The Role of Small RNAs in Human Diseases: Potential Troublemaker and Therapeutic Tools

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Abstract: Small RNAs, including short interfering RNAs (siRNAs) and microRNAs (miRNAs), are ubiquitous, versatile repressors of gene expression in plants, animals, and many fungi. They can trigger destruction of homologous mRNA or inhibition of cognate mRNA translation and play an important role in maintaining the stable state of chromosome structure and regulating the expression of protein-coding genes. Furthermore, the recent research showed that there exists close relationship between small RNAs and human diseases. Several human diseases have surfaced in which miRNAs or their machinery might be implicated, such as some neurological diseases and cancers. The specificity and potency of small RNAs suggest that they might be promising as therapeutic agents. This article will review the role of small RNAs in some human diseases etiology and investigations of taking siRNAs as therapeutic tools for treating viral infection, cancer, and other diseases. We also discuss the potential of miRNAs in gene therapy. © 2005 Wiley Periodicals, Inc.
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Key words: small RNAs; short interfering RNAs; microRNAs; RNA interference; gene therapy

1. INTRODUCTION

Small RNAs of ~22 nucleotides (nt) in length, which are ubiquitous, versatile repressors of gene expression in plants, animals, and many fungi, have attracted the attention of more and more

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biologists and biochemists. These tiny RNAs (~21–26 nt), including short interfering RNAs (siRNAs)[1] and microRNAs (miRNAs),[2–4] can control mRNA stability or translation, or target epigenetic modifications to specific regions of the genome through homologous sequence interactions.

Small RNAs can elicit at least four distinct types of responses that trigger specific gene inactivation[5]—destruction of homologous mRNA,[6–9] inhibition of translation,[10] *de novo* methylation of genomic regions that can block transcription of target genes,[11] and chromosomal rearrangement.[12] RNA-mediated silencing pathways can combat 'foreign' nucleic acids, as shown by silencing-deficient mutants, some of which unleash transposons[13] or, in plants, exhibit enhanced susceptibility to virus infection.[14]

These small RNAs play an important role in maintaining the stable state of chromosome structure and regulating the expression of protein-coding genes.[15] miRNAs can down-regulate endogenous genes which are important for implementing developmental programs in animals and plants.[16] Small RNAs and evolutionarily conserved RNA-mediated silencing pathways have established a new paradigm for understanding eukaryotic gene regulation and revealed novel host defenses to viruses and transposons.

Researchers have taken the technique of RNA interference (RNAi) as a reverse genetics tool to define gene function or biochemical pathways in the post-genomics era and a potential therapeutic treatment in viral infection, cancer, and some other diseases.

This review will discuss the etiology of human diseases related to small RNAs and RNA-mediated silencing pathways and the potential use of these RNAs in gene therapy.

A. *Short Interfering RNAs and MicroRNAs*

These small regulatory RNAs—siRNAs and miRNAs—are generated via processing of longer double-stranded RNA (dsRNA) precursors by an RNaseIII-like enzyme termed Dicer.[17] In general, siRNAs can be chemical synthesized or processed from all regions of perfect duplex RNAs derived from transgenes, transposons, heterochromatic repeats, exogenous dsRNA (i.e., experimentally introduced), or foreign nucleic acids (such as viral RNAs).[18] These long dsRNAs are diced up by Dicer[17] into short RNAs with a specific structure: 19 nucleotides of dsRNA with two unpaired nucleotides at the ends.[1] Dicer is a member of the RNase III family of nucleases that specifically cleave double-stranded RNAs, and is evolutionarily conserved in worms, flies, plants, fungi, and mammals.[17] siRNAs associate with an endonuclease-containing effector complex, RISC (RNA induced silencing complex).[6] The strand that has exactly the same sequence as a target gene is the 'sense' strand of an siRNA. This strand is removed and the 'antisense' strand, which is complementary to the target gene, is left to function in gene silencing—causing degradation of cognate mRNAs and, in plants, viral RNAs.[19] This process is termed RNAi—the silencing of cognate genes expression by double-stranded RNA molecules.[20] At least in some plants, there are two functionally distinct size classes of siRNA. A shorter class, 21–22 nt, has been implicated in mRNA degradation, and a longer size class, 24–26 nt, in systemic silencing and methylation of homologous DNA.[21] In some organisms, an RNA-dependent RNA polymerase (RdRP) is involved in gene silencing by RNAi. This enzyme uses the antisense strand of an siRNA as a primer with which to make more dsRNAs, thereby amplifying the process.[15] However, amplification is not thought to occur in vertebrates of fruitflies and mammals, as genes for a RdRP have not been found in these organisms.[19] RNAi is an evolutionarily ancient method of genome defence in many organisms. It is a way to protect the genome against invasion by viruses, mobile genetic elements such as transposable elements and repetitive genes (including transgenes), which produce aberrant RNA or dsRNA in the host cell when they become active.[1] It is crucial for the stability of the genome to silence these mobile elements, and RNAi-related process associated with the generation of small RNAs are essential to this silencing process in many organisms.

miRNAs represent a new class of highly conserved non-coding RNAs (ncRNAs). They are abundant, single-stranded RNAs, present at very high steady-state levels—more than 1,000 molecules per cell, with some exceeding 50,000 molecules per cell.[22] The *miRNA* genes are typically found in intergenic regions but can also be found in sense or antisense orientation within introns of known genes.[18] They are transcribed into long transcripts—pri-miRNAs.[23] In mammals, the biogenesis of miRNAs includes processes in nucleus and cytoplasm and dsRNAs are recognized and cleaved by RNase endonucleases. The first step is the nuclear cleavage of pri-miRNAs by the Drosha RNase III endonuclease, that release a ~60–70 nt miRNA precursor, known as the pre-miRNA.[24] Pre-miRNA can be folded into stem-loop structure and is actively tansported to cytoplasm by Ran-GTP and the export receptor Exportin-5.[25,26] Then it is processed into ~20–22 nt duplex with ~2 nt 3′overhang by enzyme Dicer.[17,27,28] This miRNA intermediate is very short-lived. Generally, only one strand acts as mature miRNA, and the other one is named miRNA*.[3] miRNAs, which also appear to associate with a RISC-like complex, can either block translation of cognate mRNA by basing pair with the 3′-untranslated region (3′-UTR) of the targets if the complementarity between a given miRNA and its target mRNA is partial,[10] or guide mRNA degradation in the manner of siRNAs if the complementarity is extensive.[16,29–31] Recently discovered miRNA functions include determination of transitions between larval stages in post-embryonic development in worm,[19] neuronal patterning in nematodes,[32] control of cell proliferation, cell death and fat metabolism in flies, modulation of hematopoietic lineage differentiation in mammals,[33] and also control of crucial developmental transitions in plants.[23] Computational approaches for finding messages controlled by miRNAs indicate that these examples represent a very small fraction of the total.[23] Little is known about human miRNA. However, as miRNAs are evolutionarily conserved, there can be little doubt about their importance in human physiology.

