

Molecular Mechanisms of RNAi: Implications for Development and Disease

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Research over the past few years has led to dramatic new discoveries on the role of double-stranded RNA (dsRNA) in the cell. RNA duplexes have been shown to orchestrate epigenetic changes, repress translation, and direct mRNA degradation in a sequence-specific manner. These diverse effects of dsRNA on gene expression have been termed RNA interference (RNAi). In addition to playing a role in viral defense and silencing transposons, RNAi also has a critical function in a number of developmental processes in the embryo. In this review, we explore these roles and discuss the molecular mechanisms behind dsRNA-mediated gene silencing. Further, we address the use of RNAi as a tool to study gene function in biology, and as a strategy for treating human disease. **Birth Defects Research (Part C) 75:28–42, 2005. © 2005 Wiley-Liss, Inc.**

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INTRODUCTION

The recently discovered phenomenon of RNA interference (RNAi) has not only revolutionized our ability to study biology, but has challenged some of the most basic assumptions behind our understanding of it. This revolution began less than 10 years ago with the observation that exogenous double-stranded RNA (dsRNA) could induce potent and sequence-specific silencing of endogenous gene expression in *Caenorhabditis elegans* (Fire et al., 1998). These humble beginnings sparked a profusion of research that has since shown the role of dsRNA in the regulation of gene expression to be highly conserved across evolution from plants through humans (*Saccharomyces cerevisiae* being a notable exception).

RNA duplexes can block translation, direct mRNA degradation, and even induce transcriptional silencing of loci. The specificity of RNAi effects derives from the nucleotide se-

quence of the dsRNA and the molecular context in which it is found. RNAi as a biological phenomenon functions in processes as diverse as defense against viral infection, cell fate specification, and regulation of developmental timing. RNAi as a biological tool has been employed to investigate gene function in vitro and in vivo. Furthermore, this technology shows much promise for therapeutic application. In this review, we summarize how RNAi was discovered and its role in cell biology, particularly with respect to embryonic development. Further, we explore how it can be used to study vertebrate development and, eventually, to treat human disease.

THE DISCOVERY OF RNA INTERFERENCE

The story of RNAi begins with a basic axiom of scientific research, the value of the control experiment. In 1995, Guo and Kemphues (1995)

were trying to understand the role of the *par-1* gene in establishing anterior-posterior polarity of the *C. elegans* embryo. At the time, a common approach to studying gene function in the nematode was to inject large amounts of antisense single-stranded RNA (ssRNA), in the hope that it would base-pair with its complementary mRNA, block translation, and lead to an effective loss-of-function (Nellen and Lichtenstein, 1993). The resulting silencing was termed RNA interference. When Guo and Kemphues (1995) tried this experiment, they found that the control sense RNA led to just as much *par-1* silencing as the antisense RNA. Further work showed that, unlike cellular mRNAs which tend to have relatively short half-lives, the silencing effects of ssRNA persisted for much longer, and could even be inherited from one generation to the next (Seydoux and Fire, 1994). This disparity suggested that there were intrinsic differences between endogenous RNA and the substrate that was inducing interference. Regardless, antisense-mediated silencing continued to be used widely as a technique, despite the fact that these observations remained largely unexplained.

Two years later, Fire et al. (1998) sought to definitively answer these questions. They noted that ssRNA samples were usually prepared with bacteriophage RNA polymerases, and often contami-

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nated with ectopic transcripts. This led them to hypothesize that perhaps the reason that both sense and antisense RNAs were able to induce silencing was because of the small amount of dsRNA that could contaminate both of these samples. To test this hypothesis, they prepared both single- and double-stranded RNA targeting the *unc-22* gene under stringent conditions, and injected these samples into worm gonads. To their surprise, they found that dsRNA was over 100-fold more effective at silencing *unc-22* than antisense ssRNA. Furthermore, these dsRNA-injected worms phenocopied null mutants of this gene. The authors went on to demonstrate that this dsRNA-mediated interference was not cell-autonomous. Even when dsRNA was injected into the adult's head, the silencing effect could cross into the gonads and be transferred to the worm's progeny. This led them to speculate that there must be an active transport mechanism for dsRNA in order to achieve these long-distance effects, and perhaps this indicated a physiological role for dsRNA-mediated silencing. In the intervening decade, work by the Fire laboratory (Andrew Fire, Stanford University, Palo Alto, CA), as well as many others, has proven many of these predictions to be true.

The discovery of this phenomenon in *C. elegans* was quickly followed by a number of genetic screens that led to the identification of the genes required for dsRNA-mediated silencing in the worm (Tabara et al., 1999). Prior to the work of Fire et al. (1998), there had been reports of gene silencing mediated by unknown substrates in organisms as diverse as insects, plants, and fungi. Various terms such as co-suppression, posttranscriptional gene silencing (PTGS), and quelling were given to the observation that transformation of plant or fungal genomes with a particular gene led to silencing of homologous genes in the genome (Napoli et al., 1990; de Carvalho et al.,

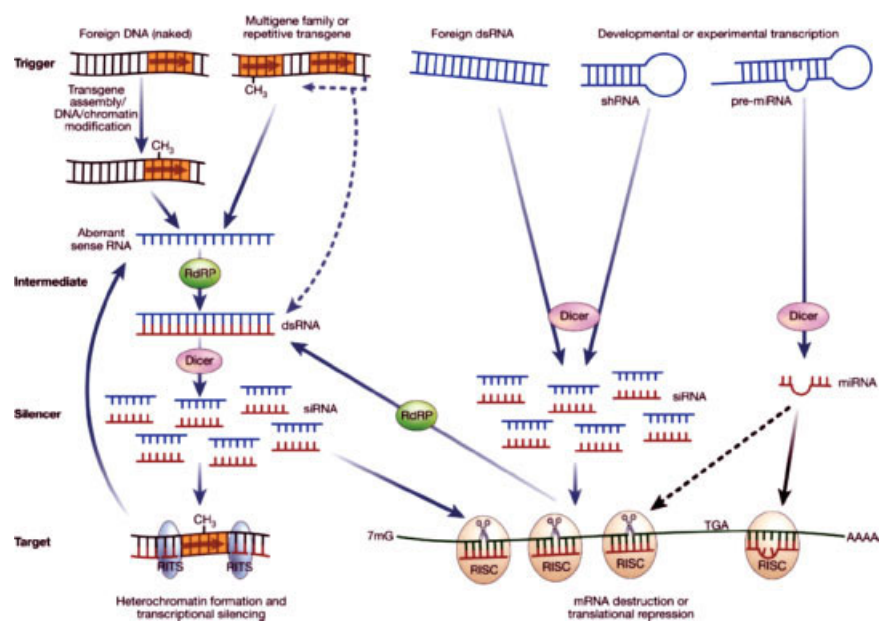


Figure 1. Overview of double-stranded RNA (dsRNA) mediated gene silencing in the cell. Endogenous triggers of this pathway include foreign DNA or dsRNA of viral origin, aberrant transcripts from repetitive sequences in the genome such as transposons, and pre-microRNA (miRNA). Alternatively, RNAi can be triggered experimentally by exogenous introduction of dsRNA or constructs which express shRNAs. All of these dsRNA substrates are then processed by Dicer into smaller RNA duplexes called short, interfering RNAs (siRNAs) or miRNAs, depending on their origin. These small dsRNAs then guide protein effectors to sequence-specific targets in order to achieve gene silencing. Transcriptional silencing at the genome level is mediated by the RNA-induced transcriptional silencing complex (RITS). Translational repression and mRNA degradation are due to the activity of another protein complex, called the RNA-induced silencing complex (RISC). [Reproduced with permission from Mello and Conte (2004).]

