Cellular transformation and leukemia development by activated tyrosine kinases

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Method Article

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Abstract

Introduction

Protein kinases are one of the largest families of the protein coding genes: constituting some 2% of the expressed proteins, regulate almost all biochemical pathways and phosphorylate up to 30% of the proteome. More than 400 human diseases have been linked, directly or indirectly, to protein kinases. Deregulated tyrosine kinases have been implicated in neoplastic development. Here, we describe biochemical procedures to monitor the activation of the tyrosine kinases (ABL, SRC, PDGFRA, PDGFRB and EGFR). These kinases and its constitutively activated variants were expressed in mammalian cells and kinase activity was measured by tyrosine phosphorylation by immunoblot analysis. We also describe the procedure for cellular transformation of BAF3 cells for IL-3 independent cell proliferation, and the development of leukemia in mice. The combination of these procedures is useful to evaluate the activating mutations in kinases and its potential to develop cancer. This protocol can be completed in 3-4 months.

Reagents

• BALB/c mice (Jackson lab; cat# 000651) • POP-PURO-Ires-GFP retroviral vectors containing various tyrosine kinases (EGFR, ABL, SRC, PDGFRA and PDGFRB) • pCL-ECO ecotropic helper plasmid • 293T cells (American Type Culture Collection, cat. no. CRL11268) • BAF3 cell (American Type Culture Collection, cat. no. • DMEM (Invitrogen, cat. no. 11965-092) • RPMI (Invitrogen, cat. no. 10829-018) • Cell proliferation reagent WST-1 (Roche; cat # 11644807001) • Heat-inactivated FBS (Gemini Bioproducts, cat. no. 100-106) • Penicillin/streptomycin solution 100X (Sigma, cat. no. P4333) • A-33 disinfectant (Ecolab Professional Products, cat. no. 61122362) • 3 M Vetbond tissue adhesive (Webster Veterinary Supply, cat. no. 480200) • 70% (vol/vol) ethanol • Isoflurane, USP (Baxter) • Gauze squares • PBS without Ca/Mg (Mediatech, cat. no. 21-040-cv) • L-Gln (Invitrogen, cat. no. 25030-081)/GlutaMAX (Invitrogen, cat. no. 35050-061) • 2-Mercaptoethanol (Sigma, cat. no. M7522) • 7.5% BSA solution (wt/vol; Invitrogen, cat. no. 15260-037) • Protamine sulfate (Sigma, cat. no. P4020) • FuGENE 6 transfection reagent (Roche Applied Science, cat. no. 1181509001) • WEHI conditioned medium • Anti-phospho tyrosine antibody (Santa Cruz Biotechnology; cat # SC-7020HRP) • Anti-ABL antibody (Santa Cruz Biotechnology c-Abl (K-12): sc-131) • Anti-ABL antibody (Cell signaling Technology; Phospho-c-Abl (Tyr245) #2868) • Anti-ABL antibody (Cell signaling Technology; Phospho-c-Abl (Tyr412) #2865) • Anti-SRC (Cell signaling Technology; Src (36D10) Rabbit mAb #2109) • Anti-SRC (Cell signaling Technology; Phospho-Src (Tyr527) Antibody #2105) • Anti-SRC (Cell signaling Technology; Non-phospho-Src (Tyr527) Antibody #2107) • Anti-SRC (Cell signaling Technology; Non-phospho-Src (Tyr416) #2102) • Anti-SRC (Cell signaling Technology; Phospho-Src Family (Tyr416) Antibody #2101) • Anti-EGFR (Cell signaling Technology; EGF Receptor (1F4) Mouse mAb #2239) • Anti-EGFR (Cell signaling Technology; Phospho-EGF Receptor (Tyr1045) #2237) • Anti-EGFR (Cell signaling Technology; Phospho-EGF Receptor (Tyr1068) #2236) • Anti-EGFR (Cell signaling Technology; Phospho-EGF

