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Lab Resource: Stem Cell Line

Generation and characterization of a human iPS cell line from a patient-related control to study disease mechanisms associated with DAND5 p.R152H alteration

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A B S T R A C T

A *DAND5*-control human iPSC line was generated from the urinary cells of a phenotypically normal donor. Exfoliated renal epithelial (RE) cells were collected and reprogrammed into iPSCs using Sendai virus reprogramming system. The pluripotency, *in vitro* differentiation potential, karyotype stability, and the transgene-free status of generated iPSC line were analyzed and confirmed. This cell line can be exploited as a control iPSC line to better understand the mechanisms involved in *DAND5*-associated cardiac disease.

Resource table.

Unique stem cell line identifier	NMSUNLi002
Alternative name(s) of stem cell line	*iUC-DAND5_455/control*
Institution	*CEDOC, NOVA Medical School*
Contact information of distributor	José A. Belo, jose.belo@nms.unl.pt
Type of cell line	*iPSC*
Origin	Human
Additional origin info	*Sex: male* *Ethnicity: Caucasian*
Cell Source	*Exfoliated renal epithelial cells isolated from urine*
Clonality	*Clonal*
Method of reprogramming	*Transgene-free (Sendai virus vector)*
Genetic Modification	*NO*
Type of Modification	*N/A*
Associated disease	*N/A*
Gene/locus	*NM_152654.2:c.455G; DAND5 c.G455G; p.R152R*
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*January 2018*
Cell line repository/ bank	*N/A*
Ethical approval	*Approved by the Ethics Committee of NOVA Medical School (Protocol N° 13/2016/ CEFCM) and by the National Committee for Data Protection (CNPD, Permit N° 8694/ 2016).*

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Resource utility

This *DAND5*-control iPSC line is essential in studying the disease related impairment in heart formation of DAND5 found in a previous study. This control cell line will allow to uncover the role of *DAND5* c.455 G > A variant in the molecular mechanisms of cardiomyocyte proliferation along with the previously established line.

Resource details

DAND5 is a Nodal antagonist that is involved in the correct left/right body axis establishment during gastrulation (Belo et al., 2017). The heart is the first organ to be formed and the first organ to be affected by improper levels of Nodal signaling throughout embryo development. Several variants of different genes involved in this signaling pathway have been associated with Congenital Heart Disease (Deng et al., 2015). Recently, we have identified and functionally characterized a c.455G > A *DAND5*-variant in a patient diagnosed with ventricular septal defect with overriding aorta, right ventricular hypertrophy, and pulmonary atresia (a case of extreme tetralogy of Fallot phenotype) (Cristo et al., 2017a). The variant was inherited from the apparently healthy mother. To further study the impact of altered DAND5 proteins in molecular pathways that are involved in cardiac development and on cardiomyocyte behavior, we have generated and characterized the NMSUNLi001-A variant cell line, which has a heterozygous non-synonymous variant in exon 2 of DAND5 gene (c.455G > A), causing an amino acid change of p.R152H in the functional domain of the DAND5 protein (Cristo et al., 2017b). However, to unveil the precise mechanism of how this variant affects early heart development, the generation of control iPSC lines is essential. Here, we generated and characterized a *DAND5* patient-related (father) control iPSC line that does not carry any alteration in the locus of the variant described in the NMSUNLi001-A cell line. This cell line will be utilized for disease modeling purpose. Hence, exfoliated renal epithelial (RE) cells were collected from urine sample collected from a healthy donor. After being grown during 7 days in culture, the RE cells were reprogrammed using CytoTune™-iPS 2.0 Reprogramming Kit (Life Technologies, Invitrogen). The kit utilizes non-transmissible, non-integrating form of Sendai Virus (SeV) vectors that deliver four key transcription factors (SOX2, OCT3/4, c-MYC and KFL-4) to reprogram the somatic cells into a pluripotent state, and the iPSC colonies appeared 17 days after the delivery of the reprogramming factors. At this time, we observed that cells assumed typical stem cell morphology. To obtain homogeneous and clonal iPSCs lines, we manually picked and expanded several single cell-derived iPSCs colonies. Among those, one sub-clone that best displayed the ESC-like morphology (Fig. 1A) was chosen for further characterization. Firstly, after 27 passages in culture, DNA Sanger sequencing and karyotype analysis proved the genotype 455G in *DAND5* exon 2 (Fig. 1D), and the number (46, XY) and arrangement of chromosomes (Fig. 1C). We assessed the transgene-free status of the iPSC line by qPCR (Fig. 1E), confirming the clearance of the viral vectors. Since the cytoplasmic nature of SeV only allows the exogenous reprogramming vectors to be cleared after several passages, we used an early passage of iPSCs as a positive control. The pluripotency of the cells was analyzed by both fluorescence immunocytochemistry (Fig. 1F) and qPCR (Fig. 1B). We confirmed the expression of the key pluripotency factors OCT4, NANOG, and SSEA4 both at protein and mRNA level. At mRNA level, we additionally confirmed the expression of pluripotency markers NODAL and SOX2. Embryoid body (EB) formation assay was performed to assess the spontaneous differentiation potential of the iPSCs *in vitro*. From this assay, we assessed that the EBs cultured for 19 days expressed markers of the three germ layers: endoderm, mesoderm, ectoderm, *i.e.*, alpha-fetoprotein (AFP), smooth muscle actin (SMA), tubulin beta-3 chain (TUBB3), respectively (Fig. 1G). Finally, STR analysis was performed showing that all the 16 loci tested matched (Table 1) .

