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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

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A B S T R A C T

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 hmz 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 hmz 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

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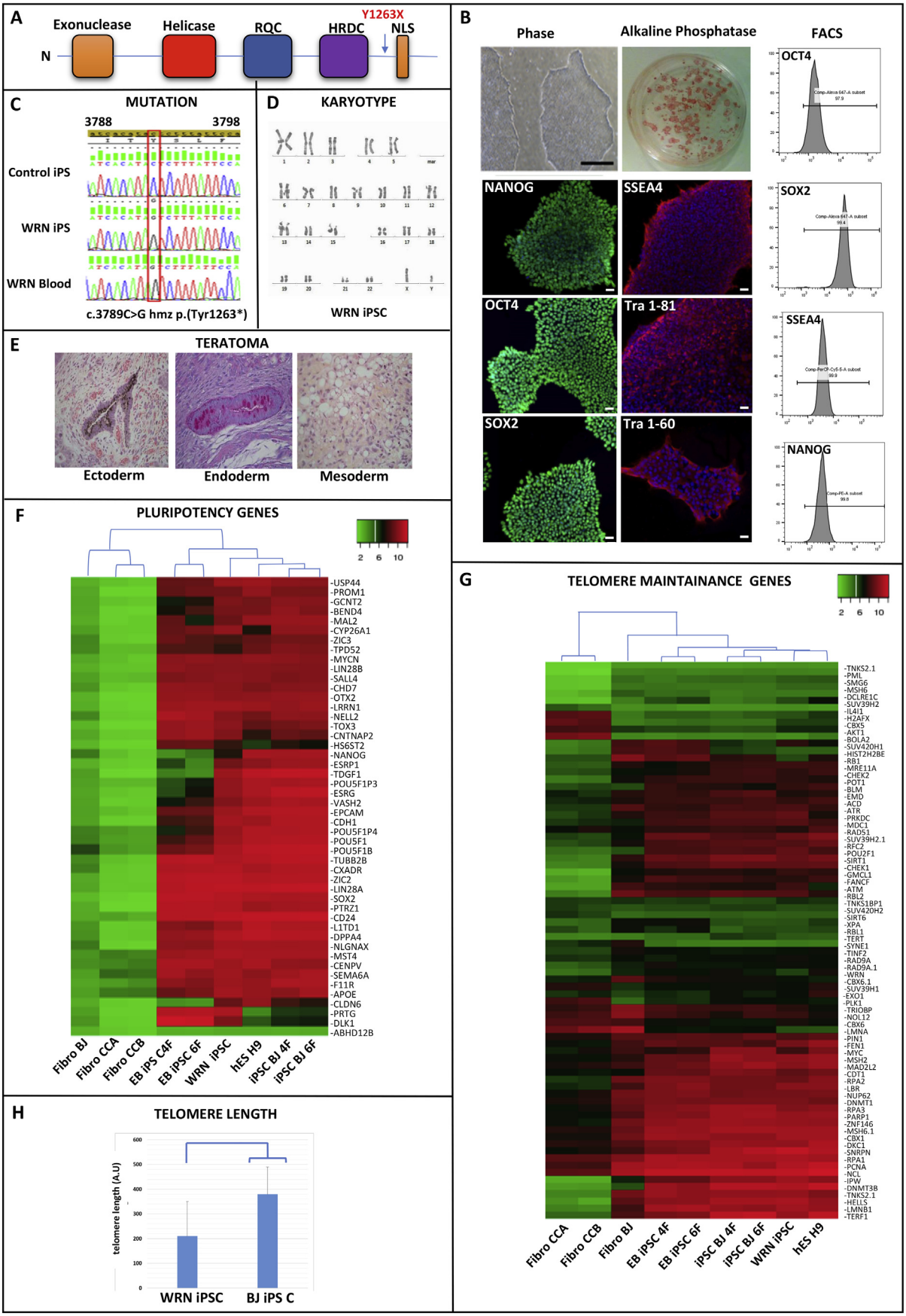
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Exonuclease
Helicase
RQC
HRDC
NLS
Y1263X
N
B
Phase
Alkaline Phosphatase
FACS
OCT4
NANOG
SSEA4
SOX2
OCT4
Tra 1-81
SSEA4
SOX2
Tra 1-60
NANOG
C
MUTATION
3788
3798
Control iPS
WRN iPS
WRN Blood
c.3789C>G hmz p.(Tyr1263*)
D
KARYOTYPE
WRN iPSC
E
TERATOMA
Ectoderm
Endoderm
Mesoderm
F
PLURIPOTENCY GENES
2 6 10
-USP44
-PROM1
-GCNT2
-BEND4
-MAL2
-CYP26A1
-ZIC3
-TPD52
-MYCN
-LIN28B
-SALL4
-CHD7
-OTX2
-LRRN1
-NELL2
-TOX3
-CNTNAP2
-HS6ST2
-NANOG
-ESRP1
-TDGF1
-POU5F1P3
-ESRG
-VASH2
-EPCAM
-CDH1
-POU5F1P4
-POU5F1
-POU5F1B
-TUBB2B
-CXADR
-ZIC2
-LIN28A
-SOX2
-PTRZ1
-CD24
-L1TD1
-DPPA4
-NLGNAX
-MST4
-CENPV
-SEMA6A
-F11R
-APOE
-CLDN6
-PRTG
-DLK1
-ABHD12B
Fibro BJ
Fibro CCA
Fibro CCB
EB iPS C4F
EB iPSC 6F
WRN iPSC
hES H9
iPSC BJ 4F
iPSC BJ 6F
G
TELOMERE MAINTAINANCE GENES
2 6 10
-TNKS2.1
-PML
-SMG6
-MSH6
-DCLRE1C
-SUV39H2
-IL4I1
-H2AFX
-CBX5
-AKT1
-BOLA2
-SUV420H1
-HIST2H2BE
-RB1
-MRE11A
-CHEK2
-POT1
-BLM
-EMD
-ACD
-ATR
-PRKDC
-MDC1
-RAD51
-SUV39H2.1
-RFC2
-POU2F1
-SIRT1
-CHEK1
-GMCL1
-FANCF
-ATM
-RBL2
-TNKS1BP1
-SUV420H2
-SIRT6
-XPA
-RBL1
-TERT
-SYNE1
-TINF2
-RAD9A
-RAD9A.1
-WRN
-CBX6.1
-SUV39H1
-EXO1
-PLK1
-TRIOBP
-NOL12
-CBX6
-LMNA
-PIN1
-FEN1
-MYC
-MSH2
-MAD2L2
-CDT1
-RPA2
-LBR
-NUP62
-DNMT1
-RPA3
-PARP1
-ZNF146
-MSH6.1
-CBX1
-DKC1
-SNRPN
-RPA1
-PCNA
-NCL
-IPW
-DNMT3B
-TNKS2.1
-HELLS
-LMNB1
-TERF1
Fibro CCA
Fibro CCB
Fibro BJ
EB iPSC 4F
EB iPSC 6F
iPSC BJ 4F
iPSC BJ 6F
WRN iPSC
hES H9
H
TELOMERE LENGTH
telomere length (A.U)
600
500
400
300
200
100
0
WRN iPSC
BJ iPS C

