Supplementary materials

Pro-survival activity of the MAK-V protein kinase in PC12 cells

Igor V. Korobko[1,2,*], Svetlana V. Kalinichenko[1], Elena V. Korobko[1,2], Natalia N. Ninkina[3], Sergey L. Kiselev[1,†], Vladimir L. Buchman[3]

[1]Institute of Gene Biology, Russian Academy of Sciences; Moscow, Russia

[2]University of Oslo, Centre for Medical Studies in Russia; Moscow, Russia

[3]School of Biosciences, Cardiff University; Cardiff, United Kingdom

[†]Present address: Vavilov Institute of General Genetics, Russian Academy of Sciences; Moscow, Russia

*Correspondence to: I.V. Korobko; Institute of Gene Biology, Russian Academy of Sciences; 34/5 Vavilov str.; Moscow 119334, Russia; Tel.: +7-499-1359970; Fax: +7-499-1354105; Email: igorvk@igb.ac.ru

Matherial and methods

Cells and cell culture

PC12TetOn cells (Clontech) were cultured on Dulbecco's modified Eagle's medium (DMEM) without sodium pyruvate (Gibco) supplemented with 10% heat-inactivated horse serum (Gibco), 5% tetracycline-screened fetal bovine serum (HyClone) and 100 μg/ml of G418 sulfate (Calbiochem). To generate clones with inducible expression of C-terminally tagged MAK-V protein kinase or its catalytically inactive mutant, respective cDNAs were cloned into pTre-Tight vector (Clontech) and used to co-transfect PC12TetOn cells with pHygro plasmid (Clontech). Clones were selected in medium containing 200 μg/ml of hygromycin B (Calbiochem) and maintained in medium containing 100 μg/ml of hygromycin B. Medium was replaced every 2 days with fresh one. To induced transgene expression, cells were treated with 1 μg/ml of doxycycline (DOX) for 2 days. Collagen IV from human placenta and mouse laminin for coating cell culture dishes were purchased from Sigma and used as recommended by manufacturer. To induce PC12 cell differentiation along the neural pathway, cells were cultured in low-serum medium (1% heat-inactivated horse serum and 0.5% tetracycline-screened fetal bovine serum) supplemented with 50 ng/ml of 7S nerve growth factor (NGF) from mouse submaxillary gland (Sigma) replacing medium every 2 days.

Cell viability determination

Cells were plated in wells of 96-well plate coated with collagen IV (10000 cells/well) and grown with or without DOX for 8 days. Viable cells at the end of incubation were quantified by their dehydrogenise activities using CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

LDH measurement

Cells were seeded in wells of 96-well plate coated by collagen IV (15000 cells/well) in medium containing or lacking DOX, and lactate dehydrogenase (LDH) activity released to cell culture medium was measured after 2.5 days of incubation with CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega).

Quantification of caspase-3/7 activity

Cells were seeded in wells of 96-well plate coated by collagen IV (15000 cells/well) in medium containing or lacking DOX, and activation of caspase-3/7 was quantified after 2 days of incubation using Caspase-Glo® 3/7 Assay (Promega). Caspase-3/7 activities were normalized to

total cell numbers determined by assaying intracellular ATP content in duplicate set of wells using CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

Western blot analysis and antibodies

Cells were lysed in sample buffer and lysates were analyzed by Western blotting with primary antibodies followed by secondary horse radish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (GE Healthcare) as appropriate. Primary antibodies used were from Sigma (rabbit polyclonal anti-FLAG® and anti-α-tubulin, clone DM1α) and Cell Signaling (rabbit polyclonal anti-p42/44 MAP kinase, anti-phospho-p42/44(Thr202/Tyr204), anti-Akt, anti-phospho-Akt(Ser473), anti-cofilin, and rabbit monoclonal anti-phospho(Ser3)-cofilin).

Flow cytometry

Cells for flow cytometry were harvested by trypsinization, pooled with culture medium and PBS used to wash cells to assure that detached cells were not lost, and washed with PBS. Cells were stained with Annexin V-FITC using Annexin V-FITC Apoptosis Detection Kit (Sigma) and immediately analyzed on Cytomics FC500 MPL flow cytometer (Beckman Coulter).

