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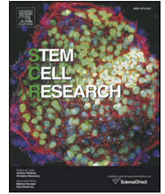
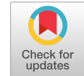


Lab Resource: Stem Cell Line

A homozygous p53 R282W mutant human embryonic stem cell line generated using TALEN-mediated precise gene editing



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ABSTRACT

The tumor suppressor gene *TP53* is the most frequently mutated gene in human cancers. Many hot-spot mutations of *TP53* confer novel functions not found in wild-type p53 and contribute to tumor development and progression. We report on the generation of a H1 human embryonic stem cell line carrying a homozygous *TP53* R282W mutation using TALEN-mediated genome editing. The generated cell line demonstrates normal karyotype, maintains a pluripotent state, and is capable of generating a teratoma *in vivo* containing tissues from all three germ layers.

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Resource table.

Unique stem cell line identifier	CDMLe003-A (https://hpscreg.eu/cell-line/CDMLe003-A)
Alternative name(s) of stem cell line	H1-p53(R282W/R282W) and 1R5
Institution	The University of Texas Health Science Center at Houston, Houston, Texas, USA
Contact information of distributor	Dung-Fang Lee dung-fang.lee@uth.tmc.edu
Type of cell line	Human embryonic stem cell line
Origin	Human
Additional origin info	Sex: Male
Cell Source	Human blastocyst
Clonality	Clonal
Method of reprogramming	N/A
Genetic Modification	Yes
Type of modification	Homozygous R282W mutation of *TP53* on exon 8.
Associated disease	Li-Fraumeni syndrome; cancers
Gene/locus	17p13.1; *TP53* exon 8
Method of modification	Transcription activator-like effector nucleases (TALEN)
Name of transgene or resistance	None
Inducible/constitutive system	None
Date archived/stock date	2017/12
Cell line repository/bank	None
Ethical approval	Cell lines were used according to institutional guidelines. UTHealth approval number: SCRO-16-01

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Resource utility

We generated a human embryonic stem cell (hESC) carrying a homozygous mutation in *TP53* to facilitate the modeling of mutant p53-related diseases and elucidate pathogenesis of mutant p53-associated cancers.

Resource details

TP53 is the most frequently mutated gene in human cancers. Several "hotspot" mutations are found in various cancer types, indicating selective advantages during cancer development and progression (Freed-Pastor and Prives 2012; Zhou et al. 2017). Missense substitutions at many "hotspot" sites confer novel cancer-promoting functions on mutant p53 and have been suggested to be attractive targets for cancer therapy (Freed-Pastor and Prives 2012; Zhou et al. 2017). We previously established a human Li-Fraumeni syndrome (LFS) disease model by using LFS patient-specific iPSCs to delineate the pathological mechanisms caused by mutant p53 in osteosarcoma. LFS iPSC-derived osteoblasts recapitulate osteosarcoma features including defective osteoblastic differentiation and tumorigenic ability (Lee et al. 2015), suggesting that pluripotent stem cells can serve as a "disease in a dish" platform for elucidating mutant p53-mediated disease pathogenesis. To provide a useful lab resource for modeling mutant p53-related cancers and diseases, we generated a H1 human embryonic stem cell line carrying a homozygous R282W mutation of *TP53* (one of the hotspot mutations) using TALEN-mediated genome editing (Fig. 1A. TALEN target sites are in bold red. Start sites of the upstream and downstream homologous arms are underlined. The design of the mutant donor vector is also illustrated. Primers used to identify correctly inserted colonies are shown with arrows).

