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Generation of induced pluripotent stem cell line from a patient suffering from arterial calcification due to deficiency of CD73 (ACDC)

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Abstract

Arterial calcification due to deficiency of CD73 (ACDC) is an adult onset, rare genetic vascular disorder signified by calcium deposition in lower extremity arteries and joints of hands and feet. Mutations in *NT5E* gene has been shown to be responsible for the inactivation of enzyme CD73 causing calcium buildup. Here, we report a iPSC line generated from a patient showing signs of ACDC and carrying a missense mutation in *NT5E* (c.1126→-G,p.T376A) gene. This iPSC line shows normal morphology, pluripotency, karyotype, and capability to differentiate into three germ layers, making it useful for disease modeling and investigating pathological mechanisms of ACDC.

Keywords

Arterial calcification due to deficiency of CD73; Induced pluripotent stem cells; NT5E

2. Resource Utility

iPSC line described in this paper was generated from a patient exhibiting signs of arterial calcification due to deficiency of CD73 (ACDC) and carrying a mutation in the *NT5E* gene

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103285.

(c.1126A → G,p.T376A). iPSCs generated from this patient provides a valuable resource to model ACDC disease *in vitro* and for prospective drug screening.

3. Resource Details

Arterial calcification due to deficiency of CD73 is a rare vascular disorder primarily affecting adults. It is common for patients diagnosed with ACDC to have calcium buildup in the joints of their hands and feet, as well as in the large vessels (mainly below the waist). ACDC is caused by an autosomal recessive gene mutation in the *NT5E* gene rendering the cell-surface protein CD73 dysfunctional. Due to the inactivity of CD73, tissue-nonspecific alkaline phosphatase (TNAP) levels rise while pyrophosphate levels decrease, thereby preventing the breakdown of calcium phosphate. This leads to calcium deposition (St Hilaire et al., 2011). ACDC patients suffer from progressive pain in their hands and/or feet and are at a higher risk of cardiovascular diseases. Current treatment options for ACDC are limited and are focused primarily on removing blood calcium and improving mobility. However, studies have shown that adenosine or phosphatase inhibitors can prevent and reverse calcification, suggesting potential treatment options (Lanzer et al., 2021).

By leveraging patient-specific induced pluripotent stem cells (iPSCs), we can develop a sustainable *in vitro* screening platform to mimic ACDC disease phenotype in-a-dish from patients with *NT5E* gene mutations. These iPSCs can then be differentiated into endothelial cells (iPSC-ECs) and vascular smooth muscle cells (iPSC-VSMCs), enabling us to conduct relevant clinical trial-in-a-dish (Sayed,Liu,and Wu, 2016).

Here, we generated an iPSC line from a 48-year old (BFVSBi001-A) male exhibiting signs of ACDC and carrying a missense mutation in *NT5E* gene (c.1126A → G,p.T376A) (Table 1) (Alsaigh Tom et al., 2023). We reprogrammed the peripheral blood mononuclear cells (PBMCs) of the patient into iPSCs by using a Sendai virus vector containing Oct3/4, Sox2, Klf4, and c-Myc (Yamanaka factors). A typical morphology was observed for this iPSC clone (Fig. 1A) and the iPSC line expressed markers of pluripotency when immunostained for OCT3/4, NANOG, and SOX2 (Fig. 1B). Further characterization of the iPSC line showed their ability to differentiate into the three germ layer lineages i.e., ectoderm, endoderm, and mesoderm when immunostained for OTX2, SOX17, and BRACHYURY respectively (Fig. 1C). Importantly, the iPSC line expressed NANOG and SOX2 at the mRNA level using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Fig. 1D) and tested negative for the Sendai virus vector (Fig. 1E). The presence of the NT5E gene missense mutation (c.1126A → G,p.T376A) was confirmed by Sanger sequencing (Fig. 1F). To assess the quality of the iPSC line, we conducted karyotyping analysis using the karyoStat assay that showed a normal karyotype (Fig. 1G) and mycoplasma testing that confirmed that the iPSC line was free of mycoplasma contamination (Supplementary Fig. 1). Lastly, Short tandem repeat (STR) analysis of the iPSC line, compared to their donor PBMCs, confirmed the same genetic origin (Supplementary Fig. 2).

