[15] High-Throughput RNA Interference Strategies for Target Discovery and Validation by Using Synthetic Short Interfering RNAs: Functional Genomics Investigations of Biological Pathways

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Abstract

During the past five years, RNA interference (RNAi) has emerged as arguably the best functional genomics tool available to date, providing direct, causal links between individual genes and loss-of-function phenotypes through robust, broadly applicable, and readily upscalable methodologies. Originally applied experimentally in *C. elegans* and *Drosophila*, RNAi is now widely used in mammalian cell systems also. The development of commercially available libraries of short interfering RNAs (siRNAs) and other RNAi silencing reagents targeting entire classes of human genes provide the opportunity to carry out genome-scale screens to discover and characterize gene functions directly in human cells. A key challenge of these studies, also faced by earlier genomics or proteomics approaches, resides in reaching an optimal balance between the necessarily high throughput and the desire to achieve the same level of detailed analysis that is routine in conventional small-scale studies. This chapter discusses technical aspects of how to perform such screens, what parameters to monitor, and which readouts to apply. Examples of homogenous assays and multiplexed high-content microscopy-based screens are demonstrated.

Introduction: The Advent of RNA Interference (RNAi)-Based Genomics

The completion of major genome sequencing projects, from those of *C. elegans*, *Drosophila melanogaster*, mouse, and rat to the human genome project itself, have marked major milestones in recent biomedical research. The enormous amounts of data that have emerged from these projects now offer huge potential for significantly advancing both basic academic research and pharmaceutical drug development. Thus, there has been the

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advent of so-called functional genomics technologies to efficiently exploit those mountains of genome sequence data, extracting new insights into the functional relevance of individual genes.

These technologies have most notably included microarray-based expression profiling, high-throughput (HT) bioinformatic tools for *in silico* data mining, and large-scale two-hybrid screens for generating protein–protein interaction maps. Although these and other first-generation functional genomics technologies have accelerated our understanding of a broad range of biological phenomena, their inherent limitations are now becoming evident. In particular, these approaches typically yield indirect evidence, at best, of a gene product's function or possible interactions with other cellular components. For instance, correlations between gene expression levels and a given disease state, as generated by comparative transcriptional profiling of diseased versus normal tissue biopsies, cannot distinguish between therapeutically relevant causes and irrelevant consequences of the diseased state. Based on this, one can argue that this approach is better adapted to identify new diagnostic biomarkers than new therapeutic drug targets. Evidence from purely *in vitro* or *in silico* analyses also tend to offer questionable pathophysiological significance and high rates of false positives, making the resulting data difficult to prioritize for eventual follow-up, especially when its sheer volume is already far beyond the scale usually faced by traditional research laboratories.

RNA-mediated interference (RNAi) has emerged over the past few years as perhaps the best way of overcoming many of these obstacles, making it arguably the most powerful second-generation functional genomics technology available to date (Carpenter and Sabatini, 2004). Its ability to induce the destruction of individual mRNAs in a targeted way with high efficacy and specificity enables the generation of direct relationships between a gene's expression level and its functional role in any biological process being studied. Furthermore, RNAi-based methodologies in several key experimental systems from *C. elegans* to cultured human cells have proven robust enough to be applied in a high-throughput manner. Combined with the availability of full genome sequences and associated gene structure predictions, these advances have enabled the systematic, genome-scale application of this technology, thus heralding the advent of RNAi-based genomics.

The development of large-scale RNAi screening, though clearly exciting, has not been without its own challenges and, like all other experimental techniques, its own limitations and caveats have emerged steadily. The feasibility of applying RNAi as a systematic genome-scale screening method was first demonstrated rather early on in *C. elegans* (Fraser *et al.*, 2000;

Gönczy *et al.*, 2000; Kamath *et al.*, 2003), although the delivery methods and readout assays chosen in these studies only allowed for low to medium throughputs. Significantly higher throughputs were only achieved once these methods were successfully implemented in cultured cell systems, in which more highly parallelized experimentation is possible, starting with *Drosophila* cells (Clemens *et al.*, 2000; Kiger *et al.*, 2003; Lum *et al.*, 2003). Although at that time many were already dreaming of high-throughput genomewide screens in cultured human cells, the experimental applicability of RNAi in vertebrate systems remained in doubt due to the uncertainty of how to trigger RNAi without also triggering interferon response in those organisms. Thankfully, the first broadly applicable solution to this problem was demonstrated in 2001 by Tuschl and colleagues (Elbashir *et al.*, 2001), whose success came from the use of chemically synthesized double-stranded RNA (dsRNA) molecules designed to mimic the size and structure of so-called short interfering RNAs (siRNAs), which had been identified in plants as apparent intermediates in the RNAi pathway (Hamilton and Baulcombe, 1999). The result of this breakthrough, not only for the RNAi field itself but also more broadly for an ever-growing range of biomedical research overall, has been nothing short of a revolution. Although most researchers began to experience success with small-scale applications of RNAi focusing on handfuls of their favorite genes, those aiming for genome-scale applications quickly began to recognize new choices and challenges inherent to this pursuit, beyond those already faced with *C. elegans* or *Drosophila*. Chief among these were the following:

1. *Building a library of silencing reagents:*
   - *Choice of source target sequences:* Using cDNA libraries or predicted gene sequences from genomic databases.
   - *Choice of reagent type:* Chemically synthesized siRNAs or vector-based constructs expressing short hairpin RNAs (shRNAs).
   - Automated selection of optimal siRNA/shRNA sequences to achieve genomewide coverage with reproducibly high silencing efficacy and specificity.
2. *Delivering the silencing reagents:* The efficient delivery of siRNAs into cultured cells, with minimal associated cytotoxicity or delivery-associated side effects.
3. Controlling the experiments adequately to ensure that not only is the RNAi treatment effective, but, most importantly, that the observed RNAi-induced phenotypes truly are target specific rather than reagent specific.
4. Designing a screening strategy to maximize cost efficiency while maintaining scientific integrity and depth of the experiment.

This chapter reviews our progression in exploring and addressing these issues, as we have developed our genomewide RNAi screening program in human cells, after having done so in *Drosophila* cells and *C. elegans*. Having made the strategic choice early on of focusing on the use of chemically synthesized siRNAs rather than exploring alternative approaches such as vector-based expression of short hairpin RNA constructs, the scope of this chapter will necessarily be restricted to the former, whereas the latter is covered in more depth in other chapters in this volume. We show basic concepts, necessities, and technical considerations. We also discuss the range of applications that are at present considered feasible and being pursued by our group and others not only in basic research but also in the more applied areas of discovery and development of new therapeutic drugs.

Building a Genome-Scale Short Interfering RNA (siRNA) Library

First Choices: Source Sequences and Reagent Types

A number of strategies either focusing on speed or comprehensive screening coverage have emerged for building genome-scale libraries of RNAi silencing reagents, involving choices for both the source sequences and the type of reagent to be used. We have opted to aim for maximally comprehensive genome coverage, believing strongly that this represents an important opportunity afforded by the combination of currently available resources, that is, fully sequenced genomes and a targeted method of silencing, which can and should be realized. To achieve this aim, we have had to focus on the use of chemically synthesized siRNAs as the best-characterized, best-performing gold standard reagents in the field to date, custom designed from available genome sequence data to target all predicted genes.

This approach has therefore required that we develop new bioinformatics tools (discussed later) to systematically apply to all known available selection criteria for effective siRNA designs in an automated manner over the entire genome sequence. Thus, the primary pitfall of this strategy is its dependence on the science of gene structure predictions, which remains in rapid evolution at present. As a result, many genes predictions, that is, the locations of intron–exon boundaries, are still constantly evolving, thus requiring the concomitant updating of RNAi libraries to keep up with the new annotations. However, the alternative—the use of cDNA libraries as source material—avoids this issue by ensuring that all silencing reagents necessarily target expressed gene sequences. However, this precludes the achievement of anything close to genomewide coverage and biases such

libraries heavily toward only those genes that showed good expression and therefore good representation in the cDNA library. This bias can be used to significant advantage in those cases in which the scope of the screen is meant to be focused on a subset of genes that may be preferentially enriched in the said cDNA library.

Chemically synthesized siRNAs and vector-encoded shRNAs are at present the most broadly used reagents for all scales of RNAi experimentation in human cells. The use of viral vector-mediated expression of shRNAs (Arts *et al.*, 2003; Rubinson *et al.*, 2003) has the primary advantage of generating RNAi-based silencing in a more sustained manner, beyond the 5- to 7-day transient effect afforded by siRNAs. The viral delivery approach also facilitates studies in certain cell types that are otherwise difficult to transfect. Nonetheless, for our purposes, these benefits have so far been outweighed by the accompanying disadvantages, which include the need for much more specialized, dedicated laboratory infrastructure (especially if one aims to carry out this work at the genome scale), the high complexity and fallibility of required large-scale cloning strategies, and the much higher variability in silencing performance exhibited by shRNA than by siRNAs. Importantly, the inability to control the effective dose of shRNA generated inside the expressing cell also emerges as a significant shortcoming of the shRNA approach, especially in view of published reports that indicate an increased risk of off-target and nonspecific effects (including the activation of interferon response genes) when using excessive concentrations of RNAi silencing reagents (Bridge *et al.*, 2003; Persengiev *et al.*, 2004; Sledz *et al.*, 2003). Although these issues clearly do not preclude the overall use of vector-based shRNA libraries for RNAi screening [as has already been successfully demonstrated, e.g., Arts *et al.* (2003), Berns *et al.* (2004), and Paddison *et al.* (2004)], they limit their breadth of applicability, making them best adapted for groups who are unconcerned with the issue of being comprehensive and who accept that some phenotypes may be missed.

Automated Design of siRNAs

In model organisms such as *C. elegans* and *Drosophila melanogaster*, libraries of relatively long dsRNAs (usually ~500–1500 bp) have been successfully built here and elsewhere through *in vitro* transcription of RNA from DNA templates generated by PCR amplification of appropriate portions of purified chromosomal DNA. Customized software algorithms were developed to efficiently screen through available genomic sequence data and select target amplicons chosen primarily to maximize their content in exon sequences from a single targeted gene, to ensure their uniqueness during the PCR amplification process, and to otherwise avoid known

problems that might hinder synthesis. These long dsRNAs proved to be highly specific and potent silencers, but the need to avoid the interferon response in mammalian cells required the development of new algorithms to select optimal 2-mer siRNA sequences.

siRNA design algorithms comprise a wide range of different criteria to maximize silencing efficacy, which in our case have included specific base compositions at defined positions along the 19 core siRNA base pairs, thermodynamic base-pairing profiles defining regional base compositions (GC content in particular), base composition of 3′ overhangs, positions along the targeted mRNA, and lack of variability of the targeted mRNA over the relevant site (avoiding known single nucleotide polymorphisms, etc.). These and other criteria were derived empirically from a large experimental dataset of siRNA-derived silencing efficacies, covering hundreds of genes. One of the most potent design features implemented in our algorithm, as well as algorithms of others, is the creation of a differential between base-pairing thermodynamics at either end of the siRNA, ensuring a significantly weaker pairing of the 5′ end of the antisense strand. As recently confirmed by Zamore and colleagues (Schwarz *et al.*, 2003), such asymmetry strongly favors the loading of the antisense strand over that of the sense strand into available RNA-induced silencing complexes (RISCs), thereby ensuring the recognition and subsequent destruction of the correct target mRNA.