Receptor \(\text{Tyr845}\) Antibody #2231) • Anti-EGFR \(\text{Cell signaling Technology; EGF Receptor Antibody #2232}\) • Anti-PDGFR\(\text{Cell signaling Technology; Phospho-PDGF Receptor } \alpha \text{ \(\text{Tyr754}\) #2992}\) • Anti-PDGFR\(\text{Cell signaling Technology; PDGF Receptor } \alpha \text{ Antibody #3164}\) • Anti-PDGFR\(\text{Cell signaling Technology; PDGF Receptor } \beta \text{ \(\text{2B3}\) Mouse mAb #3175}\) • Anti-PDGFR\(\text{Cell signaling Technology; Phospho-PDGF Receptor } \beta \text{ \(\text{Tyr751}\) #3161}\) • Sequi-Blot PVDF Membrane, \(\text{Biorad; cat \# 162-0184}\) • Precast SDS-PAGE gel \(\text{Biorad; Criterion Tris-HCl Gel, 7.5\%, cat \# 345-0006}\) • KODAK Biomax MR film \(\text{Kodak; cat \# 8701302}\) • KODAK Biomax Light film \(\text{Kodak; cat\# 178-8207}\) • Rainbow molecular weight marker \(\text{Amersham; cat\# RPN800}\) • Zeocine \(\text{Invitrogen; cat \# R250-01}\) • PCRXL-TOPO Kit \(\text{Invitrogen; cat \# K4700-10}\) • TOP10 Electrocompetent cells \(\text{Invitrogen; cat \# C4040-52}\) • TOP10 Chemical competent cells \(\text{Invitrogen; cat \# C4040-03}\) • X-GAL \(\text{Invitrogen; cat \# 15520-034}\) • Rapid DNA ligation kit \(\text{Roche; cat \# 11635379001}\) • Protease inhibitor cocktail \(\text{Roche; cat \# 11873580001}\) • EXPAND Long template PCR \(\text{Roche; cat \# 11681842001}\) • QUICKCHANGE XL mutagenesis kit \(\text{Stratagene; cat \# 200522}\) • SuperSignal West Pico Chemiluminescent Substrate \(\text{Pierce; cat \# 34080}\) • Phosphatase Inhibitor Cocktail 1 \(\text{Sigma; cat\# P2850}\) • Phosphatase Inhibitor Cocktail 1 \(\text{Sigma; cat\# P5726}\) • Ni-NTA agarose \(\text{Qiagen; cat\# 30210}\) • Carnation Non-fat dry milk \(\text{Nestle; Shaws grocery store}\) • BAF3 growth medium \(\text{RPMI medium containing 10\% FBS \(\text{vol/vol}\), 2 mM L-Gln, 50 U ml⁻¹ penicillin, 50 mg ml⁻¹ streptomycin. To prepare 500 ml of the medium, mix 50 ml inactivated FBS, 5 ml L-Gln and 5 ml penicillin/streptomycin, and then make up to 500 ml with RPMI. Filter the medium with a bottle-top 0.22- \(\mu\text{m}\) filter and store at 4 °C for 1 week.}\) • HEK293T growth medium \(\text{DMEM medium containing 10\% FBS \(\text{vol/vol}\), 2 mM L-Gln, 50 U ml⁻¹ penicillin, 50 mg ml⁻¹ streptomycin. To prepare 500 ml of the medium, mix 50 ml inactivated FBS, 5 ml L-Gln and 5 ml penicillin/streptomycin, and then make up to 500 ml with DMEM. Filter the medium with a bottle-top 0.22- \(\mu\text{m}\) filter and store at 4 °C for 1 week.}\) • Lysis Buffer \(\text{50 mM Sodium phosphate \(\text{pH 7.5}\), 50 mM NaCl, 1\% triton X100, 0.1\% SDS, 5 mM EDTA, 1mM EGTA, 1 mM sodium fluoride, 2 mM Sodium Vanadate, Protease inhibitors cocktail \(\text{Roche}\), phosphatases inhibitors cocktail 1 and 2 \(\text{Sigma}\) and 5\% Glycerol.}\) • 5X gel loading buffer \(\text{350 mM Tris-HCl \(\text{pH 6.8}\), 500 mM DTT, 15\% SDS, 10 mM Benzamidine, 5 mM EDTA, 5 mM EGTA, 5 mM Sodium Vanadate, Protease cocktail, 50\% Glycerol, and 0.001\% Bromophenol blue.}\)

