Specificity of short interfering RNA determined through gene expression signatures

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Short interfering RNA (siRNA) is widely used for studying gene function and holds great promise as a tool for validating drug targets and treating disease. A critical assumption in these applications is that the effect of siRNA on cells is specific, i.e., limited to the specific knockdown of the target gene. In this article, we characterize the specificity of siRNA by applying gene expression profiling. Several siRNAs were designed against different regions of the same target gene for three different targets. Their effects on cells were compared by using DNA microarrays to generate gene expression signatures. When the siRNA design and transfection conditions were optimized, the signatures for different siRNAs against the same target were shown to correlate very closely, whereas the signatures for different genes revealed no correlation. These results indicate that siRNA is a highly specific tool for targeted gene knockdown, establishing siRNA-mediated gene silencing as a reliable approach for large-scale screening of gene function and drug target validation.

DNA microarray | RNA interference | gene knockdown

The use of RNA interference for inhibiting gene expression represents a powerful tool for exploring gene function (1–7), identifying and validating new drug targets, and treating disease (8, 9). The process of RNA interference is mediated by double-stranded RNA, which is cleaved by the enzyme DICER into 21- to 23-nt duplexes containing a 2-nt overhang at the 3′ end of each strand. These duplexes are incorporated into a protein complex called the RNA-induced silencing complex (RISC). Directed by the antisense strand of the duplex, RISC recognizes and cleaves the target mRNA (for recent reviews, see refs. 1 and 10–13). Although long double-stranded RNAs (>30 nt) invoke an interferon response, short interfering RNAs (siRNAs) that resemble the products produced by DICER have been reported to specifically inhibit gene expression in many different mammalian cell lines (1–7). It has been shown that even single nucleotide mismatches between the antisense strand of the siRNA and target mRNA can abolish RNA interference (14). In addition, mapping of mRNA cleavage sites has revealed no cleavage sites outside of the region of complementarity (10). However, the specificity of siRNA at the cellular level remains to be comprehensively studied.

For siRNAs to be a useful tool in gene knockdown experiments, it is critical that siRNA-mediated transcriptional silencing be specific. It is not enough to simply show that a control siRNA with a scrambled nucleotide sequence fails to knock down the protein of interest or produce the same cellular phenotype. Ideally, the siRNA must not cause *any* effects other than those related to the knock down of the target gene. There are several types of nonspecific effects that siRNA could potentially display. In addition to the possibility for cross-hybridization of the antisense strand of the siRNA to different mRNAs, siRNAs could bind in a sequence-dependent manner to various cellular proteins. Indeed, antisense oligonucleotides have been shown to bind to many different proteins (15–17) and cause significant nonspecific effects (18–20). In addition, several RNA aptamers are known to bind to endogenous proteins and alter their function (21). It may also be possible for siRNAs to induce common, nonspecific changes in gene expression. These nonspecific or off-target effects could complicate the inter-

pretation of gene knockdown experiments and severely limit the utility of siRNA.

To examine the specificity of siRNAs, we used DNA microarrays, which are ideally suited to provide a global fingerprint of gene regulation (22–24). Several siRNAs were designed against different regions of the same target gene for three different targets. We hypothesized that if siRNAs elicit a specific response, then all of the siRNAs designed against the same target would be expected to produce similar gene expression signatures even though each siRNA has a different nucleotide sequence. At the same time, the siRNAs designed against different target genes would be expected to show very little overlap between their expression profiles. This test of specificity is more stringent and more comprehensive than those reported thus far that follow the knockdown of particular proteins or the observation of specific cellular phenotypes.

Materials and Methods

siRNA Design. Based on the rules suggested by Elbashir *et al.* (10), the antisense strand of siRNA was targeted against an $AA(N)_{19}$ sequence at least 100 nt downstream of the start codon. The GC content of the duplexes was kept within the 40–70% range. In addition to these rules, we used a computer program that maximizes the hybridization specificity of siRNA. In the first step, the program identifies and excludes from further consideration regions of the target mRNA highly homologous to other, unwanted sequences in RefSeq (25). Next, all possible 19-nt sequences against a given coding sequence are generated that satisfy the suggested rules (10) and a BLAST search is conducted against GenBank. The resulting sequences are sorted by the length of the homology region with the next best match. All sequences with the region of homology to the next best hit shorter than 15 nt are displayed as the final output. Additional information is also provided, such as the identity of the next best hit, the length of the homology region, and the value of $\Delta T_m = T_m(t) - T_m(bm)$, where $T_m(t)$ is the T_m for the duplex formed by the antisense strand of siRNA and the target mRNA and $T_m(bm)$ is the T_m for the duplex formed between the antisense strand of siRNA and the next best match mRNA. The T_m values were calculated as reported (26). From this output, the best sequences are selected with the shortest homology region and the highest ΔT_m. The sequences of all siRNAs used in this study are listed in Table 1, which is published as supporting information on the PNAS web site, www.pnas.org. The siRNA duplexes were obtained from Dharmacon Research (Lafayette, CO).

