Targeted Comparative RNA Interference Analysis Reveals Differential Requirement of Genes Essential for Cell Proliferation[□]

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Differences in the genetic and epigenetic make up of cell lines have been very useful for dissecting the roles of specific genes in the biology of a cell. Targeted comparative RNAi (TARCOR) analysis uses high throughput RNA interference (RNAi) against a targeted gene set and rigorous quantitation of the phenotype to identify genes with a differential requirement for proliferation between cell lines of different genetic backgrounds. To demonstrate the utility of such an analysis, we examined 257 growth-regulated genes in parallel in a breast epithelial cell line, MCF10A, and a prostate cancer cell line, PC3. Depletion of an unexpectedly high number of genes (25%) differentially affected proliferation of the two cell lines. Knockdown of many genes that spare PC3 (p53[−]) but inhibit MCF10A (p53⁺) proliferation induces p53 in MCF10A cells. EBNA1BP2, involved in ribosome biogenesis, is an example of such a gene, with its depletion arresting MCF10A at G1/S in a p53-dependent manner. TARCOR is thus useful for identifying cell type-specific genes and pathways involved in proliferation and also for exploring the heterogeneity of cell lines. In particular, our data emphasize the importance of considering the genetic status, when performing siRNA screens in mammalian cells.

INTRODUCTION

RNA interference (RNAi) was initially characterized in *Caenorhabditis elegans* as a cellular response to double-stranded RNA (Fire *et al.*, 1998). Specific gene silencing can be triggered by soaking worms or *Drosophila* cells in a solution containing RNA duplexes of target mRNAs (Tabara *et al.*, 1998; Clemens *et al.*, 2000). RNAi was used to perform large-scale screens in these organisms (Fraser *et al.*, 2000; Gonczy *et al.*, 2000; Maeda *et al.*, 2001; Kamath *et al.*, 2003; Bettencourt-Dias *et al.*, 2004; Boutros *et al.*, 2004). These screens have identified genes involved in cell division, apoptosis, and fat metabolism. In mammalian cells, however, introduction of long double-stranded RNA (>30 nucleotides) triggers the antiviral interferon response and subsequent cell death (Stark *et al.*, 1998). This problem was solved by the discovery that duplexes of 21-nucleotide RNA termed siRNAs (small interfering RNAs) can trigger RNAi pathway in mammalian cells without activating the antiviral response (Elbashir *et al.*, 2001). siRNA can be generated by chemical synthesis or enzymatic digestion of target mRNA duplexes in vitro (Yang *et al.*, 2002) or by expressing short hairpin

RNAs in mammalian cells (Brummelkamp *et al.*, 2002; Sui *et al.*, 2002).

The human genome sequencing project revealed that human cells contain ~20,000–25,000 protein-coding genes (International Human Genome Sequencing Consortium, 2004) and has encouraged genome-wide functional genomics in mammals. Several groups have constructed RNAi libraries to perform loss-of-function genetics in cultured mammalian cells (Berns *et al.*, 2004; Kittler *et al.*, 2004; Paddison *et al.*, 2004; Moffat *et al.*, 2006). Libraries of short hairpin RNAs or endoribonuclease-prepared short interfering RNAs (siRNAs) were used in selection and screening assays, respectively, to identify genes important for cancer cell phenotypes (Kolfschoten *et al.*, 2005; MacKeigan *et al.*, 2005; Westbrook *et al.*, 2005). Although the response to silencing of a few genes is different between two *Drosophila* cell lines, suggesting that cell lines from different backgrounds respond differently (Kiger *et al.*, 2003), no attention has been paid to exploiting the vast heterogeneity of mammalian cell lines in siRNA screens. We reasoned that if phenotypes of multiple cell lines after RNAi-mediated gene silencing can be compared in a quantitative and high throughput manner, we can identify genes with differential requirements between cell lines. Targeted comparative RNAi (TARCOR) reported here can be used to perform such comparative functional genomics in multiple human cell lines. The comparison required the development of criteria for quantitative reproducibility of results. In addition, instead of a global analysis on a heterogeneous collection of genes, TARCOR targets a narrower set of genes relevant to the phenotype being studied. For example, the genes can be selected from previous studies such as microarray-based gene expression analysis. We

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Abbreviations used: RNAi, RNA interference; siRNA, small interfering RNA; TARCOR, targeted comparative RNAi.