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 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 × *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 StemLightTM 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). Mices 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. 1*panel B*
Phenotype	Qualitative analysis (Immunofluorescence and Flow Cytometry)	*Oct4, Nanog, Sox2, Nanog, SSEA4, Tra-1-81, Tra-1-60*	Fig. 1*panel B*
	Quantitative analysis (Flow Cytometry)	*Oct3/4:100%, Nanog: 100%, SSEA-4: 100%, SOX-2: 100%*	Fig. 1*panel B*
Genotype	Karyotype (RHG-banding)	*46XY, Resolution 450 No*	Fig. 1*panel D*
	CNV microarray	*additional CNV*	*Supplementary Fig. 1*
Identity		*DNA profiling*	*Not performed*
	STR analysis	*16 STR were sites tested, and matched between the original cell lines and the reprogramed one*	*Supplementary Table 1*
Mutation analysis (IF APPLICABLE)	Sequencing	*homozygous, c.3789C > G hmz p.(Tyr1263*)*	Fig. 1*panel 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 Anatomo-Histopathologist)*	Fig. 1*panel E*
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*Negative by Elisa analysis*	*Not shown but available with authors*
Genotype additional info (OPTIONAL)	Blood group genotyping	*Not performed*	Not available
	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*
Alternative name(s) of stem cell line	*WRN iPSC*
Institution	*CHU Montpellier, Saint Eloi Hospital*
Contact information of distributor	*Dr. Lemaitre*
Type of cell line	*iPSC*
Origin	*human*
Additional origin info	*Age:53* *Sex: Male* *Ethnicity: Caucasien*
Cell Source	*blood*
Clonality	*mixed*
Method of reprogramming	*Sendaï*
Genetic Modification	*YES*
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 sequencage)	*WERNER mutation*	*5'TGAGCTCCCCATAAAAAGGGAA3'/ 5'TGGCCAAACTAAACTTGCTGC3'*

Declaration of Competing Interest

None.

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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.2019.101515.

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