1992; Romano and Macino, 1992). Upon identification of the genes required for dsRNA-mediated silencing in *C. elegans*, sequence comparisons immediately revealed that silencing phenomena in other organisms shared much of the same machinery (Fagard et al., 2000). Furthermore, it was shown that dsRNA could mediate silencing in many different organisms, and that PTGS was also associated with the production of small RNA duplexes (Hamilton and Baulcombe, 1999). These exciting discoveries suggested that dsRNA-mediated silencing was more than just a new tool for loss-of-function studies, but rather an ancient phenomenon likely to have an important role in biology. In this way, the results of a simple control experiment led to the coalescence of many disparate fields and sparked the RNAi revolution.

MOLECULAR MECHANISMS OF RNA INTERFERENCE

Cellular Machinery and Molecular Triggers of RNAi

RNAi is an umbrella term for any process in which a dsRNA precursor can trigger transcriptional or translational repression of a homologous gene. These dsRNA precursors are processed into small 21–28 nucleotide (nt) duplexes that guide target recognition. There are two types of naturally-occurring small RNAs that can act as gene silencers: short, interfering RNA (siRNA) and microRNA (miRNA) (Fig. 1). Each of these arises from different triggers of the RNAi pathway. siRNAs derive from long dsRNA duplexes. These duplexes are often produced during the course of viral reproduction within cells (foreign dsRNA), or by hybridization of overlapping transcripts from repetitive sequences in the genome, such as transposons

or latent viruses (foreign DNA). These long dsRNAs are then cleaved into 21–22-nt siRNAs by the enzyme Dicer (Bernstein et al., 2001). Dicer is a large protein (~220 kD) containing a dsRNA binding domain (dsRBD), two catalytic RNase III domains, and a helicase domain, as well as a piwi-argonaute-zwille (PAZ) interaction domain (Bernstein et al., 2003). This protein can unwind and cleave long dsRNA duplexes into small 21-bp duplexes with 5' phosphates and dinucleotide 3' overhangs. Some organisms, such as mammals and *C. elegans*, have only one copy of this gene. Others have several paralogs that are each responsible for processing RNA from different sources. For example, *Drosophila melanogaster* has two Dicer paralogs DCR-1 and DCR-2. Long dsRNA processing is managed by DCR-2 in association with the dsRBD-containing protein R2D2, while miRNA processing is assigned to DCR-1 (Lee et al., 2004). Another species difference with regard to Dicer activity is ATP-dependence. While human Dicer does not use ATP, *Drosophila* DCR-2 activity requires ATP hydrolysis (Nykänen et al., 2001; Zhang et al., 2002). This type of RNA-mediated silencing can protect the cell from troublesome invaders such as viruses and transposons, but a more global role for RNAi in gene regulation was not found until the discovery of miRNAs.

Almost all metazoan cells encode some transcripts that contain 20–50-bp inverted repeats of complementary sequences (Bartel, 2004). These transcripts fold back on themselves and base-pair along complementary regions to form dsRNA hairpins. Hairpin precursors are first processed into shorter pre-miRNAs (~70 nt long) within the nucleus by the enzyme Drosha, a ribonuclease III similar to Dicer (Lee et al., 2003b). These pre-miRNAs are then exported to the cytosol, where they are converted into mature 21–23-nt-long miRNAs by Dicer before they go on to inhibit translation of complementary mRNAs (Fig. 1) (Bernstein et al., 2001). As we discuss later, miRNA-

mediated silencing plays a particularly important role in developmental processes. Finally, RNAi can also be engaged by exogenous introduction of any of these substrates into the cell or whole organism. This capability has provided a rapid means of doing loss-of-function experiments in organisms that have not been amenable to genetic manipulation.

There is one final entryway into the RNAi pathway that has only been observed in plants, fungi, and *C. elegans*. Forward genetic screens in these organisms have identified a unique family of genes encoding RNA-dependent RNA polymerases (RdRPs) to be involved in transgene silencing (Cogoni and Macino, 1999; Dalmay et al., 2000; Smardon et al., 2000). Upon encountering ssRNA in the cell, RdRPs can synthesize a complementary strand of RNA in a primer-independent manner (Fig. 1) (Makeyev and Bamford, 2002). The resulting dsRNA intermediates can then be processed by Dicer and trigger silencing of homologous sequences as described above. The discovery of the role of these enzymes in RNAi solved the mystery of how dsRNA-mediated silencing is actually amplified within worms (Sijen et al., 2001).

Posttranscriptional Gene Silencing

There are as many ways to trigger RNAi as there are types of dsRNA, but they all converge on one enzyme, Dicer. How are the siRNAs and miRNAs generated by Dicer able to direct such dramatic effects on gene expression? RNA-mediated silencing can impact expression of homologous genes by three different means: translational repression, mRNA cleavage, and chromatin modification. A given RNA duplex can direct any of these programs, depending on the extent of its sequence homology to the target, the composition of the protein complex to which it is bound, and the organism in which it is found.

Both of the posttranscriptional effects of RNAi, translational repression and mRNA degradation,

begin with the assembly of siRNAs or miRNAs into ribonucleoprotein (RNP) complexes called RISC (RNA-induced silencing complex) or miRNPs, respectively (Fig. 1) (Hammond et al., 2000; Mourelatos et al., 2002). These RNP complexes consist of the unwound RNA duplex tightly bound to a member of the Argonaute (Ago) family of proteins. Ago proteins contain two conserved domains, a PAZ domain which binds small RNAs (Ma et al., 2004) as well as a PIWI domain (named for the protein piwi), which has significant homology to RNase H proteins (Song et al., 2004) and has been shown to catalyze mRNA cleavage in human cells (Liu et al., 2004; Meister et al., 2004). There are several members of the Ago family, and growing evidence suggests that rather than being functionally redundant, each member may actually be specialized for subsets of small RNAs (Okamura et al., 2004). Once bound to this complex, the single-stranded siRNA serves as a template for sequence-specific degradation of homologous mRNAs (Martinez et al., 2002). While miRNAs in plants function exactly as siRNAs, in animals they are thought to have insufficient homology to target mRNAs to catalyze cleavage (Bartel, 2004). In most cases, they tend to initiate translation repression rather than mRNA degradation. The mechanism of this miRNA-mediated translational regulation is poorly understood. miRNAs in combination with their mRNA targets have been found associated with polyribosomes, suggesting that miRNPs block translation downstream of the initiation step (Kim et al., 2004). While it is tempting to generalize that siRNAs are specialized for mRNA degradation while miRNAs act as a brake on translation, it is unlikely to hold true. Recent work has shown that miRNPs can mediate cleavage of even relatively mismatched targets with a cost in efficiency (Hutvagner and Zamore, 2002). Similarly, siRNAs which are not perfectly homologous to their targets can repress their translation (Doench et al., 2003; Saxena et al., 2003). It seems that the fate of an mRNA tar-

get in RNAi interference is likely to depend on a constellation of factors, including sequence homology and the molecular context in which it finds itself.

RNA Interference Enters the Nucleus

While RNAi in *C. elegans* and mammals so far seems limited to posttranscriptional effects, work in other organisms presents evidence that its influence may extend well into the cell nucleus where it can modulate the epigenetic status of the genome itself. RNA-mediated epigenetic modulation can occur in two different ways: RNA-directed DNA methylation (RdDM) and RNA-mediated heterochromatin formation. The former was first observed in plants, where small dsRNAs were shown to turn off transcription of target genes by inducing de novo methylation of homologous sequences in promoter regions (Mette et al., 2000). Unlike canonical DNA methylation, which occurs at CpG dinucleotides, RdDM is distinguished by its ability to methylate any cytosine residue. Furthermore, the minimum promoter target size is only 30 bp, allowing a degree of precision unparalleled by other epigenetic covalent modifications which occur at the nucleosome level (Pélissier and Wassenegger, 2000). Will RdDM be an important mechanism of transcriptional regulation in the animal kingdom? There is a lot of circumstantial evidence to suggest that it will. Non-CpG methylation has been detected at some loci in mammalian cells, and many of the important protein components of the pathway in plants are conserved (reviewed in Matzke and Birchler, 2005). Even more intriguing, recent work has demonstrated that exogenous introduction of siRNAs into human cells can trigger promoter methylation and transcriptional silencing (Kawasaki and Taira, 2004; Morris et al., 2004). These results suggest that mammalian cells do indeed have the capacity for RdDM, but it remains to be determined what

role this process plays in endogenous gene regulation.

