Efficient Modulation of TP53 Expression in Human Induced Pluripotent Stem Cells

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TP53 point mutations are found in 50% of all cancers and seem to play an important role in cancer pathogenesis. Thus, human induced pluripotent stem cells (hiPSCs) overexpressing mutant TP53 are a valuable tool for the generation of in vitro models of cancer stem cells or for in vivo xenograft models. Here, we describe a protocol for the alteration of gene expression in hiPSCs via overexpression of a mutant form of the *TP53* (R249S) gene using lentiviral transduction. A high amount of TP53 protein is detected 1 week after transduction and antibiotic selection. Differentiation of transduced hiPSCs gives insight into better understanding cancer formation in different tissues and may be a useful tool for genetic or pharmacologic screening assays. © 2019 The Authors.

Basic Protocol 1: Production and concentration of third-generation lentivirus
Support Protocol 1: Cloning of gene of interest into modulation vector
Support Protocol 2: Preparation of DMEM GlutaMAX™ with 10% fetal bovine serum and 1% penicillin-streptomycin
Basic Protocol 2: Transduction of human induced pluripotent stem cells and selection of positively transfected cells
Support Protocol 3: Preparation of Matrigel®-coated plates
Support Protocol 4: Preparation of mTeSR™1 medium

Keywords: hiPSC • lentiviral transduction • molecular alteration • TP53

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INTRODUCTION

Due to their infinite availability, human induced pluripotent stem cells (hiPSCs) offer the opportunity to perform unlimited numbers of in vitro experiments. Further, hiPSC differentiation into a desired tissue allows studying physiologic processes in human-relevant cell systems. By introducing disease-specific mutations, these cells allow for studying genetic origins of disease in a tissue-specific manner with those cells. Most tumors have TP53 mutations. Among the six hot-spot mutations of *TP53*, TP53 R249S (exchange of

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arginine for serine) is a point mutation, which causes a structural change of the protein and thereby enhances cell proliferation (Bullock & Fersht, 2001; Friedler et al., 2004; Kollareddy et al., 2015). Genetic manipulation of cells by lentiviral transduction introduces mutations in a stable and efficient manner (Naldini et al., 1996). Here, we apply this procedure to hiPSCs using a vector containing mutated TP53 (Rapti et al., 2015; Zare et al., 2016).

In this article, we report two basic protocols that have been enlisted that describe the process of hiPSC lentiviral transduction to generate genetically modified stable cell lines. In summary, Basic Protocol 1 describes the production of third-generation lentivirus with HEK 293T cells, and Basic Protocol 2 describes the generation of TP53 R249S transduced cells with the use of a lentiviral vector.

NOTE: hiPSCs are grown in standard laboratory conditions, in a humidified incubator (37°C, 5% CO_2).

NOTE: Virus production and transduction of hiPSCs requires a biosafety level 2 (BSL2) laminar flow cabinet.

NOTE: All solutions and equipment must be sterile if they come in contact with cells.

BASIC PROTOCOL 1

PRODUCTION AND CONCENTRATION OF THIRD-GENERATION LENTIVIRUS

This protocol is used for the production of concentrated third-generation lentivirus. Concentrated lentivirus is produced to transduce cells (e.g., hiPSCs). All work in our laboratory is performed in BSL2 facility. However, the level of biosafety containment should be in accordance with institutional and governmental guidelines. A proper risk assessment must be done prior to beginning the work. The equipment and laminar flow hood used must be sterilized in accordance with your local rules on risk assessment when working with lentivirus.

Materials

HEK 293T cells
Dulbecco's modified Eagle medium (DMEM) GlutaMAX™ containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (see Support Protocol 2)
DMEM (e.g., Thermo Fisher Scientific, cat. no. 41965039)
Plasmid DNA
Third-generation lentivirus plasmids:
  pMDLg/pRRE (Addgene #12251; see Fig. 1)
  pRSV-REV (Addgene #12253; see Fig. 2)
  pMD2.G (Addgene #12259; see Fig. 3)
FuGENE® HD Transfection Reagent (e.g., Promega, cat. no. 231A)
Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (e.g., Thermo Fisher Scientific, cat. no. 14190094)
DMEM GlutaMAX™ containing 10% (v/v) FBS (e.g., Sigma-Aldrich, cat. no. F7524)
50% (v/v) polyethylene glycol (PEG) in distilled, deionized water
1.5 M NaCl, prepared in distilled, deionized water

10-cm plastic dishes (e.g., Greiner Bio-One, cat. no. 664160)
Cell culture incubator (37°C, 5% CO_2)
0.5- and 1.5-ml microcentrifuge tube
15-ml conical tube

