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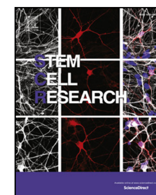

Lab Resource: Multiple Cell Lines

Generation of a gene-corrected human induced pluripotent stem cell line derived from a patient with laterality defects and congenital heart anomalies with a c.455G > A alteration in *DAND5*.

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A B S T R A C T

Human induced pluripotent stem cells (hiPSCs) from individual patient basis are considered a powerful resource to model human diseases. However, to study complex multigenic diseases such as Congenital Heart Disease, it is crucial to generate perfect isogenic controls to understand gene singularity and contribution. Here, we report the engendering of an isogenic hiPSC line with homozygous correction of c.455G > A alteration in the *DAND5* gene, using CRISPR/Cas9 technology. The characterization of a clone of this cell line demonstrates normal karyotype, pluripotent state, and potential to differentiate in vitro towards endoderm, mesoderm, and ectoderm.

Resource Table:

Unique stem cell lines identifier	NMSUNLi003
Alternative names of stem cell lines	iUC-DAND5_455/10C
Institution	CEDOC, NOVA Medical School
Contact information of distributor	José A. Belo, jose.belo@nms.unl.pt
Type of cell lines	iPSC
Origin	Human
Cell Source	Exfoliated renal epithelial cells isolated from urine
Clonality	Clonal
Method of reprogramming	Sendai virus
Multiline rationale	Isogenic cell line
Gene modification	Yes
Type of modification	Gene correction
Associated disease	Heterotaxy and Congenital Heart Disease
Gene/locus	Rs45513495:*DAND5*, c.455; p.R152
Method of modification	CRISPR
Name of transgene or resistance	No transgene
Inducible/constitutive system	N/A
Date archived/stock date	July 2019
Cell line repository/bank	N/A
Ethical approval	Approved by the Ethics Committee of NOVA Medical School (Protocol no. 13/2016/CEFCM) and by the National Committee for Data Protection (CNPD, Permit Nº8694/2016)

1. Resource utility

The molecular mechanism by which alterations on *DAND5* gene increase the risk of the complex multigenic Congenital Heart Disease is still undetermined. The generated corrected iUC-DAND5_455/10C line is the most ultimate control to establish models in order to understand the role of this gene in patients with *DAND5* c.455 G > A variant, in terms of the molecular mechanisms of cardiomyocyte proliferation, drug screening, and regenerative medicine.

2. Resource details

DAND5 is a secreted molecule that controls the bioavailability of NODAL signaling at the organizer during early embryogenesis (Inacio et al., 2013). The exact localization and temporal expression of NODAL are what determines the correct establishment of the anatomic asymmetries on the visceral organs, in particular, the anatomical development of the heart (Araujo et al., 2014; Belo et al., 2017). The c.455G > A variant in the functional domain of *DAND5* gene causes a significative decrease in the activity of this NODAL antagonist (Cristo et al.,2017a). This non-synonymous variant was first identified in a patient clinically diagnosed with a ventricular septal defect with overriding aorta, right ventricular hypertrophy, and pulmonary atresia,