While RdDM is highly sequence-specific, RNA-mediated heterochromatin formation serves as a means to silence transcription across large extents of the chromosome. In general, heterochromatin is found in highly-condensed regions of genomic DNA that are transcriptionally inactive, and often contain long regions of transposon-derived tandem repeats. The first clue that RNAi may be involved in silencing heterochromatin came from experiments in the fission yeast *Schizosaccharomyces pombe*. In this species, almost all of the key components of the RNAi pathway such as Dicer, Ago, and RdRP are present as single-copy genes. When any one of these genes is ablated, reporter genes embedded in centromeric heterochromatin become transcriptionally active, and transcripts from both strands of these repeats begin accumulating in cells (Volpe et al., 2002). Biochemists have isolated the proteins responsible for this activity and termed it the RNAi-induced transcriptional gene silencing (RITS) complex (Fig. 1) (Verdel et al., 2004). The RITS complex incorporates siRNAs and Ago, as well as several other proteins. The current model for RITS activity is that siRNAs guide this complex to homologous loci, which leads to histone modification by associated histone methyltransferases. The entire complex is then tethered to the locus by histone methylation in order to maintain silencing over the long term (Noma et al., 2004). A growing body of data suggests that this mechanism may be conserved in multicellular eukaryotes as well, including vertebrates. Heterochromatin-associated proteins, such as the H3mK9 methyltransferase and the chromodomain protein Hp1, lose centromeric localization upon RNase treatment in mouse (Maison et al., 2002) or genetic disruption of RNAi machinery in fly (Pal-Bhadra et al., 2004). Another such protein, Cohesin, also fails to be localized to

heterochromatin in human cells deficient for Dicer (Fukagawa et al., 2004). A major mystery still remaining, with respect to both RdDM and RNA-mediated heterochromatin formation, is whether small dsRNAs can actually base-pair with genomic DNA, or if they operate by pairing with nascent RNA being transcribed from the target locus (Grewal and Moazed, 2003).

Negative Regulators of RNAi

Given the potential of RNAi to affect gene expression at the genome, mRNA, and ribosome levels, it is not surprising that it itself is under the control of several regulatory mechanisms. Viruses have evolved ways of evading RNAi-based defenses, such as proteins that specifically sequester siRNAs or compromise the RISC machinery (Li and Ding, 2001). Endogenous mechanisms regulating RNAi found so far include the phenomenon of RNA editing. In RNA editing, specific adenosine residues in dsRNA are converted to inosines by dsRNA-specific adenosine deaminases (ADARs) (Bass, 2002). Not only do edited transcripts lose homology to targets, but inosine residues are poorly tolerated by Dicer (Knight and Bass, 2002). Furthermore, it has been shown that abolishing RNAi function in ADAR mutants actually rescues some phenotypes, suggesting that these two pathways are intimately related (Tonkin and Bass, 2003). Genetic screens in *C. elegans* have also identified several other molecules to be negative regulators of RNAi, such as the protein ERI-1 (Enhanced RNAi-1). ERI-1, a ribonuclease with a preference for siRNAs, is specifically expressed in neurons. Loss of ERI-1 function in *C. elegans* facilitates RNAi in the nervous system, which is normally somewhat resistant to dsRNA-mediated gene silencing (Kennedy et al., 2004). The discovery of the many ways that RNAi affects gene expression as well as the numerous control mechanisms built into the system, suggest that dsRNA-mediated silencing must indeed play a central role in cell biology.

BIOLOGICAL FUNCTIONS OF RNA INTERFERENCE

Sequence-Directed Viral Immunity

Eukaryotic cells utilize RNAi for a number of important functions, including defense against viral infection, protecting the genome, silencing heterochromatin, and developmental regulation of endogenous gene expression. As early as 1928, it was observed that while the leaves of a plant inoculated with tobacco virus become sick, secondary leaves from the same plant, which develop later, are resistant to infection (Wingard, 1928). More recently, biologists found that transgenic plants engineered to express viral proteins do not accumulate viral particles upon infection and, conversely, viral infection of wild-type plants can suppress expression of homologous plant genes (Lindbo et al., 1993; Baulcombe, 1996). In 1997, Ratcliff et al. (1997) demonstrated for the first time that all of these observations are related, and that viral defense is closely tied to gene silencing in plants. Since then, it has become clear that both phenomena operate by the RNAi pathways described above. Infection by any type of virus has the potential to produce dsRNA in the cell. RNA viruses produce dsRNA intermediates during replication, while DNA viruses can generate dsRNA upon overlapping transcription of their genomes. These long dsRNA duplexes are then processed into siRNAs, and direct a sequence-specific cellular defense program against these pathogens (Hamilton and Baulcombe, 1999). It remains unknown if RNAi represents a significant component of the antiviral response in the animal kingdom as well. While there is some evidence that it does play a role in flies and worms, work in other organisms has been limited by the innate immune response to viral infection (reviewed by Ding et al., 2004). Introduction of dsRNA duplexes longer than 30 bp into mammalian cells activates

the enzymes dsRNA-dependent protein kinase (PKR) and 2'-5' oligoadenylate synthetase (OAS), unleashing a nonspecific response leading to sequence-independent degradation of mRNAs and broad translational repression (Minks et al., 1979; Williams, 1997).

Transposon Silencing

A second role for RNAi in cell biology also relates to cellular defense, this time against active transposons, which can rapidly compromise the integrity of a cell's genome if left unfettered. Transposable elements litter the genomes of almost all organisms, and constitute 45% of the human genome and 12% of the *C. elegans* genome. The most common transposon in the worm is *Tc1*, which can jump from one location in the genome to another in somatic cells, but not the germline (Tabara et al., 1999). Genetic screens have found that many of the genes required for silencing transposition in the germline are also required for RNAi, suggesting that dsRNA may function to repress transposition (Tabara et al., 1999). Recently, Sijen and Plasterk (2003) demonstrated that siRNAs corresponding to *Tc1* can indeed be isolated from germline cells, and that the parent dsRNA duplexes are formed from read-through transcription of these elements in the genome. The authors of this study propose that germline repression of *Tc1* transposition is most likely due to the posttranscriptional effects of RNAi. However, the discovery of RNA-mediated heterochromatin formation and the fact that retrotransposable elements often interrupt heterochromatic repeats suggest that RNAi has the potential to impact transposable elements at the genome level as well.

