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Lab Resource: Stem Cell Line

Generation of human iPSC line from a patient with laterality defects and associated congenital heart anomalies carrying a *DAND5* missense alteration

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ABSTRACT

A human iPSC line was generated from exfoliated renal epithelial (ERE) cells of a patient affected with Congenital Heart Disease (CHD) and Laterality Defects carrying tshe variant p.R152H in the *DAND5* gene. The transgene-free iPSCs were generated with the human OSKM transcription factor using the Sendai-virus reprogramming system. The established iPSC line had the specific heterozygous alteration, a stable karyotype, expressed pluripotency markers and generated embryoid bodies that can differentiate towards the three germ layers in vitro. This iPSC line offers a useful resource to study the molecular mechanisms of cardiomyocyte proliferation, as well as for drug testing.

Resource table

Unique stem cell line identifier	NMSUNLi001-A
Alternative name(s) of stem cell line	iUC-DAND5_455/10
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	Age: 7 Sex: male Ethnicity: caucasian
Cell Source	Exfoliated renal epithelial cells isolated from urine
Method of reprogramming	Transgene free (Sendai Virus)
Genetic Modification	NO
Type of Modification	N/A
Associated disease	Heterotaxy and congenital heart disease
Gene/locus	rs45513495: DAND5 c.455G > A; p.R152H
Method of modification	No modification
Name of transgene or resistance	No transgene
Inducible/constitutive system	N/A
Date archived/stock date	January 2017
Cell line repository/bank	Not applicable
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).

1. Resource utility

DAND5 is the human homologue of mouse *Cerl2*/*Dand5*, a gene involved in left-right asymmetry establishment and also in heart formation. This generated iPS cell line, from a patient carrying the variant c.455G > A in the *DAND5* gene offers a useful resource to investigate the molecular mechanisms of cardiomyocyte proliferation, as well as for drug testing.

2. Resource details

Exfoliated renal epithelial (ERE) cells isolated from a urine sample were obtained from a 7-year old male child. The patient was clinically diagnosed with ventricular septal defect with overriding aorta, right ventricular hypertrophy and pulmonary atresia (a case of extreme

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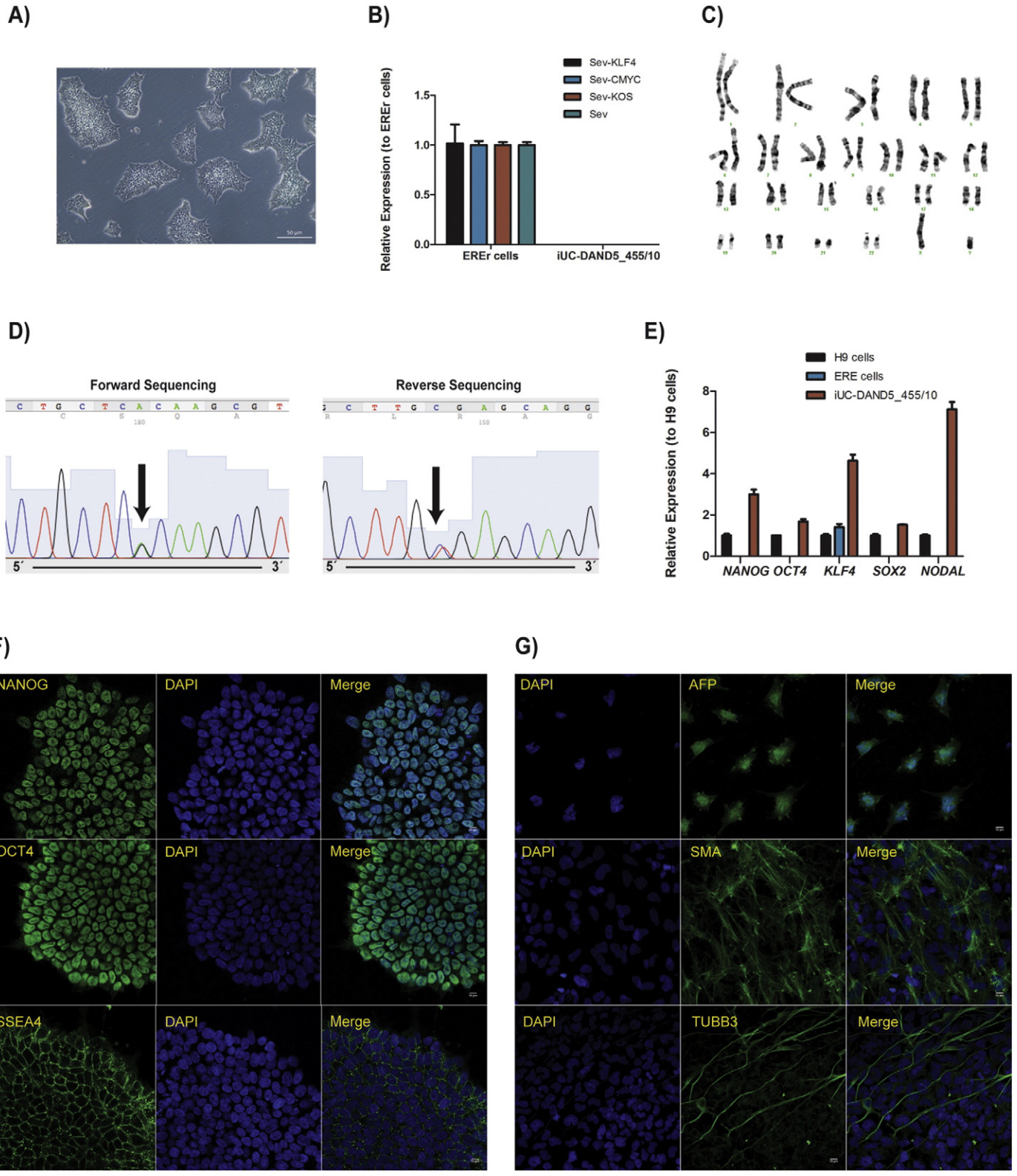

