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Lab resource: Stem Cell Line

Generation of a heterozygous p53 R249S mutant human embryonic stem cell line by TALEN-mediated genome editing

Zijun Huo[a,b,1], Jian Tu[a,c,1], An Xu[a], Yan Li[d], Donghui Wang[a,e], Mo Liu[a], Ruoji Zhou[a,f], Dandan Zhu[a], Yu Lin[a,g], Julian A. Gingold[h], Chia-Jui Yen[i], Haipeng Xiao[b,*], Ruiying Zhao[a,*]

[a] Department of Integrative Biology and Pharmacology, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX 77030, USA
[b] Department of Endocrinology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, PR China
[c] Department of Musculoskeletal Oncology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, PR China
[d] Department of Pathology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, PR China
[e] Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, PR China
[f] The University of Texas, MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, Houston, TX 77030, USA
[g] Guangdong Research Center of Metabolic Diseases of Integrated Western and Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou 510006, PR China
[h] Women's Health Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA
[i] Division of Hematology and Oncology, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan

Abstract: As one of the most essential genome guardians, p53 and its mutants have been suggested associated with many types of cancers. Many p53 mutants function induce unique phenotypes, including carcinogenesis, metastasis, and drug resistance. The p53(R249S) mutation is the most prevalent and specific mutation associated with liver cancer development. Here, we demonstrate the generation of a heterozygous p53(R249S) mutation in the H9 human embryonic stem cell line using TALEN-mediated genome editing. The generated cell line maintains a normal karyotype, a pluripotent state and the *in vivo* capacity to develop a teratoma containing all three germ layer tissues.

Resource table

Unique stem cell line identifier	WAe009-A-11
Alternative name(s) of stem cell line	H9-p53(WT/R249S) and A94–42
Institution	McGovern Medical School, The University of Texas Health Science Center Houston, Houston, Texas, USA
Contact information of distributor	Ruiying Zhao ruiying.zhao@uth.tmc.edu
Type of cell line	ESC
Origin	Human
Additional origin info	Age: N/A Sex: Female Ethnicity: N/A
Cell Source	H9 human Embryonic Stem Cell
Clonality	Clonal
Method of reprogramming	N/A
Genetic modification	Yes
Type of modification	Induced mutation
Associated disease	Liver cancer
Gene/locus	*TP53* (c.747G > T)/17p13.1
Method of modification	TALENS
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	2018/1
Cell line repository/bank	Human Pluripotent Stem Cell Registry (https://hpscreg.eu/user/cellline/edit/WAe009-A-11)
Ethical approval	Cell lines were used according to institutional guidelines. UTHealth approval number: SCRO-16-01

Resource utility

We created a human embryonic stem cell (hESC) line carrying a heterozygous p53 R249S mutation to facilitate the modeling of mutant p53-associated liver cancer.

* Corresponding authors.
E-mail addresses: xiaohp@mail.sysu.edu.cn (H. Xiao), ruiying.zhao@uth.tmc.edu (R. Zhao).
[1] These authors contributed equally to this work.

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Resource details

More than 50% of human cancers harbor the tumor suppressor gene *TP53* mutation, the most frequent genetic alteration in human cancers. The majority of the p53 mutations found in cancers are missense mutations that mostly occur at particular "hotspots". Remarkably, these mutations not only abrogate normal p53 function but also drive gain-of-function that significantly promotes cancer initiation and progression. For instance, the p53 R249S mutation is the most common hotspot mutation observed in liver cancer patients with suspected exposure to high levels of aflatoxin or infected with hepatitis virus B (HBV) (Hussain et al., 2007). Although, these findings emphasized the clinical significance of p53(R249S) in hepatocarcinogenesis, the pathological mechanism underlying how p53(R249S) contributes to the liver cancer development has not yet been elucidated. Furthermore, the limited access of primary liver cancer tissues or cell lines harboring p53(R249S) mutation impedes the study of its function. Since we have reported on using Li-Fraumeni syndrome (LFS) patient-derived induced pluripotent stem cells (iPSCs) to study the role of one mutant p53 (p53 G245D) in the osteosarcoma development, for the first time demonstrated the feasibility of using PSCs to model mutant p53-associated malignancies (Lee et al., 2015). Therefore, to provide a useful lab resource for modeling and exploring mutant p53-associated liver cancer, we generated a H9 human embryonic stem cell (hESC) line carrying a heterozygous p53(R249S) mutation by TALEN-mediated precise gene editing (Fig. 1A).

