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RNA Interference in Biology and Medicine

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***Abstract*——First discovered in plants the nematode *Caenorhabditis elegans*, the production of small interfering RNAs (siRNAs) that bind to and induce the degradation of specific endogenous mRNAs is now recognized as a mechanism that is widely employed by eukaryotic cells to inhibit protein production at a post-transcriptional level. The endogenous siRNAs are typically 19- to 23-base double-stranded RNA oligonucleotides, produced from much larger RNAs that upon binding to target mRNAs recruit RNases to a protein complexthat degrades the targeted mRNA. Methods for expressing siRNAs in cells in culture and in vivo using viral vectors, and for transfecting cells with synthetic siRNAs, have been developed and are being used to establish the functions of specific proteins in various cell types and organisms. RNA interference methods provide several major advantages over prior methods (antisense DNA or antibody-based techniques) for suppressing gene expression. Recent preclinical studies suggest that RNA interference technology holds promise for the treatment of various diseases. Pharmacologists have long dreamed of the ability to selectively antagonize or eliminate the function of individual proteins—RNAi technology may eventually make that dream a reality.**

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I. Introduction

RNA interference (RNAi[1]), as commonly defined, is a phenomenon leading to post-transcriptional gene silencing (PTGS) after endogenous production or artificial introduction into a cell of small interfering double strand RNA (siRNA) with sequences complementary to the targeted gene (Bosher and Labouesse, 2000). Whereas the transcription of the gene is normal, the translation of the protein is prevented by selective degradation of its encoded mRNA. However, PTGS is not restricted to RNAi and has emerged as a more complex mechanism that involves several different proteins and small RNAs. It is presumed that cells employ RNAi to tightly regulate protein levels in response to various environmental stimuli, although the extent to which this mechanism is employed by specific cell types remains to be discovered. However, the fact that RNAi is operative in cells of organisms ranging from plants, to nematodes and flies, and to mammals attests to its fundamental importance in the selective suppression of protein translation by targeted degradation of the encoding mRNA. Beyond its biological relevance, PTGS is emerging as a powerful tool to study the function of individual proteins or sets of proteins. User-friendly technologies for introducing siRNA into cells, in culture or in vivo, to achieve a selective reduction of single or multiple proteins of interest are rapidly evolving. The present article reviews this emerging technology, findings obtained to date using such RNAi methods, and the potential of RNAi-based therapeutics for treating human disease.

II. Principles of RNA Interference

RNA interference most likely evolved as a mechanism for cells to eliminate unwanted foreign genes. Foreign genes are often present in cells at high copy numbers, being present as viral genes, transposable elements, or as plasmids introduced experimentally in cell transfection protocols. It has been known for several decades that the level of expression of transgenes usually decreases as the number of copies present in the cell increases and that endogenous homologous genes can also be suppressed by the presence of the transgene (Napoli et al., 1990). Although such gene silencing can occur at the transcriptional level, it is now recognized that a major mechanism of gene suppression occurs post-transcriptionally, and that a major mechanism for this PTGS is RNAi, the selective degradation of mRNAs targeted by siRNAs (Van Blokland et al., 1994). Such PTGS via RNAi can occur very rapidly with proteins for many genes, being decreased within hours, and completely absent within 24 h (Pruss et al., 1997). Based upon these and other findings initially made in studies of plants (Ratcliff et al., 1997), it seems very likely that RNAi evolved as a mechanism to defend plant cells against viral infections.

A. Post-Transcriptional Gene Silencing and the Discovery of RNA Interference

PTGS and RNAi were discovered in genetic transformation studies of eukaryotic cells, principally plants and worms, wherein it was shown that mRNAs for the encoded transgene alone, or together with mRNAs for homologous endogenous genes are very low or absent despite high levels of transcription (Fire, 1999; Marathe et al., 2000). The ability to manipulate and monitor gene expression in the plant *Arabidopsis thaliana* and the roundworm *Caenorhabditis elegans* (the genomes of both species are now complete) revealed the process of RNAi and allowed the relatively rapid identification of several genes that regulate the RNAi process.

Transgenes insert into the genomes of plants by recombination in an apparently random manner so that the number of inserted copies, their chromosomal location, and their local arrangement within the chromosome vary among transformants. The observation of an inverse correlation between copy number and the level of gene expression suggested that an increased copy number of a particular gene results in silencing of that gene (Assaad et al., 1993). It was initially thought that such gene silencing was due to reduced gene transcription resulting from interactions between closely linked copies that result in the formation of secondary structures that promote methylation and inhibition of transcription (Ye and Signer, 1996). Further studies showed that transcriptional gene silencing (TGS) could also occur in trans, such that one transgene can be silenced by another transgene introduced either by crossing or transformation. It was then proposed that a silencing RNA is produced by one locus that somehow effects the silencing of the other gene by a mechanism involving RNA-mediated inhibition of transcription (Mette et al., 2000). Although some data were consistent with such mechanisms of transcriptional silencing, additional data suggested the involvement of PTGS. The presence of double-stranded RNAs (dsRNAs) and their cleavage into siRNAs of approximately 23 nucleotides was demonstrated, and it was then shown that expression of dsRNA with sequences corresponding to open reading frames in plants results in PTGS (Hamilton and Baulcombe, 1999). Similarly, expression of dsRNAs with sequences complementary to those of endogenous genes

[1]Abbreviations: RNAi, RNA interference; AIF, apotosis-inducing factor; dsRNA, double-stranded RNA; IAP, inhibitor of apoptosis protein; PTGS, post-transcriptional gene silencing; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; siRNA, small interfering RNA; IP_3, inositol 1,4,5-trisphosphate; NMD, nonsense-mediated mRNA decay; miRNA, micro-RNA; GFP, green fluorescent protein; NF-κB, nuclear factor-κB; HIV, human immunodeficiency virus; TNF, tumor necrosis factor; TGS, transcriptional gene silencing.

results in the selective silencing of those genes in *C. elegans* (Zamore et al., 2000). Collectively, the studies of *A. thaliana* and *C. elegans* showed that both TGS and PTGS can be initiated by the same RNA degradation pathway—TGS occurs when the dsRNA includes promoter sequences, whereas PTGS occurs when the dsRNA includes coding sequences. Although degradation of dsRNA is common to both mechanisms of gene silencing, the results also indicated that dsRNA-mediated TGS and PTGS involve different specific steps.

Although RNAi as a mechanism of PTGS was first discovered in plants and may have evolved as a cellular defense mechanism against foreign DNA and RNA, it is very clear that RNAi is widely employed in most if not all eukaryotic cells as a mechanism to regulate the expression of endogenous genes. In 1998, it was discovered that injection of dsRNA was much more effective for silencing of gene expression in *C. elegans* than was single-stranded antisense RNA (Fire et al., 1998). This experimentally induced PTGS, the first report of the use of RNAi as a tool in biology, was very potent, and remarkably, the PTGS occurred not only in the worms to which the dsRNA was administered, but also in their progeny. It was then demonstrated that the endogenous mRNA was the target of the injected dsRNA by a post-transcriptional mechanism and involving degradation of the targeted mRNA (Montgomery et al., 1998). Surprisingly, it was further shown that the dsRNA is effective at very low concentrations, such that the copy numbers of the targeted mRNA are far greater than the number of dsRNAs present in the cell (Fire et al., 1998; Kennerdell and Carthew, 1998). In addition, the suppression of the protein encoded by the targeted mRNA was found to persist through many rounds of cell division. The latter two observations strongly suggested that cells possess a mechanism for amplifying the RNAi mechanism. Not only can the RNAi process be maintained within cells of a common lineage, but it can also be transferred between cells, as shown in *C. elegans* where injection of dsRNA into the intestine results in silencing of the targeted gene in all cells of the F1 progeny of that worm (Fire et al., 1998). Indeed, dsRNA can enter cells and induce PTGS when worms are soaked in a solution containing the dsRNA or when the worms are fed bacteria expressing dsRNA (Tabara et al., 1998; Timmons and Fire, 1998). Recently, a transmembrane protein called SID-1 was identified as a possible mediator of intercellular transfer of RNAi (Winston et al., 2002).

Subsequently, other organisms were assayed for their capacity to induce RNAi. Evidence for RNAi in Drosophila was first demonstrated by Kennerdell and coworkers (Kennerdell and Carthew, 1998) who showed the involvement of the *frizzled* and *frizzled2* genes in the wingless pathway after introduction of dsRNA into embryos. Again, several techniques were developed in order to use dsRNA in this organism leading to the establishment of cell-free (Tuschl et al., 1999) and cell culture models (Caplen et al., 2000). A system that employed dsRNA as an extended hairpin-loop RNA was developed to induce heritable gene silencing (Kennerdell and Carthew, 2000). The *Drosophila* system has allowed the identification of several endogenous genes that play key roles in the RNAi process. An RNA nuclease activity called RISC (RNA-induced silencing complex) was discovered that is responsible for the degradation of endogenous mRNAs, as well as small nucleotide fragments (~25 nucleotides in length), which could be used as guides by RISC (Hammond et al., 2000). They later characterized RISC as a ribonucleoproteic complex (Hammond et al., 2001). These results were soon extended by showing that RNAi is an ATP-dependent and translation-independent event where the introduced dsRNA is processed into 21–23 nucleotide fragments that guide the cleavage of endogenous transcripts (Zamore et al., 2000). The enzyme responsible for the processing of the dsRNA was later discovered as a RNase III family nuclease named Dicer, a protein with high homology to the *rde-1 C. elegans* gene (Bernstein et al., 2001). To study the functions of RNAi in yeast, Volpe et al. (2002) deleted argonaute, dicer, and RNA-dependent RNA polymerase homologs; deletion resulted in the accumulation of complementary transcripts from centromeric heterochromatic repeats and de-repression of transgenes integrated at the centromere and impairment of centromere function. The authors of the latter study proposed that dsRNA arising from centromeric repeats targets the formation and maintenance of heterochromatin through RNAi.

In mammalian cells, RNAi was first employed as a tool to induce the silencing of the targeted gene (Wianny and Zernicka-Goetz, 2000). This approach was partially successful in mouse embryos (Wianny and Zernicka-Goetz, 2000; Svoboda et al., 2001) and embryonic cell lines (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002b) where specific gene silencing was achieved. On the other hand, the introduction of dsRNA into mammalian somatic cells presents a major problem because it can induce (in a manner similar to the silencing observed during viral infection) to the activation of the PKR (protein kinase R) and RNAseL pathway, resulting in the inhibition of protein synthesis and induction of apoptosis (Baglioni and Nilsen, 1983; Clarke and Mathews, 1995; Gil and Esteban, 2000). Interestingly, this shows that, in mammalian cells, the mechanisms for RNA interference are not identical to those in lower organisms although RNAi does operate in at least a subset of mammalian cell types, in a Dicer-dependent manner via post-transcriptional mechanisms (Billy et al., 2001; Paddison et al., 2002b). Elbashir et al. (2001a) had the idea of directly introducing 21–23 nucleotide dsRNA (siRNAs) into mouse and human cells to try to avoid the problems associated with the expression of longer dsRNAs. They showed that the siRNA could efficiently trigger silencing in the mammalian cells.

RNAi mechanisms may provide explanations for various biological phenomena that were previously described, but without any understanding of the possible underlying mechanism. For example, it was recently proposed that RNAi mechanisms could explain the controversial process of RNA-mediated memory transfer in planaria (Smalheiser et al., 2001). It will certainly be of interest to investigate possible roles for RNAi in the many different physiological processes that involve modulation of protein levels at a post-transcriptional level.

B. Mechanism of RNA Interference

A clearer picture of PTGS emerged from several different basic observations, including the necessity of transcriptionally active genes and the ability of RNA viruses to silence a homologous endogenous gene (English et al., 1997). Within the last 3 years, a flurry of studies have identified several of the molecules that mediate RNAi, and the mechanism whereby these molecules effect the selective degradation of targeted mRNAs. It is now clear that the production of dsRNA with sequence complementary to the mRNA being targeted is fundamental to the process of PTGS; single-stranded RNA is not sufficient to induce PTGS. The importance of dsRNA is supported by a wealth of data. Transgenes engineered to synthesize dsRNA require only a few copies of the dsRNA to achieve PTGS and can induce cosuppression. There are several ways such transgenes produce dsRNA including the synthesis of long hairpin mRNAs by transcription of an inverted repeat (Kennerdell and Carthew, 2000; Tavernarakis et al., 2000), and transcription of complementary sense and antisense strands by opposing promoters (Wang et al., 2000). Other studies have shown that although cells may initially produce very long dsRNAs, they are cleaved into smaller dsRNAs, 21–25 nucleotides in length, that actually mediate RNAi (Hamilton and Baulcombe, 1999).