These small RNA molecules directed astonishing diversity of regulatory pathways through the association with various protein partners in complexes that degrade cognate viral RNA or mRNAs, block translation or modify chromatin structure. Many components of these complexes have yet to be identified. Both biochemical and genetic studies have led to the identification of two conserved gene families that are universal components of the small RNAs-mediated silencing process. These are the Dicer family and the Argonaute gene family.[34] The latter is comprised of proteins with unclear biochemical function and the various members of which might determine whether siRNAs or miRNAs are used as substrates by RISC.[35] The dependency of both siRNA- and miRNA-mediated silencing on these two families shows that the miRNA and siRNA pathways share conserved components and likely have related mechanisms.

Although miRNAs and siRNAs have significant similarities: both are approximately 21–25 nt and have the characteristic features of Dicer or a similar protein products (5′-phosphate and 3′-hydroxyl); both require PPD (PAZ and PIWI domains) family members (members of Agonaute family) for their accumulation; both act on mRNA targets through complementary sequences; and both need Dicer and RISC in related mechanisms. They also have important differences. siRNAs come from exogenous or endogenous long dsRNA molecules or bimolecular duplexes, processed such that numerous siRNAs accumulate from both strands of these dsRNA, and the products are two complementary RNAs in equal abundance; while miRNA precursors are endogenous that can form local hairpin structures, and an miRNA is generated from one arm of the stem-loop that contains bulges and/or loops to yield a single-stranded RNA usually in vast excess over any complement. Furthermore, the general target genes or genetic elements of siRNAs are the ones that they originated from, whereas miRNAs regulate separate genes—perhaps hundreds or more per miRNA. The number of miRNAs that are bound to the target mRNA is thought to determine the degree of translational inhibition by miRNAs. Typically, such an mRNA contains many binding sites at 3′-UTR, and several different miRNAs can target the same 3′-region.[19]

2. SMALL RNAs AND HUMAN DISEASE ETIOLOGY

One kind of small RNAs, miRNAs, are naturally generated endogenous molecules and participate in many crucial processes including developmental regulation. They have been found in all multicellular organisms studied, and their encoding genes seem to make up 0.5%–1% of the predicted genes in these organisms. It has been estimated by computational methods that there are total 200–255 *miRNA* genes in human.[36] RISCs are required in the RNA-mediated silencing pathways and there have been many homologies of RISCs components identified in human. So, it can be conjecturable that the disorder or abnormal expression of components of RISC or miRNAs themselves may result in diseases. In fact, there have already been some proof-of-concept evidences that miRNAs or related machinery might be implicated in some human diseases (Table I).

A. Neurological Disease

The first suggested link between small RNAs and human disease was found in *Drosophila*. The protein dFMR1 is the *Drosophila* homolog of the fragile X mental retardation protein (FMRP) and was identified as a RISC subunit, suggesting that disruptions in RNAi-related pathways may contribute to human disease.[37,38] Fragile X syndrome is a common form of inherited mental retardation and is generally caused by transcriptional silencing of the *FMRP/FMR1* gene because of a CGG repeat expansion in the 5′-UTR resulting in abnormal DNA methylation of both a nearby CpG island and the repeat itself.[38–40] FMRP, which is produced from the human fragile X locus, is expressed not only in neuronal cells but also in numerous other tissues. Fragile X patients display additional phenotypes, including macroorchidism.[37] FMRP is involved in synapse formation and function.[40] The specific biochemical role of the human protein FMRP is still unclear; however, it is thought to negatively regulate the expression of numerous mRNAs at the level of protein synthesis. In *Drosophila*, a bona fide dFXR regulatory target has been identified as Futsch, a Map1B homolog.[37] FMRP, and its autosomal paralogs, the fragile X-related proteins FXR1P and FXR2P, constitute a small family of RNA-binding proteins (fragile X-related gene family). Each member of fragile X family contains two copies of a KH domain (hnRNP K homology) and an RGG box. All of these domains have been proposed to bind RNA.[37] Some of the mRNAs associated with FMRP have recently been identified, and a G-quartet/stem structure in these mRNAs has been shown to be involved in the FMRP–mRNA interaction.[41] Several studies showed that FMRP presents in RISC and works as part of RNAi complexes. Caudy et al.[37] identified proteins associated with RISC activity from partially purified *Drosophila* preparations, and found that dFMR1 is one of these proteins. dFMR1 coimmunoprecipitates with AGO2 and another RISC protein (VIG) from *Drosophila* cell lysates. Both a miRNA (miR-2b) and siRNAs can co-immunoprecipitate with dFMR1. Moreover, immunoprecipitates of dFMR1 from dsRNA challenged cells have RISC activity.[37,40] Ishizuka et al.[38] showed that dFMR1 is present in a complex with components of RNAi and miRNAs in cultured *Drosophila* S2 cells. They purified from *Drosophila* cell lysates a novel ribonucleoprotein (RNP) complex that contained dFMR1, as well as two ribosomal proteins, L5 and L11, 5S rRNA and, surprisingly, AGO2, a protein implicated in RNAi. dFMR1 also physically interacts with Dicer and miRNAs. These results suggest that dFMR1 is in an RNAi related apparatus. Jin et al.[41] also showed that *in vivo* mammalian FMRP interacts with miRNAs and the components of the miRNA pathways including Dicer and the mammalian ortholog of AGO1. They found that endogenous eIF2C2 (eukaryotic initiation factor 2C) could indeed be coimmunoprecipitated with FMRP, FXR1P, and FXR2P. Furthermore, using *D. melanogaster* as a model system, they demonstrated that AGO1 was critical for FMRP function in neural development and synaptogenesis.[41] These results suggested that FMRP may not directly bind its mRNA targets but rather regulate the translation of its mRNA ligands via miRNA as part of RNAi-related apparatus. Involvement or absence of FMRP may disrupt this regulatory process.[41] FMRP may be one of many distinct protein subunits that join RISCs, depending

on the tissue, subcellular localization, and the developmental stage.[37] The connection between the RNAi and fragile X fields is likely to become clear as we find out which small RNAs mediate translational repression by dFMR1 and FMRP, and how the repressive mechanism operates. Nevertheless, it is worth pondering that fragile X syndrome may be the result of protein synthesis abnormality caused by defects in a RNAi-related apparatus within neurons.[38,40]

