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Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of disease-specific and CRISPR/Cas9-mediated gene-corrected iPS cells from a patient with adult progeria Werner syndrome

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A B S T R A C T

Adult progeria Werner syndrome (WS), a rare autosomal recessive disorder, is characterized by accelerated aging symptoms after puberty. The causative gene, *WRN*, is a member of the RecQ DNA helicase family and is predominantly involved in DNA replication, repair, and telomere maintenance. Here, we report the generation of iPS cells from a patient with WS and correction of the *WRN* gene by the CRISPR/Cas9-mediated method. These iPSC lines would be a valuable resource for deciphering the pathogenesis of WS.

1. Resource Table

Unique stem cell lines identifier	CUi001-A CUi001-A-1
Alternative names of stem cell lines	WB1-10 (CUi001-A) WB1-10 WRNKI-Cre18 (CUi001-A-1)
Institution	Chiba University
Contact information of distributor	Hisaya Kato, Yoshiro Maezawa, Koutaro Yokote hisayakato@chiba-u.jp, yoshiromaezawa@chiba-u.jp, kyokote@faculty.chiba-u.jp
Type of cell lines	iPSC
Origin	Human
Cell Source	Peripheral blood mononuclear cells, CD34 positive
Clonality	Clonal
Method of reprogramming	Transgene free Sendai viral vector
Multiline rationale	Gene corrected clone
Gene modification	YES
Type of modification	Gene correction
Associated disease	Werner syndrome
Gene/locus	*WRN*:c.3139-1G > C
Method of modification	CRISPR/Cas9-mediated homology-directed repair
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	N/A
Cell line repository/bank	N/A
Ethical approval	The Ethics Committee of Chiba University School of Medicine [Approval # 974(885), # 1029(973)]

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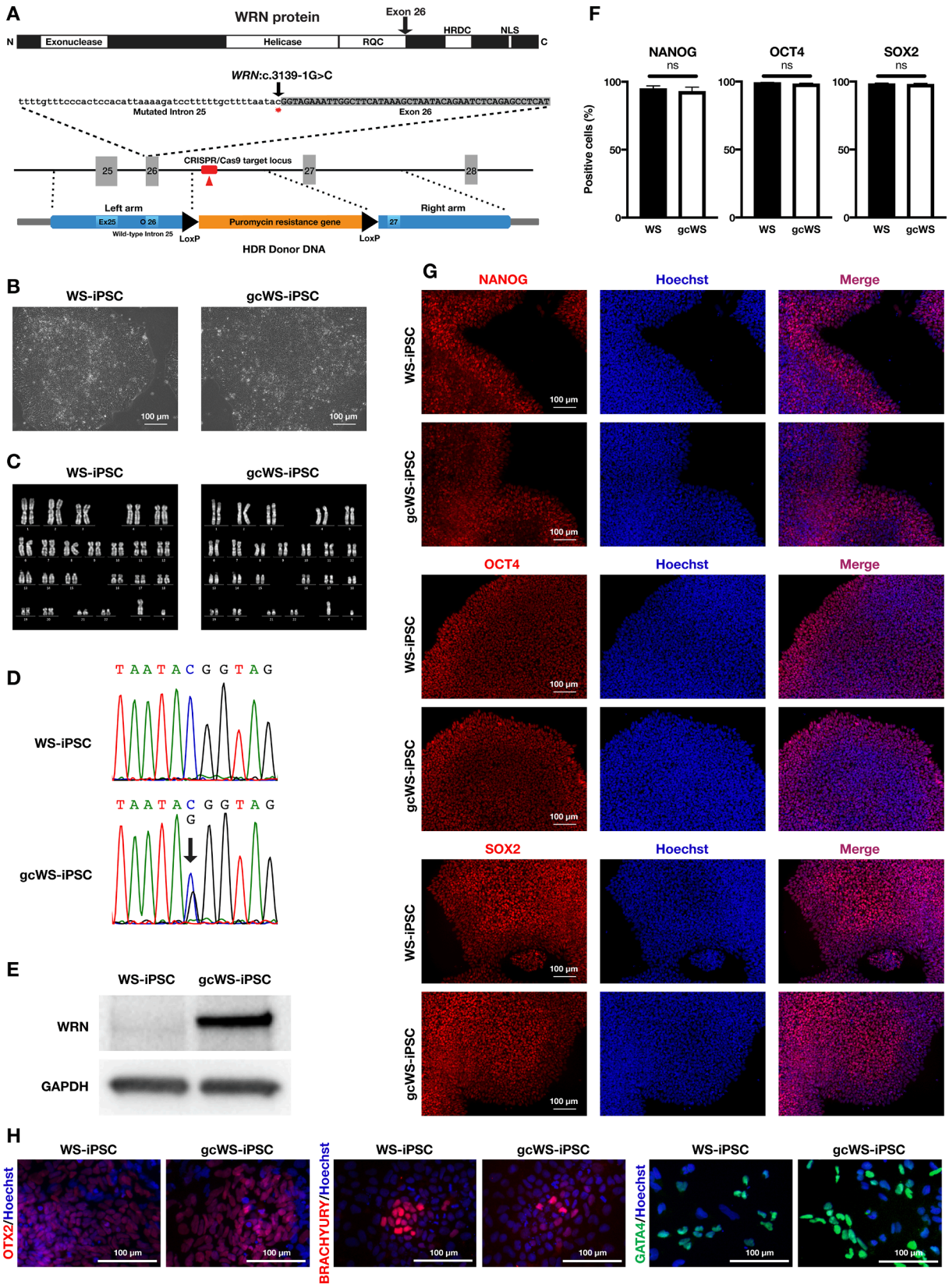