Regulation of Embryonic Development

Some of the most exciting roles discovered for RNAi to date are in embryonic development. While the epigenetic effects of dsRNA are sure to have some function, the best understood cases of RNAi in

the embryo involve miRNA-mediated posttranscriptional regulation. In fact, the first miRNA ever described was identified in a forward genetic screen designed to isolate genes that regulate developmental timing in *C. elegans* (Ambros and Horvitz, 1984). When the authors of this study cloned the gene responsible for one of these heterochronic mutations, they found that it did not encode a protein at all (Lee et al., 1993). Instead, *lin-4* encoded a hairpin precursor of a small noncoding RNA (21–22 nt). Characterization of another heterochronic mutant from the same screen led to the identification of its mRNA target. The *lin-4* miRNA can imprecisely base-pair with the 3' untranslated region (UTR) of its target mRNA, encoded by the *lin-14* gene (Lee et al., 1993; Wightman et al., 1993). *Lin-14* had long been known to form a temporal gradient of protein expression, critical for the normal progression of larval development (Ruvkun and Giusto, 1989). The discovery of *lin-4* solved the puzzle of how this gradient was generated. *Lin-4* miRNA expression leads to translational repression of *lin-14*. This landmark work was the first sign that miRNAs could have a significant impact on developmental regulation. miRNA function in embryos is not just limited to developmental timing, but also extends to other major processes such as cell fate determination. Investigators in the Hobert laboratory (Oliver Hobert, Columbia University, NY, NY) were interested in understanding how asymmetry is established in the *C. elegans* nervous system. They focused on two sensory neurons in particular: the ASE left (ASEL) and ASE right (ASER) neurons. These two neurons express distinct chemoreceptors and, therefore, recognize different stimuli in the environment (Pierce-Shimonura et al., 2001). The Hobert group initiated a forward genetic screen to try to find genes that regulate the assignment of ASE identity, and looked for mutants that either had two ASEL or two ASER neurons, rather than one of each (Chang et al., 2003). While a number of mutants were defective in various transcription factors,

they found one which did not correspond to a defect in a protein-coding gene. This mutation traced back to the gene *lisy-6* (for lateral symmetry defective), which turned out to encode the hairpin precursor for a miRNA (Johnston and Hobert, 2003). The *lisy-6* miRNA can base-pair with the 3' UTR of its target mRNA, and lead to translation repression of its protein product, the transcription factor Cog-1 (Fig. 2A). Cog-1 is at the top of a transcriptional cascade that promotes expression of the ASER chemoreceptor and blocks expression of the ASEL chemoreceptor (Chang et al., 2003). Selective expression of the *lisy-6* miRNA, only in ASEL neurons, leads to a drop in Cog-1 protein levels and a resulting upregulation of the ASEL chemoreceptor (Fig. 2B). In a fascinating addition to this story, it has been found that another miRNA named *mir-273* also acts in this pathway, several steps upstream of *lisy-6* (Fig. 2B) (Chang et al., 2004).

The limited expression of these miRNAs, both in terms of location and timing, offers compelling proof that miRNAs can influence development with exquisite specificity in *C. elegans*. miRNAs with developmental functions have now been identified in many different organisms, in addition to the nematode. In *D. melanogaster*, for example, microRNAs *bantam* and *mir-14* modulate the balance between apoptosis and cell proliferation (Brennecke et al., 2003; Xu et al., 2003). Working out the role of RNAi in mammalian development, however, has proven to be a much more formidable challenge. The early embryonic lethality of mice that lack certain components of the RNAi machinery, such as Dicer, hints that dsRNA processing is critical for normal development (Bernstein et al., 2003; Yang et al., 2005). However, identifying functional miRNAs has been difficult because mammalian systems are not amenable to forward genetics, and most miRNAs are not abundant transcripts. One mammalian miRNA that was cloned because of its relative abundance in hematopoietic cells is mouse *mir-181*. *Mir-181* is normally expressed at low

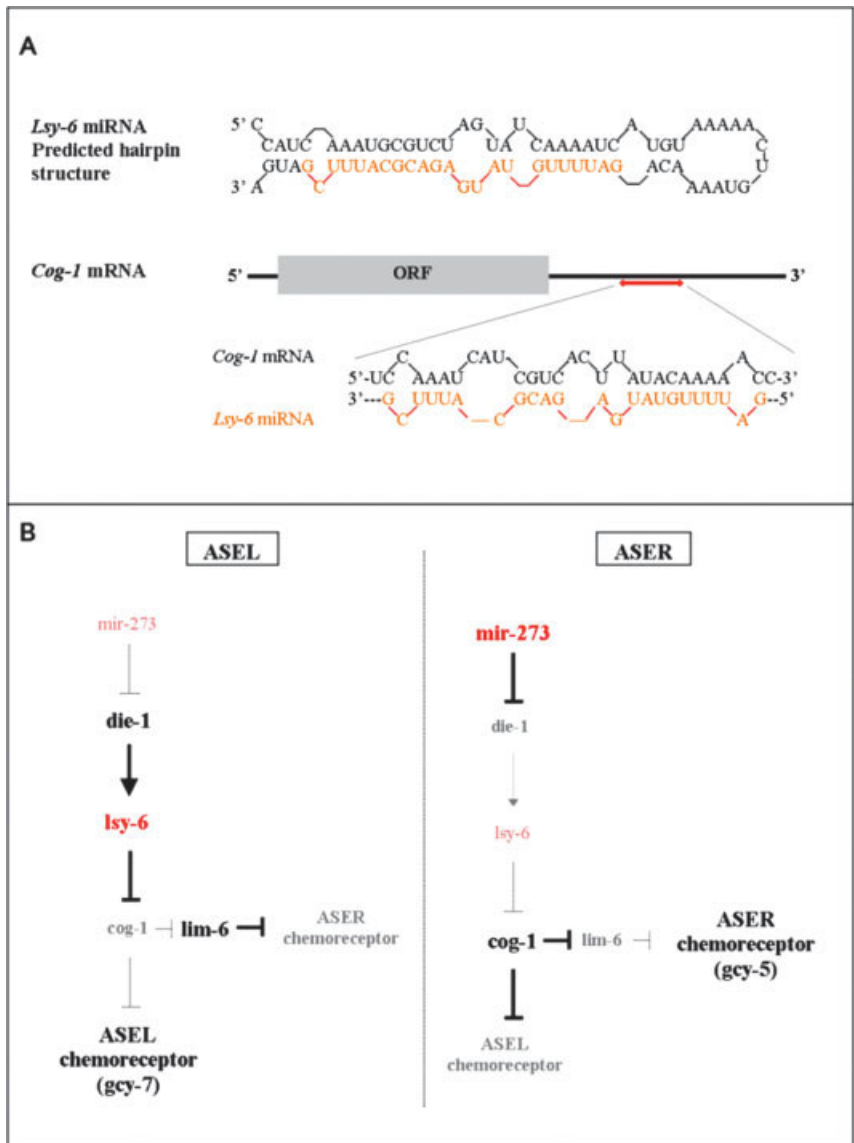


Figure 2. Regulation of cell fate specification in the *C. elegans* nervous system by differential expression of microRNAs (miRNAs). **A:** Predicted hairpin structure of *lisy-6* pre-miRNA, with proposed 23-nt miRNA in red. This miRNA silences expression of its target gene *cog-1* by binding to a site on the 3' untranslated region of the *cog-1* transcript, as shown. **B:** Genetic pathway leading to left-right specification of ASE chemosensory neurons in *C. elegans*. In ASEL, low levels of *mir-273* permit high expression of its target, the transcription factor *die-1*. Die-1 activates *lisy-6* expression, which goes on to silence its target *cog-1*, another transcription factor. Cog-1 silencing blocks expression of the ASER chemoreceptor and derepresses expression of the ASEL chemoreceptor *gcy-7*. In the ASER neuron, however, high expression of *mir-273* limits *die-1* activation of *lisy-6*, leading to increased expression of *cog-1*. Cog-1 is then able to block expression of the ASEL chemoreceptor, while disinhibiting expression of the ASER chemoreceptor (*gcy-5*).

levels in hematopoietic progenitors, and upregulated as they differentiate into B-cells in the bone marrow and thymus (Chen et al., 2004). When it is overexpressed in progenitors, they give rise to a greater fraction of B-lymphoid cells than normal, in vitro as well as in vivo. These data demonstrated that

mir-181 is capable of influencing cell fate in the hematopoietic system and could play a role in B-cell differentiation.

The relatively few known examples of miRNA regulation of development are likely to represent just the tip of the iceberg. A number of groups have undertaken bioinforma-

matics screens to try to identify miRNA-coding genes in the genome, based on the characteristics of known miRNAs (reviewed by Ambros, 2004). So far, they have focused their search on miRNAs with particular physical qualities, which have complementarity to the phylogenetically-conserved 3' UTRs of other genes. While these studies often produce vastly different sets of candidates, most agree that vertebrate genomes probably encode at least 250 miRNAs, while the genomes of *C. elegans* and *D. melanogaster* contain on the order of 100 different miRNA genes. It is important to note that most of the known miRNAs, including *lisy-6*, were not identified by cDNA cloning or these computational approaches (Ambros, 2004). Rather, they required forward genetic screens and careful examination of sometimes subtle phenotypes in order to elucidate their existence and role in development. This suggests that the vast number of remaining miRNAs with undetermined functions is sure to keep developmental biologists busy for a long time to come.