Cell Culture, siRNA Transfections, and Western Blotting. Human non-small cell lung carcinoma cells H1299 were cultured in RPMI medium 1640 (Invitrogen) supplemented with 10% FBS (Invitrogen). Transfections were performed by using TransIT-TKO reagent (Mirus, Madison, WI) according to the manufacturer's instructions. Briefly, 3 μl of 20 μM siRNA solution and 15 μl of the transfection reagent were incubated in 0.5 ml of serum-free RPMI medium 1640

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for 20 min to facilitate complex formation. The resulting mixture was added to the cells cultured in a 60-mm tissue culture dish (Corning) with 2.5 ml of RPMI medium 1640. For Western blots, cell lysates were electrophoresed on SDS/PAGE gels, and the proteins were blotted onto a nitrocellulose membrane. Primary antibodies against Rb (Santa Cruz Biotechnology), AKT1 (Cell Signaling Technology, Beverly, MA), Plk1 (Zymed), and actin (Abcam, Cambridge, U.K.) were used according to the manufacturer's recommendations.

RNA Isolation, Microarray Analysis, and RT-PCR. Total RNA was extracted by using TRIzol reagent (Invitrogen) and purified on RNeasy columns (Qiagen). Labeled cRNA was prepared according to the chip manufacturer's protocol and hybridized to Human Genome U95Av2 chips (Affymetrix, Santa Clara, CA) containing ≈12,000 genes and ESTs. Microarray data were analyzed by using RESOLVER software (Rosetta Inpharmatics, Kirkland, WA). Two-dimensional clustering was performed by using the agglomerative hierarchical clustering algorithm. All genes regulated with a P value ≤0.05 were retained in the clustering analysis. For gene clustering, pairwise similarity metrics among genes were calculated based on the expression ratios across all experiments. For experiment clustering, pairwise similarity measures among all experiments were calculated based on the expression ratios across all genes with $P \leq 0.05$. The Euclidean distance was used as a similarity metric. TaqMan real-time quantitative PCR was performed by using ABI Prism 7700 (Applied Biosystems). Reverse transcription and amplification of total RNA (100 ng) was accomplished in a one-step format with ThermoScript Plus RT/Platinum Taq DNA polymerase (Invitrogen). Gene-specific TaqMan primers and probes were designed by using the PRIMER EXPRESS program (Applied Biosystems).

Results

siRNA Design. In our initial experiments, siRNAs were designed according to Elbashir *et al.* (10). In addition to the siRNAs designed against particular target genes, control siRNAs were prepared by inverting a 4-nt fragment in the middle of the sequence and performing a BLAST search of the resulting sequence against GenBank. Interestingly, in the initial microarray experiments, some of these control siRNAs produced what appeared to be strong effects on gene expression. Although these control siRNAs did not have a perfect 19/19 match to any known mRNA sequences based on our initial design criteria, they generated significant gene expression signatures 24 h after transfection (data not shown). The expression profiles were different for each siRNA and sequence specific. On further analysis, we found that each of the control siRNAs contained 17- to 18-nt sequences that were homologous to specific mRNAs in the database. Although it has been reported (14) that even a single mismatch between an siRNA and the target mRNA abrogates silencing, we speculated that the expression signatures observed for the control siRNAs could be due to cross-hybridization and subsequent degradation of unwanted transcripts. Recent findings also suggest that such "scrambled" siRNAs may translationally repress untargeted genes by forming duplexes with bulging loops and initiating the miRNA mechanism (27). Therefore, to minimize the potential for cross-hybridization, more stringent rules were implemented in our revised design criteria. In addition to the published siRNA design guidelines (10), we selected the sequences with the shortest homology region with the best unwanted match and the highest T_m differential between the desired target silencing complex and the most likely cross-hybridizing complex (see *Materials and Methods*).

Optimization of siRNA Transfection Conditions. Our initial experimental conditions were based on an earlier study involving an siRNA-mediated knockdown of 21 genes in human cells (28). In these studies, the concentration of siRNA was 100 nM. Our
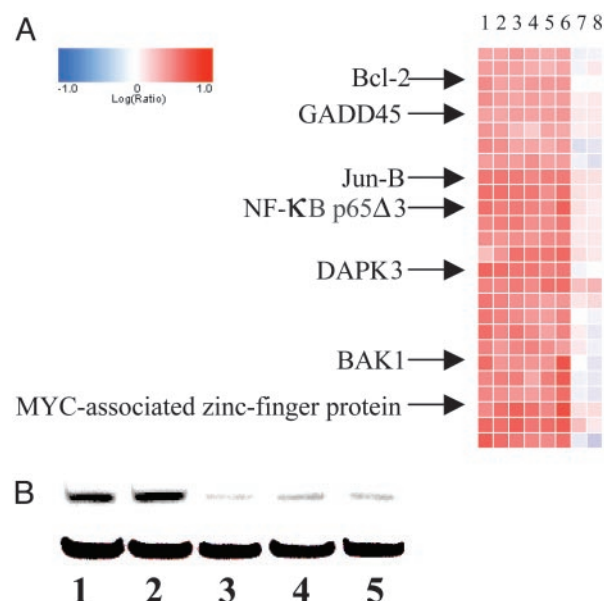

