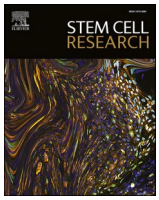
Stem Cell Research 67 (2023) 103019

Contents lists available at ScienceDirect

Stem Cell Research

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



Lab Resource: Multiple Cell Lines

Generation of two human iPSC lines with Exon 3 mutations in BCL2-Associated Athanogene 3 (*BAG3*) from dilated cardiomyopathy patients

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A B S T R A C T

Dilated cardiomyopathies (DCM) are one of the main causes of heart failure as one ages. BAG3 is a chaperone protein that is heavily implicated in the development of DCM and speed of progression toward heart failure. Here we generate two human iPSC lines from individuals with mutations in exon 3 of BAG3 and provide validation of their pluripotency and ability to differentiate toward the three primary germ layers. These two cell lines can help our understanding of BAG3 and its role in DCM by providing a good model for BAG3 inactivation and insufficiency.

Unique stem cell line identifier	1. SCVIi073-A 2. SCVIi074-A
Alternative name(s) of stem cell line	
Institution	Stanford Cardiovascular Institute Stanford, CA
Contact information of the reported cell line distributor	Joseph C. Wu, joewu@stanford.edu
Type of cell line	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 60 (SCVIi073-A) / 71 (SCVIi074-A) Sex: Female Ethnicity: White
Cell Source	PBMC
Method of reprogramming	Nonintegrating Sendai virus expression of human OCT4, SOX2, KLF4, and c-MYC
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-qPCR
Type of the Genetic Modification	Spontaneous/naturally occurred mutation
Associated disease	Dilated cardiomyopathy (DCM)

(*continued on next column*)

(*continued*)

Unique stem cell line identifier	1. SCVIi073-A 2. SCVIi074-A
Gene/locus	BAG3, Exon 3 (10q26. 11) SCVIi073-A: Homozygous at p.Tyr233Ter (c.699C > A); Heterozygous at p. Ala262Thr (c.784G > A) SCVIi074-A: Heterozygous at p. Ser185Leu (c.554C > T)
Date archived/stock creation date	SCVIi073-A: 12/8/2020 SCVIi074-A: 12/7/2018
Cell line repository/bank	https://hpscreg.eu/cell-line/SCVIi073-A https://hpscreg.eu/cell-line/SCVIi074-A
Ethical/GMO work approvals	The generation of the lines was approved by the Administrative Panel of Human Subjects Research (IRB) under IRB #29904 "Derivation of Human Induced Pluripotent Stem Cells"

1. Resource utility

The generation of these two induced pluripotent stem cell (iPSC) lines will allow investigators to generate an unlimited number of

Abbreviations: CM, Cardiomyopathy; DCM, Dilated Cardiomyopathy; BAG3, BCL2-Associated Athanogene 3; iPSC, Induced Pluripotent Stem Cell.

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https://doi.org/10.1016/j.scr.2023.103019
Received 20 December 2022; Accepted 3 January 2023
Available online 5 January 2023

cardiac-specific cell types that will aid in the characterization of the role BAG3 plays in the development and progression of a large subset of DCM.

2. Resource details

Cardiomyopathies (CM) are one of the major causes of heart failure as one ages, with prevalence among different ethnic groups ranging from 20 to 45 % after age 45 (Tsao et al., 2022). Dilated cardiomyopathy (DCM) is a subtype of these diseases, characterized by an enlarged left ventricle with decreased contractile capability. According to GWAS, a strong genetic component may underlie the speed at which people begin to present with progressively worsening symptoms, eventually leading to heart failure (Garnier et al., 2021). BAG3 was initially described as a HSP70 cofactor but is now known to be an important mediator of Z-disc stability through interactions with the CapZ actin-capping protein (Martin et al., 2021). BAG3-mediated CM make up roughly 50% of the cases of DCM in patients younger than 20, and greater than 74% in patients older than 40, with earlier prognosis in males more than females (Dominguez, 2018). After diagnosis, BAG3-mediated CM have a yearly risk rate of 4.7% for heart failure-related events (Dominguez, 2018). Here we describe the generation of two iPSC lines created from female patients with mutations in Exon 3 of BAG3. Our findings will be useful for future investigations into the effects of functional BAG3 depletion or absence on the development and progression of DCM (seeTable 1).

