RNA interference: the new somatic cell genetics?

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RNAi is evolving into a powerful tool for manipulating gene expression in mammalian cells with potential utility for investigating gene function, for high-throughput, function-based genetic screens and potentially for development as a therapeutic tool.

Introduction

Since the 1970s, the war on cancer has been based on the notion that studying the disease will lead to the discovery of vulnerabilities that can be exploited in the clinic. While many underlying genetic determinants of cancer have been identified, this knowledge has failed to translate into new therapeutic strategies, with a handful of notable exceptions. One hypothesis is that this failure has largely been due to the genetically intractable nature of cultured mammalian cells. The recent emergence of dsRNA-induced gene silencing, or RNA interference (RNAi), in mammalian systems is likely to reinvigorate the field of somatic cell genetics, and in the process revolutionize the study of human disease. During the past year, a point has been reached at which any gene in the human genome can conceivably be targeted using small, dsRNA gene-silencing triggers—small interfering RNAs (siRNAs) or expressed short hairpin RNAs (shRNAs). The application of siRNAs and shRNAs for single gene analysis is rapidly becoming standard methodology, and genome-wide reverse genetic screens are certainly on the horizon. The future of RNAi may lie in the development of highly specific, nucleic acid-based therapies for cancer and other diseases.

RNAi in invertebrate systems

RNAi first emerged as a biological oddity in *C. elegans* (Fire et al., 1998) and plants (Jorgensen et al., 1996), but was quickly harnessed as a powerful genetic tool in these systems. However, it has become clear that dsRNA-induced silencing phenomena are present in evolutionarily diverse organisms, including plants, fungi, and metazoans (reviewed in Bernstein et al., 2001b; Hammond et al., 2001). A combination of genetic and biochemical studies suggest that many of these phenomena share a common mechanism (Figure 1). The prevailing model begins with the conversion of the dsRNA silencing "trigger" into small RNAs (siRNAs) by an RNase III family nuclease, Dicer (Bernstein et al., 2001a). These small RNAs (~22–25 nt in size) become incorporated into a multicomponent nuclease complex, which uses the sequence of the siRNAs as a guide to identify and destroy homologous mRNAs (Tuschl et al., 1999; Hammond et al., 2000; Zamore et al., 2000; Nykanen et al., 2001).

So far, the only universally conserved players in RNAi are Dicer and Argonaute (Ago) gene family members. Dicer contains a tandem repeat of RNaseIII catalytic domains, a carboxyl-terminal dsRNA binding domain, an amino-terminal DExH/DEAH RNA helicase domain, and a PAZ domain (Bernstein et al., 2001a; Nicholson and Nicholson, 2002). Ago proteins, which are components of the RNA-induced silencing complex (RISC), contain a PAZ domain and a carboxyl-terminal

PIWI domain.

The RNAi pathway may have evolved early in eukaryotes as a cell-based immunity against viral and genetic parasites. Double-stranded RNA viruses or mobile genetic elements with the potential to form dsRNA structures are virtually ubiquitous and may be subject to RNAi-dependent gene silencing in *C. elegans*, plants, *Drosophila*, yeast, and mammals (reviewed in Hannon, 2002). However, the RNAi pathway is also used for the regulation of endogenous gene targets during metazoan and plant development (reviewed in Hannon, 2002).