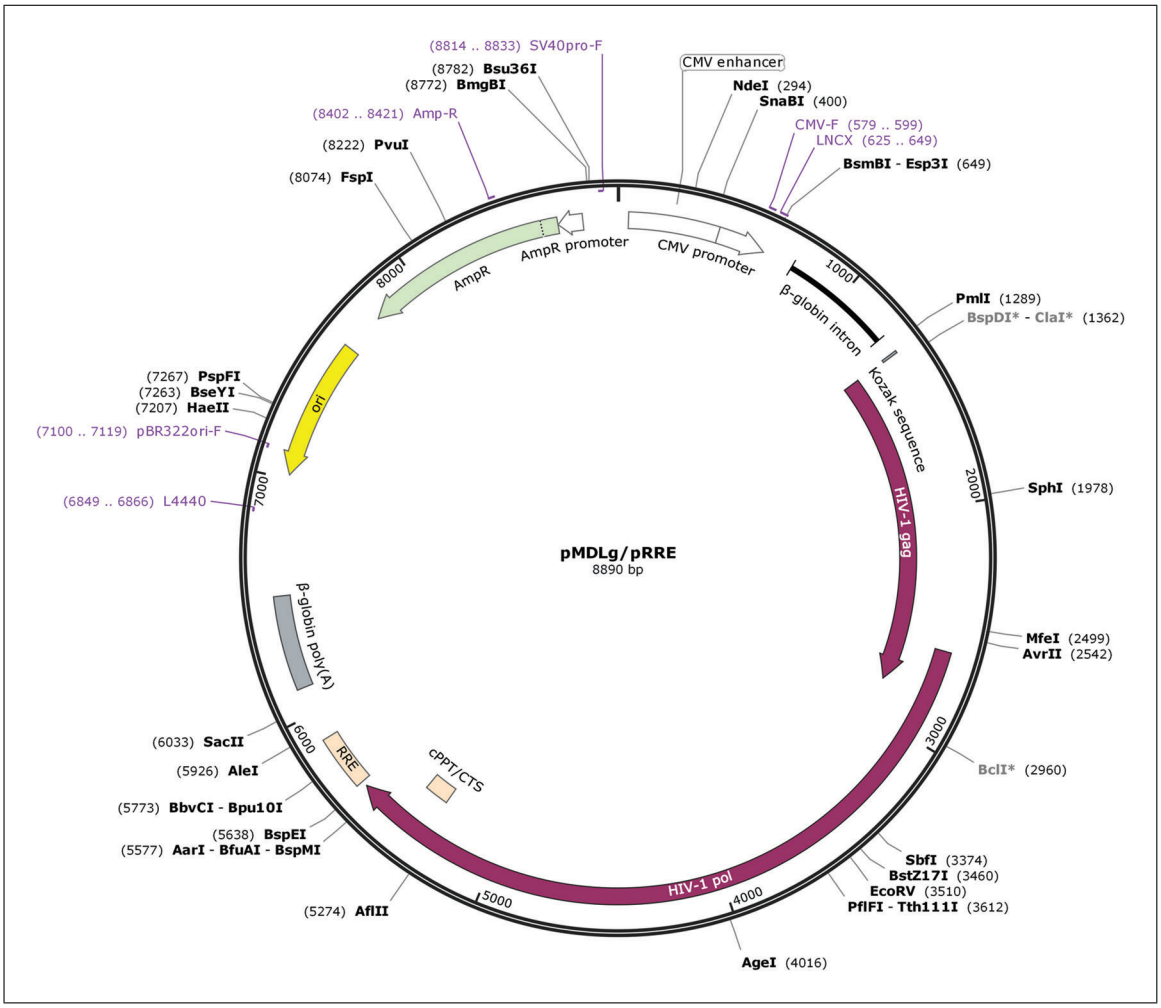

Figure 1 Vector map of the third-generation lentiviral packaging plasmid pMDLg/pRRE (Addgene #12251).

10-ml syringe
0.45-µm syringe filter (e.g., VWR, cat. no. 514-0063)
Vortex mixer
Refrigerated centrifuge (e.g., Hettich® Rotina 38R with fixed-angle rotor, cat. no.1792)

Preparation

1. Plate HEK 293T cells in a 10-cm dish with 10 ml DMEM GlutaMAX™ containing 10% FBS and 1% penicillin-streptomycin.

   HEK 293T cells used in this protocol were kindly provided by Dr. Astrid Weyerbrock, Department of Neurosurgery, University Hospital Freiburg, Freiburg, Germany.

Day 0

2. Allow cells to reach 80% to 90% confluency. Prepare infection solution in a 1.5-ml microcentrifuge tube in the following order:

   800 µl DMEM
   8 µg plasmid DNA
   4 µg pMDLg/pRRE
   2 µg pRSV-REV
   2 µg pMD2.G
   60 µl FuGENE® HD.

