

High Content Analysis of a Reverse Transfection siRNA Screen using a Cell Line Reporting G1-S Cell Cycle Transition

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Introduction

Recent developments in small interfering RNA (siRNA) techniques for specifically modulating gene expression have revolutionized the functional analysis of gene function in mammalian cells. Advances in the capabilities of siRNA have been matched by advances in sophisticated fluorescence imagers and software capable of imaging and analysing cellular events in live cells at high-throughput. Such instrumentation enables study of complex systems by allowing the combination of data from fluorescent cellular sensors with morphological parameters to provide a detailed description of the phenotypic effects of siRNAs in cellular screens. In this study we have used high-throughput sub-cellular imaging in concert with a dynamic G1/S cell cycle GFP sensor to screen the effects of a panel of siRNAs delivered to cells using reverse transfection.

Materials and Methods

A stable U2OS cell line expressing an EGFP-DNA Helicase B PSLD sensor (Fig. 1) was developed to report on G1/S cell cycle transition. Sensor expressing cells were transfected with siRNA pools (Dharmacon) using 4 siRNAs/pool directed against 79 cell cycle control genes. Additional non-targeting, RISC-free and housekeeping gene siRNAs (Dharmacon) were used as controls. siRNAs (6.25pmol) were dried in 96 well imaging plates (ViewPlate, Packard), stored at -20°C, and reconstituted immediately prior to use with 25µl DharmaFECT transfection reagent in rehydration buffer (Dharmacon). Transfections were initiated by addition of 100µl trypsinized cells (5000 cells/well) in antibiotic free medium, and cells cultured for 48 hours under standard tissue culture conditions. For analysis, cells were washed in PBS fixed with 100µl of neutral buffered 4% formaldehyde solution for 30 minutes at room temperature and nuclei stained with 100µl 2µM Hoechst 33342 or 5µM DRAQ5 in PBS. Cells were imaged on IN Cell Analyzer 3000 using excitation and emission settings for Hoechst/DRAQ5 and EGFP. Images were analyzed for EGFP intensity and distribution using IN Cell Analyzer 3000 Object Intensity Image and Nucleo-Cytoplasmic Translocation analysis modules. Morphological parameters were abstracted using IN Cell Developer Toolbox.

Results

Reverse transfection conditions were first optimised for cell viability and knockdown efficiency using varying concentrations of an EGFP siRNA pool with 4 transfection reagents (DharmaFECT 1-4) followed by analysis of cell number and EGFP intensity (Fig. 2). No significant differences in cell number, viability and knockdown were observed using 10nM, 50nM and 100nM EGFP siRNA (results not shown). Optimum transfection conditions (50nM siRNA: DharmaFECT4) were then used to examine the effects of gene targeted siRNA pools on cell number (Fig. 3) and cell cycle G1/S transition (Fig. 4).

A number of siRNAs (Cyclin A2, Cyclin B1, Cyclin B2, Cyclin D2, Cyclin E1, CDK1, and PLK) produced a significant decrease in cell proliferation (Fig 3) relative to transfection and siRNA controls. These siRNAs had very similar effects on cell proliferation in a parallel screen carried out using a U2OS cell line expressing a G2/M EGFP sensor (data not shown). Analysis of the nuclear/cytoplasmic distribution of the EGFP G1/S sensor (Fig. 4) revealed a number of siRNAs including Cyclin D1, Cyclin D2, Cyclin E1, Cyclin E2, CDK6, CDK7, MDM2 and Retinol Binding Protein 1 (RBP1) resulted in a significant increase in cells in G1. These findings correlate well with the known roles of D and E type Cyclins and associated CDKs in regulating G1/S transition, and with p53 mediated arrest at the G1/S checkpoint via increased p53 activity and consequent inhibition of retinoblastoma protein (RB) phosphorylation (Fig. 5).

