

## REPORT

# Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs)

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## ABSTRACT

RNA interference is an evolutionarily conserved process in which expression of a specific gene is post-transcriptionally inhibited by a small interfering RNA (siRNA), which recognizes a complementary mRNA and induces its degradation. Currently, RNA interference is being used extensively to inhibit expression of specific genes for experimental and therapeutic purposes. For applications in mammalian cells, siRNAs are designed to be <~30 base pairs to avoid nonspecific effects that arise from inducing the cellular double-stranded RNA (dsRNA)-dependent protein kinase (PKR) response. Here we perform expression profiling in mammalian tissue-culture cells treated under standard conditions with conventional 21-bp siRNAs and find, unexpectedly, that >1000 genes involved in diverse cellular functions are nonspecifically stimulated or repressed. The effects on gene expression are dependent upon siRNA concentration and are stable throughout the course of siRNA treatment. Our results can be explained by previous studies showing that dsRNAs can affect multiple signaling and transcription pathways in addition to PKR. The potential for this widespread, nonspecific effect on mammalian gene expression must be carefully considered in the design of siRNA experiments and therapeutic applications.

**Keywords:** RNA interference; siRNA; interferon response; PKR response

## INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved process of sequence-specific, post-transcriptional gene silencing that uses double-stranded RNA (dsRNA) as the signal to trigger the degradation of homologous mRNA (for review, see Fire 1999; Sharp 2001; Hannon 2002; Plasterk 2002; Zamore 2002). The mechanism by which dsRNA induces gene silencing involves a two-step process. First, long dsRNAs are recognized by the ribonuclease III-like enzyme Dicer, which cleaves the dsRNA into smaller RNAs of 21–23 nt. These small interfering RNAs (siRNAs) are then incorporated into a multicomponent nuclease complex known as the RNA-induced silencing complex (RISC), which recognizes and targets cognate mRNAs for destruction.

RNAi has proven to be an effective tool for studying gene function in numerous organisms, including *Caenorhabditis elegans*, *Drosophila*, and plants (Hannon 2002). In mammalian cells, however, the use of RNAi for targeted gene silencing has been limited due to nonspecific effects induced by long dsRNAs, which result in interferon (IFN) activation and induction of the cellular dsRNA-dependent protein kinase (PKR) response. Therefore, for applications in mammalian cells, siRNAs are designed to be <~30 bp to circumvent the PKR response and avoid nonspecific effects on gene silencing (see, for example, Caplen et al. 2001; Elbashir et al. 2001).

Currently, siRNAs are being used extensively in mammalian cells to inhibit expression of specific genes for experimental and therapeutic purposes. A critical assumption of this approach is that the siRNA will selectively inhibit the complementary gene. Here, by using expression profiling, we show that conventional siRNAs can in a concentration-dependent manner nonspecifically stimulate or repress expression of >1000 genes with protein products that are involved in diverse cellular functions.

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## RESULTS AND DISCUSSION

### Expression profiling reveals nonspecific effects on mammalian gene expression by siRNAs

To identify targets of transcription factors, we had been performing expression profiling after siRNA treatment of mammalian tissue-culture cells. As a nonspecific control in these studies, we used a well-characterized siRNA directed against a luciferase reporter gene (Elbashir et al. 2001; Schwarz et al. 2002). This siRNA efficiently silences luciferase expression at a concentration of 1 nM (Schwarz et al. 2002). Unexpectedly, after transfection with 200 nM luciferase siRNA under standard conditions, we found that of the 33,000 genes represented on an Affymetrix U133 chip, expression of 1154 genes increased and expression of 689 genes decreased by  $\geq 2.5$ -fold compared with untreated cells. Genes with expression that was altered by luciferase siRNA treatment encoded proteins involved in diverse cellular activities, including cell signaling, cytoskeletal organization, gene expression, metabolism, and cell adhesion (Table 1).

Twelve genes with expression that was either increased or decreased by luciferase siRNA treatment were randomly selected for further characterization. To confirm the results of the microarray experiment, we performed RT-PCR analysis. Figure 1A shows that expression of all six of the genes up-regulated in the microarray analysis were increased to varying extents by treatment with the luciferase siRNA. Likewise, expression of all six of the down-regulated genes in the microarray analysis were decreased to varying extents after treatment with the luciferase siRNA. Consistent with the microarray analysis, expression of *GAPDH* was unaffected. Significantly, treatment of cells with the transfection reagent (Oligofectamine) alone did not affect expression of any of the 12 genes analyzed, indicating the effect is attributable to the siRNA. We presume this is also the case for the other genes with expression that was affected in the microarray analysis.