hESC H1 cells were transfected by electroporation with paired TALEN plasmids and a R282W donor vector containing a Frt-EM7-Neo^R-Frt (FNF) selection cassette. The electroporated cells were selected by G418 (Geneticin). The G418-resistant clones were picked up, isolated, and expanded. The *TP53* targeted regions were analyzed by PCR (Fig. 1B) using two pairs of primers (p53_6FM13 and 3FNF-N1; and 5FNF-C1 and 3p53_16821_RM13). We confirmed that the R4–18 clone underwent precise homologous recombination and FNF cassette insertion between exons 8 and 9. The presence of exon 8/9 PCR product when using exon 8/9 paired primers (p53_8/9FM13 and p53_8/9RM13) suggested that the FNF cassette was inserted into only one allele instead of two alleles (Fig. 1B). Upon removal of the FNF cassette by Flp recombinase, PCR of the exon 8/9 region in FNF-removed clone 1R5 revealed the existence of double bands, suggesting that the lower band is the original exon 8/9 and the higher band is the TALEN-modified exon 8/9 containing a Frt fragment in between exon 8 and 9. Unexpectedly, PCR/Sanger sequencing of the exon 8/9 region revealed that clone 1R5 contains a R282W mutation in both alleles (only TGG rather than (T/C)GG in the mutated exon 8/9 region) (Fig. 1C). The possibility of additional R282W mutation in the unengineered allele is due to spontaneous homologous recombination-mediated mutagenesis by the R282W donor vector. Immunoblotting results revealed the increased level of p53 in R4-18 and 1R5 clones in comparison with that of parental H1 cells (Fig. 1D), suggesting mutant p53(R282W) is more stable than wild-type p53. We rename the 1R5 line to H1-p53(R282W/R282W).

The H1-p53(R282W/R282W) line displayed a typical round shape hESC-like morphology and exhibited positive alkaline phosphatase activity (Fig. 1E, scale bar 50 μm). H1-p53(R282W/R282W) cells also express pluripotency transcription factors NANOG and OCT4 as well as hESC surface markers SSEA4 and TRA-1-81 (Fig. 1F, scale bar 50 μm). Quantitative real-time PCR (qRT-PCR) analysis showed comparable mRNA expression of pluripotency genes (*NANOG*, *OCT4*, *SOX2*, *DPPA4*, *REX1* and *TERT*) to parental H1 cells (Fig. 1G, error bars indicate ± SEM of triplicates). The H1-p53(R282W/R282W) line maintained a normal karyotype (Fig. 1H) and pluripotency, namely the ability to differentiate into three germ layers *in vivo* (Fig. 1I, scale bar 50 μm). PCR-based mycoplasma detection assay demonstrated that the cell line is free of mycoplasma (Fig. 1J). In summary, H1-p53(R282W/R282W) cells maintain a pluripotent state with a normal karyotype. This line provides a useful resource to study p53(R282W)-associated malignancies and is of great value in exploring gain-of-function of p53(R282W) mutation.

Materials and methods

Cell culture

H1 hESCs and the generated clones were cultured on Matrigel (Corning)-coated plates in *StemMACS*™ iPS-*Brew* XF medium (Miltenyi Biotec) and maintained at 37 °C in a humidified 5% CO_2 incubator. Cells were passaged using StemMACS™ Passaging Solution XF (Miltenyi Biotec) when reaching 85% confluence. The plates were pre-coated with 1:50 diluted Matrigel at room temperature for 1 h. hESC medium with 2 μM ROCK inhibitor Thiazovivin (Calbiochem) was used to improve survival of dissociated hESCs. Culture medium was then changed to *StemMACS*™ iPS-*Brew* XF medium the following day. *StemMACS*™ iPS-*Brew* XF medium was changed every other day. Cells were passaged every 5–7 days at a 1:10 ratio (Table 1).

Generation of p53(R282W) donor vector and TALEN plasmids

The TALEN guides targeting intron 8 of *TP53* was designed using ZiFiT Targeter Version 4.2 (http://zifit.partners.org/). TALENs targeting 5′-TTTCCTTACTGCCTCT-3′ upstream, and 5′-CTATCCTGAGTAGTGGTA-3′ downstream were assembled using REAL Assembly TALEN kit (Addgene) and inserted into JDS vectors according to protocol (Sander et al. 2011).

Homologous arms were amplified using H1 hESC genomic DNA isolated by the PureLink Genomic DNA Mini Kit (Thermo Fisher). The primers used to amplify the left and the right homologous arms are shown in Table 2. PCR products were run on 0.8% agarose gel, purified and ligated into pGEM-T Easy vector. Site-directed mutagenesis was performed on right homologous arm to generate R282W mutant donor arm. Homologous arms were then digested with *Sal*I and *Eco*RI (left homologous arm) and *Bam*HI and *Not*I (right homologous arm), respectively, and ligated into the pFNF (Frt-EM7-Neo^R-Frt) vector to facilitate clone selection with G418.