Two human iPSC lines (SCVIi073-A and 74-A) were generated from peripheral blood mononuclear cells (PBMCs) of two White females (ages 60 and 71) evaluated in the clinic for early signs of heart failure. SCVIi073-A contains two notable mutations in exon 3 of BAG3: a homozygous c.699c>A (p.Tyr233Ter) and heterozygous c.784G>A (p.Ala262Thr), both characterized as likely disease-causing by the Stanford Center for Inherited Cardiovascular Disease (SCICD). SCVIi074-A contains heterozygous mutation in exon 3, c.554C>T (p.Ser185Leu) listed as uncertain significance in ClinVar. PBMC reprogramming was performed using a non-integrating Sendai virus containing Yamanaka factors. Both lines exhibited similar, normal, morphology (Fig. 1A) with brightfield microscopy (black scale bar = 180 μm). Additionally, both iPSC lines showed robust expression of pluripotency markers SOX2 (green), NANOG (red), and OCT3/4 (magenta) (white scale bar = 130 μm) (Fig. 1A). This data was replicated with qPCR, showing increased SOX2 and NANOG expression over a differentiated control though not as

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1A
Pluripotency status evidence for the described cell line	Qualitative analysis -Immunocytochemistry	Positive expression of pluripotency markers: SOX2, NANOG, OCT3/4	Fig. 1A
	Quantitative analysis *(i.e. Flow cytometry, RT-qPCR)*	SOX2 and NANOG expression from RT-qPCR	Fig. 1B
Karyotype	Karyotype	KaryostatTM Assay, resolution 1–2 Mb Normal karyotype 46, XX for both lines	Fig. 1E
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	PCR + Sanger sequencing	Fig. 1D
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	SCVIi073-A: homozygous c.699C > A, heterozygous c.784G > A SCVIi074-A: heterozygous c.554C > T	Fig. 1D
	Transgene-specific PCR (when applicable)	N/A	
Verification of the absence of random plasmid integration events	PCR/Southern	Not performed	Not performed
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	*22 Loci tested, 100 % identical* e.g. Include minimum amelogenin+ 8 loci (D5S818, D13S317, D7S820, D16S539, vWA, Th01, TPOX, CSF1PO) and specify if matched or not. Tabulate the STR results in an excel file	*Submitted in archive with journal*
Multilineage differentiation potential	Sequencing (genomic DNA PCR or RT-PCR product)	N/A	Sanger sequencing tracks with deciphered (deconvoluted if mixed tracks) sequence(s) with comparison to a reference genome, parental and engineered lines should be presented in the main figure
	PCR-based analyses	Detection of correctly-targeted and randomly-integrated selectable targeting construct status	Representative genotyping data could be presented in main or supplementary figures
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	e.g. number of insertions in the genome, off-target effects; demonstration of protein elimination in KOs	e.g. sequencing deposited at DATABASE : recordID
Off-target nuclease activity analysis	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	N/A	Sanger sequencing tracks could be presented in the Supplementary figure
Specific pathogen-free status	Mycoplasma	Negative	Supplementary Fig. 1A
Multilineage differentiation potential	e.g. Embryoid body formation OR Teratoma formation OR Scorecard OR Directed differentiation	Directed differentiation of all 3 germ layers, analyzed with immunofluoresence	Positive expression of: Ectoderm: Pax6, Otx2 Mesoderm: Brachyury, Tbx6 Endoderm: Sox17, FoxA2
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*Not performed*	Not performed
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	*Not performed*	Not performed
	HLA tissue typing	*Not performed*	Not performed