Fig. 1.

2. Resource utility

Werner syndrome (WS) is a rare autosomal recessive disorder characterized by accelerated aging-associated symptoms. Premature senescence and early exhaustion of WS patient cells make it difficult to perform extensive research *in vitro*. We provide disease-specific and gene-corrected WS-iPSCs that enable us to undertake further research to elucidate the disease pathogenesis.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
WB1-10 (CUi001-A)	WS-iPSC	Male	52	Japanese	Homozygous for *WRN*:c.3139-1G > C	Werner syndrome
WB1-10 WRNKI-Cre18 (CUi001-A-1)	gcWS-iPSC	Male	52	Japanese	Heterozygous for *WRN*:c.3139-1G > C	Werner syndrome (gene-corrected)

3. Resource details

Adult progeria Werner syndrome (WS) is a rare autosomal recessive genetic disorder in which aging accelerates after puberty and causes the early onset of aging-associated symptoms such as cataracts, graying and loss of hair, diabetes, arteriosclerosis, and malignant tumors. The responsible gene is *WRN*, which is a member of the RecQ helicase family and plays an important role in DNA replication, repair, and telomere maintenance. Approximately 60% of the cases are Japanese, and the mutation of *WRN*:c.3139-1G > C is considered a founder mutation in Japan and is the most widespread (Yokote et al., 2017). This time, we isolated CD34-positive cells from peripheral blood of a 52-year-old male WS patient harboring a homozygous mutation of *WRN*:c.3139-1G > C, introduced Yamanaka 4 factors using Sendai virus, and generated WS-iPSC. CRISPR/Cas9-mediated homology-directed repair (HDR) was performed on the WS-iPSC using a CRISPR/Cas9 expression vector and an HDR DNA donor vector equipped with the healthy genome sequence to repair the mutation (Fig. 1A, Table 1). The obtained WS-iPSC and gene-corrected WS-iPSC (gcWS-iPSC) showed iPSC-like morphology (Fig. 1B, Table 2). These showed normal karyotypes (Fig. 1C), and Sanger sequencing revealed heterozygous repair in gcWS-iPSC (Fig. 1D). In addition, Western blotting confirmed the expression of WRN protein in gcWS-iPSC (Fig. 1E). These iPSCs showed high positive rates for NANOG, OCT4, and SOX2 in immunostaining (Fig. 1F, G). Besides, these exhibited equivalent differentiation potential into three germ layers *in vitro* (Fig. 1H). In conclusion, we succeeded in creating WS-iPSC and its gene-corrected strain. These iPSCs would provide isogenic and semi-permanent materials for future WS research.

