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Lab Resource: Multiple Cell Lines

Generation of four human induced pluripotent stem cell lines from COVID-19 hospitalized patients with increased levels of cardiac Troponin in the acute infection phase developing or not myocarditis

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A B S T R A C T

Coronavirus disease (COVID-19) is an infectious disease caused by SARS-CoV-2 virus, leading to mild to severe respiratory symptoms. Cardiovascular involvement is frequent and mainly manifests with myocarditis, arrhythmias, cardiac arrests, heart failure and coagulation abnormality. We generated human induced pluripotent stem cells (hiPSCs) from four COVID-19 patients, all characterized by increased levels of high-sensitivity Troponin I (hsTnI) during the infection acute phase, who developed (n = 2) or not (n = 2) severe myocarditis, as COVID-19 complication. The established hiPSCs were characterized for pluripotency and genomic stability, and constitute a useful resource for studying the mechanisms underlying the variability in COVID-19 severe cardiac manifestations.

1. Resource Table:

Unique stem cell lines identifier	*CCMi007-A (https://hpscreg.eu/cell-line/CCMi007-A)* *CCMi008-A (https://hpscreg.eu/cell-line/CCMi008-A)* *CCMi009-A (https://hpscreg.eu/cell-line/CCMi009-A)* *CCMi011-A (https://hpscreg.eu/cell-line/CCMi011-A)*
Alternative name(s) of stem cell lines	*SAS1 C5* *SAS2 C1* *SAS3 C10* *SAS7 C6*
Institution	*Centro Cardiologico Monzino – IRCCS, 20138, Milan, Italy*
Contact information of distributor	*Rabino Martina; martina.rabino@cardiologicomonzino.it*
Type of cell lines	*hiPSCs*
Origin	*Human*
Additional origin info required	*SAS1* *Age: 50 (at blood sampling)* *Sex: Male* *Ethnicity if known: Caucasian* *SAS2* *Age: 61 (at blood sampling)* *Sex: Male* *Ethnicity if known: Caucasian* *SAS3* *Age: 84 (at blood sampling)* *Sex: Male* *Ethnicity if known: Caucasian* *SAS7* *Age: 74 (at blood sampling)* *Sex: Female* *Ethnicity if known: Caucasian*
Cell Source	*Peripheral Blood Mononuclear Cells (PBMCs)*
Clonality	*Clonal*
Method of reprogramming	*Episomal reprogramming vectors*

(continued on next page)

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(*continued*)

Genetic Modification	*No*
Type of Genetic Modification	*N/A*
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*PCR*
Associated disease	*COVID-19*
Gene/locus	*N/A*
Date archived/stock date	*December 2021*
Cell line repository/bank	*hPSCreg (https://hpscreg.eu/)*
Ethical approval	Centro Cardiologico Monzino Ethical Committee, R1492/21-CCM 1579

2. Resource utility

Nineteen percent of hospitalized COVID-19 patients have myocardial involvement with > hsTnI and 10-fold increased mortality risk *vs* patients with normal hsTnI (Shi et al., 2020). Only a subgroup of these patients developed myocarditis, but the underlying mechanisms are still unknown. Patient-specific hiPSCs are excellent models to study patient-specific molecular mechanisms involved in COVID-19 myocarditis development.

3. Resource details

For more than two years, the COVID-19 pandemic has severely challenged the world's population health. Despite countless scientific research efforts, the mechanisms underlying the great variability in COVID-19 clinical manifestations remain largely unknown. Here, we obtained and characterized induced Pluripotent Stem Cells (hiPSCs) from four COVID-19 patients (average age: 67,2 years) who displayed increased levels of high-sensitivity Troponin (hsTnI, greater than 99°p) during the acute phase of infection. Out of these patients, two developed severe myocarditis (SAS1, SAS2), while the others did not (SAS3, SAS7).

After institutional ethical committee approval and informed consent obtainment, Peripheral Blood Mononuclear Cells (PBMCs) isolated from patients' blood samples were reprogrammed to hiPSCs by electroporation with lentiviral vectors encoding the human reprogramming factors L-MYC, LIN28, SOX2, KLF4 and OCT3/4. The obtained hiPSCs, cultured in feeder-free conditions, showed the typical embryonic stem cell-like morphology (Fig. 1***A***). Once cell lines were established, the selected clones were in-depth characterized to assess their effective pluripotency and genomic stability (Table 1). Specifically, we verified: i) expression of pluripotency markers, ii) developmental competence, iii) absence of the exogenous reprogramming factors, iv) DNA profile, v) karyotype, and vi) absence of mycoplasma contamination.