An interesting and previously unreported observation is the very significant blockage of cells in G1 induced by RBP1 knockdown (Fig. 6). We postulate that this effect is mediated through intra-cellular release of retinol and subsequent inhibition of RB phosphorylation mediated through Cyclin D and Cyclin E and the CDK inhibitors p21 and p27 (Fig 7).

To further define the effects of siRNAs, images were analyzed using a custom analysis protocol written using IN Cell Developer Toolbox to extract quantitative descriptors of nuclear morphology (Fig. 8). Of those siRNAs having anti-proliferative activity Cyclins A2 and B1, CDK1 and PLK showed significant changes in nuclear morphology, while knockdown of Cyclins B2, D2 and E1 had little or no effect on nuclear size, shape or symmetry. siRNAs producing significant arrest of cells in G1, e.g. Cyclin D2, MDM2 and RBP1, did not induce significant changes in nuclear morphology (Fig. 8). This data supports segregation of siRNA activity into G1 blockers (nuclear EGFP, nuclear morphology unchanged), early G2 blockers (cytoplasmic EGFP, nuclear morphology unchanged) and late G2 blockers (cytoplasmic EGFP, significant change in nuclear morphology).

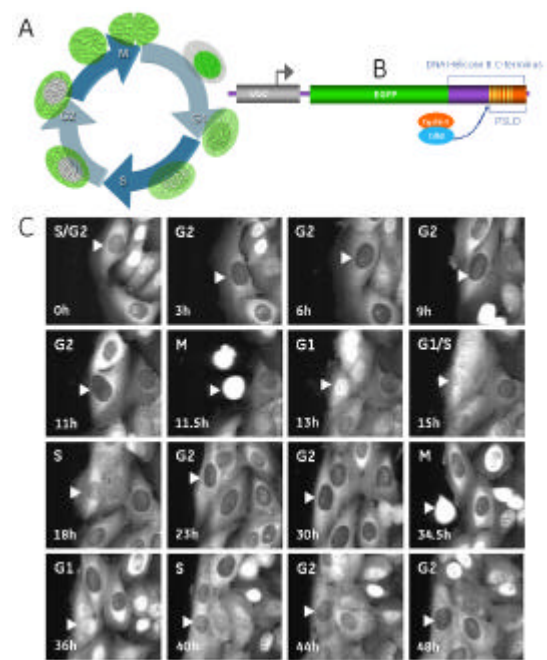


Figure 1. G1/S Cell Cycle Phase Marker. Cell cycle G1-S transition is reported by dynamic translocation (A) of an EGFP fusion protein (B) containing the phosphorylation and subcellular localization domain (PSLD) of DNA helicase B [1]. Phosphorylation of serine 967 in the PSLD masks a nuclear localisation sequence and activates a previously masked nuclear export sequence leading to translocation of the construct from the nucleus to the cytoplasm during G1/S transition. Expression of the construct in a stable U2OS cell line (C) allows monitoring of cell cycle progression via analysis of nuclear/cytoplasmic EGFP distribution.

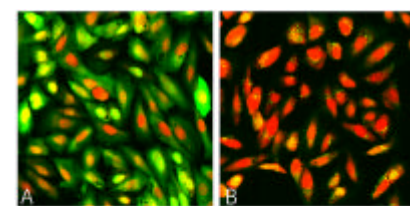


Figure 2. Analysis of EGFP knockdown. G1/S CCPM cells were reverse transfected with control (A) and EGFP (B) siRNA pools and EGFP intensity (C) determined by image analysis. Data shown is for 50nM EGFP siRNA, mean knockdown was 70.2±1.9%.