In spinal muscular atrophy (SMA), a common genetic disease characterized by progressive degeneration of motor neurons, deletions or loss-of-function mutations in the survival of motor neuron (SMN) protein are thought to be the cause of this disease.[44] The SMN complex is a key factor in the biogenesis and function of diverse RNPs and comprises SMN, Gemin2, Gemin4, Gemin5, and Gemin6. SMN is also part of a large complex that functions in the assembly/restructuring of ribonucleoprotein (RNP) complexes. Mourelatos et al.[42] identified a novel RNP that sediments as an ~15S particle on sucrose gradients and contains Gemin3, Gemin4, and human eIF2C2 along with numerous miRNAs. Hutvagner and Zamore[50] solidified the link between this complex and RNAi by showing that it can cleave substrates that are homologous to its constituent miRNAs. Thus the EIF2C2/Gemin3/Gemin4 complex may indeed represent mammalian RISC.[50] The result of Dostie et al. also suggested that Gemin3 interacts with miRNAs in various cell types including motor neuron cells, as part of miRNPs.[44] The discovery of the relationship between Gemin3 and miRNP indicates that Gemin3 may mediate RNA unwinding or RNP restructuring events during the maturation of miRNAs and/or in downstream events such as target RNA recognition.[42] The residence of the Gemin3 and Gemin4 proteins in the SMN complex raises the intriguing possibility that the SMN complex may intersect with the pathways in which miRNPs function. The binding of Gemin3 to SMN is impaired in SMN mutants found in SMA patients.[42] It will be of great interest to determine what effect Gemin3 has on miRNPs in this devastating neurodegenerative disease and, more generally, what regulates its distribution between the SMN complex and miRNPs.[42] The activity of miRNPs may also be affected by possible redistribution or other changes of Gemin3 and Gemin4. Thus, it is hinted that specific or general changes in the activity of the miRNPs may play a role in the development of SMA.[44] However, some puzzles still remained to be determined, such as whether there exists any dysregulation of miRNA biogenesis or function in SMA, and the possible effect of it.

There are additional clues that miRNAs might play a role in other neurological diseases. An fascinating finding is that the gene locus of miR-175 is related to two neurological diseases: early-onset parkinsonism (Waisman syndrome) and X-linked mental retardation (MRX3).[44] miR-175 is located on the X chromosome in humans and is conserved in *D. melanogaster* and *M. musculus*. Dostie et al.[44] found miR-175 is part of a longer human expressed sequence tag (EST) in human retinoblastoma Weri cells. This EST encodes a putative isoform of the epsilon subunit of the gamma-aminobutyric acid (GABA), a receptor that is a multisubunit chloride channel that inhibits synaptic transmission in the central nervous system. Furthermore, it has been demonstrated that the gene locus of the epsilon subunit is a candidate region for these two diseases.[44] It will be of significance to determine if there are any changes in the synthesis or activity of miR-175 and the effect of these changes in the development of these diseases.

B. Cancer

In addition to neurological diseases, another kind of diseases linked to small RNAs or their machinery is cancer. Some circumstantial evidence links members of the Argonaute family of proteins with some cancers. The region of chromosome 1p34–35 in human, on which three closely related Argonaute family members (hAgo3, EIF2C1/hAgo1, and hAgo4) reside in tandem (the orthologous genes are in the same orientation on chromosome 4 in mouse), is often lost in Wilms' tumors of the kidney[35] or altered in primitive neuroectodermal tumors and many other types of cancer.[18] Human EIF2C1/hAgo1 is associated with Golgi and endoplasmic reticulum and alternatively known as GERp95.[35] Its expression level is low to medium in most tissues, while it is particularly high in

Table I. The Relationship Between Small RNAs Pathway and Human Disease

factors relating with diseases		loci of genes	mutations	related disease	reference
Components of RISC	FMRP	the human fragile X locus	a CGG repeat expansion in the 5' UTR	fragile X syndrome	37-40
	the Survival of Motor Neuron (SMN) protein	—	deletion or loss-of-function	spinal muscular atrophy (SMA)	42
	hAgo3, EIF2C1/hAgo1, hAgo4	the region of chromosome 1p34 - 35 in human	loss	Wilms' tumors of the kidney	35
	EIF2C1	—	increase	tumors that lack the Wilm's tumor suppressor gene WT1	
			—	testicular germ cell tumors	

	Hiwi (a member of the Argonaute family in human)	the chromosome 12q24.33 in human	loss	ambiguous genitalia and hypogonadism	
			overexpression	seminomas	43
miRNAs	miR-175	the X chromosome in humans	—	early-onset parkinsonism (Waisman syndrome) and X-linked mental retardation (MRX3)	44
	miR-15 and miR-16	the chromosome 13q14 in human	deletion or down-regulation	in the majority (68%) of chronic lymphocytic leukemia (CLL) cases, 50% of mantle cell lymphoma, 16–40% of multiple myeloma, and 60% of prostate cancers	45, 46
	miR-142	the chromosome 17q22	MYC translocates into the mir-142	an aggressive B cell leukemia.	47

(Continued)

Table I. (*Continued*)

			loci, resulting in ~20nt conserved sequence element downstream of the mir-142 hairpin lost		
	miR-143 and miR-145	the chromosome 5q32-33	reduction	precancerous and neoplastic colorectal tissue	48
	miR-155/BIC	the chromosome 21	high expression	pediatric Burkitt's lymphoma	49

embryonic kidney and lung. EIF2C1 level also increase in tumors that lack the Wilm's tumor suppressor gene *WT1*.[35] Another member of the Argonaute family, hiwi, a member of the piwi gene family in human is located on chromosome 12q24.33, which displays genetic linkage to the development of testicular germ cell tumors of adolescents and adults.[43] The piwi family genes are highly conserved during evolution and play essential roles in stem cell selfrenewal, gametogenesis, and RNAi in diverse organisms. The gene hiwi is expressed abundantly in the adult testis. Qiao et al.[43] showed hiwi is specifically expressed in both normal and malignant spermatogenic cells in a matuation stage-dependent pattern, in which it might function in germ cell proliferation. Loss of the region containing hiwi has been correlated with ambiguous genitalia and hypogonadism.[35] In addition, hiwi overexpression is also detectable in seminomas, tumors derived from embryonic germ cells that maintain stem cell character, suggesting a role for Hiwi reminiscent of the role of *Drosophila* Piwi in cell-autonomously driving stem cell division.[43] These studies implicate the processes of *miRNA/RNAi* gene silencing in some aspects of oncogenesis. Although several mammalian Argonaute proteins have been identified, little is known about their functions. To clarify the relationship between Argonaute proteins and human disease will be beneficial to the study of there functions.

Links between cancer and miRNAs have also been described. Recently, Calin et al.[45] reported an association between chronic lymphocytic leukemia (CLL) and deletion of a section of chromosome 13 that contains the genes for *miR-15* and *miR-16*. These two genes are clustered and located at chromosome 13q14 within a 30-kb region of loss in CLL, and both genes are deleted or down-regulated in the majority (68%) of CLL cases.[45] This region lies between exons 2 and 5 of the *LEU2* gene. Deletions at this region also occur in approximately 50% of mantle cell lymphoma, in 16%–40% of multiple myeloma, and in 60% of prostate cancers,[46] suggesting that one or more tumor suppressor genes at 13q14 are involved in the pathogenesis of these human tumors. Since the 13q14 region contains at least four non-coding genes, including these two miRNAs and Leu-1 and Leu-2, homozygous loss of this region is particularly interesting.[46] Therefore, it is possible that the CLL gene(s) on 13q14 acts in a different way compared with the classical tumor suppressor genes. As B-CLL is characterized by a progressive accumulation of CD5+ B lymphocytes, and ubiquitous expression of *miR-15* and *miR-16* genes with the highest level is found in normal CD5+ lymphocytes, suggesting that these genes play an important role in normal CD5+ B cell homeostasis.[45] It is possible that miRNA levels are crucial in maintaining regulatory control over target genes during normal CD5+ B cell differentiation. Further studies aimed at the identification of *miR15* and *miR16* target genes will shed light on their mechanism of action and provide further clues about their role in pathogenesis of these diseases. Functional assays with the different putative targets will be required to further test the mechanisms of action for *miR15* and *miR16* genes.

