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Sets of short interfering RNAs (siRNAs) focused on a specific gene class (siRNA libraries) have the capacity to greatly increase the pace of pathway analysis and functional genomics. In the following series of experiments, 178 siRNAs targeting 178 different human kinases were individually tested to determine their effect on cellular proliferation and mitotic index. The screen identified several kinase genes that are involved in the cell cycle, confirmed the identity of some kinases known to be involved and identified a few kinases that had not previously been reported to be involved in cell cycle regulation.

Experimental Design

HeLa cells were plated at approximately 8000 cells per well in 96-well plates, 24 h prior to transfection. One hundred seventy-eight validated siRNAs from the Silencer™ Kinase siRNA Library (Ambion Inc., Austin, TX, USA) targeting 178 different kinases were individually transfected in triplicate into the cells (100 nM final concentration). An siRNA targeting cyclin B1 was used as a positive control, and Silencer Negative Control #1 siRNA (Ambion) was used as a "scrambled" sequence negative control. Forty-eight h post-transfection the cells were fixed and stained with 1) 4'-6-diamidino-2-phenylindole (DAPI) to reveal chromatin, with 2) anti-tubulin for microtubule distribution analysis and 3) with an anti-

Assessing Gene Function with siRNA Libraries: Kinases Affecting Cell Proliferation and Mitotic Index

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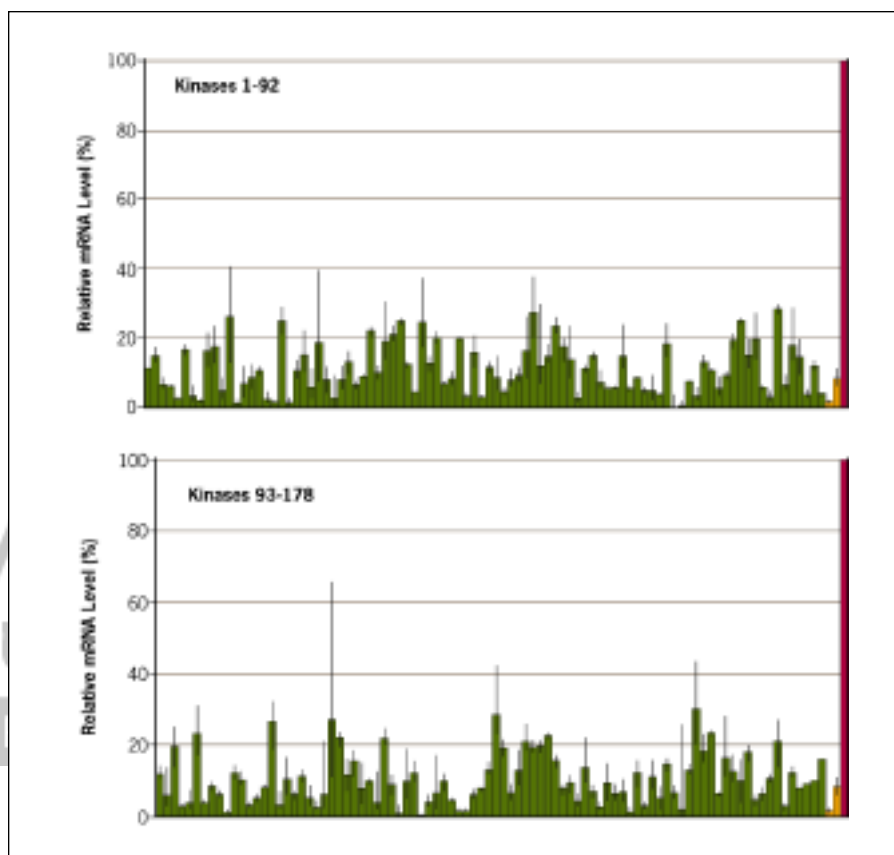