Generation of H1-p53(R282W/R282W) line by TALEN-mediated genome editing

Mouse embryonic fibroblast (MEF) culture dishes were prepared one day before electroporation. Tissue culture dishes were coated with 0.1% gelatin at room temperature for 30 min. The gelatin was aspirated after coating. 6.7×10^5 irradiated CF1 MEFs (Thermo Fisher) were seeded per 10 cm dish using MEF culture medium (DMEM (Sigma) supplemented with 10% of FBS (GenDEPOT) and 1% Penicillin/Streptomycin (Sigma)).

Fig. 1. Establishment and characterization of the homozygous p53 R282W mutant human embryonic stem cell line H1-p53(R282W/R282W). (A) Schematic diagram of the strategy for p53 (R282W) generation. TALEN target sites are indicated in bold red. Start sites of the upstream and downstream homologous arms are underlined. (B) PCR of precise homologous recombination in *TP53* genome loci. (C) Sequences of the homozygous p53(R282W) mutationin H1-p53(R282W/R282W) cell line. (D) Immunoblotting of the p53(R282W) expression in cell line R4–18 and 1R (H1-p53(R282W/R282W)). (E) Cell morphology and AP staining of H1-p53(R282W/R282W) cell line. Scale bar, 50 μm. (F) Immunofluorescence staining of pluripotency markers NANOG, OCT4, SSEA4 and TRA-1-81 in H1-p53(R282W/R282W) cell line. Scalebar, 50 μm. (G) qRT-PCR assay for expression of endogenous pluripotency genes in H1-p53(R282W/R282W) cell line. (H) Karyotype analysis of H1-p53(R282W/R282W) cell line. (I) *In vitro* teratoma assay of H1-p53(R282W/R282W) cell line. Scale bar, 50 μm. (J) Mycoplasma detection of H1-p53(R282W/R282W) cell line.

