Supplementary Materials and Methods

Cell growth assay

A total of 2000 cells were plated in 100μl medium in 96-well plates. After 24-h incubation, various concentrations of gefitinib, erlotinib, and 5mM metformin were added to each well as indicated, and cells were further cultured for 48 h. Then 10μl of 5mg/ml MTT (Sigma) in 100μl medium was added to each well. After 4 h, medium was removed and 150μl of DMSO was added to each well to dissolve the formazan crystals. Then the absorbance at 490nm was determined using a ThermoFisher Spectrophotometer1510 (Molecular Devices, Inc.). Cell viability was determined by dividing the absorbance values of treated cells to those of untreated cells. IC_{50} was defined as the concentration of gefitinib or erlotinib at which 50% growth inhibition was obtained and calculated from the dose-response curve. Experiments were conducted in triplicates.

Cell proliferation was also assessed by 5-bromo-20-deoxyuridine (BrdU) incorporation assay using a BrdU labeling and detection kit (Roche Applied Science). Briefly, cells were treated with metformin (5 mM) or TKIs (IC_{25}^{48h}), or both, for 48 hours, and then incubated for 6 hours with BrdU (10 mmol/L) and fixed. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and viewed with a live cell station (Delta Vision, API). At least 500 cells from three independent experiments were counted. Data were expressed as the mean value of the percentage of positive cells ± SEM.

Cell invasion assay

Triplicate samples of 2×10^4 tumor cells (untreated or pretreated with TKIs (IC_{25}^{48h}) and/or 5 mM metformin for 48 hours as indicated) were seeded into the upper well in serum-free medium and incubated with 10% FBS in the lower chambers. Cells were cultured for 48 hours at 37 ^oC with 5% CO_2.

Cells in the upper chamber were then removed with a cotton swab and cells that had migrated into the lower chamber were fixed in 4% paraformaldehyde and stained with 0.1% Crystal Violet. Cells on the bottom side of the filters were counted in 5 random ×100 microscope objective fields.

Cell migration assay

A total of 1×10^4 cells were seeded into a 24-well plate, treated with TKIs (IC_{25}^{48h}) and/or 5mM metformin as indicated for 48 hours, and then time-lapse videos were acquired using a live cell station (Delta Vision, API). One hundred randomly chosen cells were traced over 60 mins at 5 min intervals. Cell migration trajectories of ~30 cells were acquired using Image J (NIH imaging software), with starting positions being placed at the origin.

Cell apoptosis assay

Flow cytometric analysis was adopted to detect apoptosis via the examination of altered plasma membrane phospholipid packing by lipophilic dye Annexin V. Briefly. Cells were treated with gefitinib (IC_{25}^{48h}) and/or 5mM metformin for 48 hours, harvested by trypsin, washed twice with PBS, and then resuspended at a concentration of 1×10^7 cells/mL. Thereafter, 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added to 100 μL of cell suspension and incubated for 30 minutes at room temperature in the dark. Then labeled cells were processed by flow cytometry. All early apoptotic cells (Annexin V-positive, PI-negative), necrotic/late apoptotic cells (double positive), as well as living cells (double negative) were detected by Cytomics FC 500 (Beckman Coulter, Miami, FL, USA).

Immunostaining assay

For immunofluorescence, cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 minutes. Tumor tissues fixed in 4% paraformaldehyde for 48 hours were embedded in paraffin and cut into 4-μm thick sections. Nonspecific binding was blocked using 10% normal goat serum (Sigma). Cells or tumor sections were incubated with the following primary antibodies (diluted in PBS with 1% bovine serum albumin) at 4 °C overnight: mouse monoclonal anti E-cadherin (Abcam), and rabbit monoclonal anti-Vimentin (Abcam). Then cells or tumor sections were washed twice with PBS and incubated with secondary antibodies at 37 °C for 30 minutes as follows: FITC-conjugated goat-anti-rabbit IgG (Abcam) or TRITC-conjugated goat-anti-mouse IgG (Sigma). The slides were mounted in mounting medium with 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and viewed with a live cell station (Delta Vision, API).

Western blot analysis

Cells grown and treated as indicated were collected and total protein was extracted. The following primary antibodies were used: rabbit monoclonal anti-Akt, rabbit monoclonal anti-phosphorylated Akt, rabbit monoclonal anti-STAT3, rabbit monoclonal anti-phosphorylated STAT3, rabbit monoclonal anti-OCT1, rabbit monoclonal anti-phosphorylated AMPK, rabbit monoclonal anti-AMPK, rabbit monoclonal anti-LKB1, rabbit monoclonal anti-phosphorylated Acetyl-CoA Carboxylase (ACC), or rabbit monoclonal anti-ACC (all from Cell Signaling Technology, Inc.), mouse monoclonal anti-E-cadherin (Abcam), rabbit monoclonal anti-Vimentin (Abcam), or rabbit monoclonal anti-Snail (Cell Signaling Technology, Inc.). Horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (Thermo Scientific) was used as a secondary antibody. The control for equal protein loading was assessed with an anti-β-actin antibody (Cell Signaling Technology, Inc.).

Analysis of IL-6, insulin and glucose levels

The level of IL-6 secretion was analyzed by ELISA. The tested samples were collected from treated human lung cancer cells or mouse serum, and subjected to a 1:5 dilution, and the level of IL-6 was detected using a high-sensitivity human IL-6 kit (Sigma) and mouse IL-6 kit (Sigma), respectively. IL-6 gene transcription was analyzed by realtime RT-PCR using primers described previously (1). The serum glucose levels were examined by enzyme colorimetric (glucose-oxidase) method with kits from Cayman Chemical. The serum insulin levels were analyzed by mouse insulin ELISA Kits (Millipore, Billerica, MA).

Construction of PC-9psb388 cell lines

PC-9psb388 stable over-expressing human recombinant IL-6 cell line was established by transfecting PC-9 cells with a Lentivirus psb388 and then selected by puromycin at the concentration of 2μg/mL. Cells were further tested by short tandom repeat profiling.

References:

1. Yao Z, Fenoglio S, Gao DC, Camiolo M, Stiles B, Lindsted T, et al. TGF-beta IL-6 axis mediates selective and adaptive mechanisms of resistance to molecular targeted therapy in lung cancer. Proc Natl Acad Sci U S A. 2010;107:15535-40.