CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

siRNA, miRNA, and shRNA: *in vivo* Applications

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ABSTRACT

RNA interference (RNAi), an accurate and potent gene-silencing method, was first experimentally documented in 1998 in *Caenorhabditis elegans* by Fire *et al.*, who subsequently were awarded the 2006 Nobel Prize in Physiology/Medicine. Subsequent RNAi studies have demonstrated the clinical potential of synthetic small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) in dental diseases, eye diseases, cancer, metabolic diseases, neurodegenerative disorders, and other illnesses. siRNAs are generally from 21 to 25 base-pairs (bp) in length and have sequence-homology-driven gene-knockdown capability. RNAi offers researchers an effortless tool for investigating biological systems by selectively silencing genes. Key technical aspects—such as optimization of selectivity, stability, *in vivo* delivery, efficacy, and safety—need to be investigated before RNAi can become a successful therapeutic strategy. Nevertheless, this area shows a huge potential for the pharmaceutical industry around the globe. Interestingly, recent studies have shown that the small RNA molecules, either indigenously produced as microRNAs (miRNAs) or exogenously administered synthetic dsRNAs, could effectively *activate* a particular gene in a sequence-specific manner instead of silencing it. This novel, but still uncharacterized, phenomenon has been termed 'RNA activation' (RNAa). In this review, we analyze these research findings and discussed the *in vivo* applications of siRNAs, miRNAs, and shRNAs.

KEY WORDS: *Caenorhabditis elegans*, RNA interference, small-interfering RNAs, microRNAs, short hairpin RNAs, gene silencing, RNA activation, *in vivo*.

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INTRODUCTION

The nematode *Caenorhabditis elegans*, a model genetic organism for studying the behavior and development of humans, was well-established by the pioneering work of Robert Horvitz, Sydney Brenner, and John Sulston (Kenyon, 1988), for which they were awarded the 2002 Nobel Prize for Physiology/Medicine. Recently, two key phenomenal discoveries emerging from *C. elegans* were the identification of inherent tiny, non-coding RNA genes, lin-4 and let-7, which produce microRNAs (miRNAs) that regulate development and the establishment of RNA interference (RNAi) by long double-stranded RNAs (dsRNAs). Fire and Mello demonstrated that 21- to 25-nucleotide short interfering RNAs (siRNAs) are the key effector molecules of RNAi in *C. elegans* (Fire *et al.*, 1998). Conversely to siRNAs, miRNAs are derived from the processing of endogenously encoded short hairpin RNAs (shRNAs). In contrast, the exogenous siRNAs hypothetically represent perfect drugs for the specific blocking of unwanted or disease-causing gene products. There is no disease affecting humans that does not have a genetic component, and RNAi sequences can be tailored to block just about any gene (Aagaard and Rossi, 2007). These sequences make blocking possible by binding and then degrading the mRNA produced by the gene before that mRNA can start producing a harmful protein, which actually causes the illness (Fig.1A). Ever since the Nobel Prize-winning discovery by Fire *et al.*, in 1998, more than 12,000 articles and 1300 reviews related to RNAi technology have been published. In this review, we analyze these research findings and discuss the *in vivo* applications of siRNAs, miRNAs, and shRNAs.

MOLECULAR MECHANISMS OF RNAi

The discovery of RNAi in *C. elegans* initiated an array of biological and genetic experiments aimed at identifying the molecular components involved in the RNAi phenomenon (Grishok, 2005). The RNAi mechanism by which introduction of long dsRNAs into a cell can result in the degradation of targeted mRNAs is illustrated in Fig. 1B. Introduced dsRNAs are recognized by a dsRNA-binding protein termed 'RNAi DEfective family member-4' (RDE-4) (Grishok *et al.*, 2000; Tabara *et al.*, 2002; Tabara and Yasuda, 2003; Wang and Barr, 2005). This binding facilitates the cleavage of dsRNA into 21- to 25-base-pair siRNAs, with 2-nucleotide overhangs at both 3′-ends, by an RNase III enzyme called Dicer (Hammond *et al.*, 2000; Bernstein *et al.*, 2001a,b, 2003). These siRNAs are unwound by an ATP-dependent enzyme called helicase (Nykanen *et al.*, 2001), and the single-stranded siRNAs then act as guides in association with a protein complex to target homologous transcripts for cleavage. This RNA/Protein complex is called the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000). However, the synthetic siRNAs, which are 21-30 base-pairs in length, skip the Dicer step and are converted to single strands by ATP-dependent helicase and incorporated into the RNA-induced silencing complex for the specific cleavage of the target mRNA species (Grishok *et al.*, 2001; Victor *et al.*, 2002; Timmons *et al.*, 2003; Hutvagner *et al.*, 2004).

In vivo APPLICATIONS OF RNAi

RNAi has been used to generate model systems, to identify novel molecular targets, to study gene function in a genome-wide manner, and to create a new niche for clinical therapeutics (PY Lu *et al.*, 2005; Xie *et al.*, 2006; Martin and Caplen, 2007). We and others have recently reviewed the therapeutic potential of synthetic siRNAs in various human diseases and disorders (Pushparaj and Melendez, 2006). siRNAs have successfully been tested in various disease models of animals, and we discuss some of these studies in the following section of this review.

In vivo RNAi in Oral Diseases

Nasopharyngeal Carcinoma

The specific silencing of hyaluronan receptor (CD44) gene expression by RNAi in the nasopharyngeal carcinoma cell line (CNE-2L2) resulted in profound reduction of malignant potential of the cells, including growth, *in vitro* colony formation, tumorigenesis, and metastasis of tumors in nude mice (Jod *et al.*, 2007; Shi *et al.*, 2007a). Direct injection of the adenoviruses harboring and producing siRNA to CD44 into the tumor inoculated with CNE-2L2 cells in nude mice caused inhibition of tumor growth. Analysis of the data indicated a positive association of CD44 expression with the malignant activities of CNE-2L2 cells and suggested a possible therapeutic effect of direct introduction of siRNA to CD44 into some human solid tumors with high expression of the CD44 gene (Shi *et al.*, 2007a,b).

Head and Neck Cancer

To identify genes that could potentially serve as molecular therapeutic markers for human head and neck cancer, Chen *et al.* (2007) used differential display analysis to compare the gene expression profiles between head and neck cancer tissue and histopathologically normal epithelial tissues. They identified desmoglein 3 as differentially expressed at both the RNA and protein levels. Consistent with the clinical findings, inhibition of desmoglein 3 by RNAi significantly reduced cell growth and colony formation to 57-21% in 3 head and neck cancer cell lines. Moreover, *in vivo* xenograft experiments revealed that administration of desmoglein 3-RNAi plasmid significantly inhibited tumor growth for 2 mos in BALB/C nude mice. These findings with RNAi suggest that desmoglein 3 is a potential molecular target in the development of adjuvant therapy for head and neck cancer (Chen *et al.*, 2007).