Endogenously expressed small hairpin RNAs regulate gene expression through the RNAi pathway during *C. elegans* development (Reinhart et al., 2000; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001;
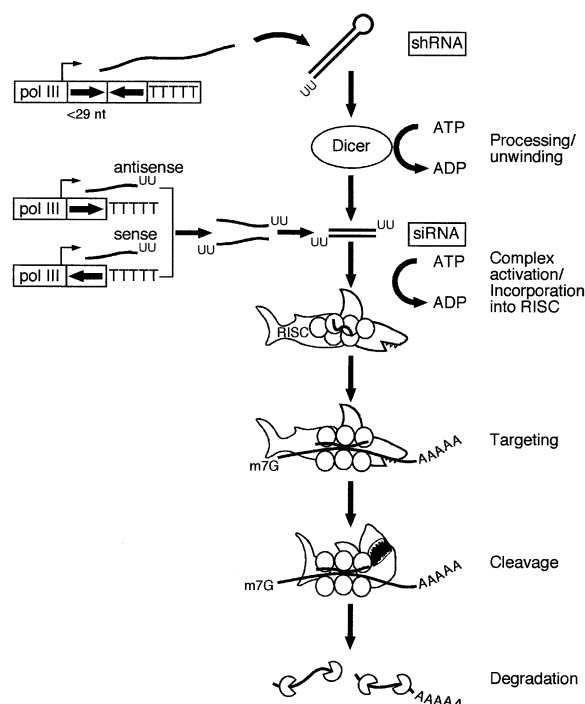

Figure 1. A model for RNA interference in mammalian cells

Small double-stranded RNA triggers of RNAi (shRNAs and siRNAs) are shown, expressed from RNA polymerase III promoters. Short hairpin RNAs (shRNAs), containing 19–29 nt dsRNA stems, are processed by Dicer and incorporated into the RNA induced silencing complex (RISC), resulting in the targeting and degradation of cognate mRNAs. Small interfering RNAs (siRNAs), containing 19 nt or 21 nt of dsRNA, presumably bypass the requirement for Dicer and are directly incorporated into RISC.

reviewed in Hannon, 2002). These small hairpin RNAs (~70 nt) are processed into a 21–22 nt mature form by Dicer and then used to seek out mRNA targets of similar sequence (generally via imperfect base-pairing interactions). For the two prototypes of this family, *C. elegans* lin-4 and let-7, silencing occurs at the level of protein synthesis (reviewed in Bernstein et al., 2001b). The first small hairpin RNAs were dubbed small temporal RNAs (stRNAs), owing to their role in developmental timing (Lee et al., 1993; Wightman et al., 1993; Ha et al., 1996; Slack et al., 2000). More recently, dozens of orphan hairpins have been identified in *C. elegans*, *Drosophila*, mouse, and humans, which are collectively referred to as microRNAs (miRNAs) (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Mourelatos et al., 2002).

RNAi and related pathways underlie many homology-dependent silencing phenomena, including cosuppression, virus-induced gene silencing, transgene-induced silencing, and quelling (reviewed in Bernstein et al., 2001b). These silencing phenomena variably involve either Post-Transcriptional Gene Silencing (PTGS), Transcriptional Gene Silencing (TGS), or both. RNAi in *C. elegans* appears to solely involve PTGS, while in plants the same dsRNA trigger can target both mRNA and chromatin.

RNAi in mammals

Given the strong conservation of RNAi-related genes in vertebrates, including Dicer and Argonaute family members, the expectation was that RNAi would operate in mammalian cells in some capacity. The first glimpse of RNAi in mammals came from injections of long dsRNAs (~500 nt, similar to those used to trigger RNAi in invertebrate systems) into mouse embryos, which resulted in sequence-specific gene silencing (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000). Several groups, including our own, extended these findings to embryonal cell lines (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002a). Biochemical and genetic evidence from these studies suggested that RNAi operates in at least a subset of mammalian cell types, in a Dicer-dependent manner via posttranscriptional mechanisms (Billy et al., 2001; Paddison et al., 2002a).

In somatic cells, however, the use of conventional dsRNA triggers (~500 nt dsRNAs) is limited by antiviral/interferon responses, including the PKR and RNaseL pathways (Baglioni and Nilsen, 1983; Clarke and Mathews, 1995; Gil and Esteban, 2000; reviewed in Williams, 1997), which trigger generalized translational repression and apoptosis in response to dsRNA of >30 bp in length. Even where PKR activity is removed from somatic cells, by either viral inhibitors or targeted disruption, long dsRNA still triggers a residual nonspecific repression of gene expression (Abraham et al., 1999; Paddison et al., 2002a; P. Paddison and G. Hannon, unpublished data).