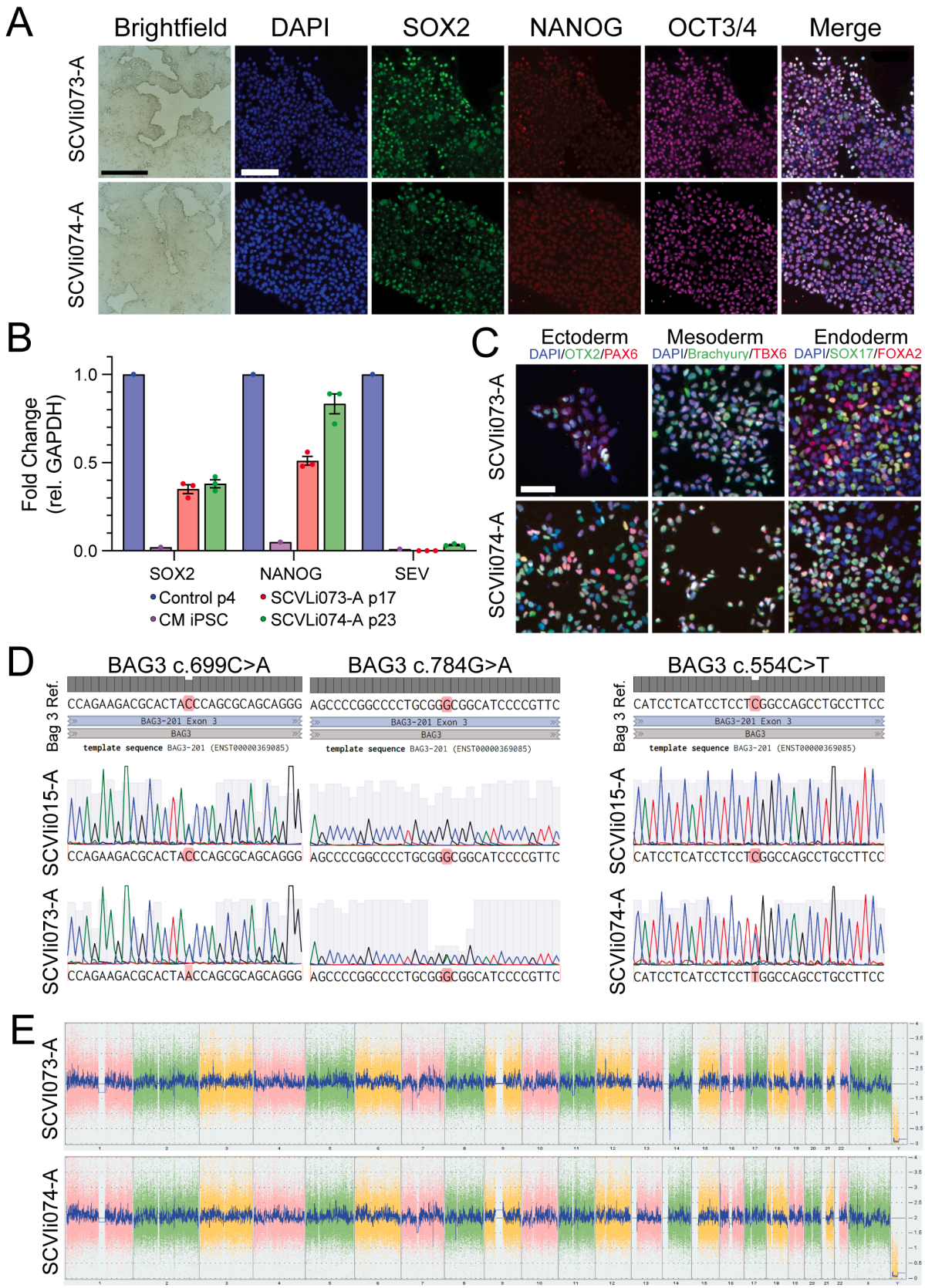

Fig. 1. Characterization of two iPSC lines derived from cardiomyopathy patients with BAG3 mutations.

