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Lab Resource: Multiple Cell Lines

Generation of 3 clones of induced pluripotent stem cells (iPSCs) from a patient affected by Crohn's disease

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A B S T R A C T

Crohn's disease is a debilitating and incurable chronic inflammatory bowel disease, affecting millions of individuals worldwide, with an increasing frequency. Surgery must be applicable in half of the cases often with a disabling course, and pharmacological treatments may have adverse complications. We generated three isogenic clones of iPSCs from peripheral blood mononuclear cells (PBMCs) of a patient with Crohn's Disease under pharmacological treatment without adverse effects. Sendai virus based vector was used and the iPSCs were characterized for genetic uniqueness, genomic integrity, pluripotency, and differentiation ability. These iPSCs will be a powerful tool to develop tailored therapies.

Resource utility

Crohn's disease is an incurable chronic inflammatory bowel disorder, affecting millions of individuals. Surgery and pharmacological treatments are the therapeutic options, but many adverse complications, such as pancreatitis frequently occur. The iPSCs can be a useful tool to understand the disease and the pharmacological susceptibility for developing new tailored therapies.

Resource details

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) affecting more than 2.5 million individuals with an increasing incidence (Molodecky et al., 2012). CD is debilitating and incurable, and is characterized by intestinal ulceration and inflammation, commonly affecting the distal small intestine. Surgery is an option in half of the patients, but post-operative complications are possible; many pharmaceutical treatments are available, but they have been shown effective only in 50% of the patients and can cause secondary deleterious effects, such as pancreatitis (Boyapati et al., 2015; Fousekisa et al., 2019). The iPSCs are definitively a flexible tool to generate tissue specific cells, for amplifying knowledge, to better understand the disease and discover susceptibility markers to complications and test more effective and safe pharmaceutical treatments.

We generated iPSCs from PBMCs of a 17 years old male suffering of CD, treated for many years with immunosuppressants. In particular, at the moment of sample collection (6/6/2017), the patient has been in therapy with azathioprine for five years without significant adverse effects. The patient was in therapy also with infliximab (62 weeks) and salazopirine and the disease was inactive. After transduction using the CytoTune-iPS 2.0 Sendai Reprogramming Kit, based on a non-transmissible and non-integrating Sendai virus (SeV) vector carrying the Yamanaka's factors *OCT4, SOX2, KLF4*, and *c-MYC*, in a feeder-free condition, we selected three independent sub-clones (UNIBSi005-A, UNIBSi005-B and UNIBSi005-C) (Table 1) positive for the Tra-1-60 stemness marker, and displaying ESC-like morphology (Fig. 1A). We established the genetic uniqueness and identity of the clones to the parental cells with STR analysis (Table 2). Karyotyping was performed three times (P6, P15, and P33) using standard QFQ-banding, showing the cell line original 46, XY pattern (Supplemental Fig. 1).

Pluripotency was assessed by both immunofluorescence staining and gene expression analysis. The stem cell markers Tra-1-60 and OCT4

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
UNIBSi005-A	C1	male	17	Caucasian	na	Crohn's disease
UNIBSi005-B	C5					
UNIBSi005-C	C20					

were expressed on the cell surface, and at nuclear level, respectively (Fig. 1B).

The expression of endogenous *NANOG, OCT4, SOX2, c-MYC*, and *KLF4* genes was investigated by SYBR qPCR, comparing the three clones (passage p21), the patient primary PBMCs, and a commercial certificated control iPSC line (CTL-hiPSC, Gibco® Episomal hiPSC Line, Cat#A18945). All clones showed a relative expression comparable to that of the certified cell line (Fig. 1C).

Viral particles clearance was assessed by endpoint RT-PCR not detecting transgenes expression (Fig. 1D).

After differentiation of iPSCs to ectoderm, mesoderm and endoderm layers, using the commercial StemMACS™ Trilineage Differentiation Kit (Miltenyi), we tested specific markers (*PAX6, SOX1, CXCR4, ACTA2, GATA4*, and *SOX17*) through TaqMan qPCR comparing undifferentiated iPSCs to the derived differentiated cells (Fig. 1E). The clones were definitively able to differentiate into cells committed to the three germ layers.

Moreover, mycoplasma contamination was excluded analysing the *16S rRNA* gene conserved among the genus *Mycoplasma* (Fig. 1F).

In conclusion, we generated three iPSC clones from a patient with CD not displaying adverse reactions to pharmacological treatments (characterization summarized in Table 2, reagents in Table 3). Three independent and isogenic clones are available to perform studies to better clarify the disease mechanisms and susceptibility markers.