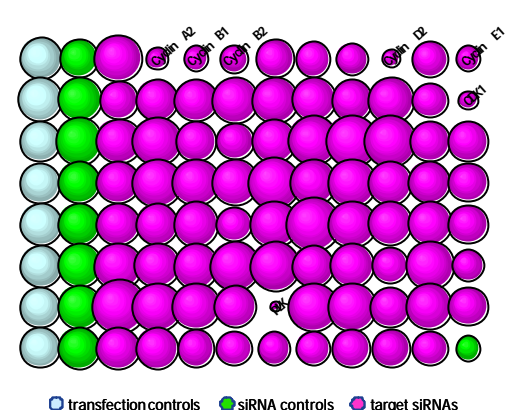


Figure 3. Effect of siRNAs on cell proliferation. G1/S CCPM cells were reverse transfected with control and target specific siRNA pools and cell numbers determined by image analysis after 48 hours. Relative cell numbers for each well are represented by the diameters of spheres on the plate map. Data are the means of 4 plates.

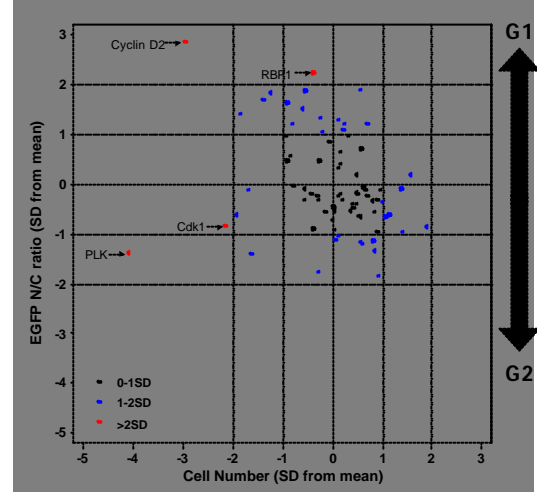


Figure 4. Effect of siRNAs on G1/S cell cycle transition. Images of cells transfected with siRNAs directed against cell cycle control genes were analysed for cell number and nuclear/cytoplasmic EGFP distribution. Data for each siRNA pool (mean of 4 replicates) are shown as divergence in standard deviations from the mean of the data set. (PLK-polo like kinase; Cdk1-cyclin dependent kinase 1; RBP1-retinol binding protein 1).

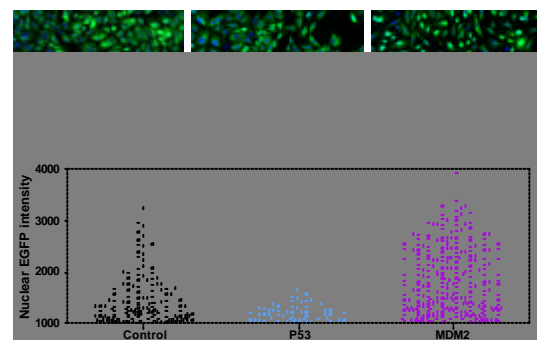


Figure 5. Effect of p53 and MDM2 siRNAs on G1/S cell cycle transition. Knockout of p53 reduced G1 cells relative to control siRNA, conversely knockout of the p53 regulator MDM2 increased G1 cells relative to the control siRNA.

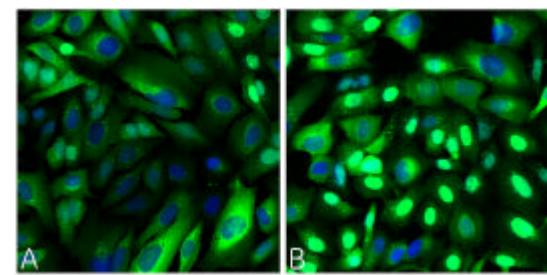


Figure 6. Effect of siRNA knockdown of Retinol Binding Protein 1 (RBP1) on G1/S transition. Cells were treated with (A) a control siRNA pool and (B) a siRNA pool directed against RBP1. Knockdown of RBP1 leads to a significant accumulation of cells in G1 with nuclear EGFP.