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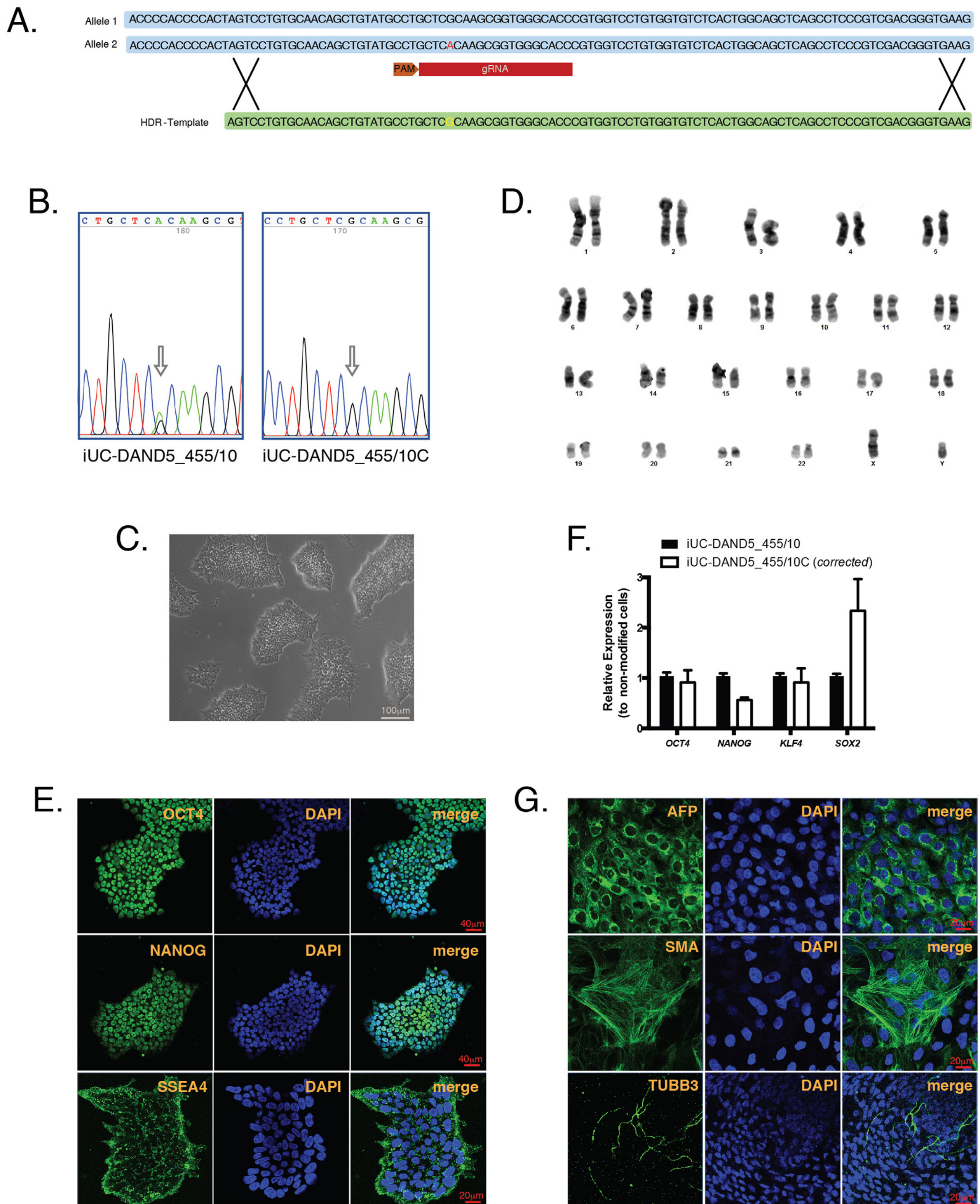

Fig. 1. Characterization of iUC-DAND5_455/10C cell line. A. Gene correction strategy. B. DNA sequence showing the c.455G > A variant in the iUC-DAND5_455/10 line and the same region of the corrected iUC-DAND5_455/10C cell line. C. Bright-field image of the iUC-DAND5_455/10C iPSC line morphology. D. Karyotype of a representative metaphase showing normal 46 chromosomes (XY). F. Real-Time PCR analysis of the endogenous pluripotency markers. E. Immunodetection of pluripotency markers of iUC-DAND5_455/10C cell line. G. Immunofluorescence for endodermal marker α-fetoprotein (AFP), ectodermal marker βIII-tubulin (TUBB3) and mesodermal marker α-smooth muscle actin (SMA). Nuclei were stained with DAPI.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	ESC-like morphology	Fig. 1, panel C
Phenotype	Qualitative analysis *(Immunocytochemistry)*	Positive staining for expression of pluripotency markers: OCT4, NANOG, SSEA4	Fig. 1 panel E
	Quantitative analysis *(RT-qPCR)*	Expression of pluripotency markers: *NANOG, OCT4, KLF4, SOX2*	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	46XY, Resolution 450–500	Fig. 1 panel D
Identity	Microsatellite PCR (mPCR) OR STR analysis*]*	N/A 16 loci analyzed, match	N/A Supplementary Figure 2
Mutation analysis (If applicable)	Sequencing	Homozygous (corrected)	Fig. 1 panel B
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Figure 1
Differentiation potential	Embryoid body formation	Positive AFP endodermal staining, positive TUBB3 ectodermal staining, positive SMA mesodermal staining	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

