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RNAi and HTS: exploring cancer by systematic loss-of-function

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Cancer develops through the successive accumulation and selection of genetic and epigenetic alterations, enabling cells to survive, replicate and evade homeostatic control mechanisms such as apoptosis and antiproliferative signals. This transformation process, however, may create vulnerabilities since the accumulation of mutations can expose synthetic lethal gene interactions and oncogene-driven cellular reprogramming ('addiction'), giving rise to new therapeutic avenues. With the completion of the human genome project, it is anticipated that the identification and characterization of genetic networks that regulate cell growth, differentiation, apoptosis and transformation will be fundamental to decoding the complexity of these processes, and ultimately, cancer itself. Genomic methodologies, such as large-scale mRNA profiling using microarrays, have already begun to reveal the molecular basis of cancer heterogeneity and the clinical behavior of tumors. The combination of traditional cell culture techniques with high-throughput screening approaches has given rise to new cellular-genomics methodologies that enable the simultaneous interrogation of thousands of genes in live cells, facilitating true functional profiling of biological processes. Among these, RNA interference (RNAi) has the potential to enable rapid genome-wide loss-of-function (LOF) screens in mammalian systems, which until recently has been the sole domain of lower organisms. Here, we present a broad overview of this maturing technology and explore how, within current technical constraints, large-scale LOF use of RNAi can be exploited to uncover the molecular basis of cancer – from the genetics of synthetic lethality and oncogene-dependent cellular addiction to the acquisition of cancer-associated cellular phenotypes.
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Introduction

From a reductionist perspective (Hanahan and Weinberg, 2000; Hahn and Weinberg, 2002), cancer is the culmination of somatic genetic alterations that cluster around the acquisition of key traits – limitless replicative potential, suppression of apoptosis, invasion, growth factor independence, sustained angiogenesis and insensitivity to antigrowth signals. These cancer phenotypes are acquired both through the increased activity of genes and their encoded proteins (oncogenes), as well as the loss of genes that normally suppress features of uncontrolled cell behavior, such as cell growth (tumor suppressor genes). Typically, these mutations are thought to confer selective advantages to the cancer cell; however, it is becoming increasingly clear that they may also create vulnerabilities that can be exploited therapeutically. Cancer cells appear to become genetically rewired or 'addicted' to the loss, deregulation or enhancement of gene expression such that reintroducing lost genes, or diminishing enhanced gene expression can lead to growth arrest or death of the cell. Moreover, the acquisition of tumor phenotypes may come at the cost of enhanced dependencies on otherwise nonessential pathways or proteins. Given that a major component of cancer development involves the acquisition and selection of somatic mutations and epigenetic modifications, RNA interference (RNAi) appears ideally suited to explore the molecular basis of cancer development and progression. Of particular interest are new procedures, which combine the power of RNAi with high–throughput screening (HTS) techniques currently being developed as genetic techniques in tissue culture (see Figure 1 for general description).

RNAi is a naturally occurring process in eukaryotes by which double-stranded RNAs (dsRNA) trigger the sequence-specific degradation of homologous mRNAs, thus preventing expression of their protein products. First described in *Caenorhabditis elegans* (Fire *et al*., 1998), RNAi has become a widespread tool for reverse genetics in invertebrate model systems and has quickly found the limelight in large-scale functional genomic screens (Fraser *et al*., 2000; Gonczy *et al*., 2000; Ashrafi *et al*., 2003; Kamath *et al*., 2003; Lum *et al*., 2003). However the tendency of dsRNA to induce the antiviral interferon response and subsequent cell death in mammalian cells (Stark *et al*., 1998) appeared to compromise its feasibility in higher organisms. This barrier was overcome with the observation that an early event in the initiation of RNAi involves the cleavage of long dsRNA stretches into small fragments of 21–23 bp, termed siRNAs (for small interfering RNAs), which can trigger the RNAi response in mammalian cells without activating the antiviral response (Elbashir *et al*., 2001).

