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THE SILENT REVOLUTION: RNA Interference as Basic Biology, Research Tool, and Therapeutic

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■ **Abstract** RNA interference (RNAi) is an evolutionarily conserved mechanism for silencing gene expression. In primitive organisms, RNAi protects the genome from viruses and other insertable genetic elements and regulates gene expression during development. The antisense (guide) strand of short double-stranded RNAs is incorporated into an RNA-induced silencing complex that can either suppress protein expression or direct degradation of messenger RNAs that contain homologous sequence(s). The discovery that RNAi works in mammalian cells has sparked intense investigation into its role in normal mammalian cell function, its use as a tool to understand or screen for genes functioning in cellular pathways in healthy and diseased cells and animals, and its potential for therapeutic gene silencing. RNAi may provide an important new therapeutic modality for treating infection, cancer, neurodegenerative disease, and other illnesses, although in vivo delivery of small interfering RNAs into cells remains a significant obstacle.

INTRODUCTION

RNA interference (RNAi) is a recently described mechanism for inhibiting gene expression. It was originally identified in plants, fungi, and worms when introduction of control sense oligonucleotides into cells unexpectedly led to reduced gene expression (1–3). In a paradigmatic experiment, petunias surprisingly developed areas of hypopigmentation when transduced with the gene encoding an enzyme required for pigment synthesis. RNAi-mediated gene silencing suppresses gene expression by several mechanisms, including the targeted sequence-specific degradation of mRNA, translational repression, and the maintenance of silenced regions of chromatin. Silencing of endogenous genes regulates basic biological processes, including the transition from one developmental stage to the next (4). In addition, RNAi is used as a form of primitive immunity to protect the genome from invasion by exogenous nucleic acids introduced by mobile genetic elements, such as viruses and transposons.

RNAi was first described in animal cells by Fire and colleagues in the nematode *Caenorhabditis elegans* (1). They found that introducing long double-stranded RNA (dsRNA) into *C. elegans* led to the targeted degradation of homologous mRNA. RNAi is related to other gene-silencing phenomena, including posttranscriptional gene silencing in plants and quelling in the fungus *Neurospora crassa* (2, 5, 6). Although these processes at first seemed unrelated, they all use dsRNA homologous to the silenced gene. Moreover, key proteins involved in RNAi in disparate organisms are highly conserved (5–7). It now appears that RNAi is a universal, omnipresent conserved mechanism in eukaryotic cells.

Interest in RNAi soared when Tuschl and colleagues showed that RNAi also occurs in mammalian cells (8). This raised the prospect of harnessing this potent and specific gene-silencing mechanism for biomedical research and therapy. In the past few years, there has been an RNAi revolution as researchers have sought to understand how RNAi works to regulate gene expression, have used it to perform reverse genetics in mammalian cells, and have begun to explore its potential therapeutic use. What we know now about the molecular basis of RNAi and how it regulates gene expression is clearly just the tip of the iceberg. This review introduces the reader to this emerging revolution in gene regulation and its implications for medicine.

THE SILENCING MECHANISM

RNAi pathways have been most fully described in *Drosophila*, but mammalian complexes and mechanisms are thought to be similar. The effector molecules that guide mRNA degradation are small [21- to 25-nucleotide (nt)] dsRNA, termed small interfering RNAs (siRNAs), produced by the cleavage of long dsRNAs (9–12) (Figure 1). These short RNAs are produced by the cytoplasmic, highly conserved Dicer family of RNase III–like enzymes, resulting in a characteristic 21–23-nt dsRNA duplex with symmetric 2- to 3-nt 3′ overhangs (7, 13). RNAi can also be initiated by introducing chemically synthesized siRNAs into cells. The siRNAs are taken up into a multisubunit ribonucleoprotein complex called RISC (RNA-induced silencing complex). The antisense (guide) strand of the siRNA directs the endonuclease activity of RISC to the homologous (target) site on the mRNA resulting in mRNA cleavage (recently reviewed in Reference 14).

Studies in *Drosophila* following the fate of introduced siRNAs have shown that they are incorporated into a series of ribonucleoprotein complexes of increasing size (15–17) (Figure 1). Although purified Dicer can process long dsRNA into siRNAs in vitro, the principal siRNA-generating enzyme is actually a Dicer-R2D2 heterodimer, which remains associated with the siRNA. The RISC siRNA is initially duplexed but unwinds in the activated, mRNA cleavage-competent form of RISC ("holoRISC") (17). In addition to Dicer, R2D2, and the single-stranded siRNA, activated RISC also contains the highly conserved Argonaute 2 (Ago2), recently identified as the RNAi endonuclease (or Slicer) (18–22). Relatively little is known about how RNAi is regulated. Recently, a highly conserved RNase,