Fig. 1. (*A*) Fragment of a heat map created by hierarchical clustering of the following gene expression profiles: H1299 cells transfected with 100 nM scrambled control siRNA (lanes 1 and 2); 100 nM siRNA against Chk1 (lane 3), AKT1 (lane 4), and Rb (lanes 5 and 6); and 5 nM (lane 7) and 20 nM (lane 8) siRNA against Rb. (*B*) Western blot of the Rb protein (upper band) in H1299 cells transfected with 5 nM (lane 3), 20 nM (lane 4), and 100 nM (lane 5) Rb siRNA 112. Untransfected H1299 cells (lane 1) and cells transfected with random-sequence scrambled siRNA (lane 2) were used as controls. Actin was used as loading control (lower band).

microarray analysis showed that under these conditions, all of the siRNAs cause a nonspecific 4- to 8-fold induction of a large number of common genes (Fig. 1*A*, lanes 1–6), including some apoptosis-related (e.g., BAK, Bcl-2, and DAPK) and stress-response (GADD, p38, and MAP kinases) genes. Based on the nature of the genes induced, we reasoned that these transcriptional effects may represent a concentration-dependent cellular response to the toxic effects of siRNA. Therefore, in subsequent experiments, we reduced the siRNA concentration to 5 and 20 nM and monitored the expression level of these "siRNA-response" genes, as well as the target knockdown. Fig. 1*A* illustrates some of the genes reproducibly regulated in the siRNA response signature. Clearly, at 5 nM (lane 7) or 20 nM (lane 8), the siRNA response genes were unaffected (revealed no statistically significant fold change). Meanwhile, the retinoblastoma protein (Rb) was knocked down to a similar extent compared with 100 nM siRNA (Fig. 1*B*). Similarly, the other siRNAs used in this study efficiently silenced their targets at 20 nM, without generating the siRNA response signature (data not shown). These data led us to use 20 nM siRNA in all subsequent experiments.

Comparison of Gene Expression Signatures for Different siRNAs Targeting the Same Gene. To compare the transcriptional effects observed for siRNAs targeting different regions of the same gene, microarray experiments were conducted in duplicate by using five different siRNAs per target gene. The target genes chosen for this study include Rb1, AKT1, and Plk1. Before the microarray experiments, all siRNAs were tested for mRNA knockdown by RT-PCR. Because mRNA degradation does not always result in protein knockdown due to a long protein half-life or other factors, we also measured the knockdown of the target proteins. Fig. 2*A* depicts the results of the RT-PCR analysis for five siRNAs against Rb that were subsequently selected for the microarray study. The mRNA knockdown for siRNAs 112, 114, 1308, 1310, and 1314 varied from 70%
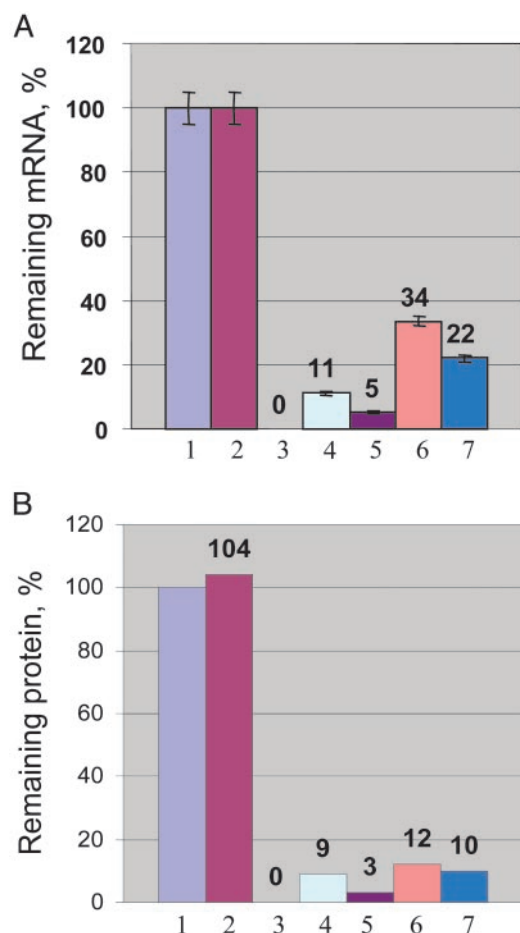
Fig. 2. Characterization of Rb siRNAs. (*A*) Q-RT-PCR analysis of Rb mRNA knockdown in H1299 cells by siRNAs 112 (lane 3), 114 (lane 4), 1308 (lane 5), 1310 (lane 6), and 1314 (lane 7). (*B*) Western blot analysis of the Rb protein knockdown in H1299 cells by siRNAs 112 (lane 3), 114 (lane 4), 1308 (lane 5), 1310 (lane 6), and 1314 (lane 7). The numbers were normalized to the no-siRNA control (lane 1). Random-sequence, or ''scrambled,'' siRNA (lane 2) was also used as a negative control.