Strong up-regulation of MYC expression caused by the translocation of MYC into the mir-142 loci results in an aggressive B cell leukemia, suggesting that translocations into oncogene loci could result in cancer. Approximately 20 nt conserved sequence element downstream of the mir-142 hairpin is lost in the translocation.[47] It suggested that the loss of this element in the putative fusion prevented the recognition of the transcript as a miRNA precursor to be properly processed, and therefore may have caused accumulation of fusion transcripts and overexpression of *myc*.[47]

Recently, it was reported that miR-143 and miR-145 consistently display reduced steady-state levels of the mature miRNA at the adenomatous and cancer stages of colorectal neoplasia.[48,51] Both of these miRNAs appear to be derived from genomic sequences within 1.7 kb of each other on chromosome 5 (5q32–33). Their gene(s) reside approximately 50 kb from the *interleukin 17* gene, within the 1.5-Mb region that is deleted in the myelodysplastic 5q-syndrome.[48] In their study, down-regulation of accumulation of miR-143 and miR-145 was showed in cells derived from breast, prostate, cervical, and lymphoid cancers as well as colorectal tumors.[48] Their studies also indicated that this reduction is because of post-transcriptional processes. The identification of miRNAs that consistently display reduced steady-state levels in tumors raises the possibility that they, or their

targets, may be directly involved in the processes that lead to neoplasia. Several gene transcripts have been identified as possible targets for repression by *miR-143* and *miR-145*. These genes encode proteins involved in signal transduction and gene expression, including RAF1 kinase, G-protein γ 7, and tumor-suppressing subfragment candidate 1. All of them have been implicated in oncogenesis.[48] However, how the observed reduction of mature miR-143 and miR-145 levels is associated with the translation of these putative targets is still unknown. More work should be done to examine the interactions between miR-143, miR-145, and their potential targets, as well as the mechanism of miRNA-induced translational repression. However, if proven, miRNA-directed regulation of the expression of these target genes will provide novel insights into possible causes for cancer progression.

Another example is the relationship between miR-155 and Burkitt lymphoma (BL). miR-155 is encoded by nucleotides 241–262 of the human BIC gene, which is on chromosome 21 (GenBank accession number: AF402776). The BIC locus was originally identified as a common retroviral integration site in avian-leukosis virus-induced B-cell lymphomas.[49] Metzler et al.[49] saw a more than 100-fold up-regulation of the hairpin precursor miR-155 RNA in BL patients. Their research suggests that miR-155 may function in cooperation with MYC or its related pathways in the transformation of B cells and may play a role in late stages of tumor progression. It can be speculated that miR-155 directly down-regulates one of the MYC antagonists. The overexpression of miR-155 may also be linked to the TP53 signaling pathway, known to be frequently inactivated in BLs with translocation t(8;14).[49] However, it is still an enigma how and to what extent this proposed interaction between the microRNA and MYC takes place.

Recently, Calin et al.[51] reported some intriguing findings. The distribution of *miRNA* genes are not random and a significant number of them are frequently located at fragile sites (FRAs) or are close to human papilloma virus (HPV) integration sites. FRAs are preferential sites of many crucial processes, including sister chromatid exchange, translocation, deletion, amplification, or integration of plasmid DNA and tumor-associated viruses such as HPV. Because HPV integration into the host cell genome can cause mutations, such as large deletions, amplification, or complex rearrangements, the expression of cellular genes at or near integration sites may be affected. Therefore, *miR* genes located near the integration sites are possible targets of such genome alterations.[51] As miRNAs play important roles during development, this kind of situations suggest potential causes of developmental defects or other human diseases. They also found that 98 of 186 (52.5%) *miR* genes are in cancer-associated regions,[51] suggesting a close relationship between miRNAs and cancers. Several *miR* genes are located in homozygously deleted regions associated with cancer without known tumor supressor genes, such as *miR-15* and *miR-16*, suggesting these *miR* genes maybe novel kind of candidate tumor supressor genes.[51] Some *miR* genes are near breakpoint regions. For example, miR-142 is located at 50 nt from the t(8,17) break and this translocation brings the *MYC* gene near the *miR* gene promoter resulting in oncogene MYC overexpression. Another intriguing findings is that there exists a strong correlation between the location of specific *miRs* and Homeobox (*HOX*) genes.[51] The miRs are located inside or near HOX clusters. The miR relating *HOX* gene, such as *HOXB4*, *HOXB5*, *HOXC9*, *HOXC10*, *HOXD4*, and *HOXD8*, are de-regulated in a variety of solid and hematopoietic cancers, suggesting the related miRs may be altered along with these *HOX* genes in human cancers. These interesting findings suggest a role of miRNA in human cancer and it may involve more than a few genes.

Small non-coding RNAs have been found to have roles in a great variety of processes, including transcription and chromosome structure, RNA processing and modifications, mRNA stability and translation, and protein stability and transport.[52–54] Whereas oncogenes involved in differentiation, such as transcription factors and cell-cycle control factors are targeted, and would therefore be subject to post-transcriptional regulation. It can be speculated that cancer might arose when miRNA related mutations occurs, including mutations in either the *miRNA* genes, the 3′-UTR miRNA binding sites, or in pathways which regulate the expression of miRNA.[55] miRs activity can be influenced either by

the reposition of other genes close to miRs promoters or regulatory regions (as is the case of miR-142s/c-*myc* translocation) or by the relocalization of an miR near other regulatory elements.[51] A speculative model for miRNA involvement in cancers was drawn where miRs could be contributors for oncogenesis working as classical tumor supressor genes (as is the case of *miR-15* and *miR-16*) or as classical oncogenes (as is the case of *miR-155*), whereas some miRs express abnormally so that it causes a post-transcriptional misregulation of a tumor suppressor gene or oncogene.[51,55] As in the case of mir-15/16, mir143/145, or mir-142, miRNAs expression might be lost or down-regulated in some cancers. In these cases, it is possible that the miRNA might act in a different way compared with the classical tumor suppressor genes.[45] In some other cases, as in miR-155/BIC in pediatric Burkitt's lymphoma, expression of the miRNA is inappropriately up-regulated, commensurate with oncogenesis,[49] leading to the idea that these miRNAs might serve the role as oncogenes. As miRNAs can repress genes expression, if some miRNA is surpressing a tumor surpressor, the net effect could be that the miRNA would be considered as an oncogene. Further stressing the importance of miRs in cancer, it was shown that mutations in genes required for miRs biosynthesis cause developmental defects and cancer, as the mutations of FMRP in fragile X syndrome, hAgo3, EIF2C1/hAgo1, hAgo4 in Wilms' tumors of the kidney, etc.

