Using RNAi to identify and validate novel drug targets

targeting human kinases with an siRNA library

Libraries of siRNAs directed against predefined subsets of genes now offer the capacity to greatly accelerate and improve the quality of functional genomics-based drug discovery by enabling a much more targeted approach that effectively integrates the discovery and validation of novel targets. By avoiding the complexities of overexpression phenotypes, the RNAi-based gene silencing approach offers clearer, more direct insights into gene function with inherently higher patho-physiological relevance. Here, the power of siRNA libraries is demonstrated through screening experiments carried out against human kinases. The initial screening experiment identified several kinase genes that are involved in the cell cycle, confirming some previously known functions and also identifying additional kinases that had not previously been reported to affect cell growth. This case study also illustrates the value of applying high content or multi-parameter readout assays to generate richer datasets more efficiently.

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RNA interference (RNAi) is a natural phenomenon in which double-stranded RNA (dsRNA) causes degradation of the complementary mRNA (**Figure 1; step 1**). Introducing dsRNAs into cells can artificially induce the RNAi response. After introduction or expression, these long dsRNAs are cleaved intracellularly into short 21-25 nucleotide small interfering RNAs, or siRNAs, by a ribonuclease known as Dicer (**step 2**). siRNAs assemble with the protein components of the RNA-induced silencing complex (RISC), unwinding in the process (**step 3**). This activated RNA-RISC complex then binds to the complementary transcript by base pairing interactions between the siRNA antisense strand and the mRNA. The bound mRNA is cleaved (**step 4**) and subsequently degraded (**step 5**). This mRNA degradation leads to decreased protein levels for the targeted gene, and therefore, gene silencing.

The first reports of RNAi were in plants, Caenorhabditis elegans and Drosophila melanogaster[1-3]. In these organisms, RNAi can be induced by introducing long dsRNA complementary to the target mRNA. In mammalian cells,