Table 1. Inhibition index and differential index for genes with differential effects

Gene name	Inhibition index (%)				Differential index ($AV_M - AV_P$)/ ($SD_M + SD_P$)
	MCF10A		PC3		
	AV_M	SD_M	AV_P	SD_P	
DDX21	110.8	10.9	5.6	5.7	6.4
MCM3	100.7	1.7	47.7	6.9	6.1
RAD51	105.7	2.5	64.1	6.0	4.9
EBNA1BP2	140.9	15.1	32.8	7.3	4.8
NET1	95.1	7.9	46.1	4.1	4.1
S1P1	117.1	24.1	-40.1	15.5	4.0
NOL5A	92.3	6.2	38.9	9.6	3.4
UMPS	74.4	14.7	-72.1	30.8	3.2
TRIM3	84.8	4.2	49.1	7.1	3.2
RFC3	82.4	15.8	10.5	12.3	2.6
C20orf1	7.0	21.1	100.0	3.9	-3.7
PLK	14.3	9.8	89.6	6.2	-4.7

AV_M , average in MCF10A; AV_P , average in PC3; SD_M , SD in MCF10A; SD_P , SD in PC3.

demonstrate that TARCOR can be used to identify genes that are differentially required for proliferation of two human cell lines. Furthermore, the differential sensitivity to several of the genes could be due to the activation of p53 in one cell line and not the other. For example, inhibition of ribosome biogenesis appears to cause a G1/S arrest by a p53-dependent pathway in the p53+ MCF10A cell. Besides emphasizing the importance of p53 in a cell's response to depletion of many genes, the results highlight how such targeted comparative screens can find biologically relevant differences between cells and conversely, how the heterogeneity of mammalian cell lines can be exploited to add value to siRNA screens.

MATERIALS AND METHODS

Cell Culture, siRNA Transfection, and FACS Analysis

MCF10A is an immortalized breast epithelial cell line derived from fibrocystic disease that was cultured in DMEM/F12 containing 5% donor calf serum, 0.02 μ g/ml EGF (Sigma, St. Louis, MO), 1 μ g/ml insulin (Sigma), 1.4 μ M hydrocortisone (Sigma), and 0.1 μ g/ml cholera toxin. PC3 prostate cancer cells were cultured in RPMI1640 containing 10% fetal calf serum. A screen of 257 siRNAs was performed using three 96-well plates with three technical replicates. For the siRNA screen, 5000 cells were transfected with 4 pmol of siRNA duplex using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 96-well plate. For real-time PCR and Western blotting, 2×10^5 cells were transfected in 6-well plates. To eliminate concerns from off-target activity of siRNA duplexes, the genes that were essential in MCF10A and focused on in Table 1 were also knocked down by SMARTpools (5 siRNA per targeted gene). Standard methods were used for flow-cytometry analysis of DNA content, and ModFit software (Verity Software House, Topsham, ME) was used for estimation of percentage of cells in various phases of the cell cycle.

Real-Time PCR, Western Blotting, and In Vitro Kinase Assay

Total RNA was isolated by RNeasy mini kit (Qiagen, Chatsworth, CA) and used for cDNA synthesis by Superscript III (Invitrogen). The cDNAs were used as templates for real-time PCR using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The primer sequences are available on request. The antibodies used for Western blotting were as follows: anti-p21 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cyclin E (HE12; Santa Cruz), anti-p53 (Cell Signaling, Beverly, MA), and anti-pRB (a gift from Dr. E. Harlow). For quantitation of Western blotting, signal intensity was measured with Scion Image (Scion, Frederick, MD), and the p53 signal was normalized to that of β -actin (loading control). In vitro kinase assays were performed as

reported previously (Machida *et al.*, 2005). Anti-cyclin E (HE111; Santa Cruz) was used for immunoprecipitation.