to 100%. The Western blot data (Fig. 2*B*) showed the complete elimination of the target protein for siRNAs 112 and 1308 and significant ($\approx$90%) knockdown for the other three siRNAs.

For all five siRNAs, gene expression signatures were generated in duplicate by using human H1299 cells 12 h after transfection. The 12-h time point was chosen based on our previous gene expression studies with Rb (data not shown) and earlier microarray studies of E2F-1 activation (29–31). At 12 h after E2F-1 activation, one can observe the induction of multiple genes involved in the G_1/S-phase cell cycle transition and DNA biosynthesis. Microarray analysis revealed a 4- to 8-fold knockdown of the Rb gene, largely consistent with the RT-PCR results. The wide range of the knockdown may be attributed to the fact that the cleavage fragments of the target mRNA may be rather stable. In this case, some of the probes on the microarray would still generate a hybridization signal. In contrast, the RT-PCR primers were designed so that they would flank the cleavage site.

We performed two-dimensional hierarchical clustering of the gene expression profiles for all five Rb siRNAs. Fig. 3*A* presents the full view of the resulting cluster. The heat map contains all 2,475 genes regulated in at least one of the experiments with a *P* value ≤0.05. Each column represents an individual siRNA transfection experiment (5 siRNAs × 2 replicates = 10 experiments), and each row represents an individual gene. As shown in Fig. 3*A*, all five Rb
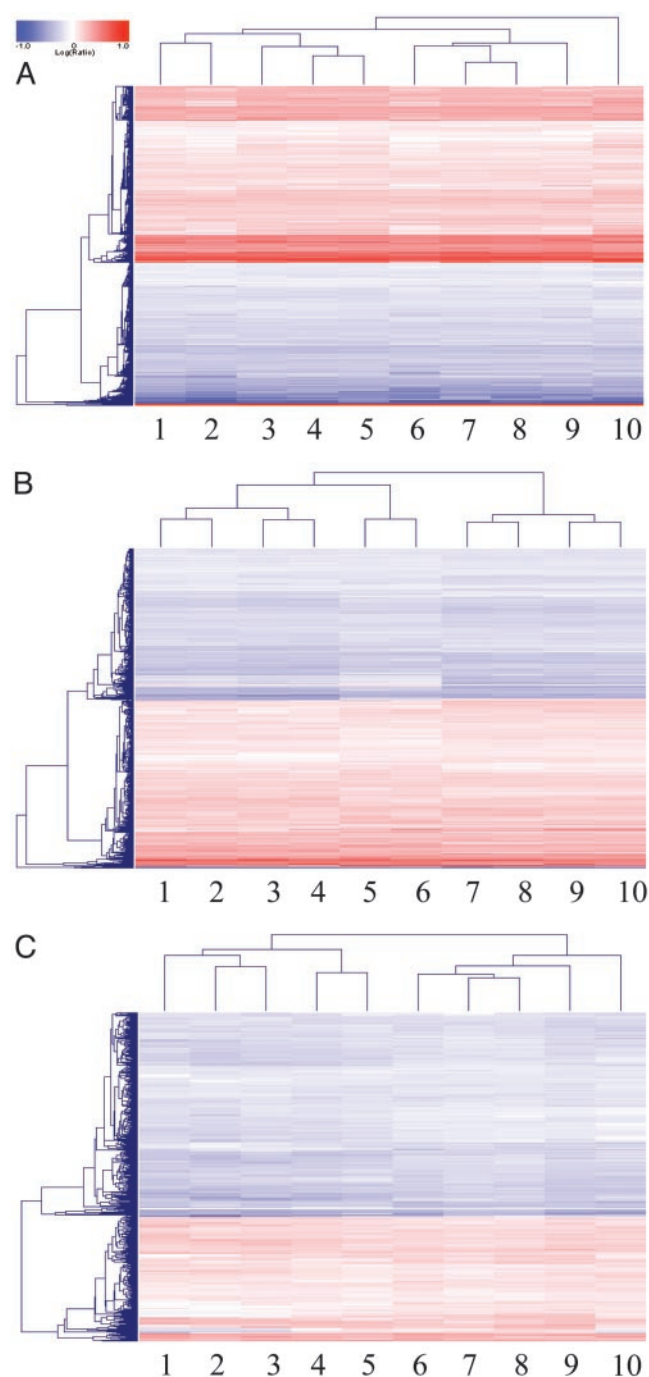