The luciferase siRNA used in the above experiments lacks significant sequence similarity to any human gene. It therefore seemed likely that the effects on gene expression we observed were independent of the specific siRNA sequence. To confirm this prediction, we analyzed expression of several genes after treatment with a randomized nonspecific control siRNA, which again lacks significant similarity to any human gene. The results of Figure 1B show that the luciferase siRNA and the nonspecific siRNA had comparable effects on all 12 genes analyzed.

### siRNA treatment affects gene expression in a concentration-dependent manner

We sought to determine whether the nonspecific effects on gene expression were dependent upon siRNA concentra-

tion. We therefore analyzed the expression of a subset of genes at siRNA concentrations ranging from 0 to 200 nM. The results of Figure 2A show, as observed above, that at 200 nM luciferase siRNA, expression of *FGG*, *IGFBP5*, and *PCDH10* were increased, whereas expression of *CDH5* and *Histone H3H* were reduced. At 100 nM luciferase siRNA, expression of *FGG*, *IGFBP5*, *PCDH10*, and *CDH5* were still significantly affected and expression of *Histone H3H* was affected modestly. When the concentration of luciferase siRNA was reduced to 50 nM, expression of *IGFBP5*, *CDH5*, and *Histone H3H* remained modestly affected. Further reduction to 25 nM still affected *CDH5* and *Histone H3H* expression, whereas *IGFBP5* expression was near normal. Thus, the nonspecific effects on gene expression are dependent upon siRNA concentration in a gene-specific manner.

### Kinetics of altered gene expression

We next analyzed the time-course of altered expression of several genes after siRNA treatment. The results of Figure 2B show that for *FGG*, *IGFBP5*, *PDK1*, and *PCDH10*, a significant increase in expression was first detected 12 to 24 h after siRNA treatment and remained constant throughout the remainder of the 72-h time-course. For *ZFP36L2* and *PCDHGC3*, a decrease in expression was first observed 24 h after siRNA treatment and was maintained, whereas a decrease in expression of *TNFSF10* and *Histone H3H* first occurred 48 h after siRNA treatment. Thus, the nonspecific effects on gene expression induced by siRNAs are not transient and, once initiated, are sustained throughout the course of siRNA treatment.

### siRNAs, long dsRNAs, and IFN affect overlapping pathways

The nonspecific effects we observed by using siRNAs raise the possibility that dsRNAs can affect gene expression through pathways other than PKR. Of particular significance, polyI.polyC, a long dsRNA homopolymer, can still activate expression of specific genes in knockout mice lacking both copies of the PKR gene (Yang et al. 1995). This result is consistent with reports that dsRNA can activate several protein kinases such as p38, JNK2, and IKK (Kumar et al. 1994; Chu et al. 1999; Williams 1999) in addition to PKR. Induction of these signaling pathways can alter gene expression by regulating the activity of transcription factors such as NF- $\kappa$ B, IRF-3, and ATF-1 (Williams 1999). Because siRNAs do not trigger the PKR response, the most likely explanation for the nonspecific effects on gene expression that we have observed is that siRNAs can affect gene expression through one of these other pathways. As an initial investigation of the pathway(s) affected by siRNAs, we compared the results of our expression profiling analysis with those obtained after treatment with type 1 IFN (Der et al. 1998) or polyI.polyC in cells lacking the type I IFN locus