USING RNA INTERFERENCE TO STUDY VERTEBRATE DEVELOPMENT

Loss-of-Function Techniques in Developmental Biology

RNAi is unique within biology because not only is it an important regulatory mechanism within cells, but it also represents a major technical advance in biomedical research. Two common approaches to the study of gene function are loss- and gain-of-function experiments. However, technical restrictions have often limited the application of these approaches to subsets of model systems, especially with respect to in vivo studies of vertebrate development. In general, techniques such as in ovo electroporation, injection of recombinant viruses, and transgenic technology have made gain-of-function strategies tractable in most systems. Loss-of-function experiments, however, have remained considerably more

difficult. Until the advent of RNAi, zebrafish had been the system of choice for these experiments, because gene expression could be silenced by injection of antisense DNA or morpholinos, chemically-modified nucleic acids. Both of these antisense strategies, however, were often plagued with nonspecific side effects and variability in penetrance from one embryo to the next (reviewed by Heasman, 2002; Kos et al., 2003). High doses of antisense DNA can be toxic to cells, and silencing effects are often short-lived because single-stranded DNA is highly susceptible to degradation by nucleases present within cells. While morpholinos are less toxic and more nuclease-resistant than DNA oligonucleotides, their use demands reliable sequence information for a specific 30-bp stretch of nucleotides spanning the start codon. Furthermore, both approaches function by inhibiting translation, requiring the generation of antibodies against the protein of interest in order to monitor loss of gene product. Researchers interested in the development of vertebrate organisms other than zebrafish were left with few options other than gene targeting in the mouse, a significant investment of time, labor, and financial resources. Given the quickly accumulating wealth of genomic information, developmental biology is in need of a rapid means of evaluating gene function.

The application of RNA interference to the study of embryonic development offers just such a possibility. In order to effectively use RNAi as a tool for genomic analysis, a number of questions need to be addressed in advance. First, what is the best dsRNA substrate for achieving maximal gene silencing with minimal off-target effects? Second, are plasmid-based systems a superior alternative to dsRNA? Third, what methods can one use to introduce these RNAi effectors in vivo? Finally, how does one go about determining loss of gene product and, if so, is there truly a resulting developmental phenotype? In the following section, we address all of these issues, and also discuss the control exper-

iments necessary to validate any observed loss-of-function phenotypes.

RNAi Effector Molecules: From RNA to DNA

After its initial characterization, RNAi became widely used to study the biology of organisms such as *C. elegans* and *D. melanogaster*, because even crude exposure to dsRNA seemed to initiate highly sequence-specific gene silencing in these systems. One simply had to transcribe a gene of interest in both directions, anneal the two strands of RNA, and introduce the resulting duplexes into embryos by injection or feeding (Kennerdell and Carthew, 1998; Timmons and Fire, 1998). While there was one report that in ovo electroporation of long duplexes was similarly effective in chick (Pekarik et al., 2003), it seemed that this technology could not be more widely applied to the study of vertebrate development, because introduction of long dsRNA duplexes into mammalian cells triggered a non-specific interferon response (Stark et al., 1998). Pioneering work by El Bashir et al. (2001), however, demonstrated that dsRNA duplexes less than 30 bp in length can elude this innate immune response. This group went on to show that transfection of mammalian cells in vitro with commercially-synthesized siRNAs leads to dramatic, sequence-specific silencing of homologous genes (Fig. 3A) (El Bashir et al., 2002). A third alternative are esiRNAs, generated by endonuclease digestion of long dsRNA duplexes into smaller 15–40-bp fragments (Fig. 3A) (Yang et al., 2002). Their small size makes them unlikely to trigger an immune response and there is some evidence that in vivo electroporation of esiRNAs leads to a transient loss-of-function in mouse embryos (Calegari et al., 2002).

The choice of which substrate to use when designing experiments (long dsRNA duplexes, esiRNAs, or siRNAs), will depend on the developmental model system as well as the nature of the investigation (Fig.

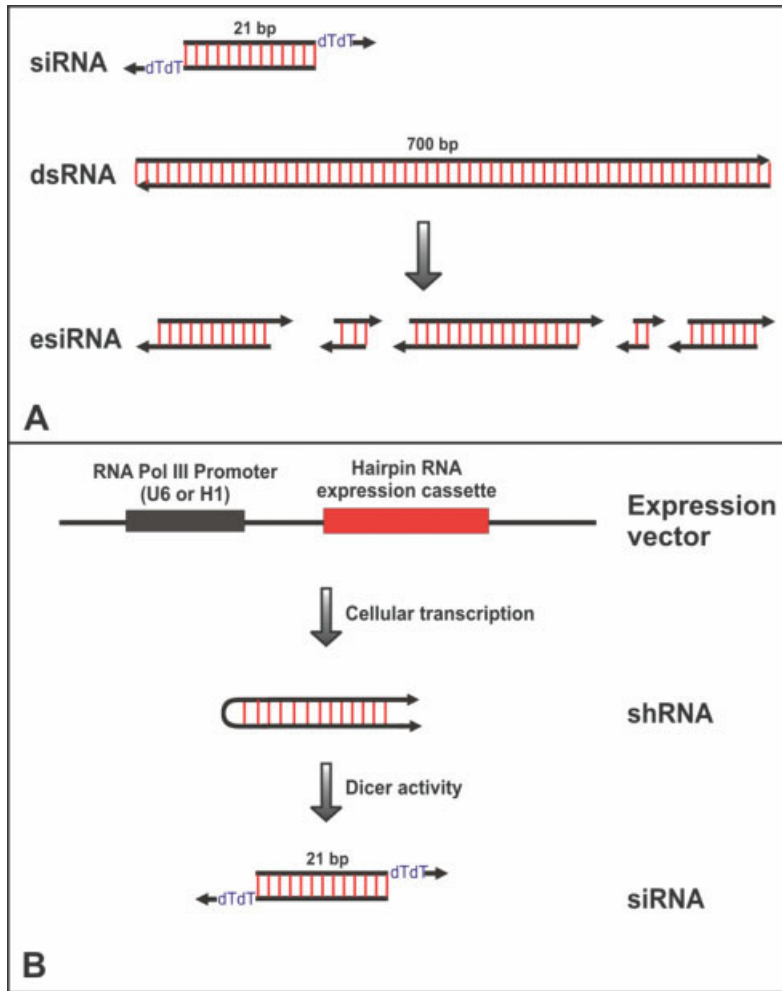


Figure 3. Experimental triggers for gene silencing. **A:** The three types of double-stranded RNA (dsRNA) that can be used to trigger RNA interference (RNAi) include short, interfering RNA (siRNA), long dsRNA duplexes (dsRNA), and endonuclease-digested siRNAs (esiRNAs). **B:** DNA constructs encoding short, hairpin RNA (shRNA) can also be used to initiate RNAi. Once these shRNAs are converted into siRNAs by the enzyme Dicer, they can go on to silence gene expression.