Thus there are multiple entrances for miRNA involvement in human disease, and to identify miRNAs and their targets will likely be a helpful way for us to understand the cooperation of miRNA pathways in diseases.[55] It can be predicted that the involvement of small RNAs in disease will become an important issue to define the functions of miRNAs. Connections between small RNAs and human diseases will only strengthen in parallel with our knowledge of small RNAs and the gene networks that they control. Furthermore, our understanding of the regulation of small RNA-mediated gene silencing is leading to the development of novel therapeutic approaches that will be likely to revolutionize the practice of medicine. It can be predicted that the involvement of ncRNAs in disease will become an important issue as we struggle to define what functions miRNAs perform.

3. SHORT INTERFERING RNAs AND HUMAN DISEASE THERAPY

The specificity and potency of siRNAs, another kind of small RNAs, suggest that they might be a kind of promising therapeutic agents, and the RNAi technology can be used to combat viral infections, as well as to curb diseases that are caused by dominantly acting mutant alleles. Although siRNAs have not been used to treat any human disease by now, an ever-increasing number of proof-of-concept studies have proved potential therapeutic uses (Table II). These studies carried out to date have focused mainly on viral infection, cancer, neurodegenerative diseases, and these are likely to be the areas of early therapeutic efforts.

A. Short Interfering RNAs and Inhibition of Virus Infection

Several groups have now shown that siRNA can be used to interfere all the stages of the whole life cycle of a number of RNA viruses relevant to human disease, including hepatitis B virus,[59,80,81] HCV,[60,82] HPV,[60] influenza,[62–64] and the SARS-associated coronavirus (SARS-CoV),[65,66] etc. RNAi is now being used to inhibit both the cellular and viral factors that perpetuate the disease caused by viruses.

One of the possible strategies is to inhibit virus entry into host cells. siRNAs that target cellular receptor or co-receptor, such as CD4,[58] CCR5,[86] and CXCR4,[56] effectively blocked these cell-surface proteins expressions and their consequent functions in a gene specific manner, thus HIV entry was impeded, cells were protected from infection and virus replication was delayed. Some other host genes that are essential in the viral life cycle, such as Tsg101, an essential host factor and required for vacuolar sorting and efficient budding of HIV-1 progeny, are also potentially good targets for RNAi, providing that they are not necessary for survival of the cell.[87]

Table II. Examples of Human Disease Therapy by RNA Interference Technology

diseases		strategies	target genes	references
virus infection	HIV	Block HIV entry and replication	Chemokine receptor gene CXCR4and CCR5	56
		Inhibit HIV-1 gene expression and replication	Regulatory proteins Tat and Rev	57
		decrease HIV-1 entry	cellular receptor CD4	58
		Inhibit replication and pre-and/or post-integration infection	p24	
	HBV	Inhibit virus transription	X ORF, core ORF	59
	HCV	Inhitit virus replication	Nonstructural(NS) protein 5B, NS4B	60
	HPV	Silence viral gene expression	Oncoprotein E6, E7	61
	influenza	Inhibit virus replication	nucleocapsid protein (NP), components of RNA transcriptase (PA and PB1),	62-64
		Inhibit viral gene expression	Spike gene	65

	SARS-associated coronavirus (SARS-CoV)	inhibite viral replication		RNA polymerase	66
cancer		Induce cancer cell apoptosis		Bcl-2	67
				Plk-1	68
		silence oncogene	cancer-causing fusion oncogenes	bcr-abl oncogene	69, 70
			point-mutated transforming oncogenes	Ras	71
		Improve the efficacy of chemotherapy and radiotherapy in cancer		MDR	72, 73
				Prkdc	74
				TEL-PDGF β R	75
		Inhibit cancer cells invasion and migration		integrin	76
				urokinase-type plasminogen activator (u-PA)	77

(Continued)

Table II. *(Continued)*

neurodegenerative diseases	spinocerebellar ataxia type 1 (SCA1)	repress mutant allele expression	Mutant polyQ proteins	mutant ataxin-1	78
	Spinobulbar muscular atrophy(SBMA)			A truncated human androgen receptor(ar)	79

RNA virus requires RNA intermediates. Therefore, some investigations have employed siRNAs targeting viral structural genes or regulatory genes, which are essential for replication or package and relatively conserved between viral strains, at multiple stages of these viruses life cycle. In the case of HIV-1, several specific genes have been successfully knockdown, including Gag, Pol, Vif, and the small regulatory proteins Tat and Rev. It is shown by these studies that RNAi can effectively trigger the destruction of not only viral mRNAs, but also genomic RNAs at both the pre- and post-integration steps of the viral lifecycle.[57,58,83] Wang et al.[66] generated plasmid-mediated siRNAs to specifically target the SARS-CoV RNA polymerase gene. The expression of siRNAs effectively inhibited viral replication and consequently blocked the cytopathic effects of SARS-CoV on Vero cell. The results demonstrated the feasibility of developing siRNAs as effective anti-SARS drugs.

B. RNA Interference and Cancer Therapy

According to the different mechanisms of cancer genesis and development, applications of RNAi technology in the field of oncology have been performed from different aspects.

siRNAs have been applied to target oncogenes including those which are characteristically mutated generically or in specific cancers, such as dominant mutant oncogenes, amplified oncogenes, cancer-causing fusion oncogenes,[69,70] and viral oncogenes[61]. For example, effective down-regulation of BCR/ABL mRNA and fusion oncoprotein has been demonstrated by multiple investigators using specific siRNAs.[69,70] The amazing sequence specificity of the RNAi mechanism may also allow for the targeting of point-mutated transcripts of transforming oncogenes, such as Ras.[71]

Apoptosis inhibitors, such as Bcl-2,[67] which make cell resistant to caspase-mediated apoptosis, are another kind of targets for siRNA-technology based tumor gene therapy to promote apoptosis of cancer cells. Polo-like kinase 1 (PLK1) is a key cell-cycle regulator that is overexpressed in various human tumors. After transfection with plasmids containing U6 promoter-driven shRNAs against human PLK1, levels of PLK1 mRNA, and protein in HeLa S3 cervical and A549 lung cancer cell lines were lower than in control. Proliferation of cells transfected with PLK1 shRNA was lower than that of cells transfected with either control plasmid, and proliferation of cells transfected with ATA-treated PLK1 shRNA plasmids was even lower. Furthermore, in mice with human xenograft tumors, PLK1 shRNA expression from ATA-treated plasmids reduced tumor growth to 18% and from untreated plasmids reduced tumor growth to 45% of that of tumors in mice treated with scrambled control PLK1S shRNA plasmids.[68] Thus inhibition of the expression of PLK1 by RNAi technology successfully induced cancer cell apoptosis.

