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Lab Resource: Multiple Cell Lines

Generation of 3 clones of induced pluripotent stem cells (iPSCs) from a patient affected by Autosomal Recessive Osteopetrosis due to mutations in TCIRG1 gene.

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A B S T R A C T

Autosomal recessive osteopetrosis (ARO) is a rare inherited disorder leading to increased bone density with impairment in bone resorption. Among the genes responsible for ARO, the TCIRG1 gene, coding for the a3 subunit of the osteoclast proton pump, is mutated in more than 50% of the cases, increasing the importance of TCIRG1-iPSCs as disease model. We generated 3 iPSC clones derived from Peripheral Blood Mononuclear Cells (PBMCs) of a patient carrying the heterozygous mutations p.Y512X and c.2236 + 1G > *A*. A Sendai virus-based vector was used and the iPSCs were characterized for genetic identity to parental cells, genomic integrity, pluripotency, and differentiation ability.

Resource Table:

Unique stem cell lines identifier	*UNIBSi010-A* *UNIBSi010-B* *UNIBSi010-C*
Alternative names of stem cell lines	iPSC-TCIRG1-C1 (*UNIBSi010-A*) iPSC-TCIRG1-C2 (*UNIBSi010-B*) iPSC-TCIRG1-C3 (*UNIBSi010-C*)
Institution	Department of Molecular and Translational Medicine, University of Brescia, 25,123 Brescia, Italy Humanitas Clinical and Research Institute, Rozzano, Italy; National Research Council-Institute of Genetics and Biomedical Research (CNR-IRGB), Milan Unit, Milan, Italy
Contact information of distributor	Gaetana Lanzi, g_lanzi@hotmail.com
Type of cell lines	iPSCs
Origin	human
Additional origin info	Age: 4,5 months Sex: male Caucasian
Cell Source	PBMCs
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	YES
Type of modification	Hereditary
Associated disease	Autosomal Recessive Osteopetrosis (ARO)
Gene/locus	TCIRG1 NM_006019 / 11q13.2
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	https://hpscreg.eu/cell-line/UNIBSi010-A https://hpscreg.eu/cell-line/UNIBSi010-B https://hpscreg.eu/cell-line/UNIBSi010-C
Cell line repository/bank	NA
Ethical approval	The study was approved by the Ethical Committee of the Humanitas Clinical and Research Institute, with approval number ICH 166-10.

1. Resource utility

Autosomal Recessive Osteopetrosis due to mutation in TCIRG1 represents the majority of the cases affected by this rare and severe disease. Production of iPSCs and their use as a model to understand the pathophysiology of the disease will be a useful and important step to the development of therapeutic approaches.