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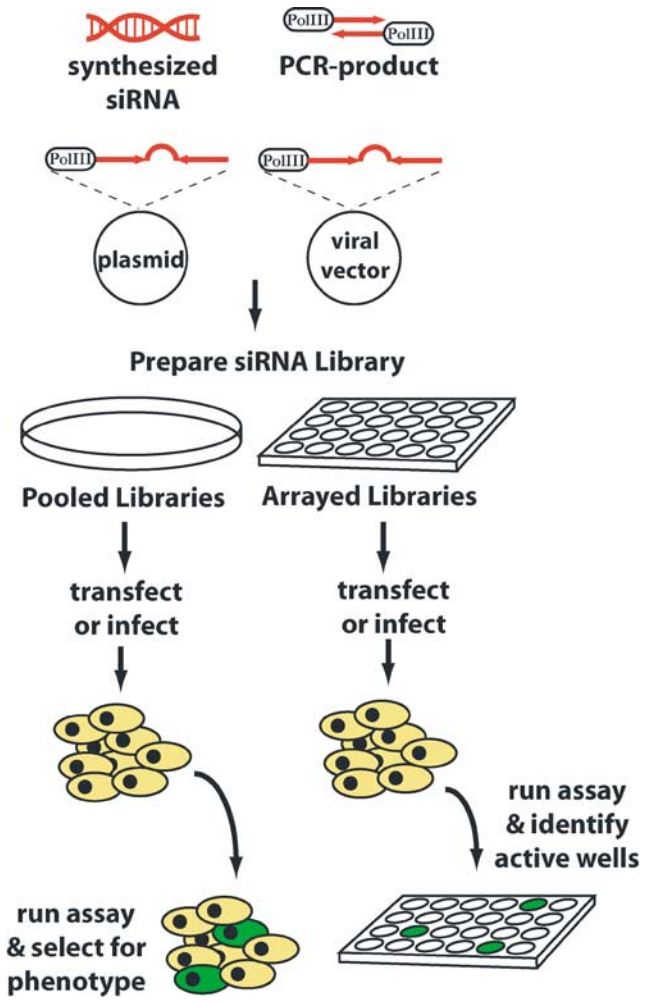

Figure 1 Summary schematic of siRNA HTS strategies. Libraries can be composed of chemically synthesized siRNAs or DNA (as plasmids or PCR fragments) with RNA polymerase III promoters driving expression of shRNAs. DNA-based libraries can be pooled together and transfected or infected into cells in bulk. A screen or assay relying on selection for a desired phenotype can then be used to isolate and identify hits from the screen. Alternatively, siRNA libraries can be spotted in an arrayed format where each RNAi inducing reagent is individually transferred into cells. Further details and references are provided within the body of this review

Since these initial observations were made, the intense efforts dedicated to understand the underlying mechanisms of RNAi are revealing not only the individual components of the system but also the existence of a previously unknown regulatory machinery controlled by small RNA molecules. Most prominent among these are micro-RNAs (miRNAs), endogenous small-hairpin-like molecules that regulate mRNA translation. miRNAs appear to be involved in a number of major cellular regulatory processes such as development and differentiation. Another activity attributed to siRNA-like molecules is the regulation of chromatin conformation and accessibility, reminiscent of transcriptional gene silencing (TGS) mechanisms previously observed in plants (recently reviewed in He and Hannon, 2004; Novina and Sharp, 2004)). Although we do not fully understand the extent of the RNA-driven regulatory machinery, RNAi-based gene silencing has rapidly become a mainstay in mammalian cell biology, used as the tool-of-choice to interrogate the consequences of specific gene silencing.

The use of RNAi in cancer biology, as in other fields, has gained considerable momentum in the past 2 years. Whereas only six studies using siRNA in cancer were published in 2001, that number has risen dramatically to 277 in the first half of 2004 (using the search terms 'siRNA and cancer', PubMed August, 2004). A significant number of basic research studies in cancer genetics routinely use RNAi to interrogate the consequences of eliminating specific genes. Here, we present an updated view of RNAi technology, explore the advantages and caveats of its application in large-scale screening mode, and discuss how RNAi-based screens can be used to gain insight into cancer-associated pathways, synthetic lethal gene interaction, oncogene 'addiction' and the molecular mechanisms that underlie cancer-associated phenotypes.

RNAi as a screening tool

Factors affecting the efficacy of RNAi as a screening tool: the problem of false positives and negatives

Although the success of RNAi as a research tool is impressive, limitations of the technology exist. Such liabilities can be mostly overcome when addressing individual genes, but have a strong impact on the success of RNAi-based screening approaches. Most notably, it has been shown that some siRNAs, but more prominently short hairpin RNAs (shRNAs), can induce the interferon-mediated antiviral response to a certain degree (Bridge *et al*., 2003; Sledz *et al*., 2003), the impact of which should be carefully evaluated before addressing any project by RNAi screening. Secondly, the potency of siRNA silencing varies significantly depending on the sequence selected within the mRNA target. Studies by several groups have shown that the efficacy of siRNA can be improved, in part, at the level of sequence design (reviewed in Mittal, 2004). Applying design rules derived from these studies improve overall siRNA performance, and in terms of siRNA screens, increase the overall probability of being able to significantly knockdown the transcription of as many genes as possible. However, there is considerable variability in siRNA performance that cannot be correlated with sequence composition and which may depend in part on secondary structure, mRNA turnover, mRNA abundance or other, as yet unknown factors. It is clear, for example, that there are inherently 'difficult genes' for which efficient siRNAs have been hard to identify (P Aza-Blanc *et al*, unpublished observations). These issues are compounded by the extent of gene silencing necessary to observe a phenotype in a given assay and cell type, as well as factors such as protein stability. Thus, although better siRNA design certainly contributes to more efficient