much as an undifferentiated control with higher native expression of GAPDH (Fig. 1B). Further, SCVIi073-A (p17) and SCVIi074-A (p23) both show loss of the Sendai virus genome compared to the p4 control line, instead are similar to the differentiated CM control line (Fig. 1B). Differentiation capability of both lines was initiated followed by immunostaining for canonical nuclear markers of ectoderm, mesoderm, and endoderm (white scale bar = 70 μm) (Fig. 1C). Sanger sequencing of BAG3 exon 3 was used to verify each iPSC line's mutation and determine homozygosity or heterozygosity (Fig. 1D). Chromosomal abnormalities were determined using Fisher Scientific KaryoStat™ which showed a partial gain in Chr. 12 for SCVIi073-A and normal for SCVIi074-A (Fig. 1E). Lastly, a mycoplasma test was used to verify lack of the bacteria (Supplement 1A) and a short tandem repeat (STR) analysis was employed to confirm the derived iPSCs were the same as the donor PBMCs (submitted in archive).

3. Materials and methods

3.1. Reprogramming

PBMCs were isolated from patients' blood by Percoll gradient separation, purified with multiple rounds of washing in DPBS, and plated in a 24-well plate. 1–2 × 10^6 PBMCs were cultured in 1 mL of StemPro-34 medium (Thermo Fisher) supplemented with 100 ng/mL SCF and 20 ng/mL IL-3 (Peprotech), 100 ng/mL FLT3, 20 ng/mL IL-6, and 20 ng/mL EPO (Thermo Fisher). Medium was replaced every other day until cell number stabilized. 2 × 10^5 PBMCs were then resuspended in 300 μl of complete PBMC medium and reprogrammed using the CytoTune®-iPSC Sendai Reprogramming Kit (Thermo Fisher). After 24 hr, media was replaced followed by every 2 days. On day 7, cells were re-plated with a 1:1 mix of Stem-MACS™ iPSC-brew XF medium with supplement (Brew) and Stem-Pro™-34 media. On d8, a full switch over to the Stem-MACS™ media was performed. Media was replaced as colonies appeared on d10–15 (Yildirim et al., 2022).

3.2. Cell culture

Human iPSCs were cultured using Brew, replaced every 2 days, until confluent. Cells were lifted using 500 μM EDTA in DPBS for 5 min and replated in Brew with 5 μM ROCK inhibitor (Y27632, SelleckChem) overnight after, new media without ROCK inhibitor was used. Cells were maintained in a 37 °C incubator with 5% CO_2 and ambient O_2.

3.3. Trilineage differentiation

For ectoderm differentiation, StemXVivo Ectoderm kit (#SC031B, Bio-Techne-R&D) was used according to the manufacturer's protocol. For mesoderm differentiation, RPMI plus B27-Insulin supplement with 12 μM CHIR 99021 (Peprotech) was incubated with the cells for 2 days. Endoderm differentiation was performed with STEMdiff™ Definitive Endoderm Kit (#05110, StemCell Tech.). Cells were all passage 16 at the time of differentiation.