3A). For example, all three types of dsRNA have proven to be effective to various degrees in chick embryos; but siRNAs produce the greatest silencing with the least toxicity (Rao et al., 2004). In mammalian systems, interferon responses make the use of long dsRNA duplexes simply impractical. In these organisms, siRNAs tend to be favored over esiRNAs because they are less likely to have nonspecific effects. esiRNAs represent a mixture of duplexes with different nucleotide sequences, some of which may target genes other than the one of interest. siRNAs, on the other hand, can be screened by sequence comparison during the design phase to minimize cross-reac-

tive silencing. Furthermore, siRNAs require minimal sequence information about a gene to do functional analysis, because they can effectively target any portion of its transcript, the open reading frame (ORF), as well as untranslated regions. It is important to note the one major drawback to using siRNAs: cost. This can represent a significant issue since different siRNAs targeting the same gene can have a range of efficacies, and one often needs to try several siRNA sequences before finding one that works well enough to use reliably. Several groups have conducted systematic analyses of the physical characteristics of highly functional siRNAs (Reynolds et al., 2004). In

addition to identifying particularly effective nucleotide combinations, these studies have found that whichever strand of the RNA duplex is most easily unwound from 5' to 3' will be preferentially assembled into the RISC complex (Schwarz et al., 2003). This has led to the conclusion that siRNAs designed to be unstable at the 5' end of the antisense strand are the most effective, and have the least likelihood of sense strand-directed silencing (Khvorova et al., 2003). These insights on RISC assembly and siRNA characteristics have been incorporated into a number of software programs, both commercial and public access, which can help identify the best siRNA targets for a user-defined sequence (<http://web.mit.edu/mmcmanus/www/home1.2files/siRNAs.htm>; <http://bioinfo.wistar.upenn.edu/siRNA/siRNA.htm>). While these advances help make siRNAs feasible for most experiments, the cost of each duplex does render these small RNAs less suitable for large screens. These types of high-throughput experiments can instead employ either long dsRNA duplexes in the chick (Pekarik et al., 2003) or endonuclease-digested pools of esiRNAs in mammalian cells (Kittler et al., 2004).

Another way to use RNAi to study development is to avoid RNA all together. Plasmid-based systems have been developed to express short, hairpin RNAs (shRNA) that can be processed by Dicer and serve as a template for sequence-specific silencing (Fig. 3B) (Paddison et al., 2004a). shRNAs are single-stranded RNA molecules containing inverted repeats, much like miRNA precursors, that allow them to form intramolecular duplexes. The first shRNA plasmids to be developed utilized RNA polymerase III promoters and transcription termination sequences from genes coding for endogenous small RNAs, such as U6 and H1 (Sui et al., 2002). A second generation of plasmids has since been developed, based on new insights on miRNA processing. These plasmids can incorporate flanking sequences that permit RNA

polymerase II transcription and shRNA expression cassettes optimized for Droscha/Dicer processing (Paddison et al., 2004a). The principal advantage of using shRNAs as effectors for RNAi is that continued expression from the DNA construct maintains shRNA levels over time. Unlike siRNAs, esiRNAs, or long dsRNA duplexes, which can only deliver transient silencing due to the limited half-life of RNA in the cell, shRNA expression vectors can produce long-lasting effects. An additional benefit is that shRNA expression vectors can be engineered to contain selectable markers to generate stable transfectants, or coexpress reporter genes such as GFP to aid identification of transfected cells (reviewed by Hannon and Conklin, 2004). shRNA-based systems are optimal for experiments that necessitate analysis of loss-of-function >72 hours after gene silencing. They are also more amenable to high-throughput screens in mammalian cells than siRNAs. While the initial investment required to generate a library of shRNA expression constructs can be significant, once generated, this library could be amplified and reused indefinitely with minimal additional expense. When using shRNA vectors, one additional caveat is that it is absolutely essential to confirm the sequence of the final expression cassette. Almost every step in the construction of these cassettes from oligonucleotide synthesis to cloning and bacterial amplification is prone to introducing errors. Given the sequence sensitivity of RNAi effects, it is important to scrutinize the composition of shRNAs to maximize target silencing and minimize cross-reactivity. Of note, many of the rules of rational target design developed for siRNAs are likely to be applicable to shRNA design as well. In fact, two recent publications have suggested that a hybrid version of these two substrates, synthetic shRNAs, are actually more potent at silencing gene expression than siRNAs (Siolas et al., 2004; Kim et al., 2005).

Methods of RNAi Effector Delivery: In Vitro and In Vivo

Once an appropriate substrate has been chosen, the next step in designing an RNAi-based experiment is deciding how this substrate will be delivered. In vitro delivery is very straightforward. dsRNA molecules of all lengths, as well as shRNA expression constructs, can be introduced into cells in culture by standard methods of transfection. siRNA transfection is significantly less efficient than plasmid DNA transfection, but can be optimized by using lipid-mediated transfection reagents specifically designed for this purpose (Spagnou et al., 2004). One advantage of an shRNA-based approach is that cells which prove difficult to transfect, such as some primary cultures, can be infected with viruses engineered to express shRNAs (Hannon and Conklin, 2004).

In vivo studies do represent more of a challenge, but many of the techniques used for gain-of-function approaches can be adapted for RNAi experiments. In the chick embryo, for example, in ovo electroporation has been widely employed to misexpress genes in various developing organ systems including the neural tube, the eye, and somites (Krull, 2004). Conversely, this technique can also be used to silence genes by electroporating shRNA-encoding plasmids or one of the three types of dsRNA directly into any of these tissues (Pekarik et al., 2003; Chestnutt and Niswander, 2004; Rao et al., 2004). Regions less accessible to electroporation can be inoculated with viruses expressing shRNAs targeting the genes of interest. Almost all of the available recombinant viral vectors have been modified to express shRNAs. Similarly, transgenic technology in mice has also been adapted for RNAi. Instead of using constructs which code for proteins, constructs are made to express shRNAs targeting the gene of interest (Hasuwa et al., 2002; Kunath et al., 2003). This provides a relatively rapid means of evaluating loss of gene function in the mouse

embryo, without the difficulties of gene targeting. In addition, genes can be conditionally silenced in a stage- or tissue-specific manner by designing these constructs with upstream stop sequences flanked by LoxP recombination sites (Fritsch et al., 2004). Just as in conditional gene targeting, crossing these shRNA transgenic mice with mice expressing Cre recombinase will yield embryos with loss of target gene product only in cells that express Cre. Regional electroporation and viral infection of shRNA expression constructs have also proven to be fruitful in mouse, particularly in the developing nervous system (Konishi et al., 2004; Matsuda and Cepko, 2004). In the case of one gene called *doublecortin*, while traditional gene targeting by several different groups did not prove very informative, viral RNAi experiments were singularly effective at uncovering gene function in vivo (Bai et al., 2003).

Analysis of RNAi-Based Loss-of-Function Experiments

The first step in analyzing an RNAi experiment is to ask, did the manipulation achieve loss of target gene product? One can check for silencing at the protein level by Western blot or immunohistochemical staining. The benefit of an RNAi approach to loss-of-function experiments is that if antibodies against the targeted protein are not available, silencing can be evaluated at the mRNA level by Northern blot or in situ hybridization. In our experience, there tends to be a notable degree of variability in the extent of silencing from one embryo to the next with in vivo applications of RNAi (M. Rao and S. Sockanathan, unpublished results). This suggests that it might be important to evaluate every experimental embryo for loss of gene product, prior to analyzing it for a loss-of-function phenotype.