Materials and methods

Reprogramming of peripheral blood mononuclear cells (PBMCs)

PBMCs from a CD patient were reprogrammed to iPSCs using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo-Fisher-Scientific) following manufacturer's instructions. Briefly, they were cultured 4 days in StemPro®-34 SFM Medium (Thermo-Fisher-Scientific) with the addition of SCF, FLT-3 (100 ng/ml), IL-3, and IL-6 (20 ng/ml) (Gibco) cytokines, and then infected with the SeV-based Vector; after 21 days, the Tra-1-60 positive colonies were seeded on Matrigel-coated plates (Corning) in daily changed Nutristem hPSC XF medium (Biological-Industries), manually picked every 5 days with a split ratio of 1:3, and incubated at 37 °C-5%CO_2.

iPSC karyotyping

Karyotype was performed at passages p6, p15, and p33. Proliferating iPSCs were blocked by 10 µg/ml of colcemid for 3 h (KaryoMax, Gibco Co. BRL), detached by trypsin–EDTA, and swollen by exposure to hypotonic KCl (0.075 M) solution for 7 min at 37 °C. Glass slides were prepared with three steps of fixation in methanol/glacial acetic acid (3:1). QFQ-banding at 400–450 bands resolution according to the International System for Human Cytogenetic Nomenclature (ISCN2016) was analyzed. A minimum of 20 metaphases and 3 karyotypes were analyzed.

In vitro germ layers differentiation

StemMACS™ Trilineage Differentiation Kit (MiltenyiBiotec) was used to commit iPSCs (p27) into differentiated cells of the three germ layers. After seven days, the cells were collected for RNA extraction and qPCR of the three germ layers specific genes.

Gene expression analysis

Total RNA was achieved by NucleoSpin® RNA II kit (Macherey-Nagel) following manufacturer instructions. A deep gDNA contamination cleaning was performed by a TURBO-DNase (Ambion), if required by the subsequent analyses. RNAs were retro-transcribed by ImProm-II™ Reverse Transcription System (Promega). Transgenes were amplified by endpoint-PCR using Taq Gold™ (LifeTechnologies), and a PE9700 thermocycler as described in the CytoTune-iPS 2.0 Sendai Reprogramming Kit.

Pluripotency was assessed by qPCR using iTaq™ Universal SYBR® Green Supermix (Bio-Rad), while gene expression of the markers characterizing the three germ layers was evaluated by TaqMan qPCR using iQ MPLX powermix (Bio-Rad). Tests were performed on the CFX96 C1000 Touch™ Real-Time PCR Detection System, and analyzed with the CFX manager software v.3.1 (Bio-Rad). The relative quantification of target genes was calculated by the $2^{-}\Delta\Delta Ct$ method, using β*ACTIN* as housekeeping gene.

Immunofluorescence

The expression of the pluripotency markers Tra-1-60 and OCT4 was verified by immunofluorescence. Briefly, cells grown on glass slides were fixed 15 min and permeabilized with the Fix & Perm-Reagents (*SIC*) at room temperature (RT). Blocking was assessed by iBind™ Buffer (Invitrogen) by incubation for 45 min at RT. The primary antibodies were incubated for 3 h at RT in blocking buffer, and the secondary antibodies for 1 h at RT. Hoechst33342 (Thermo-Fisher-Scientific) was used to stain nuclei. Mounting was performed with Glycerol/Gelatin (Sigma-Aldrich). Images were acquired by an inverted fluorescence microscope (Olympus IX70) and analyzed by the Image-Pro Plus software v7.0 (Media-Cybernetics).

Mycoplasma detection

A standard PCR was performed to amplify the 16SrRNA of the genus Mycoplasma from the supernatant of confluent cell culture and positive and negative controls.

STR analysis

DNAs from parental PBMCs and iPSCs clones were achieved by the QIAamp DNA Blood Mini kit (QIAGEN), and amplified with AmpFlSTR® Identifiler® Plus (LifeTechnologies) following instructions.