4. Materials and methods

4.1. Reprogramming

Percoll density gradient medium (#17089109, GE Healthcare) was used to isolate PBMCs from blood, purified with DPBS, and plated in a 24-well plate as previously described (Manhas et al., 2022). PBMCs were cultured in StemPro®-34 SFM medium (#10639011, ThermoFisher Scientific) and nourished with specific supplements: SCF (100 ng/mL, #300-07, Peprotech), FLT3 (100 ng/mL, #PHC9414, ThermoFisher Scientific), IL-3 (20 ng/mL, #200-3, Peprotech), IL-6 (20 ng/mL, #PHC0063, ThermoFisher Scientific), and EPO (20 ng/mL, #PHC9631, ThermoFisher Scientific). The PBMCs were reprogrammed according to the instructions provided in the CytoTune™-iPSC 2.0 Sendai Reprogramming Kit (#A16517, ThermoFisher Scientific). 2.5 x 10^5 freshly isolated PBMCs were transduced, plated with StemPro™-34 medium (Thermo Fisher Scientific) on Matrigel-coated plates. After seven days the iPS-Brew XF medium was switched over to StemMACS™ iPS-Brew medium (#130-104-368, Miltenyi Biotec), and the cells were maintained for another ten to fifteen days. A cell colony was picked, and clones were expanded according to the procedure described previously (Sayed et al., 2020).

4.2. Cell culture

iPSCs were cultured at 37° C in a humidified incubator with 5 % CO_2 in StemMACS iPS-Brew XF medium. Briefly, PBS-EDTA was used to detach the cells, centrifuged at 300g for 3 min, and 1.2 x 10^5 iPSCs were seeded per well of a 6 well pate. Cells were passaged every 4 days. The ROCK inhibitor (#Y27632, Selleck Chemicals) was used only for the first 24 h after every passage, and cells were replaced with fresh medium every other day until confluency was reached.

4.3. Trilineage Differentiation

iPSCs were differentiated at passage 12 into three germ layers-endoderm, mesoderm, and ectoderm (#05110, STEMCELL ™ Technologies) in order to validate the inherent properties of the iPSC line.

4.4. Immunofluorescence

Fixation of iPSCs or iPSC-differentiated germ layers (endoderm, mesoderm, and ectoderm) was performed using 4 % paraformaldehyde for 10–15 min. The cells were permeabilized for 10 min with digitonin (50ug/mL, #D141, Sigma-Aldrich), then blocked with Bovine Serum Albumin (#A7030, Sigma-Aldrich) and 5 % serum (Donkey Serum, #D9663, Sigma-Aldrich; Goat Serum, #31873, ThermoFisher Scientific). After overnight incubation at 4 °C with specific primary antibodies (Table 2), cells were washed and incubated at room temperature with respective secondary antibodies (Table 2). Images were captured after NucBlue staining (#R37606, ThermoFisher Scientific).

4.5. RT-PCR

TRIzol® was used to collect 2 x 10^6 iPSCs from iPSC line at passage 13, and the Direct-zol™ RNA Miniprep Kit (#R2050, Zymo Research) was used to extract total RNA. The

RT-PCR analysis of NANOG, SOX2, and SEV was conducted using primers (Table 2) and the TaqMan™ Gene Expression Assay (#4444556, Applied Biosystems™) after synthesis of cDNA using the iScript™ cDNA Synthesis Kit (#1708891, BioRad) using following protocol: 25° C for 5 min, 46° C for 20 min, and 95° C for 1 min. RT-PCR was performed using StepOnePlus™ Real-Time PCR system, Applied Biosystems™.

4.6. Karyotyping

At passage 12, 2 x 10^6 iPSCs were collected from the iPSC line and genomic DNA was extracted using PureLink™ Genomic DNA Purification Kit (#K1820-02). A total of 100 ng of gDNA was used to prepare GeneArray® for KaryoStat according to the manual and analyzed for chromosomal abnormality by the KaryoStat™ assay (ThermoFisher).

4.7. Short Tandem Repeat Analysis

DNeasy Blood & Tissue Kit (#69504, Qiagen) was used to isolate and purify genomic DNA from PBMCs and iPSCs. Using CLA IdentiFiler™ Direct PCR Amplification Kit (#A44660, Thermo Fisher), the DNA was amplified using following steps: 95° C for 11 min, 94° C for 20 sec and 59° C for 3 min(28 cycles), 60° C for 10 min, and 4° C for 2 min. The amplified products were then analyzed using capillary electrophoresis on an ABI3130xl by Stanford Protein Nucleic Acid (PAN) Facility.