One way around these nonspecific dsRNA responses is to simply create dsRNA triggers of <30 bp in length. In the past year, two short dsRNA structures have emerged, which evoke sequence specific gene silencing in somatic cells without activating antiviral responses. These are the small interfering RNAs (siRNAs) and the short hairpin RNAs (shRNAs). Both are modeled after biologically active structures in the RNAi pathway: Dicer cleavage products and small temporal RNAs, respectively.

Tuschl and colleagues and Caplen and colleagues (Elbashir et al., 2001; Caplen et al., 2001) first demonstrated that small dsRNAs, resembling siRNAs from other systems, induce sequence-specific gene silencing when transiently transfected into mammalian cells. These small interfering RNAs (siRNAs) are chemically synthesized emulations of Dicer cleavage products, which are short RNA duplexes ~19 nt in length with 2 nt 3′ overhangs on each strand. The siRNAs presumably bypass the requirement for Dicer and enter the silencing pathway by incorporation into RISC complexes (Figure 1). The use of siRNAs has been recently reviewed, in detail, and resources for the design and use of siRNAs are available from Tom Tuschl's laboratory online (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html).

As an alternative strategy, we and others have developed in vivo expression constructs for small dsRNA triggers in mammalian cells, which resemble endogenously expressed hairpin RNAs (Paddison et al., 2002b; Brummelkamp et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Zeng et al., 2002). This approach uses small inverted repeats (19–29 nt) expressed from RNA polymerase III promoter to create short hairpin RNAs (shRNAs), which can then be processed by Dicer and shunted into the RNAi pathway (Figure 1). However, siRNAs can also be produced in vivo by the expression of complementary 19 or 21 nt RNAs from separate RNA polymerase III transcription units (Lee et al., 2002; Miyagishi and Taira, 2002; Yu et al., 2002). For some studies, expressed dsRNA triggers have potential advantages over siRNAs when combined with well-worn strategies for stable and inducible gene expression in vitro and in vivo. The details of these strategies are further discussed below.

One of the major differences between mammalian cell RNAi and the response observed, for example, in *C. elegans* is the apparent lack of amplification of the RNAi effect or of "transitive RNAi" (Sijen et al., 2001). In *C. elegans,* "amplification" may contribute to heritable, systemic gene silencing. According to one model, amplification of the dsRNA signal is initially mediated by RNA-dependent RNA polymerases (RdRP). An RNA degradation product (e.g., an siRNA) may prime RdRPs along the mRNA template, resulting in the production of dsRNA homologous to sequences 5′ (i.e., upstream) of the initially targeted sequence (Sijen et al., 2001). When combined with transport, amplification results in a self-propagating silencing effect throughout the organism. In mammalian cell systems, however, transient transfection of RNAi triggers, e.g., long dsRNA, siRNAs, or shRNAs, results in a transient effect, lasting 2–7 days. The longevity of silencing is likely dependent on gene expression homeostasis (e.g., abundance of mRNA and protein, stability of the protein, transcriptional feedback loops, etc.), the half-life of the silencing complex itself, and cell division, which serves to dilute the effect over time.