How does a small dsRNA with a sequence complementary to a specific mRNA effect PTGS? The proteins that mediate the RNAi process have been identified using several approaches, most notably genetic screens for mutants resistant to RNAi in *C. elegans* and resistant to PTGS in *Neurospora* and *Arabidopsis* (Fig. 2). These studies have identified homologous genes in each species, and subsequent identification of mammalian homologs, that encode proteins required for RNAi. Three homologous genes identified in the initial screens are *rde-1* in *C. elegans* (Tabara et al., 1999), *qde-2* in *Neurospora* (Cogoni et al., 1996) and *ago-1* in *Arabidopsis* (Dalmay et al., 2000; Fagard et al., 2000). The proteins encoded by each of these genes share homologies with the eIF2C translation factors. In *C. elegans*, *rde-1* is required for germline transmission of RNAi, but not for transmission of RNAi among cells of the worm (Grishok

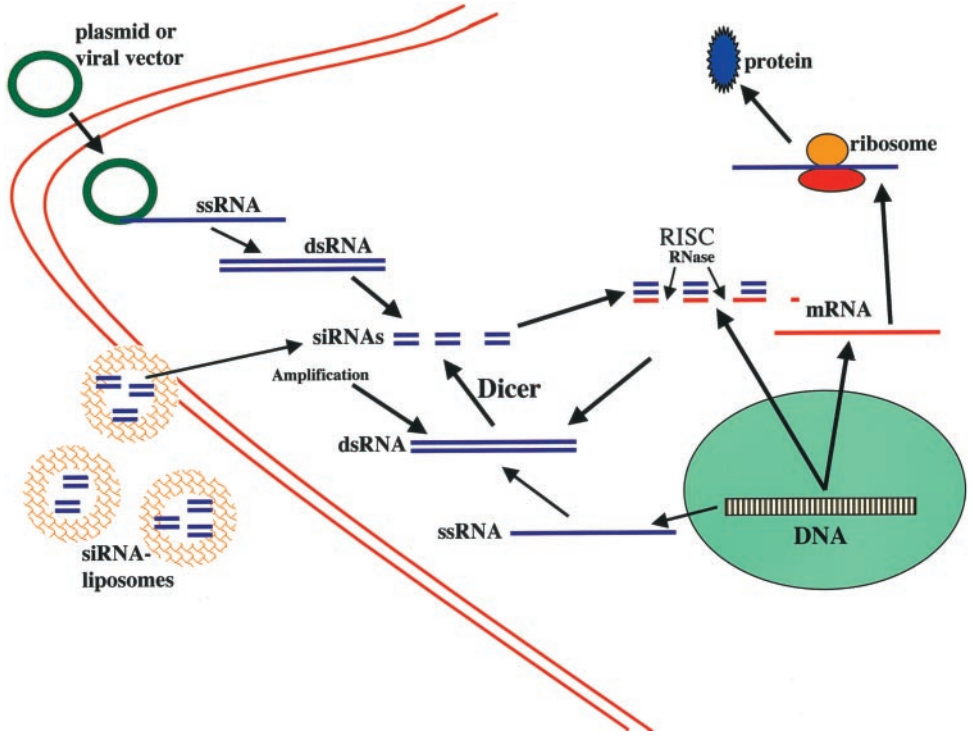

FIG. 1. Pathways of RNAi. Cells produce single-stranded RNA (ssRNA), which provide a template for the formation of dsRNA, which involves the activity of RNA-dependent RNA polymerases. The dsRNA is then cleaved by a protein called Dicer to form small 21–23 nucleotide siRNAs. The siRNAs (blue) then associate with the specific mRNA targeted by their nucleotide sequence (red) in a nucleic acid-protein complex called RISC, which includes RNase activity that degrades the mRNA at sites not bound by the siRNAs. The synthesis of the protein encoded by the mRNA targeted by the siRNAs is prevented, and that protein is selectively depleted from the cell. RNAi-mediated silencing can be induced experimentally by introducing synthetic siRNAs into cells using various transfection methods including liposomes (bottom left). Viral vectors can also be used to express dsRNAs against a specific gene, which are then acted upon by Dicer.

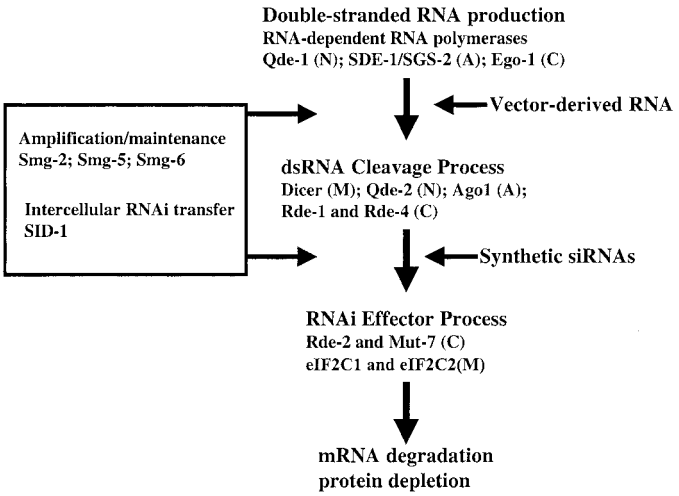

FIG. 2. Proteins involved in the process of RNAi. The production of dsRNA from a single-stranded RNA (ssRNA) template is mediated by RNA-dependent RNA polymerases such as Ode-1, SDE-1, and Ego-1. The cleavage of dsRNA to produce siRNAs is mediated by Dicer and related proteins such as Ode-2, Ago1, Rde-1, and Rde-4. The components of the RISC complex that mediate the recognition and degradation of the mRNA targeted by the siRNAs may include Rde-2, Mut-7, eIF2C1, and eIF2C2. In addition to this intrinsic RNAi pathway, mechanisms exist for amplification of RNAi (the Smg-2, Smg-5, and Smg-6 proteins appear critical for this process in *C. elegans*) and intercellular transfer of RNAi (the protein SID-1 may play a key role in this process in *C. elegans*). A, *Arabidopsis*; C, *C. elegans*; M, mammals; N, *Neurospora*.

et al., 2000) suggesting that it plays a role in the production of the RNAi signal. Another set of homologous genes involved in RNAi, identified in the screens for PTGS/RNAi mutants, include *ego-1* in *C. elegans* (Tabara et al., 1999; Smardon et al., 2000), *qde-1* in *Neurospora* (Cogoni and Macino, 1999) and *sgs2/sde1* in *Arabidopsis* (Mourrain et al., 2000). The latter proteins appear to be required for PTGS and may act by catalyzing the production of dsRNA in cells because they contain motifs similar to those of RNA-directed RNA polymerases that convert a single-stranded RNA template into a dsRNA. Further studies in *C. elegans* have identified the *smg-2*, *smg-5*, and *smg-6* genes as being involved in PTGS. However, the products of these genes are not required for the initial silencing by dsRNA, but are required for long-term maintenance of the gene suppression (Domeier et al., 2000). Smg-2 apparently amplifies the RNAi signaling such that it persists for the lifetime of the worm, whereas SMG-5 and SMG-6 are phosphatases that may facilitate Smg-2's actions by dephoshorylating it. Interestingly, Smg-2 shares a high degree of homology with yeast UPF1, a protein known to have RNA binding, ATPase and helicase activities (Page et al., 1999).

A working model for RNAi is shown in Figs. 1 and 2. The first step is the production of dsRNA directed against an mRNA. The second step involves the recognition of dsRNA and its processing to produce 21–23 nucleotide siRNAs. The "effector step" is the recognition of the target mRNA by the siRNAs and the selective degradation of that mRNA. The introduction of dsRNA into cells, whether produced endogenously from exogenous plasmids or viral vectors, results in its recognition by an enzyme that cleaves the dsRNA into 21- to 23-nucleotide double-stranded fragments in an ATP-dependent, processive manner with a 2-nucleotide 3′-overhang and a 5′-phosphorylated end (Zamore et al., 2000; Elbashir et al., 2001b). This nuclease was identified as an enzyme called Dicer that is highly conserved among plants, fungi, worms, flies, and mammals; it is a member of the RNase III family of dsRNA-specific ribonucleases (Bernstein et al., 2001). Dicer enzymes recognize and process dsRNA (Bernstein et al., 2001; Ketting et al., 2001) and are essential for RNAi (Bernstein et al., 2001; Grishok et al., 2001; Ketting et al., 2001). Dicer is thought to function as a dimer based upon knowledge of bacterial RNase III and structural evidence; crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage (Blaszczyk et al., 2001). Dicer not only processes dsRNA into siRNAs, but also processes endogenous regulatory RNAs called micro-RNAs. The *C. elegans* RNAi pathway gene *rde-4* encodes a dsRNA binding protein that interacts during RNAi with RNA identical to the trigger dsRNA; RDE-4 protein also interacts with Dicer and a conserved DExH-box helicase (Tabara et al., 2002). These and additional data obtained by the authors in the latter study suggest that RDE-4 and RDE-1 function together to detect, retain, and present dsRNA to Dicer for processing. Different domains of Dicer have been identified including a dsRNA binding domain, an RNase III activity domain, a helicase activity domain and a PAZ domain (Piwi-Argonaut-Zwille domain, a region of a hundred amino acids, which could mediate interaction with argonaute proteins) (Bernstein et al., 2001). Mouse Dicer is very similar to human Dicer with a predicted size of 1906 amino acids and molecular mass of 215 kDa, and contains a tandem repeat of RNase III catalytic domains, dsRNA binding region, a DExH/DEAH helicase motif and a PAZ domain (Nicholson and Nicholson, 2002). The mouse Dicer gene is located in chromosome 12 and the gene is widely expressed in cells throughout the body in embryonic and adult life.

Once generated, the small 21–23 nucleotide dsRNA fragments called siRNA are then recognized by a multiprotein complex called RISC and used as a guide for the recognition and degradation of the target mRNA (Tuschl et al., 1999; Hammond et al., 2000; Zamore et al., 2000; Nykanen et al., 2001). Experiments in *Drosophila* showed that RISC is present as a precursor complex that can be activated by ATP to form a complex with endonuclease activity that can cleave endogenous mRNAs (Hammond et al., 2000, 2001; Nykanen et al., 2001). The specific components of the RISC are not known, but do include members of the Argonaute family (Hammond et al., 2001) that have been implicated in many processes previously linked to post-transcriptional silencing. Moreover, RISC should include protein responsible for

endo-and *exo*-nuclease activity. Recently, RISC activity was studied in a human model. Two proteins of the Argonaute family, eIF2C1 and eIF2C2, were identified in the affinity-purified human RISC; the authors further showed that RISC uses single-stranded siRNAs as a guide to cleave the endogenous mRNA. In their studies of the mechanism of RNAi in human cells, Chiu et al. (2002) provided evidence that the status of the 5′-hydroxyl terminus of the antisense strand of a siRNA determines RNAi activity, whereas blocking the 3′ terminus does not prevent RNAi. They found that an A-form helix structure was required for the formation of antisense-target RNA duplexes. Surprisingly, RNAi still occurred when the siRNA duplex was cross-linked by psoralen, suggesting that complete unwinding of the siRNA helix is not necessary for RNAi activity. Thus, it appears that amplification of RNA by RNA-dependent RNA polymerase is not essential for RNAi in human cells.

It is likely that additional proteins modify the different steps in the RNAi process. For example, recent experiments have shown that the *Drosophila* homolog of the fragile X mental retardation protein interacts with Dicer and RISC suggesting a possible role in the RNAi machinery (Caudy et al., 2002; Ishizuka et al., 2002). The latter results also raise the possibility of a role of abnormalities in RNAi in various human diseases.

C. Other Related Phenomena

In addition to producing dsRNAs, which are cut into siRNAs and then (together with proteins of the RISC complex) target and degrade an mRNA species, some cells possess additional mechanisms for post-transcriptional gene regulation at the RNA level. Cells contain large amounts of noncoding RNA including tRNAs, snRNAs and rRNA (for review, see Eddy, 2001). Among these, a particular class called micro-RNA (miRNA) has recently received considerable attention. MiRNAs are approximately 22 nucleotides in length and are present in many different organisms from *C. elegans* to humans. Studies of the *lin-4* gene in *C. elegans* have demonstrated that miRNAs are able to block the translation of specific mRNAs from the *lin* family by binding to the 3′-untranslated region. In contrast to siRNA, the mRNA targeted by the miRNA is not destroyed during this process. First expressed as a 70-nucleotide stem loop precursor, lin-4 RNA is further processed by the same Dicer protein involved in siRNA-mediated RNAi. After processing the precursor, lin-4 RNA can bind on the target RNA region by complementary base pairing. The synthesis of lin-14 and lin-28 proteins is repressed by this miRNA mechanism to control development of the worm. A recent study showed that in human cells both siRNAs and miRNAs function concomitantly in the process of PTGS (Hutvagner and Zamore, 2002). The expression of some miRNAs, such as lin-4, are tightly regulated over time and seem to play an important role in development. Such temporal regulation has only been established for some of the miRNAs discovered so far; such RNA species are called small temporal RNA, which can be considered a subset of miRNA (Banerjee and Slack, 2002). It was recently shown that, as with siRNAs, miRNAs can be used as a tool to suppress expression of genes of interest (McManus et al., 2002).

Nonsense-mediated mRNA decay (NMD), although not strictly a PTGS phenomenon, is relevant to the general topic of RNAi. NMD is a process that appears to be a quality control mechanism that eliminates nonsense transcripts such as mRNAs with premature termination codons (Frischmeyer and Dietz, 1999). First discovered in yeast, this surveillance mechanism is ubiquitous among eukaryotes. Coupled to mRNA splicing, this pathway results in the degradation of aberrant mRNAs. There is evidence that NMD is involved in the PTGS-related degradation of the mRNA, because some *C. elegans* genes are required for both RNAi and NMD (Domeier et al., 2000). The two mechanisms are different, however, because NMD is dependent on translation of the mRNA, whereas the decrease in mRNA observed in RNAi and related PTGS phenomena is not prevented by inhibitors of translation (Holtorf et al., 1999). Also, the mRNA degradation associated with NMD begins with de-capping followed by 5′ to 3′ exonuclease degradation (Ruiz-Echevarria et al., 1996), whereas the degradation associated with PTGS appears to begin with endonucleotidic cleavage (Elbashir et al., 2001b).

III. Technical Considerations in the Use of RNA Interference

In several respects the approaches for silencing gene expression using RNAi methods are similar to those used for antisense DNA-mediated suppression of gene expression. In principle, any cloned gene can be targeted by designing RNA oligonucleotides or RNA-expressing viral vectors with sequences complementary to the mRNA transcribed from the target gene. The present section of this review article is intended to provide readers who are planning to use, or have just begun to use, RNAi technology into their experimental tool kit with practical information on designing and performing experiments using RNAi methods. In addition, we provide descriptions of emerging technical approaches for selective gene silencing in vitro and in vivo using RNAi. Examples of studies that employed methods described in the section can be found in Table 1. More detailed information on technical aspects of RNAi technology can be found on several different websites including: http://www.ambion.com; http://www.imgenex.com; http://www.genetherapysystems.com.