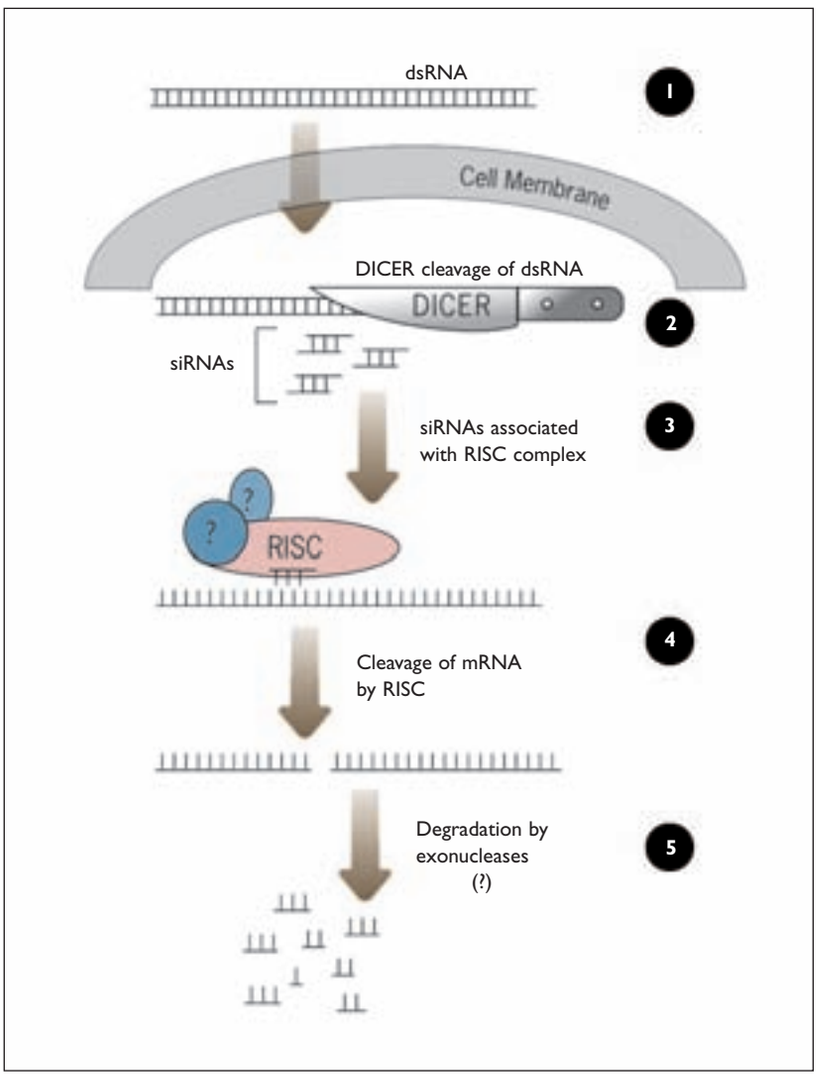

Figure 1: The mechanism of RNA interference
An overview of the RNAi pathway is depicted. For standard mammalian cell experiments, the dsRNA in 1 would be siRNA and would not be further cleaved by Dicer once it is inside the cell

however, introducing dsRNA longer than 30 base pairs activates the interferon response pathway, resulting in a general shutdown of protein synthesis and apoptosis. To circumvent this stress response, siRNAs (typically 21 base pair duplexes with two nucleotide 3' overhangs) are used to induce RNAi in mammalian cells and organisms[4]. The widespread success of this approach has fuelled the rapid acceptance of RNAi as a standard technique for functional genomics.

RNAi and target identification and validation

Artificial induction of the natural RNAi pathway in now routinely used to specifically silence target genes. Applications of RNAi include analysis of gene function, identification of molecules involved in biochemical pathways, lead optimisation, toxicological studies and functional genomics[5]. The most common use of RNAi in drug discovery is for target validation experiments. The removal, or 'knockdown', of any targeted mRNA allows a simple, reversible ablation of individual genes to unravel the biology associated with different disease states. More importantly, the complex biology surrounding most putative drug targets and their pathways can be better understood by knocking down multiple genes within a pathway in multiple cell lines. The growing use of RNAi, as well as gene expression studies using DNA microarrays and other recent functional genomic advances, seeks to increase the value of a small molecule screen before it is started.

Recent genomics initiatives have led to the identification of large numbers of potential drug targets. The downside is that most of these potential targets are poorly understood. This results in an increased amounts of basic biological research required of pharmaceutical companies to understand each target's value. RNAi experiments provide a way to perform systematic studies of gene families, signalling pathways and biochemical pathways. RNAi screens can be used to identify new drug targets and for target validation purposes. The most advanced target validation experiments combine RNAi, DNA microarrays and/or drug treatments to study the pleiotropic effects of knocking down a gene or combinations of genes. A natural progression of these target validation experiments is to systematically knockdown every gene in a family or pathway.

The first systematic RNAi screens were very large, genome-scale screens performed in the lower eukaryotes Caenorhabditis elegans and Drosophila melanogaster[6-8]. These screens have demonstrated the power of this approach from efficiently attributing new functional insights to large numbers of genes. Similar screens are also possible in mammalian cells using siRNAs, whether at the genomic or sub-genomic levels, for example, targeting entire gene families (eg kinases) or pathways (eg caspase activation). This type of systematic screen is obviously useful for new target identification, although the acquisition of highly validated targets is the true goal. A systematic RNAi screen can be used to completely dissect a pathway to increase the knowledge about a potential target and its pathway. This type of systematic screen can, and perhaps should, include knocking down multiple genes simultaneously to uncover redundant pathways and to fully confirm new components of a pathway. Also, by implementing higher content or multi-parameter readout assays, the screening data can carry significantly more contextual value, thereby further increasing the validation level of the target.