Fig. 3. Scaled-down representation of the heat map obtained by hierarchical clustering of the gene expression profiles for multiple siRNAs against Rb (*A*), AKT1 (*B*), and Plk1 (*C*). Each color patch in the matrix represents the expression fold change of the associated gene relative to the untreated control: red indicates up-regulation and blue indicates down-regulation, as shown by the color bar. (*A*) Signatures for siRNAs 112 (lanes 1 and 2), 114 (lanes 7 and 8), 1308 (lanes 3 and 10), 1310 (lanes 4 and 5), and 1314 (lanes 6 and 9) against Rb run in duplicate. (*B*) Signatures for siRNAs 1286 (lanes 1 and 3), 1288 (lanes 7 and 8), 1290 (lanes 5 and 6), 29 (lanes 2 and 4), and 31(lanes 9 and 10) against AKT1 run in duplicate. (*C*) Signatures for siRNAs 1378 (lanes 1 and 2), 1386 (lanes 7 and 8), 1406 (lanes 3 and 10), 1408 (lanes 4 and 9), and 1410 (lanes 5 and 6) against Plk1 run in duplicate.

siRNAs produced very similar gene expression patterns. The dendrogram at the top of the matrix represents the degree of similarity between the samples. It is noteworthy that the gene expression
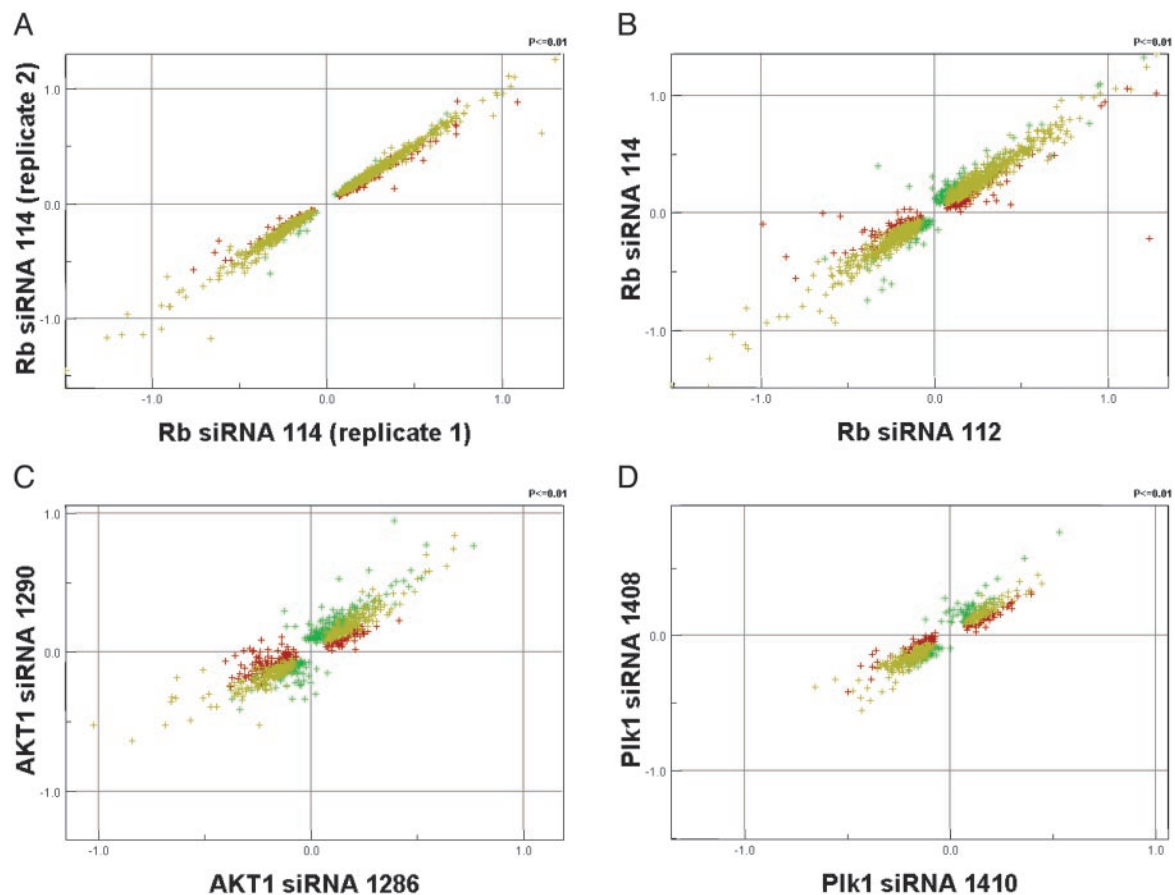
Fig. 4. Correlation between gene expression signatures. The red data points represent the signature of the x-axis experiment, and the green data points represent the signature of the y-axis experiment. The yellow data points belong to the common signature ($P \leq 0.01$). (A) Replicates of the siRNA 112 experiment. (B) Rb siRNA 112 vs. Rb siRNA 114. (C) AKT1 siRNA 1286 vs. AKT1 siRNA 1290. (D) Plk1 siRNA 1410 vs. Plk1 siRNA 1408.

signatures generated by the siRNAs were so similar that in some cases the signatures for different siRNAs clustered more closely than the biological duplicates of the same siRNA.