4. Materials and methods

4.1. Generation and culture of iPSC

Fifty ml of peripheral blood were obtained from a patient with WS and purified to mononuclear cells using Lymphoprep (STEMCELL Technologies, #07801). CD34 positive cells were enriched with CD34 Microbeads kit (Miltenyi Biotec, #130–097-047) according to the manufacturer's instruction. Cells were transfected with the Sendai viral vector expressing OCT3/4, SOX2, KLF4, and MYC as previously described (Nishimura et al., 2017). After transfection, cells were plated on a Matrigel (Corning, #356230)-coated dish with X-VIVO 10 (Lonza, #04-380Q) + 1% BSA (Stem cell technologies, #09300) supplemented with SCF (50 ng/ml, R&D Systems, #255-SC), TPO (20 ng/ml, R&D Systems, #288-TP), FL (50 ng/ml, PeproTech, #300–19), IL-6 (50 ng/ml, Miltenyi Biotec, #130–093-934), IL-3 (20 ng/ml, Miltenyi Biotec, #130–095-069), and G-CSF (10 ng/ml, Miltenyi Biotec, #130–094-265). Culture medium was half-exchanged by StemFit (Ajinomoto, #AK02N) every two days for a week and total-exchanged thereafter. Two weeks later, iPSC-like colonies were picked up onto laminin 511-E8 (Ajinomoto, #iMatrix-511)-coated 12 well-plate filled with StemFit + 10 μM of Y-27632 (Wako, #257–00614). Two days later, siRNA targeting L-gene of the Sendai viral vector was transfected using RNAiMAX (Invitrogen, #13778–150) to eliminate Sendai viruses.

For passaging, subconfluent iPSCs were treated with 0.5 × TrypLE select, consisting of TrypLE select (Thermo Fisher Scientific, #12563029) and 0.02% EDTA-2Na (DOJINDO, #345–01865) + PBS(-) diluted at 1:1 ratio, at 37 °C for 10 min, and suspended with 1 ml of StemFit by gently pipetting. Single cells of 1.5 × 10^4 were plated on a newly prepared 6-well plate.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel B
Phenotype	Qualitative analysis (Immunocytochemistry)	Positive for NANOG, OCT4, SOX2	Fig. 1 panel G
	Quantitative analysis (Immunocytochemistry counting)	NANOG: WS-iPSC 95.1 ± 2.4%, gcWS-iPSC 92.2 ± 3.4% OCT4: WS-iPSC 99.0 ± 0.4%, gcWS-iPSC 98.1 ± 0.7% SOX2: WS-iPSC 98.1 ± 0.8%, gcWS-iPSC 97.8 ± 1.0% Data are means ± SEM of four microscopic views. For statistical analysis, student *t*-test was performed (ns, not significant).	Fig. 1 panel F
Genotype	Karyotype (Q-banding) and resolution	46XY, Resolution 300–500	Fig. 1 panel C
Identity	STR analysis	Performed 24 sites were tested by using GlobalFiler PCR Amplification Kit (Applied Biosystems, #4476135) and matched for iPSCs and primary cells.	Submitted in archive with journal Submitted in archive with journal
Mutation analysis	Sequencing and Western blotting	Homozygous and heterozygous for *WRN*:c.3139-1G > C in WS-iPSC and gcWS-iPSC, respectively	Fig. 1 panel D and E
	Random integration analysis	Sequencing for random integration sites in the genome targeting the right arm of the donor vector. No significant random integration was detected. Other potential off-target effects were not evaluated.	Sequencing deposited at DDBJ Sequence Read Archive Accession numbers: DRR276516 (WS-iPSC), DRR276518 (gcWS-iPSC)
Microbiology and virology	Mycoplasma	Negative for Mycoplasma by PCR testing kit (LiliF, #25239)	Supplementary Fig. 1
Differentiation potential	Directed differentiation	Positive for specific markers of ectodermal (OTX2), mesodermal (BRACHYURY), and endodermal (GATA4) lineages	Fig. 1 panel H
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3
Reagents details.