Flow cytometry quantitative analysis revealed that more than 95% of cells expressed the human iPSC-specific surface marker SSEA4 (Fig. 1***B***). In addition, quantitative PCR demonstrated that all the generated iPSC lines expressed high level of the endogenous pluripotency transcription factors *OCT4*, *SOX2* and *LIN28* (Fig. 1***C,*** primers reported in Table 2). The generated hiPSCs were also able to differentiate into cells belonging to the three germ layers, as assessed by the *in vitro* trilineage differentiation assay we conducted to verify their developmental competence (Fig. 1***D***). The absence of the episomal iPSC reprogramming vectors in the iPSC colonies was then verified by endpoint PCR using the primers listed in Table 2. Results displayed in ***Supplementary*** Fig. 1***A*** revealed that no integration occurred, indicating that the pluripotency features shown so far are no longer due to the presence of the transgenes provided during the reprogramming process. A Short Tandem Repeat (STR) analysis was conducted to unequivocally demonstrate that the generated hiPSCs genetically matched donor's primary PBMCs. As expected, the iPSC and PBMC populations of each line displayed an identical DNA profile at seventeen polymorphic loci, with no match to any cell line in either the ATCC or Expasy STR databases (data not shown). Finally, chromosomal profile and the absence of contaminating mycoplasma in the new reprogrammed lines have been assessed through Q-banded karyotype and PCR, respectively. Results are shown in ***Supplementary*** Fig. 1***B-C*** and indicated that the established hiPSC lines displayed a normal female or male karyotype (46XX or 46XY) and were mycoplasma free.

Overall, these data demonstrated that these new hiPSC lines generated from four COVID-19 patients are genetically stable and fulfil the most stringent criteria for pluripotency. These cell lines represent an excellent *in vitro* model to investigate the mechanisms that determine the onset of myocarditis in some COVID-19 patients.

4. Materials and methods

4.1. iPSC generation

PBMCs were reprogrammed to hiPSCs as previously described (Rovina, Castiglioni et al. 2022). Briefly, the protocol consisted of electroporation with the reprogramming vectors of the Epi5 Episomal iPSC Reprogramming Kit (ThermoFisher Scientific) at day 0 (1650 V, 10 s, 3 pulse), followed by a gradual transition of the culture medium from StemSpanSFEM II (STEMCELL) to ReproTeSR (STEMCELL) for promoting iPSC colony maturation. Starting from day 7, the medium was refreshed daily until the colonies were ready to be picked. After 14–21 days of reprogramming, clones were manually picked and cultured in StemFlex medium (GIBCO) on Vitronectin-coated plates (37 °C, 5% CO_2). Non-enzymatically passages with ReLeSR™ (STEMCELL) were performed every 3–4 days. Mature hiPSCs were cryopreserved in CryoStor® (Sigma-Aldrich).

4.2. Flow cytometry

hiPSCs (P8) were harvested, washed with 0.5 mM EDTA PBS, and fixed for 20 min on ice using BD Cytofix buffer (BD Biosciences) before an overnight incubation with SSEA4 antibody (4 °C). Cells were then stained with a fluorescent secondary antibody (1 h, 4 °C) and analysed using Gallios flow cytometer and Kaluza software (Beckman Coulter). All antibodies, listed in Table 2, were diluted in 0.1% BSA, 0.5 mM EDTA PBS.

4.3. Real-Time PCR

RNA was extracted (P21) with the Total RNA Purification kit (Norgen Biotek) and quantified with Nanodrop One (ThermoFisher Scientific). RNA was reverse transcribed with SuperScript III Reverse Transcriptase (ThermoFisher Scientific) according to manufacturer's instructions. qPCR was performed using iTaq Universal SYBR® Green Supermix and specific primers (Table 2) in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Expression was calculated as ΔCt, considering GAPDH as reference gene.

4.4. Trilineage differentiation

STEMdiff™ Trilineage Differentiation kit (STEMCELL) was used for ectodermal and endodermal differentiation. For mesodermal differentiation, hiPSCs were differentiated into cardiomyocytes (Lian et al., 2013). Differentiated hiPSCs (*P*21) were fixed (4% formaldehyde, 10 min), permeabilized (0.1% Triton-X 100, 5 min), blocked (3% BSA, 1 h), and stained with specific antibodies (primary: overnight, 4 °C; secondary: 1 h, RT) (Table 2) and Hoechst 33342 (1:500, 15 min). Cells were studied using confocal microscopy (LSM710, Zeiss).