4.8. Sequencing

Genomic DNA was extracted from iPSC line using the DNeasy Blood & Tissue Kit (#69504, Qiagen), and PCR reactions with primers targeting the region of interest were performed using the HighFidelity kit (#M0541S, New England Biolabs). Sequencing of the PCR products was performed on ABI3130xl by the Stanford PAN facility using QIAquick Purification Kit (#28706, Qiagen).

4.9. Mycoplasma Detection

The MycoAlert Detection Kit (#LT07-118, Lonza) was used to assess mycoplasma contamination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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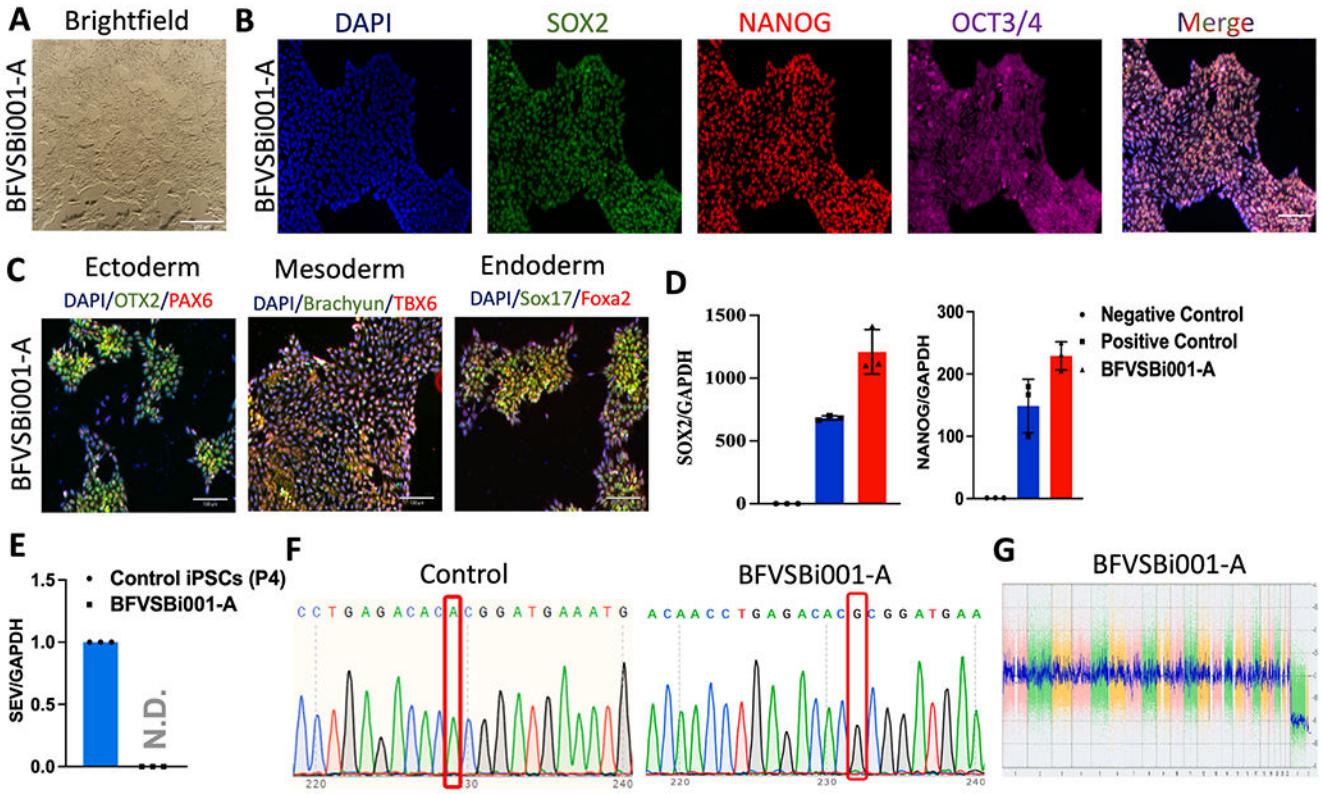

Fig. 1.

1.

