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Lab resource: Stem Cell Line

Reprogramming of Human Peripheral Blood Mononuclear Cell (PBMC) from a patient suffering of a Werner syndrome resulting in iPSC line (REGUi003-A) maintaining a short telomere length

Vincent Gatinois^{a,b,1}, Romain Desprat^{c,1}, Fabienne Becker^c, Lydiane Pichard^{a,c}, Florence Bernex^{d,e}, Carole Corsini^f, Franck Pellestor^{a,b,c,*}, Jean-Marc Lemaitre^{a,c,**}

^a Laboratory of Genome and Stem Cell Plasticity in Development and Aging, Institute for Regenerative Medicine and Biotherapy, INSERM UMR1183, Univ Montpellier, Montpellier, France

^b Laboratory of Cytogenetics, ChromoStem Facility, Univ Montpellier, CHU de Montpellier, Montpellier, France

^c SAFE-iPSC Facility INGESTEM, Univ Montpellier, CHU de Montpellier, Montpellier, France

^d Institut de Recherche en Cancérologie de Montpellier, Univ Montpellier, INSERM, U1194, Montpellier, France

^e Network of Experimental Histology, Univ Montpellier, BioCampus, CNRS, UMS3426, Montpellier, France

^f Medical Genetics Department, Univ Montpellier, CHU de Montpellier, Montpellier, France

ABSTRACT

Werner syndrome (WS) is a rare human autosomal recessive disorder characterized by early onset of aging-associated diseases, chromosomal instability, and cancer predisposition, without therapeutic treatment solution. Major clinical symptoms of WS include common age-associated diseases, such as insulin-resistant diabetes mellitus, and atherosclerosis. *WRN*, the gene responsible for the disease, encodes a RECQL-type DNA helicase with a role in telomere metabolism. We derived a stable iPSC line from 53 years old patient's PBMC, with a normal karyotype, but exhibiting a short telomere length, as a major aspect of the cellular phenotype involved in the pathology.

Resource utility

Werner syndrome is an heterogeneous genetic disease due to mutations in *WRN* helicase (Uhrhammer et al., 2006). The specific mutation of this patient, c.3789C > G hnz p.(Tyr1263*), generating a premature stop codon, does result in the absence of a nuclear translocated protein. Consequently, this iPSC line maintains a short telomere constituting a promising tool for disease modelling.

Resource details

PBMC were obtained from a (53)-years-old man harbouring a specific mutation in the *WRN* helicase gene (resource utility and Fig. 1A-Scheme for the functional domains of the Werner protein and position of the mutation), triggered in proliferation/differentiation toward the erythroid lineage and reprogrammed after a transient expression of the four reprogramming factors OCT3/4, SOX2, KLF4, and C-MYC using the integration-free Sendai virus gene-delivery method. The resulting *WRN*

iPSC line had a normal morphology (Fig. 1B-Characterization of *WRN* iPSC colonies, by morphology (bright field picture), alkaline phosphatase staining and by Immunofluorescence staining after 10 passages analyzed by microscopy and Flow Cytometry using specific pluripotency markers). Expression of pluripotency markers was revealed by immunocytochemistry staining analyzed by fluorescence microscopy and cytometry (Fig. 1B). The *WRN* iPSC identity line was confirmed and compared with parental cells by STR analysis (provided as Supplementary Table 1) and the presence of the disease-associated mutation in the *WRN* gene by DNA sequencing (Fig. 1C-Characterization of the *WRN* mutation by Sanger sequencing of the genomic DNA allowing the confirmation of the homozygous mutation detected in the *WRN* gene of the patient (c.3789C > G hnz p.(Tyr1263*) A red square is around the new guanine). The *WRN* iPSC line exhibited a normal and stable diploid karyotype (46,XY) and absence of additional CNV when compared to the parental cells (Fig. 1D-Karyotype analysis performed on Werner iPSC line showing no global genomic alteration and Supplementary Fig. 1). Pluripotency was assessed by the ability of the *WRN* iPSC to

* Corresponding author at: Pr. Franck Pellestor Laboratory of Cytogenetics, ChromoStem Facility, Montpellier University Hospital, Montpellier, France.