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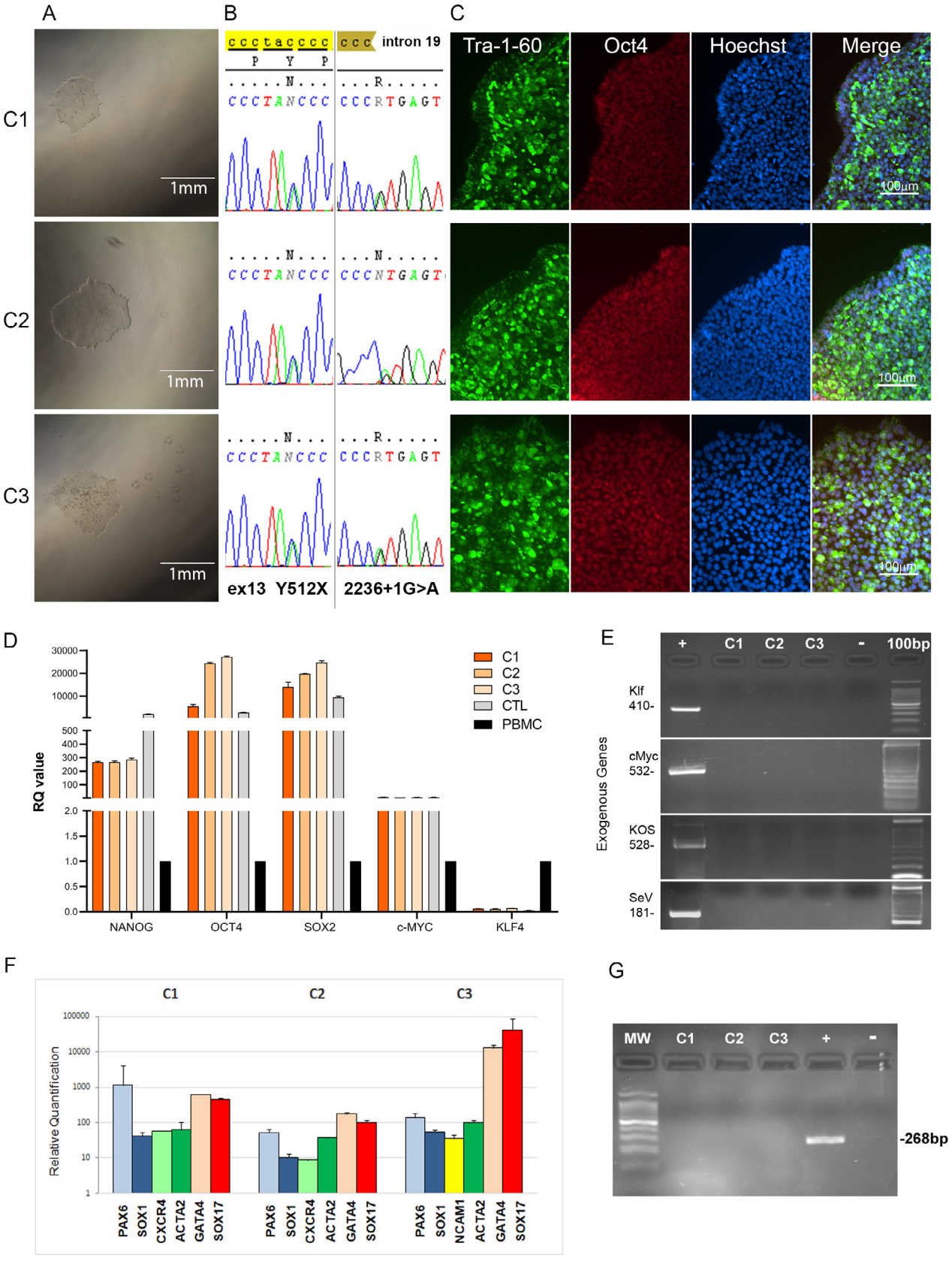

Fig. 1. A. iPSC morphology B. Genetic analyses of clones. C. Stemness markers staining by immunofluorescence: Tra1-60 (green) and Oct4 (red); nuclei staining with Hoechst33342 (blu); merged. D. Pluripotency markers analysis E. Transgenes and Sev expression F. Expression analysis of three germ layers markers G. Mycoplasma detection.