Oral Squamous Cell Carcinoma

S phase kinase-interacting protein 2, an F box protein, is required for the ubiquitination and consequent degradation of p27. It is well-known that reduced expression of p27 is frequently observed in various cancers, including oral squamous cell carcinoma, and is due to an enhancement of its protein degradation. A previous study showed that overexpression of S phase kinase-interacting protein 2 was frequently found in oral squamous cell carcinoma and inversely correlated with p27 expression (Kudo *et al.*, 2005b). Interestingly, S phase kinase-interacting protein 2 siRNA inhibited the cell proliferation of oral squamous cell carcinoma cells both *in vitro* and *in vivo*. These findings suggest that siRNA-mediated gene silencing of S phase kinase-interacting protein 2 can be a novel modality of cancer gene therapy for suppression of p27 down-regulation (Kudo *et al.*, 2005a). It has been shown that the basal transcription of p53-inducible ribonucleotide reductase small-subunit 2 could be associated with the sensitivity to anticancer agents (Yanamoto *et al.*, 2005) . Moreover, investigators have assessed the possibility that p53-inducible ribonucleotide reductase small-subunit 2 would be a good molecular target, and reported that RNAi targeting of p53-inducible ribonucleotide reductase small-subunit 2 could be

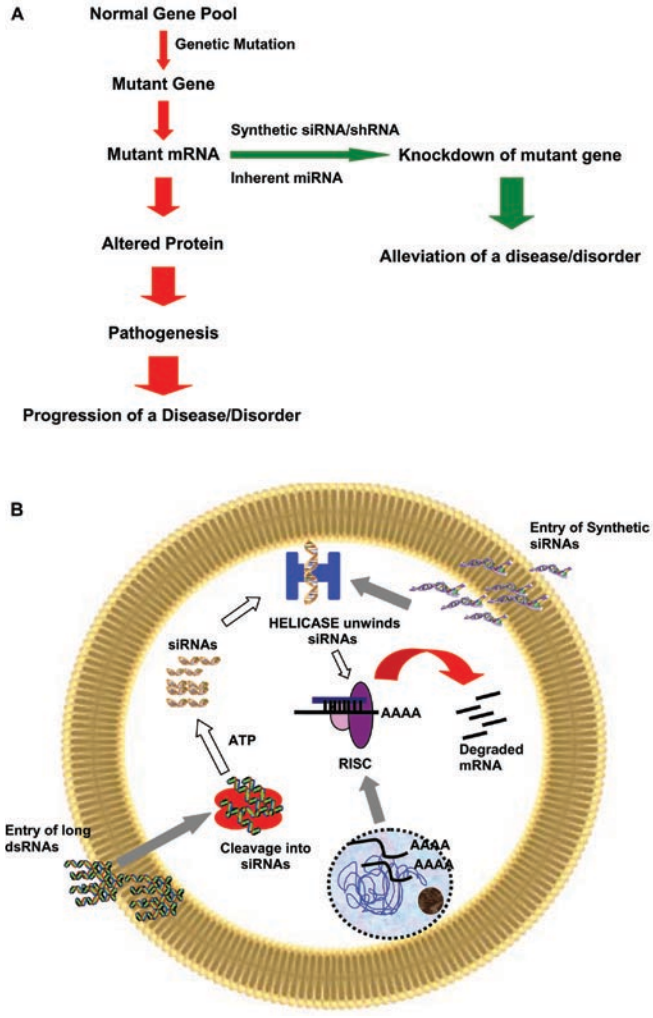

Figure 1. Mechanism of RNAi. **(A)** Synthetic siRNAs and shRNAs hypothetically represent perfect drugs for the specific blocking of unwanted or disease-causing gene products. These RNA sequences can be tailored to block just about any gene. They bind and then degrade the mRNA produced by the gene before that mRNA can start producing a harmful protein, which actually causes the illness. In contrast, miRNAs are being produced or coded by genes inherently within a cell and control the expression of various genes involved in key cellular processes. **(B)** The RNAi pathway involves the introduction of a long 'triggering' dsRNA with 2-nucleotide overhangs at the 3′ terminal end that is subsequently processed into 21- to 25-bp small interfering RNAs (siRNA) by an RNaseIII-like enzyme called Dicer. The siRNAs are unwound by a helicase before entering into a multi-subunit RNA-induced silencing complex (RISC), and the strand complementary to the target mRNA is integrated into RISC. An endonuclease present within the RISC either degrades or inhibits translation of specific mRNA transcripts. In contrast, synthetic siRNAs (generally 21-25 bp long) skip the Dicer cleavage, directly unwind by helicase, and enter into the RISC for the specific blocking of mRNA transcripts.

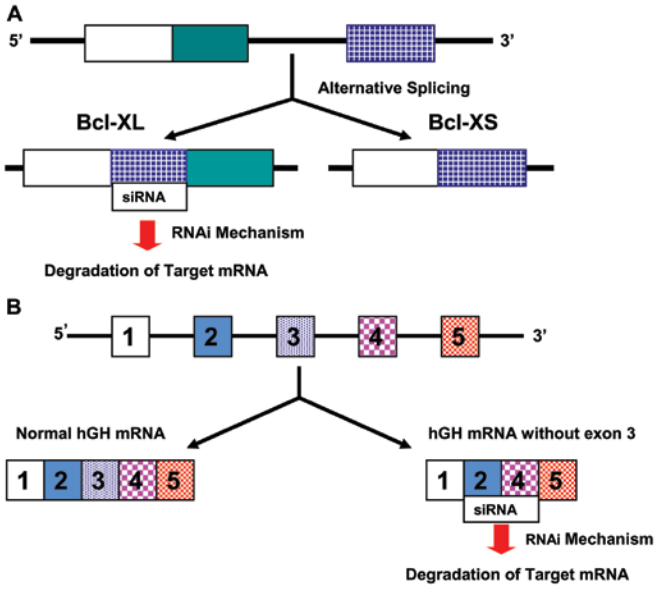

Figure 2. RNAi-mediated knockdown of splicing isoforms. **(A)** Bcl-xL-specific siRNA silences Bcl-xL protein and inhibits the proliferation of 5-fluorouracil and tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL)-resistant cells. **(B)** RNAi-mediated silencing of human growth hormone (hGH) splice variant without exon 3.

useful for oral cancer gene therapy (Yanamoto *et al.*, 2005).

Tooth Development

siRNAs can also be used to investigate the roles of key genes involved in the development of tooth. By viral-mediated RNAi knockdown of homeo box, msh-like 1, or distal-less homeobox 2 mRNAs in the dental mesenchyme, it is possible to reproduce the identical tooth phenotype seen in mice deficient for homeobox, msh-like 1, and distal-less homeobox 2 genes, respectively. It demonstrates that silencing of homeobox, msh-like 1 in the dental mesenchyme results in an arrest of tooth development at the bud stage, indicating a critical role for homeobox, msh-like 1 in tooth development (Song *et al.*, 2006).