Resource Table

Unique stem cell lines identifier	BFVSBi001-A
Institution	Baszucki Family Vascular Surgery Biobank
Contact information of the reported cell line distributor	Dr. Nazish Sayed sayedns@stanford.edu
Type of cell lines	iPSC
Origin	Human
Additional origin info (Applicable for human ESC or iPSC)	BFVSBi001-A; Age:48, Sex: Male; Ethnicity: Hispanic
Cell Source	PBMCs
Method of reprogramming	Integration-free Sendai virus expressing human OCT4, SOX2, KLF4, and c-MYC
Clonality	Clonal
Evidence of the reprogramming transgene loss	RT/q-PCR
Type of Genetic Modification	Missense mutation
Associated disease	Arterial calcification due to deficiency of CD73 (ACDC)
Gene/locus	NT5E (c.U26A → G,p.T376A); missense; pathogenic)
Date archived/stock date	BFVSBi001-A: 10-31-2021
Cell line repository/bank	https://hpscreg.eu/cell-line/BFVSBi001-A
Ethical approvals	The Administrative Panel approved the generation of the lines on Human Subjects Research (IRB) under IRB #62122, “Human Induced Pluripotent Stem Cells for Studying Cardiac and Vascular Diseases.”

Table 1

Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1A
Phenotype	Qualitative analysis (Immunocytochemistry)	Positive expression of pluripotency markers: Oct3/4, NANOG, SOX2	Fig. 1B
Genotype	Quantitative analysis (RT-qPCR)	mRNA expression of SOX2 and NANOG	Fig. 1D
	Karyotype: Whole genome array (KaryoStat™ Assay)	Normal karyotype: 46	Fig. 1G
	Resolution 1–2 Mb	XY for BFVSBi001-A	
		XY for BFVSBi002-A	
Identity	Microsatellite PCR (mPCR) or STR analysis	N/A	N/A
		16 loci tested, 100 % matching indentity	Supplementary Fig. 2
Mutation analysis	Sequencing	NT5E (C.1126A → G,p.T376A)	Fig. 1F
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Luminescence: Negative	Supplementary Fig. 1
Differentiation potential	Directed differentiation, Immunofluorescence staining for 2 markers per germ layer	Positive Immunofluorescence staining of three germ layer markers **Ectoderm:** PAX6, OTX2 **Endoderm:** SOX17, FOXA2, **Mesoderm:** BRACHYURY, TBX6	Fig. 1C
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2

Reagents details.

Antibodies used for Immunocytochemistry

	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit Anti-NANOG	1:200	Proteintech Cat# 142951-1-AP,	AB_1607719
	Mouse IgG2bκ Anti-OCT-3/4	1:200	Santa Cruz Biotechnology Cat# sc-5279,	AB_628051
	Mouse IgG1κ Anti-SOX2	1:200	Santa Cruz Biotechnology Cat# sc-365823,	AB_10842165
Ectoderm Markers	Goat Anti-OTX2	1:200	R&D Systems Cat# 963273	AB_2157172
	Rabbit Anti-Pax6	1:100	Thermo Fisher Scientific Cat# 42-6600	AB_2533534
Endoderm Markers	Goat Anti-SOX17	1:200	R&D Systems Cat# 963121	AB_355060
	Rabbit Anti-Foxa2	1:250	Thermo Fischer Scientific Cat# 701698	AB_2576439
Mesoderm Markers	Goat Anti-Brachyury	1:200	R&D Systems Cat# 963427	AB_2200235
	Rabbit Anti-Tbx6	1:200	Thermo Fischer Scientific cat # PA5-35102	AB_2552412
Secondary Antibodies	Alexa Fluor 488 Goat Anti-Mouse IgG1	1:1000	Thermo Fisher Scientific #A-21121	AB_2535764
	Alexa Fluor 488 Donkey Anti-Goat IgG (H + L)	1:1000	Thermo Fisher Scientific #A-11055	AB_2534102
	Alexa Fluor 555 Goat Anti-Rabbit IgG (H + L)	1:500	Thermo Fisher Scientific #A-21428	AB_141784
	Alexa Fluor 647 Goat Anti-Mouse IgG2b	1:250	Thermo Fisher Scientific #A-21242	AB_2535811

Primers

	Target	Forward/Reverse primer (5′-3′)
Sendai Virus	Sendai Virus genome	Mr04269880_mr
Genotyping	NT5E gene (c.1126A → G,p.T376A)	Fwd: GGCCTGACAGTATGGGAGTTT Rev: CAGAGATGAACTGAGGCTGC
House-Keeping Gene	GAPDH	HS02758991_g1
	SOX2	HS01053049_s1
	NANOG	HS02387400_g1