Fig. 1. Characterization of the iUC-DAND_455/10 iPSC line. A. Morphology of the iUC-DAND5_455/10 line. B. Absolute quantitative real-time PCR showing absence of the vectors and the exogenous reprogramming factor in iPSCs (right) and presence of the reprogramming factors in the EREr control cells (left). C. Karyotype of representative metaphase showing normal 46 chromosomes (XY). D. DNA sequence confirming the c.455G > A variant in the iUC-DAND5_455/10 line. E. mRNA expression levels of endogenous pluripotency markers in H9 cells (Black - positive control), ERE cells (Blue) and iUC-DAND5_455/10 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). F. Immunodetection of pluripotency markers of iUC-DAND5_455/10 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.

tetralogy of Fallot phenotype), defects that can be associated with early left-right establishment impairment. Genetically, the patient carries 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. *DAND5* is an essential gene in the correct establishment of the laterality of visceral organs, including the heart, functioning as an inhibitor and master regular of Nodal signalling in a temporal and spatial precise way (Inacio et al., 2013; Marques et al., 2004). *DAND5* knockout mice display a vast array of congenital cardiac malformations associated or not with extracardiac anomalies (Marques et al., 2004). Importantly, these KO mice present thickening of the left ventricle and of the IVS due to hyperproliferation of cardiomyocytes, independent of L/R defects (Araujo et al., 2014). Our previous functional analysis of DAND5 p.R152H alteration showed a significant decrease in the function of this variant protein when compared to its wild-type counterpart. These results support a model in which the imbalance in dosage-sensitive Nodal signalling is a final common way for laterality defects and associated CHDs and suggest a possible role of this variant in the risk of disease (Cristo et al., 2017). Moreover, it has been reported that variants in genes involved in the Nodal signalling pathway are associated with isolated cases of congenital heart defects and/or laterality defects in humans (Deng et al., 2015). In the work presented here, upon isolation of urine epithelial cells from the patient, we generated the iUC-DAND5_455/10 cell line using the CytoTune®-iPS 2.0 Reprogramming kit (Life Technologies, Invitrogen). This kit includes the reprogramming factors SOX2, OCT3/4, c-MYC and KLF4 and is based on a modified and non-transmissible form of Sendai virus (SeV). Seventeen days after infection, several iPSC colonies single cell-derived were picked for further expansion and characterization. After expansion, iUC-DAND_455/10 cell line, continued to display a typical small, round shape, and tightly packed ESC-like morphology with a high nucleus/cytoplasm ratio with prominent nucleoli (Fig. 1A). The clearance of the vectors and the exogenous reprogramming factor genes were confirmed by qPCR after twenty-five culture passages (Fig. 1B). The clone was karyotypically normal (46, XY) after more than twenty culture passages (Fig. 1C), and DNA Sanger sequencing confirmed the presence of a c.455G > A substitution in one of the alleles of exon 2 in the *DAND5* gene corresponding to the R152H protein alteration (Fig. 1D). Gene expression analysis was performed by qPCR to confirm the expression of pluripotency markers at mRNA level, which showed that the endogenous pluripotency genes *OCT3/4*, *NANOG*, *SOX2*, *KLF4* and *NODAL* were present at levels comparable or higher than the human embryonic stem cell line H9 (Fig. 1E). Moreover, these pluripotency genes were almost absent in ERE cells. At the protein level, immunocytochemical (ICC) analysis confirmed the expression of self-renewal transcription factors NANOG, OCT4, and the surface marker SSEA4, (Fig. 1F), characteristic markers of pluripotent ES cells which illustrate the purity of the iUC-DAND_455/10 iPSC line (Table 1).

