NR0B1 siRNA transfection of human lung alveolar epithelial adenocarcinoma carcinoma cell line (A549) using "Biontex K2® Transfection System"

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Materials and Methods

Cell culture

One day before transfection A549 cells were seeded in a 12-well plate, in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum and 5 mg/ml gentamicin. Transfection was performed in cells that had reached a confluency of 90-100%.

Cell transfection

Cells were treated with K2® Multiplier, 2 hours before siRNA transfection. For each well, 12 µl of K2® Multiplier was dripped slowly onto 800 µl of medium and mixed by gently swaying the dishes. 4,8 µl of K2® Transfection Reagent was mixed with 90 µl DMEM without serum and left at room temperature during preparation of the siRNA. 3µl of siRNA solution 30uM against NR0B1 was mixed with 90 µl DMEM without serum. siRNA solution was added to the solution containing the K2® Transfection reagent (not the other way around) and mixed by inverting the tubes, followed by 15 minutes incubation at room temperature. Transfection solution was applied to cells by slow dropwise addition to the medium followed by gently swaying the dishes to achieve mixing. Transfected cells were incubated at 37C and 5% CO2 for 24 hours, then the medium was replaced.

Evaluation of mRNA and protein expression.

mRNA and protein levels were evaluated after 48 h by quantitative PCR and western blot.

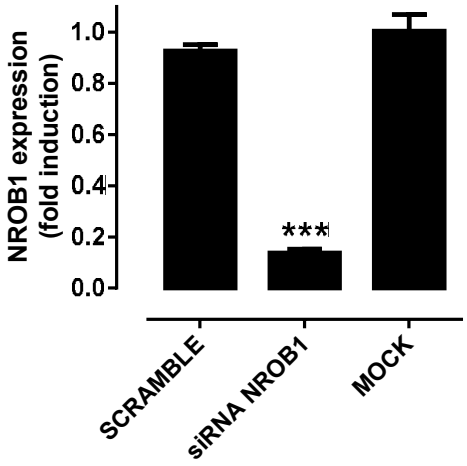
qPCR was performed in triplicate on the Rotor Gene Q cycler (Qiagen, Hilden, Germany) using the resulting cDNA, the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) for product detection, and the following primers: forward 5'-TGCTCTTTAACCCGGACGTG-3'; reverse: 5'-GCGTCATCCTGGTGTGTTCA-3'. Primers were supplied by Genbiotech (Argentina) and dissolved in water according to the supplier's instructions and kept at -20`C until use.

For western blots, total cellular proteins were extracted from A549 cells with fresh RIPA lysis buffer (1 ml/well) on ice, transferred into 1.5 ml eppendorfs, mixed and kept at -20`C until use. 5X of sample buffer was added to 10 µg of total protein, denatured for 5 min at 95`C, spinned shortly and then loaded onto 10% SDS

polyacrylamide gels. Samples were run through stacking gel at 100 V for 10 min and separating gel at 200 V for approximately 1 h. Protein transfer onto activated Hybond nitrocellulose membranes (GE Healthcare Life Sciences) was performed overnight at 4`C at 125 mA. For the detection of the NR0B1 protein levels, the blots were incubated in blocking buffer, consisted of 5% low fat milk powder in TBST solution for 1 h at RT (10 ml/membrane) and subsequently with a primary antibody against NR0B1 [Anti-NR0B1/Dax1 antibody (EP13786) – N-terminal (ab196649), abcam], or against GAPDH antibody (Santa Cruz). Following 3X washing with TBST, the blots were probed with species-specific horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and finally washed 5X with TBST. For all incubations and washes rolling shakers were used. For luminescent signal detection, membranes were incubated with 10 ml of luminol solution, supplemented with 100 ml of enhancer solution and 3.1 ml of 30% H2O2 for approximately 1 min at RT in the dark. Following film exposure, development and fixation, protein levels among samples were quantified relatively to GAPDH signal using the ImageJ software3 (Rasband, W.S., ImageJ, United States National Institutes of Health, Bethesda, MD, United States, 1997–2012).

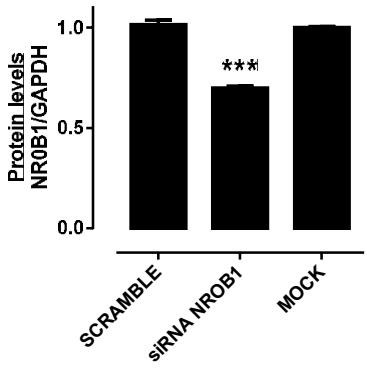
Results

Expression of NR0B1 mRNA



Expression of NR0B1 mRNA. The expression was specifically interfered by about 90% with respect to mock and scramble transfected cells. *** p<0.01 ANOVA test.

Expression of NR0B1 protein



Expression of NR0B1 protein. The expression was specifically diminished by about 30% with respect to mock and scramble transfected cells. *** $p<0.01$ ANOVA test.

Conclusions.

Our results show that A549 cells are high efficiently transfected with K2® Transfection System. Both quantitative PCR and western blot assays showed that the level of expression of NR0B1 was specifically dampened. The different outcome of PCR and western blot might due to the stability of the NR0B1 protein, which was not yet degraded at the time of measuring.