a case of extreme tetralogy of Fallot (Cristo et al.,2017a). To better understand the role of DAND5 in the human cardiac development program, we have generated and characterized the NMSUNLi001-A variant cell line, in which the c.455G > A *DAND5* variant is heterozygotic (Cristo et al.,2017b). This iPSC line was generated using Sendai-virus reprogramming system, retained a stable karyotype, expressed pluripotency markers (e.g., NANOG, OCT4, SOX2), and differentiated towards the three germ layers in vitro (Cristo et al., 2017b).

Here, using CRISPR/Cas9-mediated genome editing, we were able to generate an isogenic *DAND5*-corrected iPSC line, i.e., c.455 G homozygous. The strategy for gene correction, in which we made use of CRISPR-induced homology directed repair (HDR) technology using a single-stranded oligonucleotide (ssODN) as a repair template, is illustrated in Fig 1A. DNA Sanger sequencing analysis of NMSUNLi003 confirmed the correction of the heterozygotic c.455G > A *DAND5* alteration (Fig 1B). The genetic identity of this isogenic cell line to the parental NMSUNLi001-A variant cell line was confirmed by STR analysis, showing that all 16 loci tested matched (Table 1). The *DAND5*-corrected iPSC line showed a typical human embryonic stem cell (hESC) colony-like morphology with a high nucleus/cytoplasm ratio (Fig 1C), and was karyotypically normal (Fig 1D). Pluripotency was assessed by immunofluorescence for OCT4, NANOG, and SSEA4 pluripotency markers (Fig 1E), and by the expression of endogenous pluripotency factors *OCT4, NANOG, KLF4, SOX2* (Fig 1F). In vitro embryoid body (EB)-based differentiation, followed by immunofluorescence analysis of the endodermal marker α-fetoprotein (AFP), the mesodermal marker smooth muscle actin (SMA) and the ectodermal marker βIII-tubulin (TUBB3) revealed that the pluripotent isogenic *DAND5*-corrected iPSC line differentiates into all three germ layers (Fig 1G). The presence of mycoplasma was verified by PCR and the result was negative (Supplementary Fig 1).

Thus, the generated corrected iUC-DAND5_455/10C cell line represents the unmatched control to understand the contribution of the c.455G > A *DAND5* variant to the disease phenotype.

3. Materials and methods

3.1. iPSCs culture

Human iPSCs were cultured in StemFlex Medium (Thermo Fisher Scientific) on hESC-Qualified Geltrex (Thermo Fisher Scientific) coated plates at 37 °C in a humidified atmosphere (95% air, 5% CO_2). Medium was renewed daily and cells passaged using TrypLE Select (Thermo Fisher Scientific) upon reaching 85% confluence.

3.2. CRISPR gene correction

The heterozygous non-synonymous variant in exon 2 of *DAND5* gene (c.455G > A) was corrected using CRISPR/Cas9-induced homology directed repair technology. A specific sgRNA was designed using the CRISPR design web tool in crispr.mit.edu, and was cloned into pCAG-SpCas9-GFP-U6-gRNA plasmid (Addgene #79144). A total of 1.0×10^6 cells were co-nucleofected with 2 µg of the resulting plasmid, and 1 µL of 100 µM ssODN using Neon transfection system (Thermo Fisher Scientific), according to the manufacturer's instructions. After transfection, the cells were cultured in StemFlex Medium (Thermo Fisher Scientific) supplemented with RevitaCell Supplement at a 1X final concentration (Thermo Fisher Scientific), at 37 °C in a humidified atmosphere (95% air, 5% CO_2). 48 h post-nucleofection, cells were FACS sorted and plated at single cell density into 96-well plates. Clones were screened for the desired base correction by sequencing of amplicons spanning the target site of *DAND5* gene exon 2.