Materials and methods

Reprogramming of RE cells

RE cells were collected and expanded in culture. After ~4 days in culture, cells started to become evident and the medium was changed to REBM™ supplemented with REGM™ BulletKit (Lonza). When cells reached ~80% confluency, they were seeded on a 6-well plate and reprogrammed using CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Life Technologies). At day 8 post-transduction, cells were passaged onto a 100 mm culture dish coated with Geltrex (Gibco, Thermo Fisher Scientific) and the next day medium was changed to Essential 8™ (E8) Flex medium, replaced until the iPSCs have emerged. 17 days post-transduction, colonies that best display an ESC-like morphology were picked and expanded with daily renewal of the E8 Flex medium.

Sequencing

To confirm the absence of the c.455G nucleotide in the established *DAND5*-control cell line, genomic DNA was extracted using ISOLATE II Genomic DNA kit (Bioline). Then, using the primers indicated in Table 2, exon 2 of *DAND5* was amplified by PCR and purified using NZYGelpure kit (NZYTech). Sequencing was conducted by STAB VIDA (http://www.stabvida.com/).

RNA extraction and real time qRT-PCR

The clearance of SeV transgenes and the expression of pluripotency markers OCT4, NANOG, SSEA4 (primers listed in Table 2) were carried out using Direct-zol™ RNA MiniPrep (Zymo Research). Subsequently, reverse transcription and qRT-PCR were performed.

Embryoid body formation assay

Embryoid bodies (EBs) consisting of approximately 2000 iPS cells/20 µl drop in Essential 8™ (E8) medium with 4 mg/ml polyvinylalcohol and RevitaCell™ Supplement (Thermo Fisher Scientific) were generated using hanging drop method. After 2 days, EBs were suspended in 50% E8 medium and 50% differentiation medium (DMEM with 20% FBS, Pen/Strep, NEAA, 2 mM L-glutamine, and 0,1 mM β-mercaptoethanol) and grown 3 more days. At day 5, EBs were placed on 24-well-plate with differentiation medium changes every other day. By day 19, EBs were fixed with 4% formaldehyde and the immunocytochemistry assayed for the three germ layers AFP, SMA and TUBB3.

Fluorescent immunocytochemistry (ICC)

Cells were fixed with 4% paraformaldehyde, permeabilized, blocked and incubated with primary and secondary antibodies (listed in Table 2) overnight at 4 °C. Prior to image acquisition, DAPI was used to stain DNA. All fluorescent images were acquired with confocal microscopy.

Karyotyping

Chromosome analysis was performed using GTG high resolution banding technique, according to standard procedures with a minimum of 10 metaphase spreads analyzed. Analysis of GTG-banded chromosomes was performed at a resolution of 400 bands per haploid genome and karyotypes were established according to the International System for Human Cytogenetic Nomenclature (ISCN 2016).

Mycoplasma contamination test

The sterility of the iPSC culture from mycoplasma was verified by PCR using the primers in Table 2.