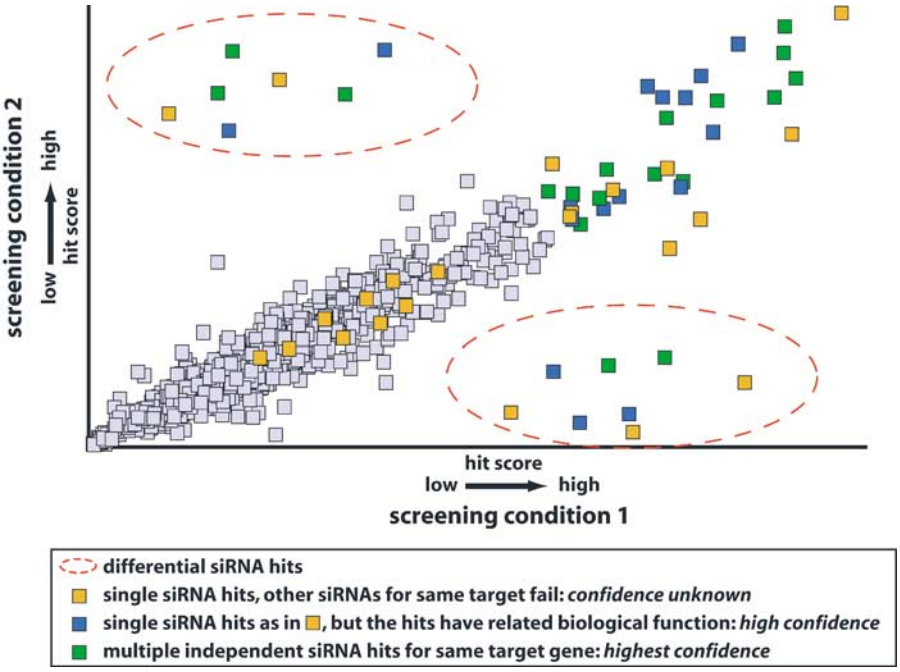

Figure 2 Representation of a hypothetical RNAi screening result highlights different classes of hits according to their significance. In this example, the hypothetical siRNA library includes more than one reagent against each target. Data from two screening conditions are graphed against each other. Such conditions could include: (1) distinct assays measuring the same biological process, (2) the same biological assay in different cell lines to address cell-specific effects, (3) different yet complementary assays, or (4) the same biological process assayed under different conditions such as the presence or absence of treatment with a drug or ligand (e.g. TRAIL). Circled in red areas are siRNAs, which are positive under one screening condition and not another (differential hits). If these screens were on different cell types, these could represent synthetic lethality screening hits (see Discussion within this review). In orange are those cases where only one siRNA for a given target is isolated as a hit but the remaining siRNAs targeting that transcript show no activity. Such hits are potentially false positives and need to be confirmed with additional, independent siRNAs against the same target. Blue data points represent siRNA hits that, like the orange hits, only have one active siRNA per targeted mRNA. However, this class is of particular note and potential biological relevance because the various genes identified can be grouped into a common cellular pathway (e.g. MDM2, p53, ARF). Lastly, in green are those hits where multiple siRNAs against the same target mRNA were identified. This class of hits is most compelling because presence of multiple siRNA hits alleviates concerns over off-target effects and other screening artifacts

mRNA reduction, the underlying reason why a particular siRNA does not exhibit activity in a given cellular assay may not be readily detected. Therefore, making biological interpretations of negative results in a high-throughput (HT) siRNA screen should be avoided and addressed outside of the screening process.

Off-target effects are perhaps the key limitation to applying RNAi technologies in mammalian cells. siRNAs are known to produce a 'signature' of inhibited transcripts in addition to the intended target (Jackson *et al*., 2003; Semizarov *et al*., 2003; Snove and Holen, 2004). Such promiscuity can yield potentially misleading results (false-positive hits) and undermine the significance of real hits in large-scale screens. Several sources of off-target effects can be considered. Jackson and collaborators have shown that some off-target effects can be associated with significant base pairing between just the 5′ region of the siRNA chains and the unintended targets (Jackson *et al*., 2003). In addition, siRNAs may also work through miRNA-like mechanisms, which can silence targets without the need of perfect base-pair matching or interfere with chromatin state and promoter activity through a TGS mechanism. Indeed, recent studies have shown that siRNAs can be used to modulate promoter activity (Kawasaki and Taira, 2004). Thus, the potential sources of off-target effects are diverse, making it unclear whether specificity filters, such as BLAST or Smith–Waterman comparisons against the transcriptome, which are most often applied during the library design process, substantially contribute to eliminating these effects.

Currently, off-target effects appear to be inherent to the use of siRNAs and produce a significant level of uncertainty concerning the activity (or inactivity) of any given siRNA in a particular biological assay. With no technical solutions on the horizon for overcoming these issues, the current remedy for raising screening accuracy is to increase the number of independent RNAi reagents directed against each target (redundancy of the library). Combined with smarter strategies such as parallel screening of complementary assays, this strategy should significantly increase the confidence level of screening results (Figure 2). In fact, most RNAi libraries available today are built with enhanced redundancy.