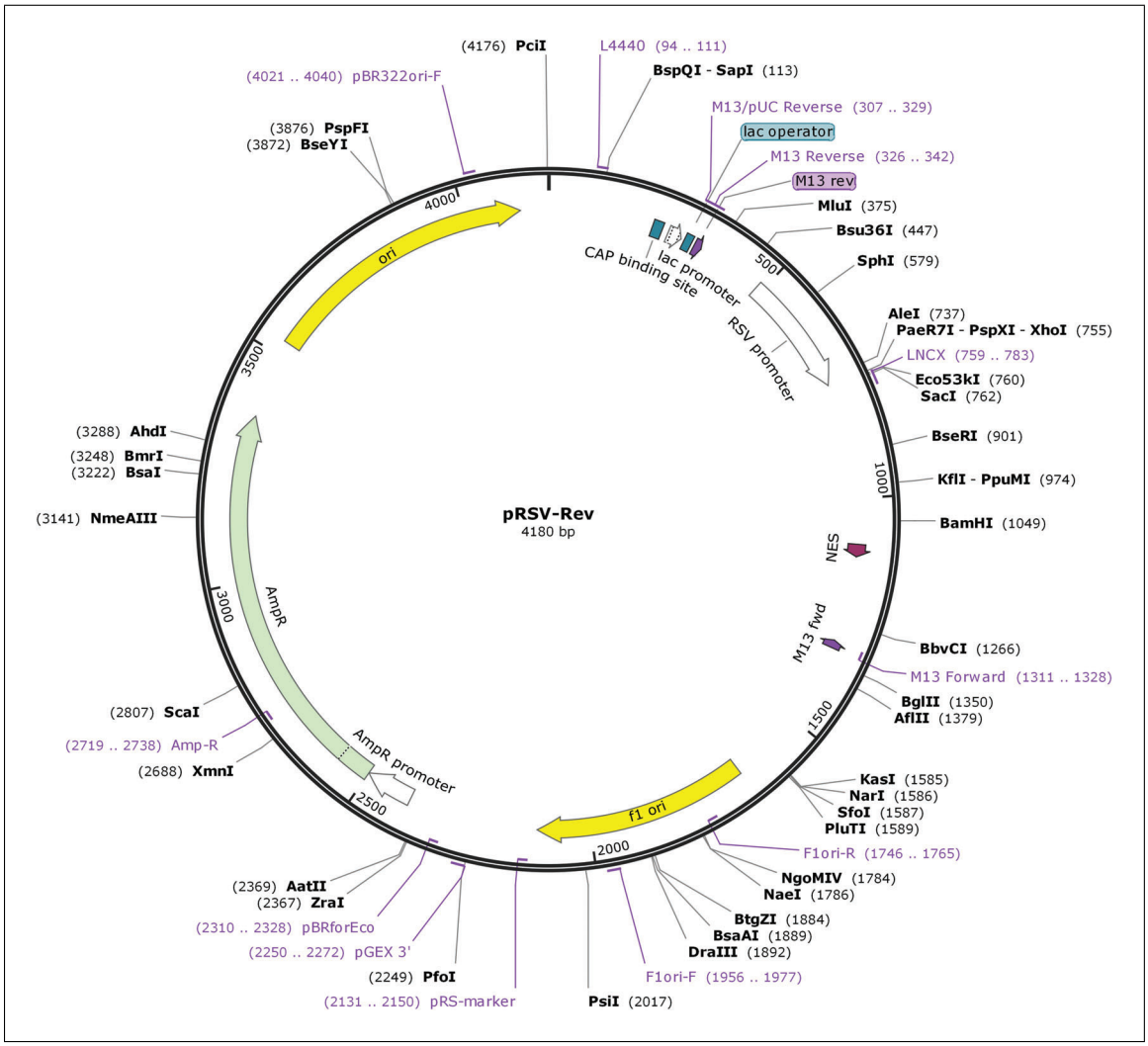

Figure 2 Vector map of the third-generation lentiviral packaging plasmid pRSV-Rev (Addgene #12253).

3. Pipet solution up and down, and incubate for at least 10 min at room temperature.
4. Meanwhile, wash HEK 293T cells with DPBS.
5. Add 10 ml DMEM GlutaMAX^{TM} containing 10% FBS without antibiotics to each dish.
6. After incubation, add infection solution dropwise to each dish. Move dish gently to distribute solution, and incubate at 37°C with 5% CO_2.

Day 1

7. Remove supernatant and add 10 ml DMEM GlutaMAX^{TM} containing 10% FBS and 1% penicillin-streptomycin.

Days 2, 3, and 4

8. To a 15-ml conical tube, add 1 ml of 50% PEG. Separately, connect syringe with a 0.45-µm filter. Pull the plunger out of the syringe out, and store it sterile in its packaging.
9. Fill syringe with supernatant, and filter supernatant into the 15-ml conical tube containing PEG by pushing the plunger.
10. Throw away syringe and filter.

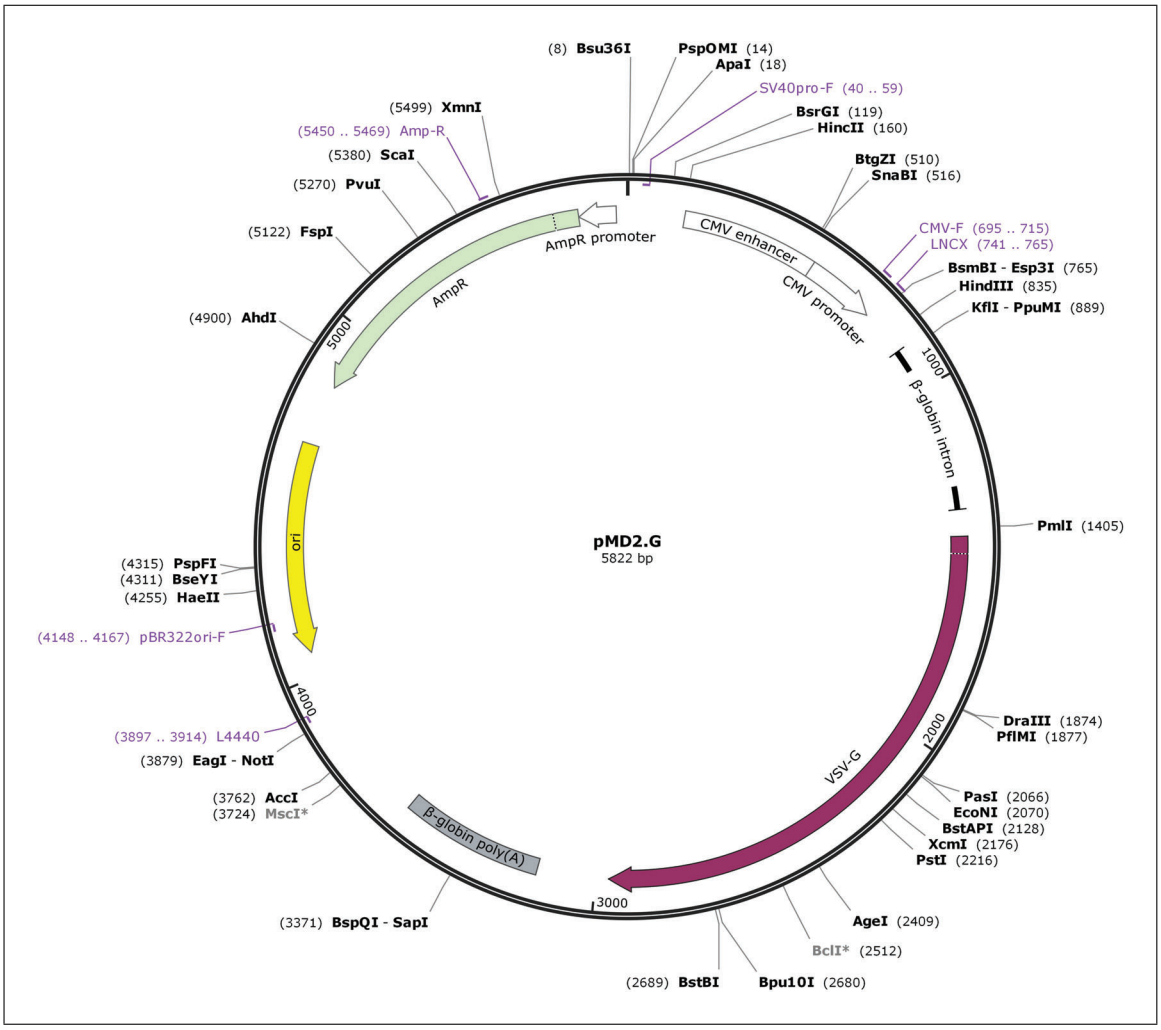

Figure 3 Vector map of the VSV-G envelope–expressing plasmid (Addegne #12259).