Statistics

Each experimental point, if applicable, was done at least in triplicate. Each experiment was repeated at least twice. Data are presented as mean ± standard deviation (SD). If normalized data are shown, SD ($\sigma_{F(x,y)}$) was calculated as $\sigma_{F(x,y)}=\sqrt{(\frac{dF(x,y)}{dx}\sigma_x)^2+(\frac{dF(x,y)}{dy}\sigma_y)^2}$. Unpaired *t*-test was used to calculate two-tailed *P*-value with GraphPad software (www.graphpad.com).

Figure S1

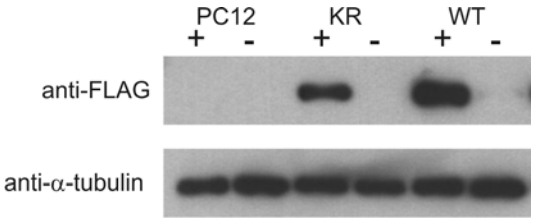

Figure S1. PC12TetOn cells with inducible expression of MAK-V-FLAG protein. Lysates were prepared from parental PC12TetOn (PC12), WT (WT) and KR cells (KR) treated (+) or untreated (-) with 1 μg/ml of DOX for 48 hrs. MAK-V-FLAG protein in WT cell lysates and MAK-V(KR)-FLAG protein in KR cell lysates were detected by Western blotting with anti-FLAG antibodies. α-tubulin contents in samples were monitored with DM1α monoclonal antibodies to control total protein loading.

Figure S2

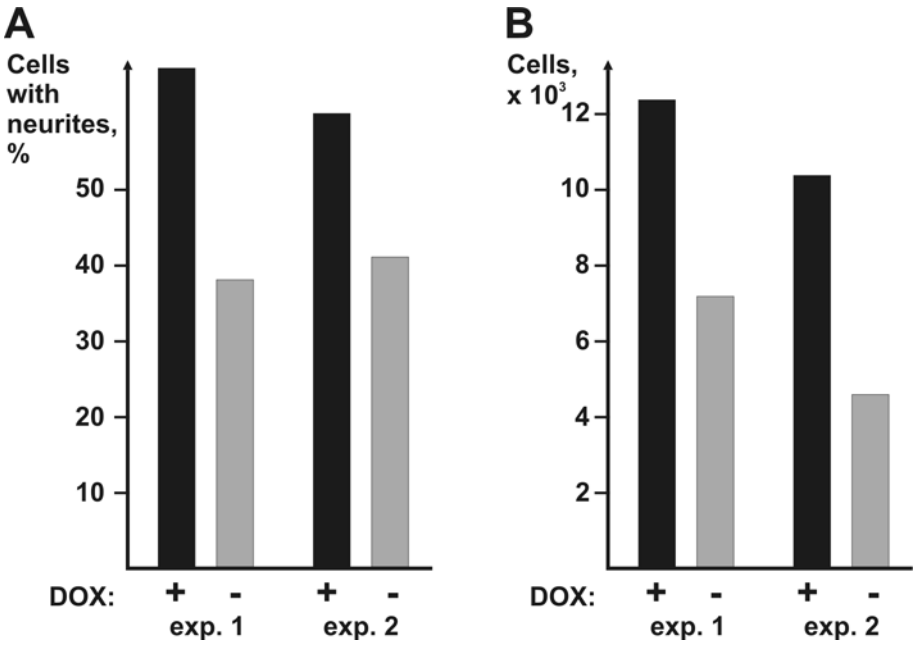

Figure S2. Effect of MAK-V expression on NGF-induced differentiation of WT cells. Equal number of cells were plated at low density on collagen IV-coated 30 mm cell culture dishes (approx. 30000 cells per dish) and cultured in the presence of NGF with (DOX+, black columns) or without (DOX-, grey columns) DOX. Cells in representative regions on each plate were counted and scored as neurite-bearing (processes longer than cell diameters) or without neuritis (no processes or processes less than cell diameter). Percentage of neurite-bearing cells (**A**) and total number of cells per dish (**B**) at day 6 of NGF treatment are shown for two independent experiments (exp.1 and exp.2).