Large-scale siRNA screens in mammalian cells

Thus far, relatively few large-scale screens have been reported, in large part, due to the prohibitive cost of RNA oligonucleotide synthesis, detection reagents and the equipment typically required for large-scale cell-based assays. However, with the development of less-expensive DNA-based RNAi screening methods, as well as the anticipated public availability of DNA-based siRNA collections, we predict a dramatic increase in the numbers of large-scale studies. Initial proof of concept for RNAi screening using both synthetic siRNAs and plasmid-encoded shRNAs were reported last year using gene-family focused libraries (Aza-Blanc *et al*., 2003; Brummelkamp *et al*., 2003). In rapid succession, Zheng *et al*. (2004) reported a PCR-based approach to generate a DNA-mediated RNAi library representing >8000 human genes and Berns *et al*. (2004) described the construction and application of a retroviral library encoding shRNAs covering 7194 targets. Finally, Paddison *et al*. (2004) reported the creation of a retroviral library encoding shRNAs targeted against 10 000 human and 5000 mouse genes. Other groups have reported alternative methods to generate RNAi libraries, although they have yet to report the results of large-scale screens using these reagents (Kawasaki *et al*., 2003; Luo *et al*., 2004; Sen *et al*., 2004; Shirane *et al*., 2004).

Factors to be assessed when considering siRNA screening technology are: (1) the vehicle used to deliver and induce RNAi (typically synthetic siRNAs *versus* DNA methods) and (2) the screening format (i.e. arrayed *versus* pooled strategies).

Making the choice between synthetic and vector-based reagents

The reagents initially used to trigger RNAi in mammalian cells were chemically synthesized siRNAs (Elbashir *et al*., 2001). More affordable RNAi reagents have since been developed, combining the hairpin method first described in plants (which use vectors that express single transcripts containing inverted repeats that fold to produce long dsRNA stretches inside cells, Smith *et al*., 2000), with structural features observed in the newly described miRNAs. These vectors typically employ PolIII-dependent promoters (U6 or H1) to express shRNAs that are processed in the cell yielding endogenous siRNAs (recently reviewed in Dorsett and Tuschl, 2004; Mittal, 2004).

Both synthetic and DNA-mediated RNAi methods are compatible with HTS methodologies. However, the relatively high cost of synthetic siRNAs has biased most public efforts toward vector-encoded siRNA libraries. Although the initial cost of acquiring whole-genome synthetic libraries is large, the screening cost thereafter is fairly low (e.g. approx. US \$0.002 per well for the screen reported in Aza-Blanc *et al*., 2003). Thus, synthetic siRNA libraries distributed as lyophilized, deprotected, arrayed reagents should eventually become a viable solution to make this resource more widely available. DNA-based methods are substantially cheaper and can be perpetuated indefinitely, although the labor required to clone and prepare these libraries for transfection is substantial. A main advantage of vector-based methods is that collections can be constructed as viral libraries that enable access to nontransfectable cell types and even tissues, overcoming a major limitation of synthetic reagents. Viral systems also enable long-term silencing of target genes, opening up the possibility of screening events such as growth in soft agar, senescence, and long-term differentiation that require weeks, rather than days. Although such collections already exist (Berns *et al*., 2004; Paddison *et al*., 2004), their application in arrayed format (see below) is still challenging due to the technical difficulties of producing uniform, high-titer viral supernatants in parallel format and in a cost-effective manner. Still, viral libraries can be screened by transfection as regular plasmids as shown by Paddison and co-workers, a flexibility that currently makes viral RNAi libraries the preferred screening reagent.

Selecting the screening format

In an array-based format, each reagent is individually used to challenge the cellular assay. A major advantage of the arrayed format is that each RNAi-inducing reagent is identifiable through its location within the screening plates; thus, the identification of 'hits' from the primary screen is immediate. In addition, no selection is required and both activators and repressors can be detected in the same experiment. This format has been used successfully in both RNA- and DNA-based screens (Aza-Blanc *et al*., 2003; Zheng *et al*., 2004). However, this format requires considerable up-front effort, including the design, synthesis and arraying of the individual reagents. New methods that combine microarray spotting technologies with cell-based assays might help overcome this caveat. By these methods, RNAi reagents (siRNAs, shRNAs) are spotted on a solid support and cells are reverse transfected on the surface (Ziauddin and Sabatini, 2001; Baghdoyan *et al*., 2004; Carpenter and Sabatini, 2004; Yoshikawa *et al*., 2004). Conceptually similar to array-based screens in multiwell format, these methods would significantly reduce costs, making the technology widely accessible.