11. Add 10 ml DMEM GlutaMAX™ containing 10% FBS and 1% penicillin-streptomycin to dish.
12. Add 1.2 ml of 1.5 M NaCl to each conical tube.
13. Mix solution containing supernatant, PEG, and NaCl well by vortexing or shaking, and store at 4°C until further use on day 5.

Day 5

14. Precool centrifuge to 4°C.
15. Centrifuge conical tubes (from step 13) 30 min at 7000 × *g*, 4°C.
16. Check if a pellet is visible.

    At times, it might be difficult to see the pellet due to the contrast. The pellet from day 3 might be smaller than from the days before.

17. After centrifugation, place tubes on ice.
18. Remove supernatant under sterile conditions, being careful not to disturb the pellet. Aspirate remaining supernatant gently with a pipette tip.
19. Resuspend pellets from all three tubes (days 2, 3, and 4) to a final volume of 400 µl DPBS (1:75 dilution of original sample volume).

20. Prepare 40-µl aliquots in 1.5-ml or 0.5-ml microcentrifuge tubes.

In order to maintain efficiency, avoid continuously freezing and thawing the virus by preparing ready-to-use aliquots.

21. Store aliquots at −80°C or use immediately.

SUPPORT PROTOCOL 1

CLONING OF GENE OF INTEREST INTO MODULATION VECTOR

This protocol describes the cloning procedure for inserting the TP53 R249S point mutation into a vector by a restriction enzyme–based process, leading to overexpression of the TP53 variant. The plasmid backbone is based on pSin-Ef2-Nanong-Pur (Addgene #16578; Fig. 4) and was modified with an EGFP tag at the N-terminal end, multiple cloning site, and removal of the Nanog gene sequence.

The free software SnapGene Viewer (GSL Biotech; available at *https://www.snapgene.com/*) was used to prepare the virtual cloning file and to design the oligomers. After cloning and purification of the plasmid from *E. coli*, the plasmid can be used for virus production.

Materials

Template for gene sequence: pLenti6/V5-p53_R249S (Addgene #22935)
Phusion high-fidelity DNA polymerase and buffer (e.g., New England Biolabs, cat. no. M0530L)
10 mM dNTP mix

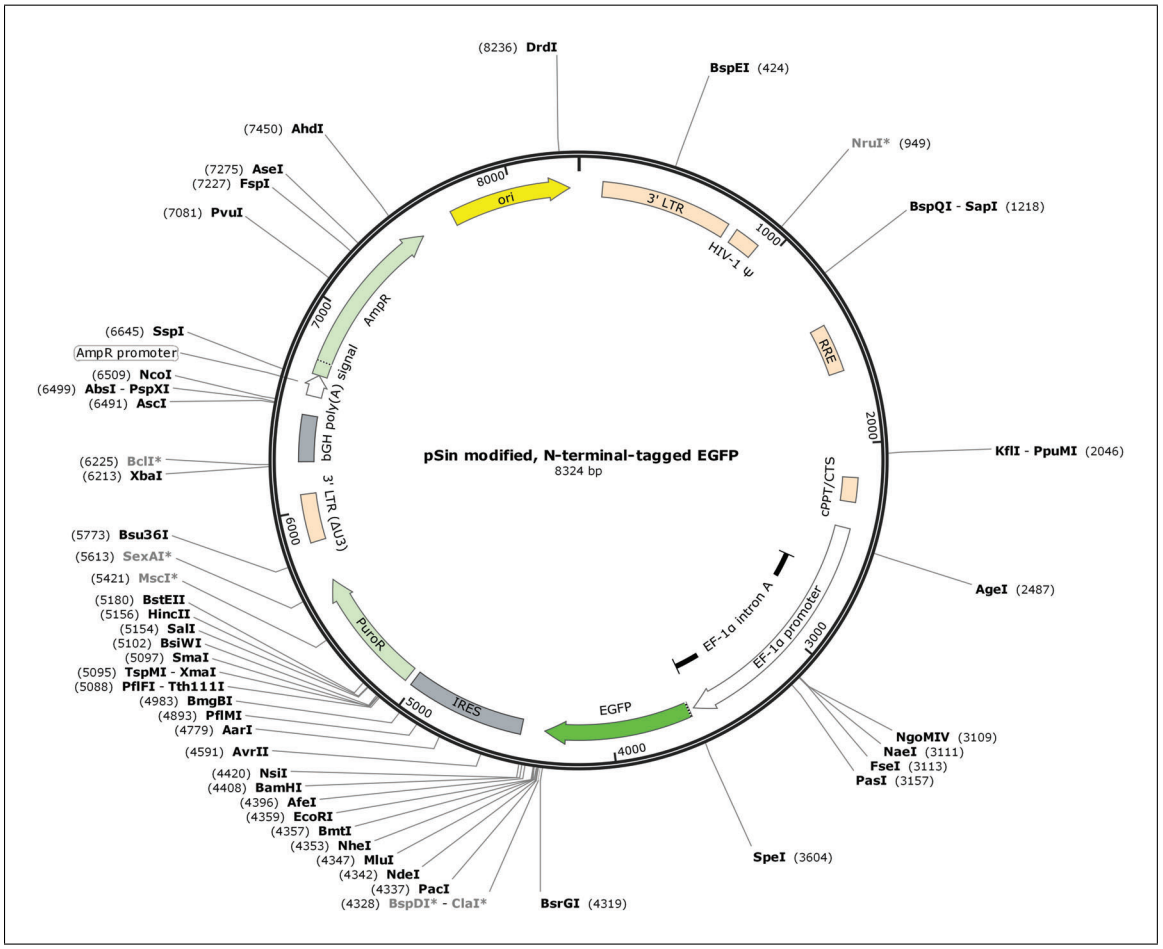

Figure 4 Vector map of the modified pSin vector containing N-terminal-tagged EGFP and based on the original pSin-EF2-Nanong-Pur vector (Addgene #16578). The vector is used for cloning the gene of interest.