Moreover, because multiple studies have reported the detection of complex sequence-dependent off-target effects at the mRNA level (Jackson *et al.*, 2003; Persengiev *et al.*, 2004), most siRNA design algorithms also include measures to maximize specificity by minimizing the risk of such off-target effects. Although this issue has led us and others to integrate design requirements that siRNAs should have minimal numbers of mismatches against any and all off-target gene sequences, the field's current understanding of these risks remains quite superficial, and most siRNAs commonly available at present are likely to have quite complex targeting footprints, at least when analyzed at the mRNA level. This is further discussed later, but it is important to point out here that this issue is readily neutralized by confirming the target-specific nature of any observed RNAi-induced phenotypes through the use of multiple, distinct siRNA designs against the same target gene.

Ultimately, no matter how good the *in silico* predictions look, the performance of the algorithm-designed siRNAs in silencing endogenously expressed genes must be demonstrated experimentally in cultured human cells under strictly standardized conditions. Furthermore, it is our experience that such tests should integrate large enough numbers of genes so as to generate a statistically relevant sample and that a suitably quantitative analysis

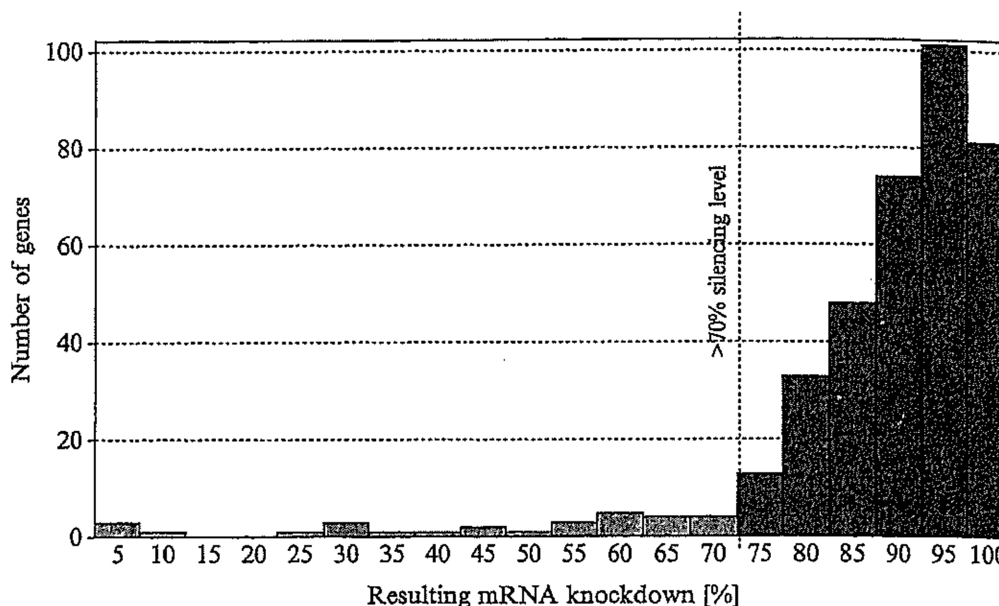

FIG. 1. Results of efficacy testing for short interfering RNAs (siRNAs) against 379 human genes. Among 379 genes (1106 siRNAs), more than 70% silencing was achieved for more than 93% of the genes (as measured by real-time RT-PCR at 48 h after transfection into HeLa cells), applying our proprietary siRNA design algorithm.

of target mRNA levels [e.g., real-time RT-PCR or quantitative (qRT-PCR)] should be applied. As an example, results of our qRT-PCR testing of silencing efficacies are shown in Fig. 1 for a set of more than 1100 siRNAs designed by using our proprietary algorithm to target 379 human genes.

Applying this type of algorithm on a genome- or transcriptomewide scale, we have completed the designs of multiple siRNAs targeting virtually every predicted gene of the human, mouse, and rat genomes. These predesigned siRNAs now enable not only genomewide screens but essentially any more focused screening scope, for example, on subsets of genes corresponding to known families [e.g., kinases, G-protein-coupled receptors (GPCRs), or "druggable genome"] or custom sets defined by expression profiling studies.

Optimizing Large-Scale siRNA-Based Experiments

Delivery of Chemically Synthesized siRNAs

Large-scale RNAi experiments require a method for delivering siRNAs into cells with high efficiency, low toxicity, very high reproducibility, as well as cost efficiency. The manipulations involved in the delivery protocols must be simple and fast enough to be amenable to highly parallelized

experimentation, usually in 96-well or even 384-well microplate formats. The most commonly used and best-characterized approaches are reviewed next.

Lipofection. For most commonly used cell lines, especially transformed cells, transfection with cationic liposomal reagents is a straightforward way of getting siRNAs into cells. Several siRNA lipofection reagents are available, which exhibit different profiles in terms of their range applicability to different cell lines, both in terms of transfection efficiency and in toxicity to the cells.

Because every cell line has its unique requirements, the type of lipofection reagent as well as the exact transfection amounts and conditions must be tested and adjusted for each cell line to determine optimal conditions. Normally, transfection optimization for a new cell line consists in testing of one or more transfection reagents and several siRNA:transfection reagent ratios. Other sensitive parameters to be optimized include the cell seeding density and the use (or omission) of serum and antibiotics during the transfection process.

To monitor delivery success, one can begin by visualizing the internalization of fluorescently labeled siRNAs. However, microscopy examinations of this type often reveal siRNAs accumulating in discrete membranous compartments (presumably endocytic vesicles or organelles) with little or no detectable signal in the cytosol. Thus, unless a high correlation can be demonstrated between the presence of labeled siRNAs in these compartments and the triggering of an RNAi response in those cells (which is not always the case), such signals should not be considered reliable predictors of silencing. Instead, we favor the direct monitoring of silencing either in individual cells or, more commonly, in screening projects over the entire cell population in each well.

Thus, two readouts are necessary: (1) monitoring for knockdown of the gene of interest, preferably by qRT-PCR, and (2) proper monitoring for cytotoxicity carried out in parallel, which is highly recommended [e.g., ToxiLight™ assay (Cambrex, Baltimore, MD), which is conveniently performed by using a sample of the culture's growth medium]. Optimal conditions are usually reached when the best compromise between transfection efficiency and toxicity is achieved. The sensitivity of the desired functional assay readouts should also be considered, as some are more highly sensitive to moderate toxicity than others. A transfection optimization example is illustrated in Fig. 2.

Electroporation. For cell types proving to be recalcitrant to lipofection-based protocols, electroporation can be a valuable alternative. Electroporation involves applying an electric field pulse to induce the formation of microscopic pores in the cell's plasma membrane, which then allow the

Protocol 1. Lipofection-Mediated siRNA Transfection of Human Cells (Oligofectamine™ Protocol for 96-Well Plates, Final siRNA Concentration 100 nM)

1. Twenty-four hours before transfection, seed cells at the appropriate density to each well of a 96-well plate (e.g., 20,000 cells/well for HepG2, 6000 cells/well for HeLa, and 13,000 cells/well for MCF-7).
2. Dilute siRNAs to a 10 μM working stock concentration.
3. Prepare siRNA mix: 1 μl of 10 μM siRNA + 16 μl Opti-MEM®.
4. Prepare Oligofectamine™ mastermix: 0.4 μl Oligofectamine (Invitrogen, Carlsbad, CA) + 2.6 μl Opti-MEM. Incubate at room temperature for 10 min.
5. Combine solutions in (3) and (4) by gently pipetting up and down (do not vortex); incubate 20 min at room temperature.
6. Remove the culture medium from the cells (*Optional:* wash with 200 μl serum-free medium).
7. Serve cells (40–50% confluent) with 80 μl Dulbecco's modified Eagle medium (DMEM; without phenol red, serum, or antibiotics) per 96 wells.
8. Carefully add 20 μl transfection mix to the center of each well; shake the plate gently.
9. After 4 h, add 50 μl of medium (containing 3× serum and antibiotics) per well, to give a total culture volume of 150 μl.
10. Incubate for 48 or 72 h.

siRNA to traverse the membrane. Under specific pulse conditions, the pores reseal quickly and the electroporated cells recover and resume growth. A distinct advantage of electroporation is that it is not dependent on cell division, and RNAi-induced mRNA reduction can be detected just a few hours after delivery.

Most existing electroporation protocols were developed to deliver plasmid DNA to cell nuclei, and these protocols often suffer from high cell mortality. For transient RNAi experiments, siRNAs need to be delivered into the cytoplasm only, and therefore milder electroporation conditions can be used that minimize cellular mortality and trauma while ensuring highly efficient siRNA delivery.

Different primary and neuronal cell types require different electroporation parameters. In our experience, varying the number of pulses is the most influential parameter and a key determinant for the mortality or viability of cells (Fig. 3). For example, HUVEC cells require one electropulse (150 μS,

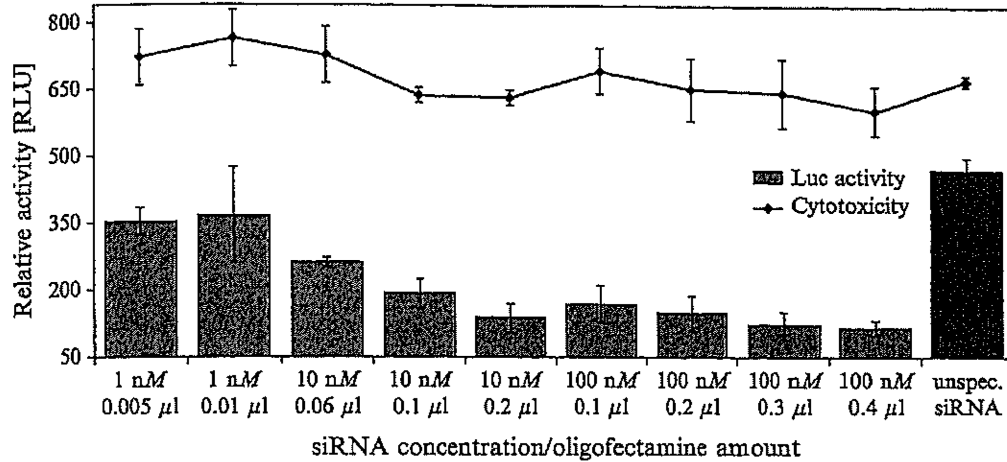

FIG. 2. Example of an siRNA transfection optimization by lipofection. An siRNA targeting luciferase was transfected into MCF-7 cells (pretransfected with a luciferase reporter plasmid) by using Oligofectamine™ (Invitrogen). Readouts were luciferase reporter activity (measured with Bright-Glo™, Promega) and cytotoxicity (detected using ToxiLight™, Cambrex). Best results were obtained with 100 n*M* siRNA/0.4 μl Oligofectamine.