Design and expression of dsRNA triggers

Tuschl and colleagues have elaborated several guidelines for designing siRNA oligos for chemical synthesis (Elbashir et al., 2002). The selection of the target sequence should avoid regions of the mRNA which might bind RNA regulatory proteins, such as 5′ and 3′ UTR and regions close to the start site (<100 nt). Between +100 nt (with the AUG referenced as +1) and the stop codon, 23 nt sequences conforming to the consensus (5′-AA[N19]UU-3′, where N is any nucleotide) are selected from the mRNA sequence. Sequences of >70% or <30% GC content or which are highly G-rich should be avoided. The siRNA is then constructed by designing sense and anti-sense (i.e., reverse complement) N19 sequences, each ending with two 3′ 2-deoxythymidine residues. Refer to Elbashir et al. (2002) for a
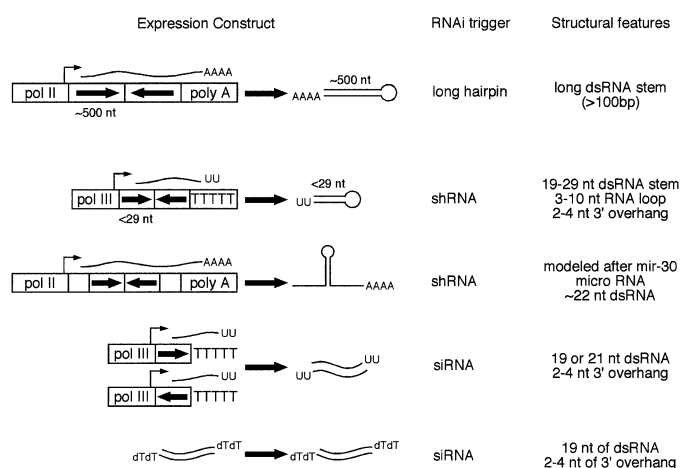
Figure 2. Expression of dsRNA triggers in mammalian cells

This figure shows the various strategies that have been used to express dsRNA triggers in mammalian cells and some of the structural features of the dsRNA triggers. Of note is that long hairpins have so far only proven effective in embryonal cell types, which lack PKR/interferon responses. Expressed shRNAs and siRNAs, however, evoke sequence-specific silencing in numerous cell types tested.

more detailed protocol. The current, average cost for chemical synthesis siRNAs is between $270 to $500 per siRNA, depending on purification and scale of synthesis.

Two less costly strategies for generating siRNAs involve in vitro transcription reactions using T7 polymerase (Paddison et al., 2002b, Yu et al., 2002; Donze and Picard, 2002; with commercial kits for this purpose available from New England Biolabs [http://www.neb.com] and Ambion [http://www.ambion.com]) and in vitro processing of long dsRNA using the Dicer enzyme (J. Myers and J. Ferrell, personal communication). T7 generated siRNAs differ from normal Dicer products in that they contain 5′-triphosphates. Despite this difference, T7-siRNAs have been shown to be biologically active. A T7-siRNA design program and detailed instructions are available at http://www.cshl.org/pub lic/SCIENCE/hannon.html. In vitro processing of long dsRNA by purified Dicer enzyme may eventually be the most effective way to generate siRNAs, since the end products will be a mixture of dozens of separate siRNAs targeting a single mRNA. However, siRNA populations will likely require purification to avoid contamination of long dsRNA, and complex siRNA populations may have a higher probability of targeting other genes than do discrete siRNAs. Regardless of the method used to generate siRNAs, the major drawback of exogenously produced siRNAs is the inability to stably or inducibly regulate gene expression.

Toward this end, we and others have developed expression strategies for dsRNA triggers in embryonal cell types (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002a) and in somatic cells (Brummelkamp et al., 2002; Lee et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002b; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Zeng et al., 2002) (Figure 2).

For cells derived from somatic tissues, a flurry of recent reports demonstrates that expression of short hairpin RNAs (shRNAs) or complementary siRNA strands leads to sequence-specific gene silencing. The basic expression schemes and expression strategies are presented in Figure 2. Most strategies use RNA polymerase III promoters (either human or mouse U6-snRNA or human RNase P [H1] RNA promoters) to drive expression of short RNAs, since RNA polymerase III can be exploited to precisely initiate and terminate RNA transcripts (Goomer and Kunkel, 1992). These promoters should be active in most if not all embryonal and somatic cell types. One group, however, successfully triggered silencing by burying a microRNA structure within an RNA polymerase II-derived transcript (Zeng et al., 2002).