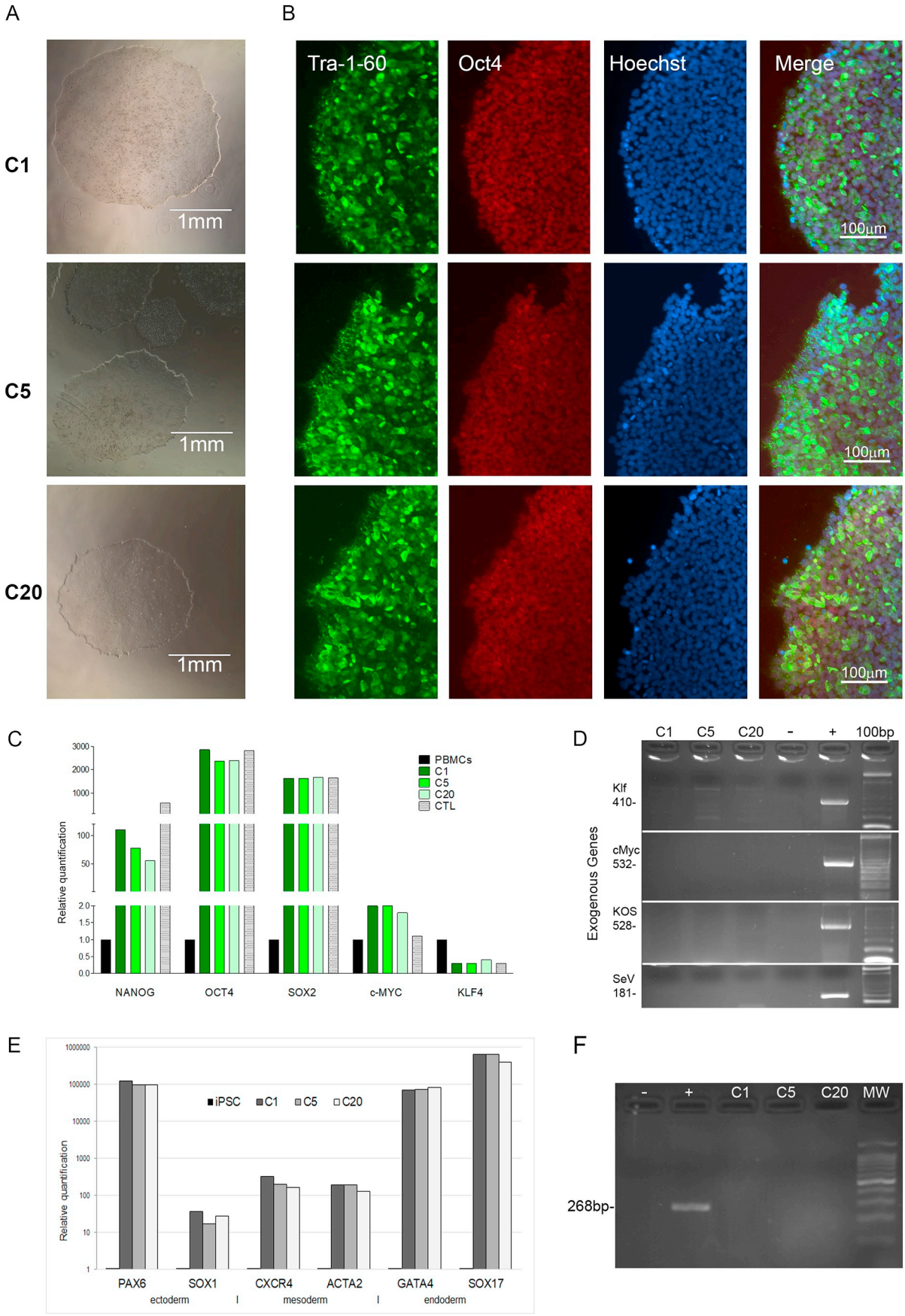

Fig. 1. A. iPSC clones morphology B. Immunofluorescence staining with the stemness markers Tra1-60 (green) and Oct4 (red). Nuclei were stained with Hoechst33342 (blu), and the three channels were merged. C. Pluripotency markers expression D. Transgenes expression and SeV clearance analyses E. Expression analysis of three germ layers markers F. Mycoplasma detection.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel A
Phenotype	Qualitative analysis Immunofluorescence	Positive for: OCT4, and Tra-1-60	Fig. 1 panel B
	Quantitative analysis Sybr Green RT-qPCR	Positive for: *NANOG, OCT4, SOX2, c-MYC, KLF4*	Fig. 1 panel C
Genotype	Karyotype (Q-banding)	46,XY Resolution: 400–450 bands	Supplemental Fig. 1
Identity	STR analysis	16 distinct loci: all matched to parental cell line	Available with the authors
Mutation analysis (If Applicable)	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Endpoint PCR for Mycoplasma 16S rRNA	Negative	Fig. 1 panel F
Differentiation potential	*In vitro* Trilineage Differentiation	Induction of selected genes expressed in the three germ layers (Ectoderm: *PAX6-SOX1*; Endoderm: *GATA4-SOX17*; Mesoderm: *ACTA2-CXCR4*).	Fig. 1 panel E
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 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-OCT4	1:400	Thermo Fisher Scientific, Cat# A-13998. RRID: AB_2534182
Pluripotency markers	Mouse anti-TRA-1-60	1:100	Thermo Fisher Scientific, Cat# 4110000. RRID: AB_2533494
Secondary antibodies	Goat anti rabbit IgG (H + L) Alexa Fluor 568	1:300	Thermo Fisher Scientific, Cat# A-11011. RRID: AB_143157
Secondary antibodies	Goat anti mouse IgG (H + L) Alexa Fluor 488	1:300	Thermo Fisher Scientific, Cat# A-11001. RRID: AB_2534069