As an alternative, the use of the pooled libraries requires significantly less up-front investment. Here, the library consists of a complex mix of clones used to challenge a cellular assay of interest. Hits are isolated after positive selection, which limits the scope of assays that can be performed. After selecting the cell population containing positive hits, clones have to be retrieved and sequenced to identify the causative si/shRNA. A shortcut was recently reported that retrieves the identity of assay hits using a barcode system followed by microarray hybridization (Berns *et al*., 2004; Paddison *et al*., 2004), similar in design to barcoding approaches described in yeast (Shoemaker *et al*., 1996). One key issue with the pooled approach is that some hits might be the result of complex interactions between different RNAi reagents (such as multiple clones targeting

different genes in the same cell), complicating the follow-up process. In addition, pooled approach outcomes might involve non cell-autonomous effects, which are essentially impossible to dissect. Despite the limitations, screening pooled libraries does not require complex automation or robotics and is possible in most laboratory settings, making it the most likely format to become popular in the short term.

Using RNAi to screen cancer cells for novel molecular targets

Clinical validation of molecularly targeted anticancer agents, such as Herceptin (trastuzamab, Genentech), targeted against the her2 receptor in breast cancer patients, and Gleevec (imatinib mesylate, Novartis), a small molecule that inhibits the enzymatic activity of the leukemia-associated protein fusion, BCR-abl, have ushered in a new era in cancer treatment. Novel agents, such as Iressa (gefitinib, AstaZeneca) and Erbitux (Imclone Systems), both of which target the epidermal growth factor receptor, have recently joined the ranks of a steadily growing arsenal of new cancer therapeutics. However, to provide a context for current cancer therapeutic efforts, it is important to note that these targets – BCR-abl, Her2/neu and EGFR – had been the subject of intense investigation for the past 20–25 years, and their validity as causal mutations in cancer development and progression was securely established before the development of pharmaceutical agents. The challenge currently facing cancer biology (and other biomedically important disciplines) is the identification of new genes and proteins that play central roles in the etiology of these diseases. Below, we discuss several ways in which loss-of-function (LOF) RNAi screens are likely to contribute to this goal – interrogation of cancer-associated pathways, targeting oncogene 'addiction' and synthetic lethality and dissecting cancer-associated phenotypes.

Dissection of cancer-associated pathways

The knowledge that alteration of a specific gene can contribute to cancer development can be used as the basis for a pathway screen. For example, stimulation of the EGFR receptor leads to increased growth rates in certain cancer cells. To understand how EGFR is connected to cell proliferation could be addressed by RNAi, either by a phenotypic screen (identify which siRNAs leads to reduction of cell growth, filtered for specificity by EGFR blocking antibodies) or by a reporter gene assay (e.g. using activation of a downstream component as a reporter). Recent examples of pathway screens in *Drosophila* and mammalian cells provide a context for this approach. The *Hedgehog* (*Hh*) and *Wingless* (*Wg*) pathways play critical roles in development, eliciting cell proliferation and differentiation, and their misregulation has been shown to contribute to tumorigenesis. Using a dsRNA library targeting 43% of predicted *Drosophila* genes, Beachy and co-workers screened for *Hh* pathway components in a wing imaginal disc-derived cell line engineered to express a *Hh*-responsive luciferase reporter (Lum *et al*., 2003). Four known *Hh* pathway components were identified, as were four genes with previously unrecognized roles in the pathway. One of these genes, casein kinase 1a, plays a role in regulating the basal activity of both *Wnt* and *Hh* pathways, raising the possibility that it may represent a tumor suppressor in a variety of cancers linked to these pathways. Phosphoinositide 3-kinases (PI3K) control a variety of important cellular functions, including proliferation and apoptosis. A small screen targeting 30 components of the human PI3K pathway with 150 siRNAs identified PTEN and PDK1, two known regulators of Akt phosphorylation (Hsieh *et al*., 2004). In a study of ubiquitin-specific proteases that mediate deubiquitination of cellular substrates, Brummelkamp *et al*. (2003) used a collection of shRNAs targeting 50 human deubiquitinating enzymes to identify modulators of TNF-dependent activation of NF-κB. Loss of the cylindromatosis tumor suppressor (CYLD) was found to enhance the activation of NF-κB, leading to apoptotic resistance. CYLD suppression was reportedly reversed by sodium salicylate and prostaglandin A1, leading the authors to test these agents as therapy for cylindromas. A larger genome-scale screen for NF-κB regulators was conducted by Schultz and co-workers using a library constructed from a novel dual-promoter expression method targeting >8000 human genes (Zheng *et al*., 2004). Of 94 genes identified as modulators of NF-κB signaling, 20 were selected as the most robust, with eight representing known components of the pathway. The remaining genes are likely to be pleiotropic effectors, such as the BCL2-like protein 13 and death-associated protein kinase, DAPK2, both of which induce apoptosis; thus, future follow-up will be required to determine whether these are NF-κB specific.

Novel components of the p53 pathway have also been identified using a library of $\sim$24 000 retroviral vector-encoded shRNAs targeting 7914 human genes (Berns *et al*., 2004). To facilitate screening on this scale, the library was condensed to 83 pools and used to infect human primary fibroblasts engineered with a temperature-sensitive p53 protein to screen for p53-dependent growth arrest. Six genes were shown to suppress the temperature induced proliferation arrest, including five new components of the p53 pathway. The isolation of shRNAs targeting p53 in the screen serves to validate the approach. Knockdown of these p53 pathway members was shown to lead to downregulation of p53 target genes, including $p21^{cip1}$, suggesting that these genes may act as tumor suppressors *in vivo*.

