Identifying novel p53-target genes and their functional analysis

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Abstract

Introduction

This protocol describes a procedure to search for novel p53 target genes. First, screen for potential p53 target gene using p53MH algorithm. Then, test these candidate genes using a series of molecular biological assays, including Chromatin immunoprecipitation assay, luciferase reporter activity assay, and regulation of the expression levels of mRNA and protein of these genes by p53 protein. This protocol also includes some assays to measure the mouse implantation and reproduction used in our study.

Reagents

Chromatin Immunoprecipitation \(ChIP) Assay Kit \(Catalog# 17-295, Millipore) Anti-p53 antibodies \(anti-p53 polyclonal antibody FL393, Catalog# sc-6243; anti-human p53 monoclonal antibody DO-1, Catalog# sc-126, Santa Cruz Biotechnology) TOPO TA Cloning kit \(Catalog# K4500-01, Invitrogen) pGL2-Basic vector \(Catalog# E1641, Promega) Lipofectamine 2000 \(Catalog#, invitrogen) Dual-luciferase reporter assay system \(Catalog# E1910, Promega) Quantikine mouse LIF ELISA kit \(Catalog# MLF00, R&D) 17-β-estradiol \(E2, Catalog# E-2257, Sigma) Chicago Sky Blue 6B dye \(Catalog# C8679, Sigma) M2 medium \(Catalog# M7167, Sigma) Recombinant mouse LIF \(Catalog# LIF2010, Millipore)

Equipment

7000 Applied Biosystems Sequence Detection System \(Applied Biosystems) TD-20/20 Luminometer \(Turner Designs) Wallac Victor 3 1420 Multilabel Counter \(Perkin Elmer) Gammacell 40 Exactor \(MDS Nordion)

Procedure

Screen for potential p53 target genes 1 Download p53MH algorithm from "http://linkage.rockefeller.edu/p53":http://linkage.rockefeller.edu/p53. 2 Save the sequence of the candidate genes in .txt file in p53MHwin fold and add the file name into file “p53 names.txt”. 3 Run the p53MH.exe. 4 View potential p53 binding sites listed in file “p53res.txt”. **Cell culture** 5 Human H1299-WTp53 cell line contains a p53 expression plasmid under the control of tetracycline and expresses the wild type p53 protein upon tetracycline withdrawal. Culture H1299-WTp53 cells with 1 μg/ml tetracycline as control or culture them without tetracycline for 24 h to express the wild type p53 protein. 6 Mouse fibroblasts Val5 and Vm10 cells contain a temperature-sensitive mutant p53 plasmid \(alanine 135 to valine) and express mutant p53 at 39°C and wild type p53 at 32°C. Culture Val5 and Vm10 cells at 32°C for 16 h to express the wild type p53 protein or at 39°C as control. **Chromatin Immunoprecipitation** 7 Use 1×10^6 cells in a 10 cm culture dish. 8 Fix cells with 1% formaldehyde and incubate for 10 min at 37°C. 9 Scrape cells into conical tube and pellet cells at 4°C. 10 Resuspend cell pellet in 200 μl of SDS lysis buffer provided with the ChIP assay kit \(Millipore) and incubate for 10 min on ice. 11 Sonicate