A. Design and Synthesis of Small Interfering RNAs

Double-stranded RNAs of 20–23 nucleotides (siRNAs), which can be synthesized in large quantities

TABLE 1
Applications of RNA interference

Gene	Organism	In vitro/In vivo	Cell Line/Tissue	RNA Species	Delivery Method	Reference
Acyl-CoA binding protein	Human	in vitro	HeLa, HopG2, Chang Cells	slRNA	Oligofectamine	Faergeman and Knudsen, 2002
Androgen receptor (polyCAG)	Human	in vitro	HEK293T cells	slRNA	CellFectin	Caplen et al., 2002
β-galactosidasa	Mouse	in vivo	Embryo	slRNA	Microinjection/ electroporalion	Calegari et al. 2002
β-tubulin	Mouse	in vitro	P19 cells	slRNA, shRNA (transcribed) shRNA (expressed)	Llpofectamine 2000	Yu et al. 2002
CD4, p24	Human, Virus	in vitro	Magl-CCR5, HoLa-CD4 cells	slRNA	Oligofectamine	Novins et al. 2002
CD4/CD8α	Mouse	in vitro	T lymphocytes primary T cells and bone marrow derived dentritic cells, hematopoietic stem cells, ES cells, zygotes, adult mice	slRNA	Electroporation/ cationic lipid	McManus et al. 2002
CD8, CD25, Bim, p53	Mouse	in vitro/in vivo		shRNA (U6 promoter-driven)	Lentivirus	Rubinson et al. 2003
Cdc14a	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Mailand et al. 2002
Cdc14A	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Mailand et al. 2002
CDH1, p53	Human	in vitro	MCF-7	shRNA (H1 promoter)	Retrovirus	Brummalkamp et al. 2002
Centrin-1	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Salisbury et al. 2002
Cep135	hamster	in vitro	CHO cells	siRNA	Lipofectamine, Fugene	Ohta et al. 2002
Dlcer	Human	in vitro	HEK293T cells	shRNA plasmid	Calcium phosphate	Paddison et al. 2002[b]
DNA-PK	Human	in vitro	Fibroblesis	siRNA	Liposomes	Peng et al. 2002
Drep1	Human	in vitro	HEK293T cells	shRNA plesmid	Calcium phosphate	Irotni et al. 2002
Fortilin, myelold cell leukemia 1 protein	Human	in vitro	U2OS	siRNA	Transit solution	Zhang et al. 2002
GalTIIA, GalTIIB	Human	in vitro	HeLa	siRNA	Oligofectamine	Bal et al. 2001
GFP	Human	in vitro	293T	shRNA construct	Lentivirus	Abbas-Terki et al. 2002
GFP	Mouse	in vivo	Brain/striatal cells	shRNA	Adenovirus	Xia et al. 2002
GFP	Mouse	in vivo (mouse)	Whole organism	shRNA (H1 promoter)	Lentivirus	Tiscomia et al. 2003
GFP, luciferase, β-catenin	Human	in vitro	HeLa S3/EBNA-1	shRNA (U6 promoter-driven)	Lipofectamine 2000	Miyagishi and Taira, 2002
GFP, mAbp1, Dok2	Human	in vitro	293T	siRNA	Lipofectamine	Mise-Omata et al. 2003
GFP, MAP-2	Rat	in vitro	Primary cortical neurons	siRNA	Lipofectamine 2000	Krichevsky and Kosik, 2002
GFP, PKR	Human (PKR)	in vitro	HeLa, HEK293	siRNA (T7 promoter-generated)	Lipofectamine, calcium phosphate	Donze and Picard, 2002
Hepatitis C-NS5B	Virus	in vitro	Huh-7.5	siRNA	Electroporation	Randall et al. 2003
HIV-1 rev	Virus	in vitro	293/EcR cells	shRNA (U6 promoter-driven)	Lipofectamine Plus	Lee et al. 2002
HIV-gag	Virus	in vitro	HEK293/T lymphocytes	siRNA/fluorine derivatized siRNA	Lipofectin or naked addition (derivatized)	Capodici et al. 2002
Human papillomavirus genes E6 and E7	Virus	in vitro	Cervical carcinoma cells	siRNA	Oligofectamine	Jiang and Milner, 2002
Karyopherin α2, α3	Porcine	in vitro	Embryo	siRNA	Microinjection	Cabol and Prether, 2003
Kinase interacting stathmin	Human	in vitro	HEK 293 cells	siRNA	Lipofectamine 2000	Boehm et al. 2002
Kinesin E15	Human	in vitro	HeLa	siRNA	Calcium phosphate, oligofectamine electroporation	Weil et al. 2002
Lamin A/C	Human	in vitro	HeLa cells	shRNA (U6 promoter-driven)	Lipofectin Plus	Paul et al. 2002

PI3 kinase, phosphatidylinositol 3-kinase

TABLE 1—*Continued*

Gene	Organism	In vitro/In vivo	Cell Line/Tissue	RNA Species	Delivery Method	Reference
Luciferase		in vitro	293T/Cos1/NIH3T3/HeLe	shRNA plasmid	Calcium phosphate	Paddison et al. 2002a
Luciferase		in vivo (mouse)	Liver, kidney, spleen, lung, pancreas	siRNA	Tall vein injection	Lewis et al. 2002
Luciferase		in vivo (mouse)	Liver	siRNA	Hydrodynamic transfection, naked RNA	McCaffrey et al. 2002
Luciferase, famin A/C	Human (lamin A/C)	in vitro	HeLa, Cos-7, HEK293, HEK293	siRNA	Oliogofectamine	Elbashir et al. 2002a
mAPH-1a and b, nicastrin, presenilin	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Lee et al. 2002
Mina53	Human	in vitro	HeLa, rat3Y1MycB cells	siRNA	Oligofectamine	Tsuneoka et al. 2002
Mps1 kinase	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Stucke et al. 2002
Myocyte enhancer factor 2A	Mouse	in vitro	Primary cerebellar granulo neurons	shRNA	Calcium phosphate	Gaudilliere et al. 2002
Nuclear Obf2-related kinase, p75	Human	in vitro	HeLa cells	shRNA	Retrovirus	Devroe and Silver, 2002
Nuclear, cytoskeletal, cell cycle	Human	In vitro	HeLa	siRNA	Oligofectamine	Harborth et al. 2001
Oct 3/4, c-mos	Mouse	in vitro	Oocytes	siRNA	Microinjection	Kim et al. 2002
Oml/Htre2	Human	in vitro	U2Os	siRNA	Effectene	Martins et al. 2002
Orc6	Human	in vitro	HeLa cells	siRNA	Liposomes	Presanth et al. 2002
p110 subunit of P13 kinase, akt1, akt2	Human	in vitro	HeLa	shRNA plasmid; siRNA	Effectene, oligofectamine	Czaudema et al. 2003
p21	Mouse	in vitro	Prostate cancer cell line	siRNA	Liposomes	Ukomadu and Dutta, 2003
p53	Human	in vitro	HEK293T cells	shRNA	Lentivirus	Barton and Medzhhitov, 2002
p53	Human	in vitro	H1299 cells	siRNA	Lipofectamine	Martinez et al. 2002
p53	Human	in vitro	SK-N-SH cells	siRNA	Oligofectamine	Kartasheva et al. 2002
p65 subunit of NF-κB	Human	in vitro	MAGI/Jurkat cells	siRNA	Oligofectamine	Surabhi and Gaynor, 2002
PKC α	Human	in vitro	HEK293T cells	siRNA and shRNA	Cationic lipid	Leirdal and Sioud, 2002
Polio-specific capsid, polymerase	Virus	in vitro	HeLa S3, mouse embryonic fibroflasts	siRNA	Lipofectamine 2000	Gitlin et al. 2002
Polio-like kinase	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Liu and Erikson, 2002
Poly (ADP-ribose) polymerase	Mouse, Rat	in vitro	Neuro 2A-derived cell line	siRNA	Lipofectamine	Gan et al. 2002
PRC1	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Mollinari et al. 2002
ront1/hUpf1 and rent2/hUpf2	Human	in vitro	HeLa cells	siRNA	Liposomes	Mendell et al. 2002
RNA-specific adenosine deaminase-1	Human	in vitro	Huh-7	siRNA	Lipofectamine 2002	Wong and Lazinski, 2002
Shc	Human	in vitro	HeLa cells	siRNA	Oligofectamine	Kisielow et al. 2002
Speedy (Spy)	Human	in vitro	HEK 293T cells	siRNA	Calcium phosphate	Porter et al. 2002
Sphingosine kinase-1	Human	in vitro	HUVE cells	siRNA	Oligofectamine	Ancellin et al. 2002
Various PKC isoforms	Human, Rat	in vitro	HeLa, Cos-7, HEK293, rat-1	siRNA	Oliogofectamine	Irle et al. 2002

PI3 kinase, phosphatidylinositol 3-kinase

and transfected into cells, are the most commonly used reagents for RNAi in cultured cells. All that is needed to implement siRNA-mediated silencing of expression of a gene of interest is the cDNA sequence of that gene, and commercially available reagents with which to perform the synthesis. Although targeting of siRNAs to any region of an mRNA would be expected to induce degradation of the mRNA and therefore abolish production of the encoded protein, empirical data suggest that the probability of achieving selective silencing can be increased by targeting the siRNAs to specific regions of the mRNA. Ambion (Houston, TX) recommends the following approach for designing an siRNA. 1) Beginning with the AUG start codon of the target gene transcript, scan downstream for AA dinucleotide sequences; each AA and the 3′ adjacent 19 nucleotides are potential siRNA targets. 2) Compare the sequences of the potential target sequences to sequences in the species-appropriate genome database (www.ncbi.nlm.nih.gov/BLAST/) and eliminate from consideration any target sequences that are homologous to other coding sequences. 3) Select 3–4 target sequences along the length of the gene for production of siRNAs. Of course it is important for all siRNA experiments to include negative control siRNAs with the same nucleotide composition but a scrambled sequence. See http://www.mpibpc.gwdg.de/abteilungen/100/105/public.html for further information.

Chemical synthesis was the first method used to produce siRNAs, but now they can be produced in any laboratory using in vitro transcription methods. One protocol involves the synthesis of DNA oligonucleotides that include an 8-base sequence complementary to the 5′ end of a T7 promotor primer. Each gene-specific oligonucleotide is annealed to the T7 promoter primer, and a fill-in reaction using Klenow fragment produces a double-stranded template for use in an in vitro transcription reaction (Ambion Silencer siRNA construction kit). The two RNA products of the in vitro transcription reactions are hybridized to each other, treated with DNase (to remove the DNA template) and RNase (to even the ends of the dsRNA), and the RNA is column purified. Another protocol for the production of siRNAs takes advantage of the availability of recombinant human Dicer. Large in vitro transcribed RNA templates are cleaved by Dicer to produce multiple species of 22 base pair siRNAs (Dicer siRNA generation kit; Gene Therapy Systems Inc., Dan Diego, CA). An advantage of the latter method is that, because it produces a mixture of different siRNAs directed against the same mRNA target, the probability of obtaining gene silencing is increased.

B. Construction of Plasmids and Viral Vectors for RNA Interference

There are several reasons why expression plasmids and viral vectors are being used in basic and applied RNAi research. One major reason is that expression vectors allow continuous production of siRNAs in cells and, therefore, sustained depletion of the protein encoded by the targeted mRNA. A second reason is that, particularly with viral vectors, the transfection efficiency of certain types of cells, particularly postmitotic cells can be greatly increased. A third advantage of viral vectors is that they are typically more effective in obtaining sustained expression (and gene silencing, in the case of RNAi) in vivo. For example, adenoviral vectors have been extensively used to express genes in postmitotic neurons in vivo (Smith and Romero, 1999).

Short hairpin RNAs (shRNAs) can be transcribed from RNA polymerase III promoters in cells in culture or in vivo allowing continuous suppression of expression of the targeted mRNA (Paddison et al., 2002a). The latter authors proposed the use of this technology in the generation of transgenic mice as an alternative approach to gene knockout mice. Brummelkamp and colleagues (Brummelkamp et al., 2002a) developed a novel vector system for the stable expression of siRNAs in mammalian cells. They used the polymerase-III H1-RNA gene promoter, which produces a small RNA transcript lacking a poly-adenosine tail and has a well defined transcription start and termination signals. The construct also allows cleavage of the transcript at the second uridine after the termination resulting in a transcript that resembles the ends of synthetic siRNAs. They designed a gene-specific insert that specified a 19-nucleotide sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same 19-nucleotide sequence resulting in the production of a 19-base pair stem-loop structure. This vector system was shown to be effective in sustained suppression of target gene expression in several different types of cultured cells.

Several laboratories have constructed plasmids that contain DNA templates for the synthesis of siRNAs under the control of the U6 promoter. For example, Sui et al. (2002) inserted DNA fragments that acted as templates for the synthesis of small RNAs under the control of the mouse U6 promoter that directs the synthesis of a Pol III-specific RNA transcript to generate an RNA composed of two identical 21-nucleotide sequence motifs in an inverted orientation, separated by a 6-base pair spacer of nonhomologous sequences. Five thymidines that function as a termination signal for Pol III were added at the 3′ end of the repeat; the resulting RNA is predicted to fold back to form a hairpin dsRNA with a 3′ overhang of several thymidines. Using this plasmid,

they were able to demonstrate the efficient inhibition of expression of three different endogenous genes (lamin A/C, CDK-2, and DNA methyltransferase) in cultured human cells. A similar approach that employed U6 promoter-driven siRNAs with four uridine 3′ overhangs was used to effectively suppress expression of ectopically expressed genes as well as the endogenous β-catenin gene (Miyagishi and Taira, 2002). Retroviral delivery systems have been developed based upon several commercially available vectors. For example, a retroviral siRNA vector was developed in which the U6 promoter and anti-target gene hairpin was subcloned into pMSCVpuro (BD Biosciences Clontech, Palo Alto, CA) at the unique NsiI site just upstream from the 3′ long terminal repeat (Devroe and Silver, 2002). Using this retroviral siRNA delivery system, they demonstrated the efficient and sustained depletion of the NDR kinase and the transcriptional coactivator p75 in cultured cells.