There are a few points to note concerning the expression strategies that have been used thus far. First, U6 derived shRNAs and siRNAs have sequence constraints, where a G residue is required for efficient initiation (Goomer and Kunkel, 1992). Second, one report suggests that adding the leader sequence of 27 nt from the U6 snRNA improves expression (Paul et al., 2002). Third, there is in vitro data to suggest that the mouse U6 promoter may be more active in human cells, given the strong affinity of the SNAP-c complex for the proximal sequence element (Chong et al., 2001). However, given the published data and our unpublished results, the minor differences amongst the reported expression strategies are unlikely to have a major impact on the efficacy of silencing.

Surprisingly, many of the structural features present in micro RNAs can be ignored in shRNAs expressed from RNA polymerase III promoters. When using the human U6 and H1 promoters, we have found that loop structures of 4 or 8 nt work equally well when comparing the same 19 nt and 29 nt stems (our unpublished data). Structured stems and loops, modeled after human let-7-like hairpins (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001), work considerably less well than perfectly matched stems with simple loops (Paddison et al., 2002b and our unpublished data). The only differences in efficiency which arise appear to be dependent upon length of the hairpin stem, where stems of 29 nt work 10%–40% more efficiently than stems of 19 nt, at least when targeting reporter genes (our unpublished data). This modest increase in efficiency must, however, be balanced against concerns that longer stems could theoretically increase the possibility of off-target effects. Thus, the optimal structure for shRNAs will likely emerge only after the accumulation of copious additional in vivo data with large numbers of genes.

Whichever method is used, we suggest selecting 3–6 shRNA sequences per gene. Empirical data suggests that one or more RNAi expression construct should give 40%–90% reduction in gene expression when used transiently. A program for constructing shRNA cloning primers, along with detailed protocols, is available at http://www.cshl.org/public/SCIENCE/hannon.html.

By default, we use rules similar to those described by Tuschl and colleagues for choosing shRNA targeting sequences. A target sequence of 5′-(Nx)C-3′, where N is any nucleotide, x is a length from 18 to 28 nt, and the targeted sequence contains between 30%–70% GC, is selected from the target mRNA. We normally engineer the 5′ stem strand as the anti-sense strand; however, either strand is effective (our unpublished data; Brummelkamp et al., 2002; Paddison et al., 2002b; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). To aid in cloning and to increase stability in bacteria, we incorporate G-U base pairs in the stem of shRNAs, which are permitted in duplexed RNA but not DNA. However, this strategy has not been used by others and is likely nonessential.

Delivery strategies
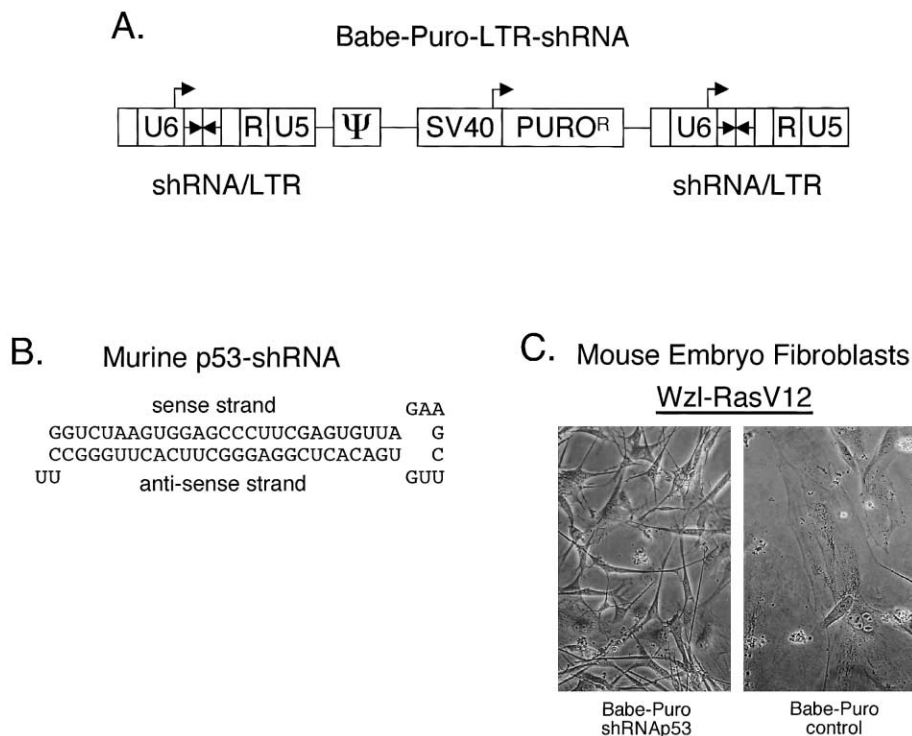
Both siRNAs and vectors containing dsRNA triggers can be

A.

