qPCR Identification of Genes Involved in Apoptosis and Cell Cycle Regulation

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Introduction

Systematic high-throughput functional genomic screens with the targeted silencing of genes by the introduction of small interfering RNA (siRNA) have emerged as an extremely powerful tool for analysis of biological functions. Such screens commonly result in a huge amount of "hits", genes that potentially play a role in the specific biological function being investigated. However, it proves difficult to distinguish true- from false-positive hits and to carefully assess the mechanisms by which the genes act in the analyzed conditions. Many of these assays employ, as a phenotypic/functional readout, changes in apoptosis or cell cycle. However, a change in these key cellular processes can be induced by a wide range of different pathways. To obtain a deeper understanding of how a gene knockdown leads to changes in apoptosis or cell cycle, an analysis of the expression of other genes involved in these processes is very useful, as this may elucidate the involved pathways and identify functional gene networks.

We carried out siRNA screens in HLR-CHOP cells to identify genes modulating cell cycle regulation and apoptosis. In both screens, genes were down-regulated by transfection with siRNAs, and the effects were analyzed by flow cytometry for DNA content after 7-AAD staining in the case of cell cycle analysis and for activation of caspase-3 in the apoptosis assay. In cell cycle analysis, down-regulation of DONSON and GDF3 via RNAi led to a block in G1-phase of the cell cycle. To characterize these two genes further regarding their involvement in cell cycle regulation, we analyzed a set of 91 cell cycle genes for endogenous expression employing qRT-PCR after knockdown of the DONSON and GDF3 genes. To this end we used the RealTime ready Cell Cycle Regulation Panel with the LightCycler® 480 Instrument. Caspase-3 activation was determined with a specific antibody and flow cytometry. In this assay we had found activation of caspase-3 increased following down-regulation of the C24ORF17 and BARD1 genes. To identify the role of the encoded proteins in the induction of apoptosis, in this study we analyzed C24ORF17 and BARD1, when down-regulated by RNAi, for their effect on a set of 378 genes involved in apoptosis regulation via qRT-PCR using the RealTime ready Apoptosis Panel, 384 (beta version), also with the LightCycler®480 Instrument.

The DONSON and GDF3 genes that had already been analyzed in the Cell Cycle Regulation Panel were further tested for potential effects on the RealTime ready Reference Gene Panel of 19 housekeeping genes and then normalized for changes in housekeeping gene expression. Moreover, for all panels the changes in endogenous expression of the analyzed genes after transfection with siRNAs was compared with expression in HeLa cells that had been transfected with siRNA targeting GFP as a control.

Materials and Methods

Cell culture

HLR-CHOP cells (Stratagene) were maintained in DMEM (GIBCO-BRL) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 10% FBS (GIBCO-BRL), hygromycin (100 µg/ml) and G418 (geneticin, 250 µg/ml) at 37°C in 5% CO_2. Cells were transfected with 40 nM siRNA and 48

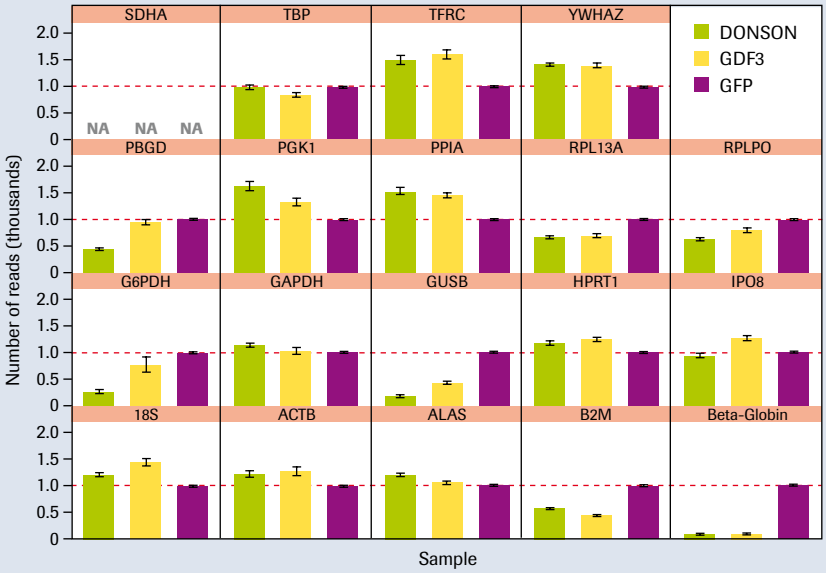

Figure 1: Changes in the expression level of housekeeping genes. Fold changes of expression are shown for all the selected housekeeping genes of the Reference Gene Panel.