Lentiviral systems for shRNA delivery have also been developed. Lentiviruses can infect noncycling and postmitotic cells, and also provide the advantage of not being silenced during development allowing generation of transgenic animals through infection of embryonic stem cells or embryos (Naldini, 1998; Lois et al., 2002; Pfeifer et al., 2002). Using this approach, silencing of green fluorescent protein (GFP) in GFP-positive transgenic mice has been shown after transduction with lentiviruses expressing shRNA directed against the GFP protein (Tiscornia et al., 2003). More recently, Rubinson et al. (2003) used lentivirus-delivered shRNA to induce silencing of CD8 and CD25 in cycling primary T cells and the pro-apoptotic molecule Bim in primary bone marrow-derived dentritic cells. Lentiviral-mediated silencing of CD8 in hematopoietic stem cells was still present after injection of the cells in lethally irradiated congenic mice. Moreover, in vivo silencing for CD8 or p53 was also observed after infection of ES cells or zygotes leading to stable and functional silencing in adult RNAi transgenic mice.

C. Transfection Methods

Several different transfection methods previously used to introduce oligodeoxynucleotides and DNA plasmids into cells have been used to successfully introduce siRNAs into cells. However, it has become clear there is no single transfection method that can be successfully applied to all cell types under all experimental conditions. It is therefore important to optimize transfection conditions so that maximum gene silencing is acheived. The following transfection parameters have been shown to affect transfection and gene silencing efficacy: cell culture conditions, including cell density and medium composition; the type and amount of transfection agent; the quality and amount of siRNA; and the length of time that the cells are exposed to the siRNA. For proliferating cells, a subconfluent cell density is preferable. For postmitotic cells such as neurons, cell densities in the range of 200 to 500 cells per mm^2 of culture surface work well (O. Milavet and M. P. Mattson, unpublished data). Because proteins in serum can bind to and/or degrade siRNAs, the transfection should be performed in serum-free medium. Differences have been reported in the ability to transfect and silence gene expression between adherent and nonadherent cells. For example, the ErbB3 gene was readily silenced in adherent carcinoma cells using liposome-mediated siRNA transfection, whereas the same transfection method was ineffective in nonadherent myeloma cells (Walters and Jelinek, 2002). Postmitotic cells such as neurons and muscle cells tend to be more difficult to transfect using liposomes compared to mitotic cells such as stem cells, fibroblasts, and tumor cells.

Calcium phosphate-mediated transfection has been used successfully by several laboratories (Donza and Picard, 2002; Weil et al., 2002). The most commonly used and effective transfection method for short-term suppression of gene expression using RNAi is to incorporate siRNAs into liposomes. There are an increasing variety of such transfection reagents including: Oligofectamine, LipofectAMINE-2000 and CellFectin from Invitrogen (Carlsbad, CA) (Caplen et al., 2002; Gan et al., 2002; Gitlin et al., 2002; Irie et al., 2002; Wong and Lazinski, 2002; Mise-Omata et al., 2003); Effectene from Qiagen (Valencia, CA) (Martins et al., 2002); and siPORT-Amine and siPORT-Lipid from Ambion (Austin, TX). Other methods that have proven effective for transfecting siRNAs into cultured cells include electroporation (Calegari et al., 2002; McManus et al., 2002; Randall et al., 2003), microinjection (Calegari et al., 2002; Kim et al., 2002), and hydrodynamic shock (McCaffrey et al., 2002). Similar transfection methods have been used to introduce RNA-expressing plasmids into cultured cells (Iratni et al., 2002; Czauderna et al., 2003). An example of the kind of results obtained with an optimized transfection protocol that employed Oligofectamine is shown in Fig. 3 where levels of the cellular prion protein are markedly decreased in mouse neural precursor cells using two different siRNAs.

Xia and coworkers (Xia et al., 2002) described the development of methods for using a viral-mediated delivery system for gene silencing in mice in vivo. They constructed a 21-base pair hairpin representing sequences directed against enhanced green fluorescent protein (eGFP), and tested its ability to reduce target gene expression in mammalian culture cells. Two different constructs were used, one in which the siRNA hairpin targeted against eGFP was placed under the control of the cytomegalovirus promoter and contained a full-length simian virus-40 (SV40) polyadenylation (polyA) cassette, and the second in which the hairpin was juxtaposed almost immediate to the cytomegalovirus transcription start site (within 6 base pairs) and was followed by a synthetic, minimal polyA cassette. Cotransfection of these constructs with an eGFP expres-

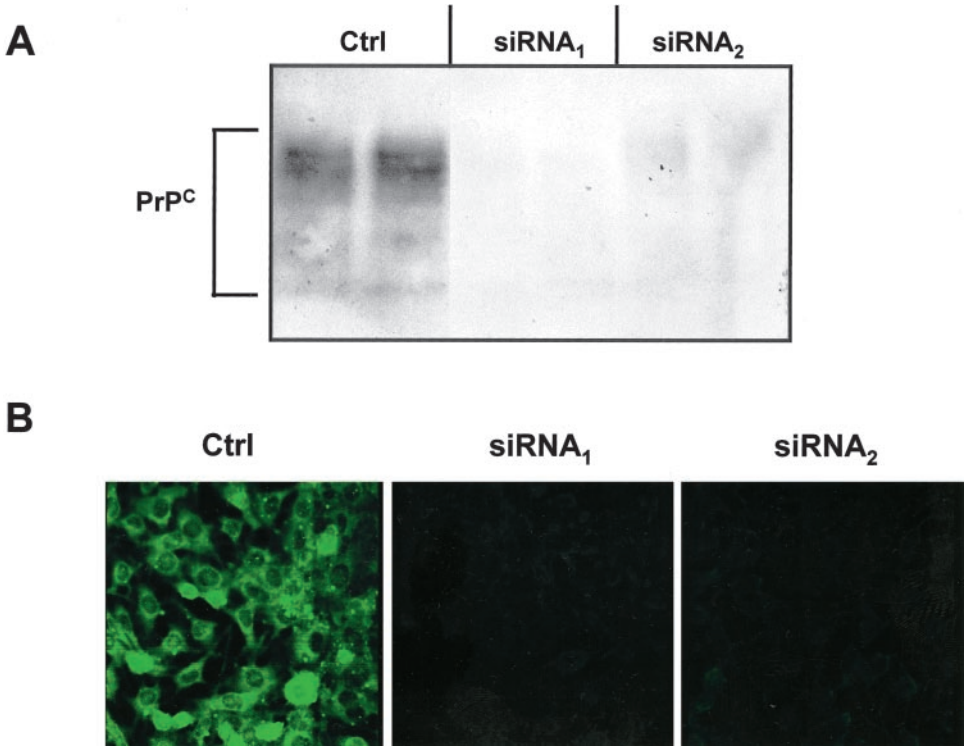

FIG. 3. Selective depletion of the cellular prion protein in neural progenitor cells by siRNA-mediated post-transcriptional gene silencing. Two chemically synthesized siRNAs were designed to target two different regions of the cellular isoform of the prion protein (PrP^C). siRNAs (50 nM) were transfected into cultured C17.2 mouse cerebellar neural progenitor cells using Oligofectamine reagent. Five days after transfection, cell lysates were subjected to immunoblot analysis using an antibody against PrP^C (panel A). Additionally, cells were fixed and immunostained with a PrP^C specific antibody before analysis by confocal microscopy (panel B). Note that both siRNAs greatly reduced the amount of PrP^C in the cells, compared to mock-transfected control cells.

sion plasmid showed that the second construct was effective in silencing eGFP expression (silencing was correlated with the generation of a 63-base pair RNA specific for eGFP). The authors then constructed recombinant adenoviruses that expressed siRNAs directed against either GFP or β-glucuronidase. These vectors were effective in suppressing expression of endogenous GFP (in GFP transgenic mice) and β-glucuronidase in liver or brain in vivo (Xia et al., 2002). Another study reported the use of a rapid injection method to deliver a large volume of physiological solution containing siRNAs into the tail vein of mice (Lewis et al., 2002). They demonstrated the effectiveness of this method for reducing target gene expression in cells throughout the body by coinjecting postnatal mice with 10 ug of a plasmid containing the luciferase gene along with 5 ug of a synthetically prepared siRNA duplex targeted against luciferase (or control siRNAs). One day after injection they collected several different organs, prepared homogenates, and screened them for luciferase activity. Luciferase activity was decreased by 80 to 90% in the liver, spleen, lung, kidney, and pancreas of mice injected with luciferase siRNA, compared with that in mice injected with control siRNAs. They further showed that inhibition of target gene expression by siRNA was dose-dependent and persisted for several days after siRNA administration. Using similar approaches, it was shown that gene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed in vivo from DNA templates (McCaffrey et al., 2002). The latter study also demonstrated the therapeutic potential of RNAi by suppressing expression of a sequence from the hepatitis C virus in the mice. The methods used to transfect cells with viral vectors that produce shRNAs are essentially identical to those used to transfect cells with similar vectors designed to express cDNAs (Abbas-Terki et al., 2002; Barton and Medzhhitov, 2002; Devroe and Silver, 2002; Xia et al., 2002). In another study, injection of liposomes containing siRNAs directed against the mRNA-encoding agouti-related peptide, a peptide known to regulate body weight, resulted in an increase in metabolic rate and reduced body weight without a change in food intake (Makimura et al., 2002).

It should be recognized that as RNAi technology advances it will likely be possible to produce RNAi "knockout" mice (or other mammals) in which the expression of a protein of interest is repressed by the expression of its corresponding RNAi related molecule (shRNA or miRNA, for example). The resulting animal could be seen as an equivalent of its knockout generated by targeted gene disruption but with much more flexibility and efficiency. For example, cell type-specific promoters could be used to effect PTGS only in cells of interest; in

many cases this may circumvent embryonic lethality resulting from gene deletion from all cells. It would also be quicker and less costly to produce RNAi transgenic animals compared with conventional knockouts.

IV. Applications of RNA Interference to Establishing Gene Function

The most widely used RNAi technology has been in cell culture and in vivo studies aimed at understanding the function of an individual (or multiple) proteins. The kinds of studies described below and listed in Table 1 demonstrate the power and flexibility of RNAi for unambiguously establishing (or excluding) a function of individual proteins in various cellular processes. It should also be noted that cells can be transfected with different combinations of multiple siRNAs, each directed against a specific mRNA of interest, to elucidate the specific contributions of the proteins to a biological process involving a multiprotein complex. For example, Wojcik and DeMartino (2002) recently took advantage of the latter feature of RNAi methods to elucidate roles for different subunits of the proteasome in its assembly and function. The power of *C. elegans* and *Drosophila* molecular genetics is providing the opportunity to use genome-wide RNAi to rapidly establish functions of genes in a specific process. For example, Ruvkun and colleagues (Lee et al., 2002c) systematically inactivated 5690 genes in *C. elegans* using RNAi to identify genes that limit lifespan. They identified a mitochondrial leucyl-tRNA synthetase gene and showed that mutations of this gene that impair mitochondrial function increase lifespan, which was associated with decreased ATP levels and oxygen consumption.

A. Signal Transduction

Intercellular messenger molecules play vital roles in the development and proper functioning of all organisms. Among the most prominent of such signals in mammals are growth factors, cytokines, cell adhesion molecules, neurotransmitters, steroids, and gases such as nitric oxide. Specific receptors located on the cell surface or within the cell transduce responses to the ligand via signaling cascades that are often complex, involving kinases and transcription factors, for example. RNAi methods provide powerful tools for establishing the roles of individual proteins in the signal transduction pathway employed by a specific ligand. Several recent studies have demonstrated the efficacy of siRNA-mediated knockdown of signal transduction proteins and have elucidated roles for those proteins in biological responses of cells. Adaptor proteins of the Shc family transduce signals from a diverse group of growth factors that signal through receptor tyrosine kinases. Liposome-mediated introduction of siRNA against a single isoform of ShcA into HeLa cells was used to selectively reduce levels of that Shc revealing its role in the regulation of cell proliferation (Kisielow et al., 2002). Neurotrophin receptors and integrins (receptors for extracellular matrix molecules such as laminin) are often coupled to a signaling pathway involving phosphatidylinositol 3-kinase and Akt kinase (Gary and Mattson, 2001; Cheng et al., 2003). Decreasing the amount of the 110 β subunit of phosphatidylinositol 3-kinase using siRNAs resulted in a marked decrease in the growth and tissue invasiveness of tumor cells (Czauderna et al., 2003). Small molecule inhibitors have been widely employed to study the functions of various protein kinases in cells. However, most such inhibitors are not specific and affect multiple kinases. RNAi has been successfully employed to unequivocally establish the roles of specific kinases in signal transduction processes. For example, Irie and coworkers (Irie et al., 2002) demonstrated the ability to knockdown levels of specific subtypes of protein kinase C in cultured human and rat cells in a species-specific manner. The existence of an extracellular system for the production of sphingosine-1-phosphate was demonstrated in a study in which siRNAs directed against the sphingosine kinase-1 enzyme were used to inhibit its production and export in cultured endothelial cells (Ancellin et al., 2002).