RNAi as a therapeutic

siRNAs are also being evaluated as potential therapeutic agents[9]. If realised, the impact on the drug discovery process would be revolutionary. Researchers have already shown that the RNAi pathway is active in mice and that siRNAs are tolerated and effective in several different tissues[10]. Synthetic siRNAs and siRNA expression vectors (both plasmid and viral) have been injected systemically and into defined tissues, and elicited target-specific responses. A number of publications have shown that siRNAs can inhibit the replication of HIV[11, 12] and Hepatitis B[13]. Additionally, a siRNA targeting a prion-prone protein was capable of inhibiting prion formation in cells, creating an alternative therapeutic approach to prion diseases[14]. Together, these advances have spurred a flurry of investments and partnering in the biotech sector, although significant hurdles remain.

The most significant hurdle for the therapeutic use of siRNA is delivery: how can siRNAs be targeted to specific cells? Delivery of nucleic acids to specific organs, tissues and cells will require significant advances in nucleic acid chemistries, including possible novel conjugations and/or formulations to specifically target certain cells. The first indication for siRNA to reach clinical trials is likely to target the VEGF receptor for wet acute macular degeneration. Early results suggest that a naked siRNA can be effective when directly injected into the eye[15]. Other indications that require systemic applications of siRNA will require new formulations to ensure targeting of the siRNA to the desired organ and tissue.

Two other hurdles for siRNA therapeutics relate to challenges faced by all nucleic acid therapeutics: drug stability and manufacturability. The *in vivo* stability of siRNAs can be increased by a variety of modifications, including alterations to the sugar moiety, the backbone and/or the base. These modifications are the result of years of research for antisense therapeutics, ribozyme therapeutics and aptamer technologies, providing a head start for siRNA therapeutics.

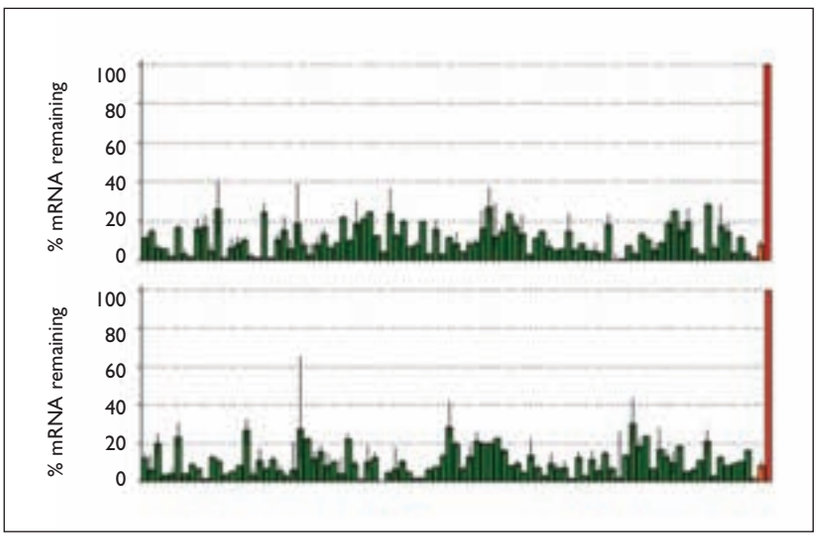

Figure 2: Knockdown efficiency after treatment with siRNAs targeting kinases
HeLa cells were transfected with individual siRNAs targeting 178 different human kinases from the Silencer Kinase siRNA Library (Ambion). Knockdown efficiency was measured 48 hours post transfection by real time PCR, using cells treated with a negative (scrambled) control siRNA as the reference. The top panel depicts results from 92 of the kinase siRNAs, while the bottom panel shows the results for kinase siRNAs 93-178. The knockdown is greater than 70% for every siRNA tested

The current technologies for manufacturing nucleic acids on solid supports are sufficient for supplying kilogramme quantities of materials for clinical trials, although new technologies will likely be required to produce the tons of RNA oligonucleotides that will be required to support a major pharmaceutical product.