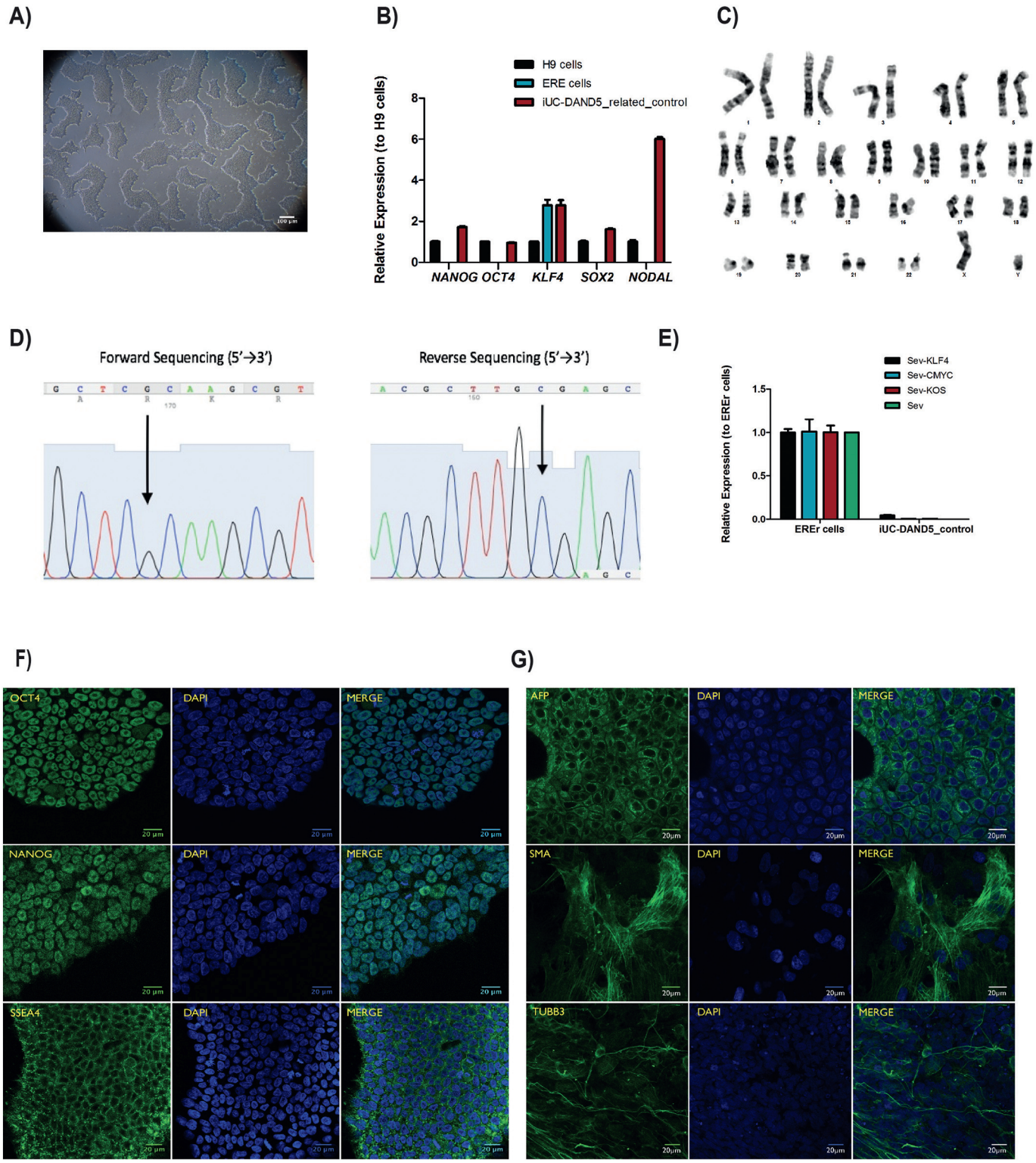

Fig. 1. Characterization of the iUC-DAND5_455/control iPSC line. A. Morphology of the iUC-DAND5_455/control line. B. mRNA expression levels of endogenous pluripotency markers in H9 cells (Black - positive control), ERE cells (Blue) and iUC-DAND5_455/control line (Red). CT-values were normalized to the geometric mean of the two housekeeping genes GAPDH and β-actin and with H9 human embryonic stem cell line as reference (set to 1). C. Karyotype of representative metaphase showing normal 46 chromosomes (XY). D. DNA sequence confirming the normal homozygous c.455G genotype in the iUC-DAND5_455/control line. E. Absolute quantitative real-time PCR showing absence of the vectors and the exogenous reprogramming factor in iPSCs (right) and presence of the reprogramming factors in the EREr control cells (left). F. Immunodetection of pluripotency markers of iUC-DAND5_455/control line. G. Immunofluorescence analyses of in vitro differentiation of EBs using specific antibodies against the endodermal marker α-fetoprotein (AFP), ectodermal marker βIII-tubulin (TUBB3) and mesodermal markers α-smooth muscle actin (SMA). Nuclei were stained with DAPI (scale bars = 20µm).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*ESC-like morphology*	Fig. 1, panel A
Phenotype	Immunocytochemistry	*Staining of pluripotency markers: Oct4, Nanog, SSEA4*	Fig. 1, panel F
	qPCR	*Expression of pluripotency markers: NANOG, OCT3/4, SOX2, KLF4 and NODAL*	Fig. 1, panel B
Genotype	Karyotype (G-banding) and resolution	*46XY, Resolution 400–500*	Fig. 1, panel C
Identity	Microsatellite PCR (mPCR)	*N/A*	
	STR analysis	*16 loci analyzed, all matching*	Supplementary Fig. S1 panel A
Mutation analysis (IF APPLICABLE)	Sequencing	*Homozygous (G > G)*	Fig. 1, panel D
	Southern Blot OR WGS	*N/A, Non-integrating reprogramming methodology*	
Microbiology and virology	Mycoplasma contamination	*Mycoplasma-free culture*	Supplementary Fig. S1 panel B
Differentiation potential	Embryoid body formation	*Proof of formation of three germ layers from Embryoid bodies: α-fetoprotein (AFP), βIII-tubulin (TUBB3), α-smooth muscle actin (SMA).*	Fig. 1, panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*N/A*	N/A
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-citometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	*Rabbit anti-NANO*	*1:200*	*Abcam Cat# ab21624, RRID:AB_446437*
	Rabbit anti-OCT4	*1:400*	*Abcam Cat# ab19857, RRID:AB_445175*
	Mouse anti-SSEA4	*1:200*	*Abcam Cat# ab16287, RRID:AB_778073*
Differentiation Markers	*Mouse anti-Human TUBB3*	*1:400*	*Sigma-Aldrich Cat# T8660, RRID:AB_477590*