In vitro RNAi in Oral Diseases

Some of the recent RNAi studies *in vitro* have shown that the siRNAs can be potentially used for the treatment of dental disorders such as ankylosis and periodontal diseases. Periodontal-ligament-associated protein-1 (PLAP-1)/asporin is involved in chondrogenesis, and its involvement in the pathogenesis of osteoarthritis has been proved (Yamada *et al.*, 2001). Overexpression of asporin in mouse periodontal-ligament-derived clone cells interfered with both naturally and bone morphogenetic protein-2-induced mineralization of the periodontal ligament cells. In contrast, knockdown of asporin transcript levels by RNAi enhanced bone morphogenetic protein-2-induced differentiation of periodontal ligament cells. These results suggest that asporin plays a specific role(s) in the periodontal ligament as a negative regulator of cytodifferentiation and mineralization, probably by regulating BMP-2 activity to prevent the periodontal ligament from developing non-physiological mineralization such as ankylosis (Yamada *et al.*, 2007). Recently, Ye *et al.* (2006) used H413 epithelial cells derived from a human oral squamous cell carcinoma and 'knocked down' the CD24 protein by RNAi to cause about 90% reduction in the mRNA level. CD24 is a heavily glycosylated peptide molecule implicated in hematogenous metastasis of carcinomas (Schindelmann *et al.*, 2002). This down-regulation of CD24 mRNA was associated with reduced E-cadherin expression and up-regulated expression of transcription factors such as snail, twist, and transforming growth factor-β3. Hence, it was concluded that CD24 could play an important role in modulating the expression of genes that regulate epithelial differentiation in the periodontium (Ye *et al.*, 2006).

In vivo RNAi in the Regulation of Splice Variants

The abnormal regulation of splice variant expression of certain genes has been recognized as the reason for several genetic disorders in humans, including cancer (Fig. 2A) (Faustino and Cooper, 2003; Garcia-Blanco *et al.*, 2004). The human growth hormone (hGH) gene contains 5 exons and 4 introns. Elimination of all 4 introns produces mRNA that encodes the full-length 22-kDa hGH protein. The exon-C-skipped mRNA encodes a 17.5-kDa protein that is linked to isolated hGH deficiency (IGHD) type II, an autosomal-dominant form of hGH deficiency (GHD) (Lee *et al.*, 2000). Therapies that specifically target the 17.5-kDa isoform might be useful in persons with IGHD II (Fig.2B) (Monson, 2003). Certainly, RNAi has the potential to be an efficient alternative to current hGH replacement therapy, which has many side-effects, such as benign intracranial hypertension and insulin resistance (Ryther *et al.*, 2004).

In vivo RNAi in Neurodegenerative Diseases/Disorders

Specific siRNA knockdown of mutant SOD 1 slows amyotrophic lateral sclerosis (ALS) in animal models (Wang *et al.*, 2008). A recent study by Alnylam Pharmaceuticals, Inc. and collaborators from the University of Massachusetts Medical School and Massachusetts General Hospital demonstrated that chemically synthesized siRNAs targeting the gene responsible for Huntington's disease provide a therapeutic benefit in an animal model of the human disease. The new pre-clinical study showed that a single injection of a cholesterol-conjugated-siRNA targeting *huntingtin*, the gene responsible for Huntington's disease, resulted in improved symptoms of disease in an animal model. These improved effects included reduction in neuronal pathology and an improvement in motor behavior. The RNAi therapy reduced expression of mutant *huntingtin* in the brain and sustained a benefit in motor behavior for at least one week. In preliminary studies, the RNAi therapy was found to be well-tolerated in the brain after direct CNS administration (DiFiglia *et al.*, 2007).

In vivo RNAi in Acute and Chronic Inflammation

Sepsis and allergy are very important diseases affecting human beings (Vlasenko and Melendez, 2005; Melendez *et al.*, 2007). Recently, we and our colleagues have shown that specific silencing of the mouse sphingosine kinase 1 isoform results in the amelioration of C5a-induced acute peritonitis (Pushparaj *et al.*, 2008b) and allergic asthma (Lai *et al.*, 2008) in mouse models of disease.

miRNAs

miRNAs are a group of small non-coding RNA molecules

produced endogenously (Grishok *et al.*, 2000). miRNAs can play important functions from *C. elegans* to higher vertebrates by base-pairing to mRNAs to regulate the expression of a specific gene (Pushparaj and Melendez, 2006; Hatfield and Ruohola-Baker, 2008). The number of miRNAs reported so far (the 2008 release of miRBase at the Sanger Institute) is in excess of 5300, several-fold as many as initial calculations, and thousands of predicted miRNAs are awaiting experimental confirmation. After the identification of several miRNAs in all sequenced genomes, one of the key questions remains the systematic identification of the targets and biological processes that are being regulated.

miRNAs: BIOGENESIS AND MOLECULAR MECHANISMS

miRNAs are encoded in the genome and are transcribed by RNA polymerase II (pol II) as long precursor transcripts, which are known as primary miRNAs (pri-miRNAs) of several kilobases in length (Lee *et al.*, 2004). Mature miRNAs are generated from pre-miRNAs by sequential processing steps (Fig.3A). The pri-miRNAs are initially recognized by the microprocessor complex in the nucleus, whose core components are the RNase-III enzyme Drosha and its binding partner DiGeorge critical region 8 protein (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Landthaler *et al.*, 2004). The microprocessor complex excises the stem–loop hairpin structure that contains the miRNA, a 60- to 80-nucleotide intermediate termed 'precursor miRNA' (pre-miRNA). The pre-miRNA is recognized by the nuclear export factor Exportin-5, which transports it to the cytoplasm (Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Lund *et al.*, 2004). Pre-miRNAs are rapidly exported to the cytoplasm by the nuclear export factor exportin 5, which uses Ran-GTP as a co-factor (Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Lund *et al.*, 2004). Further cytoplasmic processing by a second RNase III enzyme named Dicer performs a second cleavage to generate double-stranded 18- to 24-nucleotide-long miRNA (Bernstein *et al.*, 2001a; Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). One of these two strands—the guide strand—is incorporated in an ATP-independent manner into the RNA-induced silencing complex, which includes as core components the Argonaute proteins (Ago1–4 in humans) (Kim, 2005). Only one strand of the miRNA duplex remains stably associated with RISC. This strand becomes the mature miRNA. The opposite strand, known as the passenger strand, is disposed of through two alternative mechanisms. When miRNAs are loaded into RISC containing Ago2, the only human Ago protein capable of cleaving target mRNAs, the passenger strand may be cleaved. Alternatively, RISC containing any Ago protein may remove the passenger strand *via* a bypass mechanism that does not require cleavage and likely involves duplex unwinding (Gregory *et al.*, 2005; Matranga *et al.*, 2005; Rand *et al.*, 2005). The miRNA guides RISC to the target mRNA, which will then be subsequently cleaved or translationally silenced. The