Improving the efficacy of chemotherapy and radiotherapy in cancer has been also a potential application of RNAi technology in cancer therapy. Overexpression of P-glycoprotein, encoded by the *MDR1* gene, confers multidrug resistance (MDR) on cancer cells and is a frequent impediment to successful chemotherapy. Thus MDR which pumps chemotherapeutic drugs out of tumors, would be a hopeful target of siRNAs for cancer therapy.[72] Yague et al.[73] have expressed two different short hairpin RNAs against MDR1 from stably integrated plasmids in doxorubicin-resistant K562 leukaemic cells and resulted in decreased MDR1 mRNA, abolished P-glycoprotein expression, and completely reversed the MDR phenotype to that of the drug-sensitive K562 parental line. Peng et al.[74] used RNAi to target Protein kinase, DNA-activated, catalytic polypeptide (Prkdc) in human fibroblasts and found that radiosensitivity was increased particularly in low-dose region of 0–1 Gray. Another case is that Chen et al.[75] applied a retroviral delivery system to express stably siRNA against the unique fusion junction sequence of TEL-PDGF β R in transformed hematopoietic cells. Their data demonstrated that stable expression of siRNA is able to sensitize TEL-PDGF β R—transformed cells to the small molecule inhibitors imatinib and rapamycin. These investigations provided a promising way for the treatment of cancers.

Invasion and migration are characteristics of cancer cells and play important roles in cancer development. The serine protease urokinase-type plasminogen activator (u-PA) mRNA is

up-regulated in human hepatocellular carcinoma (HCC) biopsies and its level of expression is inversely correlated with patients' survival.[77] Salvi et al.[77] transfected an HCC-derived cell line at high level of u-PA expression with siRNA against u-PA. These siRNA u-PA-transfected cells showed a reduction of migration, invasion, and proliferation. Thus, stable expression of siRNA u-PA could potentially be an experimental approach for HCC gene therapy. Lipscomb et al.[76] used RNAi technology to inhibit integrin (α 6 β 4)-mediated invasion and migration of breast carcinoma cells.

As there already have had many explorations that employed RNAi technology in cancer therapy from variety of aspects and made some cheering outcomes. RNAi may be a new wave of cancer therapy.

C. RNA Interference for the Therapy of Genetic and Other Diseases

In addition to viral infection and cancer, dominantly inherited diseases would seem to be ideal candidates for siRNA-based therapy. In some cases of this kind of diseases, mutant allele is toxic, while wild type is important, such as neurodegenerative diseases due to polyglutamine-mediated cytotoxicity. At least eight human neurodegenerative diseases due to polyglutamine expansion-mediated cytotoxicity, including Huntington's disease (HD) and spinobulbar muscular atrophy (SBMA) (Kennedy's disease) are caused by expansion of trinucleotide (CAG) repeats.[79] They are dominant, progressive, untreatable disorders. In inducible mouse models of SCA1 and HD, disease phenotypes can be improved when mutant allele expression is repressed.[78] Thus, to inhibit expression of the mutant gene would be sensible therapy strategy. Miller et al.[88] demonstrated in mammalian cell models that allele-specific silencing of disease genes with siRNA could be achieved by targeting either a linked single-nucleotide polymorphism (SNP) or the disease mutation directly. Xia et al.[78] injected recombinant adeno-associated virus (AAV) vectors expressing short hairpin RNAs targeting mutant ataxin-1 into cerebelar of a mouse model of SCA1 with polyglutamine-induced neuro-degeneration caused by this mutant gene. This treatment profoundly improved motor coordination, restored cerebellar morphology, and resolved characteristic ataxin-1 inclusions in Purkinje cells of SCA1 mice. These studies demonstrated *in vivo* the potential use of RNAi as therapy for dominant neurodegenerative disease.

As target identification depends upon Watson–Crick basepairing interactions, the small RNAs-mediated silencing processes can be both flexible and exquisitely specific. Prior to the discovery of small RNAs, methodologies that have been exploited to achieve gene-specific inhibition to produce a loss-of-function phenotype included antisense technology, catalytic ribozymes, homologous recombination, or targeted mutagenesis. Although these techniques were successful in the past, they are limited by expense, inefficient annealing to target sequences, and the difficulty in transmitting mutations through the germline, respectively.[89] Compared to these previous gene expression interference strategies, RNAi technology has some obvious advantages: first, high specificity: only a single base alteration in targets can reduce the silencing effect dramatically.[1] So, it can be used to achieve allele-specific silencing. Second, high efficiency: siRNAs are able to reduce the target gene expression by more than 90%.[90] Furthermore, when compared directly to each other, siRNA may have a greater inhibitory effect than anti-sense methods in multiple cancer cell lines. Comparisons between RNAi and ribozymes in mammalian cell culture also show that siRNA are more effective gene silencers.[90] Beyond traditional drug targets such as proteins, enzymes, and receptors which all fall into the post-translational category, small RNAs induced inhibition offers post-transcriptional and translational targeting.

In theory, RNAi could be used to treat almost any disease that is caused by expression or overexpression of a native or mutated gene, providing that low expression ($\sim$10% of wild-type) will not be toxic.[91] The development of RNAi technology shows significant promise for gene therapy.

4. PERSPECTIVE

Previous data suggested that miRNAs and siRNAs can use similar mechanisms to suppress mRNA expression and that the choice of mechanism may be largely or entirely determined by the degree of complementarity between the small RNA and the RNA target.[92] The *in vivo* siRNA expression systems used at present are designed following the mechanism of miRNAs biosynthesis. Designed miRNAs were also excised from transcripts encompassing artificial miRNA precursors and could inhibit the expression of mRNAs containing a complementary target site.[93] So both natural and designed miRNAs can inhibit the expression of cognate mRNAs and novel miRNAs can be readily produced *in vivo* and can be designed to specifically inactivate the expression of selected target genes in human cells.[93] Thus, miRNAs also possess the potential in gene therapy.

miRNAs or their targets may be directly involved in the processes that lead to oncogenesis. If it is the truth, down-regulating the expression of miRNAs serving as oncogenes and up-regulating or supplementing the ones serving as tumor suppressors may be useful in tumor therapy.

It has been proven that RNAi can stably repress gene expression in stem cells and reconstituted organs derived from those cells.[94,95] Thus, this technology possesses a potential application in *ex vivo* gene therapy. Regulation of the RNAi efficiency through inducible systems should be useful for the inducible knockdown of gene expression. Reseachers have developed etracycline or doxycycline-inducible RNAi systems.[96–98] However, this system possesses a relatively high background of expression in the uninduced state in certain cell lines.[99] Another most widely used inducible mammalian system, ecdysone-inducible system, has also been combined with RNAi technology. This system is tightly regulated with no expression in the uninduced state and a rapid inductive response, and the components of the inducible system are inert with rapid clearance kinetics and, therefore, do not affect mammalian physiology.[99] Matsukura et al.[100] reported a CRE recombinase-inducible RNA interference system. These researches have broadened the way to apply RNAi technology. Creating novel methods of delivering tissue-specific expressing small RNAs or even cell differentiation-dependent expressing ones to target certain diseases are exciting goals for the future.