Finally, in vitro embryoid body (EB)-based differentiation followed by ICC analysis of the endodermal marker α-feto protein (AFP), the mesodermal marker smooth muscle actin (SMA) and the ectodermal marker Tubulin β 3 class III (TUBB3) confirmed the pluripotency of iPSCs and their ability to differentiate into all three germ layers (Fig. 1G).

3. Materials and methods

3.1. Ethical statement

All the experimental protocols in the present study 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.

3.2. Generation of iPSCs

Urine epithelial cells were collected, expanded, and reprogrammed using the 3 Sendai virus vectors included in the CytoTune-iPS 2.0 Reprogramming Kit (Life Technologies) at a 1.5 MOI (multiplicity of infection). After 24 h, medium was replaced with fresh RE proliferation medium and cells cultured for 7 days with medium changes every other day. On day 8, cells were passaged using TrypLE Select (Gibco, Thermo Fisher Scientific) and seeded onto a 100 mm culture dish (Corning) coated with Geltrex (Gibco, Thermo Fisher Scientific). In the next day, medium was replaced to Essential 8 (E8) flex (Gibco, Thermo Fisher Scientific) and renewed every day until hiPSC colonies appeared. 17 days after infection, individual colonies were picked and expanded, with daily renewing of the E8 flex medium.

3.3. Sequencing analysis

Genomic DNA was extracted from patient hiPSCs using the Isolate Genomic DNA mini kit (BIOLINE). Subsequently, amplification by PCR of the exon 2 of *DAND5* gene, containing the c.455G > A alteration, was carried out using the primers listed in Table 2. PCR products were direct sequenced at STAB VIDA (http://www.stabvida.com/).

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, Sox2	Fig. 1, panel F
	qPCR	Expression of pluripotency markers: NANOG, OCT3/4, SOX2, KLF4 and NODAL	Fig. 1 panel E
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	Heterozygous (G > A)	Fig. 1, panel D
	Southern Blot OR WGS	N/A, Non-integrating reprogramming methodology	
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	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	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-NANOG	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 - TaqMan)	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	

3.4. Real-time PCR analysis

Real time PCR was carried out with Fast SYBR Green Master Mix (Applied Biosystems) and the primers listed in Table 2 on an Applied Biosystems® 7500 Real-Time PCR machine

3.5. Test for absence of the reprogramming Sendai vectors

ERE in reprogramming (EREr) and established hiPSC cells were tested for absence of the Sendai reprogramming vectors by qRT-PCR (Table 2).

3.6. Fluorescent immunocytochemistry

Undifferentiated or differentiated iUC-DAND_455/10 cells were fixed in 4% paraformaldehyde, incubated with primary antibodies overnight at 4 °C, listed in Table 2, and then incubated with Alexa Fluor 488-conjugated secondary antibodies overnight at 4 °C. Nuclei were stained with DAPI at room temperature and cell images were acquired with Zeiss Axio Imager Z2 microscope (Carl Zeiss) or confocal microscopy.

3.7. In vitro differentiation potential by embryoid bodies formation assay

For the generation of embryoid bodies (EBs), iPS cells were collected and suspended in non-adherent tissue culture 100 mm dishes with E8 medium plus polyvinyl alcohol and Revitacell for 7 days. At this time, the EBs were transferred onto Geltrex-coated lummox 24-well plates (SARSTEDT) and cultured for another 14 days or longer. Then, cells were fixed with 4% formaldehyde and incubated with the indicated primary antibodies specific for the three embryonic germ layers.

3.8. 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).

3.9. Mycoplasma contamination detection

The absence of mycoplasma was assessed by PCR using the Primers listed in Table 2.

3.10. STR analysis

iUC-DAND_455/10 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.2017.10.019.

Author contributions

Conceived and designed the experiments: FC, 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: FC, JMI, GR; Karyotype experiment and analysis: IMC, JBM, LPA; Contributed to writing the manuscript: FC, JMI and JB.

All authors read and approved the final manuscript.

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