**TABLE 1.** List of selected genes with expression that was either increased or decreased by luciferase siRNA treatment

Name and biological function	Acc. no.	Gene product	Fold increase
<b>Stimulated genes</b>			
Cytokine signaling			
FGG	NM 000509	fibrinogen, gamma polypeptide	5.7
HFL1	X 56210	H factor (complement)-like 1	5.4
RAP2B	NM 002886	member of RAS oncogene family	4.9
TRG@	M 16768	T cell receptor gamma locus	4.2
CCL23	U 58913	chemokine (C-C motif) ligand 23	4.0
ISG20	NM 002201	interferon stimulated gene 20 kD	3.5
CXCL14	NM 004887	chemokine (C-X-C motif) ligand 14	3.3
CCL3	NM 002983	chemokine (C-C motif) ligand 3	2.9
IRF4	U 52683	interferon regulatory factor 4	2.9
FGL1	NM 004467	fibrinogen-like 1	2.8
IL5RA	M 96651	interleukin 5 receptor, $\alpha$	2.6
Cytoskeletal organization			
LAMA4	U 77706	laminin, $\alpha$ 4	4.4
BMP2	NM 001200	bone morphogenetic protein 2	3.0
GOLGA6	NM 018652	golgin subfamily a, member 6	3.0
COL15A1	NM 001855	collagen, type XV, $\alpha$ 1	2.7
COL16A1	NM 001856	collagen, type XVI, $\alpha$ 1	2.7
Apoptosis			
REPRIMO	NM 019845	p53 induced G2 arrest mediator	3.8
Extracellular matrix/cell adhesion			
IGFBP5	AW 007532	insulin-like growth factor binding protein 5	4.8
EDN2	NM 001956	endothelin 2	4.6
CDH13	NM 001257	cadherin 13	4.2
MRC1	NM 002438	mannose receptor, C type 1	3.9
SEPP1	NM 005410	selenoprotein P, plasma, 1	3.8
FGF13	NM 004114	fibroblast growth factor 13	3.6
IGF1	M 37484	insulin-like growth factor 1	3.0
Metabolism			
PDK1	NM 002610	pyruvate dehydrogenase kinase, isoenzyme 1	4.1
HPD	NM 002150	4-hydroxyphenylpyruvate dioxygenase	3.6
ARHU	NM 021205	ras homolog gene family, member U	3.4
NDUFB7	M 33374	NADH dehydrogenase 1 beta subcomplex, 7	3.2
DNMT3L	NM 013369	DNA (cytosine-5)-methyltransferase 3-like	3.1
Other genes			
CGI-60	NM 016008	CGI-60 protein	4.1
ZNF135	NM 003436	zinc finger protein 135	3.0
FKBP8	L 37033	FK-506 binding protein 8	2.8
MAGEA1	NM 004988	melanoma antigen, family A, 1	2.8
<b>Repressed genes</b>			
RNA synthesis			
18S rRNA	M 10098	18S ribosomal RNA	3.1
28S rRNA	M 11167	28S ribosomal RNA	2.8
Metabolism			
ACOX2	NM 003500	acyl-Coenzyme A oxidase 2	4.7
ALPP	NM 001632	alkaline phosphatase, placental	4.2
RODH	NM 003725	3-hydroxysteroid epimerase	4.2
MAOA	NM 000240	monoamino oxidase A	3.8
GAD2	M 81882	glutamate decarboxylase 2	3.4
DUSP6	AB 013382	dual specificity phosphatase 6	3.1

(continued)

(Table 2; Geiss et al. 2001). Interestingly, most of the genes were affected by two but not all three of the inducing agents. As expected, a number of genes were affected by treatment with IFN and long dsRNAs but not by siRNAs. This is presumably because IFN and long dsRNAs induce expression of these genes through the PKR pathway, which

is not activated by siRNAs. Consistent with this conclusion, RT-PCR analysis revealed that several genes with expression that is induced by dsRNA, IFN $\beta$ , and 2'-5' oligoadenylate synthetase (OAS), or by IFN, myxovirus-resistance-1 (MXA), and IFN-stimulated gene 54 (ISG-54), were unaffected by siRNA treatment (data not shown). Thus, the