10 µM TP53 forward oligomer: 5'-CCTTAATTAAAATGGAGGAGCCGCAGTCA-3'
10 µM TP53 reverse oligomer: 5'-GGAATTCCATATGTCAGTCTGAGTCAGGCCCTT-3'
DNA containing gene of interest (GOI)
Agarose
1× Tris-acetate-EDTA (TAE) buffer
SYBR® Safe DNA gel stain (e.g., Thermo Fisher Scientific, cat. no. S33102)
6× DNA gel loading dye (e.g., Thermo Fisher Scientific, cat. no. R0611)
1-kb DNA ladder (e.g., Thermo Fisher Scientific, cat. no. SM1331)
E.Z.N.A.® Cycle Pure Kit, V-spin (e.g., Omega Bio-Tek, cat. no. D6492)
10× CutSmart® Buffer (e.g., New England Biolabs, cat. no. B7204S)
NdeI restriction enzyme (e.g., New England Biolabs, cat. no. R0111)
PacI restriction enzyme (e.g., New England Biolabs, cat. no. R0547)
pSin-EF2 modulation vector (based on vector pSin-EF2-Nanog-Pur; Addgene #16578; see Fig. 4)
E.Z.N.A.® Gel Extraction Kit, V-spin (e.g., Omega Bio-Tek, cat. no. D2500)
10× T4 DNA ligase and buffer (e.g., New England Biolabs, cat. no. M0202)
TOP10 chemically competent cells (e.g., New England Biolabs, cat. no. C3019)
LB medium with and without 100 µg/ml ampicillin
10-cm LB agar plates with 100 µg/ml ampicillin (e.g., Applichem, cat. no. A0839)
Glycerol
E.Z.N.A.® Plasmid Mini Kit I, V-spin (e.g., Omega Bio-Tek, cat. no. D6943)
NucleoBond® Xtra Midi/Maxi (e.g., Machery-Nagel, cat. no. 740410)

Computer running SnapGene Viewer (available at *https://www.snapgene.com/*)
PCR reaction tubes
Thermal cycler
Spectrophotometer capable of measuring DNA concentration
1.5-ml microcentrifuge tube
16°C and 37°C incubators, with shaking capabilities
UV transilluminator
Scalpel
42°C heating block
Laboratory shaker
Parafilm® M (e.g., Bemis Company, cat. no. PM996)
15-ml conical tubes
Cyrovials

Preparation

Design plasmid and DNA oligomers

1. Choose a restriction site located at the multiple cloning site that cuts the vector only once. These enzymes should not cut your GOI. Use a DNA documentation program such as SnapGene Viewer to plan the cloning. To maintain the reading frame, bases might have to be added between the restriction site and the GOI. It is possible to use any base, but take care that no stop codon is generated.

   We recommend adenosine or thymidine to have a lower melting temperature.

2. Check which restriction enzymes cut your vector only once and not your GOI. If you have chosen two enzymes, check the efficiency of both enzymes in the restriction buffer, and if possible, use the same buffer for both enzymes.

   You can use the following website for help: https://nebcloner.neb.com/#!/redigest.

3. Design oligomers for cloning, which include the restriction site, additional bases, and around 20 to 30 bases of your GOI. Use the table from New England Biolabs, and add bases according to the restriction enzyme (*https://www.neb.com/-/media/nebus/files/chart-image/cleavage_olignucleotides_old.pdf?la=en*).

PCR amplify gene of interest

4. As a template for the GOI, use either a plasmid or cDNA.

   In our case, we used the pLenti6/V5-p53-R249S plasmid (Addgene #22935).

5. In PCR reaction tubes, set up a PCR as follows (50 µl total PCR volume):

   32.5 µl water
   10 µl of 5× Phusion buffer
   1 µl of 10 mM dNTP mix
   2.5 µl of 10 µM TP53 forward oligomer
   2.5 µl of 10 µM TP53 reverse oligomer
   2 µl DNA
   0.5 µl Phusion polymerase.

   In the case of cDNA, plasmid DNA, and genomic DNA, dilute to 50 to 100 ng.

   Use a high-fidelity polymerase with proofreading function to get a high accuracy of your GOI. This is especially important if you have a gene with a point mutation.

6. Run PCR using a thermal cycler and the following conditions:

1 cycle	30 s	98°C	(initial denaturation)
29 cycles	10 s	98°C	(denaturation)
	30 s	variable	(annealing)
	30 s per kb	72°C	(extension)
1 cycle	10 min	72°C	(final extension)
Final step	indefinitely	4°C	(hold).

   Use 60°C for the annealing temperature for the first time. If 60°C does not work, try a temperature gradient to detect the optimal annealing temperature.

7. Prepare a 1% (w/v) agarose gel with 1× TAE and SYBR® Safe (1:10,000 dilution) to assess PCR amplification. Run 5 µl sample mixed with 6× DNA gel loading dye. Use a 1-kb DNA ladder to identify the size of your GOI. Run gel at 130 V for 15 min.

8. Purify PCR products with a positive result using the E.Z.N.A.® Cycle Pure Kit following the manufacturer's instructions.

9. Elute sample in 25 µl water. To achieve a higher concentration, prewarm water to 50°C. Measure concentration with a spectrophotometer.

   The concentration of the sample should vary between 10 and 50 ng/µl.

10. Freeze sample at −20°C, or proceed with digestion.

Digest PCR product and vector

11. Prepare each digestion in a 1.5-ml microcentrifuge tube as follows (30 µl total volume):

    24 µl purified PCR product
    3 µl of 10× CutSmart® Buffer
    1 µl restriction enzyme NdeI
    1 µl restriction enzyme PacI
    1 µl water.