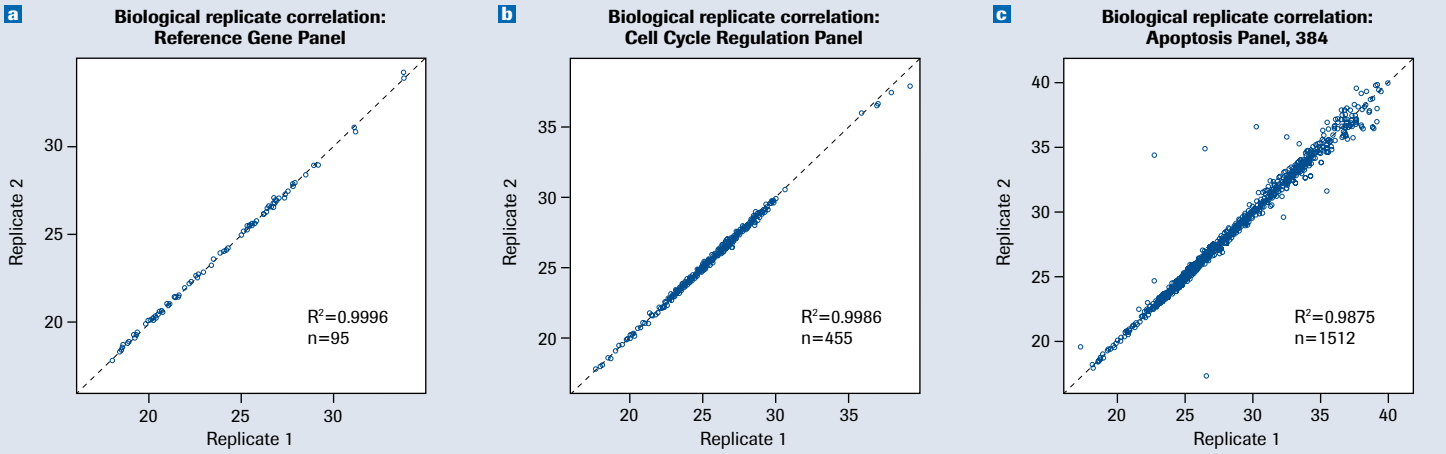

Figure 2: Correlation between two biological replicates. This plot shows the correlation between first and second replicate for each sample in **(a)** Reference Gene Panel, **(b)** Cell Cycle Regulation Panel, and **(c)** Apoptosis Panel.