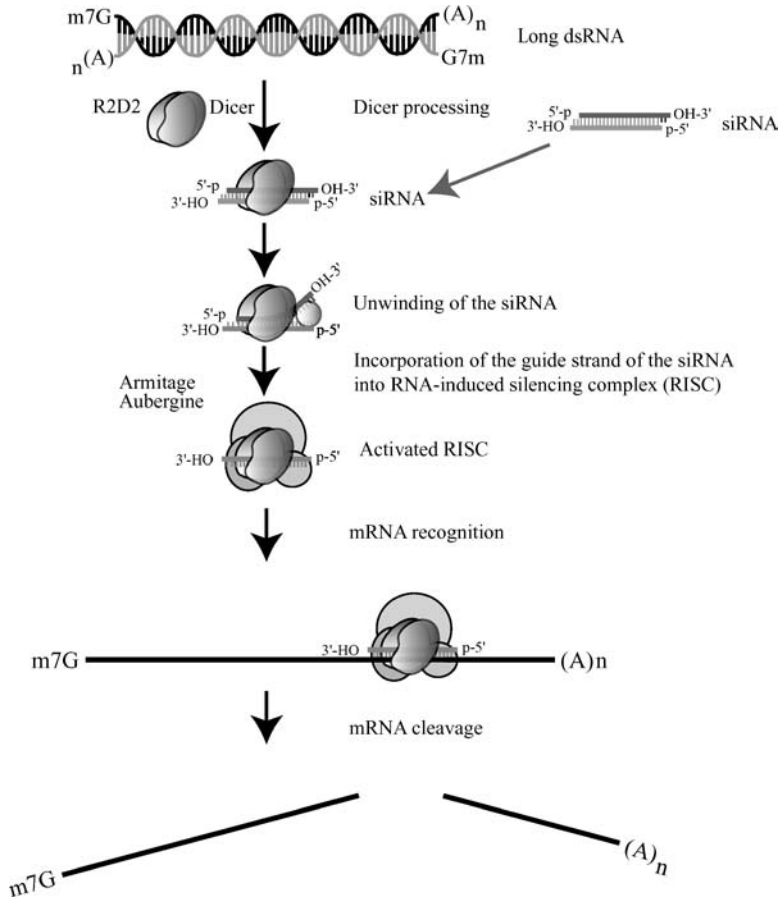

Figure 1 The classical RNA interference (RNAi) pathway in *Drosophila*. Long double-stranded RNAs (dsRNAs) are processed by the R2D2/Dicer heterodimer into small interfering RNAs (siRNAs) (15). The duplexed siRNA is unwound in an ATP-dependent manner starting at the 5′ terminus that has the lowest relative free energy of base pairing (52, 53). This strand of the siRNA, the guide strand, is also preferentially taken up by the RNA-induced silencing complex (RISC) in a step that requires the *Drosophila* Armitage and Aubergine proteins (17). RISC also contains the Vasa intronic gene (VIG), the Tudor staphylococcal nuclease (TSN), Argonaute 2 (AGO2), and the *Drosophila* homolog of the Fragile-X related protein (dFXR) (18–20). The single-stranded siRNA guides the endonuclease activity of the activated RISC ("holoRISC") to the homologous site on the mRNA, cleaving the mRNA (17).

ERI-1, that degrades siRNAs was discovered in a *C. elegans* genetic screen for mutations that had enhanced sensitivity to RNAi (23). This is the first evidence for a regulatory mechanism to limit RNAi-mediated silencing.

MULTIPLE MECHANISMS OF SMALL-RNA-MEDIATED GENE SILENCING

The endogenous RNAi pathway contributes significantly to regulating cellular gene expression. Analysis of naturally occurring small RNA species has identified several classes of them, including siRNAs, microRNAs (miRNAs), and repeat-associated siRNAs (rasiRNAs) (4, 24, 25). miRNAs are produced from pre-miRNAs through a multistep maturation process (Figure 2) (26, 27). The pre-miRNAs are hairpins with imperfect complementarity in their stems and frequent bulges, mismatches and G:U wobble base pairings. Although the function of most miRNAs remains unknown, the archetype miRNAs, let-7 and lin-4, regulate *C. elegans* larval development (4). miRNAs are expressed in a specific spatial and temporal pattern during development in *D. melanogaster* or differentiation of mouse embryonic stem cells; this observation further supports a role in development (25, 28). miRNAs appear to function by binding to sites (often in the 3′ untranslated region) on the mRNA that have only partial sequence complementarity. Instead of cleaving the target mRNA, miRNAs interfere with protein expression by an unknown mechanism that may repress translation or possibly direct nascent proteins for degradation. Although siRNAs and miRNAs are processed from different dsRNA precursors, they appear to be functionally interchangeable, since an siRNA will direct translational repression of a target site with partial complementarity, whereas a miRNA can direct cleavage of a fully complementary target mRNA (29, 30).

In addition to regulating development, small RNAs also help preserve the integrity of the genome, by suppressing invading genetic material such as transposons, retrotransposons, and viruses. This has been shown in genetic studies in *Drosophila*, *C. elegans*, *S. pombe*, and plants. Some of the small RNAs in these organisms are homologous to regions of repetitive DNA, termed rasiRNAs, including transposons, retrotransposons, centromeric repeats, and satellite and microsatellite DNA (25, 31–34). Small RNAs in *C. elegans* and *S. pombe* recruit and maintain regions of silenced chromatin containing these repetitive sequences by activating sequence-specific DNA methylation and histone methylation and by recruiting heterochromatin-associated proteins (35–39) (Figure 3). A protein complex, termed RITS (RNA-induced initiation of transcriptional gene silencing), required for heterochromatin assembly in *S. pombe*, has been recently purified and found to contain Ago1 (a known component of the RNAi pathway), the heterochromatin-associated protein Chp1, and the novel protein Tas3 (targeting complex subunit 3) (40). Although transcriptional gene silencing has not been demonstrated in any mammalian system, mammalian cells deficient in Dicer