TABLE 1. Continued

Name and biological function	Acc. no.	Gene product	Fold increase
Chromosome organization			
HIST1H2AG	NM 021064	histone 1, H2ag	3.5
HIST1H2AE	NM 021052	histone 1, H2ae	3.4
H3FH	NM 003534	histone H3 family, member H	3.1
H3FL	NM 003537	histone H3 family, member L	2.9
Extracellular matrix/cell adhesion			
CDH5	NM 001795	cadherin 5, type 2	4.1
CD5	NM 014207	CD5 antigen (p56-62)	3.3
PCDHGC3	L 11372	protocadherin gamma subfamily C, 3	3.1
CD1D	NM 001766	CD1D antigen, d polypeptide	2.5
CD38	NM 001775	CD38 antigen (p45)	2.5
PCDH11X	NM 014522	protocadherin 11 X-linked	2.5
Cell signaling			
TNFSF10	NM 003810	tumor necrosis factor superfamily, member 10	4.8
IL11	NM 000641	interleukin 11	4.5
SLC2A6	NM 017585	solute carrier family 2, member 6	4.5
ZFP36L2	NM 006887	zinc finger protein 36, C3H type-like 2	4.4
INHBB	NM 002193	inhibin $\beta$ B	3.8
HBP17	NM 005130	heparin-binding growth factor binding protein	3.5
RARRES1	NM 002888	retinoic acid receptor responder 1	3.0
FGF18	NM 003862	fibroblast growth factor 18	2.8
FGFR4	AF 202063	fibroblast growth factor receptor 4	2.8
SLC21A12	NM 016354	solute carrier family 21, member 12	2.8
SSI1	U 88326	suppressor of cytokine signaling 1	2.8
IL7	NM 000880	interleukin 7	2.5
Transcription			
EHF	NM 012153	ets homologous factor	3.6
ELF4	NM 001421	E74-like factor 4	3.3
GLI2	NM 030379	GLI-Kruppel family member	3.1
SHOX2	AF 022654	short stature homeobox 2	2.9
DLX2	NM 004405	distal-less homeobox 2	2.8
PITX2	NM 000325	paired-like homeodomain transcription factor 2	2.8
HOXC5	NM 018953	homeobox C5	2.5
Other genes			
CDC14A	NM 003672	cell division cycle 14 homolog A	6.4
PSCA	NM 005672	prostate stem cell antigen	5.5
SERPINB4	AB 046400	serine proteinase inhibitor, clade B, member 4	4.3
MDA5	NM 022168	melanoma differentiation associated protein 5	4.1
HSPA1A	NM 005345	heat-shock 70-kD protein 1A	3.8
DPT	NM 001937	dermatopontin	3.1

pathways affected by IFN, long dsRNAs, and siRNAs appear to be overlapping but nonidentical.

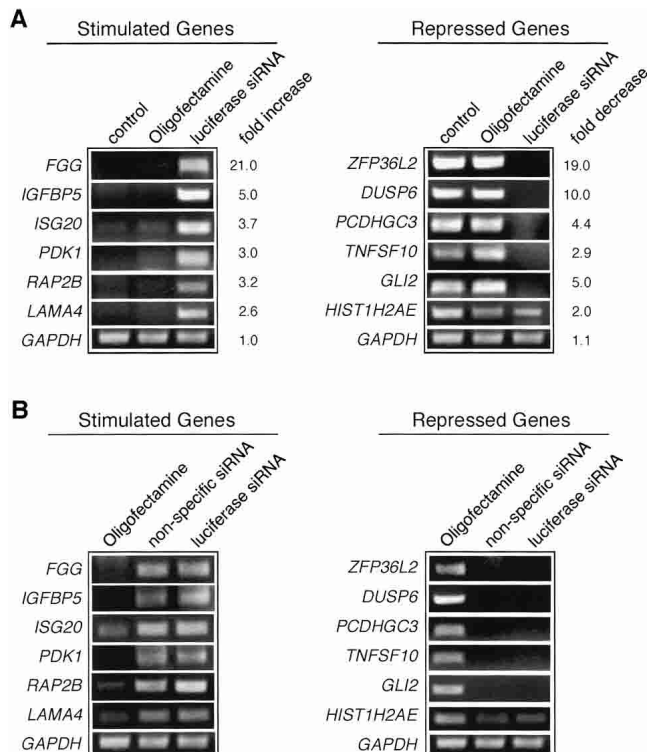
In this report, we have found that in addition to the well-known ability of siRNAs to silence specific genes, there is also a widespread nonspecific effect on mammalian gene expression. Interestingly, expression of some genes was increased, whereas others were reduced. These nonspecific effects must be considered in the design of controls for siRNA-mediated loss-of-function experiments.

Previous studies examining siRNA specificity have generally monitored expression of only a few genes and probably would not have detected the nonspecific effects described here. However, several recent reports have used expression profiling to reveal nonspecific effects on human gene expression by siRNAs (Jackson et al. 2003; Semizarov et al. 2003) or short hairpin RNAs (shRNAs; Bridge et al.

2003). Below we discuss the relationship and implications of our results and those in these related studies.

What is the mechanism(s) by which siRNAs induce non-specific effects on gene expression? Jackson et al. (2003) attributed the nonspecific effects they observed to off-target gene regulation, in which the expression of nontarget genes are suppressed due to cross-hybridization of transcripts containing regions of partial homology with the siRNA sequence. The effects we observed, however, cannot be explained by off-target regulation because the siRNAs used in our experiments lacked significant sequence similarity to any human gene. Moreover, for all of the genes tested, expression was affected by two siRNAs (luciferase and non-specific) with sequences that are completely unrelated.