Babe-Puro-LTR-shRNA

shRNA/LTR   shRNA/LTR

B.   Murine p53-shRNA

sense strand   GAA
GGUCUAAGUGGAGCCCUUCGAGUGUUA   G
CCGGGUUCACUUCGGGAGGCUCACAGU   C
UU   anti-sense strand   GUU

C.   Mouse Embryo Fibroblasts
Wzl-RasV12

Babe-Puro   Babe-Puro
shRNAp53   control

Figure 3. Stable expression of an shRNA using a retroviral system

This figure depicts one strategy for expressing shRNAs from retroviruses. A U6-p53-shRNA was inserted into the 3′ LTR of a MoMuLV pBabe-Puro retroviral construct. **A:** The predicted structure and orientation of the retrovirus as integrated into the genome of the infected cell. **B:** The predicted structure of a murine p53 shRNA. **C:** An assay for bypass of rasV12-induced senescence in early passage mouse embryo fibroblasts (P2). Cells were first transduced with Babe-Puro alone or Babe-Puro-LTR-U6-p53-shRNA and selected in puromycin for 3 days, after which cells were infected with Wzl-Hygro-rasV12 and treated with hygromycin. Only cells initially receiving Babe-Puro-LTR-p53-shRNA were morphologically transformed by rasV12 and continued to divide (see text). A time point 5 days after transduction with rasV12 is shown. Potential complications with this strategy would arise if shRNAs target viral and drug resistance transcripts in packaging or target cells, or if shRNAs target essential genes in the packaging cells. Therefore, we are currently designing inducible U6 constructs for expression from self-inactivating retroviruses.

transiently transfected into mammalian cells using commonly available transfection reagents. Chemically synthesized siRNAs are reliably effective at concentrations ranging from .05 to .5 nM in transient transfections, while U6-shRNA vectors are effective at concentrations normally used for expression of transgenes.

There are a number of well-characterized stable expression technologies currently being used in mammalian cells, which should permit permanent expression of shRNAs and siRNAs in target cells. These include systems based on retroviral integration (e.g., Hannon et al., 1999; Lois et al., 2002) (Figure 3), transposon hopping (e.g., Ivics et al., 1997), episomally replicated DNA fragments (e.g., Chittenden et al., 1989; Sedman and Stenlund, 1995), and homologous recombination (e.g., Nagy, 2000).

Among recent reports, stable RNAi has been demonstrated using random plasmid integration (Brummelkamp et al., 2002; Paddison et al., 2002b) and episomal plasmid maintenance (Miyagishi and Taira, 2002). However, based upon our observations, stable maintenance of RNAi following plasmid integration may be problematic where the phenotype itself is not positively selected (e.g., bypass of senescence). Therefore, we have begun exploring retroviral strategies for stable expression of shRNAs. We have found that MoMuLV or MSCV vectors harboring U6-shRNA cassettes can stably evoke RNAi (Figure 3A). Figure 3 shows bypass of rasV12-induced senescence in early passage mouse embryo fibroblasts using a mouse p53 shRNA (Paddison et al., 2002b) expressed from pBabe-Puro (Morgenstern and Land, 1990). MEFs cotransduced with Wzl-rasV12 and Babe-Puro alone show a flattened morphology and growth arrest consistent with cellular senescence (Serrano et al., 1997; Ferbeyre et al., 2000), while cells cotransduced with Wzl-rasV12 and Babe-Puro-U6-shRNA-p53 display a transformed morphology with little or no observable growth arrest. With retrovirus-based strategies, the expression of shRNA dur-