hESC H9 cells were electroporated with paired TALEN plasmids and the p53(R249S) donor vector containing a Frt-EM7-Neo^{R}-Frt (FNF) cassette. Transfected cells were then selected with G418. The G418-resistant clones were picked up and expanded. The *TP53* target regions were examined by PCR using two pairs of primers (p53_5FM13 and 3FNF_N1 for left arm, 5FNF_C1 and 3p53_16821_RM13 for right arm, Table 2) to confirm the precise homologous recombination and FNF cassette insertion between exons 7 and 8 (Fig. 1A). Among the examined 400 clones, 3 clones including the A94 clone demonstrated precise FNF cassette insertion into the *TP53* locus (Fig. 1B). After transfection of Flp recombinase, cells were picked up and FNF cassette removal was verified by PCR (p53_7FM13 and p53_7RM13 primers, Table 2). Successful removal of the FNF cassette was demonstrated in cells from the A94–42 clone (Fig. 1C). PCR of the endogenous *TP53* exon 7 revealed two PCR bands, the lower band representing the original exon 7 and the upper band representing the engineered exon 7 with an Frt insertion in between exons 7 and 8. Sanger sequencing confirmed the heterozygous p53(R249S) mutation in the clone A94–42 (Fig. 1D). After the above verification, clone A94–42 was expanded and cryopreserved and renamed H9-p53(WT/R249S).

The H9-p53(WT/R249S) line maintained a normal stem cell-like morphology with a tightly packed round shape and displayed a high level of alkaline phosphatase (AP) staining (Fig. 1E). Immunofluorescent staining confirmed high expression of pluripotency transcription factors (NANOG and OCT4) and hESC surface markers (SSEA4 and TRA-1-81) (Fig. 1F). The expression levels of pluripotency genes (*SOX2*, *DPPA4*, *REX1* and *TERT*) were comparable to those in the parental H9 cells by quantitative real-time PCR (qRT-PCR) (Fig. 1G). Immunoblotting showed that the expression of p53 in the H9-p53 (WT/R249S) line was comparable to that in parental H9 cells (Fig. 1H). PCR-based mycoplasma assay revealed the H9-p53 (WT/R249S) line to be free of the pathogen (Fig. 1I). The teratoma assay underscored the pluripotent ability of the H9-p53(WT/R249S) line with *in vivo* differentiation into three germ layers (Fig. 1J). Furthermore, no chromosomal abnormalities were observed in karyotype analysis (Fig. 1K). In addition, short tandem repeat (STR) analyses confirmed that the H9-p53(WT/R249S) line derived from parental H9 cells (Supplementary Table S1). In summary, the H9-p53(WT/R249S) line exhibits a normal karyotype and all characteristics consistent with a fully pluripotent state. It represents a valuable tool to study p53(*R249S*)-associated liver cancer.

Materials and methods

Cell culture

hESCs were cultured in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec) on Matrigel (Corning)-coated plates under normal tissue culture conditions (37 °C, 5% CO_2). Cells were passaged every 5–7 days at a 1:10 ratio using StemMACS™ Passaging Solution XF (Miltenyi Biotec). hESC medium (DMEM/F12 (Corning) containing 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)). 2 μM ROCK inhibitor Thiazovivin (Calbiochem) was added on the first day after passaging to improve cell survival. The medium was replaced with StemMACS™ iPS-Brew XF medium beginning day two after passaging.

p53(WT/R249S) donor vector, TALEN plasmids and pCAGGS-flpE-puro plasmid

Homologous arms were amplified using H9 hESC genomic DNA. The primers used to amplify the left and right homologous arms are listed in Table 2. PCR products were run on a 0.8% agarose gel, then purified and ligated into a pGEM-T Easy vector (Promega). Site-directed mutagenesis was performed on the left arm to generate the R249S mutant donor arm. The pair of homologous arms were then digested with *Eco*RI (left homologous arm), and *Bam*HI and *Not*I (right homologous arm), and subcloned into the pFNF vector (Addgene #22687), which introduced a FNF selection cassette (Frt-EM7-NeoR-Frt) into intron of the TP53 genomic region to facilitate clone selection.