To quantitatively assess the similarity between the expression signatures for different siRNAs, we plotted the expression fold changes for different experiments against each other and determined the correlation coefficients. Fig. 4 depicts comparison plots for the duplicates of the same siRNA (A) and for two different siRNAs against Rb (B). The degree of correlation between the experiments was quantified by calculating the correlation coefficient ρ for the population of genes regulated in either experiment at a confidence level of 99%. The tightness of the plot shown in Fig. 4A and the value of $\rho = 0.996$ indicates a high level of reproducibility between the biological replicates. The datasets obtained for the siRNAs designed against different regions of the same gene revealed a remarkable level of correlation as illustrated by the plot shown in Fig. 4B and the value of the correlation coefficient ($\rho = 0.971$), which is close to that observed for the biological duplicates.

In a more detailed analysis of the Rb1 knockdown signature, we identified the set of genes regulated by at least one of the five siRNAs against Rb1. There are 919 genes regulated at least 1.5-fold by at least one siRNA at the 95% confidence level. Of these 919 genes, 720 (78.3%) were up- or down-regulated by all five siRNAs, 146 (15.9%) were regulated by at least four siRNAs, and 37 (4%) were regulated by at least three siRNAs. Thus, 903 genes (98.2%) were regulated by at least three of five siRNAs. The overwhelming majority (98.1%) of genes regulated by three or fewer siRNAs were regulated <2-fold. Detailed analysis of the Rb1 siRNA signature revealed the induction of a significant number of genes involved in

cell cycle regulation and DNA biosynthesis (data not shown). This finding is consistent with the mechanism of Rb1 protein function (32, 33). Knockdown of Rb1 results in the release and activation of the E2F1 transcription factor, which controls the expression of many cell cycle and DNA synthesis genes. This results in activation of the DNA replication machinery and transition of the cell from G_1 into the S phase (32, 33).

We also profiled five different siRNAs that effectively silenced the AKT1 gene. A total of 840 genes were regulated in at least one experiment at the 95% confidence level. Fig. 3B presents the full view of the matrix obtained by agglomerative hierarchical clustering of the 10 experiments (5 siRNAs × 2 replicates). As in the case of Rb, the gene expression pattern is similar across all 10 experiments. To obtain a quantitative measure of the similarity between the knockdown signatures, we built comparison plots for all possible pairs of siRNAs. An example of such a plot is shown in Fig. 4C. The two siRNAs cause similar quantitative changes in the expression level of each of the genes represented in the plot, with the exception of a few outliers. The correlation coefficient between these two signatures is $\rho = 0.89$, which implies a strong agreement between the expression fold changes for the genes regulated by these two siRNAs. Although the AKT1 knockdown signature was significantly smaller than the one for Rb (840 vs. 2,475 genes at $P \leq 0.05$), the multiple siRNAs correlated very well with each other, confirming our observations on the Rb knockdown signature.