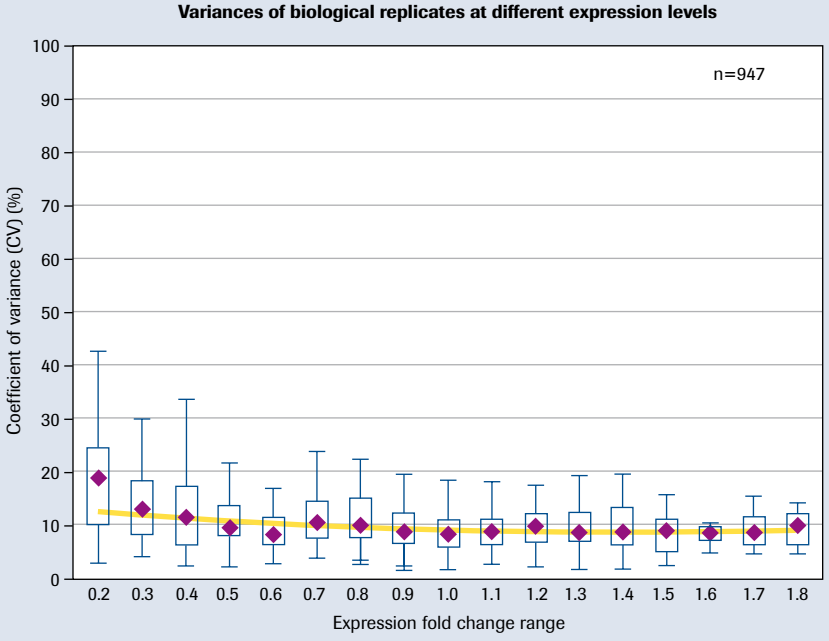

Figure 3: Variances of biological replicates at different expression levels. This plot illustrates how stable the determined relative expression level is (in form of coefficient of variance, CV) between two replicates and in what range the variance is stable. Relative expression level I is caculated by I=C*2-Ct, where Ct is the cycle number and C is a constant that we define in this case as 1. Ct values from two biological replicates are normalized by a panel of housekeeping genes as well as reference sample, to derive the relative expression level with the ddCt method. Except for sample-detector pairs with reference sample and housekeeping genes, we collect 1,432 relative expression levels and their standard deviations to draw this bandwidth plot. These 1432 experiments are divided by their relative expression level value into 18 sub-groups (from 0.1 to 2.6, on the x-axis, each group spans a relative expression level range at increment of either 0.1 or 0.5; ‚0.1' stands for [0.1, 0.2], ‚0.2' for [0.2, 0.3], and so on), The CV of each sub-group is shown in the boxes. The plot shows that a relative expression levels from 0.1 to 2.6 shares a stable CV with its median around 10%.

hours after transfection cells were stimulated with TNF-α (33 ng/ml).

RNA isolation and reverse transcription

Total RNA was extracted from 1 x 10^7 HLR-CHOP cells in two independent reactions (two biological replicates per siRNA) using the RNeasy Kit.

First–strand cDNA synthesis was performed in independent reactions using 700 ng of total RNA each and the Transcriptor First Strand cDNA Synthesis Kit using a mixture of oligo(dT)18 and random hexamer primer according to the manufacturer's instructions.

qRT-PCR

qRT-PCR using the RealTime ready assays was performed with the LightCycler® 480 Instrument and 2 x LightCycler® 480 Probes Master in a reaction volume of 20 µl in the 96-well format and a reaction volume of 10 µl in the 384-well format. In the 96-well format a cDNA/RNA concentration of 10 ng per reaction and in the 384-well format 4 ng per reaction were used. Reaction conditions for the LightCycler® 480 Instrument were: 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 second followed by 40°C for 30-second final cooling.

Results and Discussion

First, we tested the effects of knocking down GDF3 and DONSON in HLR-CHOP cells on the expression of 19 housekeeping genes. The expression results were then compared with the expression of the housekeeping genes after transfection of the cells with a control siRNA targeting GFP. As expected, expression of many of the housekeeping

genes was not significantly affected by a knockdown of GDF3 or when compared with cells treated with an siRNA targeting GFP (Figure 1). The expression of some housekeeping genes such as beta-globin, however, is strongly regulated by the knockdown. This shows the importance of analyzing which housekeeping genes are affected by a knockdown before normalizing with housekeeping genes in experiments. Therefore, we always normalized for an average of all reference genes provided on a RealTime ready Focus Panel.

To validate the reproducibility of the results, we analyzed the quantitative results of all the three RealTime ready Focus Panels (apoptosis, cell cycle and housekeeping) comparing the data from two biological replicates. A nearly perfect correlation between the first and second replicate was observed with all three tested panels (Figure 2). Next, we analyzed the stability of expression between the two biological replicates over a range of different expression levels. This provides a measure of the variance of this assay at different expression levels. To this end, two replicates (of each sample treated with siRNAs targeting BARD1, C19ORF24, GDF3 or DONSON) were normalized by a panel of housekeeping genes as well as the reference sample (siGFP treated cells), to derive the respective relative expression levels (Figure 3). This was analyzed across all three panels (representing 947 duplicate measurements in total). These results indicate that the coefficient of variance is very stable within the range of 0.1- to 2.5-fold expression and is around 10%.

We then quantified the expression of 94 genes in the Cell Cycle Regulation Panel after transfection of HLR-CHOP cells with siRNAs targeting GDF3 or DONSON and compared the resulting data with those obtained from cells that had been transfected with control siGFP. Both siRNAs against GDF3 and DONSON had shown an effect in a 7-AAD cell cycle assay. Therefore, we expected to also observe expression changes in some of the genes analyzed in the cell cycle panel. Indeed, we found 25 genes that showed more than 50% difference in expression when comparing cells transfected with siRNAs targeting GDF3 and DONSON with control cells transfected with siGFP (Figure 4). Cyclin D2 was down-regulated by 70% and 85% in GDF3 and DONSON treated cells, respectively, when compared with siGFP treated control cells (Figure 4) in the 94 genes of the Cell Cycle Regulation Panel (beta version). Cyclin E2 was 80% down-regulated only in cells treated with siRNA targeting DONSON. In GDF3-transfected cells, cyclin E2 was only slightly changed. If we analyze for genes that were up-regulated in the same samples, the strongest up-regulation in both samples was observed for p21, also termed cyclin-dependent kinase inhibitor 1a. p21 was found to be 1.81- and 3.31-fold up-regulated in GDF3 and DONSON siRNA transfected cells, respectively, when compared with control cells transfected with siGFP (Figure 4).