** Correspondence to: Lemaitre Jean-Marc, Laboratory of Genome and Stem Cell Plasticity in Development and Aging, Institute for Regenerative Medicine and Biotherapy, INSERM UMR1183, Univ Montpellier, Montpellier, France.

E-mail addresses: f-pellestor@chu-montpellier.fr (F. Pellestor), jean-marc.lemaitre@inserm.fr (J.-M. Lemaitre).

¹ authors contribute equally: Vincent Gatinois and Romain Desprat.

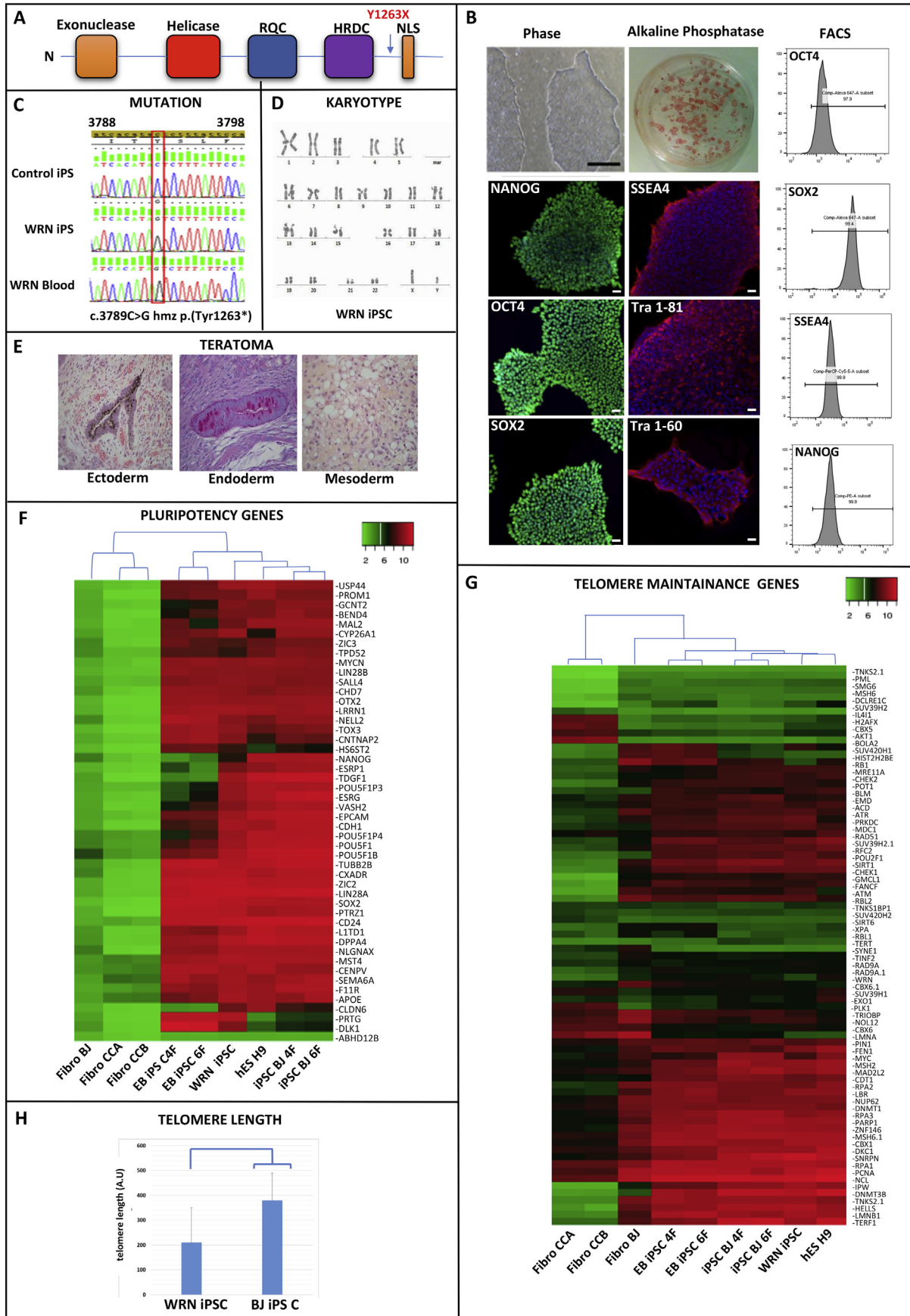


Fig. 1.