Figure S3

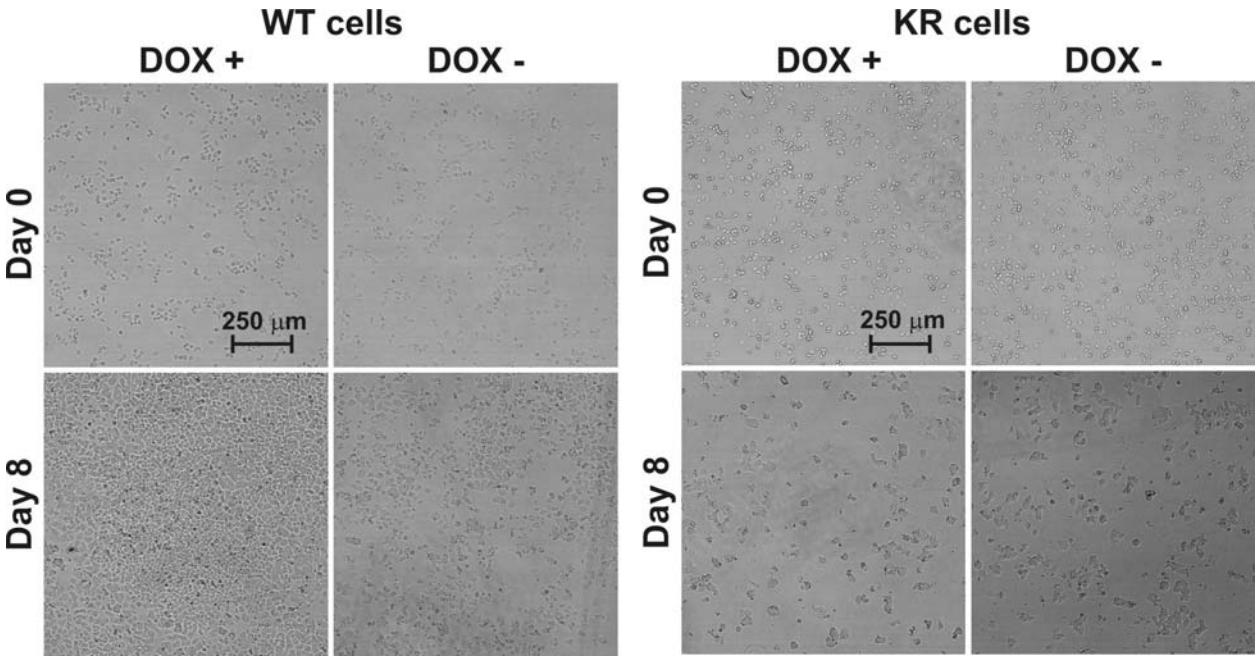

Figure S3. Collagen IV results in impaired WT and KR cell growth that can be rescued by expression of wild type but not catalytically inactive MAK-V protein kinase. WT or KR cells as indicated were plated in wells of 96-well plate coated with collagen IV (10000 cells/well) and grown with (DOX+) or without (DOX-) DOX for 8 days. Representative images of wells were taken several hours after plating (Day 0) and at the end of incubation (Day 8).