The paired TALEN constructs (targeting 5′-tccaggtcaggagccact-3′ upstream and 5′-ccggacgacacggggt-3′ downstream) were designed by ZiFiT Targeter software (http://zifit.partners.org/ZiFiT/TALZiFiTNuclease.aspx) and then assembled using the REAL Assembly TALEN kit (Addgene) and ligated into the JDS74 vector (Addgene #32288) following the manufacture's protocol (Sander et al., 2011) to generate the TALEN vectors, in which Fok1 nucleases were fused to engineered TALEN domains to induce a double strand break (DSB) precisely at the targeted endogenous genomic location.

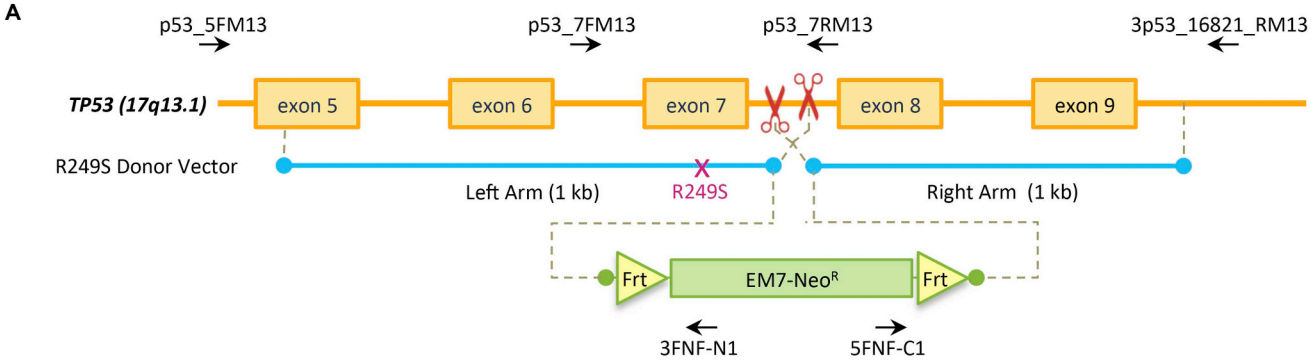
The pCAGGS-flpE-puro plasmid (Addgene #20733) carrying Flp recombinase was used to excise the FNF cassette by transfection.

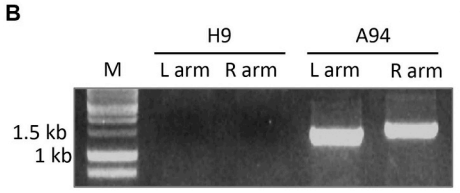
Generation of H1-p53(WT/R249S) line by TALEN-mediated genome editing

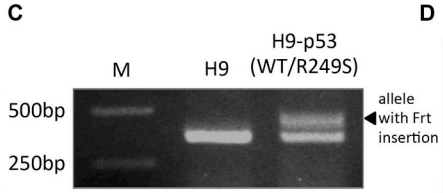
10^7 cells were mixed with 50 μg of p53(R249S) donor vector and 5 μg of each TALEN encoding plasmid in 600 μl Embryo Max Electroporation Buffer (Millipore). Electroporation was performed at 300 V/500 μF in Bio-Rad Gene PulserXcell System. Cells were then immediately plated on 10 cm mouse embryonic fibroblast (MEF)-coated dishes in hESC medium containing 2 μM ROCK inhibitor Thiazovivin. The medium was replaced 2 days after the electroporation with fresh hESC medium supplemented with 50 μg/ml G418. Cells were maintained for 2 to 3 weeks and medium was changed every 2 to 3 days for colony growth. Resistant clones were picked up, expanded and identified by PCR using primers (p53_5FM13 and 3FNF-N1 Primer for left homologous arm; 5FNF-C1 and 3p53_16821_RM13 primer for right homologous arm, Table 2). The wild-type (WT) clone and mutant clone were further confirmed by Sanger sequencing.