lysate to shear DNA to lengths between 200 to 1000 bp. \(Sonication conditions have to be optimized first). 12 Dilute the sonicated cell supernatant 10 fold in ChIP dilution buffer provided with the ChIP assay kit. 13 Pre-clear cell supernatant with Salmon Sperm DNA/Protein A Agarose-50% Slurry provided with the ChIP assay kit for 30 min at 4°C with rotation to reduce nonspecific background. 14 Add anti-p53 antibody \(FL393 for mouse cells and DO-1 for human cells) to the pre-cleared supernatant fraction and incubate overnight at 4°C with rotation. 15 Add Salmon Sperm DNA/Protein A Agarose-50% Slurry and rotate for 1 h at 4°C to collect the antibody/histone complex. 16 Pellet agarose by gentle centrifugation. 17 Wash the protein A agarose/antibody/histone complex for 3-5 min with 1 ml of each of the buffers provided with the ChIP assay kit listed in the orders as given below: a) Low salt immune complex wash buffer, one wash b) High salt immune complex wash buffer, one wash c) LiCl immune complex wash buffer, one wash d) TE buffer, two wash 18 Elute the histone complex with 250 µl freshly prepared elution buffer \(1% SDS, 0.1M NaHCO3) with rotation at room temperature for 15 min for 2 times. 19 Add 20 µl 5M NaCl to the combined eluates and reverse histone-DNA crosslinks by heating at 65°C for 4 h. 20 Add 10 µl of 0.5M EDTA, 20 µl 1M Tris-HCl, pH6.5 and 2 µl of 10 mg/ml Proteinase K to the elutes and incubate at 45°C for 1 h. 21 Recover DNA by phenol extraction and ethanol precipitation. 22 The sequences of primer sets for PCR are as follows: For human LIF gene: 5’-ACCCCGGCCCCCACCATCTTC-3’ and 5’-ACTCATCCGCTGTCCCTTGTCC-3’. For mouse LIF gene: 5’-TGACCTGGGGGAGAGCAACCTAAC-3’ and 5’-AGCCAACAGCCCCAGCATCAC-3’. **Construct the luciferase plasmids containing the potential p53 binding elements** 23 Amplify PCR fragments containing the potential p53 binding elements in LIF genes by using PCR primers for ChIP assays. 24 Clone PCR fragments into pCRII-TOPO vector \(Invitrogen). 25 Subclone the sequence-confirmed clones into pGL2 luciferase reporter plasmids at _XhoI_ and _HindIII_ sites. 26 Screen clones by _XhoI_ and _HindIII_ digestion to find the clones with the right insertion and confirm them by sequencing. **Transfection** 27 Plate 4×10^5 cells per well in 6-well culture plates and culture them for overnight in culture medium without antibiotics. 28 Transfect cells using a set of three plasmids: 200 ng pGL2-luciferase plasmid \(empty or containing potential p53-responsive elements); 1 µg p53 expression plasmid \(wild type or mutant); and 0.5 ng Renilla luciferase internal control. 29 Dilute the plasmid mix and the Lipofectamine 2000 reagent with Opti-MEM and incubate separately for 5 min at room temperature 30 Combine the plasmids and the lipofectamine 2000, and incubate for 20 min at room temperature. 31 Add the mixture to each well and mix gently. 32 Incubate cells at 37°C for 4-6 h. 33 Aspirate the culture medium, replace with fresh medium. 34 Incubate cells for another 24 h **Measure Luciferase activity** 35 Aspirate the culture medium. 36 Add 400 µl Passive lysis buffer provided with dual-luciferase reporter assay system to each well. 37 Scrape the cells and transfer into 1.5 ml tubes. 38 Apply 2 freeze/thaw cycles to complete lysis of cells. 39 Predispense 50 µl of luciferase assay reagent II \(Promega) into tubes. 40 Add 10 µl of cell lysate into each tube and read the firefly luciferase activity in a luminometer. 41 Add 50 µl Stop & Glo buffer \(Promega) into the same tube and read the Renilla luciferase activity in a luminometer. 42 Normalize the firefly luciferase activity from each sample with the Renilla luciferase activity from the internal control plasmid. **Measure the levels of RNA expression by real-time PCR** 43 Extract total RNA from cells or mouse tissues with the RNeasy kit \(Qiagen). 44 Treat RNA samples with DNase I to remove residual genomic DNA. 45 Prepare cDNA with random primers by using the Taqman reverse transcription kit \(Applied Biosystems). 46 Perform real-

