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Title: Identification of simvastatin-regulated targets associated with JNK activation in DU145 human prostate cancer cell death signaling

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SUPPLEMENTARY METERIALS AND METHODS

Materials

DU145 human prostate cancer cell line (HTB-81^{TM}), fetal bovine serum (FBS) and eagle's minimum essential medium (EMEM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Penicillin/streptomycin was from Life Technologies (Carlsbad, CA, USA). Simvastatin, docetaxel, 0.4% trypan blue solution and Coomassie Brilliant Blue (CBB) G were from Sigma-Aldrich (St Louis, MO, USA). SP600125 was from Calbiochem (Billerica, MA, USA). Cell counting kit-8 (CCK-8) was from Dojindo (Kumamoto, Japan). DMSO, acrylamide/bis-acrylamide 37.5:1 solution (40%), urea, Tween-20 and ammonium sulfate were from Amresco (Solon, OH, USA). IPG strip (pH 4-7) 17 cm was from Bio-Rad (Quarry Bay, Hong Kong). Recombinant DNase I (RNase-free) was from Takara (Shiga, Japan). Sequencing-grade modified trypsin was from Promega (Madison, WI, USA). SuperSignal West Pico stable peroxide solution was from Pierce (Rockford, IL, USA). The following antibodies were used in this study: phospho-S473-Akt (Cell signaling, Danvers, MA, USA), phospho-Y204-ERK1/2 (Bioworld Technology, Louis Park, MN, USA), ERK1 (Transduction Laboratories, San Jose, CA, USA), phospho-T183/Y185-JNK1/2/3 (Bioworld Technology), JNK1 (D-6, Santa Cruz, Dallas, TX, USA), phospho-S63-c-Jun II (Cell signaling), PKCζ (C-20, Santa Cruz), IKKα (M-110, Santa Cruz), β-actin (Sigma-Aldrich), hnRNP K (D-6, Santa Cruz), TXNDC5 (2E7/7, Abcam, Cambridge, MA, USA), cHMGCS (H-9, Santa Cruz), NDRG1 (A-5, Santa Cruz), RAB1B (G-20, Santa Cruz), IDI1 (H-66, Santa Cruz), goat anti-rabbit horse-radish peroxidase conjugate (Bio-Rad, Hercules, CA, USA) and goat anti-mouse horse-radish peroxidase conjugate (Thermo Scientific, Rockford, IL, USA).

Cell culture

DU145 cells were maintained in medium A (10% FBS, 1% penicillin/streptomycin, EMEM) in a humidified 5% CO_2 incubator at 37ºC.

Cell viability analysis

DU145 cells were split in a 24-well dish (3 X 10^4 cells) and treated as indicated. The cells were incubated with a medium A containing 10% CCK-8 reagent for 1.5 h at 37ºC in a CO_2 incubator. The reaction solution (100 μl each) was then transferred to a 96-well dish and was analyzed by measuring absorbance at $OD_{485\ nm}$ using a CHAMELEON microplate reader (Hidex).

Western blot analysis

Whole cells were extracted with sodium dodecyl sulfate (SDS) sample buffer and were boiled for 5 min at 95ºC followed by protein quantification using the Bradford reagent. Total proteins were separated using 10% or 13.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an NC membrane. The blot was blocked for 1 h at room temperature in blocking buffer (3% skim milk, 0.1% Tween-20, phosphate buffered saline (PBS)) and then incubated with primary antibody at 4ºC overnight. The blot was then washed with PBST (0.1% Tween-20, PBS) three times for 10 min and incubated with a horseradish peroxidase-conjugated secondary antibody in blocking buffer for 1.5 h. After being washed with PBST, the blot was analyzed with the SuperSignal ECL detection system.

Cell morphology analysis

DU145 cells were split in a six-well dish (1 X 10^5 cells) or 10 cm dish (2.5 X 10^6 cells) and cultured with the indicated drug(s) for the indicated time. The cell morphology was analyzed

using phase-contrast light microscopy (EVOS XL Core, Life Technologies) with X 150 amplification.

Detection of dead cells

DU145 cells were split in a six-well dish (1 X 10^5 cells) and treated as indicated. Without removing the medium, the cells were stained with a 0.04% trypan blue staining solution for 30 min. Dead cells changed to a dark blue color were visualized using bright-field phase-contrast light microscopy (EVOS XL Core, Life Technologies) with X 150 amplification. The color contrast of the images was adjusted with a Photoshop program.

Sample preparation, 2D electrophoresis, CBB staining and image analysis

Whole cells (attached and floating cells) were washed with PBS and prepared for 2D electrophoresis as previously described (33). Protein samples (500 μg each) were subjected to one-dimensional isoelectric focusing, followed by 2D electrophoresis on a 8.5-14% sucrose gradient polyacrylamide gel. The gels were fixed in fixing solution (40% methanol, 5% ortho-phosphoric acid) at room temperature for 3 h and were stained with CBB staining solution (17% ammonium sulfate, 3% phosphoric acid, 34% methanol, 0.1% CBB G) for 24 h. After destaining with H_2O, gel images were obtained using a UMAX scanner (PowerLook 2100XL). A quantitative analysis of 8 CBB-stained images (DMSO- and simvastin-treated 2 groups) was performed using PDQuest software (Bio-Rad) according to the manufacturer's instructions.

Mass spectrometric analysis and database search

The CBB-stained protein spots were excised and finely chopped, and the gel pieces were destained, reduced, alkylated and in-gel tryptic digested as previously described (33). The

tryptic digested peptides were collected by extraction with 66% ACN/0.1% trifluoroacetic acid and were dried in a vacuum centrifuge. The peptides were resolved with 50% acetonitrile/0.1% trifluoroacetic acid solution, and 1.5 μl of the sample was mixed with 1.5 μl of matrix solution (10 mg of α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, 0.1% trifluoroacetic acid). The mixed tryptic peptides (1 μl) were immediately spotted onto a 384-well target plate for mass spectrometric analysis. Peptide mass spectra were obtained using a ABI 4800 Plus TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) and were analyzed as previously described (34).

Statistical significance

Statistical significance was determined using Student's *t*-test ($P<0.05$ was considered statistically significant).

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