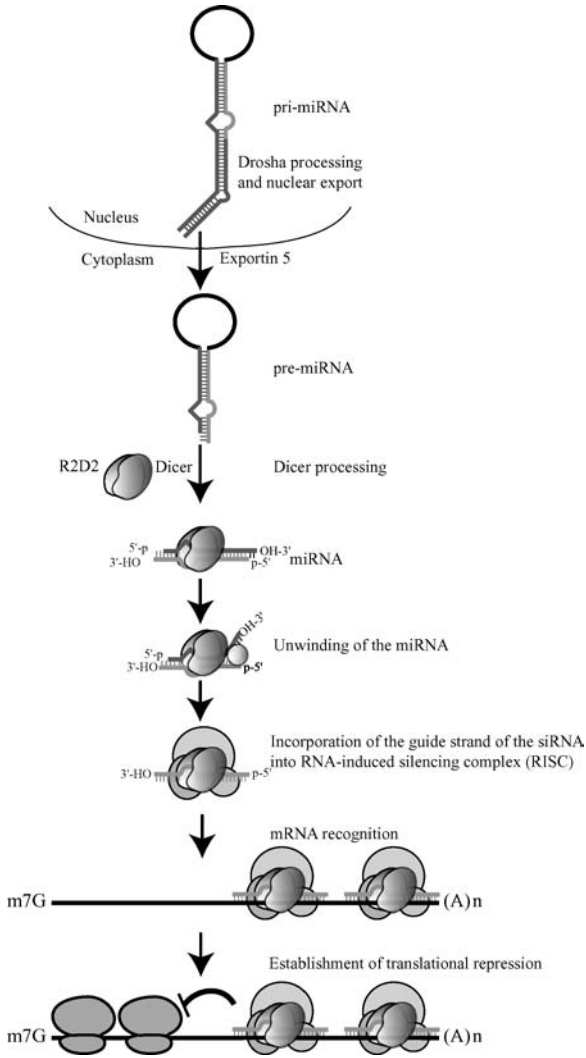

Figure 2 The miRNA pathway. The pre-miRNA is expressed in the nucleus from endogenous long transcripts and is processed into ~70-nt hairpins by the RNase III family member Drosha to become the pre-miRNA (26, 27). The pre-miRNA is exported to the cytoplasm by Exportin 5 (114) and is further cleaved by the R2D2/Dicer heterodimer into the mature miRNA. The miRNA is loaded into RISC and guides it to sites on the mRNA that have only partial sequence complementarity to the miRNA, leading to repression of translation.

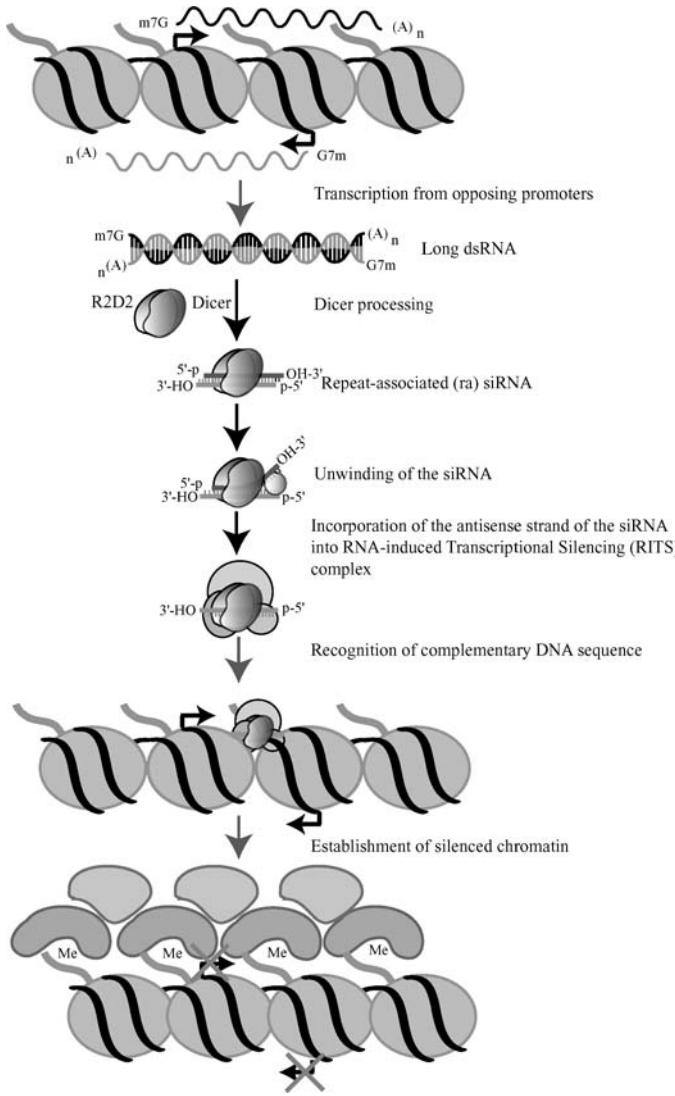

Figure 3 The repeat-associated siRNA (rasiRNA) pathway (25). Transcription from opposing promoters found in repetitive DNA elements, such as centromeric repeats and satellite DNA, leads to the formation of long dsRNAs. These long dsRNAs are cleaved by Dicer, presumably the R2D2/Dicer heterodimer, into siRNAs. These are unwound and taken up by the RNA-induced transcriptional silencing complex (RITS) (40), which directs the establishment of silenced chromatin over the region of DNA homologous to the siRNAs. This silenced chromatin is characterized by methylation of the Lysine 9 residue of histone H3 and the recruitment of heterochromatin-associated proteins (HP1 and HP2) in *Drosophila* polytene chromosomes (39).

aberrantly accumulate transcripts from normally silenced centromeric repeat DNA, and exogenously introduced siRNAs homologous to a promoter sequence can inhibit transcription and direct DNA methylation of the targeted sequence in human cells (41, 41a). These results suggest that it is likely that RNAi is also naturally used to maintain regions of heterochromatin in mammalian cells.

SPECIFICITY OF RNAi

In an initial study, transfection of dsRNAs shorter than 30 nt effectively silenced gene expression by RNAi in a sequence-specific manner without activating interferon pathway genes (8). In addition, introducing a single nucleotide change to the siRNA sequence could abrogate siRNA-mediated silencing; this observation implies that efficient silencing is highly sequence specific. The specificity of RNAi has been best demonstrated by using RNAi to silence the pathogenic allele of a gene while leaving the wild-type allele unaffected, the two alleles differing by a single nucleotide (42–44). siRNA-mediated suppression of viral gene expression can lead to viral RNAi escape variants with a single nucleotide change in the siRNA binding site (45–47).

Nonetheless, some changes between the siRNA and the mRNA target sequences can be tolerated without disrupting silencing (48, 49). Changes at the 3′ end of the guide siRNA strand are relatively well tolerated, whereas changes in the middle and at the 5′ end have an adverse effect (50). Although these conclusions were initially based on a single siRNA:target mRNA, they have been validated by other studies (45–47, 51).