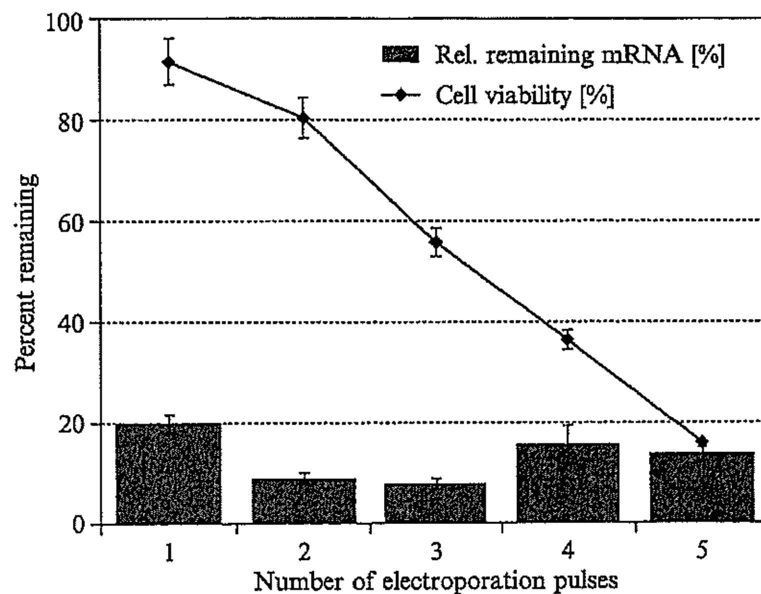

FIG. 3. Example of an siRNA transfection optimization by electroporation. An siRNA targeting GAPDH was electroporated into NHDF-Neo primary cells, using a varying number of electroporation pulses. In a 1-mm electroporation cuvette, 1.5 μg siRNA was transfected in 75 μl at 150 μS and 900 V, using a Gene Pulser Xcell™ (Bio-Rad). Twenty-four hours posttransfection, total RNA from cells was analyzed by real-time RT-PCR for target mRNA levels (normalized against 18S rRNA). Remaining mRNA was calculated as a percentage of mRNA compared with the negative control sample that was transfected with unspecific siRNA.

250 V; Ovcharenko *et al.*, 2004) whereas primary NHDF-Neo cells respond optimally to two pulses (70 μS, 900 V, 1-mm electroporation cuvette). Parameters that can further be optimized are the composition of electroporation buffer (which ideally facilitates rapid pore resealing) and the electro-pulse generator itself [e.g., Gene Pulser Xcell™ (Bio-Rad, Hercules, CA) or ECM® 830 (BTX, Holliston, MA)]. However, the biggest technical limitation of electroporation has been the lack of a commercially available 96-well electroporation device to enable high-throughput application with good well-to-well reproducibility. A number of prototypic devices are now appearing on the market, offering the hope that this may be overcome in the near future.

Validation of siRNA Efficacy

One crucial prerequisite for performing RNAi experiments is the need to link any observed phenotype to a demonstrable degree of siRNA-mediated knockdown of the target, either at the mRNA or the protein level. Despite any and all assurances offered by siRNA vendors, experimental validation of siRNA performance in the particular cell line of interest and with the transfection reagent of choice should always be part of any RNAi project. There are two scenarios:

1. *Low-throughput assays:* Monitoring RNAi-mediated knockdown for all siRNAs is feasible (e.g., preferably by qRT-PCR).
2. *High-throughput screens:* Monitoring knockdown for all siRNAs of a library of hundreds or thousands of siRNAs is not feasible. Instead, transfection optimization and siRNA validation should be done for a set of relevant control siRNAs, and this protocol be taken for the screen. Any positive hits coming out of the screen should then be tested for silencing efficacy as outlined previously.

Although in our experience protein and corresponding mRNA levels often mirror each other well, the choice of which to monitor (both is always preferable) depends primarily on the questions being asked: mRNA levels offer the most direct measure of success of the RNAi silencing and the protein level the most direct link to loss of function. In either case, the level of target mRNA or protein reduction can only offer an indirect and imperfect predictor of loss of function, as, in some cases, even as little as 5–10% remaining protein might be enough to maintain wild-type functions at levels that are not detectably different from controls. Conclusions drawn from RNAi experiments must therefore take this into account, which is why negative RNAi results are always very difficult to interpret.

siRNA Validation at the mRNA Level. For monitoring target mRNA levels, Northern blotting, branched-DNA (bDNA), or real-time RT-PCR (also known as qRT-PCR) are all valid assays. However, in view of its high sensitivity, upscalability, and reproducibility, qRT-PCR has become the method of choice (Fig. 4). It can be performed either with gene-specific primers (SybrGreen method), in which case monitoring melting curves represents an additional necessary quality control step, or with dual-labeled fluorescent probes (TaqMan® probes), which has the advantage that predesigned ready-to-run probes for most human, mouse, and rat genes are available commercially (Assays-on-Demand™, ABI, Foster City, CA).

Protocol 2. Validation of siRNA Efficacy in Human Cells by Real-Time RT-PCR (qRT-PCR) (SybrGreen Method, 384-Well Plate Setup, ABI-7900-HT Real-Time PCR Machine)

1. Perform siRNA transfection experiment according to Protocol 1.
2. At 48 h or 72 h after transfection, extract total RNA from cells (e.g., using RNAqueous™, Ambion, Austin, TX, or Invisorb® kits, Invitek, Germany), following the manufacturer's protocol.
3. Check quality and quantity of total RNA on an agarose gel.
4. Produce cDNA (e.g., using TaqMan RT reagents, ABI), following the manufacturer's instructions.
5. Check quality and quantity of cDNA on an agarose gel.
6. Run real-time qPCR with gene-specific primers:
   5.5 μl 2× SybrGreen PCR mix (e.g., from ABgene, Surrey, UK, or ABI)
   3.0 μl cDNA
   2.5 μl 2 μ*M* F/R primers
   11 μl total
7. *Real-time qPCR program:* 50° for 2 min, 95° for 10 min, 45 cycles (95° for 15 sec and 60° for 1 min), 95° for 15 sec, 60° for 15 sec, 95° for 15 sec (melting curve).
8. *Normalization:* 18S rRNA or GAPDH as a housekeeper; run for each sample.
9. *Degree of knockdown:* This is calculated by comparing the amplification level of the gene of interest, normalized through the level of the housekeeper gene, between samples transfected with a specific siRNA and negative control samples (e.g., transfected with Negative 1 nonsense siRNA (Ambion), also at a final concentration of 100 n*M*).

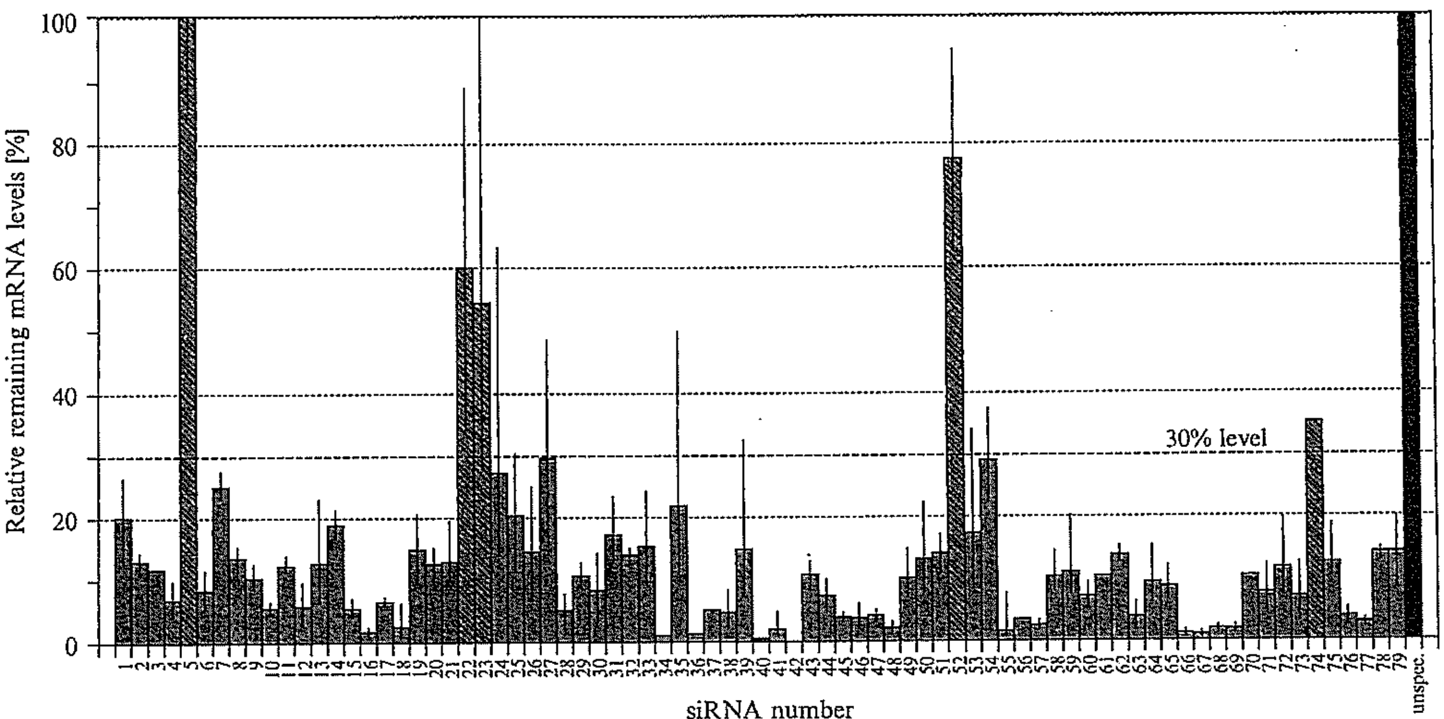

FIG. 4. Real-time RT-PCR-based validation of RNA interference (RNAi) efficacy. Transfection was performed in HeLa cells, with 79 individual siRNAs (each at 100 n*M* final concentration) against endogenously expressed human kinases. Forty-eight hours after transfection, total RNA was extracted from cells and cDNA was subjected to real-time RT-PCR (see also Protocol 2). siRNAs above the 30% level (i.e., below 70% silencing) are indicated by light bars. For 74 of 79 (94%) siRNAs, the mean remaining mRNA level was less than 30% compared with the unspecific control siRNA.

siRNA Validation at the Protein Level. To monitor RNAi knockdown at the protein level, several options are principally possible:

- Western blotting or ELISA-based methods are most commonly used for analyzing protein levels (Fig. 5). However, potential differences in the specificities of the antibody and the siRNAs should be taken into account as they can otherwise lead to misleading results.
- An enzyme activity assay can also give a direct answer about the amount of a particular protein or enzyme left after RNAi treatment.
- If a reporter gene construct is cotransfected, the readout for the degree of its depletion can be performed [e.g., luciferase reporter activity or green fluorescent protein (GFP) fluorescence]. On the other hand, extrapolation from such cotransfection assay results to the eventual performance of the same siRNA on the endogenously expressed target is not always reliable, especially when the cotransfection is in a cell type that is significantly different from the natural one.

However, the limitations common to most of these methods are that they are only applicable for monitoring the levels of a few or even only one protein at a time and that most are only applicable in a relatively low-throughput scale.