Calcium plays important roles as an intracellular signal that mediates a variety of responses of cells to environmental stimuli. Mechanisms for regulating levels of calcium in the cytoplasm are complex and involve movements of calcium ions across the plasma membrane, as well as into and out of endoplasmic reticulum and mitochondria. A key role for inositol 1,4,5-trisphosphate (IP_3)-mediated release of intracellular calcium in the maturation of mouse oocytes was demonstrated in which siRNAs against the IP_3 receptor-1 were injected into germinal vesicle-intact oocytes (Xu et al., 2003). The siRNAs reduced IP_3 receptor-1 levels by 90% and, following insemination, blocked the intracellular calcium oscillations that play a critical role for the first steps in development. In another study, RNAi-mediated depletion of the endoplasmic reticulum calcium-ATPases resulted in lethality in *C. elegans* (Cho et al., 2000), demonstrating a pivotal role for calcium uptake by this organelle in cell functions and survival.

B. Cell Cycle Regulation

Because of their fundamental importance for development, tissue homeostasis, and stem cell biology and their centrality to the field of cancer research, genes that regulate the cell cycle have been a prominent focus of studies that employ RNAi. Examples of the kinds of findings obtained using RNAi technologies to knockdown the expression of cell cycle genes are as follows. A requirement of the Mps1 kinase for the spindle assembly checkpoint was established, whereas a role for this kinase in centrosome duplication was ruled out (Stucke et al., 2002). A key role for a novel centrosomal protein called Cep135 in microtubule organization was revealed

(Ohta et al., 2002), and a role for centrin-2 in centriole duplication was established (Salisbury et al., 2002). RNAi was used to show that the regulation of cell cycle progression in response to mitogens is controlled by the cyclin-dependent kinase inhibitor p27 (Kip1) (Boehm et al., 2002). Depletion of Plk1 using siRNAs results in activation of cyclin B and inhibits centrosome amplification in hydroxyurea-treated U2OS cells (Liu and Erikson, 2002). RNAi was used to show that the protein PRC1 is a microtubule-associated protein that facilitates bundling of microtubules (Mollinari et al., 2002) and that the processes of centrosome separation and chromosome segregation require the phosphatase Cdc14A in culture human cells (Mailand et al., 2002). Depletion of the origin recognition complex subunit ORC6 using RNAi resulted in cells with decreased DNA replication, multipolar spindles and aberrant mitosis (Prasanth et al., 2002). A novel human protein called Speedy, expressed only during the G_1/S phase of the cell cycle, was shown to enhance cell proliferation by enhancing the activity of Cdk2 (Porter et al., 2002).

C. Development

The complex and remarkably rapid events that occur during development of the fertilized egg into an adult organism remain largely a mystery. There would appear to be a great potential for RNAi technology to unravel the cellular and molecular events that regulate developmental processes. Methods for silencing single or multiple selected genes in developing embryos in vivo and stem cells in culture (see *Section III.C.*) are beginning to reveal the functions of specific proteins in developmental processes. Nodal is a secreted factor that plays a key role in the formation and patterning of the mesoderm during gastrulation. RNAi was used to demonstrate that the transcriptional corepressor DRAP1 inhibits the transcription factor FoxH1 and thereby regulates signaling by Nodal during mouse embryogenesis (Iratni et al., 2002). Depletion of karyopherins α2 and α3 in cleavage stage porcine embryos revealed different requirements of these two proteins in embryogenesis (Cabot and Prather, 2003). In another study of embryogenesis, siRNAs directed against the mRNAs encoding Oct-3/4 and c-mos resulted in depletion of the encoded proteins, and phenotypes similar to those observed in Oct-3/4 and c-mos knockout mice (Kim et al., 2002). A key role for microtubule-associated protein-2 in the regulation of dendrite outgrowth in developing brain neurons was demonstrated using siRNAs (Krichevsky and Kosik, 2002). The transcription factor myc is known to play a fundamental role in the regulation of cell proliferation. A key role for the novel myc target gene mina53 in the regulation of cell proliferation by myc was demonstrated using RNAi technology (Tsuneoka et al., 2002).

D. Macromolecular Synthesis and Degradation

Regulated synthesis of nucleic acids, proteins, and lipids, and the turnover of those macromolecules is essential for cell survival and functions. Although biochemical technologies have allowed the identification of various biosynthetic pathways and mechanisms for the degradation of proteins and other macromolecules, the functions of specific proteins in such processes are not well understood. Recent studies have employed RNAi to advance the understanding of the regulation of macromolecular synthesis and degradation. For example, depletion of galactosyltransferase II using siRNAs revealed a critical role for this enzyme in the biosynthesis of the linkage region of glycosaminoglycans (Bai et al., 2001). The regulation of the processing of RNAs transcribed from coding and noncoding regions of the genome is an active area of investigation because of the recent realization of its importance in the regulation of gene expression. Mendell and coworkers (Mendell et al., 2002) used RNAi to show that rent1/Upf1 plays distinct roles in the regulation of splicing and decay of nonsense transcripts. Transfection of cultured HeLa and HepG2 cells with siRNAs directed against the mRNA encoding the acyl-CoA binding protein resulted in cessation of cell proliferation, cell detachment, and death, demonstrating that acyl-CoA binding protein is essential for cell survival (Faergeman and Knudsen, 2002).

E. Cell Motility

The migration of cells within and among tissues, and extensions of cells such as the axons and dendrites of neurons, is central to the structural and functional organization of all tissues. Although there are a few pharmacological agents that have proven useful in studying cell motility (the actin-depolymerizing agent cytochalasin D and the microtubule-stabilizing agent taxol, for example), a detailed understanding of the molecular regulation of cell motility is lacking. Recent studies have taken advantage of RNAi methods to elucidate roles for specific proteins in regulating cell motility. Depletion of the cytoskeletal linker protein trypanin using siRNAs resulted in a loss of directional cell motility in African trypanosomes that is caused by impaired coordination of the flagellar beating (Hutchings et al., 2002). The growth and guidance of axons in developing neurons is regulated by substrate-associated and soluble ligands encountered by the axonal growth cone. RNAi-mediated depletion of the integrin-interacting protein MIG-15 resulted in a dysregulation of commissural axon navigation in *C. elegans* (Poinat et al., 2002). RNAi has also shown that although actin binding protein 1 is essential for the processes of endocytosis, it is not necessary for lamellipodia formation in human embryonic kidney cells (Mise-Omata et al., 2003). Membrane microdomains called lipid rafts and clathrin-associated domains are increasingly recognized as sites of signal transduction

and endocytosis in eukaryotic cells. Proteins critical for the function of the signaling and endocytic functions of these membrane domains are being identified using RNAi approaches. For example, RNAi methods were used to establish an essential role for a J-domain protein called auxilin in clathrin-mediated endocytosis in *C. elegans* (Greener et al., 2001).

F. Cell Death

In many tissues throughout the body, cells have a finite life span and then undergo apoptosis, a form of programmed cell death in which the cell dies in a well controlled manner and is removed from the tissue without adversely affecting adjacent healthy cells. Apoptosis also plays a key role in sculpting the cellular structure of tissues during embryonic and postnatal development (Baehrecke, 2002). Of course, abnormal cell death is a major problem in a variety of diseases, including neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, and ischemic vascular conditions (Mattson, 2000). Considerable progress is being made in understanding the molecular mechanisms that regulate cell death, with the goal of identifying key targets for therapeutic intervention. Drug development efforts have resulted in several exciting classes of agents that either prevent the death of cells such as neurons, or induce selective death of cancer cells. For example, inhibitors of the pro-apoptotic protein p53 (Duan et al., 2002; Zhu et al., 2002) and caspases (Eldadah and Faden, 2000) are being developed for use in neurodegenerative disorders. RNAi might be used in lieu or in combination with such drugs.

RNAi has recently been employed to establish roles for specific genes in apoptotic and anti-apoptotic pathways. For example, a critical role for p73δ in p53-mediated apoptosis was demonstrated by showing that depletion of p73δ using siRNA prevents cell death (Kartasheva et al., 2002). Depletion of the catalytic subunit of DNA-dependent protein kinase using siRNAs increased the sensitivity of human fibroblasts to radiation-induced death because of an impaired ability to sense and repair the DNA damaged by the radiation (Peng et al., 2002). RNAi was used to establish a role for the calcium-binding protein calreticulin in necrotic cell death in *C. elegans* (Xu et al., 2001). Many cells express one or more inhibitor-of-apoptosis proteins (IAPs) that can prevent apoptosis by directly binding to and inhibiting caspases. RNAi was used to identify the serine protease Omi/HtrA2 as a mammalian XIAP-binding protein that sensitizes cells to apoptosis (Martins et al., 2002). In a study of cell death in neurons, RNAi was used to show that myocyte enhancer factor 2A is required for activity-dependent cell survival (Gaudilliere et al., 2002). In another study, RNAi was used to deplete cells of apoptosis-inducing factor (AIF), thereby preventing apoptosis (Wang et al., 2002).

Associations between expression of a gene and a particular biological process are often taken as evidence for a major role for the protein encoded by that gene in that biological process. However, correlations do not establish cause-effect relationships. A well known example of this fact is found in the record of studies of the transcription factor NF-κB. Because NF-κB was shown to be activated in several different types of cells during the process of apoptosis, it was assumed that NF-κB functioned in the cell death process. However, subsequent studies in which NF-κB activity was selectively blocked revealed that this transcription factor actually induced the expression of anti-apoptotic proteins and that cells died more readily when NF-κB function was blocked (Mattson and Camandola, 2001). Thus, in addition to revealing the function of a specific protein, RNAi can also be used to establish that a protein is not involved in a particular process

G. Viral Invasion/Replication

It is believed that endogenous RNAi mechanisms evolved, at least in part, to protect cells against infectious pathogens such as viruses. For example, by producing siRNAs directed against genes required for viral replication, the infected cells prevented propagation of the virus (Lindenbach and Rice, 2002). The development of RNAi technology has verified the importance of RNAi mechanisms in viral invasion and is revealing the underlying molecular interactions. Treatment of various types of cultured cells with siRNAs directed against both viral and cellular targets has revealed specific roles for the targeted proteins in the processes of viral invasion or replication. When cells were treated with siRNAs directed against the HIV-1 tat or reverse transcriptase genes, or against the NF-κB p65 subunit of the host cells, the expression of these viral and cellular proteins were decreased and HIV-1 replication was inhibited, demonstrating the ability of RNA interference to elucidate the biological roles of cellular and viral regulatory factors involved in the control of HIV-1 gene expression (Surabhi and Gaynor, 2002). When human and mouse cells were pretreated with siRNAs to the poliovirus genome, the replication of the virus in those cells was dramatically reduced (Gitlin et al., 2002). In another study it was shown that siRNAs can efficiently silence both cellular lamin AC and hepatitis C virus RNAs in Huh-7 hepatoma cell lines resulting in an 80-fold decrease in HCV RNA within 4 days (Randall et al., 2003). The latter study further showed that the same siRNAs could be used to almost completely eliminate the virus from cells with an established infection. The expression of the human papilloma virus E6 and E7 genes was silenced resulting in the accumulation of cellular p53 protein, transactivation of the cell cycle control p21 gene and reduced cell growth (Jiang and Milner, 2002). A final example of the use of RNAi in the discovery of mechanisms of viral infection is a study of hepatitis

delta virus (HDV) which uses a host-encoded RNA-editing machinery to express two essential proteins from the same coding sequence (Wong and Lazinski, 2002). The authors employed siRNAs to deplete either a small or long form, or both forms, of an adenosine deaminase that acts on RNA (ADAR)1. They found that editing during viral replication was only inhibited when both forms of the enzyme were depleted, demonstrating a cooperative interaction between the short and long forms of ADAR1 in viral replication.

V. Therapeutic Applications of RNA Interference

The most obvious clinical uses of RNAi are for diseases in which selective depletion of one or a few specific proteins would be expected to slow or halt the disease process in the affected cells. Ideally this would be accomplished with no or tolerable side effects. Although there are candidate gene targets for many different diseases, we will focus on four different types of diseases that are very common and for which RNAi approaches are currently being tested in preclinical studies.

A. Cancer

There are two general abnormalities in cancer cells—they exhibit dysregulation of the cell cycle resulting in uncontrolled growth and they are resistant to death as a result of abnormalities in one or more proteins that mediate apoptosis (Nam and Parang, 2003). The goals for RNAi approaches for cancer therapy are therefore to knock out the expression of a cell cycle gene and/or an anti-apoptotic gene in the cancer cells thereby stopping tumor growth and killing the cancer cells. To selectively eliminate cancer cells without damaging normal cells, the RNAi would be targeted to a gene specifically involved in the growth or survival of the cancer cell, or the siRNAs would be selectively delivered into the cancer cells.

For many years antisense oligodeoxynucleotide technology was pursued in preclinical studies of cancer therapies but with discouraging results overall (Jansen and Zangemeister-Wittke, 2002). Recent studies have clearly demonstrated advantages of RNAi methods for the growth suppression and killing of cancer cells. In one study, siRNA was shown to be greater than an order of magnitude more potent than antisense DNA in suppressing gene expression in human hepatoma and pancreatic cancer cell lines (Aoki et al., 2003). In another study four different myeloid leukemia cell lines (HL-60, U937, THP-1, and K562) were transfected with dsRNA duplexes corresponding to the endogenous c-raf and bcl-2 genes (Cioca et al., 2003). Levels of Raf-1 and Bcl-2 proteins were markedly decreased in each of the transfected cell lines; combined RNAi for c-raf and bcl-2 induced apoptosis in HL-60, U937, and THP-1 cells and increased their sensitivity to the DNA-damaging agent etoposide. Activation of tumor necrosis factor (TNF) receptors and related death receptors can induce death of some cancer cells, but may simultaneously activate pathways that promote cell survival; one protein that inhibits the TNF cell death pathway is called FLIP (FLICE-like inhibitory protein). When FLIP expression was suppressed in cancer cells using siRNAs, the cells were more sensitive to being killed when death receptors were activated (Siegmund et al., 2002).