differentiate into the three germ layers by the formation of teratoma in immune-compromised mice (Fig. 1E-Teratoma assay on the WRN iPSC line analyzed after HES staining of histological sections) and by transcriptomic analysis of pluripotency genes comparing fibroblasts/embryoid bodies and different iPSC lines (Fig. 1F-Heatmap of WRN iPSC line transcriptome, analyzed by supervised clustering on genes involved in pluripotency: The list of genes involved in pluripotency was described in Guenther et al., 2015 and transcriptomic analysis was performed on fibroblasts BJ (Fibro BJ), fibroblasts from GEO Samples GSM1566208 (Fibro CCA and CCB), iPS derived from BJ fibroblasts with 4 reprogramming factors OSKM or 6 factors OSKMNL (Lapasset et al., 2011), respectively (iPSC BJ 4F and iPSC BJ 6F) and the corresponding Embryoids Bodies harvested at 15 days of differentiation (EB iPSC 4F and EB iPSC 6F, iPSC obtained from the WS patient (WRN iPSC), the human embryonic pluripotent stem cell line H9 (hES H9) and Fig. 1G-Heatmap of WRN iPSC line transcriptome, analyzed by supervised clustering on genes involved in telomeres maintenance: The list of genes was from Vaziri et al., 2010 and transcriptomic data used were as in Fig. 1F). Telomere length was evaluated by QFISH shorter than a control iPSC BJ line, which is consistent with the absence of functional WRN protein. (Fig. 1H-Telomere length in Werner iPSC line, evaluated by Q FISH analysis using specific fluorescent PNA is smaller than in iPSC derived from fibroblast BJ).

Materials and methods

Reprogramming PBMC into iPSCs

PBMC were isolated from blood using standard procedures and were cultured in SFEM II (StemSpan™ SFEM II Stemcell, cat# 09605) medium with cytokines (StemSpan™ Erythroid Expansion Supplement (100×) Stemcell, Catalog cat#02692) at a density of 5.10^5 cells per ml, during 7 days. The cells were transduced by Sendai-virus using the CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific, cat#A34546), delivered at an MOI of 10-10-6 (KOS MOI = 10, hc-Myc MOI = 10, hKlf4 MOI = 6) without polybrene, within a final total volume between 1 and 1.5 ml. Cells and viruses are centrifuged at $1000 \times g$ for 30 min at room temperature. PBMC pellet is re-suspended complete in 1 ml of SFEM II with cytokines to the tube, the cells, and transfer them to a well of a 12-well plate (total volume should now be between 2 and 2.5 ml). The plate was incubated overnight in a 37 °C incubator with a humidified atmosphere of 5% CO₂, 5% O₂. Later changes of medium were following instructions of the Thermo fisher scientific protocol for iPSC production. After 3 weeks, the colonies with an ES-like appearance were manually isolated.

iPSC culture and genomic DNA extraction

iPS cells were maintained on extracellular matrix Matrigel (Fisher Scientific, no.354277) in Essential 8™ culture media (Thermo Fisher Scientific, no. A15169-01), according to the manufacturer's instruction at 37 °C in 5% O₂ and 5% CO₂. DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen), following manufacturer's instructions.

Telomere length analysis

Fluorescently labelled PNA probes were used to estimate the telomere length on iPSC line. Werner iPSC line was showing a telomere length significantly smaller (Fig. 1H). The y-axis is in arbitrary unit (A.U.).

Karyotyping

It was performed on actively dividing cells on RHG-banding using standard procedures at ChromoStem facility of Montpellier, France (Fig. 1D). A minimum of 10 to 15 metaphases were counted (iPS cells

used were at passage 10–15) and scored, up to 50 when a mosaicism was suspected. Image acquisition was performed with an Axio Imager Z1 (ZEISS) Apotome, and analyzed with IKAROS software (Metasystems).