To provide an additional test of specificity for a different gene, we profiled five siRNAs against Plk1 that efficiently silenced the target (data not shown). At the P value cut-off of 0.05, 451 genes were regulated in at least one of the 10 experiments (5 siRNAs ×
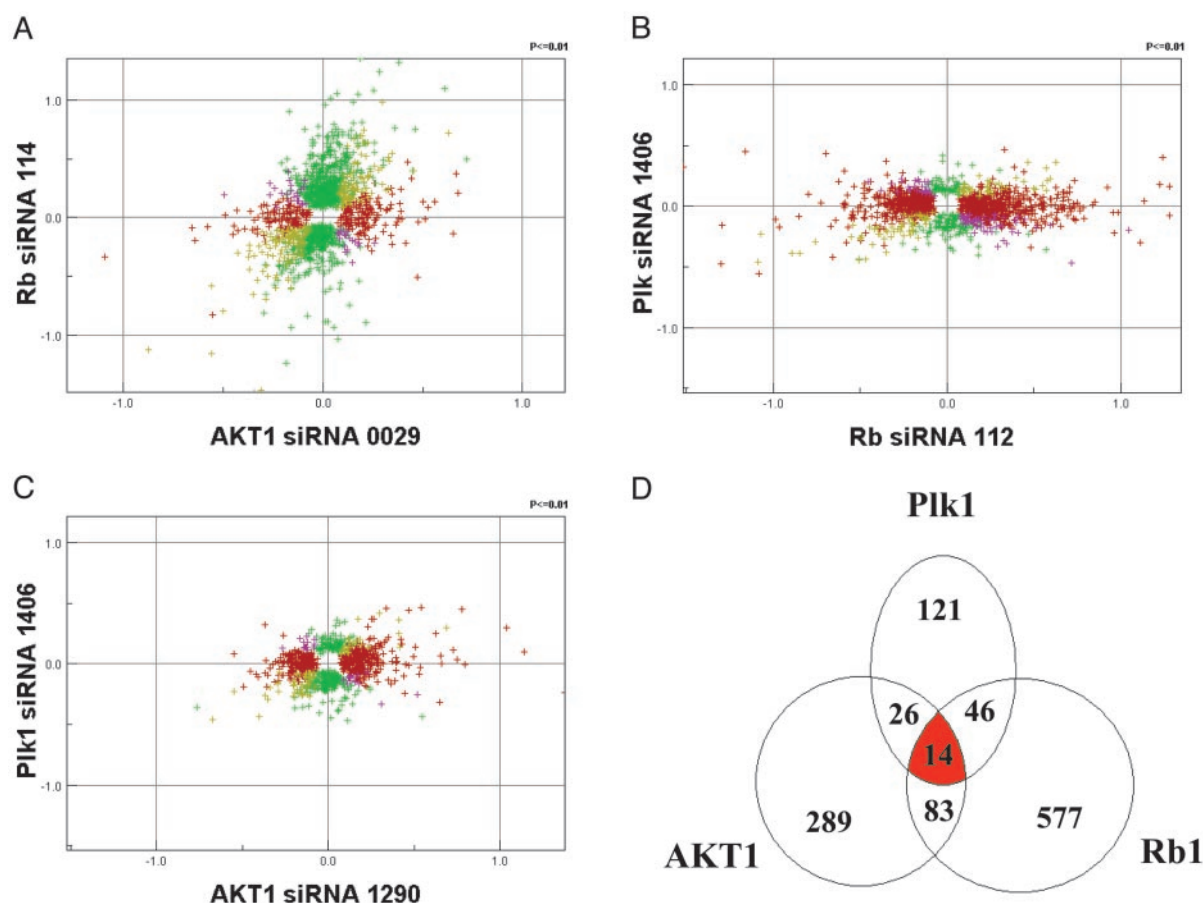
Fig. 5. Correlation between gene expression signatures for siRNAs against Rb and AKT1 (*A*), Rb and Plk1 (*B*), and AKT1 and Plk1 (*C*). (*D*) Venn diagram representing the overlap between the gene expression signatures for the Rb, AKT1, and Plk1 siRNAs.

2 biological replicates). Hierarchical two-dimensional clustering of the 10 experiments and the 451 genes (Fig. 3*C*) reveals a remarkable similarity between the expression signatures as previously observed for Rb and AKT1. The pairwise comparison plots for these experiments demonstrated a close quantitative correlation between the expression fold changes for all of the genes regulated by the Plk1 siRNAs, as illustrated by the example shown in Fig. 4*D*. The correlation coefficient between the signatures for siRNAs 1408 and 1410 was $\rho = 0.94$, which is close to that observed for the biological duplicates of the Plk1 siRNA 1408 ($\rho = 0.98$). Overall, silencing of the Plk1 gene caused fewer transcriptional effects than the Rb knockdown (451 vs. 2,475 genes at $P \leq 0.05$), but the correlation between the different siRNAs was very strong, confirming our conclusions on the specificity of siRNA under the optimized conditions.

Comparison of Gene Expression Signatures for siRNAs Designed Against Different Genes. Theoretically, the observed qualitative and quantitative similarities between different siRNAs against the same gene could be due to a nonspecific cellular response to double-stranded RNA. If this were the case, then the signatures for siRNAs against different genes would be very similar to each other as well. Therefore, we compared the Rb, AKT1, and Plk1 knockdown signatures by using the procedure described above. The comparison plots for Rb and AKT1 (Fig. 5*A*), Rb and Plk1 (*B*), and AKT1 and Plk1 (*C*) siRNAs clearly indicate the lack of correlation between the signatures generated for siRNAs against different genes. This finding is consistent with the correlation coefficients for these pairs: $\rho = 0.358$ (Rb and AKT1), $\rho = 0.04$ (Rb and Plk1), and $\rho = 0.21$

(AKT1 and Plk1). These data suggest that the knockdown signatures are unique to each target, ruling out the possibility that our previous findings are due to a nonspecific response to siRNA or the transfection reagent.