Small RNAs and related-machinery have close relations with the cause of some human diseases, and they are new hopes of human disease therapy. However, there still exist so many mysteries in the processes that small RNAs are involved in. For example, the exact buildup of RISCs is not very clear, not even their functions. Researchers have not made out the mechanism of small RNAs clearing away. Little is known about how miRNAs are regulated, much less about what polymerase transcribes them. Furthermore, little is known about what signals convey the temporal and/or spatial expression of miRNAs. This can be predicted to become an active area of research that will be highly important in development and disease. Until all the puzzles are resolved, the detailed relations between small RNAs and disease will be uncovered, and the application of small RNAs will be pushed forward.

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REFERENCES

1. Elbashir SM, Lendeckel W, Tusch T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Gene Dev 2001;15:188–200.
2. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science 2001;294:853–858.

3. Lau N, Lim L, Weinstein E, Barte D. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 2001;294:858–862.
4. Lee R, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. Science 2001;294:862–864.
5. Agami R. RNAi and related mechanisms and their potential use for therapy. Curr Opin Chem Biol 2002;6:829–834.
6. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 2000;404:293–296.
7. Parrish S, Fleenor J, Xu S, Mello C, Fire A. Functional anatomy of a dsRNA trigger: Differential requirement for the two trigger strands in RNA interference. Mol Cell 2000;6:1077–1087.
8. Yang D, Lu H, Erickson JW. Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. Curr Biol 2000;10:1191–1200.
9. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 2000;101:25–33.
10. Olson P, Ambros V. The lin-4 regulator RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 1999;216:671–680.
11. Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J 2000;19:5194–5201.
12. Mochizuki K, Fine NA, Fujisawa T, Gorovsky MA. Analysis of a *piwi* related gene implicates small RNAs in genome rearrangement in tetrahymena. Cell 2002;110:689–699.
13. Plasterk RHA. RNA silencing: The genome 's immune system. Science 2002;296:1263–1265.
14. Vance V, Vaucheret H. RNA silencing in plants—Defense and counterdefense. Science 2001;292:2277–2280.
15. Hannon GJ. RNA interference. Nature 2002;418:244–251.
16. Carrington J, Ambros V. Role of microRNAs in plant and animal development. Science 2003;30:336–338.
17. Bernstein E, Caudy A, Hammond S, Hannon G. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001;409:363–366.
18. Nelson P, Kiriakidou M, Sharma A, Maniataki E, Mourelatos Z. The microRNA world: Small is mighty. Trends Biochem Sci 2003;28:534–540.
19. Novina CD, Sharp PA. The RNAi revolution. Nature 2004;430:161–164.
20. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998;391:806–811.
21. Hamilton A, Voinnet O, Chappell L, Baulcombe D. Two classes of short interfering RNA in RNA silencing. EMBO J 2002;21:4671–4679.
22. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP. The microRNAs of *Caenorhabditis elegans*. Gene Dev 2003;17:991–1008.
23. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297.
24. Lee Y, Ahn C, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S, V. Kim N. The nuclear RNase III Drosha initiates microRNA processing. Nature 2003;425:415–419.
25. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Gene Dev 2003;17:3011–3016.
26. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science 2004;303:95–98.
27. Hutvágner G, McLachlan J, Pasquinelli A, Bálint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 2001; 293:834–838.
28. Grishok A, Pasquinelli A, Conte D, Li N, Parrish S, Ha I, Baillie D, Fire A, Ruvkun G, Mello C. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* and developmental timing. Cell 2001;106:23–34.
29. Llave C, Xie Z, Kasschau K, Carrington J. Cleavage of scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. Science 2002;297:2053–2056.
30. Kasschau K, Xie Z, Allen E, Llave C, Chapman E, Krizan K, Carrington J. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Dev Cell 2003;4:205–217.
31. Boutet S, Vasquez F, Liu J, Béclin C, Fagard M, Gratias A, Morel JB, Crété P, Chen X, Vaucheret H. *Arabidopsis* HEN1: A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr Biol 2003;13:843–848.
32. Johnston RJ, Hobert OA. MicroRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. Nature 2003;426:845–849.

33. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. Science 2004;303:83–86.
34. Okamura K, Ishizuka A, Siomi H, Siomi MC. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev 2004;18:1655–1666.
35. Carmell MA, Xuan Z, Zhang MQ, Hannon GJ. The Argonaute family: Tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Gene Dev 2002;16:2733–2742.
36. Lim L, Glasner M, Yekta S, Burge C, Bartel D. Vertebrate microRNA genes. Science 2003;299:1540.
37. Caudy AA, Myers M, Hannon GJ, Hammond SM. Fragile X-related protein and VIG associate with the RNA interference machinery. Gene Dev 2002;16:2491–2496.
38. Ishizuka A, Siomi MC, Siomi H. A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. Gene Dev 2002;16:2497–2508.
39. Bardoni B, Mandel JL. Advances in understanding of fragile X pathogenesis and FMRP function and in identification of X linked mental retardation genes. Curr Opin Genet Dev 2002;12:284–293.
40. Carthew RW. RNA interference: The fragile X syndrome connection. Curr Biol 2002;12:R852–R854.
41. Jin P, Zarnescu DC, Ceman S, Nakamoto M, Mowrey J, Jongens TA, Nelson DL, Moses K, Warren ST. Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. Nat Neurosci 2004;7:113–117.
42. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G. miRNPs: A novel class of ribonucleoproteins containing numerous micro-RNAs. Genes Dev 2002;16: 720–728.
43. Qiao D, Zeeman AM, Deng W, Looijenga LH, Lin H. Molecular characterization of hiwi: A human member of the piwi gene family whose overexpression is correlated to eminomas. Oncogene 2002;21: 3988–3999.
44. Dostie J, Mourelatos Z, Yang M, Sharma A, Dreyfuss G. Numerous microRNPs in neuronal cells containing novel microRNAs. RNA 2003;9:180–186.
45. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. Frequent deletions and down-regulation of micro-RNA genes *miR15* and *miR16* at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002;99:15524–15529.
46. Dong JT, Boyd JC, Frierson HF, Jr. Loss of heterozygosity at 13q14 and 13q21 in high grade, high-stage prostate cancer. Prostate 2001;49:166–171.
47. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002;12:735–739.
48. Michael MZ, O'Connor SM, Pellekaan NGH, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 2003;1:882–891.
49. Metzler M, Wilda M, Busch K, Viehmann S, Borkhardt A. High expression of precursor microRNA-155/ BIC RNA in children with Burkitt lymphoma. Gene Chromosome Cancer 2004;39:167–169.
50. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science 2002; 297:2056–2060.
51. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 2004;101:2999–3004.
52. Ambros V. MicroRNAs: Tiny regulators with great potential. Cell 2001;107:823–826.
53. Storz G. An expanding universe of noncoding RNAs. Science 2002;296:1260–1263.
54. Schwarz DS, Zamore PD. Why do miRNAs live in the miRNP? Genes Dev 2002;16:1025–1031.
55. McManus MT. MicroRNAs and cancer. Semin Cancer Biol 2003;13:253–258.
56. Martínez MA, Gutiérrez A, Armand-Upón M, Blanco J, Parera M, Gómez J, Clotet B, Esté JA. Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. AIDS 2002;16:2385–2390.
57. Coburn GA, Cullen BR. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J Virol 2002;76:9225–9231.
58. Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P, Sharp PA. siRNA-directed inhibition of HIV-1 infection. Nat Med 2002;8:681–686.
59. Sholmai A, Shaul Y. Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology 2003;37:764–770.
60. Randall G, Grakoui A, Rice CM. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc Natl Acad Sci USA 2003;100:235–240.
61. Jiang M, Milner J. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treatment with siRNA: A primer of RNA interference. Oncogene 2002;21:6041–6048.

62. Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen HN, Chen J. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc Natl Acad Sci USA 2003;100:2718–2723.
63. Ge Q, Filip L, Bai A, Nguyen T, Eisen HN, Chen J. Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc Natl Acad Sci USA 2004;101:8676–8681.
64. Tompkins SM, Lo CY, Tumpey TM, Epstein SL. Protection against lethal influenza virus challenge by RNA interference *in vivo*. Proc Natl Acad Sci USA 2004;101:8682–8686.
65. Zhang Y, Li T, Fu L, Yu C, Li Y, Xu X, Wang Y, Ning H, Zhang S, Chen W, Babiuk LA, Chang Z. Silencing SARS-CoV Spike protein expression in cultured cells by RNA interference. FEBS Lett 2004;560: 141–146.
66. Wang Z, Ren L, Zhao X, Hung T, Meng A, Wang J, Chen YG. Inhibition of severe acute respiratory syndrome virus replication by small interfering RNAs in mammalian cells. J Virol 2004;78:7523–7527.
67. Cioca DP, Aoki Y, Kiyosawa K. RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. Cancer Gene The 2003;10:125–133.
68. Spankuch B, Matthess Y, Knecht R, Zimmer B, Kaufmann M, Strebhardt K. Cancer inhibition in nude mice after systemic application of U6 promoter-driven short hairpin RNAs against PLK1. J Natl Cancer Inst 2004;96:862–872.
69. Wilda M, Fuchs U, Wossmann W, Borkhardt A. Killing of leukemic cells with a *BCR/ABL* fusion gene by RNA interference (RNAi). Oncogene 2002;21:5716–5724.
70. Scherr M, Battmer K, Winkler T, Heidenreich O, Ganser A, Eder M. Specific inhibition of bcr-abl gene expression by small interfering RNA. Blood 2003;101:1566–1569.
71. Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2002;2:243–247.
72. Nieth C, Priebsch A, Stege A, Lage H. Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). FEBS Lett 2003;545:144–150.
73. Yague E, Higgins CF, Raguz S. Complete reversal of multidrug resistance by stable expression of small interfering RNAs targeting MDR1. Gene Ther 2004;11:1170–1174.
74. Peng Y, Zhang Q, Nagasawa H, Okayasu R, Liber HL, Bedford JS. 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 2002;62:6400–6404.
75. Chen J, Wall NR, Kocher K, Duclos N, Fabbro D, Neuberg D, Griffin JD, Shi Y, Gilliland DG. Stable expression of small interfering RNA sensitizes TEL-PDGFbetaR to inhibition with imatinib or rapamycin. J Clin Invest 2004;113:1784–1791.
76. Lipscomb EA, Dugan AS, Rabinovitz I, Mercurio AM. Use of RNA interference to inhibit integrin mediated invasion and migration of breast carcinoma cells. Clin Exp Metastas 2003;20:569–576.
77. Salvi A, Arici B, De Petro G, Barlati S. Small interfering RNA urokinase silencing inhibits invasion and migration of human hepatocellular carcinoma cells. Mol Cancer Ther 2004;3:671–678.
78. Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, Orr HT, Paulson HL, Yang L, Kotin RM, Davidson BL. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat Med 2004;10:816–820.
79. Caplen NJ, Taylor JP, Statham VS, Tanaka F, Fire A, Morgan RA. Rescue of polyglutamine-mediated cytoxicity by double-stranded RNA-mediated RNA interference. Hum Mol Genet 2002;11:175–184.
80. McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, Wieland SF, Marion PL, Kay MA. Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol 2003;21:639–644.
81. Konishi M, Wu CH, Wu GY. Inhibition of HBV repliation by siRNA in a stable HBV-producing cell line. Hepatology 2003;38:842–850.
82. McCaffrey AP, Meuse L, Karimi M, Contag CH, Kay MA. A potent and specific morpholino antisense inhibitor of hepatitis C translation in mice. Hepatology 2003;38:503–508.
83. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. Nature 2002;418:435–438.
84. Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, Rossi J. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat Biotechnol 2002;20:500–505.
85. Martínez MA, Clotet B, Esté JA. RNA interference of HIV replication. Trends Immunol 2002;23: 559–561.
86. Qin XF, An DS, Chen IS , Baltimore D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci USA 2003;100:183–188.
87. Gitllin L , Karelsky S, Andino R. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 2002;418:430–434.

88. Miller VM, Xia H, Marrs GL, Gouvion CM, Lee G, Davidson BL, Paulson HL. Allele-specific silencing of dominant disease genes. Proc Natl Acad Sci USA 2003;100:7195–7200.
89. Brantl S. Antisense-RNA regulation and RNA interference. Biochim Biophys Acta 2002;1575:15–25.
90. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002;296:550–553.
91. Lieberman J, Song E, Lee SK, Shankar P. Interfering with disease: Opportunities and roadblocks to harnessing RNA interference. Trends Mol Med 2003;9:397–403.
92. Zeng Y, Yi R, Cullen BR. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proc Natl Acad Sci USA 2003;100:9779–9784.
93. Zeng Y, Wagner EJ, Cullen BR. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol Cell 2002;9:1–20.
94. Hemann MT, Fridman JS, Zilfou JT, Hernando E, Patrick PJ, Cordon-Cardo C, Hannon GJ, Lowe SW. An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes *in vivo*. Nat Genet 2003;33:396–400.
95. Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Zhang M, McManus MT, Gertler FB, Scott ML, Parijs LV. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells, and transgenic mice by RNA interference. Nat Genet 2003;33:401–406.
96. Wetering M, Oving I, Muncan V, Fong MTP, Brantjes H, Leenen D, Holstege FCP, Brummelkamp TR, Agami R, Clevers H. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO R 2003;4:609–615.
97. Wiznerowicz M, Trono D. Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible RNA interference. J Virol 2003;77:8957–8961.
98. Matsukura S, Jones PA, Takai D. Establishment of conditional vectors for hairpin siRNA knockdowns. Nucleic Acids Res 2003;31:e77.
99. Gupta S, Schoer RA, Egan JE, Hannon GJ, Mittal V. Inducible, reversible, and stable RNA interference in mammalian cells. Proc Natl Acad Sci USA 2004;101:1927–1932.
100. Tiscornia G, Tergaonkar V, Galimi F, Verma IM. CRE recombinase-inducible RNA interference mediated by lentiviral vectors. Proc Natl Acad Sci USA 2004;101:7347–7351.

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