Once embryos have been generated with sufficient gene silencing to warrant further analysis, one can proceed with the phenotypic analysis. Before drawing any conclusions

about gene function, however, one has to consider the potential for on- as well as off-target effects of RNAi. On-target effects are the desired effects, resulting from sequence-specific silencing of targeted genes. Off-target effects include any undesired effects on the remainder of the transcriptome, and can be sequence-dependent as well as sequence-independent. Sequence-dependent effects result from homology between the dsRNA or shRNA and untargeted transcripts in the cell. Large scale microarray experiments have found that siRNAs can downregulate expression of transcripts that have as few as 11 out of 21 bp in homology (Jackson et al., 2003). These effects are largely preventable by careful design of siRNA and shRNA sequences. Once a list of candidate siRNAs is generated for a particular gene target, basic local alignment search tool (BLAST) searches against both the nonredundant (nr) and expressed sequence tag (EST) databases on national center for biotechnology information (NCBI) can help identify those which have homology to untargeted genes. While it may be impossible to identify a candidate that has less than 11 bp of homology to every untargeted gene in the genome, one should try to use one which at least meets the following criteria: less than 17 bp in consecutive matches between the antisense strand of the duplex and untargeted mRNAs (Huppi et al., 2005), and minimal homology at the 5' end of the antisense strand (nts 2–12) (Haley and Zamore, 2004). Sequence-independent off-target effects of dsRNA-mediated silencing are much more difficult to avoid. Even small RNA duplexes, which can elude the interferon response, can potentially interact with other dsRNA binding proteins leading to off-target effects. While many studies have shown that siRNAs and shRNA can have a global effect on gene expression, it remains unclear what pathway mediates these effects (Persengiev et al., 2004). The only known variable is dsRNA concentration, as these effects seem to be dose-dependent in every case. One can therefore minimize off-target effects by designing siRNAs and shRNAs

with great care, and limiting the dose as much as possible.

Regardless of how specific a siRNA or shRNA is predicted to be, the only way to conclude that a phenotype is truly due to loss of gene function is by empirical evidence. A general consensus has developed on requiring the following controls for RNAi experiments (Hannon and Rossi, 2004). First and foremost, there should be proportionate decreases in the mRNA and protein levels of the target gene. Second, siRNAs or shRNAs targeting different regions of a gene's transcript should lead to a similar phenotype. Third, this phenotype should be absolutely dependent on the sequence of the dsRNA. There are a number of approaches to address this issue. One can generate siRNAs or shRNAs that differ from the experimental dsRNA at 2–3 bp and show that these are no longer capable of eliciting gene silencing or the null phenotype. Alternatively, some choose to generate control RNA duplexes targeting reporter genes or endogenous genes known to lack a role in the pathway of interest. The advantage of this approach is that control gene silencing indicates that the RNAi machinery is in action, yet the absence of a phenotype in this context suggests that the phenotype is critically dependent on the sequence of the dsRNA template. While it remains worthwhile to try to match the physical characteristics of control and experimental duplexes, scrambled duplexes have lost favor as controls because it is impossible to determine whether they have any biological activity. A fourth control is to demonstrate that the extent of gene silencing is correlated with the dose of dsRNA delivered. Not only does this ensure that dsRNA doses are titrated down to the lowest levels required to trigger an effect, but confirms that gene silencing is indeed dsRNA-dependent. Finally, the gold standard for RNAi controls is the rescue experiment. If coexpression of a version of the gene that is insensitive to the siRNA or shRNA can rescue the loss-of-function phenotype, this offers almost conclusive proof of

specificity. One can engineer such a rescue experiment in several ways. Some have designed siRNAs or shRNAs targeting untranslated regions of the gene of interest and cotransfected them with an expression construct containing only the ORF of this gene. Alternatively, silent mutations can be introduced into the cDNA that abolish homology with the RNA duplex but preserve protein function. Cotransfection of this mutant expression construct along with the RNA duplexes should abolish the loss-of-function phenotype. While all of these controls might not be feasible in every case, it is expected that most of these criteria will be met prior to drawing conclusions about gene function based on RNAi experiments.

Functional Genomic Screens Using RNAi

A number of groups have already begun taking advantage of RNAi to develop high-throughput reverse genetic screens to study gene function. The greatest advances have been made in *C. elegans* because RNAi can be easily induced, and the worm genome is relatively small. Since silencing can be induced simply by feeding worms bacteria expressing dsRNA, libraries of bacterial strains have been generated targeting over 85% of the genes in the *C. elegans* genome (Kamath and Ahringer, 2003). These libraries have already been utilized to study gene function in a number of processes including viability, fat metabolism, and embryogenesis (Piano et al., 2000; Ashrafi et al., 2003; Lee et al., 2003a;). Results from these screens will certainly prove informative to researchers interested in the development of any organism, because many genes are likely to be conserved. So far, the nematode is the only system in which high-throughput reverse genetics have been applied in vivo. This approach in vertebrates continues to face formidable technical challenges. Until experimental manipulation in vertebrate systems becomes more compatible with au-

tomation, this frontier is unlikely to move forward very quickly.

While large in vivo screens have remained intractable in vertebrates, a number of in vitro screens have been developed. So far, most of these studies have focused on identifying new molecules involved in the regulation of cell death and proliferation (Aza-Blanc et al., 2003). In the process, several groups have generated libraries of retroviral shRNA and plasmid siRNA vectors targeting over 9,000 mammalian genes (Berns et al., 2004; Paddison et al., 2004b; Zheng et al., 2004). These libraries can be applied to the analysis of any biological problem that can be modeled in vitro. This possibility is sure to bring exciting advances in our understanding of developmental processes amenable to these experimental conditions, such as growth factor signaling and cell fate specification.

THE THERAPEUTIC POTENTIAL OF RNA INTERFERENCE

Historically, pharmacological therapy for human disease has largely been based on serendipitous discoveries and anecdotal evidence of benefit. In the past few decades, however, the field has taken a dramatic turn away from this precedent and towards the development of rational therapeutics and evidence-based medicine. This is the simple idea that clinicians should try to use drugs that directly target the disease process, and for which there is empirical evidence of therapeutic benefit. This philosophical shift has been fueled by major advances in molecular biology that have shed new light on the pathophysiology of disease. While these advances have improved our understanding of disease, development of drugs to treat these diseases continues to rely on high-throughput screens of small molecules for biological activity. The application of RNAi to clinical medicine has the potential to bridge this gap and translate molecular advances directly into therapy, the ultimate realization of rational therapeutics.

Promising developments in animal models of cancer, infectious disease, and neurodegenerative disorders suggest that this potential may be realized in the near future.

Inhibiting Tumorigenesis with RNAi

Antisense technologies have already set the precedent for the utility of inhibiting gene expression in preventing tumor growth. For example, an oligonucleotide targeting the antiapoptotic gene *Bcl2* has shown promise for treating metastatic skin cancer in clinical trials (Buchele, 2003). Given the potency of dsRNA-mediated silencing relative to antisense technologies, RNAi has the potential to significantly improve upon these effects. It is first being explored for the treatment of chronic myelogenous leukemia (CML), caused by a chromosomal translocation that generates a constitutively active form of the Abl tyrosine kinase. siRNAs targeting the fusion transcript can specifically silence the oncogenic fusion protein while preserving expression of the wild-type form (Scherr et al., 2003; Wohlbold et al., 2003). Pancreatic and colon carcinomas are also neoplasms associated with specific genetic changes, often point mutations in *Ras* oncogenes. Retroviral vectors expressing shRNAs targeting the mutant transcripts have been shown to selectively silence the *K-RAS^{V12}* mutant, and limit tumor growth (Brummelkamp et al., 2002). Rapidly accumulating data on the types of mutations associated with various types of cancer will provide a long list of targets for this type of RNAi-based approach.

Another strategy for applying RNAi to cancer therapy is to use it to improve existing therapies. For example, tumors often become resistant to standard pharmacological treatments over time by initiating expression of MDR1, a multidrug transporter that cells can use to expel chemotherapeutic drugs. One group has transfected tumor cells with siRNAs targeting MDR1 to demonstrate that RNAi can be used to maximize the benefit of existing therapies (Nieth et al., 2003).