Oncogene 'addiction' and synthetic lethality as a basis for genome-wide RNAi screens in cancer cells

Oncogene 'addiction' has been used to describe the genetic reprogramming of cells that occurs in the presence of a causal oncogenic mutation (Reddy and Kaelin, 2002; Weinstein, 2002). Much of this thinking

derives from observations that oncogenes, such as *myc*, not only drive tumor formation but subsequent loss of their expression can cause terminal differentiation or even death of the malignant cells (Felsher and Bishop, 1999; Pelengaris *et al*., 1999, 2002). Thus, oncogene-driven cells can become survival-dependent ('addicted') to the presence of the oncoprotein. Clinically exploited examples include inhibition of c-abl kinase activity in BCR-abl-transformed 32D cells, which leads to a loss of viability, whereas inhibition of that activity in the parental cells has no effect (Druker, 2002). This is an unexpected observation because by definition the parental cells, which do not express the oncoprotein, survive in culture. Such cellular rewiring has been noted for other mutant tyrosine kinases, such as FLT3 (Weisberg *et al*., 2002). The fact that the induction of cell death by selective small molecule or biologic inhibitors can be effectively mimicked by diminution of the transcript via RNAi (Wilda *et al*., 2002) provides a rationale for the validation of candidate oncogenes in a cellular context. One can envision the development of a bank of isogenic cells harboring a large set of oncoproteins whose inhibition can be interrogated by siRNA transfection. Those whose inhibition leads to selective death of the derivate line(s) would provide strong evidence to further explore that gene's role in cancer progression.

This basic approach can be broadened to discover cancer-associated genes (with or without mutations). Systematic gene silencing in cancer cells with large numbers of siRNAs is likely to reveal unforeseen cellular dependencies that may be pharmaceutically tractable (reviewed in Deveraux *et al*., 2003). Experimental support for this approach comes from a number of anecdotal examples, such as fatty acid synthase (FASN), whose inhibition by natural synthetic FASN inhibitors in cancer cells that overexpress the FAS protein leads to selective tumor cell apoptosis (Kuhajda, 2000). Notably, inhibition of FASN in normal cells does not demonstrate any appreciable toxic effect, revealing a tumor-selective therapeutic window. Other cancers appear to be highly susceptible to inhibitors of HSP90 (e.g. geldanamycin), which is critical to normal protein folding (Neckers, 2002), as well as inhibitors of the proteosome (e.g. PS341), which plays a central role in normal protein degradation (Kuhajda, 2000). Although these genes may not be considered oncogenes *per se*, they become essential to at least some oncogene-driven cancer phenotypes. We expect that genome-wide RNAi-based screens will expose a large number of such dependencies in cancer cells.

Although synthetic lethal interactions were first described for genetic studies in flies in the early 1900s, Hartwell *et al*. in 1997 proposed synthetic lethality as a way to search for novel anticancer drugs, based on the idea that chemical compounds could be identified, which bind to/inhibit a protein that is synthetically lethal to a cancer-associated mutation (Hartwell *et al*., 1997). The concept is well suited to current high-HTS paradigms, where one can assay large numbers of compounds against cancer cells in microculture. Although parallel interrogation of one or more normal and cancer cells for compounds that lead to selective tumor cell death is perhaps the simplest embodiment of this approach, there are many caveats, not least of which is the genetic variation of cells isolated from different subjects. In contrast, isogenic cell pairs, engineered to harbor specific cancer-associated mutations, are better suited to the identification of synthetic lethality. This approach is particularly attractive in cases where the oncoprotein of interest is not pharmaceutically biddable (e.g. c-*myc* or b-catenin, where one would typically have to rely on the disruption of protein–protein interactions). The synthetic lethal approach affords the opportunity to antagonize chemically tractable proteins that genetically and/or physically interact with the cancer-associated oncoprotein.