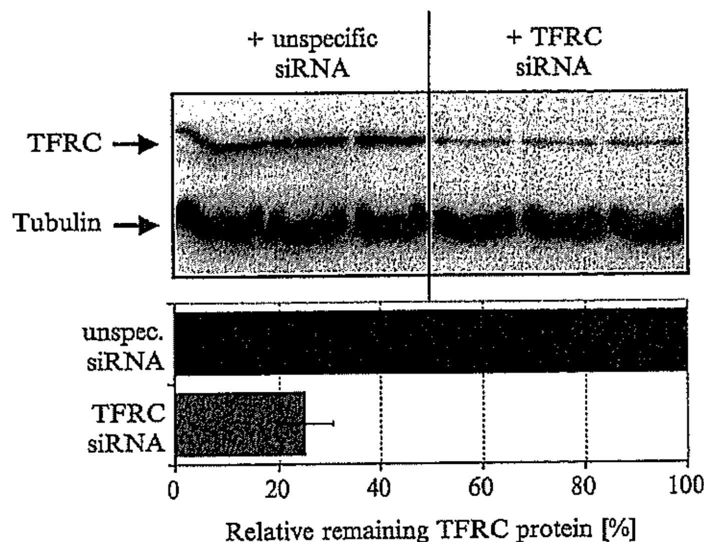

FIG. 5. RNAi-mediated knockdown of transferrin receptor (TFRC) measured by Western blot. Protein extracts from HeLa cells (24-well plates) were prepared 72 h after transfection; quantification was done on an ECL reader, with two different exposure times to account for the different abundance of the two proteins. TFRC expression levels were normalized against tubulin and compared to the unspecific control.

Kinetics of siRNA-Based Screens

The kinetics of the RNAi response derive from a complex multistep enzymatic process in which the target mRNA is specifically degraded, resulting in depletion of the target protein pool through natural turnover. Thus, the relative kinetics of degradation of the mRNA versus the protein should be taken into account when monitoring RNAi phenotypes. Whereas the mRNA degradation, as the first step of the RNAi mechanism, is believed to start almost immediately following delivery of siRNAs into cells, the reduction of the protein and accompanying loss of function completely depend on the protein's half-life, progressing more slowly over the hours and days that follow delivery of the siRNA. The half-life differs markedly from protein to protein, thus making the choice of time points for monitoring RNAi-induced phenotypes a particularly important issue. Although the ideal solution, that is, to collect data at multiple time points, can allow one to document the progression of increasingly severe hypomorphic phenotypes, this is rarely feasible in the context of large-scale screening projects. Thus, a single time point must usually be chosen, which inevitably represents a compromise to minimize the risk of leaving any phenotypes undetected.

In our experience, whether monitoring target mRNA levels by qRT-PCR or examining functional readouts, the time window of 36–72 h posttransfection (with 48 h being the most convenient) has yielded the most informative results in siRNA-based experiments.

In this context, also note that the relatively slow kinetics of the RNAi response represent a major difference with compound screening paradigms. As seen in Fig. 6, the RNAi effect needs 1–2 days to become detectable, but is then stable over several days after transfection. Although a second transfection could be an option to prolong that time (although many cells show adverse effects from such repeated transfection protocols), an assay window of up to 5 days is normally convenient and flexible enough for most assays. Assays requiring sustained gene silencing lasting longer than 5–7 days reach the limits of what is advisable with siRNAs and at present would be best addressed by using vector-based shRNA expression (discussed previously). Also, in pathway analysis screens, transduction of the signal that is triggered by a silenced constituent of the pathway can take time to effect in an actual signal (Fig. 7).

Assay Screenability and Evaluation of Hits

Assessing Assay Screenability. As a statistic value of assessing the quality of an assay, the screening window coefficient or Z-factor introduced by Zhang *et al.* (1999) can be used to describe the assay's suitability for

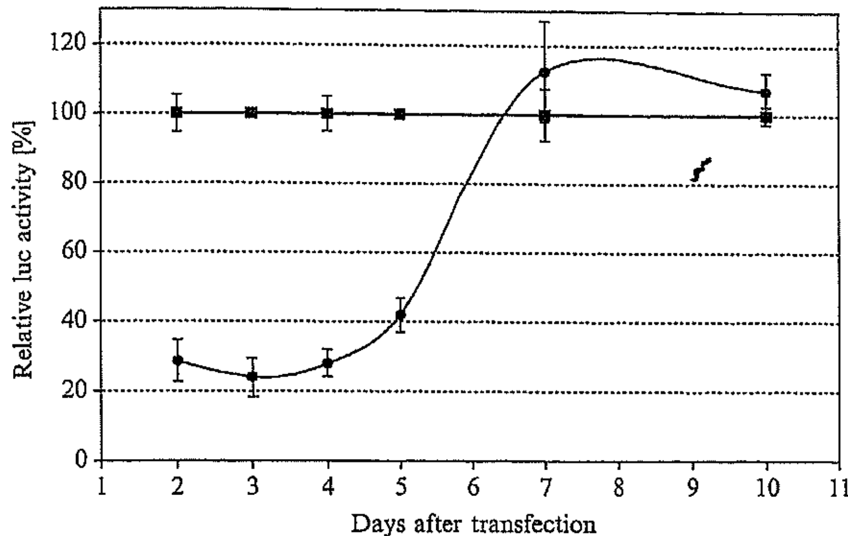

FIG. 6. Time course of siRNA-mediated RNAi knockdown. An siRNA against luciferase was transfected at 100 nM into MCF-7 cells (cotransfected with luciferase reporter plasmid). Luciferase activity readout (Bright-Glo™, Promega) was normalized by Wst activity (Wst reagent, Roche). Relative luciferase activity for samples treated with luciferase targeting siRNA is shown in circles, and samples treated with unspecific siRNAs are indicated by squares.

large-scale screening (see equation). It is a measure of the separation band between data variability of positive controls (for RNAi screens, samples transfected with a positive control siRNA) and of negative controls (for RNAi screens, samples transfected with an unspecific scrambled control siRNA).

$$Z = 1 - \frac{(3\times \mathrm{SD}_{\mathrm{treated}} + 3\times \mathrm{SD}_{\mathrm{unspecific}})}{|\mathrm{mean}_{\mathrm{treated}} - \mathrm{mean}_{\mathrm{unspecific}}|}$$

The suggestions of Zhang and coworkers on how to interpret Z-factors are useful indicators:

- $Z = 1$ describes an ideal assay (either no variation or an unlimited distant signal)
- $1 > Z > 0.5$ reflects an excellent assay with a large separation band
- $0.5 > Z > 0$ reflects a double assay with a small separation band
- $Z = 0$ describes a yes/no assay (sample and control variations touch)
- $Z < 0$ does not allow proper screening, because sample and control variations overlap.

The same rules can also be applied to RNAi-based assays (Fig. 8). Therefore, before an RNAi assay is implemented as a large-scale screen, it is highly advisable to monitor its robustness by using the Z-factor method (positive

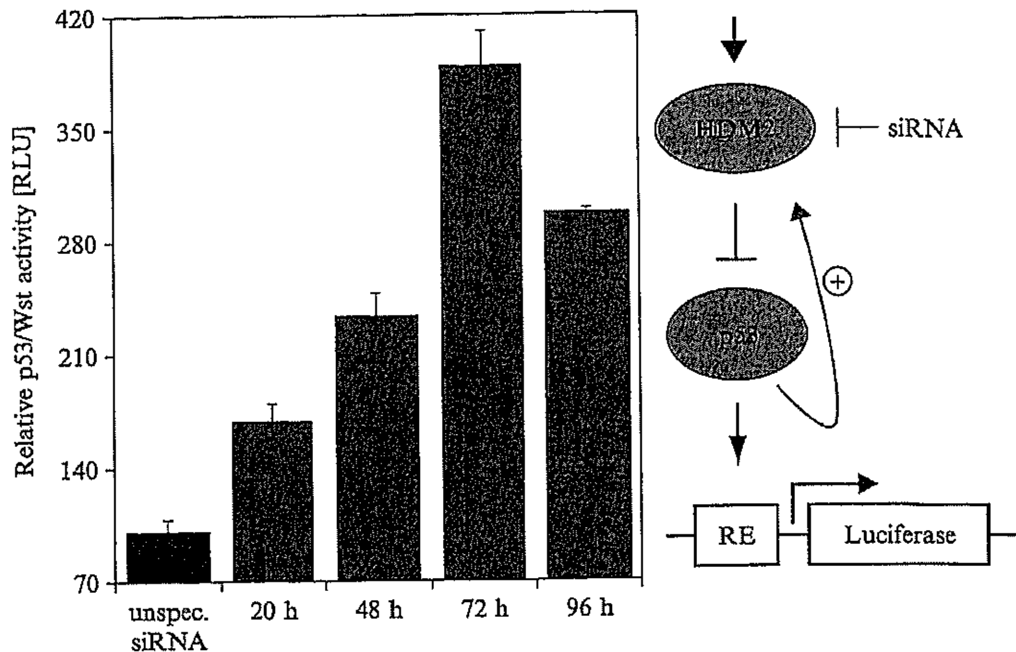

FIG. 7. Pathway analysis: time course of p53 activation after HDM2 silencing. Transfection of an siRNA against HDM2 (in MCF-7 cells cotransfected with p53 reporter plasmid) results in a downstream p53 activation, proving the applicability of RNAi for pathway dissection. The reporter construct consists of a TK basal promoter, a p53-responsive element (RE), and a firefly luciferase gene downstream. RNAi-based inhibition of HDM2 first activates p53, which is seen through the induction of luciferase expression. Later, a feedback loop (induction of endogenous HDM2 that also has a p53 response element in its promoter) results in the decrease of luciferase signal at 96 h.

versus negative controls). This is ideally done also to determine intraplate, interplate, interoperator, and interexperiment sources of variability.

Evaluation and Confirmation of Hits. To evaluate screening results from homogenous and microscopy-based RNAi screens, a threshold of three standard deviations (SDs) from the negative control (cells transfected with an unspecific siRNA) is generally considered to be the hit limit, equivalent to a 99.73% confidence limit to the sample data (Zhang *et al.*, 1999). Depending on the screen, this threshold can be adjusted to modulate the size of the resulting hit collection to be further examined in secondary screening.