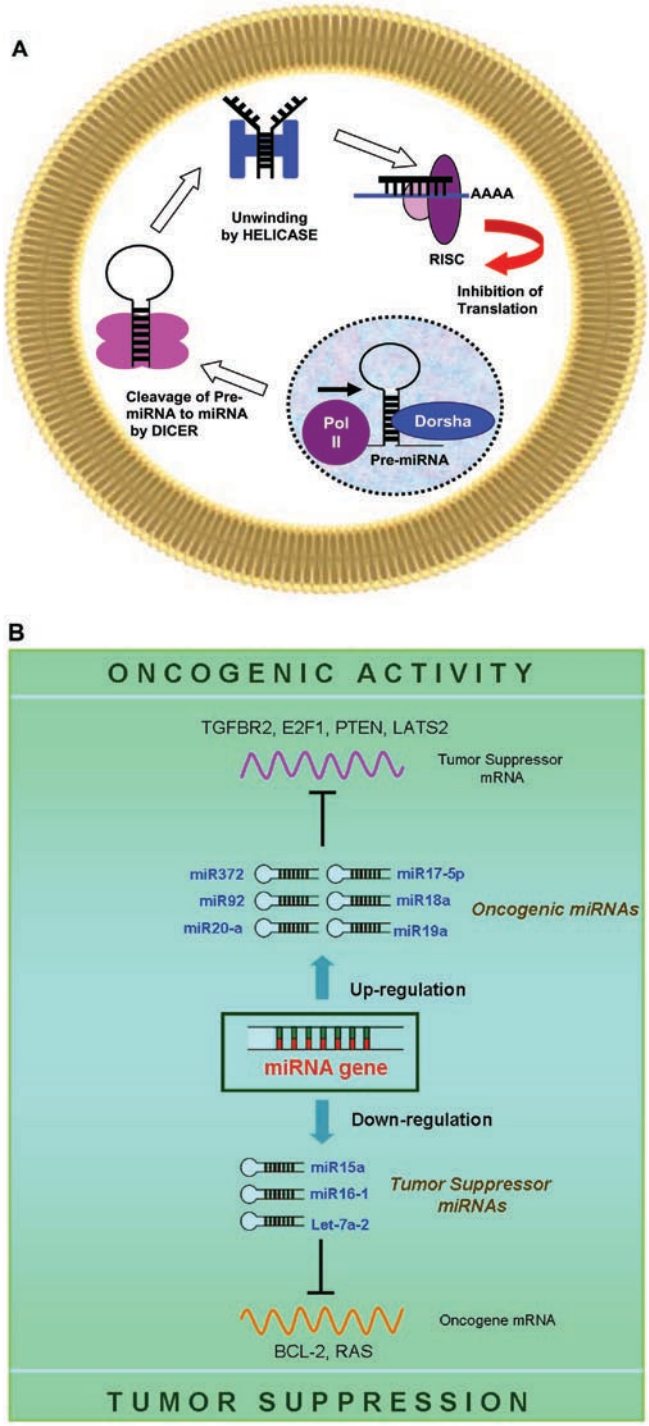

Figure 3. Biogenesis of miRNA. **(A)** The biogenesis of miRNA involves several enzymatic steps. Following transcription by RNA polymerase II (Pol II), capped and polyadenylated primary miRNA transcripts (pri-miRNAs) are processed in the nucleus by the endonuclease Drosha into one or more pre-miRNAs (1). This pre-miRNA is exported from the nucleus to the cytoplasm and processed by another RNase enzyme called Dicer, which produces a transient 19- to 24-nucleotide duplex (2). The duplex is cleaved (3), and only one strand of the miRNA duplex (mature miRNA) is incorporated into the RISC (RNA-induced silencing complex), which retains only the single-stranded mature miRNA (4). This miRNA-programmed RISC negatively regulates the stability and/or translation of target mRNAs, depending on the degree of complementary sites between the miRNA and its target. **(B)** In the proposed model, amplification or overexpression of a miRNA that down-regulates a tumor suppressor or other important genes involved in differentiation might also contribute to tumor formation by stimulating proliferation, angiogenesis, and invasion. For example, amplifications of the oncogenic miRNAs, miR-17–92 cluster, miR-21, and miR-372 have been clearly associated with tumor initiation and progression, whereas miRNAs that normally down-regulate an oncogene can act as a tumor suppressor gene when lost in a tumor. Any abnormalities in miRNA biogenesis might result in an abnormal expression of the target oncogene, which subsequently contributes to tumor formation.

degree of complementarity between an miRNA and its target determines the mechanism of binding and silencing.

miRNAs: Biological Significance

miRNAs have been shown to be involved in crucial biological processes, including development, differentiation, apoptosis, and proliferation (Bartel, 2004; Harfe, 2005). A current challenge is to elucidate the function of miRNAs in normal physiologic processes and in disease states. In addition to providing critical functions during normal development and cellular homeostasis, it has become more and more clear that abnormalities in miRNA activity contribute to human disease pathogenesis (Esau and Monia, 2007).

miRNAs in Cancer and Inflammation

Several studies have recently indicated the potential role of miRNAs in cancer and suggest that aberrations in miRNAs may be important in tumor progression (McManus, 2003; Ambros, 2004). Recent studies also showed that amplification or overexpression of a miRNA that down-regulates a tumor suppressor or other important genes involved in differentiation might contribute also to tumor formation by stimulating proliferation, angiogenesis, and invasion (Fig. 3B). In addition, it was demonstrated that more than 50% of miRNA genes are located in fragile sites and cancer-associated genomic regions, suggesting that miRNAs may play a more important role in the pathogenesis of human cancers (Calin *et al.*, 2004). The first proof that miRNAs are involved in cancer came from the finding that miR-15a and miR-16–1 are down-regulated or deleted in most persons with chronic lymphocytic leukemia (CLL) (Calin *et al.*, 2002). The use of miRNA microarrays made possible large profiling studies in cancer patients, confirming that miRNAs are differentially expressed in normal and tumor samples (Calin and Croce, 2006a). Recent studies showed that several pro-inflammatory mediators can up-regulate miRNA expression, thereby finding a potential link between inflammation and cancer. Many studies now report a role for miRNA-155 (miR-155) in regulating T-cell-dependent antibody responses and in immune-cell function. miR-155 is generated from the non-coding transcript of the BIC gene. It is known to be expressed in human B-cell lymphomas, in activated mature and T-cells, and in activated macrophages and dendritic cells. bic/miR-155 shows overexpression in activated B- and T-cells (Eis *et al.*, 2005), as well as in activated macrophages and dendritic cells (Taganov *et al.*, 2006, 2007; O'Connell *et al.*, 2007, 2008). Overexpression of bic/miR-155 has been reported in B-cell lymphomas and solid tumors, suggesting that the locus may also be linked to cancer (Calin and Croce, 2006b).

miRNAs in Endocrine Disorders

Proof-of-concept studies have recently shown that miRNAs play an important role in endocrine function, and their differential expression may be responsible for aberrations in hormone regulation. The discovery of a pancreatic islet-specific miRNA, miR-375, which inhibits insulin secretion in mouse pancreatic cells, revealed a novel component of the insulin secretion system (Poy *et al.*, 2004; 2007). miR-375 is thought to act by inhibiting the expression of myotrophin (also known as V-1), which induces the exocytosis of insulin granules (Poy *et al.*, 2004). Another miRNA that has been shown to be associated with adipocyte differentiation is miR-143 (Esau *et al.*, 2004). Since excess adiposity contributes to type 2 diabetes, hypertension, and coronary heart disease, new insights provided by the study of miRNAs in adipocyte biology could have a significant clinical impact.

miRNAs in Cardiovascular Development and Pathogenesis

The global role of miRNA function in the heart has been addressed by the conditional inhibition of miRNA maturation in the murine heart, and it has been found that miRNAs play an important role during its development (Zhao *et al.*, 2007). miRNA expression profiling studies have shown that expression levels of specific miRNAs change in diseased human hearts, suggesting their role in cardiomyopathies (van Rooij *et al.*, 2006). Moreover, *in vivo* studies on specific miRNAs have identified distinct roles for miRNAs both during heart development and under pathological conditions, including the regulation of key factors important for cardiogenesis, the hypertrophic growth response, and cardiac conductance (Xiao *et al.*, 2007; Yang *et al.*, 2007; Zhao *et al.*, 2005, 2007). Recent technological developments in the areas of bead-based flow cytometry, single-molecule detection, and massively parallel sequencing, coupled with the miRAGE approach, may help to launch a high-speed automatable process for miRNA profiling in the near future (J Lu *et al.*, 2005; Neely *et al.*, 2006; Service, 2006).