Bridge et al. (2003) reported that shRNAs can affect the expression of many genes, including several IFN targets,



**FIGURE 1.** Analysis of siRNA-induced alterations in gene expression by RT-PCR. (A) Representative set of up-regulated genes (left) or down-regulated genes (right) after treatment of HeLa cells with transfection reagent (Oligofectamine) alone or with luciferase siRNA. Untreated HeLa cells were used as a control. Fold differences in expression as measured by RT-PCR is indicated on the right. (B) siRNA-induced alteration in gene expression is independent of siRNA sequence. Comparison of luciferase siRNA and a random nonspecific siRNA on the expression of a selected set of stimulated genes (left) or repressed genes (right). Oligofectamine-treated cells were used as a control.

and therefore suggested that shRNAs induce an IFN response. Because shRNAs are processed to siRNAs in the cell, these observations raised the possibility that siRNAs might also induce an IFN response. However, a comparison of genes affected by siRNAs and IFN (Table 2) revealed that siRNAs affect pathways that are overlapping but not identical to those regulated by IFN. Thus, siRNAs do not trigger a true IFN response. More importantly, Bridge et al. (2003) reported that the nonspecific effects occurred with an shRNA expressed from an RNA polymerase III promoter but not with a synthetic siRNA. In contrast, the nonspecific effects we observed occurred with synthetic siRNAs.

A characteristic feature of the nonspecific effects on gene expression we observed was dependence on siRNA con-

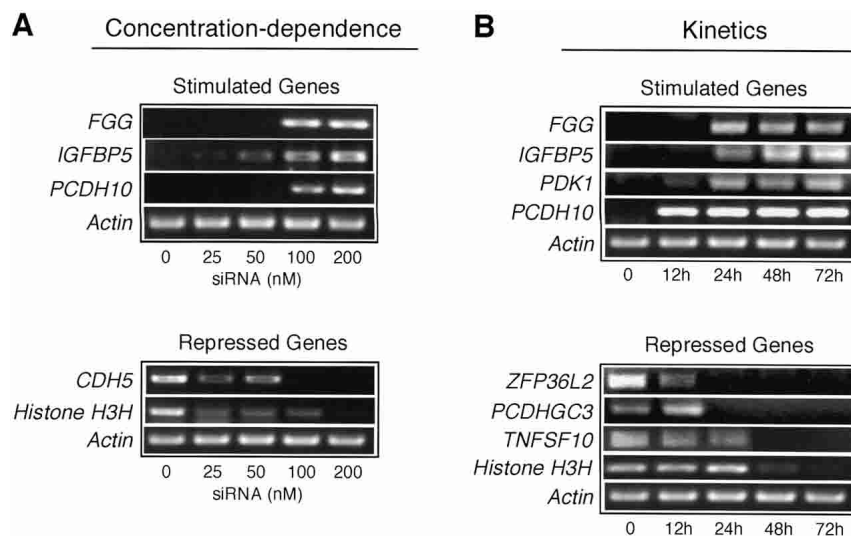
centration. Semizarov et al. (2003) reported nonspecific effects that occurred at an siRNA concentration of 100 nM but not at 20 nM. Thus, we suspect that the nonspecific effects reported by Semizarov et al. (2003) are related to our results. However, for several reasons it is not yet clear that the basis for altered gene expression in our study is identical to that of Semizarov et al. (2003). Specifically, unlike our study, Semizarov et al. used only microarray analysis to study expression, did not test whether different unrelated siRNAs comparably altered the expression of specific genes, and did not analyze the kinetics of altered expression. Finally, Semizarov et al. (2003) did not report genes with expression that was nonspecifically reduced by siRNAs in a concentration-dependent fashion.

Gene silencing experiments in mammalian cells have used siRNAs at varying concentrations, typically ranging from 20 nM (Semizarov et al. 2003) to 200 nM (Wu et al. 2003). Many published studies report using siRNAs at 100 nM, a concentration that is also suggested by manufacturers of siRNAs and siRNA-related products. Significantly, 100 nM siRNA is a concentration at which we found nonspecific effects occur. Unfortunately, however, lowering the concentration of a particular siRNA <100 nM can reduce silencing efficiency. These considerations underscore the importance of determining the rules for siRNA design so that optimal silencing efficiency can be achieved at minimal siRNA concentrations to avoid nonspecific effects.

## MATERIALS AND METHODS

### siRNA preparation

The siRNAs were chemically synthesized by the UMass CFAR (Center for AIDS Research) Molecular Biology Core using 2'-



**FIGURE 2.** Concentration-dependence and kinetics of siRNA-induced alteration in gene expression. (A) Concentration-dependence. Comparison of varying concentrations of luciferase siRNA on the expression of a selected set of genes. (B) Kinetics. Time-course analysis by RT-PCR of selected genes with expression that increased or decreased after luciferase siRNA treatment.