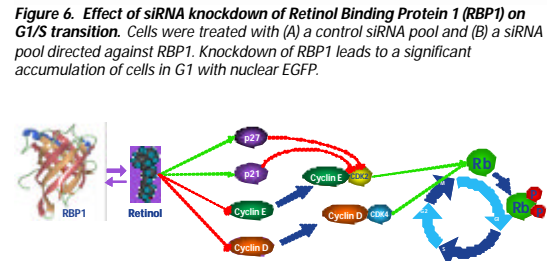


Figure 7. Hypothesis for RBP1 siRNA G1 arrest. Knockdown of RBP1 leads to increased cellular retinol and metabolite levels leading to dual mode inhibition of G1/S transition via down-regulation of expression of Cyclin D and E [2,3] and a synergistic up-regulation of the CDK inhibitors p21 and p27.

Conclusions

- Reverse transfection of siRNAs is an efficient high-throughput method for gene knockdown in cultured cells.
- High content analysis provides an information rich environment to investigate the role of genes in controlling key cellular processes. The combination of high-throughput fluorescent imaging of fluorescent sensors with automated image analysis of morphological parameters enables large numbers of siRNAs to be screened and characterised for phenotypic effects in cellular assays.

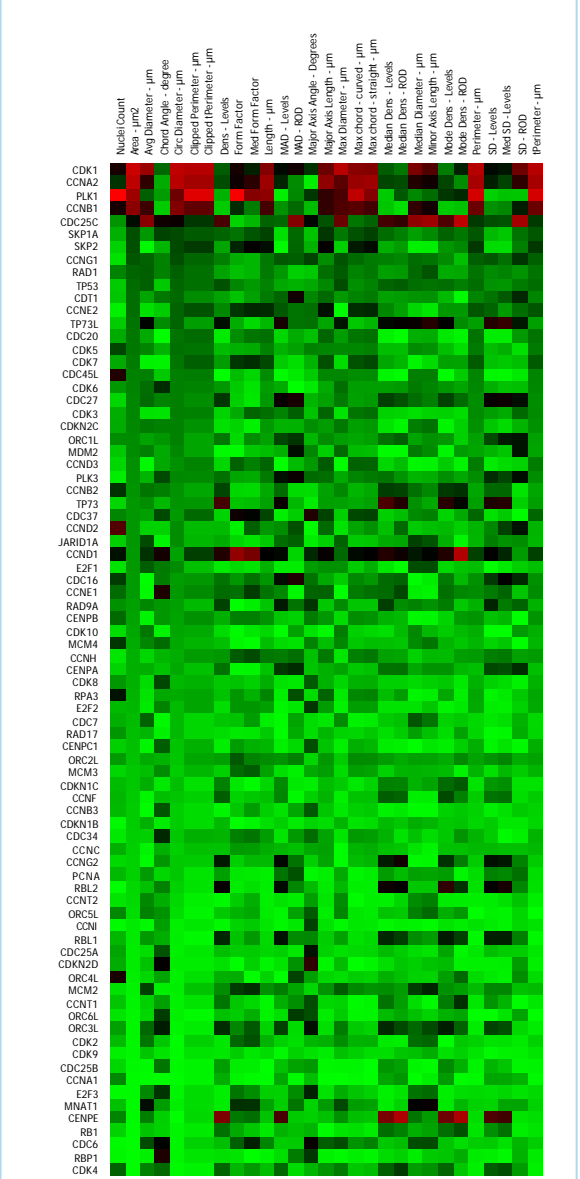


Figure 8. Nuclear morphology analysis. Images of G1/S CCPM cells treated with siRNA pools were processed using an analysis protocol written with IN Cell Developer Toolbox to abstract a range of nuclear morphology descriptors. Data for each descriptor on the heatmap is shown as variance in SD from the mean value of the descriptor across the entire screen (green-low/red-high). siRNA data is ordered according to nuclear area (top-high; bottom - low).

References:

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- Yu Z. et al. Induction of cell-cycle arrest by all-trans retinoic acid in mouse embryonic palatal mesenchymal (MEPM) cells. *Toxicol Sci*. 2005 83(2):349-54.
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