Figure 1. mRNA silencing by 178 kinase siRNAs from the Silencer™ Kinase siRNA Library. HeLa cells were plated at approximately 8000 cells per well in 96-well plates. Twenty-four hours later the cells were transfected in triplicate with each siRNA (100 nM). Forty-eight hours post-transfection, RNA was isolated, converted to cDNA and analyzed by real-time PCR. Shown are the relative mRNA levels compared to cells transfected with a scrambled negative control siRNA (red bar, Silencer Negative Control #1 siRNA).

phosphohistone H3 antibody to identify mitotic cells. The extent of cell proliferation was monitored by counting the number of cells in each well, and the percentage of cells undergoing mitosis (mitotic index) was evaluated by fluorescence microscopy. RNA was isolated from parallel samples, reverse transcribed to produce cDNA, and the cDNA was used to measure target mRNA levels by

real-time polymerase chain reaction (PCR).

Verifying siRNA Efficacy

An important component of data analysis in any siRNA experiment is to monitor the extent of mRNA degradation, or knockdown, elicited by a particular siRNA. Target mRNA remaining 48 h after siRNA transfection was monitored by real-time PCR. For each kinase siRNA, target

mRNA levels were reduced by 70% or greater compared to mRNA levels in negative control siRNA transfected cells (Figure 1).

Effects on Cell Proliferation

Wells transfected with the negative control siRNA contained approximately 1250 cells per microscopic image after 48 h (Figure 2). Inhibition of cyclin B1, which is known to be critical for initiation of mitosis, results in growth arrest. As expected, cell proliferation was dramatically inhibited by the siRNA targeting cyclin B1. Wells transfected with siRNA targeting cyclin B1 contained fewer than 700 cells per microscopic image.

Figure 2 also shows several kinase siRNAs that inhibit cell proliferation. This inhibition can result from a number of causes, including cell necrosis, apoptosis and cell cycle deregulation. Interestingly, a few kinase siRNAs appear to have a stimulatory effect on cell proliferation.

Changes in Mitotic Index

A good way to understand a gene's role in cell cycle progression is to monitor the percent of cells undergoing mitosis at any given point in time, with and without siRNA treatment. For asynchronously growing cultures, such as those used in these experiments, the mitotic index reflects the fraction of time that cells spend in mitosis versus the rest of the cell cycle. For exam-