2. Resource details

Autosomal Recessive Osteopetrosis (ARO, OMIM # 259700) is a rare and severe genetic disease with an incidence of 1 in 250,000 births. It involves bones remodeling with early onset in the first few months of life. ARO is characterized by macrocephaly and typical facies together with respiratory problems and blindness. One of the complications is bone marrow insufficiency, leading to cytopenia and immunodeficiency (Sobacchi et al., 2013). The first gene identified as cause of ARO was *TCIRG1*, encoding the osteoclast specific a3 subunit of the vacuolar proton pump accountable for bone resorption (Frattini et al., 2000), and responsible for more than 50% of the cases of ARO (Pangrazio et al., 2012). We reprogrammed peripheral blood mononuclear cells (PBMCs) from the blood of a compound heterozygous male carrying a nonsense mutation (c.1536C > *A* p.Y512X) and a splice site mutation (c.2236 + 1G > *A*). Using the CytoTune-iPS 2.0 Sendai Reprogramming Kit, based on a non-integrating form of Sendai virus (SeV) carrying the Yamanaka's factors *OCT4, SOX2, KLF4*, and *c-MYC*, in a feeder-free condition, we obtained iPSCs colonies. We selected three independent clones (*UNIBSi010-A, UNIBSi010-B, UNIBSi010-C*) displaying ESC-like morphology (Fig. 1A) and we confirmed the mutation by sequencing (Fig. 1B) and the genetic uniqueness with STR analysis (Supplemental Table1). 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 firstly assessed by qualitative immunofluorescence staining showing the presence of two stem cell markers, Tra-1-60 and OCT4, expressed on cell surface and at nuclear level, respectively (Fig. 1C). Then, pluripotency was investigated at p10 by gene expression analysis of endogenous OCT4, SOX2, c-MYC, and KLF4 transcription factors in SYBR Green quantitative PCR (SYBR qPCR), comparing the newly generated iPSC clones with a commercial certificated control iPSC line (CTL-hiPSC, Gibco® Episomal hiPSC Line, Cat#A18945) and with the parental PBMCs, set as calibrator (Fig. 1D). The gene expression levels were comparable to those of the certified cell line. Moreover, clearance of viral vectors was confirmed by end point PCR showing no amplification for both transgenes and SeV (Fig. 1E).

The clones were finally investigated for their ability to differentiate into cells committed to the three germ layers. After the induction of iPSCs differentiation at passage 30, using the commercial StemMACS™ Trilineage Differentiation Kit (Miltenyi), and TaqMan qPCR, we tested the expression of ectodermal, mesodermal and endodermal markers (PAX6, SOX1, NCAM1, CXCR4, ACTA2, GATA4, SOX17) (Fig. 1F).

Mycoplasma contamination was tested by PCR amplification of the *16S rRNA* gene conserved among the genus *Mycoplasma*. No contamination was detected in the investigated clones (Fig. 1G).

In conclusion, we generated three independent iPSC clones carrying mutations in the *TCIRG1* gene, the most frequently mutated gene in ARO (characterization is summarized in Table 2, reagents in Table 3). Three clones are the optimum number of independent but isogenic samples to perform studies of differentiation to osteoclasts to better clarify the disease mechanisms.

3. Materials and methods

3.1. Reprogramming of peripheral blood mononuclear cells (PBMCs)

PBMCs from ARO patient were cultured for 4 days in StemPro® − 34 SFM Medium (Thermo-Fisher Scientific) supplemented with SCF, FLT-3 (100 ng/ml), IL-3, and IL-6 (20 ng/ml) (Gibco) cytokines. Then, PBMCs were infected using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo-Fisher Scientific) following manufacturer's instructions. Colonies positive to Tra-1-60 staining were manually picked 21 days post-transduction, and seeded on matrigel-coated plates (Corning) with daily renewal of the Nutristem hPSC XF medium (Biological Industries). Manual picking was subsequently performed with a splitting ratio of 1:3. Cell culture was performed at 37 °C with 5%CO_2.

3.2. iPSC karyotyping

Genomic stability was assessed by karyotyping performed at passages p6, p15, and p33 for all the clones. Briefly, proliferating iPSCs were blocked at metaphase by 10 μg/ml of colcemid (Karyo Max, Gibco Co. BRL), detached by trypsin–EDTA, and subsequently swollen by exposure to hypotonic KCL (0,075 M) solution. Glass slides preparative were performed with cells after three steps fixation in methanol/glacial acetic acid (3:1). Cytogenetic analysis was performed using QFQ-banding at 400–450 bands resolution according to the International System for Human Cytogenetic Nomenclature (ISCN 2016). A minimum of 20 metaphase spreads and 3 karyotypes were analysed, in 3 independent experiments.

3.3. iPSCs in vitro differentiation to the three germ layers

iPSCs were dissociated into single-cell suspension and cultured in StemMACS™ Trilineage Differentiation Kit specific media (MACS Miltenyi Biotec), according to the manufacturer's protocol. After seven days, the differentiated cells were collected for total RNA extraction and qPCR analysis of markers specific for the three germ layers.