3.4. Immunofluorescence

Passage 16 cells were fixed, permeabilized and stained according to Abcam's immunocytochemistry and immunofluorescence protocol. Blocking occurred in a 1% BSA plus 5% normal Donkey serum for 30 min at room temperature (RT). Cells were incubated overnight at 4°C with primary antibodies and for 1 hr at RT for secondary antibodies (Table 2). Finally, nuclei were counterstained with NucBlue (R37606, Invitrogen) before imaging.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse IgG2b kanti-OCT-3/4	1:100	Santa Cruz Biotechnology Cat# sc-5279, RRID: AB_628051
	Rabbit anti-NANOG	1:100	Proteintech Cat# 14295–1-AP, RRID: AB_1607719
	Mouse IgG1 kanti-SOX2	1:100	Santa Cruz Biotechnology Cat# sc-365823, RRID: AB_10842165
Differentiation Markers (Ectoderm)	Goat anti-OTX2	1:200	R and D Systems Cat# AF1979, RRID: AB_2157172
	Rabbit anti-PAX6	1:200	Thermo Fisher Scientific Cat# 42–6600, RRID: AB_2533534
Differentiation Markers (Mesoderm)	Goat anti-Sox17	1:200	R and D Systems Cat# AF1924, RRID: AB_355060
	Rabbit anti-FOXA2	1:200	Thermo Fisher Scientific Cat# 701698, RRID: AB_2576439
Differentiation Markers (Endoderm)	Goat anti-Brachyury	1:200	R and D Systems Cat# AF2085, RRID: AB_2200235
	Rabbit anti-TBX6	1:200	Thermo Fisher Scientific Cat# PA5-35102, RRID: AB_2552412
Secondary antibodies	Alexa Fluor 488 Goat anti-Mouse IgG1	1:1000	Thermo Fisher Scientific Cat# A-21121, RRID: AB_2535764
	Alexa Fluor 647 Goat anti-Mouse IgG2b	1:250	Thermo Fisher Scientific Cat# A-21242, RRID: AB_2535811
	Alexa Fluor 488 Donkey anti-Goat IgG (H + L)	1:500	Thermo Fisher Scientific Cat# A-21428, RRID: AB_2535849
	Alexa Fluor 555 Donkey anti-Rabbit IgG (H + L)	1:500	Abcam Cat# ab150074, RRID: AB_2636997
Nuclear stain	NucBlue™ Live ReadyProbes™ Reagent (Hoechst33342)	2 drops/mL of wash buffer	Invitrogen Cat#: R37605

Primers and Oligonucleotides used in this study

	Target	Forward/Reverse primer (5′-3′)
Sendai Virus Plasmid (qPCR)	Sendai Virus genome	Mr042698800_mr
Pluripotency Markers (qPCR)	SOX2	Hs04234836_s1
	NANOG	Hs02387400_g1
House-Keeping Genes (qPCR)	GAPDH	Hs02786624_g1
Genotyping (PCR and Sanger)	Exon 3 of BAG3	Forward: CCGCCACACACCCAGCTAATTTTT Reverse: ACTTGCCTGTAGGTACACGCAT Band Size: 845 bp Sequencing: TATGGATTGCCCTGAGGAGGTG

3.5. RT-qPCR

RNA was extracted using Direct-zol™ RNA Microprep Kit (ZYMO #R2062). To generate cDNA, iScript™ cDNA Synthesis Kit (BioRad #1708891) was performed according to manufacturer recommendation. Expression of SOX2, NANOG, and SEV was amplified using commercially available probes (Table 2) and TaqMan™ Gene expression Assay (ABI™ #4444556).

3.6. Mutation sequencing

PCR primers were designed in Benchling to flank exon 3 (Table 2) and used with Q5® Hot Start High-Fidelity DNA polymerase (M0494L, NEB). The PCR was performed according to manufacturer recommendation with a 69°C annealing temp. PCR products were separated on a 1% agarose gel and extracted using the QiaQuick gel extraction kit (Qiagen). Amplicon sequencing was performed using Azenta Life Sciences Sanger Sequencing Service using a novel primer.

3.7. Mycoplasma Detection

Contamination was evaluated using a MycoAlert Detection kit (LT07-318, Lonza) at p16 for both lines.

3.8. Short tandem repeat analysis

Genomic DNA from PBMCs and p10 iPSCs was purified using DNeasy Blood and Tissue kit (Qiagen). STR analysis was performed using CLA IdentiFiler™ Plus and Identifiler™Direct PCR amplification kit (Thermo Fisher) by the Stanford PAN facility.

Declaration of Competing Interest

J.C.W. is a co-founder of Khloris Biosciences; however, the work done here is completely independent. The other 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.

Acknowledgments

We thank Carlos D. Vera, James WS Jahng, Gema Mondéjar-Parreño, and Kiran A. Vaidya for their technical support with these projects. This work was supported by National Institutes of Health (NIH) 75N92020D00019, R01 HL126527, R01 HL130020, R01 HL146690, and P01 HL141084 (JCW) and T32 EB009035 (PjHZ).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103019.

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