Because the key goal of the primary screening pass is always to reduce the scale of the task to something more manageable, longer assay time points and higher siRNA concentrations are advisable to ensure maximal inclusiveness and minimal risk of missing positives (high detection sensitivity but low specificity or accuracy). Although this approach inevitably yields a high rate of false positives, these can be readily weeded out during

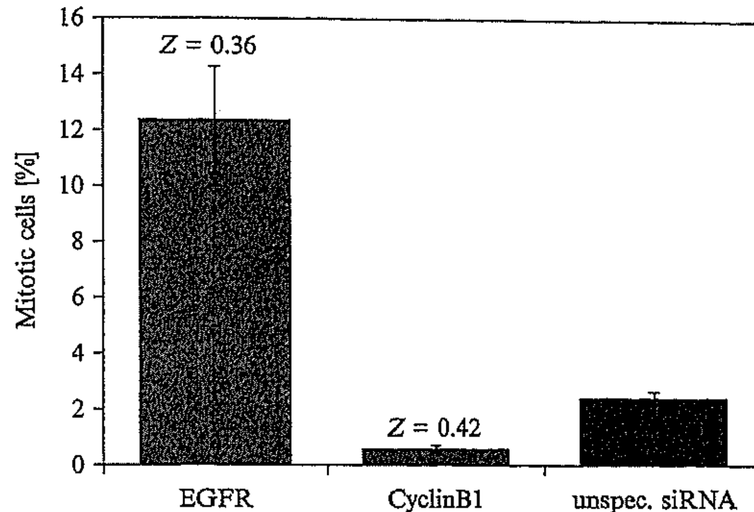

FIG. 8. Calculation of Z-factor. For a mitotic index assay in HeLa cells, the Z-factor was calculated for two positive controls, EGFR and cyclin B1. Both positive controls, for increase and decrease of mitotic index, gave Z-factors around 0.4, which means that this assay can be classified as being a *double assay* according to the terminology of Zhang *et al.* (1999) (see text).

the secondary screening pass, which we recommend to design for maximum specificity, and thereby to increase the accuracy and confidence in a putative hit (confirmatory pass). Therefore, to verify primary hits from an RNAi screen, the secondary screen should not only involve retransfection of the same siRNA against all putative hits but also the validation of those results through the use of at least one additional siRNA targeting the same gene. Ultimately, one must always link the observed phenotypes to the knockdown of the targeted gene, but this is usually not feasible during the primary screening pass, and therefore can and should be integrated in the secondary screen. Thus, in our view, a positive hit should be one that displays the same phenotype by using at least two distinct siRNAs in two separate experiments, showing a clear correlation with reduced expression levels.

Common Caveats of siRNA-Based Screens

Interpreting Unexpected Negatives

The most basic caveat of all RNAi-based screens is that negative results, that is, the absence of a detectably altered phenotype, is nearly always impossible to interpret conclusively. This is because of the inescapable fact that RNAi is inherently a knockdown rather than a knockout technology: siRNAs inevitably cause varying degrees of loss of function, and although the best siRNA design algorithms produce very efficient

siRNAs, the level of silencing is rarely 100%. Thus, it is always expected that some level of the target protein will remain, and, depending on the type of protein (e.g., enzymes versus structural components), this may offer a possible justification for maintaining wild-type readings (a situation comparable to human genetics, in which the carrier of an autosomal recessive disorder does not exhibit the disease). Indeed, the threshold (i.e., the degree of silencing) required for achieving a detectable loss-of-function phenotype differs markedly between proteins and depends on the detection sensitivity of the assay. Conversely, if a detectable phenotype is generated, the ability of RNAi to titrate silencing by performing dose–response experiments is a significant strength of RNAi technology that can provide allelic series and often yields further insights into a gene's functions. These considerations underscore the importance of documenting the silencing efficacy in these experiments.

Specificity

As a clear reminder of how young the RNAi field is, a number of studies over the past year have documented instances of direct off-target effects caused by reagents, including siRNAs, designed to trigger RNAi responses (Jackson *et al.*, 2003; Scacheri *et al.*, 2004; Snove and Holen, 2004). Of course, these findings have challenged the initial hope that these reagents could exquisitely mediate gene-specific silencing, with clear implications on the design of such reagents, as well as the associated experimental strategies, screening paradigms, and data analysis criteria. Although the general understanding that emerges to date remains far from complete, it does suggest that many siRNAs in use at present not only recognize the intended, perfectly matched target mRNA but also direct the destruction of other imperfectly matched secondary target mRNAs, though usually to a significantly lesser extent. These sequence-dependent off-target effects are proving difficult to predict, as the underlying stretches of base-pair complementarity have not exhibited readily recognizable thresholds in size, composition, or other obvious patterns. Thus, the top BLAST hits for a given siRNA do not necessarily represent the most likely secondary targets. Although some have worried that imperfect siRNA-target pairings showing discrete mismatches may be triggering translational suppression, as natural miRNAs do, the finding that secondary target mRNA levels actually decrease in these cases suggests that these types of off-target effects are in fact mediated through RNAi-like modulation of mRNA stability. Nonetheless, the sequence dependence of this type of off-target effect can be used to completely and easily neutralize this problem. Because each individual siRNA's off-target footprint is defined by its sequence, one can quickly ascertain the target specificity of any observed phenotype simply by eliciting it by using multiple distinct siRNAs targeting the same gene of interest (Anon, 2003).

A second type of off-target effect that has been noted in RNAi experiments consist of the concentration-dependent modulation of the expression of nontargeted stress response genes, including factors of the interferon response pathway (Persengiev *et al.*, 2004; Sledz *et al.*, 2003). Although still poorly understood, these effects are thought to be dependent on such factors as cell type and delivery method, and, unlike the sequence-dependent off-target effects, their risk is known to be significantly increased by the use of excessively high concentrations of silencing reagents such as siRNAs (Persengiev *et al.*, 2004). Thus, titration of the siRNA concentration (e.g., from 100 n*M* down to about 10 p*M*) has the potential of attenuating or even eliminating these effects. Having a powerful design algorithm that generates very efficient siRNAs (see Automated Design of siRNAs) allows transfection of siRNAs at concentrations lower than 100 n*M* without losing efficiency (which is, however, only recommended for confirmatory experiments). In addition, parallel qRT-PCR monitoring of the interferon response markers such as OAS1, OAS2, and STAT1 at each step of the dose–response experiment can provide a further valuable control. One or two scrambled or unspecific siRNAs, that is, siRNAs that do not exhibit significant matches to any gene of the targeted genome, are usually included in most RNAi experiments to control for this issue. This is a more appropriate control than a so-called mock control (with transfection reagent but without siRNA), because the latter often yields toxic effects that are irrelevant to the experimental samples. Moreover, untransfected samples, though necessary to control for transfection effects, are also inadequate negative controls if used on their own for RNAi experiments. A test of several unspecific siRNAs is advisable. In fact, in essentially all RNAi screens, the vast majority of tested genes yield negative results and therefore provide a huge abundance of baseline control values.

Beyond these basic precautions, the following controls may also be considered in the validation procedure for primary hits (one siRNA) or confirmed hits (at least two distinct siRNAs) coming out of an RNAi screen:

- mRNA/protein/phenotype controls: If an observed loss-of-function phenotype is observed with multiple distinct siRNAs against the same target, the ultimate proof of a valid RNAi experiment is to relate that phenotype to a significant knockdown of both the mRNA and protein for each of the effective siRNAs.
- Functional controls: The ultimate proof of validity of an RNAi experiment is to rescue expression of the target in an siRNA-refractory manner. Although these experiments currently remain too laborious and technically complex for wide application, the development of new tools in this area is expected to facilitate this very powerful approach.

Screening Automation and Data Handling

Screening Automation. The availability of liquid handling robots makes automation of various screening steps affordable. Nonetheless, these instruments do not necessarily solve all problems, and careful thought and planning should therefore be invested to determine whether individual tasks in a screening project would best benefit from automation, or perhaps only mechanization, or from being maintained strictly under human manual control. Although the gain in throughput from liquid handling is sometimes not so significant compared to manual work with multichannel pipettes, the gain in robustness, reproducibility, and the reduction of possible errors caused by manual intervention are the strongest arguments for automation. Furthermore, some processes associated with screening, namely, rearraying, reformatting, and hit-picking of siRNAs, cannot be performed reliably enough by applying a manual approach. For reasons of further improving reliability, avoiding contamination, and streamlining the screening process, the use of several individual robots that are dedicated for particular defined steps of the protocol is highly recommended. In our experience, all automated liquid handling systems (e.g., from Tecan, Switzerland; Perkin Elmer, Wellesley, MA; Hamilton, Reno, NV; Beckman, Fullerton, CA) have their own pros and cons, dependent on the particular project, and there is no general recommendation for the best system.

Lastly, to deploy the full power of automation, the establishment of a laboratory information management system (LIMS) to coordinate task scheduling, controlling, and monitoring of all the automated processes is highly recommended, as discussed next.

Data Handling. One key requirement that is widely underestimated for successfully carrying out large-scale or genomewide RNAi screens is the necessity for an appropriate LIMS and data-handling setup with a robust, well-structured database at its core, in addition to the use of robotic liquid handlers. Especially in academic environments, the scientific ambitions to perform genomewide screens often reach far higher than the bioinformatics and informatics infrastructure that are available to enable them. Grant funding review boards are slowly recognizing this reality now.

The absence of this biocomputing infrastructure can potentially cause serious problems at every stage of such screens, creating very significant risks of misattribution or even complete loss of large datasets, severely compromising the overall quality and comprehensiveness of the screen. Even if errors occur only 1% of the time, because of the repetitive, systematic nature of these projects, these few mishaps often bear the potential to cast doubt on huge, entire datasets, which risk being rendered useless.

Furthermore, those few mishaps can be extremely difficult and time consuming to track down. Thus, crucially important is putting in-depth thought into the design of a data flow tracking system, be it electronic form or based on a well-conceived paper records (or better yet both), before starting such a large scale project. The following are some important aspects:

- *Gene/siRNA information and screening results:* The handling of multiple siRNAs targeting thousands of genes requires a proper database system for storing the gene/siRNA information and linking it to the screening results.
- *Rearraying and reformatting reagent stocks and experimental plates:* Trying to coordinate these processes solely based on the use of Excel sheets is highly error prone and should be avoided. Worklists, output lists, and reformatted plate layouts cannot be generated or handled manually.
- Scheduling and monitoring screening plate processing steps are highly desirable features of a good LIMS/database system.
- *Data acquisition, mining, and storage:* Because of the amount and complexity of data and their file size, these issues require major IT support, especially before starting microscopy-based screens.

Although tight integration of the LIMS interfaces with laboratory hardware can deliver the most optimal streamlining of data flow management and lowest chances of error, we have found this to be a particularly time-consuming development task. In addition, this aspect of a LIMS significantly reduces the versatility of the system, locking it in with specific instrument choices, which only makes sense if these are to be extensively used in the long run. We therefore recommend approaching the issue of tight hardware integration carefully and selectively, focusing initially perhaps more on those developing instrument-independent functionalities that maximally streamline repetitive and error-prone tasks. For example, we have found the careful implementation of a well-thought-out barcoding system, covering not only the full range of tubes, plates, and other sample types involved throughout the experiment process but also essentially any other types of data that must be entered repetitively (such as technicians' names, if this is being tracked, which we also highly recommend). Data entries by using barcode scanners should be considered whenever possible to replace the much more error-prone keyboard entries. (Mild dyslexia is more prevalent than one might expect.)

Thus, the overall combination of laboratory and computing infrastructures, reagent libraries, and detailed expertise needed to effectively run such screens represents a very significant investment of money and in most

cases at least 1–2 years of development time. It is not surprising then that most major research funding organizations have now recognized the wisdom of entrusting such studies to specialized core facilities and service providers, thereby minimizing unnecessary and very costly duplication of effort.

Readout Assays for siRNA-Based Screens

We describe some examples of commonly used homogenous and microscopy-based high-throughput assays. Although the choice may seem restricted to oncology-related assays, their underlying principles can be applied widely and adapted to other research fields of interest. Our guiding principle in designing such assays is, wherever it is feasible and appropriate, to strive toward implementing the richest possible readouts, such that the resulting dataset offers maximal depth and breadth in its phenotypic classification of screened genes.

