) RT-PCR to confirm transcription of pluripotency and absence of Sendai virus. (F) KASP genotyping results of each iPSC line for the *ADGRL3* SNP rs1397547.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Light microscopy	Typical hESC-like morphology	Fig. 1 panel A
Phenotype	Qualitative analysis using RT-PCR and immunofluorescence	Transcript (DPPA5, NANOG, OCT4, SOX2) and protein expression (OCT4, SSEA4, TRA-1–60) of pluripotency markers	Fig. 1 panel B and E
	Quantitative analysis using immunofluorescence colocalisation	High colocalisation of nuclear stain and pluripotency markers	Fig. 1 panel C
Genotype	Global Screening Array-24 BeadChip	High rate of genetic correlation between fibroblast cells and derived iPSC clones, no significant increase in DNA abnormalities	Supplementary Figure 1A; further data available with the author
Identity			
	STR analysis	21 STR sites tested and profiled	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	KASP™ assay	Presence or absence of *ADGRL3* rs1397547 SNP	Fig. 1 panel F
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	LookOut(R) Mycoplasma PCR Detection Kit showed all cultures were negative	Supplementary Figure 1B
Differentiation potential	Directed differentiation	Expression of βIII-Tubulin, α-feto protein, and α-smooth muscle actin	Fig. 1 panel D
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	Not shown but available with author
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-OCT4	1:500	Thermo Fisher Scientific Cat# 710,788	RRID:AB_2633097
	Mouse anti-SSEA4	1:200	Thermo Fisher Scientific Cat# MA1-021	RRID:AB_2536687
	Mouse anti-TRA-1-60	1:100	Novus Cat# NB100–730	RRID:AB_10001809
Differentiation Markers	Rabbit anti-TUJ1	1:700	Thermo Fisher Scientific Cat# A25532	RRID:AB_2651003
	Mouse anti-AFP	1:700	Thermo Fisher Scientific Cat# A25530	RRID:AB_2651004
	Mouse anti-SMA	1:200	Thermo Fisher Scientific Cat# A25531	RRID:AB_2651005
Secondary Antibodies	Alexa Fluor 488 goat anti-mouse IgG3	1:250	Thermo Fisher Scientific Cat# A-21151	RRID:AB_2535784
	Alexa Fluor 488 goat anti-mouse IgM	1:250	Thermo Fisher Scientific Cat# A-21042	RRID:AB_2535711
	Alexa Fluor 488 goat anti-mouse IgG1	1:250	Thermo Fisher Scientific Cat# A25536	RRID:AB_2651011
	Alexa Fluor 555 goat anti-mouse IgG2a	1:250	Thermo Fisher Scientific Cat# A25533	RRID:AB_2651012
	Alexa Fluor 647 donkey anti-rabbit	1:250	Thermo Fisher Scientific Cat# A25535	RRID:AB_2651010

	Primers		
	Target	Size of band	Forward/Reverse primer (5′-3′)
Pluripotency markers (RT)-PCR	hSOX2	278 bp	AACCAGCGCATGGACAGTTA/GACTTGACCACCGAACCCAT
	hNANOG	325 bp	ACCAGTCCCAAAGGCAAACA/AAAGGCTGGGGTAGGTAGGT
	hOCT4	646 bp	GTTGATCCTCGGACCTGGCTA/GGTTGCCTCTCACTCGGTTCT
	hDPPA5	215 bp	CGGCTGCTGAAAGCCATTTT/AGTTTGAGCATCCCTCGCTC
Sendai virus (SeV) detection (RT)-PCR	SeV	181 bp	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC
	c-Myc	532 bp	TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG
	KOS	528 bp	ATGCACCGCTACGACGTGAGCGC/ATGCACCGCTACGACGTGAGCGC
	Klf4	410 bp	TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA
Housekeeping gene (RT)-PCR	hGAPDH	701 bp	ATCACCATCTTCCAGGAGCGA/AAGTGGTCGTTGAGGGCAAT

with DAPI (ThermoFisher) and images obtained using the Eclipse Ti2-E inverted widefield epifluorescence microscope (Nikon). Colocalisation analysis was performed using the Colocalisation function on the NIS Elements software.

4.8. Molecular karyotyping

Genomic DNA was extracted using the DNeasy Kit (Qiagen) and analysed using the Infinium Global Screening Array-24 + v3.0, GSA v3.0 + MD by the Institute of Human Genetics, LIFE&BRAIN GmbH, University of Bonn. SNPs were called using the GenomeStudio Software v2.0. Due to the low sample number no SNPs were excluded due to genotyping rate. All samples had a genotyping rate above 95 %. No gender mismatch was observed. Only variants with a MAF < 0.02 based on the SNP154 annotation were included for the relatedness matrix. All SNPS were included for the CNV calling. For the relatedness analysis genotyping files (.bed format) were exported and analyzed in plink version 1.9. Relatedness between samples was calculated using the –genome flag without any modifiers. The PI HAT score was used as similarity measure and visualized as heatmap using the heatmap2 function with hierarchical clustering in R (RVersion 4.2). CNV based karyotyping was based on the logR Ratio and the B-allele frequency extracted per SNP per sample from the Genomestudio software as FinalReport Files. CNV calling was performed implementing the previously published CNVision pipeline. *ADGRL3* rs1397547 genotype was determined using competitive allele-specific PCR (KASP™) assay (LGC), which uses two different allele-specific primers conjugated to different dyes to enable bi-allelic scoring of SNPs.

4.9. Mycoplasma testing

All clones were tested for Mycoplasma contamination using the LookOut® Mycoplasma PCR Detection Kit (Sigma-Aldrich). An internal positive control was included.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

This publication was supported by the Open Access Publication Fund of the University of Würzburg.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103016.

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