DESIGN AND SYNTHESIS OF TARGET-SPECIFIC siRNAs

Designing of mRNA-specific siRNAs

The siRNA selection methods represent a critical first step that can have a significant impact on the downstream processes governing the efficiency and efficacy of the RNAi of a particular gene target (Elbashir *et al.*, 2002). Several algorithms have been published, but they remain inefficient, obscure, or commercially restricted. Holen (2006) described an open-source JAVA program that is surprisingly efficient at predicting active siRNAs (Pearson correlation coefficient r = 0.52, n = 526 siRNAs). Furthermore, the version 1.0 sets the stage for further improvement of the free code by the open-source community (http://sourceforge.net) (Holen, 2006). There are many online algorithms available for proper siRNA design. We have listed various Web resources for the proper design of siRNAs (Table 1).

Conventional siRNA Design

The conventional methods of siRNA design begin by the identification and proper selection of the coding sequence for regions that are free of translational or regulatory proteins (*e.g.*, ~115 bases downstream of the start codon). The sequence motifs characterized by an AA (or NA) dinucleotide preceding about 20 base sequences with 35-75% G/C content are then chosen. The dinucleotide leader defines the sequence composition of the antisense 3′ overhangs so that 20-base duplexes targeting AA (N_{20}) would have 3′ termini of UU or dTdT. The subsequent anti-sense strand would be completely complementary to the target mRNA sequence. On an average, 65-75% of the siRNA duplexes designed by conventional methods cause 50- 65% gene silencing, but manifest variability in gene knockdown efficacy (Bernstein *et al.*, 2001a). In many cases, less than 70% of gene knockdown may not be biologically or therapeutically significant. This, in turn, calls for additional techniques to sort out this lesser degree of gene-silencing ability by the conventionally designed siRNAs.

Table 1. Web Resources for Proper siRNA Design and their Designing Rules.

Developer	Web Site ID	Designing Rules	Critical Analysis
Ambion	www.ambion.com/techlib/misc/siRNA_finder.html	Tuschl (T)	No score/rank, links with NCBI Blast
Dharmacon	http://design.dharmacon.com/	Reynolds (R)	Score/Rank; Automatically runs Blast
Clontech	http://bioinfo2.clontech.com/rnaidesigner/	T	No score/rank; link to NCBI Blast
Deqor	http://cluster-1.mpi-cbg.de/Deqor/deqor.html	Own algorithm	Score/Rank; runs Blast automatically
dsCheck	http://alps3.gi.k.u-tokyo.ac.jp/~dscheck/main/index2.php	Own algorithm	Not for humans and mice
EMBOSS	http://inn.weizmann.ac.il/EMBOSS	T	Score/Rank; No Blast
GenScript	www.genscript.com/ssl-bin/app/rnai	T, R &Ui-Tei (UT)	Score/Rank; with NCBI Blast
Hannon Lab	http://katahdin.cshl.org:9331/siRNA/	Own algorithm	No Score/Rank; No description of method
IDTDNA	http://biotools.idtdna.com/rnai/	T, Fire	No score; link to NCBI Blast
Interagon	www.interagon.com/demo/	Own algorithm	Perform Blast automatically (only mouse)
Invitrogen	https://rnaidesigner.invitrogen.com/sirna/	T or Proprietary	Rank No score; Automatically runs Blast
Jack Lin	www.sinc.sunysb.edu/Stu/shilin/rnai.html	T	Link to NCBI Blast; by overhang and %GC
OptiRNAi	http://bioit.dbi.udel.edu/rnai/	T	Score/Rank; Link to Genome Blast
Promeg	www.promega.com/siRNADesigner/program/	Mixed	Score/Rank;Link to NCBI
Qiagen	www.qiagen.com/Products/GeneSilencing/	T	Score/Rank; Link to NCBI; no control
SFold	http://sfold.wadsworth.org/sirna.pl	R& T	No rank; No Blast
SiDE 20	http://side.bioinfo.ochoa.fib.es/	Mixed	Built for high-throughput; Blast against Ensembl
siDirect	http://design.rnai.jp/sidirect/	UT	Option for custom rules; No Score/Rank
siRNA wizard	http://www.sirnawizard.com/design_advanced.php	Mixed rules	Gives top 10, Auto-Blast
siSearch	http://sonnhammer.cgb.ki.se/siSearch/siSearch_1.6.html	Mixed rules	Customizable, Various output formats; Blast
TROD	http://websoft2.unige.ch/sciences/biologie/bicel/RNAi	Own algorithm	Score/Rank; unigene, Blast
Whitehead	http://jura.wi.mit.edu/siRNAext/	T & R	Blast of candidates; sorted by thermodynamics
Wistar	http://hydra1.wistar.upenn.edu/Projects/siRNA	R&Thermodymanics	No score; link to NCBI Blast

Rational siRNA Design

When the conventional approach was applied to 22 genes of the insulin-signaling pathway, only 25% of the 120 siRNAs designed produced more than 80% of the gene silencing (Hsieh *et al.*, 2004). In contrast, 48 of the 120 siRNAs designed based on the functional parameters using an algorithm yielded more than 80% gene-silencing capacity (Huang *et al.*, 2004). The benefit of the method was found unexpectedly when the rationally designed siRNAs were pooled (Holen *et al.*, 2002; Martinez *et al.*, 2002). The unanticipated results provided by the rationally designed siRNA was that, on average, the efficacy required to silence a particular gene target was greater than that of conventionally designed siRNAs. Significant levels of silencing can be attained with sub-nanomolar concentrations both *ex vivo* and *in vivo* (Harborth *et al.*, 2003).

FACTORS DETERMINING siRNA EFFICACY

Proof-of-concept experiments have precisely demonstrated that siRNA efficacy depends largely on multiple factors, such as the thermodynamic stability of the duplex at the 5′ antisense end, ability to form internal hairpins in the RISC complex (reduces the silencing ability), GC content, base preference for an adenosine, not a cytosine, at the 19th or 20th base position of the sense strand (required for the unwinding/activation step in the RISC), and preferences for uridine at position 10 and adenosine at position 3 and any base at position 13 except guanosine (Khvorova *et al.*, 2003). siRNAs have been shown to be more stable in mammalian cells and physiological fluids than antisense oligodeoxyribonucleic acids (AS-ODNs). However, differences in efficacy at different gene targets remain problematic for both siRNA- and AS-ODN-based gene targeting (Lewis *et al.*, 2002). There have been attempts to prolong the gene-silencing activity of siRNAs by introducing various chemical modifications that aim to increase the stability of the molecules while maintaining their gene-silencing potency, such as 2′-O-methylation (Chiu and Rana, 2003). It is noteworthy that phosphorothioate modifications (replacement of one of the non-bridging oxygen atoms in the phosphodiester bond by sulfur), similar to those introduced in the AS-ODNs that are currently in clinical trials, appear to be compatible with siRNAs (Amarzguioui *et al.*, 2003). Currently, companies such as Dharmacon RNAi technologies (Thermo Scientific) supply multiple siRNAs for silencing of target genes (www.dharmacon.com). It has been shown that single- or multiple-siRNA duplexes directed against a single as well as multiple target genes yielded more than 80% gene silencing, compared with the pooling of conventionally designed siRNAs (Reynolds *et al.*, 2004). Recently, two independent research groups reported that relatively long double-stranded RNAs (25-30 bp in length) have significantly higher gene-silencing capability than the conventional 21-bp siRNAs. The enhanced efficacy of RNAi has been demonstrated with synthetic RNAs of 27 bp in length with blunt ends (Kim *et al.*, 2005). It has been shown that dsRNAs of 29 bp with a hairpin loop structure significantly improved gene-silencing ability (Siolas *et al.*, 2005). The longer dsRNAs are 100-fold more potent than the conventional siRNAs, and the dsRNAs of 25-30 bp in length can be exploited for gene silencing in laboratory experiments and clinical therapeutics.