Bromodeoxyuridine ELISA

Cells were incubated with 10 μ M bromodeoxyuridine (BrdU) for 15 min to 1 h. Cells were washed once with PBS and fixed with FixDenat (Roche, Indianapolis, IN) for 30 min. After washing once with PBS, the plates were blocked with 3% BSA in PBS for 1 h. The plates were then incubated with HRP-coupled anti-BrdU antibody (Roche) diluted in 3% BSA in PBS for 1 h. After washing three times with PBS containing 0.1% TX-100, the plates were incubated with TMB substrate (Pierce, Rockford, IL) for 5–10 min. The reactions were stopped by adding 1 M H_2SO_4 , and the absorbance at 450 nm was measured.

Data Preprocessing and Analysis

Each assay plate contained four wells of negative control (luciferase; GL2) and two wells of positive control (ORC2) siRNAs for normalization. To normalize values of BrdU incorporation, we calculated the inhibition index (%) using the following equation: Inhibition index of gene X (%) = $(GL2_{av} - X)/(GL2_{av} - ORC2_{av}) \times 100$, where X, $GL2_{av}$, and $ORC2_{av}$ represent BrdU incorporation (absorbance at 450 nm) of gene X and average of GL2 and ORC2, respectively. Genes with a SD of inhibition indices (from 3 technical replicates) greater than a cutoff value were eliminated to select technically reproducible data. To select biologically reproducible data, inhibition indices from two screens were plotted and the distance of each gene from the $y = x$ line, representing ideal behavior, was calculated and plotted. Genes with distances in the top 5% or bottom 5% of the distribution were eliminated to select biologically reproducible data. The cutoff value that eliminates 10% of the worst performing genes in the biological replicates was used as the cutoff to eliminate the worst performing genes in the technical replicates. Hierarchical clustering was performed on the response of cells to the silencing of individual genes. To select genes that have reliably differential effects on MCF10A and PC3, we calculated the differential inhibition index using the following equation: Differential inhibition index of a gene = $(AV_M - AV_P)/(SD_M + SD_P)$, where AV_M and AV_P represent the average of inhibition indices of a gene in MCF10A and PC3, respectively, and SD_M and SD_P represent the SD of inhibition indices for each cell line.

RESULTS

TARCOR Analysis of Genes Required for Proliferation of Human Cells

Two hundred twenty-eight genes potentially relevant to cell survival, growth, and the cell cycle were selected from previous studies that identified serum-stimulated genes (Iyer *et al.*, 1999) or E2F-regulated genes (Ren *et al.*, 2002; No. 1-228 in Supplementary Table S1). Besides many well-characterized cell cycle-related genes, the gene-set contains many uncharacterized genes so that we have a chance to identify new genes involved in proliferation control. In addition, we included 29 uncharacterized ATPases to test if we can expand TARCOR analysis to gene sets selected by other criteria (No. 229-257 in Supplementary Table S1).

Figure 1A represents the scheme of TARCOR analysis of these genes in human cell lines. siRNAs were transfected in 96-well plates and incorporation of BrdU measured after 72 h to quantitate the proliferation of the cells. Because we compare BrdU incorporation per well, we could identify genes essential for viability as well as cell proliferation. In this study, we compared a breast epithelial cell line, MCF10A, and a prostate cancer cell line, PC3.

Each experiment was performed with three technical replicates for each gene, and a representative result is shown in Figure 1B. To compare results between cell lines, we developed analytical methods that ensured the quantitative reproducibility of results and avoided confounding factors such as differences in RNAi efficacy in the two cell lines. First, BrdU incorporation after knockdown of each gene was normalized by the inhibition seen after knockdown of a positive control gene. We chose ORC2, a gene involved in replication initiation, as a positive control because its basal and inhibited mRNA levels are similar in the two cell lines (Figure 1C), and RNAi reduced BrdU incorporation to sim-