**TABLE 2.** Comparison of selected genes affected by siRNA, poly(I):poly(C), and interferon type 1

Gene description	siRNA	long dsRNA	IFN- $\alpha/\beta$
Guanylate binding protein 1, interferon inducible (GBP1)	●	●	●
Cell division cycle 14 homolog A (CDC14A)	●	●	
Chemokine (C-C motif) ligand 2 (CCL2)	●	●	
Fibroblast growth factor 2 (FGF2)	●	●	
Proteoglycan 1, secretory granule (PRG1)	●	●	
Caspase 8 (CASP8)	●		●
CCAAT/enhancer binding protein delta (C/EBPD)	●		●
Chemokine (C-X-C motif) ligand 11 (CXCL11) <sup>a</sup>	●		●
Collagen, type XVI, alpha 1 (COL16A1)	●		●
Glucose monophosphate reductase (GMPR)	●		●
Interferon stimulated gene 20kDa (ISG20) <sup>a</sup>	●		●
Zinc finger protein 36, C3H type-like 2 (ZFP36L2)	●		●
Adenosine deaminase, RNA-specific (ADAR)		●	●
Complement component 1, s subcomponent (C1S)		●	●
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)		●	●
Interferon regulatory factor 1 (IRF1)		●	●
Interferon regulatory factor 2 (IRF2)		●	●
Mothers against decapentaplegic homolog 4 (MADH4)		●	●
N-myc (and STAT) interactor (NMI)		●	●
Plasminogen activator, urokinase receptor (PLAUR)		●	●
Transporter 1, ATP-binding cassette, subfamily B (TAP1)		●	●

Genes found to be up- or down-regulated twofold or more by microarray analysis after treatment with a nonspecific siRNA (this study), long dsRNA [poly(I):poly(C)] (Geiss et al. 2001), or IFN $\alpha$  or IFN $\beta$  (Der et al. 1998).

<sup>a</sup>Genes affected by siRNA treatment that have previously been reported to be regulated by IFN (*ISG20*, Gongora et al. 2000; *CXCL11*, Cole et al. 1998) although they were not reported in the microarray analysis carried out by Der et al. (1998).

TOM-RNA phosphoramidites. The luciferase siRNA oligoribonucleotides (5'-CGUACGCGAAUACUUCGATT-3' and 5'-UCGAAGUAUCCGCGUACGTT-3') correspond to the coding region 153–173 relative to the first nucleotide of the start codon of the *Photinus pyralis* luciferase gene (Elbashir et al. 2001). The nonspecific siRNA oligoribonucleotides (5'-AAUUUUUUUCCCCCAAAGGGGG-3' and 5'-AACCCCCUUUGGGGAAAAAAA-3') were randomly synthesized and did not correspond to any known gene in the human genome database. To anneal the siRNAs, 20  $\mu$ M single-stranded RNAs were incubated in annealing buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM Hepes-KOH at pH 7.4) for 1 min at 90°C followed by incubation for 2 h at 37°C. DsRNAs were stored at –20°C until transfection.

### Cell culture and transfection

HeLa S3 cells were propagated at 37°C in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cells were regularly passed to maintain exponential growth. Transfections were carried out by using Oligofectamine (Life Technologies) and 200 nM duplex siRNA per well, as per the manufacturer's instructions. Cells were transfected one to three times at ~24-h intervals.

### Microarray analysis

Total RNA was prepared from test (siRNA treated) and control (untreated) cells by using the RNeasy kit (Qiagen). Targets for

hybridization to the microarrays were prepared as described (Eisen and Brown 1999). Hybridization and scanning of human U133 GeneChip Arrays (Affymetrix) were performed as recommended by the manufacturer. The complete list of genes induced or repressed by luciferase siRNA treatment is available upon request.

### RNA isolation and RT-PCR analysis

RT-PCR analysis was performed according to standard protocols (Ausubel et al. 2001), except that total RNA was prepared by using the RNeasy extraction kit (Qiagen). RT-PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The density of the ethidium bromide-stained bands was quantitated by using NIH Image analysis software. Fold differences were calculated by comparing the intensity of the signal in the luciferase siRNA-treated sample relative to that obtained by using transfection reagent alone. Primer sequences used for RT-PCR analysis are available upon request.

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