Functional profiling: from microarrays *via* cell-based assays to novel tumor relevant modulators of the cell cycle

CAN-05-0642

Materials and Methods – supporting figures

Erk1/2 activation assay

NIH 3T3 cells, cultured in Dulbecco´s modified Eagle medium (DMEM, high glucose) with 1% penicillin/streptomycin, 1 % L-glutamine, 1% non-essential amino acids and 10% FBS (GIBCO-BRL, Life technologies) were seeded in 24 well plates (Falcon, BD Biosciences, USA) at 32,000 cells/well. After 24 hours, cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer´s instructions and incubated at 37°C, 5% CO_2. Cells were allowed to express the proteins for 24 hrs and then serum starved in DMEM-high glucose with 1% FBS for additional 24 hrs to reduce basal Erk1/2 activity. The cells were then washed 1x with D-PBS and trypsinized. Cells were fixed with 2% PFA for 10 min at 37°C. Fixed cells were washed once with D- PBS and then permeabilized with ice cold 90% methanol for 30 min on ice. After washing once with wash buffer (0.5% BSA in PBS) and incubation for 30 min at RT with rabbit anti-phospho p44/42 (New England Biolabs, Beverly, MA) diluted 1:100 in wash buffer. Cells were washed once more and then incubated with goat anti-rabbit APC labeled antibody (Molecular Probes, Karlsruhe) that had been diluted 1:250 in wash buffer. Cells were finally washed twice, resuspended in 250µl wash buffer and measured with a *FACS Calibur* (BD Biosciences, Heidelberg).

TaqMan Quantitative Real-Time PCR

Caki-1 and Caki-2 cells were grown as described above, and total cellular RNA was extracted. Fifty µg of RNA were reverse transcribed (RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas GmbH)). TaqMan real-Time PCR was carried out in 384well

plates using the Absolute™ QPCR Rox Mix (Abgene) together with Assays-on-demand (Applied Biosystems) containing a quenched-fluorescence probe and two primers, which spanned at least one intron, using an ABIPrism® 7900 HT (Applied Biosystems) at standard conditions. Primers and probes were designed for four genes with the help of the QuantiProbeTM Design Software (Qiagen), assays were carried out with the QuantiTec™ Probe PCR Kit (Qiagen). Thermal cycling conditions were 2 min at 50°C and 15 min at 95°C followed with 45 cycles at 94°C for 15 s, 56°C for 30 s and 76°C for 30 s. Each sample was analyzed in ten replicates. Data analysis was with the SDS 2.1 software from Applied Biosystems. The relative quantification of the mRNA expression level of the genes was estimated using the comparative CT method (2-ΔΔCT) described by Livak and Schmittgen (1). TaqMan assays were normalized to the expression of COPB, Qiagen assays were normalized for the expression of the MEK2 gene. RNA from a cell line pool (Universal Human Reference RNA, Stratagene) was taken as reference for expression.

Assay for quantification of anchorage-independent growth

Flat bottom 96-well plates were coated with poly(HEMA) (Poly(2-hydroxylethylmethacrylate, 120mg/ml 95% ethanol) as described(2). Kidney clear cell carcinoma cells Caki-1 (ATCC HTB-46) and Caki-2 (ATCC HTB-47) cells were cultured in RPMI 1640 with 1% penicillin/streptomycin, 1 % L-glutamine, 1% non-essential amino acids and 10% FBS (GIBCO-BRL, Gaithersburg,MD, USA) and transfected with Lipofectamine 2000 (Invitrogen, Karlsruhe) according to the manufacturer´s instructions. Proteins found to activate DNA replication were transfected into Caki-2 cells to measure their potential to stimulate anchorage-independent growth of this cell line. Repressors of DNA replication were analyzed in Caki-1 cells for their potential to reduce anchorage-independent growth. 24 hours after transfection, 3,500 Caki-1 or Caki-2 cells were spread in each well of the poly(HEMA)

coated plates in a total volume of 200 µl RPMI 1640. Measurement of cell growth was performed as described (2).

Additional References for supplementary material

1. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.
2. Fukazawa H, Mizuno S, Uehara Y. A microplate assay for quantitation of anchorage-independent growth of transformed cells. Anal Biochem 1995;228:83-90.