Figure 1. TARCOR analysis in human cells. (A) Experimental scheme of TARCOR analysis of genes essential for cell proliferation. (B) BrdU ELISA in MCF10A cells. Values are mean $\pm$ SD (n = 3). Black and white columns represent negative (GL2) and positive (ORC2) controls, respectively. (C) mRNA levels of a standard gene, ORC2, in MCF10A and PC3. Cells were transfected with control (GL2) or ORC2 siRNAs for 48 h, and the relative amount of ORC2 mRNA was measured by real-time PCR. Amount of ORC2 mRNA was normalized to that of β -actin. Values are mean $\pm$ SD (n = 3). (D and E) Elimination of biologically nonreproducible genes. Inhibition indices from two screens (Experiments 1 and 2) in MCF10A (D) and PC3 (E) are plotted. Dashed lines show the cutoff for elimination of biologically nonreproducible genes. Eliminated genes are shown by white circles.

ilar levels ($19.2 \pm 10.1\%$ for MCF10A and $11.9 \pm 5.5\%$ for PC3; n = 36). siRNAs targeting luciferase (GL2) were used as negative controls. The decrease in BrdU incorporation after each siRNA transfection was converted to an inhibition index where GL2 siRNA gave 0% inhibition and ORC2 siRNA gave 100% inhibition. Second, we ensured the technical reproducibility of data. Genes with a high SD between the three technical replicates in a single experiment were eliminated from further analysis (see *Materials and Methods*) to reduce noise from technical variability (Figure 1A). Two hundred sixteen and 243 genes gave technically reproducible inhibition indices in MCF10A and PC3, respectively. Third, we ensured the biological reproducibility of the data by repeating the entire experiment in each cell line and limiting our study to genes that gave reproducible results in

the two biological replicates (Figure 1A). Inhibition indices from two independent screens in MCF10A (or PC3) were plotted against each other, and the 10% of genes that showed maximal discordance between the screens was eliminated (Figure 1, D and E). One hundred ninety-one and 223 genes gave reproducible inhibition indices in the biological replicates of MCF10A and PC3, respectively.

Because the primary screen was performed on very small populations of cells in 96-well plates, [3 H]thymidine incorporation in 24-well plates was used to confirm a subset of the results (Supplementary Figure S1A). RT-PCR confirmed the reduction of target mRNAs after RNAi of a subset of the genes (Supplementary Figure S1B). For genes that were followed up, we eliminated the possibility of off target activity of siRNAs by confirming the inhibition of cell growth by a

Figure 2. Comparison of genes essential for proliferation between MCF10A and PC3. (A and B) Scatter plot of inhibition indices of selected genes in MCF10A cells (A) and PC3 (B). (C) Scatter plot of inhibition indices of selected genes in MCF10A and PC3. Average of two biological replicates in each cell line were plotted. Genes (n = 172) that showed reproducible inhibition indices in both MCF10A and PC3 are plotted

Figure 3. mRNA levels after RNAi-mediated gene knockdown. (A–L) MCF10A and PC3 cells were transfected with control (GL2) or indicated siRNAs for 48 h. Relative amount of target mRNAs was quantitated by real-time PCR and shown after normalization to β -actin mRNA levels. *Cell line where RNAi did not inhibit BrdU incorporation.

second RNA duplex against a different sequence of the gene or by transfecting SMARTpools of siRNAs (Dharmacon; Supplementary Figure S1C and unpublished data).

Comparison of Gene Requirement in MCF10A and PC3

We could now compare the requirement of individual genes in the two cell lines. By intersecting gene sets that passed the reproducibility screen (191 and 223 genes in MCF10A and PC3, respectively), we obtained 172 genes with reproducible inhibition indices in both MCF10A and PC3. A scatter plot of the inhibition indices of the 172 targeted genes from two screens in the same cell line shows that the inhibition indices

are close to a $y = x$ diagonal, confirming that data are highly reproducible in a given cell line (correlation coefficients of 0.930 and 0.958 in MCF10A and PC3, respectively; Figure 2, A and B). The inhibition indices are more scattered when MCF10A and PC3 data were compared with each other ($r = 0.630$; Figure 2C). Seventy-three percent of the genes were within the cutoff lines of biological reproducibility for MCF10A (Figure 2C, genes between two dashed lines), suggesting that a large group of genes behave similarly in this assay in the cells from two different lineages. In contrast, 27% of the genes (46 genes) were outside the cutoff lines, suggesting that RNAi against a subset of genes affects the two cell lines differently. A hierarchical cluster analysis of the inhibition indices for 172 genes identified clusters of genes whose knockdown had differential effects in the two cell lines (Figure 2D and data in Supplementary Table S2).