Synthetic lethality has been demonstrated in PTEN null tumors, which are highly sensitive to the mTOR (*t*arget *o*f *r*apamycin) inhibitor, CCI-779, a rapamycin derivative (Neshat *et al*., 2001). mTOR signals the protein translation machinery downstream of akt, a branch of the PI3K pathway associated with antiapoptotic and proproliferative signaling. Thus, inhibition of mTOR can be described as synthetically lethal to the loss of PTEN, a tumor-specific LOF mutation that cannot be approached pharmaceutically. Evidence that this concept might be generalizable comes from experiments targeting ras mutation. Using isogenic colon carcinoma cell derivatives with and without v12ras mutation, Torrance *et al*. (2001) reported the identification of small organic compounds capable of selectively inhibiting the ras-dependent tumor cell *in vitro*, as well as ras-dependent tumorigenicity *in vivo*. Stockwell and co-workers have described the identification of genotype-selective molecules specifically cytotoxic to genetically defined malignant cells (containing hTERT, SV40 small and large T-antigens and v12ras), sparing their normal isogenic counterparts (Dolma *et al*., 2003). Several of these are known anticancer agents; others (e.g. erastin) are novel, leading to cell death via nonapoptotic cell death. Such strategies, combined with the development of automated microscopy screening tools to compare differential growth rates between isogenic cells lines (with and without specific oncogenic mutations), can be readily adapted to HT-siRNA screens for genes whose LOF induces cell death or growth arrest in the absence of tumor suppressors (p53, pRB, PETN, etc.), the presence of oncogenic mutations (v-myc, k-ras, BCR-abl, her2/nue, etc.) or antiapoptosis genes (BCL2 and IAP families). While encouraging, the chemical approach suffers from a lack of target(s) definition, a critical (and typically difficult) step required to bring chemistry efforts forward into the realm of lead molecule development.

In their synthetic lethality proposal, Hartwell and co-workers also noted that the genetic changes giving rise to cancer can be liabilities, and therefore the key to tumor cell sensitivity. Lamenting the state of human cell genetics in the 1990s, and the notable lack of LOF tools to exploit genetics in human cell lines, Hartwell *et al*. proposed conducting synthetic lethal screens in model

organisms. The advent of RNAi in mammalian cells, however, provides a systematic, addressable and (possibly) more specific method to achieve the same goal, that is, revealing synthetic lethal interactions where the identity of the target is known *a priori*. The development of siRNA technologies allows for efficient, genome-wide LOF screens, opening the door for synthetic lethal screening paradigms in human cancer cell models. LOF screens could adopt a variety of strategies including (1) synthetic lethality in combination with known oncogenic lesions such as mutation of p53, or k-ras, amplification of c-*myc*, or BCR-abl fusion; (2) synergy with known chemotherapeutic agents; (3) selective killing of tumor cells *versus* wild-type cells from their tissue of origin; or (4) a reversal of the aforementioned approaches, which would search for suppressors of these effects in an effort to unravel the underlying regulatory mechanisms of cell proliferation.

We have begun to explore the application of synthetic lethality in cancer genetics for modulators of apoptosis induced by the *t*umor necrosis factor receptor-*r*elated *a*poptosis-*i*nducing *l*igand (TRAIL) (Aza-Blanc *et al*., 2003). Using a chemically synthesized library of siRNAs targeting 510 human genes (including much of the predicted kinome and 130 other proteins), cell viability in combination with TRAIL treatment was assayed, leading to the first demonstration of an RNAi-based screening paradigm in mammalian cells. Owing to the tumor selectivity of the TRAIL ligand, this cytokine and its receptors have been the subject of much investigation and current biomedical interest (reviewed in Ashkenazi and Dixit, 1999). Known mediators of TRAIL-induced apoptosis (e.g. DR4 TRAIL receptor and CASP8) as well as modulators of apoptosis, which had not previously been associated with TRAIL (e.g. MYC, JNK3 and SRP72; Ren *et al*., 2004) were identified, thus validating the screening approach. Importantly, genes with no previously described role in apoptosis were identified (one of which was shown to be an effector of the cell death signaling factor, BID, which we term DOBI, for *d*ownstream *o*f *BI*D). Future genome-wide screens, which survey the entire transcriptome in an unbiased manner promise to identify a host of important new modulators (such as SRP72, a component of the protein secretion machinery), which would not be intuitively selected as a modulator of apoptosis. Lastly, while this RNAi-based screening strategy was successful in identifying known and new components of the TRAIL-induced cell death pathway, other key modulators, such as the DR5 receptor, and caspase-9 were not identified, underscoring the issue of 'false negatives'.

Studies of the EGFR inhibitor, gefitinib (Iressa, AstraZeneca), reveal a specific selectivity for EGFR proteins with either a missense mutation (L858R) or an 18-bp deletion from positions 747–753 (Lynch *et al*., 2004; Paez *et al*., 2004). Although increased activity of the receptor via these mutations was proposed to account for the increased gefitinib potency, it appears that selective signaling to antiapoptotic pathways, specifically phosphorylation of Akt and STAT-5, may account for the targeted susceptibility (Sordella *et al*., 2004). Thus, the wild-type EGFR receptor does not appreciably engage these pathways, whereas the mutant receptors do. It follows that a parallel, genome-wide siRNA screen of mammary epithelial cells engineered with the mutant or wild-type receptor would be expected to reveal synthetic lethality following inhibition of Akt, STAT-5 and other proteins engaging the apoptotic pathway.