4.5. PCR

DNA (P20) was extracted with QIAamp DNA Mini kit (QIAGEN) and quantified by Nanodrop One. PCR was performed with GoTaq® G2 Flexi DNA Polymerase (PROMEGA) and specific primers (Table 2) following

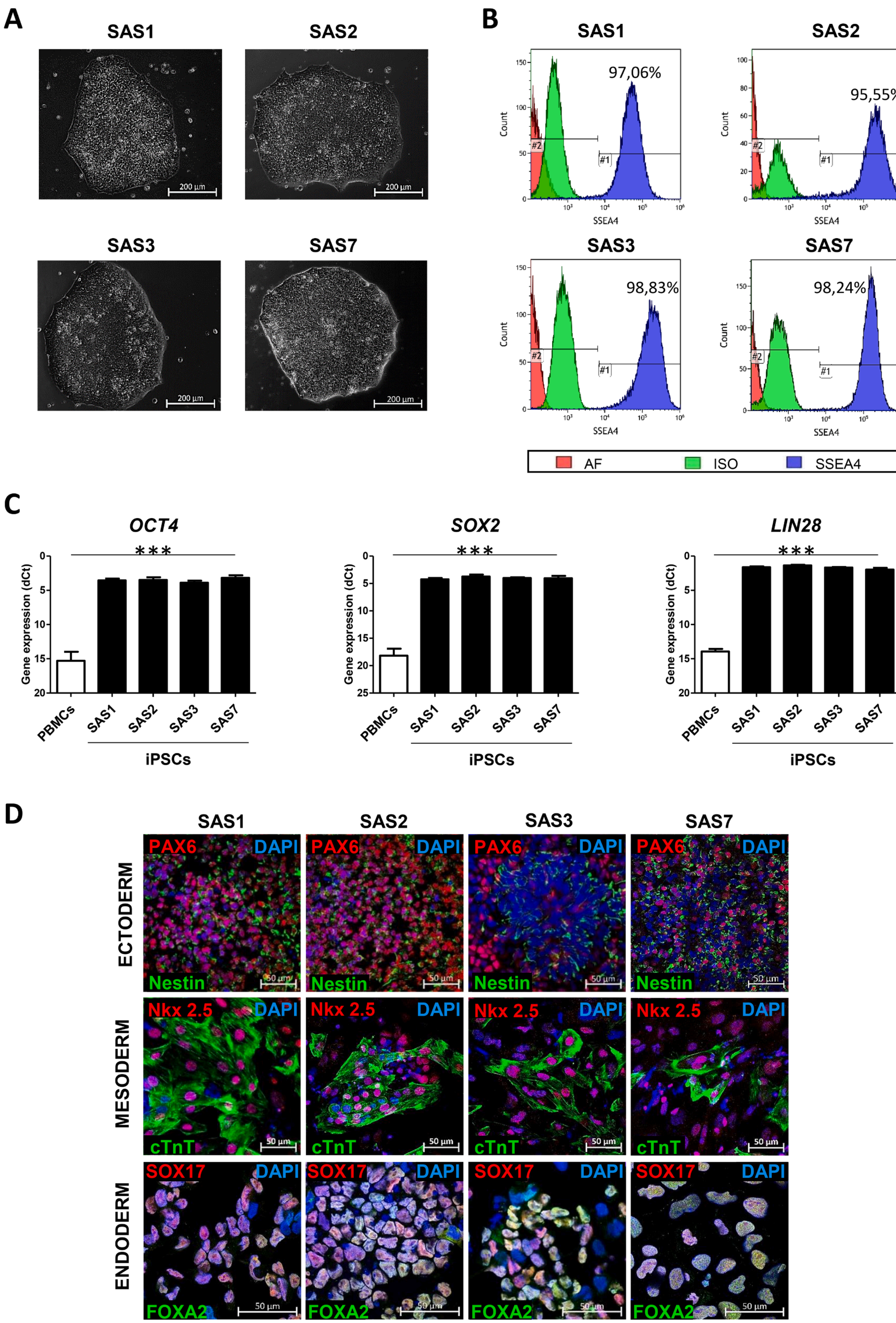

Fig. 1. Characterization of COVID-19 iPSCs.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1 A
Phenotype	Qualitative analysis: Immunocytochemistry	Determined the expression of markers for each of the three germ layers	Fig. 1D
	Quantitative analysis: Flow cytometry	Positive cells for cell surface marker SSEA-4 (SAS1 97,06%; SAS2 95,55%; SAS3 98,83%; SAS7 98,24%)	Fig. 1B
	Real Time- PCR	mRNA expression level of pluripotency markers: *OCT3/4, SOX2* and *LIN28*	Fig. 1C
Genotype	Karyotype (Q-banding) and resolution	SAS1 46XY, SAS2 46XY, SAS3 46XY, SAS7 46XX Resolution 400	Supplementary Fig. 1B
Identity	Microsatellite PCR (mPCR)	*N/A*	N/A
	STR analysis	17/17 loci + Amelogenin matched	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	*N/A*	N/A
	Southern Blot OR WGS	*N/A*	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Fig. 1 C
Differentiation potential	Directed differentiation	Determined the expression of markers for each of the three germ layers	Fig. 1D
List of recommended germ layer markers	Expression of these markers demonstrated at protein (IF) levels	Protein expression of PAX6 and Nestin (ectoderm), SOX17 and FOXA2 (endoderm), cTnT and Nkx2.5 (mesoderm)	Fig. 1D
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 cytometry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Mouse anti-SSEA4	1:100 for FACS	Abcam Cat# ab16287	RRID: AB_778073
Differentiation Markers	Rabbit anti-SOX17	1:300	Cell Signaling Inc. Cat# 81778	RRID: AB_2650582
	Mouse anti-FOXA2	1:200	Abcam Cat# ab60721	RRID: AB_941632
	Mouse anti-cardiac troponin T (cTnT)	1:300	Thermo Fisher Scientific Cat# MA-512960	RRID: AB_11000742
	Rabbit anti-Nkx2.5	1:200	Thermo Fisher Scientific Cat# PA5-49431	RRID: AB_2634885
	Mouse anti-NESTIN	1:150	Abcam Cat# ab22035	RRID: AB_446723
	Rabbit anti-PAX6	1:300	BioLegend Cat# PRB-278P	RRID: AB_291612