12. Prepare digestion of modulation vector as follows (20 µl total volume):

    2 µg undigested pSin-EF2 modulation vector
    2 µl of 10× CutSmart® Buffer
    1 µl restriction enzyme NdeI
    1 µl restriction enzyme PacI
    Bring to 20 µl with water.

13. Incubate both digestions overnight at 37°C. Do not incubate longer than 15 to 16 hr.

14. Prepare a 1% (w/v) agarose gel with 1× TAE and SYBR® Safe (1:10,000 dilution). Use a comb with a 30-µl volume capacity in order to load the entire digest.

15. Mix whole digestion with 6× loading dye, and load on gel. As a control, load 200 ng undigested vector. Use a 1-kb DNA ladder as a reference for the sample size. Leave one empty well between samples and DNA ladder to prevent contamination.

16. Perform electrophoresis at 130 V for 15 min.

17. Use a UV transilluminator to visualize bands of digestion. Wear protective equipment to protect from UV light.

18. Cut out respective band with a scalpel, and place gel containing GOI in a prelabeled microcentrifuge tube.

19. Purify sample using the E.Z.N.A.® Gel Extraction Kit according to manufacturer's instructions.

20. Measure concentration.

21. Freeze sample at −20°C, or proceed with ligation.

Ligate GOI and modulation vector

22. Calculate amount of insert required for the ligation reaction using the free calculator available at *http://www.insilico.uni-duesseldorf.de/Lig_Input.html*. Use the following details for calculation:

    Vector size: 8324 bp
    Vector amount: 50 ng
    Insert size: 1182 bp
    Molar ratio: 1:3.

    If the insert size is more than 4 kbp use a molar ratio of 1:3 and 1:5 in the software.

23. Prepare ligation as follows (10 µl total volume):

    50 ng digested vector
    21.3 ng digested insert
    1 µl of 10× T4 DNA ligase buffer
    1 µl T4 DNA ligase
    Bring to 10 µl with water.

24. Prepare ligation control as follows (10 µl total volume):

    50 ng digested vector
    1 µl of 10× T4 DNA ligase buffer
    1 µl T4 DNA ligase
    Bring to 10 µl with water.

25. Incubate ligation mixture overnight at 16°C. Do not incubate longer than 16 hr.

26. The next day, proceed with transformation.

Transform

27. Let TOP10 chemically competent cells thaw on ice for 10 min. Use one vial of competent cells per ligation reaction.
28. Add 10 µl ligation mixture to chemically competent cells in a 1.5-ml microcentrifuge tube. Mix gently by tapping tube.
29. Incubate on ice for 10 min.
30. Place reaction tube in a 42°C heating block for 1 min (heat shock).
31. Stop reaction by placing cells on ice for 2 min (cold shock).
32. Add 300 µl LB medium without antibiotics.
33. Shake at 37°C for 40 min at 800 rpm.
34. Spread 150 µl cells on 10-cm LB agar plates containing ampicillin.
35. Incubate plate overnight at 37°C. Do not incubate longer than 16 hr.

    The ligation control should technically have no clones. The ligation control is done to determine the experimental background. If colonies grow on the control plate, it might be undigested or contain religated vector. There should be no or a significantly lower number of colonies on the ligation control plate compared with the ligation plate of your GOI.

36. Wrap plate with Parafilm® M, and store at 4°C until further use.

Analyze plasmid

37. Pick 3 to 5 clones from each plate, and individually add to 15-ml conical tubes containing 5 ml LB medium with ampicillin.

    For each tube pick a separate colony (e.g., with a sterile pipet tip).

38. Incubate culture overnight at 37°C with shaking at 250 rpm. Do not incubate longer than 16 hr.
39. Prepare a glycerol stock of the cultures in a cryovial by adding 500 µl culture to 500 µl glycerol. Store at −80°C for further use.
40. Perform a mini-prep with the remaining culture using the E.Z.N.A.® Plasmid Mini Kit I following manufacturer's instructions.
41. Perform a digest of plasmid to control integration of the insert. As a control, digest 200 to 300 ng plasmid as described above. Stop digestion after 2 hr.
42. Check digestion on agarose gel to see whether two bands of the correct size are visible. Discard any plasmids that do not contain inserts or have fragments of the wrong size.
43. Prepare sample for sequencing according to requirements.

    Check how many micrograms of vector DNA are required by the chosen sequencing company and if the primer should be added or sent separately. Inform yourself how long the sequenced area is or if different lengths can be booked. For sequencing, you can use the forward or reverse primer from the PCR amplification. If you have a point mutation in your GOI, make sure the sequenced area covers the mutation. If the forward or reverse primer does not cover the point mutation, you can design your own sequencing primer. Choose a site around 20 to 30 bp before or after your point mutation because sequencing is poor at the beginning. The primer should have a length of ∼20 bp.

44. Compare sequencing results with your own vector, which contains the GOI. If it matches, proceed with the next step.

45. Prepare a midi- or maxi-prep from the positive plasmid to obtain enough material for virus production.

    We recommend using a NucleoBond® Xtra Midi/Maxi Kit for plasmid purification and following manufacturer's instructions.

46. Measure concentration.

47. Freeze plasmid at −20°C until further use.

PREPARATION OF DMEM GlutaMAX™ WITH 10% FETAL BOVINE SERUM AND 1% PENICILLIN-STREPTOMYCIN

SUPPORT PROTOCOL 2

This protocol describes the preparation of DMEM GlutaMAX™ containing a final concentration of 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin for culturing HEK 293T cells.