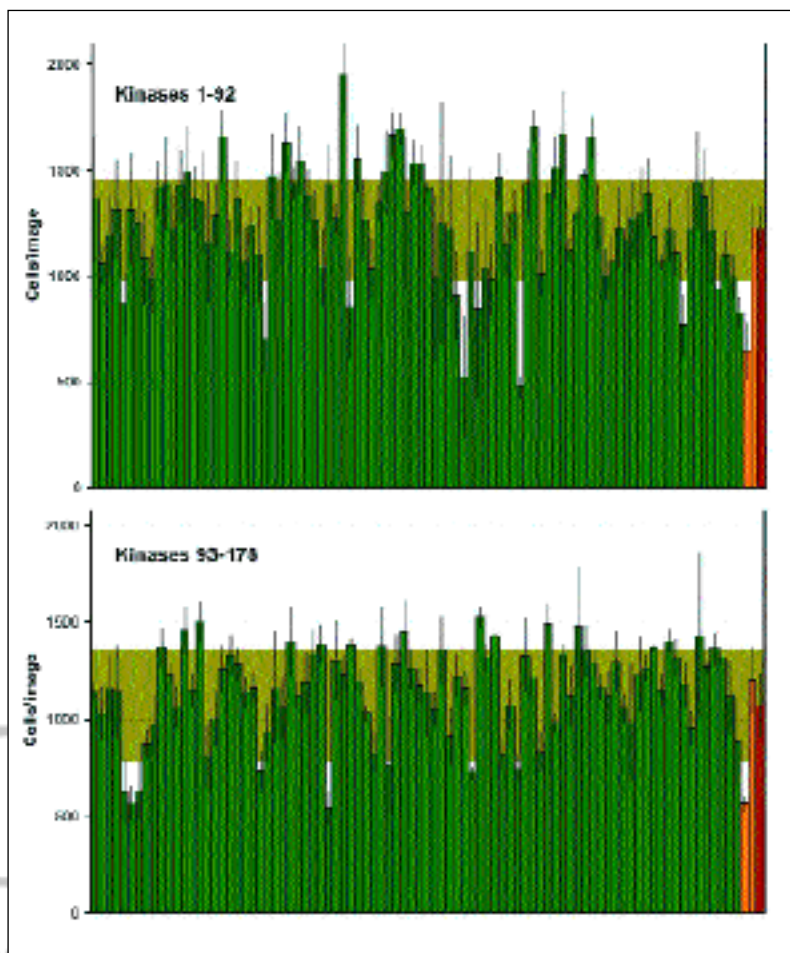


Figure 2. Changes in cell proliferation induced by 178 kinase siRNAs from the Silencer™ Kinase siRNA Library. HeLa cells were transfected with individual siRNAs targeting 178 different kinases as described in Figure 1. Cells were counted 48 hours post-transfection.

ple, in Figure 3, the approximately 2.5% mitotic index value observed for control cells transfected with the negative control siRNA indicates that cells spent 2.5% of the cell cycle in mitosis. Several siRNAs induced dramatic changes in the mitotic index when monitored 48 h after siRNA transfection. Decreases in mitotic index reflect either a shortening of mitosis or a lengthening of interphase (arrest). Conversely, increased mitotic index results from a lengthening of mitosis (e.g., by arrest) or a shortening of interphase. Both of these results were observed among the siRNAs tested. Two of the siRNAs increased the mitotic index more than five-fold.

The cause of the mitotic arrest phenotypes then was examined by

immunofluorescent staining of multiple markers, including tubulin, to reveal microtubule distribution. This revealed one kinase siRNA that triggered a prometaphase-like arrest state (nearly 16% mitotic index, or approximately seven-fold higher than normal) wherein spindles were clearly bipolar and chromosomes were well condensed but there was no evidence of successful chromosome alignment (Figure 3, top panel, left inset). This could correspond to activation of the spindle assembly checkpoint, which monitors the correct, functional interactions between spindle microtubules and kinetochores in preparation for

metaphase and subsequent anaphase onset. A second kinase siRNA that also generated cells in mitotic arrest induced the formation of aberrant spindles displaying either too many or too few apparent poles (e.g., Figure 3, top panel, right inset).

Silencing kinases necessary for passage of the cell through G1, S and G2 phases of the cell cycle may be expected to lengthen interphase and therefore decrease the percentage of cells in mitosis. It also will decrease the overall cell proliferation rate. Several of the siRNAs investigated here induced this phenotype (Figure 4). Similarly, inhibition of kinases required for passage through mitosis would be expected to cause an increase in mitotic index and a decrease in cell proliferation.