Among genes with high differential indices (see *Materials and Methods*), those with high inhibition indices ($>70\%$) in one cell line were selected for further analysis (Table 1). First, we wanted to rule out the simple explanation that the

Figure 2 (cont). in A–C. (D) A heat map of a hierarchical cluster analysis of the inhibition indices of 172 genes. Yellow and blue represent inhibition and stimulation of BrdU incorporation after RNAi, respectively. Clusters of genes whose knockdown have differential effects between MCF10A and PC3 are indicated by green and red lines.

knockdown left more residual mRNA in the nonaffected cell line compared with the affected cell line (Figure 3). The residual mRNA of DDX21, EBNA1BP2, NOL5A, S1P1, and RFC3 was less in the nonaffected cell line, PC3, compared with the affected cell line, MCF10A (Figure 3, A–E), suggesting that the resistance of the PC3 cells was not due to failure of knockdown. In contrast, MCM3, RAD51, NET1, UMPS, TRIM3, C20orf1, and PLK RNAi left higher residual levels of mRNA in the nonaffected cell line (Figure 3, F–L). We do not know why the latter class of siRNAs selectively failed to adequately reduce the target mRNA in one cell line, but possible reasons include the expression of splicing variants that lack the siRNA target sequence or the presence of proteins that bind to and protect the target sequence. On the basis of these results, we conclude that for 5 of the 10 genes that spared PC3, DDX21, EBNA1BP2, and NOL5A (involved in ribosome biogenesis), S1P1 (sphingosine-1-phosphate receptor 1), and RFC3 (a component of the clamp-loader in DNA replication and repair) the differential toxicity cannot be explained by failure of knockdown in PC3. In contrast, for two of the two genes that spared MCF10A (C20orf1 and PLK) the result could be explained by the higher residual levels of the target in MCF10A after knockdown.

Knockdown of Many Genes That Differentially Inhibit MCF10A Induces p53

Most of the 10 siRNAs that were selectively toxic to MCF10A-targeted genes involved in RNA or DNA metabolism. When searching for a common thread accounting for this, we noticed that MCF10 has wild-type p53, whereas PC3 cells are p53 null (Carroll *et al.*, 1993). Because p53 is induced by a variety of cellular stresses, we wondered whether the positive p53 status of MCF10A might contribute to the selective toxicity seen upon depletion of the genes listed above. Of the five siRNAs that spared PC3 cells despite knockdown to levels below that in MCF10A, four (DDX21, EBNA1BP2, NOL5A, and S1P1) induced p53 protein more than fivefold in MCF10A (Figure 4). In addition, three more of the siRNAs that spared PC3 (MCM3, NET1, and UMPS) induced p53 protein more than fivefold. Therefore p53 is activated by 7 of the 10 genes that were selectively toxic to MCF10A.

To test whether the induced p53 was functional, we focused on the depletion of EBNA1BP2, a gene involved in ribosome biogenesis. RNAi against EBNA1BP2 in MCF10A-induced p53 and a known target of p53, p21, while arresting cells in G1 phase (Figure 5, A and B). Consistent with the induction of p21, an inhibitor of cyclin-dependent kinases, pRB, is hypophosphorylated, and cyclin E-associated kinase activity was inhibited (Figure 5, B and C), suggesting that knockdown of EBNA1BP2 causes CDK inhibition and decreases S phase entry in MCF10A. Identical results were obtained with another differentially toxic siRNA targeting DDX21 (unpublished data). To test whether the G1 arrest after EBNA1BP2 depletion is dependent on p53 induction, we pretreated MCF10A cells with p53 siRNA followed by EBNA1BP2 siRNA. p21 induction and G1 arrest after EBNA1BP2 depletion is suppressed in cells pretreated with p53 siRNA (Figure 5, D and E), suggesting that MCF10A cells undergo p53-dependent p21 induction and G1 arrest upon EBNA1BP2 depletion. Thus, p53 status contributes to the cellular response to EBNA1BP2 depletion, providing an example where the genetic background of a cell line determines the cellular response in an siRNA screen.