Materials

FBS
DMEM GlutaMAX™
Penicillin-streptomycin
0.45-µm bottle-cap filter

1. Under sterile conditions, filter 50 ml FBS with a 0.45-µm bottle-cap filter. Add 445 ml DMEM GlutaMAX™.

2. Mix medium by swirling flask.

3. Remove 10 ml per 10-cm dish to be used later for virus preparation, and store at 4°C.

4. Add to the remaining medium the appropriate amount of penicillin-streptomycin. Store medium at 4°C.

TRANSDUCTION OF HUMAN INDUCED PLURIPOTENT STEM CELLS AND SELECTION OF POSITIVELY TRANSFECTED CELLS

BASIC PROTOCOL 2

This protocol can be used for transduction of hiPSCs with highly concentrated third-generation lentivirus. All work should be performed in accordance with the appropriate biosafety containment level, which is determined by institutional and governmental guidelines. The equipment and laminar flow hood used must be sterilized in accordance with local rules and risk assessment with regard to use of lentivirus.

Materials

hiPSC (e.g., IMR90-04; WiCell Research Institute)
mTeSR™1 culture medium with and without puromycin (see Support Protocol 4)
0.5 mM EDTA in DPBS
Highly concentrated lentivirus (see Basic Protocol 1)

Matrigel®-coated 12-well plate (see Support Protocol 3)
Cell culture incubator

1. Culture hiPSCs in a Matrigel®-coated 12-well plate using mTeSR™1 culture medium.

   For a detailed description of culturing hiPSCs, see Chen (2012).

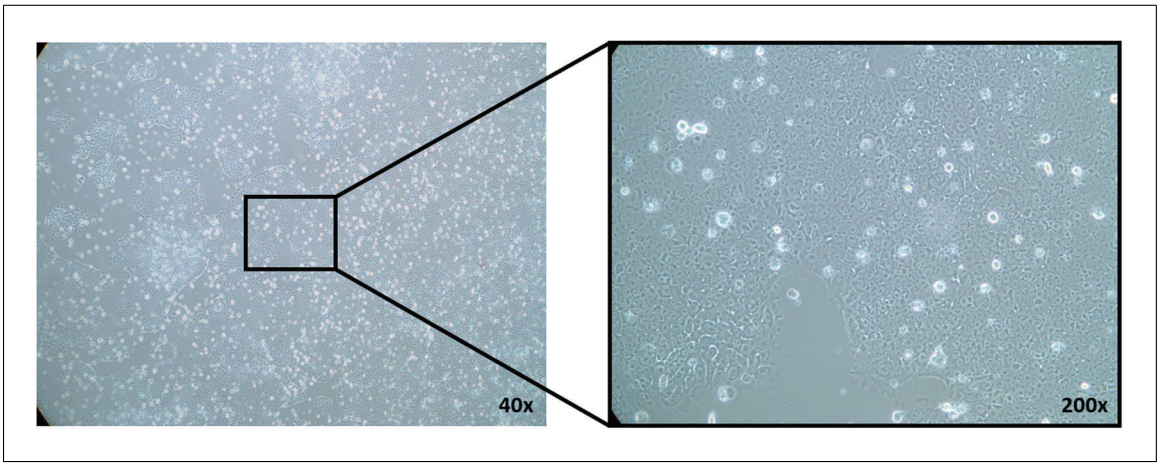

Figure 5 IMR90-04 cells on the day of transduction. Cells should show a similar confluency of 60% to 80%.

2. Split hiPSCs with 0.5 mM EDTA.
3. When cells reach 60% to 80% confluency (Fig. 5), change medium (1 ml per well), and add 1 vial (40 µl) concentrated virus to each well.
4. The next day, remove 500 µl supernatant, and add 500 µl fresh medium.
5. After 48 hr, remove all medium, and add 1 ml fresh medium.
6. Perform antibiotic selection after 72 hr using culture medium containing 1 µg/ml puromycin.

   The concentration of the antibiotic should be determined beforehand by performing a killing curve for each cell line. After 2 days of selection, it is possible to see if cells survive. Cells that do not survive selection will detach.

7. Split cells at least once in a plate containing medium with puromycin to achieve a more efficient selection.

   You can use a 6-well plate to expand the cells. On the next day, you can check if cells have attached and survived the splitting.

8. After 1 week stop antibiotic selection, and culture cells without antibiotics.
9. Expand cell culture, and use for assays.

SUPPORT PROTOCOL 3

PREPARATION OF MATRIGEL®-COATED PLATES

This protocol explains in short how to prepare Matrigel®-coated 6- or 12-well plates.

Materials

Matrigel®, hESC-qualified matrix (e.g., Corning, cat. no. 354277)
KnockOut DMEM (e.g., Invitrogen, cat. no. 10829018)

15-ml conical tubes
12-well plate (e.g., Greiner Bio-One, cat. no. 657160)
6-well plate (e.g., Greiner Bio-One, cat. no. 665180)
Parafilm® M (e.g., Bemis Company, cat. no. PM996)

1. Thaw Matrigel® and precool pipet tips to 4°C overnight.
2. For an even distribution, gently swirl Matrigel®, and keep on ice at all times.

3. Dilute 10 ml Matrigel® with 10 ml KnockOut DMEM. Gently pipet up and down with a precooled pipet. Prepare 0.5-ml aliquots in 15-ml conical tubes, and store at −20°C until further use.
4. To coat a plate, retrieve an aliquot from the freezer. Under sterile conditions, add an additional 1 ml KnockOut DMEM to tube, and allow to thaw.
5. Mix tube well by inverting.
6. Add 13.5 ml KnockOut DMEM, and gently mix by pipetting up and down. Prevent air bubbles and ensure everything is mixed.
7. Add 0.5 ml Matrigel®-containing solution to each well of a 12-well plate or 1 ml for a 6-well plate.
8. Swirl plate for an even distribution, and ensure the entire surface is covered. Seal plate with Parafilm® M, and incubate at room temperature for 1 hr.

   Plates can be stored at 4°C for up to 2 weeks.

   Under sterile conditions, Matrigel® solution is removed before use, and an appropriate volume of mTeSR™1 medium is added for cell culture. For 12-well and 6-well plates, 1 ml and 2 ml of mTeSR™1 medium is used for each well, respectively.
9. If stored at 4°C, remove plate from refrigerator 5 min before use, and equilibrate at room temperature.