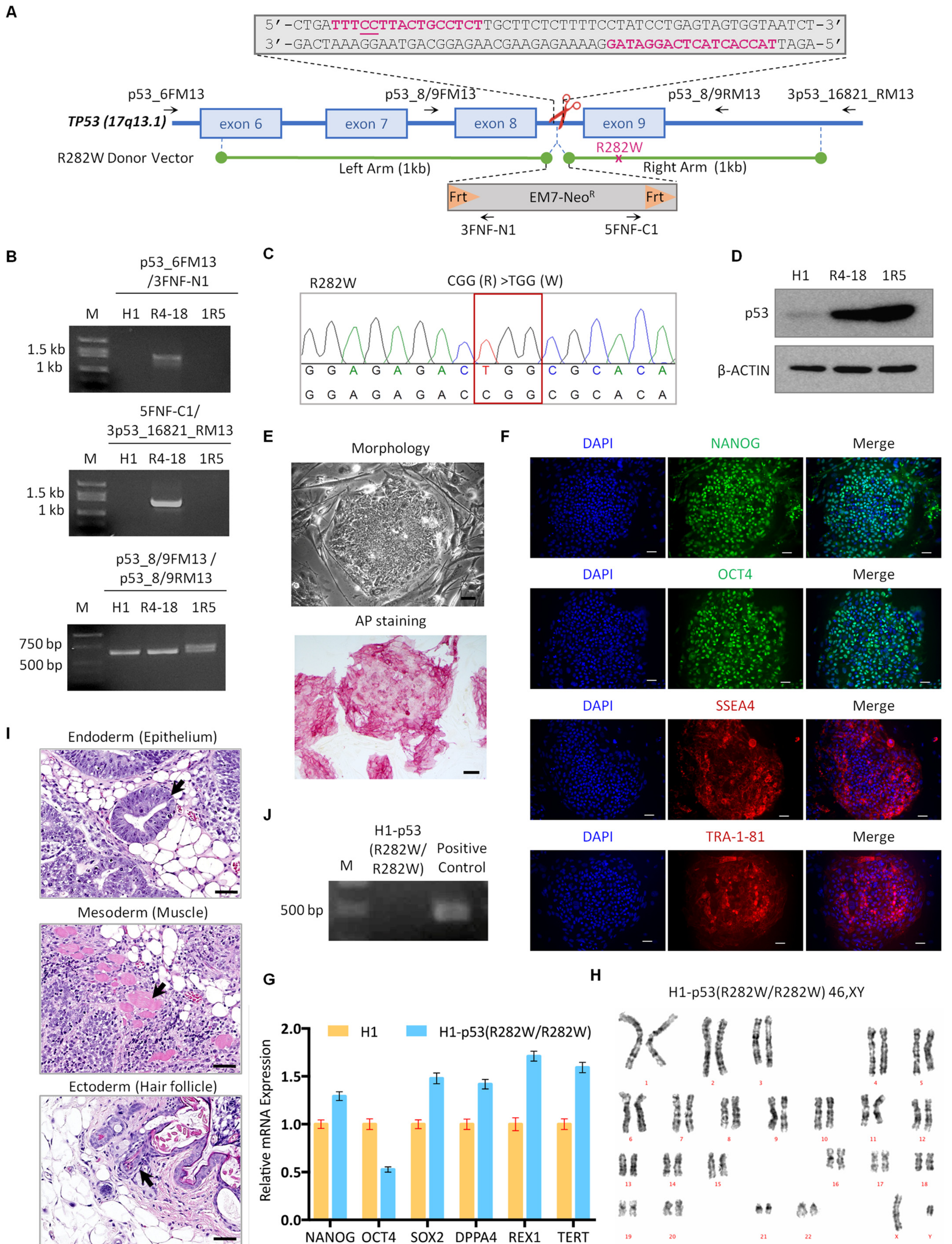
A
5′-CTGATTTCCTTACTGCCTCTTGCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCT-3′
3′-GACTAAAGGAATGACGGAGAACGAAGAGAAAAGGATAGGACTCATCACCATTAGA-5′
p53_6FM13
p53_8/9FM13
p53_8/9RM13
3p53_16821_RM13
TP53 (17q13.1)
exon 6
exon 7
exon 8
exon 9
R282W Donor Vector
R282W
Left Arm (1kb)
Right Arm (1kb)
Frt
EM7-NeoR
Frt
3FNF-N1
5FNF-C1
B
p53_6FM13 /3FNF-N1
M H1 R4-18 1R5
1.5 kb
1 kb
5FNF-C1/ 3p53_16821_RM13
M H1 R4-18 1R5
1.5 kb
1 kb
p53_8/9FM13 / p53_8/9RM13
M H1 R4-18 1R5
750 bp
500 bp
C
R282W
CGG (R) >TGG (W)
D
H1 R4-18 1R5
p53
β-ACTIN
E
Morphology
AP staining
F
DAPI NANOG Merge
DAPI OCT4 Merge
DAPI SSEA4 Merge
DAPI TRA-1-81 Merge
I
Endoderm (Epithelium)
Mesoderm (Muscle)
Ectoderm (Hair follicle)
J
M
H1-p53 (R282W/ R282W)
Positive Control
500 bp
G
H1
H1-p53(R282W/R282W)
Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
NANOG OCT4 SOX2 DPPA4 REX1 TERT
H
H1-p53(R282W/R282W) 46,XY

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	hESC morphology	Fig. 1 panel E
Phenotype	Immunocytochemistry	NANOG, OCT4, SSEA4, TRA-1-81 and AP-positive	Fig. 1 panel E, F
	RT-qPCR	Comparable of *NANOG*, *OCT4*, *SOX2*, *DPPA4*, and *REX1* expression with H1	Fig. 1 panel G
Genotype	Karyotype (G-banding) and resolution	46 XY Resolution: 450–500	Fig. 1 panel H
Identity	Microsatellite PCR (mPCR) OR	N/A	N/A
	STR analysis	14 sites tested, STR profile match human embryonic cell line H1	Available with authors
Mutation analysis (if applicable)	Sequencing	Homozygous R282W mutation of *TP53*.	Fig. 1 panel C
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma test shows negative.	Fig. 1 panel J
Differentiation potential	*In vivo* teratoma	Teratoma comprises tissues of ectoderm, mesoderm, and endoderm.	Fig. 1 panel I
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