Viral vectors have also been used to express siRNAs and inhibit cancer cell growth and tumorogenicity. For example, a retroviral vector was used to specifically and stably inhibit expression of the oncogenic K-RAS(V12) allele in human tumor cells (Brummelkamp et al., 2002b). Depletion of K-RAS(V12) resulted in loss of anchorage-independent growth and tumorigenicity. In addition to blocking the expression of normal genes that are required for cancer cell growth and survival, RNAi can be used to target specific cancer-causing mutations. For example, dsRNA was employed to target the M-BCR/ABL fusion site to kill leukemic cells with such a rearrangement (Wilda et al., 2002). Leukemic cells without BCR/ABL rearrangement were not killed by M-BCR/ABL-dsRNA. Several other studies have demonstrated efficacy of liposome-mediated or viral vector-mediated transfection of cancer cells in suppressing their growth and/or inducing their death (Zhang et al., 2002a). The next step in the development of RNAi technology for cancer therapy will be to establish methods for targeting tumor cells in vivo. Another approach might be to target genes that promote angiogenesis. Tumor cells require a rich supply of blood and achieve this by stimulating the process of angiogenesis; it may therefore be possible to inhibit tumor growth by targeting the vascular endothelial cells involved in angiogenesis. As evidence, it was shown that depletion of the crk adaptor protein using RNAi inhibited the migration of cultured vascular endothelial cells (Nagashima et al., 2002).

B. Infectious Diseases

Diseases caused by viruses and bacteria continue to be major causes of death worldwide and are an increasing concern because of the emergence of resistant strains and the potential use of infectious pathogens by terrorists (Tan et al., 2000; Franz and Zajtchuk, 2002). Currently, HIV infection has reached epidemic proportions in many African countries and also continues to be a major cause of morbidity and death among homosexuals and intravenous drug users. Other prominent infectious diseases include influenza, hepatitis, Lyme disease, and West Nile virus. Many deaths also result from bacterial infections, with pneumonia and sepsis being prominent examples. The ability of RNAi to inhibit the replication or cellular uptake of viruses and other infectious agents has been clearly demonstrated in cell culture studies and, therefore, holds promise for the treatment of human patients. The ability of HIV-1 to infect cells and replicate can be severely compromised by targeting of

viral genes using siRNAs. Examples include the suppression of HIV-1 replication in human cells transfected with siRNA directed against tat and the rev gene (Capodici et al., 2002; Jacque et al., 2002; Lee et al., 2002a; Novina et al., 2002). Transfection of human cells with siRNAs directed against different genes in the poliovirus genome resulted in resistance of the cells to infection with poliovirus (Gitlin et al., 2002). The ability of siRNAs targeting the gene encoding the death receptor Fas to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis was tested by Song and colleagues (Song et al., 2003). Intravenous injection of Fas siRNA specifically reduced Fas protein levels in the livers of mice during a 10-day period. Fas siRNA treatment abrogated hepatocyte necrosis and inflammatory infiltration and markedly reduced serum concentrations of transaminases demonstrating a clear hepatoprotective effect of the siRNA therapy.

C. Cardiovascular and Cerebrovascular Diseases

Cardiovascular disease is the leading cause of death in the United States and many other industrialized countries. It most commonly results from the progressive occlusion of arteries in a process called atherosclerosis, which can ultimately culminate in a myocardial infarction or stroke. Atherosclerosis involves damage to vascular endothelial cells, local production of inflammatory cytokines, and the recruitment of macrophages to the site forming foam cells; in addition, apoptosis of foam cells and vascular smooth muscle cells occurs (Geng and Libby, 2002). The severe ischemia that occurs in heart or brain cells during a myocardial infarction or stroke results in the death of cardiac muscle cells or neurons. Although some of the cells die rapidly by necrosis, many other cells die more slowly by apoptosis; data from animal studies suggest that such cardiac myocytes and brain neurons that die by apoptosis can be saved (Mattson et al., 2000; Zhao and Vinten-Johansen, 2002).

It may be possible to use RNAi technology to intervene in the process of atherosclerosis or to reduce the damage to heart tissue and brain cells that patients suffer following a myocardial infarction or stroke. A key step in the process of atherosclerosis is the up-regulation of cell adhesion molecules in vascular endothelial cells, which play an essential role in the recruitment of macrophages to the site of endothelial damage. The production of cell adhesion molecules can be selectively suppressed in cultured cells (Jarad et al., 2002). In another study relevant to the pathogenesis of atherosclerosis, it was shown that mevastatin, an inhibitor of cholesterol synthesis, suppresses cell proliferation by inhibiting cyclin-dependent kinase-2 (Ukomadu and Dutta, 2003).

D. Neurodegenerative Disorders

Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis are examples of relatively common age-related neurodegenerative disorders that are increasing as average life expectancy increases. Each disorder is characterized by the dysfunction and death of specific populations of neurons: hippocampal and cortical neurons involved in learning and memory processes in Alzheimer's disease, dopamine-producing neurons in the substantia nigra that control body movements in Parkinson's disease, and spinal cord motor neurons in amyotrophic lateral sclerosis. Specific genetic mutations are responsible for a small percentage of cases of Alzheimer's and Parkinson's disease and amyotrophic lateral sclerosis (Hardy, 2001), whereas all cases of Huntington's disease result from mutations (polyglutamine expansions) in the huntingtin protein (Rubinsztein, 2002). Studies of patients, and of animal and cell culture models of each disease, have revealed shared biochemical cascades that result in neuronal death. Those cascades include increased oxidative stress, dysregulation of cellular calcium homeostasis and apoptosis (Mattson, 2000). There have therefore been two different strategies for preventative and therapeutic interventions in neurodegenerative disorders. One strategy is to block the disease-specific events that are believed to initiate the neurodegenerative process, whereas the second strategy targets downstream events in the neurodegenerative cascade. For example, an abnormality in the proteolytic processing of the amyloid precursor protein is believed to be a key early event in Alzheimer's disease pathogenesis, and two enzymes called β- and γ-secretases that are responsible for cleaving of amyloid precursor protein to generate the neurotoxic amyloid β-peptide are being targeted for drug development. RNAi has recently been used to identify additional proteins, such as APH-1, that are critical for production of amyloid β-peptide (Lee et al., 2002b). Downstream targets include proteins involved in the production of reactive oxygen species, in the regulation of calcium homeostasis, and in the process of apoptosis (Mattson, 2000, 2003).

Recent studies have shown that cultured neurons can be efficiently transfected with siRNAs and that the targeted genes are effectively silenced. In one study it was shown that cultured neurons can be depleted of the p75 neurotrophin receptor, a protein in the TNF receptor family that has been implicated in neuronal apoptosis in certain settings (Higuchi et al., 2003). Pro-apoptotic members of the Bcl-2 family (Colussi et al., 2000) and caspases (Quinn et al., 2000) have been effectively targeted and neuronal death prevented, using RNAi methods. Caplen and colleagues (Caplen et al., 2002) performed studies aimed at determining whether RNAi could be used to target the pathogenic process in inherited neurodegenerative disorders caused by polyglutamine expansions. They used *Drosophila* and human tissue culture models of spinobulbar muscular atrophy, a disease caused by CAG expansions in the gene encoding the androgen receptor. They assessed the abilities of different siRNAs to selectively inhibit expression of

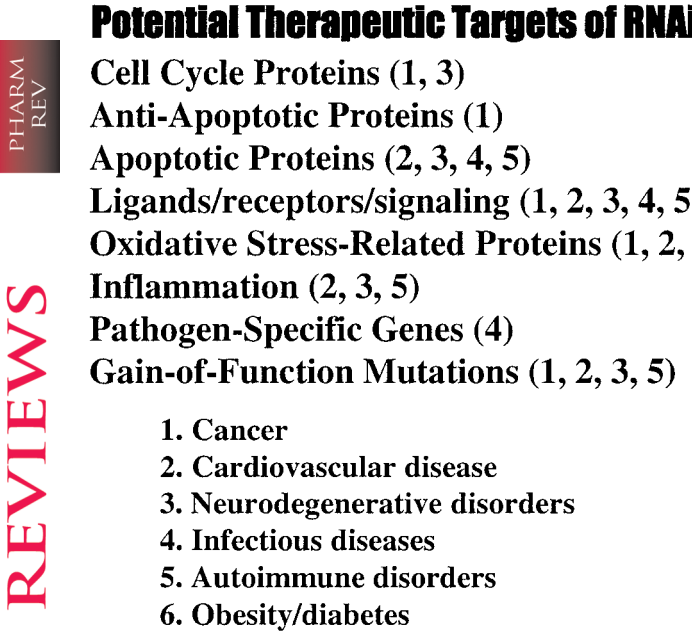

FIG. 4. Examples of therapeutic targets of RNAi in medicine. The ability to target a specific gene or genes using siRNAs or vector-mediated RNA expression methods, suggests the potential of RNAi to block the disease process or relieve symptoms of the disease. Depletion of proteins critical for the cell cycle, such as cyclins, cyclin-dependent kinases, or telomerase, might be effective in treating cancers and some neurodegenerative disorders. Blocking the production of anti-apoptotic proteins such as Bcl-2, inhibitor of apoptosis proteins and antioxidant enzymes may be used to kill cancer cells. Conversely, RNAi-mediated suppression of expression of apoptotic proteins (Bax, Par-4, p53, AIF, and caspases, for example) may slow or stop the degenerative processes in degenerative myocardial, neurological, and autoimmune disorders. Ligands, receptors, and downstream signal transduction proteins critical for a specific disease process might also be targeted. For example, it might be possible to suppress the appetite of obese patients by targeting neuropeptide Y- or ghrelin-producing cells using shRNA-expressing viral vectors. Genes that encode proteins involved in oxidative stress and inflammation (nitric-oxide synthase, cyclooxygenases, and tumor necrosis factor, for example) might be targeted in autoimmune and infectious or inflammatory diseases. Viral and bacterial genes are obvious targets for RNAi-based therapeutic intervention in infectious diseases.

transcripts that included a truncated human androgen receptor gene containing different CAG repeat lengths (16–112 repeats). They found that RNA duplexes containing CAG repeat tracts only induced gene-specific inhibition when flanking androgen receptor sequences were included. Sequence-specific small dsRNAs of 22 nucleotides rescued the toxicity and caspase-3 activation induced by plasmids expressing a transcript encoding an expanded polyglutamine tract. Thus, it is possible, at least in cell culture, to selectively silence a transcript associated with an important group of genetic diseases by RNAi.

Several aspects of neuronal cell biology provide opportunities for novel uses of RNA. Neurons possess complex morphologies with long axons and dendrites, and synapses that are often located at considerable distances from the cell body (for example, the presynaptic terminals of the axons of the lower motor neurons that innervate muscles in the foot in humans can be more than a meter from their cell bodies in the spinal cord). Recent findings suggest that the neurodegenerative cascades that occur in different neurodegenerative disorders may be activated first in synapses. Indeed, it has been shown that apoptotic biochemical cascades can be activated in synapses causing their degeneration (Mattson et al., 1998). Accordingly, pharmacological agents (p53 and caspase inhibitors; Glazner et al., 2000; Gilman et al., 2003) and antisense treatments (Par-4 antisense; Duan et al., 1999) that target apoptotic cascades have been shown to protect synapses in cell culture models of neurodegenerative disorders. RNAi technology would seem to be an ideal approach to target synaptic proteins involved in the pathogenesis of neurodegenerative disorders.

VI. The Future of RNA Interference in Biology and Medicine

Even at this early stage of understanding the molecular mechanisms of RNAi and in the development of methods for the use of RNAi technology for selective gene silencing, it is clear that RNAi will be a widely used tool for establishing the functions of genes. The ability to selectively deplete a single protein of interest in cultured cells using siRNAs, and plasmids and viral vectors, is now established. Improvements on the currently available protocols for RNAi are being made, and the methods are being applied by thousands of investigators in diverse fields. With the advent of these methods has come an explosion of studies that have employed RNAi. Indeed, although there were only nine publications listed on Medline in the years 1987 through 1998 inclusive, there are 631 listed from 1999 to the time of writing of this article. The current status of RNAi as an experimental tool is such that many investigators are now aware of the technology, but most have not yet implemented it in their own studies. The development of RNAi kits by several companies will facilitate the implementation of RNAi methods by essentially any investigator, regardless of their knowledge of RNAi mechanisms. However, major hurdles remain to be crossed, including the application of RNAi methods to in vivo studies. Once it becomes possible to reliably target a specific gene and deplete the protein encoded by that gene from one or all cell types in an organism, a wealth of information will flow from studies of processes ranging from embryogenesis to the function of organ systems.

As for uses of RNAi in medicine, its potential remains to be established. The application of gene therapy approaches for the treatment of specific diseases has progressed much more slowly than initially anticipated. There are, of course, many potential gene targets for therapeutic intervention using RNAi (Fig. 4). Studies that employ RNAi to counteract a disease process in vivo are emerging. For example, RNAi that targeted the Fas gene (Song et al., 2003) or the hepatitis C virus genome

(Wilson et al., 2003) protected mice from hepatitis. One might expect that the next demonstrations of successful treatment of disease in mice will come from models of cancer and neurodegenerative disorders. Because of the potential of RNAi for therapeutic intervention, major efforts should be placed on preclinical studies using this technology.

References

Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, and Aebischer P (2002) Lentiviral-mediated RNA interference. *Hum Gene Ther* **13:**2197–2201.