	Mouse anti-Human SMA	*1:600*	*Dako Cat# M0851, RRID:AB_2223500*
	Rabbit anti-Human AFP	*1:200*	*Dako Cat# A0008, RRID:AB_2650473*
Secondary antibodies	*Alexa Fluor 488-conjugated Donkey anti-Mouse IgG (H + L)*	*1:300*	*Jackson ImmunoResearch Labs Cat# 715–545-150, RRID:AB_2340846*
	Alexa Fluor 488-conjugated Donkey anti-Rabbit IgG (H + L)	1:300	Jackson ImmunoResearch Labs Cat# 711–545-152, RRID:AB_2313584

Primers

	Target	Forward/Reverse primer (5′-3′)
Elimination of Sendai Virus transgenes (qPCR)	*Sev*	*GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTT AAGAGATATGTATC*
	Sev-KLF4	*TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTG CTCAA*
	Sev-C-MYC	*TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCT GGATGATGATG*
	Sev-KOS	*ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATC CTGATGTGG*
Pluripotency Markers (qPCR)	*NANOG*	*CATGAGTGTGGATCCAGCTTG/CCTGAATAAGCAGATCCATGG*
	OCT3/4	*GACAGGGGGAGGGGAGGAGCTAGG/CTTCCCTCCAACCAGTTGCCCCAAAC*
	SOX2	*GGGAAATGGGAGGGGTGCAAAAGAGG/TTGCGTGAGTGTGGATGGGATTGGTG*
	KLF4	*ACCAGGCACTACCGTAAACACA/GGTCCGACCTGGAAAATGCT*
	NODAL	*GGGCAAGAGGCACCGTCGACATCA/GGGACTCGGTGGGGCTGGTAACGTTTC*
House-Keeping Genes (qPCR)	*GAPDH*	*CTGGTAAAGTGGATATTGTTGCCAT/TGGAATCATATTGGAACATGTAAACC*
	β-actin	*GCAAAGACCTGTACGCCAAC/AGTACTTGCGCTCAGGAGGA*
Mycoplasma detection	*Pair 1*	*CTGCAGATTGCAAAGCAAGA/CCTCCTTCTTCACCTGCTTG*
	Pair 2	*GGCGAATGGGTGAGTAACACG/CGGATAACGCTTGCGACCTATG*
Targeted mutation analysis/sequencing	*DAND5 exon 2*	*GGAAGTGGACAGGTGATTATCC/CAC GTCTTTCTTGGTCCATCTC*

STR analysis

iUC-DAND5_455/control cells and the corresponding ERE cells were authenticated by STR analysis performed by STAB VIDA (http://www.stabvida.com/).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.04.015.

Author contributions

Conceived and designed the experiments: FC, SP, JMI, JB; Diagnosis of patients: PM, JM, RA; Patient recruitment, sample collection and clinical data collection: FC, JMI, PM, JM, RA, DM; Analyzed the data: FC, JMI, GR, JB; Performed the experiments: SP, FC, JMI, GR; Karyotype experiment and analysis: IMC, JBM, LPA; Contributed to writing the manuscript: SP, FC, JMI and JB. All authors read and approved the final manuscript.

Ethical statement

All the experimental protocols were approved by the Ethics Committee of the NOVA Medical School (Protocol N.°13/2016/CEFCM) and by the National Committee for Data Protection (CNPD, Permit N.° 8694/2016), according to European Union legislation. Written informed consent was obtained from patient guardian prior to sample collection.

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