Secondary antibodies	Rabbit anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa®Fluor 488	1:400 (for SSEA4) 1:300 (for FOXA2) 1:300 (for NESTIN) 1:200 (for cTnT)	Thermo Fisher Scientific Cat# A11059	RRID: AB_2534106
	Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 546	1:300 (for Sox17) 1:300 (for PAX6) 1:300 (for Nkx2.5)	Thermo Fisher Scientific Cat# A11010	RRID: AB_2534077
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids (qPCR)	All 5 episomal plasmid containing *OCT3/4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28* genes	666 bp	ATCGTCAAAGCTGCACACAG/ CCCAGGAGTCCCAGTAGTCA	
Pluripotency Markers (qPCR)	*SOX2*	151 bp	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG	
Pluripotency Markers (qPCR)	*OCT3/4*	144 bp	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC	
Pluripotency Markers (qPCR)	*LIN28*	129 bp	AGCCATATGGTAGCCTCATGTCCGC/ TCAATTCTGTGCCTCCGGGAGCAGGGTAGG	
House-Keeping Genes (qPCR)	*GAPDH*	89 bp	CCACCCATGGCAAATTCC/TCGCTCCTGGAAGATGGTG	

manufacturer's instructions, and run in a C1000 Touch Thermalcycler (Bio-Rad). PCR products were visualized on agarose gel.

4.6. *Mycoplasma detection*

The absence of mycoplasma contamination (P20) was verified with EZ-PCR Mycoplasma Detection kit (Biological Industries) according to manufacturer's instructions. PCR products were visualized on agarose gel.

4.7. Karyotype

Cells at P12 were treated overnight with 10 μg/ml Colcemid and 0.05% Tryspin-EDTA, then incubated in 0.56% KCl solution for 6 min, washed with 5% acetic acid (3 min), and fixed with methanol/acetic acid (3:1). 20 Q-banded metaphases were acquired at 100X and karyotyped using CytoVision software (Leica).

4.8. STR

Seventeen STR loci plus Amelogenin were amplified using the PowerPlex®18D Kit (Promega). Samples were processed using the Prism®3500xl Genetic Analyzer and data were analysed with GeneMapper®ID-X v1.2 software (Applied Biosystems). Appropriate positive and negative controls have been used.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103018.

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