Optimised siRNA design and validation

The optimal length for a siRNA is 21 base pairs; therefore, hundreds to thousands of different siRNAs can be designed for every gene. The sequence design of a siRNA is a crucial factor in the effectiveness of a siRNA as a silencing reagent. Design rules have been proposed by a number of academic groups and biotechnology siRNA suppliers (Ambion, Dharmacon and Qiagen). Designing an effective siRNA is similar to designing effective PCR primer sets: basic rules can be defined, tested and used to build a siRNA design algorithm.

siRNA design algorithms incorporate multiple criteria related to the sequence. Standard criteria such as the melting temperature of the duplex, the melting temperature of the ends of the duplex, nucleotide content of the 3' overhangs, base composition at certain locations, and position within the mRNA are included in most algorithms The best siRNA design algorithms also incorporate strict specificity requirements to minimise the risk of so-called off-target effects, ensuring that only the desired gene is targeted. However, the most important attribute of any algorithm is ultimately its performance as tested experimentally. Unlike testing PCR primer design algorithms, which requires a simple PCR assay, siRNA design algorithm testing requires the standardised transfection of siRNAs into living cells, isolation of RNA at one or many time points, followed by a measurement of the endogenous mRNA by quantitative methods such as real time PCR.

As more is learned about siRNAs, it is becoming increasingly clear that using highly potent siRNAs that efficiently silence their target gene at low siRNA concentrations is advantageous. Transfection of low siRNA concentrations reduces the potential for off target effects or induction of the antiviral response[16-18]. Low siRNA concentrations also allow the use of less siRNA per experiment, saving reagent costs and permitting the use of multiple siRNAs targeting different genes in a single experiment.

Delivery of nucleic acids into cells is not only important for therapeutic uses of siRNA, it is also crucial for a successful tissue culture experiment. Chemically synthesised siRNAs can be delivered into cells by a number of methods, including most prominently transfection and electroporation. Transfection methods typically include the non-covalent combination of the siRNA with a lipid reagent. Each cell type has unique requirements for optimal siRNA transfection, including different lipid compositions, siRNA concentrations and lipid: siRNA ratios, each of which needs to be optimised. The side effects of these lipid reagents can be significant (toxicity, activation of stress responses, etc) and need to be taken into account for any RNAi experiment. Many cell lines, especially primary cell lines, hematopoetic cell lines and neuronal cell lines are very difficult to transfect using a lipid reagent. For these recalcitrant cell lines, electroporation offers an exciting alternative to deliver siRNAs into these cells. However, the biggest technical challenges of electroporation include the need to compromise between high rates of cell death and delivery, as well as the lack of commercially available 96-well electroporation devices to enable high throughput applications.

siRNA libraries

siRNA libraries are collections of siRNAs designed against a group of similar targets, including the entire drugable genome. These libraries allow analysis of entire classes of genes simultaneously for both target identification and target validation experiments. These siRNA libraries are best divided by either molecular function (eg kinases, GPCRs, nuclear hormone receptors), or by pathway classifications. Pathway-specific libraries have the difficulty that some genes appear in dozens of pathways and new members of pathways are being added over

time. A simple method to circumvent these issues is to stock siRNAs against every gene in-house.

This latter suggestion is an important step to alter the early phases of the current drug development process to identify higher value drug targets at an earlier stage in the development process. Genome-wide RNAi screens can now be coupled with the gene expression data from DNA microarrays, genetic variability data, whole genome protein-protein interaction maps and knockout mouse models. Together, these data represent an important paradigm shift from the days of identifying hundreds of 'targets' from one source of data to our current ability to have multiple, complementary data sets. Importantly, RNAi screens are not cost prohibitive compared to large DNA microarray data sets or other technologies that are very early stage.