Antibodies used for immunocytochemistry/Western blotting			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-NANOG	1:200	Cell Signaling Technology Cat# 4903, RRID:AB_10559205
Pluripotency Markers	Rabbit anti-OCT4	1:200	Cell Signaling Technology Cat# 2840, RRID:AB_2167691
Pluripotency Markers	Rabbit anti-SOX2	1:200	Cell Signaling Technology Cat# 3579, RRID:AB_2195767
Differentiation Markers	Goat anti-OTX2 NL557-Conjugated	1:20	R&D Systems Cat# SC022, RRID:AB_2889887
Differentiation Markers	Goat anti-Brachyury NL557-Conjugated	1:20	R&D Systems Cat# SC022, RRID:AB_2889887
Differentiation Markers	Goat anti-GATA4 NL493-Conjugated	1:20	R&D Systems Cat# SC022, RRID:AB_2889887
WRN protein	Mouse anti-WRN	1:1000	Abcam Cat# ab66606, RRID:AB_1143919
GAPDH protein	Rabbit anti-GAPDH	1:1000	Cell Signaling Technology Cat# 5174, RRID:AB_10622025
Secondary antibody for immunocytochemistry	Donkey anti-Rabbit IgG, Alexa Fluor 568	1:400	Thermo Fisher Scientific Cat# A10042, RRID:AB_2534017
Secondary antibodies for Western blotting	Sheep Anti-Mouse IgG, HRP Conjugated	1:2500	GE Healthcare Cat# NA931, RRID:AB_772210
Secondary antibodies for Western blotting	Donkey Anti-Rabbit IgG, HRP Conjugated	1:2500	GE Healthcare Cat# NA934, RRID:AB_772206
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Left arm of donor vector	chr8:31140949 + 31141854	GTTTCACCATGTTAGCCATGATGG/ CTATAAACTGGTATAATTTGAATCAC	
Right arm of donor vector	chr8:31142133 + 31143226	GACAGGGTCTTCTAGTGCTTCCTAG/ AAAATGTAATTCACTATGCTTGCC	
CRISPR/Cas9 sgRNA-complementary DNA sequence	Intron 26 of *WRN* gene	TACTTGGGCCCACGAGATCG	
Targeted mutation PCR	*WRN*:c.3139-1C > G	GGTGGAAGGCTTTTTCCCGTCAGC/ GACTTATCCTTTCCTCACAGATCC	
Targeted mutation sequencing	*WRN*:c.3139-1C > G	GGTGGAAGGCTTTTTCCCGTCAGC	
Random integration analysis forward primer 1	Right arm of donor vector	ATCTTCGGGCACCAAAGAGCATT	
Random integration analysis forward primer 2	Right arm of donor vector	AATTCGGCCAGGGAAGTGATGTG	
Random integration analysis forward primer 3	Right arm of donor vector	AATCCACTGGCAGACTTCAGC	
siRNA #1	L gene of Sendai virus	GGUUCAGCAUCAAAUAUGAAG	
siRNA #2	L gene of Sendai virus	UCAUAUUUGAUGCUGAACCAU	

4.2. Gene-correction of WRN mutation using CRISPR/Cas9-mediated HDR

CRISPR/Cas9 expression vector (pX458, #48138) was obtained through Addgene (https://www.addgene.org/). sgRNA design and insertion of sgRNA-complementary oligo DNA into pX458 was conducted as previously reported (Ran et al., 2013). The predicted on– and off-target scores were 62.8 and 96.3, respectively (calculated by Benchling; https://benchling.com. Score is from 0 to 100. Higher is better). HDR DNA donor vector was built using the In-Fusion HD Cloning Kit (Clontech, 639648). One million iPSCs were electroporated with 15 μg of CRISPR/Cas9 expression vector and 15 μg of donor vector by using Neon Transfection System (Invitrogen, #MPK5000) at 1300 V, 10 ms, 3 times, then seeded on a 6-well plate. After the puromycin selection, the puromycin resistance gene was removed by Cre recombinase (Clontech, #631449) according to the instruction. After reaching subconfluency, cells were re-plated to form single-cell colonies, and genotyping was performed based on the previous report (Li et al., 2016).

4.3. Karyotyping

According to the manufacturer's protocol, the subconfluent iPSCs were treated with the colcemid kit (Chromocenter, Japan) and fixed with Carnoy's solution. Q-banding was performed by Chromocenter, Japan.

4.4. Western blotting

The western blotting analysis was performed using a standard protocol. The image was taken by ChemiDoc (Bio-Rad, USA). The antibodies are listed in Table 3.

4.5. Immunofluorescent analysis and differentiation into three germ layers

Immunostaining of iPSCs was performed according to StemLight Pluripotency Antibody Kit (Cell Signaling Technologies, #9656). Differentiation into three germ layers was performed using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems, #SC027B) based on the protocol. The Human Three Germ Layer 3-Color Immunocytochemistry Kit (R&D Systems, #SC022) was used to stain the differentiated cells. For staining of the nuclear DNA, Hoechst 33,342 (DOJINDO, 346–07951) was used.

4.6. Random integration analysis

The random integration analysis was performed by FASMAC, Japan, based on the previously described method (Saito et al., 2020).

Author contributions

H.K., Y.M., and K.Y. designed the study, analyzed the data, and wrote the manuscript; H.K. carried out the experiments and composed the figures; Y.O., N.T., M.S., K.S., A.T.W., K.T., S.N., H.S., and M.O. carried out the experiments; M.K., M.N., H.T., A.S., A.I., and K.E. discussed the data; all authors approved the final version of the manuscript.

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.2021.102360.

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