Figure 4. p53 protein levels after knockdown of genes that affect MCF10A but not PC3. (A) MCF10A cells were transfected with indicated siRNAs for 48 h and cell lysates analyzed by Western blotting for p53 and β-actin (loading control). (B) Quantitation of Western blot performed in triplicate. p53 signal normalized with β-actin is shown.

DISCUSSION

In this article, we demonstrated the utility of targeted comparative RNAi analysis for discovering genes whose knockdown shows different effects on proliferation of two human cell lines. By comparing two cell lines, we showed that high throughput RNAi can identify different rate-limiting genes among multiple cell lines. Although we used cell proliferation as an assay in this study, TARCOR analysis is easily applicable to any cellular processes that can be studied by *in vitro* cell culture.

Comparative Analysis of Cell Lines

The quantitative comparison of the cellular responses to knockdown of genes in two human cell lines allowed the systematic discovery of different rate-limiting steps in cells of different lineages and different genetic backgrounds. The key to a successful comparison of the cell lines was the establishment of a highly reproducible screening method involving high throughput siRNA transfection and measurement of BrdU incorporation. Furthermore, we applied a strict quality control filter so that only highly reproducible data were used for the subsequent comparison analysis. An unexpectedly high percentage of genes (>25%) showed a differential effect on survival of the two cell lines upon depletion by RNAi. This result suggests that cells of different lineages and genetic backgrounds have different rate-limiting genes even for the fundamental phenotype of cell proliferation. Thus TARCOR is particularly suited for discovering genes that are differentially rate-limiting among multiple cell lines.

The difference in the genetic background of cells is certainly responsible for some of the differential effects of siRNAs. Knockdown of many genes that affect MCF10A (p53+) but not PC3 (p53-) induces p53 in MCF10A cells. The cell cycle arrest after EBNA1BP2 depletion was indeed dependent on p53 induction in MCF10A. Thus, p53 status of cell

Figure 5. Analysis of cells after RNAi against genes involved in ribosome biogenesis. (A) FACS analysis of DNA content after RNAi against EBNA1BP2 (indicated as EBP2) and DDX21. MCF10A was transfected with indicated siRNAs and examined by FACS after 48 h. (B) Western blot analysis of p53 and p21 after RNAi (48 h) against EBNA1BP2 and DDX21 in MCF10A. (C) Cyclin E-associated kinase activity after EBNA1BP2 RNAi. Cyclin E protein levels in cell lysates and incorporation of 32 P in the substrate (pRB-C) in the in vitro kinase assay is shown. (D) p53-dependent p21 induction after EBNA1BP2 RNAi. MCF10A cells were transfected with GL2 or p53-1 siRNAs for 48 h followed by transfection with GL2 or EBNA1BP2-1 siRNAs (48 h). Cell lysates were analyzed by Western blotting for indicated proteins. (E) p53-dependent G1 arrest in EBNA1BP2-depleted cells. MCF10A cells treated as in D were analyzed by FACS, and the percentage of cells in the indicated phases of the cell cycle is shown.

lines seems to be a major determinant of differential cellular response to depletion of a growth-related genes.