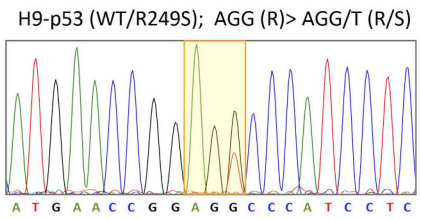
Standard PCR

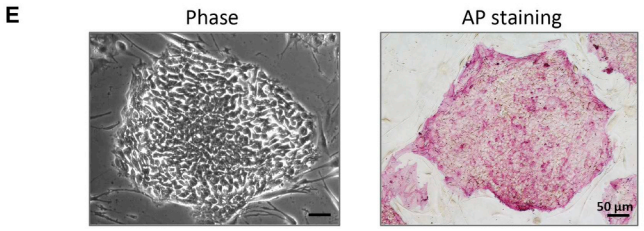
Genomic DNA was obtained using Easy-DNA gDNA purification kit (Invitrogen) by the manufacture's protocol. OneTaq Quick-Load 2 × Master Mix (New England Biolabs) was applied for PCR following the manufacture's instructions. The PCR reaction condition was as follows: 94 °C for 1 min; 35 cycles of reaction: 94 °C for 30 s, 56 °C for 45 s and 68 °C for 105 s; and 68 °C for 5 min on a Biometra TRIO Thermal