Case study: Using a kinase siRNA library to identify novel kinase functions

To illustrate the point that a broad RNAi screen can identify high-value drug targets, a case study was initiated to identify new kinases involved in cellular proliferation and cell division. Kinases represent a very popular class of genes for new drug targets, demonstrated by the recent commercialisation of Gleevec, an anti-tyrosine kinase drug from Genentech. The identification of which kinases participate in the cell cycle and in proliferation is one of the most obvious knowledge gaps that can be filled using a siRNA library targeting kinases. The case study shown here highlights the rich data that can be obtained from a systematic screen combining a siRNA library with a relatively simple multi-parameter readout.

The overall concept is simple: the goal is to identify which kinases are involved in proliferation and the cell cycle. siRNAs were prepared against every human kinase using a well-defined siRNA design algorithm. The experimental plan was to identify kinases that are expressed in HeLa cells, define a set of siRNAs that individually knock down mRNA levels corresponding to each kinase, perform the actual siRNA transfections, confirm the knockdown percentage for each gene, then measure cellular proliferation and the mitotic index.

178 kinases were identified that were both expressed in HeLa cells and have a siRNA that has been shown to knockdown the targeted mRNA by at least 70%. These kinases represent a broad array of kinases, with members of each of the families described in the 'kinome'[19]. A siRNA targeting cyclin B1 was used as a positive control because of the well-known role of cyclin B1 in the G2/M checkpoint. An additional 'negative' control, which targets no known gene, was used to control for general effects of nucleic acid transfections. Forty-eight hours post-transfection, RNA was isolated from one group of cells, while an identical group of cells was fixed and stained with DAPI to reveal DNA localisation, anti-tubulin for microtubule distribution, and an anti-phosphohistone H3 to identify mitotic cells. Cell proliferation was measured by counting the number of cells in each well. The percentage of cells undergoing mitosis, or 'the mitotic index', was evaluated by fluorescence microscopy using the number of mitotic cells divided by the total number of cells.

Verifying siRNA efficacy

An important component of data analysis in many siRNA experiments is to monitor the extent of mRNA degradation, or 'knockdown', elicited by a particular siRNA. This siRNA experiment was designed to generate target validation-quality data, rather than to perform a quick screen to identify kinases whose inhibition leads to newly defined phenotypes. Real-time PCR was used to show that the amount of mRNA remaining for each target gene was less than 30% of the original mRNA for every siRNA tested. **Figure 2** shows not only excellent knockdown, it also highlights the importance of spending time to optimise the transfection procedures before starting the experiment.

Effects on cell proliferation

A standard assay for oncology and cell cycle research is to measure the proliferation rate after a drug treatment. **Figure 3** shows that treatment of cells with gene-specific siRNAs identified