ing virus packaging may result in reduced virus production. For example, shRNAs may target viral genomic transcripts in packaging cells or the markers used for selecting infected cells. Furthermore, targeting essential genes is likely to have adverse effects on packaging cells. The movement to inducible RNA pol III promoters should ameliorate these problems, and we have recently derived an activator-dependent, U6-based expression system for shRNAs (P. Paddison, E. Julien, W. Herr, and G. Hannon, unpublished data).

The above results suggest that retroviral vectors may represent potent delivery strategies for shRNA expression, although more work is needed to determine an optimal viral configuration. Of particular interest is the potential to create transgenic animals through the transduction of preimplantation embryos or ES cells with suitably modified lentiviral vectors (Lois et al., 2002; Pfeifer et al., 2002).

RNAi-based screens: applications in cancer cells
In other model systems, the use of genetic screens to explore functional dependencies has been an enabling feature of countless discoveries. For example, analysis of temperature sensitive mutants in bacteriophage T4 led to the discovery of viral morphogenesis modules (Edgar and Wood, 1966). Similar approaches in yeast revealed functional hierarchies among genes regulating cell cycle progression (Hartwell et al., 1974; Hartwell and Weinert, 1989). The key to such discoveries has been the ability to create recessive, genetically defined lesions in molecular pathways.

Since cancer arises from genetic lesions in somatic cells, the concept of synthetic lethality has been heralded as way to functionally define vulnerabilities in cancer cells (Hartwell et al., 1997). Synthetic lethal interactions occur when mutations in two or more nonallelic genes synergize to kill cells. For example, a mutation in gene A or gene B may be tolerated when singly present in cells, but when combined may result in a loss of viability. Thus, synthetic lethal interactions reveal situations in which cellular homeostasis is altered by a molecular lesion so that the
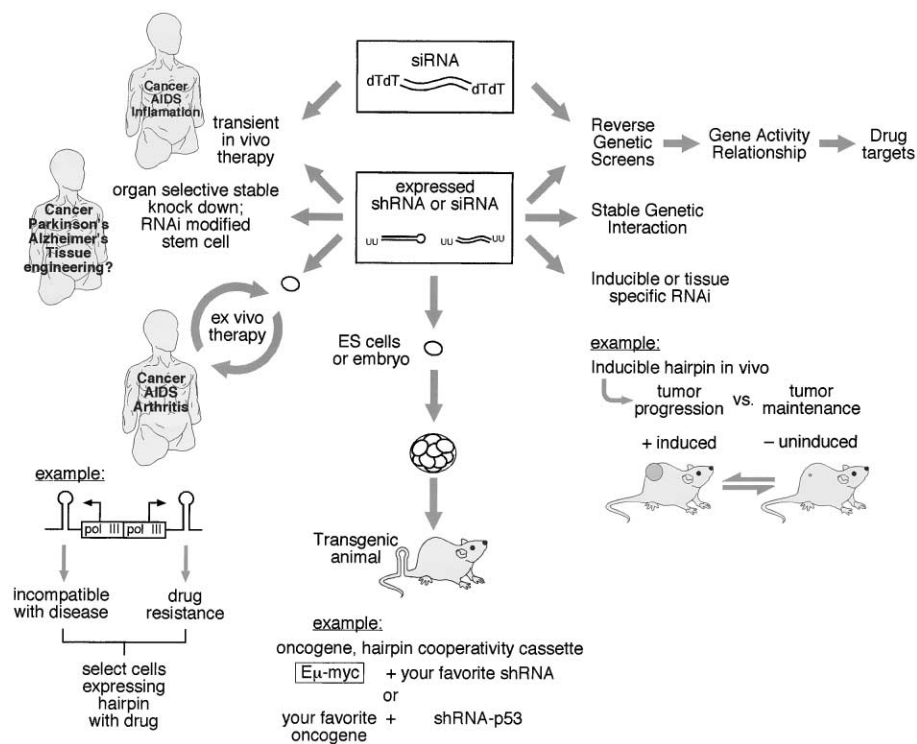
Perspectives