Thermodynamic profiling of siRNAs targeting several genes showed that siRNAs with lower thermodynamic stability for base pairing at the 5′ end of their antisense (guide) strand and in the middle of the siRNA were more effective at RNAi than siRNAs that had stronger base pairing in these regions (52, 53). The lower stability of the 5′ end affects the uptake of the guide strand into RISC and may also enhance RISC binding to the target mRNA, since mutations in this region decrease the affinity of the siRNA for the target mRNA (29, 53, 54). Mutations around the center of the siRNA:mRNA recognition site, between nucleotides 9 and 14 relative to the 5′ end of the siRNA guide strand, also have a significant effect on silencing.

Although RNAi mediated by the introduction of long dsRNA has been used to silence gene expression in a wide variety of organisms including *C. elegans*, plants, *Drosophila*, mosquito, and mouse oocytes, their use in vertebrate cells has been limited owing to global suppression of gene expression by dsRNA-induced activation of the interferon response (55) (Figure 4).

Recently, several studies have shown that under some conditions even short dsRNAs may activate PKR and induce a subset of interferon response genes (56–58). However, no cytopathic effects were seen. In fact, gene expression profiles of HeLa cells transfected with siRNAs showed only a partial overlap when compared with treatments known to induce an interferon response (56). The nonspecific

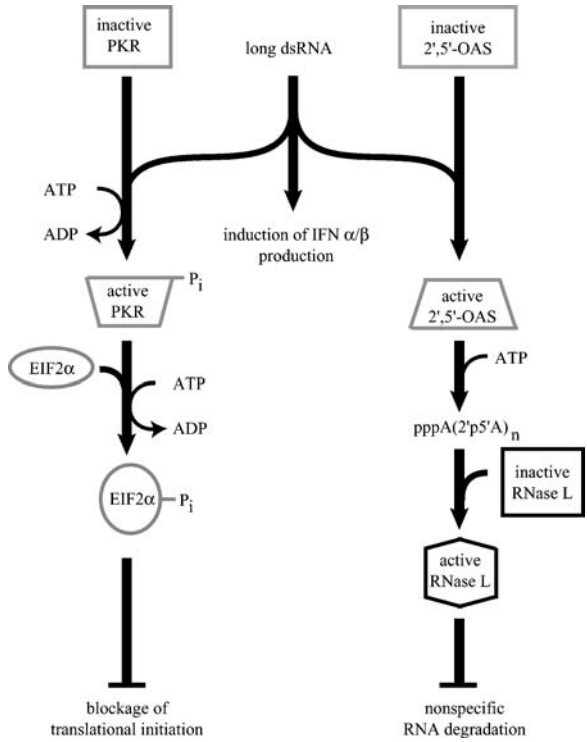

Figure 4 The interferon response. In mammalian cells, long dsRNA elicits a sequence-nonspecific inhibition of gene expression resulting from activation of the protein kinase PKR and 2′,5′-oligoadenylate synthetase (2′,5′-OAS). Activation of PKR induces phosphorylation of the translation initiation factor (EIF2α) and inhibits translation initiation; activation of 2′,5′-OAS catalyzes the conversion of ATP into long oligoadenylate chains [pppA(2′p5′A)$_n$] that in turn activate RNase L to degrade cellular RNA. Long dsRNA can also activate cells to express and release interferons that act in a paracrine and autocrine fashion to induce cell growth arrest and possible apoptosis (55).

effects of siRNAs on gene expression depend on the siRNA concentration, specific sequence, and delivery method, as well as the cell type tested (56–58). High siRNA concentrations consistently enhance nonspecific effects. Identifying highly active siRNAs and decreasing siRNA concentrations can alleviate some nonspecific effects while maintaining efficient silencing.

Sequence-specific off-target silencing of mRNA sequences that have partial complementarity to the siRNA also occurs, either by siRNA-directed cleavage of mRNAs or by repression of translation (29, 30, 54, 59). In some instances, siRNAs inhibit off-target gene expression when 15 nt, with as few as 11 contiguous nucleotides, of the siRNA are complementary to an off-target mRNA sequence

(54). Either the sense or the antisense strand can cause off-target silencing. These off-target effects are specific to the siRNA sequence and independent of the intended target, since different siRNAs against the same target inhibit different genes (54). Screening candidate siRNAs for homology with available sequence databases can, in principle, predict and avoid many off-target effects. These studies have illustrated that siRNA sequences must be chosen very carefully to maximize the efficiency of gene silencing while minimizing nonspecific and off-target effects.

RNAi AND VIRUSES

Mammalian pathogens or diseases may either utilize or circumvent RNAi in their pathogenesis. Plants use viral-induced gene silencing, an RNAi-related phenomenon, as a primitive "immune system" to silence viral gene expression and replication in response to dsRNAs produced in the life cycle of most plant viruses. This has led some plant viruses to produce proteins that inhibit RNAi (reviewed in Reference 60). In addition, pathogenic viroids (infectious RNAs that do not encode any proteins) express miRNAs that probably cause disease by suppressing host gene expression (61, 62). It is likely that some animal viruses also hijack or harness the RNAi machinery as part of their life cycle. Epstein Barr virus–infected cells were recently shown to contain unique miRNAs, the profile of which changed with different latency states (63). Moreover, dsRNA binding proteins encoded by vaccinia and influenza viruses interfere with RNAi-mediated antiviral effects in *Drosophila* cells (64).

RNAi AND THE REGULATION OF DEVELOPMENT

Biochemical and bioinformatic approaches have identified several hundred human miRNAs (reviewed in Reference 65). These miRNAs bind to sites, usually in the 3′ untranslated region, on the target mRNA with incomplete complementarity leading to inhibition of protein expression by an unknown mechanism (29). Because miRNAs bind with low affinity to their target sequence, multiple binding sites, possibly for more than one miRNA, may have to work in concert to silence gene expression efficiently. Although the target genes of most miRNAs are unknown, bioinformatic approaches have predicted putative mammalian miRNA targets (66). In lower organisms, the target genes are often fundamental regulators of cellular development that determine body patterning or transition from one developmental stage to another and include genes that encode for transcription factors and apoptosis regulators (67, 68).