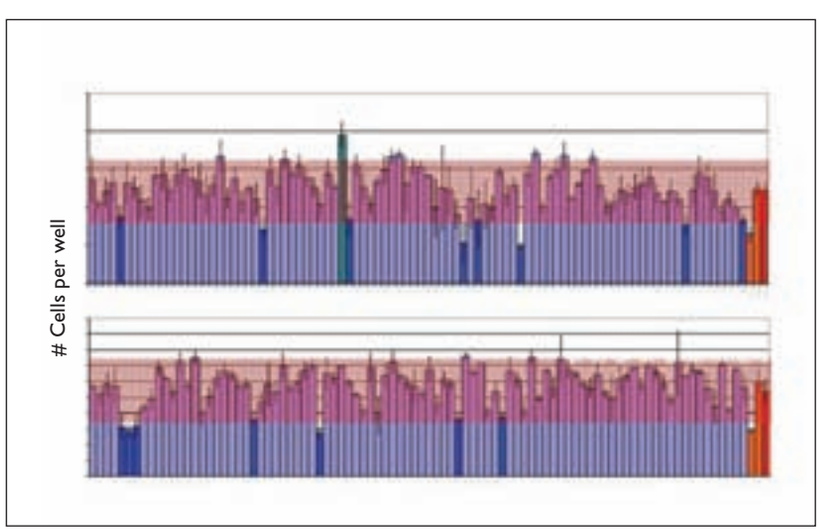

Figure 3: Proliferation assay after treatment with siRNAs targeting kinases
HeLa cells were transfected with individual siRNAs targeting 178 different human kinases from the Silencer Kinase siRNA Library (Ambion). Cell numbers were measured 48 hours post transfection. The top panel depicts results from 92 of the kinase siRNAs, while the bottom panel shows the results for kinase siRNAs 93-178. Results from the three control siRNAs are on the right of the graphs; the number of negative controlsiRNA treated cells are shown in red and cyclinB1siRNA treated cells are shown third from the right in orange. The shaded, horizontal bar represents the expected normal range for the cell number

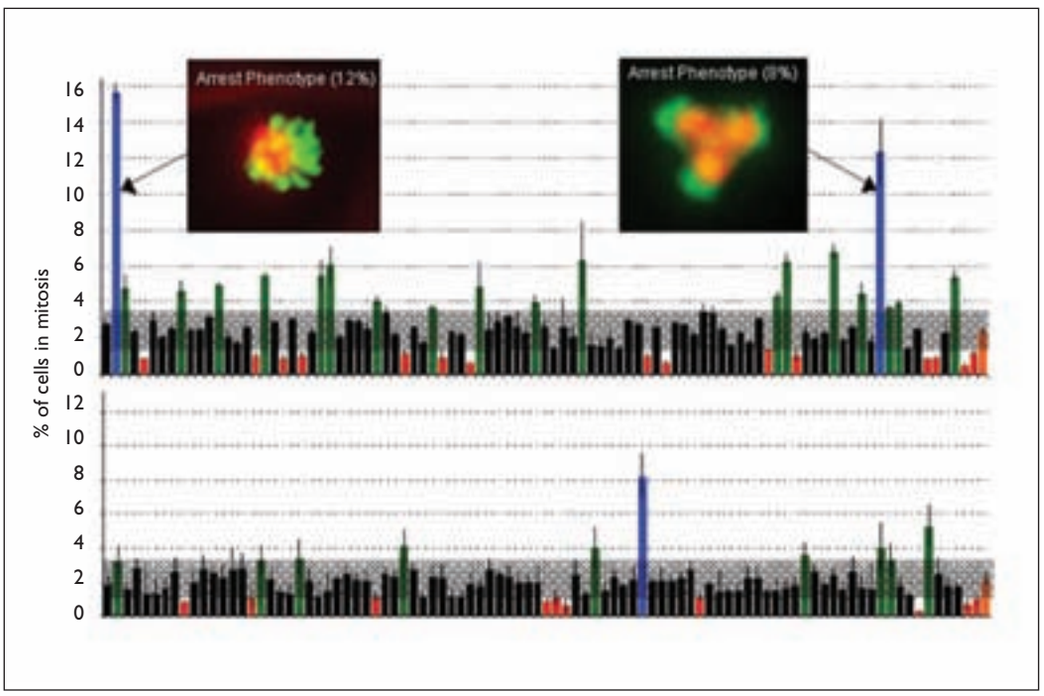

Figure 4: Mitotic index after treatment with siRNAs targeting kinases
HeLa cells were transfected with individual siRNAs targeting 178 different human kinases from the Silencer Kinase siRNA Library (Ambion). The mitotic index was measured 48 hours post transfection using an antibody to stain phosphorylated histone H3. The top panel depicts results from 92 of the kinase siRNAs, while the bottom panel shows the results for kinase siRNAs 93-178. The red bars, excluding the rightmost control bars, indicate kinase knockouts with a pre-mitotic arrest. The blue bars indicate kinase knockouts with a mitotic arrest. Results from the three control siRNAs are on the right of the graphs; mitotic index of negative control siRNA treated cells is shown in red and that of cyclinB1siRNA treated cells is shown third from the right in orange. The shaded, horizontal bar represents the expected normal range for the mitotic index