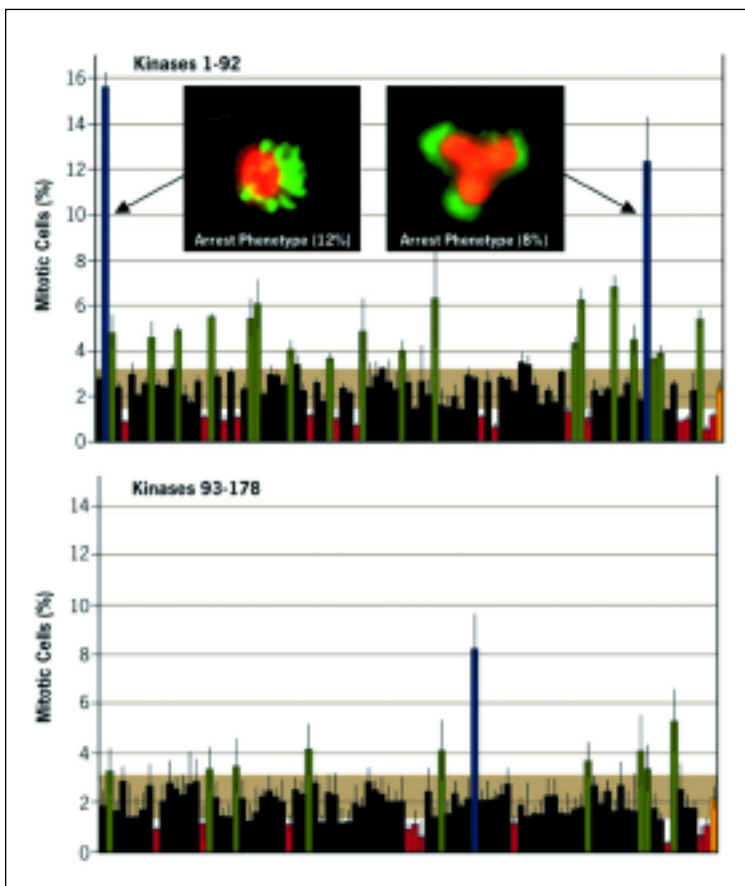


Figure 3. Mitotic index of cells transfected with 178 siRNAs from the Silencer™ Kinase siRNA Library. HeLa cells were transfected with individual siRNAs targeting 178 different kinases as described in Figure 1. Mitotic index was measured 48 hours post-transfection. The orange bar shows the percent of negative control cells, which were transfected with a scrambled negative control siRNA, undergoing mitosis (mitotic index range of 2.1 to 2.3). The semitransparent brown rectangles highlight the 95th percentile range of normal mitotic index. The inset shows two interesting mitotic arrest phenotypes induced by siRNAs targeting two different kinases (green = chromatin; orange = tubulin).

Several siRNAs also appeared to induce this phenotype.

Conclusions

This screening experiment illustrates the power of higher content, multiparameter assays combined with libraries of effective siRNAs. The data set allows the researcher to go beyond a primary readout (effects on cell proliferation) to efficiently and directly address underlying causes such as cell cycle deregulation at several levels with a single screening experiment. In this example, by comparing the cell proliferation and mitotic index data, one can relate the antiproliferative effects observed for several ki-

nase-targeting siRNAs back to underlying effects on cell cycle regulation.

In collaboration with Cenix, Ambion has developed a set, or library, of siRNAs corresponding to the human druggable genome. siRNA library subsets divided by gene functional class (e.g., cytochrome P450, phosphatase, ion channel, oxygenase, ATP binding, caspase, protease, phosphodiesterase, nuclear hormone receptor, etc.) also have been created. These types of reagents can greatly accelerate drug target identification and validation efforts, as well as help realize the promise of functional genomics. Contact Ambion for details at libraries@ambion.com.

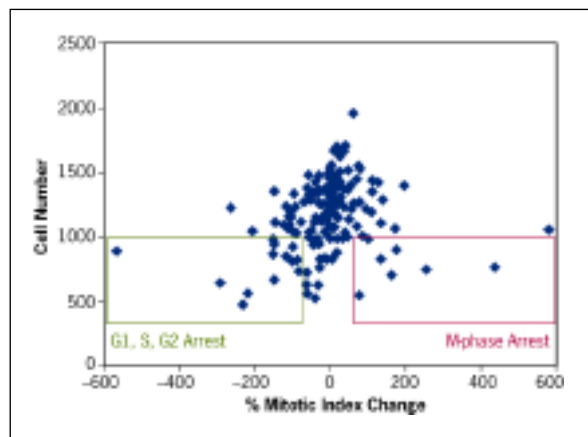


Figure 4. Deviations in cell number and mitotic index induced by siRNAs targeting 178 kinases. Plot of the data from Figure 2 vs. Figure 3 demonstrating that silencing of certain kinases dramatically affects the cell cycle. Multiple categories of data can be defined, such as 1) no change, 2) increase in cell growth with no change in mitotic index, 3) no change in cell growth, increase in mitotic index, etc. Shown in red are cells exhibiting arrest in G1, S or G2 (left) and cells exhibiting arrest in M (right) phases of the cell cycle.

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