The first example of a miRNA functioning in a mammalian system is miRNA-181 (69). It is highly expressed in hematopoietic tissues (bone marrow, spleen, thymus), particularly in B lymphocytes. When hematopoietic stem cells transduced with a retrovirus that expresses miRNA-181 are adoptively transferred into irradiated mice, they develop preferentially into B cells. Although little is known about miRNA-181, including its targets, it is likely to be the first of many examples of miRNA regulation of mammalian development and lineage-specific

differentiation. The coordinate expression of miRNAs acting on the 3′ untranslated regions of mRNA may turn out to have as great an effect on regulating expression of key developmental, activation, and survival genes as the transcription factors that bind to DNA at 5′ promoter regions. Because miRNAs are so important in development, it is reasonable to assume that the misregulation of miRNAs will perturb normal developmental pathways, potentially leading to certain types of cancers.

RNAi AS A TOOL TO UNDERSTAND GENE FUNCTION IN VITRO AND IN VIVO

RNAi is a powerful research tool for reverse genetic studies, which determine the function of a gene by its disruption. Target genes can be silenced by transfection of chemically or enzymatically synthesized siRNAs or by DNA-based vector systems that encode short hairpin RNAs (shRNAs) that are processed intracellularly into siRNAs (Figure 5). Typically, RNAi-mediated silencing is incomplete (a "knockdown," not a "knockout"), although in some cases, the targeted mRNA is undetectable even with ultrasensitive PCR assays (70). Transfection of siRNAs into rapidly dividing cells has a maximal silencing effect 2–3 d posttransfection, with silencing lasting up to a week. This is presumably due to dilution of the siRNA with each cell division. However, siRNA-mediated silencing can persist for several weeks in terminally differentiated, nondividing cells, such as macrophages or neurons (71, 72). To prolong silencing, DNA vectors have been developed that effectively express siRNAs or siRNA precursors (e.g., shRNAs), using expression systems based on adenovirus, adeno-associated virus, oncoretroviruses, and lentiviruses (14, 73). Viral expression systems also have the advantage of being able to infect and deliver siRNAs efficiently into cells that are refractory to traditional lipid-based transfection protocols.

The sequencing of the human genome has catalogued most of the genes expressed in humans and some other organisms. This sequencing information and the ubiquitous nature of the RNAi machinery have opened up the potential to silence practically any gene in the human genome, as well as the genomes of many other organisms. This approach has been pursued most extensively in *C. elegans* but more recently in *Drosophila* and human cells (74–77). Since gene silencing can be induced systemically in *C. elegans* by feeding bacteria that express long dsRNAs (78), bacterial expression libraries that silence practically all *C. elegans* genes have been produced and used to screen for genes involved in fundamental worm processes, such as early embryogenesis (74), longevity, or fat metabolism. In flies, which lack an interferon response, comprehensive libraries of ~500 base-pair dsRNAs representing sequences from >90% of all *Drosophila* mRNAs have been used for powerful high-throughput screening to identify unknown genes involved in cell viability (75).

Although large-scale mammalian siRNA screens are more challenging, the first RNAi screens in mammalian cells have recently been accomplished (76, 77). These

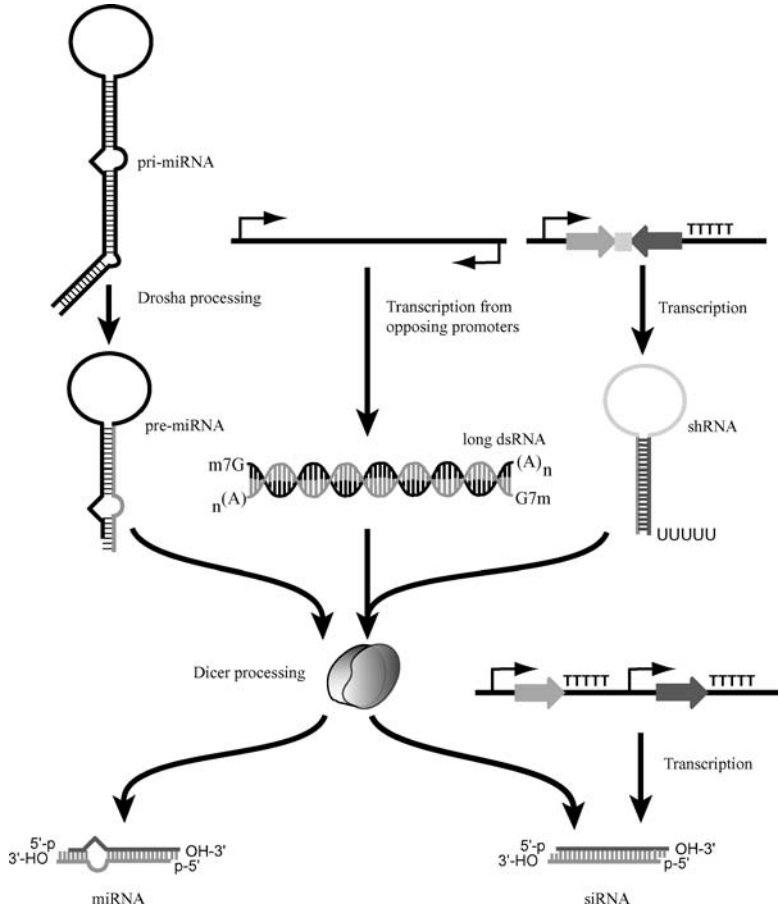

Figure 5 Strategies to introduce or express siRNA in cells. Dicer can cleave both endogenously expressed and exogenously introduced substrates. The endogenous substrates include the hairpin-structured pre-miRNA and long dsRNA produced by transcription from opposing promoters, as well as hairpin RNA that can be introduced directly into the cells or can be produced by transcription from plasmid or viral vectors. An RNAi response can also be generated by introducing in vitro synthesized siRNAs or by expressing individual sense and antisense strands of the siRNA from a vector containing tandem promoters (14).