Human Genome CGH Array

Genomic DNA extraction was performed as described above. Genome variation profiling by chromosomal microarray was conducted at ChromoStem facility (Montpellier, France, <http://www.chu-montpellier.fr/fr/chercheurs/plateformes/les-plateformes-recherche/chromostem/>) with a SurePrint G3 Human CGH Microarrays 8x60k (Agilent®), (Supplementary Fig. 1) and no additional CNV was detected in comparison to parental cells.

Detection of disease-causing mutations in WRN gene

WRN mutations were confirmed by Sanger sequencing of PCR amplicons. Sanger sequencing was achieved using the BigDye® Terminator v1.1 Cycle Sequencing Kit on the Applied Biosystems 3130xl. (Fig. 1C).

Short tandem repeat analysis (STR)

Analysis was carried out with GeneMarker V2.6.7 (SoftGenetics) on 13 STR (Supplementary Table 1).

Flow cytometry analysis

BD Stemflow Human Pluripotent Stem Cell Transcription Factor Analysis Kit. Cells were analyzed on a CANTO II Becton Dickinson and analysis was made with Flow-JO. Results are presented in Fig. 1B and antibodies used are in Table 1.

Immunofluorescence

Cells grown on coverslips were fixed in 4% paraformaldehyde in PBS and labelled overnight at room temperature, after 0,1% Saponin permeabilization in the blocking buffer (5% goat serum) for 60 min according to the standard protocol of StemLight™ Pluripotency Antibody Kit (Cell Signaling, no.9656). Antibodies panel, listed in Table 1, included: Oct-4A, Sox2, Nanog, SSEA4, TRA-1-60, TRA-1-81. Appropriate fluorochrome-conjugated anti primary antibodies with Alexa Fluor® 488 and Alexa Fluor® 555 dyes were applied 60 min. DNA was stained with DAPI (ImmunoChemistry, no.6244) 15 min and coverslips mounted in Vectashield (Vector, no.H-1400). Image acquisition was performed with an Axio Imager Z1(ZEISS) Apotome, X10 objective (Table 2).

Transcriptomic analysis

Total RNA isolation was performed using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. RNA was hybridized on a Affymetrix GeneChip Human Genome U133 Plus 2.0 array (shown in Fig. 1F and G).

Teratoma formation

The differentiation potency was performed by in vivo teratoma derivation. Clusters corresponding to approximately 3×10^6 of iPSC cells were injected into anesthetized NOD SCID gamma (NOD.CgPrkdcscidIl2rg tm1Wjl/SzJ). Mice were transplanted subcutaneously in dorso-lateral area on both sides at 8 weeks old. After 4–8 weeks of latency and a 100% derivation efficiency, teratomas were fixed, embedded in paraffin blocks, stained by HES and analyzed by a pathologist for the presence of structures within the 3 embryonic germ layers (shown in Fig. 1.G).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal colonies	Fig. 1panel B
Phenotype	Qualitative analysis (Immunofluorescence and Flow Cytometry)	Oct4, Nanog, Sox2, Nanog, SSEA4, Tra-1-81, Tra-1-60	Fig. 1panel B
Genotype	Quantitative analysis (Flow Cytometry)	Oct3/4:100%, Nanog: 100%, SSEA-4: 100%, SOX-2: 100%	Fig. 1panel B
	Karyotype (RHG-banding)	46XY, Resolution 450 No additional CNV	Fig. 1panel D
Identity	CNV microarray	DNA profiling	Supplementary Fig. 1
	STR analysis	16 STR were sites tested, and matched between the original cell lines and the reprogramed one	Not performed Supplementary Table 1
Mutation analysis (IF APPLICABLE)	Sequencing	homozygous, c.3789C > G hmz p.(Tyr1263*)	Fig. 1panel C
	Southern Blot or WGS	Not performed	Not available
Microbiology and virology	Mycoplasma	Tested by luminescence, as Negative	Supplementary Table 2
	Differentiation potential	Teratoma formation]	Detection of the presence of the three embryo germ layer by histochemistry (validated by a certified Anatomopathologist)
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative by Elisa analysis	Fig. 1panel E
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	Not shown but available with authors
	HLA tissue typing	Not performed	Not available

Mycoplasma detection

Mycoplasma is detected with MycoAlert® Detection Kit (Lonza) according to manufacturer's instructions (Supplementary Table 2).