Cell Proliferation Assays

One assay that is most broadly applied in cell cycle research and oncology is quantifying the number of living cells to measure growth arrest or induction of proliferation. Numerous colorimetric, luminescent, or fluorescent homogenous assay kits based on different assay principles are available:

- Quantification of mitochondrial reductase activity by tetrazolium salts (MTT, XTT), or WST-1.
- ELISA-based measurement of BrdU incorporation into chromosomal DNA during S-phase.
- Detection of ATP levels in cell lysates (e.g., ATPlite™, Perkin Elmer) (Fig. 9).
- Staining of nucleic acids (e.g., CyQUANT™, Molecular Probes, Eugene, OR).
- Quantification of the reduction potential of intact cell membranes (e.g., CytoLite™, Perkin Elmer).
- Live/dead staining assays.

The most notable differences in the performance of these assays come from the degree of linearity, sensitivity and stability, the signal-to-noise ratio, and the complexity of the protocol and its costs. For high-throughput screens, systems with sustained stable emission and with an easy-to-follow protocol make most sense.

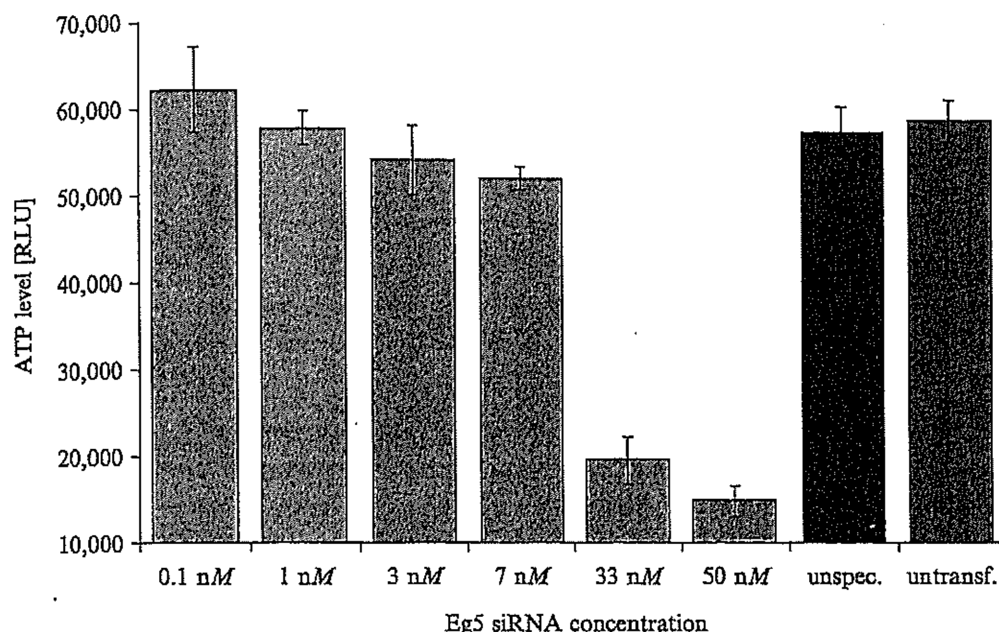

FIG. 9. Assay examples: proliferation assay. Hela cells were transfected with increasing concentrations of a siRNA targeting Eg5 (RefSeq name KIF11) in a mixture that was brought to 100 n*M* with unspecific negative control siRNA. Seventy-two hours after transfection, ATP levels were measured on a multilabel reader, using the ATPlite™ kit (Perkin Elmer).

When observing proliferation inhibition, one major limitation is that the underlying causes, namely, necrosis, apoptosis, or cell cycle deregulation, cannot be distinguished from each other by applying these assays on their own. This dilemma can be solved either by the combination of homogenous proliferation, apoptosis, and necrosis assays, or, more compellingly, through a microscopy-based approach whereby these multiple parameters can be detected simultaneously in so-called multiplexed assays (see later).

Mitotic Index Assay

Another example of an oncology-relevant assay is the mitotic index assay, which can reveal a gene's role in cell cycle progression by quantifying the proportion of cells undergoing mitosis at a given time point. The protocol, to be run as a microscopy assay, involves fixation of cells, followed by staining of chromosomes [6-diamidino-2-phenylindole (DAPI) or Hoechst], microtubules (anti-tubulin antibody) and with a mitosis-specific marker such as anti-phosphohistone H3 antibodies. In a large-scale, high-throughput scenario, data can then be acquired with an automated

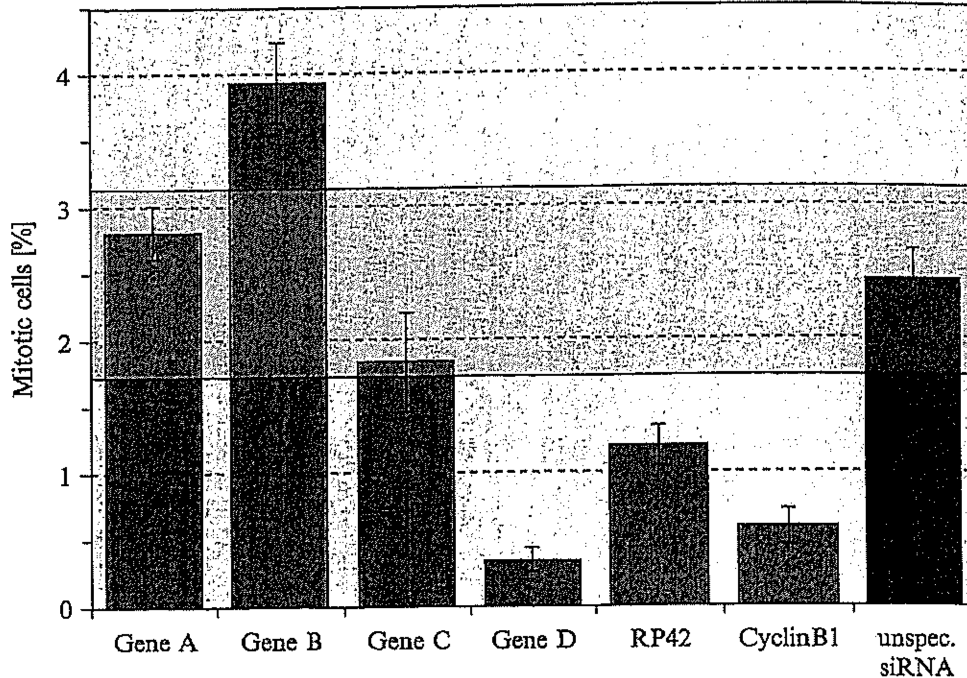

FIG. 10. Assay examples: Mitotic index assay. HeLa cells were transfected with 100 n*M* of validated siRNAs targeting several genes. Forty-eight hours after transfection, cells were fixed and stained with DAPI, anti-tubulin and anti-phosphohistone H3 (see text). The mitotic index (mitotic cells/total cells) was evaluated by fluorescence microscopy. The $\pm 3\times$ SD range of the negative control (hit limit) is indicated by a shaded box.

fluorescence microscope (e.g., the Discovery-1™ system, Molecular Devices, Sunnyvale, CA), using image processing software (e.g., MetaMorph®, Molecular Devices, or Cellenger®, Definiens, Germany).

In the example illustrated in Fig. 10, targets with a significantly increased or decreased mitotic index are shown, thus revealing their impact on cell cycle regulation. From the perspective of an oncology-focused target discovery screen, for example, cases in which silencing of a gene caused a decreased mitotic index (i.e., in which the gene is needed for passage through G1, S, and G2 phases) could point to a potential novel antiproliferative target. The combination of mitotic index screens with proliferation and apoptosis assays is highly advisable to derive even more compelling and comprehensive conclusions about potential antiproliferative targets.

Apoptosis Assays

Apoptosis, the multistep process of programmed cell death, is one of the most intensively studied processes in cell cycle research as well as in applied cancer research and drug development. Most notably from an assay

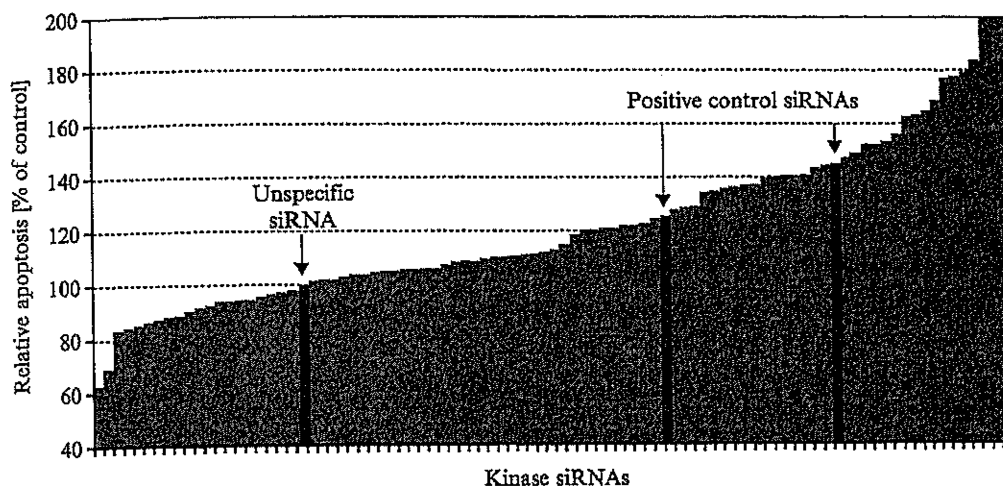

FIG. 11. Assay examples: apoptosis assay. Eighty-eight kinases were targeted, using individual siRNAs of validated efficiency (from the *Silencer*™ Kinase siRNA Library, Ambion). HeLa cells were seeded in 96-well plates at 8000 cells/well. Twenty-four hours after seeding, siRNAs were transfected (100 n*M*, see Protocol 1). Forty-eight hours after transfection, cells were subjected to the apoptosis Apo-One assay (Promega), measured on a Victor-2 multilabel reader (Perkin Elmer). siRNAs against cyclin B1 and RP42 were used as positive controls; the Negative 1 siRNA (Ambion) was taken as the negative control. Arrows indicate negative and positive controls (black). Experiments were performed in triplicates, but for better visability graphs are shown without error bars.

point of view, apoptosis involves the activation of caspase cascades, loss of mitochondrial membrane potential, acidification, cell membrane permeability, chromosome condensation, DNA fragmentation, and the formation of apoptotic bodies.

Homogenous assays are established for different stages of apoptosis, roughly divided into early and late ones: very common assay principles with numerous commercially available kits are caspase 3/7 assays, as well as ELISA assays of cytochrome C release or DNA fragmentation (TUNEL assay). Figure 11 gives an example of an apoptosis screen through a large set of human kinases, using the ApoOne™ kit (Promega, Madison, WI).

Alternatively, antibodies against poly (ADP-ribose) polymerase (PARP), phosphorylated Akt-1, caspase-3, lamin A, cytokeratin 18, or cytochrome C are tools for use as part of microscopical high-content assays. An example is discussed in High-Content Microscopy Assays.