Pluripotency Primers for RT-qPCR with SYBR Green chemistry

	Target	Forward/Reverse primer (5′-3′)
Pluripotency markers	*NANOG*	TGAACCTCAGCTACAAACAG/TGGTGGTAGGAAGAGTAAAG
Pluripotency markers	*OCT4*	CCTCACTTCACTGCACTGTA/CAGGTTTTCTTTCCCTAGCT
Pluripotency markers	*SOX2*	CCCAGCAGACTTCACATGT/CCTCCCATTTCCCTCGTTTT
Pluripotency markers	*C-MYC*	TGCCTCAAATTGGACTTTGG/GATTGAAATTCTGTGTAACTGC
Pluripotency markers	*KLF4*	GATGAACTGACCAGGCACTA/GTGGGTCATATCCACTGTCT
House-keeping genes	*βACTIN*	CGCCGCCAGCTCACCATG/CACGATGGAGGGGAAGACGG

Transgenes Primers for endpoint PCR

	Target	Forward/Reverse primer (5′-3′)
Sendai virus detection	*KOS transgene*	ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTGATGTGG (528 bp)
Sendai virus detection	*SeV transgene*	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC (181 bp)
Sendai virus detection	*C-MYC transgene*	TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG (532 bp)
Sendai virus detection	*KLF4 transgene*	TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA (410 bp)

Differentiation RT-qPCR assays with TaqMan chemistry

	Target	Probe
Ectoderm	*PAX6*	Hs.PT.58.25914558
	SOX1	Hs.PT.58.28041414.g
Mesoderm	*ACTA2*	Hs.PT.56a.2542642
	CXCR4	Hs00607978_s1
Endoderm	*GATA4*	Hs.PT.58.259457
	SOX17	Hs.PT.58.24876513
Housekeeping gene	*ACTB*	Hs.PT.39a.22214847

Mycoplasma detection

	Target	Forward/Reverse primer (5′-3′)
Genus Mycoplasma (GSO/MGSO)	*16S rRNA*	GGGAGCAAACAGGATTAGATACCCT/TGCACCATCTGTCACTCTGTTAACCTC (268 bp)

Key resources table

Unique stem cell lines identifier	*UNIBSi005-A* *UNIBSi005-B* *UNIBSi005-C*
Alternative names of stem cell lines	iPSC-AG-Crohn-C1 (*UNIBSi005-A*) iPSC-AG-Crohn-C5 (*UNIBSi005-B*) iPSC-AG-Crohn-C20 (*UNIBSi005-C*)
Institution	Department of Molecular and Translational Medicine, University of Brescia, 25,123 Brescia, Italy Institute for Maternal and Child Health IRCCS Burlo Garofolo, 34,128 Trieste Department of Life Sciences, University of Trieste, 34,127 Trieste Department of Medical, Surgical and Health Sciences, 34,149 University of Trieste, Trieste
Contact information of distributor	Gaetana Lanzi, g_lanzi@hotmail.com
Type of cell lines	iPSC
Origin	human
Additional origin info	Age: 17 Sex: male Ethnicity: Caucasian
Cell Source	Blood
Clonality	Clonal
Method of reprogramming	CytoTune™-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher Scientific), expressing the four Yamanaka factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC*
Multiline rationale	isogenic clones
Gene modification	NO
Type of modification	No modification
Associated disease	Crohn's disease
Gene/locus	NA
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	Feb-2018
Cell line repository/bank	https://hpscreg.eu/cell-line/UNIBSi005-A https://hpscreg.eu/cell-line/UNIBSi005-B https://hpscreg.eu/cell-line/UNIBSi005-C
Ethical approval	*The study (internal ID RC 07/14) was approved by the Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo, with approval number 1556.*

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101548.

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References

Boyapati, Ray, Satsangi, Jack, Ho, Gwo-Tzer, 2015. Pathogenesis of Crohn's disease. In: F1000Prime Reports. 7. pp. 44.

Fousekisa, Fotios S., Theopistosa, Vasileios I., Mitselosa, Ioannis V., Skamnelosa, Alexandros, Kavvadiasa, Athanasios, Katsanosa, Konstantinos H., Christodouloua, Dimitrios K., 2019. Specific features of patients with inflammatory bowel disease and primary sclerosing cholangitis. J. Clin. Med. Res. 11 (2), 81–88.

Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A., Ferris, M., Chernoff, G., Benchimol, E.I., Panaccione, R., Ghosh, S., Barkema, H.W., Kaplan, G.G., 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 142, 46–54.