Ancellin N, Colmont C, Su J, Li Q, Mittereder N, Chae SS, Stefansson S, Liau G, and Hla T (2002) Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. *J Biol Chem* **277:**6667–6675.

Aoki Y, Cioca D, Oidaira H, Kamiya J, and Kiyosawa K (2003) RNA interference may be more potent than antisense RNA in human cancer cell lines. *Clin Exp Pharmacol Physiol* **30:**96–102.

Assaad FF, Tucker KL, and Signer ER (1993) Epigenetic repeat-induced gene silencing (RIGS) in Arabidopsis. *Plant Mol Biol* **22:**1067–1085.

Baehrecke EH (2002) How death shapes life during development. *Nat Rev Mol Cell Biol* **3:**779–787.

Baglioni C and Nilsen TW (1983) Mechanisms of antiviral action of interferon. *Interferon* **5:**23–42.

Bai X, Zhou D, Brown JR, Crawford BE, Hennet T, and Esko JD (2001) Biosynthesis of the linkage region of glycosaminoglycans: cloning and activity of galactosyltransferase II, the sixth member of the beta 1, 3-galactosyltransferase family (beta 3GalT6). *J Biol Chem* **276:**48189–48195.

Banerjee D and Slack F (2002) Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. *Bioessays* **24:**119–129.

Barton GM and Medzhitov R (2002) Delivery of small interfering RNA into primary cells. *Proc Natl Acad Sci USA* **99:**14943–14945.

Bernstein E, Caudy AA, Hammond SM, and Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature (Lond)* **409:**363–366.

Billy E, Brondani V, Zhang H, Muller U, and Filipowicz W (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci USA* **98:**14428–14433.

Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL, and Ji X (2001) Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. *Structure (Camb)* **9:**1225–1236.

Boehm M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ, and Nabel EG (2002) A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. *EMBO (Eur Mol Biol Organ) J* **21:**3390–3401.

Bosher JM and Labouesse M (2000) RNA interference: genetic wand and genetic watchdog. *Nat Cell Biol* **2:**E31–E36.

Brummelkamp TR, Bernards R, and Agami R (2002a) A system for stable expression of short interfering RNAs in mammalian cells. *Science (Wash DC)* **296:**550–553.

Brummelkamp TR, Bernards R, and Agami R (2002b) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2:**243–247.

Cabot RA and Prather RS (2003) Cleavage stage porcine embryos may have differing developmental requirements for karyopherins alpha2 and alpha3. *Mol Reprod Dev* **64:**292–301.

Calegari F, Haubensak W, Yang D, Huttner WB, and Buchholz F (2002) Tissue-specific RNA interference in postimplantation mouse embryos with endoribonuclease-prepared short interfering RNA. *Proc Natl Acad Sci USA* **99:**14236–14240.

Caplen NJ, Fleenor J, Fire A, and Morgan RA (2000) dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. *Gene (Amst)* **252:**95–105.

Caplen NJ, Taylor JP, Statham VS, Tanaka F, Fire A, and Morgan RA (2002) Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference. *Hum Mol Genet* **11:**175–184.

Capodici J, Kariko K, and Weissman D (2002) Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J Immunol* **169:**5196–5201.

Caudy AA, Myers M, Hannon GJ, and Hammond SM (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev* **16:**2491–2496.

Cheng A, Wang S, Yang D, Xiao R, and Mattson MP (2003) Calmodulin mediates brain-derived neurotrophic factor cell survival signaling upstream of Akt kinase in embryonic neocortical neurons. *J Biol Chem* **278:**7591–7599.

Chiu YL and Rana TM (2002) RNAi in human cells: basic structural and functional features of small interfering RNA. *Mol Cell* **10:**549–561.

Cho JH, Bandyopadhyay J, Lee J, Park CS, and Ahnn J (2000) Two isoforms of sarco/endoplasmic reticulum calcium ATPase (SERCA) are essential in Caenorhabditis elegans. *Gene (Amst)* **261:**211–219.

Cioca DP, Aoki Y, and Kiyosawa K (2003) RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. *Cancer Gene Ther* **10:**125–133.

Clarke PA and Mathews MB (1995) Interactions between the double-stranded RNA binding motif and RNA: definition of the binding site for the interferon-induced protein kinase DAI (PKR) on adenovirus VA RNA. *RNA* **1:**7–20.

Cogoni C, Irelan JT, Schumacher M, Schmidhauser T, Selker EU, and Macino G (1996) Transgene silencing of the al-1 gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. *EMBO (Eur Mol Biol Organ) J* **15:**3153-3163.

Cogoni C and Macino G (1999) Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. *Nature (Lond)* **399:**166–169.

Colussi PA, Quinn LM, Huang DC, Coombe M, Read SH, Richardson H, and Kumar S (2000) Debcl, a proapoptotic Bcl-2 homologue, is a component of the Drosophila melanogaster cell death machinery. *J Cell Biol* **148:**703–714.

Czauderna F, Fechtner M, Aygun H, Arnold W, Klippel A, Giese K, and Kaufmann J (2003) Functional studies of the PI(3)-kinase signalling pathway employing synthetic and expressed siRNA. *Nucleic Acids Res* **31:**670–682.

Dalmay T, Hamilton A, Rudd S, Angell S, and Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101:**543–553.

Devroe E and Silver PA (2002) Retrovirus-delivered siRNA. *BMC Biotechnol* **2:**15.

Domeier ME, Morse DP, Knight SW, Portereiko M, Bass BL, and Mango SE (2000) A link between RNA interference and nonsense-mediated decay in Caenorhabditis elegans. *Science (Wash DC)* **289:**1928–1931.

Donze O and Picard D (2002) RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Res* **30:**e46.

Duan W, Rangnekar V, and Mattson MP (1999) Par-4 production in synaptic compartments following apoptotic and excitotoxic insults: evidence for a pivotal role in mitochondrial dysfunction and neuronal degeneration. *J Neurochem* **72:**2312–2322.

Duan W, Zhu X, Ladenheim B, Yu QS, Guo Z, Oyler J, Cutler RG, Cadet JL, Greig NH, and Mattson MP (2002) p53 inhibitors preserve dopamine neurons and motor function in experimental parkinsonism. *Ann Neurol* **52:**597–606.

Eddy SR (2001) Non-coding RNA genes and the modern RNA world. *Nat Rev Genet* **2:**919–921.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature (Lond)* **411:**494–498.

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, and Tuschl T (2001b) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO (Eur Mol Biol Organ) J* **20:**6877–6888.

Eldadah BA and Faden AI (2000) Caspase pathways, neuronal apoptosis and CNS injury. *J Neurotrauma* **17:**811–829.

English JJ, Davenport GF, Elmayan T, Vaucheret H, and Baulcombe DC (1997) Requirement of sense transcription of homology-dependent virus resistance and trans-inactivation. *Plant J* **12:**597–603.

Faergeman NJ and Knudsen J (2002) Acyl-CoA binding protein is an essential protein in mammalian cell lines. *Biochem J* **368:**679–682.

Fagard M, Boutet S, Morel JB, Bellini C, and Vaucheret H (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi and RNA interference in animals. *Proc Natl Acad Sci USA* **97:**11650–11654.

Fire A (1999) RNA-triggered gene silencing. *Trends Genet* **15:**358–363.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, and Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis Elegans. *Nature (Lond)* **391:**806–811.

Franz DR and Zajtchuk R (2002) Biological terrorism: understanding the threat, preparation and medical response. *Dis Mon* **48:**493–564.

Frischmeyer PA and Dietz HC (1999) Nonsense-mediated mRNA decay in health and disease. *Hum Mol Genet* **8:**1893–1900.

Gan L, Anton KE, Masterson BA, Vincent VA, Ye S, and Gonzalez-Zulueta M (2002) Specific interference with gene expression and gene function mediated by long dsRNA in neural cells. *J Neurosci Methods* **121:**151–157.

Gary DS and Mattson MP (2001) Integrin signaling via the PI3-kinase-Akt pathway increases neuronal resistance to glutamate-induced apoptosis. *J Neurochem* **76:**1485–1496.

Gaudilliere B, Shi Y, and Bonni A (2002) RNA interference reveals a requirement for myocyte enhancer factor 2A in activity-dependent neuronal survival. *J Biol Chem* **277:**46442–46446.

Geng YJ and Libby P (2002) Progression of atheroma: a struggle between death and procreation. *Arterioscler Thromb Vasc Biol* **22:**1370–1380.

Gil J and Esteban M (2000) Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* **5:**107–114.

Gilman CP, Chan SL, Guo Z, Zhu X, Greig NH, and Mattson MP (2003) p53 is present in synapses where it mediates mitochondrial dysfunction and synaptic degeneration in response to DNA damage and oxidative and excitotoxic insults. *Neuromolecular Med* **3:**159–172.

Gitlin L, Karelsky S, and Andino R (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature (Lond)* **418:**430–434.

Glazner GW, Chan SL, Lu C, and Mattson MP (2000) Caspase-mediated degradation of AMPA receptor subunits: a mechanism for preventing excitotoxic necrosis and ensuring apoptosis. *J Neurosci* **20:**3641–3649.

Greener T, Grant B, Zhang Y, Wu X, Greene LE, Hirsh D, and Eisenberg E (2001) Caenorhabditis elegans auxilin: a J-domain protein essential for clathrin-mediated endocytosis in vivo. *Nat Cell Biol* **3:**215–219.

Grishok A, Tabara H, and Mello CC (2000) Genetic requirements for inheritance of RNAi in C. Elegans. *Science (Wash DC)* **287:**2494–2497.

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, and Mello CC (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* **106:**23–34.

Hamilton AJ and Baulcombe DC (1999) A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science (Wash DC)* **286:**950–952.

Hammond SM, Bernstein E, Beach D, and Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature (Lond)* **404:**293–296.

Hammond SM, Boettcher S, Caudy AA, Kobayashi R, and Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science (Wash DC)* **293:**1146–1150.