3.4. Gene expression analysis

Total RNA was extracted using NucleoSpin® RNA II kit (Macherey-Nagel). Genomic DNA contamination was removed by RNA treatment with a TURBO-DNase (Ambion), if required by the gene expression analysis. RNA was retro-transcribed by ImProm-II™ Reverse Transcription System (Promega), following the manufacteurer's instructions. Pluripotency was assessed by qPCR using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). Gene expression of the markers characterizing the three germ layers differentiation was assessed by TaqMan qPCR performed using iQ MPLX powermix (Bio-Rad). Tests were performed on 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^− ΔΔCt method, using *bACTIN* as housekeeping gene.

3.5. Immunofluorescence staining

Immunofluorescence was performed to analyze the expression of the pluripotency markers Tra-1-60, and OCT4. Briefly, cells grown on

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
UNIBSi010-A	C1	male	4.5 months	Caucasian	C/A	Autosomal Recessive Osteopetrosis
UNIBSi010-B	C2				G/A	
UNIBSi010-C	C3				Compound heterozygous	

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 C
	Quantitative analysis SYBR Green RT-qPCR	Positive for: NANOG, OCT4, SOX2, c-MYC, KLF4	Fig. 1 panel D
Genotype	Karyotype (Q-banding)	46,XY Resolution 400–450	Supplemental Figure 1
Identity	STR analysis	16 distinct loci: all matched to parental cell line	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	c.1536C > *A* p.Y512X and c.2236 + 1G > *A*	Fig. 1 panel B
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Endpoint PCR for Mycoplasma 16S rRNA	Negative	Fig. 1 panel G
Differentiation potential	*In vitro* Trilineage Differentiation	Induction of selected genes expressed in the three germ layers (Ectoderm: PAX6-SOX1; Endoderm: GATA4-SOX17; Mesoderm: ACTA2-NCAM1 or CXCR4).	Fig. 1 panel F
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

glass slides were fixed for 15 min and permeabilized with the Fix & Perm-Reagents (SIC) at room temperature (RT). Unspecific staining was reduced by a blocking solution (iBindTM Buffer, Invitrogen) for 45 min. The primary antibodies incubation was performed for 3 h at RT in blocking buffer, followed by the secondary antibodies staining for 1 h at RT. Nuclei were counterstained with Hoechst 33,342 (Thermo–Fisher Scientific). Mounting was performed with Glycerol/Gelatin (Sigma–Aldrich) at 4 °C in the dark overnight. Cells were observed with an inverted fluorescence microscope (Olympus IX70), and images were acquired with the Image-Pro-Plus software v7.0 (Media Cybernetics).

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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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-13,998. RRID: AB_2,534,182
Pluripotency Markers	Mouse anti–TRA–1–60	1:100	Thermo Fisher Scientific, Cat# 4,110,000. RRID: AB_2,533,494
Secondary antibodies	Goat anti rabbit IgG (*H* + +*L*) Alexa Fluor 568	1:300	Thermo Fisher Scientific, Cat# A-11,011. RRID: AB_143,157
Secondary antibodies	Goat anti mouse IgG (*H* + +*L*) Alexa Fluor 488	1:300	Thermo Fisher Scientific, Cat# A-11,001. RRID: AB_2,534,069
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 Gene	*βACTIN*	CGCCGCCAGCTCACCATG/ CACGATGGAGGGGAAGACGG	
Transgenes Primers for endpoint PCR			
Vector detection	*KOS transgene* (528 bp)	ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTGATGTGG	
vector detection	*SeV transgene* (181 bp)	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC	
Vector detection	*C-MYC transgene* (532 bp)	TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG	
Vector detection	*KLF4 transgene* (410 bp)	TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA	
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	
	NCAM1	Hs.PT.58.39694135	
	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	*16S rRNA* (268 bp)	GGGAGCAAACAGGATTAGATACCCT/TGCACCATCTGTCACTCTGTTAACCTC	

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101660.

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