screens use retroviral vectors to express shRNAs in cells, using high-throughput synthesis and cloning methods to produce libraries designed to silence expression of much of the mammalian genome (Figure 6). In lower organisms libraries of long dsRNAs can be used for screening, whereas mammalian screens require expression of short dsRNAs so as not to trigger an interferon response. Algorithms can be used to design shRNAs that are likely to silence any known gene. To maximize

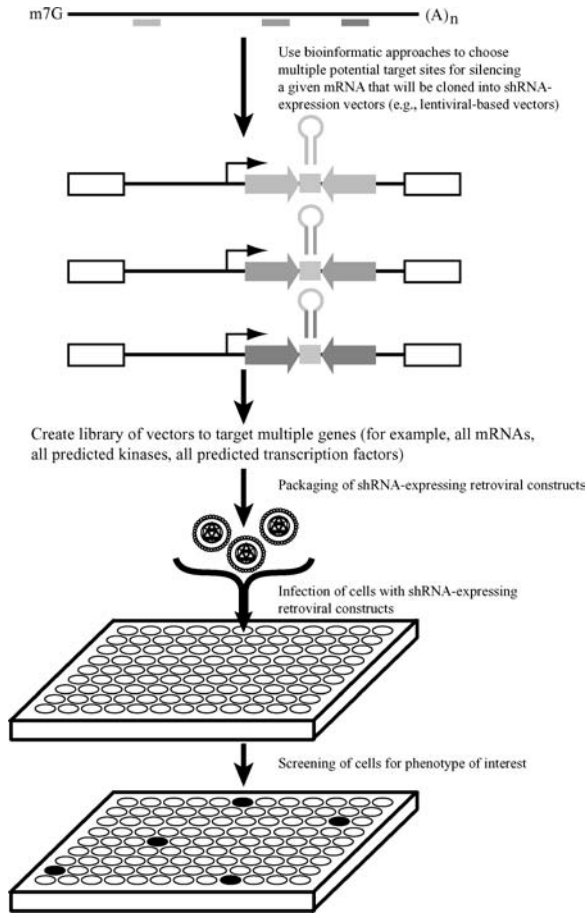

Figure 6 Genomic RNAi screens in mammalian cells. Several shRNAs (typically 2–5 sequences) designed to target a large subset of genes are cloned into an shRNA expression vector. These are packaged into retroviral particles and used to infect the cell of interest. This is usually accomplished in a large-format (for example, 96 well plate) approach. Cells are then screened for particular phenotypes. For example, Berns and colleagues used an RNAi library containing 23,742 shRNAs targeting 7914 human genes to identify novel proteins that modulate p53-mediated cell cycle arrest (77).

silencing and distinguish off-target effects, several siRNA sequences (usually 3–6) are chosen for each target. The library of shRNA-expressing infected cells can then be screened to identify candidate genes in any biological pathway and potential therapeutic drug targets. One of the first mammalian screens identified new candidate genes involved in p53-mediated cell cycle arrest (77). Better understanding of the mechanism of RNAi will facilitate development of more effective algorithms

for designing shRNAs and of expression vectors for more efficient gene-silencing libraries.

Lentiviral vectors that express shRNAs have been used to produce transgenic animals with stably silenced gene expression (79, 80). This approach has been utilized mostly in mice but can be applied to other organisms not amenable to traditional, homologous recombination-based gene knockout approaches (81). Embryonic stem cells or eight-cell embryos infected with shRNA-expressing lentiviruses can generate animals in which gene expression is permanently silenced in all cells or in certain cell lineages by appropriate choice of promoters driving shRNA expression (Figure 7*a*). The use of inducible promoters and tissue-specific promoters allows for the controlled expression of the shRNA in response to an inducer (for example, tetracycline) or in specific cell types or tissues, respectively (82–84). Moreover, because RNAi sequences vary in the extent of silencing, it is possible to produce mice with graded degrees of silencing of a particular gene (85). Because producing a knockdown mouse only requires inserting a single transgene, whereas making a knockout mouse involves removing a part of the gene on both chromosomes, silenced mice can be generated with a few months of work, compared to the few years generally required to produce knockout mice. When hematopoietic stem cells are infected with shRNA-encoding lentiviruses in vitro and transplanted into lethally irradiated mice, silencing of gene expression is limited to hematopoietic cells (Figure 7*b*) (80).

RNAi AS A THERAPEUTIC

Some have touted RNAi as the next new class of therapeutics. Because all cells are thought to contain the machinery to carry out RNAi and all genes are potential targets, the possible applications for medicine are, in principle, unlimited. This widespread applicability, coupled with relative ease of synthesis and low cost of production (especially compared to proteins such as antibodies or recombinant growth factors at the concentration needed for therapeutic effects), makes siRNAs an attractive new class of small-molecule drugs. In addition, siRNAs are chemically stable and can be stored lyophilized without refrigeration. Once in cells, the anticipated duration of silencing is predicted to vary from ∼5 d to several weeks, so dosing as an injectable drug seems feasible. RNAi drug development builds on the lessons learned from clinical studies administering antisense oligonucleotides. Antisense suppression of gene expression is generally much less effective (∼100–1000-fold less active on a molar basis) (86), works for only some genes, and achieves less efficient silencing than siRNAs do (73).