High-performance-purity (HPP)-grade siRNA Synthesis and Quality Control (QC)

High-purity, full-length siRNA increases the specificity and

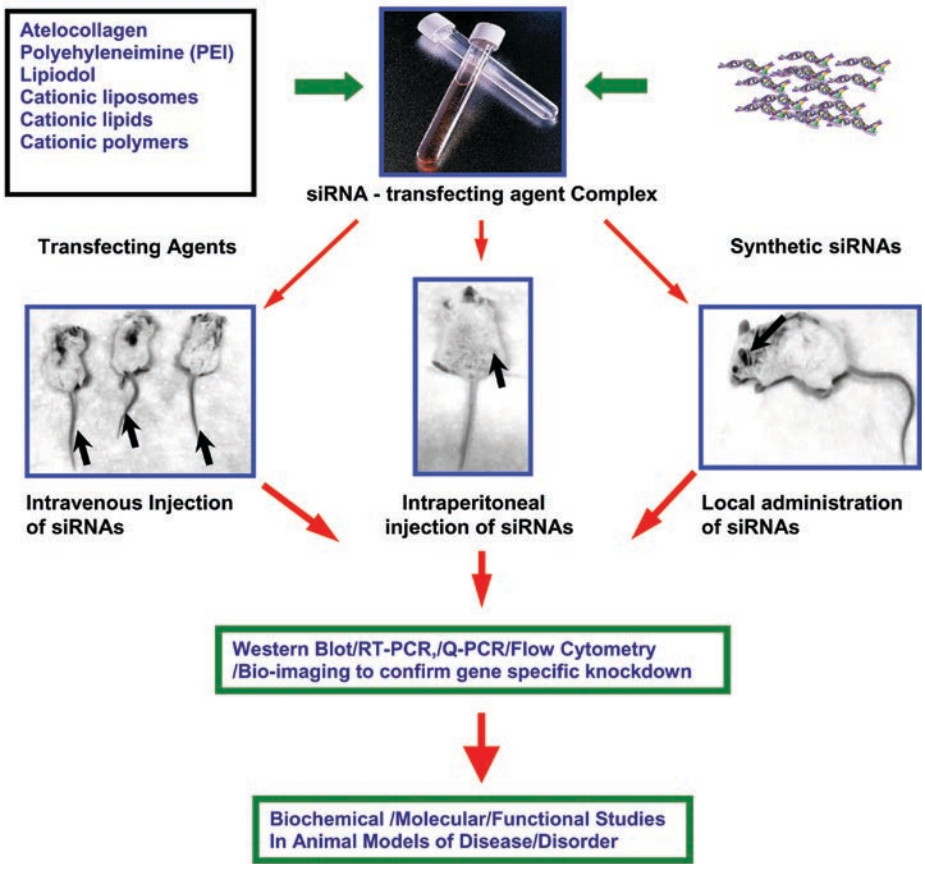

Figure 4. Schematic representation of non-viral *in vivo* transfecting agent-mediated transfer of siRNA duplex for functional genomics and therapeutics. The transfecting agents are useful for both local and systemic delivery of siRNA, since most of the siRNA-transfecting agent complexes are stable *in vivo*. Subsequently, for the evaluation of the *in vivo* efficacy and stability of siRNAs, Western blot/RT-PCR/Q-PCR/flow-cytometry/non-invasive bio-imaging can be adopted. Based on the degree of knockdown, the specific siRNA sequence can be used to silence the specific disease-causing gene in various animal models of disease/disorder.

efficiency of gene silencing. HPP siRNA can be synthesized by patented methods, such as TOM amidite chemistry (Pitsch and Weiss, 2001). The siRNAs synthesized are > 90% pure and can be used without further high-performance liquid chromatography or polyacrylamide gel electrophoresis purification (Pitsch *et al.*, 1999). The chemical synthesis of RNA is more difficult than that of DNA, because the 2′ OH group of RNA nucleotides must be protected during synthesis. Additional steps in the synthesis process introduce 2′-OH protecting groups into monomers and remove them once RNA is assembled. With TOM-protected monomers, high coupling efficiencies of > 99.5% are routinely achieved. The coupling efficiency and easy, clean de-protection allows RNA of up to 100 bases in length to be synthesized with the same quality and yield as for DNA. TOM chemistry is fully compatible with all available modifications, including fluorescent labels, sequence and terminus modifiers, backbone modifications, and unnatural nucleobases (Pitsch and Weiss, 2001; Pitsch *et al.*, 2001). Qiagen (www.qiagen.com) has developed HPP-grade siRNA synthesis that combines high-throughput RNA synthesis and high-throughput purification. HPP-grade siRNA duplexes have excellent yields and a reproducible purity of > 90%. An integrated tracking system monitors siRNA production from the data entry of an mRNA target sequence to chemical synthesis in 96-well plates, quality control (QC), and final processing and packaging. siRNA is synthesized in 96-well format and is purified by automated, high-throughput HPP purification.

DELIVERY OF siRNAs

Several types of vectors for siRNA delivery have been developed, including viral vectors and non-viral vectors. Among them, non-viral vectors have the advantages of low toxicity, ease of synthesis, and low immune response (Zhang *et al.*, 2007). Unlike plasmids, viral vectors have the advantage of delivering siRNAs to non-dividing cells such as neurons: They are the most commonly used carriers for gene transfer because of their high *in vitro* transfection efficiency. An advantage of lentiviral vectors, in comparison with adenovirus, is that they allow for the introduction of short RNAs into blood and bone marrow cells (Hannon and Rossi, 2004). Although expression vectors are widely used to induce short RNA-mediated RNAi *in vitro* and *in vivo*, it is anticipated that the toxicity of viral vectors will deter their use in humans (Reid *et al.*, 2002; McCaffrey *et al.*, 2003). There are still some major issues that need to be addressed before the gene-silencing approach with viral vectors proceeds to the clinic for treating patients (Azzouz, 2006). Recently, it has been shown that the lentivirus-mediated RNAi can be used to study gene function in mammalian tooth development (Song *et al.*, 2006). Much research is currently focused on improving expression vectors, to diminish the side-effects, and to enhance the delivery of siRNAs into specific target tissues. The simplest strategy relies on tet-operable polymerase III-promoted shRNAs and co-expression of the tetracycline regulatory protein, TetR. The construction of shRNA libraries has been discussed in detail (Bernards *et al.*, 2006). Chang *et al.* (2006) have constructed first-generation shRNA libraries modeled after precursor miRNAs and second-generation libraries modeled after primary miRNA transcripts (the Hannon-Elledge libraries). These libraries were arrayed and sequence-verified, and cover a substantial portion of all known and predicted genes in the human and mouse genomes. Comparison of first- and second-generation libraries indicates that RNAi triggers that enter the RNAi pathway through a more natural route yield more effective silencing. These large-scale resources are functionally versatile, since they can be used in transient as well as stable studies, and for constitutive or inducible silencing. Library cassettes can be easily shuttled into vectors that contain different promoters and/or that provide different modes of viral delivery (Chang *et al.*, 2006). Recently developed genome-wide shRNA- and miRNA-adapted short hairpin RNA (shRNAmir) libraries incorporate advances in shRNA design and molecular