Harborth J, Elbashir SM, Bechert K, Tuschl T, and Weber K (2001) Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J Cell Sci* **114:**4557–4565.
Hardy J (2001) The genetic causes of neurodegenerative diseases. *J Alzheimers Dis* **3:**109–116.
Higuchi H, Yamashita T, Yoshikawa H, and Tohyama M (2003) Functional inhibition of the p75 receptor using a small interfering RNA. *Biochem Biophys Res Commun* **301:**804–809.
Holtorf H, Schob H, Kunz C, Waldvogel R, and Meins F Jr (1999) Stochastic and nonstochastic post-transcriptional silencing of chitinase and beta-1, 3-glucanase genes involves increased RNA turnover-possible role for ribosome-independent RNA degradation. *Plant Cell* **11:**471–484.
Hutchings NR, Donelson JE, and Hill KL (2002) Trypanin is a cytoskeletal linker protein and is required for cell motility in African trypanosomes. *J Cell Biol* **156:**867–877.
Hutvagner G and Zamore PD (2002) A microRNA in a multiple-turnover RNAi enzyme complex *Science (Wash DC)* **297:**2056–2060.
Iratni R, Yan YT, Chen C, Ding J, Zhang Y, Price SM, Reinberg D, and Shen MM (2002) Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. *Science (Wash DC)* **298:**1996–1999.
Irie N, Sakai N, Ueyama T, Kajimoto T, Shirai Y, and Saito N (2002) Subtype- and species-specific knockdown of PKC using short interfering RNA. *Biochem Biophys Res Commun* **298:**738–743.
Ishizuka A, Siomi MC, and Siomi H (2002) A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev* **16:**2497–2508.
Jacque JM, Triques K, and Stevenson M (2002) Modulation of HIV-1 replication by RNA interference. *Nature (Lond)* **418:**435–438.
Jansen B and Zangemeister-Wittke U (2002) Antisense therapy for cancer–the time of truth. *Lancet Oncol* **3:**672–683.
Jarad G, Wang B, Khan S, DeVore J, Miao H, Wu K, Nishimura SL, Wible BA, Konieczkowski M, Sedor JR, and Schelling JR (2002) Fas activation induces renal tubular epithelial cell beta 8 integrin expression and function in the absence of apoptosis. *J Biol Chem* **277:**47826–47833.
Jiang M and Milner J (2002) Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* **21:**6041–6048.
Kartasheva NN, Contente A, Lenz-Stoppler C, Roth J, and Dobbelstein M (2002) p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene* **21:**4715–4727.
Kennerdell JR and Carthew RW (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 Act in the wingless pathway. *Cell* **95:**1017–1026.
Kennerdell JR and Carthew RW (2000) Heritable gene silencing in Drosophila using double-stranded RNA. *Nat Biotechnol* **18:**896–898.
Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, and Plasterk RH (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. Elegans. *Genes Dev* **15:**2654–2659.
Kim MH, Yuan X, Okumura S, and Ishikawa F (2002) Successful inactivation of endogenous Oct-3/4 and c-mos genes in mouse preimplantation embryos and oocytes using short interfering RNAs. *Biochem Biophys Res Commun* **296:**1372–1377.
Kisielow M, Kleiner S, Nagasawa M, Faisal A, and Nagamine Y (2002) Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. *Biochem J* **363:**1–5.
Krichevsky AM and Kosik KS (2002) RNAi functions in cultured mammalian neurons. *Proc Natl Acad Sci USA* **99:**11926–11929.
Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, and Rossi J (2002a) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* **20:**500–505.
Lee SF, Shah S, Li H, Yu C, Han W, and Yu G (2002b) Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch. *J Biol Chem* **277:**45013–45019.
Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, and Ruvkun G (2002c) A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. *Nat Genet* **33:**40–48.
Leirdal M and Sioud M (2002) Gene silencing in mammalian cells by preformed small RNA duplexes. *Biochem Biophys Res Commun* **295:**744–748.
Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, and Herweijer H (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet* **32:**107–108.
Lindenbach BD and Rice CM (2002) RNAi targeting an animal virus: news from the front. *Mol Cell* **9:**925–957.
Liu X and Erikson RL (2002) Activation of Cdc2/cyclin B and inhibition of centrosome amplification in cells depleted of Plk1 by siRNA. *Proc Natl Acad Sci USA* **99:**8672–8676.
Lois C, Hong EJ, Pease S, Brown EJ, and Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science (Wash DC)* **295:**868–872.
Mailand N, Lukas C, Kaiser BK, Jackson PK, Bartek J, and Lukas J (2002) Deregulated human Cdc14A phosphatase disrupts centrosome separation and chromosome segregation. *Nat Cell Biol* **4:**317–322.
Makimura H, Mizuno TM, Mastaitis JW, Agami R, and Mobbs CV (2002) Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci* **3:**18.
Marathe R, Anandalakshmi R, Smith TH, Pruss GJ, and Vance VB (2000) RNA viruses as inducers, suppressors and targets of post-transcriptional gene silencing. *Plant Mol Biol* **43:**295–306.
Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, and Tuschl T (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110:**563–574.
Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, and Downward J (2002) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem* **277:**439–444.
Mattson MP, Keller JN, and Begley JG (1998) Evidence for synaptic apoptosis. *Exp Neurol* **153:**35–48.
Mattson MP (2000) Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* **1:**120–129.
Mattson MP (2003) Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Med* **3:**65–94.
Mattson MP and Camandola S (2001) NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Investig* **107:**247–254.
Mattson MP, Culmsee C, and Yu ZF (2000) Apoptotic and antiapoptotic mechanisms in stroke. *Cell Tissue Res* **301:**173–187.
McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, and Kay MA (2002) RNA interference in adult mice. *Nature (Lond)* **418:**38–39.
McManus MT, Petersen CP, Haines BB, Chen J, and Sharp PA (2002) Gene silencing using micro-RNA designed hairpins. *RNA* **8:**842–850.
Mendell JT, Rhys CM, and Dietz HC (2002) Separable roles for rent1/hUpf1 in altered splicing and decay of nonsense transcripts. *Science (Wash DC)* **298:**419–422.
Mette MF, Aufsatz W, van der Winden J, Matzke MA, and Matzke AJ (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO (Eur Mol Biol Organ) J* **19:**5194–5201.
Mise-Omata S, Montagne B, Deckert M, Wienands J, and Acuto O (2003) Mammalian actin binding protein 1 is essential for endocytosis but not lamellipodia formation: functional analysis by RNA interference. *Biochem Biophys Res Commun* **301:**704–710.
Miyagishi M and Taira K (2002) U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* **20:**497–500.
Mollinari C, Kleman JP, Jiang W, Schoehn G, Hunter T, and Margolis RL (2002) PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. *J Cell Biol* **157:**1175–1186.
Montgomery MK, Xu S, and Fire A (1998) RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis Elegans. *Proc Natl Acad Sci USA* **95:**15502–15507.
Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K, Sanial M, Vo TA, Vaucheret H (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101:**533–542.
Nagashima K, Endo A, Ogita H, Kawana A, Yamagishi A, Kitabatake A, Matsuda M, and Mochizuki N (2002) Adaptor protein Crk is required for ephrin-B1-induced membrane ruffling and focal complex assembly of human aortic endothelial cells. *Mol Biol Cell* **13:**4231–4242.
Naldini L (1998) Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol* **9:**457–463.
Nam NH and Parang K (2003) Current targets for anticancer drug discovery. *Curr Drug Targets* **4:**159–179.
Napoli C, Lemieux C, and Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2:**279–289.
Nicholson RH and Nicholson AW (2002) Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference. *Mamm Genome* **13:**67–73.
Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P, and Sharp PA (2002) siRNA-directed inhibition of HIV-1 infection. *Nat Med* **8:**681–686.
Nykanen A, Haley B, and Zamore PD (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107:**309–321.
Ohta T, Essner R, Ryu JH, Palazzo RE, Uetake Y, and Kuriyama R (2002) Characterization of Cep135, a novel coiled-coil centrosomal protein involved in microtubule organization in mammalian cells. *J Cell Biol* **156:**87–99.
Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, and Conklin DS (2002a) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16:**948–958.
Paddison PJ, Caudy AA, and Hannon GJ (2002b) Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci USA* **99:**1443–1448.
Page MF, Carr B, Anders KR, Grimson A, and Anderson P (1999) SMG-2 is a phosphorylated protein required for mRNA surveillance in Caenorhabditis elegans and related to Upf1p of yeast. *Mol Cell Biol* **19:**5943–5951.
Paul CP, Good PD, Winer I, and Engelke DR (2002) Effective expression of small interfering RNA in human cells. *Nat Biotechnol* **20:**505–508.
Peng Y, Zhang Q, Nagasawa H, Okayasu R, Liber HL, and Bedford JS (2002) Silencing expression of the catalytic subunit of DNA-dependent protein kinase by small interfering RNA sensitizes human cells for radiation-induced chromosome damage, cell killing and mutation. *Cancer Res* **62:**6400–6404.
Pfeifer A, Ikawa M, Dayn Y, and Verma IM (2002) Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci USA* **99:**140–145.
Poinat P, De Arcangelis A, Sookhareea S, Zhu X, Hedgecock EM, Labouesse M, and Georges-Labouesse E (2002) A conserved interaction between beta1 integrin/PAT-3 and Nck-interacting kinase/MIG-15 that mediates commissural axon navigation in C. elegans. *Curr Biol* **12:**622–631.
Porter LA, Dellinger RW, Tynan JA, Barnes EA, Kong M, Lenormand JL, and Donoghue DJ (2002) Human Speedy: a novel cell cycle regulator that enhances proliferation through activation of Cdk2. *J Cell Biol* **157:**357–366.
Prasanth SG, Prasanth KV, and Stillman B (2002) Orc6 involved in DNA replication, chromosome segregation and cytokinesis. *Science (Wash DC)* **297:**1026–1031.
Pruss G, Ge X, Shi XM, Carrnington JC, and Vance VB (1997) Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9:**1749–1759.

Quinn LM, Dorstyn L, Mills K, Colussi PA, Chen P, Coombe M, Abrams J, Kumar S, and Richardson H (2000) An essential role for the caspase dronc in developmentally programmed cell death in Drosophila. *J Biol Chem* **275:**40416–40424.
Randall G, Grakoui A, and Rice CM (2003) Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. *Proc Natl Acad Sci USA* **100:**235–240.
Ratcliff F, Harrison BD, and Baulcombe DC (1997) A similarity between viral defense and gene silencing in plants. *Science (Wash DC)* **276:**1558–1560.
Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Zhang M, McManus MT, Gertler FB, Scott ML, and Van Parijs L (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* **33:**401–406.
Rubinsztein DC (2002) Lessons from animal models of Huntington's disease. *Trends Genet* **18:**202–209.
Ruiz-Echevarria MJ, Czaplinski K, and Peltz SW (1996) Making sense of nonsense in yeast. *Trends Biochem Sci* **21:**433–438.
Salisbury JL, Suino KM, Busby R, and Springett M (2002) Centrin-2 is required for centriole duplication in mammalian cells. *Curr Biol* **12:**1287–1292.
Siegmund D, Hadwiger P, Pfizenmaier K, Vornlocher HP, and Wajant H (2002) Selective inhibition of FLICE-like inhibitory protein expression with small interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis. *Mol Med* **8:**725–732.
Smalheiser NR, Manev H, and Costa E (2001) RNAi and brain function: was McConnell on the right track? *Trends Neurosci* **24:**216–218.
Smardon A, Spoerke JM, Stacey SC, Klein ME, Mackin N, and Maine EM (2000) EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans. *Curr Biol* **10:**169–178.
Smith GM and Romero MI (1999) Adenoviral-mediated gene transfer to enhance neuronal survival, growth and regeneration. *J Neurosci Res* **55:**147–157.
Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, and Lieberman J (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* **9:**347–351.
Stucke VM, Sillje HH, Arnaud L, and Nigg EA (2002) Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. *EMBO (Eur Mol Biol Organ) J* **21:**1723–1732.
Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC, Shi Y (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* **99:**5515–5520.
Surabhi RM and Gaynor RB (2002) RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. *J Virol* **76:**12963–12973.
Svoboda P, Stein P, and Schultz RM (2001) RNAi in mouse oocytes and preimplantation embryos: effectiveness of hairpin dsRNA. *Biochem Biophys Res Commun* **287:**1099–1104.
Tabara H, Grishok A, and Mello CC (1998) RNAi in C. elegans: soaking in the genome sequence. *Science (Wash DC)* **282:**430–431.
Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, and Mello CC (1999) The Rde-1 gene, RNA interference and transposon silencing in C. Elegans. *Cell* **99:**123–132.
Tabara H, Yigit E, Siomi H, and Mello CC (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1 and a DExH-box helicase to direct RNAi in C. elegans. *Cell* **109:**861–871.
Tan YT, Tillett DJ, and McKay IA (2000) Molecular strategies for overcoming antibiotic resistance in bacteria. *Mol Med Today* **6:**309–314.
Tavernarakis N, Wang SL, Dorovkov M, Ryazanov A, and Driscoll M (2000) Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat Genet* **24:**180–183.
Timmons L and Fire A (1998) Specific interference by ingested dsRNA. *Nature (Lond)* **395:**854.
Tiscornia G, Singer O, Ikawa M, and Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci USA* **100:**1844–1848.
Tsuneoka M, Koda Y, Soejima M, Teye K, and Kimura H (2002) A novel myc target gene, mina53, that is involved in cell proliferation. *J Biol Chem* **277:**35450–35459.
Tuschl T, Zamore PD, Lehmann R, Bartel DP, and Sharp PA (1999) Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev* **13:**3191–3197.
Ukomadu C and Dutta A (2003) Inhibition of cdk2 activating phosphorylation by mevastatin. *J Biol Chem* **278:**4840–4846.
Van Blokland R, Van Der Geest N, Mol JMN, and Kooter JM (1994) Transgene-mediated expression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. *Plant J* **6:**861–877.
Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, and Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science (Wash DC)* **297:**1833–1837.
Walters DK and Jelinek DF (2002) The effectiveness of double-stranded short inhibitory RNAs (siRNAs) may depend on the method of transfection. *Antisense Nucleic Acid Drug Dev* **12:**411–418.
Wang X, Yang C, Chai J, Shi Y, and Xue D (2002) Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. *Science (Wash DC)* **298:**1587–1592.
Wang Z, Morris JC, Drew ME, and Englund PT (2000) Inhibition of Trypanosoma brucei gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J Biol Chem* **275:**40174–40179.
Weil D, Garcon L, Harper M, Dumenil D, Dautry F, and Kress M (2002) Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. *Biotechniques* **33:**1244–1248.
Wianny F and Zernicka-Goetz M (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol* **2:**70–75.
Wilda M, Fuchs U, Wossmann W, and Borkgardt A (2002) Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene* **21:**5716–5724.
Wilson JA, Jayasena S, Khvorova A, Sabatinos S, Rodrigue-Gervais IG, Arya S, Sarangi F, Harris-Brandts M, Beaulieu S, and Richardson CD (2003) RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. *Proc Natl Acad Sci USA* **100:**2783–2788.
Winston WM, Molodowitch C, and Hunter CP (2002) Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. *Science (Wash DC)* **295:**2456–2459.
Wojcik C and DeMartino GN (2002) Analysis of Drosophila 26 S proteasome using RNA interference. *J Biol Chem* **277:**6188–6197.
Wong SK and Lazinski DW (2002) Replicating hepatitis delta virus RNA is edited in the nucleus by the small form of ADAR1. *Proc Natl Acad Sci USA* **99:**15118–15123.
Xia H, Mao Q, Paulson HL, and Davidson BL (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* **20:**1006–1010.
Xu K, Tavernarakis N, and Driscoll M (2001) Necrotic cell death in C. elegans requires the function of calreticulin and regulators of Ca(2+) release from the endoplasmic reticulum. *Neuron* **31:**957–971.
Xu Z, Williams CJ, Kopf GS, and Schultz RM (2003) Maturation-associated increase in IP(3) receptor type 1: role in conferring increased IP(3) sensitivity and Ca(2+) oscillatory behavior in mouse eggs. *Dev Biol* **254:**163–171.
Yang S, Tutton S, Pierce E, and Yoon K (2001) Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Mol Cell Biol* **21:**7807–7816.
Ye F and Signer ER (1996) RIGS (repeat-induced gene silencing) in Arabidopsis is transcriptional and alters chromatin configuration. *Proc Natl Acad Sci USA* **93:**10881–10886.
Yu JY, DeRuiter SL, and Turner DL (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* **99:**6047–6052.
Zamore PD, Tuschl T, Sharp PA, and Bartel DP (2000) RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101:**25–33.
Zhang D, Li F, Weidner D, Mnjoyan ZH, and Fujise K (2002a) Physical and functional interaction between myeloid cell leukemia 1 protein (MCL1) and Fortilin. The potential role of MCL1 as a fortilin chaperone. *J Biol Chem* **277:**37430–37438.
Zhang H, Kolb FA, Brondani V, Billy E, and Filipowicz W (2002b) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO (Eur Mol Biol Organ) J* **21:**5875–5885.
Zhao ZQ and Vinten-Johansen J (2002) Myocardial apoptosis and ischemic preconditioning. *Cardiovasc Res* **55:**438–455.
Zhu X, Yu QS, Cutler RG, Culmsee CW, Holloway HW, Lahiri DK, Mattson MP, and Greig NH (2002) Novel p53 inactivators with neuroprotective action: syntheses and pharmacological evaluation of 2-imino-2,3,4,5,6,7-hexahydrobenzothiazole and 2-imino-2,3,4,5,6,7-hexahydrobenzoxazole derivatives. *J Med Chem* **45:**5090–5097.