The sequence specificity of RNAi, even when off-target effects are considered, promises potent therapies with little toxicity due to off-target gene silencing. This high specificity also implies that the application of RNAi in some instances, such as to treat viral infections or cancer, might lead to resistance due to sequence mutations. This has proved to be the case in several in vitro studies suppressing viral replication by RNAi [e.g., poliovirus (45) and HIV-1 (46, 47)]. However,

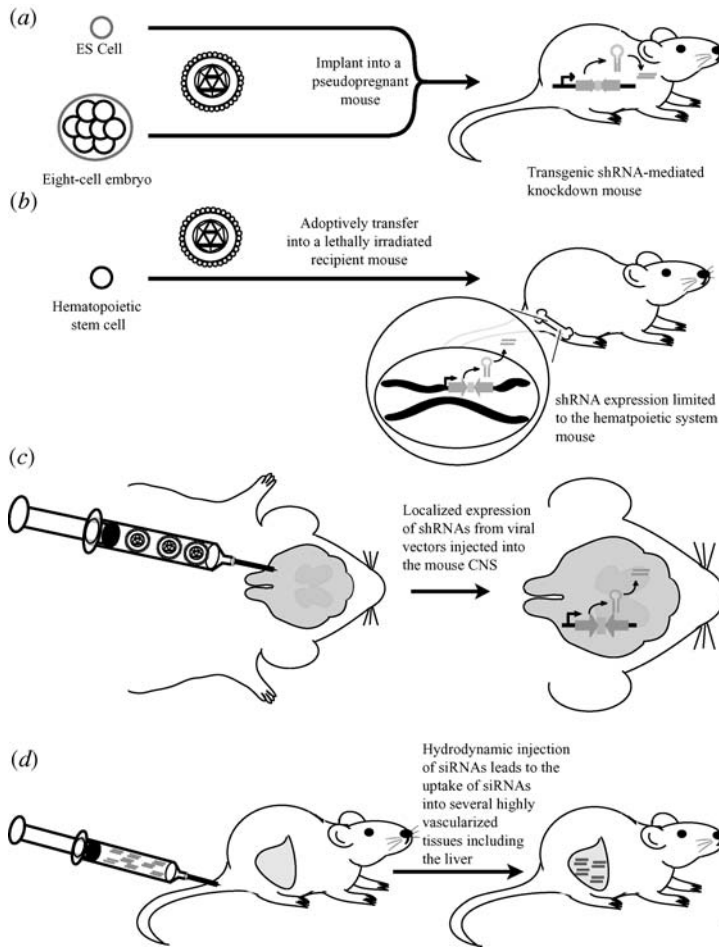

Figure 7 Some strategies for in vivo RNAi. (*a*) Transgenic shRNA-mediated knockdown mice. The transduced embryonic stem cells or embryos can be implanted into pseudopregnant female mice; progeny will express the shRNA and silence the gene of interest. (*b*) Reconstitution of the mouse hematopoietic system with shRNA-expressing stem cells. (*c*) The injection of viral constructs into the central nervous system of mice can lead to localized gene silencing. For example, the intracerebellar injection of adeno-associated viruses expressing shRNAs against ataxin-1 led to a loss of inclusion body formation and improved motor coordination (112). (*d*) The hydrodynamic (high-pressure, high-volume, rapid) injection of siRNAs into the tail vein of mice leads to the uptake ("hydroporation") of siRNAs into a variety of tissues including the liver, pancreas, lung, and spleen (99).

unlike resistance to other small molecules, which leads to an expensive and time-consuming search for new therapeutic agents, resistance to RNAi may be overcome by introducing a new siRNA that targets a different site on the same mRNA. Moreover, siRNAs that target conserved sequences or multiple sequences at once may provide a way around this problem.

Several recent studies using highly sensitive microarray analyses have shown that siRNAs can have off-target effects by silencing unintended genes (54, 87). Although differences in expression for most genes are well within the normal variations of the assay, a few genes may vary in expression by >2–3-fold in a sequence-dependent manner. These off-target effects can be minimized by modifying the siRNAs to prevent incorporation of the sense strand into RISC and by choosing sequences with minimal complementarity to known genes in the database, particularly at the 5′ end of the guide strand (88). In addition, at high concentrations or when siRNAs are expressed as shRNAs from viral vectors, some interferon response genes may be triggered. siRNAs may also activate the dsRNA Toll-like receptor TLR3 on macrophages and dendritic cells, inducing these cells to mature and produce Type I interferons (89). Because these effects are concentration dependent, finding siRNAs that are active at low concentrations should help to abrogate some of them. It is unclear whether these off-target effects will prove a significant source of in vivo toxicity.

Delivery remains a major hurdle for RNAi therapy, since siRNAs do not cross the mammalian cell membrane unaided and since many of the transfection methods used for in vitro studies cannot be used in most in vivo settings. There are two strategies for delivering siRNAs in vivo (reviewed in Reference 90). One is to stably express siRNA precursors, such as shRNAs, from viral vectors (Figure 5) using gene therapy; the other is to deliver synthetic siRNAs by complexing or covalently linking the duplex RNA with lipids and/or delivery proteins.

RNAi-based gene therapy may make the most sense for correcting dominant genetic defects, such as inherited neurodegenerative diseases like Huntington's disease or amyotrophic lateral sclerosis (91). Several papers have demonstrated silencing of a pathogenic, dominant allele that differs by a single nucleotide from the wild-type allele (42–44, 92). Gene therapy provides the opportunity for long-term correction of an underlying and persistent problem by using lentiviral vectors that integrate into the genome and can be used to transduce hematopoietic stem cells and other slowly dividing progenitor cells in a variety of tissues (Figure 7*b*). As gene therapy vectors continue to improve, the infection efficiency and level and persistence of gene expression will probably improve, as will the ability to transduce progenitor cells, while maintaining progenitor cell pluripotency. The pseudotyping of lentiviral vectors with different envelope glycoproteins may be used to target particular cell types. Alternatively, the expression of shRNAs in particular tissues or cell types can be achieved by expressing shRNAs from tissue-specific or inducible promoters. One of the main concerns with current gene therapy approaches is insertional mutagenesis from preferential integration of retroviral vectors into actively transcribed genes, including protooncogenes.