'barcodes' to facilitate more complex RNAi screens and provide the opportunity to progress to more complex genetics in whole animals (Fewell and Schmitt, 2006). Various types of transfecting agents are used to deliver the siRNAs *in vivo* (Fig. 4). The non-viral delivery system, such as atelocollagen for siRNA, which could be useful for functional screening of the genes *in vitro* and *in vivo*, will provide a foundation for further development of RNAi therapeutics (Beertsen *et al.*, 1997; Minakuchi *et al.*, 2004; Takeshita *et al.*, 2005; Takeshita and Ochiya, 2006; Honma *et al.*, 2007; Ochiya *et al.*, 2007). Polymer particles and cationic liposomes, major varieties of non-viral vectors used for gene delivery, have been shown to be suitable for the delivery of siRNA (Zhang *et al.*, 2007). Cationic liposomes bearing siRNAs have been intravenously injected into mice (Hannon and Rossi, 2004), and electroporation has been used to deliver siRNAs and shRNAs to post-implantation embryos and post-natal retinas (Reid *et al.*, 2002; Sorensen *et al.*, 2003; Hannon and Rossi, 2004). The lactosylated cationic liposome 5 that contained the most lactose residues, shown to introduce the most siRNA into a human hepatoma cell line, inhibited replication of HCV replicons. In mice, the siRNA/lactosylated cationic liposome 5 complexes accumulated primarily in the liver, were widespread throughout the hepatic parenchymal cell, and specifically and dose-dependently reduced intra-hepatic HCV expression in transgenic mice without interferon response (Watanabe *et al.*, 2007). The potential of apolipoprotein A-I (apo A-I) for the systemic delivery of nucleic acids to the liver has been demonstrated with real-time *in vivo* imaging (Kim *et al.*, 2007). As a proof-of-concept experiment, synthetic siRNAs against HBV were formulated into complexes of apo A-I and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/cholesterol (DTC-Apo) and injected intravenously (i.v.) into a mouse model carrying replicating HBV. The administration of these nanoparticles can significantly reduce viral protein expression by receptor-mediated endocytosis. The advantages of the apo A-I-mediated siRNA delivery method are its liver specificity, its effectiveness at low doses ($\leq$ 2 mg/kg) in only a single treatment, and its persistent antiviral effect up to 8 days. A hydrophobically modified protein transduction domain, cholesteryl oligo-d-arginine (Chol-R9), stabilized and enhanced tumor regression efficacy of the VEGF-targeting siRNA. The non-covalent complexation of a synthetic siRNA with Chol-R9 efficiently delivered siRNA *in vitro*. Moreover, in a mouse model bearing a subcutaneous tumor, the local administration of complexed VEGF-targeting siRNA, but not of scrambled siRNA, led to the regression of the tumor (Kim *et al.*, 2006). Delivery of siRNAs relevant to lung diseases has been attempted through multiple routes and the use of various carriers in animal models (Thomas *et al.*, 2007). However, the clinical success of siRNA-mediated interventions for any disease/disorder in humans critically depends upon the safety and efficacy of the delivery methods and agents.

MAJOR CHALLENGES OF RNAi THERAPEUTICS

The major obstacle is the development of effective delivery systems for the direct delivery of siRNAs into target tissues. However, alternative approaches—such as electropulsation (Golzio *et al.*, 2005; Escoffre *et al.*, 2008; Golzio and Teissie, 2008), antibody-mediated endocytosis (Song *et al.*, 2005; Vornlocher, 2006), or repetitive administration of siRNA in lesser volume and speed—should be considered, instead of the lethal hydrodynamics-based transfection *in vivo* (Pushparaj *et al.*, 2008b). Hence, the clinical utility of siRNAs and DNA therapeutics will depend, at least in part, on the development of safe and effective delivery systems which are pivotal for the advancement of future therapy for human diseases. Preliminary studies on RNAi with long dsRNA yielded only partial success in both *in vitro* and *ex vivo* studies (Zhao *et al.*, 2001). Researchers later found that this was in fact due to the dsRNA-dependent activation of interferon production, which subsequently resulted in a global inhibition of translation and cell death (Baglioni, 1979; Minks *et al.*, 1979). However, proof-of-concept studies later demonstrated that the cytotoxic interferon response can be bypassed by the introduction of chemically synthesized siRNAs that result in the targeted gene-silencing phenomenon (Caplen *et al.*, 2001). It has been made clear that the RNAi effect triggered by endogenous or exogenous siRNAs is transient and dose-dependent. However, there is little information on the regulation of RNAi. Various studies have shown that siRNA degradation generally peaks 36 to 48 hrs after introduction and begins to diminish at around 96 hrs. This can be prolonged with repeated siRNA delivery and obviously depends on the rate of target turnover (Pushparaj and Melendez, 2006). Recently, Hong *et al.* (2005) tested the expression inhibition of the transgene of hepatitis B virus surface antigen by different amounts of siRNA in the presence or absence of a mouse orthologue of enhanced RNAi1 (eri-1). Dose-response analyses carried out *in vitro* and *in vivo* with *Escherichia coli*-expressed and enzyme-digested siRNA (esiRNA) confirmed the dose-dependent effect. However, the results revealed an unexpected decrease in the inhibition effect that was associated with the introduction of a higher dose of esiHBVP, compared with a lower dose of esiHBVP (Hong *et al.*, 2005). Moreover, we have recently shown that the amount of siRNA delivered is crucial, since using too little or too much siRNA minimizes the knockdown effect (Pushparaj *et al.*, 2008b).

By targeting the promoter region of various genes, Li *et al.* (2006) identified several dsRNAs that induce gene transcription in a sequence-specific manner. This RNA-mediated process requires the Ago2 protein and is associated with histone changes linked to gene activation. Although the exact mechanism is unknown at present, the identification of RNA activation (RNAa) may still have significant therapeutic applications (Li *et al.*, 2006). It was also proved that, like proteins, hormones, and small molecules, siRNAs interact at promoters and can trigger or suppress gene expression (Janowski *et al.*, 2006, 2007). The use of RNAi is currently being proposed as a gene-specific approach for molecular medicine (Pushparaj and Melendez, 2006; Manikandan *et al.*, 2007). By the same principle, the specific activation of silenced tumor suppressor genes such as p21 or other common dysregulated genes, such as E-cadherin, by RNAa may further add to the growing therapeutic prospects for dsRNAs-based drugs in the treatment of cancer and other diseases (Check, 2007). Interestingly, RNAi typically silences genes for 5 to 7 days, but RNAa boosted gene activity for up to 13 days (Garber, 2006). How small dsRNAs could activate genes, especially for a very long period, has not been well-characterized. This information also indicates a new obstacle for RNAi, in which siRNAs may undesirably stimulate the expression of off-target genes. Hence, it can be stated that both RNAa and RNAi constitutes the Yin and Yang of the RNAome in living organisms (Pushparaj *et al.*, 2008a).