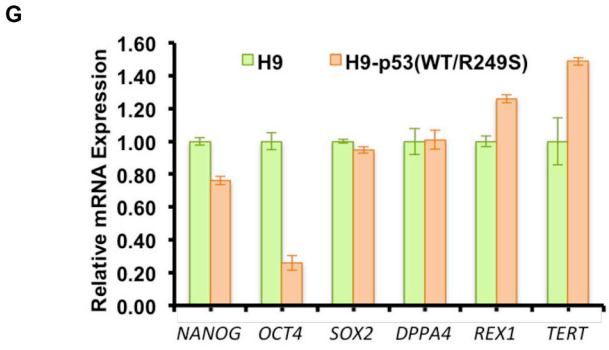


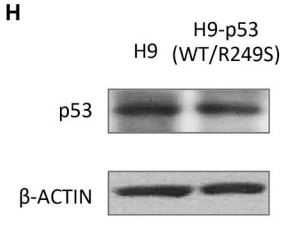


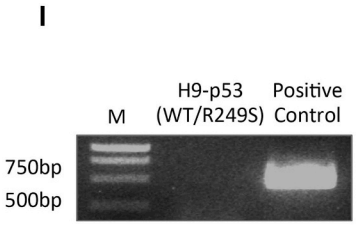


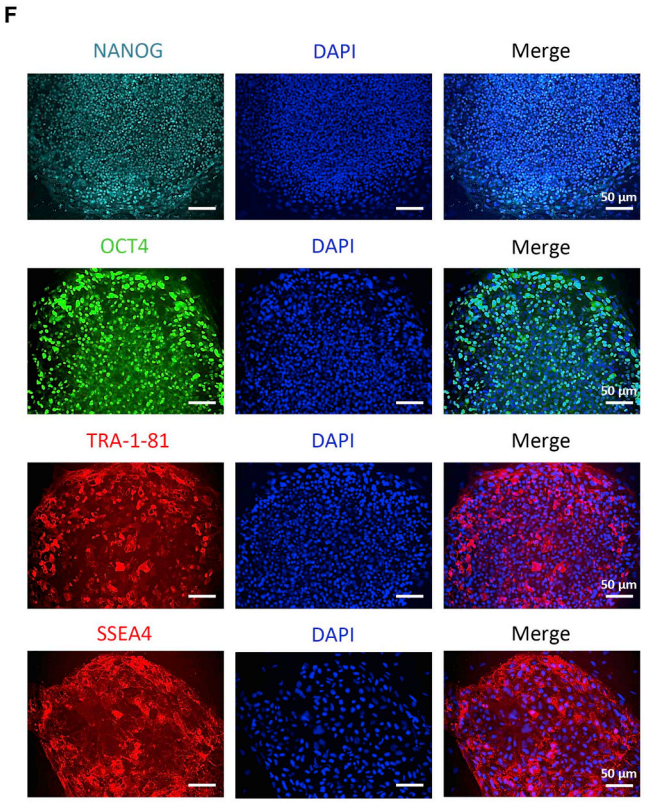


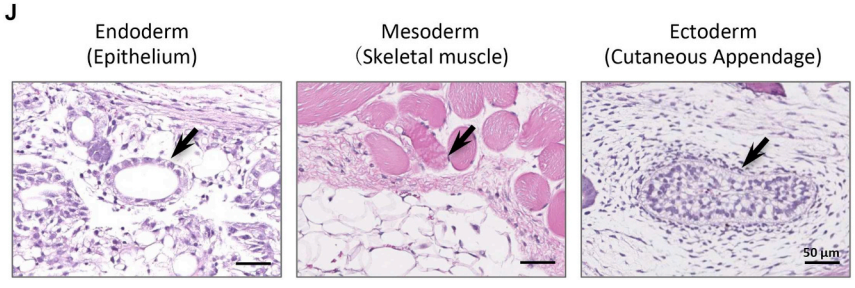


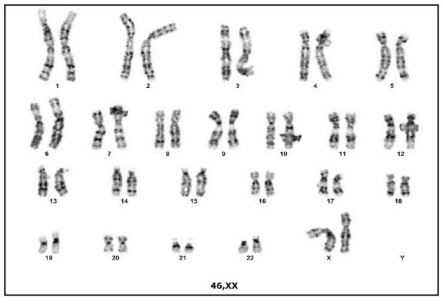












(caption on next page)

Fig. 1. Establishment and characterization of the heterozygous p53(R249S) mutant human embryonic stem cell line H9-p53(WT/R249S). (A) Schematic diagram of the gene targeting strategy for p53(R249S) generation by TALEN. (B) PCR reveals precise homologous recombination and FNF cassette insertion in *TP53* genome loci. (C) PCR confirms removal of the FNF cassette. (D) Sequencing results of the heterozygous p53(R249S) mutation in the H9-p53(WT/R249S) cell line. (E) Cell morphology and AP staining shows typical stem cell characteristics of the H9-p53(WT/R249S) cell line. Scale bar, 50 μm. (F) Immunofluorescence staining indicates the H9-p53(WT/R249S) cell line expresses pluripotency markers NANOG, OCT4, SSEA4 and TRA-1-81. Scale bar, 50 μm. (G) qRT-PCR assay shows comparable expression levels of endogenous pluripotency genes *SOX2*, *DPPA4*, *REX1*, and *TERT* in the H9-p53(WT/R249S) cell line to those of H9 cells. (H) Immunoblotting indicates p53 expression in the H9-p53(WT/R249S) cell line is similar to that in H9 cells. (I) Mycoplasma testing confirms that the H9-p53(WT/R249S) cell line is mycoplasma-free. (J) *In vivo* teratoma assay demonstrates that the H9-p53(WT/R249S) cell line is capable of differentiating into endodermal (epithelium), mesodermal (skeletal muscle) and ectodermal (cutaneous appendage) lineages. Scale bar, 50 μm. (K) Karyotype analysis shows a normal chromosomal complement in the H9-p53(WT/R249S) cell line.

Cyclers (Analytik Jena).

Immunofluorescent staining

Cells were fixed by incubation with 4% paraformaldehyde in 1 × DPBS for 10 min at room temperature. After 3 washes with 1 × DPBS, cells were blocked and permeabilized by incubation with 10% serum in 0.1% DPBST for 30 min at room temperature. Then cells were incubated with indicated primary antibodies (Table 2) at 4 °C overnight. After 3 washes with 1 × DPBS, cells were incubated with corresponding secondary antibodies at room temperature for 1 h and then stained with 3 μM DAPI (Thermo Fisher) at room temperature for 3 min. The staining results were imaged by Leica DMi8.

qRT-PCR

Total mRNA was isolated using TRIzol (Invitrogen) by the manufacturer's protocol. 1 μg mRNA was used for reverse transcription using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using a CFX96 machine (Bio-Rad). The 20 μl PCR solution is composed of 10 μl SYBR Green PCR Master Mix, 1 μl of 10 μM forward and reverse primers, 1 μl cDNA with a concentration of about 400 ng/μl and 7 μl H_2O. All reactions were run in triplicate and normalized to GAPDH expression. Primer sequences were listed in Table 1.

Immunoblotting

Immunoblotting was performed as described (Zhao et al., 2011).

Mycoplasma test

Regular testing for mycoplasma was performed using PCR mycoplasma test kit (abm G238) following the manufacturer's instructions.