multiple kinases that are important for cellular proliferation. The positive control, inhibition of cyclin B1, results in complete growth arrest (no increase in the number of cells post-transfection). In addition to the kinases required for proliferation, the knockdown of a few kinases actually stimulated cell growth (**Figure 3**). The more commonly-observed inhibitory effect, however, can result from a number of different underlying causes, all of which fall into four broad categories: effects reducing metabolism, effects causing cell necrosis, effects causing apoptosis and effects causing cell cycle deregulation.

Changes in mitotic index

A gene's role in the cell cycle can be monitored by measuring the percentage of cells undergoing mitosis at any given point in time with and without siRNA treatment. The average mitotic index for cells grown under the case study conditions was 2.5%. As expected, treatment with the cyclinB1 siRNA control resulted in a severely altered mitotic index resulting in a four-fold decrease in the percent of cells in mitosis (**Figure 4**).

In addition to the cyclinB1 positive control, several kinase siRNAs caused a decrease in the mitotic index (red bars in **Figure 4**). These data are consistent with a pre-mitotic (interphase) arrest phenotype induced by the transfected siRNAs. The blue bars in **Figure 4** show the opposite phenotype, in which the mitotic index was increased in siRNA treated cells. In the most severe case, more than 15% of the cells are in mitosis. When these cells were analysed further by DNA and tubulin staining, the chromosomes demonstrated proper chromosomal condensation, but the chromosomes were not successfully aligned. In the other (**Figure 4**, right most micrograph), aberrant spindle formation was noted. Both defects apparently resulted in mitotic arrest.

Putting it all together and future directions

This screening experiment illustrates the power of combining RNAi with higher content, multi-parameter assays. These data allow the

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researcher to go beyond single data point readouts as in a typical screen. Rather, when this type of in-depth information is garnered, it can be used to efficiently evaluate the functions of hundreds or even thousands of genes. For example, a comparison of the cell proliferation data to the mitotic index data shows that the cell cycle phenotypes and proliferation phenotypes when knocking down specific kinases have every possible combination of phenotypes (not shown). To fully understand each kinase's role in proliferation and the cell cycle, additional time points and a verification of the induced phenotype by the use of additional siRNAs to the same target gene will be required. Even without that verification, however, these data show the incredible power of a siRNA library for target identification, target validation, and pathway analysis applications.

The full value of RNAi technologies requires the combination of data from various sources, including multiple RNAi experiments, published research articles, gene expression data, proteomics data, and protein-protein interaction data. In this case study, one of the most compelling phenotypes is from the knockdown of a kinase with only one published research article describing any biological results (blinded names pending IP filings). The true value of the results for this interesting kinase is not only the fact that this is a novel result for this kinase. Rather, it is the large amount of functional data that surrounds these kinase results. Additional RNAi experiments in different cell lines, expression profiling and other functional genomics experiments will unravel whether this kinase is a high value drug target. RNAi alone will not solve every aspect of target identification and validation, but RNAi does provide real functional data for putative drug target and not just a snapshot of a single datum point.

RNAi screening is not limited to the early stages of the drug development process. For example, siRNAs can be used in toxicology experiments to test the metabolic rate of lead compounds. The lead discovery process can also include RNAi to knockdown certain genes to differentiate between potential chemical leads (eg specificity of leads). Almost every scientist performing target validation experiments wants to perform siRNA experiments in animals to test for phenotypic effects after knockdown of their putative drug targets. Although this future work requires technological advances to siRNAs, feasibility experiments already show promise[10]. When these uses for RNAi become commonplace, RNAi technologies will provide a real decrease in the expenditures required to get a drug into clinical trials. DDW

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