Table 2. siRNAs in Clinical Trials

Pharmaceutical Company	Project and Strategy	Disease/Disorder	Clinical Trial Status
Allergan	AGN211745	Wet age-related macular degeneration (AMD)	Phase II
Alnylam	ALN-RSV01	Respiratory syncytial viral (RSV) infections	Phase II
Opko Health	Cand5/bevasiranib	Wet AMD	Phase III
Silence Therapeutics, Quark Biotech, Pfizer	RTP801i-14	Wet AMD	Phase II
	AKIi-5	Acute renal failure	Phase I
	CTPi-1	Chronic obstructive pulmonary disease	Pre-clinical studies
	BT16	Dyslipidemia	Pre-clinical studies
	AHLi-11	Acute hearing loss	Pre-clinical studies

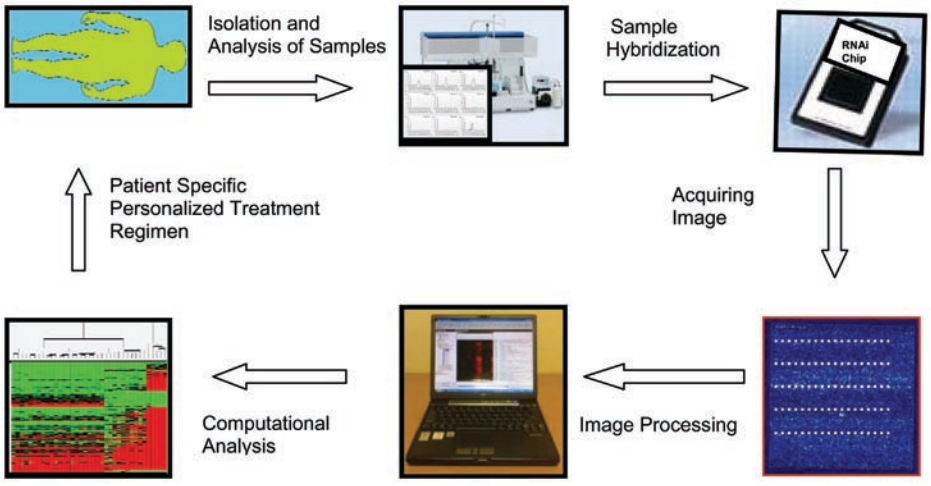

Figure 5. The disease-causing genes in humans can be identified by molecular diagnosis, and the treatment testimonial could be formulated based on the expression analysis in an RNAi chip for specific genes using computational analysis in a typical clinical milieu.

RNAi AS NOVEL THERAPEUTICS – IS IT IMMINENT?

Over the past two decades, scientific and technical breakthroughs have significantly advanced the field of ribonucleic-acid-based therapeutics. The first siRNA, bevasiranib (Opko Health; formerly named Cand5 from Acuity Pharmaceuticals), has recently entered a key Phase III trial for the treatment of wet age-related macular degeneration (AMD). In a Phase II trial, bevasiranib was established to be safe and well-tolerated and showed benefits against several endpoints, including near vision and lesion size. However, the leader in the siRNA field is Alnylam Pharmaceuticals, and its most advanced drug candidate, ALN-RSV01, is in a Phase II trial for the treatment of respiratory syncytial virus infections. The trial will involve adult humans experimentally infected with respiratory syncytial virus. A Phase II trial in naturally infected persons is planned for 2008. Respiratory syncytial virus infection, the leading cause of pediatric hospitalization in the US and a prevalent infection in immune-compromised adults, currently does not have a viable treatment option. Alnylam also expects to file an investigational new drug application (IND) for either ALN-PCS01, for the treatment of hypercholesterolemia, or ALN-VSP01, for the treatment of liver cancers and potentially other solid tumors. This application would be particularly significant, since it would be the first time a systemically delivered siRNA is brought into clinical development. The list of siRNAs currently in clinical trials is shown in Table 2. Alnylam's ALN-RSV01 was tested in a Phase I human clinical trial, and the results were announced at the 18th Annual Drug Delivery to the Lungs meeting, held in Edinburgh, UK. Alnylam Pharmaceuticals, Inc. has announced that the European Patent Office has granted the previously allowed Tuschl II patent. The European patent (EP 1407044) broadly covers compositions, methods, and uses of siRNAs. In addition, the company announced that the German Patent Office has granted a new patent (DE 10066235) in the Kreutzer-Limmer I patent series, broadly covering methods, uses, and medicaments for siRNAs with a length of 15 to 49 nucleotide pairs expressed *via* vectors. PARI's eFlow, an advanced electronic nebulizer, has been optimized and used to deliver the siRNAs. Polyplus-transfection is developing several *in vivo* siRNA formulation solutions based on its lead compounds *in vivo*-jetPEI and INTERFERin, as well as on an original RNA modification, sticky siRNA. Recently, Polyplus-transfection has signed a collaboration agreement with Alnylam Pharmaceuticals to provide Alnylam with its RNAi therapeutic delivery solutions and share its expertise in formulation for siRNAs.

Given the blockbuster potential of RNA-based therapeutics, some of the most strategically and financially significant deals between biotechnology and pharmaceutical companies in the past two years have been signed around the globe. Alnylam has entered into non-exclusive licensing agreements with Novartis and Roche—deals potentially worth US$700 million and $1 billion, respectively. Silence Therapeutics partnered with AstraZeneca ($400 million) to develop RNAi drugs for respiratory diseases. Isis licensed its pre-clinical antisense programs in diabetes, obesity, and metabolic disease to Bristol-Myers Squibb ($192 million) and Ortho-McNeil/Johnson and Johnson ($460 million). Archemix, a leading aptamer company, has partnered with Elan ($360 million), Merck Serono, Pfizer, and Takeda. Also, Merck paid a 100% premium to acquire Sirna for $1.1 billion. These transactions generated tremendous value for pioneering biotechnology companies and their investors, and provided further validation of the potential of RNA-based drugs to become an important next generation class of medicine.

CONCLUSIONS

In this review, we have provided insights into the *in vivo* applications of RNAi and its huge untapped potential for treating various diseases, including oral diseases/disorders The advantages of efficient, economical knockdown offered by RNAi and the large amount of data it provides will ensure that it remains a technology of choice for functional genomics and drug discovery research. Recent advances in RNAi microarrays promise to increase the efficiency, economy, and ease of genome-wide RNAi screening, and these new approaches will be vital for the development of RNAi technology into a robust therapeutic approach (Fig. 5). Overcoming the systemic delivery obstacle remains one of the most crucial challenges on the road of bringing RNAi drugs to the market. Although there has been significant progress in this area to date, creating delivery solutions for RNAi therapeutics remains an important component in realization of the full potential of this promising technology. However, the molecular machinery of RNAome, involving both RNAi/RNAa, still needs to be characterized to answer the question of how the same enzymes of the RNAi pathway can sometimes switch genes off, and sometimes on. Hence, a thorough understanding of the molecular machinery of RNAa and RNAi is required before the RNAi/RNAa strategies can be adapted from bench to bedside.

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