Fighting Infection and Inflammation with dsRNA

The greatest promise for RNAi in clinical medicine may be for the treatment of infectious disease and its inflammatory sequelae. This is hardly surprising, given the evolutionary role for dsRNA-mediated silencing in defense against viral infection. To date, viruses that infect the liver have received the most attention, because of the ease of nucleic acid delivery to this organ in mice by tail injection and hydrodynamic shock (Lewis et al., 2002; McCaffrey et al., 2002). Viral hepatitis is a major health problem that can lead to acute liver failure, cirrhosis, and liver cancer as a result of chronic infection. While a vaccine is available to prevent infection with hepatitis B virus (HBV), once infected there is no treatment (Poland and Jacobson, 2004). Similarly, although 3% of the world's population is infected with hepatitis C virus (HCV), there is no vaccine and few options for treatment (Davis, 1997). To date, two studies using tail vein injection of shRNAs targeting the HBV and HCV replicons have demonstrated the ability of RNAi to selectively repress viral protein expression in vivo (McCaffrey et al., 2002, 2003). These findings offer new hope for hepatitis as well as other viral diseases with poor prognoses.

One limitation of this strategy is that viruses such as HIV, which can evolve very rapidly, could modify their genomes to elude sequence-dependent silencing. An alternate approach is to use RNAi to either target host proteins required for pathogen invasion or signaling pathways that initiate the inflammatory response, which is often more damaging than the infection itself. One exciting study used shRNAs targeting a host protein to try to alleviate hepatitis in an animal model of acute liver failure (Song et al., 2003). In this in vivo study, siRNAs targeting the cell death receptor Fas not only reached the liver, entered hepatocytes, and blocked Fas expression, but were able to limit disease severity and extend the survival of treated animals.

New Hope for Genetic Disease and Developmental Disorders

More than any others, patients with dominant genetic disorders may stand to benefit the most from RNAi-based approaches. Despite decades of research into these diseases, most have remained immune to standard therapies. Moreover, their pathology usually derives from small changes in the nucleotide sequences of genes. The sequence-specificity of RNAi, therefore, makes it an ideal candidate for treating these conditions. dsRNA can be engineered to selectively target mutant transcripts, while leaving the function of wild-type alleles unaltered. The most obvious strategy is to generate dsRNA targeting the disease mutation itself. This approach has shown some promise in cellular models of amyotrophic lateral sclerosis (ALS) (Ding et al., 2003). An alternative strategy is to design siRNAs targeting single-nucleotide polymorphisms (SNPs) in mutant alleles. Systematic analysis of siRNA composition has found that single-nucleotide mismatches are the most compromising when located in the center of the siRNA (Miller et al., 2003). One group has demonstrated that siRNAs designed this way to target SNPs in mutant alleles can inhibit expression of the mutant allele and decrease disease pathology in vitro (Miller et al., 2003, 2004). Testing these principles at the in vivo level is still in its infancy. Recent work by Xia et al. (2004) showed that intracerebral delivery of adenoviral vectors encoding shRNAs targeting a mutant allele can diminish intranuclear inclusions and restore motor coordination in a mouse model of spinocerebellar ataxia type I. Their results bring new hope for the treatment of a vast spectrum of polyglutamine expansion diseases. Although most of the work thus far applying RNAi to genetic disease has been in neurodegenerative conditions, it could in theory be used to treat any disease associated with inappropriate or harmful gene expression. For example, some developmental disorders such as Down's syndrome, caused by trisomy 21, are attributed to gene dosage imbal-

ances (Antonarakis et al., 2004). Once investigators can hone in on the duplicated genes responsible for phenotypic effects, RNAi could be utilized to diminish their expression to normal levels.

While only dominant or overexpression disorders can be directly targeted by RNAi, indirect strategies in other types of disorders make its application almost limitless. The following represent some of our ideas for these potential applications. First, many loss-of-function disorders can be mitigated by limiting the activity of upstream pathways. For example, lysosomal storage diseases are a group of recessive genetic disorders characterized by their catastrophic effects on human development (reviewed by Futerman and van Meer, 2004). Each of these is caused by a deficiency in a specific lysosomal enzyme, which leads to accumulation of upstream metabolites within cells. One of the few interventions found to work in these disorders is to limit dietary consumption of metabolites that could serve as substrates for these pathways. In this same vein, RNAi could be utilized to limit the activity of metabolic pathways that give rise to these substrates and slow disease progression. A second possibility is to apply rapidly advancing research on the role of dsRNA in biological processes, which is uncovering more and more pathways regulated by endogenous miRNAs. These findings could provide new handles for therapeutic manipulation of cell physiology in the context of disease. For example, miRNAs in *C. elegans* have been found to regulate developmental timing and stage-specific processes (Lee et al., 1993). If similar mechanisms are conserved in humans, then exogenous introduction of miRNAs might allow us to reactivate developmental processes to promote repair. Along these lines, one group has found a miRNA expressed in pancreatic β -islet cells that can regulate insulin secretion, representing a novel target for diabetes therapy (Poy et al., 2004). Finally, the marriage of RNAi to emerging stem cell therapies could make a powerful

combination. Investigators have already shown that miRNAs are capable of influencing the differentiation of hematopoietic stem cells (Chen et al., 2004). Misexpression of miRNAs could be used to generate pure populations of differentiated B-cells or other cell types for use in ex vivo approaches. While all of these are exciting possibilities for the therapeutic potential of RNAi, there are clearly many hurdles to overcome before they can move from the bench to the bedside.

Challenges to RNAi-Based Therapies

The obstacles to using RNAi in clinical medicine are two-fold: delivery and the potential for nonspecific effects. Just as with antisense oligonucleotide-based approaches, the primary challenge in bringing RNAi to the bedside is achieving efficient delivery of nucleic acids into cells. In mice, large volumes of fluid injected into the tail vein cause a rapid increase in intravascular pressure that can drive siRNAs into cells in vivo (Lewis et al., 2002; McCaffrey et al., 2002). While this hydrodynamic delivery approach is not feasible for systemic treatment of humans, it could be used for local delivery of siRNAs into organs such as the liver, kidney, heart, and lungs (Dillon et al., 2004). A promising development is that intravenous and intraperitoneal administration of siRNAs in mice does lead to systemic distribution, suggesting that parenteral administration may work in humans as well (Filleur et al., 2003; Sorensen et al., 2003). However, an important limitation to consider is that the lifespan of RNA duplexes in the bloodstream is likely to be only a few days (Lewis et al., 2002). An alternative to siRNAs is to use viral vectors coding for shRNA transcripts. This is the approach taken by gene therapy and, therefore, conjures up all of the same safety concerns. Viral integration into the genome has the potential for insertional mutagenesis, leading to inappropriate activation or inactivation of endogenous genes. Given the shaky nature of gene therapy clinical trials in the

past, the focus has remained on optimizing RNA duplex delivery. Many groups are actively exploring methods of transfection such as cationic liposomes, as well as modifying the structure of siRNAs to improve bio-distribution and limit nuclease sensitivity (reviewed by Paroo and Corey, 2004).

The second obstacle is one that must be considered for any therapeutic measure, the potential for nonspecific toxicity. Since RNAi is an endogenous pathway with its own cellular machinery, it is possible that utilizing this machinery for therapeutic siRNAs could disrupt its intrinsic functions. Moreover, there is some evidence that even small RNA duplexes such as siRNAs and shRNAs can trigger an interferon response or broad transcriptional silencing (Sledz et al., 2003). Further work into the basic biology of RNAi should improve siRNA design and curb the potential for any non-specific effects.

CONCLUSION

A series of observations on the activity of dsRNA in model organisms such as the nematode has led to a revolution in cell biology. This RNAi revolution, in turn, has a number of implications for the study of vertebrate development. First, dsRNA-mediated silencing represents a novel regulatory mechanism that needs to be studied in the context of developmental gene expression. Second, RNAi as a technique offers an unparalleled degree of ease and specificity for conducting loss-of-function experiments in vitro and in vivo. When used with the appropriate controls, it can be invaluable as a tool to investigate gene function in embryonic development on any scale, from studying single genes to surveying entire genomes. Finally, RNAi represents a whole new prospect for treating human disease, providing a template that the cell's own machinery can use to limit expression of harmful genes. In the coming years, new insights into the basic biology of RNAi are sure to yield ways of improving the specificity and delivery of dsRNA, which will advance all of these frontiers.

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