Reporter Gene Assays

Applying reporter gene technology is a powerful way of monitoring pathways of interest. The idea is simple and widely applied in small molecule compound screening: a target gene, a target's promoter, or a

responsive element is positioned upstream of a reporter gene (e.g., luciferase) carried within an appropriate expression vector. This plasmid is either transiently transfected into the desired cell line (see Protocol 3) or a stable cell line is generated. The cells can then be used for large-scale projects, screening libraries of siRNAs, or chemical compounds. The adaptation of such an assay for RNAi is illustrated in Fig. 12. The strength of this kind of assay is the ease of the protocol, and the weakness the narrowness of the window afforded by this approach into the biology resulting from the experimental perturbation.

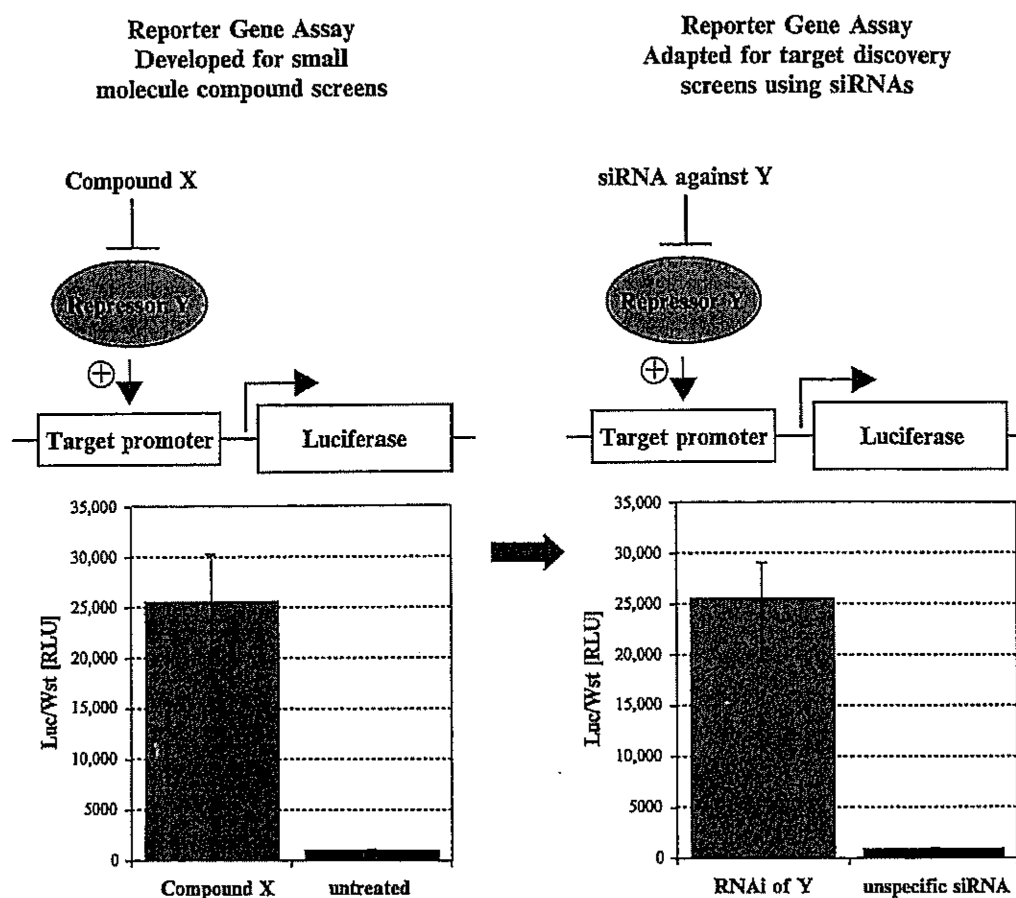

FIG. 12. Development of a typical RNAi reporter gene assay. Reporter gene assays are widely used for small molecule compound screening. The principle of converting a compound screen into an RNAi target discovery screen is illustrated. Instead of screening for small molecule inhibitors of a known target repressor Y, an siRNA-based target discovery screen can reveal novel repressors of a known target. Variants of this principle can come from different target promoters, responsive elements, or from screening for inducers rather than repressors (i.e., having an off-assay instead of an on-assay).

Although having the disadvantage of being more expensive than the plasmid-based reporter-gene assays, a version similar to reporter gene assays, in a wider sense, can be the direct monitoring of mRNA levels of two or more targets by qRT-PCR. For example, when monitoring for two or three key activity markers of a particular pathway, this strategy could reveal novel components of that pathway with high sensitivity.

Protocol 3. Cotransfection of Plasmid DNA and siRNA into Human Cells (Two Separate Transfections)

1. Twenty-four hours before transfection, seed cells at the appropriate density into each well of a six-well plate (e.g., 700,000 for MCF-7).
2. Next day, with 50–80% confluent cells, transfect 2 μg plasmid vector with 7 μl FuGene (Roche Diagnostics, Pleasanton, CA) per well, following the steps given next.
3. Per well, mix 7 μl FuGene with 93 μl serum-free medium and incubate for 5 min at room temperature.
4. Per well, prepare 10 μl plasmid vector at 0.2 μg/μl in sterile water and place into fresh tubes at 10 μl each.
5. Per tube, add dropwise the 100 μl of solution in Step 3 and gently tap the tube to mix. Incubate for 15 min at room temperature.
6. Remove the old medium from cells, and overlay with 2 ml fresh serum-containing growth medium.
7. Per well, add dropwise the 110 μl of solution in Step 5 onto the cells and gently shake the plate.
8. Incubate for 6 h, and then harvest and pool the cells from all transfected wells. Seed cells into a 96-well plate at the appropriate density (e.g., 13,000 cells/well for MCF-7). Two wells of a six-well plate are normally sufficient for one 96-well plate. Incubate cells overnight.
9. Next day, continue with Step 2 of the siRNA transfection method as outlined in Protocol 1.

High-Content Microscopy Assays

As previously noted, it is our view that the full value of cell-based screens in general, and of RNAi screens in particular, can best be realized by acquiring rich, multiparameter readouts. One strategy of doing that could be the combination of multiple homogenous assays, but an even

more elegant approach is to carry out rich *in situ* analyses by establishing so-called high-content microscopy assays. Ideally, this effort to broaden our viewing window onto the biological consequences of a silencing experiment should integrate, whenever possible, both spatial and temporal information, as illustrated by the time-lapse microscopy readout used by our group and others in studying cell division genes in *C. elegans* embryos (Gönczy *et al.*, 2000; Sönnichsen *et al.*, 2004). However, such an endeavor inevitably poses a much more considerable challenge not only for the overall size and complexity of the study but most notably in terms of the analysis and annotation of resulting datasets. The development of automated image analysis algorithms and associated image handling software tools remains in its infancy, especially in cases in which kinetic data such as time-lapse recordings are concerned. Nonetheless, with the development of increasingly sophisticated automated microscopy instruments in recent years from such established vendors as Molecular Devices/Universal Imaging and Cellomics, these goals are becoming feasible and clearly well worth the extra effort. Indeed, by enabling more contextual analyses, genome-scale discovery efforts are becoming smarter, allowing for significantly more informative phenotypic classifications of genes and thereby reducing much of the guesswork that has so far been required in their follow-up.

As one very striking example, the screen illustrated in Fig. 13 revealed very complex data on proliferation, mitotic index, apoptosis, and necrosis: RNAi-mediated knockdown of both Eg5 (RefSeq name KIF11) and Cenix target X led to similar reductions in cell proliferation, as measured by the density of nuclei in imaged fields. Thus, the cellular response to these two siRNAs would be hard to distinguish by classical reader-based cell proliferation assays, which would require subsequent extra rounds of experimental follow-up to do so. In the present case though, our microscopy-based screen vastly expanded the available "discovery space" in a single round of screening, allowing us to further classify and interpret these cellular phenotypes with only modest extra effort invested. The silencing of Eg5 yielded a strong accumulation of mitotically arrested cells exhibiting condensed chromosomes and aberrant monopolar spindles (as reported previously by Mayer *et al.*, 1999). Many cells exit this arrest by entering apoptosis, which can be detected by increased levels of a caspase-cleaved fragment of PARP, a chromatin-associated protein used as an apoptotic marker. The unchanged interphase morphology of Eg5-silenced cells is consistent with the established function of Eg5 as a protein exclusively required during mitosis. The knockdown of target X, in contrast, did not cause a mitotic arrest and no increase in apoptosis was detected, thus initially suggesting that the observed reduction in cell proliferation might come from increased necrosis or a general, uniformly slowed progress