Dissecting cancer-associated phenotypes

Cancer cells exhibit diverse cellular phenotypes as a consequence of mutations acquired during malignant progression. These include increased cellular growth, extracellular matrix-independent survival and spread (anoikis), increased cellular motility, morphological change (e.g. epithelial-to-mesenchymal transition, EMT), substrate invasiveness, anchorage independence and metastatic spread. With the advent of high-content cellular screening tools, which range from HT-CCD-based photography to multiparametric cellular readouts, one can now consider the development of RNAi screens that interrogate cancer-associated phenotypes. Identifying factors that modulate cell viability constitutes the most straightforward phenotypic assessment. Currently, most systematic RNAi-based screens for cell growth have been conducted in model organisms such as *C. elegans* and *Drosophila*. Such studies in a multicellular model organism afford the opportunity to use genome-wide LOF to investigate complex phenotypes such as development, sterility and embryonic lethality.

Kamath *et al*. performed the first systematic functional analysis of a metazoan genome, by building a library targeting 86% of the predicted *C. elegans* genome and screening for RNAi-induced phenotypes. More than 1500 genes exhibited viability, growth-related or developmental phenotypes, with 33 human disease gene homologues, including NF2 (neurofibromatosis), APC (adenomatous polyposis of the colon) and MADH4 (pancreatic carcinoma), inducing an observable phenotype. Using a similar screening paradigm, Tijsterman and co-workers identified 61 genes that protect the *C. elegans* genome against mutations (including dsDNA breaks) (Pothof *et al*., 2003). Many of these genes can be classified as components of DNA repair/replication, cell cycle checkpoint and chromatin organization/remodeling pathways. As malignant transformation only occurs after the accumulation of multiple somatic mutations, it follows that genes, which protect DNA sequence integrity, including the nearly 20 novel genes discovered in this screen, may well represent novel tumor suppressors. Perrimon and co-workers conducted an RNAi analysis of growth and viability using a dsRNA library targeting >90% of predicted *Drosophila* genes to screen two different embryonic cell lines (Boutros *et al*., 2004). A quantitative assay of cell number identified more than 400 essential genes, including 50 with homology to human disease genes. Notably, 10 of these genes are implicated in blood-cell leukemia, an enrichment explained by the fact that the

Drosophila cell lines screened are embryonic hemocyte lines (Echalier and Ohanessian, 1970; Yanagawa *et al*., 1998).

Morphological changes characteristic of EMT may also be captured by automated HT microscopy. Such screens in *Drosophila* using simultaneous labeling of actin, cytoskeleton and DNA revealed genes whose inhibition led to growth without cell division, inhibition of progression through mitosis as well as cell shape (determined by a combination of phenotypic annotations) (Kiger *et al*., 2003). Notably, this screen identified *polo*, the *Drosophila* counterpart of human polo-like kinase 1 (plk-1), as affecting mitotic spindle integrity. Plk-1 inhibition in cancer cells leads to a similar phenotype, arresting cells prior to cell division and leading to the induction of tumor cell-selective apoptosis. The authors also report the results of a co-RNAi screen, designed to identify dsRNAs that could revert cells to a wild-type morphology following inhibition of the *Drosophila* PTEN gene; this approach identified two known members of the pathway, Akt and PI3K(92e), suggesting a rapid way in which to dissect pathways that affect a given phenotype.

Historically, functional genetic screens of model organisms have provided valuable insights into cell cycle, developmental regulations and other signal transduction pathways, yet critical limitations exist. Foremost is the fact that key tumor suppressors and oncogenes, such as MYC and components of the p53 pathway, are not conserved in lower eukaryotes. Other cancer-related phenotypes not conserved in worms and flies include telomere shortening and its regulation of cellular senescence and oxygen sensing and their relation to tumor angiogenesis and metastasis. Conducting genetic screens for modulators of cancer development and progression in mammalian cell lines stands to address some of these unresolved issues. We suggest caution should be exercised, however, in that our own experience has shown that human cell growth may also be modulated by assay-dependent artifacts, such as augmentation of transfection agent toxicity, induction of interferon responses or translational interference; thus, a significant investment in follow-up for a broad phenotype may offset its initial simplicity. Nonetheless, such screens in multiple cancer cells, counter-screened against 'normal' cells may provide the degree of specificity necessary to ensure a robust set of viability/survival-associated genes.

Future directions

The application of RNAi as a tool to selectively diminish gene expression is a maturing technology and carries limitations yet to overcome. However, RNAi has the potential to significantly contribute to large-scale biology, from the dissection of genetic pathways where one or more members are known, to 'black-box' experiments, in which a phenotype or behavior of interest is queried to gain insights into the underlying biology. Systematic genome-wide screens have already been conducted in model organisms and, given the recent development of mammalian siRNA libraries, are now underway in human and mouse cell models for cancer. There are an estimated 3000 disease-modifying genes present in the human genome, yet to date, the pharmaceutical industry has only focused their efforts on about 500 (reviewed in Hopkins and Groom, 2002). As technical limitations are resolved and siRNA design is enhanced to the extent possible, we look forward to the availability of validated, redundant genome-wide siRNA collections and the interrogation of complex phenotypes. These advancements will likely lead to novel targets and therapeutic strategies based on new insights.

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