time PCR in triplicate with Taqman PCR Mix \(Applied Biosystmes) for 15 min at 95°C for initial denaturing, followed by 40 cycles of segments of 95°C for 15 sec and 60°C for 1min in a 7000 Applied Biosystems Sequence Detection System. **Measure the levels of protein expression by ELISA** 47 Collect cell pellets and prepare cell lysates by adding RIPA buffer. 48 Measure the LIF protein levels by using LIF ELISA kit \(R & D). 49 Add 50 µl of Assay Diluent RD1W provided with the kit to each well. 50 Add 50 µl of standard, control or sample per well, and incubate for 2 h at room temperature. 51 Aspirate each well and wash with Wash Buffer provided with the kit for 5 times. 52 Add 100 µl of diluted mouse LIF conjugate to each well and incubate for 2 h at room temperature. 53 Repeat aspirate/wash as in step 51. 54 Add 100 µl of Substrate solution to each well and incubate for 30 min at room temperature. Protect from light. 55 Add 100 µl of Stop solution to each well. 56 Determine the optical density of each well within 30 min, using a microplate reader set to 450 nm. **Estrogen treatment** 57 Culture cells in phenol red-free culture medium supplemented with 10% charcoal-stripped FBS for 3 days. 58 Add various concentrations of 17-β-estradiol \(E2, sigma) to cells cultured in phenol red-free culture medium supplemented with 10% charcoal-stripped FBS. γ-irradiation of mice or uterine tissues from mice 59 For whole body irradiation, mice were put into chamber of the irradiator. 60 Irradiate mice for 5 Gy with a 137Cs gamma source. 61 Move mice back to regular cage. 62 Sacrifice mice at 6 h after IR by CO_2, dissect mice and collect various tissues, snap freeze the tissues and keep them in -80 °C. 63 For in vitro uterine tissue irradiation, sacrifice mice by CO_2, collect uterine tissues and put tissues on top of an absorbable gelatin sponge soaked with DMEM supplemented with 10% FBS. 64 Irradiate uterine tissues for 5 Gy, collect tissues at 6 h after IR. **Set up breeding experiment** 65 8-week old mice were used for breeding experiment. 66 Co-house one male mouse and one female mouse in one breeding cage for at least 6 weeks. 67 Check for new litters in each breeding cage every morning from19 days after breeding cages were set up. 68 Pregnancy rate is calculated as the ratio of the number of females with confirmed pregnancy to the number of female mice co-housed with male mice. **Collect uterine tissues at different day of pregnancy** 69 Co-house female mice with male mice 1-2 h before the start of the dark cycle. 70 Each morning, 1-2 h after the end of the dark cycle, check female mice for the presence of a vaginal plug. The day that a plug is observed is designated as pregnancy day 1. 71 Collect uterine tissues at different day of pregnancy. **Blue sky staining to examine the early implantation sites** 72 Anesthetize the pregnant female mice. 73 Rinse the tail three or four times with paper towels soaked with warm water to dilate the tail veins. 74 Inject 0.1 ml of 1% blue dye solution through a tail vein using a 1 ml syringe with a 27-gauge needle. 75 Sacrifice mice 3-5 min after the dye injection by CO_2. 76 Dissect the uterus and count the number of implantation sites appeared as distinct blue bands along the uterine horns. **Uterine flushing to recover blastocysts** 77 Sacrifice the pregnant female mice by CO_2. 78 Open the abdominal cavity. Remove the uterus. Trim the mesometrium away. Cut between the oviduct and the ovary, keeping the utero-tubal junction intact. 79 Place the uterus in a small volume of M2 medium in a 35-mm plastic tissue culture dish. 80 Cut lengthwise in utero-tubal junction. 81 Insert a 26-gauge needle fitted to a 1 ml syringe into the cut cervix and slide it into the base of each horn to flush with ~0.2 ml of M2 medium. 82 Count the number of blastocysts under a dissecting microscope. **IP injection** 83 Pick up a mouse by the scruff of its neck as close to the ears as possible. 84 Use the hypodermic needle to pierce the skin and

abdominal muscles to inject 5 μg recombinant mouse LIF \(Millipore) into the intraperitoneal cavity. Repeat injection of LIF after 5 h. Inject same volume of PBS as control.