Karyotype analysis

Standard G-band karyotype analysis was performed in the Genetics/Genomics Division, Sonora Quest Laboratories Core Facility in Tempe, Arizona. Twenty metaphase chromosome spreads were classified with G-band resolution of 400. Karyotypes were analyzed according to the International System for Human Cytogenetic Nomenclature (ISCN).

In vivo teratoma formation assay

2×10^7 H9-p53(WT/R249S) cells were harvested and resuspended in 100 μl of ice cold 1 × DPBS. Then the cells were mixed with an equal volume of thawed phenol red-free Matrigel (Corning) and maintained on ice until the time of injection. The cell/Matrigel mixture was then loaded into a 1 ml syringe and injected subcutaneously into both sides of flanks of immunocompromised *NU/NU* nude mice (Charles River), with 100 μl cell mixture each side. The mice were monitored weekly and the teratoma was excised 6 weeks after injection, fixed in 10% formalin, embedded and processed for H&E immunohistochemical detection of three germ layer markers by HistoWiz (Brooklyn, NY).

STR analysis

H9 and H9-p53(WT/R249S) cells were authenticated using STR analysis performed by the Characterized Cell Line Core Facility in The University of Texas M.D. Anderson Cancer Center. 14 STR loci of H9-p53(WT/R249S) were analyzed and compared with the STR profile of parental H9 cells (Supplementary Table S1).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.101360.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel E
Phenotype	Qualitative analysis immunocytochemistry	NANOG, OCT4, SSEA4, TRA-1-81 and AP-positive	Fig. 1 panel E, F
	Quantitative analysis RT-qPCR	Lower levels of expression of NANOG and OCT4 compared with H9	Fig. 1 panel G
Genotype	Karyotype (G-banding) and resolution	46 XX, Resolution 400	Fig. 1 panel K
Identity	Microsatellite PCR (mPCR) OR	DNA Profiling not performed	N/A
	STR analysis	14 sites tested, all sites matched human embryonic stem cell line H9	Submitted in archive with journal
Mutation analysis (if applicable)	Sequencing	Heterozygous R249S mutation of TP53.	Fig. 1 panel D
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR: Negative	Fig. 1 panel I
Differentiation potential	Teratoma formation	Proof of three germlayers formation	Fig. 1 panel J
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

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 cat# 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 Blotting)	Rabbit anti-p53 (7F5)	1:1000	Cell signaling technology cat# 2527 RRID:AB_331211
β-ACTIN(Western Blotting)	Mouse anti-β-ACTIN	1:5000	Proteintech group cat# 66009–1-Ig RRID:AB_2687938
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
Housekeeping genes (qPCR)	*GAPDH*	CCACTCCTCCACCTTTGAC/ACCCTGTTGCTGTAGCCA
Targeted mutation analysis/ sequencing	p53_5FM13	TGTAAAACGACGGCCAGTCTAGCTCGCTAGTGGGTTGC
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_7FM13	TGTAAAACGACGGCCAGTGCCTCCCCTGCTTGCCACAG
Targeted mutation analysis/ sequencing	p53_7RM13	CAGGAAACAGCTATGACCGGGAGCAGTAAGGAGATTCC
Targeted mutation analysis/ sequencing	5p53-L001–1 kb/EcoRI for left homologous arm	GAATTCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAG
Targeted mutation analysis/ sequencing	3p53-L001–1 kb/EcoRI for left homologous arm	GAATTCAGGCCAGTGTGCAGGGTGGCAAGTGGCTCCTGACCT
Targeted mutation analysis/ sequencing	5p53-R001–1 kb/BamHI for right homologous arm	GGATCCGCTGTGCCCCAGCCTCTGCTTGCCTCTGACCCCTGG
Targeted mutation analysis/ sequencing	3p53-R001–1 kb/NotI for right homologous arm	GCGGCCGCCAGGCTAGGCTAAGCTATGATGTTCCTTAGATTAGG
Targeted mutation analysis/ sequencing	5p53-R249S for R249S mutagenesis	TGCATGGGCGGCATGAACCGGAGTCCCATCCTCACCATC
Targeted mutation analysis/ sequencing	3p53-R249S for R249S mutagenesis	GATGGTGAGGATGGGACTCCGGTTCATGCCGCCCATGCA

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