PREPARATION OF mTeSR™1 MEDIUM

SUPPORT PROTOCOL 4

This protocol describes the preparation of mTeSR™1 medium for culturing hiPSCs.

Materials

mTeSR™1 basal medium and 5× supplement (e.g., StemCell Technologies, cat. no. 85850)
10,000 U/ml penicillin-streptomycin (e.g., Pan Biotech, cat. no. P06-07100)
50-ml conical tubes

1. Thaw mTeSR™1 5× supplement at room temperature or at 4°C overnight.
2. On a sterile work bench, add 100 ml mTeSR™1 5× supplement to 400 ml mTeSR™1 basal medium.
3. Add 5 ml of 10,000 U/ml penicillin-streptomycin.
4. Mix well by shaking, and prepare 40-ml aliquots in 50-ml conical tubes. Store at −20°C.
5. Thaw frozen aliquots at room temperature or at 4°C overnight. Store at 4°C after thawing.

COMMENTARY

Background Information

Human cell cultures are being used to study diseases in various contexts. Frequently, patient-derived cell systems, such as cancer cell lines, are the only biological matrix used in biomedical research. Recent evidence demonstrated the limitations of cancer cell lines as a tool to use in the field of pharmacology (Ben-David et al., 2018). The low rate of success to reproduce findings of drug response when using cancer cell lines has been previously highlighted (Begley & Ellis, 2012; Haibe-Kains et al., 2013; Prinz, Schlange, & Asadullah, 2011). Recently, alternative donor-derived cancer modeling technologies, based on the introduction of defined molecular alterations in non-neoplastic cells, have emerged (Hegde, Karanikas, & Evers, 2016;

Sancho-Martinez et al., 2016). Those synthetic cancer systems, as an alternative to classic cancer cell lines, may be of use in basic and translational cancer research. Here we present a detailed description of the initial steps for generation of synthetic cancer systems using hiPSCs. The use of hiPSCs is ethically accepted, and cancer models arising from a single cell of origin may be useful for fundamental basic science, as well as for translational and screening applications.

Critical Parameters and Troubleshooting

There are several critical parameters during the generation of TP53 R249S–mutated hiPSCs. Use of a highly standardized, quality-controlled hiPSC culture is of importance. The culture should be regularly checked for the expression of hiPSC markers (e.g., OCT3/4, SSEA4, Nanog, and Sox2), a normal karyotype (Steichen, Hannoun, Luce, Hauet, & Dubart-Kupperschmitt, 2019), and typical stem cell morphology with high nuclei to cytoplasm ratio and prominent nucleoli (Fig. 5; for details see Wakui et al. 2017). Successful generation of transduced hiPSCs depends further on the efficiency of the third-generation lentivirus and the ratio between cells and virus. Reducing the number of cells by reducing medium or increasing the quantity of virus by using more than one vial per experiment could improve the efficiency of the final outcome. At times, the high viral titer can be toxic to the cells; therefore, a reduction of the viral burden by dilution should be tested first (Abbasalipour et al., 2019). After transduction, select hiPSCs to obtain a homogenous culture. For antibiotic selection, a killing curve should be done to determine the optimal antibiotic dosage. Splitting/passaging cells into antibiotic-containing medium should be done on the second or third day of selection. Some promotors can be silenced over time or show no activity in stem cells (Chung et al., 2002; Xia, Zhang, Zieth, & Zhang, 2007). If cell transduction does not work, it would be best to try different promotors (e.g., EF1α; Zhang et al., 2017). At first, we used a cytomegalovirus promoter; however, after cloning the genes into an EF1α promoter–driven vector, we could transduce the hiPSCs.

Understanding Results

With the help of this protocol, it is possible to transduce hiPSCs with TP53 R249S. After 1 week of antibiotic selection, the cells can be expanded, and overexpression of TP53 can be detected (Fig. 6). These protocols have been replicated in another stem cell line (data not shown).

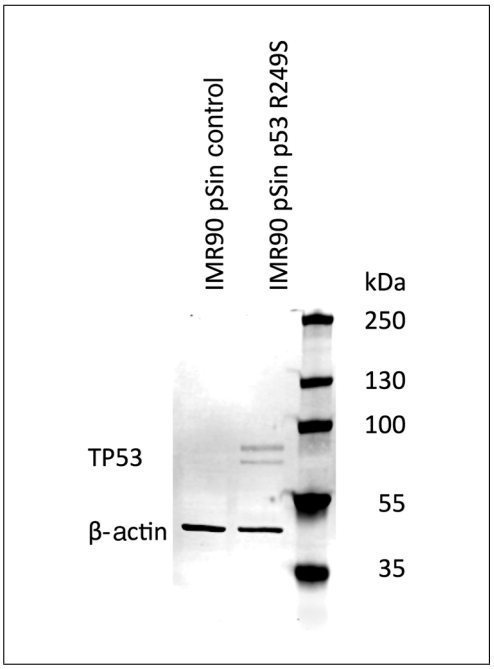

Figure 6 Western blot depicting TP53 overexpression. A total of 40 µg protein was loaded on the gel. The primary antibodies used were anti-p53 (Abcam, cat. no. ab28) and β-actin (13E5) (Cell Signaling Technology, cat. no. 4970). The TP53 band for pSin TP53 R249S runs at ∼80 kDa due to the fusion of EGFP and TP53 R249S.

Time Considerations

Preparation of the lentivirus and transduction of hiPSCs takes about 2.5 weeks. Lentivirus production takes ∼1 week if the vector and HEK 293T cells are ready to use. After 1 week the virus is concentrated and can be used for transduction of hiPSCs. Transduction of hiPSCs requires 10 days. The virus is incubated with the hiPSCs for 3 days, and subsequently antibiotic selection begins and continues for the next 7 days. After selection, depending on the cell material, it can take up to 1 week to obtain enough cell material for the first experiments to validate expression of the molecular alteration.

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