Thus, knockdown of GDF3 and DONSON induced down-regulation of Cyclin D2 and up-regulation of p21. It is known that Cyclin D2 forms a complex with the regulatory subunits of CDK4 or CDK6 (1). This activity of this complex is required for cell cycle G1/S transition [1]. Moreover, it has been described that induction of p21 leads predominantly to cell cycle arrest, and while p21 has been described to stabilize interactions between cdk4/cdk6 and D-cyclins, thus promoting

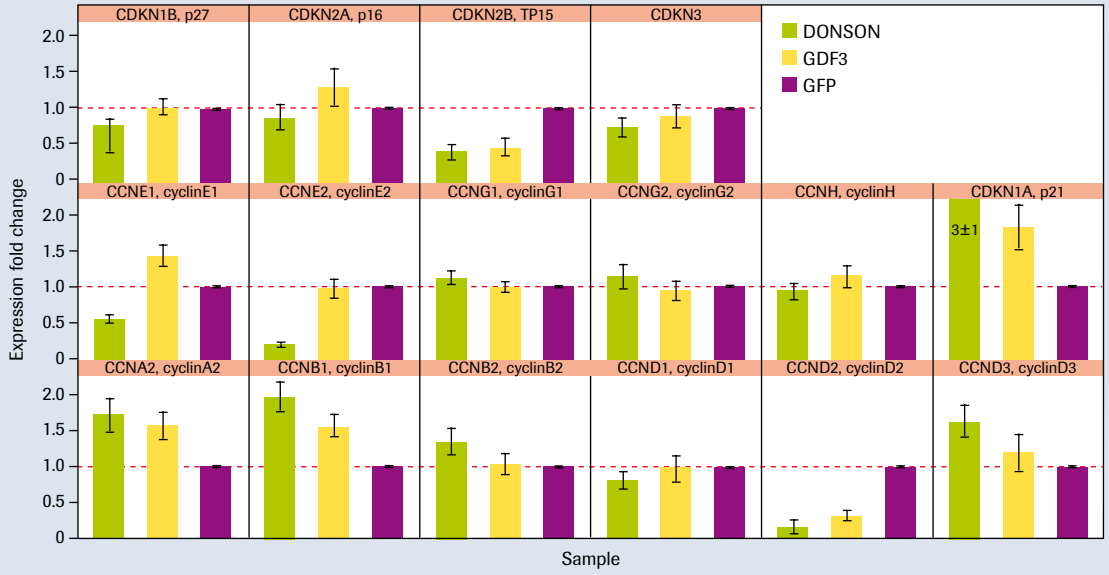

Figure 4: Changes in the expression level of cell cycle genes. Shown are expression levels of selected genes (cyclins and cyclin-dependent kinases) that were tested in the RealTime ready Cell Cycle Regulation Panel.

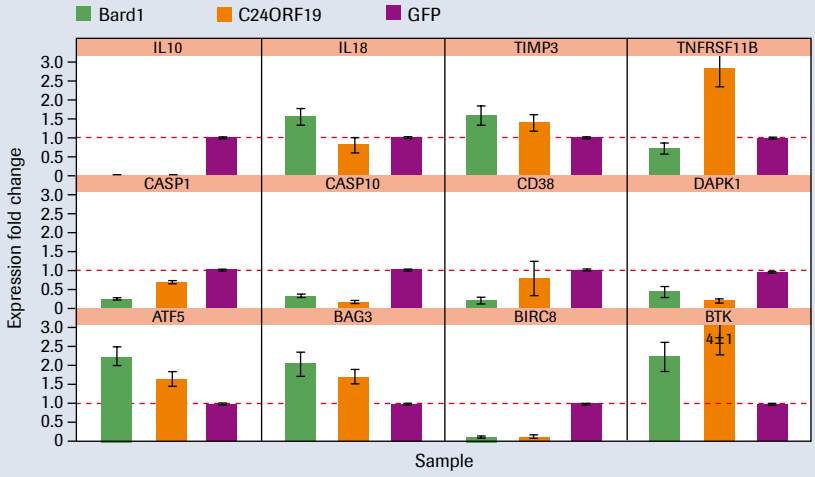

Figure 5: Changes in the expression level of apoptosis genes. Shown are expression levels of selected genes that were tested in the apoptosis panel.

the formation of active complexes, that negatively modulates cell cycle progression [2]. Thus, knockdown of GDF3 and DONSON induces up-regulation of p21 and down-regulation of cycline D2, explaining an arrest in G1-phase.