Key resources table

Unique stem cell line identifier	REGUI003-A	Additional origin info	Age:53 Sex: Male Ethnicity: Caucasian
Alternative name(s) of stem cell line	WRN iPSC	Cell Source	blood
Institution	CHU Montpellier, Saint Eloi Hospital	Clonality	mixed
Contact information of distributor	Dr. Lemaire	Method of reprogramming	Sendai
Type of cell line	iPSC	Genetic Modification	YES
Origin	human	Type of Modification	Hereditary.
		Associated disease	Werner syndrome
		Gene/locus	(NM_000553) Werner syndrome ATP-dependent helicase
		Method of modification	No
		Name of transgene or resistance	None
		Inducible/constitutive system	None
		Date archived/stock date	None
		Cell line repository/bank	SAFE-iPS facility IRMB
		Ethical approval	CPP CHU MONTPELLIER. 2014-A00178-39

Table 2
Reagents details-Werner iPSC.

Antibodies used for immunocytochemistry/flow-Cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers flow cytometry	Oct-4A Rabbit mAb (Clone C30A3) IgG	1:200	Cell signaling technology cat# 2840, RRID:AB_2167691
Pluripotency markers flow cytometry	Sox2 XP® Rabbit mAb (Clone D6D9) IgG	1:200	Cell signaling technology cat# 3579, RRID:AB_2195767
Pluripotency markers flow cytometry	Nanog XP® Rabbit mAb (Clone D73G4) IgG	1:200	Cell signaling technology cat# 4903, RRID:AB_10559205
Pluripotency markers flow cytometry	SSEA4 Mouse mAb (Clone MC813) IgG3	1:200	Cell signaling technology cat# 4755, RRID:AB_1264259
Pluripotency markers flow cytometry	TRA-1-60(S) Mouse mAb (Clone TRA-1-60(S)) IgM	1:200	Cell Signaling Technology Cat# 4746, RRID:AB_2119059
Pluripotency markers flow cytometry	TRA-1-81 Mouse mAb (Clone TRA-1-81) IgM	1:200	Cell signaling technology cat# 4745, RRID:AB_2119060
Pluripotency markers immunostaining	PE Mouse anti-human Nanog (Clone: N31-355)	1:5	BD Biosciences Cat# 560791, RRID:AB_1937305
Pluripotency Markers Immunostaining	PerCP-CyTM 5.5 Mouse anti-Oct3/4 (Clone: 40/Oct-3)	1:5	BD Biosciences Cat# 560794, RRID:AB_1937313
Pluripotency Markers Immunostaining	Alexa FluorR 647 Mouse anti-Sox2 (Clone: 245610)	1:5	BD Biosciences Cat# 560301, RRID:AB_1645308
Pluripotency markers immunostaining	Alexa FluorR 647 Mouse anti-SSEA-4 (Clone: MC813-70)	1:5	BD Biosciences Cat# 560796, RRID:AB_2033991
Pluripotency markers immunostaining	PE Mouse IgG1, κ Isotype Control (Clone MOPC-21)	1:5	BD Biosciences Cat# 554121, RRID:AB_395252
Pluripotency markers immunostaining	PerCP-Cy5.5 Mouse IgG1, κ Isotype Control (Clone: X40)	1:5	BD Biosciences Cat# 347202, RRID:AB_400265
Pluripotency markers immunostaining	Alexa Fluor® 647 Mouse IgG2a, κ Isotype Control (Clone: MOPC-173)	1:5	BD Biosciences Cat# 558020, RRID:AB_396989
Pluripotency markers immunostaining	Secondary Antibody Alexa Fluor® 488 conjugate Goat anti-Rabbit IgG	1:400	Invitrogen-thermo fisher scientific cat# A-11034, RRID:AB_2576217
Pluripotency markers immunostaining	Secondary Antibody Alexa Fluor® 555 conjugate Goat anti-Rabbit IgG	1:400	Invitrogen-thermo fisher scientific cat# A-21424, RRID:AB_141780
Primers			
	Target	Forward/Reverse primer (5'→3')	
Werner mutation (Sanger sequencing)	WERNER mutation	5'TGAGCTCCCATAAAAAGGAA3'/ 5'TGGCCAACTAAACTTGCTGC3'	

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

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