This has led to hematological malignancies in young patients with severe combined immunodeficiency (SCID) disease treated with retroviral-based gene therapy (93). It may be possible to address this problem by designing vectors that integrate into specific, well-defined regions of the genome, as has been accomplished in mice.

Treatment with synthetic siRNAs as small-molecule drugs would avoid the problems inherent in current retroviral-based gene therapy. The main obstacle to the use of siRNA drugs is delivery into the cytosol, where they need to be for silencing. Naked siRNAs are not efficiently taken up even by mammalian cells, such as macrophages and dendritic cells, that are actively sampling their environment by pinocytosis (70, 94). A further obstacle is the extremely short in vivo half-life of siRNAs (measuring seconds to minutes) due to rapid renal clearance. In addition, although most endogenous RNases are inactive against dsRNAs, some serum RNases can degrade siRNAs, leading to an in vitro serum half-life of ~1 h. The in vivo retention time of the siRNAs can be increased by complexing with lipids or protein carriers to limit renal filtration. In principle, complexes can be designed to enhance the rate of uptake into the cell and, potentially, direct the siRNAs to specific cell types. Coupling of siRNAs to basic fusogenic peptides has been reported to facilitate the transport of siRNAs across the cell membrane (95). siRNAs complexed to polyethyleneimine administered intranasally inhibited pulmonary influenza infection (96). Effective cell-specific delivery by coupling siRNAs to cell surface receptor ligands or antibodies could greatly reduce potential toxicity. Chemical modification, such as capping the ends of the siRNA strands or substitution of the 2′-hydroxyl groups of the ribose moieties with other chemical groups, has been shown to protect siRNAs from digestion by endogenous RNases (49, 50, 97). Although these modifications can increase the stability of the siRNAs, they may reduce the efficacy of silencing (98).

Several studies have demonstrated efficient in vivo delivery of siRNAs and therapeutic benefit in mice. The first demonstration of effective in vivo siRNA delivery took advantage of a high-pressure injection method (called hydrodynamic delivery) originally developed to deliver antisense oligonucleotides and plasmid DNA. When siRNAs are rapidly injected intravenously in large volumes, cells in the liver and other highly vascular tissues, such as the kidney and lung, efficiently take up siRNAs (99, 100). It is postulated that the sudden volume load induces right-sided heart failure and the resulting high venous pressures "hydroporate" siRNAs into the cells (101). Hydrodynamic delivery was used to deliver siRNAs that targeted Fas to protect mice in two models of autoimmune hepatitis (71). siRNAs were taken up by ~90% of hepatocytes and silenced both Fas mRNA and protein in the liver by ~80%–90%. Gene expression was silenced without diminution for 10 d, began to return at 2 weeks, and had returned to normal within 3 weeks, which suggests that infrequent dosing might be feasible for treating liver disease. In fulminant hepatitis induced by intraperitoneal injection with Fas antibody, all the control mice died within a few days, whereas >80% of Fas-siRNA treated mice survived (Figure 7*d*). Similarly, expression of hepatitis B

proteins from transduced hepatitis B replicons can be reduced in the liver of mice by hydrodynamic delivery of siRNAs targeting viral genes (102, 103).

Although effective in mice, hydrodynamic delivery is unlikely to be applicable to human therapy. Nonetheless, regional delivery of siRNAs via small-volume local injection into a tissue via catheterization of regional veins or local injection into the cerebrospinal fluid may be a clinically viable alternative. Intrathecal injection of siRNAs targeting the gene for a pain-related cation channel protein alleviated chronic neuropathic pain in a rat model (104). Injection of Fas siRNAs in a small volume into the renal vein in mice provides protection from ischemia-reperfusion injury of the kidney comparable to hydrodynamic delivery (105).

Some lipid-mediated transfection methods that work in vitro can also be used for in vivo delivery. In one study, pretreatment by intraperitoneal injection of cationic liposomes containing siRNAs that targeted TNF-α provided protection from sepsis induced by lipopolysaccharides (106). Intravenous immunoliposome delivery has shown efficacy in delivering siRNAs across the blood-brain barrier into glioma cells in the central nervous system (101). Lipid-complexed siRNAs also can be introduced locally, e.g., to silence gene expression in the vagina (D. Palliser, J. Lieberman, unpublished). For localized delivery, electrical currents have been used to introduce siRNAs into the retina and muscle cells by electroporation (107). A beneficial effect on tumor growth has also been demonstrated by local injection of siRNAs mixed in an atelocollagen gel (108). In addition, parasites in the blood take up siRNAs and could be potential therapeutic targets (109, 110).

The therapeutic potential of RNAi achieved through vectored expression of shRNAs has been shown in mouse models using adeno-associated viruses, retroviruses, and lentiviruses (83, 111, 112). Both adenoviral and lentiviral vectors expressing shRNAs have been injected into the central nervous system and shown to silence a CAG repeat gene implicated in spinocerebellar atrophy with reduced pathogenic protein aggregation (Figure 7*c*) (44, 91, 112). Targeted silencing of the mutant gene but not its wild-type allele slowed the development of ataxia. In vitro silencing of genes implicated in other progressive neurodegenerative diseases, including Alzeimer's disease, Huntington's disease, and amyotrophic lateral sclerosis, suggests that this group of challenging diseases may be fruitful targets for early clinical studies. For a gene therapy approach to engineer lymphocytes and macrophages resistant to HIV-1 infection, hematopoietic stem cells infected in vitro with lentiviruses expressing sequences designed to silence HIV *rev* were injected into SCID mice transplanted with human thymus and fetal liver grafts (113). The differentiated macrophages and T cells were resistant to in vitro HIV-1 challenge, which suggests that lentiviral vector-based siRNA delivery can be adapted for clinical use.

Development of RNAi-based therapeutics is in its infancy. It is too soon to predict whether the promise of harnessing RNAi for treatment will be realized. However, early clinical studies are likely soon to begin assessing the use of this new class of therapeutics to tackle some of the more challenging diseases in medicine, including chronic infection, cancer, and neurodegenerative disease.

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