To generate the H1-p53(R282W/R282W) line, 10^7 H1 hESCs were re-suspended with 0.6 ml Embryo Max Electroporation Buffer (Millipore), mixed with 50 μg of p53(R282W) donor vector and 5 μg of each TALEN encoding plasmid and electroporated at 300 V/500uF in BIO-RAD Gene Pulser Xcell System. The electroporated cells were immediately dispensed into 10 cm MEF plates in hESC medium (DMEM/F12 (Corning) with 20% KnockOut Serum replacement (Life Technologies), 1% Gibco GlutaMax (Life Technologies), 1% NEAA (Corning), 0.0007% β-mercaptoethanol (Sigma) and 10 ng/ml FGF2 (EMD Millipore)) supplemented with 2 μM ROCK inhibitor Thiazovivin for 2 days. Cells were then selected with 50 μg/ml G418 for 2–3 weeks and medium was changed every two days until colonies emerged. Individual clones were then picked and expanded. The correctly inserted colonies were identified by PCR using specific primer sets (p53_6FM13 and 3FNF-N1 Primer for left homologous arm; 5FNF-C1 and 3p53_16821_RM13 primer for right homologous arm, Table 2).

Immunoblotting

Immunoblotting was performed as described (Lee et al. 2007).

Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde for 8 min at room temperature, blocked with 10% serum in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.01% Tween-20 (Sigma) (DPBST) at room temperature for 1 h. After blocking, cells were incubated with the indicated primary antibodies (Table 2) at 4 °C overnight. Cells were then washed with DPBST 3 times at room temperature, and incubated with corresponding secondary antibodies at room temperature for 1 h. DAPI counterstaining was performed by incubating cells with 3 μM DAPI (Thermo Fisher) diluted in DPBS at room temperature for 5 min. The results were visualized by Leica DMi8.

Table 2
Reagents details.

Antibodies used for immunocytochemistry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Goat anti-NANOG	1:500	R and D Systems Cat# AF1997 RRID:AB_355097
Pluripotency Markers	Rabbit anti-OCT4	1:300	Santa Cruz Biotechnology Cat# sc-9081 RRID:AB_2167703
Pluripotency Markers	Mouse anti-SSEA4 PE-conjugated	1:600	R and D Systems FA1435P-025
Pluripotency Markers	Mouse anti-TRA-1-85 Alexa Fluor 555-conjugated	1:600	R and D Systems Cat# FAB3195A RRID:AB_663789
p53(Western Blot)	Mouse anti-p53 (DO-1)	1:1000	Santa Cruz Biotechnology Cat# sc-126
Secondary antibodies	Goat anti-rabbit IgG (Alexa Fluor 488 conjugate)	1:500	Jackson ImmunoResearch Labs Cat# 111–545-144 RRID:AB_2338052
Secondary antibodies	Donkey Anti-Goat IgG (Alexa Fluor488 conjugate)	1:500	Jackson ImmunoResearch Labs Cat# 705–545-003 RRID:AB_2340428