Through the use of reverse genetic approaches, RNAi has developed into a powerful tool for probing gene function in *C. elegans* and other invertebrate systems. In worms, RNAi is currently being used to systematically target ~19,000 predicted genes (J. Ahringer, personal communication). Similar approaches are underway in plants (D. Baulcombe and P. Waterhouse, personal communication). With the added capacity of RNAi, somatic mammalian cells will hopefully gain admittance into the pantheon of model genetic systems. In practical terms, the use of RNAi in cultured cells may deliver new insights into a host of disease-related processes, including concrete information on potential drug targets.

action of another gene or pathway is required to compensate. The fact that cancer cells arise from genetic alterations makes synthetic lethality ideally suited for identifying cellular targets required by cancer cells for viability.

Our group is currently in the process of undertaking large-scale RNAi-based screens for lethal targets in cancer cell lines. The biggest question in regard to designing mammalian cell screens is whether to use forward or reverse genetic approaches. Randomized, forward genetic screens have been used with some degree of success in mammalian systems for gain of function genetic lesions. Such screens generally consist of expressing, in mass, cDNAs or genomic fragments in receipt cell populations and screening for a positively selectable phenotype (e.g., Deiss and Kimchi, 1991; Wong et al., 1994; Maestro et al., 1999; reviewed in Gudkov and Roninson, 1997). Perhaps the best example of this type of approach came early on with the cloning of the ras oncogene from genomic libraries in rodent cells (Goldfarb et al., 1982; Shih and Weinberg, 1982). While such approaches are compatible with RNAi-induced phenotypes (e.g., bypass of senescence), a well-to-well, reverse genetic approach has two major advantages. First, neutral or negatively selected phenotypes (e.g., apoptosis, growth arrest) can be scored in each well for single and multiple gene targeting events. Second, RNAi expression constructs can be assembled into restricted functional sets a priori based on known or inferred function of gene targets (e.g., DNA replication, DNA damage repair, etc.). Comparing phenotypic readouts among different restricted sets may give rise to "epistasis signatures," or maps of functional dependencies underlying a particular phenotype in a particular genetic background (e.g., transformed versus nontransformed cells). Such signatures would be comparable to transcript array patterns, except that epistasis signatures would be functionally defined and thus, although less precise, potentially more suggestive of cause and consequence.

RNAi also holds promise for in vivo genetic applications in mammals. Perhaps the most immediate question is whether expressed RNAi triggers can be combined with transgenic approaches for stably knocking down gene expression in rodents (Figure 4). Studies of ex vivo modified cells can also benefit from RNAi, where primary or transformed cells are stably engineered with shRNAs and then implanted into mice. Inducible RNAi triggers may ultimately prove to be key components of both in vivo and ex vivo approaches in rodent systems (Figure 4).

In humans, there are many scenarios in which RNAi could be enlisted to combat disease. These include targeting viral pathogens, targeting disease- or symptom-causing genes (or alleles), modifying primary cells ex vivo to remove undesirable gene products, expressing shRNAs from replication competent viruses to selectively kill cancer cells, and so on (Figure 4). With regard to target specificity, dsRNA triggers of gene silencing are well-suited as therapeutic molecules, since gene products are targeted based on mRNA sequence rather than protein activity, and are thus not limited by the ability of medicinal chemistry to target a protein class or interaction. However, at present, effective delivery strategies present a significant barrier to therapeutic applications of RNAi.

RNAi shows tremendous promise as a new technology for manipulating gene expression for both experimental and therapeutic purposes. However, we are still in the very early stages of understanding both the mechanistic basis and biological roles of these gene-silencing pathways. Thus, we will undoubtedly see both spectacular successes and notable failures of RNAi before we fully understand the power and limitations of this new tool.

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