3.3. Fluorescent immunocytochemistry

Undifferentiated or differentiated iUC-DAND5_455/10C 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 Zeiss LSM710 confocal microscope.

3.4. In vitro differentiation potential by embryoid bodies formation assay

Embryoid bodies (EBs) consisting of 2000 cells/20 µL in E8 (Thermo Fisher Scientific) medium plus polyvinyl alcohol (SIGMA) and RevitaCell (Thermo Fisher Scientific) were generated using hanging drop method. Two days after, 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) (Thermo Fisher Scientific) and grown 3 more days on non-adherent Petri dishes (LABBOX). 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.

3.5. Real-time PCR analysis

Real time PCR was carried out with SensiFAST SYBR Lo-ROX Mix

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:400	Abcam Cat# ab19857, RRID:AB_445175
	Mouse anti-SSEA4	1:200	Abcam Cat# ab16287, RRID:AB_778073
	Rabbit anti-NANOG	1:100	Abcam Cat# ab21624, RRID:AB_446437
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_2,223500
	Rabbit anti-Human AFP	1:200	Dako Cat# A0008, RRID:AB_2,650473
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′)
Pluripotency Markers (qPCR)	*NANOG*	CATGAGTGTGGATCCAGCTTG/CCTGAATAAGCAGATCCATGG
	OCT3/4	GACAGGGGGAGGGGAGGAGCTAGG/CTTCCCTCCAACCAGTTGCCCCAAAC
	SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG/TTGCGTGAGTGTGGATGGGATTGGTG
	KLF4	ACCAGGCACTACCGTAAACACA/ GGTCCGACCTGGAAAATGCT
House-Keeping Genes (qPCR)	*GAPDH*	CTGGTAAAGTGGATATTGTTGCCAT/TGGAATCATATTGGAACATGTAAACC
Mycoplasma detection	*Pair 1*	CTGCAGATTGCAAAGCAAGA/CCTCCTTCTTCACCTGCTTG
	Pair 2	GGCGAATGGGTGAGTAACACG/CGGATAACGCTTGCGACCTATG
Targeted mutation analysis/sequencing	*DAND5 exon 2*	GGAAGTGGACAGGTGATTATCC/CACGTCTTTCTTGGTCCATCTC

(BIOLINE) and the primers listed in Table 2 on an ABI QuantStudio5 Real-Time PCR machine.

3.6. Karyotyping

Chromosome analysis was performed using GTG high resolution banding technique at 400–500 band resolution, 30 metaphases analyzed (Service of GenoMed, Diagnósticos de Medicina Molecular, SA, Lisboa, Portugal).

3.7. Mycoplasma contamination detection

The absence of mycoplasma was assessed by PCR using two pairs of Primers listed in Table 2, and Mycoplasmacheck test (Barcode 84397997) by Eurofins Genomics (http://www.eurofinsgenomics.eu/).

3.8. STR analysis

Genomic DNA was extracted (Qiagen) from iUC-DAND_455/10C cells (gDNA #10C) and the parental iUC-DAND_455/10 cells (gDNA #7.39) and the cell line authentication (16 loci) was analyzed by STAB VIDA (http://www.stabvida.com/).

Ethical statement

All the experimental protocols were approved by the Ethics Committee of the NOVA Medical School (Protocol No. 13/2016/ CEFCM) and by the National Committee for Data Protection (CNPD, Permit No. 8694/ 2016), according to European Union legislation.

CRediT authorship contribution statement

José M. Inácio: Validation, Writing - original draft, Investigation, Formal analysis, Conceptualization. **Micael Almeida:** Validation, Writing - original draft, Investigation, Formal analysis, Conceptualization. **Fernando Cristo:** Formal analysis, Investigation, Writing - original draft, Validation, Conceptualization. **José A. Belo:** Formal analysis, Investigation, Writing - original draft, Validation, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101677.

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