Equipment

• PCR Thermal cycler \(\text{BIORAD}\) • DNA gel electrophoresis system \(\text{BIORAD}\) • Protein gel electrophoresis system \(\text{BIORAD}\) • Protein gel transfer apparatus \(\text{BIORAD}\) • Microcentrifuge \(\text{Eppendorf}\) • 37°C incubator shaker for bacterial culture \(\text{New Brunswick}\) • Inverted tissue culture microscope with phase contrast \(\text{4, 10, 20, 40 objectives}\) • Stereomicroscope \(\text{Nikon SMZ-1500 or similar}\) • Biosafety cabinet with aspirator for tissue culture • CO₂ incubator, 37 °C, humidity • Tissue culture centrifuge • Tissue culture dish, 35, 100 and 150 mm • Tissue culture plates, 6-well, 12, well and 96 well • Conical tubes, 15 and 50 ml • Glass Pasteur pipettes, 9 inches—sterilized using autoclave • Cryovials, 2.0 ml \(\text{Corning, cat. no. 430488}\) • Plastic disposable transfer pipettes, 1, 5, 10, 25 and 50 ml • Disposable sterile filter system, 0.22 \(\mu\text{m}\), 500 ml \(\text{Corning, cat. no. 430758}\) • Disposable syringes, 10, 5 and 1 ml • Hypodermic needle, 27-30G • Acrodisc filter, 0.2 \(\mu\text{m}\), low protein binding \(\text{Pall Corporation, cat.}\)

no. 4602) • Acrodisc filter, 0.2 m, DMSO safe \ (Pall Corporation, cat. no. 4433) • Aspirator tube assembly \ (Sigma, cat. no. A5177) • Sterile instruments \ (scalpel, scissors and forceps) • Sterile Petri dishes • Anesthesia machine/chamber \ (VetEquip) • Class 2 \ (Type A/B3) biological safety cabinet \ (Baker) • Coulter counter \ (Beckman Coulter) or hemocytometer

Procedure

• Steps 1-15: Expression of tyrosine kinases in 293T cells and retrovirus production • Steps 16-21: Retrovirus infection of BAF3 cells • Steps 22-24: IL-3 independent growth of BAF3 cells • Steps 25-33: BAF3 injection through tail vein and leukemia development • Steps 34-41: Extract preparation and western blotting

****Expression of tyrosine kinases and retrovirus production****

1. Quickly thaw a vial of 293T cells by swirling in 37 °C water bath.
2. Spray vial with 70% ethanol and wipe dry before placing in tissue culture hood.
3. Gently add 1 ml 293T medium, mix with contents of cryovial and transfer into 15-ml conical tube containing 4 ml 293T medium.
4. Centrifuge the cells at 1200 rpm at room temperature for 5 min.
5. Discard the supernatant, resuspend the cells with 5 ml 293T medium into 50-ml conical tube.
6. Determine the number of 293T cells using a hemacytometer and adjust the concentration to 2×10^5 cells ml^{-1} .
7. Transfer 3 ml of cell suspension \ (5×10^5 cells) into a 60-mm dish.
8. Incubate the cells at 37 °C, 5% CO_2 overnight. Cells will be 60–70% confluent in 1–2 d.
9. Aspirate medium from 293T cells and replace with 10 ml fresh 293T medium.
10. For each 60 mm plate, add 6 μl FuGENE 6 transfection reagent, 90 μl of DMEM, and mix; incubate at room temperature for 5 min.
11. Add 1-2 μg retroviral vector, 1 μg of pCLECO plasmid.
12. Mix and incubate at room temperature for 15 min.
13. Add the transfection reagent: DNA complex to the cells in a dropwise manner. Swirl the dish to ensure distribution over the entire plate surface.
14. Incubate at 37 °C, 5% CO_2 for 48-72 h.
15. Day 3: collect retrovirus-containing medium and pass through 0.45-mm filter unit.