For C24ORF17 and BARD1, which induce caspase-3 activation upon down-regulation, we analyzed a set of 378 genes using the RealTime ready Apoptosis Panel, 384 (beta-version). 125 of the analyzed genes showed a more than 50% change in their expression level after treatment with either of the siRNAs as compared with siGFP-treated control cells. Interestingly, knockdown of the two genes induced many similar effects, but we also observed some clear differences for genes that were up- or down-regulated. For example, IL-10, BIRC8, Caspase 1 and Caspase 10 showed a strong down-regulation, whereas TIMP3, ATF5, BAG3, and BTK showed a significant up-regulation in samples transfected with siRNAs targeting C24ORF17 or BARD1 when compared with siGFP-treated control cells (Figure 5). Other mRNAs coding for TNFRSF11B showed a strong up-regulation only in cells transfected with siC24ORF19, but not in siBARD1 transfected cells, whereas IL-18 showed significant up-regulation only in siBARD1-transfected cells but not in siC24ORF19-transfected cells. In contrast, CD38 showed strong down-regulation only in siBARD1-transfected cells, while DAPK1 showed strong down-regulation in cells transfected with siRNAs targeting C24ORF17 and weak down-regulation in cells transfected with siRNAs targeting BARD1.

In summary, different patterns of the affected genes were found for the two genes tested in the apoptosis panel. The relevance of the up- and down-regulation of these genes for apoptosis remains to be elucidated.

Conclusions

All three RealTime ready Focus Panels (Reference Gene, Cell Cycle Regulation, and Apoptosis) performed well in respect to reproducibility over different ranges of expression. Almost identical Ct values were measured in more than 98% of all biological replicates and across all panels. Our experiments demonstrate that these panels are efficient tools for the quantitative analysis of gene expression for genes related to cell cycle and apoptosis as well as for housekeeping genes. Because of different housekeeping genes that can be used as internal controls on apoptosis and cell cycle panels, it is easily possible to normalize expression differences between two samples. The apoptosis and cell cycle panels delivered very informative and reliable data on how the expression of apoptosis- and cell-cycle-related genes are altered in a specific sample. This leads to a better understanding of how the analyzed condition induces an effect on cell cycle or apoptosis. ■

Order INFO

Product	Pack Size	Cat. No.
LightCycler® 480 Instrument	1 instrument (96-well version) 1 instrument (384-well version)	05 015 278 001 05 015 243 001
LightCycler® 480 Probes Master	5 x 1 ml (2x conc.) 10 x 5 ml (2x conc.) 1 x 50 ml (2x conc.)	04 707 494 001 04 887 301 001 04 902 343 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit	04 379 012 001
RealTime ready Human Reference Gene Panel, 96	2x 96-well plates	05 339 545 001
RealTime ready Human Reference Gene Panel, 384	2x 384-well plates	05 467 675 001
RealTime ready Human Nuclear Receptor Panel, 96	2x 96-well plates	05 339 332 001
RealTime ready Human Nuclear Receptor Panel, 384	2x 384-well plates	05 467 691 001
RealTime ready Human Cell Cycle Regulation Panel, 96	2x 96-well plates	05 339 359 001
RealTime ready Human Cell Cycle Regulation Panel, 384	2x 384-well plates	05 467 683 001
RealTime ready Human ABC Transporter Panel, 96	2x 96-well plates	05 339 324 001
RealTime ready Human ABC Transporter Panel, 384	2x 384-well plates	05 467 713 001
RealTime ready Human GPCR Panel, 96	2x 96-well plates	05 353 068 001
RealTime ready Human GPCR Panel, 384	2x 384-well plates	05 467 705 001
RealTime ready Human Apoptosis Panel, 96	2x 96-well plates	05 392 063 001
RealTime ready Human Apoptosis Panel, 384	2x 384-well plates	05 339 316 001