Primers

	Target	Forward/Reverse primer (5′–3′)
Pluripotency Markers (qPCR)	OCT4	AACCTGGAGTTTGTGCCAGGGTTT/TGAACTTCACCTTCCCTCCAACCA
Pluripotency Markers (qPCR)	SOX2	AGAAGAGGAGAGAGAAAGAAAGGGAGAGA/GAGAGAGGCAAACTGGAATCAGGATCAAA
Pluripotency Markers (qPCR)	NANOG	TTTGTGGGCCTGAAGAAAACT/AGGGCTGTCCTGAATAAGCAG
Pluripotency Markers (qPCR)	DPPA4	GACCTCCACAGAGAAGTCGAG/TGCCTTTTTCTTAGGGCAGAG
Pluripotency Markers (qPCR)	REX1	GCCTTATGTGATGGCTATGTGT/ACCCCTTATGACGCATTCTATGT
Pluripotency Markers (qPCR)	TERT	TGAAAGCCAAGAACGCAGGGATG/TGTCGAGTCAGCTTGAGCAGGAATG
House-Keeping Genes (qPCR)	GAPDH	CCACTCCTCCACCTTTGAC/ACCCTGTTGCTGTAGCCA
Targeted mutation analysis/sequencing	p53_6FM13	TGTAAAACGACGGCCAGTCCACCATGAGCGCTGCTCAG
Targeted mutation analysis/sequencing	3FNF-N1	TCCAGACTGCCTTGGGAAA
Targeted mutation analysis/sequencing	5FNF-C1	GGGGAGGATTGGGAAGACAA
Targeted mutation analysis/sequencing	3p53_16821_RM13	CAGGAAACAGCTATGACCGCCCAGGAGGGTATAATGAGCTA
Targeted mutation analysis/sequencing	p53_8/9FM13	TGTAAAACGACGGCCAGTACCTCTTAACCTGTGGCTTC
Targeted mutation analysis/sequencing	p53_8/9RM13	CAGGAAACAGCTATGACCTACAACCAGGAGCCATTGTC
Targeted mutation analysis/sequencing	5p53-L003-1 kb/SalI for left arm	GTCGACCCTATGAGCCGCCTGAGGTCTGGTTTGCAA
Targeted mutation analysis/sequencing	3p53-L003-1 kb/EcoRI for left arm	GAATTCGAAATCAGGTCCTACCTGTCCCATTTAAAAAACCAGGCTC
Targeted mutation analysis/sequencing	5p53-R003-1 kb/*Bam*HI for right arm	GGATCCCTTACTGCCTCTTGCTTCTCTTTTCCTATCCTGAGTAGTG
Targeted mutation analysis/sequencing	3p53-R003-1 kb/NotI for right arm	GCGGCCGCCAAGGCAGGCAGATCACAAGGTCAGGAGTTCGAGAC
Targeted mutation analysis/sequencing	5p53-R282W for mutagenesis	GCCTGTCCTGGGAGAGACTGGCGCACAGAGGAAGAGAAT
Targeted mutation analysis/sequencing	3p53-R282W for mutagenesis	ATTCTCTTCCTCTGTGCGCCAGTCTCTCCCAGGACAGGC

Quantitative real-time PCR

Total mRNA was isolated using TRIzol (Invitrogen) following the manufacturer's instruction. 1 μg of RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Real-time PCR analysis was performed on a CFX96 machine (Bio-Rad) using the SYBR Green PCR Master Mix (Bio-Rad). The PCR reaction consisted of 10 μl SYBR Green PCR Master Mix, 1 μl of 10 μM forward and reverse primers, and 1 μl of diluted template cDNA (1:3 dilution in water). The RT-PCR reaction was performed using the following protocol: 50 °C for 10 min, 95 °C for 5 min, 40 cycle of 95 °C for 10 s and 60 °C for 30 s, respectively, and 95 °C for 10 s. Samples were analyzed in triplicate and normalized to GAPDH expression. The primer sequences are shown in Table 2.

Karyotype analysis

The G-banding karyotype was performed in Department of Pediatrics, Baylor College of Medicine, Texas Children's Cancer and Hematology Centers. Twenty metaphase chromosome spreads were classified according to the standard G-banding technique (450–500 nucleotide resolution).

In vivo teratoma formation assay

2×10^7 H1-p53(R282W/R282W) cells were resuspended in 100 μl of ice cold 1 × DPBS and then mixed with 100 μl of phenol red-free Matrigel (Corning). The Matrigel-mixed cells were injected subcutaneously into both sides of flanks of immunocompromised nude mice (Charles River). Teratomas were excised around 6 weeks after injection and fixed in 10% neutral buffer formalin. Tissue embedding and H&E staining were performed by HistoWiz (Brooklyn, NY).

Mycoplasma detection

Mycoplasma detection was performed using PCR Mycoplasma Detection Kit (Applied Biological Materials Inc) according to the manufacturer's instructions.

Short tandem repeat (STR) analysis

STR analysis was performed by Characterized Cell Line Core Facility The University of Texas M.D. Anderson Cancer Center. The number of STRs at 14 loci, namely AMEL, CSF1PO, D13S317, D16S539, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, TH01, TPOX and vWA, was assessed for H1-p53(R282W/R282W) cells. This STR profile was compared with the STR profile of parental H1 cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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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.2018.01.035.

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