****Viral transduction of BAF3 cells****

16. Plate 1 million BAF3 cells in each well of a 6 well plate using fresh BAF3 growth media with 5% WEHI conditioned medium as a source of exogenous IL-3.
17. Add 3 μl of 5mg ml^{-1} of protamine sulphate and 1 ml of filtered viral supernatant that usually has viral titer of 10^6 .
18. Centrifuge at 2500 rpm for 90 min at 33°C in a Sorvall RT6000 table centrifuge.
19. Incubate at 37 °C, 5% CO_2 , for 24 h.
20. Transduced cells are selected by addition of 3 microliters of 2.5 mg ml^{-1} puromycin.
21. After 4-6 days, live cells were harvested by washing the cells with 10 ml of RPMI medium and used for further analysis.

Oncogenic transformation of BAF3 cells

22. Five thousands transduced BAF3 cells were plated in quadruplicate in each well of 96 well plate in RPMI media containing only 10% IFBS. Cell growth in the absence of IL-3 is scored as oncogene mediated signaling complementation or cellular transformation.
23. Plates were incubated at at 37 °C, 5% CO_2 , 14 days.
24. Plates were monitored everyday for cell survival and proliferation. Confluent wells were scored as transformed cells.

****Tail vein injection and leukemia development****

25. 10 million transformed cells were washed in PBS and resuspended in PBS containing 0.1 % BSA. Quantitate the number of cells present using a Coulter counter or a hemocytometer.
26. Centrifuge cells at 225g and 20 °C for 5 min. Remove the supernatant, and re-suspend the cells in PBS with 0.1% BSA at a concentration of 1×10^7 cells per ml.
27. Place the recipient BALB/c mice in the

anesthesia chamber following the manufacturer's recommended settings. 28. After a mouse reaches sufficient anesthetic depth, remove it from the chamber and place it (ventral side down) in a properly sized nose cone, configured according to the manufacturer's recommended settings, with the dorsal side facing upwards. To confirm anesthetic depth, pinch the rear foot lightly. If no kicking response is present, continue with the procedure. 29. Agitate the cell suspension to prevent the cells from settling, and withdraw from the sterile tube into a 1-cc TB syringe with the needle removed. 30. Inject, with a 26 G needle, 0.1 ml of the cell suspension (1×10^6 cells) through tail vein. 31. Place the animal in a clean cage and observe for 10–15 min to ensure recovery from the anesthetic. 32. Monitor the mice for leukemia development and their survival. 33. Blood were withdrawn from each mice and analysed for cell count using blood smear slide. ****Extract preparation and western blotting**** 34. Harvest approximately 4-6 millions of either transfected 293T or transduced BAF3 cells by centrifugation at 2000 RPM for 5 min. Aspirate media and resuspend cell pellet with 1 ml of ice cold PBS containing protease inhibitors and phosphatase inhibitors. 35. Centrifuge the cell suspension and aspirate the PBS, and then resuspend in lysis buffer. 36. Cell lysates are then homogenized and sonicate for 1 minute. Crude extracts are then centrifuged at 12000 g for 5 min. 37. Ni-NTA affinity purification— Rinse Ni-NTA resin in lysis buffer. 38. 200 ml of crude cell lysates were added to resin and allowed to bind for 4-6 hrs at 4°C. 39. The resins were centrifuged and washed in lysis buffer twice. 40. Resin bound proteins were resuspended in gel loading buffer. 41. Crude extracts and affinity purified proteins were analysed by standard western blotting assays using phospho-specific antibodies

Timing

3-4 months