Figure S4

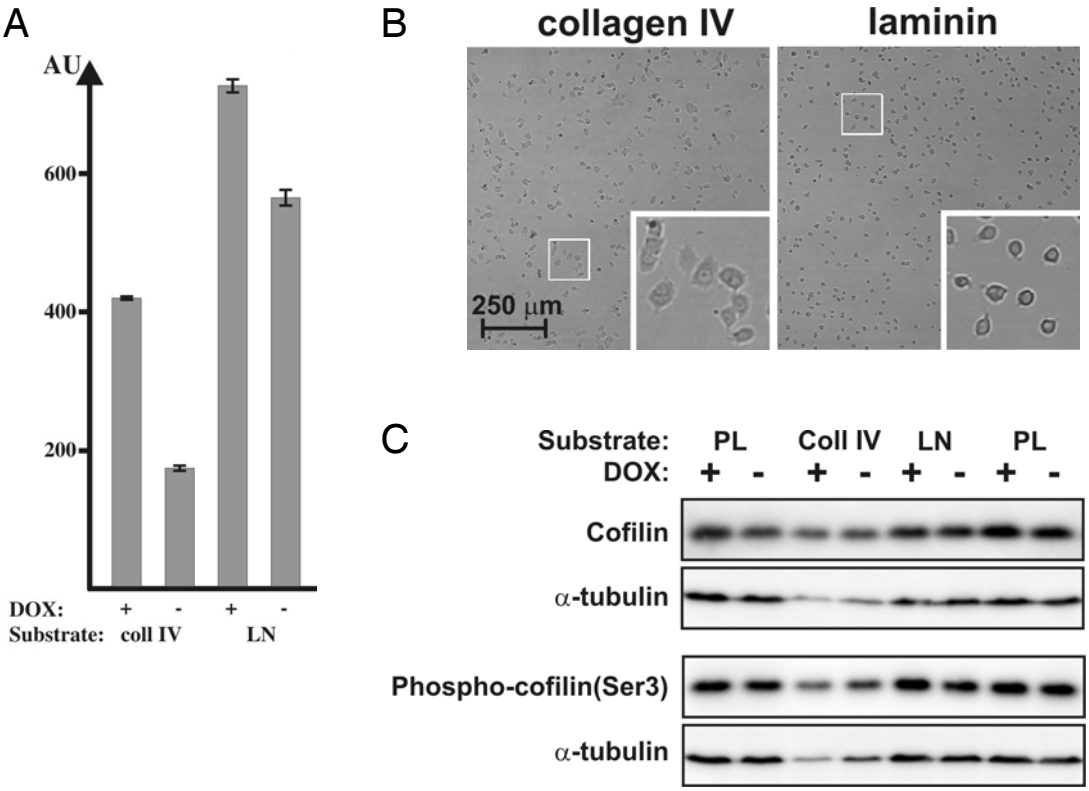

Figure S4. A - Growth of WT cells on different substrates. WT cells (10,000/well) were plated in 96-well plates treated with collagen IV (coll IV) or laminin (LN). In 2 hrs, medium was changed to fresh one either supplemented with 1 μg/ml of DOX (+) or not (-). Medium was changed every 2 days. Viable cells were quantified on day 7 in culture. Data are presented in arbitrary units (AU) of cellular dehydrogenase activities as average of measurements in three independent wells ± standard deviation. **B** – Images of WT cells plated on collagen IV- or laminin-coated surfaces with insets demonstrating that when plated on collagen IV but not on laminin, WT cells acquire spreaded morphology. **C** – Cofilin phosphorylation in WT cells grown for 2 days on tissue culture-treated plastic support (PL) or on collagen IV- (Coll IV) or laminin-treated (LN) surfaces in the presence (+) or absence (-) of DOX. Total cell lysates were analysed by Western blotting with anti-cofilin or anti-phospho(Ser3)-cofilin antibodies. To monitor total protein loading, membranes were re-probed with anti-α-tubulin monoclonal antibodies.

Figure S5

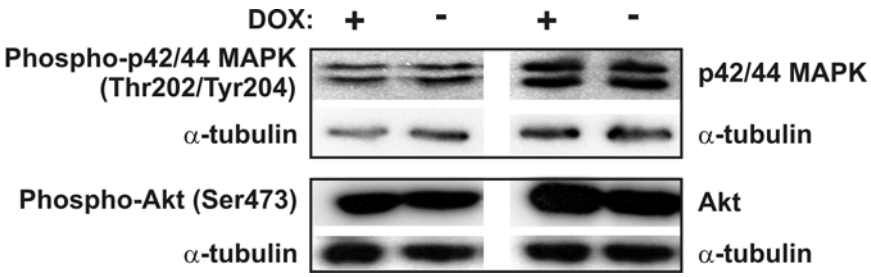

Figure S5. Analysis of ERK and Akt signal transduction pathways in WT cells. Cells were cultured on collagen IV in presence (+) or absence (-) of DOX for 2 days and activation states of p42/p44 MAPK (ERK1/2) and Akt were assessed by Western blot analysis of whole cell lysates with indicated pan- or phospho-specific antibodies. To monitor total protein loading, membranes were re-probed with anti-α-tubulin monoclonal antibodies. Western blot analysis was done with two different sample preparations and was repeated at least twice. Representative results of Western blot analysis are shown.