We also identified the individual genes regulated by >1.5-fold ($P \leq 0.05$) by the siRNAs against Rb, AKT1, and Plk1 and determined the overlap between these datasets (Fig. 5*D*). Only 14 genes were common for the three signatures (see Table 2, which is published as supporting information on the PNAS web site). The common set included the gene encoding an enzyme involved in RNA metabolism (uridine phosphorylase), several genes implicated in the control of cell proliferation and stress response (FYN oncogene and c-Fos gene), and a number of genes encoding hypothetical proteins. With the exception of GenBank entries U73682 and AB007896, all of the genes from this set were also regulated by control random-sequence siRNAs. However, we were not able to establish a connection between the common transcriptional effects and the proposed siRNA mechanisms. We are currently attempting to gain further insight into the siRNA mechanism by analyzing transcriptional regulation by siRNA.

Discussion

siRNA shows tremendous promise as a tool for targeted gene silencing. However, its utility will depend on its specificity, i.e., the ability to specifically knock down the target gene without interfering with the expression or function of other genes or proteins. In cell-based knockdown experiments, siRNA-mediated gene silencing allows for the rapid analysis of the resulting phenotype and thus provides insight into the target gene's function in a high-throughput

manner. However, if an siRNA produces a phenotype such as apoptosis or cell cycle arrest because of cross-hybridization, sequence-specific protein binding, or a general dsRNA response, then the target gene may be erroneously associated with that phenotype. Before the discovery of RNA interference, antisense oligonucleotides were the primary tools for targeted gene silencing (for review, see refs. 16–18 and 34). However, they have been shown (17–20) to cause significant nonspecific effects. In particular, the affinity to cellular proteins has been shown to be an essential factor that can influence and complicate the interpretation of antisense oligonucleotide-mediated gene silencing experiments (15).

There are several nonspecific effects that could be induced by siRNAs, including (*i*) degradation of mRNA other than the target due to cross-hybridization followed by downstream effects, (*ii*) binding to cellular proteins in a sequence-specific manner (aptamer effect) and all of the downstream transcriptional effects, (*iii*) translational silencing through miRNA effect (27), and (*iv*) induction of "dsRNA response" nonspecific with respect to the siRNA sequence. In this study we examined the specificity of siRNA in a comprehensive manner by using DNA microarrays. Our approach involved the use of transcriptional profiling as a measure of the cellular effects of siRNA. We chose microarrays as a tool for this study because they provide a global view of the gene expression in the cell, often referred to as the "molecular phenotype" or "gene expression signature." Previously, gene expression signatures have been extensively used for molecular classification of tumors (22–24). In our study, transcriptional signatures were generated for multiple siRNAs designed against different regions of the same target gene. We hypothesized that if siRNA only produced specific (on-target) effects, then different siRNAs against the same gene would generate highly similar gene expression signatures. At the same time, the siRNAs designed against different genes would have very little overlap between their signatures.

To reduce the possibility for cross-hybridization as a source of nonspecificity, we minimized the length of the homology region with the best unwanted match (≤ 15 nt) and maximized the T_m differential between the desired target silencing complex and the most likely nonspecific complex. Although we do not have conclusive proof that siRNA cross-hybridization leads to aberrant gene regulation, our observations imply that such nonspecific silencing may occur. Unlike aptamer effects, cross-hybridization is a potential source of nonspecificity that can be controlled by applying rational siRNA design. Therefore, we suggest that stringent design

rules, such as those described above, be used in gene silencing experiments in addition to published siRNA design guidelines (10).

Our optimization experiments also showed that the specificity of siRNA is concentration dependent. At concentrations of ≈ 100 nM, siRNA nonspecifically induces a significant number of genes, many of which are known to be involved in apoptosis and the stress response. However, reduction of the siRNA concentration to 20 nM eliminated this nonspecific response. We believe that this finding has direct implications for how siRNA knockdown experiments should be designed. According to Schwarz *et al.* (35), effective siRNA duplexes typically produce potent silencing at 1- to 10-nM concentrations.

The optimized siRNA design rules and transfection conditions were used to generate gene expression signatures for multiple siRNAs directed against different regions of the same target. These experiments were performed for a total of three targets. Our data indicate a very close qualitative and quantitative correlation between the expression signatures for multiple siRNAs against the same gene. This correlation implies that, under the optimized conditions, the effects of siRNA are limited to specific target knockdown, and suggests that, when properly designed and used, siRNA does not undergo cross-hybridization. Our data also indicate that siRNA does not appear to interact with cellular proteins. The interactions with cellular proteins may cloud the silencing effect of an agent by limiting its availability for targeting the mRNA and cause nonspecific effects in the cell (15–17). Furthermore, the expression signatures generated by siRNA-mediated gene knockdown are unique to each target, ruling out the possibility that the observed correlation is due to a nonspecific cellular response to RNA.

In summary, we have demonstrated that siRNA is a highly specific tool for targeted gene knockdown. A number of questions related to siRNA-mediated gene knockdown remain to be addressed. For example, the observed differences in potency of siRNAs targeted to different regions of the same mRNA suggest that target accessibility is an important factor governing the siRNA response. Nevertheless, our data establish siRNA-mediated gene silencing as a reliable and valuable approach for large-scale screening of gene function and drug target identification and validation.

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