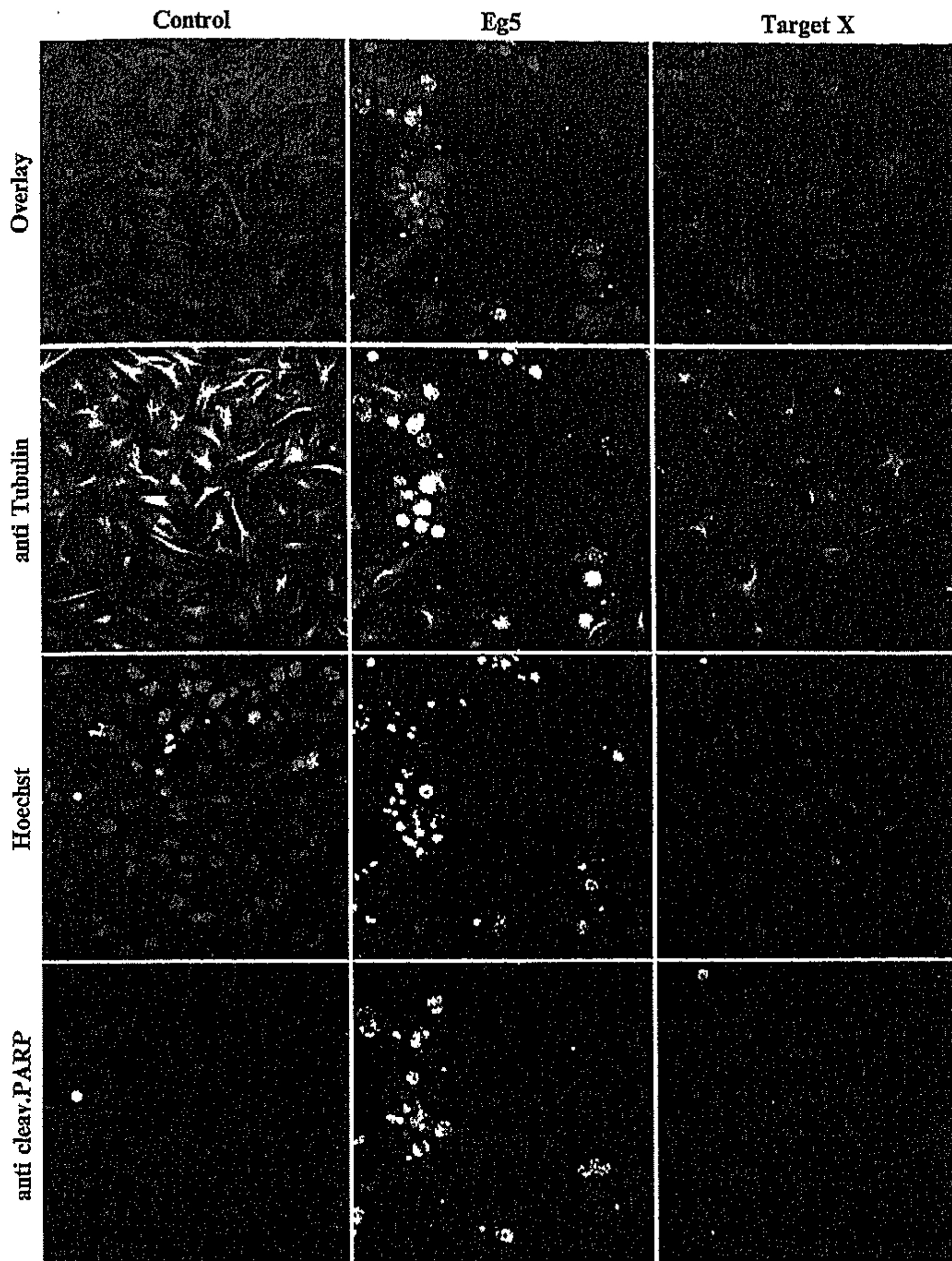

Fig. 13. High-content screening example. Triple-channel images of HeLa cells fixed and stained 48 h after treatment with three different siRNAs: unspecific control siRNA (left column), Eg5 (central column), and Cenix target X (right column). After fixation, cells were stained with specific antibodies against cleaved poly (ADP-ribose) polymerase (PARP; fourth row) and tubulin (second row). Nuclei were stained with Hoechst 33342 (third row). The three-channel overlays (first row) show cleaved PARP in red, tubulin in green, and Hoechst in blue. See text for a further discussion of this study. (See color insert.)

through all cell cycle stages (thus not affecting mitotic index). Our more detailed analysis of the same dataset also revealed a reproducible increase in the incidence of binucleated cells, suggesting a possible role of target X in the progression through cytokinesis, a deficiency that could explain the increased necrosis and lower proliferation without seeing an effect on mitotic index.

Thus, the use of high-content assays allows the identification of hits whose phenotypes may not represent drastic changes in a single parameter but the combination of subtle changes in several parameters. This is especially valuable in the context of RNAi screens, in which subtle phenotypes may result from partial gene silencing, and therefore a multiparameter approach can greatly improve not only the depth and breadth of the readout but also the overall sensitivity of the screen.

Case Study: RNAi Screening with a Kinase siRNA Library

The following case study serves as an example of a focused screen by using transient transfection of chemically synthesized siRNAs to illustrate the opportunities afforded by a multiplexed readout. The goal of this screen was to identify kinases that are involved in apoptosis, cell proliferation, and cell cycle control. The study itself was focused on 88 human kinases known to be expressed in HeLa cells, using prevalidated siRNAs known to trigger silencing of their target mRNAs by at least 70% (Cenix predesigned siRNAs, Ambion).

Forty-eight hours after transfection, cells were fixed and stained with DAPI, anti-tubulin and anti-phosphohistone H3 antibodies. Data were obtained by using an automated fluorescence microscope (Discovery-1, Molecular Devices) with automated image processing software (MetaMorph), in combination with homogenous readouts obtained with a Victor-2 multilabel reader (Perkin Elmer). This enabled the simultaneous high-throughput acquisition of proliferation (cell numbers), mitotic index (percentage of cells in mitosis), apoptosis, and cytotoxicity, as well as cytoskeletal organization and overall cell morphologies. As a result (Fig. 14), several siRNAs were identified as causing dramatic increases or reductions in mitotic index, compared to the ~2.5% mitotic cells observed in control samples. First, this analysis, together with the proliferation data, gave indications on the underlying cell cycle regulation effects, namely, whether a higher mitotic index was elicited by mitotic arrest or a shorter interphase and whether a lower mitotic index points to an interphase arrest or a shorter mitosis. Second, it can clearly be distinguished between kinases whose inhibition leads to apoptosis and necrosis, or both. Third, the immunofluorescence staining of cell components (chromatin, microtubules)

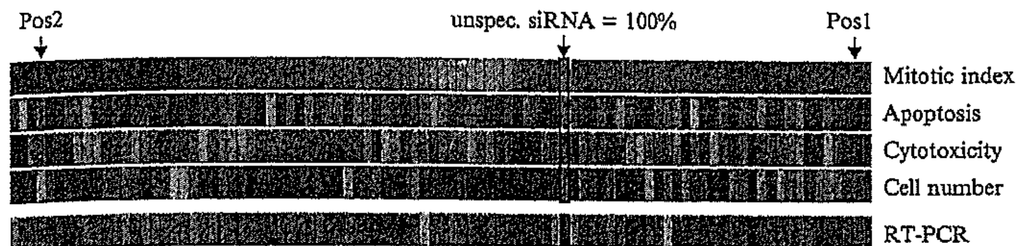

FIG. 14. Case study: mitotic index, apoptosis, necrosis, and proliferation. Multiple functional screening data illustrated for a subset of 88 kinases whose apoptosis data are shown in Fig. 11. The 88 kinases (plus two positive controls and one negative control) were targeted with individual validated siRNAs (part of the *Silencer* Kinase siRNA Library, Ambion). In 96-well plates, HeLa cells were transfected with individual siRNAs at a final concentration of 100 n*M* (Oligofectamine transfection reagent; see Protocol 1). Forty-eight hours after transfection, cells were subjected to different assays. To determine mitotic index (percentage of mitotic cells) and cell numbers, cells were fixed and stained with DAPI as well as with anti-tubulin and anti-phosphohistone H3 antibody, and data were acquired by automated fluorescence microscopy (Discovery-1 microscope, Molecular Devices). Other readouts were apoptosis (ApoOne™ kit) and cytotoxicity (ToxiLight kit), both measured on a Victor-2 multilabel reader (Perkin Elmer). The degree of RNAi silencing triggered by each siRNA, measured as the relative remaining mRNA level in real-time RT-PCR (see Protocol 2), is also depicted (always at the <30% level). Data are given in a heatmap format, illustrating the relative changes compared with the negative control (unspecific siRNA). Red indicates increasing values and green decreasing values. Data are ordered by the mitotic index (bright red to bright green). All data points show the average of a triplicate. (See color insert.)

allowed a direct evaluation of microscopy data to further classify mitotic arrest phenotypes. Taken together, the data lead to new, detailed insights into the function of a broad set of human kinases. The concept that was applied here as well as in an earlier study (Krönke *et al.*, 2004) serves as a proof of principle for the use of siRNA libraries and the value of combining large-scale siRNA-based screening with multiparametric readout assays.

Outlook: Reagents, Techniques, and Overall Strategies

In this chapter, we have reviewed and discussed the opportunities and challenges of genome-scale RNAi-based screens, using transient transfection of chemically synthesized siRNAs. We now look forward to the emergence of new reagents and techniques that will help further extend the applicability of RNAi-based functional genomic screens both horizontally, that is, using cell-based methods to study a broader range of biological processes, and vertically, applying RNAi screens to advance downstream applications in the development of new therapeutic drugs.

The latter will proceed through the discovery and characterization of gene functions that are not measurable in cell systems by using RNAi screening in animals. Furthermore, those RNAi screening methodologies currently being used in cells can and will be used, with minor adjustments, to gain new insights into the mechanisms of action, sources of toxicities, and side effects of therapeutic compounds currently under development.

One particularly elegant technique, pioneered by Sabatini and colleagues (Carpenter and Sabatini, 2004), and now known as retrotransfection or solid-phase optimized transfection (SPOT), is being further developed by several groups, allowing the creation of SPOT-RNAi arrays of siRNAs or even viruses, arranged as discrete spots on microscopically compatible growth supports (slides or plates) onto which cells can be seeded. Although it remains unclear whether, in practice, this will truly allow the use of smaller amounts of reagents, as is promised, this approach does offer the potential of greatly accelerating the throughput of compatible screening assays. The range of compatibility of screening assays for SPOT-RNAi will also depend on the method's overall experimental robustness, particularly in accommodating different cell types exhibiting varying levels of motility, as well as the numbers of cells required within each sample (i.e., over each siRNA spot) to yield statistically significant results from the desired assay.

Another up-and-coming development of note will likely be the emergence of new types of RNAi reagents, both for silencing and for better delivery. In the former category, we take particular note the discovery of so-called endonuclease-derived siRNAs, or esiRNAs, by Yang *et al.* (2002). These pools of siRNA-like molecules are generated through the controlled RNase III digestion of long dsRNA molecules (usually >200 bp) produced by *in vitro* transcription. The resulting pool of heterogeneous sequences, all targeting the same transcript, is thought to increase the potency of the reagent while diluting out the off-target effects of each individual molecule. In view of the method's inherently low production costs this sounds very promising, and therefore a more detailed characterization of esiRNA's actual experimental performance, especially in screening applications, is keenly awaited.

In the meantime, although siRNAs at present represent the gold quality standard, the associated cost factor is limiting its widespread adoption by academic groups for large-scale studies, some of whom are opting for vector-based screening strategies. Although renewal costs of shRNA vector libraries are thought to be generally lower than for siRNA libraries, the initial investments are considerable in both cases, the general quality and accuracy of vector libraries are notoriously variable (in large part due

to the complexities of large-scale cloning), and the subsequent updating of vector libraries is much more cumbersome. It is most heartbreaking to note the acceptance by some of almost undoubtedly higher rates of false negatives (i.e., missed positives) afforded by shRNA vector libraries compared to what siRNAs could yield in the same experiments, as an acceptable trade-off, because the laboratory "will get a good number of genes to follow-up on anyway." Although this attitude reflects a commonly shared pragmatic wisdom necessary to keep successful research programs moving forward, because funding is unlikely to be granted for a second group to repeat the same screen, this is precisely the type of scenario whereby missed positive genes remain undiscovered by the research community for years or decades to follow. Thus, the obvious need to be pragmatic notwithstanding, we suggest that the motivation driving the design and implementation of these screens should, whenever possible, reach beyond the mere feeding of project pipelines of individual groups rather striving more explicitly toward the most comprehensive coverage possible of the targeted "discovery space." In this context then, a more defensible justification for choosing shRNA vectors for RNAi screening, in our view, is found in those cases in which delivery of siRNAs into the cells of interest is simply not feasible or when the silencing effect must be sustained for longer periods than what is afforded by siRNAs. Then, at least as RNAi technology stands at the moment, the choice of shRNAs makes clear sense. Ideally, the cost arguments should only offer stronger motivation to request and insist on higher grant funding levels for this type of research or to improve the availability of specialized outsourcing facilities or service providers, such that the scientific quality of the investigations is not compromised.

Finally, beyond these considerations, the chapter has hopefully illustrated the value of combining genome-scale siRNA-based screening with the application of rich, high-content readout assays. The potential of effectively generating complex, gene-specific phenotypic signatures, fingerprints, or perhaps phenoprints offers an important step forward in the evolution of functional genomics, advancing both systems biology and many more traditional cell, molecular and developmental investigations.

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