Genes Essential for Proliferation of Human Cells

Most of the current screens for essential genes have been performed in model organisms such as yeasts, flies, or *C. elegans*, where the genetic homogeneity has led to the assumption that the same genes will be uncovered as essential. The genetic and epigenetic variability of mammalian cells, however, must be taken into account when devising screens for essential genes in human cells. An unexpected benefit of TARCOR on mammalian cells was a significant expansion of the number of hit genes when the analysis was expanded to two cell lines. We obtained different hit genes in the two cell lines, with the total pool of hit genes increasing by 45% (116 vs. 80 genes) by pooling results from two cell lines. Thus it is important to use multiple cell lines when screening genes in human cell lines. The list of hit genes included many expected genes involved in the cell cycle and in DNA, RNA, and protein metabolism. Thus the 15 genes for which no function has been ascribed and which were identified as essential for proliferation in this study have the potential to be involved in these critical processes.

SiRNA screens are hypomorphic genetic screens and not null screens. In such screens a gene could be deemed essential for proliferation, either because the lower level of the gene product is insufficient for an essential function in cell growth or because the lower level triggers cellular checkpoint mechanisms that arrest the cell cycle or induce apoptosis. One way to distinguish the two mechanisms is to test if impairment of a checkpoint pathway can restore cell proliferation. In this article, EBNA1BP2 is an example of a gene that scores as essential for proliferation because of the second type of mechanism. Codepletion of p53 by RNAi can rescue the cell cycle defect of EBNA1BP2-depleted cells, indicating that the decrease in the EBNA1BP2 is not sufficient to be the direct cause of the cell cycle arrest in MCF10A cells.

How does EBNA1BP2 depletion activate the p53 pathway? It has been recognized that any stress on rRNA production arrests the cell cycle through a p53-mediated pathway (Pestov *et al.*, 2001). Because the yeast homolog of EBNA1BP2 is involved in rRNA processing (Huber *et al.*, 2000; Tsujii *et al.*, 2000), the p53 induction could be in response to stress on rRNA production. Another possibility is suggested by the fact that EBNA1BP2 binds to the Epstein-

Barr virus nuclear antigen 1 (EBNA1) protein, which is known to destabilize p53 by inhibiting the latter's interaction with HAUSP/USP7, a deubiquitination enzyme for p53 (Saridakis *et al.*, 2005). Although the cells used in our studies do not contain EBNA1, an intriguing possibility is that EBNA1BP2 (or a cellular interaction partner) is required to destabilize p53 by similar mechanisms.

Targeted Screen

DNA microarrays have successfully identified large sets of differentially expressed genes. Focusing on cell proliferation-related genes previously identified by microarray studies allowed us to obtain a significantly higher hit rate in our analysis, suggesting that the high throughput targeted RNAi analysis is particularly useful for following up on the accumulating microarray data. Hundreds of genes are differentially expressed in specific cancer cells in microarray studies, but there is no easy method for sorting through these genes to identify those relevant to cancer cell phenotypes. Our results suggest that the screens targeted on genes differentially expressed in cancer cells might be an efficient way of finding those that are critical for cancer cell proliferation.

TARCOR in Drug Discovery

The key issue for cancer drug development is selectivity for the target cell types. One way to add selectivity to drugs is to choose a target protein that is selectively rate-limiting for the target cells. Thus TARCOR can be an effective screening method for discovery of cancer-specific drug targets among genes that are differentially expressed in cancer cells in microarray studies. Another application will be the comparison of two cell lines that are isogenic except for a single cancer-causing mutation. The potential for such a screen is revealed by our discovery that inhibition of ribosome biogenesis is selectively inhibitory to p53⁺ but not p53[−] cells. TARCOR screens for genes that are synthetically lethal with cancer-related loss-of-function mutations can be executed, for example, by comparison of cells with functional and nonfunctional BRCA1 (Scully *et al.*, 1999) or of cells that are p53^{+/+} and p53^{−/−} (Bunz *et al.*, 1998). Such screens are expected to identify drug targets that are specific to cancer cells with those mutations.

So far, drugs are mostly small chemicals that bind to receptors or enzymes. However, siRNAs themselves might be used directly as drugs in future (Soutschek *et al.*, 2004; Zimmermann *et al.*, 2006). In that case TARCOR will become even more useful because the differentially inhibitory siRNA is immediately viable as a drug.

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